A Quantitative Real-time Polymerase Chain Reaction Assay for *Botrytis aclada* in Onion Bulb Tissue

Timothy W. Coolong¹

Department of Horticulture, The University of Georgia, Athens, GA 30602

Ronald R. Walcott

Department of Plant Pathology, The University of Georgia, Athens, GA 30602

William M. Randle

Department of Horticulture and Crop Science, The Ohio State University, Columbus, OH 43210

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Abstract. A real-time polymerase chain reaction (PCR) assay has been developed for the detection and quantification of *Botrytis aclada* (Fresenius), a causal agent of neck rot in onion (*Allium cepa* L.) bulbs. The assay uses TaqMan probe-based chemistry to detect an amplicon from the L45-550 region of *B. aclada* while using a DNA sequence from the onion serine acetyl transferase gene (*SAT1*) as a control. The assay detection limits for *B. aclada* and onion were 10 pg μL^{-1} of genomic DNA. The detection limit for lyophilized *B. aclada* mycelium was 1 μg . The presence of onion tissue in the samples did not affect the performance of the real-time PCR assay. The assay distinguished among different amounts of *B. aclada* mycelium growing on onion disks that were inoculated with 0, 10², or 10⁴ *B. aclada* conidia. Visual observations during the incubation period corresponded with changes in real-time PCR results. This assay could be used to determine the amount of *B. aclada* mycelium in bulbs during growth, harvest, and storage, thus giving researchers an objective and efficient tool by which to quantify the growth rate and virulence of *B. aclada* strains in vivo.

Onion (*Allium cepa* L.) is an economically important vegetable, accounting for nearly \$1 billion in farm gate income annually in the United States [United States Department of Agriculture National Agricultural Statistics Service (USDA NASS)]. In 2006, 44,000 ha of summer onions were grown for storage (USDA NASS). Postharvest rots can contribute to significant losses for growers and the primary storage disease of onion is neck rot. Losses to neck rot during storage can be as high as 35% in some years (Tietjen and Ceponis, 1981; Williams-Woodward, 2001).

Five *Botrytis* species have been linked to neck rot in onion (Yohalem et al., 2003). Three species, however, are considered exclusively associated with symptoms of neck rot in onion. These are *B. aclada* (Fresenius), *B. allii* (Munn), and *B. byssoides* (Walker) (Yohalem et al., 2003). Based on restriction fragment length polymorphism analysis, Nielsen et al. (2002) suggested that there were two distinct groups of *B. aclada*, types AI and AII, with the latter containing *B. allii*. It has been proposed that all three species are distinct and that *B. allii* was the

result of an interspecific hybridization between *B. aclada* and *B. byssoides* (Nielsen and Yohalem, 2001; Yohalem et al., 2003). Nonetheless, *B. aclada* and *B. allii* remain difficult to differentiate as a result of morphological similarities (Chilvers and duToit, 2006). Despite being pathogenic to onion bulbs, *B. byssoides* is infrequently detected relative to *B. aclada* or *B. allii* (Chilvers et al., 2004; du Toit et al., 2004; Nielsen et al., 2002).

Botrytis aclada can infect onions at any stage during the growing season. Potential sources of *B. aclada* inoculum include infected seeds, onion debris, and alternate crops (Maude, 1976; Walcott et al., 2004). Culling infected bulbs at harvest is challenging because onion plants may become infected without displaying visual symptoms of neck rot (Kritzman, 1983; Maude, 1990). With a latency period of 8 to 10 weeks, infected bulbs may appear asymptomatic at harvest but develop symptoms during storage, resulting in significant economic loss (Maude, 1990). Because visual inspection of intact bulbs in the field is ineffective, alternative methods have been developed to detect *B. aclada* in onion bulbs. These include culturing samples on semiselective media, enzyme-linked immunosorbent assay tests, and conventional polymerase chain reaction (PCR) detection (Kritzman and Netzer, 1978; Linfield et al., 1995; Nielsen et al.,

2002). Although all of these methods are useful in identifying the presence or absence of *B. aclada* in bulb tissue, none of them can be used to easily quantify the level of *B. aclada* inoculum in infected tissue. The ability to quantify inoculum in bulb tissue is of potential importance because it could allow for the objective assessment of disease severity and strain aggressiveness. Although other factors such as plant resistance, storage temperature, and relative humidity interact to determine the severity of neck rot infection, it would be useful to quantify the level of *B. aclada* inoculum present in bulbs at harvest (Alderman and Lacy, 1984; Bertolini and Tian, 1997). Potentially this could allow for the prediction of storage rot based on inoculum levels at harvest. Conventional diagnostic assays do not have the capacity to reliably quantify mycelial mass in onion tissue; however, quantitative real-time PCR represents one technique by which this can be accomplished.

Unlike traditional PCR, quantitative real-time PCR assays can simultaneously amplify and estimate the concentration of specific template DNA sequences (Wilhelm and Pingoud, 2003). The ability to estimate the amount of sequence-specific template DNA or RNA in a sample allows for the identification and quantification of pathogens in crops of interest. Real-time PCR uses fluorescent dyes that emit light of a specific wavelength during amplification (Schna et al., 2004). Fluorescence can be produced through nonspecific methods such as the fluorescence emitted by SYBR green as it is intercalated into double-stranded DNA or sequence-specific methods such as the light-emitting probes used in TaqMan or Scorpion PCR systems (Wilhelm and Pingoud, 2003). Regardless of the system, the amount of fluorescence increases as the product is amplified. In general, a threshold value for fluorescence is established, below which samples are considered negative. The number of cycles required for a sample to reach the threshold is the cycle threshold (Ct) value. The higher the concentration of template DNA, the fewer the number of amplification cycles required to reach the fluorescence threshold and the lower the Ct value will be for that sample (Wilhelm and Pingoud, 2003). Because the amount of pathogen template DNA should be proportional to the level of pathogen present, real-time PCR can be used to estimate fungal mass, viral load, or bacterial counts in a given sample.

Suarez et al. (2005) developed a TaqMan-based real-time PCR assay for the quantification of *B. cinerea* in *Pelargonium* species. This assay facilitated quantification of *B. cinerea* inoculum over four orders of magnitude. Furthermore, there was a positive relationship between estimates of inoculum concentration and the visible symptoms on leaf discs. Quantitative real-time PCR has been used for a range of pathogens in agronomic and horticultural crops including *Fusarium* spp. in wheat, *Rhizoctonia* spp. in tomato, and *Phytophthora ramorum* in

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¹To whom reprint requests should be addressed; e-mail timcoolong@uky.edu

Quercus spp. (Leivens et al., 2006; Schena, et al., 2006; Schnerr, et al., 2001; Tooley et al., 2006).

Recently Chilvers et al. (2007) developed a real-time PCR assay for the detection *Botrytis* spp. in onion seed. Using primers developed for the intergenic space region of *Botrytis* spp., the authors detected *Botrytis* DNA at levels of $10 \text{ fg} \cdot \mu\text{L}^{-1}$. However, the real-time PCR assay was applied for pathogen detection in onion seed and did not consider estimation of mycelial colonization in bulb tissue (Chilvers et al., 2007).

Our objective in this study was to demonstrate that real-time PCR could be used to reliably quantify the level of *B. aclada* colonization in inoculated onion tissue while using a reference gene, serine acetyl transferase (*SAT1*), from onion to normalize data.

Materials and Methods

Fungal isolates. Fungal isolates *B. aclada* 703-308 and *B. cinerea* 870-404A, 870-404B, 870-404C were routinely maintained on potato dextrose agar (PDA) (Beckton-Dickinson, Sparks, MD). The mycelia used for DNA extraction was produced by culturing *B. aclada* 703-308 in potato dextrose broth with agitation for 7 d at 20°C . Mycelia were harvested by passing the liquid culture through a $0.45\text{-}\mu\text{m}$ filter disk and freezing the mycelial mat in liquid nitrogen. The mycelia was then lyophilized and ground into a fine powder using a mortar and pestle. Cultures from two common onion bacterial pathogens, *Pantoea ananatis* 99-8 and *Burkholderia cepacia* 92-1, were maintained at -80°C and routinely grown on Luria-Bertani broth ($25 \text{ g} \cdot \text{L}^{-1}$). Onion bulbs cv. 'Pegasus' were greenhouse grown until maturity. Approximately 100 g fresh tissue from mature bulbs was frozen in liquid nitrogen, lyophilized, and ground into a fine powder using a mortar and pestle.

DNA extraction. DNA was extracted from lyophilized fungal and onion tissue using a modified phenol extraction method (Ellington and Pollard, 1999). To a $100\text{-}\mu\text{L}$ suspension containing 1 mg of fungal or onion tissue, 0.4 mL extraction buffer containing 200 mM Tris HCl, 250 mM NaCl, 25 mM EDTA, and 0.5 w/v sodium dodecyl sulfate was added and heated at 65°C for 10 min. Then 0.5 mL of 25:24:1 phenol/chloroform/isoamyl alcohol was added, and the mixture was centrifuged at $10,000 \text{ g}$ for 60 s. The aqueous phase was removed and reextracted in 0.4 mL of 25:24:1 phenol/chloroform/isoamyl alcohol. The DNA was precipitated with one-tenth volume of 3 M sodium acetate (pH 5.2) followed by the addition of two volumes of 70% ethanol and incubation at -20°C for 15 min. The precipitated DNA was then centrifuged for 5 min at $10,000 \text{ g}$ and the pellet washed twice with 1 mL of 100% ethanol and centrifuged for 5 min at $10,000 \text{ g}$. The pellet was dried at 25°C for 10 min and redissolved in $50 \mu\text{L}$ sterile water.

Fig. 1. Polymerase chain reaction amplification of the *Botrytis aclada* L45-550 fragment and onion (*Allium cepa* L.) *SAT1* gene using primers and probes described in Table 1. Lanes 1 and 8: the 100-base pair (bp) ladder ranging from 100 to 1000 bp; lanes 3 and 4: 200-bp amplicons for the onion *SAT1* gene; lanes 6 and 7: 200-bp amplicons from the L45-550 fragment of *B. aclada*, lanes 2 and 5: negative control lanes of onion and *B. aclada* template DNA, respectively, without Taq polymerase.

Primer and probe design for *B. aclada*. Conventional PCR primers previously described for detection of *B. aclada* in onion were used in this study (Nielsen et al., 2002). The oligonucleotide BA2F (Table 1) developed in that study was used as the forward primer. A new reverse primer (BA3R) and probe were designed using PrimerQuest software [Integrated DNA Technologies (IDT), Coralville, IA] based on the L45-550 region from *B. aclada* (Nielsen et al., 2002). For real-time PCR, the TaqMan system was used and the Taqman probe, BaProbe (Table 1), was dual-labeled with Texas Red-X at the 5' end and Black Hole Quencher 2 (BHQ2) at the 3' end (IDT).

Primer and probe design for an internal control using the onion *SAT1* gene. A second real-time PCR assay was developed as a negative control targeting the onion *SAT1* gene (McManus et al., 2005). This was necessary to ensure that DNA extraction and amplification efficiency was similar among samples and to guard against false-negative signals resulting from poor DNA quality. Onion *SAT1* was chosen because it is a well-characterized onion gene (McManus et al., 2005). Primers and probe were designed as previously described (McManus et al., 2005) (Table 1). The TaqMan probe, SatProbe, was dual-labeled with 6-carboxy-fluorescein (6-FAM; IDT) and Black Hole Quencher 1 (BHQ1; IDT) at the 5' and 3' ends, respectively (Table 1).

Polymerase chain reaction conditions. Real-time PCR assays of *B. aclada* and onion DNA were performed in separate $25\text{-}\mu\text{L}$ reactions using the Cepheid Smart Cycler (Cepheid, Sunnyvale, CA). Routinely, real-time PCR was performed using the commercially available Eppendorf RealMasterMix Probe containing HotMaster Taq DNA polymerase with self-adjusting magnesium buffer (Eppendorf, Hamburg, Germany). Final primer and probe concentrations for the real-time PCR assays were 300 nM and 200 nM, respectively. The thermal profile used for DNA amplification included an initial denaturation at 95°C for 105 s followed by

40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 68°C for 30 s. Fluorescence was measured during the extension step in each cycle and samples were considered to be positive when fluorescence exceeded 30 fluorescence units. For quantitative PCR, the partial cycle number at which fluorescence for a reaction surpassed the background fluorescence, the C_t value, was recorded.

To confirm identities of the L45-550 region and *SAT1* sequences, PCR amplicon lengths were confirmed using gel electrophoresis (Fig. 1). PCR products were separated in a 1% agarose gel containing $0.5 \mu\text{g} \cdot \text{mL}^{-1}$ ethidium bromide in 0.04 M Tris acetate buffer with 2 mM EDTA (Ellington and Pollard, 1999) buffer at 90 V for 60 min. PCR products were cloned using the TOPO-TA cloning kit (Invitrogen Corp., Carlsbad, CA) following the manufacturer's directions. Cloned DNA from *B. aclada* and onion was purified using the Qiagen Plasmid Mini Kit (Qiagen, Valencia, CA) and sequenced on an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA) at The University of Georgia Integrated Biotech Sequence and Synthesis Facility (University of Georgia, Athens, GA).

Specificity of the *B. aclada* and onion TaqMan polymerase chain reaction assay. The specificity of the real-time PCR assays for *B. aclada* and the onion control gene were tested using DNA from *B. cinerea*, *Pantoea ananatis*, the causal agent of center rot, and *Burkholderia cepacia*, the causal agent of sour skin. DNA was extracted from isolates of *B. cinerea* 870-404A, 870-404B, 870-404C as well as isolates of the bacterial pathogens *P. ananatis* 99-8 and *B. cepacia* 92-1. Five nanograms of DNA were used as a template and reactions were designed as described previously. Each reaction was carried out in triplicate, and samples that yielded a C_t value greater than 39 were considered to be negative.

Relationship between DNA concentration cycle threshold value and *B. aclada* mycelial mass. Real-time PCR was performed on *B. aclada* and onion samples of known DNA concentrations to confirm that C_t values and DNA concentrations had a statistically significant negative linear relationship and to determine the detection limits for the assay. Tenfold serial dilutions of *B. aclada* and onion genomic DNA were generated from $100 \text{ ng} \cdot \mu\text{L}^{-1}$ to $1 \text{ pg} \cdot \mu\text{L}^{-1}$ and real-time PCR was conducted on four replicates of each dilution as described previously. To determine if the presence of onion DNA interfered with the amplification of *B. aclada* DNA and vice versa, 50 ng of onion DNA and 5 ng of *B. aclada* DNA were added to the serial dilutions of *B. aclada* and onion DNA, respectively. The DNA mixtures were tested using real-time PCR. Regression analyses were performed on data from *B. aclada* DNA with and without onion DNA to determine the precision of the test and whether the presence of nontarget DNA altered the results. This experiment was replicated four times.

Table 1. Nucleotide sequences of TaqMan primers and probes used to detect *Botrytis aclada* and onion (*Allium cepa* L.) DNA used in this study.

Name	Sequence 5'-3'	Function	Source
SatF1	GAT CGG TGA AAC TGC AGT CAT AGG	Onion <i>SAT1</i> ² forward primer	This study
SatR1	CAT CGA TCA GAA CAA CAG ACC CTG	Onion <i>SAT1</i> reverse primer	This study
SatProbe	6-FAM-AGG GAA TAT CAG AAT CGG TGC TGG G-BHQ-1	Onion <i>SAT1</i> probe for TaqMan assay	This study
BA2F	GTG GGG GTA GGA TGA GAT GAT G	<i>B. aclada</i> L45-550 forward primer	Nielsen et al., 2002
BA3R	TTG AAT TGG GAG AGC GTT CCT TCG	<i>B. aclada</i> L45-550 reverse primer	This study
BaProbe	TexRed-TCC GCC CTT GTT GAT GAA GTC GAG AA-BHQ2	<i>B. aclada</i> L45-550 probe for TaqMan [®] assay	This study

²SAT1 = serine acetyl transferase gene in onion.

To determine the detection threshold of the real-time PCR assay, tenfold serial dilutions of *B. aclada* mycelia suspended in water were made from 10 to 0.001 mg·mL⁻¹ lyophilized mycelia. DNA extractions and real-time PCR analysis were carried out as previously described. To determine the effects of adding onion tissue to the assay, 5 mg of lyophilized onion tissue was added to each dilution of *B. aclada* mycelium. This experiment was replicated four times.

To determine the accuracy and precision of the real-time PCR method, 1 mg lyophilized *B. aclada* mycelia and 5 mg lyophilized onion tissue were added to the same vial, suspended in 1 mL of sterile distilled deionized water, and DNA extracted as described previously. This was replicated seven times and real-time PCR was used to estimate *B. aclada* and onion DNA concentrations. The results of the *B. aclada* assay were normalized by multiplying the estimated DNA concentration by a normalization factor obtained by dividing Ct values obtained for the mean of all onion samples by the Ct value determined from the onion DNA in each replicate.

Detecting *B. aclada* in onion bulb tissue. To determine if results from the real-time PCR assay corresponded to visual observations of fungal growth, an assay was conducted using onion disks inoculated with *B. aclada* conidia. Asymptomatic greenhouse-grown onion bulbs, cv. Pegasus, were peeled and the first fleshy scale removed. Seven-millimeter diameter disks were taken from the second fleshy scale of each bulb and washed three times for 10 s each in 70% ethanol and once for 30 s in sterile water. The average weight of each disk was 193.7 mg (± 2.3 mg). Three randomly chosen disks were then plated onto individual petri dishes containing PDA. Each disk on a dish was stab-inoculated with 10 μ L of sterile deionized water containing 0, 10², or 10⁴ *B. aclada* conidia. Conidial suspensions were obtained from 7- to 10-d-old PDA cultures of *B. aclada* 703-308 by flooding the colony with sterile water and gently rubbing the colony surface with a glass rod to dislodge conidia. Each conidial suspension was collected in a 50-mL tube and conidial concentrations enumerated using a hemacytometer. Onion disks with each of the three inoculum levels were included in each dish and each dish served as a single replication for one of three incubation times (0, 72, 120 h). After inoculation, dishes were sealed with parafilm (Pechiney Plastic Packaging, Chicago, IL) and incubated in the dark at 20 °C and 80% relative

humidity. After incubation, the disks were visually examined, photographed, and subjected to DNA extraction and the real-time PCR assay as previously described.

Statistical analyses. Results were subjected to linear regression and the Proc GLM procedure using SAS statistical software (SAS v.9.1; SAS Institute, Cary, NC). Data were considered significant at $P < 0.05$.

Results

Specificity of the *B. aclada* and onion TaqMan assays. The sequences of the 200-base pair (bp) PCR products from the L45-550 and *SAT1* regions of the *B. aclada* and onion genomes, respectively, were confirmed by gel electrophoresis and sequencing (Fig. 1). Blast searches of the National Center for Biotechnology Information database for the *B. aclada* amplicon revealed 99.5% homology with the 546 bp L45-550 randomly amplified polymorphic DNA fragment from *B. aclada* AI strain BA8 (GenBank accession AJ291477). Blast searches of the onion amplicon showed a 99% match to the 1094 bp complete cDNA sequence for the onion *SAT1* gene (GenBank accession AF212156). Specificity of primer and probe combinations were confirmed for both *B. aclada* and onion PCR assays using template DNA from a variety of sources. Both the *B. aclada* and onion real-time PCR assays were negative for samples containing 5 ng· μ L⁻¹ template DNA from *B. cinerea* 870-404A, 870-404B, 870-404C, *P. ananatis*, and *B. cepacia*. The real-time PCR assay for *B. aclada* was also negative when only onion template DNA was provided and vice versa.

Relationship between DNA concentration and cycle threshold values. The real-time PCR assay showed a significant negative linear relationship between *B. aclada* DNA concentration and Ct value ($R^2 = 0.97$) and successfully detected *B. aclada* DNA across five orders of magnitude at concentrations ranging from 100 ng· μ L⁻¹ to 10 pg· μ L⁻¹ genomic DNA (Fig. 2A). The addition of 50 ng onion DNA to the substrate did affect the detection limit or overall performance of the TaqMan assay for quantifying *B. aclada* DNA (Fig. 2A). A negative linear relationship was also observed between onion DNA concentrations and Ct value (Fig. 2B). The onion assay successfully amplified and quantified onion genomic DNA across five orders of magnitude from 100 ng· μ L⁻¹ to 10 pg· μ L⁻¹ (Fig. 2B). The addition of 5 ng *B. aclada* DNA to onion DNA did not affect the

Fig. 2. (A) Detection of *Botrytis aclada* DNA using a real-time polymerase chain reaction (PCR) assay based on the L45-550 DNA sequence. —●— *B. aclada* DNA, ...○... *B. aclada* DNA with 50 ng onion genomic DNA in each sample. (B) Detection of onion DNA using the real-time PCR assay for the *SAT1* genomic region. —●— Onion DNA, ...○... onion DNA with 5 ng *B. aclada* DNA in each sample. Analyses at each concentration (A and B) were replicated four times. In each sample, the cycle threshold (Ct) value for a positive response was a fluorescence value of 30.

detection limit or trend of the real-time PCR assay (Fig. 2B).

Relationship between mycelial weight and cycle threshold value. The *B. aclada* real-time PCR assay was able to amplify DNA from *B. aclada* mycelium across five orders of magnitude, from 10 mg to 1 μ g lyophilized mycelium. The relationship between mycelial mass and Ct values was negative and linear (Fig. 3). When 5 mg lyophilized onion tissue was added to each sample, the slope of the standard curve changed significantly, but the detection limit was not significantly different from results without onion tissue ($P = 0.288$) (Fig. 3). The standard curve obtained for mycelial weight plotted against

Fig. 3. Standard curves for detection of *Botrytis aclada* mycelium using a real-time polymerase chain reaction assay for the *L45-550* region of the *B. aclada* genome. —●— *B. aclada* mycelium, ...○... *B. aclada* mycelium with 5 mg lyophilized onion tissue in each sample. Each mycelial weight was replicated four times with and without onion tissue. In each sample the cycle threshold (Ct) value for a positive response was a fluorescence value of 30.

Ct value in the presence of onion tissue was: Ct value = $-1.47 \ln(\text{mycelia weight}) - 23.42$. This standard curve was used to calculate recovery of lyophilized mycelia and infection levels in subsequent experiments.

Using the curve developed for the relationship between mycelial weight and Ct value, the percentage recovery for *B. aclada* mycelium was determined. After normalizing with the response from the onion *SAT1* real-time PCR assay, samples containing 1 mg lyophilized mycelium and 5 mg lyophilized onion tissue displayed a mean recovery of $0.992 \text{ mg} \pm 0.202 \text{ mg}$ mycelium. This equated to a mean recovery of 99.2% with a cv of 20.2% for the seven 1-mg samples of mycelium.

Quantitative real-time polymerase chain reaction assay of inoculated onion bulb disks.

The ability of the real-time PCR assay to detect and quantify *B. aclada* mycelium in inoculated onion bulb tissue was tested. As expected, *B. aclada* was not detected in noninoculated onion disks. The estimated *B. aclada* mycelial mass was significantly higher for disks inoculated with 10^4 conidia than those inoculated with 10^2 conidia ($P < 0.001$). Furthermore, mycelial mass increased significantly with incubation time of the inoculated disks ($P < 0.001$) (Fig. 4A). Immediately after inoculation (0 h), *B. aclada* was only detected in disks inoculated with 10^4 conidia (Fig. 4A). However, after 72 h of incubation, *B. aclada* was detected in disks inoculated with 10^2 and 10^4 conidia with average mycelial masses of 0.022 and 0.486 mg mycelium/disk $^{-1}$, respectively. After 120 h of incubation, mean fungal mycelium was estimated at 1.5 and 2.4 mg for disks inoculated with 10^2 and 10^4 conidia, respectively (Fig. 4A). The real-time PCR assay results for conidial number and dura-

Fig. 4. (A) Average level of *Botrytis aclada* mycelial weight with se bars of onion disks stab inoculated with *B. aclada* conidia and incubated for 0, 72, and 120 h before mycelial weights were determined. Each bar represents the mean of three replicates. (B) The average amount of onion DNA present in onion disks inoculated with conidia.

tion of incubation were similar with Ct values decreasing as both conidia and incubation time increased. The results obtained from the real-time PCR assay correspond well to visual observations of fungal growth on the onion disks at the time of sampling. Fungal mycelia appeared on onion disks after 72 h and were most easily seen on disks inoculated with 10^4 conidia. By 120 h of incubation, profuse mycelial growth was observed in disks inoculated with 10^2 and 10^4 conidia (Fig. 5).

Onion template DNA concentrations were significantly different for each incubation time ($P < 0.001$) decreasing by more than 50% during the 120 h incubation. However, onion DNA concentrations were unaffected by conidia treatment levels ($P = 0.133$). Because concentration of onion template DNA significantly decreased in the onion disks over time, average onion DNA concentrations at each sampling time were used for normalization of *B. aclada* data (Fig. 4B).

Discussion

Neck rot is one of the most economically important diseases of stored onion bulbs. Although the open wound resulting from the removal of foliage at harvest is an important site for bulb infection, many plants are infected early in the growing season without displaying visible disease symptoms (Maude, 1990). Conidia produced during the season can infect plants at the seedling stage (du Toit, et al., 2004; Maude et al., 1982; Maude and Presly, 1977), resulting in asymptomatic plants, which can develop symptoms of neck rot during storage (Maude, 1990). The ability to quantify the level of inoculum in bulbs at different sampling times either during growth or during storage could yield insight into the growth patterns or virulence of the pathogen. In turn, this might be used to predict the level of rot during storage based on the amount of inoculum present in bulbs at harvest.

Although quantitative PCR using SYBR green technology (Wilhelm and Pingoud, 2003) is common, it has several pitfalls that make it less appropriate for disease quantification than sequence (probe)-based systems. First, a probe-based system adds another level of specificity to the assay, because both primers and a probe must hybridize to the template DNA sequence before amplification and signal generation. Second SYBR green-based assays require postamplification dissociation curve analysis (melting point analysis) to ensure that nonspecific amplicons did not contribute to fluorescence (Wilhelm and Pingoud, 2003). Additionally, a probe-based system could allow for the detection and quantification of multiple target sequences in a single reaction cell, thereby increasing the efficiency of the assay. Although multiplex real-time PCR assays have been successfully developed for pathogen quantification (James et al., 2006; Schena et al., 2006) attempts to develop such a system in this study were limited by competition between the amplification reactions of *B. aclada* and the *SAT1* gene. Hence, PCR reactions for the two targets were conducted separately.

The assay developed in this study was as sensitive as the conventional PCR assay for *B. aclada* (Nielsen et al., 2002) and was unaffected by the presence of onion DNA. The assay detected DNA extracted from lyophilized *B. aclada* mycelium over five orders of magnitude with a detection limit of 1 μg of mycelia. This is important, because a primary goal of this study was to use real-time PCR to accurately measure mycelial mass. The high percentage recovery observed further indicates that this method is accurate. Although a cv value for quantification of 20.2% may seem high, it is considered close to the minimum relative error for quantification for real-time PCR assays (Wilhelm and Pingoud, 2003). The explanation for the high cv observed is that very small differences in Ct value can be magnified when amounts of starting template are calculated

Fig. 5. Images of 7-mm diameter onion (*Allium cepa* L.) disks stab-inoculated with 0, 100, and 10,000 conidia of *Botrytis aclada* and incubated on plates of potato dextrose agar for 0, 72, and 120 h.

using standard curves. For example, in this test, the average Ct value for 1 mg of *B. aclada* mycelia was 22.1 cycles with a SD of 0.52 cycles. This equated to a cv 2.3% when calculated based on Ct values alone. Because Ct values are based on logarithmic units, they do not represent true variation and should not be used to calculate variation in real-time PCR assays (Wong and Medrano, 2005).

Using the *SAT1* gene as a control in *B. aclada* mycelium quantification should prevent false-negative results with real-time PCR. During incubation for 120 h, onion template DNA concentrations decreased significantly. This was likely the result of degradation of DNA in the onion tissue while at room temperature but could have been the result of decreasing extraction efficiencies at each sampling time. Therefore, the average DNA concentration obtained at each sampling time was used to normalize the mycelial weights obtained for duration of incubation assessed. Failure to do so would have resulted in overestimating mycelial growth at longer incubation times. Other studies have reported similar decreases in recovery of DNA from degraded plant tissues incubated at room temperature for extended periods of time (Ceccherini et al., 2003; Rostag, 1992; Thomas and Stoddart, 1980).

A positive relationship between visual symptoms of disease in *Pelargonium* leaf disks infected with *B. cinerea* spores and detection of DNA using a real-time PCR assay has been reported previously (Suarez et al., 2005). Leaf disks infected with 10^3 *B. cinerea* spores showed more severe colonization and earlier symptom development than leaf disks infected with 10 spores. The more severely infected leaves also showed a

concomitant rise in Ct value when assayed using a TaqMan real-time PCR assay for *B. cinerea*. In this study, those disks that visually appeared to be more heavily colonized also were determined to have greater weights of mycelium by the real-time PCR assay. Although the difference in mycelium weight was greater between the 10^2 and 10^4 conidia inoculation treatments after 72 h of incubation than after 120 h, there was 1.6 times more mycelium present in the disks treated with 10^4 conidia than those treated with 10^2 conidia when measured at 120 h. The reason for this smaller difference in mycelium weight at 120 h of incubation is that the onion disks in the 10^2 and 10^4 conidia treatments were heavily colonized (saturated) after 120 h. Additionally, although the diameters of the mycelial colonies on the plates after 120 h were greater for the 10^4 conidia treatments, DNA was only extracted from the onion disks. This might partially explain the small differences in mycelial weights between the two conidia treatments at 120 h. Longer-term studies may require the use of larger onion disks.

In future studies, the real-time PCR assay developed in this study could be used to reliably quantify the level of *B. aclada* mycelium growth in onion tissue. This could allow for the investigation of growth patterns of *B. aclada* during storage or throughout the growing season. It would also allow for comparisons of the relative aggressiveness of different strains of *B. aclada* in onion tissue. These data suggest that real-time PCR could eventually be used to predict neck rot incidence in storage based on initial bulb inoculum levels at harvest. Further research is needed to evaluate the applicability of real-time PCR for whole bulb assessment.

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