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Molecular Diagnostics in Tomato: Chip Digital PCR Assays Targeted to Identify and Quantify *Clavibacter michiganensis* subsp. *michiganensis* and *Ralstonia solanacearum in planta*

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Abstract: *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) and *Ralstonia solanacearum* (Rs) are important bacterial pathogens of tomatoes (*Solanum lycopersicum*), are included in A2 list in the EPPO (European and Mediterranean Plant Protection Organization) region and are recommended for regulation as quarantine pests. The control of quarantine pathogens requires accurate and rapid detection tools. In this study, a method based on chip digital PCR (cdPCR) was developed to identify and quantify Cmm and Rs. The assays were tested on pure bacteria samples and on tomato samples naturally contaminated or spiked with bacteria DNA. For a better estimation of infection level in host plants, duplex assays that are able to simultaneously amplify plant and bacteria DNA were developed. The two cdPCR assays proposed can be used for the rapid and timely detection of this group of high-risk quarantine bacteria to prevent the spread of pathogens and the occurrence of disease in other areas.

Keywords: tomato pathogens; molecular diagnostics; digitalPCR

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1. Introduction

Clavibacter michiganensis subsp. *michiganensis* (Cmm) and *Ralstonia solanacearum* (Rs), causes of black canker and bacterial wilt disease, respectively, are the most devastating pathogens in tomatoes (*Solanum lycopersicum*) cultivated in both greenhouses and fields [1,2]. They can reduce tomato yield and quality, causing significant economic losses worldwide. Tomato is the second most important vegetable crop after potato, with an estimated production of 187 million tons [3], of which more than 40 million tons are grown in Mediterranean countries [4]. These two soil-borne pathogens infect plant roots and invade xylem vessels and the aerial parts of the vascular system. During colonization, they secrete cell-wall-degrading enzymes and destroy xylem and parenchyma cells to acquire nutrients, causing rapid wilt and plant death [5,6]. The high lethality of these pathogens and their capacity to survive in soil for long periods make consequent infections difficult to eliminate. For these reasons, these bacteria are considered quarantine microorganisms in Europe and are listed as A2 pests by the EPPO (European and Mediterranean Plant Protection Organization) [7]. Cmm is a Gram-positive soil-borne bacterium able to epiphytically infect tomatoes via natural openings and wounds or from infected seeds. Once inside a susceptible host, the pathogen multiplies in the xylem vessels where it produces extensive biofilm-like structures, promoting colonization [8]. Cmm is a serious threat to tomato processing and fresh market industries, having caused huge epidemics in several tomato-growing areas.

It inhibits tomato production and causes the premature death of plants and, for these reasons, is one of the principal deleterious plant diseases [9]. The Gram-negative Rs is considered the most destructive plant bacterium worldwide and is the second pathogen, after *Pseudomonas syringae* pathovars, enlisted in the "Top 10" plant pathogenic bacteria index based on scientific and economic importance [10]. This bacterium colonizes the xylem, where it can quickly multiply and produce great amounts of cell biomass in tissues, clogs vessels resulting in water transport disruption and cause wilting. After host plant death, the bacterium is released into the environment. In areas where Rs is considered a quarantine organism, the regulation dictates eradication measures and restrictions on further production in infected lands. In fact, Rs can survive for several years in wet soil, water ponds, crop residues and asymptomatic reservoir plants [10,11].

The timely and proper identification of pathogens from affected plants is achieved by specific control measures, and the quarantine of produce relies heavily on accurate detection methods. Standard protocols for plant pathogen detection consist of isolation and the further identification of microorganisms, which is time consuming and not always sensible or specific enough. Sometimes traditional methods have low reproducibility due to the need to identify by phenotypic traits and false negatives results. Compared to traditional methods, advanced molecular diagnostic tools allow easy, rapid, precise and accurate detection and quantification. In the case of PCR diagnostics, no preliminary enrichment of the target microorganism by culture is, in fact, required. This is an advantage because of the shorter analytical times and because of the reduced possible accidental leaks of quarantine bacteria. Furthermore, the high sensitivity of DNA-based diagnostics allows the identification of the target pathogen even in complex samples. An efficient and rapid molecular diagnosis of infectious microorganisms allows for effective management strategies such as applying ideal treatments, undertaking correct agronomic measure or proceeding with eradication.

Several molecular diagnostic approaches were proposed as an alternative strategy to traditional microbiological methods for the identification and quantification of Cmm and Rs. For example, in the work of Penanzova et al. [12], a multiplex real-time PCR method based on fluorescent TaqMan® probes was employed for the simultaneous detection and quantification of Cmm, *Pseudomonas syringae* pv, tomato and Xanthomonas. Recently Wang et al. [13] proposed a droplet digital PCR (ddPCR) for low Cmm load detection, which could facilitate both inspection for quarantine pathogen and routine controls of black canker in tomato. The purpose of this study was to develop an accurate and rapid detection tool based on chip digital PCR (cdPCR) to identify and quantify Cmm and Rs presence. Digital PCR (dPCR) is an important new tool in plant pathology diagnostics and laboratories. Advantages in the use of dPCR are absolute quantification without relying on a standard curve, greater precision and accuracy, and more accurate quantitation in comparison to other molecular tools. Moreover, the observed reduction in false negatives is of critical importance for the diagnosis of infections to be included in certification programs [14]. The two cdPCR-developed assays were evaluated in tomato samples naturally contaminated or spiked with bacteria.

2. Materials and Methods

2.1. Bacterial Samples

DNA of *Clavibacter michiganensis* subsp *michiganensis* (Cmm), *Ralstonia solanacearum* (Rs), *Xanthomonas vesicatoria* (Xv), *Xanthomonas axonopodis* pv. *phaseoli* (Xap), and *Pseudomonas syringae* pv. tomato (Pst) was obtained from Università Cattolica del Sacro Cuore, Piacenza, Italy. DNA was quantified with Qubit® fluorimeter (Life Technologies™, Invitrogen, Monza, Italy) using Qubit™ dsDNA BR assay Kit (Invitrogen by Thermo Fischer Scientific, OR, USA). Table 1 reports information on the sources and the isolation procedures of the bacterial strains and their morphology when plated on different media.

Table 1. The bacterial strains used, the plant tissues from which the strains were collected and their isolation procedure and morphology on different media. All the strains are conserved at Università Cattolica del Sacro Cuore (Piacenza, Italy) repository and identified by the abbreviated name of the species to which they belong followed by an internal code: *Clavibacter michiganensis* subsp *michiganensis* (Cmm), *Ralstonia solanacearum* (Rs), *Xanthomonas vesicatoria* (Xv), *Xanthomonas axonopodis* pv. *phaseoli* (Xap), and *Pseudomonas syringae* pv. tomato (Pst). The following isolation media were used: Phyto Cmm Agar Base (SCM agar, HiMedia Laboratories GmbH, Einhausen, Germany); yeast extract-dextrose-CaCO3 (YDC, DUCHEFA BIOCHEMIE B.V, Haarlem, The Netherlands); semi-selective medium from South Africa (SMSA, [15]); phyto Xcv Agar Base (mTMB, HiMedia Laboratories GmbH, Einhausen, Germany); Milk Tween agar (MT, DUCHEFA BIOCHEMIE B.V, Haarlem, The Netherlands); King's B agar (KB, DUCHEFA BIOCHEMIE B.V, Haarlem, The Netherlands).

Strain	Source	Isolation Procedure		Morphology	Ref.
		Isolation Media	Incubation Temperature/Time		
Cmm UCSCC	Symptomatic tomato stem	Isolation on semi-selective SCM agar; purification on YDC medium	28 °C/7–10 days	On SCM: translucent grey, mucoid, often irregularly shaped with a variable grey to almost black center. On YDC: yellow, mucoid, confluent and convex, becoming deeper yellow with longer incubation.	[16]
Rs UCSCR	Symptomatic tomato stem	Isolation on semi-selective SMSA agar; purification on semi-selective SMSA agar	28 °C/2–6 days	On SMSA: fluidal, confluent, irregular and creamy-white with pinkish center.	[15]
Xv UCSCV	Symptomatic tomato berries	Isolation on mTMB agar; purification on YDC agar	28 °C/3–6 days	On mTMB: yellow, slightly mucoid, raised and circular. On YDC: pale or bright yellow, circular, mucoid and slightly raised.	[17]
Xap UCSCX	Bean seeds	Isolation on MT agar medium; purification on YDC agar	28 °C/3–5 days	On MT: yellow, with two zones of hydrolysis, i.e., a large clear zone of casein hydrolysis and a smaller milky zone of Tween ™ 80 lysis. On YDC: yellow and mucoid.	[18]
Pst UCSCP	Symptomatic tomato leaves	Isolation on KB agar medium; purification on KB agar medium	25 °C/2–4 days	On KB: production of a pale-blue pigment fluorescent under UV light. Flat, clear and cream-colored colonies.	[19]

2.2. Plant Samples

Plant genomic DNA was extracted in triplicate from 100 mg samples of *Solanum lycopersicum* leaves and stems using DNeasy® Plant Mini Kit (Qiagen® Italia, Milano, Italy) according to the manufacturer's instructions. Two tomato varieties, Cuore di bue d'Albenga and Passenger, for direct consumption and for industrial transformation, were used. Tomato DNA concentrations were determined using Qubit® fluorimeter (Life Technologies™, Invitrogen, Monza, Italy) and Qubit™ dsDNA BR assay Kit (Invitrogen by Thermo Fischer Scientific, OR, USA). Plant DNA samples, previously verified for the absence of bacterial contamination, were spiked with Cmm and Rs DNA. For the preparation of such samples, batches of 3 ng tomato DNA were spiked with dilutions of Rs and Cmm DNAs.

2.3. Naturally Infected Plant Samples

Six samples of naturally infected tomato plants (3 infected by Cmm, and 3 infected by Rs) were used. The positivity of the infections was verified by visual symptom evaluation, according to the procedures described in EPPO Standards PM 7/42 (3) [16] and PM 7/21 (3) [15]. The infection levels were not quantified. DNA was extracted from xylem sap obtained by grinding approximately 2 cm stems directly in 20 mL of extraction buffer AP1 (DNeasy® Plant Mini Kit, Qiagen® Italia, Milano, Italy). After 10 min in incubator at 65 °C, the extraction from 400 µL of lysate was conducted according to manufacturer's instructions. DNA concentrations were determined using Qubit® fluorimeter, as previously reported.

2.4. Design of Primers and Probes

Table 2 reports the primers and probes sequences. Primer Express 3.0.1 Software (Life Technologies™, Invitrogen, Monza, Italy) was used to design Cmm-dig assays. Multiple Primer Analyzer (Thermo Fisher Scientific, Monza, Italy) was used to verify the absence of self-complementarity and primer dimer formation.

Table 2. Primers and probes sequences used in the study to target bacteria and tomato.

Assay Code	Probe and Primers ID	Probe and Primers Sequences	Biological Target	Target Gene	Amplicon Size	Reference
Cmm-dig	Cmm-digF	tctgggtgtgtctggtttcttg	*Clavibacter michiganensis* subsp *michiganensis*	16S-23S GenBank: HM18741.1	61 bp	This Work
	Cmm-digR2	ccccaccaccatccacaa				
	Cmm-Pr	FAM-cggacccttttccgtcgt-MGB				
Rs-dig	RS-I-F	gcatgccttacacatgcaagtc	*Ralstonia solanacearum*	16S GenBank: OP269681.1	93 bp	[20]
	RS-II-R	ggcacgttccgatgtattactca				
	RS-Pr	FAM-agcttgctacctgccggcgagtg-MGB				
Tom-dig	Tom-F	gcaatatcaagagccccgtc	*Solanum lycopersicum*	Prosystemin GenBank: M84800.1.1	91 bp	[21]
	Tom-R	ggagcgcttagcacacat				
	Tom-Pr	VIC-tgcaacatccttctttcttctcgtg-MGB				

2.5. Real-Time qPCR

The primer amplification efficiency was evaluated in Real-Time qPCR using six tenfold dilution series of bacterial DNA in duplicate. Real-time qPCR was prepared in a final volume of 20 µL obtained by mixing 10 µL of Sybr Green GoTaq® qPCR Master Mix 2X (Promega Italia, Milan, Italy), 0.2 µL of Reference Dye ROX 100X (Promega Italia, Milan, Italy), 0.3 µL of each primer at 10 µM (final concentration 150 nmol), 2 µL of DNA template and 7.4 µL of water. PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems by Life Technologies, Monza, Italy) using the following cycling protocol: 95 °C for 10 min (activation), 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing and extension). To evaluate the specificity of the amplified product, a melting curve analysis was included and a negative control without DNA template was run with each assay. Using the direct method of calibration standard dilution curve and slope calculation, real-time qPCR efficiency (E) was determined as $E = 10^{(-1/\text{ slope})} - 1$.

2.6. Chip Digital PCR

QuantStudioTM 3D Digital PCR System (Applied Biosystems by Life Technologies, Monza, Italy) was used for Chip digital PCR assays. The reaction was carried out in a final volume of 16 µL containing 8 µL of QuantStudioTM 3D Digital PCR 2X 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), 3 µL of DNA and 1.48 µL of nuclease free-water. Nuclease-free water as template was used in the negative control. A total of 15 µL of reaction mixture was loaded onto the QuantStudioTM 3D Digital PCR chips using QuantStudioTM 3D Digital chip loader, according to manufacturer' instructions. Amplifications were performed in ProFlexTM 2Xflat PCR System Thermocycler (Applied Biosystems by Life Technologies, Monza, Italy) using the following cycling protocol: 96 °C for 10 min, 47 cycles of 98 °C for 30 s (denaturation) and 55 °C for 2 min (annealing and extension). End-point fluorescence data were collected by QuantStudioTM 3D Digital PCR Instrument and files generated were analyzed using cloud-based platform QuantStudioTM 3D AnalysisSuite dPCR software, version 3.1.6. Each sample was analyzed in duplicate.

3. Results

Three sets of primers/probe were used in this study with the final aim to develop a chip digital PCR based assay panel for *Clavibacter michiganensis* and *Ralstonia solanacearum* detection and quantification in tomato plants. This workflow was followed:

Identification of specific primers/probe sets and optimization;
Evaluation of primers/probe specificity and efficiency in a real-time qPCR system;
Transfer of the assays from real-time qPCR to chip digital PCR;
Validation of chip digital PCR assays in naturally infected tomato plants.

3.1. Primers/Probe Sets

The primer/probe set used for *Ralstonia solanacearum* detection was derived from the study of Weller et al. [20], designed on the 16S rRNA region and suggested by the authors as useful for the broad-range detection of all the *R. solanacearum* biovars. Its transferability to a droplet digital PCR system was evaluated by Dreo et al. [22] in potato tubers. In our study, this same primer/probe set was evaluated in tomatoes using a different PCR platform, i.e., chip digital PCR.

The primer/probe set used for *Clavibacter michiganensis* was optimized, starting with the assay designed by Peňázová et al. [12] on the 16S-23S intergenic region. The assay was redesigned to obtain a shorter amplicon.

A third set of primers/fluorogenic probe, designed with the tomato prosystemin gene sequence, was also used as an internal PCR control. The set was previously used by Collier et al. [21] in a droplet digital PCR system.

3.2. Assays Efficiency, Specificity and Repeatability Evaluation in Real-Time PCR

The efficiency and specificity of the assays were initially evaluated in a real-time PCR system. The efficiencies of the reactions, tested on pure Cmm and Rs DNA ten-fold serial dilutions, were near 100%, as shown in Table 3. Both the assays showed a linear correlation between the amount of bacterial DNA and the Ct values, with a strong determination coefficient equal to $R^2 = 0.999$ for Cmm and $R^2 = 0.993$ for Rs.

Table 3. Efficiency calculation by real-time PCR standard curve analysis from ten-fold serial dilution of pure DNA of Cmm amplified with Cmm-dig assay and Rs amplified with Rs-dig assay. Ten-fold serial dilutions were used, starting from the highest concentration of 10 ng.

Bacteria DNA Amount	Mean Ct Cmm-dig Assay ± dev.stnd	Mean Ct Rs-dig Assay ± dev.stnd
10 ng	12.96 ± 0.21	13.26 ± 0.08
1 ng	16.05 ± 0.014	16.68 ± 0.12
0.1 ng	19.4 ± 0.14	20.01 ± 0.18
0.01 ng	22.82 ± 0.23	23.19 ± 0.052
0.001 ng	25.76 ± 0.035	27.34 ± 0.011
0.0001 ng	29.36 ± 0.26	30.09 ± 0.19
Slope	−3.27	−3.4
Y-intercept	17.13	18.25
Efficiency	102%	96%

The specificity of the two assays was evaluated on a panel of three bacterial species other than Cmm and Rs and on a panel of Cmm and Rs isolates collected from different northern Italian regions. The Cmm assay amplified all the DNAs extracted from the different Cmm isolates, and the Rs assay amplified all the DNAs from the Rs isolates. However, both assays gave no amplification signals in the presence of DNA extracted from the tomato pathogens *Pseudomonas syrigae pv. tomato*, *Xanthomonas vesicatoria* and *Xanthomonas axonopodis pv. Phaseoli.*

The repeatability of the assays was evaluated by replications of the same DNA samples in replicated amplification reactions, i.e., PCR replicates run under repeatability conditions. Standard deviations were found ≤25% for both assays, complying with the acceptance criterion reported by Hough et al. [23].

3.3. Chip Digital PCR for Cmm and Rs Diagnostics

The Cmm and RS assays evaluated in real-time PCR were transferred to the chip digital PCR system. The specificity of the assays was evaluated on the same DNA already extracted from *Pseudomonas syrigae* pv. tomato, *Xanthomonas vesicatoria*, *Xanthomonas axonopodis* pv. *phaseoli*, *Ralstonia solanacearum* and *Clavibacter michiganensis* subsp *michiganensis* using the real-time PCR platform. The specificity was confirmed for both assays, as shown in Figures 1 and 2.

To evaluate the assays' efficiency, DNA templates were prepared by spiking a fixed amount of tomato DNA with Cmm DNA or with Rs DNA dilutions. Figures 3 and 4 show the results obtained by cdPCR regarding the bacteria and tomato copies/µL in tomato samples spiked with different concentration of Cmm and Rs. A correlation between the bacteria DNA amount and bacteria copies/µL obtained by cdPCR had a strong determination coefficient equal to R^2 = 0.99 for both assays.

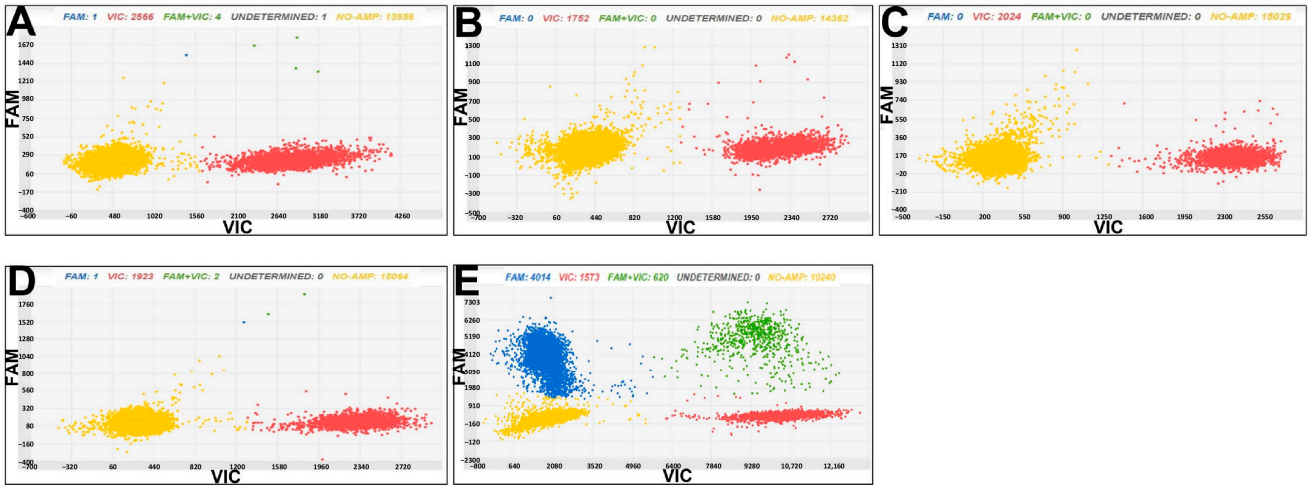

Figure 1. Two-dimensional scatter graphs obtained after chip digital PCR (cdPCR) analysis using the Cmm-dig assay (which generates a FAM-positive signal, visualized as a blue dot) and the Tom-dig assay (which generates a VIC-positive signal, visualized as red). Scatter plots show the signals from FAM reporter dye on the Y-axis and VIC reporter dye on the X-axis. All data points from every well on the chip are shown in the figure. The DNA samples analyzed are as follows: (**A**) Pst (*Pseudomonas syrigae* pv. tomato) plus tomato; (**B**) Xv (*Xanthomonas vesicatoria*) plus tomato; (**C**) Xap (*Xanthomonas axonopodis* pv. *phaseoli*) plus tomato; (**D**) Rs (*Ralstonia solanacearum*) plus tomato; (**E**) Cmm (*Clavibacter michiganensis* subsp *michiganensis*) plus tomato. Amplifications were obtained in all samples for tomato (red dots) and for Cmm in sample E only (blue dots). Green dots are partitions in which both amplifications (for tomato and for Cmm DNA) occurred, whereas yellow dots are negative PCR partitions without any target amplification.

The sampling of stem xylem fluids from infected plants is a well-known, robust and fast method to identify pathogens and to estimate the bacterial load. The rapid extraction of xylem sap is crucial for diagnosing the xylem-colonizing bacteria as Rs and Cmm [24]. Starting from the availability of naturally infected tomato plants, the potentialities of the assays in practical applications were tested. The chip digital PCR quantification of Cmm and Rs was conducted on DNA samples extracted from the stem xylem fluids of the plants. Figure 5 shows the scatter graphs obtained by cdPCR regarding the bacteria's presence in the xylem fluids of infected tomato plants.

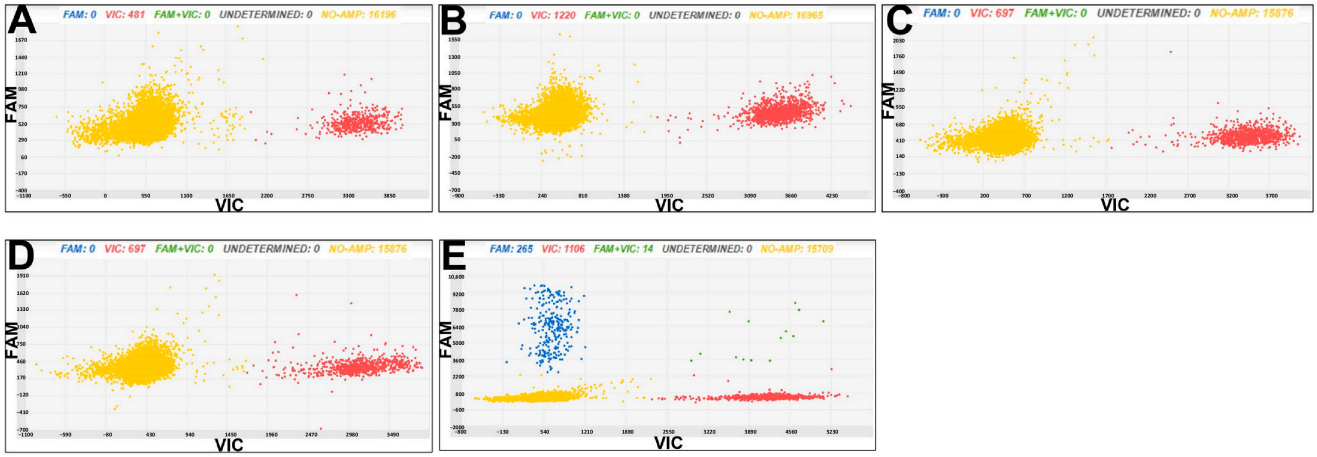

Figure 2. Two-dimensional scatter graphs obtained after chip digital PCR (cdPCR) analysis using the Rs-dig assay (which generates a FAM-positive signal, visualized as a blue dot) and the Tom-dig assay (which generates a VIC-positive signal, visualized as a red dot). Scatter plots show the signals from FAM reporter dye on the Y-axis and VIC reporter dye on the X-axis. All data points from every well on the chip are shown in the figure. The DNA samples analyzed are as follows: (**A**) Pst (*Pseudomonas syrigae* pv. tomato) plus tomato; (**B**) Xv (*Xanthomonas vesicatoria*) plus tomato; (**C**) Xap (*Xanthomonas axonopodis* pv. *phaseoli*) plus tomato; (**D**) Cmm (*Clavibacter michiganensis* subsp *michiganensis*) plus tomato; (**E**) Rs (*Ralstonia solanacearum*) plus tomato. Amplifications were obtained in all samples for tomato (red dots) and for Rs in sample E only (blue dots). Green dots are partitions in which both amplifications (for tomato and for Cmm DNAs) occurred, whereas yellow dots are negative PCR partitions without any target amplification.

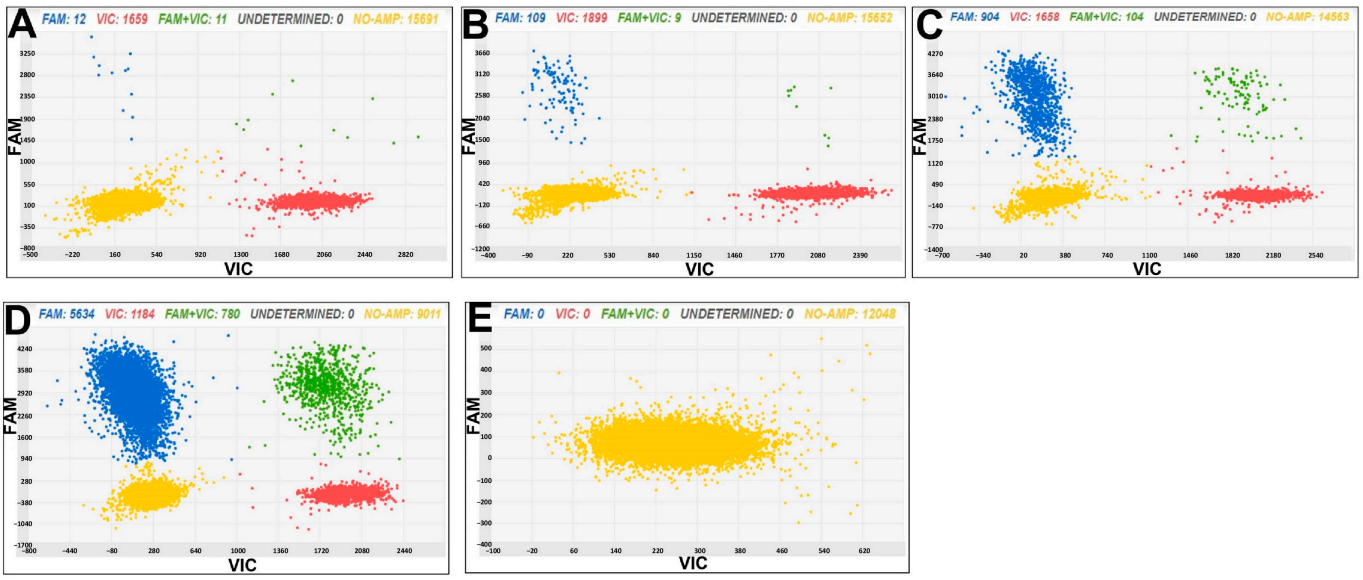

Figure 3. Two-dimensional scatter graphs obtained by chip digital PCR amplification with Cmm-dig assay (which generates a FAM-positive signal, visualized as a blue dot) and the Tom-dig assay (which generates a VIC-positive signal, visualized as a red dot). Scatter plots show the signals from FAM reporter dye on the Y-axis and VIC reporter dye on the X-axis. Fixed amounts of tomato DNA were spiked with increasing quantities of Cmm DNA as follows: (**A**) 3 ng tomato DNA spiked with 0.05 pg Cmm DNA; (**B**) 3 ng tomato DNA spiked with 0.5 pg Cmm DNA; (**C**) 3 ng tomato DNA spiked with 5 pg Cmm DNA; (**D**) 3 ng tomato DNA spiked with 50 pg Cmm DNA; (**E**) no template control-NTC

(blank sample without DNA). Blue dots are PCR partitions with a FAM-positive result, indicating an amplification of Cmm DNA target. Red dots are PCR partitions, which are VIC-positive to the tomato target. Green dots are the partitions in which co-amplification of both targets occurred. Yellow dots are negative PCR partitions without target amplification.

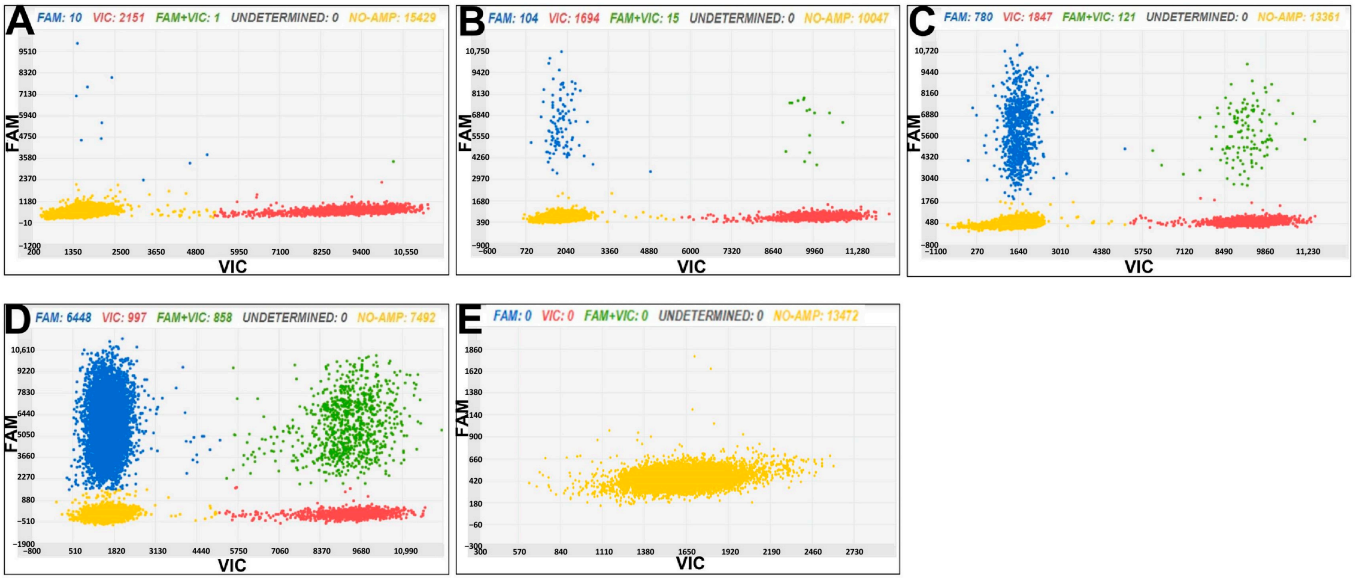

Figure 4. Two-dimensional scatter graphs obtained by chip digital PCR amplification with Rs-dig assay (which generates a FAM-positive signal, visualized as a blue dot) and the Tom-dig assay (which generates a VIC-positive signal, visualized as a red dot). Scatter plots show the signals from FAM reporter dye on the Y-axis and VIC reporter dye on the X-axis. Fixed amounts of tomato DNA were spiked with increasing quantities of Rs DNA as follows: (**A**) 3 ng tomato DNA spiked with 0.05 pg Rs DNA; (**B**) 3 ng tomato DNA spiked with 0.5 pg Rs DNA; (**C**) 3 ng tomato DNA spiked with 5 pg Rs DNA; (**D**) 3 ng tomato DNA spiked with 50 pg Rs DNA; (**E**) no template control-NTC (blank sample without DNA). Blue dots are PCR partitions with a FAM-positive result, indicating an amplification of Rs DNA target. Red dots are PCR partitions, which are VIC-positive to the tomato target. Green dots are the partitions in which co-amplification of both targets occurred. Yellow dots are negative PCR partitions without target amplification.

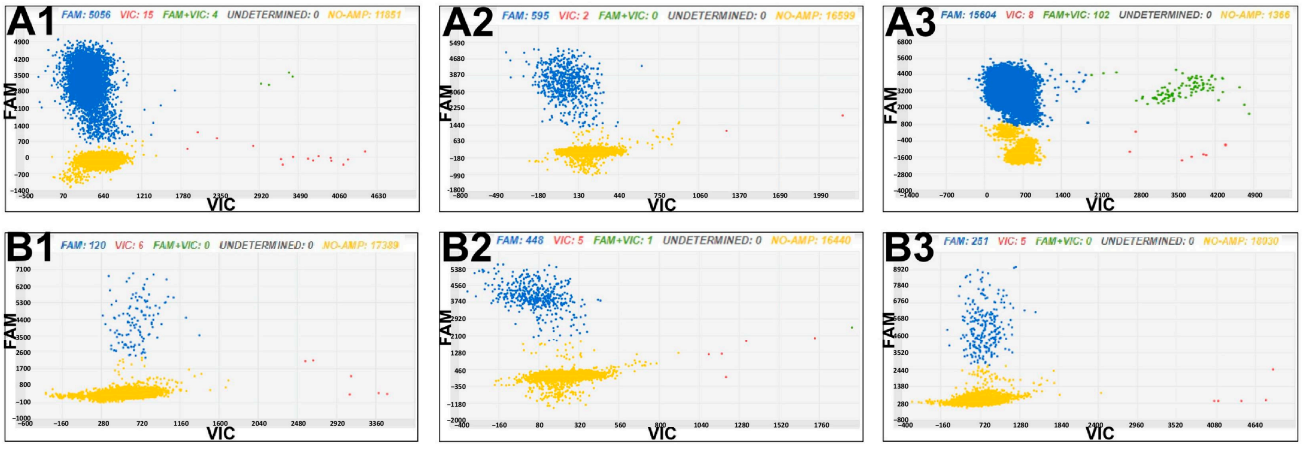

Figure 5. Two-dimensional scatter graphs obtained by chip digital PCR amplification of DNA extracted from xylem sap of naturally infected tomato plants with Cmm-dig assay or with Rs-dig assay (which both generate a FAM-positive signal, visualized as a blue dot) and the Tom-dig assay (which generates a

VIC-positive signal, visualized as a red dot). Scatter plots show the signals from FAM reporter dye on the Y-axis and VIC reporter dye on the *X*-axis. (**A1**–**A3**) show scatter plots obtained after the amplification of DNA extracted from Cmm-infected tomato plants with Cmm-dig and Tom-dig assays. Blue dots are PCR partitions positive for Cmm presence, whereas red dots are PCR partitions that are positive for the tomato target. Green dots are the partitions in which the co-amplification of both targets occurred. Yellow dots are negative PCR partitions without any target amplification. (**B1**–**B3**) show scatter plots obtained after the amplification of DNA extracted from Rs-infected tomato plants with Rs-dig and Tom-dig assays. Blue dots are PCR partitions positive for Rs presence, whereas red dots are PCR partitions that are positive for the tomato target. Green dots are the partitions in which the co-amplification of both targets occurred. Yellow dots are negative PCR partitions without any target amplification.

The highest colonization level among Cmm-infected plants was found in sample A3, Figure 5, with 3400 copies/µL of Cmm target sequence, whereas sample B2, Figure 5, showed the highest level of Rs infection. The low tomato copies/µL found are coherent with the starting matrix from which the DNA was extracted, i.e., xylem fluids, verisimilarly poor in tomato cells and rich in Cmm and Rs. Lowe-Power et al. [25], in fact, demonstrated how both Cmm and Rs can grow to high cell densities in this niche.

4. Discussion

A major factor in the long-distance spread of diseases and insect pests that could be dangerous to the agriculture of importing countries is the interchange of germplasm and the international trade of horticultural products. Protecting countries from the unauthorized influx of novel insect pests and illnesses falls within the purview of the National Plant Protection Organizations [26], which combine regulatory and technical strategies to achieve the exclusion of pests and pathogens, guaranteeing biosecurity for a country or region. The International Standards for Phytosanitary Measures (ISPMs), which offer standards on pest prevention, detection and eradication, are developed by the International Plant Protection Convention (IPPC) of the Food and Agriculture Organization of the United Nations [27].

To comply with Council Directive 2000/29/EC, National Plant Protection Organisations (NPPO) and inspection services use detection methods as their first line of defense to uncover incursions of quarantine plant pathogens or pests (Q-pests) across a border. During the early phases of detection, visual methods are implemented with the assistance of a laboratory for test confirmation and follow-up monitoring. The reliance on laboratory testing could result in considerable delays and, hence, the proliferation of the pest or pathogen. Thus, there is a need for quick, easy and reliable detection techniques that NPPOs can apply in the field in conjunction with inspection services to enable the early detection of Q-pests [26].

From the simplest detection of symptoms appearing on leaves to nucleic acid detection techniques, many sensing methods have been used throughout the years to produce sensitive and selective detection systems. Two primary categories of traditional analytical approaches for the identification of plant diseases have been categorized as direct and indirect methods of detection [28–31]. One of the direct procedures is polymerase chain reaction.

To standardize practices throughout the EPPO territory, the EPPO has implemented a work program in the diagnostics area since 1998. This program is primarily concerned with detecting bugs that are classified as quarantine pests [7].

Since 1998, EPPO has created more than 140 diagnostic standards. These are all archived in the EPPO Global Database and have been published in the EPPO Bulletin.

The two EPPO Standards PM 7/21 (3) [15] and PM 7/42 (3) [16] have been developed, respectively, for the *Ralstonia solanacearum/R. pseudosolanacearum* species complex and for *Clavibacter michiganensis* subsp. *michiganensis*. Both standards describe their diagnostic procedures as flow diagrams, which are applicable in both symptomatic and asymptomatic samples. The procedures include a combination of isolation and identification steps includ-

ing morphological, serological and molecular tests. A panel of PCR-based assays has been validated and reported in the PM 7/21 (3) [15] and PM 7/42 (3) [16]. Standard PM 7/42 (3) [16] for identification in tomatoes includes a direct PCR test on IF-positive tomato seed extracts and conventional and real-time PCR tests on isolates. PM 7/21 (3) Standard [15] for Ralstonia diagnostics include several assays based on conventional PCR, loop-mediated isothermal amplification, TaqMan real-time PCR and duplex PCR. Both standards suggest that, for a robust identification, a combination of molecular tests targeting different parts of the genome should be used. Moreover, the EPPO is open to new achievements in the molecular diagnostic field; therefore, in this study, the use of digitalPCR technology has been evaluated for the identification and quantification of Ralstonia and Clavibacter in tomato.

A droplet digital (ddPCR) assay has been already developed for Clavibacter identification by Wang et al. [13] based on the *pat-1* gene sequence of *C. michiganensis* subsp. *michiganensis*. Real-time PCR (qPCR) and ddPCR have been used to test this new primer/probe pair. According to the detection results, the ddPCR technique developed in this work was quite specific for the target strains and 100 times more sensitive than qPCR. The primers and probes developed by Weller et al. [20] were evaluated in a droplet digital PCR system by Dreo et al. [22] to quantify Ralstonia in potato samples. The authors concluded that ddPCR is suitable to detect low concentrations of Ralstonia in potato tuber samples.

In comparison with the above cited, previous studies, the novelty of our work is in the target sequence for Clavibacter and in the analytical technology for Ralstonia. Moreover, in previous works, the assays were applied to potato samples, whereas in the present study they were applied to tomato samples.

In our study new primers/probe were therefore developed and evaluated with both qPCR and chip digital PCR for Clavibacter quantification in tomatoes. The assay was designed on the 16S-23S gene sequence, targeting a different region of the bacterial genome in comparison with the assay developed by Wang et al. [13], which was designed on the *pat-1* gene sequence. On the contrary, for Ralstonia detection, the same primers/probe, developed by Weller et al. [20] for qPCR and evaluated by Dreo et al. [22] with a ddPCR approach, was used with chip digital PCR technology.

Both the qPCR and cdPCR assays used for the detection of Clavibacter and Ralstonia proved to be specific to each. However, a criticality can derive from the fact that they have been evaluated on a limited number of bacterial strains. For Ralstonia detection, a single isolate was tested in our work, similarly to the testing conducted by Dreo et al. [22]. A broader testing of the 16S primer/probe set was conducted by Weller et al. [20]. In the Weller dataset, the 16S primer/probe set did not give positive results for all *Ralstonia solanacearum* isolates. However, this isolate collection was established before phylotype designations were developed.

On the other hand, the in silico evaluation of the 16S primer/probe set sequences using a BLAST approach [32] confirms the specificity of the assay for all the Ralstonia strains for which sequences are deposited at NCBI [33] (data not shown). This same result has been obtained for primers and probe designed for Clavibacter detection: even in this case, the BLAST analysis confirms the specificity of the assay for all the Clavibacter strains for which sequences are deposited in the database (data not shown).

Although encouraging, these results cannot ensure that the assays may be able to detect all the *Ralstonia solanacearum* and *Clavibacter michiganensis* subsp. *michiganensis* isolates. A validation step on a large number of isolates from different origins could further strengthen the assay reliability. Alternatively, it can be speculated that the specificity level can be strengthened via multiplexing assays designed on different genic regions of the same target pathogen.

Behind this aspect, the two cdPCR assays evaluated in this work have characteristics of efficiency and repeatability such that they can be proposed for the fast and timely detection of this group of high-risk quarantine bacteria to prevent the spread of pathogens and the occurrence of diseases in other areas.

The two assays showed similar levels of efficiency using qPCR or cdPCR technology; however, several reasons are suggested to shift to the dPCR format. DigitalPCR is becoming an important new tool in plant pathogens diagnostics and crop protection. Many examples of diagnostic assays ex novo developed or translated from similar qPCR assays have been recently published [34]. Several classes of pathogens have been targeted, ranging from fungi and bacteria to viruses and phytoplasma. All authors found several advantages over qPCR diagnostic assays, as reviewed by Demeke et al. [35]. including improved precision and accuracy and more accurate quantitation. Moreover, the observed reduction in false negatives is critically important for the diagnosis of infections to be included in certification programs. Furthermore, a major advantage is the absolute quantification of a target without reference to a standard/calibration curve. DigitalPCR, in fact, works without the need for calibrants. The errors potentially deriving from the comparison of different matrices, i.e., the calibrant and the test sample, are therefore reduced. The loss of certified reference materials for the construction of calibration curves is not a bottleneck, as it is for qPCR. On the contrary, dPCR can be used to produce certified materials as a higher order reference measurement method. This has been suggested by several authors such as Mehle et al. [36] for plant pathogen detection, Dong et al. [37] for environmental microbiology and Pavšič et al. [38] for microbial diagnostics. Debski et al. [39] underlined the advantages deriving from the synergic use of qPCR and dPCR in human diagnostics. Finally, thanks to the sample partitioning, dPCR is less sensitive to inhibitors in comparison to qPCR.

Digital PCR's disadvantages in comparison to qPCR are the limited dynamic range of detection, the problems with very large amplicons and the more complex workflow. It is questionable whether dPCR analysis is more expensive compared to qPCR analysis. Undoubtedly, dPCR instrumentation is more expensive than qPCR, and the cost of reagents for a single reaction is higher. However, qPCR needs several replicates, reference materials and standard curves that increase the analytical cost of a sample in terms of reagents and working time.

In conclusion, the assays developed for Ralstonia and Clavibacter diagnoses in the cdPCR format could be considered contributions in implementing new methods for quarantine pests' detection and control after further validation steps.

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References

1. Tripathi, R.; Vishunavat, K.; Tewari, R.; Kumar, S.; Minkina, T.; De Corato, U.; Keswani, C. Defense Inducers Mediated Mitigation of Bacterial Canker in Tomato through Alteration in Oxidative Stress Markers. *Microorganisms* **2022**, *10*, 2160. [CrossRef] [PubMed]
2. Xu, C.; Zhong, L.; Huang, Z.; Li, C.; Lian, J.; Zheng, X.; Liang, Y. Real-time monitoring of *Ralstonia solanacearum* infection progress in tomato and Arabidopsis using bioluminescence imaging technology. *Plant Methods* **2022**, *18*, 7. [CrossRef]
3. FAOSTAT. Available online: https://data.un.org/Data.aspx?d=FAO&f=itemCode%3A388 (accessed on 14 March 2023).
4. Panno, S.; Davino, S.; Caruso, A.G.; Bertacca, S.; Crnogorac, A.; Mandić, A.; Noris, E.; Matić, S. A Review of the Most Common and Economically Important Diseases That Undermine the Cultivation of Tomato Crop in the Mediterranean Basin. *Agronomy* **2021**, *11*, 2188. [CrossRef]
5. Yadeta, K.A.; Thomma, B.P. The xylem as battleground for plant hosts and vascular wilt pathogens. *Front. Plant Sci.* **2013**, *4*, 97. [CrossRef]
6. Huet, G. Breeding for resistances to *Ralstonia solanacearum*. *Front. Plant Sci.* **2014**, *5*, 715. [CrossRef]
7. EPPO (European and Mediterranean Plant Protection Organization). Available online: https://www.eppo.int/ (accessed on 10 March 2023).

8. Tancos, M.A.; Chalupowicz, L.; Barash, I.; Manulis-Sasson, S.; Smart, C.D. Tomato fruit and seed colonization by *Clavibacter michiganensis* subsp. *Michiganensis through external and internal routes. Appl. Environ. Microbiol.* **2013**, *79*, 6948–6957. [CrossRef] [PubMed]
9. Nandi, M.; Macdonald, J.; Liu, P.; Weselowski, B.; Yuan, Z.-C. *Clavibacter michiganensis* ssp. *Michiganensis: Bacterial canker of tomato, molecular interactions and disease management. Mol. Plant Pathol.* **2018**, *19*, 2036–2050. [CrossRef]
10. Mansfield, J.; Genin, S.; Magori, S.; Citovsky, V.; Sriariyanum, M.; Ronald, P.; Dow, M.; Verdier, V.; Beer, S.V.; Machado, M.A.; et al. Top 10 plant pathogenic bacteria in molecular plant pathology. *Mol. Plant Pathol.* **2012**, *13*, 614–629. [CrossRef]
11. Álvarez, B.; Biosca, E.G.; López, M.M. On the life of *Ralstonia solanacearum*, a destructive bacterial plant pathogen. In *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*; Méndez-Vilas, A., Ed.; Formatex: Badajoz, Spain, 2010; pp. 267–279.
12. Peňázová, E.; Dvořák, M.; Ragasová, L.; Kiss, T.; Pečenka, J.; Čechová, J.; Eichmeier, A. Multiplex real-time PCR for the detection of *Clavibacter michiganensis* subsp. Michiganensis, Pseudomonas syringae pv. Tomato and pathogenic Xanthomonas species on tomato plants. *PLoS ONE* **2020**, *15*, e0227559. [CrossRef] [PubMed]
13. Wang, D.; Liu, E.; Liu, H.; Jin, X.; Niu, C.; Gao, Y.; Su, X. A droplet digital PCR assay for detection and quantification of *Verticillium nonalfalfae* and *V. albo-atrum*. *Front. Cell. Infect. Microbiol.* **2023**, *12*, 1110684. [CrossRef]
14. Morcia, C.; Ghizzoni, R.; Delogu, C.; Andreani, L.; Carnevali, P.; Terzi, V. Digital PCR: What Relevance to Plant Studies? *Biology* **2020**, *9*, 433. [CrossRef]
15. PM 7/21 (3) *Ralstonia solanacearum*, *R. pseudosolanacearum* and *R. syzygii* (*Ralstonia solanacearum* species complex). *EPPO Bull.* **2022**, *52*, 225–261. [CrossRef]
16. PM 7/42 (3) *Clavibacter michiganensis* subsp. *michiganensis*. *Eppo Bull.* **2016**, *46*, 202–225. [CrossRef]
17. PM 7/110 (1) Xanthomonas spp. (*Xanthomonas euvesicatoria*, *Xanthomonas gardneri*, *Xanthomonas perforans*, *Xanthomonas vesicatoria*) causing bacterial spot of tomato and sweet pepper. *EPPO Bull.* **2013**, *43*, 7–20. [CrossRef]
18. ISTA 7-021 (2022), International Rules for Seed Testing. 7-021: Ver 3.2 Detection of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* on *Phaseolus vulgaris*. In *Annexe to Chapter 7: Seed Health Testing Methods*; International Seed Testing Association: Bassersdorf, Switzerland, 2022.
19. Mohan, S.K.; Schaad, N.W. An improved agar plating assay for detecting *Pseudomonas syringae* pv. *syringae* and P. s. pv. *phaseolicola* in contaminated bean seed. *Phytopathology* **1987**, *77*, 1390–1395. [CrossRef]
20. Weller, S.A.; Elphinstone, J.G.; Smith, N.C.; Boonham, N.; Stead, D.E. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Appl. Environ. Microbiol.* **2000**, *66*, 2853–2858. [CrossRef] [PubMed]
21. Collier, R.; Dasgupta, K.; Xing, Y.P.; Hernandez, B.T.; Shao, M.; Rohozinski, D.; Kovak, E.; Lin, J.; de Oliveira, M.L.P.; Stover, E.; et al. Accurate measurement of transgene copy number in crop plants using droplet digital PCR. *Plant J.* **2017**, *90*, 1014–1025. [CrossRef]
22. Dreo, T.; Pirc, M.; Ramšak, Ž.; Pavšič, J.; Milavec, M.; Zel, J.; Gruden, K. Optimising droplet digital PCR analysis approaches for detection and quantification of bacteria: A case study of fire blight and potato brown rot. *Anal. Bioanal. Chem.* **2014**, *406*, 6513–6528. [CrossRef]
23. Hougs, L.; Gatto, F.; Goerlich, O.; Grohmann, L.; Lieske, K.; Mazzara, M.; Narendja, F.; Ovesna, J.; Papazova, N.; Scholtens, I.; et al. Verification of analytical methods for GMO testing when implementing interlaboratory validated methods. In *Testing and Analysis of GMO-Containing Foods and Feed*; EUR 29015 EN; Publication Office of the European Union: Luxembourg, 2017; ISBN 978-92-79-77310-5. [CrossRef]
24. Longchar, B.; Phukan, T.; Yadav, S.; Senthil-Kumar, M. An efficient low-cost xylem sap isolation method for bacterial wilt assays in tomato. *Appl. Plant Sci.* **2020**, *8*, e11335. [CrossRef]
25. Lowe-Power, T.M.; Hendrich, C.G.; von Roepenack-Lahaye, E.; Li, B.; Wu, D.; Mitra, R.; Dalsing, B.L.; Ricca, P.; Naidoo, J.; Cook, D.; et al. Metabolomics of tomato xylem sap during bacterial wilt reveals *Ralstonia solanacearum* produces abundant putrescine, a metabolite that accelerates wilt disease. *Environ. Microbiol.* **2018**, *20*, 1330–1349. [CrossRef]
26. NPPO. Available online: https://www.fao.org/documents/card/en/c/CA6375EN (accessed on 14 March 2023).
27. Chalam, V.C.; Gupta, K.; Sharma, R.; Sharma, V.D.; Maurya, A.K. Pest Risk Analysis and Plant Quarantine Regulations. In *Emerging Trends in Plant Pathology*; Singh, K.P., Jahagirdar, S., Sarma, B.K., Eds.; Springer: Singapore, 2021. [CrossRef]
28. Gautam, A.K.; Shashank, K. Techniques for the detection, identification, and diagnosis of agricultural pathogens and diseases. In *Natural Remedies for Pest, Disease and Weed Control*; Academic Press: Cambridge, MA, USA, 2020; pp. 135–142.
29. Fang, Y.; Ramasamy, R. Current and prospective methods for plant disease detection. *Biosensors* **2015**, *5*, 537–561. [CrossRef]
30. Khater, M.; de la Escosura-Muñiz, A.; Merkoçi, A. Biosensors for plant pathogen detection. *Biosens. Bioelectron.* **2017**, *93*, 72–86. [CrossRef] [PubMed]
31. Patel, R.; Mitra, B.; Vinchurkar, M.; Adami, A.; Patkar, R.; Giacomozzi, F.; Lorenzelli, L.; Baghini, M.S. A review of recent advances in plant-pathogen detection systems. *Heliyon* **2022**, *8*, e11855. [CrossRef]
32. Basic Local Alignment Search Tool. Available online: https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome (accessed on 18 April 2023).
33. National Center for Biotechnology Information. Available online: https://www.ncbi.nlm.nih.gov/ (accessed on 18 April 2023).

34. Morcia, C.; Tumino, G.; Gasparo, G.; Ceresoli, C.; Fattorini, C.; Ghizzoni, R.; Carnevali, P.; Terzi, V. Moving from qPCR to Chip Digital PCR Assays for Tracking of some Fusarium Species Causing Fusarium Head Blight in Cereals. *Microorganisms* **2020**, *8*, 1307. [CrossRef]
35. Demeke, T.; Dobnik, D. Critical assessment of digital PCR for the detection and quantification of genetically modified organisms. *Anal. Bioanal. Chem.* **2018**, *410*, 4039–4050. [CrossRef] [PubMed]
36. Mehle, N.; Gregur, L.; Bogožalec Košir, A.; Dobnik, D. One-step reverse-transcription digital PCR for reliable quantification of different *Pepino mosaic virus* genotypes. *Plants* **2020**, *9*, 326. [CrossRef]
37. Dong, X.; Gao, D.; Dong, J.; Chen, W.; Li, Z.; Wang, J.; Liu, J. Mass ratio quantitative detection for kidney bean in lotus seed paste using duplex droplet digital PCR and chip digital PCR. *Anal. Bioanal. Chem.* **2020**, *412*, 1701–1707. [CrossRef] [PubMed]
38. Pavšič, J.; Žel, J.; Milavec, M. Assessment of the real-time PCR and different digital PCR platforms for DNA quantification. *Anal. Bioanal. Chem.* **2016**, *408*, 107–121. [CrossRef]
39. Debski, P.; Gewartowski, K.; Bajer, S.; Garsteki, P. Calibration-free assays on standard real-time PCR devices. *Sci. Rep.* **2017**, *7*, 44854. [CrossRef]