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CROP PROTECTION

European apple canker: morphophysiological variability and pathogenicity in isolates of *Neonectria ditissima* in southern Brazil

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ABSTRACT: *European apple canker (EC) is caused by Neonectria ditissima, a pathogen officially registered as a quarantine pest in 2012. Thirty-five isolates of N. ditissima of different geographical regions of southern Brazil from apple branches showing symptoms of EC were identified by the specific pair primers Ch1 and Ch2 and analyzed concerning the virulence on Gala apple cultivar and morphophysiological characteristics. The disease symptoms were characterized and the isolates compared based on average mycelium growth (AMG), mycelium growth index (MGI), colony color, conidia type, dimensions and growth on potato dextrose agar (PDA), malt agar (AM), and synthetic SNAY (SN) culture media. Nineteen isolates showed the greatest AMG on PDA, forming three growth groups of 35.56 (GI), 52.71 (GII), and 62.67mm (GIII). Seven isolates showed MGI greater than 4.0mm diameter on PDA compared with that on AM and SN. The highest conidia production was on SN, and the predominant colony color in all media was white to beige with central pigmentation of brown and borders colored in shades of beige. There were significant differences among the average dimensions of micro- and macroconidia on PDA, AM, and SN. The pathogenicity was confirmed for all isolates despite of different morphophysiological characteristics. There was no correlation among isolates morphophysiological variability, virulence, and geographical origin.*
Key words: *Malus domestica L., culture medium, mycelial and conidial morphophysiology.*

Cancro europeu da macieira: variabilidade morfofisiológicas e patogenicidade de isolados de *Neonectria ditissima* no sul do Brasil

RESUMO: *O cancro europeu (CE) da macieira é causado pelo fungo Neonectria ditissima e foi oficialmente registrado como praga quarentenária em 2012. Foram avaliados 35 isolados de N. ditissima provenientes de ramos de macieira com sintomas típicos de CE de diferentes regiões do sul do Brasil. Os isolados foram identificados pelos primers específicos Ch1 e Ch2 e analisados quanto as características morfofisiológicas e virulência na cultivar de macieira Gala. Os sintomas foram caracterizados e os isolados comparados com base no crescimento micelial médio (CMM), índice de crescimento micelial (ICM), coloração das colônias e tipos, dimensões e produção de conídios em meios de cultura batata-dextrose-ágar (BDA), malte-ágar (MA) e meio sintético SNAY (SS). Dezenove isolados tiveram os maiores CMM sobre BDA, formando três grupos de crescimento de 35.56 (GI), 52.71 (GII) e 62.67mm (GIII). Sete isolados tiveram ICM maior de 4mm de diâmetro sobre BDA quando comparados com os meios MA e SS. A maior produção de conídios ocorreu sobre o meio SS e a coloração predominante foi de branco-à-bege com pigmentação central marrom e bordas de colônia em tons de bege. Foram observadas diferenças significativas nas dimensões médias dos micro- e macroconídios sobre os meios de culturas BDA, MA e SS. Todos os isolados foram patogênicos na cultivar de macieira gala, independentemente das diferenças morfofisiológicas. Não foram observadas correlações entre a variabilidade morfofisiológicas, virulência e origem geográfica dos isolados.*
Palavras-chave: *Malus domestica, meios de culturas, morfofisiologia de micélios e conídios.*

INTRODUCTION

Neonectria ditissima (Tul. & C. Tul.) [anamorph *Cylindrocarpon heteronema* (Berk. & Broome)] is the causal agent of European canker (EC) in apple [*Malus domestica* (Borkh.) Borkh.] (CASTLEBURY et al., 2006; CHAVERRI et al., 2011; WEBER, 2014). The causal agent was originally named *Nectria ditissima* in 1865 (FLACK & SWINBURNE, 1977) and is already well spread worldwide (SANHUEZA, 1998; BOGO et al., 2008). In Brazil, EC was first reported in a nursery in Vacaria municipality in the state of Rio Grande do Sul at 1998 (SANHUEZA, 1998). In the absence of disease inspection and management, EC was spread through infected commercial seedling to Rio Grande do Sul, Santa Catarina and Parana States, and only in 2012 was EC caused by *N. ditissima* officially registered by the Brazilian Agriculture, Livestock and Supply Ministry (MAPA) as a quarantine pest (BRASIL, 2013).

N. ditissima infection occurs via natural as well as pruning-induced wounds, foliar abscission, bud-base entrance, and/or grafting, and may occur throughout the year. Also, *N. ditissima* can cause fruit

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calyx infection at the beginning of the growing season and/or pre- or postharvest fruit calyx decay. The first trunk or branch symptoms are reddish to dark-brown spots in the bark. These lesions increase in size, developing into a wood tissue slump, and form a canker with exposure of internal tissue (SANHUEZA, 1998). These cankers can be used as infection routes for secondary pathogens that exacerbate the disease (BRAYFORD et al., 2004). EC symptoms in apple fruits begin as dark lesions near the floral apparatus followed by a red pigmentation around the calyx. The fruit rot is brown, superficial, and with dry texture or cork, and fruiting bodies can usually be found under high relative humidity (LANGRELL, 2002; CHAVERRI et al., 2011).

The pathogen overwinters as ascospores in perithecia (SANHUEZA, 1998). Although macroconidia are highly infectious, the epidemiological role of microconidia is uncertain (WEBER, 2014). Morphological variation in *Neonectria* resulted in the subdivision of species into five informal groups, mostly based on perithecial characteristics, as *N. coccinea/galligena*-group, *N. mammoidea*-group, *N. rugulosa* group, *N. radicicola*-group, and *N. veuillotiana*-group. However, species that have been placed in *Neonectria* and species of *Nectria* having a *Cylindrocarpon* anamorph vary greatly in the morphology of their perithecia and degrees of ascospore ornamentation. Some species of *Neonectria* are similar in perithecial morphology with differences seen only in the anamorph (CHAVERRI et al., 2011).

Ascospores of *N. ditissima* are ellipsoidal, one-septate, and slightly constricted at the septum. Ripe perithecia may be identified by a whitish cirrus of ascospores (WEBER, 2014). Two types of conidia are produced in nature and in culture. Macroconidia are straight or slightly curved and often 5-septate, although on agar there may be predominantly 1- to 3-septate forms. Microconidia are short-cylindrical or ellipsoidal and aseptate or 1-septate. It is difficult to distinguish microconidia from immature macroconidia (BOOTH, 1966; WEBER, 2014). FLACK & SWINBURNE (1977) had noted differences in both host range and symptoms produced by *Nectria* spp. from different source hosts. BARNARD et al. (1988) indicated no substantive intraspecific variability with respect to spore morphology among many *N. galligena* isolates. Additionally, they suggested little evidence of host specificity among isolates because all isolates were pathogenic to different hosts.

There are no references to EC disease or *N. ditissima* characterization in Brazil. Thus, the purpose of this study was the morphophysiological characterization of 35 isolates of *N. ditissima* in different culture media and their pathogenicity on 3-year-old Gala apple trees in southern Brazil.

MATERIALS AND METHODS

Isolates and inoculum production

Branches of the Gala apple cultivar with typical EC symptoms were collected from 12 and 23 commercial orchards in Santa Catarina State (Água Doce/SC municipality: 26° 59′ 52″ S, 51° 33′ 22″ W) and Rio Grande do Sul State (Vacaria/RS municipality: 28° 30′ 44″ S, 50° 56′ 02″ W), respectively. Fragments of epidemic tissue branches were disinfected with 92 ethyl alcohol, placed in acidified potato dextrose agar (PDA) medium in Petri dishes, and incubated in a biochemical oxygen demand (BOD) chamber under a 24h light photoperiod at 20±0.5°C. The 35 pure colonies were gently scraped with a scalpel to remove the sporodochia and agitated in test tubes containing 5mL of sterile distilled water (SDW) plus one drop of Tween 80. The suspensions were filtered through gauze and the concentrations of conidia were measured with a Neubauer chamber and adjusted to $1x\ 10^4$ conidia mL^{-1}. A 1mL aliquot of each spore solution was placed on PDA in a Petri dish and incubated in a BOD chamber for 5 days under a 24h light photoperiod at 22±0.5°C. Mycelium plugs (5mm diameter) from single-spore cultures of the isolates were transferred to PDA test tubes, incubated under the same conditions, and stored at 4°C. The 35 isolates were identified by the Agronômica laboratory (Laboratório de Diagnóstico Fitossanitário e Consultoria – NIRE 43.205.056.551) using a pair of Ch1 (5'-AAC CCC TGT GAA CAT ACC CAT C-3') and Ch2 (5'-GTG GCC GCG CTG CTC TTC CG-3') specific primers plus SYBR-Green and designed from comparisons of the internal transcribed spacer (ITS) regions as *N. ditissima*.

Colonies and conidia characterization

The 35 *N. ditissima* isolates were evaluated for average mycelium growth (AMG), mycelium growth index (MGI), colony color, conidia type, dimension, and sporulation in three culture media: potato-dextrose-agar (PDA), malt extract agar (MA), and SNAY synthetic (SN). Mycelial plugs (5mm diameter) from 12-day-old cultures of the 35 isolates were placed in the centers of Petri dishes containing PDA and incubated in a BOD for 12 days under a 24h light photoperiod at 22±0.5°C. Colony diameter was assessed in two different ways every 24h by digital pachymeter by orthogonal dimension and by two

perpendicular dimensions. Results were converted to AMG (mm h^{-1}) according to LEHNER et al. (2014) and MGI according to the adapted formula described by MAGUIRE (1962). Isolates with AMG values ≥60.0mm were assigned as the highest-mycelium-growth group at the end of the evaluation time. The experiment was performed twice. MGI values ≥4.0mm were assigned as the largest interaction between isolates and culture media. The experiments followed a completely randomized 3x35 factorial design (three culture media and 35 isolates) with five replicates per isolate in each culture medium. A colony growing in a plate was treated as an experimental unit. Central and border colony color (mycelium color in the adaxial and abaxial Petri dish) and pigmentation were visually evaluated using the Pantone® color standard range after 12 days of incubation. The 35 *N. ditissima* isolates were cultured on PDA, AM, and SN culture media during 20 days under a 24h light photoperiod at 22±0.5°C until sporodochia formation. One sporodochium was macerated in 5mL of SDW with one drop of Tween 80 and the conidial concentration was measured with a Neubauer chamber to estimate conidia production (sporulation). The experiments followed a completely randomized design with five replicates per isolate in each culture medium.

Conidial morphology

Conidial morphology on PDA, AM and SN culture media was assessed by the length and width of 50 conidia of each isolate on each medium. Measurements were converted to micrometers (µm). The experiments followed a completely randomized 3x35 factorial design (three culture media and 35 isolates) with four replicates per isolate in each medium. The data were transformed for analysis of variance by addition of 0.5 to each data point.

Pathogenicity testin

The EC symptoms were reproduced by inoculation of 3-year-old Gala apple cultivar plants in 10L pots filled with soil and organic matter. Five buds from different plant were removed with a scalpel and replaced with 5.0mm PDA mycelium plugs of the 35 *N. ditissima* isolates and covered with adhesive tape. The control test was inoculated with a 5.0mm pure PDA plug. Plants were kept in a climate chamber with 12h light photoperiod at 22±0.5°C for 14 days. The symptoms were evaluated by visual assessment of typical EC symptoms. The experiments followed a completely randomized design with four replicates of one plant with five experimental wounds.

Statistical analyses

Data for all experiments were analyzed by analysis of variance using ASSISTAT 7.7 Beta (SAS Institute Inc., Cary, NC) to determine the significance of treatment effects. When effects were significant, the means were analyzed by the Scott Knock test.

RESULTS

Colony characterization and conidia production in different culture media

There were significant differences in mycelium growth among the 35 *N. ditissima* isolates and among the PDA, AM, and SN culture media. The majority of isolates belonged to group II, which included isolates with AMG of 40.00-59.99m (Table 1). Seven (NR02, NR03, NR05, and NR10), three (NR02, CPi02 and FROL08), and two (CPi06 and CPi17) isolates showed growth over 60.0mm diameter in PDA, AM, and SN, respectively. Seven isolates (NR02, NR03, NR10, FR04, AD07, FROL08, and FROL09) showed the highest MGI (MGI>4.0mm) on the PDA culture medium and the MGI were statistically different compared with that on AM and SN culture media (Table 1). Five of seven isolates with highest AMG also showed the highest MGI (Table 1). The AM culture medium showed no isolates with MGI greater than 4.0mm diameter (Table 1). There were significant differences (P<0.05) in the macro- and microconidia production of the 35 *N. ditissima* isolates on PDA, AM, and SN (Table 1). Sporulation was higher ($73.7x10^4$ conidia $sporodochium^{-1}$) on SN than on PDA or AM. The CPi09, AD06, and AD10 isolates showed the highest conidial production on PDA, SN, and AM, respectively (Table 1). PDA was the only medium that showed the formation of three growth groups (group I, 20.00-39.99mm; group II, 40.00-59.99mm; and group III, ≥60.00mm) based on the AMG (Table 2). There was broad variation in colony color and aspect in the *N. ditissima* isolates on PDA, AM and SN culture media. The colonies showed smooth borders and little aerial mycelium to slightly cottony mycelium in the three culture media. Some colonies showed dense aerial mycelium and flocculates with the presence of concentric zones. Aerial mycelium color varied from white through tones of beige to orange–brown. There were a formation of four different colony color group (group A, B, C, and D) on PDA culture medium, according to the Pantone® color scale (Table 3). Colonies were white, pale cream, and light orange with tones of beige or without borders and fluffy on plain PDA but produce soluble beige, yellowish to brown with white, beige or orange borders (Table 3). Colony color varied from white to light beige, with absent borders and pigmentation in tons of white,

Table 1 - Average mycelium growth (AMG), mycelium growth index (MGI) and average sporulation on the three different culture media of the 35 *Neonectria ditissima* isolates from five orchards of Gala apple cultivar of two regions of southern Brazil after 20 days of incubation under 24h light photoperiod at temperature of 22°C ±0.5°C.

Isolates	AMG			MGI			Average sporulation (conidia x 10^4 sporodochium^{-1})		
	Culture media[1]								
	BDA[1]	SN[2]	AM[3]	BDA	SN	AM	BDA	SN	AM
NR02	60.32b*	63.94a	39.75m	4.06cB	4.29 bA	2.39iC	6.25eA	0.00fB	6.25eA
NR03	67.89a	59.13c	59.20c	4.61aA	3.84dB	3.89aB	18.75cB	35.00bA	15.00dB
NR05	62.68b	43,73j	38,79m	3,95dA	2,53iB	2,25iC	2,00fB	21,25cA	22,50cA
NR10	61,08b	52,54f	50,16g	4,30bA	3,88dB	3,53dC	12,5dA	20,2cA	17,5dA
FR04	60,32b	48,40h	52,58f	4,09cA	3,02gC	3,40eB	9,0eB	15,0dA	20,0cA
FR10	44,63j	54,40e	49,83g	2,97iC	3,77dA	3,20fB	31,2bA	16,2dB	10,0eB
FR14	56,90d	49,19g	41,64 l	3,64eA	3,08 gB	2,43iC	12,5dB	38,7bA	15,0dB
FR15	46,53i	54,30e	46,12i	3,09hB	3.70dA	3.10gB	21.2cA	26.2cA	11.2dB
CPi01	46.21i	55.29d	58.46c	3.09hA	1.68mC	2.06jB	0.00gC	55.0aA	40.0bB
CPi02	59.76c	61.97b	57.06d	2.22 lB	3.24fA	1.81 lC	8.7eB	0.00fC	33.7cA
CPi03	36.63n	38.14n	39.89m	3.32gA	2.43iB	2.29iB	3.7fA	0.00fB	6.2eA
CPi05	32.23p	49.45g	49.11g	3.12hB	3.33fA	2.04jC	45.0aA	60.0aA	22.5cB
CPi06	37.14n	39.02m	62.08b	3.41fA	2.49iB	2.33iC	7.5eA	0.00fB	8.75eA
CPi09	58.61c	37.80n	56.35d	3.27gA	3.37fA	2.49hB	47.5aA	16.2dB	16.2dB
CPi10	35.28o	34.92o	49.49g	3.21gA	2.14 lB	1.94 lC	16.2dA	0.00fB	0.00gB
CPi14	64.35a	48.52h	58.04c	2.82iC	3.41eB	3.73bA	0.00gB	27.5bA	0.00gB
CPi16	41.44 l	49.93g	55.47d	3.95dB	4.55aA	3.62cC	33.7bB	48.7aA	25.0cB
CPi17	37.83n	56.96d	61.36b	2.10mB	2.31jA	2.30iA	16.2dA	12.5dB	10.0dB
AD03	50.32g	32.15p	36.42n	2.06mC	2.91hB	3.07gA	23.7cB	41.2bA	20.0cB
AD04	34.26o	46.79i	29.69p	2.25lC	2.45iB	3.94aA	21.2cA	28.7bA	10.0eB
AD05	53.30e	42.36 l	39.15m	3.71eA	2.36jC	3.45dB	0.00gC	24.2cA	10.0eB
AD06	44.75j	47.55h	32.44p	2.28 lB	2.25jB	3.01gA	21.2cA	73.7aA	16.2dA
AD07	54.35e	41.63 l	39.59m	4.16cA	2.91hC	3.65cB	18.7cA	15.0dA	13.7dA
AD08	47.44h	48.65h	38.15n	2.48jC	2.88hB	3.46dA	13.7dB	25.0cA	11.2dB
AD10	51.62f	37.81n	33.94o	2.33 lC	3.50eB	3.87aA	43.7aA	33.7bA	50.0aA
FROL01	52.82f	41.18 l	37.24n	3.40fA	2.43iB	2.21jC	15.0dB	38.7bA	7.5eC
FROL02	55.25d	43.58j	38.37m	3.44fA	2.49iB	2.15jC	18.7cA	21.2cA	15.0dA
FROL03	53.13e	40.84 l	38.01n	3.44fA	2.52iB	2.26iC	7.5eB	21.2cA	6.2eB
FROL04	55.76d	34.55o	34.41o	3.62eA	1.98 lB	2.07jB	11.2dA	8.2dA	13.7dA
FROL05	59.38c	51.24f	44.43j	3.74eA	3.07gB	2.67hC	15.0dA	14.0dA	0.00gB
FROL06	56.81d	38.73m	36.75n	3.60eA	2.18 lB	2.15jB	21.5cA	30.0bA	27.5cA
FROL07	54.11e	47.48h	42.04 l	3.44fA	2.96hB	2.55hC	4.2fA	6.2eA	3.0fA
FROL08	62.02b	61.56b	48.26h	4.19cA	4.16bA	3.07gB	6.5eB	15.0dA	11.2dA
FROL09	58.84c	59.22c	48.23h	4.04cA	4.02cA	3.22fB	22.5cA	20.0cA	14.0dA
FROL10	57.63c	55.47d	48.28h	3.91dA	3.71dB	3.09gC	0.00gB	11.2dA	13.7dA
CV %		2.71			3.5			13.87	

[1]PDA = potato-dextrose-agar; SN[2] = synthetic SNAY; AM[3] = malt extract agar. *Means followed by the same small letter in the column and the capital letter in the row are not significantly different by Scott-Knock test (P<0.05).

Table 2 - The distribution of the 35 *Neonectria ditissima* isolates in groups according to ascending order of average mycelium growth (AMG) on potato-dextrose-agar medium after 20 days of incubation under 24h light photoperiod at temperature of 22°C±0.5°C.

Size groups (mm)	Isolates	AMG	Groups AMG average (mm)
Group I 20.00 – 39.99	CPi05	32.23 p	35.56
	AD04	34.26 o	
	CPi10	35.28 o	
	CPi03	36.63 n	
	CPi06	37.14 n	
	CPi17	37.83 m	
	CPi16	41.44 l	
	FR10	44.63 j	
	AD06	44.75 j	
	CPi01	46.21 i	
	FR15	46.53 i	
	AD08	47.44 h	
	AD03	50.32 g	
	AD10	51.62 f	
Group II 40.00 – 59.99	FROL01	52.82 f	52.71
	FROL03	53.13 e	
	AD05	53.30 e	
	FROL07	54.11 e	
	AD07	54.35 e	
	FROL02	55.25 d	
	FROL04	55.76 d	
	FROL06	56.81 d	
	FR14	56.90 d	
	FROL10	57.63 c	
	CPi09	58.61 c	
	FROL09	58.84 c	
	FROL05	59.38 c	
	CPi02	59.76 c	
	FR04	60.32 b	
	NR02	60.32 b	
	NR10	61.08 b	
Group III >60.00	FROL08	62.02 b	62.67
	NR05	62.68 b	
	CPi14	64.35 a	
	NR03	67.89 a	

Means followed by the same small letter are not significantly different by Scott Knock test (P<0.05).

Table 3 - Distribution of the 35 ***Neonectria ditissima*** isolates in groups accordance to colony color, border and culture medium pigmentation according to the Pantone® color scale on potato-dextrose-agar medium after 20 days of incubation under 24h light photoperiod at temperature of 22°C±0.5°C.

Groups	Isolates	Colony color	Border	Pigmentation
A	NR02, NR03, NR10, CPi02	White	Absent	Tones of yellow
B	FR04, FR10, FR14, FR15, CPi01, CPi05, CPi06, CPi09, Cpi16, Cpi17, AD03, AD04, AD06, FROL03, FROL04, FROL 05, FROL07, FROL08, FROL09, FROL10	Tones of beige	Tones of beige	Brown with edges in tones of beige
C	CPi03, CPi10, FROL01, FROL06, AD05	White to tones of beige	Absent	Tones of beige
D	NR05, CPi14, AD07, AD08, AD10, FROL02	Light orange	White	Orange with white edges

beige, and yellow on AM culture medium, and colony color and pigmentation in tones of translucent white on SN culture medium. Three distinct color groups were formed in PDA, according to the Pantone® color scale. The largest group B, with 15 isolates, showed colony color in tones of beige and brown pigmentation with borders in tones of beige (Table 3).

Conidia morphology, size and pathogenicity testing

All *N. ditissima* isolates produced micro- and macroconidia in at least one of the culture media evaluated. There were significant differences among the average dimensions of micro- and macroconidia on PDA, AM, and SN Table 4). The three media produced macroconidia that were slightly curved, hyaline, and multicellular varying from 1 (immature conidia) to 7 septa, with rounded extremities and dimensions varying in the range 9.5-11.0x48.3-69.8µm. Microconidia were straight, elliptic to cylindrical, hyaline, and unicellular, with dimensions varying in the range 4.5-6.8x30.9-44.2µm. The average micro- and macroconidia length and width on PDA, AM, and SN were 35.4x4.8, 35.2x5.2, and 34.8x5µm and 63.5x10.1, 62.3x10.6, and 59.7 x 10.3µm, respectively (Table 4). The largest micro- and macroconidia were produced on the AM and SN culture media; however, with no significant differences between the media (Table 4). All 35 *N. ditissima* isolates were pathogenic to 3-year-old Gala apple cultivar plants despite of isolates origin and virulence groups were not observed after 20 days post-inoculation. Within 8-12 days after inoculation, most plants began to exude clear to amber-colored gum droplets at the points of inoculation. This host response was exhibited by plants inoculated with each of the 35 *N. ditissima* and lacking in the control inoculations. Most of the young branches began to show the first symptoms of swallow elliptical point with an exposure of bark internal tissue typical of canker after 12-14 days after *N. ditissima* isolates inoculation. Cankers showed a reddish-brown spot around a leaf scar, spur, and bark. Young cankers produced small, whitish sporodochial fruiting bodies after 14 days after *N. ditissima* isolates inoculation. Over time, all inoculated plants displayed varying degrees of bark tissue discoloration/ necrosis, bark fissuring, and/or stem deformation at or near the points of artificial inoculation. Comparable symptoms were lacking in control inoculations.

DISCUSSION

European canker is an important and recent quarantine pest in Brazil that demands special attention to the correct characterization and pathogenicity test of the isolates populations present in southern Brazil. Our data indicate no substantive intraspecific variability with respect to spore morphophysiological parameters among 35 *N. ditissima* isolates collected from different apple orchards in Rio Grande do Sul and Santa Catarina States. They also suggested little evidence of host specificity among isolates, given that all isolates were pathogenic to the Gala apple cultivar. These results parallel reported that morphophysiological isolates of *N. ditissima* were capable of infecting the most popular and wide spread cultivar in southern Brazil, giving a confident interpretation that the 35 isolates belonging to the same group or variety.

One of the objectives of the study was to the comparison of the influences of three different media, including synthetic, semi-synthetic, and natural on the growth of *N. ditissima*. The fungus grew on all culture media tested. However, natural solid media were more favorable for growth. The differences observed in the AMG and MGI of the isolates on PDA, AM, and SN may have been due to qualitative and quantitative aspects of these media. PDA has a simple formulation and the highest nutrient contents, supporting the best mycelial growth of the fungus (BOGO et al., 2008; SAHA et al., 2008). The genera *Neonectria* and *Cylindrocarpon* usually produce pigmentation with tones of light brown and white borders on potato sucrose agar medium, but on SN medium the colonies

Table 4 - Microconidia and macroconidia average length and width (mm) of the 35 *Neonectria ditissima* isolates on different culture media after 20 days of incubation under 24h light photoperiod at temperature of 22°C±0.5°C.

Isolates	Microconidia						Macroconidia					
	Length (mm)			Width (mm)			Length (mm)			Width (mm)		
	Culture media[1]											
	PDA	SN	AM	PDA	SN	AM	PDA	SN	AM	PDA	SN	AM
NR02	32.1eA*	-	-	4.8eA	-	-	-	-	48.3gA	-	-	10.0cA
NR03	33.0eA	33.2eA	32.8fA	5.0dA	5.0eA	4.9dA	-	-	-	-	-	-
NR05	36.2cA	-	-	5.4cA	-	-	-	64.7cA	62.8cB	-	10.7aA	10.2cB
NR10	-	33.1eA	-	-	4.8eA	-	62.9dA	58.3eB	54.7eC	10.7bA	10.1bB	9.6dC
FR04	36.6cA	30.5fC	32.3fB	4.9dA	4.5fB	4.5eB	-	-	-	-	-	-
FR10	31.7eA	32.3eA	30.9fA	4.6eA	4.5fA	4.5eA	-	-	-	-	-	-
FR14	32.6eA	-	-	5.1dA	-	-	-	66.8bA	62.4cB	-	10.8aA	10.2cB
FR15	30.5eA	31.5fA	31.1fA	4.5eA	4.5fA	4.5eA	-	-	-	-	-	-
CPi01	-	36.1dA	37.1cA	-	5.9cA	5.2cB	-	-	-	-	-	-
CPi02	-	-	-	-	-	-	64.4cB	-	69.8aA	10.1dB	-	10.5bA
CPi03	39.1bB	-	41.3bA	5.5cB	-	5.9aA	-	-	-	-	-	-
CPi05	36.3cA	33.6eB	34.2eB	5.5cA	5.1dB	5.2cB	-	-	55.5eA	-	-	10.5bA
CPi06	-	-	36.1dA	-	-	5.5bA	65.8bA	-	-	10.4bA	-	-
CPi09	39.9bA	37.5cB	-	5.5cA	5.7cA	-	64.8cA	-	58.6dB	10.5cA	-	10.5bA
CPi10	33.6dA	-	-	5.1dA	-	-	-	-	-	-	-	-
CPi14	-	36.8cA	-	-	5.4dA	-	-	-	-	-	-	-
CPi16	39.8bB	32.6eC	42.3aA	5.5cB	4.9eC	6.0aA	-	-	-	-	-	-
CPi17	36.9cA	34.5dB	38.1cA	5.1dB	5.3dB	5.6bA	-	-	-	-	-	-
AD03	37.3cA	-	37.9cA	6.0bA	-	5.7bB	66.7aB	68.1aA	-	9.8dB	11.0aA	-
AD04	32.2eB	37.1cA	36.2dA	4.6eB	5.7cA	5.5bA	-	-	-	-	-	-
AD05	-	-	-	-	-	-	-	65.2cA	64.8cA	-	10.7aA	10.7aA
AD06	31.5eA	30.5fA	31.8fA	4.5eA	4.5fA	4.6eA	-	-	-	-	-	-
AD07	41.5aA	-	-	6.8aA	-	-	66.8aA	65.0cB	66.1bA	10.7bA	10.7aA	11.0aA
AD08	34.9dA	35.5dA	31.2fB	5.1dA	4.6fB	4.5eB	-	-	-	-	-	-
AD10	42.5aA	-	-	6.0bA	-	-	62.9dB	65.0cA	64.6cA	10.5cB	10.8aA	10.5bB
FROL01	-	42.1bA	31.9fB	-	6.6bA	4.7dB	66.5aA	57.6eB	-	11.6aA	9.9bB	-
FROL02	40.8aB	45.5aA	-	5.6cB	6.5bA	-	-	54.8fB	63.3cA	-	10.1bB	10.6bA
FROL03	-	44.2aA	40.3bB	-	6.8aA	5.6bB	65.6bA	59.8dB	-	10.9bA	10.3bB	-
FROL04	34.2dA	35.4dA	35.5dA	5.0dA	5.1dA	5.2cA	-	-	-	-	-	-
FROL05	-	-	-	-	-	-	64.7cA	64.8cA	-	10.8bA	10.7aA	-
FROL06	-	-	-	-	-	-	64.9cA	60.0dB	64.2cA	10.7bA	10.4bB	10.9aA
FROL07	32.6eA	30.5fB	32.5fA	4.9dA	4.5fB	5.1cA	-	-	-	-	-	-
FROL08	39.1bA	35.0dB	-	5.8bA	4.6fB	-	-	-	51.7fA	-	-	10.0cA
FROL09	-	-	36.2dA	-	-	5.6bA	52.0eB	59.2dA	49.6gC	10.5cA	10.8aA	9.5dB
FROL10	-	31.9eA	32.0fA	-	4.5fA	4.5eA	-	-	-	-	-	-
CV %		3.42			4.58			2.31			4.14	
average	35.4c	35.2c	34.8d	4.8c	5.2c	5.2c	63.5a	62.3a	59.7b	10.1a	10.6a	10.3a
SD[2]	3.1	3.2	3.1	0.4	0.6	0.4	2.3	3.6	5.7	0.3	0.3	0.3

[1]PDA= potato-dextrose-agar; SN= synthetic SNAY; AM= Malt extract agar. [2]Standard deviation. *Means followed by the same small letter in the column and the capital letter in the row are not significantly different by Scott Knock test (P<0.05).

were not pigmented (hyaline) and the mycelium was aerial (LANGRELL, 2002). Four distinct color groups were formed in PDA. SN and AM were excellent media for production of large numbers of multi-celled conidia for most isolates. SN showed the lowest AMG and MGI values but the highest conidial production. Culture media with vegetable extract, plant material, and low carbohydrate content usually stimulate the sporulation of many fungi (NOZAKI et al., 2004). The *N. ditissima* conidial morphology and sporulation *in vitro* are in agreement with the results of AMPONSAH et al. (2014) who reported that *N. ditissima* conidia produced *in vitro* had morphological characteristics and pathogenicity similar to those produced in the field. Variation in colony and substrate color, colony border, topography of mycelia, and number of septa on the three different solid media adds information that may be helpful in the taxonomic identification and genetic variability of *N. ditissima*. There is large conidial diversity between the *Neonectria* and *Cylindrocarpon* genera (BRAYFORD, 2004; CHAVERRI et al., 2011), with respect to presence or absence of microconidia, macroconidia, chlamydospores, and number of septa. Variation in number of septa can be associated with factors such as nutrition, relative humidity, and temperature (FLACK & SWINBURNE, 1977). Conidium length and number of septa may vary with glucose, carbon concentration and initial medium pH, nitrogen source, and culture age (HARDING, 1975). The few one septum conidia reported on the three culture media were probably immature conidia. Thus, on the basis of some differences in the conidial morphology and physiological variation, *N. ditissima* can be considered a pathogen with not a large variability. This report has described the use of PDA, AM, and SN culture media to study and produce *N. ditissima* conidia *in vitro* reliably and on demand for use in infection studies. The conidia produced in this way were equally as pathogenic as those obtained from the field, and the conidia produced on these media resulted in faster development of disease symptoms, which were visible as soon as 14 days after inoculation. Our study also showed that the verification of the efficacy and performance of a selected medium reported in the literature, using well-characterized type and reference strains, is a crucial step before it is adopted for enumeration. This method for production of *N. ditissima* conidia *in vitro* will be particularly useful for EC resistance research in Brazil. In summary, the present study showed that despite of PCR identification of the 35 isolates as *N. ditissima*, there are morphophysiological variations between them despite them been pathogenic to the Gala apple cultivar. There was no evidence of correlation between isolates virulence and geographical origin. The non-existence of isolates clusters from the two distant geographical regions evaluated suggests that there was no evidence of genetic differentiation in their recent introduction in southern Brazil.

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