

Revista Brasileira de Ciências Agrárias (Agrária)

Revista Brasileira de Ciências Agrárias

ISSN: 1981-1160

editorgeral@agraria.pro.br

Universidade Federal Rural de
Pernambuco
Brasil

dos Santos, Ricardo Feliciano; Durigon, Miria Rosa; Blume, Elena
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Revista Brasileira de Ciências Agrárias, vol. 10, núm. 1, 2015, pp. 49-53
Universidade Federal Rural de Pernambuco
Pernambuco, Brasil

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Aggressiveness of *Ilyonectria* spp. and “*Cylindrocarpon*” *pauciseptatum* associated with black foot disease of grapevine

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ABSTRACT

Since the 90's, black foot disease threatens grape production in many countries. Recently, new species associated to this disease have been described, but more studies related to their aggressiveness are necessary. Isolates of *Ilyonectria macrodidyma*, *Ilyonectria robusta* and “*Cylindrocarpon*” *pauciseptatum* obtained from grapevines with black foot symptoms collected in Rio Grande do Sul, Brazil, were inoculated on rooted cuttings of *Vitis labrusca* cv. Bordô and its root and aerial symptoms were evaluated. All isolates caused symptoms typical of the disease, including necrosis of the root and crown, as well as mass reduction of the canopy and of the root system. The isolates were reisolated from necrotic wood fragments, thus confirming their potential for damage on *Vitis labrusca*.

Key words: grapevine decline, pathogenicity, soil pathogens, *Vitis* spp.

Agressividade de *Ilyonectria* spp. e “*Cylindrocarpon*” *pauciseptatum* associados com a doença pé-preto da videira

RESUMO

Desde a década de 90, a doença pé-preto ameaça a produção de uva em vários países. Recentemente, novas espécies associadas a esta doença têm sido descritas, necessitando de estudos avaliando sua agressividade. Isolados de *Ilyonectria macrodidyma*, *Ilyonectria robusta* e “*Cylindrocarpon*” *pauciseptatum*, obtidos de videiras com sintomas de pé-preto, foram coletados no Rio Grande do Sul, Brasil, e inoculados em estacas enraizadas de *Vitis labrusca* cv. Bordô e sintomas radiculares e aéreos foram avaliados. Todos os isolados originaram sintomas típicos da doença incluindo necrose em raízes e base da planta, além de redução de massa de parte aérea e raízes. Os isolados foram reisolados de fragmentos lenhosos necróticos confirmando, assim, seu potencial de dano em *Vitis labrusca*.

Palavras-chave: declínio da videira, patogenicidade, patógenos de solo, *Vitis* spp.

Introduction

The cultivation of grapevines has great importance at the global level due to its field extension, occupying an area of 7.528 million ha (OIV, 2013). In Brazil, grapevines are one of the most important fruit trees in the agricultural sector. According to the same author cited before, in 2012 it occupied an area of 91,000 ha vineyards, with an annual production of 291.7 million liters of wine. With regard to trunk diseases in grapevines, black foot disease has been gaining notoriety in the winegrowing sector. It was first detected in vines in France, in 1961 (Maluta & Larignon, 1991). Since 1999, the death of American grapevines (*Vitis labrusca* L.) has been observed in Rio Grande do Sul state, and after pathogenicity tests and morphological characterization, the presence of “*Cylindrocarpon*” *destructans* (Zinssm.) Scholten was confirmed in vineyards of the Serra Gaúcha Region. This was considered the first report of black foot disease in Brazil (Garrido et al., 2004). The disease has already been described in other countries of South America such as Argentina (Gatica et al., 2001), Chile (Auger et al., 2007) and Uruguay (Abreo et al., 2010), as well as in other countries like the United States of America (Petit & Gubler, 2005), Portugal (Rego et al., 2000), South Africa, New Zealand (Halleen et al., 2004), Turkey (Özben et al., 2012) and Canada (Petit et al., 2011).

Black foot disease in grapevines affects mostly young vines causing typical symptoms in the canopy and the root system. Garrido et al. (2004) report that three-year-old vines cv. Bordô presented trunk darkening. Other symptoms include the biomass reduction of root system, roots necrosis, vascular discolouration, slow grow and reduced vigour (Halleen et al., 2006a; Cabral et al., 2012). This disease is caused by species of *Ilyonectria* P. Chaverri & C. Salgado (anamorph: “*Cylindrocarpon*” Wollew.), *Campylocarpon* Halleen, Schroers & Crous and *Cylindrocladiella* Boesew. In Brazil, besides the “*C.*” *destructans* associated with the disease (Garrido et al., 2004), the presence of *Ilyonectria liriodendri* (Halleen, Rego & Crous) Chaverri & Salgado (Russi et al., 2010), *Ilyonectria macrodidyma* (Halleen, Schroers & Crous) P. Chaverri & C. Salgado (Santos et al., 2014a), “*Cylindrocarpon*” *pauciseptatum* Schroers & Crous (Santos et al., 2014b), *Ilyonectria robusta* (A.A. Hildebr.) A. Cabral & Crous (Santos et al., 2014c), *Campylocarpon fasciculare*

Schroers, Halleen & Crous and *C. pseudofasciculare* Halleen, Schroers & Crous (Correia et al., 2013) were also reported.

A recent study described *Ilyonectria lusitanica* A. Cabral, Rego & Crous, *Ilyonectria estremocensis* A. Cabral, Nascimento & Crous and *Ilyonectria europaea* A. Cabral, Rego & Crous as more aggressive to grapevine than other species previously accepted as the main causal agents of black foot disease, such as *I. liriodendri* e *I. macrodidyma* (Cabral et al., 2012). Alaniz et al. (2007) verified that *I. macrodidyma* and *I. liriodendri* isolates significantly reduced the dry root weight if compared to control treatment, however, there were no differences between species under evaluation of the disease severity in the root system.

The objective of this work was to evaluate the aggressiveness of isolates of *Ilyonectria* spp. and “*Cylindrocarpon*” *pauciseptatum* associated with black foot disease in *Vitis labrusca* cv. Bordô.

Materials and Methods

In this study, nine isolates of *Ilyonectria* spp. and two isolates of “*Cylindrocarpon*” *pauciseptatum* were used. They were obtained from grapevines with black foot disease symptoms, from georeferenced vineyards in the state of Rio Grande do Sul, Brazil (Table 1), in 2012. Symptomatic plants showed root mass reduction, roots and crown necrosis, delayed sprouting, reduced vigour, wilting of the canopy and death of the plant. Fungal cultures were isolated from the roots and basal region of the symptomatic plants. Fragments of the necrotic tissue (~3x3x3 mm) were washed with running water and disinfected with 70% alcohol and 1% sodium hypochlorite, followed by three baths in distilled and sterilised water for one minute each. After drying, these fragments were plated in Petri dishes with Potato-Dextrose-Agar culture medium complemented with 0.5 g L⁻¹ of streptomycin sulphate and incubated for seven days at 25 °C, in the dark. After this period, morphological characteristics were observed according to Booth (1966), Garrido et al. (2004) and Halleen et al. (2004; 2006b). Single spore cultures were obtained and stored in PDA slants at 5 °C in the fungi collection of the Plant Pathology Laboratory at Federal University of Santa Maria (Universidade Federal de Santa Maria) in Santa Maria, Brazil and also at Embrapa Grape and Wine (Embrapa Uva e Vinho) in Bento Gonçalves, Brazil.

Table 1. List of isolates studied with collection details

Isolate	Origin (Region)	Rootstock/Cultivar	GenBank accession Histon H3
<i>Ilyonectria macrodidyma</i>			
Cy4UFSM	Erechim	VR 043-43/Isabel	KF633167
Cy5UFSM	Garibaldi	Niágara Branca*	KF633168
Cy7UFSM	Garibaldi	16149/ Isabel	KF633170
Cy8UFSM	Garibaldi	16149/ Isabel	KF633171
Cy10UFSM	Bento Gonçalves	1103 P/Burdin	KF633154
Cy11UFSM	Bento Gonçalves	Gravesac/Merlot	KF633155
Cy15UFSM	Flores da Cunha	Niágara Branca*	KF633159
Cy16UFSM	Flores da Cunha	Bordô*	KF633160
<i>Ilyonectria robusta</i>			
Cy9UFSM	Nova Pádua	Bordô*	KF633172
“ <i>Cylindrocarpon</i> ” <i>pauciseptatum</i>			
Cy12UFSM	Dois Lajeados	Concord*	KF633156
Cy13UFSM	Dois Lajeados	Bordô*	KF633157

* Own-rooted cultivar.

In preliminary studies the histone H3 region of the isolates used in this assay was sequenced and deposited in Genbank (Santos et al., 2014a; 2014b; 2014c). These were grown in PDA plates, incubated at 25 °C, in the dark, for 30 days prior to inoculation. The inoculum was prepared through superficial scraping of the culture using 10 mL of distilled and sterilised water in each plate with the help of a Drigalski spatula. The resulting suspension was filtered in two layers of cheesecloth and conidial concentration was adjusted to 10^6 conidia mL⁻¹ with distilled and sterilised water, using a Neubauer chamber.

Four-month-old rooted cuttings of *Vitis labrusca* cv. Bordô were used. Before inoculation, the cuttings were removed from bags, the root system was washed in running water and slightly trimmed with sterilised scissors. Roots of ten plants per isolate were immersed in a conidial suspension for 60 min (Cabral et al., 2012). After inoculation, the plants were transplanted to 1 L plastic bags containing a commercial substrate Mec Plant® and kept in a greenhouse at 25 ± 2 °C with a photoperiod of 12 h and daily watering to near field capacity. After one month, a substrate re-inoculation was performed with a 40 mL (10^6 conidia mL⁻¹) of suspension in each plant, with the aim of ensuring infection of the roots (Alaniz et al., 2007). The control plants only received distilled and sterilised water during inoculation and re-inoculation.

After four months, the plants were removed from packaging, and root systems were washed in running water to eliminate the substrate. To evaluate the symptoms on the canopy a grading scale was used, in which 0 = healthy plant (control treatment), 1 = 0 to 20% reduction of leaf mass, 2 = 20 to 40% reduction of leaf mass, 3 = 40 to 60% reduction of leaf mass, 4 = 60 to 80% reduction of leaf mass, 5 = reduction of leaf mass above 80%, dried out or dead canopy. Root system symptoms were evaluated according to the scale adapted from Alaniz et al. (2007), with grades from 0 to 5: 0 = healthy plant without necrotic lesions, 1 = 0 to 10% reduction of root mass, 2 = 10 to 25% reduction of root mass, 3 = 25 to 50% reduction of root mass, 4 = reduction of root mass by more than 50%, and 5 = plant death. The weight of dry mass in the aerial and root systems used for the symptom grading was obtained after

drying the plant material in a forced ventilation oven at 60 °C, until constant weight.

For the re-isolation of the pathogen, 10 fragments of wood tissue were extracted from the base region (2 cm above the inferior end of the cutting) of each plant and were superficially disinfected once with alcohol (70%) and sodium hypochlorite (1%) and three times in distilled and sterilised water for one minute each. The fragments were dried up in sterilised filters paper and transferred to Petri dishes containing PDA medium complemented with 0.5 g L⁻¹ of streptomycin sulphate. The plates were incubated in the dark at 20 °C for 14 days. After this period, the percentage of wood tissue fragments from which the pathogen was recovered in relation to the total number of fragments was recorded.

Trial design was entirely randomised. Statistical analysis was comprised of analysis of variance of the data transformed to $\sqrt{(x + 1)}$, and when significant effects were found, a means comparison was performed through the Scott-Knott test at 5% probability. The software SISVAR 5.3 (System for the Analysis of Variance of Balanced Data) was used for the analyses (Ferreira, 2011).

Results and Discussion

Results for the aggressiveness of isolates under study are presented in Table 2. The inoculated plants presented a reduction of root mass, roots and crown necrosis, darkened vessels, necrosis on leaf ribs, mass reduction and canopy drying and, in some cases, plant death (Figure 1). The necrosis on leaf ribs were observed only in *Ilyonectria macrodidyma* isolates possibly by the action of toxins. All the isolates under study showed to be pathogenic to *Vitis labrusca* cv. Bordô producing a negative effect on the inoculated plants canopy and differing statistically from the control plants. Isolate, Cy9UFSM (*Ilyonectria robusta*) presented the lowest value (3.2) for aerial symptoms. On the other hand, no significant differences were observed among the isolates of the three species evaluated (Table 2).

Table 2. Aggressiveness of isolates of *Ilyonectria* spp. and "*Cylindrocarpon*" *pauciseptatum* in *Vitis labrusca* cv. Bordô

Specie/Isolate	Shoot disease severity ^{1,3}	Root disease severity ^{2,3}	% Reisolation
Control	0.0 a ⁴	0.0 a ⁴	0.0 a ⁴
<i>Ilyonectria macrodidyma</i>			
Cy4UFSM	5.0 b	4.8 b	47.5 b
Cy5UFSM	4.8 b	4.0 b	45.0 b
Cy7UFSM	5.0 b	4.0 b	77.5 c
Cy8UFSM	5.0 b	3.8 b	55.0 b
Cy10UFSM	5.0 b	4.8 b	75.0 c
Cy11UFSM	4.6 b	3.6 b	87.5 c
Cy15UFSM	4.6 b	3.8 b	70.0 c
Cy16UFSM	4.2 b	4.6 b	70.0 c
<i>Ilyonectria robusta</i>			
Cy9UFSM	3.2 b	2.6 b	70.0 c
" <i>Cylindrocarpon</i> " <i>pauciseptatum</i>			
Cy12UFSM	4.8 b	4.4 b	72.5 c
Cy13UFSM	5.0 b	4.6 b	60.0 b

¹ Shoot symptoms were rated using the following scale: 0 = healthy (control treatment), 1 = 0 to 20% shoot mass reduction, 2 = 20 to 40% shoot mass reduction, 3 = 40 to 60% shoot mass reduction, 4 = 60 to 80% shoot mass reduction, 5 = > 80% shoot mass reduction or drying or plant death.

² Root symptoms were rated using the following scale: 0 = healthy with no lesions, 1 = 0 to 10% root mass reduction, 2 = 10 to 25% root mass reduction, 3 = 25 to 50% root mass reduction, 4 = > 50% root mass reduction, 5 = death: adapted from Alaniz et al. (2007).

³ Transformed means ($\sqrt{(x + 1)}$).

⁴ Means in the same column followed by the same letter are not significantly different (Scott-Knott, $p < 0.05$).

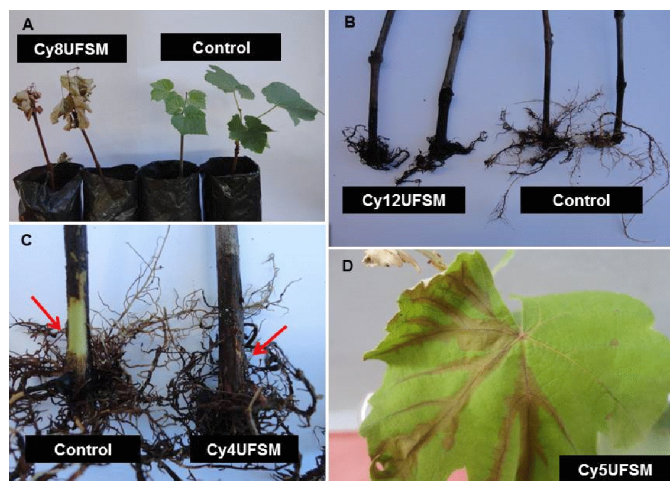


Figure 1. Symptoms of black foot disease in *Vitis labrusca* cv. Bordô: A) drying of shoots after inoculation with Cy8UFSM versus control; B) root mass reduction after inoculation with Cy12UFSM versus control; C) crown necrosis after inoculation with Cy4UFSM versus control; D) necrosis of the leaf ribs after inoculation with Cy5UFSM

No differences among the three species were recorded concerning root system symptoms. Only control plants differed from the inoculated plants. A similar result was obtained by Petit & Gubler (2005) when evaluating "*C.*" *destructans* and *I. macrodidyma* and also by Mohammadi et al. (2009) with *I. liriodendri* isolates, with no variation between the different isolates. The values attributed to the root system symptoms varied from 2.6 for isolate Cy9UFSM (*Ilyonectria robusta*) to 4.8 for isolate Cy10UFSM (*Ilyonectria macrodidyma*) (Table 2).

The variation on aggressiveness of isolates can be explained by differences in enzymatic activity and in the production of mycotoxins. Rahman & Punja (2005) verified that highly virulent isolates of "*Cylindrocarpon*" *destructans* obtained from ginseng (*Panax quinquefolius* L.) produced higher levels of enzymes, pectinase and polyphenoloxidase, than less virulent isolates. Histological studies carried out by the same authors in inoculated ginseng roots with highly virulent isolates showed the direct penetration of hyphae through the epidermis, followed by intracellular colonisation in the cortex, cellular disintegration and accumulation of phenolic compounds. Lyr & Kluge (1968) when evaluating pathogenic isolates of *Nectria radicola* pathogenic to *Pinus sylvestris* L. concluded that these presented higher pectinase and cellulase activity when compared to non-pathogenic isolates. Studies of sections diseased tissue revealed that the majority of xylem vessels showed tyloses with thick walls or brown gum and that functional elements of phloem were obstructed with gum (Halleen et al., 2006a) impeding the flux of sap, thus resulting in the decline and death of the plant. Garrido et al. (2004) observed complete internal darkening in the basal region of the plant, with dark-brown to black discolouring, differing from symptoms of grapevine fusariosis (*Fusarium oxysporum* Schl. f. sp. *herbemontis* Tocchetto) which are restricted to the xylem region. Similar symptoms were also observed in this work. In pathogenicity studies using rootstock 1103 P and 60 *Ilyonectria* spp. isolates of grapevines and other hosts, isolates obtained from other

hosts proved to be more aggressiveness than those obtained from grapevine (Cabral et al., 2012).

Inoculated pathogens were not recovered from the control plants. A percentage re-isolation higher than 45% was observed for all isolates. Isolates Cy4UFSM, Cy5UFSM, Cy8UFSM and Cy13UFSM presented the lowest percentage of re-isolation.

Conclusions

All isolates from the three species studied, *Ilyonectria macrodidyma*, *I. robusta* and "*Cylindrocarpon*" *pauciseptatum*, were highly aggressive to the aerial and root system, demonstrating their virulence for *Vitis labrusca* cv. Bordô. This is the first work evaluating the severity of damage caused by different species associated with black foot disease in Brazil.

Acknowledgments

The authors thank the Coordination for the Improvement of Higher Education Personnel (CAPES) for the master's scholarship granted to the first author.

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