

Differentiation of *Corynespora cassiicola* and *Cercospora* sp. in leaf-spot diseases of *Hydrangea macrophylla* using a PCR-mediated method

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Mmbaga, M. T., Kim M.-S., Mackasmiel, L. and Klopfenstein, N. B. 2015. **Differentiation of *Corynespora cassiicola* and *Cercospora* sp. in leaf-spot diseases of *Hydrangea macrophylla* using a PCR-mediated method.** Can. J. Plant Sci. **95**: 711–717. *Corynespora cassiicola* and *Cercospora* sp. have been identified as the most prevalent and destructive leaf-spot pathogens of garden hydrangea [*Hydrangea macrophylla* (Thunberg) Seringe] in the southeastern USA, but they are often difficult to accurately detect and distinguish because they often occur together in a disease complex with other pathogenic leaf-spot fungi and produce very similar symptoms. This study was conducted to provide diagnostic PCR primers for detecting and distinguishing *Corynespora cassiicola* and *Cercospora* sp. among other leaf-spot pathogens of garden hydrangea. Two primer pairs showed specificity to *Corynespora cassiicola* and one primer pair showed specificity to *Cercospora* sp., and these primers did not amplify DNA from any other common fungal pathogens associated with hydrangea leaf-spot diseases. Results from this study show that DNA-based diagnostic primers provide a useful tool for pathogen detection/identification in hydrangea leaf-spot disease, which is an essential step toward understanding disease etiology and developing/applying appropriate disease-management practices in the southeastern USA.

Key words: *Cercospora* leaf spot, garden hydrangea, molecular diagnostics, plant disease complex, diagnostic primers

Mmbaga, M. T., Kim M.-S., Mackasmiel, L. et Klopfenstein, N. B. 2015. **Différenciation de *Corynespora cassiicola* et de *Cercospora* sp. dans les maladies à tache foliaire d'*Hydrangea macrophylla* grâce à une méthode recourant à la PCR.** Can. J. Plant Sci. **95**: 711–717. *Corynespora cassiicola* et *Cercospora* sp. sont les agents pathogènes les plus courants et les plus destructeurs à l'origine des taches foliaires chez l'hydrangée [*Hydrangea macrophylla* (Thunberg) Seringe], dans le sud-est des États-Unis. Cependant, il est souvent difficile de les dépister et de les différencier, ces champignons produisant des symptômes très semblables et se retrouvant fréquemment avec d'autres pathogènes des feuilles dans un regroupement de maladies. Les auteurs ont recouru à la PCR pour créer des amorces diagnostiques susceptibles de faciliter la détection et la différenciation de *Corynespora cassiicola* et de *Cercospora* sp. parmi les autres agents pathogènes responsables de la tache foliaire chez l'hydrangée. Deux paires d'amorces étaient spécifiques à *Corynespora cassiicola* et une autre à *Cercospora* sp.; aucune n'amplifiait l'ADN des autres champignons pathogènes couramment associés à la tache foliaire chez l'hydrangée. Les résultats de cette étude indiquent que les amorces diagnostiques à base d'ADN constituent un outil utile pour dépister et identifier les agents pathogènes lorsque l'hydrangée est atteinte de tache foliaire, étape essentielle vers une meilleure compréhension de l'étiologie de la maladie et l'élaboration puis l'application de pratiques culturales qui faciliteront la lutte contre elle dans le sud-est des États-Unis.

Mots clés: Cercosporiose, hydrangée, diagnostic moléculaire, ensemble de maladies végétales, amorces diagnostiques

Garden hydrangea [*Hydrangea macrophylla* (Thunberg) Seringe], also known as the bigleaf, French or florist hydrangea, is a popular landscape shrub. Leaf-spot diseases impact the aesthetic value and market value of hydrangea by causing unsightly appearance associated with discolored plant foliage, severe defoliation, and blighted flowers. Although severe defoliations are sporadic, repeated defoliations impact plant vigor and overall plant health. Frequent rain showers and hot weather favor hydrangea leaf-spot diseases; plants may also sustain severe damage when overhead irrigation is used in nursery production systems (Williams-Woodward and

Daughtrey 2001). Fungal genera associated with leaf-spot diseases in garden hydrangea include *Ascochyta*, *Botrytis*, *Cercospora*, *Colletotrichum*, *Corynespora*, *Phyllosticta*, and *Myrothecium* (Sinclair et al. 1987; Hagan and Mullen 2001; Williams-Woodward and Daughtrey 2001; Mmbaga et al. 2010). Although several fungal pathogens are associated with hydrangea leaf-spot diseases in the southeastern USA, *Corynespora cassiicola* and *Cercospora* sp. were previously found to be the most frequently isolated pathogens of the leaf-spot disease complex on garden hydrangea (Mmbaga et al. 2012). Although other pathogens such as *Phoma exigua*, *Myrothecium roridum*,

Botryotinia fuckeliana (Anamorph *Botrytis cinerea*), *Glomerella cingulata* (Anamorph: *Colletotrichum gloeosporioides*), *Glomerella acutata* (Anamorph: *Colletotrichum acutatum*) and *Alternaria alternata* were isolated at low frequency, these co-occurring pathogens can contribute to the disease complexes and further complicate disease diagnosis and resistance evaluations (Mmbaga et al. 2012).

Cercospora leaf spot caused by *Cercospora* sp. is the best-known, leaf-spot disease of hydrangea (Sinclair et al. 1987; Hagan and Mullen 2001; Williams-Woodward and Daughtrey 2001; Hagan et al. 2004; Mmbaga et al. 2009; Vann 2010; Mmbaga et al. 2012). As a group, fungi in the genus *Cercospora* are nearly universally pathogenic and are among the most prevalent and destructive plant pathogens on a wide range of hosts in almost all major dicot families, most monocot families, and even some gymnosperm and fern families (Pollack 1987). However, symptoms of *Cercospora* leaf spot described in the literature are identical to those described for *Corynespora* leaf spot (Hagan et al. 2004; Zaher et al. 2005; Schlub and Smith 2007). Although some isolates of *Corynespora cassiicola* have exhibited host specificity (Dixon et al. 2009), most isolates have a wide host range and more than 50 ornamental plants have been reported as hosts of *Corynespora cassiicola* (Ellis 1957; Ellis and Holliday 1971; Farr et al. 1989; Chase et al. 1995). Both *Cercospora* and *Corynespora* leaf-spot diseases tend to have annual outbreaks once the pathogen has become established in an area (Hagan and Mullen 2001; Hagan et al. 2004; Zaher et al. 2005; Schlub and Smith 2007; Mmbaga et al. 2012). When the two pathogens co-occur in a disease complex, it becomes difficult to determine the main cause of the disease because neither fungus readily sporulates in culture (Mmbaga et al. 2012). Furthermore, morphological similarities between *Corynespora* spp., *Alternaria* spp., and *Helminthosporium* spp. may contribute to misdiagnosis when *Corynespora cassiicola* is the primary pathogen, which would result in an underestimation of its economic importance (Zaher et al. 2005; Schlub and Smith 2007), especially in less-studied plants such as ornamentals. Thus, *Corynespora cassiicola* could potentially be misidentified, and may be erroneously reported as better known fungal pathogens, such as *Cercospora*, *Helminthosporium* and *Alternaria* species.

Accurate identification of pathogens that cause plant diseases is critical for recognizing their economic importance, understanding disease etiology, and developing successful disease management strategies including disease-resistance evaluations. Typically, detection methods are based on isolation and growth of the disease-causing organism via culture of the diseased tissue, followed by morphology-based identification of the organism using microscopy (Weiland and Sundsbak 2000). The objective of this study was to design and test diagnostic PCR primers for detecting and differentiating *Corynespora cassiicola* and *Cercospora* sp. among other leaf-spot pathogens of garden hydrangea in the southeastern USA.

MATERIALS AND METHODS

This study was conducted at Tennessee State University, Otis L. Floyd Nursery Research Center (TSU-NRC) in McMinnville, Tennessee, USA. Pathogenic fungi were isolated from a large group of *H. macrophylla* commercial cultivars (69 *H. macrophylla* ssp. *macrophylla*, 18 *H. macrophylla* ssp. *serrata*, and three hybrids between the two subspecies) following methods described in Mmbaga et al. (2012). In brief, ca. 50 infected leaves were randomly collected from different plants and surface-disinfested, leaf pieces were then placed on potato dextrose agar (PDA), and conidia or sub-culturing were used to establish pure cultures. In addition, ca. 50 infected leaves were incubated in a moist chamber at room temperature for 24–48 h and conidia were transferred to water agar and sub-cultured on PDA. Fungal cultures were maintained on PDA for DNA-based analysis. Fungal isolates were characterized morphologically using dichotomous keys (Ellis 1957, 1971; Barnett and Hunter 1998).

Genomic DNA was extracted from pure cultures of *Corynespora cassiicola*, *Cercospora* sp., and six other hydrangea leaf-spot pathogens (*Glomerella cingulata*, *G. acutata*, *Alternaria alternata*, *Myrothecium roridum*, *Phoma exigua* and *Botrytis cinerea*) using DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) following standard protocols with some modifications (Takamatsu and Kano 2001). DNA amplification with primers ITS1F (or ITS1)/ITS4 (White et al. 1990; Gardes and Bruns 1993) and diagnostic primers was performed following standard PCR protocols used by Mmbaga et al. (2012). Each 50-μL reaction mixture contained ca. 25 ng template DNA (or no DNA template for negative controls), 2.5 U Taq DNA polymerase (Applied Biosystems, Foster City, CA), 4 mM MgCl₂, 200 μM dNTPs, and 0.5 μM of each primer. Primers ITS1F (or ITS1) and ITS4 were used for PCR amplification of the internal transcribed spacer 1–5.8S–internal transcribed spacer 2 region of rDNA (ITS1–5.8S–ITS2; hereafter called ITS). PCR thermal profiles consisted of an initial denaturation (2 min 30 s) at 94°C followed by 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 48°C for ITS1F/ITS4 and 1 min at 67.6°C for diagnostic primers for *Corynespora cassiicola* and *Cercospora* sp.), and extension (1 min 30 s at 72°C; 10 min at 72°C for a final extension cycle). PCR products for ITS region were purified using QIAquick PCR Purification Kit (Qiagen) and sequenced using an ABI 377XL PRISM automatic sequencer (Davis Sequencing Inc., Davis, CA). The sequences obtained were compared with all ITS region sequences in GenBank using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

The ITS sequences for *Corynespora cassiicola* (GenBank accession No. HQ845386) and *Cercospora* sp. (GenBank accession No. HQ845387) were used to design diagnostic primers using software Primer3 (<http://frodo.wi.mit.edu/>). Two primer pairs, CoryITS-f1 (5'-GGCCTCGCCCCCTTCGAGAT-3')/CoryITS-r1 (5'-CCGACC CGCAGCCACTTCAG-3') and CoryITS-f2 (5'-CGGG GACCCACCACAAACCC-3')/CoryITS-r2 (5'-CTCGT

GGCCTGCTGGGAACC-3'), were designed for *Corynespora cassiicola* and one primer pair, CerITS-f1 (5'-GCCCGGAGGCGCTTCAAAC-3')/CerITS-r2 (5'-GAACACCGCGGCGCCCAATA-3'), was designed for *Cercospora* sp. Primer pairs were evaluated on pure cultures of *Corynespora cassiicola*, *Cercospora* sp., and their host (*H. macrophylla*). To test for their specificity, the primer pairs were also evaluated on six other hydrangea leaf-spot pathogens (*G. cingulata*, *G. acutata*, *A. alternata*, *M. roridum*, *P. exigua*, and *B. cinerea*) collected from the local area. In addition, GenBank Primer-Blast (<http://www.ncbi.nlm.gov/tools/primer-blast/>) was used to ensure that the primer pairs did not match sequences from other fungi reported to be associated with hydrangea leaf-spot disease.

The utility of diagnostic primer pairs in diagnosis of *Corynespora cassiicola* and *Cercospora* sp. in hydrangea leaf-spot disease complexes was also evaluated preliminarily using direct PCR (without prior DNA purification) of leaf-spot disease samples of from naturally infected hydrangea leaves collected from central and eastern Tennessee (six samples) and central/northeastern Arkansas (four samples). Plant Direct PCR Kit (Thermo Fisher Scientific Inc., Waltham, MA) and each putative diagnostic primer sets were used to analyze infected leaves following protocols provided by the manufacturer. Disease samples from different leaf-spot symptom types were derived from fresh and pressed dry leaves that provided sources of DNA template for the pathogens. In addition, universal primers ITS1F/ITS4 were also used on direct PCR to analyze infected leaves and compare results with those obtained from pure cultures of the two pathogens.

RESULTS

Pure cultures of *Corynespora cassiicola* were obtained via direct plating of surface-disinfested, leaf pieces with leaf-spot disease with subsequent subculture and from isolated conidia from incubated leaves; however, *Cercospora* sp. could only be isolated from conidia produced on incubated leaves. Colonies of *Corynespora cassiicola* and *Cercospora* sp. were morphologically distinct from each other (Fig. 1). *Cercospora* colonies were snow white to greyish white in color and slow growing (Fig. 1a–d); while *Corynespora cassiicola* colonies were whitish grey in color and darkened to olive green with culture age (Fig. 1e–g). *Corynespora cassiicola* cultures were fast growing and somewhat resembled *Alternaria* spp. (Fig. 1e–g). All isolates of *Cercospora* produced a purplish/yellowish colored exudate that diffused into the culture medium (Fig. 1d). Single spore-derived cultures of *Cercospora* sp. showed some morphological variability in the shade of the purplish/yellowish exudate, with some culture exudates having a more purplish color whilst others had a yellowish color. No morphological variability was apparent in *Corynespora cassiicola* (data not shown).

When infected leaves were incubated in a moist chamber for 24–48 h, conidia of *Corynespora cassiicola*

and/or *Cercospora* sp. were observed in most of the infected leaves. Furthermore, both *Corynespora cassiicola* and *Cercospora* sp. were often present in the same lesions. Conidia of *Cercospora* sp. were hyaline, long and thin, cylindrical to filiform, several celled and tapered on one side; they were borne terminally on dark conidiophores that often occurred in clusters (Fig. 2d–f). *Cercospora* spores measured $1.8\text{--}2.5 \times 39\text{--}62\text{ }\mu\text{m}$ in size and were produced by a sympodial proliferation of conidiogenous cells (Fig. 2d–e). Conidia of *Corynespora cassiicola* were also several celled, but dark colored with a thick, colorless exospore and a prominent dark basal scar (Fig. 2a, c). Conidia of *Corynespora cassiicola* were large in size and measured $12.5\text{--}14.5 \times 95\text{--}98\text{ }\mu\text{m}$ (Fig. 2c); they were borne terminally and singly or in short chains, on dark several-celled conidiophores that often had bulbous swollen tips (Fig. 2a, b). Conidia of *Corynespora cassiicola* showed a superficial resemblance to those of *Helminthosporium* species. However, neither *Corynespora* nor *Cercospora* species sporulated readily in culture.

The ITS sequence of *Corynespora cassiicola* and *Cercospora* sp. isolates from this study had 100% identity with our previously reported *Corynespora cassiicola* (GenBank accession no. HQ845386) and *Cercospora* sp. (HQ845387). Amplified DNA products corresponding to the ITS region were ca. 600 bp for *Corynespora cassiicola* (Fig. 3; lane 1) and ca. 570 bp for *Cercospora* sp. (Fig. 3; lane 2). The diagnostic primer pairs, CoryITS-f1/CoryITS-r1 and CoryITS-f2/CoryITS-r2, produced amplification products for *Corynespora cassiicola* only and produced DNA amplicons of ca. 430 bp and ca. 320 bp, respectively (Fig. 3; lane 3 and lane 5); they did not amplify DNA from *Cercospora* sp. (Fig. 3; lane 4 and lane 6) or six other common leaf-spot pathogens (*G. cingulata*, *G. acutata*, *A. alternata*, *M. roridum*, *P. exigua*, and *B. cinerea*) of hydrangea (data not shown). One diagnostic primer pair, CerITS-f1/CerITS-r2 produced an amplification product, which was ca. 250 bp in size, for *Cercospora* isolates only (Fig. 3; lane 8), and did not amplify DNA from *Corynespora cassiicola* (Fig. 3; lane 7) or other hydrangea leaf-spot pathogens tested (data not shown). These diagnostic primer pairs were effective in distinguishing *Corynespora cassiicola* and *Cercospora* sp. among other pathogens associated with the leaf-spot disease complexes of hydrangea. The BLAST search with diagnostic primer sets (CerITS-f1/CerITS-r2 for *Cercospora* sp.; CoryITS-f1/CoryITS-r1 and CoryITS-f2/CoryITS-r2 for *Corynespora cassiicola*) did not find significant alignments for other common foliar and aerial fungi associated with hydrangea leaf spots/blights (data not shown).

Using universal ITS primers (ITS1/ITS4), direct PCR with naturally occurring hydrangea leaf-spot tissue resulted in multiple DNA amplicons, including those with the expected sizes for *Corynespora cassiicola*, *Cercospora* sp., and other amplicons that are likely associated with other uncharacterized fungi (data not shown). Using diagnostic primer pairs designed for *Corynespora cassiicola*

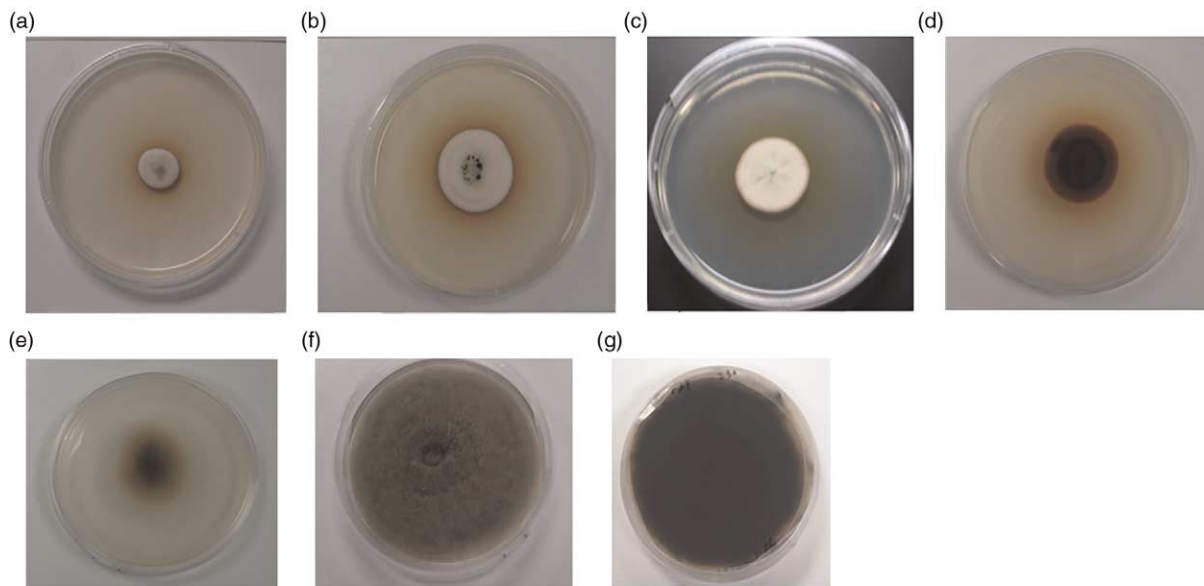


Fig. 1. Colonies of *Cercospora* sp. (a–d) and *Corynespora cassiicola* (e–g) isolated from leaf-spot lesions in garden hydrangea (*Hydrangea macrophylla*) showing upper side (a, b, c, f) and underside view (d, e, g) on potato dextrose agar after 7 d (a, e) and 14 d (b, c, d, f, g).

(CoryITS-f1/CoryITS-r1 and CoryITS-f2/CoryITS-r2) or *Cercospora* sp. (CerITS-f1/CerITS-r2), direct PCR of the 10 leaf-spot samples resulted in the same amplicon sizes found with genomic DNA from the cultured *Corynespora cassiicola* and *Cercospora* sp. Out of the 10 leaf samples, six samples (60%) had amplicons corresponding to a mixture of *Cercospora* and *Corynespora cassiicola*, three samples (30%) had *Cercospora* sp. alone and only one sample (10%) had *Corynespora cassiicola* alone. These results further confirm that *Corynespora cassiicola* and *Cercospora* sp. can co-occur in leaf-spot samples.

DISCUSSION AND CONCLUSIONS

Accurate identification of pathogens that cause plant diseases is critical in determining disease etiology, assessing pathogen prevalence, and implementing appropriate disease-management strategies, such as germplasm resistance screening. Detection methods for plant pathogens have typically relied on growth of the disease-causing organisms isolated from the diseased tissue, followed by morphology-based identification of the organisms using microscopy (Weiland and Sundsbak 2000). This identification method requires considerable expertise and time, especially for slow-growing fungi, such as *Cercospora* sp. that can also be obscured by fast-growing fungi (e.g., *Corynespora cassiicola* and *Alternaria* spp.). Pathogen identification can be further complicated, when multiple fungal pathogens co-occur within a disease complex. The identification of specific pathogens associated with hydrangea leaf-spot diseases is frequently based on previously described disease symptoms (Hagan and Mullan 2001). However, such pathogen identification practices are inadequate because similar foliar symptoms observed during the growing season may be caused by different

pathogens or complex biotic/abiotic interactions. For example, *Corynespora cassiicola* spreads to stems and fruits and causes severe dieback, defoliation, and flower blight (Mmbaga et al. 2012), which closely reflects symptoms described for *Cercospora* leaf blight/spot (Schlub and Smith 2007; Mmbaga et al. 2012).

The scarcity of *Cercospora* and *Corynespora cassiicola* spores and morphological similarity between colonies of *Corynespora* and *Alternaria* saprophytes can also contribute to misdiagnosis when *Corynespora cassiicola* is a primary pathogen. Previous studies reported a higher isolation frequency of *Corynespora cassiicola* as compared with *Cercospora* (Mmbaga et al. 2012), which suggested that, *Corynespora cassiicola* was the most prevalent pathogen and more important than *Cercospora* sp. within the study area of Tennessee, USA. Notably, the presence of *Cercospora* sp. was revealed by direct PCR amplifications of infected leaves with different symptom types, which included symptomatic leaves for which *Cercospora* sp. was not recovered by direct isolations or observed as conidia after leaf incubation in high moisture for 24–48 h (data not shown). These observations suggest that frequency of *Cercospora* occurrence may be higher than that observed from direct isolations and/or induced spore-production, which further confirms the need for DNA-based tools to confirm leaf-spot disease diagnosis in hydrangea.

As expected, the BLAST search with diagnostic primer pair (CerITS-f1/CerITS-r2) for *Cercospora* sp. resulted in significant alignments with *Cercospora hydrangea*, a cause of leaf-spot disease in hydrangea. In addition, the BLAST search also revealed potential primer pair alignments with several unrelated fungal genera (e.g., *Mycosphaerella*,

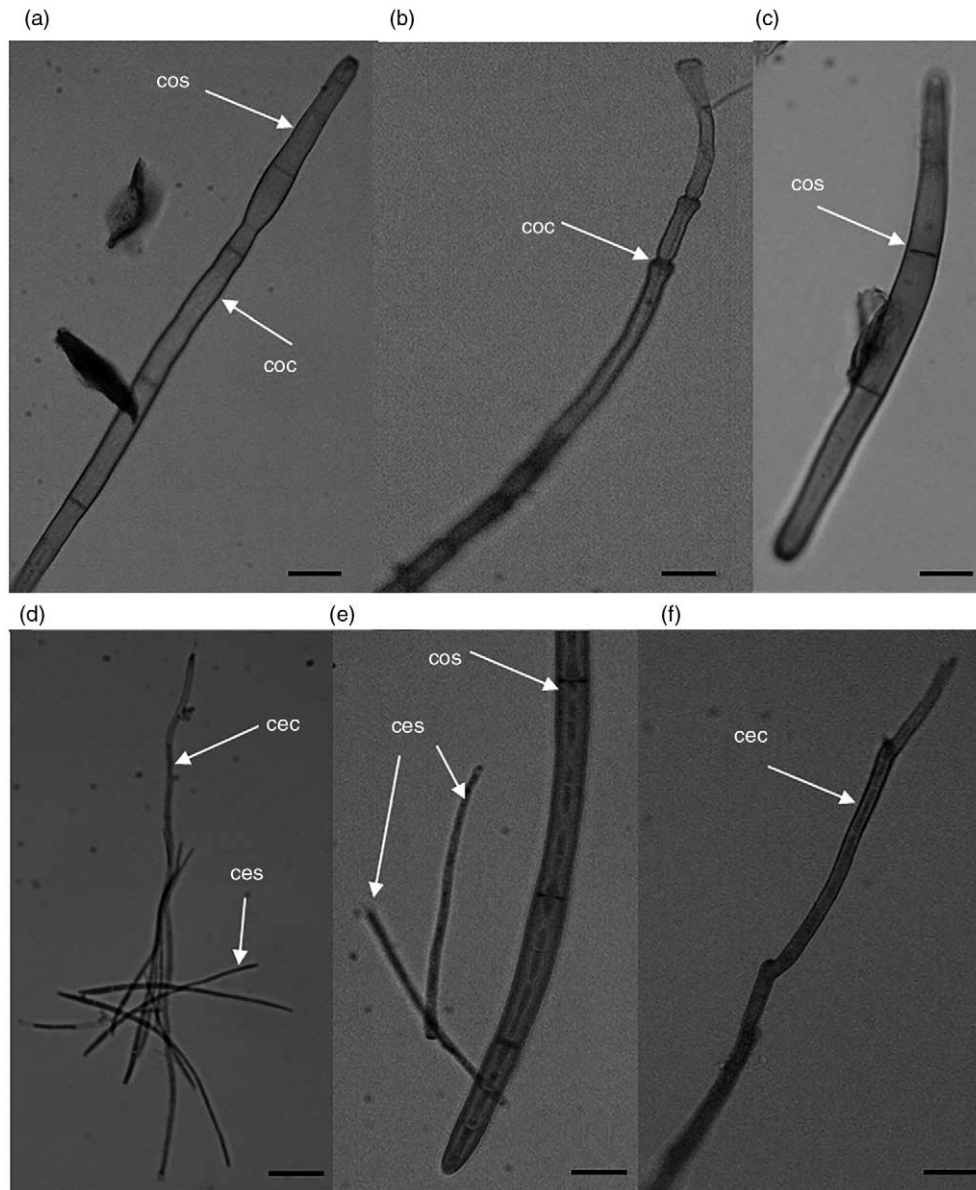


Fig. 2. Morphological features of conidia of *Corynespora cassiicola* (a–c), and *Cercospora* sp. (d, f); mixed spores of *Cercospora* sp. and *Corynespora cassiicola* (e) observed on same lesion and differentiated by their relative sizes and morphological features; arrows point to *Corynespora* spores (c, cos), and conidiophores (b, coc); *Cercospora* spores (d, ces) and conidiophores (f, cec). Scale bars is 12.5 μ m.

Pseudocercospora, *Passalora*, and *Sphaerulina*); however, these unrelated fungi have not been conclusively documented as causing disease in garden hydrangea (Fungal Database: <http://nt.ars-grin.gov/fungaldatabases/>) and have not been found in association with hydrangea leaves (Mmbaga et al. 2012).

Based on rDNA sequences, the *Cercospora* sp. isolated from this study appears to be *Cercospora hydrangeae* (JF495458: 100% similarity of partial DNA sequences of ITS1-5.8S-ITS-2), but this taxonomic issue is not fully resolved. Due to the concept of host specificity for

Cercospora species, more than 3000 names have been listed for *Cercospora* species according to the host species (Pollack 1987); *Cercospora hydrangeae* is the species associated with hydrangea (Hagan and Mullen 2001; Williams-Woodward and Daughtrey 2001, <http://www.plantpath.caes.uga.edu/extension>). However, continued studies will contribute a better understanding of *Cercospora* pathogens and their appropriate taxonomy in association with leaf-spot disease of ornamentals.

The primary objective of this study was to develop and test methods to detect and distinguish two leaf-spot

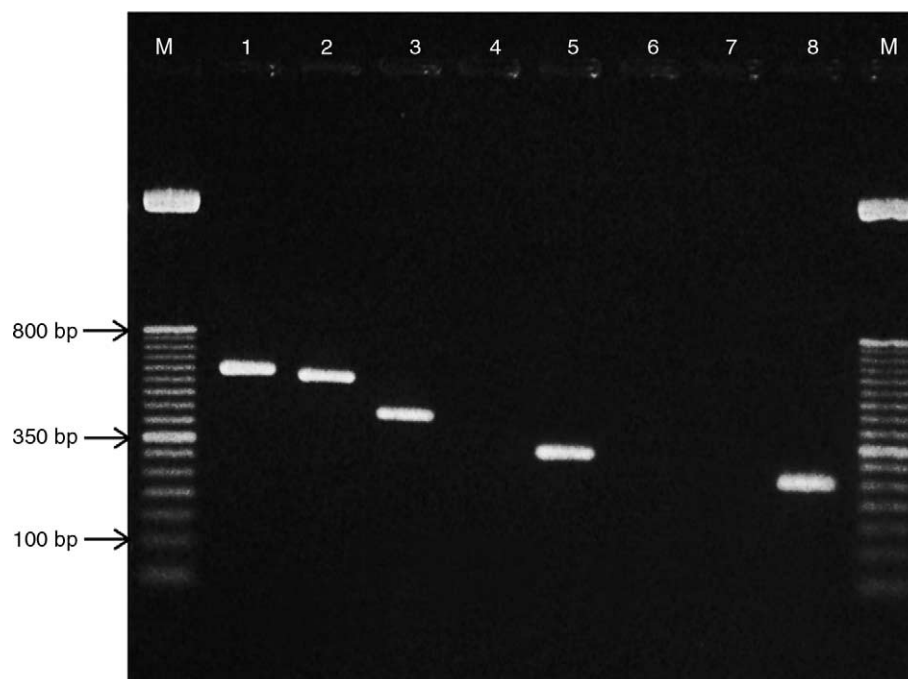


Fig. 3. Comparison of PCR products of *Corynespora cassiicola* and *Cercospora* sp. Lanes 1–2: PCR products using ITS1F/ITS4 primer set, *Corynespora cassiicola* (lane 1) and *Cercospora* sp. (lane 2); Lanes 3–4: PCR products using diagnostic primer set (CoryITS-f1/CoryITS-r1), *Corynespora cassiicola* (lane 3) and *Cercospora* sp. (lane 4); Lanes 5–6: PCR products using diagnostic primer set (CoryITS-f2/CoryITS-r2), *Corynespora cassiicola* (lane 5) and *Cercospora* sp. (lane 6); Lanes 7–8: PCR products using diagnostic primer set (CerITS-f1/CerITS-r2), *Corynespora cassiicola* (lane 7) and *Cercospora* sp. (lane 8); M: 50 bp-molecular ladder. Sequence information of diagnostic primer sets are described in Materials and Methods.

fungi (*Corynespora cassiicola* and *Cercospora* sp.) that are not easily differentiated by isolation techniques and produce very similar symptoms in garden hydrangea. Based on these results, the diagnostic primer sets are a useful tool to differentiate *Cercospora* sp. and *Corynespora cassiicola* among other leaf-spot causing fungal pathogens of garden hydrangea in the southeastern USA. Although these diagnostic primers are associated with the same limitations of all diagnostic primers (e.g., potential matches with undescribed fungal taxa and unavailable sequence data), all current evidence supports that these diagnostic primers are suitable for their intended purpose.

Although results from this study showed that morphological features including colony characteristics and spore morphology can differentiate *Cercospora* sp. from *Corynespora cassiicola*, diagnostic primers reliably distinguished the two pathogens among six other common leaf-spot pathogens even when the pathogens co-occurred and even in the absence of sporulation on hydrangea tissue within the study area of Tennessee, USA. Furthermore, the use of these diagnostic primers will allow fast and efficient detection/differentiation of *Cercospora* sp. and *Corynespora cassiicola* in hydrangea leaf-spot diseases, which is essential for determining disease etiology, assessing economic impacts, and implementing appropriate disease management practices. Further studies are

needed to determine whether the diagnostic primers for *Cercospora* sp. and *Corynespora cassiicola* have utility for leaf-spot diseases of other less-studied plants.

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