

**Identification and characterization of a fungus that causes leaf spot on Japanese  
tree lilac in the Upper Midwest**

by

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This thesis is dedicated to Steve, for all your support and encouragement.

## TABLE OF CONTENTS

ABSTRACT	v
CHAPTER 1. GENERAL INTRODUCTION	1
Thesis organization	1
Literature Review	1
Literature Cited	10
Figures	15
CHAPTER 2. A LEAF SPOT DISEASE ON JAPANESE TREE LILAC IS CAUSED BY A <i>CERCOSPORA</i> SPECIES	16
Abstract	16
Introduction	17
Materials and Methods	18
Results	21
Discussion	23
Literature Cited	26
Tables	29
Figures	30
CHAPTER 3. TEMPERATURE OPTIMA OF A <i>CERCOSPORA</i> SP. THAT CAUSES LEAF SPOT ON JAPANESE TREE LILAC	31
Abstract	31
Introduction	31
Materials and Methods	33
Results	35
Discussion	36
Literature Cited	37
Tables	39
Figures	40
CHAPTER 4. SPORULATION OF A <i>CERCOSPORA</i> SPECIES PATHOGEN OF JAPANESE TREE LILAC	44
Introduction	44
Materials and Methods	45
Results and Discussion	48
Literature Cited	50
Tables	52
CHAPTER 5. GENERAL CONCLUSIONS	53
ACKNOWLEDGMENTS	55

## ABSTRACT

An unidentified pathogen caused symptoms of angular necrotic lesions on leaves, leaf chlorosis, and premature leaf drop on Japanese tree lilacs in the Upper Midwest and Idaho. We isolated the putative pathogen and confirmed pathogenicity twice through the use of Koch's postulates. Greenhouse-grown Japanese tree lilacs were inoculated with a mycelial suspension in water. Symptoms identical to those on naturally infected trees appeared 2 wk after inoculation on 1 of 8 (12.5%) of trees in the first trial and 2 of 8 (25 %) of trees in the second trial. The isolated pathogen was identified using both genetic and morphological characteristics. Amplification and sequencing of the ITS region of ribosomal DNA had high (97%) homology to several known species of *Cercospora* and one *Septoria* sp. in Genbank. Morphological characteristics included indistinctly multiseptate, hyaline spores produced within 2-5 mm necrotic lesions, delimited by leaf veins. The temperature optimum, for *in vitro* growth of the *Cercospora* sp. pathogen was determined by incubating six isolates at 10, 15, 20, 25, 30, and 37 °C. Colony diameter was greatest after 18 days of incubation at 20 or 25 °C. Linear regression of the results estimated the optimum temperature at 22°C. *In vitro* sporulation of the pathogen was studied by studying the affect of growth medium, mycelial transfer technique, and photoperiod on sporulation of six pathogen isolates. Treatments were incubated for 2 wk, after which spore counts were made by removing plugs of mycelium from each treatment, vortexing the plugs in distilled water for 30 s and using a hemacytometer to count spores. Spores were counted in only a few of the treatments, but there was some consistency in media and isolate associated with the production of spores.

## CHAPTER 1. GENERAL INTRODUCTION

### Thesis Organization

This thesis is divided into five chapters. The first chapter gives an introduction to leaf spot disease on Japanese tree lilac, an overview of Japanese tree lilac, and a description of pests and diseases found on lilacs. The second chapter describes the research I did to identify the Japanese tree lilac leaf spot pathogen. The third chapter describes an experiment that determined the optimum temperature for mycelial growth of the pathogen *in vitro*. The fourth chapter describes an experiment to determine how media, photoperiod, and plating technique affected sporulation of the pathogen. The final chapter summarizes the research and overall conclusions that were obtained from this research.

### Literature Review

Japanese tree lilac, *Syringa reticulata* ssp. *reticulata* (Blume) Hara, is a small tree that has become increasingly popular in the Upper Midwest. In this region, there is strong consumer demand for landscape trees that have aesthetic appeal, low maintenance requirements, and cold-hardiness. Japanese tree lilac is compact enough to serve as a street tree and is an acceptable choice for planting under aerial power lines. It is considered low maintenance and more disease resistant than other *Syringa* spp. (Dirr, 1998). Several cultivars of Japanese tree lilac have a compact form and produce dense panicles of white flowers in late May to early June (Dirr, 1998).

A leaf spot disease of unknown etiology has appeared on Japanese tree lilac in the Upper Midwest and Idaho over the past 5 years. This disease causes scattered chlorotic spots

on leaves, followed by 2- to 10 mm-diameter necrotic lesions that are angular, interveinal and often coalesce (Fig. 1A). Infected leaves prematurely fall from the tree, and severe infections result in a tree nearly barren of leaves by late Aug. to early Sept. (Fig. 1B). Until this study, no information was available about the identity, characteristics, or control of the pathogen causing this leaf spot disease.

### **Taxonomic classification of Japanese tree lilac**

Japanese tree lilac originated in Japan (Green and Chang, 1995) and was first classified as *Ligustrum reticulatum* by Blume in 1850. Since then, it has been renamed no less than 10 times (Hara, 1941). It is morphologically similar to two other lilac species found in eastern Asia: the Amur lilac, from Northeastern China and Korea, and the Pekin (Peking) lilac, from Northern China and Mongolia (Green and Chang, 1995). The taxonomic status of these three morphologically and genetically similar lilacs has been in flux since their first descriptions as species. In 1995, all three were defined as subspecies of *Syringa reticulata*: *S. reticulata* ssp. *reticulata* (Japanese tree lilac); *S. reticulata* ssp. *amurensis* (Rupr.) P.S. Greene & M.C. Chang (Amur lilac); and *S. reticulata* ssp. *pekinensis* (Rupr.) P.S. Greene & M.C. Chang (Pekin tree lilac). However, *S. reticulata* is still an accepted name for Japanese tree lilac (ITIS, 2005). A recent study (Li et al., 2001) suggested that the Pekin lilac differs considerably in genetics and morphology from Amur and Japanese tree lilacs. Li and co-workers suggested reorganization of the three lilacs into two species, *Syringa pekinensis* and *Syringa reticulata*, and one variety, *Syringa reticulata* var. *amurensis*. However, this system has not yet been verified and adopted.

Leaves of *Syringa reticulata* ssp. *reticulata* are typically >7 cm long with noticeable hairs on the leaf undersurface (especially along the midrib), short petioles compared to the leaves of the Amur and Pekin lilacs, and seed capsules with rounded tips and numerous lenticels (Green and Chang, 1995; Li et al., 2001). Japanese tree lilac is adapted to USDA hardiness zones 3 to 7 (Dirr, 1998).

Because of the recent increase in popularity of Japanese tree lilac in the urban landscape, more cultivars are being developed. One of the most popular cultivars, 'Ivory Silk', was selected in 1973 by Sheridan Nursery, Ontario, Canada. This cultivar is grafted onto *Syringa vulgaris* L. rootstock (Leiss, 1981; Dirr, 1998). It is a heavy-flowering cultivar that flowers at a young age and has a compact form. Other popular cultivars include 'Summer Snow' and 'Regent'. Additionally, there are a few variegated cultivars, including 'Chantilly Lace' and 'Cameo Jewel' (Dirr, 1998).

### **Pests and diseases of Japanese tree lilac**

Dirr (1998) describes Japanese tree lilac as being "possibly the most trouble-free lilac." It is considered more resistant to powdery mildew, scale insects, and lilac borers than many other lilacs. Diseases and pests noted previously on this species include bacterial blight, *Phytophthora* blight, ringspot virus, witches'-broom, graft blight (on those lilacs grafted onto *Ligustrum* L. spp.), leaf roll necrosis, leopard moth borer, caterpillars, giant hornet, lilac leaf miner, and scale insects (Dirr, 1998).

**Insect Pests of Japanese tree lilac.** There are three major insect pests of Japanese tree lilac. Although less common than on other *Syringa* spp., lilac borers (*Podosesia syringae syringae*

[Harr]) cause wilting and death of affected Japanese tree lilac branches. Another pest, lilac leaf miner (*Gracillaria syringella* [Fabricius]), tunnels into leaves in early June and later emerges to roll and skeletonize leaves. Lilac leaf miners are most common in the northeastern U.S. and parts of Canada (Felt and Rankin, 1932; Wisc. Dept. of Agriculture, n.d.). The third major insect pest of Japanese tree lilac is scale, which often is concentrated in lower stems of the plant. Two scales are equally injurious on Japanese tree lilac: oyster-shell scale (*Lepidosaphes ulmi* L.) and euonymous scale (*Unaspis euonymi* Cornstock) (Felt and Rankin, 1932).

### **Diseases of Japanese tree lilac**

**Witches'-Broom.** Witches'-broom is caused by a phytoplasma. Lilacs in the Midwest and Eastern U.S. are affected by this disease, which is transferred by unknown insect vectors or through grafting with infected cuttings. It occurs on many species of lilac, and severe occurrences have been found on Japanese tree lilac. The disease causes clusters of thin short twigs that resemble brooms at the tops of plants. Leaves on these brooms are distorted and chlorotic and no flowers are produced on these brooms. Affected branches experience dieback (Sinclair et al., 1987; Jones and Benson, 2001).

**Bacterial Blight.** Bacterial blight is caused by *Pseudomonas syringae* pv. *syringae*, which affects most lilac species. Symptoms of the disease start as brown leaf spots, often with a yellow halo. As the disease progresses, the spots enlarge and turn black, with discoloration usually starting at leaf margins and progressing towards the midrib in a wedge-like pattern, killing the leaf. Younger leaves and shoots are most susceptible. Succulent stems are girdled

and the shoot withers and dies. Infected flower clusters are blackened, and the buds die (Jones and Benson, 2001; Sinclair et al., 1987).

### **Damage caused by environmental conditions**

Several environmental factors cause disease-like symptoms on Japanese tree lilac. The species is sensitive to ozone, and daily atmospheric concentrations of greater than 0.05 ppm during the growing season or hourly concentrations of >0.1 ppm can result in rapid cell death. Subterminal leaves of growing shoots are the first to display symptoms. Symptoms of cell death from sensitivity to ozone are visible as flecks or stippling on upper leaf surfaces, sometimes followed by chlorosis and leaf drop. If severe injury occurs, the leaves may appear bleached or darkly pigmented on upper leaf surfaces (Sinclair et al., 1987).

Japanese tree lilac is also sensitive to the herbicide dicamba, which causes bleached leaves followed by leaf browning (Sinclair et al., 1987). Japanese tree lilac has moderate tolerance to salt and sulfur dioxide (Sinclair et al., 1987).

### **Leaf spots on lilacs**

Several species of fungi can cause leaf spots on *Syringa* spp. Two species of *Cercospora* attack lilacs: *Cercospora lilacis* (Desmaz.) Saccardo and *Cercospora macromaculans* Heald & F.A. Wolf (Farr et al., 1989). *Cercospora lilacis* produces tan circular lesions with reddish-purple margins <15 mm in diameter (Sobers and Gill, 1972). Additionally, Greene (1967) noted an unidentified species of *Cercospora* on *Syringa ×hyacinthiflora* (Hort. Lemoine) Rehd. 'Blue Hyacinth' in Wisconsin. He described

symptoms as 1-cm-diameter, mottled brown spots, producing conidia that were pallid olivaceous and 3-septate (Greene, 1967).

Other fungal leaf spots on *Syringa* spp. are caused by *Pleospora herbarum* (Pers.) Rabenh., *Alternaria alternata* (Fr.) Keissl., *Alternaria brassicae* (Berk.) Sacc., *Macrophoma halstedii* Ellis & Everh., *Phyllosticta porteri* Tehon & E.Y. Daniels, *P. syringae* Westend., and *Phyllosticta* sp. (Farr et al., 1989). *Phyllosticta syringae* Westend. is a common cause of leaf spot on lilacs. Symptoms of infection by this pathogen include gray spots surrounded by a darker border. Pycnidia are produced on the upper leaf surface and contain one-celled, lens-shaped spores measuring 5-8 by 3-3.5  $\mu\text{m}$  (Phillips, 1982).

### ***Mycosphaerella* and *Cercospora***

Many fungi cause leaf spots on a wide range of hosts worldwide. Many leaf spots are caused by the asomycete *Mycosphaerella* Johans., or one of the > 43 anamorph genera associated with *Mycosphaerella* (Crous and Braun, 2003; Goodwin et al., 2001). Some of these asexual fungi rarely or never produce sexual fruiting structures; they apparently exist only in the asexual stage. In such cases, it can be difficult to determine the teleomorph genus to which the species belongs. Adding to the complexity, some *Mycosphaerella* spp., like other sexual fungi, produce multiple asexual fruiting stages (Goodwin et al., 2001).

Molecular techniques have been helpful in determining associations between asexual and sexual stages of fungi. Goodwin et al. (2001) sequenced the ITS1 and ITS2 regions of ribosomal DNA and found evidence to support the contention that *Mycosphaerella* and *Cercospora* are monophyletic genera. Although few species of *Cercospora* have a confirmed sexual stage, those that have been identified have all been placed in

*Mycosphaerella* (Chupp, 1953; Goodwin et al., 2001). Molecular research has supported the theory that the genus *Cercospora* evolved from *Mycosphaerella* lineage (Goodwin et al., 2001).

The teleomorph genus *Mycosphaerella* is a member of the Dothideales and has more than 27 different Coelomycete or Hyphomycete anamorphs. Use of genetic techniques that sequence the ITS1, 5.8s, and ITS2 rDNA of fungal DNA has clarified the relationship between *Mycosphaerella* and its anamorphs. This genetic analysis has provided evidence that the cercosporoid fungi have evolved more than once within *Mycosphaerella* (Goodwin et al., 2001).

### ***Cercospora***

*Cercospora* is a large genus of anamorphic fungi associated with the teleomorph genus *Mycosphaerella*. *Cercospora* is one of the most diverse genera in the Hyphomycetes. Species are associated with leaf spots on numerous hosts, and can be found in nearly all climatic regions. Members of the genus can cause lesions on flowers, fruits, bracts, seeds, and pedicels (Chupp, 1953; Crous and Braun, 2003).

Previously, *Cercospora* spp. were considered to be mostly host specific (Chupp, 1953), and each time a *Cercospora* sp. was found on a new host it was considered a new species. However, results of cross-inoculation experiments, lack of morphological differences, and differences of less than 2 nucleotides in the ITS2 sequences of rDNA among many taxa have led to the current assumption that there is less host specificity than previously thought, and the taxonomy of *Cercospora* spp. is being re-examined (Crous and Braun, 2003; Goodwin et al., 2001).

Another change since Chupp's (1953) monograph of the genus is that many of the fungi Chupp described as *Cercospora* spp. have since been transferred into new genera based on morphology and genetics. Deighton (1967, 1973, 1976, 1979) reclassified many of the *Cercospora* species into new genera. The Cercosporoid complex refers to the true *Cercospora* genus and all fungi that were formerly classified as *Cercospora* spp. The genera in this complex include *Cercospora*, *Pseudocercospora*, *Stenella*, and *Passalora* (Crous and Braun, 2003). Genera in the Cercosporoid complex were more recently combined into three groups (Crous and Braun, 2003; Goodwin et al., 2001).

*Cercospora* spp. are characterized by having long, multiseptate, hyaline, acicular conidia with a conspicuous hilum, and solitary to fasticulate conidiophores that are unbranched, septate, smooth, long, and brown (Crous and Braun, 2003; Goodwin et al., 2001).

In nature many *Cercospora* pathogens are known to thrive in warm, humid conditions (Bair and Ayers, 1986; Windels et al., 1998). These conditions are favorable for all aspects of pathogenicity including sporulation, germ tube growth, and mycelial growth.

**Cercosporin.** Some *Cercospora* spp. can produce the photosensitizing compound cercosporin, which is involved in pathogenesis. Cercosporin is a light-activated molecule and damages plant cells through activated oxygen species. In the presence of light, the cercosporin molecule absorbs energy and is converted to an activated state. The activated cercosporin molecule then reacts with molecular oxygen to form radical and nonradical species of activated oxygen, which target macromolecules in the host plant cell such as lipids, proteins and nucleic acids, leading to host cell death (Daub and Ehrenshaft, 2000).

### **Sporulation of *Cercospora* in culture**

Sporulation of *Cercospora* spp. is affected by light, temperature, and composition of media. Beckman and Payne (1983) tested the influence of these factors on isolates of *Cercospora zeae-maydis* Tehon & E.Y.Daniels. They found that varying light treatment, culture medium, and the interaction of these factors resulted in differences in spore production. Overall, conidia production was greatest on V-8 agar, whereas colonies on PDA produced the most rapid mycelial growth but little to no sporulation. Colonies grown under diurnal light sporulated more prolifically than when exposed to constant light or constant darkness. Colony morphology, sporulation, and growth of the isolates varied depending on treatment, with some treatments resulting in reduced colony growth or increased production of sterile mycelia (Beckman and Payne, 1983).

Many *Cercospora* spp. are reluctant to sporulate in culture. Isolates of *Cercospora zeae-maydis* that initially produce sporulating gray mycelium when grown in culture eventually become overrun with sterile white mycelium (Weaver and Elliot, 1977).

Sporulating colonies are often maintained only by transferring conidia from sporulating colonies (Latterell and Rossi, 1974). However, Weaver and Elliott (1977) found that homogenizing sterile mycelium was also effective at triggering sporulation

**Temperature optima of *Cercospora*.** Temperature can influence sporulation, disease development, growth, and cercosporin production of *Cercospora* spp. Jenns et al. (1989) found that incubation at 20 to 30° C produced the highest cercosporin production in most isolates tested. They determined that potato dextrose agar and malt extract agars were most favorable for cercosporin production, and potato dextrose agar generally produced the

highest colony growth. Beckman and Payne (1983) researched optimal conditions for growth and sporulation of the gray leaf spot pathogen of corn, *Cercospora zeae-maydis*. They found that the optimal temperature for both growth and sporulation of this pathogen was 22 to 30° C; however, constant light inhibited both mycelial growth and sporulation.

## Objectives

The newly discovered leaf spot disease is a threat to the continued popularity of Japanese tree lilac as a landscape tree in the Upper Midwest. Information about the identity of the pathogen and the etiology of the disease is necessary to develop effective control strategies.

The goal of this research was to isolate and identify the pathogen causing leaf spot disease on Japanese tree lilac, and to take the first steps towards understanding the etiology of the disease. The objectives were to:

- 1) Isolate the pathogen producing leaf spot symptoms on Japanese tree lilac.
- 2) Confirm pathogenicity of the isolated pathogen by fulfilling Koch's postulates.
- 3) Determine the temperature optima for growth of the isolated pathogen.
- 4) Determine the effects of media, light and plating technique on sporulation in culture.

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**Figure 1.**

**A.**



**B.**



**Figure 1.** (A) Japanese tree lilac leaves showing symptoms of leaf spot disease, and (B) defoliation of Japanese tree lilac resulting from severe infection with leaf spot disease. Both trees were located at the Iowa State University Horticulture Farm, Gilbert IA, and symptoms were photographed during the summer of 2004.

## CHAPTER 2. A LEAF SPOT DISEASE ON JAPANESE TREE LILAC IS CAUSED BY A *CERCOSPORA* SPECIES

A manuscript prepared for submission to *Plant Disease*

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### Abstract

A disease of unknown etiology has been observed on Japanese tree lilac (*Syringa reticulata* ssp. *reticulata* [Blume] Hara) in the Upper Midwest and Idaho during the past five years. The disease causes brown, 0.3- to 1.0-cm diameter, angular lesions on leaves. Heavily infected leaves turn yellow and abscise prematurely. A putative fungal pathogen was isolated from symptomatic leaves. A pathogenicity test was performed twice by inoculating leaves of greenhouse-grown 'Ivory Silk' Japanese tree lilacs with a suspension of mycelial fragments from 2-week-old cultures of the fungus grown on potato dextrose agar. Characteristic symptoms appeared on leaves of trees in both trials, and reisolation from symptomatic leaves was successful. Polymerase chain reaction was used to amplify the internal transcribed spacer region of ribosomal DNA of four pathogenic isolates obtained from naturally infected leaves. The amplified DNA was sequenced and compared with sequences of known species in Genbank (National Center for Biotechnical Information, Bethesda, MD). Sequences had 97% homology with three *Cercospora* spp. (*C. sorghi* f. *maydis* Ellis & Everh., *C. asparagi* Sacc., and *C. nicotianae* Ellis & Everh.) and one *Septoria* sp. (*S. glycines* Hemmi). Naturally infected leaves collected in Ames, IA in 2003 and 2004 included asexual fruiting structures that contained multiseptate hyaline conidia. Based on

genetic and morphological evidence, the pathogen causing leaf spot on Japanese tree lilac was identified as a *Cercospora* sp. Identification of the pathogen is an essential first step toward development of control strategies for leaf spot of Japanese tree lilac.

## Introduction

There is strong demand for landscape trees that have both aesthetic appeal and compact size. One small tree, Japanese tree lilac, is cold-hardy in the northern U.S., blooms prolifically, and may be more disease-resistant than are other members of the genus *Syringa* (Dirr, 1998). Because of these features, Japanese tree lilac is increasingly popular as a landscape tree in the Upper Midwest.

A disease of unknown etiology has appeared on Japanese tree lilacs during the past five years in Iowa, Minnesota, and Idaho. This disease results in angular leaf spots in mid- to late summer, starting on lower leaves and then spreading throughout the tree. As the disease progresses, premature leaf abscission results in partial to complete defoliation. Disease development may be more severe during relatively rainy growing seasons. Information about the identity, ecology, and management of the pathogen causing this leaf spot disease is needed in order to develop effective control strategies.

Several fungi can cause leaf spots on *Syringa* spp., including *Cercospora lilacis* (Desmaz.) Saccardo and *C. macromaculans* Heald & F.A. Wolf (Farr et al., 1989). Additionally, Greene (1967) noted an unidentified species of *Cercospora* causing leaf spots on *Syringa ×hyacinthiflora* (Hort. Lemoine) Rehd. ‘Blue Hyacinth’ in Wisconsin. Other leaf spot diseases on *Syringa* spp. are caused by *Pleospora herbarum* (Pers.) Rabenh, *Alternaria alternata* (Fr.) Keissl., *Alternaria brassicae* (Berk.) Sacc., *Macrophoma halstedii* Ellis &

Everh., *Phyllosticta porteri* Tehon & E.Y. Daniels, *P. syringae* Westend., and *Phyllosticta* sp. (Farr et al., 1989).

Our objective was to identify the pathogen that causes leaf spot disease on Japanese tree lilac by 1) proving pathogenicity of the putative causal fungus by fulfilling Koch's postulates and 2) analysis of genetic and morphological characteristics.

## **Materials and Methods**

### **Koch's postulates**

'Ivory Silk' Japanese tree lilacs, 60 to 90 cm in height, were obtained from Bailey Nurseries, Inc. (St. Paul, MN) in Feb. 2003. Each lilac was planted in a 38-L plastic pot in a soilless mix (Sungro<sup>™</sup> BB300, 40 to 55% pine bark, Canadian sphagnum peat moss, perlite, dolomite, limestone, gypsum, and wetting agent; Sungro Horticulture, Bellvue, WA) and grown in a greenhouse with temperature ranging from 22 to 32 °C. Trees were fertilized every 3 wk in the spring and summer from April through September with liquid fertilizer (20N-10P-20K) (Nutriculture<sup>®</sup>, Plant Marvel Laboratories Inc., Chicago Heights, IL). Lilacs were pruned every 2 wk to maintain height of <1.0 m and width of <0.5 m.

Isolates of the leaf spot pathogen were obtained from naturally infected trees located in Ames, Des Moines, and Gilbert, IA, during Aug and Sept of 2003 and 2004. Collected leaves were sealed in plastic crispers and stored at 4 °C until use.

Leaves were surface-sterilized for 3 min in 50 ml of 70% ethyl alcohol amended with two to three drops of 0.5% NaOCl, followed by triple rinsing for 3 min in sterile water. Isolations were made under a dissecting microscope, using a sterile razor to excise small sections at the margins of diseased tissue. Four pieces of diseased tissue were placed at equal

spacing in 9-cm-diameter plastic petri dishes containing potato dextrose agar amended with antibiotics (0.15 g streptomycin and 0.15 g tetracycline), 40 ml of 50% lactic acid, and 18 drops Tergitol in 1 L of deionized water. Plates were sealed with Parafilm<sup>®</sup> (Pechiney Plastic Packaging, Chicago, IL) and placed in covered plastic 'crisper' storage containers at room temperature (24 to 27 °C).

As inoculum, we used a suspension of mycelial homogenate in sterile water. Mycelium was obtained from 2-wk-old lawn colonies (made by homogenizing mycelium in a blender with sterile water, then spreading mycelial fragments evenly over the agar surface) of six isolates that were obtained from naturally infected leaves and cultured on potato dextrose agar. A sterile spatula was used to remove four fungal colonies and all agar from the petri dish. Contents were blended with 90 ml of sterile water for a total of 45 s, pausing the blender at 15 and 30 s to allow settling of unblended material. Mycelial homogenate was filtered through two layers of cheesecloth and then diluted with 300 ml of sterile water. Concentration of mycelial fragments in the suspension was estimated to be 150 to 200 fragments/ml by using a hemacytometer.

Fresh inoculum was prepared on the day of each inoculation trial. Before each inoculation, a subsample of the inoculum suspension was sprayed onto a petri dish containing potato dextrose agar, using the same hand-held plastic sprayer as for inoculation of the plants, and incubated at 27° C to verify viability of the mycelial fragments.

Inoculation trials were initiated on 10 Feb and 13 July 2004. Both trials were conducted in a growth chamber with a photoperiod of 12 h, and temperature was maintained at 27 ±1 °C. Lilacs were assigned randomly to growth chamber positions, which were maintained throughout the experiment.

Ten lilac plants were used in each inoculation trial. Before placing them in the growth chamber, all pots were watered with tap water to saturate the soil. Two lilacs were randomly assigned to each of four incubation periods (24, 36, 48, or 72 h). One control plant received a sterile water spray and incubation of 72 h, whereas the other control plant received no spray and was kept unbagged in the growth chamber for 72 h. Lilacs were inoculated with the mycelium suspension by using a hand-held plastic sprayer to wet both sides of the leaves to runoff. A 208-L black polyethylene garbage bag was placed over each plant immediately, and the open ends were secured under the pot base to retain leaf wetness. After the assigned incubation period, bags were removed. All lilacs remained in the growth chamber until the end of the longest incubation time (72 h), after which the plants were arbitrarily placed on a greenhouse bench (temperature range estimated at 22 to 32 °C) for symptom observation.

Inoculated lilacs were observed for symptom development twice per week for 8 wk. Samples of leaves displaying symptoms consistent with leaf spot disease on naturally infected Japanese tree lilacs were collected to represent different levels of symptom development. Leaves from control plants and from asymptomatic inoculated plants also were collected and photographed. Collected leaves were examined microscopically, and pathogen isolation was attempted as previously described. Cultures were compared with cultures previously isolated from naturally infected leaves.

### **DNA extraction, sequencing, and phylogenetic analysis**

The internal transcribed spacer (ITS) region (ITS-1, 5.8S rDNA, and ITS-2) was sequenced from four isolates of the pathogen obtained from naturally infected leaves

collected from different trees in Ames, IA during Aug 2003. Template DNA was obtained by scraping mycelium from 6-wk-old cultures of these isolates. Both amplification and sequencing were done by using the methods described by Batzer et al. (2005), using the primers ITS-1F/ITS-4 and annealing at 55 °C. Automated sequencing was performed at the Iowa State University DNA Sequencing and Synthesis Facility.

BioEdit (Hall, 1999) was used to import sequences, and the 5'- and 3'-ends were trimmed to aid in alignment. Aligned sequences were corrected by using lower-case letters to denote a correction or 'n' to denote a missing sequence. FAFSTA (Pearson and Lipman, 1988) was used to conduct Basic Local Alignment Search Tool (BLAST) searches that downloaded partial sequences from GenBank (National Center for Biotechnical Information, Bethesda, MD) with high homology to the corrected pathogen sequences.

## Results

**Examination of naturally infected leaves.** Examination of diseased leaves revealed scattered mottling of leaf tissue, followed by the development of pinpoint necrotic spots that merged into 0.3- to 1-cm-diameter, tan to brown, angular, necrotic lesions (Fig. 1A). Lesions were sometimes bordered by yellow tissue and delimited by veins. On severely infected leaves, lesions often coalesced to encompass large areas of leaf surface. Necrotic lesions were visible on both upper and lower leaf surfaces. Brown fruiting structures were visible under 10× magnification and densely scattered on both upper and lower surfaces within necrotic tissue. Diameter of these structures ranged between 110 and 150 µm. A few conidia were found in the most mature fruiting structures. The conidia were curved, hyaline, multiseptate, and appeared to contain oil droplets. Lengths ranged from 25 to 55 µm with an

average of  $41 (\pm 10) \mu\text{m}$ . Tips of conidia appeared acute, whereas bases appeared subacute. Average width at spore base was  $1 \mu\text{m}$  and average width at spore tip was  $0.5 \mu\text{m}$ . Number of septa in each conidia was observed ranging from three to seven septa per spore. Isolates of the fungus grown on potato dextrose agar or potato dextrose agar amended with antibiotics produced a pigment that turned the agar yellow when cultures were incubated for 1.5 wk at temperatures of  $\geq 20^\circ\text{C}$ .

### **Koch's postulates**

Leaf spot symptoms indistinguishable from those observed on naturally infected leaves appeared 4 to 5 wk after inoculation (Figure 1A, 1B). One of eight plants in the first trial and two of eight plants in the second trial were positive for symptoms. No control plants were symptomatic. Symptoms on plants appeared first on lower leaves and spread to upper leaves over 2 wks. Symptoms appeared first as scattered areas of chlorotic tissue that later coalesced into 4- to 6-mm-diameter lesions that became tan to brown. Lesions were angular and delimited by veins.

In both trials, the pathogen was reisolated from lesions on symptomatic leaves on greenhouse-inoculated plants. Attempts to isolate the pathogen from control plants or from asymptomatic, inoculated plants were unsuccessful.

Microscopic analysis of lesions from inoculated lilacs revealed close similarity to diseased tissue on naturally infected leaves. Dark brown fruiting structures, possibly clusters of conidiophores, were observed, but no mature conidia were found.

**Genetic analysis.** Basic local alignment search tool (BLAST) search results with ITS ribosomal sequences obtained from four isolates of the pathogen from naturally infected lilacs showed 97% homology of base pairs with sequences from three *Cercospora* spp. and one *Septoria* sp. (Table 1). The four Japanese tree lilac pathogen isolates had 99% base pair homology when aligned.

### Discussion

The pathogen causing symptoms of leaf spot on Japanese tree lilacs in the Upper Midwest and Idaho was provisionally identified as a *Cercospora* sp. Both genetic and morphological characterization supported this identification. Although we determined that characteristics of the pathogen were consistent with those of *Cercospora* spp., a species match was not made. A more thorough comparison of morphological features with other *Cercospora* spp. may aid in determination of species.

*Cercospora* is an anamorphic genus including leaf spot pathogens on many agricultural and ornamental plants (Goodwin et al., 2001). Taxonomy of this group of fungi can be difficult to determine because there are few distinguishing morphological characteristics (Goodwin et al., 2001). Although identification can be aided by examining the sexual stage, many *Cercospora* spp. have no known sexual stage and overwinter as mycelia on plant debris (Bair and Ayers, 1986; Chupp, 1953). We did not observe any sexual fruiting structures on naturally infected leaves, and none formed in culture. In addition, no sexual fruiting structures were identified on seed clusters that had remained on trees through the winter.

Four species of fungal pathogens had high homology with the pathogen. Unlike the pathogen we isolated, *Septoria glycines* produces conidia in pycnidia, a characteristic feature of members of the genus *Septoria* (Punithalingham and Holliday, 1972). In the genus *Cercospora*, however, there are many morphological similarities to the Japanese tree lilac pathogen we isolated. Like our pathogen, *Cercospora* spp. are characterized by hyaline or very pale conidia, conidiophores that were often darkly colored and frequently produced in fascicles (Chupp, 1953). These similarities led us to identify the leaf spot pathogen provisionally as belonging to the genus *Cercospora*.

Goodwin et al. (2001) conducted bootstrap analysis on the ITS region of rDNA of anamorphic species associated with *Mycosphaerella*, including *Cercospora*. The three *Cercospora* spp. with high homology to our pathogen were part of a subgroup of 12 species of the *Cercospora* cluster. This cluster was grouped with 88% bootstrap support. *Mycosphaerella* is the only known sexual stage produced by members of this cluster (Goodwin et al., 2001).

Several species of *Cercospora* are known to infect *Syringa* spp. Greene (1967) reported a species of *Cercospora* on *Syringa ×hyacinthiflora* 'Blue Hyacinth' in Wisconsin. Unlike the putative *Cercospora* spp. occurring on Japanese tree lilac, which has indistinctly multiseptate hyaline conidia, the *Cercospora* described by Greene (1967) produced conidia that were pallid-olivaceous and 3-septate. Although leaf spots on *S. ×hyacinthiflora* were rounded, whereas those on Japanese tree lilac were conspicuously angular, this morphological distinction could reflect differences in leaf morphology between the hosts.

Another pathogen of lilac, *Cercospora lilacis*, causes leaf spot on common lilac (*S. vulgaris* L.) and Persian lilac (*Syringa ×persica* L.) in the southern U.S. as well as Minnesota

Germany, Belgium, and Russia (Farr et al., 1989). Fruiting of this pathogen is visible on both leaf surfaces, like the pathogen on Japanese tree lilac; however, *C. lilacis* leaf spots are circular to oblong, and dull brown to almost gray with a rust-colored edge. Conidia of both *Cercospora* spp. were indistinctly multiseptate, wider at the base than the tip, and curved. *C. lilacis* spores are subhyaline to very pale olivaceous brown (Chupp, 1953).

A third species of *Cercospora* on lilacs, *Cercospora macromaculans*, causes leaf spot on common lilac (*S. vulgaris*) in Iowa, Oklahoma, Texas, and Wisconsin (Farr et al., 1989). *Cercospora macromaculans* produces zonate leaf spots, and epidemics often result in defoliation. As for our pathogen, fruiting occurs on both sides of the leaves, and spores are hyaline, multiseptate, and straight or mildly curved, with spore base somewhat truncate and tips acute. The morphological similarities between the two fungi warrant further investigation into the possibility that they belong to the same species.

Many *Cercospora* species produce a photosensitizing agent, cercosporin, which appears as a red pigment when grown in culture (Daub and Ehrenshaft, 2000). Goodwin et al. (2001) found that cercosporin-producing *Cercospora* spp. formed a phylogenetic group, the *Cercospora* cluster, and that only species in this group produced cercosporin. *Cercospora sorghi* f. *maydis* Ellis & Everh., *C. nicotianae* Ellis & Everh., and *C. asparagi* Sacc. are all in this cluster and produce cercosporin. Our leaf spot pathogen produced a yellow pigment on potato dextrose agar. We tested for cercosporin by placing mycelial plugs in acetone (M. Daub, N.C. State University, personal communication) but did not confirm cercosporin production.

Schlosser (1971) first described the characteristics of a yellow toxin produced by *C. beticola*. This toxin was later found to be a group of several phytoalexin toxins called the

beticolins, which all have similar chemical structures biological activity (Milat et al. 1992). Beticolin-1 has been shown to cause plant cell disruption by targeting the plasma membran  $H^+$ -ATPase (Gomez et al., 1996).

Provisional identification of the genus of this damaging leaf spot pathogen on Japanese tree lilac is a critical first step toward development of control recommendations. Our isolation of the pathogen and reproduction of characteristics under controlled conditions has set the stage for studies characterizing the environmental conditions associated with infection, assessment of cultivar resistance, and evaluation of fungicides for disease management.

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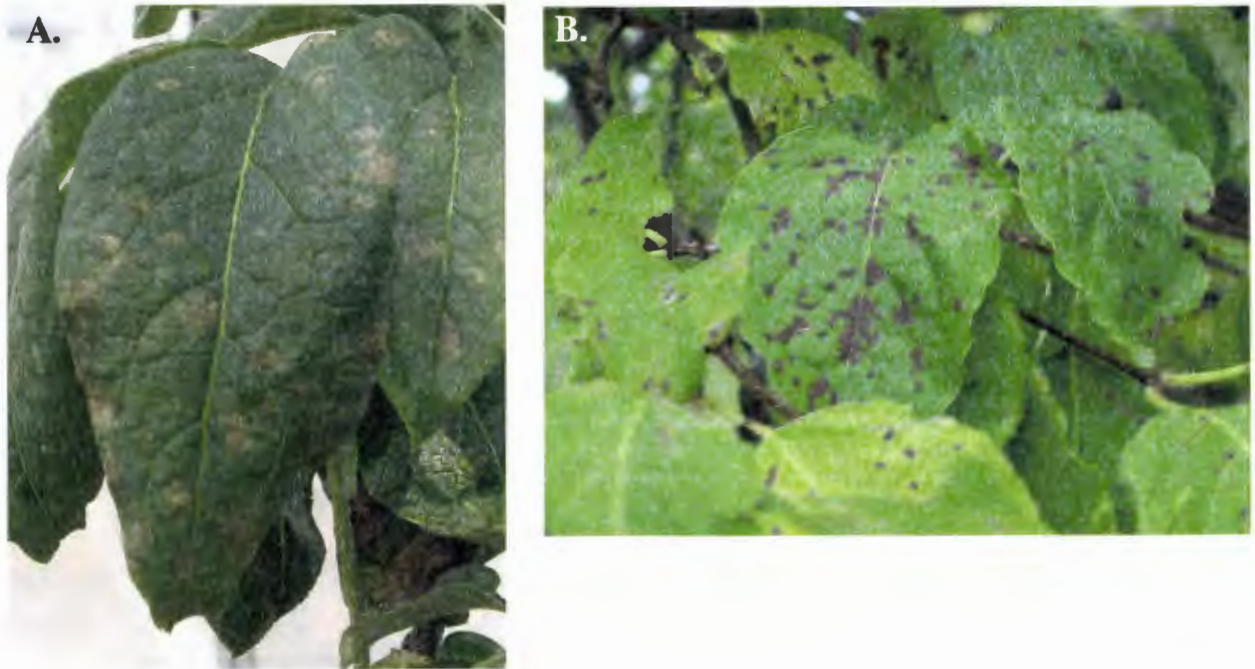
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**Table 1.** Basic Local Alignment Search Tool (BLAST) search results for base-pair homology of sequences of the ITS (Internal transcribed spacer) region of ribosomal DNA between four isolates of the Japanese tree lilac (*Syringa reticulata* ssp. *reticulata* [Blume] Hara) leaf spot pathogen and sequences of species downloaded from GenBank (National Center for Biotechnical Information, Bethesda, MD). Isolates of the pathogen were obtained from naturally infected leaves collected from different Japanese tree lilacs located in Ames, IA. The five most homologous pairings are listed with the number of positive base-pair identities out of total base pairs aligned. The level of homology between isolate and known species was the same (97%) for all five pairings.

Species /accession number	Isolate			
	Isolate 1	Isolate 2	Isolate 3	Isolate 4
<i>Septoria glycines</i> / AY826767.1	530/544	542/555	542/555	539/553
<i>Cercospora sorghi</i> f. <i>maydis</i> / AF297232	523/537	538/551	538/551	536/550
<i>Cercospora sorghi</i> f. <i>maydis</i> / AF297233	523/539	538/553	538/551	536/552
<i>Cercospora asparagi</i> / AF297229	522/538	537/552	537/552	535/551
<i>Cercospora nicotianae</i> / AF297230	523/537	538/551	538/551	536/550

**Figure 1.**



**Figure 1.** Japanese tree lilac leaf showing angular lesions on leaves four wk after inoculation (A) with the *Cercospora* sp. mycelial suspension, and naturally infected Japanese tree lilac leaves (B). Inoculated leaves received inoculation treatments of 24, 36, 48 and 72 h in a growth chamber at  $27 \pm 1$  °C followed by incubation in a greenhouse at 22 to 32 °C.

### **CHAPTER 3. TEMPERATURE OPTIMUM *IN VITRO* OF A PUTATIVE *CERCOSPORA* SP. THAT CAUSES LEAF SPOT ON JAPANESE TREE LILAC**

#### **Abstract**

A putative *Cercospora* sp. was recently identified as the causal agent of leaf spot disease on Japanese tree lilac [*Syringa reticulata* ssp. *reticulata* (Blume) Hara], a small tree whose popularity is increasing in managed landscapes in the Upper Midwest. Our objective was to determine optimum temperature for mycelial growth of this pathogen *in vitro*. Mycelial plugs of six isolates from 2-wk-old lawn cultures were incubated for 20 days on potato dextrose agar at temperatures of 10, 15, 20, 25, 30, or 37°C. The experimental design was a randomized complete block with seven replicates. Two measurements of colony diameter, at 90° angles, were made every 3 or 4 d. Optimum growth of isolates occurred at 20 or 25 °C; slower growth occurred at 10, 15 and 30 °C, and no growth occurred at 37 °C. The experiment was repeated once. Knowledge of the environmental biology of the pathogen will aid in developing effective control measures for this important disease of Japanese tree lilac.

#### **Introduction**

Japanese tree lilac is a small landscape tree commonly planted in the Upper Midwest. In addition to a flowering period that is later than other lilacs, it has a reputation as being low-maintenance because it has few disease and insect problems. Recently, a leaf spot disease appeared on Japanese tree lilacs in the Upper Midwest, causing angular lesions and

premature defoliation. A *Cercospora* sp. was provisionally identified as the pathogen through inoculation experiments, morphological features, and genetic analysis. This fungal pathogen causes angular leaf spots, leaf chlorosis, and premature leaf drop on infected Japanese tree lilacs.

The effect of temperature on radial growth of fungal colonies has been studied on other pathogenic *Cercospora* spp. (Calpouzos and Stallknecht, 1965; Stavely and Nimmo, 1969). Stavely and Nimmo (1969) incubated four isolates of *C. nicotianae* Ellis & Everh., a pathogen of tobacco (*Nicotiana tabacum* L.), for 7 days at 12, 18, 20, 23, 26, 29, 32, and 35 °C. Following incubation, measurements of colony diameter were made. They also tested growth of the isolates on V-8 medium, *C. nicotianae* growth medium (Stavely and Nimmo, 1969), and potato dextrose agar. Optimum radial growth of *C. nicotianae* occurred at 26 °C on all three media tested.

It is helpful to have vigorously growing colonies for use in experiments comparing growth of fungal colonies. One problem that can occur with experiments on growth or sporulation of *Cercospora* spp. is that the fungi often do not grow well when plugs of mycelium are serially transferred. Therefore, alternative transfer methods, such as transfer of homogenized mycelium, are preferred in order to maintain vigorously growing and sporulating colonies (Beckman and Payne, 1983; Latterell and Rossi, 1974).

There is no published information about temperature optima for mycelial growth of the putative *Cercospora* sp. that infects Japanese tree lilac. Information is needed about the temperature response of this pathogen *in vitro* so that colonies can be grown quickly for future experiments. This information may also provide clues about environmental conditions affecting infection of the host plant. The objective of this experiment was to determine

temperature optima for mycelial growth of the leaf spot pathogen of Japanese tree lilac *in vitro*.

### **Materials and Methods**

Six isolates of the putative *Cercospora* sp. were used in this experiment. Four isolates were obtained from diseased leaves of Japanese tree lilacs in Ames, IA in Aug and Sept 2004 but were not used in previous inoculation experiments. Two isolates were from reisolations of greenhouse-inoculated Japanese tree lilacs (cv. 'Ivory Silk') in July 2004. These isolates had originally been isolated from diseased leaves collected in Ames and Gilbert, IA during summer 2003.

**Preparation of Inoculum.** Six *Cercospora* sp. isolates were grown on potato dextrose agar amended with 0.15 g streptomycin, 0.15 g tetracycline, 40 ml of 50% lactic acid, and 18 drops of Tergitol in 1 L of deionized water. A flame-sterilized was used spatula to cut around the perimeter of each fungal colony, removing both the colony and the media directly under it. These colonies were placed in a 100-ml blender cup, and sterile water was added to fill the container. This mixture was homogenized at high speed for 45 s, stopping the blender for approximately 3 s at 15 and 30 s to allow settling of unblended material. Two to 3 ml of this homogenate were then transferred using a sterile pipette to 9-cm-diameter Petri plates containing potato dextrose amended with 150 µg/ml each of streptomycin and tetracycline, 40 ml of 50% lactic acid, and 18 drops Tergitol. A bent glass rod was used to spread the homogenate over the entire surface of the media. After 20 to 30 min, excess liquid was drained off. Plates were labeled, wrapped in Parafilm<sup>®</sup> (Pechiney Plastic Packaging,

Chicago, IL), enclosed in plastic 'crispers', and stored in incubators at  $25 \pm 0.5$  °C with constant light. Each well in each plate was randomly assigned a number between 1 and 6 and marked with a permanent marker. The bottom side of the plate was marked with horizontal and vertical lines, at 90° angles to each other, across the center of each well.

**Transfer of inoculum.** After plates had been incubated for 2 wk, 4.5-mm-diameter plugs containing mycelium were removed and transferred to PDA in 6-well plates (Costar<sup>®</sup>, Corning Incorporated, Corning, NY) for incubation at the assigned temperatures. One plug was placed in the middle of each well with the mycelium side down. This process was repeated with the remaining isolates. Plates were wrapped in Parafilm and stacked in the crispers. Seven plates were numbered 1 through 7 and placed in one crisper per incubator. Stack order was determined randomly and maintained throughout the experiment. Crispers were wrapped in two layers of aluminum foil to keep the plates in darkness during incubation.

**Incubators.** Incubators set at 10, 15, 20, 25, 30, and 37 °C were used for the experiment. A temperature sensor (Watchdog<sup>®</sup> Model 450, Spectrum Technologies Inc., Plainfield, IL) was placed in each incubator to monitor temperature at 15-min intervals. Temperature data was collected from the sensors twice per wk; all incubators maintained their target temperatures  $\pm 1$  °C at all times.

**Data collection.** Colony diameter was measured after 4 d. Measurements were made at a 90-degree angle to each other using the lines drawn on the underside of the plates as guides.

The diameter of the original plug (4.5 mm) was subtracted from each measurement. Each plate was measured twice per wk for 3 wk. Measurements were made using a ruler ( $\pm 1$  mm) and a dissecting microscope lit from below. The experiment was conducted twice using the same growth chambers for each trial.

**Data analysis.** Data analysis was done with analysis of variance (ANOVA) using the General Linear Models procedure (PROC GLM) of SAS version 8.02 (SAS Institute Inc., Cary, NC) with repetition and replication as random variables, and temperature and isolate as fixed variables. Preliminary analysis was done to evaluate the effect of trial  $\times$  treatment interaction to determine whether data from separate trials could be combined.

## Results

There was significant ( $P < .0001$ ) interaction between trial and treatment, so data from the two trials were analyzed separately (Table 1). The statistical analysis also showed significant ( $P < 0.001$ ) interaction between temperature and isolate. There was little obvious interaction between isolate and temperature, however (Fig. 1). The high F value for temperature may have resulted in a significant interaction between isolate and temperature.

Mycelial growth was most rapid at 20 and 25 °C (Fig. 2). Growth at 25 °C slowed after day 18 of incubation (Fig. 2), possibly due to colony growth at this temperature reaching the edge of the well. Consequently, we made comparisons of data on day 18 of incubation in both trials rather than day 21.

There were significant ( $P < 0.05$ ) differences in mean colony diameter on day 18 at six temperatures in both trials. Radial growth decreased at lower and higher temperatures;

colonies incubated at 37 °C had almost no growth (Fig 1). Linear regression (Fig. 3) indicated that the optimum temperature was 21.9 °C and 21.3 °C in trials 1 and 2, respectively.

There were differences ( $P < 0.05$ ) in colony diameter among isolates at each temperature on day 18. In trial one, isolates 3 and 4 had significantly more growth on day 18 than the other isolates (data not shown). Isolate 4 again had the greatest mean radial growth in trial 2. Isolate 3 could not be compared because it was not included in the second trial due to contamination. Isolate 6 had the smallest mean colony diameter on day 18 in trial two. In the first trial, however, radial growth of this isolate was not significantly different from isolates 1, 2, or 5.

We also noted production of a yellow pigment by all isolates at 20, 25, and 30 °C during the temperature experiment (Fig. 3).

## Discussion

The results indicate that the optimum temperature for radial growth of the putative *Cercospora* sp. pathogen of Japanese tree lilac was approximately 22 °C. This differs somewhat from findings on the optimum temperature for growth of other *Cercospora* spp. For example, Staveland and Nimmo (1969) found 26 °C to be the optimum temperature for growing *C. nicotianae*.

In nature, many *Cercospora* pathogens thrive in mild to warm, humid conditions. These conditions are favorable for all aspects of pathogenicity, including mycelial growth (Bair and Ayers, 1986; Windels et al., 1998). The putative *Cercospora* sp. in our study grew

relatively rapidly at 30 °C, was almost unable to grow at 37 °C, and grew at an intermediate rate at 10 and 15 °C.

Although the amount of radial growth at each temperature was similar among isolates, some isolates grew more rapidly than others over the entire range of temperatures. The differences in growth rate were small but statistically significant among several isolates. In the second trial, however, isolate 6 showed much less rapid growth than the other isolates. It is likely that the lack of growth of isolate 6 in the second trial was not a result of temperature. Instead, culture plates of isolate 6 from which plug transfers were obtained for trial two were not growing as vigorously as cultures of the other isolates, even though all cultures were started on the same day and grown under the same conditions.

We noted production of a yellow pigment by all isolates during the experiment at 20, 25, and 30 ° C. There are two known groups of pigments produced by *Cercospora* spp. One pigment, cercosporin, is a bright red phytotoxin produced by many *Cercospora* spp. When placed in acetone, cercosporin-producing fungi will appear blood red (Jenns et al., 1989). We placed several plugs of mycelium in acetone, and no red color was observed. Another group of toxins produced by *Cercospora* spp., called beticolins, are yellow pigments produced by *Cercospora beticola* Sacc. and have similar chemical composition. Both cercosporin and beticolins are believed to aid in pathogenicity.

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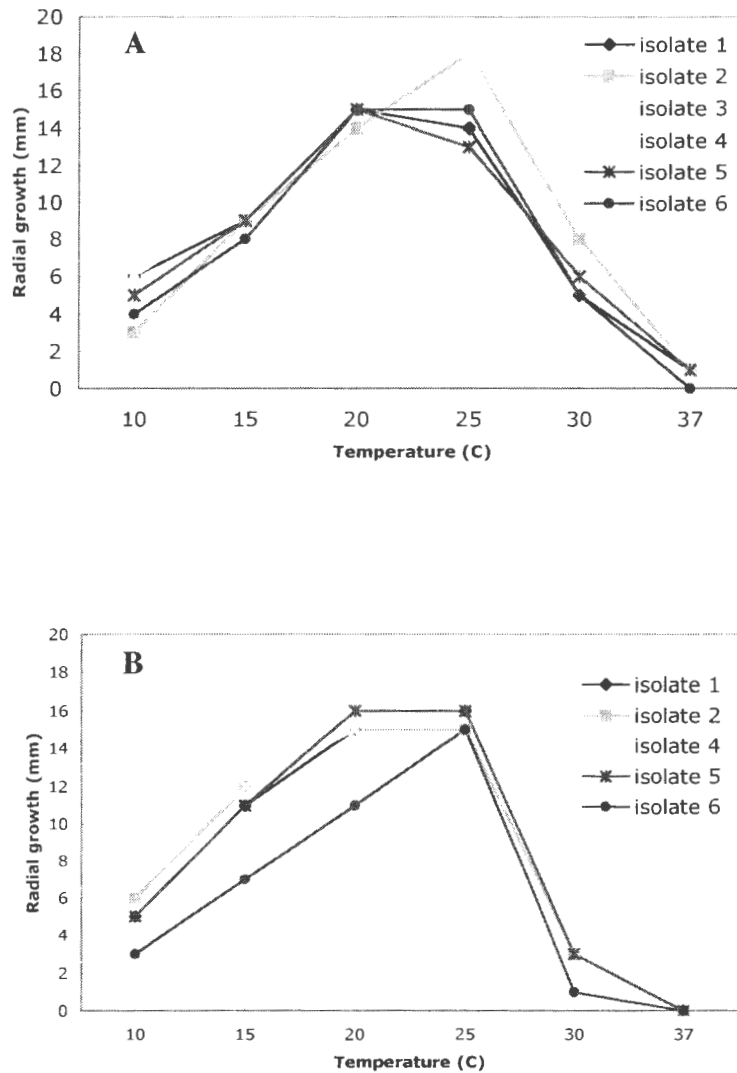
**Table 1a.**

Trial 1			
	Degrees of freedom	F value	P value
Repetition	6	0.42	0.8623
Isolate	5	17.99	< 0.0001
Temperature	5	983.58	< 0.0001
Isolate x temperature	25	5.36	< 0.0001

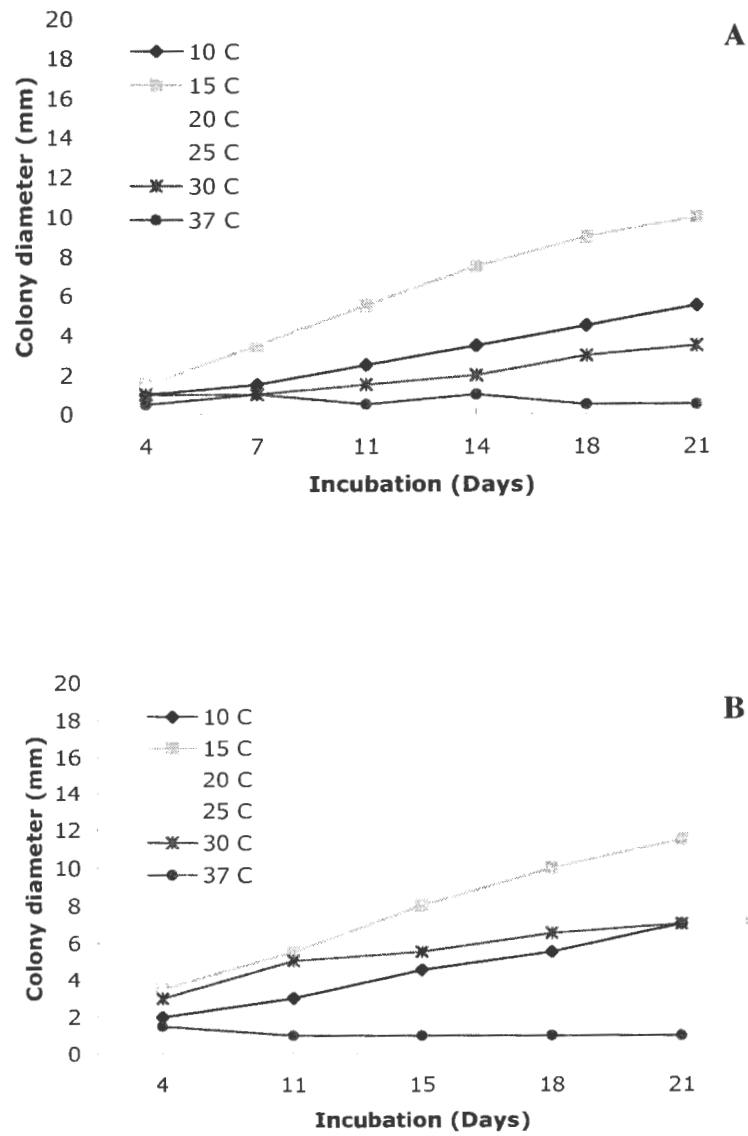
**Table 1b.**

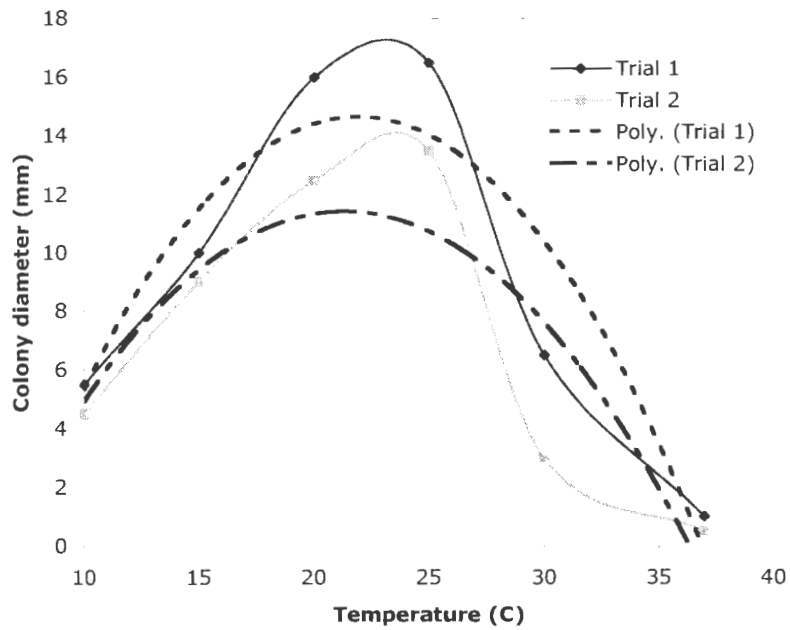
Trial 2			
	Degrees of freedom	F value	P value
Repetition	6	2.97	0.0087
Isolate	4	100.74	< 0.0001
Temperature	5	2580.16	< 0.0001
Isolate x temperature	20	12.51	< 0.0001

**Tables 1 a and b.** Analysis of variance for six isolates in the first trial and five isolates in the second trial incubated for 21 days at temperatures of 10, 15, 20, 25, 30, or 37 °C. Colony diameter was the average of two measurements at 90° angles.

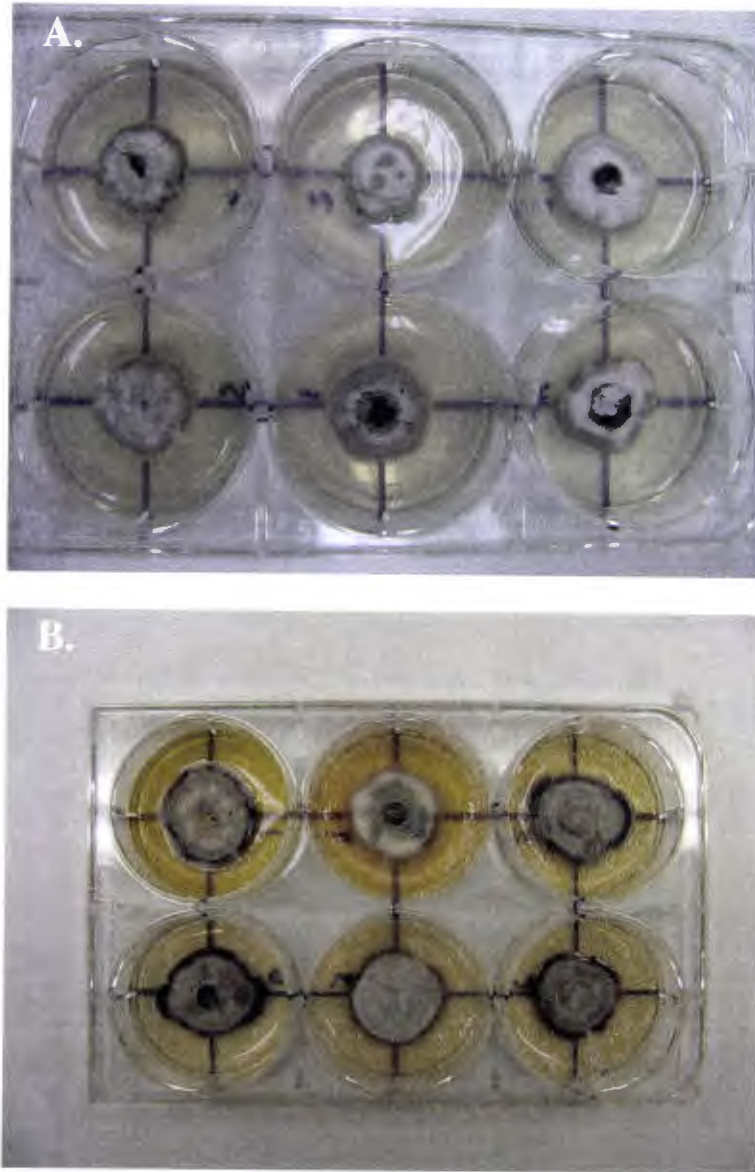
**Figure 1.**

**Figure 1.** Average radial growth of six isolates in trial 1 (A) and five isolates in trial 2 (B) after 18 d. of incubation at six different temperatures. Measurement of radial growth was achieved by averaging two measurements of colony diameter and subtracting the original diameter of the plug that was transferred at the start of the experiment. Isolate 3 was excluded from the second trial due to contamination.

**Figure 2.****Figure 2.** Radial growth averaged for all isolates at each of six incubation temperatures.



**Figure 3.** Polynomial linear regression for average colony diameter on day 18 in both trials for all isolates averaged. Trial 1 regression:  $y = -0.655x^2 + 2.871x - 16.818$ . Trial 2 regression:  $y = -0.0508x^2 + 2.1663x - 11.663$ . Using regression, the optimal temperature for radial colony growth was estimated at 21.9 °C in trial 1 and 21.3 °C in trial 2.

**Figure 4.**

**Figure 4.** Plates containing six isolates of the putative *Cercospora* sp. leaf spot pathogen on Japanese tree lilac that incubated at 15 °C (A) and 20 °C (B) for 18 days. Yellow pigment was produced at temperatures of 20, 25, and 30 °C.

## CHAPTER 4. ATTEMPTS TO INDUCE *IN VITRO* SPORULATION OF A PUTATIVE *CERCOSPORA* SPECIES PATHOGEN OF JAPANESE TREE LILAC

### Introduction

A leaf spot disease has appeared on Japanese tree lilac (*Syringa reticulata* ssp. *reticulata* [Blume] Hara) in the Upper Midwest and Idaho during the past 5 years. This disease causes angular, necrotic lesions, 0.2 to 1.0 cm in diameter, on leaves. The disease appears in mid- to late July and can cause complete defoliation of trees that become severely infected. The pathogen causing this disease was recently isolated and provisionally identified, through genetic and morphological characteristics, as a *Cercospora* sp.

Poor sporulation *in vitro* is common among *Cercospora* spp. plant pathogens and can inhibit experimental work with these pathogens (Beckman and Payne, 1983; Calpouzos and Stallknecht, 1967). The putative *Cercospora* pathogen of Japanese tree lilac has been reluctant to sporulate when grown in culture.

Research on the effects of various factors, including light, media, mycelial transfer technique, and variability among isolates, has been explored with several *Cercospora* spp. Latterell and Rossi (1974) determined that growing isolates of *Cercospora zeae-maydis* Tehon & E.Y. Daniels on different media affected growth habit, formation of specialized structures, and pigment production. They found that some cultures produced conidia in dark or diurnal light, but no conidia were produced if cultures were incubated in continuous light. Beckman and Payne (1983) also studied the effects of light duration and media on sporulation of *C. zeae-maydis* *in vitro*. Several media were included in their study: V-8 juice

agar, potato dextrose agar, green-leaf decoction agar, and senescent-corn-leaf decoction agar. Their results showed that incubation in a diurnal regime of light and darkness resulted in greater spore production than incubation in constant dark, or constant light followed by a period of dark. Spores were produced abundantly under diurnal light on all media except potato dextrose agar. A study on sporulation of *C. beticola* Sacc. by Calpouzos and Stallknecht (1967) found that constant light or diurnal light produced significantly greater sporulation in culture than constant darkness. Additionally, they determined that ultraviolet light (301 to 355 nm) produced the highest sporulation.

Previous studies have shown that the method used to transfer some *Cercospora* spp. colonies can affect growth and sporulation *in vitro*. Serial transfer of mycelium often results in slow growth, low conidia production, and production of sterile white mycelia. However, homogenizing mycelium in a blender before transferring will produce both white, sterile colonies and gray, sporulating colonies. These sporulating colonies can then be subcultured to obtain colonies that will sporulate for several months (Beckman and Payne, 1983; Weaver and Elliot, 1977).

A method to achieve vigorously and consistently sporulating colonies *in vitro* would greatly benefit future research on the leaf spot pathogen of Japanese tree lilac. The objective of this research was to determine the effect of photoperiod, media, and mycelial transfer technique on sporulation *in vitro* of six isolates of the Japanese tree lilac leaf spot pathogen.

## Materials and Methods

**Preparation of inoculum.** Mycelium was obtained from 2-wk-old cultures of six different isolates. Four isolates were obtained from diseased Japanese tree lilac leaves collected in

two locations in Ames, Iowa during the summer of 2004. Two isolates were reisolates obtained from an inoculation trial with the pathogen. Isolates were labeled as MG6, MG9, Hort1, Hort2, IS1-1, and IS1-3.

### **Description of media**

Six media were used in this experiment: carrot agar, malt extract agar, V-8 juice agar, lilac leaf agar, carnation leaf agar, and potato dextrose agar. Carrot agar was prepared by blending twice-autoclaved carrot roots with sterile water at high speed until pureed. Carrot puree was then divided into 200-ml aliquots and stored in the freezer at  $-5^{\circ}\text{C}$  until needed. Media were prepared by thawing one 200-ml aliquot of media in a microwave oven, then combining it with 25 g of bacto-agar in 800 ml deionized water. This mixture was autoclaved for 40 minutes (Edmunds et al., 2003). Malt extract agar was prepared by combining 15 g Difco<sup>®</sup> malt extract agar with 19 g bacto-agar in 1 L of water. V-8 juice agar was prepared from 200 ml 'V-8 100% juice' (Campbell Soup Company, Camden, NJ), 3 g  $\text{CaCO}_3$ , and 15 g bacto-agar in 800 ml distilled water. Lilac leaf agar was prepared from 120 g of fresh green leaves and 35 g of dried leaves, both obtained from greenhouse-grown tree lilacs (cv. Ivory Silk). Leaves were immersed in boiling tap water for 20 min. Decoctions were filtered through two layers of cheesecloth and combined with 19 g of Difco<sup>®</sup> agar and 4 g of  $\text{CaCO}_3$  per L (Beckman and Payne, 1983). Carnation leaf agar was prepared by combining 15 g of agar and 6 g of potassium chloride in 1 L distilled water. After autoclaving, four to six  $\gamma$ -irradiated, 2- to 4-mm-diameter carnation leaf pieces (119 Buckhout Laboratory, University Park, PA) were sprinkled on each plate after the media

were poured. Potato dextrose agar was prepared by combining 39 g of Difco<sup>®</sup> potato dextrose agar in 1 L water.

Prepared plates of media were randomly assigned to a plating-technique treatment and light treatment, labeled with the assigned treatment using a Sharpie marker, and stored in sealed plastic bags.

### **Transfer of mycelium onto plates**

Plates were prepared using two techniques. The first used mycelial plugs obtained with a #2 (5-mm-diameter) cork borer. Plugs from each isolate were placed upside down on the agar with the mycelium side down. A single plug was placed in the center of each plate. The second technique used homogenized mycelium that was spread over the entire surface of the agar. Mycelium was removed from plates by using a sterile spatula to cut around the circumference of the fungal colony. Three to four of these colonies were then placed into a 100-ml stainless steel blender cup, and sterile water was added to fill nearly to the top. This mixture was homogenized at high speed for three 15-s periods, with a 3-s stop between each period to allow for settling of unblended material. The homogenate was pipetted onto the designated agar in petri plates and spread with a bent glass rod. After 30 to 40 min, excess liquid was decanted from the plates.

We used three light treatments: complete darkness, constant light, and a diurnal alternation of 12 h each of light and dark. One incubator was used for each light treatment and plates were randomly assigned to plastic ‘crisper’ storage containers and stacked on shelves in the incubators. Positions in crispers and incubators were maintained for the duration of the incubation period. The light sources in each incubator were four 40-watt

Philips® fluorescent lamps (Philips Electronics N.V., Eindhoven, Netherlands). Plates were between 3 and 15 cm from the bulbs, depending on their position in the crispers. One plate of potato dextrose agar, containing no fungal colonies, was placed on top of each stack of plates for shading.

Plates were incubated at 25 °C for 14 d, after which spore counts were made. Each treatment had two replications and the experiment was performed twice. A temperature sensor (Watchdog® Model 450, Spectrum Technologies Inc., Plainfield, IL) was placed in each incubator to monitor temperature.

### **Collection and analysis of data**

Spore counts were made by removing two 1-cm-diameter disks of mycelium and attached agar from each dish, placing each in a 10-ml aliquot of deionized water, and shaking vigorously for 1 min. Six spore counts were made from each suspension using a hemacytometer (Calpouzos and Stallknecht, 1965).

## **Results and Discussion**

Vigorous sporulation was not consistently achieved with any treatment in this experiment. Spores were found on six of 432 plates in each of the first trial and three of 432 plates in the second trial (Table 1). Sporulation did not occur on enough plates to make the effect of light, media, plating technique or isolate significantly different from zero. More research will be necessary to determine factors affecting sporulation of the Japanese tree lilac pathogen in culture.

Studies that determine the effect of light intensity or the effect of exposure to different light wavelengths may be necessary to determine conditions that cause sporulation of this pathogen *in vitro*. Calpouzos and Stallknecht (1967) found light intensity and wavelength to be important factors influencing the sporulation of *C. beticola* Sacc. In our experiment with the pathogen of Japanese tree lilac, light was provided by cool white 40-watt Philips® fluorescent lamps and light intensity was not monitored. A more detailed experiment could explore the effect of light intensity and wavelength on sporulation of this pathogen.

Modifications to the media may induce sporulation of the pathogen. Other media made specifically for certain *Cercospora* spp. pathogens have yielded good results in both growth and sporulation. *C. nicotianae* Ellis & Everh. Produced rapid growth and abundant sporulation when grown on *C. nicotianae* sporulation medium (Stavely and Nimmo, 1968; Stavely and Nimmo, 1969). They also determined optimum pH levels for achieving sporulation on several media. Optimum pH varied with media type, and was 4.5-5 on V-8 juice agar and 6.5 on *C. nicotianae* sporulation medium (Stavely and Nimmo, 1968).

Another factor that may affect sporulation is temperature. In this experiment, all cultures were incubated at 25 °C, a temperature that was found to produce rapid mycelial growth of the Japanese tree lilac pathogen. However, this temperature may not be the optimum for achieving sporulation. Stavely and Nimmo (1969) found 15 °C or 22.5 °C, depending on isolate, to produce the most sporulation of *C. nicotianae*.

Further research is necessary to determine conditions that will induce vigorous sporulation of the Japanese lilac leaf spot pathogen *in vitro*. More focused experiments that

look only at one factor, such as light intensity, light wavelength, temperature, or medium may be necessary to clarify the effect these factors have on sporulation of this pathogen.

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**Table 1.**

Trial	Isolate	Media	Light treatment	Plating technique	Average spores/ml
1	ISI-3	Potato dextrose	Diurnal	Plug	$2.2 \times 10^4$
1	ISI-3	Potato dextrose	Light	Plug	$1.7 \times 10^4$
1	ISI-3	Potato dextrose	Light	Plug	$3.3 \times 10^4$
1	MG9	Carnation leaf	Diurnal	Lawn	$2.3 \times 10^4$
1	ISI-3	Carnation leaf	Dark	Lawn	$1.3 \times 10^4$
1	MG6	Malt extract	Light	Lawn	$3.0 \times 10^4$
2	ISI-3	Potato dextrose	Light	Plug	$1.5 \times 10^4$
2	MG9	Carnation leaf	Diurnal	Lawn	$3.3 \times 10^4$
2	MG9	Carnation leaf	Light	Lawn	$1.8 \times 10^4$

**Table 1.** Average spores/ml water for trials 1 and 2. Plates for which no spores were found are not listed. Spore counts were made by removing two 5-mm plugs from each plate, placing in 10 ml H<sub>2</sub>O, and vortexing for 30 s. Six counts of spores were taken using a hemocytometer and averaged.

## CHAPTER 5. GENERAL CONCLUSIONS

Leaf spot disease on Japanese tree lilac is a disease that causes necrotic lesions and premature leaf drop on infected trees. The disease could threaten the tree's popularity with consumers. This research provides the first steps towards management recommendations for this disease.

Through this research, the fungal pathogen causing leaf spot on Japanese tree lilac was isolated and provisionally identified as belonging to *Cercospora*, a genus of asexual fungi that causes disease on many different species of plants. Pathogenicity of the isolated pathogen was confirmed using Koch's postulates, and genetic and morphological analysis identified genus. The ideal temperature of near 25 °C for growing the pathogen *in vitro* was determined through the temperature-optima experiment. This information will prove useful in growing colonies for future experiments and may give clues about temperature preferences in nature. Attempts at achieving vigorously sporulating colonies failed. The pathogen is reluctant to sporulate *in vitro*. Several factors that may affect sporulation were tested, including isolate, photoperiod, and growth medium, but the results did not reveal a definitive factor that affects sporulation. Future experiments will be needed to determine factors affecting sporulation of this pathogen.

More research is needed to develop control tactics that will be effective at treating this disease. Information about the effect of the disease on plant health is needed to determine if the disease is merely aesthetic or is detrimental to overall plant health. Some cultivars of Japanese tree lilac may prove more resistant to this disease than others. Additionally, information about how the pathogen overwinters will aid in developing control

recommendations. Developing effective control strategies will help prevent this disease from causing the popularity of Japanese tree lilac as a landscape tree to dwindle in the future.

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