Simultaneous Detection of *Acidovorax avenae* subsp. *citrulli* and *Didymella bryoniae* in Cucurbit Seedlots Using Magnetic Capture Hybridization and Real-Time Polymerase Chain Reaction

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ABSTRACT

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To improve the simultaneous detection of two pathogens in cucurbit seed, a combination of magnetic capture hybridization (MCH) and multiplex real-time polymerase chain reaction (PCR) was developed. Single-stranded DNA hybridization capture probes targeting DNA of *Acidovorax avenae* subsp. *citrulli*, causal agent of bacterial fruit blotch, and *Didymella bryoniae*, causal agent of gummy stem blight, were covalently attached to magnetic particles and used to selectively concentrate template DNA from cucurbit seed samples. Sequestered template DNAs were subsequently amplified by multiplex real-time PCR using

pathogen-specific TaqMan PCR assays. The MCH multiplex real-time PCR assay displayed a detection threshold of *A. avenae* subsp. *citrulli* at 10 CFU/ml and *D. bryoniae* at 10⁵ conidia/ml in mixtures of pure cultures of the two pathogens, which was 10-fold more sensitive than the direct real-time PCR assays for the two pathogens separately. Although the direct real-time PCR assay displayed a detection threshold for *A. avenae* subsp. *citrulli* DNA of 100 fg/μl in 25% (1/4 samples) of the samples assayed, MCH real-time PCR demonstrated 100% detection frequency (4/4 samples) at the same DNA concentration. MCH did not improve detection sensitivity for *D. bryoniae* relative to direct real-time PCR using conidial suspensions or seed washes from *D. bryoniae*-infested cucurbit seed. However, MCH real-time PCR facilitated detection of both target pathogens in watermelon and melon seed samples ($n = 5,000$ seeds/sample) in which 0.02% of the seed were infested with *A. avenae* subsp. *citrulli* and 0.02% were infested with *D. bryoniae*.

Cucurbits are widely cultivated throughout the United States, with the major production areas in California, Florida, Georgia, and Arizona (30). In 2007, the United States produced ≈4.4 million metric tons of cucurbits valued at \$1.5 billion USD (30). Of the commercial cucurbits, watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) is the most important, with an estimated value of \$476 million USD in 2007 (30). Cucurbit production is constantly threatened by plant pathogens that can incite devastating disease epidemics under favorable environmental conditions (52). For some diseases of cucurbits, seed are the primary source of inoculum, which makes disease management especially difficult (12). Two important seedborne pathogens posing threats to cucurbit production are bacterial fruit blotch (BFB) and gummy stem blight (GSB) (52).

BFB is caused by *Acidovorax avenae* subsp. *citrulli*, a gram-negative bacterium in the β-Proteobacteria (39,50). Infested seedlots are the most important source of primary inoculum for BFB outbreaks, particularly in transplant seedling production systems (29,36). Seedlings typically develop water-soaked lesions along the veins of cotyledons and leaves 10 to 14 days after planting (29). The lesions turn reddish-brown and serve as inoculum sources for secondary disease cycles and fruit infection. Fruit symptoms start as irregularly shaped, water-soaked lesions that

can rapidly expand to cover the entire fruit surface (29) and lead to fruit rot. Currently, BFB is one of the most serious diseases facing U.S. cucurbit seed producers because it can cause up to 100% yield loss (29) (R. Walcott, *personal observation*). Because there is zero-tolerance for the BFB pathogen in cucurbit seedlots, all watermelon and cantaloupe seed sold in the United States must be tested and found to be free of *A. avenae* subsp. *citrulli*.

GSB is caused by the ascomycete fungus *Didymella bryoniae* (Fuckel) Rehm (= *Mycosphaerella citrullina* (C. O. Sm.) Grossenb.), anamorph *Phoma cucurbitacearum* (Fr.) Sacc. (7). The fungus infects stems, leaves, and flowers of a range of cucurbits, including watermelon, melon (*Cucumis melo*), and pumpkin (*Cucurbita* sp.) (7,35). On cucurbit foliage, the fungus causes brown lesions that become covered with pycnidia or pseudothecia (22). Flower infection leads to subsequent internal fruit rot or symptomless fruit infection at harvest, and seed from these fruit may be infested or infected with the fungus (7,24). Under warm and humid weather conditions, *D. bryoniae* can rapidly kill cucurbit vines and, in severe outbreaks, up to 15% fruit loss can occur (16). *D. bryoniae* survives on cucurbit debris (17), and seedborne inoculum is an important means of long-distance pathogen dissemination (24).

Currently, commercial hybrid watermelon seed are routinely produced in cool and arid countries around the world, including India, Mexico, and Chile. The need for reduced production costs and to avoid climates that are conducive to disease development make international hybrid seed production feasible. However, this strategy significantly increases the risk of introducing and disseminating nonindigenous plant pathogens or new strains of

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established plant pathogens. Additionally, commercial cucurbit fruit production increasingly involves transplants produced under conditions that are highly conducive to disease development. Hence, the U.S. commercial cucurbit production system is vulnerable to the introduction of exotic seedborne pathogens. With few effective chemical control options available for BFB and GSB, exclusion of seedborne inoculum through seed health testing is a highly feasible management strategy (12).

Currently, the most widely employed seed health assays for *A. avenae* subsp. *citrulli* and *D. bryoniae* are seedling grow-out and blotter assays, respectively (2). Both methods require planting seed samples under conditions conducive for disease development and subsequently observing seedlings for symptoms or signs. Unfortunately, these techniques are time, space, and labor intensive and require knowledge of the characteristic pathogen signs and disease symptoms. Additionally, subsequent confirmatory tests (e.g., serology-based assays) are required, which further extend the time for completion of the assays. These assays are also expensive and only a limited number of commercial seed-testing agencies have the capacity to conduct them. The ability to detect multiple pathogens simultaneously in seed samples would greatly enhance efficiency and provide useful tools for the exclusion of important phytopathogens such as *A. avenae* subsp. *citrulli* and *D. bryoniae*. One approach that could facilitate simultaneous detection of multiple target pathogens is the polymerase chain reaction (PCR) (37), an in vitro, primer-directed process of amplifying nucleic acid sequences specific to the target organisms.

Diagnostic PCR assays have been successfully employed in medical, veterinary, and food microbiology and are highly applicable for seed health testing (13,40,45). Unfortunately, seed of many plant species contain compounds that inhibit PCR and can lead to false negative results (8,14,47). To circumvent this problem, modifications including nucleic acid purification, neutralization of PCR inhibitors, target pathogen enrichment, and immunomagnetic separation have been attempted with varying levels of success (11,43,47). Currently, however, few commercial seed-testing agencies routinely employ PCR-based seed health assays (R. Walcott, *personal observation*). Moreover, due to the difficulty in extracting PCR-quality DNA from seed, PCR has had limited application for seedborne fungi. Pryor et al. (34) successfully employed an enrichment PCR assay to detect *Alternaria radicina* in carrot seed and, more recently, Chilvers et al. (6) reported a quantitative real-time PCR assay for the detection of *Botrytis* spp. in onion seed. However, there is still a need to develop effective, commercially viable, high-throughput PCR assays for the array of seedborne fungi that can affect seed trade and crop production.

One strategy by which the full potential of PCR might be realized for seed health testing is magnetic capture hybridization (MCH). MCH involves the use of a single-stranded biotinylated oligonucleotide probe, conjugated to streptavidin-coated magnetic beads (SCBs) to hybridize with single-stranded target DNA from crude samples (1,4,5,15). Target nucleic acids that hybridize to the capture probes can then be concentrated by magnetic force, and nontarget DNA and PCR inhibitors can be eliminated by rinsing. After elution, the enriched target DNA can be amplified and detected by real-time PCR. MCH-PCR has previously been applied to detect microorganisms in soil, food, and plant tissue (1,5,9). Walcott et al. (48) demonstrated its applicability for the detection of *Botrytis aclada* in onion seed. The objective of this research was to assess the applicability of MCH multiplex real-time PCR for the simultaneous detection of *A. avenae* subsp. *citrulli* and *D. bryoniae* in cucurbit seed.

MATERIALS AND METHODS

Pathogen growth and DNA extraction. *A. avenae* subsp. *citrulli* and other bacterial strains evaluated in this study were

grown in nutrient broth (NB) (Beckton-Dickinson, Sparks, MD), nutrient agar, or King's B medium (20) at 28°C, and stored long-term in 15% glycerol at -80°C. Isolates of *D. bryoniae* were grown at 28°C on potato dextrose agar (PDA) (Beckton-Dickinson) and stored long-term on sterile filter paper discs at -80°C. Briefly, this was done by placing sterilized Whatman no. 1 filter paper (Whatman, Springfield Mill, U.K.) on the surface of PDA plates, on which each isolate was growing, and incubating at room temperature for 8 to 14 days. After colonization by the fungus, the filter paper was removed with sterile forceps and stored at -80°C in paper envelopes. Fungal isolates were recovered by incubating pieces of colonized filter paper on PDA at room temperature. Bacterial genomic DNA was extracted from cells of an overnight NB culture (adjusted to $\approx 0.5 \times 10^8$ CFU/ml as determined with a spectrophotometer [Spectronic 20; Bausch and Lomb, Rochester, NY] at an absorbance at 600 nm = 0.3) using commercially available DNA extraction kits (Promega Wizard, Promega Corp., Madison, WI or MO BIO Laboratories, Inc., Carlsbad, CA). Fungal genomic DNA was routinely extracted from 72-h potato dextrose broth (PDB) mycelial cultures of *D. bryoniae* by grinding the mycelia in liquid nitrogen, followed by ammonium acetate and ethanol precipitation (49), or from conidia using a commercial DNA extraction kit (MO BIO Laboratories).

Development of a TaqMan real-time PCR assay for *A. avenae* subsp. *citrulli*. Using repetitive extragenic palindromic PCR fingerprinting profiles generated for a population of *A. avenae* subsp. *citrulli* strains (46), a conserved 480-bp DNA fragment was identified. This fragment was purified as described by Sambrook and Russell (38), cloned using a TOPO TA Cloning Kit (Invitrogen Corp., Carlsbad, CA), and sequenced using the Big Dye Terminator 3.1 Sequencing kit (Applied Biosystems, Foster City, CA). Sequence data were edited manually, and the identity of the sequence was inferred by locating the taxon-specific primers that were used to generate the amplicon. Based on this sequence data, forward and reverse primers (BOXAACF1 and BOXAACR2) as well as a TaqMan probe (AACPROBE) were designed (Fig. 1A; Table 1). The AACPROBE was labeled at the 5' and 3' ends with 6-carboxyfluorescein (FAM) and black hole quencher (BHQ1) (Integrated DNA Technologies, Coralville, IA), respectively (Table 1).

Specificity of the *A. avenae* subsp. *citrulli* TaqMan real-time PCR assay. The specificity of the real-time PCR assay was evaluated using a range of bacteria that included 45 *A. avenae* subsp. *citrulli* strains (Table 2). Genomic DNA from each strain (≈ 2 ng/ μ l) was used as template, and amplification was performed using a commercially available PCR mastermix (Eppendorf Real Master Mix Probe; Eppendorf, Hamburg, Germany or Bio-Rad iQ Supermix; Bio-Rad Laboratories, Hercules, CA) and a real-time PCR machine (Smart Cycler II; Cepheid, Sunnyvale, CA). The following thermal profile was used for amplification: denaturation at 95°C for 180 s, followed by 35 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 40 s. A baseline of 30 fluorescence units was set as the background threshold, and reactions in which FAM-based fluorescence exceeded this value were considered positive for *A. avenae* subsp. *citrulli*. Cycle threshold (Ct) values (PCR cycle number at which fluorescence exceeded the detection threshold) were recorded for each reaction. For confirmation, 10 μ l of amplified DNA was loaded on a 1% agarose gel containing ethidium bromide and subjected to electrophoresis in Tris acetic acid EDTA buffer at 90 V for 1 h. After electrophoresis, the gel was examined with ultraviolet illumination.

Amplification efficiency of the *A. avenae* subsp. *citrulli* TaqMan real-time PCR assay. To determine the amplification efficiency of the *A. avenae* subsp. *citrulli* TaqMan assay, 10-fold serial dilutions of pure genomic DNA were generated ranging from 1 ng/ μ l to 1 pg/ μ l in sterile deionized distilled water

(sddH₂O). Genomic DNA was extracted as described above and quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Using the *A. avenae* subsp. *citrulli* TaqMan real-time PCR assay, Ct values were generated for each dilution of genomic DNA. This was repeated four times and a standard curve was obtained by plotting mean Ct values against log₁₀ (genomic *A. avenae* subsp. *citrulli* DNA concentration). Linear regression analysis was conducted on the data, and the slope of the regression line was used to estimate amplification efficiency according to the following formula (3): Efficiency (E) (%) = (10^(-1/slope) - 1) × 100.

Development of TaqMan real-time PCR assay for *D. bryoniae*. To develop a TaqMan real-time PCR assay for *D.*

bryoniae, we employed a previously described primer set that was specific for the random amplified polymorphic DNA (RAPD) group I (RGI) isolates (41). The original primer set amplified a unique 739-bp RAPD fragment (19,42). To convert this assay to real-time PCR format, the original forward primer (GSBF1) (Table 1) was used but new sequences for a TaqMan probe (DBPROBE) and a reverse primer (DBRG1R) were selected from the amplicon sequence with the aid of the web-based primer selection program, Primer Quest (Integrated DNA Technologies, available online) (Table 1). DBPROBE was labeled with Texas Red and BHQ2 at the 5' and 3' ends, respectively.

Specificity of the *D. bryoniae* TaqMan real-time PCR assay. To determine the specificity of the *D. bryoniae* TaqMan PCR assay, genomic DNA from 143 fungal isolates, including *D. bryoniae* RGI and RGII isolates (41), *Phoma* spp., *Alternaria cucumerina*, *Colletotrichum orbiculare*, and *Fusarium oxysporum* f. sp. *niveum* race 2 (Table 3), was extracted using the Qiagen DNeasy Plant Mini kit (Qiagen, Valencia, CA). DNA concentrations were measured using a spectrophotometer (NanoDrop ND-1000), and DNA was stored at -20°C until used. DNA was routinely amplified using a commercial PCR master-mix (Bio-Rad iQ Supermix; Bio-Rad) and a Smart Cycler II real-time PCR machine (Cepheid). The following thermal profile was employed for PCR amplification: denaturation at 95°C for 120 s, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. A baseline of 30 fluorescence units was set as the background threshold and reactions in which Texas Red-based fluorescence exceeded this value were considered positive for *D. bryoniae*. The Ct value was recorded for each positive reaction.

Amplification efficiency of the *D. bryoniae* TaqMan real-time PCR assay. To determine the amplification efficiency of the *D. bryoniae* TaqMan real-time PCR assay, 10-fold serial dilutions of genomic *D. bryoniae* DNA ranging from 1 ng/μl to 1 pg/μl were amplified as described above. The following thermal profile was used for amplification: denaturation at 95°C for 180 s, followed by 35 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 40 s. Each assay was repeated four times and a plot of Ct value versus log₁₀ (*D. bryoniae* DNA concentration) was used to determine amplification efficiency as previously described.

Multiplex real-time PCR amplification of *A. avenae* subsp. *citrulli* and *D. bryoniae* DNA. The optimal concentrations of *A. avenae* subsp. *citrulli* and *D. bryoniae* primers and probes were empirically determined by testing a matrix of primer concentration and probe concentration combinations ranging from 50 to 600 nM for primers and 25 to 200 nM for TaqMan probes. Mixtures of 10-fold serial dilutions of *A. avenae* subsp. *citrulli* and *D. bryoniae* DNA, ranging from 1 ng/μl to 1 fg/μl, were

Fig. 1. DNA sequences of **A**, *Acidovorax avenae* subsp. *citrulli* and **B**, *Didymella bryoniae* loci targeted for hybridization capture and amplification, showing the relative positions of hybridization capture probes, primers, and TaqMan probes. The sequences to which the hybridization capture probes anneal are shaded with bold letters, the sequences to which the forward and reverse primers anneal are underlined, and the sequences to which the TaqMan probes anneal are indicated by a rectangular outline.

TABLE 1. Sequences and functions of oligonucleotides used as components of a magnetic capture hybridization and multiplex real-time TaqMan polymerase chain reaction (PCR) assay for the simultaneous detection of *Acidovorax avenae* subsp. *citrulli* and *Didymella bryoniae* in cucurbit seed

Oligonucleotide	DNA sequence (5'→3')	Function ^a	Source
BOXAACF	GCGTATGAGTCCCGA AGA AAT	<i>A. avenae</i> subsp. <i>citrulli</i> forward primer	This study
BOXAACR2	GCA TGCCTGTATTACGTAT	<i>A. avenae</i> subsp. <i>citrulli</i> reverse primer	This study
AACPROBE	6-FAM-CCGAAATCCGTA TTGGACGGATCGAA-BHQ1 ^b	<i>A. avenae</i> subsp. <i>citrulli</i> TaqMan probe	This study
ACCAPRW	BIO-TEG-GGCGAATTGCACGGTCGG CCCAGCCCTACGGGGTTATG GTGTATGTCGCTATGAACCTGATC ^c	<i>A. avenae</i> subsp. <i>citrulli</i> HCP	This study
GSBF1	TGTCGTTGACATCATTCCAGC	<i>D. bryoniae</i> forward primer	19,42
DBRG1R	GAAATGCTTCTATCGTACGTTAATAAC	<i>D. bryoniae</i> reverse primer	This study
DBPROBE	Texas Red-AAA TCG CAC CTG AAGATCGTGATTGTCC - BHQ2 ^d	<i>D. bryoniae</i> TaqMan probe	This study
DBCAPRW	BIO-TEG-TCCTCAGCTGTA ACG TTGTTGAAGTAGCCA AAT TTT GAGCCAGCGGCAAAGATACTT TTCCCA ^c	<i>D. bryoniae</i> HCP	This study

^a HCP = hybridization capture probe.
^b The *A. avenae* subsp. *citrulli* TaqMan probe was labeled at the 5' and 3' ends with 6-carboxyfluorescein (6-FAM) and Black Hole Quencher (BHQ) 1 (Integrated DNA Technologies, Coralville, IA), respectively.
^c Hybridization capture probes were labeled at the 5' end with biotin (BIO) and a triethylene glycol (TEG) spacer molecule.
^d The *D. bryoniae* TaqMan probe was labeled at the 5' and 3' ends with Texas Red and BHQ 2 (Integrated DNA Technologies), respectively.

amplified with each primer–probe combination to determine the optimal primer/probe ratios. Multiplex real-time PCR was routinely conducted in 25- μ l reactions containing 12.5 μ l of iQ Supermix or iQ Multiplex Powermix (Bio-Rad Laboratories), 300 nM of each primer, and 200 nM of each TaqMan probe. The amplification thermal profile for multiplex real-time PCR reactions included denaturation at 95°C for 180 s, followed by 35 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 40 s. A baseline of 30 fluorescence units was set as the threshold, and reactions in which fluorescence (FAM or Texas Red) exceeded this value were considered to be positive for *A. avenae*

subsp. *citrulli* or *D. bryoniae*, respectively. Ct values were recorded for each positive reaction.

MCH. SCBs (Dynal M-280 beads; Invitrogen Corp.) were coated with the biotinylated hybridization capture probes, AACCAPRW and DBCAPRW (Table 1), according to the manufacturer's instructions. The hybridization capture probes were empirically selected from regions upstream and downstream of the loci targeted for amplification in *A. avenae* subsp. *citrulli* and *D. bryoniae* genomic DNA, respectively (Fig. 1A and B). SCBs (150 μ l; $\approx 6.7 \times 10^8$ beads) were placed in a 2 ml-tube and washed three times with 1 ml of 2 $\times$ binding-wash (BW) buffer (10 mM

TABLE 2. Identity, origin, and source of bacterial strains used to assess the specificity of the *Acidovorax avenae* subsp. *citrulli* TaqMan real-time polymerase chain reaction (PCR) assay used in this study

Bacterium	Strain	<i>SpeI</i> PFGE group ^a	Haplotype ^b	Host	Origin	Real-time PCR result
<i>A. avenae</i> subsp. <i>citrulli</i>	00-1	II	A (A1)	Watermelon	Georgia, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	94-36, 94-50	II	B (A2)	Watermelon	Georgia, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	94-19, 52-4	II	C (A3)	Watermelon	Georgia, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	92-3; 92-17	II	D (A4)	Watermelon	Georgia, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	206-101	II	E (A5)	Unknown	China	+
<i>A. avenae</i> subsp. <i>citrulli</i>	94-87; 205-33	II	G (A6)	Watermelon	Georgia, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	92-302, 94-12	II	H	Watermelon	Georgia, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	94-39	II	J (A7)	Watermelon	Georgia, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	202-68	II	W (A11)	Watermelon	Khon Kaen, Thailand	+
<i>A. avenae</i> subsp. <i>citrulli</i>	205-22	II	W (A11)	Watermelon	Texas, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	94-48	II	U (A9)	Watermelon	Georgia, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	201-12	II	T (A10)	Watermelon	South Carolina, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	203-65	II	Z (A12)	Unknown	Unknown	+
<i>A. avenae</i> subsp. <i>citrulli</i>	205-14	II	... (A16)	Watermelon	Indiana, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	206-102	II	... (A13)	Unknown	China	+
<i>A. avenae</i> subsp. <i>citrulli</i>	206-103	II	... (A14)	Unknown	China	+
<i>A. avenae</i> subsp. <i>citrulli</i>	Saticoy B	II	Q (A8)	Watermelon	Florida, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	92-305	I	I (B2)	Watermelon	Georgia, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	AU-2	I	L (B4)	Cantaloupe	Australia	+
<i>A. avenae</i> subsp. <i>citrulli</i>	92-301	I	F (B1)	Watermelon	Georgia, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	98-17	I	N (B6)	Pumpkin	Georgia, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	99-5	I	O (B7)	Cantaloupe	Georgia, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	200-30	I	S (B10)	Cantaloupe	California, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	201-13	I	V (B11)	Watermelon	South Carolina, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	202-1, 6	I	M (B5)	Muskmelon	Nanjing, China	+
<i>A. avenae</i> subsp. <i>citrulli</i>	AU-9	I	M (B5)	Muskmelon	Australia	+
<i>A. avenae</i> subsp. <i>citrulli</i>	200-18	I	M (B5)	Cantaloupe	Georgia, USA	+
<i>A. avenae</i> subsp. <i>citrulli</i>	202-66	I	X (B12)	Melon	Israel	+
<i>A. avenae</i> subsp. <i>citrulli</i>	203-16	I	Y (B13)	Cantaloupe	Honduras	+
<i>A. avenae</i> subsp. <i>citrulli</i>	206-2	I	... (B16)	Watermelon	Unknown	+
<i>A. avenae</i> subsp. <i>citrulli</i>	206-75	I	... (B18)	Unknown	China	+
<i>A. avenae</i> subsp. <i>citrulli</i>	206-79	I	... (B19)	Unknown	China	+
<i>A. avenae</i> subsp. <i>citrulli</i>	206-80	I	... (B22)	Unknown	China	+
<i>A. avenae</i> subsp. <i>citrulli</i>	206-95	I	... (B19)	Unknown	China	+
<i>A. avenae</i> subsp. <i>citrulli</i>	ATCC 296295	I	K (B3)	Watermelon	Georgia, USA	–
<i>A. avenae</i> subsp. <i>citrulli</i>	IA58-1	I	P (B8)	Muskmelon	Iowa, USA	–
<i>A. avenae</i> subsp. <i>citrulli</i>	200-23	I	P (B8)	Watermelon	Oklahoma, USA	–
<i>A. avenae</i> subsp. <i>citrulli</i>	200-6	I	R (B9)	Cantaloupe	Georgia, USA	–
<i>A. avenae</i> subsp. <i>citrulli</i>	206-1	I	... (B17)	Watermelon	Unknown	–
<i>A. avenae</i> subsp. <i>avenae</i>	78-5	NA	NA	Maize	Georgia, USA	–
<i>A. avenae</i> subsp. <i>avenae</i>	81-2	NA	NA	Maize	Georgia, USA	–
<i>A. avenae</i> subsp. <i>avenae</i>	99-2	NA	NA	Maize	Georgia, USA	–
<i>A. avenae</i> subsp. <i>cattleyae</i>	98-1	NA	NA	Orchid	Unknown	–
<i>A. konjaci</i>		NA	NA	Konjac	Florida, USA	–
<i>A. facilis</i>	94-1	NA	NA	Unknown	Unknown	–
<i>Agrobacterium tumefaciens</i>	LBA4301	NA	NA	Unknown	Unknown	–
<i>Burkholderia cepacia</i>	83-1	NA	NA	Unknown	Unknown	–
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	TC-1	NA	NA	Unknown	Unknown	–
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i>	ECA 96	NA	NA	Unknown	Unknown	–
<i>Pseudomonas fluorescens</i>	A506	NA	NA	Unknown	California, USA	–
<i>P. fluorescens</i>	01	NA	NA	Unknown	California, USA	–
<i>P. syringae</i>	99-5	NA	NA	Tomato	Unknown	–
<i>Pantoea ananatis</i>	G-10	NA	NA	Onion	Georgia, USA	–
<i>Ralstonia solanacearum</i>	AW-1	NA	NA	Tomato	Georgia, USA	–
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	001	NA	NA	Unknown	Georgia, USA	–
<i>X. campestris</i> pv. <i>vesicatoria</i>	04-46	NA	NA	Tomato	Georgia, USA	–

^a Group determined based on digestion of genomic DNA with *SpeI* followed by pulsed-field gel electrophoresis (PFGE) (46). NA = not applicable.

^b Designation in parenthesis represents new names based on the recent discovery of new *SpeI* PFGE haplotypes (R. Walcott, unpublished data). NA = not applicable.

Tris-HCl, pH 7.5, 1 mM EDTA, and 2.0 M NaCl). For binding, washed SCBs were mixed with 400 ng of each hybridization capture probe (20 ng/μl) separately in 400 μl of 1× BW buffer. SCBs were incubated with capture probes for 1 h at 25°C and then rinsed with 1× BW buffer to remove unbound hybridization capture probe. Immobilized capture probe was denatured by incubation in 400 μl of denaturation buffer (0.125 M NaOH and 0.5 M NaCl) for 15 min, washed three times, and resuspended in 150 μl of 1× BW buffer. Coated SCBs were stored at 4°C until they were used. To facilitate washing of SCBs, magnetic particle concentrators (Invitrogen Corp.) were used.

To capture single-stranded target DNA, MCH was conducted as previously described (15). Briefly, after denaturation at 100°C for 5 min, crude DNA was hybridized in DIGEASY hybridization buffer (Roche Applied Science, Indianapolis, IN), with 20 μl (≈9 × 10⁷ beads) of SCBs coated with each capture probe. Hybridization conditions included incubation with gentle agitation in a hybridization oven (Fisher Scientific, Suwanee, GA) at 37°C for 2 h. After hybridization, the SCBs were rinsed using a KingFisher ML (Thermo Scientific Inc., Waltham, MA) automatic magnetic particle processor and resuspended in 10 μl of sddH₂O. Captured DNA was released from the SCBs by incubation at 95°C for 10 min followed by centrifugation for 5 s at 15,000 × g. Eluted DNA (5 μl) was routinely used as template for real-time PCR.

Comparison of direct and MCH real-time PCR for the detection of *A. avenae* subsp. *citrulli* in cell suspensions. To compare the sensitivity and reliability of MCH real-time PCR to

direct real-time PCR for the detection of *A. avenae* subsp. *citrulli* cells, 10-fold serial dilutions of the bacterium were generated from NB cultures of AAC 00-1 grown for 12 h at 28°C. Cells were harvested by centrifugation, washed, and resuspended in 0.1 M phosphate buffered saline (PBS). Using a spectrophotometer, the suspension was then adjusted to an optical density of 0.3 at a wavelength of 600 nm (≈0.5 × 10⁸ CFU/ml). Using this cell suspension, 10-fold serial dilutions were generated in lysis buffer (200 mM Tris-HCl [pH 8.5], 250 mM NaCl, 25 mM EDTA, and 0.5% [wt/vol] sodium dodecyl sulfate) with ≈1 × 10⁶, 1 × 10⁵, 1 × 10⁴, 1 × 10³, 1 × 10², 1 × 10¹, and 0 CFU/ml. Cell suspensions were transferred to 2-ml screw-capped centrifuge tubes half-filled with acid-washed glass beads (0.5 μm in diameter) (Biospecs Products, Bartlesville, OK) and physically disrupted with a Bead-beater apparatus (Biospecs Products) for 3 min. The tubes were then centrifuged at 16,000 × g for 30 s and the supernatant was transferred to new microcentrifuge tubes. Total nucleic acids were precipitated by adding 1/10 volume of 3 M sodium acetate (pH 7.2) and an equal volume of isopropanol, followed by centrifugation at 16,000 × g for 20 min (38). The DNA was then centrifuged at 16,000 × g for 5 min and the pellet was resuspended in 400 μl of sddH₂O or DIGEASY hybridization buffer. The pellet resuspended in DIGEASY hybridization buffer was subjected to MCH real-time PCR using SCBs coated with AACCAPRW and the *A. avenae* subsp. *citrulli* TaqMan assay, as described above. For comparison, 5 μl of the DNA pellet resuspended in 400 μl of sddH₂O was used as template DNA for direct real-time PCR

TABLE 3. Identity, origin, and source of fungal isolates used to assess the specificity of the *Didymella bryoniae* TaqMan real-time polymerase chain reaction (PCR) assay used in this study

Fungus	Isolate	RAPD group ^a	Host	Origin	Real-time PCR
<i>D. bryoniae</i>	W109; W110; W111; DBASCO1; DBASCO2	RGI	Watermelon	South Carolina, USA	+
<i>D. bryoniae</i>	GSB 1	RGI	Muskmelon	Florida, USA	+
<i>D. bryoniae</i>	C30	RGI	Honeydew	Allendale Co., SC, USA	+
<i>D. bryoniae</i>	W20	RGI	Watermelon	Calhoun Co., SC, USA	+
<i>D. bryoniae</i>	C14; C06	RGI	Muskmelon	Colleton Co., SC, USA	+
<i>D. bryoniae</i>	R-A3; R-A6	RGI	Watermelon	Alachua Co., FL, USA	+
<i>D. bryoniae</i>	R-H3; R-D7	RGI	Watermelon	Alamo, TX, USA	+
<i>D. bryoniae</i>	W06B; W279; W282; W499; W501; W47; W48	RGI	Watermelon	Allendale Co., SC, USA	+
<i>D. bryoniae</i>	W235; W236	RGI	Watermelon	Bamberg Co., SC, USA	+
<i>D. bryoniae</i>	C174	RGI	Cucumber	Bamberg Co., SC, USA	+
<i>D. bryoniae</i>	C101; C105; C111; C113; W253; W259; W488; W494	RGI	Muskmelon	Barnwell Co., SC, USA	+
<i>D. bryoniae</i>	R-V34	RGI	Muskmelon	Bowen, Australia	+
<i>D. bryoniae</i>	R-F2; R-F4	RGI	Muskmelon	Brooks Co., GA, USA	+
<i>D. bryoniae</i>	W200; W205; W206; W207; W208	RGI	Watermelon	Unknown	+
<i>D. bryoniae</i>	W25	RGI	Muskmelon	Charleston Co., SC, USA	+
<i>D. bryoniae</i>	DBPUM12	RGI	Pumpkin	Chesterfield, SC, USA	+
<i>D. bryoniae</i>	W85; W87	RGI	Watermelon	China	+
<i>D. bryoniae</i>	W03R; W23; W78; D4; W274; W260; W272; W273	RGI	Watermelon	Colleton Co., SC, USA	+
<i>D. bryoniae</i>	R-V3; R-V7, R-V8; R-V9; R-V10	RGI	Cucumber	Crete, Greece	+
<i>D. bryoniae</i>	C68; C79; F18	RGI	Cucumber	Edisto Is., SC, USA	+
<i>D. bryoniae</i>	R-D10	RGI	Muskmelon	Hidalgo, TX, USA	+
<i>D. bryoniae</i>	W241	RGI	Watermelon	Imperial Co., CA, USA	+
<i>D. bryoniae</i>	Israel D1	RGI	Muskmelon	Israel	+
<i>D. bryoniae</i>	R-T1; R-T3; R-T4; R-T6; R-T7	RGI	Watermelon	Knox Co., IN, USA	+
<i>D. bryoniae</i>	C118A; C119	RGI	Muskmelon	Lane, OK, USA	+
<i>D. bryoniae</i>	C152	RGI	Cucumber	Lexington Co., SC, USA	+
<i>D. bryoniae</i>	R-15	RGI	Unknown	Missouri, USA	+
<i>D. bryoniae</i>	AcSq5	RGI	Acorn squash	Clayton, NC, USA	+
<i>D. bryoniae</i>	GSB 23	RGI	Muskmelon	Tompkins Co., NY, USA	+
<i>D. bryoniae</i>	R-B2	RGI	Muskmelon	Oklahoma, USA	+
<i>D. bryoniae</i>	R-D6	RGI	Muskmelon	Pharr, TX, USA	+
<i>D. bryoniae</i>	W472; W473	RGI	Watermelon	Sarasota, FL, USA	+
<i>D. bryoniae</i>	R-D1	RGI	Muskmelon	Rio Grande Co., TX, USA	+
<i>D. bryoniae</i>	R-D3	RGI	Muskmelon	Starr, TX, USA	+
<i>D. bryoniae</i>	R-T5	RGI	Watermelon	Sullivan Co., IN, USA	+
<i>D. bryoniae</i>	R-J6; R-J7; R-J8; R-J9	RGI	Watermelon	Sussex Co., DE, USA	+
<i>D. bryoniae</i>	R-V2	RGI	Cucumber	Sweden	+
<i>D. bryoniae</i>	R-B10	RGI	Muskmelon	Texas, USA	+

(continued on next page)

^a Random amplified polymorphic DNA group (41). ND = not determined and NA = not applicable.

using the same TaqMan assay. PCR reagents and run conditions were similar to those described above except that a simplex reaction rather than a multiplex reaction was employed. This experiment was repeated four times. Ct values were determined for each reaction and used to compare MCH real-time PCR to direct real-time PCR.

Comparison of direct real-time PCR and MCH real-time PCR for the detection of *D. bryoniae* conidia in suspension. To compare the sensitivity and reliability of MCH real-time PCR with direct real-time PCR, assays were conducted on 10-fold serial dilutions of a suspension of *D. bryoniae* conidia. To generate conidial suspensions, *D. bryoniae* strain C156 was grown on quarter-strength PDA at room temperature under conditions of 12 h of fluorescent light and 12 h of darkness daily to induce sporulation. After 14 days, a conidial suspension was prepared (18). The concentration of conidia in the suspension was estimated using a hemacytometer and adjusted to 10^6 conidia/ml with sddH₂O. Using lysis buffer, 10-fold serial dilutions were generated to obtain suspensions with 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 , 1×10 , and 0 conidia/ml. Acid-washed glass beads (0.5 μ m in diameter) and a Beadbeater were used to extract genomic DNA from the *D. bryoniae* conidial suspensions as described above (38). MCH was performed as described above for *A. avenae* subsp. *citrulli*, except that hybridization capture was conducted with SCBs coated with DBCAPRW (Table 1) and the *D. bryoniae* TaqMan PCR assay was employed. PCR reagents and run conditions were similar to those described above, except

that a simplex rather than a multiplex reaction was employed. This experiment was repeated four times. Ct values were determined for each positive reaction and used to compare MCH real-time PCR with direct real-time PCR.

Evaluation of MCH multiplex real-time PCR for detection of *A. avenae* subsp. *citrulli* and *D. bryoniae* in vitro. To determine the sensitivity of MCH multiplex real-time PCR, purified *A. avenae* subsp. *citrulli* and *D. bryoniae* genomic DNA were mixed and adjusted to a concentration of DNA of each pathogen at 25 pg/ μ l. Six 10-fold serial dilutions of this solution were then generated in sddH₂O and DIGEASY hybridization buffer to yield solutions ranging from 25 pg/ μ l to 25 ag/ μ l of DNA for each pathogen. DNA was denatured by incubation at 100°C for 10 min, followed by rapid chilling on crushed ice. For the 10-fold serial dilutions of DNA generated in DIGEASY hybridization buffer, MCH and multiplex real-time PCR were conducted as previously described using SCBs coated separately with AACCAPRW and DBCAPRW. For comparison, direct multiplex real-time PCR were conducted using 5 μ l of each 10-fold serial dilution of mixtures of DNA generated in sddH₂O. For all dilutions, a threshold of 30 fluorescence units was employed and the Ct values were recorded for each reaction. This experiment was repeated four times.

To evaluate the sensitivity and reliability of MCH multiplex real-time PCR for simultaneously detecting *A. avenae* subsp. *citrulli* and *D. bryoniae* cells, known concentrations of bacterial cells and fungal conidia were mixed and 10-fold serial dilutions

TABLE 3. (continued from preceding page)

Fungus	Isolate	RAPD group ^a	Host	Origin	Real-time PCR
<i>D. bryoniae</i>	R-V1	RGI	Cucumber	The Netherlands	+
<i>D. bryoniae</i>	R-F3; R-F5	RGI	Muskmelon	Tifton, GA, USA	+
<i>D. bryoniae</i>	GSB 11	RGI	Muskmelon	Wisconsin, USA	+
<i>D. bryoniae</i>	C03; C123; C125; C128; C133	RGI	Muskmelon	Wadmalaw Is., SC, USA	+
<i>D. bryoniae</i>	DBPUM2	RGI	Pumpkin	Wadmalaw Is., SC, USA	+
<i>D. bryoniae</i>	R-J1; R-J3; RJ-10	RGI	Watermelon	Wicimico, MD, USA	+
<i>D. bryoniae</i>	C154B	RGI	Muskmelon	Chesterfield Co., SC, USA	+
<i>D. bryoniae</i>	C137	RGI	Muskmelon	Dorchester Co., SC, USA	+
<i>D. bryoniae</i>	C140; C143	RGI	Muskmelon	Bamberg Co., SC, USA	+
<i>D. bryoniae</i>	C152	RGI	Cucumber	Lexington Co., SC, USA	+
<i>D. bryoniae</i>	W41	RGI	Watermelon	Hampton Co., SC, USA	+
<i>D. bryoniae</i>	W178; W182	RGI	Watermelon	Horry Co., SC, USA	+
<i>D. bryoniae</i>	R-T2	RGI	Butternut squash	Michigan, USA	–
<i>D. bryoniae</i>	R-U1; R-U2	RGI	Gourd	Indiana, USA	–
<i>D. bryoniae</i>	R-J2	RGI	Watermelon	Wicimico, MD, USA	–
<i>D. bryoniae</i>	R-G3; R-G5	RGI	Watermelon	California, USA	–
<i>D. bryoniae</i>	R-G1; R-G4	ND	Watermelon	California, USA	–
<i>D. bryoniae</i>	W26	ND	Muskmelon	Florida, USA	–
<i>D. bryoniae</i>	R-V16	ND	Cucumber	Canada	–
<i>D. bryoniae</i>	GSB6	RGII	Butternut squash	Oneida Co., NY, USA	–
<i>D. bryoniae</i>	GSB 10	RGII	Muskmelon	Woodland, CA, USA	–
<i>D. bryoniae</i>	GSB19; GSB 20	RGII	Cucumber	Tompkins Co., NY, USA	–
<i>D. bryoniae</i>	GSB19	RGII	Pumpkin	Ontario Co., NY, USA	–
<i>D. bryoniae</i>	GSB25; GSB26	RGII	Muskmelon	Tompkins Co., NY, USA	–
<i>D. bryoniae</i>	GSB27; GSB28	RGII	Butternut squash	Tompkins Co., NY, USA	–
<i>D. bryoniae</i>	W201	RGII	Watermelon	Unknown	–
<i>D. lycopersici</i>	Israel D2	NA	Tomato	Israel	–
<i>Phoma</i> sp.	W19	NA	Watermelon	Calhoun Co., SC, USA	–
<i>Phoma</i> sp.	W202	NA	Watermelon	Unknown	–
<i>Phoma</i> sp.	W185; W186	NA	Watermelon	Colleton Co., SC, USA	–
<i>Phoma</i> sp.	1196	NA	Muskmelon	Horry Co., SC, USA	–
<i>Phoma</i> sp.	GSB 9	NA	Cucumber	Clinton, NC, USA	–
<i>Phoma</i> sp.	GSB 18	NA	Cucumber	Duplin Co., NC, USA	–
<i>Phoma</i> sp.	GSB 7	NA	Squash	Oneida Co., NY, USA	–
<i>P. exigua</i>	ATCC 14728	NA	Unknown	Unknown	–
<i>P. medicaginis</i>	ATCC 64481	NA	Clover	Australia	–
<i>Alternaria cucumerina</i>	AC3	NA	Muskmelon	South Carolina, USA	–
<i>A. cucumerina</i>	95-11	NA	Watermelon	South Carolina, USA	–
<i>Colletotrichum orbiculare</i>	COLL2	NA	Unknown	South Carolina, USA	–
<i>Fusarium oxysporum</i>					
f. sp. <i>niveum</i> race 2	SG1	NA	Watermelon	Colleton Co., SC, USA	–

were generated. *A. avenae* subsp. *citrulli* and *D. bryoniae* suspensions containing 10^7 CFU/ml were generated separately, as previously described, and mixed together in equal proportions. Ten-fold serial dilutions were generated in lysis buffer to obtain suspensions containing each pathogen at 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 , 1×10 , and 0 propagules/ml. Total genomic DNA was extracted from each serial dilution using the Bead-beater technique, as previously described, and resuspended in 400 μ l of DIGEASY hybridization buffer. MCH multiplex real-time PCR with AACCAPRW and DBCAPRW was used to assay the DNA solutions as previously described. For comparison, DNA extracted in a similar manner from 10-fold serial dilutions of propagules of both pathogens was suspended in 400 μ l of sddH₂O, and 5 μ l of each suspension was used as template for direct multiplex real-time PCR. This experiment was repeated four times. Ct values were determined for each reaction and used to compare MCH real-time with direct real-time PCR.

Preparation of watermelon seed artificially infested with *A. avenae* subsp. *citrulli* and *D. bryoniae*. To evaluate the ability of MCH multiplex real-time PCR to detect *A. avenae* subsp. *citrulli* and *D. bryoniae* in watermelon seed, samples ($n = 5,000$ seeds) were prepared with 0, 0.02, or 0.1% of the seed infested with each pathogen separately. *A. avenae* subsp. *citrulli*-infested seed were collected from symptomatic watermelon fruit (cv. Crimson Sweet) infected with BFB in a field in Reidsville, GA in June 2006. The BFB outbreak was induced by spray inoculating 2-week-old watermelon seedlings with *A. avenae* subsp. *citrulli* cell suspensions (10^7 CFU/ml) and allowing the fruit to develop to harvest maturity. Seed were extracted from mature, symptomatic fruit without rinsing or fermenting. After air drying, the seed were stored at 4°C until use. Because of the unavailability of watermelon seedlots with adequate levels of natural *D. bryoniae*-infection or infestation at the time this study was conducted, infested seed were artificially generated. To do this, watermelon seed (cv. Crimson Sweet) were autoclaved and incubated on PDA plates on which plugs of agar colonized by the fungus (from a 7-day-old culture) were placed. Ten seeds were arranged radially around each *D. bryoniae* plug, and plates were incubated at 25°C for 7 days with 12 h of fluorescent light daily. Seed colonized by the fungus were then removed from the agar plate, air dried at 25°C for 48 h, and stored at 4°C until use. To create seedlots with 0.02% infestation, 1 *A. avenae* subsp. *citrulli*-infested seed and 1 *D. bryoniae*-infested seed were added to 5,000 uninfested watermelon seeds of the cv. Sugar Baby. The uninfested seed samples were obtained from a commercial source (Syngenta Vegetable Seeds, Woodland, CA). Similarly, seedlots with 0.1% infested seed were generated by adding 5 *A. avenae* subsp. *citrulli*-infested and 5 *D. bryoniae*-infested seeds to 5,000 uninfested seeds of the same source.

Comparison of direct and MCH multiplex real-time PCR for the detection of *A. avenae* subsp. *citrulli* and *D. bryoniae* in watermelon seedlots. Pathogen propagules were extracted from the composite seed sample using a vacuum, as previously described (47,48). Briefly, seed samples were placed in a sterile side-arm flask containing 500 ml of sterile PBS. Using a magnetic stir bar and a stir plate, seed were continuously agitated for 1 h while a vacuum was applied. The vacuum was interrupted briefly at 15-min intervals to facilitate the extraction of pathogen propagules from under the seed coat. After vacuum extraction, the seed extract was passed through one layer of cheesecloth and centrifuged at 8,000 rpm for 15 min, and the pellet was resuspended in 2 ml of lysis buffer. Total DNA was extracted from the pellet using the Beadbeater as previously described. The sample was then centrifuged at $15,000 \times g$ for 6 min, and ≈ 2 ml of supernatant was passed through a 0.45- μ m filter on a hypodermic syringe (Fisher Scientific) to remove seed debris. This crude DNA extract (5 μ l) was used as template for direct real-time PCR. The remaining solution was divided into two equal portions

(≈ 900 μ l), each of which was used for total DNA purification by sodium acetate and isopropanol precipitation as described above. The DNA extracted from one of the subsamples was resuspended in 400 μ l of DIGEASY hybridization buffer and used for MCH multiplex real-time PCR as described above. For comparison, DNA extracted from the second subsample was resuspended in 400 μ l of sddH₂O and 5 μ l was used as template for direct multiplex real-time PCR. Each treatment (0, 0.02, and 0.1% infestation with each pathogen) was replicated four times, and the experiment was repeated twice. Ct values were determined for each reaction and used to compare MCH, crude DNA extraction, and isopropanol precipitation for the detection of *A. avenae* subsp. *citrulli* and *D. bryoniae* in watermelon seed by real-time PCR.

Evaluation of MCH multiplex real-time PCR for pathogen detection in naturally infested melon seed. Because watermelon seed naturally infested (mycelia on the seed coat) or infected (mycelia under the seed coat) with *D. bryoniae* were unavailable at the time of this study, mixtures of melon seed naturally infested with *A. avenae* subsp. *citrulli* and *D. bryoniae* were used to evaluate MCH multiplex real-time PCR. Melon seed naturally infested with *A. avenae* subsp. *citrulli* were obtained from a commercial source (anonymously provided) and found to be infested at an incidence of 0.1% (5 loci of infection in 5,000 seeds) using the greenhouse seedling grow-out assay (2). Melon seed naturally infested with *D. bryoniae* were generated as follows. Three types of melon—*Cucumis melo* subsp. *melo* cv. Ananas Yokneam, *C. melo* var. *conomon* cv. Baj-Gua, and *C. melo* var. *acidulous*—were planted on 11 May 2007 at the Clemson University Coastal Research and Education Center, Charleston, SC. Young fruit longer than 5 cm were inoculated with a 23-gauge hypodermic syringe by injecting ≈ 50 μ l of a suspension of *D. bryoniae* strain C154B at 10^7 conidia/ml into the flesh. Inoculated fruit were harvested ≈ 3 weeks after inoculation. The seed were removed, rinsed thoroughly in a sieve to remove pieces of flesh, and air dried on paper towels at ambient temperature. The level of *D. bryoniae* contamination was determined by the standard blotter assay (2). All seedlings developed typical GSB symptoms, including brown spots on the hypocotyls, pycnidia, or orange droplets of exudate. Nongerminated seed were surface disinfested by rinsing in NaOCl for 1 min, rinsed three times in sddH₂O, and incubated on PDA for 7 days in the dark. Real-time PCR with the *D. bryoniae*-specific TaqMan assay confirmed the identity of the fungal mycelia growing from the seed (data not shown).

To generate seedlots naturally infested with both pathogens, 0, 1, 5, or 10 *D. bryoniae*-infested melon seeds were mixed with 5,000 seeds from the *A. avenae* subsp. *citrulli*-infested commercial melon lot. The level of *A. avenae* subsp. *citrulli* contamination was estimated to be 13% based on a modified seedling grow-out assay conducted on 400 seeds. Briefly, the sweatbox assay involved germinating and growing seedlings on blotter paper in transparent germination boxes maintained at 28°C and 100% relative humidity for 14 days followed by visually observing the seedlings for typical BFB symptoms. As a negative control treatment, uninfested seed from a commercial source, cv. Joaquin Gold (Rogers, Gilroy, CA) ($n = 5,000$ seeds), were included. MCH multiplex real-time PCR was conducted on each seed sample as described above. The experiment was repeated four times and mean Ct values were recorded for each pathogen in each seedlot.

RESULTS

Specificity of the *A. avenae* subsp. *citrulli* TaqMan PCR assay. As expected, PCR amplification with the *A. avenae* subsp. *citrulli*-specific TaqMan assay developed in this study produced FAM-based fluorescence and the predicted 121-bp fragment with template genomic DNA (data not shown). No amplicon or FAM-

based fluorescence was observed by conventional or real-time PCR, respectively, on genomic DNA from closely related bacteria, including *A. avenae* subsp. *avenae*, *A. avenae* subsp. *cattleyae*, *A. konjaci*, *A. facilis*, and *Pseudomonas fluorescens* (Table 2). Of 45 *A. avenae* subsp. *citrulli* strains screened, genomic DNA from 40 strains was amplified by the TaqMan assay. DNA from the following group I strains was not amplified: ATCC 296295, IA58-1, 200-23, 200-6, and 206-1. These represented the *Spe*I pulsed-field gel electrophoresis (PFGE) haplotypes K (B3), P (B8), P (B8), R (B9), and B17, respectively (46).

Specificity of the *D. bryoniae* TaqMan PCR assay. The *D. bryoniae*-specific TaqMan assay produced the predicted 86-bp fragment and Texas Red-based fluorescence with *D. bryoniae* template DNA but did not amplify DNA from *D. lycopersici* or other fungal genera tested, including *Phoma* spp., *A. cucumerina*, *Colletotrichum orbiculare*, and *F. oxysporum* f. sp. *niveum* race 2 (Table 3). Similar to the conventional PCR assay from which it was derived, the TaqMan real-time PCR assay was specific to the *D. bryoniae* RGI isolates but did not amplify DNA from 10 RGII isolates and 4 uncharacterized *D. bryoniae* isolates tested (R-G1, R-G4, W26, and R-V16). Genomic DNA from a majority (93%) of the *D. bryoniae* RGI isolates was amplified with the TaqMan assay (Table 3). However, the assay failed to amplify DNA from six RGI isolates (R-G3, R-G5, R-J2, R-T2, R-U1, and R-U2) that

previously were amplified with the conventional PCR assay (26,42).

Amplification efficiency for *A. avenae* subsp. *citrulli* and *D. bryoniae* TaqMan real time PCR assays. Amplification efficiencies for the real-time PCR assays developed in this study were obtained from linear regression analyses of log₁₀ (DNA concentration) versus Ct value conducted separately for *A. avenae* subsp. *citrulli* and *D. bryoniae* (Fig. 2). For both the *A. avenae* subsp. *citrulli* and the *D. bryoniae* assays, there was a strong correlation ($R^2 > 0.99$) between genomic DNA concentration and Ct values tested. The slopes of the linear regression lines for the relationships between Ct values and DNA concentration for *A. avenae* subsp. *citrulli* and *D. bryoniae* were -3.71 and -3.01 , respectively (Fig. 2). Based on these slopes, amplification efficiencies were determined to be ≈ 80 and 115% for the *A. avenae* subsp. *citrulli* and *D. bryoniae* TaqMan assays, respectively.

Comparison of direct real-time PCR and MCH real-time PCR for the detection of *A. avenae* subsp. *citrulli* in cell suspensions. *A. avenae* subsp. *citrulli* was not detected in negative control samples by either direct or MCH real-time PCR (Table 4). Direct real-time PCR consistently (100% of samples) detected the bacterium in suspensions containing $\geq 10^3$ CFU/ml, yielding a mean Ct value of 31.2 cycles (Table 4). In comparison, MCH real-time PCR yielded a 100-fold increase in sensitivity (as low as 10 CFU/ml), with a mean Ct value of 33.3. Relative to direct real-time PCR, MCH real-time PCR reduced the mean Ct values observed at each serial dilution. Interestingly, the differences in Ct values between direct real-time PCR and MCH real-time PCR increased as the concentration of bacterial cells decreased, with the greatest difference observed for suspensions containing *A. avenae* subsp. *citrulli* at $\approx 10^2$ CFU/ml (Table 4). For example, the difference in Ct value between direct real-time PCR and MCH real-time PCR at 1×10^2 CFU/ml was 4.48 while the difference at 1×10^6 CFU/ml was 0.66.

Comparison of direct real-time PCR and MCH real-time PCR for the detection of *D. bryoniae* conidia. *D. bryoniae* was not detected in negative control samples by direct real-time PCR or MCH real-time PCR (Table 5). A detection threshold for *D. bryoniae* of 10^5 conidia/ml was observed for both direct real-time PCR and MCH real-time PCR (Table 5). Additionally, the Ct values associated with positive reactions were similar for both techniques, suggesting that MCH had no effect on real-time PCR sensitivity.

Comparison of MCH and direct multiplex real-time PCR for the simultaneous detection of *A. avenae* subsp. *citrulli* and *D. bryoniae* DNA. After optimization, the multiplex real-time PCR assay facilitated the simultaneous detection of both pathogens in mixtures of *A. avenae* subsp. *citrulli* and *D. bryoniae* DNA (Table 6). In all experiments, the mean Ct values increased proportionately as the concentration of template DNA from both pathogens decreased (Table 6). The threshold at which DNA from

Fig. 2. Standard curves of *Acidovorax avenae* subsp. *citrulli* and *Didymella bryoniae* genomic DNA against corresponding cycle threshold (Ct) values generated by real time polymerase chain reaction (PCR) using specific TaqMan assays for both pathogens in separate simplex reactions. Each data point represents the mean Ct value for DNA at each concentration for four independent replicates and the error bars represent the standard error of the means. Lines represent the linear regression analysis of the relationship between DNA concentration and Ct values generated by TaqMan real-time PCR.

TABLE 4. Comparison of direct real-time polymerase chain reaction (PCR) and magnetic capture hybridization (MCH) combined with real-time PCR for the detection of 10-fold serial dilutions of a suspension of *Acidovorax avenae* subsp. *citrulli* cells

Conc. (CFU/ml) ^a	Direct real-time PCR		MCH real-time PCR		Difference in Ct values (cycles)
	Ct $\pm$ SD ^b	Frequency ^c	Ct $\pm$ SD ^b	Frequency ^c	
1×10^6	19.08 $\pm$ 0.04	4/4	20.42 $\pm$ 0.53	4/4	0.66
1×10^5	23.52 $\pm$ 0.09	4/4	22.69 $\pm$ 0.61	4/4	0.83
1×10^4	27.84 $\pm$ 0.27	4/4	25.90 $\pm$ 1.04	4/4	1.94
1×10^3	31.18 $\pm$ 0.25	4/4	27.90 $\pm$ 0.82	4/4	3.28
1×10^2	34.54	1/4	30.06 $\pm$ 0.35	4/4	4.48
1×10^1	ND	0/4	33.28 $\pm$ 0.32	4/4	...
0	ND	0/4	ND	0/4	0

^a *A. avenae* subsp. *citrulli* concentration (CFU/ml).

^b Mean cycle threshold value (Ct) $\pm$ standard deviation. ND = samples that did not test positive for *A. avenae* subsp. *citrulli* by real-time PCR assay (TaqMan probe-based fluorescence did not exceed the background threshold after 35 PCR cycles).

^c Detection frequency = the number of positive assays (those for which the fluorescence generated by the TaqMan real-time PCR assay exceeded the threshold) divided by the number of samples tested.

both pathogens could be reliably detected (100% of samples) by direct real-time PCR was 10 pg/μl, which yielded mean Ct values of 29.4 and 33.1 for the bacterium and the fungus, respectively (Table 6). In suspensions containing DNA at 1 pg/μl, *A. avenae* subsp. *citrulli* was detected in all four samples but *D. bryoniae* was only detected in 25% of the samples (1/4). In contrast, MCH resulted in a 10-fold increase in detection sensitivity (as low as 1 pg/μl) for both pathogens compared with direct real-time PCR. For *A. avenae* subsp. *citrulli* alone, the detection threshold of

100 fg/μl was similar to that of direct real-time PCR but MCH increased the detection frequency from 25 to 100% and lowered the mean Ct value from 34.6 to 33.6 (Table 6). MCH resulted in at least a 100-fold increase in detection sensitivity for *D. bryoniae*, from 100 pg/μl to 1 fg/μl. MCH also facilitated detection of *D. bryoniae* DNA in solutions with as little as 1 fg/μl; however, the frequency of detection at this level was 25% and the Ct value was 34.9 (out of 35 cycles).

Comparison of direct and MCH real-time PCR for detection of mixtures of *A. avenae* subsp. *citrulli* and *D. bryoniae* propagules. Neither direct multiplex real-time PCR nor MCH multiplex real-time PCR detected *A. avenae* subsp. *citrulli* or *D. bryoniae* CFU in negative control samples (Table 7). Similar to experiments in which simplex real-time PCR was employed, direct multiplex real-time PCR displayed repeatable (100% of samples) detection thresholds for *A. avenae* subsp. *citrulli* at 10² CFU/ml and *D. bryoniae* at 10⁵ conidia/ml (Table 7). However, both pathogens were simultaneously detected only in suspensions containing each pathogen at 10⁵ or 10⁶ CFU/ml (for 100% of samples). In contrast, MCH multiplex real-time PCR facilitated the detection of both pathogens in cell mixtures containing as little as 10³ CFU/ml but only at 10⁵ CFU/ml for 100% of the samples. *A. avenae* subsp. *citrulli* detection was consistent at all dilutions (100% of samples) and the detection threshold for MCH multiplex real-time PCR was 10-fold greater (10 CFU/ml) than for the simplex direct real time PCR (Table 4). In contrast, the frequency of detection for *D. bryoniae* was 50 and 25% for dilutions with 10⁴ and 10³ conidia/ml, respectively (Table 7). However, the mean Ct values for the fungus were relatively high and almost identical (34.7 for both dilutions).

TABLE 5. Comparison of a direct real-time polymerase chain reaction (PCR) assay to magnetic capture hybridization (MCH) combined with a real-time PCR assay for the detection of 10-fold serial dilutions of a suspension of conidia of *Didymella bryoniae*

Conidia/ml ^a	Direct real-time PCR		MCH real-time PCR	
	Ct ± SD ^b	Freq. ^c	Ct ± SD ^b	Freq. ^c
1 × 10 ⁶	32.86 ± 0.18	4/4	32.68 ± 0.90	4/4
1 × 10 ⁵	33.66 ± 0.17	4/4	33.87 ± 0.72	4/4
1 × 10 ⁴	ND	0/4	ND	0/4
1 × 10 ³	ND	0/4	ND	0/4
1 × 10 ²	ND	0/4	ND	0/4
1 × 10 ¹	ND	0/4	ND	0/4
0	ND	0/4	ND	0/4

^a *D. bryoniae* concentration.

^b Mean cycle threshold value (Ct) ± standard deviation. ND = samples that did not test positive for *D. bryoniae* by real-time PCR assay (TaqMan probe-based fluorescence did not exceed the background threshold after 35 PCR cycles).

^c Detection frequency = the number of positive assays (those for which the fluorescence generated by the TaqMan real-time PCR assay exceeded the threshold) divided by the number of samples tested.

TABLE 6. Comparison of direct real-time polymerase chain reaction (PCR) to magnetic capture hybridization (MCH) combined with real-time PCR for the simultaneous detection of *Acidovorax avenae* subsp. *citrulli* and *Didymella bryoniae* DNA in 10-fold serial dilutions of the two cucurbit pathogens

Conc. (ng/μl) ^a	Direct real-time PCR				MCH real-time PCR			
	<i>A. avenae</i> subsp. <i>citrulli</i>		<i>D. bryoniae</i>		<i>A. avenae</i> subsp. <i>citrulli</i>		<i>D. bryoniae</i>	
	Ct ± SD ^b	Freq. ^c	Ct ± SD ^b	Freq. ^c	Ct ± SD ^b	Freq. ^c	Ct ± SD ^b	Freq. ^c
1	24.68 ± 0.34	4/4	26.22 ± 0.07	4/4	22.55 ± 0.19	4/4	25.60 ± 0.16	4/4
10 ⁻¹	26.78 ± 0.30	4/4	30.43 ± 0.30	4/4	25.93 ± 0.05	4/4	29.38 ± 0.19	4/4
10 ⁻²	29.43 ± 0.31	4/4	33.13 ± 0.40	4/4	28.31 ± 0.97	4/4	30.88 ± 1.83	4/4
10 ⁻³	32.59 ± 0.42	4/4	34.86	1/4	30.91 ± 0.27	4/4	32.32 ± 0.24	4/4
10 ⁻⁴	34.64	1/4	ND	0/4	33.60 ± 0.37	4/4	32.81 ± 0.03	4/4
10 ⁻⁵	ND	0/4	ND	0/4	ND	0/4	33.20 ± 0.81	2/4
10 ⁻⁶	ND	0/4	ND	0/4	ND	0/4	34.88	1/4

^a Concentration of 1:1 mixtures of *A. avenae* subsp. *citrulli* and *D. bryoniae* DNA.

^b Mean cycle threshold value (Ct) ± standard deviation. ND = samples that did not test positive for *A. avenae* subsp. *citrulli* and *D. bryoniae* by real-time PCR assay (TaqMan probe-based fluorescence did not exceed the background threshold after 35 PCR cycles).

^c Detection frequency = the number of positive assays (those for which the fluorescence generated by the TaqMan real-time PCR assay exceeded the threshold) divided by the number of samples tested.

TABLE 7. Comparison of direct real-time polymerase chain reaction (PCR) versus magnetic capture hybridization (MCH) combined with real-time PCR for the detection of *Acidovorax avenae* subsp. *citrulli* and *Didymella bryoniae* CFU (cells and conidia, respectively) in 10-fold serial dilutions of mixtures of the two cucurbit pathogens

Conc. (CFU/ml) ^a	Direct real-time PCR				MCH real-time PCR			
	<i>A. avenae</i> subsp. <i>citrulli</i>		<i>D. bryoniae</i>		<i>A. avenae</i> subsp. <i>citrulli</i>		<i>D. bryoniae</i>	
	Ct ± SD ^b	Freq. ^c	Ct ± SD ^b	Freq. ^c	Ct ± SD ^b	Freq. ^c	Ct ± SD ^b	Freq. ^c
1 × 10 ⁶	22.55 ± 2.22	4/4	33.25 ± 0.18	4/4	20.17 ± 0.17	4/4	32.97 ± 0.46	4/4
1 × 10 ⁵	24.70 ± 2.50	4/4	33.59 ± 0.62	4/4	22.86 ± 0.32	4/4	33.33 ± 0.28	4/4
1 × 10 ⁴	27.70 ± 0.27	4/4	ND	0/4	25.82 ± 0.88	4/4	34.72 ± 0.16	2/4
1 × 10 ³	29.81 ± 0.85	4/4	ND	0/4	27.38 ± 0.19	4/4	34.7	1/4
1 × 10 ²	33.35 ± 0.95	4/4	ND	0/4	29.70 ± 0.27	4/4	ND	0/4
1 × 10 ¹	34.65 ± 1.15	2/4	ND	0/4	31.70 ± 0.33	4/4	ND	0/4
0	ND	0/4	ND	0/4	ND	0/4	ND	0/4

^a Equal concentration of *A. avenae* subsp. *citrulli* CFU/ml and *D. bryoniae* conidia/ml.

^b Mean cycle threshold value (Ct) ± standard deviation. ND = samples that did not test positive for *A. avenae* subsp. *citrulli* and *D. bryoniae* were not detected by real-time PCR assay (TaqMan probe-based fluorescence did not exceed the background threshold after 35 PCR cycles).

^c Detection frequency = the number of positive assays (those for which the fluorescence generated by the TaqMan real-time PCR assay exceeded the threshold) divided by the number of samples tested.

Evaluation of MCH real-time PCR for the detection of *A. avenae* subsp. *citrulli* and *D. bryoniae* in artificially infested seed. Neither *A. avenae* subsp. *citrulli* nor *D. bryoniae* was detected in negative control seedlots regardless of the technique employed (Table 8). For watermelon seedlots with 0.02 and 0.1% infestation by both pathogens, crude DNA extraction and DNA purification followed by multiplex real-time PCR failed to detect either pathogen (Table 8). In one of eight samples, crude DNA extraction followed by real-time PCR yielded a positive reaction for *A. avenae* subsp. *citrulli* from seed with 0.1% infestation. In contrast, for lots with 0.1% *A. avenae* subsp. *citrulli*- and *D. bryoniae*-infested seed, both pathogens were simultaneously detected in 100% of the attempts by MCH multiplex real-time PCR (Table 8). Mean Ct values for *A. avenae* subsp. *citrulli* and *D. bryoniae* were 18.1 and 24.5, respectively. MCH multiplex real-time PCR also consistently (100% of attempts) facilitated the detection of both pathogens in seedlots with 0.02% infestation. For the latter, the mean Ct values were 21.0 and 26.7 for *A. avenae* subsp. *citrulli* and *D. bryoniae*, respectively (Table 8).

Evaluation of MCH real-time PCR for the detection of *A. avenae* subsp. *citrulli* and *D. bryoniae* in naturally infested melon seed. MCH multiplex real-time PCR consistently facilitated the simultaneous detection of *A. avenae* subsp. *citrulli* and *D. bryoniae* in naturally infested melon seedlots (Table 9). In *A. avenae* subsp. *citrulli*-infested melon seed samples with 0% *D. bryoniae*-infested seed, MCH multiplex real-time PCR detected the bacterium in 100% of the attempts, with a mean Ct value of 28.8, and, as expected, *D. bryoniae* was not detected. For samples to which 1, 5, or 10 *D. bryoniae*-infested seed were added, both pathogens were consistently detected, with mean Ct values of 34.0, 32.8, and 31.8, respectively. In contrast, crude DNA extraction and direct multiplex real-time PCR failed to detect either pathogen in naturally infested melon seedlots (data not shown). In the negative control melon seedlot, to which no *A. avenae* subsp. *citrulli*- or *D. bryoniae*-infested seed were added, neither pathogen was detected by MCH multiplex real-time PCR (Table 9).

DISCUSSION

Molecular-based pathogen detection assays, and PCR in particular, have great potential for seed health testing. However, low pathogen population and inhibitory compounds can severely limit the application of PCR for seed health testing (8). In this study, we demonstrated the potential for MCH to overcome some of these limitations. The *A. avenae* subsp. *citrulli* TaqMan PCR assay described in this study was highly specific, because it did not amplify template DNA from 13 nontarget bacterial strains, including several species closely related to *A. avenae* subsp.

citrulli. However, the assay failed to amplify DNA from five *A. avenae* subsp. *citrulli* strains. These strains have DNA fingerprint patterns similar to *SpeI* PFGE haplotypes B3 (K), B8 (P), B9(R), and B17, which are members of the group I subpopulation (46). Based on the genome sequence for AAC00-1, the *A. avenae* subsp. *citrulli* TaqMan assay developed in this study targets an unidentified gene. However, it is unclear why DNA from five of the *A. avenae* subsp. *citrulli* strains failed to amplify in the real-time PCR assay. Based on previous studies (46), strains in two of the haplotypes that were not amplified, B3 (K) and B8 (P), are closely related and have been associated with melon and watermelon plant introductions at the United States Department of Agriculture Plant Introduction Station at Griffin, GA, Ames, IA, and Lane, OK (39) (R. Walcott, *personal observation*). A strain with the PFGE haplotype R (B9) was recovered from a BFB outbreak on melon in Cordele, GA in 2000. However, at present, there is no obvious link between this strain and other strains that failed to amplify with the TaqMan assay. One possible explanation is that the strains might represent a distinct group among the group I subpopulation (46). In particular, ATCC 29625, IA58-1, and 200-23 differed from other group I strains in their inability to utilize L-leucine as a substrate (46). Although there is still a need to develop a real-time PCR assay that can amplify all pathogenic

TABLE 9. Evaluation of magnetic capture hybridization and multiplex real-time polymerase chain reaction (PCR) for the simultaneous detection of *Acidovorax avenae* subsp. *citrulli* and *Didymella bryoniae* in mixtures of melon seed naturally infested with each pathogen

Ratio ^a	<i>A. avenae</i> subsp. <i>citrulli</i>		<i>D. bryoniae</i>	
	Ct $\pm$ SD ^b	Freq. ^c	Ct $\pm$ SD ^b	Freq. ^c
0:5,000 ^d	ND	0/8	ND	0/8
0:5,000 ^e	28.75 $\pm$ 1.27	8/8	ND	0/8
1:5,000	29.31 $\pm$ 1.76	8/8	33.99 $\pm$ 1.03	8/8
5:5,000	28.25 $\pm$ 2.42	8/8	32.77 $\pm$ 1.01	8/8
10:5,000	29.45 $\pm$ 1.88	8/8	31.83 $\pm$ 0.79	8/8

^a Ratio of *D. bryoniae* to *A. avenae* subsp. *citrulli*-infested melon seed per lot.

^b Mean cycle threshold value (Ct) $\pm$ standard deviation. ND = samples that did not test positive for *A. avenae* subsp. *citrulli* and *D. bryoniae* were not detected by real-time PCR assay (TaqMan probe-based fluorescence did not exceed the background threshold after 35 PCR cycles).

^c Detection frequency = the number of positive assays (those for which the fluorescence generated by the TaqMan real-time PCR assay exceeded the threshold) divided by the number of samples tested.

^d Negative control; the 5,000 seeds used were sampled from a commercial seedlot cv. Joaquin Gold that was tested and not found to be infested with *A. avenae* subsp. *citrulli* and *D. bryoniae*.

^e The 5,000 seeds used for all seed mixtures except the negative control were sampled from a commercial melon seedlot naturally infested with *A. avenae* subsp. *citrulli* ($\approx$ 13%).

TABLE 8. Sensitivity of magnetic capture hybridization (MCH) compared with crude DNA extraction and DNA purification in combination with multiplex real-time polymerase chain reaction (PCR) for detection of *Acidovorax avenae* subsp. *citrulli* (naturally infested) and *Didymella bryoniae* (artificially infested) in watermelon seedlots ($n = 5,000$ seeds)

Infest. (%) ^a	<i>A. avenae</i> subsp. <i>citrulli</i>						<i>D. bryoniae</i>					
	Direct real-time PCR					MCH real-time PCR	Direct real-time PCR					MCH real-time PCR
	DNA extraction ^b		DNA purification ^c		DNA extraction ^b		DNA purification ^c					
	Ct ± SD ^d	Freq. ^e	Ct ± SD ^d	Freq. ^e	Ct ± SD ^d		Freq. ^e	Ct ± SD ^d	Freq. ^e			
0/0	ND	0/8	ND	0/8	ND	0/8	ND	0/8	ND	0/8	ND	0/8
0.02/0.02	ND	0/8	ND	0/8	19.03 ± 1.03	8/8	ND	0/8	ND	0/8	26.65 ± 1.52	8/8
0.1/0.1	34.95	1/8	ND	0/8	18.11 ± 1.01	8/8	ND	0/8	ND	0/8	24.46 ± 1.28	8/8

^a Seedlot infestation (percent seed infested with *A. avenae* subsp. *citrulli* and *D. bryoniae*); 0, 1, or 5 infested seeds were added to 5,000 uninfested seeds to generate each seedlot.

^b DNA prepared by physical disruption of seed extract using a Beadbeater apparatus.

^c DNA purified from physically disrupted cells in the watermelon seed extract by sodium acetate and isopropanol precipitation.

^d Mean cycle threshold value (Ct) $\pm$ standard deviation. ND = samples that did not test positive for *A. avenae* subsp. *citrulli* and *D. bryoniae* by real-time PCR assay (TaqMan probe-based fluorescence did not exceed the background threshold after 35 PCR cycles).

^e Detection frequency = the number of positive assays (those for which the fluorescence generated by the TaqMan real-time PCR assay exceeded the threshold) divided by the number of samples tested.

A. avenae subsp. *citrulli* strains, the real-time PCR assay using the BOX primer set in this study was suitable for evaluating MCH real-time PCR as a seed health assay.

With regards to the detection of *D. bryoniae*, a suitable TaqMan probe was designed to facilitate real-time PCR. As predicted, the TaqMan assay did not amplify DNA from 10 *Phoma* spp. or from 10 *D. bryoniae* RGII isolates. The assay also did not amplify DNA from four *D. bryoniae* isolates of unknown RAPD group status. However, of 114 RGI *D. bryoniae* isolates tested in this study, 6 did not yield a positive result with the TaqMan assay. This was unexpected, because the TaqMan assay was developed using the original *D. bryoniae* forward primer (GSBF1) (42) and a new reverse primer to create a smaller amplicon (86 bp) from the same locus. It is possible that the new reverse primer or probe hybridized to a locus that was absent or mutated in the four *D. bryoniae* RGI isolates that did not amplify. To our knowledge, there are no other obvious common characteristics linking these six isolates because they were isolated from a diverse range of cucurbits, including watermelon, butternut squash, and gourds. To address this problem, a new *D. bryoniae*-specific TaqMan assay has been designed that amplifies DNA from both RGI and RGII strains, and it is currently being evaluated (data not shown). This assay should be more suited to routine high-throughput testing for *D. bryoniae* (44). As with the *A. avenae* subsp. *citrulli* assay, however, we determined that the specificity of the *D. bryoniae* TaqMan assay was adequate to allow evaluation of MCH with multiplex real-time PCR as a seed health assay.

An important aspect of PCR assay development is amplification efficiency, because it directly impacts PCR product yield (4). Amplification efficiency is routinely determined based on the slopes of linear regression analysis of relationships between Ct values and template DNA concentrations over a dynamic range. These values are useful in determining whether the PCR assay design is optimal and, whereas 100% amplification efficiency is ideal, efficiencies between 80 and 115% are considered acceptable (51). Although the factors that contribute to high amplification efficiency are unknown, low efficiency (<80%) is influenced by poor primer design, inadequate real-time PCR conditions, inhibitor effects, and the length and secondary structure of amplicons (3,25,32). Pruvost and Geigl (33) demonstrated that thermolabile molecules inhibit amplification during early stages, resulting in higher Ct values. In many cases, these PCR inhibitory problems can be resolved by diluting samples. However, sample dilution also decreases DNA template copy number; therefore, it cannot be concluded that dilution always increase amplification efficiency. For applications in which quantification of nucleic acid copy number is necessary, amplification efficiency is critical for determining the accuracy of estimates and quality of the experiments. On the other hand, in cases where real-time PCR is used to generate qualitative or absence or presence data, amplification efficiency is not as relevant to the end result. In the latter case, however, amplification efficiency estimates may still be useful for estimating the likelihood that an assay might be influenced by inhibitory compounds and low-quality DNA (25). In this study, the amplification efficiencies for the *A. avenae* subsp. *citrulli* and *D. bryoniae* assays were within acceptable ranges (51). However, the *A. avenae* subsp. *citrulli* TaqMan assay displayed higher levels of sensitivity with and without MCH than the *D. bryoniae* real-time PCR assay. Although this may be attributable to the differences in amplification efficiencies, it might also be due to unique aspects of the loci selected for amplification.

In this study, MCH enhanced the detection sensitivity for *A. avenae* subsp. *citrulli* at least 100-fold in simplex reactions. Previous reports have shown similar improvements in detection sensitivity using MCH (1,10,15). The difference between Ct values obtained for MCH real-time PCR versus direct real-time PCR was not significant for cell suspensions containing 10^5 to

10^6 CFU/ml. However, as the bacterial concentration decreased, differences in Ct values for MCH real-time PCR versus direct real-time PCR increased. It is possible that, at high *A. avenae* subsp. *citrulli* cell concentrations ($>10^5$ CFU/ml), the hybridization capture probes may become saturated with target DNA. At lower cell concentrations, MCH may efficiently sequester template DNA, leading to enhanced sensitivity in the form of lower Ct values compared with direct real-time PCR.

Unfortunately, similar improvements in detection sensitivity were not observed for *D. bryoniae*. In general, the MCH real-time PCR assay was 100-fold less sensitive for *D. bryoniae* DNA than for *A. avenae* subsp. *citrulli* DNA. As previously suggested, this difference in sensitivity could be due to differences in amplification efficiencies associated with the loci for which the primers and probes were designed for each target pathogen. The MCH real-time PCR assay displayed a detection threshold of 100 pg/ μ l or 10^5 conidia/ml, which was significantly less than that reported for other fungal pathogens. Langrell et al. (28) reported that MCH combined with conventional PCR resulted in a 10- to 100-fold increase in detection sensitivity for *Nectria galligena*, the causal agent of Nectria canker, in woody apple tissues. Walcott et al. (48) demonstrated a similar increase in sensitivity when MCH-PCR was used to detect *B. aclada* in onion seed. The Ct values observed for *D. bryoniae* detection with MCH real-time PCR were similar to those of the direct real-time PCR assay. Therefore, the probe used for hybridization capture may not have efficiently captured *D. bryoniae* DNA. Efforts to improve hybridization capture, including optimization of the hybridization temperature (55 to 65°C) and evaluation of various hybridization buffers (sodium chloride or sodium citrate, phosphate buffered saline, and other low-salt buffers) (27) were unsuccessful (data not shown). Attempts to enhance fungal DNA capture using a peptide nucleic acid (31) hybridization capture probe also failed to improve detection sensitivity (data not shown).

Another possible explanation for failure of MCH to enhance detection sensitivity of *D. bryoniae* is the design of the capture probe. In this study, three different hybridization capture probes were designed and screened (data not shown). Because it was necessary to locate the hybridization capture probes close to the amplification loci, this limited the sequences that could be selected. This was the most challenging aspect of developing a MCH real-time PCR assay for *D. bryoniae*, because efficient capture probes are critical to the success of this technique. Efforts are in progress to seek more effective capture probes based on sequences upstream and downstream of the amplification loci for the new *D. bryoniae* primers that amplify both RGI and RGII isolates.

Despite the lack of enhancement in detection sensitivity by MCH real-time PCR for *D. bryoniae* in vitro, MCH multiplex PCR facilitated the simultaneous detection of both *A. avenae* subsp. *citrulli* and *D. bryoniae* in cell suspensions as well as from artificially and naturally infested seed samples. MCH multiplex real-time PCR consistently facilitated the detection of *A. avenae* subsp. *citrulli* and *D. bryoniae* in watermelon seed samples naturally infested with *A. avenae* subsp. *citrulli* and artificially infested with *D. bryoniae*. Additionally, *D. bryoniae* was consistently detected in melon seed samples with 0.02% naturally infested seed. The observation that the mean Ct values for melon seedlots with 0.02% *D. bryoniae* infestation was seven cycles higher than that for watermelon seedlots with the same level of contamination can be explained by the fact that the artificially infested watermelon seed contained higher levels of mycelia. It is also possible that, due to artificial inoculation, the mycelia in the watermelon seed may have been on the surface of the seed coat, which would allow for better extraction and fungal DNA yield compared with deep-seated, natural seed infection.

Routinely, commercial seed health testing for BFB is performed using a greenhouse seedling grow-out test which requires

large seed samples ($n = 10,000$ to $50,000$ seeds). However, it is infeasible to process such a large quantity of watermelon or melon seed samples at once by MCH real-time PCR. PCR-based seed assays for *A. avenae* subsp. *citrulli* employ smaller sample sizes ($n = 5,000$ to $10,000$ seeds) (2,45). For GSB, the standard blotter assay is applied to a sample of 400 seeds but a larger sample size will ensure higher confidence in the test result. In this study, we demonstrated that MCH multiplex real-time PCR is applicable for a sample size of 5,000 watermelon or melon seeds and can simultaneously detect *A. avenae* subsp. *citrulli* and *D. bryoniae* in naturally infested seedlots within 48 h. Hence, research to optimize the assay for commercial-scale application is not needed.

To date, molecular-based tests such as PCR enzyme-linked immunosorbent assay and dot-blot hybridization have been developed for *D. bryoniae* (21–23,42). Although these approaches have been used to confirm the identity of the fungus from pure cultures or infected plant tissues, there have been no reports of a PCR-based assay to test seed directly. Hence, this study represents the first successful attempt to develop a PCR-based seed assay for *D. bryoniae*. Based on this study, we conclude that the MCH real-time PCR assay developed in this study is applicable for watermelon and melon seed for the two pathogens evaluated, and is more amenable to high-throughput testing of seed samples than other standard assays. This technique could be improved further by the development of more specific and efficient TaqMan assays and more effective hybridization capture probes. Although the need for specialized equipment and training might limit widespread adoption of this technique, laboratories involved in regulatory testing for quarantined pests might significantly increase their capacity to detect and exclude pathogens by employing MCH multiplex PCR-based assays.

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LITERATURE CITED

- Amagliani, G., Omiccioli, E., del Campo, A., Bruce, I. J., Brandi, G., and Magnani, M. 2006. Development of a magnetic capture hybridization PCR assay for *Listeria monocytogenes* direct detection in milk samples. *J. Appl. Microbiol.* 100:375-383.
- Anonymous. 2004. National Seed Health System, Seed Health Methods, Vegetable Crop Methods. Published online by the NSHS-USDA-APHIS. <http://www.seedhealth.org>.
- Arezi, B., Xing, W., Sorge, J. A., and Hogrefe, H. H. 2003. Amplification efficiency of thermostable DNA polymerases. *Anal. Biochem.* 321:226-235.
- Chen, J., and Griffiths, M. W. 2001. Detection of *Salmonella* and simultaneous detection of *Salmonella* and Shiga-like toxin-producing *Escherichia coli* using the magnetic capture hybridization polymerase chain reaction. *Lett. Appl. Microbiol.* 32:7-11.
- Chen, J. R., Johnson, R., and Griffiths, M. 1998. Detection of verotoxigenic *Escherichia coli* by magnetic capture hybridization PCR. *Appl. Environ. Microbiol.* 64:147-152.
- Chilvers, M. I., du Toit, L. J., Akamatsu, H., and Peever, T. L. 2007. A real-time, quantitative PCR seed assay for *Botrytis* spp. that cause neck rot of onion. *Plant Dis.* 91:599-608.
- Chiu, W. F., and Walker, J. C. 1949. Physiology and pathogenicity of the cucurbit black rot fungus. *J. Agric. Res.* 78:589-615.
- DeBoer, S. H., Ward, L. J., Li, X., and Chittaranjan, S. 1995. Attenuation of PCR inhibition in the presence of plant compounds by addition of Blotto. *Nucleic Acids Res.* 23:2567-2568.
- de Moraes, R. R., Maruniak, J. E., and Funderburk, J. E. 1999. Methods for detection of *Anticarsia gemmatilis* nucleopolyhedrovirus DNA in soil. *Appl. Environ. Microbiol.* 65:2307-2311.
- Dupont, K., Vogensen, F. K., and Josephsen, J. 2005. Detection of lactococcal 936-species bacteriophages in whey by magnetic capture hybridization PCR targeting a variable region of receptor-binding protein genes. *J. Appl. Microbiol.* 98:1001-1009.
- Enroth, H., and Engstrand, L. 1995. Immunomagnetic separation and PCR for detection of *Helicobacter pylori* in water and stool specimens. *J. Clin. Microbiol.* 33:2162-2165.
- Gitaitis, R., and Walcott, R. R. 2007. The epidemiology and management of seedborne bacterial diseases. *Annu. Rev. Phytopathol.* 45:371-397.
- Guillemette, T., Iacomini-Vasilescu, B., and Simoneau, P. 2004. Conventional and real-time PCR-based assay for detecting pathogenic *Alternaria brassicae* in cruciferous seed. *Plant Dis.* 88:490-496.
- Hayward, C. 1996. Molecular biology in systematics and diagnosis of phytopathogenic prokaryotes. *Phytoparasitica* 24:271-275.
- Jacobsen, C. S. 1995. Microscale detection of specific bacterial DNA in soil with a magnetic capture hybridization and PCR amplification assay. *Appl. Environ. Microbiol.* 61:3347-3352.
- Keinath, A. P. 2000. Effect of protectant fungicide application schedules on gummy stem blight epidemics and marketable yield of watermelon. *Plant Dis.* 84:254-260.
- Keinath, A. P. 2002. Survival of *Didymella bryoniae* in buried watermelon vines in South Carolina. *Plant Dis.* 86:32-38.
- Keinath, A. P., Farnham, M. W., and Zitter, T. A. 1995. Morphological, pathological, and genetic differentiation of *Didymella bryoniae* and *Phoma* spp. isolated from cucurbits. *Phytopathology* 85:364-369.
- Keinath, A. P., Somai, B. M., and Dean, R. A. 2001. Method of diagnosing gummy stem blight in plants using a polymerase chain reaction assay. U.S. Patent 6,258,537 B1.
- King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301-307.
- Koch, C. A., Li, P. C. H., and Utkhede, R. S. 2005. Evaluation of thin films of agarose on glass for hybridization of DNA to identify plant pathogens with microarray technology. *Anal. Biochem.* 342:93-102.
- Koch, C. A., and Utkhede, R. S. 2002. Diagnosis and identification of *Didymella bryoniae*, causal agent of gummy stem blight of greenhouse cucumbers, using a dot blot technique. *J. Hortic. Sci. Biotechnol.* 77:62-66.
- Koch, C. A., and Utkhede, R. S. 2004. Development of a multiplex classical polymerase chain reaction technique for detection of *Didymella bryoniae* in infected cucumber tissues and greenhouse air samples. *Can. J. Plant Pathol.* 26:291-298.
- Koike, S. T. 1997. First report of gummy stem blight, caused by *Didymella bryoniae*, on watermelon transplants in California. *Plant Dis.* 81:1331.
- Kontanis, E. J., and Reed, F. A. 2006. Evaluation of real-time PCR amplification efficiencies to detect PCR inhibitors. *J. Forensic Sci.* 51(4):795-804.
- Kothera, R. T., Keinath, A. P., Dean, R. A., and Farnham, M. W. 2003. AFLP analysis of a worldwide collection of *Didymella bryoniae*. *Mycol. Res.* 107:297-304.
- Krasilnikova, M. M., and Veselkov, A. G. 1996. Enhancing the specificity of peptide nucleic acid binding with DNA. *Mol. Biol.* 30:226-230.
- Langrell, S. R. H., and Barbara, D. J. 2001. Magnetic capture hybridization for improved PCR detection of *Nectria galligena* from lignified apple extracts. *Plant Mol. Biol. Rep.* 19:5-11.
- Latin, R. X., and Hopkins, D. L. 1995. Bacterial fruit blotch of watermelon—the hypothetical exam question becomes reality. *Plant Dis.* 79:761-765.
- National Agricultural Statistics Service. 2008. Vegetables 2007 Summary. NASS, USDA, Vg 1-2 (08):7-35.
- Nielsen, P. E., and Christensen, L. 1996. Strand displacement binding of a duplex forming homopurine PNA to a homopyrimidine duplex DNA target. *J. Am. Chem. Soc.* 118:2287-2288.
- O'Sullivan, C. E., Kasai, M., Francesconi, A., Petraitis, V., Petraitiene, R., Kelaher, A. M., Sarafandi, A. A., and Walsh, T. J. 2003. Development and validation of a quantitative real-time PCR assay using fluorescence resonance energy transfer technology for detection of *Aspergillus fumigatus* in experimental invasive pulmonary aspergillosis. *J. Clin. Microbiol.* 41:5676-5682.
- Pruvost, M., and Geigl, E.-M. 2004. Real-time quantitative PCR to assess the authenticity of ancient DNA amplification. *J. Arch. Sci.* 31:1191-1197.
- Pryor, B. M., and Gilbertson, R. L. 2001. A PCR-based assay for detection of *Alternaria radicina* on carrot seed. *Plant Dis.* 85:18-23.
- Punithalingam, E., and Holliday, P. 1972. *Didymella bryoniae*. CMI Description of Pathogenic Fungi and Bacteria. No. 332. Commonwealth Mycol. Inst., Kew, England.
- Rane, K. K., and Latin, R. X. 1992. Bacterial fruit blotch of watermelon—association of the pathogen with seed. *Plant Dis.* 76:509-512.
- Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn,

- G. T., Mullis, K. B., and Erlich, H. A. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487-491.
38. Sambrook, J., and Russell, D. W., eds. 2001. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
39. Schaad, N. W., Sowell, G., Goth, R. W., Colwell, R. R., and Webb, R. E. 1978. *Pseudomonas pseudoalcaligenes* subsp. *citrulli* subsp. nov. *Int. J. Syst. Bacteriol.* 28:117-125.
40. Shin, G. Y., Manuel, R. J., Ghorri, S., Brecker, S., and Breathnach, A. S. 2005. Molecular technique identifies the pathogen responsible for culture negative infective endocarditis. *Heart* 91:47-48.
41. Somai, B. M., Dean, R. A., Farnham, M. W., Zitter, T. A., and Keinath, A. P. 2002. Internal transcribed spacer regions 1 and 2 and random amplified polymorphic DNA analysis of *Didymella bryoniae* and related *Phoma* species isolated from cucurbits. *Phytopathology* 92:997-1004.
42. Somai, B. M., Keinath, A. P., and Dean, R. A. 2002. Development of PCR-ELISA for detection and differentiation of *Didymella bryoniae* from related *Phoma* species. *Plant Dis.* 86:710-716.
43. Song, W. Y., Kim, H. M., Hwang, C. Y., and Schaad, N. W. 2004. Detection of *Acidovorax avenae* ssp. *avenae* in rice seeds using BIO-PCR. *J. Phytopathol.* 152:667-676.
44. Straub, T. M., Dockendorff, B. P., Quinonez-Diaz, M. D., Valdez, C. O., Shutthanandan, J. I., Tarasevich, B. J., Grate, J. W., and Bruckner-Lea, C. J. 2005. Automated methods for multiplexed pathogen detection. *J. Microbiol. Methods* 62:303-316.
45. Walcott, R. R., Castro, A. C., Fessehaie, A., and Ling, K. 2006. Progress towards a commercial PCR-based seed assay for *Acidovorax avenae* subsp. *citrulli*. *Seed Sci. Technol.* 34:101-116.
46. Walcott, R. R., Fessehaie, A., and Castro, A. C. 2004. Differences in pathogenicity between two genetically distinct groups of *Acidovorax avenae* subsp. *citrulli* on cucurbit hosts. *J. Phytopathol.* 152:277-285.
47. Walcott, R. R., and Gitaitis, R. D. 2000. Detection of *Acidovorax avenae* subsp. *citrulli* in watermelon seed using immunomagnetic separation and the polymerase chain reaction. *Plant Dis.* 84:470-474.
48. Walcott, R. R., Gitaitis, R. D., and Langston, D. B. 2004. Detection of *Botrytis aclada* in onion seed using magnetic capture hybridization and the polymerase chain reaction. *Seed Sci. Technol.* 32:425-438.
49. Weiland, J. J. 1997. Rapid DNA preparation from fungal spores and mycelia. *Fungal Genet. Newsl.* 44:60-63.
50. Willems, A., Goor, M., Thielemans, S., Gillis, M., Kersters, K., and Deley, J. 1992. Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov. comb. nov. *Acidovorax avenae* subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax konjaci*. *Int. J. Syst. Bacteriol.* 42:107-119.
51. Zhang, T., Herbert, H., and Fang, P. 2006. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. *Appl. Microbiol. Biotechnol.* 70:281-289.
52. Zitter, T. A., Hopkins, D. L., and Thomas, C. E., eds. 1996. *Compendium of Cucurbit Diseases*. The American Phytopathological Society, St. Paul, MN.