Eur J Plant Pathol (2011) 131:171–178
DOI 10.1007/s10658-011-9796-0

Phoma glomerata (Corda) Wollenw. & Hochapfel a new threat causing cankers on shoots of peach trees in Greece

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Accepted: 18 April 2011 / Published online: 18 May 2011

Abstract Shoot blights are common diseases of peach trees in Greece. This study is the first report of a shoot blight and canker disease of peach in Greece caused by the fungus *Phoma glomerata* (Corda) Wollenw. & Hochapfel. The pathogen caused distinct cankers with abundant gumming on shoots of peach trees. The rate of development of *P. glomerata* in vitro was reduced as temperatures increased from 25°C to 30°C, decreased from 25°C to 15°C, and was totally inhibited at 35 and 10°C. The rate of conidial germination and the germ tube elongation in vitro was reduced as temperatures increased from 25°C to 35°C, decreased from 25°C to 10°C, and was totally inhibited at 2–4°C. Pathogenicity tests showed that 24 peach and nectarine cultivars grown in Imathia Perfecture, Greece, were equally susceptible to *P. glomerata.* The fungicides thiophanate methyl, carbendazim, and tebuconazole were evaluated against the development of *P. glomerata* and disease symptoms. All fungicides inhibited the growth and conidial germination of *P. glomereta* and disease symptoms, and all 30 isolates tested were sensitive to the above fungicides. The disease caused by *P. glomerata* could be a threat to peach cultivation in Greece and its management should be investigated in the field.

Keywords Cultivars · Fungicides · *Prunus persicae* · Sensitivity · Susceptibility · Temperatures

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Introduction

Shoot blight is a common disease of peach occurring worldwide. The disease is most often observed in early spring following bud break. Symptoms include wilting and death of leaves on new shoots. Wilting and death of blossoms and young fruit on fruiting wood also occur. A diffuse canker, often with a concentric appearance and exuding gum, can be found on the fruiting wood at the base of the blighted shoots. Fungi of the genus *Monilinia*, *Phomopsis*, *Cytospora*, *Fusicoccum,* and *Colletotrichum* have been reported as causes of shoot blights in peach orhards (Bernstein et al. 1995; Uddin et al. 1997; Luo and Michailides 2001; Beckman et al. 2003; Pokharel and Larsen 2008). Species of genus *Phoma*, such as *P. pomorun* and *P. fimeti*, have been reported as pathogens causing "shot hole" in peach leaves, but no *Phoma* species has been found to infect peach shoots.

Knowledge of the optimum temperatures for development of a pathogen is essential to determine the best method and period to manage this pathogen. Most

Phoma species grow well at temperatures ranging from 20°C to 25°C (Itaya et al. 1996; Zhao and Shamoun 2006; Sempere Ferre et al. 2007). In general, determining the cardinal temperatures for growth of pathogens also help in gaining knowledge on when a pathogen is active.

Using of resistant cultivars is the best method for controlling a disease because growers need not expend funds on additional control measures such as fungicides. Even where fungicides must be used, resistance is a useful complementary control measure.

Thus far, chemical control is the most effective method to manage diseases in fruit trees. In Greece, the fungicides thiophanate methyl, carbendazim, and tebuconazole have been commonly used to control most of the pathogens causing fruit rots and shoot blights in fruit trees. The effectiveness of the above fungicides against fungi of the genus *Phoma* has been reported in previous works (Kruse and Verreet 2005; Tirado Lara and Mattos Calderon 2005; Schmitz et al. 2006).

The objectives of this study were: a) to investigate the effect of temperatures on development and conidial germination of *P. glomerata*, the pathogen causing shoot blight of peach in Greece; b) to evaluate the susceptibility of 24 peach—nectarine cultivars to *P. glomerata*: c) to evaluate the effectiveness of carbendazim, thiophanate methyl, and tebuconazole against *P. glomerata*; and d) to examine the sensitivity of 30 *P. glomerata* isolates in vitro to the above fungicides.

Materials and methods

Isolation and identification of *Phoma glomerata*

In July and through summer 2007, shoots of the cling peach cv. Andross were wilted and blighted in commercial orchards of the Prefecture of Imathia, in northern Greece. Close examination of these shoots revealed distinct cankers covered with abundant gumming. Isolations from the lower margins of the cankers were made by plating onto potato dextrose agar (PDA) acidified 3-mm-long sections of 3-mm-diameter twigs that had been superficially sterilized. Sterilization was made in domestic chlorine bleach 10% (sodium hypochloride; 4.8%) for 15 min and then rinsed three times with sterile water. PDA was acidified by adding 2.5 ml of 85% lactic acid per liter after autoclaving. The plates were incubated at 23°C for 5 to 7 days, and consistent colonies with dark-brown mycelia developed from the majority of the plated woody tissues. Identification of the genus was based on morphological characteristics of septate mycelia, pycnidia, and pycnidiospores (White and Morgan 1987; Boerema et al. 2004). The fungal species was identified by sequencing the internal transcribed spacer (ITS) with the forward (SSU/1609–1627: TTAAGTCCCTGCCCTTTGTA→) (De Hoog and van den Ende 1998) and reverse (LSU/287–266: ←GCATTCCCAAACAACTCGACTC) (Masclaux et al. 1995) primers by CBS Fungal Biodiversity Centre, Identification Service (Utrecht, Netherlands) (Accession Number CBS 125539).

To complete Koch's postulates, 20 segments (6 cm in length and 1.5–2 cm in diameter) of 1-year-old woody shoots of peach cultivar 'Andross' were inoculated in the laboratory. Using a cork borer, a wound of 7 mm in diameter was created in the middle of each shoot segment by removing the bark and a 6-mm diameter agar plug bearing mycelia and spores from a single spore, 15-day-old, culture was inserted in each wound. The wound was covered with petroleum jelly and wrapped with parafilm to prevent desiccation. Ten control segments were similarly wounded and inoculated with an agar disk without mycelium of the fungus. All inoculated and non-inoculated shoot segments were incubated at 25°C in moist (>95% relative humidity) chambers and the resulting necrosis was recorded after 20 days incubation. In addition, mature fruit of the peach cultivar "Tasty Free" were inoculated after they had been disinfested by dipping in a 10% sodium hypochlorite solution for 15 min. Inoculations were made by wounding the fruit with a scapel and placing a mycelial disk (5 mm diam) over the wound. Inoculated fruit were lightly enclosed in special plastic dishes (40×20×15 cm) which were then placed in a growth chamber at 24–26°C. Control fruit were inoculated with an agar disk without mycelium. Five days later, inoculated fruit were checked for the development of symptoms.

Effect of temperatures on the mycelial growth and conidial germination

Mycelial growth An agar disk, 6 mm in diameter, taken from an active colony of *P. glomerata*, which originated from a blighted shoot of the cv. "Catherine" was placed in the center of each of five replicated dishes (9 cm diam) containing PDA. Three single-spore isolates

were used. Subsequently, the dishes were incubated in a growth chamber at 5, 10, 15, 20, 25, 30, or 35°C for 6 days and the diameter of the resulting colony was recorded. Results were recorded a second time 8 days after first recording for the dishes incubated at 5, 10 and 35°C. This experiment was repeated once.

Conidial germination The same three single-spore isolates were used. To harvest the conidia from the PDA slants, a solution of Tween 20 wetting agent (BDH Ltd., Poole, UK) was prepared by mixing 50 μl of Tween 20 into 100 ml of sterile distilled water in a sterile glass beaker and mixing with a glass rod. Five ml of this solution was pipetted into each of the agar slants and the conidia agitated gently by rubbing the surface of the culture with a flamed loop. The resulting suspension was then filtered through two layers of moistened sterile cheesecloth to remove any mycelium and agar fragments.

The suspension was added to 10 ml potato dexrose broth (PDB) to produce a final conidial density of 6.34× 104 conidia/ml as determined with a Neubauer improved haemocytometer. The lids were fully tightened, each container was shaken gently to thoroughly mix the contents, and the bottles were placed in a growth chamber at 5, 10, 15, 20, 25, 30, or 35°C for 48 h. There were 5 replicate bottles for each temperature. To determine the percentage of conidial germination, 100 conidia per replicate were counted randomly under the microscope (x400). A conidium was considered germinated when the germ tube was equal to the greatest diameter of the swollen conidia (Dhingra and Sinclair 1985; Dantigny *et al*., 2006). Germ tube elongation was measured by measuring 20 germ tubes selected randomly. This experiment was conducted twice.

Pathogenicity and virulence of *P. glomerata* to peach and nectarine cultivars

This experiment was conducted in the experimental field of the Pomology Institute, Naoussa, Greece. All trees were 10 years old and were grafted onto the peach rootstock GF677 (a hybrid of peach x almond). Trees were pruned to a vase shape. Five to six irrigations were provided yearly. The spraying program included an application of Bordeaux mixture at the leaf fall stage, an application with ziram at the break of dormancy stage and 3 applications of wettable sulphur during the spring.

The stem inoculation method described by Thomidis (2000) was used to examine the pathogenicity of three single spore *P. glomerata* isolates (recovered from blighted shoots of peach) on 18 peach cultivars (Romea, S. Sun Glo, Sun Gloud, O'Henry, Gold Grest, June Gold, Early Gold, E45, Fayette, First Gold, Spring Crest, Maria Bianca, Fortuna, Everts, Crest Haven, Andross, Symphony) and seven nectarine cultivars (Caltesse 2000, Big Top, Fire Bright, Red Gold, May Grand, Kakamas, Andriana). Thirty annual woody shoots, 1.5–2 cm in diameter, were randomly selected from each cultivar and 10 were inoculated with each isolate. The inoculum, which consisted of a 4-mm-diam plug taken from a 10-day-old culture, was inserted under the bark in the middle of each shoot. The wound was covered with petroleum jelly and wrapped with adhesive tape to prevent desiccation. Results were collected by recording the length of the resulting necrosis 45 days after inoculation. This experiment was conducted in May 2008 and again in May 2009. During the period of experimentation, temperatures ranged between 15°C and 30°C and were therefore conducive for the growth of the fungus.

Sensitivity of *P. glomerata* to fungicides

In a first experiment, 30 single-spore isolates of *P. glomerata* that originated from blighted shoots of peach were tested for sensitivity to the fungicides thiophanate methyl, carbendazim, and tebuconazole which are widely used against shoot blight and fruit rots in Greece. Each isolate was grown on PDA supplemented with the appropriate fungicide. Stock suspensions were prepared by mixing the fungicide in sterile distilled water at label rates recommended by the manufacturer (PILAZIN 60WP=0.75 g l^{-1}, thiophanate methyl 70WP=0.7 g l^{-1}, and Folicur 25WG= 0.33 ml l^{-1}). This solution was added to the PDA, after autoclaving and cooling at 45°C. A 6-mm-diam agar plug, taken from the margins of an actively growing colony of each isolate, was placed in the centre of the fungicide-containing agar plates and the plates were incubated at 25°C. Colony diameter measurements were taken 7 days after initial transfer. Growth on agar without fungicide was also recorded to be used as control for determining the degree of mycelial inhibition attributable to the fungicides. Five replicated plates were used for each combination of isolate and fungicide. This experiment was conducted twice.

In a second experiment, conidia were harvested from two single spore isolates using methods described above. The conidial suspension was added to tubes containing 10 ml PDB amended test fungicides at concentrations recommended by the producers (reported above). The conidial density was 6×104 conidia/ml as determined as described above. The lids were fully tightened and each container shaken gently to thoroughly mix the contents. The bottles were placed in a growth chamber at 25°C. After 48 h, conidial germination was recorded as described above. Five replicated bottles were used for each combination of isolate and fungicide and the experiment was conducted twice.

In a third experiment, a 4-mm-diam plug of two single-spore isolates of *P. glomerata* from blighted shoots of peach were transferred onto PDA supplemented with different concentrations of the tested fungicides (PILAZIN 60WP at 0.3, 0.5 and 0.75 g l^{-1}, Thiophanate methyl 70WP at 0.3, 0.5, and 0.7 g l^{-1}, and Folicur 25WG at 0.2, 0.26 and 0.33 ml l^{-1}). Five replicated plates were used for each treatment. The methodology described in the first experiment was followed and the experiment was conducted twice.

In a fourth experiment, a single-spore isolate of *P. glomerata* from a blighted shoot of peach was used. Annual shoots of the cultivar Andross were collected at harvesting time and transferred to the laboratory. They were disinfected by dipping in a 10% domestic bleach (4.86%) solution for 10 min, washed with sterile-distilled water, and dried at room temperatures. Segments 20 cm in length and about 5–10 mm in diameter were collected from the middle of each shoot, wounded by using a flamed scalpel and then dipped in a solution containing one of the tested fungicide at the concentrations recommended by the producers. After drying, shoot segments were inoculated by placing a 4-mm-diam mycelium plug taken from the edge of a 6-day-old colony onto the wound, and the shoot segments were then incubated at 25°C for 20 days. There were 60 shoot segments, 20 for each fungicide treatment. Twenty other shoot segments inoculated with agar without mycelium and 20 shoot segments dipped in tap water were used as control. Results were collected by recording the length of necrotic areas 15 days after inoculation. This experiment was conducted twice.

In a fifth experiment, experiments were conducted in the experimental field of Pomology Institute, Naoussa, Greece. One single spore isolate of the fungus was used. Annual shoots of the cultivar Andross were wounded in the middle and sprayed with one of the test fungicides at the concentration recommended by the producers (Table 1). Two hours later, the shoots were inoculated using the method described in the pathogenicity experiments. There were 20 inoculated shoots for each fungicide tested. Twenty unsprayed shoots inoculated with the fungus were used as control. Results were collected by recording the length of the resulting necrosis 30 days later. This experiment was conducted in July 2010.

Statistical analysis

To analyse data for significant differences at $\alpha=0.05$, the Generalized Linear Model (Wald's Chi-Square) was applied (SPSS Grad Pack 16 for Windows). BETE analysis was used to fit the data in curves. $Y = a \cdot Tegb \cdot (1 - Teg)c$, where $Teg = (T - Tmin) / (Tmax - Tmin)$ and T = temperature. To estimate the coefficients a, b and c, the logarithmic form $\log Y = \log a + b \cdot \log Teg + c \log (1\text{-}Teg)$ was used

Results

Isolation and identification of *Phoma glomerata*

The fungus *P. glomerata* was isolated from blighted shoots of peach cultivar "Catherine". Large pycnidia, round to pyriform, dark in color, bearing phialides in

Table 1 Effect of fungicides on development on excised peach shoots of *Phoma glomerata* isolated from blighted shoots of peach grown in Greece

Fungicides	Rates[x]	Length of necrosis (cm)	
Thiophanate methyl	0.7 g/l	0.58	b
Carbendazim	0.75 g/l	0.97	b
Tebuconazole	0.33 ml/l	0.91	b
Control		6.11	a

[x] Label recommended rates.

[y] Values are the means of two experiments.

[z] Values followed by the same letters are not significantly different according to Wald's Test at $P<0.05$.

their inner lining were formed on blighted shoots. Pycnidia had one to several openings (ostioles) on their upper surface from which the conidia oozed. The pathogen was identified as *P. glomerata* based on the ITS sequence. Koch's postulates were satisfied after re-isolating the fungus from inoculated shoots that developed symptoms similar to those observed on shoots collected from commercial orchards.

Artificially inoculated fruit developed typical symptoms of fruit rot, although this rot has not been encountered in the field.

Effect of temperature on growth and conidial germination

The optimum temperature for mycelial growth of *P. glomerata* on PDA was 24°C, whereas mycelial growth was inhibited at 35 and 10°C. However, very little mycelial growth was observed at 5 and 10°C after 14 days incubation. Mycelial growth was significantly less at 20 than 25°C. The effect of temperature on mycelial growth of *P. glomerata* is best described with a BETE equation (Fig. 1).

The results also showed that the the rate of conidial germination and the germ tube elongation was reduced as temperatures increased from 25 to 35°C, as they decreased from 25 to 10°C. Germination and growth were totally inhibited at 2–4°C. The effect of temperature on conidial germination and germ tube elongation are shown in Figs 2 and 3 and each are best described with a BETE equation.

Pathogenicity and virulence of *P. glomerata* to peach and nectarine cultivars

Pathogenicity tests showed that all peach and nectarine cultivars artificially inoculated with the fungus *P. glomerata* developed symptoms of necrosis around the inoculation site. The length of necrosis ranged from 7 to 8 cm and there were no significant differences among the cultivars. Furthermore, no differences in virulence were found among the three isolates of *P. glomerata*.

Testing of *Phoma glomerata* isolates for their sensitivity to four fungicides

In the first experiment, all isolates of *P. glomerata* were sensitive to all fungicides and showed no mycelial development on any of the fungicide-amended agar. The mycelial growth of all isolates grown on PDA without fungicide ranged from 30 to 36 mm.

In the second experiment, all fungicides used at the concentration recommended by producer inhibited the conidial germination of *P. glomerata* for all three isolates tested. In control bottles, the percentage of conidial germination ranged from 55% to 70%, while the germ tube elongation ranged from 38 to 52 μm.

In the third experiment, thiophanate methyl, carbendazim and tebuconazole inhibited mycelial growth of all *P. glomerata* isolates used at all concentrations tested including concentrations lower than those recommennded by producers. The mycelial growth

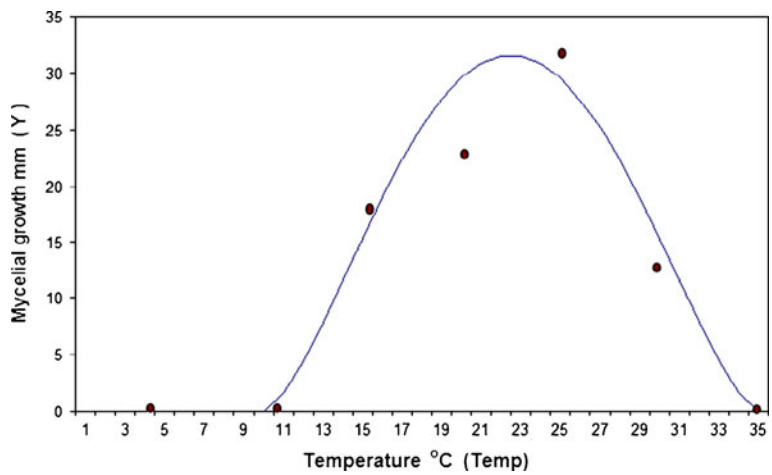

Fig. 1 Effect of temperature on mycelial growth of *Phoma glomerata* isolates collected from blighted shoots of peach in Greece

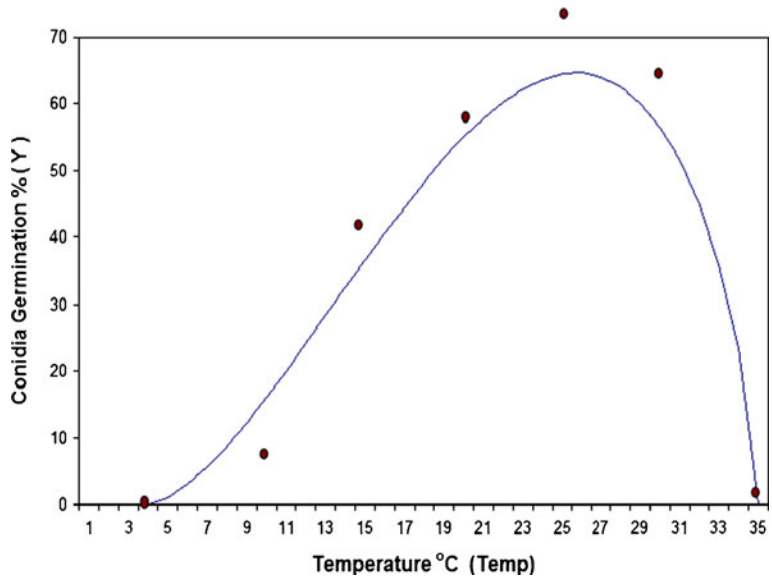

Fig. 2 Effect of temperature on conidial germination of *Phoma glomerata* isolates collected from blighted shoots of peach in Greece

of all isolates grown on PDA without fungicide ranged from 27 to 32 μm.

In the fourth experiment, a small canker developed in shoot segments treated with thiophanate methyl, carbendazim and tebuconazole at concentrations recommended by producer (Table 1). There were no significant differences among the fungicides used. In contrast, control shoot segments developed the typical symptoms of the disease (Table 1).

In the fifth experiment, all fungicides tested inhibited the growth of fungus. In contrast, control shoots developed the typical symptoms of the disease with the length of necrosis ranging from 7 to 8 cm.

Discussion

Although restriction canker and other shoot blights of peach were attributed to the fungal genera *Monilinia, Phomopsis, Colletotrichum, Cytospora*, etc., to our knowledge, this is the first report worldwide of the occurrence of *P. glomerata* causing severe canker and

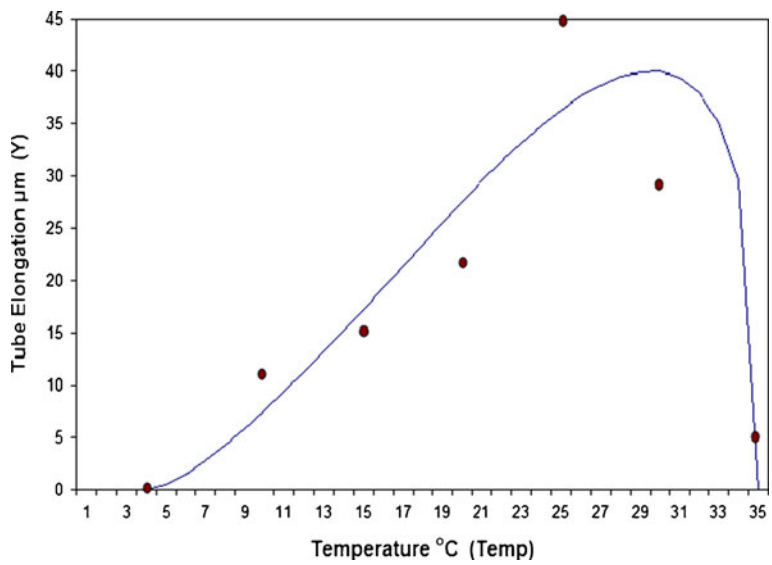

Fig. 3 Effect of temperature on germ tube elongation of conidia of *Phoma glomerata* isolates collected from blighted shoots of peach in Greece

shoot blight on peach. Other species of the genus *Phoma* have been previously reported as causal agents of stone fruit trees in other Mediterranean countries. According to Tuset and Portilla (1985) two species of *Phoma*, *P. pomorum* Thum (= *Phoma purnicola* (Opiz) Wollenw and Hochapfel) and *P. fimeti* Brun (= *Phoma armeniaceae* Thum) were identified in stone fruit trees (almond, peach, apricot, plum and cherry) in the Mediterranean area of Spain. They also reported that *P. pomorum* was more widely spread producing a small "shot-hole" in leaves that increases in abundance by the end of autumn in certain areas and humid environmental conditions. In contrast, *P. fimeti* was isolated from apricot wood only and appears to behave as a saprophyte.

In this study, *P. glomerata* was found overwintering as pycnidia on blighted shoots on trees. Studies with other species of *Phoma*, such as *P. asparagi*, showed that they also overwinter on diseased tissues as pycnidia (Liu and Hwang 1988). It is most likely that conidia released from pycnidia developed on the previous year's blighted shoots and diseased plant debris cause the primary infections of young developing shoots of peach in the spring. New pycnidia in current-season infected shoots develop during the summer and fall and may contribute inoculum for secondary infections late in the season. However, details on the disease cycle need to be investigated further.

The results of this study also showed 25°C as the best temperature for the mycelial growth and conidial germination of *P. glomerata*. The results agree with those produced by Sempere Ferre et al. (2007) who tested the effect of temperatures on growth of *P. glomerata* and found that 25°C was the optimum temperature for mycelial growth. Temperatures close to 35 and 10°C inhibited mycelial growth and reduced greatly conidial germination. Therefore, when temperatures fluctuate close to 25°C, protective methods should be taken against the disease since *P. glomerata* can cause serious damage in peach.

The isolates of the *P. glomerata* used were pathogenic to all peach and nectarine cultivars tested without any differences in the levels of their susceptibility. However, the levels of susceptibility could not be compared to a resistant cultivar as one has not been yet identified.

In all experiments, carbendazim, thiophanate methyl, and tebuconazole inhibited the development and conidial germination of *P. glomerata,* suggesting that these fungicides can be effective in controlling the disease. The effectiveness of thiophanate methyl against other *Phoma* species has been previously reported (Schmitz et al. 2006). Barbetti (1992) showed that carbendazim was effective in controlling phoma black stem of *Medicago polymorpha* var *brevispina*. According to Pethybridge et al. (2005) fungicides that belong to the demethylation inhibitor group, including prochloraz, tebuconazole, difenoconazole, and cyproconazole, reduced greatly the mycelial growth of *P. ligulicola* in vitro as compared to control media without fungicides. Kruse and Verreet (2005) controlled infections of oilseed rape from the fungus *P. lingam* with the use of tebuconazole.

In this study, all isolates of *P. glomerata* from peach in Greece tested were sensitive to benzimidazole (thiophanate methyl and carbendazim) fungicides and to triazole tebuconazole. In contrast, Van de Graaf et al. (2003) found that more than one third of the 14 isolates of *P. clematidina* originated from Clematis plants with symptoms of leaf spot and wilt, tested on fungicide-amended agar plates were highly resistant to products containing carbendazim, benomyl, or thiophanate-methyl. They also found that the fungicide tebuconazole could be a good alternative method to control this pathogen.

In conclusion, *P. glomerata* can cause shoot blights in diseased peach trees. This study showed that the pathogen can overwinter in the blighted shoots in the form of pycnidia. Conidia released the following spring are possibly the primary inocula of the pathogen. Based on in vitro studies, it appears that the best period for infections are when temperatures are around 25°C. Pathogenicity tests indicated that the main peach—nectarine cultuvars used in Greece had similar level of susceptibility to this pathogen and, therefore, selection of resistant cultivars is not a method to control this disease. In contrast, the fungicides tebuconazole, carbendazim and thiophanate methyl are very effective in areas where this pathogen could be a problem for peach trees.

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