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TaqMan real-time PCR method for detection of *Discula destructiva* that causes dogwood anthracnose in Europe and North America

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Abstract Dogwood anthracnose, caused by the asexual filamentous fungus *Discula destructiva* Redlin, is a disease of several *Cornus* L. species. First reported in the 1970s in the United States, the pathogen has devastated North American dogwood populations causing widespread loss that has greatly impacted forest ecosystems. In the early 2000s, the disease was discovered in Italy and Germany, spread probably through the ornamental nursery trade. The origin of *D. destructiva* in North America remains a mystery. To facilitate studies on its origin and dispersal, a fast and accurate method using real-time PCR was developed in this study to detect and quantify *D. destructiva*. The assay was validated with samples from the U.S., Italy, and Switzerland as well as phylogenetically closely related fungal species, and other fungi and oomycetes commonly found on *Cornus*. This method allows for fast and sensitive detection of *D. destructiva* in host tissue and should be useful in disease management and pest interception to prevent further spread of the pathogen.

Keywords Diagnosis · Fungi · Invasive species · Molecular detection · Plant disease · qPCR

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Introduction

Dogwood anthracnose is a disease of several dogwood species native to North America. It is caused by the asexual fungus *Discula destructiva* Redlin (Zhang and Blackwell 2001). Phylogenetic studies placed this species in the Diaporthales, an ascomycete order containing a number of woody plant pathogens, including the chestnut blight pathogen *Cryphonectria parasitica* (Murrill) Barr (Anagnostakis 1987; Milgroom et al. 1996; Milgroom 1995; Milgroom and Lipari 1995). Dogwood anthracnose disease symptoms, which usually start to develop in spring and early summer, include leaf spots, leaf blights, twig dieback and trunk cankers. Infected trees may be killed in one to three years from exposure or exhibit symptoms of varying severity depending on environmental conditions (Daughtrey and Hibben 1994). The disease was first reported on the Pacific

dogwood (*Cornus nuttallii* Audubon ex Torr. & Gray) in the northwestern United States in the late 1970s. It also appeared on the flowering dogwood (*C. florida* L.) in southeastern New York and southwestern Connecticut at almost the same time. Since then, the disease has spread rapidly through North American native dogwood populations in the forests, as well as ornamental plantings. Infections have been confirmed from British Columbia to northern California in western North America and from Vermont to the higher elevations of Georgia and Alabama in the southeastern U. S (Daughtrey et al. 1996). The disease has caused more than 90% dogwood tree mortality and significant forest succession in some forest types (Holzmueller et al. 2006; Pierce et al. 2008). The widespread loss of *Cornus* species threatens the ecological integrity of forest ecosystems, including nutrient cycling, and calcium and food source availability for wildlife (Jenkins et al. 2007; Schrope 2001). The disease also causes economic loss in the ornamental nursery industry, especially in the southeastern U. S (Anderson et al. 1990).

Populations of *D. destructiva* in North America are morphologically indistinguishable. Previous studies have revealed a very low population genetic diversity based on multilocus sequence typing and DNA fingerprinting (Trigiano et al. 1995; Zhang and Blackwell 2002). However, isolates from the eastern and western U. S. populations can be distinguished by a fixed G-A transition at the 5′ end of the translation elongation factor-1α gene (EF-1α) (Zhang and Blackwell 2002).

The origin of *D. destructiva* in North America is unknown. The sudden appearance of dogwood anthracnose near several U. S. ports (Daughtrey and Hibben 1994), its low population genetic diversity (Zhang and Blackwell 2002) and the fact that the Asian native dogwood species *C. kousa* Hance is naturally resistant to the fungus suggest that *D. destructiva* was introduced from Asia, probably carried by the Kousa dogwood host (Daughtrey et al. 1996; Trigiano et al. 1995). In its native range, *D. destructiva* is likely an endophyte or latent pathogen that does not cause typical anthracnose symptoms to the host plant. However, these hypotheses regarding the pathogen's origin have not been tested, partly due to lack of a fast and accurate detection tool for *D. destructiva*. Until nine years ago, *D. destructiva* had not been reported from outside of North America. In 2002, it was observed on *C. florida* in Germany and in 2003 on *C. florida* and *C. nuttallii* in Italy (Stinzing and Lang 2003; Tantardini et al. 2004). Then in June 2009, leaf spot and leaf blight symptoms were observed on *C. florida* in Switzerland.

To date, dogwood anthracnose identification has relied on disease symptoms and pathogen culture morphology. *Discula destructiva* is slow growing in culture and the identification process may take more than two weeks. It does not sporulate readily on conventional media, making morphological identification challenging (Daughtrey and Hibben 1994). In addition, similar leaf spot and twig dieback symptoms can be caused by other dogwood pathogens, such as *Colletotrichum acutatum* Simmonds, and therefore specialized skills are required to perform an accurate diagnosis (Farr 1991; Redlin 1991).

Real-time, or quantitative, polymerase chain reaction (real-time PCR or qPCR) is a powerful molecular analysis technique that has been used for a broad range of research, such as genotyping, quantitative gene expression analysis, drug target validation, and pathogen detection (Mackay 2007). It has been applied in the diagnosis of plant pathogens since the late 1990 (Schaad et al. 1999). This molecular technique offers a fast, accurate and culture-independent method for the detection of microbial pathogens from soil, water, and host plants. In real-time PCR, fluorescence data are collected during the log phase of PCR when the quantity of the PCR product is directly proportional to the amount of initial template nucleic acid. No post-PCR processing is needed. Therefore, real-time PCR is faster and it quantifies target DNA more accurately than many other molecular techniques such as conventional PCR and DNA diagnostic array. With the demand for rapid and precise disease diagnostic techniques being the driving force, the interest in real-time PCR technique in phytopathological research has increased exponentially in the past decade (Schaad and Frederick 2002; Mackay 2007; McCartney et al. 2003; Okubara et al. 2005). The main limitations of this method are its relative high cost compared to conventional PCR and lower detection throughput compared to DNA diagnostic array techniques (Okubara et al. 2005; Gachon et al. 2004; Daughtrey and Hibben 1994).

To facilitate disease management and studies on the origin and biogeography of the dogwood anthracnose pathogen, we developed a real-time PCR assay, to directly detect *D. destructiva* from both fresh and

dried plant tissue. TaqMan and SYBR green are the most widely used chemistries of real-time PCR. In this study, we chose TaqMan because of its higher detection specificity and sensitivity (Okubara et al. 2005).

Materials and methods

Microorganisms Isolates of *D. destructiva* and other microorganisms used in this study are shown in Table 1. *Discula destructiva* strains were isolated from *Cornus florida* and *C. nuttallii* in eastern U. S. (n=7), western U.S. (n=2), Italy (n=1), and Switzerland (n=4), between 1990 and 2009. Reference organisms used to evaluate the specificity of the real-time PCR method included (1) fungal species phylogenetically close to *D. destructiva* and (2) species frequently isolated from *Cornus* leaves or other hosts inhabiting the same environment. Dogwood powdery mildew *Erysiphe pulchra* (Cooke & Peck) U. Braun & S. Takam. conidia and mycelium were scraped directly from the host tissue. All other microbial isolates were grown on potato dextrose agar (PDA, BD Diagnostic Systems, MD, USA) under 22°C in cycles of 12 h light and 12 h dark for 3–7 days before DNA extraction. All isolates were maintained in 10% glycerol at −80°C.

Natural and inoculated samples Twenty-eight plant samples from natural forests, public parks or nurseries with various symptoms, or no symptoms, were used in this study (Table 2). Among them, 17 *Cornus* leaf samples were collected in the U. S. (New Jersey, Maryland, Indiana, Ohio, and Oregon) from April to September 2009. An infected Italian *Cornus* leaf sample (Italy-SubE) was collected in June 2010 and shipped to the U.S. under ambient temperature. Two European *D. destructiva* isolates (D52 and D53) were inoculated on detached *C. florida* leaf disks, which also were tested. Inoculation followed previously published procedures (Zhang and Blackwell 2002). Healthy *C. florida* leaves (New Jersey) were cut into 22-mm diameter disks, surface disinfested, and inoculated with *D. destructiva* mycelium in agar plugs (4×4 mm). The inoculated leaf disks were incubated at 22°C with a 12 h light/dark cycle for 3 days before assay. In addition, a *Carya illinoinensis* (Wangenh.) K. Koch sample showing leaf spot and powdery mildew symptoms was included. Seven New Jersey samples (D30dry-D36dry) were air dried at 30°C for 14 days before assay to determine if the detection method can be applied to dried specimens. Except for Italy-SubE that was partially rotten due to shipment under ambient temperature, all other leaf samples were used immediately after collection or kept fresh in 4°C for 1–7 days before assay.

Conventional detection of D. destructiva from leaves The 28 leaf samples were tested for the presence of *D. destructiva* using traditional culture methods. Surface disinfested leaves were cut into 5 mm squares and placed onto 2% malt extract agar (MEA, BD Diagnostic Systems, MD). The leaves were then incubated at 22°C with a 12 h light/dark cycle for 21 days, and were examined with a stereomicroscope (up to 63X magnification) every 1–2 days. Filamentous fungi that grew from the leaf samples were transferred to PDA plates and were purified with a hyphal tipping method. Purified fungal cultures were then identified by colony and conidial morphology and were further confirmed by determining DNA sequences of the internal transcribed spacer region (ITS) of the ribosomal RNA genes.

DNA extraction, PCR, and sequencing Genomic DNA was extracted from 5–10 mg of fresh mycelium from each of the isolates (Table 1), and from10 mg of the fresh or 5 mg of the dry plant samples (Table 2) using the UltraClean Soil DNA Isolation Kit (MoBio, CA) following the manufacturer's protocols. For the microbial isolates, the ITS region was amplified with primers ITS4 and ITS5 (White et al. 1990) or ITS1-F (Gardes and Bruns 1993). PCR was carried out in a 2720 Thermo Cycler (Applied Biosystems, CA) with 1 unit AmpliTaq® DNA Polymerase (Applied Biosystems, CA), 1× buffer II (Applied Biosystems, CA), 2.5 mM $MgCl_2$, 0.8 mM dNTPs, 0.2 μM each primer, and 5 ng of template DNA in a 50 μl reaction. Cycling conditions consisted of an initial 5 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C; and a final step of 10 min at 72°C. PCR products were purified using QIAquick PCR purification kit (Qiagen, CA) following the manufacturer's protocol. The purified amplicons were sequenced with the primers ITS5, ITS1-F or ITS4 and the sequence data were used to confirm the morphological identification.

Table 1 List of fungal species, isolates and characteristics used to develop and evaluate the real-time PCR method for *Discula destructiva*

Species	Isolate number [a]	Host	Location	Year of collection	Collector [b]	qPCR Mean Ct value (standard deviation) [c]
Alternaria alternata	D30 (E1-8)	*Cornus florida*	New Jersey, USA	2009	NZ	–
Amphiporthe hranicensis	AR3651 = CBS119289	*Tilia platyphyllos*	Austria	2001	AR	–
Apiognomonia errabunda	AR2813 = CBS109747	*Fagus sylvatica*	Switzerland		AR	–
Colletotrichum acutatum	D33-10 (4E-1)	*Cornus florida*	New Jersey, USA	2009	NZ	–
Cryptosporella femoralis	AR3868 = CBS121076	*Alnus rugosa*	New York, USA		AR	–
Diaporthe eres	D54	*Cornus florida*	Vertemate con Minoprio, Italy	2009	AT	–
Discula destructiva	D24-1	*Cornus florida*	Maryland, USA	2009	RL	16.7 (0.03)
Discula destructiva	D33-1 (E4-1)	*Cornus florida*	New Jersey, USA	2009	NZ	18.5 (0.02)
Discula destructiva	D49	*Cornus florida*	Sonvico, Switzerland	2009	AT	17.6 (0.08)
Discula destructiva	D50	*Cornus florida*	Sonvico, Switzerland	2009	AT	19.3 (0.09)
Discula destructiva	D51	*Cornus florida*	Sonvico, Switzerland	2009	AT	18.5 (0.05)
Discula destructiva	D52	*Cornus florida*	Sonvico, Switzerland	2009	AT	16.0 (0.05)
Discula destructiva	D53	*Cornus florida*	Vertemate con Minoprio, Italy	2009	AT	13.4 (0.20)
Discula destructiva	MD235 = ATCC76231	*Cornus florida*	Maryland, USA	1990	SCR	20.4 (0.32)
Discula destructiva	MD237-1	*Cornus florida*	Maryland, USA	1990	SCR	20.4 (0.05)
Discula destructiva	MD304	*Cornus florida*	Maryland, USA	1990	SCR	22.3 (0.31)
Discula destructiva	NC009	*Cornus florida*	North Carolina, USA	2000	NZ	17.7 (0.48)
Discula destructiva	PA03082004	*Cornus florida*	Pennsylvania, USA	2004	NZ	19.8 (0.05)
Discula destructiva	WA220-1	*Cornus nuttallii*	Washington, USA	1990	SCR	19.2 (0.37)
Discula destructiva	WAP272-1	*Cornus nuttallii*	Washington, USA	1990	SCR	22.2 (0.12)
Epicoccum nigrum	D33-4 (4P-6)	*Cornus florida*	New Jersey, USA	2009	NZ	–
Erysiphe pulchra	D22-1	*Cornus florida*	New Jersey, USA	2009	NZ	–
Gnomonia gnomon	AR4071				AR	–
Pestalotiopsis sp.	D30-7 (1P-4)	*Cornus florida*	New Jersey, USA	2009	NZ	–
Phomopsis sp.	D33-3 (4P-5)	*Cornus florida*	New Jersey, USA	2009	NZ	–
Plagiostoma fraxini	AR2789 = CBS109498	*Fraxinus pennsylvanica*	Maryland, USA		AR	–
Pleuroceras pleurostylum	CBS906.79	*Salix lapponum*	Sweden		AR	–
Pythium sp.	D36 (7E-1)	*Cornus florida*	New Jersey, USA	2009	NZ	–
Rhizoctonia solani	98-1	*Lolium perenne*	New Jersey, USA	2009	NZ	–
Waitea circinata	1-1	*Poa annua*	New Jersey, USA	2009	NZ	–
Xylaria sp.	P70-3	*Cornus florida*	Ohio, USA	2009	KB	–

[a] *ATCC* American Type Culture Collection, Manassas, VA, USA, *CBS* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

[b] *AR* Amy Rossman, *AT* Andrea Tantardini, *KB* Ken Bauman, *NZ* Ning Zhang, *RL* Rebecca Loncosky, *SCR* Scott Redlin

[c] - = undetected

Design of real-time PCR probe and primers The ITS sequences of the microbial species listed in Table 1 were aligned using the CLUSTAL X program (Thompson et al. 1997). The primers and probe were designed manually based on the alignment and were evaluated with Primer3 (Rozen and Skaletsky 2000) and Oligonucleotide Properties Calculator (Rozen and Skaletsky 2000; Kibbe 2007) programs. The designed primers for *D. destructiva* were DdITS_F1, CCAGAAACCCATTGTGAATC, and DdITS_R1, GATGCCAGAACCAAGAGATC. The probe was DdITS_Probe1, FAM-TCGGAGGTCCCTTTCGTAAGAAAGG-Iowa Black FQ, labelled at the 5′ end with the fluorophore FAM and at the 3′ end with the Iowa Black FQ quencher (see

Table 2 Comparison of real-time PCR and conventional culturing methods for detecting *Discula destructiva* in dogwood (*Cornus* sp.) leaf tissue

Sample	Plant species	Symptom	Location	qPCR mean Ct value (standard deviation)	Culture detection
D23	*Cornus* sp.	Leaf spot	New Jersey, USA	26.4 (0.50)	+
D26	*C. florida*	Leaf spot	Maryland, USA	–	–
D27	*C. florida*	Leaf spot	Maryland, USA	31.1 (0.62)	+
D28	*C. florida*	Leaf spot	Maryland, USA	31.0 (0.42)	+
D30	*C. florida*	Non-symptomatic	New Jersey, USA	–	–
D30dry	*C. florida*	Non-symptomatic	New Jersey, USA	–	–
D31	*C. florida*	Non-symptomatic	New Jersey, USA	–	–
D31dry	*C. florida*	Non-symptomatic	New Jersey, USA	–	–
D32	*C. florida*	Leaf spot	New Jersey, USA	–	–
D32dry	*C. florida*	Leaf spot	New Jersey, USA	–	–
D33	*C. florida*	Leaf spot	New Jersey, USA	31.7 (0.37)	+
D33dry	*C. florida*	Leaf spot	New Jersey, USA	32.6 (0.76)	–
D34	*C. florida*	Leaf spot	New Jersey, USA	–	–
D34dry	*C. florida*	Leaf spot	New Jersey, USA	–	–
D35	*C. florida*	Leaf spot	New Jersey, USA	–	–
D35dry	*C. florida*	Leaf spot	New Jersey, USA	–	–
D36	*C. florida*	Leaf spot	New Jersey, USA	32.7 (0.53)	+
D36dry	*C. florida*	Leaf spot	New Jersey, USA	33.3 (0.30)	–
D43	*C. nuttallii*	Non-symptomatic	Oregon, USA	–	–
D52inc	*C. florida*	Non-symptomatic	Switzerland	19.3 (0.08)	+
D53inc	*C. florida*	Non-symptomatic	Italy	14.4 (0.21)	+
Italy-SubE	*C. florida*	Leaf spot and powdery mildew, partially rotten	Italy	23.8 (0.40)	–
P25	*C. mas*	Non-symptomatic	Indiana, USA	–	–
P26	*C. florida*	Non-symptomatic	Indiana, USA	–	–
P105	*C. florida*	Leaf spot	Ohio, USA	34.7 (1.13)	+
P106	*C. florida*	Leaf spot and leaf blight	Ohio, USA	29.6 (0.40)	+
P109	*C. florida*	Powdery mildew	Ohio, USA	–	–
P129	*Carya illinoinensis*	Leaf spot and powdery mildew	Missouri, USA	–	–

Supplementary Figure). Sequences of the primers and probe were BLAST searched in the GenBank database (http://www.ncbi.nlm.nih.gov) to ensure their specificity. All primers and probe were commercially synthesized by Integrated DNA Technologies (http://www.idtdna.com).

Real-time PCR assay conditions Real-time PCR reactions were conducted in the StepOnePlus real-time PCR system (Applied Biosystems, CA, USA) using the program of an initial cycle of 3 min at 95°C, followed by 45 cycles of 15 s at 95°C and 40 s at 60°C. Amplifications were carried out in a total volume of 20 µl, which consisted of 10 µl iTaq Supermix with ROX (Bio-Rad, CA, USA), 250 nM probe, 500 nM each primer, and 4 µl template DNA. To test the specificity of the method, 31 fungal and oomycete isolates, belonging to 18 species (Table 1) were included in the assay. A standard curve was also constructed using genomic DNA of *D. destructiva* isolate MD237-1. Each sample was tested in triplicate. A Ct value less than 35 was counted as positive detection of *D. destructiva*.

Results

Detection efficiency and sensitivity A 7-point semi-log standard curve of *D. destructiva* genomic DNA

serial dilutions (4 fg to 4 ng) showed a linear relationship between the threshold cycle value (Ct) and the log input template DNA amount. The slope of the standard curve was –3.2614, and the correlation coefficient (R^2) was 0.9994. The real-time PCR efficiency was 102.6% based on the formula: Efficiency $= (10^{(-1/\text{slope})} - 1) \times 100\%$. The method can detect as low as 4 fg *D. destructiva* genomic DNA.

Detection specificity The designed real-time PCR primers DdITS_F1 and DdITS_R1, and the dual-labelled probe DdITS_Probe1 were tested with genomic DNA of *D. destructiva* and a series of other species (Table 1). All *D. destructiva* isolates produced the expected 191 bp amplicons, the identities of which were confirmed by sequencing. The positive detection was unambiguous and inclusive because low Ct values were obtained consistently for all the tested *D. destructiva* isolates from various locations, hosts, and collection dates. No amplification was detected from the closely related diaporthalean fungi or the wide range of other species tested (Table 1).

Detection in artificially inoculated and natural plant samples Twelve of the 28 leaf samples yielded positive real-time PCR detection. Both of the detached leaf samples, D52inc and D53inc that were inoculated with European isolates had positive real-time PCR results at 3-days after inoculation, prior to when symptoms were shown. D52inc yielded a mean Ct value of 19.3, while D53inc's mean Ct was 14.4. *Discula destructiva* was detected from two dried samples, D33dry and D36dry, using real-time PCR, the mean Ct values of which were 0.9 and 0.6 higher than their fresh sample counterparts collected from a New Jersey forest. Real-time PCR also yielded positive detection of *D. destructiva* from *Cornus* samples collected from Italy, and Maryland and Ohio of the U. S.

Comparison of real-time PCR and culture-based detection methods In this study, all samples that had negative real-time PCR results were also negative when using the culture identification method. *Discula destructiva* was cultured from nine leaf samples, all of which were positively detected by the real-time PCR method (Table 2). Two *D. destructiva* infected leaf samples were not detected by the culture method after a 2-week heat-drying process but still were detected with real-time PCR. Similarly, *D. destructiva* was not cultured from the partially rotten Italy-SubE leaf sample but it was detected with the real-time PCR. Poor condition of the host plant material is a very common reason for false-negative culture detection of slow growing pathogenic fungi. The time required for the real-time PCR detection was 3–5 h, including the DNA extraction step, while it took 7–14 days for the culture-based method.

Discussion

One of the major challenges of developing real-time PCR detection methods is achieving both high specificity and sensitivity. In this study, we chose the probe-based TaqMan chemistry because it is inherently more specific and sensitive than SYBR green, the other widely used chemistry of real-time PCR (Heid et al. 1996; Okubara et al. 2005). However, compared to SYBR green, the relatively higher cost of the TaqMan reagents and occasionally encountered difficulty in probe design, remain the limiting factors for its application.

In some cases, lack of DNA sequence variability between the target and non-target species makes it difficult to design target-specific primers and probes. In addition, sequence diversity among strains within a target species must be considered in probe design, in order for the detection to remain inclusive. For *D. destructiva*, we designed primers and probes based on the ITS region as the DNA sequence of this region is uniform within this species and it has relatively low sequence similarity with non-target species. The phylogenetically close relatives of *D. destructiva,* including *Amphiporthe hranicensis*, *Apiognomonia errabunda*, and *Cryptosporella femoralis*, have a 94% or lower ITS sequence similarity, which enables target-specific primer and probe design.

Our data showed that this TaqMan real-time PCR assay was highly specific. None of the closely or distantly related species tested produced detectable signals in the real-time PCR assay.

The results also showed that the presented method was highly sensitive. The detection limit for pure culture was 4 fg genomic DNA. It successfully detected *D. destructiva* from fresh, dried, and partially rotten leaf samples. Therefore, it can potentially be

used in detecting the fungal species in herbarium specimens, in which the pathogen is nonviable. For the inoculated samples, the pathogen was detected before the typical anthracnose symptoms appeared.

A number of variables can affect the efficiency of real-time PCR, such as primer secondary structure, the length of the amplicon, pipetting errors, and the amount of enzyme or other reagents (Yuan et al. 2006; Peters et al. 2004). Ideally, the efficiency of PCR should be 0.9–1 (slope between −3.6 and −3.1) (Dorak 2006). In this study, we achieved 102.6% efficiency in the 7-point standard curve, which is comparable to useful real-time PCR assays developed for pathogenic bacteria and fungi (Chilvers et al. 2007; Lambertz et al. 2008).

It has been hypothesized that the dogwood anthracnose fungus, as with the chestnut blight pathogen, was introduced from Asia to North America (Daughtrey et al. 1996; Zhang and Blackwell 2002). The pathogen was then dispersed to Europe, possibly through trade. In this research, a fast and accurate detection method using real-time PCR was developed to facilitate studies on the origin and biogeography of the dogwood anthracnose fungus. The culture-independent method requires only small amounts of host tissue and can be used to directly detect and quantify *D. destructiva* in both fresh and dried plant materials, which enables a fast screening of herbarium specimens and fresh field collections.

Traditional disease diagnosis for dogwood anthracnose requires expertise and is time-consuming because *D. destructiva* is slow growing on artificial media and similar symptoms can be caused by other pathogens. The real-time PCR procedure presented here is fast, accurate, and sensitive. It provides an alternative and efficient diagnostic method that would be valuable for disease management and dogwood breeding.

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References

Anagnostakis, S. L. (1987). Chestnut blight—the classical problem of an introduced pathogen. *Mycologia, 79*, 23–37.

Anderson, R. L., Knighten, J. L., & Dowsett, S. E. (1990). Dogwood anthracnose: a Southeastern U.S. perspective. Paper presented at the *23rd annual Tennessee nursery short course*, Nashville, TN.

Chilvers, M. I., du Toit, L. J., Akamatsu, H., & Peever, T. L. (2007). A real-time, quantitative PCR seed assay for *Botrytis* spp. that cause neck rot of onion. *Plant Disease, 91*, 599–608.

Daughtrey, M. L., & Hibben, C. R. (1994). Dogwood anthracnose—a new disease threatens two native *Cornus* species. *Annual Review of Phytopathology, 32*, 61–73.

Daughtrey, M. L., Hibben, C. R., Britton, K. O., Windham, M. T., & Redlin, S. C. (1996). Dogwood anthracnose—understanding a disease new to North America. *Plant Disease, 80*, 349–358.

Dorak, M. T. (2006) *Real Time PCR (BIOS Advanced Methods)*. Taylor & Francis.

Farr, D. F. (1991). *Septoria* species on *Cornus*. *Mycologia, 83*, 611–623.

Gachon, C., Mingam, A., & Charrier, B. (2004). Real-time PCR: what relevance to plant studies? *Journal of Experimental Botany, 55*, 1445–1454.

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for Basidiomycetes—application to the identification of mycorrhizae and rusts. *Molecular Ecology, 2*, 113–118.

Heid, C. A., Stevens, J., Livak, K. J., & Williams, P. M. (1996). Real time quantitative PCR. *Genome Research, 6*, 986–994.

Holzmueller, E., Jose, S., Jenkins, M., Camp, A., & Long, A. (2006). Dogwood anthracnose in eastern hardwood forests: what is known and what can be done? *Journal of Forestry, 104*, 21–26.

Jenkins, M. A., Jose, S., & White, P. S. (2007). Impacts of an exotic disease and vegetation change on foliar calcium cycling in appalachian forests. *Ecological Applications, 17*, 869–881.

Kibbe, W. A. (2007) OligoCalc: an online oligonucleotide properties calculator, *Nucleic Acids Research, 35 (Webserver Issue): May 25*.

Lambertz, S. T., Nilsson, C., Hallanvuo, S., & Lindblad, M. (2008). Real-time PCR method for detection of pathogenic *Yersinia enterocolitica* in food. *Applied and Environmental Microbiology, 74*, 6060–6067.

Mackay, I. (2007) *Real-time PCR in microbiology: from diagnosis to characterization*. Caister Academic Press.

McCartney, H. A., Foster, S. J., Fraaije, B. A., & Ward, E. (2003). Molecular diagnostics for fungal plant pathogens. *Pest Management Science, 59*, 129–142.

Milgroom, M. G. (1995). Population biology of the chestnut blight fungus, *Cryphonectria parasitica*. *Canadian Journal of Botany-Revue Canadienne De Botanique, 73*, S311–S319.

Milgroom, M. G., & Lipari, S. E. (1995). Population differentiation in the Chestnut Blight Fungus, *Cryphonectria parasitica*, in eastern North America. *Phytopathology, 85*, 155–160.

Milgroom, M. G., Wang, K. R., Zhou, Y., Lipari, S. E., & Kaneko, S. (1996). Intercontinental population structure of the chestnut blight fungus, *Cryphonectria parasitica*. *Mycologia, 88*, 179–190.

Okubara, P. A., Schroeder, K. L., & Paulitz, T. C. (2005). Real-time polymerase chain reaction: applications to studies on soilborne pathogens. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie, 27*, 300–313.

Peters, I. R., Helps, C. R., Hall, E. J., & Day, M. J. (2004). Real-time RT-PCR: considerations for efficient and sensitive assay design. *Journal of Immunological Methods, 286*, 203–217.

Pierce, A. R., Bromer, W. R., & Rabenold, K. N. (2008). Decline of *Cornus florida* and forest succession in a Quercus-Carya forest. *Plant Ecology, 195*, 45–53.

Redlin, S. C. (1991). *Discula destructiva* sp. nov. cause of dogwood anthracnose. *Mycologia, 83*, 633–642.

Rozen, S., & Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. In S. Krawetz & S. Misener (Eds.), *Bioinformatics methods and protocols: Methods in molecular biology* (pp. 365–386). Totowa: Humana.

Schaad, N. W., & Frederick, R. D. (2002). Real-time PCR and its application for rapid plant disease diagnostics. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie, 24*, 250–258.

Schaad, N. W., Berthier-Schaad, Y., Sechler, A., & Knorr, D. (1999). Detection of Clavibacter michiganensis subsp sepedonicus in potato tubers by BIO-PCR and an automated real-time fluorescence detection system. *Plant Disease, 83*, 1095–1100.

Schrope, M. (2001). Fungus fingered as dogwood disappears from forests. *Nature, 411*, 7–7.

Stinzing, A., & Lang, K. (2003). Dogwood anthracnose. Erster fund von *Discula destructiva* an *Cornus florida* in Deutschland. *Nachrichtenbl. Deut. Pflanzenschutzd, 55*, S1–S5.

Tantardini, A., Calvi, M., Cavagna, B., Zhang, N., & Geiser, D. (2004). Primo rinvenimento in Italia di antracnosi causata da *Discula destructiva* su *Cornus florida* e *C. nuttallii*. *Informatore Fitopatologico, 12*, 44–47.

Thompson, J. D., Gibson, T. J., & Higgins, D. G. (1997). The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 25*, 4876–4882.

Trigiano, R. N., Caetanoanolles, G., Bassam, B. J., & Windham, M. T. (1995). DNA amplification fingerprinting provides evidence that *Discula destructiva*, the cause of dogwood anthracnose in North America, is an introduced pathogen. *Mycologia, 87*, 490–500.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. Innis, D. Gelfand, J. Sninsky, & T. White (Eds.), *PCR protocols: A guide to methods and applications*. New York: Academic.

Yuan, J. S., Reed, A., Chen, F., & Stewart, C. N. (2006) Statistical analysis of real-time PCR data, *Bmc Bioinformatics, 7*.

Zhang, N., & Blackwell, M. (2001). Molecular phylogeny of dogwood anthracnose fungus (*Discula destructiva*) and the diaporthales. *Mycologia, 93*, 355–365.

Zhang, N., & Blackwell, M. (2002). Population structure of dogwood anthracnose fungus. *Phytopathology, 92*, 1276–1283.