RESEARCH ARTICLE

# Multiplex TaqMan qPCR Assay for Detection, Identification, and Quantification of Three *Sclerotinia* Species

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**ABSTRACT**

White mold (or Sclerotinia stem rot), caused by *Sclerotinia* species, is a major air, soil, or seed-transmitted disease affecting numerous crops and wild plants. Microscopic or culture-based methods currently available for their detection and identification are time-consuming, laborious, and often erroneous. Therefore, we developed a multiplex quantitative PCR (qPCR) assay for the discrimination, detection, and quantification of DNA collected from each of the three economically relevant *Sclerotinia* species, namely, *S. sclerotiorum*, *S. minor*, and *S. nivalis*. TaqMan primer/probe combinations specific for each *Sclerotinia* species were designed based on the gene sequences encoding aspartyl protease. High specificity and sensitivity of each probe were confirmed for sclerotium and soil samples, as well as pure cultures, using simplex and multiplex qPCRs. This multiplex assay could be helpful in detecting and quantifying specific species of *Sclerotinia*, and therefore, may be valuable for disease diagnosis, forecasting, and management.

## 1. Introduction

*Sclerotinia* species cause white mold (Sclerotinia stem rot), a widespread and destructive plant disease; for example, *Sclerotinia sclerotiorum* (Lib.) de Bary, one of the most devastating fungal pathogens, affects over 400 plant species worldwide [1]. They can survive in the soil for several years by producing a dormant structure called sclerotium to over-summer and overwinter, which is a key source of inoculum. Sclerotia germinate *via* apothecium formation and direct mycelial germination [2]. In the former case (e.g., *S. sclerotiorum*), sclerotia produce an apothecium similar to the fruiting body of a mushroom. Each apothecium may produce millions of airborne ascospores that can travel to other fields *via* air currents. In the latter case (e.g., *S. minor*), sclerotia germinate directly through the formation of hyphae. Thus, *Sclerotinia* species can function as both air- and soil-borne pathogens. In addition, the pathogen can be transmitted by seeds, which are considered a vital dissemination agent [3,4].

*Sclerotinia* species are identified mainly by traditional morphological traits, including cultural characteristics, sclerotia size, ascus, and ascospore dimensions, host range, and disease symptoms [5]. However, such approaches are often unreliable because of their relatively unstable and variable characteristics, and thus, often overlap between different species, according to multigenic involvement and responses to environmental stimuli [6]. Nonetheless, these characteristics are still widely used for routine identification, which is a major impediment to the early, rapid, and accurate detection of the initial inoculum of *Sclerotinia* species essential for controlling and managing disease outbreaks. In addition, seed health testing, often used to detect *Sclerotinia* species, is laborious, time-consuming, and lacks sensitivity.

The internal transcribed spacer (ITS) rDNA region is widely used as a barcode marker for fungal identification; however, it is not useful for species identification of Sclerotiniaceae, due to low interspecific variation in the nucleotides [7]. Instead, other multi-loci, including the glyceraldehyde-3-phosphate dehydrogenase, heat-shock protein 60, laccase 2 (*lcc2*), and DNA-dependent RNA polymerase subunit II, have been used successfully for the identification of *Sclerotinia* species [8,9]. Unlike these housekeeping loci, the *asp* gene encoding an aspartyl protease was associated with the pathogenicity of *S. sclerotiorum* but exhibited a high interspecific nucleotide divergence [10,11]. Freeman et al. [7] have developed a polymerase chain reaction (PCR)-based assay to detect *S. sclerotiorum* in air samples. Njambere et al. [12] designed microsatellite markers for *Sclerotinia trifoliorum*. A single-nucleotide polymorphism (SNPs) protocol was developed by

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**Table 1.** Information of *Sclerotinia* isolates used in this study.

| Species | Isolate no. | Date of isolation | Source | Location of isolation |
|---|---|---|---|---|
| *Sclerotinia sclerotiorum* | KACC 40172 | Feb 17 1996 | *Citrus sinensis* | Seogwipo-si, Jeju-do, Korea |
| | KACC 40457 | Unknown | *Lactuca sativa* | Unknown, Korea |
| | KACC 40922 | Feb 09 2001 | *Pimpinella brachycarpa* | Namyangju-si, Gyeonggi-do, Korea |
| | KACC 41064 | May 04 1998 | *Brassica napus* | Jeju-do, Korea |
| | KACC 41065 | Jan 31 2000 | *Capsicum annuum* | Hadong-gun, Gyeongsangnam-do, Korea |
| | KACC 41069 | May 04 1998 | *Solanum tuberosum* | Jeju-do, Korea |
| *Sclerotinia minor* | KACC 41066 | Feb 17 1996 | *Brassica campestris* ssp. pekinensis | Namyangju-si, Gyeonggi-do, Korea |
| | KACC 41067 | Apr 08 1996 | *Cichorium endivia* | Namyangju-si, Gyeonggi-do, Korea |
| | KACC 41068 | Mar 02 1994 | *Lycopersicon esculentum* | Iksan-si, Jeollabuk-do, Korea |
| *Sclerotinia nivalis* | KACC 45150 | Feb 20 2006 | *Aralia elata* | Gapyeong-gun, Gyeonggi-do, Korea |

Andrew and Kohn [13] to identify *S. sclerotiorum*, *S. minor*, *S. trifoliorum*, and an undescribed species of *Sclerotinia*. A multiplex PCR assay was developed to discriminate some fungal members of Sclerotiniaceae [9], which specifically detected *S. sclerotiorum*, *S. minor*, *S. trifoliorum*, and *S. homoeocarpa* [14]. However, both the single species-specific and multiplex PCR assays developed to date employ endpoint PCR, and thus, have limited potential for forecasting and epidemiological studies, compared to quantitative diagnostic techniques. Quantitative PCR (qPCR)-based methods have been developed to detect *S. sclerotiorum* [15,16]; however, these methods do not allow specific and quantitative detection of other *Sclerotinia* species that often coexist in the field.

In Korea, white mold has been reported to be caused by four species, namely, *S. sclerotiorum*, *S. minor*, *S. trifoliorum*, and *S. nivalis* [17]; however, there is no additional record of *S. trifoliorum* after its first record [18]. The above-mentioned traditional traits are not always accurate in identifying them at the species level. In addition, a high-throughput method is required to detect and quantify DNA from *Sclerotinia* species. In the present study, we developed a new multiplex qPCR assay that can simultaneously and specifically quantify the DNA collected from *S. sclerotiorum*, *S. minor*, and *S. nivalis*.

## 2. Materials and methods

### 2.1. Sclerotinia *isolates*

All isolates used in this study were obtained from the Korean Agricultural Culture Collection (KACC, Jeonju, Korea; Table 1). They were grown on a cellophane membrane placed on potato dextrose agar (PDA; Difco, Detroit, MI) at 25 °C for 3–5 d before the extraction of genomic DNA.

### 2.2. DNA extraction, PCR, and sequencing for identification

Genomic DNA (G-DNA) of *Sclerotinia* isolates was extracted using the NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) after placing the mycelium grown for 5 d in a 2 mL tube and crushing with a pellet pestle. For identification, PCR amplification was performed for the ITS region with primers ITS1 and ITS4 [19], β-tubulin gene with two primer sets, Bt2a/Bt2b [20] and TU1/TU2 [21], glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene with G3PDHfor and G3PDHrev [8], and aspartyl protease (*asp*) using two primer sets: ASPSaF/ASPSaR and ASPSbF/ASPSbR [10]. PCR was conducted in a 25 μL total volume of AccuPower PCR Premix (Bioneer, Daejeon, Korea), including 1 μL of G-DNA, 0.4 μM of each primer, and 0.8 μg/μL of bovine serum albumin (Biosesang, Seongnam, Korea), and the remaining volume was filled with nuclease-free water (Sigma-Aldrich; Merck, St. Louis, USA). PCR amplification of the ITS regions was performed with the following conditions: initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 54.5 °C for 40 s, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 4 min. For β-tubulin gene, TU1/TU2 primers was performed by initial denaturation at 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, a final elongation at 72 °C for 5 min, and Bt2a/Bt2b primers by 35 cycles of 95 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, and a final elongation at 72 °C for 5 min. *GAPDH* gene was amplified by initial denaturation at 95 °C for 3 min, followed by 32 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, and a final elongation at 72 °C for 5 min.

### 2.3. Primer and probe design for TaqMan assay

Among the four multi-loci sequences for identifying *Sclerotinia* species, *asp* gene sequences were selected for designing primers and probes because of the high number of SNPs compared to the other three markers. The *asp* sequences of all *Sclerotinia* isolates were aligned with the reference sequences available at NCBI GenBank and the genome sequence of *S. minor* LC41 (acc. No. CNP0001962) [22]. The alignment was analyzed for differences and similarities using the SeqMan software, version 2.09 (DNAStar,
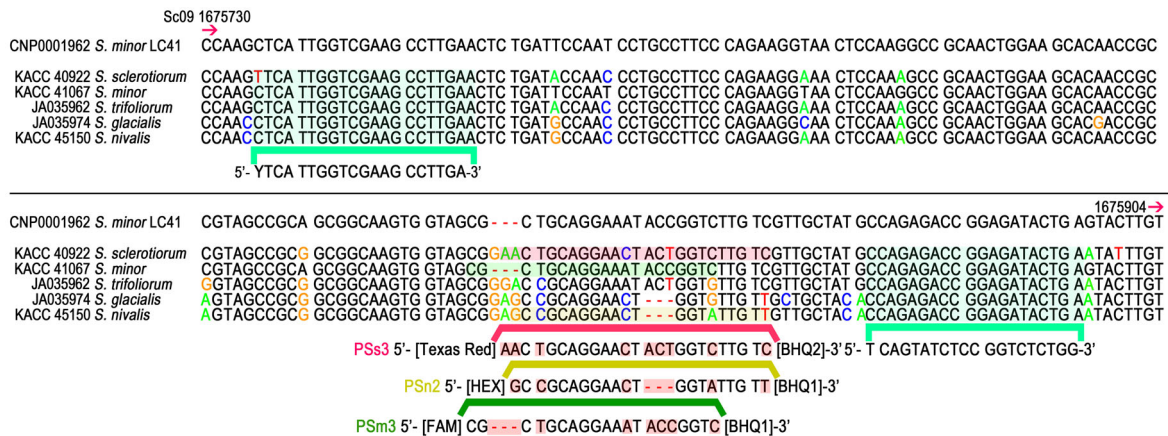
**Figure 1.** Design of TaqMan assay primers and probes for detection of *Sclerotinia* species. The sequences shown in the alignment are fragments of the DNA sequences encoding the *asp* gene. In the reference sequence, the forward primer located at 1,675,730 bp and reverse primer located 1,675,897 bp were indicated in blight green. PSm3 = *S. minor*-specific TaqMan® probe at 1,675,843–1,675,862 bp in green; PSs3 = *S. sclerotiorum*-specific TaqMan® probe at the 1,675,845 (with 2 gaps)–1,675,867 bp in red; PSn2 = *S. nivalis*-specific TaqMan® probe at the 1,675,845 (with 1 gap)–1,675,867 bp in yellow; Sasp1-1F = nonspecific forward primer (marked with a mint bar); Sasp1-1R = nonspecific reverse primer (marked with a mint bar). SNPs were highlighted in red within the probe sequence.

Inc., Madison, WI). The nonspecific forward primer Sasp1-1F (5′-YTCATTGGTCGAAGCCTTGA-3′) and reverse primer Sasp1-1R (5′-TCAGTATCTCC GGTCTCTGG-3′) were designed using Primer3Plus [23] (https://www.primer3plus.com/). The resulting partial sequences of the *asp* gene (166 characters) exhibited 20 SNPs among the three *Sclerotinia* species (Figure 1). Twelve SNPs were selected to design TaqMan probes in which species specificity was enhanced by incorporating mismatched nucleotides and gaps. Three probes were labeled differently with 6-FAM, HEX, or Texas Red reporter dyes for use in multiplexing with quencher BHQ1 or BHQ2: namely, PSs3 specific for *S. sclerotiorum* with Texas Red dye (5′-Texas Red-AACTGCAGGAACT ACTGGTCTTGTC-BHQ2-3′), PSm3 for *S. minor* with FAM™ dye (5′-FAM-CGCTGCAGGAAAT ACCGGTC-BHQ1-3′), and PSn2 for *S. nivalis* with HEX dye (5′-HEX-GCCGCAGGAACTGGTAT TGTT-BHQ1-3′). The species-specific probes were combined with nonspecific primers for simplex and multiplex qPCRs (Table 2).

95 °C for 25 s, annealing at 58 °C for 45 s, and a final elongation at 72 °C for 30 s.

Optimal concentrations of the two primers and three probes were selected using simplex qPCR. Five concentrations of primers (50, 100, 150, 200, and 300 nM) and probes (50–1000 nM) were tested for each target pathogen, and combinations of primers and probes with lower Cq values were selected. The multiplex qPCR conditions were the same as described above, and DNA samples were individually diluted at a concentration of 20 ng/μL at a volume of 1 μL per reaction. The reaction mixture was consisted of 20 ng of DNA, 300 nM of the two universal primers (Sasp1-1F/Sasp1-1R), 300 nM of the three probes (PSs3/PSm3/PSn2), and 10 μL of THUNDERBIRD™ Probe qPCR mix (Toyobo) Nuclease-free water (Sigma-Aldrich, Merck) was added to a final volume of 20 μL. Since the Cq values of multiplex qPCR tests were similar to the simplex qPCR using individual probes for the target pathogen, multiplex qPCR tests were performed using probe triads. All reactions were performed in four replicates to control for mechanical and technical errors.

## 2.4. Specificity tests of TaqMan qPCR assay

Genomic DNA of ten *Sclerotinia* isolates was tested for the sensitivity and specificity of each probe to its expected target. qPCR was performed according to the manual of THUNDERBIRD™ Probe qPCR mix (Toyobo, Osaka, Japan) using a CFX Opus 96 machine (Bio-Rad, CA, USA). The quantification cycle (Cq) values were assessed for each reaction using the CFX Maestro software (Bio-Rad). Simplex qPCR conditions were initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at

## 2.5. Standard curves of TaqMan qPCR assay

Genomic DNA from each *Sclerotinia* species was used to construct standard curves from qPCR quantifications by seven-serial dilutions (200 ng/μL–200 fg/μL). Four technical replicates were performed for each dilution using the multiplex qPCR conditions described above. Standard curves were used for the accurate quantification of the target DNA. Sensitivity was calculated by the limit of detection (LoD) of each assay, which is defined as the lowest DNA concentration detectable at least 95% of the qPCR runs.

**Table 2.** Information of TaqMan qPCR primers and probes used or designed in this study.

| Primer/probe name | Sequence (5′-3′) | Tm (°C) | GC (%) | Reference |
|---|---|---|---|---|
| ASPSaF | GGTGCYGGTACMAGAG | 55 | 56 | Andrew et al. [10] |
| ASPSaR | GGCTTRACRGTGTTSAG | 49 | 41 | |
| ASPSbF | ACATYGGWGGDGYVACTGT | 62 | 42 | |
| ASPSbR | TTRAACATRATGTCRCCGTA | 51 | 30 | |
| Sasp1-1F | YTCATTGGTCGAAGCCTTGA | 58.3 | 45 | In this study |
| Sasp1-1R | TCAGTATCTCCGGTCTCTGG | 56.7 | 55 | |
| PSs3 | [Texas Red]- AACTGCAGGAACTACTGGTCTTGTC -[BHQ2] | 61.7 | 48 | |
| PSm3 | [FAM]- CGCTGCAGGAAATACCGGTC -[BHQ1] | 60.5 | 60 | |
| PSn2 | [HEX]- GCCGCAGGAACTGGTATTGTT -[BHQ1] | 60.2 | 52.3 | |

## 2.6. Sensitivity of qPCR detection for sclerotia and soil samples

Sensitivity evaluations were conducted for sclerotia and soil samples. Soil samples, which were autoclaved at 121 °C for 45 min, were artificially inoculated with a representative isolate of each of three target *Sclerotinia* species (41065 for *S. sclerotiorum*, 41067 for *S. minor*, and 45150 for *S. nivalis*) separately and together. Inoculation was carried out by depositing a mycelium plug (5-mm diameter) taken from 3-d-old PDA cultures per pot and a sclerotium per pot on the top of the soil, then incubating in growth chamber at 25 °C under a 12/12 h (day/night) cycle for 7 d. In addition, mycelium plugs of the three species were inoculated together in a pot, and the sclerotia of the three species were inoculated in a pot. Three replicates (pots) were inoculated for each fungal isolate. As a control, an agar plug was transferred to the soil surface. Soil DNA was extracted using the SPINeasy DNA Kit for Soil (MP Biomedicals, Santa Ana, CA). A total of 300–400 mg of the inoculated soil was harvested from the surface of the soil, placed in a lysis tube, and homogenized at 30 Hz/s for 10 min using an MM400 mixer mill (Retsch GmbH, Haan, Germany). Sclerotia were harvested from colonies formed on the PDA medium, crushed with a pellet pestle, and homogenized at 30 Hz/s for 30 min using an MM400 mixer mill (Retsch GmbH). DNA was extracted using a NucleoSpin Plant II Kit (Macherey-Nagel).

## 3. Results

### 3.1. Sequence analysis for TaqMan probe and primer design

Sequence analysis was performed to develop three target *Sclerotinia* species-specific probes and a non-specific primer set. Among the four multi-loci (ITS region, β-tubulin, *GAPDH*, and *asp*) of three *Sclerotinia* species, the highest number of SNPs was found in *asp* gene alignment (Table 3). In addition, sequence alignments of the ITS region, β-tubulin, and *GAPDH* gene sequences exhibited no or few potential probe sites that could uniformly distinguish *Sclerotinia* species, but *asp* gene revealed potential target sequences that could be used in

**Table 3.** Length of alignments and the number of SNPs of the ITS, β-tubulin, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and aspartyl protease (*asp*) sequences of three *Sclerotinia* species.

| | ITS | β-tubulin | GAPDH | asp |
|---|---|---|---|---|
| Alignment (bp) | 490 | 530 | 889 | 985 |
| No. of SNPs | 7 | 54 | 47 | 95 |

probe and primer development to differentiate among *Sclerotinia* species. Therefore, the eight SNPs, depicted in Figure 1 in red color, were incorporated into the sequences of the probe (PSs3) to generate an *S. sclerotiorum*-specific TaqMan assay. The *S. minor*- (PSm3) and *S. nivalis*-specific probes (PSn2) were prepared in a similar manner. The three species-specific probes were intentionally designed to overlap (Figure 1 and Table 2).

### 3.2. Specificity testing of the TaqMan assays

The specificity of the qPCR method was confirmed with DNA extracted from six isolates of *S. sclerotiorum*, three isolates of *S. minor*, and one isolate of *S. nivalis* (Table 1). Simplex PCR assays were performed to validate the possibility of multiplexing the two primers and three probes. In the simplex assays, the designed proves, PSs3, PSm3, and PSn2, showed amplification specific to only detect *S. sclerotiorum*, *S. minor*, and *S. nivalis*, respectively, and could be included in the multiplex assays. The primer/probe concentrations were optimized for simplex qPCR assays for each *Sclerotinia* species. The optimum concentration for both primers and probes was 300 nM. In multiplex qPCR, it was possible to amplify the DNA of the three *Sclerotinia* species simultaneously and separately (Figure 2(A)) with slight variation in Cq values, compared with amplifications in simplex PCR, enabling the combination of primers and probes and qPCR condition to detect three *Sclerotinia* species. The sensitivity and specificity for each species were similar to those observed in simplex qPCR reactions.

### 3.3. Standard curves of TaqMan qPCR assay

Efficiency of the multiplex qPCR toward each target was estimated by constructing standard curves based
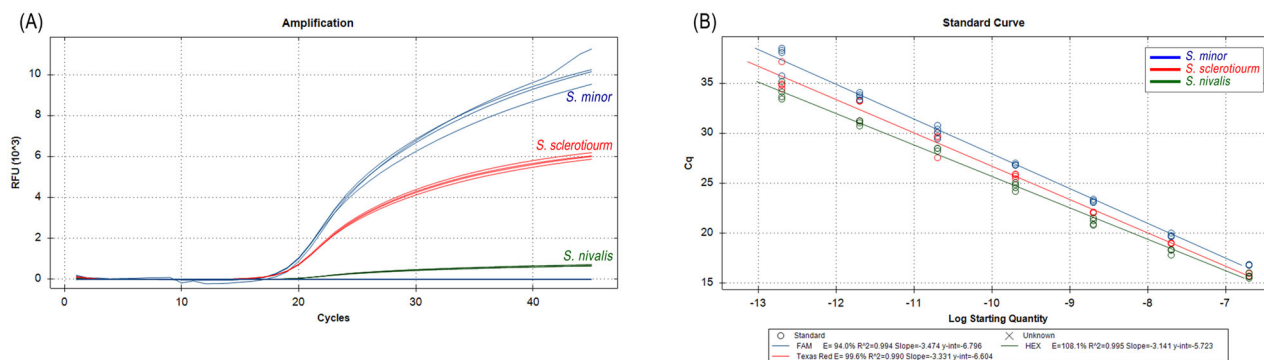
**Figure 2.** Concomitant amplification (A) and standard curves (B) of three DNA targets by multiplex qPCR: *Sclerotinia sclerotiorum* (red), *S. minor* (blue), and *S. nivalis* (green). The log rDNA copy number is plotted against the quantification cycle (Cq) values. All data points are from an average of four technical replicates.

**Table 4.** Cq values and detection of limit based on G-DNA dilutions of *Sclerotinia* species by TaqMan qPCR assay.

| DNA amount | Cq values[a] | | |
|---|---|---|---|
| | *S. sclerotiorum* | *S. nivalis* | *S. minor* |
| 200 ng | 15.78 ± 0.12 | 15.73 ± 0.19 | 16.84 ± 0.02 |
| 20 ng | 19.01 ± 0.03 | 18.21 ± 0.22 | 19.79 ± 0.13 |
| 2 ng | 22.08 ± 0.03 | 21.13 ± 0.28 | 23.22 ± 0.12 |
| 200 pg | 25.73 ± 0.17 | 24.67 ± 0.32 | 26.87 ± 0.08 |
| 20 pg | 29.22 ± 0.97 | 28.4 ± 0.12 | 30.18 ± 0.49 |
| 2 pg | 32.73 ± 0.87 | 31.07 ± 0.2 | 33.73 ± 0.26 |
| 200 fg | 35.47 ± 1.19[b] | 33.73 ± 0.3[b] | 37.51 ± 1.11[b] |

[a]Mean quantification cycle (Cq) value of three technical replicates ± standard deviation.
[b]Limit of detection (LoD): the lowest DNA concentration detected in at least 95% of the qPCR runs.

on the seven-fold serial dilutions of genomic DNA (200 ng/μL–200 fg/μL). The standard curves showed linearity for all *Sclerotinia* species (Figure 2(B)), with high efficiencies of 99.6% for *S. sclerotiorum*, 94.0% for *S. minor*, and 108.1% for *S. nivalis*. The analytical sensitivity was evaluated by the LoD (Table 4), and all target species were detected at the minimum DNA concentration of 200 fg ($2 \times 10^{-4}$ ng), corresponding with Cq values of 35.47 ± 1.19 for *S. sclerotiorum*, 37.51 ± 1.11 for *S. minor*, and 33.73 ± 0.3 for *S. nivalis*.

### 3.4. Sensitivity evaluation for soil samples and sclerotia

The multiplex assay employed in this study was highly sensitive in detecting the target pathogens at very low incidence levels through evaluating fungal DNA in the inoculated soils, regardless of the inoculation source (mycelium plug or sclerotium) (Table 5; Figure 3). All species were successfully detected in each pot inoculated with mycelium plugs of each *Sclerotinia* species (Figure 3(A-C)). However, for the pot together with mycelium plugs of the three species, the assay detected only *S. minor* and *S. sclerotiorum*, but not *S. nivalis* (Figure 3(D)). For all pots inoculated with a sclerotium of each *Sclerotinia* species individually and together, all species were detected (Figure 3(E-H)).

The sensitivity was also confirmed for only one sclerotium of each species and a mixture of sclerotia of three *Sclerotinia* species (Figure 3(I-L)).

## 4. Discussion

Despite the importance of white mold disease associated with *Sclerotinia* species on various agronomic and horticultural crops, molecular identification and detection methods developed to date have been limited to endpoint multiplex PCR or a single species-specific quantitative PCR. The former method fails to quantify the initial inoculation amount, which is essential for rapid disease diagnosis and the establishment of control measures. The drawback of the latter approach is that it may be difficult to discriminate among *Sclerotinia* species that are not distinguishable by morphological and cultural characteristics, and thus, not applicable when different species coexist in air, soil, or seeds. Therefore, the primary objective of this study was to develop a multiplex qPCR assay with *Sclerotinia* species-specific probes and test the assay for *Sclerotinia* species in soil samples. For this purpose, the *asp* gene sequences were compared to develop TaqMan probes to differentiate among *Sclerotinia* species, and triplex qPCR probes and conditions successfully detected each species from the inoculated soil samples, as well as pure cultures.

The TaqMan assay developed herein is a highly specific and sensitive detection assay for the three *Sclerotinia* species. Notably, this study describes the first *S. minor*- and *S. nivalis*-specific qPCR methods, and the sensitivity limits of 0.2 pg for *S. minor* and *S. nivalis* suggested that the amounts of genomic DNA corresponding to a sclerotium were detectable. For *S. sclerotiorum*, the present sensitivity (0.2 pg) is better than or comparable to other reports, which were previously estimated at 5 [15], 0.5 [16], 0.05 [24], or 0.07 pg [25] in qPCR assays.

By multiplexing three species-target probes while maintaining a high sensitivity, the present assay provides

**Table 5.** Multiplex qPCR detection of *Sclerotinia* species for soil and sclerotium samples.

| Study sample | S. sclerotiorum DNA Con. (ng/uL) | Mean Cq[a] | S. minor DNA Con. (ng/uL) | Mean Cq | S. nivalis DNA Con. (ng/uL) | Mean Cq | S. sclerotiorum + S. minor + S. nivalis DNA Con. (ng/uL) | Mean Cq |
|---|---|---|---|---|---|---|---|---|
| Soil with an agar plug | 12.85 ± 2.58 | 21.72 ± 0.62 | 14.79 ± 2.51 | 25.36 ± 0.08 | 30.21 ± 11.32 | 21.87 ± 0.92 | 29.29 ± 6.97 | S. sclerotiorum = 25.41 ± 2.83 S. nivalis = N.A. S. minor = 24.55 ± 1.62 |
| Soil with a sclerotium | 13.69 ± 2.03 | 22.16 ± 0.14 | 27.14 ± 4.11 | 26.26 ± 3.13 | 18.25 ± 2.17 | 22.22 ± 0.31 | 20.7 ± 0.3 | S. sclerotiorum = 24.47 ± 1.95 S. nivalis = 26.39 ± 0.19 S. minor = 25.25 ± 0.32 |
| A sclerotium | 2.02 ± 0.19 | 20.3 ± 0.17 | 3.51 ± 0.32 | 21.39 ± 0.11 | 1.03 ± 0.23 | 20.95 ± 0.31 | _[b] | S. sclerotiorum = 20.89 ± 0.16 S. nivalis = 18.76 ± 0.44 S. minor = 21.01 ± 0.09 |

[a]Mean quantification cycle (Cq) value of three technical replicates ± standard deviation.
[b]Mixed with 1 uL of DNA aliquot extracted from a sclerotium of each species.
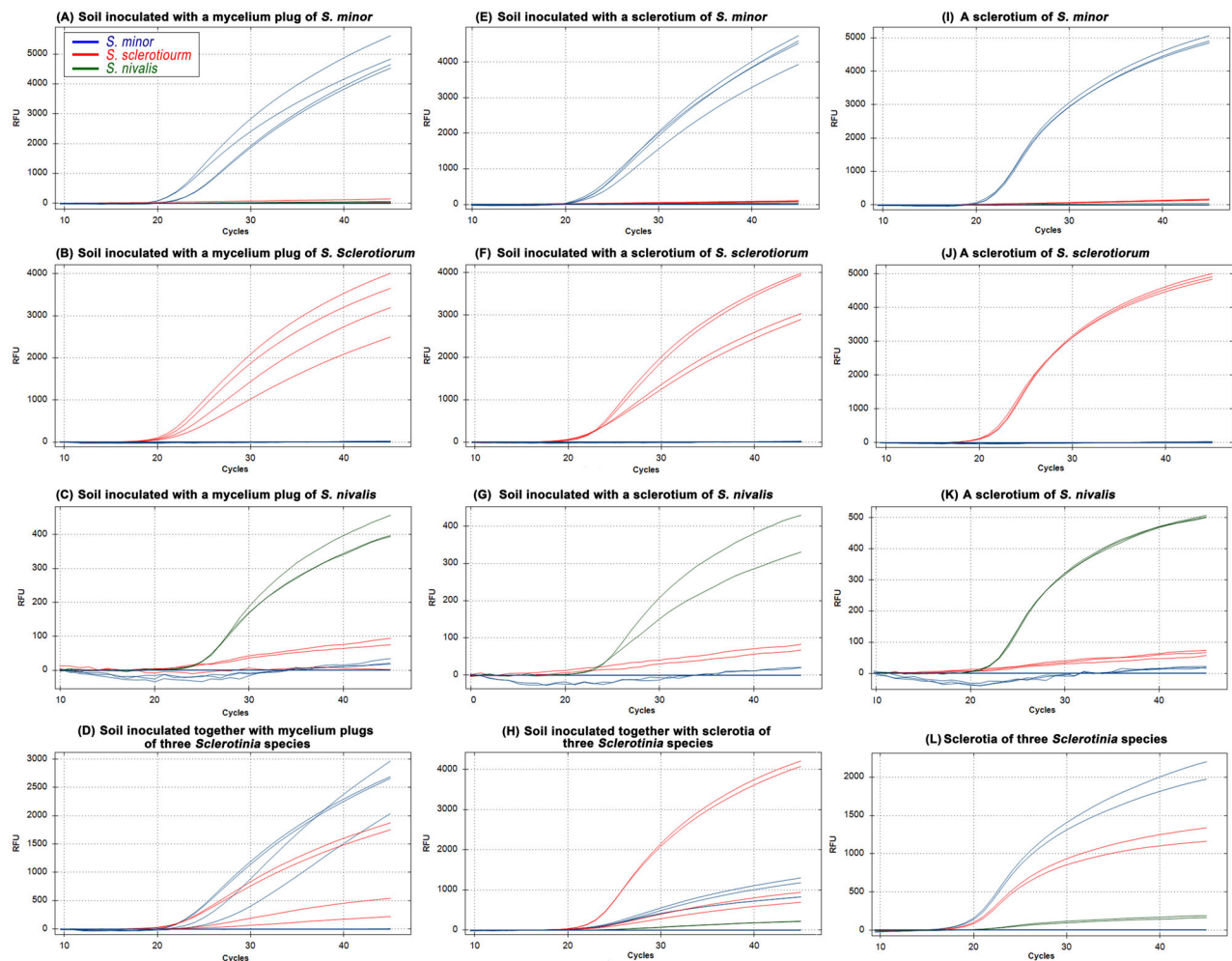


**Figure 3.** Multiplex qPCR assays of three target species for soil (A-H) and sclerotia (I-L) samples. Autoclaved soil was inoculated with a mycelium plug of *Sclerotinia minor* (A), *S. sclerotiorum* (B), *S. nivalis* (C), and three *Sclerotinia* species (D) or a sclerotium of *S. minor* (E), *S. sclerotiorum* (F), *S. nivalis* (G), and three *Sclerotinia* species (H). Each sclerotium was harvested the PDA media of *S. minor* (I), *S. sclerotiorum* (J), *S. nivalis* (K), and three *Sclerotinia* species (L). Colored lines mean the qPCR amplification curves specific for *S. sclerotiorum* (red), *S. minor* (blue), and *S. nivalis* (green).

an improvement over existing assays for *Sclerotinia* species. In addition, even in a sample containing three different species of *Sclerotinia*, this multiplex assay detected and quantified each pathogen, enabling a high-throughput analysis of agricultural samples. Thus, the developed probes have several potential downstream applications in pathogen detection in air, soil, or seed, and disease forecasting, which could be employed to reduce fungicide treatment and crop losses.

## Disclosure statement

## Funding

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