ARTIGOS

Aggressiveness of *Fusarium oxysporum* and *Fusarium solani* isolates to yerba-mate and production of extracellular enzymes

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ABSTRACT

Mezzomo, R.; Rolim, J.M.; Santos, A.F.; Poletto, T.; Walker, C.; Maciel, C.G.; Muniz, M.F.B. Aggressiveness of *Fusarium oxysporum* and *F. solani* isolates to yerba-mate and production of extracellular enzymes. ***Summa Phytopathologica,*** v.45, n.2, p.141-145, 2019.

The yerba-mate (*Ilex paraguariensis*) has great socioeconomic importance on family farming in Southerm Brazil. One of the main yerba-mate disease is root rotting, caused by *Fusarium* spp. Little is known about the pathogen physiology, especially regarding the aggressiveness associated with the production of extracellular enzymes. On this work, the aggressiveness of isolates of *F. oxysporum* and *F. solani* pathogenic to yerba-mate was evaluated and it was determined the activities of extracellular enzymes catalase, laccase, cellulase, caseinase, amylase, protease, lipase and pectinases produced by *Fusarium* spp. in culture medium. Six isolates of *F. solani* and one isolate of *F. oxysporum* pathogenic to yerba-mate were used. The *F. oxysporum* isolate proved to be less aggressive in relation to the other *F. solani* isolates. All isolates of *Fusarium* spp. produced, on a semiquantitative manner, the extracellular enzymes catalase, laccase, cellulase, caseinase, amylase, protease, lipase and pectinases (polygalacturonase and pectate lyase). However, the quantity produced for each enzyme was significantly different among the isolates. With the exception of the laccase and polygalacturonase enzymes, the M7C1 isolate showed the highest enzymatic index and was also responsible for the highest percentage of yerba-mate seedlings death.

Keywords: exoenzymes, root rotting, fungus.

RESUMO

Mezzomo, R.; Rolim, J.M.; Santos, A.F.; Poletto, T.; Walker, C.; Maciel, C.G.; Muniz, M.F.B. Agressividade de isolados de *Fusarium oxysporum* e *F. solani* à erva-mate e produção de enzimas extracelulares. ***Summa Phytopathologica,*** v.45, n.2, p.141-145, 2019.

A erva-mate (*Ilex paraguariensis*) tem grande importância socioeconômica na agricultura familiar do Sul do Brasil. A principal doença da erva-mate é a podridão-de-raízes causada por *Fusarium* spp. Pouco se sabe a respeito da fisiologia deste patógeno, principalmente quanto à agressividade associada à produção de enzimas extracelulares. Neste trabalho, avaliou-se a agressividade de isolados de *F. oxysporum* e *F. solani* patogênicos à erva-mate e determinou-se as atividades das enzimas extracelulares catalase, lacase, celulase, caseinase, amilase, protease, lipase e pectinases produzidas por *Fusarium* spp. em meio de cultura. Foram utilizados seis isolados de *F. solani* e um isolado de *F. oxysporum* patogênicos à erva-mate. O isolado de *F. oxysporum* mostrou-se menos agressivo em relação aos demais isolados de *F. solani*. Todos os isolados de *Fusarium* spp. produziram, de maneira semiquantitativa, as enzimas extracelulares catalase, lacase, celulase, caseinase, amilase, protease, lipase e pectinases (poligalacturonase e pectato liase). Entretanto, a quantidade produzida de cada enzima foi significativamente diferente entre os isolados. Com exceção das enzimas lacase e poligalacturonase o isolado M7C1 de *F. solani* exibiu as maiores médias do índice enzimático e foi também o responsável pelo maior percentual de morte de mudas de erva-mate.

Palavras-chave: exoenzimas, podridão-de-raízes, fungo.

The yerba-mate (*Ilex paraguariensis* St. Hil.) is a native forest species from Southern Brazil, Paraguay and Argentina. In Brazil, it is cultivated intercropped with agroforestry systems, in pure plantations and native crops. Its leaves and twigs are used in a wide variety of purposes such as cosmetics, medicinal products and herbal medicines; however, the main consumption is by means of teas, tererê and chimarrão (23).

The plantations are subject to pathogens attack, especially fungi of the *Fusarium* genus, which cause diseases such as the root rotting. The root rotting directly interferes in the plant process of nutrients absorption and the symptoms reflect in the aerial part, causing yellowing and leaf fall, growth reduction, wilting and drought, leading, in most cases, to

the death of the seedling (10).

Despite the root rotting disease causing damage in yerba-mate plantations, there is still a lack of information about the pathogen and host relationship. Basic studies that seek knowledge about this interaction, such as aggressiveness and production of extracellular enzymes, are fundamental.

The production of extracellular enzymes plays an important role in pathogenesis. Thus, the analysis of the enzymatic production of fungi in solid medium is considered a simple and rapid method for the identification of genetic variations in a population of fungi, by the presence or absence of specific enzymes, with the possibility of association between the pathogen production of extracellular enzymes and its degree of virulence with the host. Additionally, the production of extracellular enzymes in specific environment can also be used in the aggressiveness differentiation of *Fusarium* spp. isolates (18).

Given the above, this work evaluated the aggressiveness of isolates of *Fusarium oxysporum* and *Fusarium solani* pathogenic to yerba-mate and it was determined the activities of extracellular enzymes catalase, laccase, cellulase, caseinase, amylase, protease, lipase and pectinases produced by *Fusarium* spp. in culture medium.

MATERIALS AND METHODS

The experiments were conducted in the greenhouse and in the Laboratory of Plant Pathology Elocy Minussi of Rural Sciences Center of Federal University of Santa Maria, Santa Maria, RS, Brazil. Seven isolates proven pathogenic to yerba-mate were used, being one of *F. oyxsporum* (I6AR2) and six of *F. solani* (I1AR1, I8AR1, M3AR2, M4, M5AR2 and M7C1) belonging to the Universidade Federal de Santa Maria collection of fungi.

For the test of aggressiveness in yerba-mate seedlings, the seven isolates were initially grown on potato-dextrose agar (PDA) for seven days in the dark and then in maize grains, as described below. The grains of maize were soaked in water at ambient temperature for eight hours, then, the excess water was discarded. Eighty grams of these grains were placed in 100 mL glass flasks and autoclaved for 40 min, twice, in an interval of 24 hours. After cooling, five PDA culture medium discs (7 mm in diameter), containing the pathogen mycelium, were transferred to each flask. The flasks were incubated at 24 °C, with 12 h of photoperiod, during 14 days, according to methodology of Klingelfuss et al. (15) modified by Lazarotto et al. (16). For the control, five discs of culture medium without the pathogen were placed in flasks, and the other procedures were performed as described previously. Subsequently, plastic pots (3.6 L capacity), with holes in the bottom, were filled with commercial substrate. Then, 80 cm^3 of inoculum produced in maize grains were mixed to the substrate and moistened with sterile distilled water Lazarotto et al. (16). After 15 days, the yerba-mate seedlings were transplanted and divided in four replications and three seedlings, where they were kept in a greenhouse for 180 days when the phytomass of the aerial part and roots were determined as described by Junges et al. (14). It was also evaluated the percentage of seedlings killed by *Fusarium* spp.

To test the production of extracellular enzymes, the seven *Fusarium* spp. isolates were grown on PDA medium for seven days in the dark. Subsequently, 5 mm diameter discs containing fungal mycelium in active growth were transferred to five Petri plates containing the media culture specific to each enzyme. For control, discs containing only PDA culture medium were used.

For the determination of catalase activity, the isolates of *Fusarium* spp. were grown on PDA medium as a methodology adapted by Bueno et al. (4). The catalase was measured by means of symbols, based on the intensity of oxygen bubbles formed in the culture medium: +++ (intense); ++ (moderate); + (low) and - (absent) (3). For the laccase enzyme production, the formation of "Bavendamm's reaction" was observed (5). For the evaluation the laccase enzyme production the perpendicular diameters of the mycelial growth (mm) were measured with the aid of a digital pachymeter. For the detection of amilolytic activity the isolates were transferred to the minimal medium containing starch (MMA) as described by Marchi et al. (19). The production of caseinase was verified in the casein-agar medium containing 1% casein (27). The enzymatic activity was revealed as described by Fuentefria (9). For the determination of cellulase, the isolates were cultured on carboxymethylcellulose-agar following the methodology described by Nogueira & Cavalcanti (20). The production of exoenzymes protease and lipase were determined according to the method of Pereira (21). For the assessment of proteolytic activity, agar nutrient medium supplemented with gelatin powder was used. For determination of the lipase enzyme, isolates were transferred to Petri dishes with culture medium containing Tween-20 as substrate. To detect the pectinolytic activity, culture medium containing citrus pectin as substrate was used. The medium at pH 7.0 was used to detect the production of pectate lyase and at pH 5.0 to assess the activity of polygalacturonase as described by Hankin & Anagnostakis (11). With the exception of catalase and laccase, other enzymes were evaluated by calculating the enzymatic index (E.I) (diameter of the colony plus halo of degradation (mm)/ colony diameter (mm)) (2).

The experimental design was completely randomized, with five repetitions. The data were subjected to variance analysis, taking as variation sources the different isolates. The mean variables were compared by the Scott-Knott test ($p \leq 0.05$), using the Sisvar 5.3 statistical software (8).

RESULTS AND DISCUSSION

Analyzing the ADP averages (Table 1), the formation of four groups of isolates can be perceived. The first, and more aggressive, formed by isolate M7C1 of *F. solani* that differed from the others and control, followed by M3AR2 isolate of *F. solani* that maintained the same characteristic of M7C1. At the third group composed by isolates I1AR1, I8AR1, M4 and M5AR2, these did not differ among themselves but from others and from control, and the last group, less aggressive, integrated by isolated I6AR2, which exhibited the lowest average, statistically differing from *F. solani* isolates and control. For the variable RDP, the averages followed the same trend of observed in ADP, except for the isolate M4 embedded in the least aggressive group along with the isolate I6AR2. For % DS, the isolate M7C1 stood facing the other with 66.67%, followed by M5AR2 (50.00), M3AR2 (41.67), R1AR1, R6AR2, (both with 16.67). The lower %DS (8.33) was revealed by isolates I8AR1 and M4.

According to Poletto et al. (22) the inoculation of *F. oxysporum* in the substrate hindered the development of the yerba-mate seedlings. Also according to these authors, when analyzed the variables of root biomass and total biomass, the averages obtained with the inoculation of *Fusarium* spp. were smaller than of those not inoculated. These results affirm the pathogen potential to cause damage to the host species. Reinforcing the results obtained in the present study, Rocha et al. (24)

Table 1. Averages for dry phytomass of the aerial part (ADP) and roots (RDP) and the percentage of dead seedlings (% DS) of yerba-mate 180 days after inoculation with *Fusarium oyxsporum* (isolated I6AR2) and *F. solani* isolates (I1AR1, I8AR1, M3AR2, M4, M5AR2 and M7C1).

Isolate	ADP (g)	RDP (g)	% DS
I1AR1	6.05 c*	1.95 c	16.67 d
I6AR2	10.53 d	2.66 d	16.67 d
I8AR1	6.51 c	2.19 c	8.33 e
M3AR2	4.46 b	1.21 b	41.67 c
M4	7.35 c	2.76 d	8.33 e
M5AR2	5.55 c	1.81 c	50.00 b
M7C1	1.79 a	0.53 a	66.67 a
CONTROL	19.98 e	9.54 e	0.00 f
C.V. %	11.39	8.60	18.16

Averages followed by the same letter did not differ statistically by the Scott-Knott mean test, at 5% significance.

observed, 21 days after inoculation, the dryness and reduction of the root system of black pepper (*Piper nigrum* L.) in addition to the incidence of 100% fusariosis caused by *F. solani* f. sp. *piperis*.

From the specific culture medium, it was possible to detect the production of all enzymes by *Fusarium* spp. isolates (Table 2). The catalase enzyme production by *Fusarium* spp. isolates has followed the same trend of the percentage of dead seedlings revealed by the aggressiveness test in yerba-mate seedlings. Thus, the formation intensity of oxygen bubbles was moderate in the isolates M7C1, M5AR2, M3AR2 and weak in the others. Bueno et al. (4) reports that the catalase was produced weakly and equally by nine isolates of *F. solani* from yellow passion fruit. According to Hansberg et al. (12), on ascomycetes, the catalase has relevance in the cell growth and differentiation processes, in addition to acting on the spores germination, which corroborates to the results of the present study where the highest percentages of dead seedlings were triggered by isolates that produced catalase.

The control of all enzymes tested statistically differed from the isolates. Furthermore, it has not been possible to show the enzymatic activity in control, reason by which was attributed zero average to the enzymatic index.

For the production of the laccase enzyme, only the I1AR1 and M7C1 isolates did not differ statistically among themselves, unlike the other isolates. It is noted that the I6AR2 isolated showed the highest mycelial growth (77.62 mm) although it has displayed the greatest averages of aerial part and root dry phytomass. In this case, it is evident that the largest averages of mycelial growth were verified in the isolates that presented the lowest indexes of stagnation in the growth of seedlings. Despite the relationship between mycelial growth and the percentage of dead seedlings be inversely proportional, the development of the pathogen in specific medium suggests the laccase participation in the pathogenesis process. To Leonowicz et al. (17) the laccase activity is directly related to the degradation of lignin, one of the aromatic compounds responsible for the natural resistance of plants. During the attack of *Botrytis cinerea* in cucumber fruits, the laccase secretion, besides reducing the process of lignification, protected the pathogen of the action of toxic metabolites present in the host (25).

The highest values of enzymatic index (E.I.) of the cellulase (Table 2) were observed in M7C1 and I1AR1 isolates, which did not differ statistically among themselves. Bueno et al. (4) emphasize that all pathogenic isolates of *F. solani* degraded cellulose in culture medium. However, Stamford et al. (28), showed the production of the enzyme

Table 2. Extracellular enzyme production, in specific medium, of *Fusarium oyxsporum* (I6AR2) and *F. solani* (I1AR1, I8AR1, M3AR2, M4, M5AR2 and M7C1) isolates.

Isolates	Extracellular enzymes								
	Cat[1]	Lac[2]	Cel[3]	Cas[3]	Amyl[3]	Prot[3]	Lip[3]	Pol[3]	Pec[3]
I1AR1[4]	+	62.39 c*	1.204 a	1.178 c	1.058 b	1.180 a	1.136 b	1.078 c	1.020 b
I6AR2[5]	+	77.62 a	1.130 c	1.074 d	1.214 b	1.156 a	1.116 b	1.052 d	1.012 b
I8AR1[4]	+	47.99 d	1.164 b	1.134 c	1.040 b	1.072 b	1.082 b	1.158 a	1.020 b
M3AR2[4]	++	38.12 e	1.110 c	1.148 c	1.070 b	1.100 b	1.098 b	1.042 d	1.038 b
M4[4]	+	68.88 b	1.126 c	1.228 b	1.062 b	1.126 a	1.164 b	1.106 b	1.032 b
M5AR2[4]	++	29.15 f	1.102 c	1.252 b	1.078 b	1.050 b	1.222 a	1.126 b	1.066 a
M7C1[4]	++	59.21 c	1.236 a	1.342 a	1.856 a	1.158 a	1.282 a	1.092 c	1.074 a
CONT	-	0.00 g	0.00 d	0.00 e	0.00 c	0.00 c	0.00 c	0.00 e	0.00 c
C.V. (%)	-	10.94	2.77	5.34	20.79	4.53	6.06	2.28	1.77

Where: Cat: catalase; Lac: laccase; Cel: cellulase; Cas: Caseinase; Amyl: amylase; Prot: protease; Lip: lipase; Pol: polygalacturonase; Pec: pectate lyase; (1): symbolic evaluation: (+) = weak; (++) = moderate; (+++) = intense; (2): mycelial growth; (3): Enzymatic index (I.E) = diameter of the halo formed (cm)/colony diameter (cm); (4) = *F. solani*; (5): *F. oxysporum*. CONT: Control. Averages followed by the same letter in the column did not differ statistically by the Scott-Knott mean test, at 5% significance. C.V. (%): Coefficient of variation.

by isolates of *Fusarium* endophytic to yam bean (*Pachyrhizus erosus*). M7C1 isolate, which was higher in the mortality of seedlings of yerba-mate (66.67%), was also the largest producer of this enzyme.

The I6AR2 isolate enabled the lower production of caseinase, differing statistically from the others. The intermediate production of the enzyme led to the formation of two groups (I1AR1, I8AR1, M3AR2 and M4, M5AR2) that did not differ within each group. The M7C1 isolate ensured the highest average of the E.I., differing from the other indicating the participation of caseinase on the aggressiveness of the *F. solani* isolate in yerba-mate seedlings. Stating the results of the present study, Doria (7) describes significant differences in enzymatic indices of caseinase measured in *Fusarium* spp. isolates obtained from rubber tree (*Hevea brasiliensis*).

There was a significant difference between the enzymatic indices of amylase. The largest E.I. was provided by M7C1 isolate , therefore, differing statistically from the other isolates that showed median indices, and not differing among themselves. Doria (7) mentions the production of amylase by all *Fusarium* spp. isolates used in this research. As Machi et al. (19) *Alternaria solani* isolates, classified as good amylase synthesizers, were described as weak producers of pectinases. Sun et al. (28) claim that the starch degradation from the host tissue can contribute to the growth and sporulation processes of phytopathogenic fungi, which reinforces the results of the present study, since the isolate which showed the highest I.E. (M7C1) was also responsible for the highest percentage of yerba-mate seedlings killed.

Regarding the gelatin decomposition in specific medium for protease detection (Table 3) the I6AR2 isolate positively surprised, showing the second highest average of the E.I., not statistically differing from M7C1 and R1AR1 isolates, respectively, first and third highest averages. The remaining isolates did not differ among themselves. Adejuwon & Olutiola (1) reported the protease activity in extracts of tomato fruit (*Solanum lycopersicum)* degraded by *F. oxysporum* infection. Singh & Saxena (26) linked to the protease production and other exoenzymes to cauliflower (*Brassica oleracea*) wilt, by *F. solani*. In the present study, the I6AR2 isolate figured satisfactory E.I. averages, which would usually refute the hypothesis of the protease action in the aggressiveness of *F. oxysporum* in yerba-mate seedlings. In contrast, the highlight of the M7C1 isolate indicates the possible bond of protease in the pathogenesis of *F. solani* in yerba-mate seedlings.

For the lipase enzyme activity, the highlight was again for the M7C1 isolate, which did not differ statistically from the M5AR2 isolate (Table 2). The remaining isolates did not differ among themselves. Voigt et al. (29) characterized the FGL1 gene, responsible for the encoding of some lipases in *F. graminearum* associated with cereals. According to the authors, there is sufficient evidence to prove the gene connection with the pathogen virulence. The best performance in the degradation of the lipid-rich substrate, in this case, the "Tween-20" advocates the efficiency of enzymatic arsenal of M7C1 and M5AR2 isolates, responsible for the death of 66.67 and 50.00% of the yerba-mate seedlings, respectively. The production of lipolytic enzymes allows aggressive fungi its penetration into the host tissues through the decomposition of the cell wall, aiding in aggressiveness and dissemination (6).

There was disagreement on pecnilolitic activity measured by polygalacturonase and pectate lyase enzymes. For polygalacturonase, for the first time, the I8AR1 isolate figured the greater relationship between the halo of degradation and the mycelial growth differing from the other averages. The remaining isolates were arranged in pairs formed by isolates M5AR2, M4, M7C1, I1AR1 and M3AR2, R6AR2, which did not differ statistically within the pairs. For pectate lyase the highest enzymatic indices were observed in M7C1 and M5AR2, respectively, not differing among themselves. Following in descending order M3AR2, M4, I1AR1 and R8AR1 tied. The lowest performance in E.I. stayed with the I6AR2 isolate.

In *A. solani* isolates obtained from solanaceous plants, Marchi et al. (19) narrate that good pectinase producers have proved to be more aggressive and developed larger lesions on tomato plants. According to Jorge et al. (13) the production cell wall degradation enzymes (CWDE), in which are the polygalacturonase (PG) and pectate-lyase (PL) is strongly linked to the rotting of the roots of chickpea (*Cicer arietinum*) caused by *F. oxysporum* f. sp. *ciceris* (Foc). Those authors tested the Foc-0 (slightly less virulent, associated with symptoms of yellowing) and Foc-5 (highly virulent associated with symptoms of wilt) isolates, and concluded that the higher index of PG was associated to the Foc-0, as well as the highest value of PL was related with the Foc-5 isolate. The results obtained in the present study meet the above conclusions, once the symptoms of wilt and, consequently, the high percentage of dead seedlings were caused by isolates M7C1 and M5AR2, respectively, which are related with the highest averages of pectate lyase enzymatic indices.

Based on the data obtained, it is possible to conclude that the extracellular enzymes produced by *Fusarium* spp. isolates play a prominent role in aggressiveness in yerba-mate seedlings. The isolates that exhibited the highest enzymatic index were also responsible for the highest percentage of yerba-mate seedlings death.

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