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ESTABLISHMENT OF REAL-TIME PCR FOR QUANTITATIVELY MONITORING OF *PSEUDOPERONOSPORA CUBENSIS* IN CUCUMBER (*CUCUMIS SATIVUS*)

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Abstract

Cucumber downy mildew caused by *Pseudoperonospora cubensis* is an important airborne disease in China and greatly threatens cucumber production. Early accurate quantitative detection is the foundation of forecasting and control. Here, we designed a pair of specific primers according to the rDNA-ITS sequence of *P. cubensis* in GenBank. These primers could amplify the DNA of *P. cubensis* isolates by conventional PCR but not the DNA of other pathogens.The sensitivity of real-time PCR was higher than that of conventional PCR.The real-time PCR detection results of infected cucumber samples were significantly positively correlated with disease indexes and inocula. In conclusion, real-time PCR assays for *P. cubensis* are fast, highly specific, sensitive, and reproducible. We therefore successfully established a SYBR Green I real-time PCR method for *P. cubensis*. This method can be used to detect *P. cubensis* in cucumber and help guide the prediction and control of cucumber downy mildew.

Key words: Cucumber downy mildew, SYBR Green I, Early detection.

Introduction

Cucumber (*Cucumis sativus*) is an important vegetable crop worldwide. Its production is threatened by attack from downy mildew, target spot, powdery mildew, angular leaf spot and so forth. Among these diseases, owing to its rapid spread and wide endemicity characteristics, downy mildew is economically the most important and devastating one worldwide. Downy mildew is caused by *Pseudoperonosporacubensis* (Berkeley. & M.A. Curtis) Rostovzev, which is an obligate oomycete (Liu *et al*., 2017; Zhang *et al*., 2012).

The symptoms of cucumber downy mildew are similar to those of angular leaf spot, especially during the early stage. With the development of the disease, the lesions become necrotic, and complete crop failure ensues in the absence of adequate control measures (Lebeda & Cohen, 2011; Palti & Cohen, 1980). Fungicides are still the primary management tools for the control of cucumber downy mildew in China (Miao *et al*., 2018). The similarities of the symptoms of downy mildew and angular leaf spot during the early stages of infection are easily confusable. The traditional identification method of pathogenicity is cumbersome and cannot yield rapid and accurate diagnoses, which creates difficulty for prevention and control. Accurate disease prediction becomes important for estimating the risk of disease development and for helping growers make decisions on the timing of fungicide applications (Luo *et al*., 2007). The prediction and risk assessment of disease development via a decision support system require an accurate estimation of inoculum potential in cucumber leaves (Luo & Michailides, 2001). Therefore, the establishment of simple, fast and accurate early detection techniques is highly important and would efficiently help in the diagnosis, prediction and control of this disease.

In recent years, conventional PCR, real-time fluorescence quantitative PCR and other molecular identification methods have been extensively applied for the detection and quantitative study of plant diseases. Quantitative real-time PCR (qPCR) was introduced for the monitoring of plant pathogens directly within plant tissues at the end of the 1990s, and since then, it has been adapted for use in many plant pathosystems. Real-time PCR has the advantages of rapidity, accuracy, strong specificity, high sensitivity, and good stability. Real-time PCRhas become an important and mature technical tool in modern molecular biology research and has been applied for the rapid diagnosis, quantitative detection, and epidemic research of plant diseases (Martins *et al*., 2016; Paziewska-Harris *et al*., 2016). Some airborne, seedborne and soilborneinocula of fungi, bacteria, oomycetes, and nematodes have been successfully quantified by this method (Bates *et al*., 2001; Cao *et al*., 2005; Cullen *et al*., 2005; Mavrodieva *et al*., 2004). In the case of oomycetes,qPCR assays for *Pythium*, *Phytophthora*, *Plasmoparaviticola* and some *Peronospora* spp. have been developed (Atallah & Stevenson, 2006; Belbahri *et al*., 2005; Bilodeau *et al*., 2007; Böhm *et al*., 1999; Schena *et al*., 2006; Silvar *et al*., 2005; Valsesia *et al*., 2005). A real-time PCR detection protocol was standardized for the detection of *Peronosclerospora sorghi* isolated from infected maize seeds, and the detection limit was 32 fg of DNA per reaction (Sireesha & Velazhahan, 2018). Miguel Montes-Borrego developed a qPCR assay based on the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines for the quantification of *Peronospora arborescens* in infected downy mildew–symptomless opium poppy tissues and commercial seed stocks. A biomass ranging from 0.0003 to 0.007‰ or from 0.110 to 5,557 ppm was determined in symptomless capsules in commercial seed stocks or in stem samples from symptomless opium poppy plants that were systemically infected by the pathogen, respectively (Montes-Borrego *et al*., 2011). Lee compared the nucleotide sequences of ribosomal DNA-internal transcribed spacer (ITS), cytochrome oxidase II, and β-tubulin genes of *P. cubensis*and *Pseudoperonospora humuli*. One specific primer set was designed, which could be used specifically to identify *P. cubensis* in cucumber leaves with downy mildew (Lee *et al*., 2016).

The objective of this study was to develop molecular diagnostic tools to monitor *P. cubensis*. A special primer for *P. cubensis* was selected, and a quantitative detection system was developed that was used to identify and detect

the amount of *P. cubensis* in cucumber plants. By artificial inoculation, the relationships between the fluorescent quantitative detection results and both the number of inoculations and the final disease index were further studied to provide an effective and rapid technical means for early detection. The results will provide a theoretical basis for disease prediction, risk assessment and effective prevention and control.

Materials and Methods

Plant and pathogen materials: Isolates of *P. cubensis*, *Peronospora manshurica*, *Sphaerotheca cucurbitae*, *Phytophthora infestans*, *Phytophthora sojae*, *Alternaria cucumerina*, *Pseudomonas syringae*, *Corynespora cassiicola*, and *Fusarium oxysporum* were provided by the Plant Pathology Laboratory of Northeast Agricultural University, Harbin, Heilongjiang, China. Four isolates of *P. cubensis* were provided: pathotype 3, pathotype 5, pathotype 6 and pathotype 7.

Seeds of cucumber (*C. sativus* L.) cultivar D0328, which is susceptible to *P. cubensis*, were provided by the cucumber breeding laboratory of Northeast Agricultural University.

DNA extraction: Genomic DNA was extracted by the traditional cetyl-trimethylammonium bromide (CTAB) method (Zhou *et al*., 2020). The quality and concentration of the DNA were determined using a SMA3000 UV spectrophotometer (Beijing, China). The DNA concentration was subsequently diluted to 10 $ng{\cdot}\mu L^{-1}$, and the extracted DNA was frozen at −20°C for later use.

Primer specificity tests: The *P. cubensis* specific primer pair PCi-3 5′-TTGCACTTCCGGGTTAGTCC-3′/5′-GGTCACATGGACAGCCTTCA-3′ was designed based on the rDNA-ITS sequence (GenBank: AY198306) using Primer Premier 5.0 software. The specificity of the primers was detected by normal PCR and real-time PCR amplification.

The normal PCR amplifications were carried out in reaction volumes of 20 µL comprising 2.0 µL (10 $ng{\cdot}\mu L^{-1}$) of genomic DNA,10.0 µL of Taq-Plus PCR Forest Mix, 0.4 µL of both forward and reverse primer sets (each primer at 10 $mmol{\cdot}L^{-1}$) and 7.2 µL of water. The reactions were performed under the following conditions: an initial denaturation step for 90 s at 94°C; 35 cycles of 94°C for 30 s, 63.5°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 5 min.

The real-time PCR mixture contained 2.0 µL (10 $ng{\cdot}\mu L^{-1}$) of genomic DNA, 10.0 µL of Taq SYBR® Green qPCR Premix, 0.2 µL of primer sets (10 $mmol{\cdot}L^{-1}$) and 7.8 µL of water. The following conditions were used: 1 cycle of 94°C for 3 min; 40 cycles of 94°C for 10 s, 60°Cfor 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 5 min. Real-time PCR was performed in an iQ5 (Bio-Rad) thermocycler. All real-time PCR was performed under the same conditions previously described in this study.

Establishment of a standard curve: A series of positive standard amplification templates of 7.86×10^{1} $ng{\cdot}\mu L^{-1}$, 7.86×10^{0} $ng{\cdot}\mu L^{-1}$, 7.86×10^{-1} $ng{\cdot}\mu L^{-1}$, 7.86×10^{-2} $ng{\cdot}\mu L^{-1}$, 7.86×10^{-3} $ng{\cdot}\mu L^{-1}$, and $7.86\times10^{-4}ng{\cdot}\mu L^{-1}$ were subjected to a 10-fold gradient dilution. Each concentration was repeated 3 times. The copy number was then calculated using the following formula: copy number = DNA concentration ($ng{\cdot}\mu L^{-1}$) × Avogadro's constant × 10^{-9}/ [660×the target gene base number (215 bp)]. A standard curve was constructed with the logarithm of the copy number as the X-axis and the Ct value as the Y-axis. The standard curve was automatically generated by the fluorescence quantification software (Kelly *et al*., 2013).

Pathogen inoculum preparation and inoculation methods: The susceptible cucumber line D0328 was prepared, and inoculation was performed at the cotyledon stage. The sporangial suspensions were counted using a hemocytometer and adjusted to 2.0×10^{3} $sporangia{\cdot}mL^{-1}$ using sterile tap water. Fifteen milliliters of the sporangial suspension was inoculated onto the cucumber cotyledons (Liu *et al*., 2017). Ten plants per replicate and three replicates were tested per treatment. The culture conditions included a 26°C/18°C day/night temperature. Sterile water was used as the control. The inoculation treatments and the water controls were sampled every 2 h at intervals of 0-24 h, after which they were samples once every 12 h until the leaves showed definitive symptoms. Three experimental replicates were included. The severity scale was as follows: 0, no symptoms were visible; 1, the inoculation point was slightly small, and the diameter was less than 0.5 cm; 2, the lesion was obvious, the diameter was 0.5 to 1.0 cm, and the lesion area accounted for 1/3 of the area of the cotyledons; 3, the lesions accounted for 1/3 to 2/3 of the area of the cotyledons; and 4, the lesions accounted for more than 2/3 of the area of the cotyledons.

Results

Specificity of *P. cubensis* quantitative primers: To determine the specificity of the PCi-3 primer sets for *P. cubensis*, a PCR assay using genomic DNA from *P. cubensis* and from the other pathogens of cucumber and oomycetes was performed. The results of the ordinary PCR showed that specific PCR products were observed in *P. cubensis* only at 215 bp, while no amplification bands were detected in the DNA of other pathogens (Fig. 1). This result indicated that PCi-3 primers could be used specifically for *P. cubensis*.

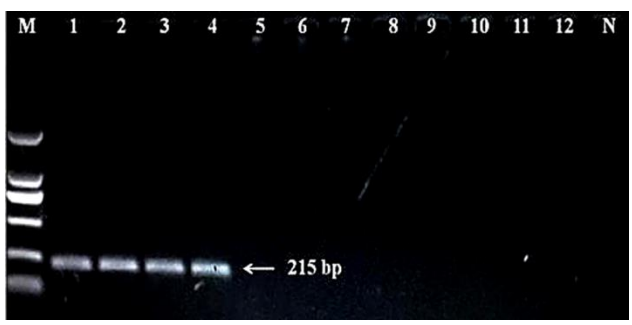

Fig. 1. The specificity of primer PCi-3 to *P.cubensis*. M: 2000 bp DNA Ladder Marker, N: Negative control, 1-4: pathotype 3, pathotype 5, pathotype 6 and pathotype 7 of P.cubensis, 5-12: *P. manschurica*, *P. infestans*, *P. sojae*, *A. cucumerina*, *P. syringaepv*, *E. cichoracearum*, *C. cassiicola*, *F. oxysporum.*

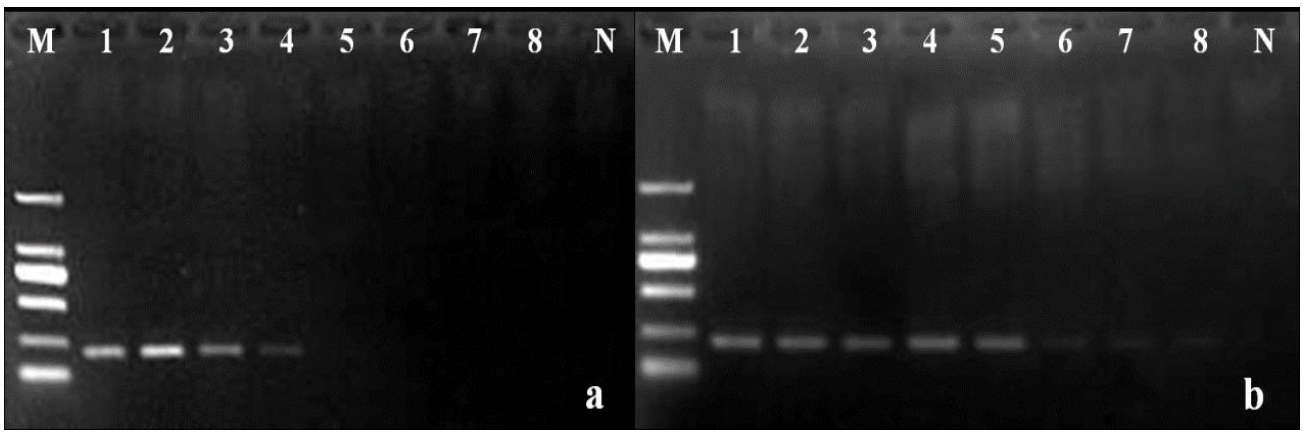

Fig. 2. Detection sensitivity of primer PCi-3inreal-time PCR and normal PCR. a: Detection sensitivityin normal PCR ; b: Detection sensitivity inreal-time PCR; M: 2000-bp DNA Ladder Marker, N: negative control, 1-8: *P. cubensis* genomic DNA samples (the 8 concentrations were 7.86×10^{1} ng.μL^{-1}, 7.86×100 ng.μL^{-1}, 7.86×10^{-1} ng.μL^{-1}, 7.86×10^{-2} ng.μL^{-1}, 7.86×10^{-3} ng.μL^{-1}, 7.86×10^{-4} ng·μL^{-1}, 7.86×10^{-5} ng·μL^{-1} and 7.86×10^{-6} ng·μL^{-1}, respectively.)

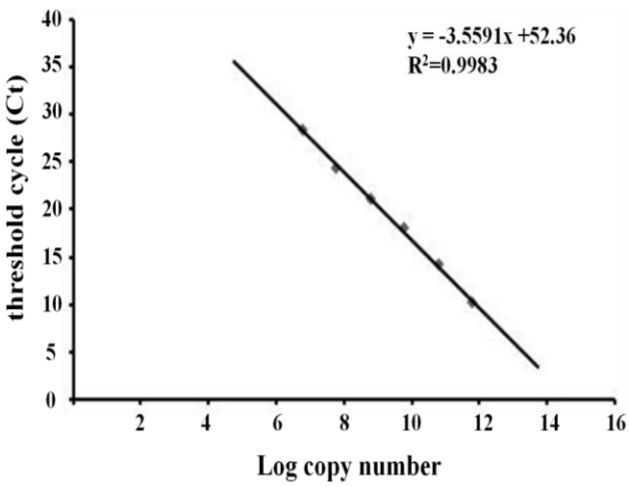

Fig. 3. Standard curve of *P.cubensis* using real-time PCR.

Primer sensitivity: A series of positive standard amplification templates of 7.86×10^{1}ng·μL^{-1}, 7.86×10^{0} ng·μL^{-1}, 7.86×10^{-1} ng·μL^{-1}, 7.86×10^{-2} ng·μL^{-1}, 7.86×10^{-3}ng·μL^{-1}, 7.86×10^{-4}ng·μL^{-1}, 7.86×10^{-5} ng·μL^{-1}, and 7.86×10^{-6} ng·μL^{-1} were subjected to a 10-fold gradient dilution. Eight concentrations of *P. cubensis* genomic DNA samples were subjected to both real-time PCR and conventional PCR and then measured via agarose gel electrophoresis (Fig. 2). The results showed that the sensitivity of real-time PCR was 7.86×10^{-6}ng·μL^{-1}, while the normal PCR sensitivity was 7.86×10^{-2}ng·μL^{-1}. The sensitivity of real-time PCR under the PCi-3 primers was 10^{4} times greater than that of the normal PCR.

Establishment of real-time PCR standard curves: Real-time PCR was performed with a 10-fold gradient dilution positive standard, and a standard curve was established by primer PCi-3 amplification. The correlation of coefficient (R^2) was 0.9983 (>0.95), and the slope was -3.5591. The standard curve formula of the relationship between the logarithm of the copy number and the threshold cycle (Ct) values was y = -3.5591x +52.36 (Fig. 3).

Table 1. The amount of leaves in diseased plants by real-time PCR.

Time after inoculation	Ct	Concentration (ng/μL)	Severity
2h	31.52	-	0
4h	31.33	-	0
6h	31.21	-	0
8h	30.64	-	0
10h	29.62	5.7755E-04	0
12h	28.72	1.0339E-03	0
14h	28.36	1.3052E-03	0
16h	27.67	2.0393E-03	0
18h	27.33	2.5415E-03	0
20h	26.92	3.3135E-03	0
22h	26.41	4.6088E-03	0
24h (1d)	25.55	8.0387E-03	0
36h (1.5d)	24.66	1.4298E-02	0
48h (2d)	22.18	7.1135E-02	1
60h (2.5d)	21.3	1.2571E-01	1
72h (3d)	16.82	2.2808	1
84h (3.5d)	16.54	2.7332	1
96h (4d)	15.74	4.5865	2
108h (4.5d)	15.57	5.1202	2
120h (5d)	14.45	10.5678	2
132h (5.5d)	13.89	15.1805	2
144h (6d)	13.43	20.4449	3
156h (6.5d)	13.07	26.8279	3
168h (7d)	12.74	31.9438	3
180h (7.5d)	12.25	43.8617	3
192h (8d)	10.36	148.9656	4
204h (8.5d)	9.24	307.4581	4
216h (9d)	8.59	468.2588	4
CK	-	-	-

Dynamic changes in the latent infection of *P. cubensis* detected by real-time PCR: We detected the dynamic changes in *P. cubensis* in cucumber cotyledons by real-time PCR after inoculation. Furthermore, the severitywas investigated after inoculation. The results showed that *P. cubensis* could be detected at 10 h after inoculation in cucumber cotyledons, and the amount of infection increased gradually with time. However, the severity was 0

before 48 h, and the cucumber did not show any visible symptoms. The amount of *P. cubensis* and severity tended to increase from 1-9 days after inoculation. This finding indicated that real-time PCR can provide rapid detection in the early stages of disease and can detect the development of disease. All the data are presented in Table 1.

Discussion

Cucumber downy mildew is the most common airborne disease in cucumber production, and it is common in cucumber greenhouses and open fields. Moreover, cucumber downy mildew often occurs in conjunction with airborne diseases such as angular leaf spot, anthracnose, brown spot, and scab, causing leaf spots in the early stages. Directly distinguishing among the symptoms of these diseases is difficult, especially during the early stage. Traditional methods of pathogen detection depend on symptom observations, spores or colony counts; however, it is impossible to quantify these accurately. Therefore, the establishment of a rapid and accurate quantitative detection technology is necessary to monitor the development and guide the scientific and reasonable prevention and control of cucumber downy mildew.

Compared with traditional methods, real-time quantitative PCR technology has the advantages of speed, sensitivity, and specificity. This technology is not limited by plant symptoms and can distinguish subtle differences. It has been widely used in the detection and monitoring of plant pathogens such as fungi, bacteria, viruses, oomycetes, and nematodes. Min *et al*., (2002) used real-time quantitative PCR (SYBR) to measure the biomass of *Magnaporthe grisea* during different periods of rice infection, demonstrating that this method was a good tool for analyzing the pathogenicity and host resistance of fungal pathogens (Qi & Yang, 2002). Airborne inocula of *Pyrenopeziza brassicae* in winter oilseed rape crops in Poland and the UK have been measured by real-time PCR assays. β-Tubulin gene-based primers and dual-labeled fluorescent probes have been developed, and the effectiveness of traditional visual identification and enumeration by light microscopy and qPCR techniques have been compared; the qPCR method used was very sensitive and highly correlated with spore counts from Melinex tapes under a light microscope (Karolewski *et al*., 2012). Several researchers have developed real-time qPCR assays for the identification and quantification of oomycetes, mainly *Phytophthora* and *Pythium* (Böhm *et al*., 1999; Schena *et al*., 2006; Silvar *et al*., 2005; Atallah & Stevenson, 2006; Bilodeau *et al*., 2007); however, to the best of our knowledge, especially less studies have developed real-time qPCR protocols for the identification or quantification of the obligate biotroph *P. cubensis*, which causes downy mildew disease. Lee developed a real-time PCR and high-resolution melting (HRM) analytical method to identify *P.cubensis* in cucumber leaves that displayed signs of downy mildew. One specific primer set was designed based on variations in the β-tubulin sequences of *P. cubensis* and *P. humuli* isolates. The results showed that the primer set could be used specifically to identify *P. cubensis* in cucumber leaves infected with downy mildew (Lee *et al*., 2016).

In this study, a *P. cubensis* specific primer, PCi-3, was designed based on the rDNA-ITS sequence (GenBank: AY198306). When the DNA templates of *P. cubensis*, *P. manshurica*, *S. cucurbitae*, *P. infestans*, *P. sojae*, *A. cucumerina*, *P. syringae*, *C. cassiicola*, and *F. oxysporum* was used to test the specificity of the primer pair PCi-3F/R, only *P. cubensis* isolates showed the expected 215-bp PCR product. No products were generated by this primer pair from other pathogen isolates, which demonstrated that this primer pair was specific to *P. cubensis*. The sensitivity of real-time PCR with the PCi-3F/R primer was 10^4 times greater than that of normal PCR. Using real-time PCR, we measured the dynamic changes in *P. cubensis* in cucumber cotyledons after inoculation. In addition, the severity was investigated after inoculation. The results showed that *P. cubensis* could be detected in cucumber cotyledons at 10 h after inoculation, and the amount of infection increased gradually with time. However, the severity was 0 before 48 h, and the cucumber plants did not show any visible symptoms. The amount of *P. cubensis* and severity tended to increase for 1-9 days after inoculation. The results of this study therefore demonstrate that real-time PCR has advantages over traditional methods of visual detection, even in the absence of macroscopic disease symptoms, and this method can be used to detect the latent infection of *P. cubensis*. In summary, the real-time qPCR protocol developed in this study for the identification and quantification of *P. cubensis* in cucumber plant tissues will be of great use for studies aimed better understanding downy mildew epidemiology and efficient management via disease prediction, risk assessment, disease resistance breeding, and efficacy assessments of disease control measures. In addition, this protocol will help to inhibit disease outbreaks and to apply suitable control means during the early stage of infection.

Conclusion

In the present study, we designed PCi-3 primers specific for *P. cubensis* and successfully established a SYBR Green I real-time PCR method for *P. cubensis*.This method can be used to detect *P. cubensis* in cucumber and can help guide the prediction and control of cucumber downy mildew.

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