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Silviculture

Original Article

Susceptibility of Pecan Cultivars to *Cladosporium cladosporioides* Species Complex

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ABSTRACT

Recent studies have observed symptoms of leaf spot in pecan (*Carya illinoinensis*) associated with species of the *Cladosporium cladosporioides* complex (*C. cladosporioides*, *C. pseudocladosporioides*, and *C. subuliforme*). Therefore, the present study aimed to investigate the pathogenicity of these *Cladosporium* spp. isolated from two pecan cultivars. Twenty-six isolates were used to inoculate leaflets of the cultivars, 'Barton' and 'Shawnee', with a spore suspension. The suspension was applied through drops on the surface of the leaflets, the material was kept in boxes, "gerbox", in the incubation room. After 22 days, leaf spots varied from small circular brown to black stains, which indicated the advancement of the disease. The three *Cladosporium* sp. were pathogenic to pecan cultivars and there was a difference in susceptibility to *C. pseudocladosporioides* species between the 'Barton' and 'Shawnee' cultivars, where 'Shawnee' showed greater disease severity.

Keywords: *Carya illinoinensis*, susceptibility, inoculation, breeding program, scab.

1. INTRODUCTION

The pecan [*Carya illinoinensis* (Wangenh.) K. Koch], of the Juglandaceae family, is considered, among the 20 species of the genus *Carya*, the most valuable native species of North America. Orchards formed by superior cultivars, propagated by cloning, are more productive and produce higher quality nuts compared to native orchards or those formed by seed propagation. In this way, breeding programs have sought genetic materials with higher yields and nut quality, as well as possessing disease and insect resistance (Thompson & Conner, 2012).

In Brazil, there has been an increase in the area planted with pecan, mainly in the Rio Grande do Sul, Santa Catarina, and Paraná states (IBGE, 2015). However, genetic improvement to obtain cultivars resistant to diseases and with fruit characteristics desirable for the market, has received little attention. According to the Ministry of Agriculture, Livestock and Food Supply (Brasil, 2015), only two pecan cultivars were grown in Rio Grande do Sul state by seedling nurseries, and practically all *C. illinoinensis* stock used in commercial orchards, is from the USA.

Pecan trees are attacked by several fungal, bacterial and nematode species, all of which affect the crop. Scab, caused by the *Fusicladium effusum* fungus (sin *Cladosporium caryigenum*), is the best-known disease, however, many other diseases can cause significant losses in production (Johnson & Black, 2012). Recently in Brazil, Lazarotto et al. (2014a) identified *Fusarium* sp. species pathogenic to pecan causing bottlenecks, root rot, and foliar necrosis associated with root rot. The occurrence of *Pestalotiopsis* sp. species causing leaf spot was also observed by Lazarotto et al. (2014b).

Leaf spots caused by *Cladosporium* sp. in pecan are small when on leaves, branches, and fruits but can expand and coalesce leading to defoliation. Abortion and losses in fruit quality and consequent decreases in productivity can also occur.

The use of resistant cultivars is one of the most important disease control strategies, as it is applicable over large areas, as well as presenting a low environmental impact compared to pesticides (Camargo, 2011). One strategy that can be applied is the incorporation of a mixture of cultivars containing different resistance genes within the orchards. Thus, the development of new cultivars with similar silvicultural characteristics, but with different resistance genes, may facilitate the use of this strategy in pecan orchards (Conner & Stevenson, 2004).

The 'Barton' cultivar was the first to be introduced by the USDA (United States Department of Agriculture), a result of a cross-breeding between 'Moore' and 'Success' in 1937 by L.D. Romberg at John Barton's orchard in Utley, Texas, whereas the 'Shawnee' cultivar was selected from a cross between 'Schley' × 'Barton' and introduced in 1968 by the USDA (CAES, 2015).

In a study by Yates et al. (1996), the authors evaluated 10 pecan cultivars for susceptibility to scab. 'Caddo', 'Choctaw', 'Desirable', and 'Farley' cultivars were more susceptible in Louisiana, while 'Curtis' and 'Stuart' were more susceptible in Florida. Thus, variable responses between cultivars in distinct regions may be due to different races of *Cladosporium caryigenum*.

The disadvantage of field evaluation methods for resistant germplasm to pecan scab is the dependence on favorable environmental conditions for pathogen development (Conner, 2002). To overcome the difficulties in conducting field resistance tests, Yates et al. (1996) developed a methodology that employs a detached-leaf method to assess cultivar resistance to pecan scab. This method has the advantage of standardized conditions under which the inoculation is performed, facilitating reproducibility between tests. Conner (2002) developed a modified protocol, testing multiple *Cladosporium caryigenum* isolates on detached leaves with pathogen development evaluations under an optical microscope. This methodology was efficient to evaluate resistance to specific races of pecan scab. In a recent study, species of the same genus (*C. cladosporioides*, *C. pseudocladosporioides*, *C. subuliforme*) were isolated from leaf spot of pecan, however their pathogenicity was not proven (Walker et al., 2016a). Thus, the objective of the present study was to confirm the pathogenicity of three *Cladosporium* sp. in causing pecan scab as well as to verify the resistance of two pecan cultivars after inoculation of pathogenic isolates.

2. MATERIALS AND METHODS

In the period from January to April 2014, collection was carried out of leaves with symptoms of leaf spot caused by *Cladosporium* spp. in pecan orchards, in

the states of Rio Grande do Sul (RS), Santa Catarina (SC), and Paraná (PR). The selection of the isolates for the pathogenicity test was based on the phylogenetic dendrogram constructed with 40 isolates of *Cladosporium*, identified by sequencing the elongation factor region (TEF-1α) (Walker et al., 2016b). Within each group formed in the clades, an isolate was selected, among these were: *Cladosporium pseudocladosporioides* (12 isolates), *C. cladosporioides* (13 isolates), and *C. subuliforme* (one isolate) (Table 1).

To confirm the pathogenicity of the isolates, the detached leaf method proposed by Conner (2002) was used, with some modifications. Leaflets were collected from the 'Barton' and 'Shawnee' cultivars, with no apparent symptoms of phytopathogen attack, obtained from an experimental orchard at three years of age, located at the Fundação Estadual de Pesquisa Agropecuária (FEPAGRO), 29" 40' 19 S and 53" 54' 35 W, county of Santa Maria, RS. The leaflets were disinfected for 30 seconds with 70% ethanol, 1% sodium hypochlorite and rinsed twice in distilled and sterilized water. Subsequently, the petiole was wrapped with moistened cotton and the leaves were arranged in gerbox boxes containing two sheets of moistened filter paper. Two glass slides were placed under each detached sheet in order to avoid the leaf coming into direct contact with the filter paper and consequently preventing its waterlogging and rotting.

The spore suspension for inoculation was obtained from *Cladosporium* sp. isolates which were grown in Potato-Dextrose-Agar (BDA) medium at 24 °C (12 h photoperiod), after 10 days growth in the incubation chamber. Subsequently, 10 mL of distilled water was added to each plate and, with the help of a Drigalski handle, the fungal mycelium was scraped from the surface of the medium. The suspension was filtered through a double layer of gauze in to a beaker. The spore concentration was evaluated with a Neubauer chamber and adjusted to 1×10^6 spores mL^{-1}. The suspension was applied using 0.1 ml distributed in 6 drops on each surface of the leaflets (in the control sterile distilled water was used). The samples were kept in gerbox boxes, in an incubation room with a temperature of 24 ± 1 °C (12 h of photoperiod). At 22 days after inoculation, leaf spot incidence was evaluated via the presence or absence of leaf symptoms and severity was observed in each leaflet at the place where the spore suspension drops were applied, noting the number of lesions, and transforming them into a percentage. A diagrammatic scale was used and grades 1 to 7 were assigned, where 1 = 0% (no symptoms); 2 = 16.6% (one drop with symptoms); 3 = 33.3% (two drops with symptoms); 4 = 50% (three drops with symptoms); 5 = 66.6% (four drops with symptoms); 6 = 83.3% (five drops with symptoms); 7 = 100% (six drops with symptoms). Cultivars with grades between 1 and 4 were considered resistant and grades above 4 were considered susceptible. After the evaluations, pathogen re-isolation was carried out to fulfill Koch's postulates.

The pathogenicity and reaction tests of the genetic material, conducted simultaneously, were carried out in a completely randomized design in a factorial scheme composed of two factors (3 *Cladosporium* spp. species × 2 pecan cultivars). Eight replicates were used, with each leaflet representing one sample unit. Data were submitted to analysis of variance and Tukey test ($p \leq 0.05$), using the statistical software Sisvar version 5.3 (Ferreira, 2008).

3. RESULTS AND DISCUSSION

According to Conner (2002), the appropriate period to assess the development of *C. caryigenum* lesions on pecan leaves is 21 days, with the author recommending that the evaluation be performed at 22 days. At this stage, leaf spots were observed with small brown to black circular scoring, which enlarged with disease progression (Figure 1 - A, B, and F).

Differences in symptoms caused by the three inoculated species were not verified. It should be noted that in the present study, inoculation with *Cladosporium* species (*C. cladosporioides*, *C. pseudocladosporioides* and *C. subuliforme*), pathogenic to pecan and belonging to the *C. cladosporioides* complex, presented very close morphological characteristics, corroborating the descriptions of Bensch et al. (2010). Until the present study, these identified species had not been reported in pecan in Brazil, nor anywhere else in the world where the species is cultivated.

Leaf spot has previously been reported in India on *Helianthus annuus* L. caused by *C. cladosporioides* (Anilkumar & Seshadri, 1975). Other species of the same genus have also been reported causing leaf spot on other hosts, such as *C. herbarum* on *Centaurea solstitialis* L. in Greece (Berner et al., 2007); *C. perangustum* in

Table 1. *Cladosporium* sp. isolates obtained from pecan leaf spots in southern Brazil, selected for the pathogenicity test.

Isolate	Access number GenBank[1]	Species	Collection date	County/State	Coordinates (GMS)
1PR	KT991540	*Cladosporium cladosporioides*	Jan/14	Missal – PR	-25°04'50" S, -54°16'03" W
2/1PR	KT991541	*Cladosporium cladosporioides*	Jan/14	Toledo – PR	-24°75'24"S, 53°66'45"W
2/2PR	KT965277	*Cladosporium pseudocladosporioides*	Jan/14	Toledo – PR	-24°75'24"S, 53°66'45"W
3PR	KT991542	*Cladosporium cladosporioides*	Jan/14	Assis Chateaubriand - PR	-24°41'00" S, -53°52'13" W
4PR	KT991568	*Cladosporium cladosporioides*	Jan/14	Pato Branco - PR	-26°27'37" S, -5260'34" W
12/7PR	KT991547	*Cladosporium cladosporioides*	Mar/14	Porto Amazonas - PR	-25°32'22" S, -49°54'29" W
12/14PR	KT991550	*Cladosporium cladosporioides*	Mar/14	Porto Amazonas - PR	-25°32'22" S, -49°54'29" W
12/8PR	KT991548	*Cladosporium cladosporioides*	Mar/14	Porto Amazonas - PR	-25°32'22" S, -49°54'29" W
6SC	KT991538	*Cladosporium pseudocladosporioides*	Jan/14	Palmitos-SC	-27°12'00" S, -53°22'10" W
7SC	KT991557	*Cladosporium pseudocladosporioides*	Jan/14	São João do Oeste - SC	-27°05'01" S, -53°58'37" W
8RS	KT991558	*Cladosporium pseudocladosporioides*	Feb/14	Alegrete-RS	-29°48'53" S, 55°49'15" W
9RS	KT991543	*Cladosporium cladosporioides*	Feb/14	Uruguaiana –RS	-29°26'11" S, 56°41'05" W
10RS	KT991559	*Cladosporium pseudocladosporioides*	Feb/14	Itaqui- RS	-29°12'07" S, 56°29'34 W
11RS	KT991560	*Cladosporium pseudocladosporioides*	Feb/14	São Borja - RS	-28°63'39" S, -55°84'83" W
13/10RS	KT991562	*Cladosporium pseudocladosporioides*	Mar/14	Santa Maria (Pains) - RS	-29°45'25" S, -53°40'00" W
13/12RS	KT991563	*Cladosporium pseudocladosporioides*	Mar/14	Santa Maria (Pains) - RS	-29°45'25" S, -53°40'00" W
14/21RS	KT948967	*Cladosporium cladosporioides*	Mar/14	Santa Maria (Fepagro) - RS	-29°40'19" S, -53°54'35" W
14/25RS	KT991567	*Cladosporium pseudocladosporioides*	Mar/14	Santa Maria (Fepagro) - RS	-29°40'19" S, -53°54'35" W
14/26RS	KT991554	*Cladosporium cladosporioides*	Mar/14	Santa Maria (Fepagro) - RS	-29°40'19" S, -53°54'35" W
15/5RS	KT991537	*Cladosporium pseudocladosporioides*	Apr/14	Dilermando de Aguiar -RS	-29°47'52" S, -54°05'45" W
15/23RS	KT991556	*Cladosporium cladosporioides*	Apr/14	Dilermando de Aguiar -RS	-29°47'52" S, -54°05'45" W
15/7RS	KT991555	*Cladosporium cladosporioides*	Apr/14	Dilermando de Aguiar -RS	-29°47'52" S, -54°05'45" W
16/2RS	KT995114	*Cladosporium subulifore*	Apr/14	Cachoeira do Sul - RS	-30°00'20" S, -52°53'00" W
16/13RS	KT991536	*Cladosporium pseudocladosporioides*	Apr/14	Cachoeira do Sul - RS	-30°00'20" S, -52°53'00" W
17/2RS	KT991533	*Cladosporium pseudocladosporioides*	Apr/14	Cachoeira do Sul - RS	-30°00'16" S, -52°53'25" W
17/18RS	KT991539	*Cladosporium cladosporioides*	Apr/14	Cachoeira do Sul - RS	-30°00'16" S, -52°53'25" W

[1]Identification of *Cladosporium* spp. species through the sequencing of the elongation factor region (TEF-1α).

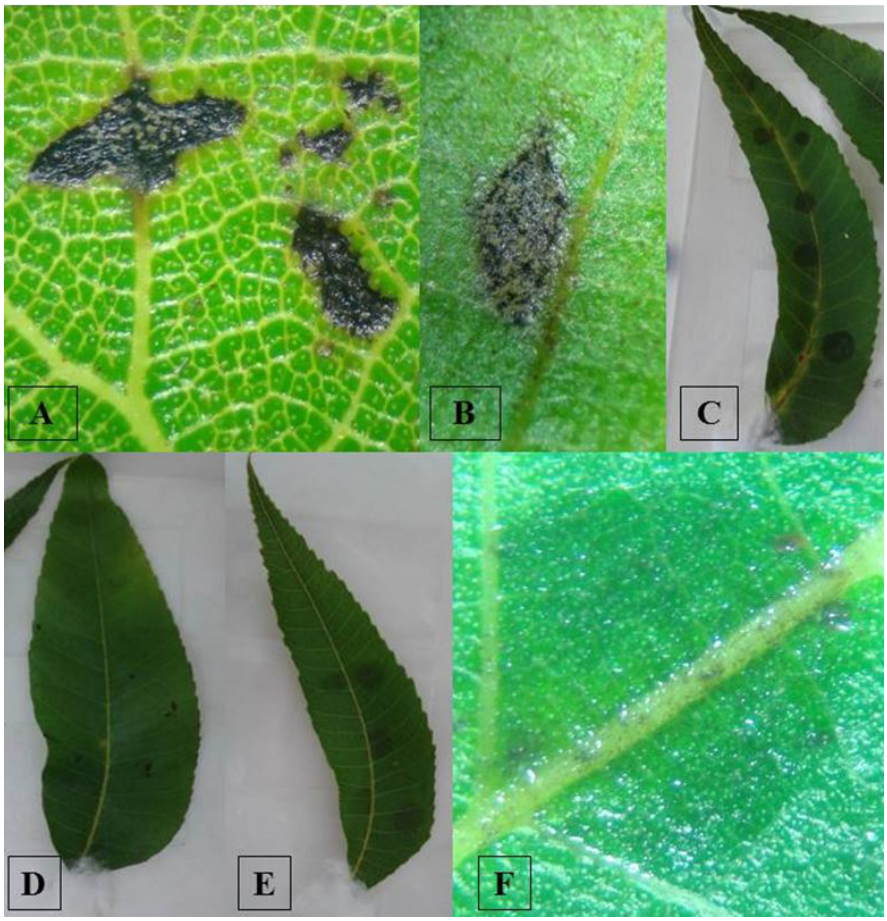

Figure 1. Typical leaf spot symptoms induced by artificial *Cladosporium* sp. isolates inoculation in pecan cultivars. Observations made by stereoscopic microscope showing dark brown to black stains with the presence of pathogen sign (A, B, and F); Symptoms visually observed (C, D, and E).

Syagrus oleracea, a palm species of the Brazilian central region (Oliveira et al., 2014); and *C. oxysporum* in *Solanum melongena* L., China (Zheng et al., 2014). In the same genus, *C. caryigenum* (Sin *Fusicladium effusum*) is reported in several studies as the pathogen responsible for causing pecan scab (Bock et al., 2014; Gottwald, 1982; Demaree, 1924).

The three species of *Cladosporium* inoculated (*C. cladosporioides*, *C. pseudocladosporioides* and *C. subuliforme*) were pathogenic in the 'Barton' and 'Shawnee' cultivars, showing significant differences compared to the control (without inoculation of the pathogen) (Table 2). When the three *Cladosporium* spp. species were inoculated on the 'Barton' cultivar, the mean ranged from 4.9 to 5.5 according to the grading scale used (degree of disease severity from 50 to 66.6%), showing no significant differences between species. Differences were observed however, when compared to the control. Thus, the cultivar 'Barton' was considered susceptible (severity score above 4) to leaf spot caused by *Cladosporium* sp. Similar results were observed in the 'Shawnee' cultivar, in which severity scores were found between 5.1 and 5.8 among the *Cladosporium* spp. species inoculated, without significant differences between them. Thus, in the same way, the 'Shawnee' cultivar was also considered susceptible to leaf spot.

In relation to the severity scores (Table 2), it was possible to observe the interaction between the *Cladosporium* spp. factors and pecan cultivars. When the *C. pseudocladosporioides* species was inoculated, a significant effect was observed among the cultivars, causing a greater degree of disease severity and was more aggressive in the 'Shawnee' cultivar (average

Table 2. Average of leaf spot severity scores induced by *Cladosporium* spp. species artificial inoculation in detached leaves from the 'Barton' and 'Shawnee' pecan cultivars.

Species	Cultivars	
	'Barton'	'Shawnee'
Cladosporium pseudocladosporioides	4.9* Ab**	5.4 Bb
Cladosporium subuliforme	5.1 Ab	5.8 Ab
Cladosporium cladosporioides	5.5 Ab	5.1 Ab
Control	1.0 Aa	1.0 Aa
C.V. (%)	16.63	

*Severity scores 1 = 0 lesions; 2 = 1 lesion; 3 = 2 lesions; 4 = 3 lesions; 5 = 4 lesions; 6 = 5 lesions; 7 = 6 lesions. **Means followed by different letters, upper case in the row and lower case in the column, differ statistically from each other by the Tukey test ($p \leq 0.05$). C.V. (%) = Coefficient of Variation.

score of 5.4) than the 'Barton' cultivar (average score 4.9). However, the two cultivars were both susceptible to the disease, since they have mean values of severity scores above 4. Demaree (1924) has previously reported a wide variation in the degree of susceptibility to pecan scab among the different cultivars. On the other hand, other cultivars are highly resistant and can be considered immune. Thus, it is probable that there is also a variation in the susceptibility of the cultivars in relation to leaf spot, the object of the present study, with the species included in the *C. cladosporioides* complex.

In a field study carried out over five years, Goff et al. (2003) found that the 'Barton' cultivar had no incidence of scab caused by *C. caryigenum* and was considered by the authors to be a resistant cultivar. Conner & Stevenson (2004) also examined the resistance of 19 pecan cultivars to 12 *C. caryigenum* isolates in the field and found that all cultivars showed differences in susceptibility to isolates. 'Barton', 'Curtis', and 'Summer' were resistant to most isolates; however, 'Wichita' and 'Schley' were susceptible to most isolates.

Conner & Stevenson (2004) reported that the presence of numerous breeds of *Cladosporium* sp., associated with pecan scab in most cultivars, including those considered highly susceptible, show resistance to multiple strains of this pathogen.

Screening in other pecan cultivars is important to verify their susceptibility to leaf spot caused by *Cladosporium* sp. as evaluated in the present study. The methodology used in the pathogenicity test allows standardization in the conditions of the tested environment and facilitates testing with a large number of isolates and cultivars.

4. CONCLUSIONS

1. *Cladosporium cladosporioides*, *C. pseudocladosporioides*, and *C. subuliforme* are pathogenic to pecan, causing leaf spot;
2. The two pecan cultivars are susceptible to leaf spot caused by the three *Cladosporium* sp. tested, and there is a difference in susceptibility to *C. pseudocladosporioides*. The 'Shawnee' cultivar has a higher disease severity compared to 'Barton', therefore the first is more susceptible.

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