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

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ABSTRACT

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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 (United States Department of Agriculture National Agricultural Statistics Service [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 crops annually, with a value of about $5.7 million (53,65). Approximately 50% of the seed produced in Washington is exported, contributing up to 20% of the world supply of onion seed (53,65). 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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percentage of bulbs that developed neck rot in storage (28). The incidence of neck rot in bulbs was proportionally greater during wet and humid growing seasons than in drier years (28). However, Maude and Presly (29), Stewart and Franicevic (61), and Chilvers (6) reported that the level of *B. allii* infection detected in seedlings was significantly lower than that in the planted seed, suggesting that environmental conditions under which plants emerge may influence seed transmission. In the semiarid Columbia Basin of Washington, du Toit et al. (14) found no significant relationship between the incidence of planted seed infected with *B. allii* and the incidence of neck rot that developed in storage, in any of three seasons. Tylkowska and Dorna (68) demonstrated a stronger correlation between the incidence of neck rot in storage and the incidence of seed infected "internally" by *B. allii* compared with the incidence of seed "infested" with the pathogen. The incidence of infected seed was determined by surface-disinfesting seed in 1% NaOCl for 10 min, rinsing the seed thoroughly in sterile water, and then plating the seed onto potato dextrose agar (PDA) or prune lactose agar (PLA). The incidence of seed infested with *B. allii* was determined similarly but without the surface-disinfestation step.

Onion seed assays currently employed by seed testing agencies (blotter assays and plating seed on agar media) differ widely in their specificity and sensitivity for detection of *Botrytis* spp. There is currently no certified, internationally accepted protocol for assaying onion seed for neck rot fungi, which compounds the controversy over the relative role of infected seed lots in neck rot outbreaks. The specific parameters used in onion seed assays can also affect results of the assays: e.g., the blotter or agar medium on which the seed is placed; whether or not the seed is treated with a disinfestant, and if so, the concentration of disinfestant and duration of surface-disinfestation used; the temperature and light parameters under which the seed is incubated; and so on (7,21,32). Furthermore, conventional blotter and agar seed assays require several weeks for completion, as well as technical training and resources for differentiating among *Botrytis* spp. that can be seedborne in onion (7). Therefore, a rapid, specific, sensitive, and robust onion seed assay for *Botrytis* spp. is needed.

Recent advances in molecular biology, including real-time PCR, have provided opportunities for development of rapid and precise assays for detection and quantification of microorganisms based on nucleic acid sequences and concentrations. Although there is much literature on the detection of microorganisms using PCR (5,27,40,45,52,63,75), few reports have been published to date demonstrating quantification of seedborne pathogens using real-time PCR (1,16,24,31,64). Walcott et al. (72) developed a magnetic capture hybridization (MCH)-PCR protocol for seedborne *Botrytis* spp. The MCH-PCR assay reduced the time required to complete onion seed assays from 10 to 14 days when using selective agar media to less than 24 h. However, the conventional agar assay yielded higher detection frequencies of *B. aclada* compared with the MCH-PCR assay for seed lots with low infection levels (2.1%), and the MCH-PCR lacked adequate reproducibility or the ability to quantify the incidence of infection in individual seed lots.

The objectives of this study were to: (i) develop sensitive and specific real-time PCR primers for detection of the primary *Botrytis* spp. associated with neck rot of onion, *B. aclada*, *B. allii*, and *B. byssoidea*; (ii) identify a DNA extraction procedure suitable for *Botrytis* spp. present in or on onion seed; (iii) evaluate the primers in a real-time PCR assay using SYBR Green chemistry (34); and (iv) compare sensitivity of the real-time PCR assay to a conventional agar assay for detection of neck rot species of *Botrytis* in/on onion seed.

MATERIALS AND METHODS

Fungal cultures and DNA extraction from mycelium. Isolates of *Botrytis* spp. and other genera used in this study are listed in Table 1. Cultures of each isolate were maintained on PDA at 22°C with a 12 h/12 h day/night photoperiod, and stored on sterile filter paper at –20°C using the protocol described by Peever et al. (44). To obtain mycelium of each isolate for DNA extraction, conidia (approximately a 1 mm^3 clump collected from the surface of a sporulating culture) or approximately 20 pieces of agar (each 0.5 mm^3) with mycelium were transferred to 25 ml of potato dextrose broth (PDB) in 50-ml conical tubes. Still cultures of the *Botrytis* spp. were maintained at 20°C. Other taxa were grown in 50 ml of PDB in 250-ml Erlenmeyer flasks at 20°C on a rotary shaker

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

Species	n[b]	Host	Code	Collector[c]
Botrytis aclada	21	Onion	BA5, B328, B330, B346, B351, B352, B363, B364, B368, B369, B390, B393, B403, B436, B463, B511, B514, B519, B523, B528, B614	L. J. du Toit
B. allii	21	Onion	BA3, B331, B332, B358, B365, B374, B377, B381, B385, B414, B423, B442, B454, B518, B520, B529, B627, B638, B678, B679, B683	L. J. du Toit
B. byssoidea	1	Onion	ATCC 60837	A. H. Presly
B. cinerea	4	Onion	BC1, BC2, BC3, BC4	L. J. du Toit
B. porri	2	Onion	BP1, BP2	L. J. du Toit
B. porri	1	Onion	BP4	J. W. Lorbeer
Fusarium oxysporum f. sp. *cepae*	2	Onion	FOC8, FOC201A	H. F. Schwartz
Acremonium sp.	1	Onion	-	L. J. du Toit
Alternaria sp.	1	Onion	-	L. J. du Toit
Aspergillus niger	1	Onion	-	L. J. du Toit
Aspergillus sp.	1	Onion	-	L. J. du Toit
Cladosporium sp.	1	Onion	-	L. J. du Toit
Epicoccum sp.	1	Onion	-	L. J. du Toit
Fusarium sp.	1	Onion	-	L. J. du Toit
Penicillium sp.	3	Onion	-	L. J. du Toit
Rhizopus sp.	1	Onion	-	L. J. du Toit
Stemphylium sp.	1	Onion	-	L. J. du Toit
Unidentified hyphomycete	1	Onion	-	L. J. du Toit
Sclerotinia sclerotiorum	1	Potato	-	C. Hammond
Allium cepa	1	-	Control	-

[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.

[c] 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 (Qbiogene, 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 ethidium bromide–stained gels with that of a λ-DNA standard (New England Biolabs, 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. byssoidea* isolate ATTC 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 PCR primer, LR12R-GAACGC CTCTAAGTCAGAATCC (R. Vilgalys, *personal communication*), was designed from the LSU 28s rDNA, and the reverse primer, CNS1-GAGACAAGCATATGA CTACTG (T. Bruns, *personal communication*), was designed from the SSU 18s rDNA. Cycling conditions consisted of an initial step of 3 min at 95°C; followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 3 min at 72°C; then a final step of 10 min at 72°C. Reactions were performed in an Omn-E thermal cycler (Thermo Electron Corp., Waltham, MA), and amplicons were separated on 0.8% agarose gels using MassRuler (MBI Fermentas, Hanover, MD) as a size standard, and visualized as described above. Amplicons of expected size were purified using Qiaquick purification columns (Qiagen, Valencia, CA). Amplicons consisting of more than a single DNA product were excised according to expected size and purified according to the manufacturer's instructions. Purified amplicons were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequence reads were performed on a PE Biosystems Model 3700 Automated DNA Sequencer (Applied Biosystems). To complete sequencing of the entire IGS region, several inward nested primer sets were designed using Primer 3 software (50). (Primer sequences are available upon request.)

Real-time PCR primer design. Contiguous sequences of the IGS region for *B. aclada*, *B. allii*, *B. byssoidea*, and *B. cinerea* were constructed and aligned using Vector NTI Suite v.10.0.1 (Invitrogen, Carlsbad, CA), with at least 2× total coverage of the locus consisting of a 1× pass in 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. byssoidea*, 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 2× 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 $MgCl_2$, 20 nM fluorescein SYBR Green I, and stabilizers; Bio-Rad), 800 nM each primer, and 1 µl of template DNA. To test sensitivity of the primer set and determine a standard curve of cycle threshold (C_t) 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 infested 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/10-cm-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 100× 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 (Qbiogene) for tissue maceration, and either the CTAB phenol:chloroform DNA extraction procedure described above or the DNeasy Plant Mini Kit from Qiagen for DNA extraction. To determine the amount of seed from which to extract DNA using the DNeasy 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 DNeasy 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 assays, 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 (C_t) 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 seed and nondisinfested 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 Statistix 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

Table 2. Results of an assay of surface-disinfested seed and an assay of nondisinfested seed on a semiselective agar medium to detect *Botrytis* spp. in each of 23 commercial onion seed lots, for comparison with the amount of DNA of neck rot *Botrytis* spp. quantified in each seed lot using a SYBR Green real-time polymerase chain reaction (PCR) assay[a]

Seed lot		Mean real-time PCR estimate of neck rot *Botrytis* DNA (fg/seed) (proportion of replicates of 25 seed in which DNA was detected)[c]		Mean incidence (%) of seed infected											
				Assay of surface-disinfested seed						Assay of nondisinfested seed					
					All other fungi[d]						All other fungi[d]				
No.	Region of production[b]			*Botrytis* spp.[e]	Total	*Aspergillus* spp.	*Cladosporium* spp.	*Penicillium* spp.	*Rhizopus* spp.	*Botrytis* spp.[e]	Total	*Aspergillus* spp.	*Cladosporium* spp.	*Penicillium* spp.	*Rhizopus* spp.
1	OR	92.5	(5/10)	3.75	15.75	0.75	4.00	3.00	0.25	9.75	99.00	17.50	86.50	67.50	0.50
2	ID	0.0	(0/10)	0.00	68.75	58.25	6.75	22.25	0.25	0.00	100.00	96.25	53.75	96.00	5.50
3	ID	31,040.0	(10/10)	2.25	49.75	12.25	1.25	3.75	0.50	0.00	100.00	84.25	65.25	47.00	9.25
4	ID	19,568.0	(10/10)	16.25	81.50	19.00	16.00	14.75	0.75	11.50	100.00	38.75	98.50	75.25	5.75
5	WA	12,360.8	(10/10)	4.50	31.25	9.00	4.25	1.25	3.00	8.00	100.00	87.75	78.75	69.00	8.50
6	WA	2,049.2	(9/10)	1.25	58.00	36.25	10.50	8.25	1.75	1.25	100.00	97.25	89.50	86.75	1.75
7	AZ	13.3	(1/10)	0.00	54.00	53.00	0.25	0.50	0.00	0.00	100.00	100.00	43.00	9.50	0.25
8	AZ	0.2	(1/10)	0.00	81.25	80.50	0.50	1.50	0.25	0.00	99.75	99.75	22.50	17.00	1.50
9	CA	0.0	(0/10)	0.00	60.00	55.75	6.75	0.25	0.25	0.00	100.00	99.75	99.50	36.50	1.50
10	CA	0.0	(0/10)	0.00	22.50	14.25	5.50	0.50	0.75	0.25	98.25	90.50	66.25	7.00	1.50
11	CA	0.0	(0/10)	0.00	6.50	5.00	1.00	0.00	0.50	0.00	98.75	88.25	59.25	7.75	5.25
12	CA	58.9	(4/10)	0.00	98.00	88.00	23.75	32.25	9.50	0.25	100.00	98.00	92.25	80.00	12.50
13	CA	7,112.8	(10/10)	0.25	25.25	23.25	1.25	0.00	0.75	0.75	99.75	99.50	50.50	3.25	4.00
14	OR	21,581.6	(10/10)	0.50	13.75	0.00	1.75	0.50	0.00	13.00	99.75	3.00	92.75	33.75	3.75
15	OR	19,524.8	(10/10)	4.25	27.25	0.50	8.25	17.25	0.00	7.00	100.00	2.00	68.50	91.00	1.25
16	WA	644.1	(10/10)	0.50	86.00	0.75	6.25	82.50	3.75	0.00	100.00	2.00	87.25	99.50	10.00
17	CA	45.5	(4/10)	0.00	38.50	37.25	0.00	0.00	0.25	0.50	100.00	96.25	2.50	2.75	0.25
18	CA	8.4	(2/10)	0.00	80.00	64.00	38.75	3.00	0.50	0.00	100.00	99.75	100.00	27.00	0.75
19	CA	244.4	(6/10)	0.25	96.75	86.25	22.50	21.00	8.25	0.00	100.00	98.25	85.50	81.25	8.75
20	ID	911.4	(10/10)	0.75	56.00	0.50	36.75	19.50	0.25	2.00	100.00	33.25	99.00	98.25	0.75
21	-	9,691.2	(10/10)	2.50	22.50	0.00	5.00	15.00	0.00	9.50	100.00	0.75	69.50	99.50	0.00
22	NZ	37,400.0	(10/10)	45.25	7.50	0.00	0.50	4.25	2.25	99.25	95.25	0.00	46.25	85.50	2.75
23	Italy	7.5	(1/10)	0.00	81.00	56.75	10.50	1.50	0.00	0.00	100.00	93.75	64.00	15.00	0.00

[a] 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 as for the surface-disinfested seed assay.

[b] 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.

[c] 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 of onion) based on the intergenic spacer (IGS) region of ribosomal DNA (refer to the text for details).

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

[e] 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′-GAGCTAGCGCATTTGAAAGC-3′ and 5′-TCACCGGGAGCTATCATAGGC-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 = $10^{(1/\text{slope})} - 1$, 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 Kritzman'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 ranged 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 infested or infected seed 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 (Qbiogene) (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[a]

Botrytis species (isolate no.)[b]	GenBank accession no.[c]	*B. allii*	*B. aclada*	*B. byssoidea*
B. allii (BA3)	DQ462236	100.0	-	-
B. aclada (BA5)	DQ462237	79.6	100.0	-
B. byssoidea (BB1)	DQ462238	99.7	79.8	100.0
B. cinerea (BC1)	DQ462239	78.7	77.9	78.8

[a] 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.
[b] Refer to the text and Table 1 for information on the source of each *Botrytis* isolate.
[c] IGS sequence information is available at GenBank under the accession numbers listed.

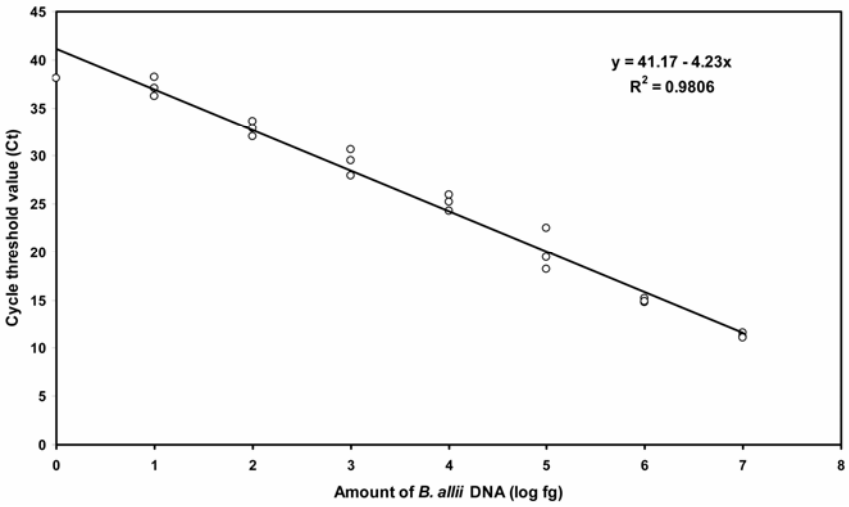

Fig. 1. Real-time polymerase chain reaction (PCR) standard curve of the log of the amount of *Botrytis allii* DNA, ranging from 1 fg to 10 ng per PCR versus the corresponding cycle threshold (C_t) values. Data was a composite of three separate runs of the real-time PCR assay. Only one of the 1-fg standard DNA concentrations of the three PCR runs crossed the C_t value.

and the samples which amplified a PCR product, had a single disassociation 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. byssoidea, 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.

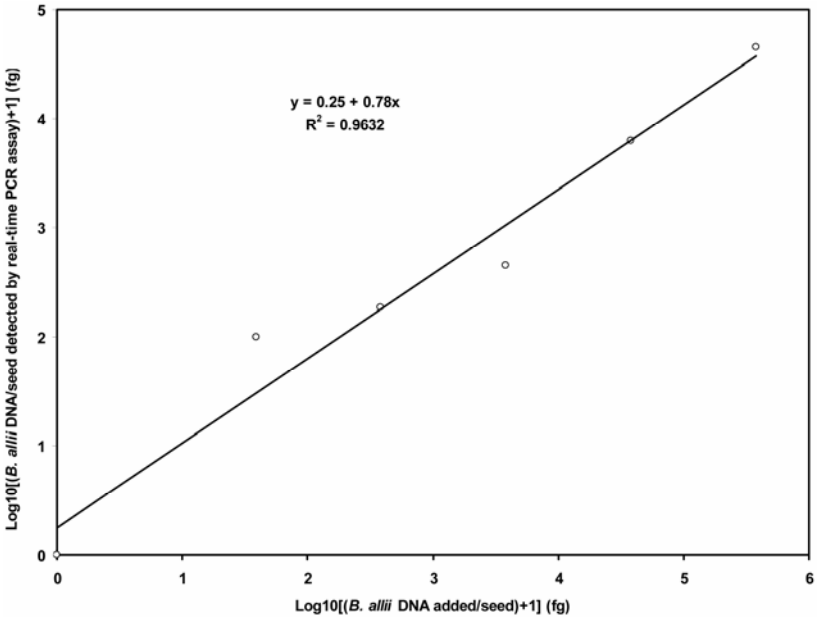

Fig. 2. Scatter plot of the quantity of *Botrytis allii* DNA detected per seed using a real-time polymerase chain reaction (PCR) versus the estimated quantity of *B. allii* DNA added per seed for each DNA extraction.

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

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

[a] 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] Observed 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: [(observed quantity of DNA)/(expected quantity of DNA)]*100.

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 C_t value greater than that of the 10-fg standard.

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

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

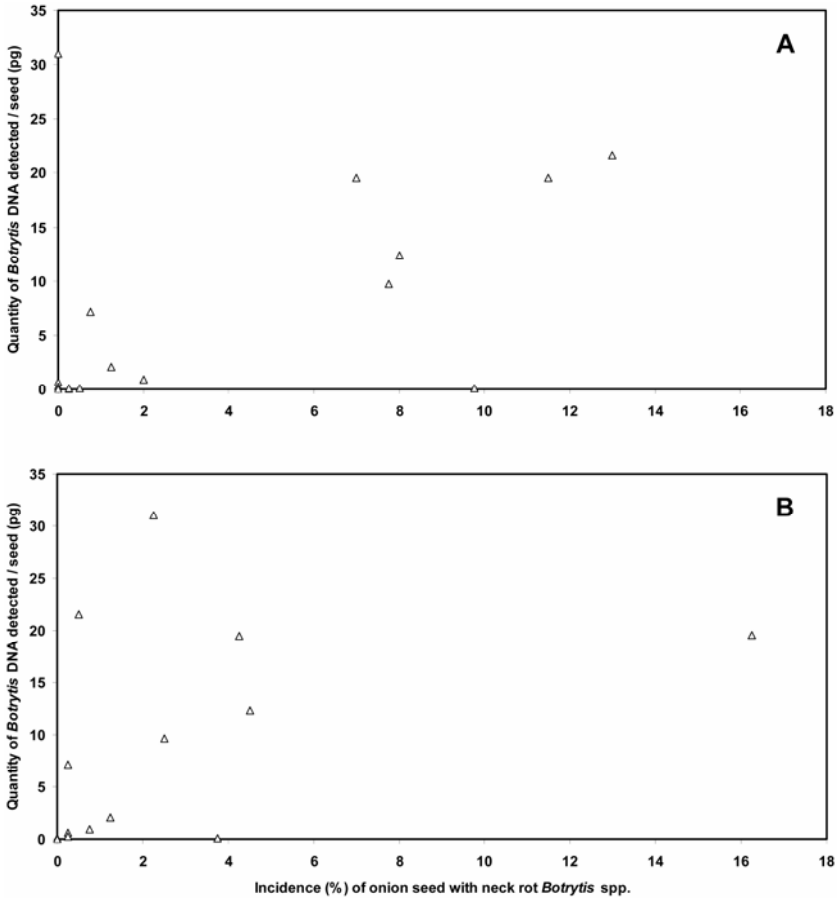

Fig. 3. Scatter plot of the amount (pg) of DNA of *Botrytis aclada*, *B. allii*, and *B. byssoidea* detected in each of 23 commercial onion seed lots using a real-time polymerase chain reaction (PCR) assay compared with the percentage of seed in each lot infested **(A)** or infected **(B)** with these neck rot fungi. The incidence of seed infested or infected with neck rot *Botrytis* spp. was determined using an assay of nondisinfested seed and an assay of surface-disinfested seed, respectively, on Kritzman and Netzer's agar medium (21) as described in the text and by du Toit et al. (13). Seed lot 22 was excluded from the analysis because of the high level of infection and infestation of this lot by neck rot fungi, to facilitate scaling of the plot.

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[a]

Infested or infected seed (type of agar assay)[b]		Real-time PCR assay[c]		Fisher's exact test (probability)[d]
		Positive	Negative	
Infested seed (assay of nondisinfested seed)	Positive	12/23 (52%)	1/23 (4%)	0.200 NS
	Negative	7/23 (30%)	3/23 (13%)	
Infected seed (assay of surface-disinfested seed)	Positive	13/23 (57%)	0/23 (0%)	0.024 *
	Negative	6/23 (26%)	4/23 (17%)	

[a] 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.

[b] "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.

[c] 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.

[d] 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.

analysis with each run. However, this option was not pursued in the study because of additional expenses for such assays (45,52,75).

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 disinfestant 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 a 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 DNeasy extraction kit from Qiagen resulted in less inhibition of PCR than a conventional CTAB phenol:chloroform extraction method (33). It may be possible to improve sensitivity of the real-time PCR assay further by optimizing the 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* 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 in this study between the percentage of onion seed on which *Botrytis* spp. were detected using the agar assays and the amount of *Botrytis* 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* DNA by real-time PCR assay in seed lots that test negative by agar assay may be associated with detection of dead or nonviable (nonculturable) *Botrytis* by the PCR assay but not by the agar assay, because real-time PCR primers cannot distinguish DNA from living versus dead cells of the target organisms (2,29). Another complicating factor in determining the relationship between the amount of DNA detected in a seed sample and the percentage of seed infected or infested with *Botrytis* is that the PCR assay cannot distinguish between seed lots with a large amount of *Botrytis* present in a few seed versus seed lots with a low amount of *Botrytis* present in many seed. Both types of seed lots would result in a similar level of DNA detected using the real-time PCR assay, but may have different epidemiological significance when planted into fields. It is also important to recognize that the assay of nondisinfested seed is an estimate of the incidence of seed that is infected and/or infested with *Botrytis* spp., whereas the assay of surface-disinfested seed is an estimate of the number of seed infected with *Botrytis* spp. However, for seed lots with high levels of colonization by other fungi, the assay of nondisinfested seed can potentially result in lower incidences of *Botrytis* detected because of greater competition from these other fungi (e.g., lots 3 and 4 in this study) compared to seed that has been surface-disinfested (13). A number of other species of fungi were observed at relatively high incidences 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 blotch (caused by *Cladosporium alliicepae*), blue mold (caused by *Penicillium* spp.), and mushy rot (caused by *Rhizopus* spp.) (55).

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 seed infested or infected with *Botrytis* using a sampling strategy of 25 seed per extract, 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 onion seed infected or infested with *Botrytis* spp. and the amount of *Botrytis* DNA present in the seed may be achieved.

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* spores 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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