Phomopsis Blight: A New Disease of *Pieris japonica* Caused by *Phomopsis amygdali* in the United States

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Abstract

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Pieris japonica, also known as Japanese andromeda, is an economically valuable broadleaf evergreen used in landscapes across the United States. From spring 2010 to 2012, *P. japonica* 'Mountain Fire' plants growing in Maryland nurseries were observed with a high incidence of stem canker, shoot dieback, and blight symptoms. Necrosis was evident on shoot tips and often advanced into lateral shoots, as well as to the crowns, leading to plant death. *Phomopsis amygdali*, known as a destructive pathogen of peach and almond, was consistently isolated from symptomatic plants. *P. amygdali* also caused similar symptoms on Mountain Fire test plants following inoculations. *P.*

amygdali was consistently recovered and its identity was confirmed with both morphological and molecular tools, thus fulfilling Koch's postulates. In addition, nursery sampling in 2012 revealed that *P. amygdali* could also be isolated from asymptomatic plants. In all instances, infected plants were shipped from a West Coast nursery, indicating that this pathogen was inadvertently introduced to new locations. *P. amygdali* may be emerging as an important pathogen in nurseries because this is the first known association of this pathogen with an ornamental plant species.

Pieris japonica (Thunb.) D. Don ex G. Don, also known as Japanese andromeda or Japanese pieris, is a broadleaf evergreen commonly used in landscapes across the United States (11). *P. japonica* is valued for its attractive white flowers and, as with some cultivars, such as 'Mountain Fire', fiery-red new foliage that adds aesthetic value. *P. japonica* is commonly planted in landscapes with other broadleaf evergreens, such as rhododendrons and azaleas (11). *P. japonica* is reported in the literature as susceptible to multiple *Phytophthora* spp., similar to other ericaceous plants, but there is little information available regarding other *P. japonica* diseases (28). Plants belonging to the genus *Pieris* have been listed as one of five groups of "high-risk plants" in the United States due to the prevalence of nursery plants found infected with the quarantine plant pathogen *Phytophthora ramorum* (33).

As part of a study surveying Maryland nurseries for the presence of plant pathogens, such as *P. ramorum*, introduced to the state through nursery trade, we sampled *Pieris japonica* cultivars imported from West Coast nurseries exhibiting symptoms of stem cankers and severe dieback (Fig. 1). Affected plants had leaves that turned a bronze color and remained attached to stems. Necrosis was evident on senescing floral panicles and shoot tips and, in severe cases, advanced into lateral shoots as well as to crowns, leading to death of the plants. Isolations from lesions on stems and shoots consistently yielded fungal isolates with similar morphology, but no *Phytophthora* was isolated. Fungal cultures had morphological characteristics consistent with those of *Phomopsis* spp. Little is known about the association and potential role of *Phomopsis* spp. as pathogens of *P. japonica*. The objective of this study was to investigate the association of *Phomopsis* spp. with *P. japonica* plants in nurseries and validate their potential as plant pathogens through various inoculation experiments. A portion of this research was recently reported (3).

Materials and Methods

Field sampling. *P. japonica* plants in two Maryland nurseries were inspected during spring 2010, 2011, and 2012. Plants were inspected upon arrival or once they were placed at a block. Blocks of plants ranged in size from approximately 200 to 1,100 *P. japonica* plants, with Mountain Fire as the most common cultivar. Other cultivars sampled were 'Brouwer's Beauty', 'Scarlett O'Hara', and 'Valley Valentine'. Most plants were in bloom when they were inspected and all blocks were irrigated with overhead sprinklers. In each block, the symptomatic plants were counted and 5 to 10% of the plants in each block were arbitrarily collected.

Additional field sampling was conducted during summer 2012. Three separate blocks of Mountain Fire plants were sampled, as well as one block each of Scarlett O'Hara and Valley Valentine. Thirty shoots were arbitrarily collected from asymptomatic plants in each block, such that both the current year's growth and the previous year's growth were collected. In addition, 30 symptomatic shoots were arbitrarily collected from the same blocks of Mountain Fire plants. Before isolation, each shoot was evaluated whether it was a new growth or the prior year's growth.

Isolation and identification. Shoots with dieback symptoms were collected from *P. japonica* Mountain Fire plants, placed in 3.8-liter (1-gal.) plastic bags, and transported back to the laboratory in a cooler. Samples were processed within a day of sampling. Tissue was washed with tap water, surface disinfested in a 0.57% NaOCl solution for 2 min, rinsed in distilled water, and blotted dry. Sections taken from the edges of necrotic lesions were embedded in potato dextrose agar (PDA; Difco) amended with 0.1% streptomycin sulfate. Emerging fungal colonies were identified based on colony pattern and morphological characteristics on PDA.

For molecular identification, isolates were grown in potato dextrose broth (Difco) for 7 days at 22°C on a rotary shaker (New Brunswick Scientific) at 200 rpm. Mycelia were harvested and genomic DNA was extracted according to the manufacturer's instructions using a DNeasy Plant Mini Kit (Qiagen) and stored at -20°C. The internal transcribed spacer (ITS) region and a portion of the translation elongation factor 1- α (TEF) were amplified and sequenced using primers ITS6/ITS4 and EF1-728F/EF1-986R, respectively (6). Sequences were edited using Geneious Pro 5.5.6 Software (Biomatter Ltd.) and submitted for BLAST searches for

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comparison to known DNA sequences in the National Center for Biotechnology Information (NCBI) database.

Inoculation experiments. Mountain Fire plants in 20-liter (5-gal.) pots were obtained from a Maryland nursery. Prior to inoculation, plants were kept in the greenhouse for 2 months to determine whether plants potentially had latent infections and would develop disease symptoms. Following the waiting period, plants were subjected to three different types of inoculations, as described below. Isolate MF-3C was used for all inoculation experiments. This culture was isolated from symptomatic Mountain Fire plants sampled in 2011. Following inoculations, plants were incubated in the greenhouse with an average daily temperature of 25°C (minimum, 16°C, and maximum, 35°C) and were watered daily.

Stem inoculations. Five stems were inoculated on each of four Mountain Fire plants. A U-shaped cut was made aseptically and a 4-mm plug that included mycelia from a 2-week-old, nonsporulating *Phomopsis* culture was inserted beneath the bark (2). The inoculation point was covered with sterile moistened cotton, sealed with Parafilm, and incubated for 4 weeks (Fig. 2A). Sterile PDA plugs were used as controls to mock inoculate an additional 20 stems. After the incubation period, the Parafilm and cotton were removed and any lesions above and below the inoculation point (excluding the wound) were measured.

Shoot tip inoculations. A modified technique described by Bradley and Li (5) was used to inoculate shoot tips. Before inoculation, approximately 5 cm of the tip of a shoot was removed (Fig. 2B). Two mycelial plugs of a nonsporulating, 2-week-old culture grown on PDA were placed into the large ends of 200- μ l pipet tips. Pipet tips containing *Phomopsis* plugs were then placed over excised shoot tips of container-grown Mountain Fire plants. To reduce desiccation, pipet tips were bent (Fig. 2B). Forty shoot tips were inoculated with the *Phomopsis* isolate and the same number inoculated similarly for controls with pipet tips containing plugs of noninfested PDA. At 2 and 4 weeks after inoculation, pipet tips were removed and the length of stem lesions that developed from the wounded ends of the cut shoot tips of the live plants was measured.

Conidial inoculations. Conidia were washed from the surface of a 4-week-old *Phomopsis* culture grown on PDA and suspended in sterile distilled water. Suspension concentrations were adjusted to 3×10^8 conidia/ml (high) and 3×10^4 conidia/ml (low) using a hemacytometer. Conidial suspensions were applied on shoot tips (about a 10-cm section) using a paintbrush, such that stems and both surfaces of leaves were inoculated (Fig. 2C). Twenty Mountain Fire shoots of four plants were inoculated for each conidial

concentration. Sterile distilled water was applied to 20 shoots on each of four plants for controls. All shoots were covered with plastic bags for 24 h to increase relative humidity (Fig. 2D). Four weeks following inoculation, symptom presence was evaluated.

Reisolations. Following the 4-week incubation period, reisolations were attempted by taking small sections from the edges of emerging necrotic lesions on inoculated tissues, including the controls. If no necrosis developed, then sections were made from 5-mm stem pieces proximal to the point of inoculation. Isolations were performed as described in isolation methods. The identification of putative *Phomopsis* isolates recovered was confirmed by examining morphological characteristics of each culture and sequencing the ITS of one representative isolate from each inoculation experiment.

Statistical analysis. Differences in isolation frequency of *Phomopsis amygdali* among previous and new years' growth for the 2012 sampling season were analyzed using *t* tests. Lesion lengths on inoculated shoots and stems were compared with the controls using analysis of variance, and Tukey's multiple comparison test was used to detect significant differences between the two incubation periods using JMP 5.0 (SAS Institute Inc.).

Results

Field sampling. The disease incidence was 50% (600 inspected plants) in 2010 and 80% (850 inspected plants) and 85% (3200 inspected plants) in 2012. *Phomopsis* spp. were consistently isolated from Mountain Fire plants sampled but were not isolated from any Brouwer's Beauty or Scarlett O'Hara. However, *Phomopsis* spp. were isolated from symptomatic Valley Valentine plants that were part of a single shipment in 2012. *Phomopsis* spp. were isolated at higher frequencies from symptomatic Mountain Fire shoots compared with asymptomatic shoots, regardless of the age of shoot growth (Table 1). When age of symptomatic Mountain Fire shoots was compared, *Phomopsis* spp. were isolated at a significantly higher ($P < 0.001$) frequency from the previous year's growth compared with the new year's growth, although *Phomopsis* spp. were also isolated from symptomatic new growth. No significant differences were detected between ages of growth when isola-

Fig. 1. Symptoms of *Phomopsis* infection on *Pieris japonica* 'Mountain Fire' plants from Maryland nurseries: **A–D**, *P. japonica* plants with severe shoot dieback and mortality; **E–G**, necrotic lesions on symptomatic shoots and stem.

Fig. 2. Methods used to inoculate *Pieris japonica* 'Mountain Fire' plants with *Phomopsis amygdali*: **A**, stem inoculations; **B**, shoot tip inoculations; **C**, conidial inoculations; and **D**, plastic bags covering conidial inoculations to increase relative humidity.

tions were conducted from asymptomatic Mountain Fire shoots. Other fungal genera, such as *Fusarium* and *Pestalotiopsis*, were also isolated from *Pieris japonica* samples at low frequencies (<1%).

Isolation and identification. Isolations from symptomatic and asymptomatic Mountain Fire plants as well as symptomatic Valley Valentine sampled in Maryland nurseries yielded cultures with morphological characteristics similar to *Phomopsis amygdali* (De-lacr.) J.J. Tuset & M.T. Portilla (Fig. 3) (30). Cultures on PDA produced dark-brown, globose picnidia, as well as ovoid to ellipsoid α conidia, 0 to 2 guttulate, 6.2 by 2.3 μm ($n = 100$). PDA cultures grown from *P. japonica* isolates also produced hyaline, elongate, slightly curved β conidia, 23.5 by 1.2 μm ($n = 100$). BLAST analyses of the sequences in the NCBI database resulted in a 100 and 99.8% match with *Phomopsis amygdali* for the ITS (e.g., AF102998 and GU133063) and TEF (e.g., GQ250339 and GQ250340) loci, respectively.

Inoculation experiments. No inoculated plants died during the 4-week incubation period. However, symptoms that developed on inoculated plants resembled those of nursery plants. These included shoot tip dieback, bronzing of foliage, and stem cankers (Fig. 4).

Stem inoculations. Two measurements were conducted after excluding the wound size: one that included lesions produced above the inoculation point and the other that included lesions produced below the inoculation point toward the roots. Stems inoculated with *P. amygdali* developed necrotic stem lesions, which extended an average 7.7 ± 6.2 mm (1 to 20 mm) above and 9.4 ± 4.8 mm (2 to 18 mm) below the point of inoculation. The lesion sizes were significantly different from controls for both measurements, above the inoculation point ($P = 0.003$) and below the inoculation point ($P = 0.0002$). However, the lesion sizes produced above and below the inoculation points did not differ ($P = 0.504$). In addition, 3 of 20 inoculated stems were completely girdled (Fig. 4A). Control plants developed, on average, 1 ± 1.8 mm (0 to 8 mm) of necrosis beyond the point of inoculation (Fig. 4B). Isolations from each of

the inoculated stems yielded cultures with morphological characteristics consistent with the *P. amygdali* isolate used for inoculations. One representative isolate was sequenced to confirm the identification of the fungus recovered and the sequence resulted in a 100% match with *P. amygdali*. No *Phomopsis* sp. was recovered from stems of control plants.

Shoot tip inoculations. Shoot tips inoculated with *P. amygdali* consistently developed necrotic lesions significantly greater ($P < 0.001$) in length compared with controls (Fig. 5). Lesions averaged 18.6 ± 8.6 mm (3 to 35 mm) in length at 2 weeks post inoculation and nearly doubled to 32.6 ± 12.1 mm (13 to 76 mm) in length at 4 weeks post inoculation (Figs. 4C and 5). In some cases, the fungus was observed sporulating on the inoculated shoot tips. Shoot tips inoculated with plugs from uncolonized PDA developed 1 mm of necrosis or less, on average (Figs. 4D and 5). Isolations from each of the 40 inoculated shoot tips yielded cultures with morphological characteristics consistent with the *P. amygdali* isolate used for inoculations. One representative isolate was sequenced to confirm the identification of the fungus recovered and the sequence resulted in a 100% match with *P. amygdali*. No *Phomopsis* sp. was recovered from shoot tips of control plants.

Conidial inoculations. Mountain Fire shoots inoculated with the low dose of *P. amygdali* conidia or water did not develop visible disease symptoms and no *Phomopsis* sp. was recovered from these shoots. However, two shoots inoculated with the high dose of conidia on one plant developed dieback symptoms similar to those observed in nurseries. The leaves turned bronze and remained attached to the stems as the dieback developed (Fig. 4E). The dieback extended to the shared main stem at the base of the two shoots, and progressed an additional 28 cm to the base of the plant (Fig. 4E). Internal necrosis was observed at the base of the stem and *P. amygdali* was recovered from this tissue (Fig. 4E inset). *P. amygdali* was also reisolated from two asymptomatic shoots on different plants that were inoculated with the high dose of conidia. One representative isolate recovered from symptomatic tissue was sequenced to confirm the identification of the fungus recovered and the sequence resulted in a 100% match with *P. amygdali*.

Table 1. Frequency of isolation of *Phomopsis amygdali* from different aged symptomatic (S) and asymptomatic (AS) growth on three different cultivars of *Pieris japonica* collected during a Maryland nursery survey in summer 2012

Cultivar	Isolation results ^a	
	Current year's growth (%)	Previous year's growth (%)
Mountain Fire (S)	17.8	88.9*
Mountain Fire (AS)	8.3	15.6
Scarlett O'Hara (AS)	0	0
Valley Valentine (S)	0	16.7

^a Asterisk (*) indicates that the frequency of isolation from the previous year's growth was significantly greater ($\alpha = 0.05$) than that of the current year's growth.

Fig. 3. Morphological characteristics of *Phomopsis amygdali* isolates: **A**, colony pattern of a 2-month-old culture on potato dextrose agar and **B**, α and β conidia ($\times 40$).

Discussion

Phomopsis spp. are generally regarded as secondary or opportunistic pathogens that affect plants that are stressed, dying, or infected by other pathogens (4,13,15,21,28). However, several reports indicate that *Phomopsis* spp. are serious pathogens on a wide range of plants, including rhododendron, poplar, sugarcane, ficus, gardenia, sunflower, soybean, multiple conifer species, and a variety of nursery hosts (14,28–30). *Phomopsis* spp. have been previously reported in association with *Pieris japonica* but the isolates in those studies were not identified to species and were regarded as secondary pathogens or endophytes (13,25). Our evaluation of morphological characteristics and sequence data indicates that the *Phomopsis* sp. isolated from *P. japonica* plants exhibiting dieback symptoms is *Phomopsis amygdali*. Additionally, *P. amygdali* isolated from symptomatic *Pieris japonica* Mountain Fire plants in Maryland nurseries caused similar symptoms in three

Fig. 4. Symptoms on *Pieris japonica* 'Mountain Fire' plants following inoculations with *Phomopsis amygdali*. **A**, Necrotic lesion on a shoot after stem inoculation with *P. amygdali* and **B**, potato dextrose agar (PDA) as control; **C**, shoot tip inoculation with *P. amygdali* and **D**, PDA controls; and **E**, shoot blight and dieback after inoculation with the high dose of *P. amygdali* conidia and internal stem necrosis (inset).

different pathogenicity experiments and was reisolated from necrotic tissue of inoculated plants, thus fulfilling Koch's postulates. To our knowledge, this is the first report of *Phomopsis amygdali* as a pathogen of *Pieris japonica*.

Due to its ability to cause high levels of disease, *Phomopsis amygdali* is considered one of the more aggressive *Phomopsis* spp. (28,31). This species has been reported in multiple countries, including the United States, as an economically important pathogen of almond and peach (1,9,10,12,17–20,22,30–32,35). On these two hosts, *P. amygdali* causes symptoms of twig cankers, shoot blight, fruit rot, and, in severe cases, may lead to loss of larger tree limbs or even tree death (7,17). *P. amygdali* caused devastating epidemics, which resulted in severe losses in peach orchards in the United States during the 1940s and 1950s (18). It has also been reported as a pathogen on grapevine stock in South Africa (24,35). It is possible that *P. amygdali* has been isolated from nursery crops previously but not been identified to species or misidentified as another *Phomopsis* sp. To our knowledge, *P. amygdali* has not been reported as a pathogen of nursery crops.

Previous studies investigating the modes of infection and colonization of hosts by *P. amygdali*, as well as other *Phomopsis* spp., have indicated that infections occur through buds, bud scale scars, blossoms, leaf scars, and fruit scars (14,18,31,32). When Mountain Fire plants arrive in Maryland nurseries from West Coast nurseries, they often lack visible disease symptoms and do not develop symptoms until at least 1 to 2 months later (*personal observation*). However, in some instances, we have sampled shipments of Mountain Fire upon arrival in Maryland nurseries and found both symptomatic and asymptomatic plants that yielded *P. amygdali*. This indicates that the plants are likely infected prior to shipment. It is unknown if the delay in symptom development is due to stressors or differences in environmental conditions on the West Coast and the East Coast.

During our surveys, we also noticed higher levels of disease on shipments of plants arriving with wounds, possibly due to loading and shipment conditions. As demonstrated during the inoculation experiments, wounding appears to have a significant effect on symptom development and results in lesion development over a relatively short period of time. This could potentially explain why so many plants develop serious dieback symptoms shortly after their arrival but not at the source nursery. This finding suggests the need for better packaging and shipment practices that reduce plant injuries, which can potentially predispose plants to diseases. In addition, inoculations using a high concentration of conidia resulted in shoot dieback, suggesting that environmental conditions that favor pathogen sporulation should be avoided.

Fig. 5. Mean lesion length of *Pieris japonica* 'Mountain Fire' shoots 2 and 4 weeks after inoculations with pipet tips containing agar plugs of *Phomopsis amygdali* or noninfested potato dextrose agar. Bars labeled with the same letter are not significantly different ($\alpha = 0.05$) according to Tukey's multiple comparison test.

In 2012, we isolated *P. amygdali* from symptomatic Valley Valentine plants in addition to Mountain Fire plants. Stem cankers and dieback symptoms were observed on a small number of plants upon arrival in a nursery. This has been the only occurrence in which we have observed symptoms of *P. amygdali* infection and isolated the pathogen on a cultivar other than Mountain Fire. Differences in disease incidence and severity have been reported among cultivars of almond and grapevine infected with *P. amygdali* (34,35). Mountain Fire appeared to be the most susceptible cultivar of those we tested. Additional studies should evaluate the range of *P. japonica* cultivars and their susceptibility to *P. amygdali*. Furthermore, other plant species should be evaluated as potential hosts. Given that the previously known hosts of *P. amygdali* were *Prunus* spp. and that this pathogen has been reported on grapevine and now *Pieris japonica*, the host range may be larger than previously understood.

Future research is needed to investigate effective management strategies for *Phomopsis* blight of *P. japonica*. Based on what is known from other *Phomopsis* diseases, it may be possible to implement control strategies in nurseries that could help to minimize losses. Clean stock for propagation, sterilization of potting media, and well-timed chemical control may be effective (8,20,23,28). However, chemical control alone for management of *Phomopsis amygdali* may not reduce disease severity to acceptable levels (7). Removal of infected plants, pruning out infected branches, use of drip irrigation, and making environmental conditions less favorable for sporulation and infection may aid in managing this disease (17,19,20,23,28). Use of resistant cultivars, biological control, and hot-water treatments are options that should also be explored (8,16).

Finally, future work should investigate the relationship between isolates of *P. amygdali* obtained in this study with isolates found associated with other hosts. The pathogenic and genetic differences among *P. amygdali* populations that infect *Pieris japonica* plants and those that infect other hosts, such as *Prunus* spp., could provide us with new insights about the migration and host preferences of this pathogen. Previous descriptions of *Phomopsis amygdali* isolates specify that β conidia are rarely produced, if at all (30), whereas our isolates readily produced β conidia in culture. This could be an indicator of broader differences among *P. amygdali* populations. Another important gap in our knowledge is the considerable lack of available sequence data for the genus *Phomopsis*. Efforts have been proposed to provide a taxonomic revision to the genus *Phomopsis* to gain a better understanding of members of this genus (26,27,31).

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