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A Real-Time, Quantitative PCR Seed Assay for Botrytis spp. that Cause Neck Rot of Onion

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ABSTRACT


A real-time fluorescent polymerase chain reaction (PCR) assay was developed using SYBR Green chemistry to quantify the Botrytis spp. associated with onion (Allium cepa) seed that are also able to induce neck rot of onion bulbs, i.e., B. aclada, B. allii, and B. byssoidea. The nuclear ribosomal intergenic spacer (IGS) regions of target and nontarget Botrytis spp. were sequenced, aligned, and used to design a primer pair specific to B. aclada, B. allii, and B. byssoidea. Primers and amplification parameters were optimized to avoid amplifying the related species B. cinerea, B. porri, and B. squamosa, as well as Sclerotinia sclerotiorum and isolates of 15 other fungal species commonly found associated with onion seed. The primers reliably detected 10 fg of genomic DNA per PCR reaction extracted from pure cultures of B. aclada and B. allii. Conventional assays of surface-disinfested and nondisinfested seed on an agar medium were used to determine the incidence of neck rot Botrytis spp. associated with each of 23 commercial onion seed lots, and the real-time PCR assay was used to determine the quantity of DNA of neck rot Botrytis spp. in each seed lot. A linear relationship could not be found between the incidence of seed infected with the neck rot Botrytis spp. using the conventional agar seed assays and the quantity of DNA of the neck rot Botrytis spp. detected by the real-time PCR assay. However, the real-time PCR assay appeared to be more sensitive than the conventional agar assay, allowing detection of neck rot Botrytis spp. in 5 of the 23 seed lots that tested negative using the conventional agar seed assay.

Additional keywords: kinetic PCR, scape blight, umbel blight

In 2005, a total of 65,141 ha of storage and nonstorage onions (Allium cepa) were harvested in the United States, representing a farm-gate value of $922 million (USDA NASS). In 2005, in the Pacific Northwest alone (Idaho, Oregon, and Washington), a total of 19,947 ha of storage and nonstorage onions were harvested, representing a farm-gate value of $298 million (USDA NASS). Washington State ranks third in the United States for production of storage bulbs, with more than 50% of the bulbs exported to Pacific Rim countries (USDA NASS). Washington State also produces approximately 800 acres of onion seed every year, with a value of about $5.7 million ($3.65). Approximately 50% of the seed produced in Washington is exported, contributing up to 20% of the world supply of onion seed ($3.65). Dependence of the onion bulb and seed industries on export markets necessitates the production of high-quality, pathogen-free onion bulbs and seed.

Seven species of Botrytis have been associated with diseases of Allium crops (19), five of which have been associated with neck rot (Common Names of Plant Diseases, APS website). However, three species appear to be most commonly associated with neck rot, namely B. aclada (Fresenius) Yohalem, B. allii (Munn) Yohalem, and B. byssoidea J.C. Walker (8,9,13,29,30,39). Until the recent taxonomic and nomenclatural clarification of these neck rot species by Yohalem et al. (76), B. aclada was considered by many to be synonymous with B. allii, while B. byssoidea was regarded by some as conspecific with B. aclada (23). The lack of distinction of these species was due, in part, to limitations at differentiating these species using classical morphological and cultural methods (47,48). Nonetheless, Owen et al. (42) demonstrated that B. byssoidea and B. aclada are valid species.

Two subgroups within B. aclada (AI and AII) can be distinguished based on chromosome number and conidial dimensions (20,58). Polymorphic polymerase chain reaction (PCR) alleles and internal transcribed spacer restriction fragment length polymorphisms (ITS-RFLPs) have been used to demonstrate that isolates in subgroups AI and AII are distinct from B. byssoidea (36,39). In fact, Nielsen and Yohalem (38) concluded that the larger-spored subgroup developed as a result of a hybridization event between a small-spored isolate of B. aclada and an isolate of B. byssoidea. Yohalem et al. (76) proposed that B. aclada be reserved for the small-spored subgroup (AI), and B. allii for the larger spored subgroup (AII) of B. aclada. A recent molecular phylogeny of the genus Botrytis, based on three nuclear protein-coding genes (RPB2, G3PDH, and HSP60), supports this proposed hybrid status (60).

Neck rot species of Botrytis are found in all areas of the world where onions are produced, but the greatest losses have been reported in temperate regions, where B. allii and B. aclada appear to be the predominant onion neck rot pathogens (8,11,13,28,36). However, the two species are difficult to distinguish morphologically, with similar growth patterns on agar media and overlapping spore sizes (7,76). B. byssoidea is occasionally reported as causing neck rot (47,73). However, the true impact of B. byssoidea may be underestimated because of difficulty in isolating and identifying this species, which sporulates sparsely on most common media used to isolate fungi from plant tissues (7).

The potential for Botrytis infected or infested onion seed to initiate an epidemic of neck rot has been established (2,28,29,61), although the relative significance of seedborne inoculum versus alternative sources of inoculum (infected culled bulbs, infected volunteers, infested debris, and soilborne sclerotia) in the development of neck rot remains highly controversial (14,28,35,41,68,69). Transmission of B. allii (undifferentiated from B. aclada) from seed to seedling was reported by Tichelaar (67), who demonstrated microscopically that the fungus is able to invade the tip of the cotyledon from the seed coat. Transmission of the fungus from seed to seedling is enhanced particularly because the cotyledon tip remains attached to the seed coat via a haustorium during germination and emergence, when the haustorium absorbs nutrients from the endodermis (4). In the United Kingdom, a linear relationship was observed between the percentage of planted seed infected with B. allii (undifferentiated from B. aclada) and the

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Aspergillus niger

Alternaria

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Unless noted otherwise, strains were isolated by L. J. du Toit from onion plants or onion seed produced in the Columbia Basin of central Washington (13).

Table 1. Fungal and plant species used to develop a real-time polymerase chain reaction (PCR) assay for neck rot Botrytis spp. of onion

<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Code</th>
<th>Collectora</th>
</tr>
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<tr>
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<td>Onion</td>
<td>B5, B328, B330, B346, B351, B352, B363, B364, B368, B369, B390, B393, B403, B436, B463, B511, B514, B519, B523, B528, B614</td>
<td>L. J. du Toit</td>
</tr>
<tr>
<td>B. allii</td>
<td>Onion</td>
<td>B3, B331, B332, B358, B365, B374, B377, B381, B385, B414, B423, B442, B454, B518, B520, B529, B627, B638, B678, B679, B683</td>
<td>L. J. du Toit</td>
</tr>
<tr>
<td>B. byssoidea</td>
<td>Onion</td>
<td>ATCC 60837</td>
<td>A. H. Presly</td>
</tr>
<tr>
<td>B. cinerea</td>
<td>Onion</td>
<td>BC1, BC2, BC3, BC4</td>
<td>L. J. du Toit</td>
</tr>
<tr>
<td>B. porri</td>
<td>Onion</td>
<td>BP1, BP2</td>
<td>L. J. du Toit</td>
</tr>
<tr>
<td>B. porri</td>
<td>Onion</td>
<td>BP4</td>
<td>J. W. Lorbeer</td>
</tr>
<tr>
<td>Fusarium oxysporum f. cepae</td>
<td>Onion</td>
<td>FOC8, FOC201A</td>
<td>H. F. Schwartz</td>
</tr>
<tr>
<td>Alternaria</td>
<td>Onion</td>
<td>-</td>
<td>L. J. du Toit</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>Onion</td>
<td>-</td>
<td>L. J. du Toit</td>
</tr>
<tr>
<td>Aspergillus sp.</td>
<td>Onion</td>
<td>-</td>
<td>L. J. du Toit</td>
</tr>
<tr>
<td>Cladosporium sp.</td>
<td>Onion</td>
<td>-</td>
<td>L. J. du Toit</td>
</tr>
<tr>
<td>Epicoccum sp.</td>
<td>Onion</td>
<td>-</td>
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</tr>
<tr>
<td>Fusarium sp.</td>
<td>Onion</td>
<td>-</td>
<td>L. J. du Toit</td>
</tr>
<tr>
<td>Penicillium sp.</td>
<td>Onion</td>
<td>-</td>
<td>L. J. du Toit</td>
</tr>
<tr>
<td>Rhizopus sp.</td>
<td>Onion</td>
<td>-</td>
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<tr>
<td>Stemphylium sp.</td>
<td>Onion</td>
<td>-</td>
<td>L. J. du Toit</td>
</tr>
<tr>
<td>Unidentified hyphomycete</td>
<td>Onion</td>
<td>-</td>
<td>L. J. du Toit</td>
</tr>
<tr>
<td>Sclerotinia sclerotiorum</td>
<td>Potato</td>
<td>-</td>
<td>C. Hammond</td>
</tr>
<tr>
<td>Allium cepa</td>
<td>-</td>
<td>Control</td>
<td>-</td>
</tr>
</tbody>
</table>

a Strains of Botrytis and other fungi isolated from onion plants or seed were used to design real-time PCR primers based on the intergenic spacer (IGS) region of ribosomal DNA, for an onion seed assay for neck rot Botrytis spp. Refer to the text for details on the PCR primers and parameters of the real-time PCR assay. B. aclada, B. allii, and B. byssoidea are the primary causal agents of neck rot of onion, but B. cinerea, B. porri, and B. squamosa are also onion pathogens (7, 23).

b n = number of isolates.

Unless noted otherwise, strains were isolated by L. J. du Toit from onion plants or onion seed produced in the Columbia Basin of central Washington (13).
(175 rpm) for 3 to 4 days (Table 1). Mycelium of each isolate was harvested by vacuum filtration, frozen, and lyophilized in sterile 2-ml screw cap centrifuge tubes. Freeze-dried mycelium was ground to a powder for 4 s in 2-ml screw cap tubes using ceramic spheres and a FastPrep FP120 Bio101 Savant machine (Qiogene, Carlsbad, CA). DNA was extracted from the mycelium according to the CTAB method of Lee and Taylor (25), using 600 μl of modified CTAB extraction buffer (0.02 M EDTA, pH 8.0, 0.1 M Tris, pH 8.0, 1.4 M NaCl, 2% CTAB, and 2.5% PVP). The DNA pellet was then eluted in 50 μl of buffer (10 mM Tris, pH 8.0). The concentration of DNA was determined by visual comparison of the band intensity on 50 µl of buffer (10 mM Tris, pH 8.0, 1.4 M NaCl, 2% CTAB, and 2.5% PVP). The DNA pellet was then eluted in 8.0, 1.4 M NaCl, 2% CTAB, and 2.5% (0.02 M EDTA, pH 8.0, 0.1 M Tris, pH 8.0). The DNA pellet was then eluted in 8.0, 1.4 M NaCl, 2% CTAB, and 2.5% (0.02 M EDTA, pH 8.0, 0.1 M Tris, pH 8.0).

Carlsbad, CA). DNA was extracted from FP120 Bio101 Savant machine (Qbiogene, Beverly, MA), and visualized using a digital imaging system (Ultra Violet Products, Upland, CA) (51). The estimated DNA concentrations were confirmed using a Bio-Rad VersaFluor fluorometer (Bio-Rad, Hercules, CA).

**Intergenic spacer region PCR and sequencing.** The intergenic spacer (IGS) region of nuclear ribosomal DNA, located between the large subunit (LSU) 28s rDNA and the small subunit (SSU) 18s rDNA repeats, was sequenced in both directions for *B. allii* isolate BA3, *B. aclada* isolate BA5, *B. byssodesia* isolate ATCC 60837, and *B. cinerea* isolate BC1. A 20-μl PCR mix was prepared for each isolate, containing 1× PCR buffer (New England Biolabs), 300 μM dNTPs (New England Biolabs), 500 nM of each of two PCR primers, 1 unit of Taq polymerase (New England Biolabs), and 10 ng of template DNA. The forward primer, *B. allii* forward primer, was designed using a 10-fold dilution series of the locus consisting of a 1× pass of each of the forward and reverse directions. The primer pair was designed visually based on alignment of the IGS sequences to selectively amplify a 114-bp product from each of *B. aclada*, *B. allii*, and *B. byssodesia*, but not from *B. cinerea*, an onion pathogen and common contaminant of onion seed which is not frequently associated with neck rot (23).

**Real-time PCR assay parameters and primer screening.** All real-time PCR assays were performed on a Bio-Rad I-cycler (Bio-Rad, Hercules, CA) using a program of one initial cycle of 10 min at 95°C; followed by 50 cycles of 10 s at 95°C, 15 s at 65°C, and 15 s at 72°C with fluorescence data collection; 10 min at 72°C; 30 s at 95°C; 1 min at 55°C; then 80 cycles starting with 5 s at 55°C and increasing the temperature by 0.5°C every cycle thereafter, with melt curve data collected; and a final step of 30 s at 20°C. Real-time PCR was performed in a total volume of 25 μl which consisted of 12.5 μl IQ SYBR Green 2x Supermix (100 mM KCl, 40 mM Tris-HCl at pH 8.4, 0.4 mM each dNTP, 50 units/ml iTaq DNA polymerase, 6 mM MgCl₂, 20 μM fluorescein SYBR Green I, 25 μM each forward and reverse primer, and 1 μl of template DNA. To test sensitivity of the primer set and determine a standard curve of cycle threshold (Cₚ) versus the log of the DNA concentration, an eight-point standard curve was constructed using a 10-fold dilution series of DNA extracted from pure fungal cultures of each of *B. allii* isolate BA3 and *B. aclada* isolate BA5, ranging from 10 ng/μl to 1 fg/μl. To test specificity of the real-time PCR primers for neck rot *Botrytis* spp., the primers were tested against 100 pg of template DNA per PCR, extracted from pure fungal cultures of each species shown in Table 1, and against DNA extracted directly from an onion leaf as a control treatment. The fungi represent a range of target and nontarget species commonly associated with onion seed and plants. An isolate of *Sclerotinia sclerotiorum* from potato was also included.

**Conventional agar assay for Botrytis spp. on or in onion seed.** Twenty-three commercial onion seed lots were tested for *Botrytis* spp. using a conventional agar assay (13) (Table 2). In this study, the incidence of infected seed indicates the incidence of seed from which *Botrytis* spp. were detected using an assay of non-surface-disinfested seed, while the incidence of infected seed indicates the incidence of seed from which *Botrytis* spp. were detected using an assay of surface-disinfested seed. For the assay of non-surface-disinfested seed, four replicates of 100 seed/lot were rinsed under running deionized water for 60 min to remove spores of fast-growing fungi present as contaminants on the seed surface. The seed was then dried in a laminar flow hood on sterile paper towel, and plated onto Kritzman’s agar (21) with 20 seed/cm² diameter petri plate. The seed were then incubated at 20°C with a 12 h/12 h day/night cycle (cool-white fluorescent light and near-ultraviolet light by day) for 14 days, during which time the seed were examined microscopically (up to 100x magnification) at approximately 4-day intervals for development of *Botrytis* spp. (13). For the assay of surface-disinfested seed, four replicates of 100 seed/lot were surface-disinfested in 1.2% NaOCl for 60 s, triple-rinsed in sterile deionized water, and then dried, plated, and examined as described for the assay of nondisinfested seed (13).

**DNA extraction from onion seed, and real-time PCR seed assay.** To determine an appropriate DNA extraction procedure from onion seed for the real-time PCR assay, various seed sample sizes, maceration techniques, and DNA extraction procedures were tested. This included the use of ceramic spheres or stainless steel ball bearings in conjunction with garnet in a FastPrep FP120 Bio101 Savant machine (Qiogene) for tissue maceration, and either the CTAB phenol/chloroform DNA extraction procedure described above or the DNasey Plant Mini Kit from Qiagen for DNA extraction. To determine the amount of seed from which to extract DNA using the DNasey kit, DNA was extracted from five replicate samples of 10 and 25 seed of each of lots 4 and 6 (Table 2).

To assess sensitivity and efficiency of the DNA extraction procedure used in conjunction with the real-time PCR assay, a seed lot determined to be “free” of *Botrytis* using the conventional agar assay (lot 2, Table 2) was placed in a 10-μl volume of sterile water containing 0, 25, 250, 2,500, 25,000, or 250,000 conidia of *B. allii* (determined using a hemocytometer) for each of four replicates of 25 seed. The seed were then frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. DNA was extracted from each sample of 25 seed using the DNasey Plant Mini Kit following the manufacturer’s instructions, including an optional step in which 500 μl of AP1 buffer was added to the ground tissue, the mixture spun at 20,000 × g for 5 min, and the supernatant used in the subsequent DNA extraction steps. DNA was eluted in a final volume of 200 μl of AE buffer.
Each of the 23 seed lots tested using the conventional agar assay was also subjected to the real-time PCR assay to determine the amount of DNA of neck rot *Botrytis* spp. that could be detected. Ten replicates of 25 seed/lot (250 total/lot) were frozen in liquid nitrogen, ground to a fine powder, and DNA was extracted and eluted in a final volume of 200 µl of AE buffer as described above. An eight-point standard curve was constructed with DNA extracted from a pure culture of *B. allii* (BA3) as described above, and at least two negative controls were always included for each run of the real-time PCR machine.

**Data analyses.** The real-time PCR assay detected 10 fg of target DNA in the standard dilution series for 100% of the seed lots, but only detected the 1-fg standard in approximately 50% of the runs. Therefore, any DNA extract for which the real-time PCR assay produced a cycle threshold (Ct) greater than the 10-fg standard was regarded as nonquantifiable, and a value of 0 was noted for DNA quantity. Efficiency of the DNA extraction procedure and real-time PCR assay was calculated for seed lot 2, to which different numbers of conidia of *B. allii* had been added, by dividing the observed quantity of *B. allii* DNA per seed by the estimated quantity of *B. allii* DNA expected per seed. The expected quantity of *B. allii* DNA was estimated by assuming an average of one nucleus per conidium (58), a genome size of 36 Mb (22), and an average DNA base pair weight of 635 Da (51). To determine if the relationship between observed and expected quantities of *B. allii* DNA was linear, both data sets were transformed [ log(DNA quantity) + 1] and a scatter plot was constructed.

A 2 × 2 contingency table was used to compare results of the conventional agar seed assays with results of the real-time PCR seed assay, based on the number of real-time PCR assays and conventional assays in which neck rot *Botrytis* spp. were detected. To test concordance between results of the real-time PCR assay and results of the agar assays of disinfested and nondisinfected seed and between the two versions of the agar assay, Fisher’s exact test was performed (15). The null hypothesis was that results were not correlated. Linear regression and correlation analyses were performed using Statistica Version 7.1 (Analytical Software, Tallahassee, FL) for the amount of DNA of onion neck rot *Botrytis* spp. detected and the percentage of onion seed infected or infested with these fungi. Seed lot 22 was dropped from the regression analyses because this lot had a very high incidence of infected and/or infested seed and therefore functioned as an outlier in the regression analyses.

**RESULTS**

**Intergenic spacer (IGS) region.** Contiguous sequences of the entire IGS region, including partial 28s and 18s ribosomal sequences, were constructed for an isolate of each of *B. allii*, *B. byssoidea*, and *B. cinerea*, and measured 3,585, 3,598, and 3,662 bp in length, respectively (data not shown).

<table>
<thead>
<tr>
<th>No.</th>
<th>Seed lot</th>
<th>Region of productionb</th>
<th>Mean real-time PCR estimate of neck rot <em>Botrytis</em> spp. (DNA fg/seed)</th>
<th>Mean incidence (%) of seed infected</th>
<th>Aspergillus spp.</th>
<th>Cladosporium spp.</th>
<th>Penicillium spp.</th>
<th>Rhizopus spp.</th>
</tr>
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<tbody>
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<td>1</td>
<td>OR</td>
<td>92.5 (5/10)</td>
<td>3.75</td>
<td>All other fungi</td>
<td>7.5</td>
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<td>2.50</td>
<td>All other fungi</td>
<td>1.0</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>22</td>
<td>NZ</td>
<td>37,400.0 (10/10)</td>
<td>45.25</td>
<td>All other fungi</td>
<td>9.0</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>23</td>
<td>Italy</td>
<td>7.5 (1/10)</td>
<td>0.00</td>
<td>All other fungi</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* For the agar assay of surface-disinfested seed, 400 seed/lot were rinsed in 1.2% NaOCl for 60 s with agitation, triple-rinsed in sterile deionized water, dried on sterile paper towel, and plated onto Kritzman’s agar (21). The seed were then incubated and examined microscopically as described by du Toit et al. (13). For the agar assay of non-surface-disinfested seed, 400 seed/lot were rinsed under running deionized water for 60 min, then dried, plated, incubated, and examined for the surface-disinfested seed assay.

* Commercial onion seed lots produced in the United States (AZ = Arizona, CA = California, ID = Idaho, OR = Oregon, or WA = Washington), Italy, or New Zealand (NZ). = information on the region of production was not available.

* Real-time PCR assay of 250 seed/lot based on SYBR Green chemistry (34) with PCR primers for detection of *B. byssoidea*, *B. aclada*, and *B. allii* (causal agents of neck rot on onion) based on the intergenic spacer (IGS) region of ribosomal DNA (refer to the text for details).

* All other fungi = percentage of seed infected with any fungus other than *Botrytis* spp. Most seed were infected with multiple fungi.

* Unless stated otherwise, all *Botrytis* isolates resembled either *B. aclada* or *B. allii*, which could not be distinguished morphologically (7). For the assay of surface-disinfested seed of lot 16, *B. cinerea* and *B. aclada/B. allii* were each observed on 1 seed (2/400 seed = 0.50%). For the assay of nondisinfested seed of lot 21, *B. cinerea* was observed on 7 seed and *B. aclada/B. allii* on 31 seed (38/400 seed = 9.50%). For the assay of nondisinfested seed of lot 22, *B. cinerea* was observed on 6 seed, and *B. aclada/B. allii* on 391 seed (397/400 seed = 99.25%).
shown). Approximately 95% of the IGS region was sequenced for B. aclada, including part of the 28s ribosomal subunit, resulting in a 3,389-bp contiguous sequence. IGS sequences of these isolates have been deposited in GenBank (accession numbers DQ462236 to DQ462239). Multiple alignment of the partial IGS sequence data revealed that B. allii and B. byssoidea were 99.7% similar (data not shown). However, greater differences were detected between the other species, ranging from 77.9% similarity between the IGS sequences of B. aclada and B. cinerea to 79.8% similarity between the IGS sequences of B. aclada and B. byssoidea (Table 3).

Real-time PCR assay parameters and primer screening. The real-time PCR primers, 5’-GAGCTAGCGCATTTGAAA GC-3’ and 5’-TCACCGGGAGCTATCATATA GGC-3’, amplified a 114-bp product from B. aclada, B. allii, and B. byssoidea. The real-time PCR assay did not amplify DNA of any of the 15 nontarget fungal species evaluated, nor of the healthy onion leaf extract tested at 100 pg of genomic DNA (data not shown). However, DNA of the nontarget species B. porri and B. squamosa was detected at C_{T} values of 27.4 and 26.7, respectively, when an initial annealing temperature of 60°C was used, compared to a C_{T} value of 18.2 for the B. allii isolate. To overcome this nonspecific amplification, the annealing temperature was raised to 65°C, which successfully eliminated amplification of B. porri and B. squamosa without reducing sensitivity of the assay for the target Botrytis spp., B. aclada, B. allii, and B. byssoidea (data not shown).

The primers successfully amplified target DNA from all isolates of B. allii, B. aclada, and B. byssoidea evaluated, with a PCR efficiency of approximately 70%, based on the formula: Efficiency = 10^{1/slope} - 1, where the slope equals that of the standard curve (Fig. 1). The fluorescent signal of the nontarget Botrytis spp. and other nontarget fungi did not exceed the background fluorescence. The real-time PCR primers were highly sensitive, reliably detecting 10 fg of genomic DNA per PCR in 100% of the assays, and 1 fg of genomic DNA per PCR in approximately 50% of the assays on pure cultures of B. allii. The C_{T} values ranged from 18 to 20 for target Botrytis spp. at 100 pg of genomic DNA. The PCR assay proved robust, as demonstrated by exponential amplification curves and single dissociation peaks of the final PCR product at approximately 79.5 ± 0.5°C for isolates of B. aclada, B. allii, and B. byssoidea. A single PCR product of the expected size was verified by electrophoresis of each sample on an agarose gel.

Conventional agar seed assay. The percentage of onion seed from which Botrytis spp. were detected using the assays of surface-disinfested and nondisinfested seed on Krizman’s agar ranged from 0 to 45.25% and 0 to 99.25%, respectively (Table 2). A majority of the Botrytis spp. resembled B. aclada or B. allii morphologically, with only three seed lots showing infection by B. cinerea. For the assay of nondisinfested seed, seed lots 21 and 22 had 1.75 and 1.50% B. cinerea, respectively; and for the assay of surface-disinfested seed, lot 16 had 0.25% B. cinerea (Table 2). For both assays, 10 seed lots had no detectable Botrytis, although the specific lots that tested free of Botrytis spp. were not identical for the assay of surface-disinfested seed (lots 2, 7 to 12, 17, 18, and 23) and the assay of nondisinfested seed (lots 2, 3, 7 to 9, 11, 16, 18, 19, and 23) (Table 2). The highest incidence of seedborne Botrytis was detected with lot 22 (99.25% and 45.25% seedborne Botrytis for the assays of nondisinfested and surface-disinfested seed, respectively).

Diverse fungi were detected on the seed lots. For the assay of surface-disinfested seed, the incidence of seed infected ranging from 0 to 88.00% for Aspergillus spp. (mean of 30.49%), 0 to 38.75% for Cladosporium spp. (mean of 9.22%), 0 to 82.50% for Penicillium spp. (mean of 10.99%), and 0 to 8.25% for Rhizopus spp. (mean of 1.47%). For the assay of nondisinfested seed, the incidence of seed infected ranged from 0 to 100% for Aspergillus spp. (mean of 66.37%), 2.50 to 100% for Cladosporium spp. (mean of 70.47%), 2.75 to 99.50% for Penicillium spp. (mean of 53.74%), and 0 to 12.50% for Rhizopus spp. (mean of 3.74%) (Table 2).

Real-time PCR seed assay for neck rot Botrytis spp. Adequate maceration of onion seed could not be achieved using ceramic spheres or stainless steel ball bearings in conjunction with garnet in a FastPrep FP120 Bio101 Savant machine (Qiogene) (data not shown). However, macerating onion seed manually in liquid nitrogen using a mortar and pestle proved adequate for release of Botrytis DNA for the real-time PCR protocol evaluated, and was the method adopted for the real-time PCR seed assay. A significantly higher amount of DNA was detected in seed lot 22 using the DNeasy kit than the CTAB method, but no difference was found between the two methods using seed lot 4. One of the five replicates of the CTAB extraction failed to amplify DNA in the real-time PCR assay on both of these seed lots. However, this did not occur in any of the replicates of DNA extraction prepared with the DNeasy kit (data not shown). All reactions, including those of the standards

Table 3. Nucleotide identity (%) of Botrytis spp. associated with neck rot of onion, based on ClustalW multiple alignment of the partial intergenic spacer (IGS) regions

<table>
<thead>
<tr>
<th>Botrytis species (isolate no.)</th>
<th>GenBank accession no.</th>
<th>B. allii</th>
<th>B. aclada</th>
<th>B. byssoidea</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. allii (BA3)</td>
<td>DQ462236</td>
<td>100.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. aclada (BA5)</td>
<td>DQ462237</td>
<td>79.6</td>
<td>100.0</td>
<td>-</td>
</tr>
<tr>
<td>B. byssoidea (BB1)</td>
<td>DQ462238</td>
<td>99.7</td>
<td>79.8</td>
<td>100.0</td>
</tr>
<tr>
<td>B. cinerea (BC1)</td>
<td>DQ462239</td>
<td>78.7</td>
<td>77.9</td>
<td>78.8</td>
</tr>
</tbody>
</table>

- The IGS region of each of the four Botrytis isolates was sequenced and aligned using ClustalW (66). Refer to the main text for details on sequencing and contiguous sequence construction.
- Refer to the text and Table 1 for information on the source of each Botrytis isolate.
- IGS sequence information is available at GenBank under the accession numbers listed.

Fig. 1. Real-time polymerase chain reaction (PCR) standard curve of the log of the amount of Botrytis allii DNA, ranging from 1 fg to 10 ng per PCR versus the corresponding cycle threshold (C_{T}) values. Data was a composite of three separate runs of the real-time PCR assay. Only one of the 1-fg standard DNA concentrations of the three PCR runs crossed the C_{T} value.
and the samples which amplified a PCR product, had a single dissociation peak of 79.5 ± 0.5°C under melting curve analysis.

Significant differences were not found between the amount of target DNA detected in DNA extracts prepared with the DNeasy kit on samples of 10 seed versus 25 seed for either seed lot 4 or 6. Therefore, subsequent DNA extractions using the DNeasy kit were carried out using 25 seed per extraction. The relationship between quantity of B. allii DNA detected in DNA extracts with the real-time PCR assay, and the estimated quantity of B. allii DNA added to each sample of 25 seed, was demonstrated to be linear when the variables were scaled using a log transformation ($R^2 = 0.9622$; Fig. 2). The nontransformed regression relationship between quantity of B. allii DNA detected and estimated quantity of B. allii DNA added per seed was described by the equation: $Y = 370.37 + 0.1192X$, where $Y =$ the quantity of B. allii DNA detected per seed (fg), and $X =$ estimated quantity of B. allii DNA added per seed (fg). However, the efficiency of DNA extraction was not consistent among treatments, and ranged from 12 to 260% with a general decrease in efficiency the greater the number of conidia added to the seed (Table 4).

The real-time PCR assay detected DNA of the target Botrytis spp. in 19 of the 23 seed lots (Table 2). Correlation coefficient analyses revealed a significant positive relationship between the quantity of neck rot Botrytis spp. DNA detected and the incidence of onion seed infested and/or infected with B. byssoides, B. aclada, and B. allii, with correlation coefficients of 0.674 ($P = 0.004$) and 0.702 ($P = 0.002$), respectively. However, significant linear regression models describing the relationship between results of the real-time PCR seed assay and the two versions of the agar seed assay were not found (Fig. 3), even when the data were transformed (data not shown). Contingency analysis (Table 5) demonstrated that results of the agar assay of nondisinfested seed and the agar assay of disinfested seed concurred with results of the real-time PCR assay for 65 and 74% of the seed lots, respectively. Fisher’s exact test indicated that results of the agar assay of nondisinfested seed and the real-time PCR assay were uncorrelated ($P = 0.200$), i.e., the incidence of seed infested with neck rot Botrytis spp. was not correlated with the amount of DNA of neck rot Botrytis spp. estimated by real-time PCR. In contrast, Fisher’s exact test rejected the null hypothesis for the agar assay of surface-disinfested seed and the real-time PCR assay ($P = 0.024$), i.e., there was significant positive correlation between neck rot Botrytis spp. detected in seed lots using the agar assay of surface-disinfested seed and the real-time PCR assay.

**DISCUSSION**

A real-time PCR assay specific for neck rot Botrytis species in onion seed was developed. The assay entailed maceration of onion seed in liquid nitrogen using a mortar and pestle, followed by use of a silica-based DNA adsorption and extraction kit from Qiagen, and application of primers specifically designed to detect multicopy DNA of the target Botrytis spp. in real-time PCR using SYBR Green chemistry. The assay was sensitive, reliably detecting 10 fg of genomic DNA per PCR of the target fungi. The assay also was highly specific when evaluated against template DNA from a range of closely related and unrelated fungal species, including common fungal onion seed microflora. Additionally, the assay did not amplify onion DNA. A twofold or greater increase in the standard deviation of DNA concentration detected was only observed when 10 fg of target DNA was present, compared with the average standard deviations for DNA quantities ranging from 10 ng to 100 fg (data not shown). Schroeder et al. (54) reported that, although quantification of Pythium DNA by real-time PCR assays was possible starting at a target DNA quantity of 10 fg, the standard deviation was greater at this amount than at higher DNA quantities, which is expected close to the limit of quantification. Although a 1-fg target DNA standard was included in every real-time PCR assay in this onion neck rot seed assay study, DNA of this amount was only amplified in 50% of the assays. This provides evidence of a lower limit of quantification using this real-time PCR assay. Therefore, results may need to be “trimmed” by assigning a 0 value to any samples with a Ct value greater than that of the 10-fg standard.

PCR assays for detection of Botrytis spp. that cause neck rot of onion have pre-

**Table 4. Efficiency of a real-time polymerase chain reaction (PCR) assay determined by adding different numbers of conidia of Botrytis allii to samples of 25 seed from a Botrytis “free” onion seed lot, followed by seed maceration, DNA extraction, and real-time PCR assay.**

<table>
<thead>
<tr>
<th>Conidia added/seed*</th>
<th>Mean observed neck rot Botrytis DNA detected/seed (fg)b</th>
<th>Expected amount of neck rot Botrytis DNA/seed (fg)c</th>
<th>Efficiency (%)d</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>99</td>
<td>38</td>
<td>260</td>
</tr>
<tr>
<td>10</td>
<td>185</td>
<td>380</td>
<td>49</td>
</tr>
<tr>
<td>100</td>
<td>446</td>
<td>3,800</td>
<td>12</td>
</tr>
<tr>
<td>1,000</td>
<td>6,288</td>
<td>38,000</td>
<td>17</td>
</tr>
<tr>
<td>10,000</td>
<td>45,520</td>
<td>380,000</td>
<td>12</td>
</tr>
</tbody>
</table>

* Number of B. allii conidia added/seed prior to grinding the seed in liquid nitrogen and extracting DNA. Refer to the text for details.

b Estimated quantity of Botrytis DNA/seed detected using a real-time PCR assay.

c Estimated B. allii genome weight based on the assumption of one nucleus per conidium and an average ascomycete genome size of 36 Mb, which is approximately 38 fg (22,51,58).

d Estimated DNA extraction and real-time PCR efficiency, calculated per extract as: $[(\text{observed quantity of DNA})/\text{(expected quantity of DNA)}]*100$. 

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**Fig. 2.** Scatter plot of the quantity of Botrytis allii DNA detected per seed using a real-time polymerase chain reaction (PCR) versus the estimated quantity of B. allii DNA added per seed for each DNA extraction.
viously been reported. However, to our knowledge this is the first real-time PCR seed assay for neck rot *Botrytis* species. Nielsen et al. (37) developed a PCR assay specifically for detection of *B. aclada* and *B. allii* based on the ribosomal internal transcribed spacer (ITS) region. In that assay, a primer specific to *B. aclada* was designed with the 3’ terminus ending on a unique nucleotide position for that species. However, to achieve specificity for *B. aclada*, the annealing temperature had to be raised to 70°C, with a resulting detection limit of 1 to 10 pg of pure fungal DNA (37). Nielsen et al. (39) developed a PCR-RFLP system based on a sequence characterized amplified region (SCAR) from a random amplified polymorphic DNA (RAPD) fragment, for differentiation of *Botrytis* spp. associated with neck rot. The SCAR appears to be based on a region of DNA with a low copy number, as the detection limit by conventional PCR was between 1 and 10 pg of pure fungal DNA, whereas the detection limit for high copy number regions of DNA (such as ribosomal DNA repeats and noncoding regions) is typically as low as 10 fg of DNA (3,10,18,40), as demonstrated in this study with DNA primers based on the IGS regions of the target fungi.

Walcott et al. (72) developed an MCH-PCR assay for *Botrytis* spp. in onion seed based on the SCAR reported by Nielsen et al. (39). The MCH-PCR assay displayed a 10-fold increase in sensitivity over the PCR-RFLP assay of Nielsen et al. (39), but reproducibility of the MCH-PCR assay was low, with 40% detection frequency compared with 100% using a direct PCR with 10 pg of template DNA (72). Because of the limited number of nucleotide differences in the ITS region of the onion neck rot fungi (only four base pair differences; 39), the ITS region was not suitable for design of specific primers for a SYBR Green-based assay. Instead, the IGS region of ribosomal DNA was employed in this study to design real-time PCR primers because the IGS region is less conserved than the ITS region, as demonstrated in this paper for *Botrytis* spp. and previously for *Metarhizium* and *Verticillium* spp. (43,46). This enabled development of an onion seed assay that is sensitive, specific, and able to detect as little as 10 fg of pure template DNA from *B. aclada*, *B. allii*, and *B. byssoidea*. The real-time PCR primers proved to be sensitive and robust for detection of DNA from all isolates of *B. aclada*, *B. allii*, and *B. byssoidea* evaluated, and the primers did not amplify DNA of a diversity of nontarget organisms assessed. In an initial screening with 100 pg of pure fungal DNA at an annealing temperature of 60°C, weak amplification of *B. porri* and *B. squamosa* DNA was detected. Raising the annealing temperature to 65°C prevented nonspecific amplification of *B. porri* and *B. squamosa* without adversely affecting sensitivity of the assay.

The SYBR Green system was employed in this study to detect double-stranded DNA, and melting point profiles were assessed to check specificity of each real-time PCR run. To enhance specificity of the assay, a probe-based detection system could be developed, which would negate the need to carry out melting point profile

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**Table 5.** Contingency table of results of an agar seed assay on 23 commercial onion seed lots tested for neck rot *Botrytis* spp. using an assay of nondisinfested seed and an assay of surface-disinfested seed, compared to results of a real-time polymerase chain reaction (PCR) assay to detect DNA of neck rot *Botrytis* spp. in onion seed.

<table>
<thead>
<tr>
<th>Infected or infected seed (type of agar assay)</th>
<th>Real-time PCR assay&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Fisher’s exact test (probability)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected seed (assay of nondisinfested seed)</td>
<td>Positive</td>
<td>12/23 (52%)</td>
</tr>
<tr>
<td>Infected seed (assay of surface-disinfested seed)</td>
<td>Positive</td>
<td>13/23 (57%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Twenty-three commercial onion seed lots were assayed for the incidence (%) of seed infested or infected with neck rot *Botrytis* spp. as described in the text and Table 2.

<sup>b</sup> “Positive” and “negative” indicate the number of seed lots from which neck rot *Botrytis* spp. were detected and not detected, respectively. Infested seed and infected seed are defined in the text.

<sup>c</sup> Real-time PCR seed assay based on SYBR Green chemistry for detecting DNA of *B. aclada*, *B. allii*, and *B. byssoidea*, the primary causal agents of neck rot of onion. Refer to the text for details. “Positive” and “negative” indicate the number of seed lots from which DNA of these *Botrytis* spp. was detected and was not detected, respectively.

<sup>d</sup> Fisher’s exact test (15) was used to assess the null hypothesis that results of the seed assays were not significantly correlated. NS and * = nonsignificant and significant chi-squared test at *P* < 0.05, respectively.
Adequate maceration of onion seed and Botrytis propagules on or in the seed is essential for detection of seedborne Botrytis spp. using PCR assays. Maude (28) observed hyphae under the onion seed coat. Various reports have demonstrated that treatment of onion seed with a disinfectant such as hypochlorite reduces, but may not eliminate, Botrytis spp. from onion seed, providing further evidence that seedborne Botrytis may reside within or under the seed coat (29,31,61). In this study, maceration of onion seed manually in liquid nitrogen using a mortar and pestle proved adequate for release of Botrytis DNA for the real-time PCR assay. However, it should be possible to develop more efficient tissue maceration method that facilitates quicker sample preparation and higher throughput while preventing cross-contamination among seed samples. For example, there are many 96-well bead mill machines available, some of which allow the sample and reaction vessel to be cooled in liquid nitrogen to enhance tissue maceration. Such a system would allow higher throughput of samples than the system used in this study. In addition, some tissue maceration devices operate at higher frequency than the systems evaluated in this study, and may prove more effective for maceration of onion seed.

Another requirement of DNA extraction procedures is the elimination or reduction of PCR inhibitors, which are commonly found in DNA extracts from seed (49,71,72). In this study, the DNA extractions resulted in less inhibition of PCR than a conventional CTAB phenol:chloroform extraction method (53). It may be possible to improve sensitivity of the real-time PCR assay further by optimizing DNA extraction procedure, e.g., by adding a magnetic capture hybridization (MCH) step to improve target DNA extraction while removing nontarget DNA and PCR inhibitors (57,72). Although no significant difference could be found in the amount of target DNA detected in extractions from samples of 10 seed versus 25 seed, further evaluation may resolve a more optimal number of seed from which to extract DNA. Optimization of the DNA extraction technique may further improve sensitivity and cost-efficiency of the sampling procedure.

An estimate of overall efficiency of the DNA extraction and real-time PCR assay was determined by adding different numbers of conidia of B. allii to an onion seed lot “free” of Botrytis. A single conidium per seed could be detected, and the relationship between quantity of observed DNA and the estimated B. allii DNA added to the seed was demonstrated to be linear. Overall efficiency of both the DNA extraction and the real-time PCR assay ranged from 12 to 260%, with a decrease in efficiency the greater the number of conidia added per seed. This efficiency may be affected by a number of factors, such as adequate maceration of fungal tissue, the DNA extraction procedure, efficiency of DNA extraction, removal of PCR inhibitors, efficiency of the real-time PCR assay, and estimates of the number of conidia added and the genome weight of B. allii used to calculate efficiency of the assay.

The real-time PCR assay evaluated in this study appeared to be more sensitive than the conventional seed assay on an agar medium. Botrytis DNA was detected in 19 of 23 commercial seed lots assayed using the real-time PCR, compared to 16 seed lots using the agar assay. For only one seed lot (no. 10) did the agar assay detect seedborne Botrytis, whereas the real-time PCR assay did not detect the target pathogens. For this lot, Botrytis spp. were detected in the assay of nondisinfested seed at 0.25% (1 out of 400 seed). The lack of Botrytis spp. DNA detection by real-time PCR assay is probably due to differences in sample size for the two assays, as the agar assay was carried out using a sample of 400 seed but the real-time PCR assay was carried out using 250 seed, reflecting natural variation among the samples of seed assayed by real-time PCR versus agar assay. The potential for false positive reactions with the real-time PCR assay as a result of contamination while setting up the reactions was ruled out by including negative control samples with each assay, which tested negative in every real-time PCR assay completed.

A significant linear relationship was not observed between the percentage of onion seed on which Botrytis spp. were detected using the agar assays and the amount of Botrytis spp. DNA detected using the real-time PCR assay. An important consideration when comparing results of the two types of seed assays is that detection of Botrytis spp. DNA by real-time PCR assay is probably due to differences in the amount of DNA detected in seeds that test negative by agar assay may be associated with detection of dead or nonviable (nunculturable) Botrytis spp. isolated from agar plates. In this study, the real-time PCR assay was less sensitive than the conventional seed assay on an agar plate.

Although a strong linear relationship between the amount of DNA of neck rot Botrytis spp. detected using the PCR assay could not be found with the incidence of neck rot Botrytis spp., the PCR assay proved to be a valuable qualitative test for seed infection or infestation by these pathogens. Therefore, the real-time PCR assay could be used as an initial screen of onion seed lots to identify those lots that need further testing and/or seed treatments for control of neck rot Botrytis spp. By using a sampling regime of one seed per DNA extraction, a more detailed assessment of the relationship between the incidence of neck rot Botrytis spp. on many of the seed lots evaluated in this study using the agar assays. Pathogenicity of these fungi on onion was not assessed in this study, but some of these fungi could be pathogenic, causing onion diseases such as black mold (caused by Aspergillus niger), leaf blight (caused by Cladosporium alliceps), blue mold (caused by Penicillium spp.), and mushy rot (caused by Rhizopus spp.) (55).

The real-time PCR assay developed in this study for detection of neck rot Botrytis spp. has several advantages over conventional agar seed assays. The real-time PCR assay is less labor- and time-intensive than agar assays, as the real-time PCR assay can be completed within 24 h versus 2 weeks for the agar assay; a larger number of samples can be processed at one time using the real-time PCR assay compared to the agar assays, allowing a greater throughput of samples; and mycological skills are not needed for the real-time PCR assay to differentiate among Botrytis spp. (although molecular biology skills and resources are needed for the former). This real-time PCR assay may also be adapted for epidemiological studies, such as quantifying Botrytis infection in onion leaf or bulb tissue, or quantifying Botrytis spp. in spore traps (17,56,59,62,70,74). The real-time PCR assay could be used in conjunction with the PCR assay developed by Nielsen et al. (39) to differentiate these Botrytis species. Alternatively, the IGS
sequence information of the four *Botrytis* species, which has been deposited in GenBank, could be used to develop additional species-specific primers. The assay may prove valuable as a tool for disease management decisions, such as whether or not to treat specific onion seed lots for *Botrytis* infection, or identifying appropriate regions in which to plant specific onion seed lots based on the relative risks of *Botrytis* seed transmission associated with regional environmental conditions (12, 13, 26, 29, 30, 68).

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