

Open Access

Identification of *Alternaria alternata* as a Causal Agent for Leaf Blight in *Syringa* Species

Margaret T. Mmbaga^{1*}, Ainong Shi² and Mee-Sook Kim³

¹Otis A. Floyd Nursery Research Center, School of Agriculture and Consumer, Tennessee State University, McMinnville, TN37110, USA

²Syngenta Seeds Inc., IA 50244, USA

³Department of Forestry, Environment, and Systems, Kookmin University, Seoul 136-702, Korea

(Received on November 30, 2010; Accepted on April 1, 2011)

While many isolates of *Alternaria alternata* are common saprophytes on trees and shrubs, this study clearly demonstrated that *A. alternata* is a primary pathogen in lilac (*Syringa* sp.), causing a leaf-blight that affects different *Syringa* species. Isolates of *Alternaria* sp. were collected from leaf blight samples of lilacs in the field. The internal transcribed spacer (ITS) region and morphological characterization were used to identify lilac blight pathogen. Based on 100% ITS nucleotide sequence identities to the *Alternaria* genus in the GenBank and morphological features, these isolates were identified as *A. alternata*. Disease symptoms were reproduced in lilac plants inoculated with *A. alternata* mycelial plugs and sprayed with a fungus-free culture filtrate, indicating that pathogenesis in lilac involves secondary metabolites or toxins. Diagnostic primers were developed to detect *Alternaria* sp. and *A. alternata* in lilac leaf blight based on ITS region and four known genes associated with pathogenesis in *A. alternata*: mixed-linked glucanase precursor, endopolygalacturonase, hsp70, and histone genes. The results from our study indicated *A. alternata* is a primary pathogen in lilac leaf blight, and these diagnostic primers can be used as a tool for the fast detection of *A. alternata* associated with lilac leaf blight.

Keywords : *Alternaria alternata*, lilac leaf blight, *Syringa* species, pathogen detection, toxins

Many isolates of *Alternaria alternata* are common saprophytes on plants and plant debris (Ellis, 1971; Rotem, 1994; Sinclair et al., 1987). *Alternaria alternata* is also one of the most important allergenic molds in the USA (Pharmacia Diagnostics, 1992). However, strains of *A. alternata* include some of the most destructive plant pathogens that

affect a wide range of host plants, causing leaf spots, blights, blossom rots, and fruit rots. More than 380 hosts, including lilacs, have been recorded in the USDA Systematic Botany and Mycology Fungus-Host Distribution Database (<http://nt.ars-grin.gov>). Some isolates of *A. alternata* cause severe diseases in different ornamental crops, including trees and shrubs (Chase, 1998; Jones, 2001; Masangkay et al., 1999; Pryor et al., 2002; Sinclair et al., 1987). However, only a few studies have been conducted on trees and shrubs (Sinclair et al., 1987). The leaf-blight disease caused by *A. alternata* was first observed in 1996, and it was reported as one of the most serious diseases of lilac in middle Tennessee, USA (Mmbaga and Sheng, 1997; Mmbaga et al., 2005). Out of 56 cultivars within 10 *Syringa* species evaluated in field environment, only 10 cultivars were consistently resistant to this disease (Mmbaga et al., 2005).

Members of the genus *Alternaria* frequently cause quiescent infections in which the fungus enters the tissue and remains dormant until changes in environmental conditions favor infection. *Alternaria*-blight symptom development in lilac is consistent with that from quiescent infections. This disease has remained a persistent problem over multiple years, and healthy-looking plants can suddenly develop infection in late June to early July resulting in severe defoliations. The mode of infection was typical of plant pathogens that persist between seasons on infected plants, with symptoms starting on the upper or middle part of susceptible plants and subsequently spreading over the entire plant (Mmbaga et al., 2005; Pschedt and Moorman, 2001). Infections by *Alternaria* species typically cause the formation of necrotic lesions, which sometimes have a target-like appearance surrounded by an un-invaded chlorotic halo. This halo is created by the diffusion of fungal metabolites, which act as toxins (Agarwal et al., 1997; Tewari, 1983).

Like many fungal pathogens, *A. alternata* is known to produce a spectrum of cell wall-degrading enzymes. These

*Corresponding author.

Phone) +1-931-815-8143, FAX) +1-931-668-3134

E-mail) mmbaga@tnstate.edu

chemically diverse secondary metabolites are employed by fungal invaders during pathogenesis and are not required for normal growth or reproduction (Eshel et al., 2000, 2002a, 2002b). The secondary metabolites enable the fungus to degrade different types of cell wall polymers, such as cellulose, hemicellulose, and pectin (Walton, 2000). Previous studies suggested the involvement of endo- and exoglucanases in pathogenicity and a correlation between enzyme production and symptom development during the interaction between *A. alternata* and its host plants (Eshel et al., 2002a). Eshel et al. (2002b) demonstrated that the production of endoglucanases in *A. alternata* was triggered by a pathogen-induced pH increase on the host. Correlation-studies between enzyme production and symptom development suggested that endo- and exo-glucanases were involved in *A. alternata* pathogenicity (Eshel et al., 2000, 2002a, 2002b). Enzymatic processes in *Alternaria* infections are essentially similar to those in other diseases.

Although *A. alternata* of citrus was found to be dependent on endopolygalacturonase activity for establishing an infection, the tangerine pathotypes of *A. alternata* did not show this dependency, possibly because this pathogen largely depends on toxin production for the colonization of its host (Isshiki et al., 2001). In general, these substances are host-specific or non-host-specific; host-specificity is associated with high toxicity to a narrow host range while the non-host-specific toxins have relatively mild phytotoxic effects to a broad spectrum of plant species. Typically, tissues weakened by stresses, senescence, or wounding are more susceptible to *Alternaria* infections than healthy tissues. Previous reports also suggest that a saprophytic *Alternaria* species can become parasitic when they encounter a weakened host. Thus, a clear distinction between saprophytic and parasitic *Alternaria* is not always evident.

Detection of *Alternaria* fungus by PCR-based assays was reported in previous studies (Johnson et al., 2000; Konstantinova et al., 2002; Zur et al., 2002). A PCR-assay reported by Konstantinova et al. (2002) can detect *Alternaria* at the genus level, but it cannot detect any specific species. Zur et al. (2002) developed PCR primers for the internal transcribe spacer (ITS) region that could detect *A. alternata* or *A. solani*, but failed to distinguish between these two species. Johnson et al. (2000) developed a PCR-assay to amplify the AMT toxin gene from a pathogenic *A. alternata* isolate that causes Alternaria blotch of apple, and they used this assay to distinguish pathogenic and non-pathogenic pathotypes of *A. alternata*. The objectives of this study were to 1) confirm the pathogenicity of *A. alternata* as a primary pathogen in lilac causing leaf blight, 2) determine if *A. alternata* pathogenicity in lilac is associated with secondary metabolites or toxin production, and 3) develop PCR-based assays to detect *Alternaria* spp. and *A.*

alternata associated with leaf blight disease of lilacs.

Materials and Methods

Diagnosis of the disease. Leaf blight samples were collected from naturally infected lilac (*Syringa* spp.) leaves in the lilac-germplasm evaluation plots at Tennessee State University, Nursery Crops Research Center in McMinnville, TN, USA (Fig. 1A). The fungal spores were observed under a compound microscope, and displayed morphological features typical of *A. alternata* (Fig. 1B). Although *Alternaria* spores were observed on infected leaves, the pathogen was isolated from surface sterilized leaves to eliminate *Alternaria* spp. epiphytic saprophytes growing on the leaf surfaces. Pure cultures and fungus colonies were grown in 2% potato dextrose agar (PDA) at room temperature (20–23°C). Pathogenicity tests were conducted using an *in-vitro* assay technique with detached leaves (Loladze et al., 2005; Park et al., 2008). Disease-free leaves were collected from *S. vulgaris* (unnamed cultivar of common lilac) and maintained in clear plastic containers/moist chambers lined with paper towels soaked with 20 ml of sterile water. Four leaves were placed in each moist chamber and inoculated with 5.0-mm plugs of 7-day-old cultures of the *A. alternata*. The inoculum was placed in the middle of each leaf. Leaves for control were mock inoculated with a plug of sterile PDA. Inoculated leaves were then maintained at room temperature (20–23°C). Two isolates (T13 and T14) were evaluated and each was replicated four times with each replicate consisting of one clear plastic containers/moist chambers containing four leaves. The pathogenicity test was repeated. At the end of each experiment, the pathogen was re-isolated from infected leaves to complete Koch's postulate. Pathogenic isolates were identified using morphological characteristics and DNA sequence analysis of the ITS region.

Determining the potential role of secondary metabolites in pathogenicity of *A. alternata*. This study was conducted to confirm previous observations in the field that had

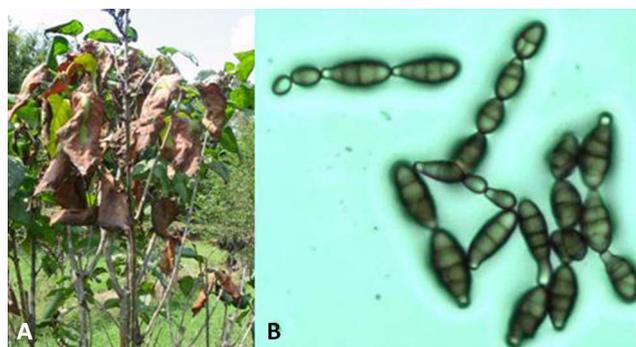


Fig. 1. Field-grown lilac plants of *Syringa prestoniae* infected with lilac leaf blight disease (A) and *Alternaria alternata* spores (B).

suggested the involvement of a toxin in disease symptom development. Pure cultures of *A. alternata* were macerated in a sterile blender and filtered through double layers of cheese cloth to remove large particles. The filtrate was then passed through a separation funnel to remove spores and hyphal pieces; a spore-free and mycelium-free filtrate was collected. Healthy lilac plants of *S. prestoniae* 'Isabella', *S. prestoniae* 'James McFarlane', and *S. meyeri* 'Dwarf Korean' were sprayed with the fungus-free filtrate using an atomizer; control plants were sprayed with filtrate derived from sterile PDA. A replication of four individual plants per cultivar was used, with half of each plant sprayed with the fungus filtrate and the other half sprayed with PDA filtrate. A clear plastic sheet was used to separate the treatments during spraying. Sprayed plants were maintained at 25±3°C greenhouse. Another set of plants was sprayed with an *A. alternata* spore suspension containing 10⁶ spores/ml. Plants were sprayed to run-off and leaves were allowed to dry before incubation at 100% Relative Humidity in a moist chamber for 48 hours at room temperature (23–25°C). Plants were arranged in a randomized, complete-block design and maintained at 25±3°C in a greenhouse.

DNA sequence analysis and development of specific primers. Using DNeasy Plant Mini Kits (Qiagen Inc, Valencia, CA, USA) and manufacturer's recommendations, genomic DNA was extracted from pure cultures and from spores obtained directly from infected leaves that had been incubated in a moist chamber for 48 hours to allow abundant spore production. PCR amplification was performed using a Touchgene Thermal Cycler (Barloworld Scientific Ltd, United Kingdom). Each 50-µl PCR reaction mixture consisted of 36 µl sterile ddH₂O, 5 µl 10X PCR buffer

(Promega), 3 µl MgCl₂ (25 mM), 1.5 µl dNTP (10 mM total, 2.5 mM each), 1.5 µl each primer (20 ng/µl), 0.2 µl Taq polymerase (Promega) (5 U/ul), and 1.3 µl template DNA (20 ng/µl). PCR cycles consisted of an initial denaturation step at 94°C for 5 min followed by 42 cycles of 1 min at 93°C (denaturation), 1 min at 30 to 60°C (annealing), and 2 min at 72°C (extension). The annealing temperature was based on the primer T_m (Table 1), typically five-degree less than the lower primer T_m (T_m-5). A final extension cycle at 72°C for 5 min was followed by a 4°C soak. The PCR products were visualized with UV light after 1.0–1.5% agarose-gel electrophoresis in 1X TBE stained with ethidium bromide.

ITS universal primer pair ITS1-F/ITS4 were used to amplify the ITS region in *A. alternata* by using standard PCR procedure described above (White et al., 1990). PCR products amplified from the primer pair ITS1-F/ITS4 were sequenced (Davis Sequencing Inc., Davis, CA, USA) and blasted to GenBank database. Sequence of *A. alternata* isolates was deposited into GenBank.

Molecular diagnostic primers to detect *Alternaria* spp. and *A. alternata* were developed using several loci. A PCR primer pair Al-f1/Al-r1 was designed from the ITS sequence of the *Alternaria* genus using Primer3 software (<http://frodo.wi.mit.edu/primer3/>) (Table 1). In addition, other diagnostic primers were designed using information obtained from the literature and the GenBank. These diagnostic primers, including those for various genes associated with pathogenesis, were used to identify *Alternaria* pathogens from lilacs. These genes included hsp70, a histone gene, endopolygalacturonase gene, and *A. alternata* mixed-linked glucanase precursor gene. Three primers, aa-hsp-f1, aa-hsp-r1 and aa-hsp-r2, were designed from the *A. alternata*

Table 1. Primers and their properties for *Alternaria* species in this study.

Primer name ^a	Sequence (5' to 3')	Size (bp)	T _m (°C)	Location ^b	GenBank accession ^c
Al-f1	CCCACCACTAGGACAAACA	19	60.16	167–185	DQ023279
Al-r1	GCTTAATGGATGCTAGACCT	20	38.35	536–517	DQ023279
aa-hsp-f1	ATCTCTGCTAAGAACGCTCTCG	22	62.67	13–34	U87808
aa-hsp-r1	GCTGAGACTCGTACTCGTCCTT	22	64.54	193–172	U87808
aa-hsp-r2	ACCAGCTCCGTAGAACTTCATC	22	62.67	249–228	U87808
aa-his-f1	TCATCGCATCGCAACATACT	20	58.35	58–77	AF404640
aa-his-r1	TCATCGCATCGCAACATACT	20	62.45	413–394	AF404640
aa-endo-f1	GTCCCTTCAGGCACAACCTT	20	59.19	19–38	AY629233
aa-endo-r1	GCTGGAGCCAATATCGAAAC	20	59.67	426–407	AY629233
aa-gp-f1	CGGCAACAACACTACATCATCG	20	60.40	129–148	AF282319
aa-gp-r1	CTCCTGGTCAAAAGGAGCTG	20	62.45	792–773	AF282319

^a Al-f1, aa-gp-f1, aa-hsp-f1, aa-his-f1, and aa-endo-f1 are forward primers and Al-r1, aa-gp-r1, aa-hsp-r1, aa-hsp-r2, aa-his-r1, and aa-endo-r1 are reverse primers.

^b Location signifies the primer is located at the position in the sequence of the corresponding GenBank accession, such as the Al-f1 is located at the 167 to 185 of the sequence of DQ023279 of GenBank accession.

^c DQ023279: *A. alternata* ITS, 608 bases; U87808: *A. alternata* hsp70 mRNA, partial cds; AF404640: *A. alternata* s.l. STE-U4267 isolate CR26 histone gene, partial cds; AY629233: *A. alternata* strain PR325 endopolygalacturonase gene; and AF282319: *A. alternata* mixed-linked glucanase precursor gene, partially cds.

hsp70 gene (GenBank accession no. U87808); the primers, aa-his-f1 and aa-his-r1 were designed from the *A. alternata* histone gene (GenBank accession no. AF404640); the primers, aa-endo-f1 and aa-endo-r1 were designed from the *A. alternata* endopolygalacturonase gene (GenBank accession no. AY629233); and the primers, aa-gp-f1 and aa-gp-r1 were designed from the *A. alternata* mixed-linked glucanase precursor gene (GenBank accession no. AF282319) (Table 1). Primers were prepared by Qiagen Inc. and they were used to evaluate our isolates using standard PCR protocols described above. A total of 28 DNA samples were tested including eight *A. alternata* isolates, 11 from lilac leaves infected with *Alternaria* leaf blight, two from healthy lilac leaves, and seven from other fungi including *Acremonium alternatum*, *Botryosphaeria dothidea*, *Botrytis cinerea*, *Cladosporium* sp., *Colletotrichum acutatum*, *C. gloeosporioides*, and *Phomopsis eucommicola*.

Results

Fungal species identification and diagnosis of the disease. All isolates of *A. alternata* produced a 608-bp fragment using the primer pair ITS1-F/ITS4. All isolates were determined to have identical sequences in the ITS region (GenBank Accession No. DQ023279). The ITS region and morphological characterization (Ellis, 1971, Fig. 1B) were used to identify lilac blight pathogen. Based on 100% ITS nucleotide sequence identities to the *Alternaria* genus in the GenBank (accession nos. GU566303 and FJ904919) and morphological features, these isolates were identified as *A. alternata*. Leaf blight symptoms developed on detached leaves inoculated with fungal mycelial plugs of two *Alternaria* isolates (T13 and T14). Three to four days after inoculation, initial development of small dark lesions, indicating infection establishment, were apparent on the underside of the leaf, underneath the mycelia plugs. Lesions expanded to cover the whole leaf in about 21 days, but leaves inoculated with sterile PDA plugs remained disease-free (Fig. 2); spray inoculation with *Alternaria* spores

produced similar symptoms. Of the two isolates evaluated, one (T14) was slightly more virulent than the other (T13) isolates and produced larger lesions in both experiments (Fig. 3). Colonies of *Alternaria* sp. identical to those used for inoculation were re-isolated from infected leaves, thereby confirming that this fungus was the primary pathogen that caused leaf blight disease in lilac. Fungus colonies were dark to grey-black and conidiophores arising singly or in small groups produced spores in chains. Conidiospores were large with longitudinal and transverse septa and a short beak typical for *Alternaria* spp. (Fig. 1B).

Determining the potential role of secondary metabolites in pathogenicity of *A. alternata*.

The fungus-free, culture filtrate was evaluated for the ability to induce wilting and produce necrotic lesions similar to those produced by the pathogen. Our results clearly demonstrated that the filtrate contained a substance that caused cell death in *S. prestoniae* ‘Isabella’ and *S. prestoniae* ‘James McFarlane’, but not on *S. meyeri* ‘Dwarf Korean’. Both *S. prestoniae* ‘Isabella’ and *S. prestoniae* ‘James McFarlane’ developed necrotic blotches when sprayed with fungus-free, culture filtrate (Fig. 4A). Development of symptoms on *S. prestoniae* ‘James McFarlane’ leaves sprayed with culture filtrate showed symptoms within 4 days, leaves subsequently wilted and

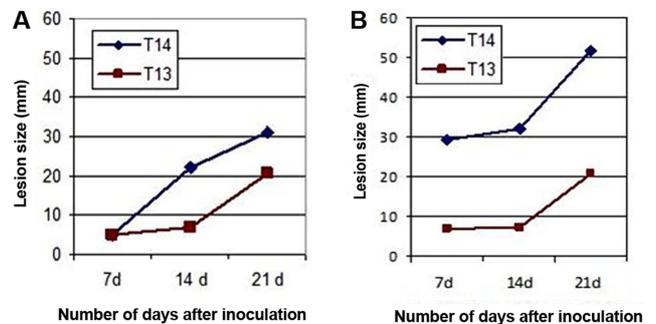


Fig. 3. Development of necrotic lesions on detached leaves of *Syringa vulgaris* inoculated with two isolates of *Alternaria alternata* (T13 and T14) in two experiments (A, B).



Fig. 2. Lilac leaf blight disease development on detached leaves pathogenicity tests on *Syringa vulgaris*; leaf inoculated with sterile growth medium (far left) remained free of symptoms 21 days after inoculation.

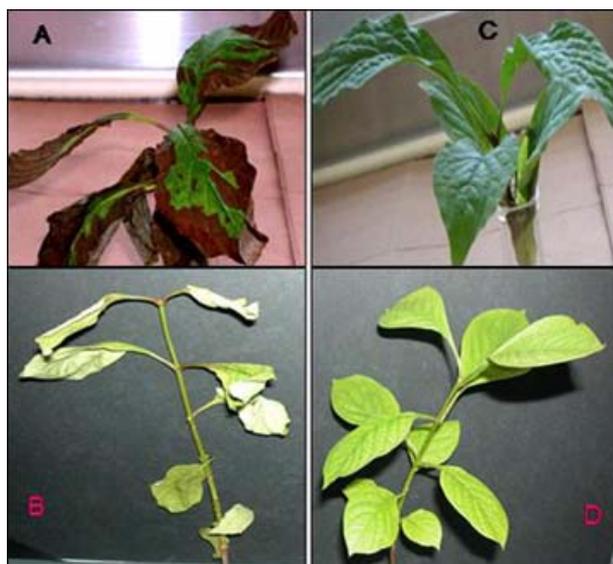


Fig. 4. Leaf wilting and necrotic blotches reproduced by a fungus-free culture filtrate of *Alternaria alternata* on *Syringa prestoniae* ‘Isabella’ (A) and *S. prestoniae* ‘James McFarlane’ (B) as compared to symptom-free plants sprayed with PDA filtrate (C and D).

died (Fig. 4B). However, symptom development in ‘Isabella’ was delayed to 6–7 days. All plants sprayed with PDA filtrate remained symptom-free (Figs. 4C and 4D). Although the *Alternaria* filtrate was toxic to *S. prestoniae* ‘Isabella’ and *S. prestoniae* ‘James McFarlane’, it showed no signs of toxicity on *S. meyeri* ‘Dwarf Korean’.

Development of diagnostic PCR primers for *A. alternata*.

The diagnostic primer pair Al-f1/Al-r1 produced a 370-bp amplification product in all *Alternaria* isolates (Fig. 5). All DNA samples extracted from pure cultures of *Alternaria* isolates from lilac leaf blight and from lilac leaf blight tissue produced the PCR product; however, the DNA samples from non-related fungi, such as *Acremonium alternatum*, *Botryosphaeria dothidea*, *Botrytis cinerea*, *Cladosporium* sp., *Colletotrichum acutatum*, *C. gloeosporioides*, and *Phomopsis eucommicola*, did not yield this PCR product. The sequence of the PCR product amplified from the primer pair Al-f1/Al-r1 showed 100% (370/370) identical similarity with the corresponding region of the *Alternaria* ITS sequence, indicating that the 370-bp PCR product from the primer pair Al-f1/Al-r1 is a useful diagnostic tool to identify or detect *Alternaria* pathogen in lilacs.

Different *Alternaria* species can have the same sequences in the ITS region, for example, ITS sequences of GenBank accession AY154682 of *A. alternata*, AY154684 of *A. longipes*, and AY154683 of *A. mali* are identical, and these species cannot be distinguished by their ITS region. Thus,

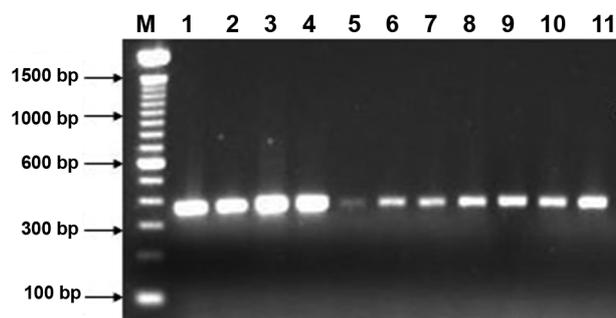


Fig. 5. Eleven PCR fragments with the same size of 370 bp amplified from the primer pair Al-f1/Al-r1 in eleven DNA samples of *Alternaria* sp. Lane 1 to 4 from *A. alternata* and Lane 5 to 11 from lilac leaves infected with *Alternaria* leaf blight. The left lane is a 100-bp, molecular weight marker. Primer pair is described in Table 1.

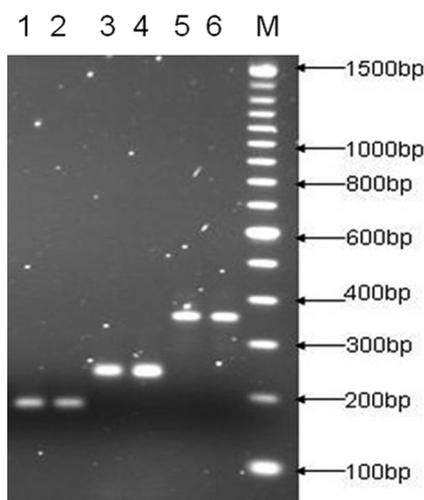


Fig. 6. DNA patterns amplified from three primer pairs in *Alternaria alternata*: (1) aa-hsp-f1/aa-hsp-r1 (Lane 1 & 2); (2) aa-hsp-f1/aa-hsp-r2 (Lane 3 & 4), and (3) aa-his-f1/aa-his-r1 (Lane 5 & 6). Lane M is a 100-bp, molecular weight marker. Primer pairs are described in Table 1.

we examined other loci for developing diagnostic tools to identify and distinguish *Alternaria* species that cause lilac leaf blight. Five diagnostic primer pairs were designed based on four loci in *A. alternata* and used to identify the pathogen (Table 1). The five diagnostic primer pairs produced a PCR product with the *A. alternata* DNA samples either from pure cultures or from the infected lilac leaves, but no PCR product was produced with DNA samples from other fungal species. PCR products amplified from the five primer pairs: aa-hsp-f1/aa-hsp-r1, aa-hsp-f1/aa-hsp-r2, aa-his-f1/aa-his-r1, aa-endo-f1/aa-endo-r1, and aa-gp-f1/aa-gp-r1 are shown in Figs. 6 and 7, and their sizes are 181 bp, 237 bp, 356 bp, 408 bp, and 679 bp, respectively. Sequences of the PCR products amplified

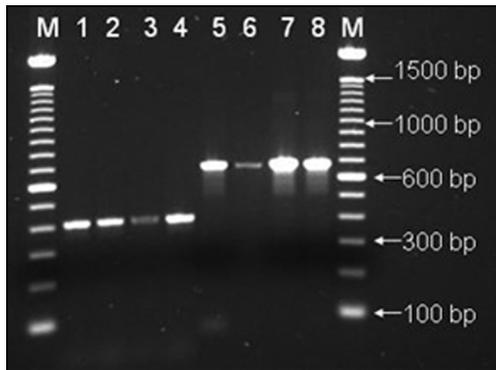


Fig. 7. DNA patterns amplified from two primer pairs in *Alternaria alternata*: (1) aa-endo-f1/aa-endo-r1 (Lane 1 to 4) and (2) aa-gp-f1/aa-gp-r1 (Lane 5 to 8). Lane M is a 100-bp, molecular weight marker. Primer pairs are described in Table 1.

from the primer pairs, aa-gp-f1/aa-gp-r1, aa-hsp-f1/aa-hsp-r2, aa-his-f1/aa-his-r1 and aa-endo-f1/aa-endo-r1 are available in the GenBank (accessions nos. DQ026789, DQ026790, DQ026791, and DQ026792, respectively). The sequence of DQ026789 amplified from aa-gp-f1/aa-gp-r1 showed 100% (659/664, 15 base gap) identity with the locus of *A. alternata* mixed-linked glucanase precursor (AF282319 in GenBank). The sequence amplified from aa-hsp-f1/aa-hsp-r2 showed 100% (237/237) identity to those in accession AAU87807 and AAU87808 in GenBank for the locus of *A. alternata* hsp70 mRNA gene. The sequence amplified from aa-his-f1/aa-his-r1 showed 100% (356/356) identity with the sequence in accession AF404640 in GenBank for the locus of *A. alternata* s1 STE-U4267 isolate CR26 histone gene. The sequence amplified from aa-endo-f1/aa-endo-r1 showed 100% (408/408) identity with the sequence in accession AY629216 to AY629234 in GenBank for the loci of *A. alternata* endopolygalacturonase gene. Thus, from this work we postulate that *A. alternata* is the primary causal pathogen for lilac leaf blight, and the diagnostic primers may be useful to distinguish the pathogenic *A. alternata* isolates in lilac.

Discussion

Of 10 *Alternaria* species pathogenic to plants, *A. alternata* (syn. *A. tenuis*), *A. citri*, *A. panax*, and *A. tenuissima* have been reported to cause disease on trees and shrubs (Sinclair et al., 1987). In most reports where *Alternaria* isolates were associated with foliar infections, the *Alternaria* sp. were not shown to be the primary or secondary pathogens and the *Alternaria* sp. was assumed to be a secondary invader of foliage (Sinclair et al., 1987). Current and previous reports indicated that *A. alternata* is a primary pathogen in lilac causing destructive leaf blight on susceptible *Syringa* species (Mmbaga et al., 2005). *Syringa prestoniae* 'Isabella',

and *S. prestoniae* 'James McFarlane' were shown to be highly resistant to powdery mildew and resistant or moderately resistant to bacterial blight caused by *Pseudomonas syringae* (Mmbaga and Sheng, 1997). However, these plants were highly susceptible to the *Alternaria* blight that causes more damage to the plants than the powdery mildew or bacterial blight. In contrast, *S. meyeri* 'Dwarf Korean' was resistant to all three diseases, while some *S. vulgaris* (common lilac) cultivars were susceptible to both bacterial blight and powdery mildew, and did not develop *Alternaria* blight symptoms (Mmbaga and Sheng, 1997; Mmbaga et al., 2005).

The development of lilac blight symptoms from the fungus-free culture filtrate confirmed that some secondary metabolites from the fungus are involved in pathogenesis. While both *S. prestoniae* 'Isabella' and *S. prestoniae* 'James McFarlane' wilted and developed necrotic blotches from the culture filtrate, *S. meyeri* 'Dwarf Korean' was not affected. A previous report showed that *S. prestoniae* 'James McFarlane' was highly susceptible, *S. prestoniae* 'Isabella' was slightly less susceptible to *Alternaria* blight, and *S. meyeri* 'Dwarf Korean' was resistant to this disease (Mmbaga et al., 2005). The same cultivars that were susceptible to the fungus were also sensitive to the toxin, indicating host specificity. Correlation of host resistance and susceptibility with sensitivity to *A. alternata* host specific toxins has been reported in citrus diseases (Gardner et al., 1986; Kohmoto et al., 1979). Other reports on *A. alternata* have shown that isolates that produce host-specific toxins generally display severe effects on a rather narrow range of species, and the toxins are essential for pathogenicity (Isshiki et al., 2001; Thomma, 2003). Saprophytic isolates of *A. alternata* were not associated with the production of host-specific toxins (Thomma, 2003). Of *A. alternata* isolates recovered from citrus, pathogenic and saprophytic isolates were morphologically and genetically similar, and the causal agents of citrus diseases could only be differentiated using pathogenicity tests, toxin assay or genetic markers (Akimitsu et al., 2003; Kohmoto et al., 1979, 1991, 1993). The ability to produce endopolygalacturonase is essential for *A. alternata* strains to cause citrus black rot (Isshiki et al., 2001). In our studies, fungus-free, culture filtrates caused necrotic lesions similar to those produced by the fungus, indicating that a toxin is involved in host colonization and infection establishment. The role of toxin in pathogenicity is supported by other reports on *Alternaria* toxin involvement in all stages of infection, from initial penetration of the tissue to establishment, colonization, and death of plant tissue (Isshiki et al., 2001; Kohmoto et al., 1979, 1991, 1993; Rotem, 1994).

Alternaria alternata is known to secrete several host-

specific toxins associated with pathogenesis, it is most commonly associated with leaves of trees and shrubs as a secondary pathogen and not as a primary pathogen as demonstrated in this study. The most important *Alternaria* disease reported on woody plants is caused by *A. panax*, on members of the aralia family (Sinclair et al., 1987). Depending on the taxon, *Alternaria* infected leaves developed brown, necrotic lesions with or without chlorotic bands and with or without concentric rings (Mmbaga and Sheng, 1997; Mmbaga et al., 2005). Some cultivars such as *S. prestoniae* 'James McFarlane' and *S. prestoniae* 'Isabella' are promoted for nursery production because of their resistance to powdery mildew; however, these cultivars were susceptible to a highly destructive *Alternaria* blight that occurred in mid summer (Mmbaga et al., 2005). The *Alternaria* blight resistance in *S. meyeri* 'Korean Dwarf' was confirmed in this study; other resistance sources including multiple disease resistance have also been previously reported (Mmbaga et al., 2005).

Specific primer pairs developed in this study can amplify specific gene loci for the *A. alternata* pathogen of lilac. These specific primers allow PCR-based detection and identification of the *A. alternata* pathogen, even without *Alternaria* spores. The PCR-based diagnostic detection in this study provides a fast and reliable tool to identify and detect *A. alternata* pathogenic isolates that cause lilac leaf blight.

References

- Agarwal, A., Garg, G. K., Devi, S., Mishra, D. P. and Singh, U. S. 1997. Ultrastructural changes in *Brassica* leaves caused by *Alternaria brassicae* and destruxin B. *J. Plant Biochem. Biotechnol.* 6:25–28.
- Akimitsu, K., Peever, T. and Timmer, L. W. 2003. Molecular, ecological and evolutionary approaches to understanding *Alternaria* diseases of citrus. *Mol. Plant Pathol.* 4:435–446.
- Chase, A. R. 1998. *Alternaria* diseases of ornamentals. *Western Connection Turf & Ornamentals* 1(3) (www.westernfarmservice.com/newsletters/turf/alternaria.pdf).
- Ellis, M. B. 1971. Demataceous hyphomycetes. CAB International, Wallingford, Oxon, England. 608 pp.
- Eshel, D., Ben-Arie, R., Dinoor, A. and Prusky, D. 2000. Resistance of gibberellin-treated persimmon fruit to *Alternaria alternata* arises from the reduced ability of the fungus to produce endo-1,4- β -glucanase. *Phytopathology* 90:1256–1262.
- Eshel, D., Lichter, A., Dinoor, A. and Prusky, D. 2002a. Characterization of *Alternaria alternata* glucanase genes expressed during infection of resistant and susceptible persimmon fruits. *Mol. Plant Pathol.* 3:347–358.
- Eshel, D., Miyara, I., Ailing, T., Dinoor, A. and Prusky, D. 2002b. pH regulates endoglucanase expression and virulence of *Alternaria alternata* in persimmon fruit. *Mol. Plant-Microbe Interact.* 15:774–779.
- Gardner, J. M., Kono, Y. and Chandler J. L. 1986. Bioassay and host-selectivity of *Alternaria citri*: The major toxin specific to rough lemon plants. *Phytochemistry* 24:2861–2868.
- Isshiki, A., Akimitsu, K., Yamamoto, M. and Yamamoto, H. 2001. Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. *Mol. Plant-Microbe Interact.* 14:749–757.
- Jones, R. K. and Benson, D. M. 2001. Diseases of Woody Ornamentals and Trees in Nurseries. APS PRESS, the American Phytopathological Society, St. Paul, Minnesota, USA. 350 pp.
- Johnson, R. D., Johnson, L., Kohmoto, K., Otani, H., Lane, C. R. and Kodama, M. 2000. A polymerase chain reaction-based method to specifically detect *Alternaria alternata* apple pathotype (*A. mali*), the causal agent of *Alternaria* blotch of apple. *Phytopathology* 90:973–976.
- Kohmoto, K., Scheffer, R. P. and Whiteside, J. O. 1979. Host selective toxins from *Alternaria citri*. *Phytopathology*, 69:667–671.
- Kohmoto, K., Akimitsu, K. and Otani, H. 1991. Correlation of resistance and susceptibility of citrus to *Alternaria alternata* with sensitivity to host specific toxins. *Phytopathology* 81:719–722.
- Kohmoto, K., Itoh, Y., Shimomura, N., Kondoh, Y., Otani, H., Kodama, M., Nishimura, S. and Nakatsuka, S. 1993. Isolation and biological activities of two host-specific toxins from tangerine pathotype *Alternaria altanata*. *Phytopathology* 83:495–502.
- Konstantinova, P., Bonants, P. J. M., van Gent-Pelzer, M. P. E., van der Zouwen, P. and van den Bulk, R. 2002. Development of specific primers for detection and identification of *Alternaria* spp. in carrot material by PCR and comparison with blotter and plating assays. *Mycol. Res.* 106:23–33.
- Loladze, A., Garland Campbell, K. A. and Chen, X. 2005. Development of a detached leaf assay for stripe rust resistance screening. *Agronomy Abstracts*. <http://crops.confex.com/crops/2005am/techprogram/P3298.HTM>
- Masangkay, R. F., Mabbayad, M. O., Paulitz, T. C. and Watson, A. K. 1999. Host range of *Alternaria alternata* f.sp. *sphenocleae* causing leaf blight of *Sphenoclea zeylanica*. *Can. J. Bot.* 77:103–112.
- Mmbaga, M. T. and Sheng, H. 1997. Evaluation of lilac (*Syringa* spp.) for multiple disease resistance to powdery mildew and bacterial blight in McMinnville, TN. *Proc. South. Nurserymen's Assoc. Res. Conf.* 42:512–518.
- Mmbaga, M. T., Sauvé, R. J., Nnodu, E. and Zhou, S. 2005. Multiple disease resistance to powdery mildew, bacterial blight, and *Alternaria* blight in lilac (*Syringa* spp.). *J. Arboriculture* 31:1–9.
- Park, D.-S., Sayler, R. J., Hong, Y. G., Nam, M. H. and Yang, Y. 2008. A method for inoculation and evaluation of rice sheath blight disease. *Plant Dis.* 92:1.
- Pharmacia Diagnostics. 1992. M6 *Alternaria alternata* (*A. tenuis*). <http://www.immunocapinvtrosight.com/templates/Allergens.asp?id=2204>
- Pryor, B. M. and T.J. Michailides, T. J. 2002. Morphological,

- pathogenic, and molecular characterization of *Alternaria* isolates associated with *Alternaria* late blight of pistachio. *Phytopathology* 92:406–416.
- Pschedt, J. W. and Moorman, G. W. 2001. Lilac diseases. In: *Diseases of Woody Ornamentals and Trees in Nurseries*, ed. by R. K. Jones and D. M. Benson, pp. 221–224. APS Press, St. Paul, MN, USA.
- Rotem, J. 1994. The genus *Alternaria*: Biology, epidemiology and pathogenicity. The American Phytopathological Society, St. Paul MN, USA. 322 pp.
- Sinclair, W. A., Lyon, H. and Johnson, W. T. 1987. *Diseases of Trees and Shrubs*. Cornell University Press, Ithaca, NY, USA. 575 pp.
- Tewari, J. P. 1983. Cellular alterations in the blackspot of rapeseed caused by *Alternaria brassicae*. *Phytopathology* 73:831.
- Thomma, B. P. H. J. 2003. Pathogen profile *Alternaria* spp.: from general saprophyte to specific parasite. *Mol. Plant Pathol.* 4:225–236.
- Walton, J. D. 2000. Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: a hypothesis. *Fungal Genet. Biol.* 30:167–171.
- White, T. J., Bruns, T., Lee, S. and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications*, eds. by M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White, pp. 315–332. Academic Press, New York, NY, USA.
- Zur, G., Shimoni, E., Hallerman, E. and Kashi, Y. 2002. Detection of *Alternaria* fungal contamination in cereal grains by a polymerase chain reaction-based assay. *J. Food Prot.* 65:1433–1440.