PCR Detection and RFLP Differentiation of *Botrytis* Species Associated with Neck Rot of Onion

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ABSTRACT

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Botrytis aclada and other *Botrytis* spp. can cause neck rot on onions, a storage disease that normally is very difficult to detect at harvest using traditional isolation techniques. Sequence characterized amplified region primers (BA2f/BA1r) were designed based on a previously cloned and amplified DNA fragment for direct amplification of isolates of *Botrytis* spp. associated with neck rot of onions. Digestion of the polymerase chain reaction (PCR) amplification product with the restriction enzyme *ApoI* makes it possible to distinguish the five groups: *Botrytis aclada* types AI and AII (*B. allii*); *B. byssoidea*; *B. squamosa*; and *B. cinerea*. The detection limit was 1 to 10 pg of pure fungal DNA. It was possible to detect *B. aclada* with the PCR method in artificially inoculated onion bulb tissue and in mature onion leaves showing no symptoms of the disease. The availability of a sensitive and specific PCR detection and identification method for *Botrytis* onion neck rot pathogens should facilitate ecological studies of this group of onion pathogens.

Additional keywords: *Allium*, *Botryotinia*, SCAR, UP-PCR

Onions (*Allium cepa* L.) are attacked by at least seven *Botrytis* species (5,6). Of these, Walker (27) distinguished three that cause neck rots: *B. aclada* Fresen (syn. *B. allii* Munn), causal agent of sclerotial neck rot; *Botrytis byssoidea* J.C. Walker (teleomorph *Botryotinia allii* (Sawada) W. Yamamoto), causal agent of mycelial neck rot (27); and *Botrytis squamosa* J.C. Walker (teleomorph *Botryotinia squamosa* Vien.-Bourg), causal agent of small sclerotial neck rot of white onion cultivars (27). *B. squamosa* is better known as the causal agent of onion leaf blight. *Botrytis cinerea* Pers.:Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) can cause leaf spots and discoloration of onion bulbs (7), and is occasionally found in rotted neck tissues. Of these onion pathogens, *B. aclada* is most commonly associated with onion neck rot disease (10-12). However, in some years *B. byssoidea* has been more frequently isolated than *B. aclada* from diseased onion bulbs in the United Kingdom (23). *B. aclada* may be seedborne, but most infections by *B. aclada* are reported to occur in the field (11,12). Tichelaar (26)

demonstrated that *B. aclada* can grow asymptotically in apparently healthy onion leaves and is not normally observed in the field until close to harvest. First symptoms of neck rot normally occur after 1 to 2 months of storage (11,12,26).

Traditional identification of *Botrytis* spp. relies on isolation followed by morphological examination (11,22,23). Both time and mycological expertise are required for identification, due to frequently observed variation in the genus. It has been demonstrated, with the aid of universally primed polymerase chain reaction (UP-PCR) DNA fingerprints that more than half of the *B. byssoidea* isolates obtained from culture collections were incorrectly identified (17). Most of the misidentified isolates had DNA fingerprints that matched those of *B. squamosa*. An enzyme-linked immunosorbent assay (ELISA) detection method has been developed for detection of *B. aclada* (8). However, the antibody strongly cross-reacts with *B. cinerea*, and the two other *Botrytis* pathogens responsible for onion neck rot, *B. byssoidea* and *B. squamosa*, are not detected using this method. Moreover, *B. aclada* consists of two genetically distinct subgroups (AI and AII). Morphological, cytological, and molecular data show that *B. aclada* type AII is a polyploid hybrid of *B. aclada* AI and *B. byssoidea* (17,19,25).

PCR-based methods have been successfully applied for sensitive and rapid detection of plant pathogenic fungi (2,14,15). To

facilitate the rapid appraisal of interactions between *Botrytis* spp. and biocontrol agents in field experiments (D. S. Yohalem, unpublished data), we developed a rapid and reliable PCR-based method for detection of latent infections of *Botrytis* spp. in onion tissues. This detection method would also be useful for epidemiological studies of the neck rot disease. Recent epidemics of neck rot attributed to infested seed lots have been reported in the United States, and the lack of standardized procedures for detection and differentiation of the causal agents has been noted (1). A PCR-based detection system could be used for evaluation of the storage potential of an onion crop and to test onion seed and onion sets for latent infection by *Botrytis* spp. to ensure healthy sowing-planting material.

A UP-PCR DNA fragment from *B. aclada* associated with neck rot of onions was cloned and sequenced (19). Based on these sequences, a PCR detection method for rapid and reliable monitoring of *Botrytis* neck rot organisms was designed and is presented here. The method exclusively detects *Botrytis* spp. neck rot-associated isolates, and it can distinguish among the five neck-rotting types of *Botrytis*.

MATERIAL AND METHODS

Fungal isolates. Fungal isolates (Table 1) were maintained on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI). For long-term preservation, isolates were either stored in a 15% glycerol suspension at -80°C, or they were grown on PDA in sealed tubes and stored at 4°C. For production of conidia, *Botrytis* isolates were grown in 9-cm plastic petri dishes containing PDA for 14 days at 21°C under near UV light. Spores were harvested by adding 2 ml of sterile H₂O to the plates and gently scraping off the spores with a sterile scalpel. Spore suspensions were filtered through a 34-µm nylon mesh to remove fungal mycelium. All neck rot-associated *Botrytis* isolates used in this study are virulent on onion bulbs (16).

Plant material and inoculation methods. Onion bulbs ('Centurion') were obtained from a local onion grower. Bulbs were sliced into disks, 0.5 cm thick, with a sterile knife, and the two outermost scales were discarded. The remaining scales were cut into pieces 1 cm in length. Four pieces were placed in a 9-cm petri dish, inocu-

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Table 1. Fungal isolates used in this study

Isolates	Source ^a	Original no.	Origin	Isolated from	Diagnostic band(s) of restriction digested PCR amplification product	
					ITS1-4 # <i>Sph</i> I ^b	BA2f-BA1r # <i>Apo</i> I ^c
<i>Botrytis aclada</i> (AI) ^d BA1	1	BA1	Denmark	Bulb of <i>A. cepa</i>	3	413 bp
BA2	1	BA2	Denmark	Bulb of <i>A. cepa</i>	3	413 bp
BA3	2	MUCL 8	Belgium	Bulb of <i>A. cepa</i>	3	413 bp
BA8	2	MUCL 3037	Canada	Bulb of <i>A. cepa</i>	3	413 bp
BA9	2	MUCL 3106	USA	Bulb of <i>A. cepa</i>	3	413 bp
BA13	2	MUCL 8415	Germany	Bulb of <i>A. cepa</i>	3	413 bp
BA17	2	MUCL 18967	England	<i>A. ascalonicum</i>	3	413 bp
BA18	3	B4065	UK	Bulb of <i>A. cepa</i>	3	413 bp
BA19	3	B4090	UK	Bulb of <i>A. cepa</i>	3	413 bp
BA21	1	3-DK99	Denmark	Bulb of <i>A. cepa</i>	3	413 bp
BA22	1	8-DK99	Denmark	Bulb of <i>A. cepa</i>	3	413 bp
BA28	2	MUCL 3810	Belgium	Bulb of <i>A. cepa</i>	3	413 bp
BA29	2	MUCL 20329	Belgium	<i>A. schoenoprasum</i>	3	413 bp
BA30	4	CBS 260.71	Denmark	Bulb of <i>A. cepa</i>	3	413 bp
BA33	5	SAL003	Japan	<i>A. cepa</i>	3	413 bp
BA35	5	SAL005	Japan	<i>A. cepa</i>	3	413 bp
BA36	5	SAL006	Japan	<i>A. cepa</i>	3	413 bp
<i>B. aclada</i> (AII) BA4	2	MUCL 403	Netherlands	Bulb of <i>A. cepa</i>	2	413 + 298 bp
BA5	2	MUCL 99	USA	Bulb of <i>A. cepa</i>	2	413 + 298 bp
BA6	2	MUCL 961	Belgium	Bulb of <i>A. cepa</i>	2	413 + 298 bp
BA7	2	MUCL 1150	Norway	Bulb of <i>A. cepa</i>	2	413 + 298 bp
BA10	2	MUCL 3109	Japan	Leaf of <i>A. cepa</i>	2	413 + 298 bp
BA11	2	MUCL 3614	Egypt	Bulb of <i>A. cepa</i>	2	413 + 298 bp
BA12	2	MUCL 494	Denmark	<i>A. ascalonicum</i>	2	413 + 298 bp
BA14	2	MUCL 9114	Netherlands	Leaf of <i>A. cepa</i>	2	413 + 298 bp
BA16	2	MUCL 18868	Scotland	Bulb of <i>A. cepa</i>	2	413 + 298 bp
BA20	6	61-2	USA	<i>A. cepa</i>	2	413 + 298 bp
BA23	1	13-DK99	Denmark	Bulb of <i>A. cepa</i>	2	413 + 298 bp
BA24	2	MUCL 1105	USA	...	2	413 + 298 bp
BA25	2	MUCL 3528	...	...	2	413 + 298 bp
BA26	2	MUCL 3537	...	...	2	413 + 298 bp
BA27	2	MUCL 3556	UK	Bulb of <i>A. cepa</i>	2	413 + 298 bp
BA31	5	SAL001	Japan	<i>A. cepa</i>	2	413 + 298 bp
BA32	5	SAL002	Japan	<i>A. cepa</i>	2	413 + 298 bp
BA34	5	SAL004	Japan	<i>A. cepa</i>	2	413 + 298 bp
<i>B. byssoides</i> BB1	2	MUCL 94	USA	Bulb of <i>A. cepa</i>	2	298 bp
BB5	2	MUCL 9113	Netherlands	<i>A. cepa</i>	2	298 bp
BB6	3	B3730	UK	<i>A. porrum</i>	2	298 bp
<i>B. cinerea</i> BC1	1	BC1	Denmark	Leaf of <i>A. cepa</i>	+	250 bp
BC3	2	MUCL 3107	USA	Leaf of <i>A. cepa</i>	+	250 bp
BC4	2	MUCL 3647	Kenya	<i>Coffea arabica</i>	+	250 bp
BC5	2	MUCL 9115	Netherlands	Leaf of <i>A. cepa</i>	+	250 bp
BC6	2	MUCL 10117	Israel	Bulb of <i>A. cepa</i>	+	250 bp
BC7	2	MUCL 21596	Netherlands	Leaf of <i>A. cepa</i>	+	250 bp
BC8	6	61-34	USA	...	+	250 bp
<i>B. squamosa</i> BS2	2	MUCL 3821	Belgium	Leaf of <i>A. cepa</i>	+	269 bp
BS6	2	MUCL 28710	USA	Leaf of <i>A. cepa</i>	+	269 bp
BS7	3	B3808	UK	<i>A. cepa</i>	+	269 bp
BS8	3	B3834	UK	<i>A. cepa</i>	+	269 bp
BS9	6	MD-16	USA	<i>A. cepa</i>	+	269 bp
BS10	6	64A	USA	<i>A. cepa</i>	+	269 bp
BS11	2	MUCL 1107	USA	Bulb of <i>A. cepa</i>	+	269 bp
<i>B. porri</i>	7	1883	...	...	+	...
<i>B. calthae</i>	7	1673	...	...	+	...
<i>B. tulipae</i>	7	1890	...	...	+	...
<i>B. convoluta</i>	7	1375	Norway	<i>Iris pseudacorus</i>	+	...
<i>Ciboria caucis</i>	7	1572	Norway	<i>Salix cyprea</i>	+	...
<i>Cladosporium</i> sp.	1	C01	Denmark	Bulb of <i>A. cepa</i>	+	...
<i>Clonostachys rosea</i>	1	201	Denmark	<i>Lycopersicon esculentum</i>	+	...
<i>C. rosea</i>	8	IK726	Denmark	Soil	+	...
<i>Fusarium</i> sp.	1	F01	Denmark	Bulb of <i>A. cepa</i>	+	...
<i>Monilinia fructicola</i>	7	782	Canada	<i>Prunus persica</i>	+	...
<i>M. fructigena</i>	7	1079	Norway	<i>P. domesticus</i>	+	...
<i>M. laxa</i>	7	1083	Norway	<i>P. avium</i>	+	...
<i>Myriosclerotinia ciborium</i>	7	1831	...	...	+	...
<i>Penicillium</i> sp.	1	P03	Denmark	Bulb of <i>A. cepa</i>	+	...
<i>Sclerotinia sclerotiorum</i>	7	926	Norway	<i>Ranunculus ficaria</i>	+	...
<i>Sclerotium cepivorum</i>	7	1878	...	...	+	...
<i>Trichoderma harzianum</i>	8	T3	Denmark	...	+	...
<i>Ulocladium atrum</i>	9	385	Holland	Leaf of <i>A. cepa</i>	+	...
<i>U. atrum</i>	1	302	Denmark	<i>Daucus carota</i>	+	...
<i>U. consortiale</i>	1	303	Denmark	<i>Chrysanthemum</i> seed	+	...
<i>U. botrytis</i>	1	304	Denmark	<i>Chrysanthemum</i> seed	+	...

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^b Polymerase chain reaction (PCR) amplification of the ITS region with primer ITS1 and TS4 was performed as positive control of the DNA. A *Sph*I restriction digest of the PCR product was done for *B. aclada* AI, *B. aclada* AII, and *B. byssoides* isolates.

^c Size of diagnostic band after *Apo*I restriction digest of PCR amplification product with primer BA2f and BA1r.

^d *B. aclada* AII is a polyploid hybrid with the ancestors *B. aclada* AI and *B. byssoides* (17,19).

lated with 5 µl of *B. aclada* (BA1) spore suspension (10^5 spores per ml), and incubated at 21°C under near UV light. Samples for DNA extraction were collected after 7 days of incubation.

For production of onion leaf pieces, three onion sets ('Hyton F1') were planted per 15-cm pot containing a soil-peat-sand mix, and grown in a greenhouse for 8 weeks. They were watered as necessary. At harvest, leaves were cut into 1- to 2-cm pieces with a knife, freeze-dried, and leaf pieces placed in plastic bags and sealed. Plastic bags with leaf pieces were irradiated (absorbed dose: 1.1×10^6 rad) for sterilization and stored in the dark at $22 \pm 2^\circ\text{C}$ until use. Five leaf pieces were aseptically placed in a 9-cm petri dish containing a sterile glass filter paper (MN615; Macherey-Nagel, Düren, Germany), and 1 ml of sterile H_2O was added. Each leaf piece was inoculated with 5 µl of *B. aclada* (BA1) spore suspension (10^5 spores ml^{-1}), except for noninoculated controls. At 0, 24, 48, 96, and 144 h after inoculation, five leaf pieces were randomly collected for DNA extraction. Control leaves that had not been inoculated were collected after 6 days (144 h). The experiment was performed twice.

DNA extraction from fungal mycelium. Fungi were grown for 5 to 7 days at 21°C in the dark on PDA covered with a dry-sterilized (120°C for 12 h) disk of cellophane. Mycelia were scraped from the cellophane with a sterile scalpel and ground in liquid nitrogen using a mortar and pestle. Powdered mycelium (30 mg) was transferred to an Eppendorf tube, and DNA was extracted using the CTAB method (13). DNA was dissolved in 50 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer and stored at -20°C until use. DNA concentration of *B. aclada* isolate BA1 was estimated with the aid of agarose gels and adjusted to $0.1 \mu\text{g } \mu\text{l}^{-1}$. Concentra-

tions for all other isolates were within the range of $0.01 \mu\text{g } \mu\text{l}^{-1}$ to $0.1 \mu\text{g } \mu\text{l}^{-1}$. For use in PCR analysis, DNA samples were diluted 10-fold. For determination of sensitivity of the PCR method, dilution series of BA1 DNA were made in the range from $100 \text{ ng } \mu\text{l}^{-1}$ to $0.1 \text{ pg } \mu\text{l}^{-1}$.

DNA extraction from plant tissue. *B. aclada*-infected onion tissues (bulbs, mature leaves) were collected with sterile forceps and scalpels. Each tissue sample (0.05 – 0.15 g) was placed in a plastic tube containing FastDNA Lysing Matrix, a 6.4-mm (0.25-in) ceramic sphere and a 6.4-mm (0.25-in) ceramic cylinder (BIO 101, Inc., Vista, CA), and frozen at -20°C until use. For DNA extraction, 600 µl of PEX buffer (100 mM Tris/HCl (pH 8.5), 10 mM EDTA, 700 mM NaCl, 6.25 mM potassium ethyl xanthogenate) (2) was added to the tube, and the onion tissue was then homogenized in a FastPrep Instrument (FP120; BIO 101) at speed setting 5 for 20 s. After the homogenization, the tubes were incubated at 65°C for 1 h. Phenol-chloroform-isoamylalcohol (12:12:1) was added (600 µl), and the tubes were hand mixed for 5 s, then placed on ice for 5 min. After centrifugation for 10 min ($10,000 \times g$ at 4°C), the supernatant carrying the DNA was transferred to a new tube. DNA was precipitated by adding 60 µl of 3 M Na-acetate, pH 4.8, and 600 µl of isopropanol. The contents were mixed by inverting the tubes, incubated 10 min on ice, and centrifuged for 10 min ($13,000 \times g$ at 4°C). The supernatant was removed and the pellet was rinsed with 500 µl of 70% EtOH, vacuum dried for 10 to 15 min, dissolved in 100 µl of TE buffer (1 mM Tris/HCl, pH 7.8, 0.1 mM EDTA), and stored at -20°C until use. DNA was used directly for PCR analysis without dilution.

Design of primers and restriction analysis. In a previous study of *Botrytis*

isolates, a UP-PCR DNA fragment, *L45-550*, was cloned and sequenced (19). Based on these sequences (GenBank accession numbers: AJ291476–AJ291487), two sequence characterized amplified region (SCAR) primers (BA2f/BA1r; Table 2) were designed using the program PrimerSelect in the program package DNASTAR (Windows 32 PrimerSelect 4.00, 1993-1999 DNASTAR Inc.) that amplify a 413-bp PCR product in the five *Botrytis* groups associated with onion diseases. An analysis of restriction enzyme cleavage sites in the sequences of *B. cinerea*, *B. squamosa*, *B. byssoides*, and *B. aclada* type AI revealed that a restriction digest with *ApoI* would make it possible to differentiate among the four *Botrytis* species (Fig. 1).

PCR amplification. PCR amplification was performed in a total reaction volume of 20 µl. The following compounds were added, with final concentrations indicated: MgCl_2 (2.5 mM), dNTP (0.25 mM of each dATP, dGTP, dCTP, dTTP), *Taq* DNA polymerase (1 unit) (Promega, Madison, WI), KCl (50 mM), Tris-HCl, pH 9.0 (10 mM), Triton X-100 (0.1%), $0.5 \text{ pmol } \mu\text{l}^{-1}$ of each primer (Table 2), and 10 ng – 100 ng of fungal DNA were added as template. A GeneAmp PCR System 9700 (Perkin Elmer, Norwalk, CT) thermo-cycler device was used with the following program: 94°C (3 min), 1 cycle; (94°C [30 s], 53°C or 60°C [30 s], 72°C [1 min]), 29 cycles; 72°C (3 min), 1 cycle. The annealing temperatures of 53°C and 60°C were used for amplification of the internal transcribed spacer (ITS) region and for the *Botrytis*-specific amplification, respectively. The PCR product (3 µl) was separated in 1.5% agarose (Sigma) gels in $1 \times \text{TAE}$ (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide, and visualized under UV light. PCR amplification of fungal isolates with the *Botrytis*-specific primer pair BA2f/BA1r was performed at least twice.

Restriction analysis. Presence of an ITS amplification PCR product was used as a control of the DNA quality for all isolates. *B. aclada*, *B. byssoides*, *B. squamosa*, and *B. cinerea* isolates were further tested by restriction analysis of the

Table 2. Nucleotide sequences of primers used in this study

Name	Application	Nucleotide sequence (5' – 3')	Product	Reference
ITS1	ITS amplification	TCCGTAGGTGAACCTGCGG	≈550 bp	28
ITS4	ITS amplification	TCCTCCGCTTATTGATATGC		28
BA2f	<i>Botrytis</i> amplification	GTGGGGGTAGGATGAGATGATG	≈413 bp	This paper
BA1r	<i>Botrytis</i> amplification	TGAGTGCTGCGGAAACAAA		This paper

Fig. 1. Restriction map of the BA2f-BA1r polymerase chain reaction (PCR)-amplified fragment for *Botrytis* spp. causing disease in onions. *B. aclada* type AI isolates contain both the *B. aclada* type AI and the *B. byssoides* DNA types. Arrows indicate primers (BA2f and BA1r) used for PCR amplification. A: *ApoI* restriction endonuclease cleavage sites.

Fig. 2. Polymerase chain reaction (PCR) amplification with BA2f-BA1r. M: 100-bp ladder, with uppermost band 1,000 bp; 1: *Ulocladium atum* 302; 2: *U. consortiale* 303; 3: *U. botrytis* 304; 4: *U. atum* 385; 5: *Clonostachys rosea* 201; 6: *C. rosea* IK726; 7: *Trichoderma harzianum* T3; 8: *Penicillium* sp. P03; 9: *Cladosporium* sp. C01; 10: *Fusarium* sp. F01; 11: *Botrytis aclada* BA1; 12: negative control.

ITS amplicon. The PCR amplification product (2 µl) was digested, without prior purification, with *SphI* (New England Biolabs Inc., Beverly, MA) in a total volume of 10 µl according to the manufacturer's recommendation. After restriction digestion, the DNA products (5 µl) were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. BA2f/BA1r PCR amplification product (2 µl) was digested with *ApoI* (New England Biolabs) without further purification, as described above. The restriction analysis of *Botrytis* isolates was performed several times.

RESULTS

PCR amplification. *Botrytis* neck rot-associated isolates, originating from Europe, the United States, Egypt, and Asia were amplified with the *Botrytis*-specific primer pair (BA2f/BA1r), giving an amplification product of 413 bp. *Botrytis* isolates nonpathogenic to onions and isolates of other fungi were amplified with ITS primers but not with the BA2f/BA1r primer pair (Fig. 2). An *ApoI* restriction digest of the PCR amplification product (Fig. 3) separated the five groups of *Botrytis* causing disease in onions. All *B. aclada* (AII) isolates contained the sum of the bands found in *B. aclada* (AI) and *B. byssoides*. To determine if the mixed restriction pattern is a result of the presence of *B. aclada* (AII) alone or is the result of a mixture of *B. aclada* (AI) and *B. byssoides*, a restriction analysis of the ITS region was performed. *B. aclada* (AI) yielded three fragments (223, 200, and 116 bp) after a *SphI*

digest of the ITS amplification product, whereas *B. aclada* (AII) and *B. byssoides* both yielded two fragments of 316 and 223 bp (Fig. 4).

DNA extraction. Use of the PEX buffer for DNA extraction from inoculated onion tissue avoided foaming during tissue maceration and allowed for easy mixing with phenol-chloroform afterwards. No differences in DNA concentration or purity between the PEX-extracted DNA and the CTAB-extracted DNA were observed (data not shown).

Sensitivity of BA2f/BA1r amplification. Dilution series of the *B. aclada* isolate BA1 DNA showed that 1 to 10 pg of pure fungal DNA could be detected (Fig. 5). *B. aclada* was amplified using the BA2f/BA1r primer pair in all 20 samples of onion scales inoculated with *B. aclada*. No PCR product was observed in the non-inoculated controls. At 48 h postinoculation, there were no visible signs of *Botrytis* infection on leaf pieces, whereas *B. aclada* was detected by PCR in six of six dead leaf pieces (Fig. 6).

DISCUSSION

The *Botrytis* specific primer pair BA2f/BA1r amplified all neck rot-associated *Botrytis* isolates used in this study. A restriction digest of the amplification product with *ApoI* separated the *Botrytis* isolates into five groups: *B. aclada* types AI and AII; *B. byssoides*; *B. squamosa*; and *B. cinerea*. DNA was not amplified using the BA2f/BA1r primers from any other species tested. Among the other species tested were isolates of: *Sclerotium cepivorum*, causal agent of white rot in onions; *Penicillium* spp., represent-

ing many common saprophytes and onion pathogens associated with blue mold of onions; *Fusarium* spp., which cause damping-off and basal rot in onions; *Cladosporium* sp., which causes leaf blotch on onions; and *Sclerotinia sclerotiorum*, causing rot of onions, as well as other non-neck rot-associated *Botrytis* species demonstrating the specificity of the primers to *Botrytis* onion neck rot-associated pathogens.

Different approaches (dependent on the fungal species) can be used to select suitable DNA sequences for designing specific primers. Nucleotide differences in the ITS region of the ribosomal genes have been used with success to design species-specific primers (3,4,14). However, the variation in the ITS region within the genus *Botrytis* is very low (17,18) and thus not suitable for designing species-specific primers. The β -tubulin gene has been used as a source of primers that differentiate between *B. cinerea* isolates that are susceptible or resistant to the fungicide benomyl (9). A third approach, and the strategy used in this paper, has been the use of sequence characterized amplified regions of random amplified DNA fragments for primer design (15,20,21).

The detection limit for our primer pair was between 1 and 10 pg of pure fungal DNA. This corresponds to the detection limit found by others using the SCAR procedure for designing specific primers (24). *B. aclada* was detected in onion leaf pieces 48 h after inoculation with conidia, at which time there was no visible infection. Moricca et al. (14) reported detection of 50 to 100 fg of *Fusarium oxysporum* f. sp. *vasinfectum* DNA, an apparent sensitivity 100 times greater than ours. However, their primers are based on ITS-rDNA sequences of which there are at least 100 copies within a genome.

Latent *Botrytis* infections in onions grown in microplots were detected in three of 60 leaf pieces using the PCR method, where standard isolation showed only one (D. S. Yohalem, unpublished data). The method also allows for assessment of infection prior to storage, which allows us to more quickly evaluate the efficacy of management strategies as well as the storage potential of the harvested crop. In a field experiment with high disease pressure, preliminary results based on 320 samples indicate a two- to threefold higher detection rate at harvest using the PCR-method

Fig. 3. *ApoI* restriction digest of BA2f-BA1r polymerase chain reaction (PCR) amplification product. M: 100-bp ladder, where uppermost band is 500 bp; 1: *Botrytis aclada* BA4 (type AII); 2: BA1 (type AI); 3: BA5 (type AII); 4: *B. byssoides* BB1; 5: BB5; 6: BB6; 7: *B. squamosa* BS2; 8: BS6; 9: BS7; 10: *B. cinerea* BC1; 11: BC3.

Fig. 5. Sensitivity of BA2f-BA1r polymerase chain reaction (PCR) amplification of *Botrytis aclada* BA1 DNA. M: 100-bp ladder; 1: 100 ng; 2: 10 ng; 3: 1 ng; 4: 100 pg; 5: 10 pg; 6: 1 pg; 7: 100 fg.

Fig. 4. *SphI* restriction digest of the internal transcribed spacer (ITS) polymerase chain reaction (PCR) amplification product. M: 100-bp ladder, where uppermost band is 600 bp; 1: *Botrytis cinerea* (BC1); 2: BC3; 3: BC4; 4: BC6; 5: BC7; 6: *B. byssoides* (BB1); 7: BB5; 8: BB6; 9: *B. squamosa* (BS2); 10: BS6; 11: *B. aclada* (BA1, type AI); 12: BA2 (type AI); 13: BA4 (type AII); 14: BA8 (type AI); 15: BA13 (type AI).

Fig. 6. Polymerase chain reaction (PCR) amplification of *Botrytis aclada* BA1 inoculated (500 spores) on 1.5 × 1.5 cm onion leaf pieces and prior DNA extraction incubated in a moist chamber for 0, 24, 48, 96, and 144 h. Control = uninoculated leaf piece, M: 100-bp DNA ladder, where uppermost band is 600 bp.

compared with conventional culturing of adjacent tissues from onion necks (D. S. Yohalem, *unpublished data*), which suggests that the PCR detection method is more sensitive than isolation. The method may also prove useful in the evaluation of seed lots for infestation with neck rot pathogens.

In addition to detecting the presence of very small amounts of neck rot-associated *Botrytis* spp. in onion tissues, the present method enhances our ability to correctly diagnose the causal agent, particularly since both *B. byssoides* and *B. squamosa* frequently fail to sporulate in culture. Differentiation among the various pathogens has important epidemiological implications and may affect management strategies.

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LITERATURE CITED

1. Anonymous. 2001. Testing onion seed for diseases. Onion World May/June:11.
2. Edwards, S. G., Fitter, A. H., and Young, J. P. W. 1997. Quantification of an arbuscular mycorrhizal fungus, *Glomus mosseae*, within plant roots by competitive polymerase chain reaction. Mycol. Res. 101:1440-1444.
3. Förster, H., and Adaskaveg J. E. 2000. Early brown rot infections in sweet cherry fruit are detected by *Monilinia*-specific DNA primers. Phytopathology 90:171-178.
4. Hamelin, R. C., Bérubé, P., Gignac, M., and Bourassa, M. 1996. Identification of root rot fungi in nursery seedlings by nested multiplex PCR. Appl. Environ. Microbiol. 62:4026-4031.
5. Hennebert, G. L. 1963. Les *Botrytis* des *Allium*. Meded. LandbHoogesch. OpzoekStns. Gent 28:851-876.

6. Hennebert, G. L. 1973. *Botrytis* and *Botrytis*-like genera. Persoonia 7:183-204.
7. Lacy, M. L., and Lorbeer, J. W. 1995. *Botrytis* neck rot. Pages 18-19 in: Compendium of Onion and Garlic Diseases. H. F. Schwartz and S. K. Mohan, eds. American Phytopathological Society, St. Paul, MN.
8. Linfield, C. A., Kenny, S. R., and Lyons, N. F. 1995. A serological test for detecting *Botrytis allii*, the causal agent of neck rot of onion bulbs. Ann. Appl. Biol. 126:259-268.
9. Luck, J. E., and Gillings, M. R. 1995. Rapid identification of benomyl resistant strains of *Botrytis cinerea* using the polymerase chain reaction. Mycol. Res. 99:1483-1488.
10. Maude, R. B. 1988. Ascomycetes V: *Botrytis allii*. Pages 431-432 in: European Handbook of Plant Diseases. I. M. Smith, J. Dunez, R. A. Lelliott, D. H. Phillips, and S. A. Archer, eds. Blackwell Scientific Publications, Oxford.
11. Maude, R. B., and Presly, A. H. 1977. Neck rot (*Botrytis allii*) of bulb onions. I. Seed-borne infection and its relationship to the disease in the onion crop. Ann. Appl. Biol. 86:163-180.
12. Maude, R. B., and Presly, A. H. 1977. Neck rot (*Botrytis allii*) of bulb onions. II. Seed-borne infection in relationship to disease in store and the effect of seed treatment. Ann. Appl. Biol. 86:181-188.
13. Möller, E. M., Bahnweg, G., Sandermann, H., and Geiger, H. H. 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Res. 20:6115-6116.
14. Moricca, S., Ragazzi, A., Kasuga, T., and Mitchelson, K. R. 1998. Detection of *Fusarium oxysporum* f. sp. *vasinfectum* in cotton tissue by polymerase chain reaction. Plant Pathol. 47:486-494.
15. Nicholson, P., and Parry, D. W. 1996. Development and use of a PCR based assay to detect *Rhizoctonia cerealis*, the causal agent of sharp eyespot in wheat. Plant Pathol. 45:872-883.
16. Nielsen, K. 2000. Molecular characterisation and biological control of grey mould (*Botrytis* spp.) in onion. Ph.D. thesis. The Royal Veterinary and Agricultural University, Copenhagen, Denmark.
17. Nielsen, K., Justesen, A. F., Jensen, D. F., and Yohalem, D. S. 2001. Universally primed polymerase chain reaction alleles and internal transcribed spacer restriction fragment length polymorphisms distinguish two subgroups in *Botrytis aclada* distinct from *B. byssoides*. Phytopathology 91:527-533.
18. Nielsen, K., Justesen, A. F., and Yohalem, D. S. 1999. PCR based detection of latent infections of *Botrytis aclada* Fres. in onion bulbs. Petria 9:105-108.
19. Nielsen, K., and Yohalem, D. S. 2001. Origin of a polyploid *Botrytis* pathogen through interspecific hybridization between *Botrytis aclada* and *B. byssoides*. Mycologia 93:264-271.
20. Paran, I., and Michelmore, R. W. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor. Appl. Genet. 85:985-993.
21. Parry, D. W., and Nicholson, P. 1996. Development of a PCR assay to detect *Fusarium poae* in wheat. Plant Pathol. 45:383-391.
22. Presly, A. H. 1985. Methods for inducing sporulation of some *Botrytis* species occurring on onions and leeks. Trans. Br. Mycol. Soc. 85:621-624.
23. Presly, A. H. 1985. Studies of *Botrytis* spp. occurring on onions (*Allium cepa*) and leeks (*Allium porrum*). Plant Pathol. 34:422-427.
24. Schena, L., Sialer, M. F., and Gallitelli, D. 2002. Molecular detection of strain L47 of *Aureobasidium pullulans*, a biocontrol agent of postharvest diseases. Plant Dis. 86:54-60.
25. Shirane, N., Masuko, M., and Hayashi, Y. 1989. Light microscopy observation of nuclei and mitotic chromosomes of *Botrytis* species. Phytopathology 79:728-730.
26. Tichelaar, G. M. 1967. Studies on the biology of *Botrytis allii* on *Allium cepa*. Neth. J. Plant Pathol. 73:157-160.
27. Walker, J. C. 1925. Two undescribed species of *Botrytis* associated with the neck rot disease of onion bulbs. Phytopathology 15:708-713.
28. White, T. J., Bruns, T., Lee, S., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols, A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego.