Characterization of *Alternaria alternata* causal agent of brown spot in *Citrus* spp

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ABSTRACT

The purpose of this study was to characterize culturally, enzymatically and pathogenically *Alternaria alternata* isolates obtained of tangerine/tangor (TP) and rough lemon (RLP). Significant differences were observed regarding mycelial growth speed and sporulation of isolates when cultivated in starch-agar (SA), potato-dextrose-agar (PDA) and tomato juice agar (V8) media. SA and PDA media promoted better mycelial growth and sporulation, respectively. Eight genetic similarity groups were defined through isoenzymatic characterization but without correlation between isolates and host or site of origin. All isolates produced amylase, cellulase, polygalacturonase and pectynase; however, no lipolytic or proteolytic activity was observed. Disease incubation period varied between 24 to 48 h for all isolates in all phenological stages of the inoculated fruit. Symptomatic fruit incidence in most tangerine and tangor isolates was higher in stage F3 compared to stages F4 and F5. *Alternaria alternata* have the ability to perform hyphal anastomosis indicating that this can be a mechanism used by the fungus to increase genetic variability.

Key words: Variability, Enzymes production, Isoenzymes, Fungi, Citrus disease.

INTRODUCTION

Alternaria alternate (Fr) Keissl is the causal agent of three citrus diseases: alternaria brown spot (ABS) found in tangerines and rough lemons, the postharvest rottenness in citrus fruit and the "Galego" lemon foliar spot (Timmer et al., 2003). Fungi of this species show pathological differences among them (Kusaba and Tsuge 1994) and produce host-selective toxins (HST) (Masunaka et al., 2005), referred as pathotype due to its host. The tangerine pathotype (TP) of *A. alternata* is the causal agent of ABS, producing a HST known as ACT toxin, specific for tangerines (*Citrus reticulata* Blanco), tangors (*Citrus sinensis* × *C. reticulata*) and tangelos (*Citrus paradisi* × *C. reticulata*) (Kohmoto et al., 1991). The 'Rough' lemon pathotype (RLP) of *A. alternata*, causal agent of alternaria foliar spot (AFS) produces the ACRL toxin, specific for the 'Rough' lemon (*Citrus jambhiri* Lush) and "Clove" lemon (*Citrus limonia* Osbeck). *Alternaria alternata* isolates capable of producing the endopolygalacturonase enzyme can cause the black rottenness, and the internal rottenness in the fruit (Timmer et al., 2003).

The knowledge of genetic variability among isolates of a pathosystem and the mechanisms that generate this variability are essential to the disease management. The sexual phase of *A. alternata* has not been identified yet and the assexual recombination, which starts with the fusion of encountering vegetative hyphae (hyphal anastomosis) followed by the heterokariosis, could represent a significant source of variability in this species (Huang et al., 1996).

Although morphocultural, enzymatic, molecular and pathogenic methods have been used to characterize phytopathogens (Marchi et al., 2006) only a few of these studies were carried out with *A. alternata* of *Citrus* spp. This work provides information about the genetic variability of *A. alternata* isolates, based on the cultural, isoenzymatic, exoenzymatic and pathogenic characterizations of *A. alternata* isolates from citrus. The occurrence of hyphal anastomosis among isolates of this pathogen is also described, which can contribute to the expansion of the genetic variability in the species.

MATERIAL AND METHODS

The *A. alternata* isolates showed in the Table 1, originated from "Dr Victoria Rossetti" Mycology Collection (UPDSorocaba/APTA/SAA), were previously identified by Dini-Andreote et al. (2009). For the assessment of mycelial growth and sporulation, mycelial disks of 5 mm of diameter taken from colonies with seven days of cultivation in potato-dextrose-agar (PDA) medium were transferred to starch-agar (SA), PDA and tomato-juice-agar (V8) media and incubated at 25 ± 1 ºC, for seven days with photoperiod of 12 h. Colonies average diameter measurements were made every 24 h and radial mycelial growth average speed (mm/day) was obtained from average of the growth speed measured daily. Colonies pigmentation and mycelial thickness (measured with a millimeter

ruler) and sporulation rate (estimated in Newbauer chamber) were assessed at seven days of cultivation. An entirely randomized statistical design with four replications per treatment was adopted. The values were expressed as means ± SD (Standard Deviation).

Enzymatic characterization followed the methodology described by Paccola-Meirelles et al. (1988) in polyacrylamide gel for esterase. Electrophoretic profiles were established based on number, intensity and position of bands and their relative mobility (Rf) (Santos et al., 2004). The NTSYS-PC 21 program (Rohlf 2000) and the Jaccard coefficient were used for the genetic similarity analysis (Sneath and Sokoal 1973). The UPGMA grouping method (Unwerighted Pair-Group Method with arithmetical Average) was used for construction of the genetic similarity dendrogram. The analysis "bootstrap" by BOOD program (Coelho 2000) determined the consistency of the groups formed.

Amylolytic, cellulolytic, proteolytic, lipolytic pectinolytic and polygalacturolytic activities of 18 isolates from TP and three from RLP were assessed according to Hankin and Anagnostakis (1975). Statistical design was entirely randomized with four replications per treatment. The data were submitted to analysis of variance by SISVAR program and the means of treatments were compared by Scott-Knott test (1974), with $P \leq 0.05$.

For the pathogenic characterization, isolates were cultivated in PDA medium at 25 ± 1 ºC, under photoperiod of 12 h for 10 cultivation days. Aliquots of 15 µL of aqueous suspension (50×10^4 conidia/mL) were deposited on the surfaces of the 'Murcott' tangor fruit and 'Ponkan' tangerine, in four stages of development, F3, F4, F5 – fruit with ¼, ½ and ¾ of the final size, respectively – and F8 – ripe fruit – and packed in plastic trays in a humid chamber at 25 ± 4 ºC, photoperiod of 12 h. Fruit inoculated only with sterilized distilled water were used as controls. Five fruits were inoculated per isolate for each phenological stage of development. Disease incidence and incubation period (period between inoculation and the onset of symptoms) were assessed.

To show the occurrence of hyphal anastomosis, disks of 5 mm of diameter with mycelium of seven days old were paired up on microscope slides covered with a thin layer of water-agar medium and incubated at 25 ± 1 ºC in the dark. After finding the colonies, they were fixed in lactophenol and the occurrence of hyphal anastomosis between and within isolates was observed by an optical microscope.

Table 1. Denomination, pathotype, isolation organ, host and origin of *Alternaria alternata* isolates of citrus used in the experiment.

Isolates[1]	Patothype[2]	Organ	Host	Collection site
LRS04/03	TP	Leaves	'South African'Tangerine	Campanha-MG
LRS08/03	TP	Leaves	Tangor 'Murcott'	Aguaí-SP
LRS11/03	TP	Fruits	Tangor 'Murcott'	São Miguel Arcanjo-SP
LRS14/03	TP	Fruits	Tangor 'Murcott'	Casa Branca-SP
LRS16/03	TP	Fruits	Tangor 'Murcott'	Mogi Mirim-SP
LRS20/03	TP	Fruits	Tangor 'Murcott'	Limeira-SP
LRS23/03	TP	Leaves	Tangor 'Murcott'	São Miguel Arcanjo-SP
LRS24/03	TP	Branches	Tangor 'Murcott'	São Miguel Arcanjo-SP
LRS25/03	TP	Branches	Tangerine 'Ponkan'	Montenegro-RS
LRS26/03	TP	Fruits	Tangor 'Murcott'	Conchal-SP
LRS35/03	TP	Fruits	Tangerine 'Nova'	Capão Bonito-SP
LRS36/03	TP	Fruits	South African Tangerine	Capão Bonito-SP
LRS38/03	TP	Fruits	Sunburst Tangerine	Capão Bonito-SP
LRS39/03	TP	Fruits	'Ortanique'Tangor	Capão Bonito-SP
LRS40/03	TP	Fruits	'Cravo'Tangerine	Capão Bonito-SP
LRS41/03	TP	Fruits	'Murcott'Tangor	Capão Bonito-SP
LRS42/03	TP	Fruits	'Murcott Tangor'	Capão Bonito-SP
LRS43/03	TP	Fruits	Tangerine 'de Wildt'	Capão Bonito-SP
LRS23/04	RLP	Leaves	Florida 'Rough' Lemon	Capão Bonito-SP
LRS25/04	RLP	Branches	Florida 'Rough' Lemon	Capão Bonito-SP
LRS26/04	RLP	Leaves	Florida Rough' Lemon	Capão Bonito-SP
LRS27/04	RLP	Leaves	'Cravo' Lemon	Capão Bonito-SP

[1]Number of isolates in "Dr Victoria Rossetti" Mycology Collection (UPDSorocaba/APTA/SAA).
[2]Tangerine pathotype (TP) and 'Rough' lemon pathotype (RLP).

RESULTS AND DISCUSSION

Although all culture media were adequate for mycelial production of *A. alternata* isolates, mycelial growth was not a parameter to differentiate isolates, since the average mycelial growth speed was similar (Figure 1). No phenotypical variability was observed among isolated colonies, since both upper surface and lower surface of the colonies showed gray and brown coloration, respectively, regardless of the isolate and cultivation medium. Sporulation rate varied from 0 to 7 × 10^4 conidia/cm^2 of colony and it was dependent on the culture medium and isolate (Figure 1), indicating the existence of physiological variability. This variability was generated probably due to different capability of absorption or utilization of nutrients available in the cultivation media. Although Rotem (1994) described that *A. alternata* sporulates easily, our results showed low production of spores for all *A. alternata* isolates of citrus.

The esterase polymorphism revealed the existence of genetic diversity among isolates; however, similar to results found by Petrunak and Christ (1992), no correlation was observed between the isolates and their host or their local of origin. Number of bands for esterase varied from one to five, considering that 19% of the isolates presented a single band; 28.6% two bands; 19% three bands, 28.6% four bands and only isolate LRS35/03 showed five bands. Eight groups of genetic similarity (Figure 2) were differentiated: group A (LRS14/03, LRS23/04), group B (LRS25/03, LRS36/03, LRS38/03, LRS42/03, LRS43/03), group C (LRS35/03), group D (LRS08/03, LRS16/03, LRS24/03, LRS41/03), group E (LRS26/04 e LRS20/03), group F (LRS04/03, LRS11/03, LRS23/03, LRS26/03), group G (LRS40/03) and group H (LRS27/04, LRS39/03). It is important to highlight that isolate LRS27/04 from pathotype LRP was grouped with isolate LRS39/03 from pathotype TP.

All isolates were positive for amylolytic, cellulolytic, polygalacturonase and pectinolytic activities (Table 2) and negative for proteolytic and lipolytic activities. The amylolytic activity index (AAI), enzyme that may be required for infection of vegetal organs, with starch in their composition (Marchi et al., 2006), allowed the classification of 66.7% of isolates in group A (AAI of 1.08 to 1.27) and 33.3% in group B (AAI of 1.31 to 1.45) of genetic similarity. The cellulolytic activity index (CAI) classified 38.1 % of the isolates in group A (CAI from 1.10 to 1.17); 38.1% in group B (CAI from 1.04 to 1.08) and 23.8% in group C (CAI from 1.00 to 1.02) of genetic similarity. Isolate LRS27/04 was the greatest producer of pectinase (Table 2) with a pectinolytic activity index (PAI) of 1.39. Around 23.8% of the isolates showed PAI between 1.16 and 1.22; 38.1% showed PAI between 1.07 to 1.14 and 33.33% showed PAI between 1.0 and 1.06.

All isolates had polygalacturonase activity (PoAI) (Table 2). Isolate LRS27/04 showed greater PoAI (1.29). Two isolates showed PoAI between 1.15 and 1.17; 33.3% with PoAI between 1.08 and 1.12; and the remaining 52.4% showed PoAI next to 1.00. According Isshiki et al. (2001) the polygalacturonase, an endo-polygalacturonase capable of degrading the cell wall, can have different functions in the pathogenicity of *Alternaria citri* and *A. altenata.* However enzymes as lipases, proteases and amylases are related with the virulence level, playing an important role in the pathogen-host interaction (Bocchese et al., 2003). The results presented here have not allowed correlate the production of these enzymes with the pathogenicity of *A. alternata.* The enzymatic profile obtained with this type of study can help future characterization works since works of this nature in *A. alternata* are scarce in the literature.

Disease incubation period and the onset of the first symptoms varied between 24 to 48 h, regardless of the isolates and the phenological stage of the fruit. Typical lesions were observed (Figure 3), however, there was no expansion of the affected area, even seven days after inoculation. The 'Ponkan' and 'Murcott' varieties were susceptible only to TP isolates in all phenological stages assessed (Table 3). For most tangerine and 'Murcott tangor isolates, stage F3 promoted greater incidence of symptomatic fruit when compared to stages F4 and F5. Fruit in the beginning of their development, in general, are more susceptible to the disease (Vicent et al., 2004). However, with the exception of isolate LRS20/03, all the other TP isolates developed symptoms of the disease in ripe fruit, with high incidence at stage F8 Isolate LRS43/03 favored the high incidence of symptomatic fruit in both varieties, showing greater virulence compared to the other TP isolates.

Three isolates from TP (LRS04/03, LRS14/03, LRS23/03) and four from RLP (LRS23/04, LRS25/04, LRS26/04 e LRS27/04) were selected to verify the occurrence of anastomoses between and within the same isolate, and anastomoses were observed in all combinations (Figure 4), except in crossings between RLP isolates with TP isolates. The development of heterocarions generates recombinant individuals, potentially more pathogenic. Natural occurrence, observed by Masunaka et al. (2005) of an isolate obtained from a 'Rough' lemon, also pathogenic to the Iyokan" tangor, capable of producing both toxins, ACT and ACRL, suggests that this characteristic originated from the horizontal transference, via hyphal anastomosis, of genes between isolates of distinct pathotypes.

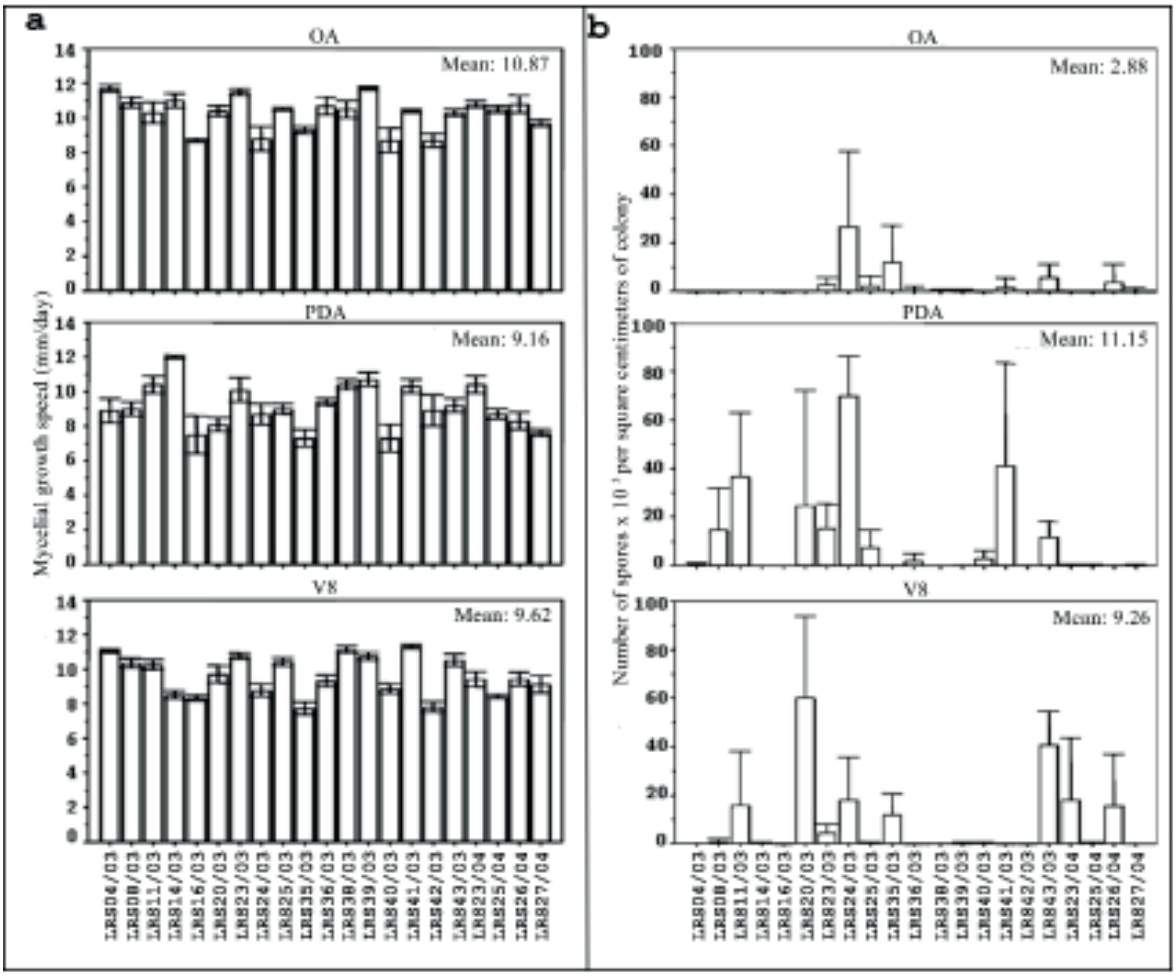

Figure 1. Mycelial growth speed (a) and number of spores per square centimeters of colony (b) of *Alternaria alternata* isolates, causal agent of brown spot in *Citrus* spp, grown in oatmeal-agar (OA), potato-dextrose-agar (PDA) and tomato juice-agar (V8) media.

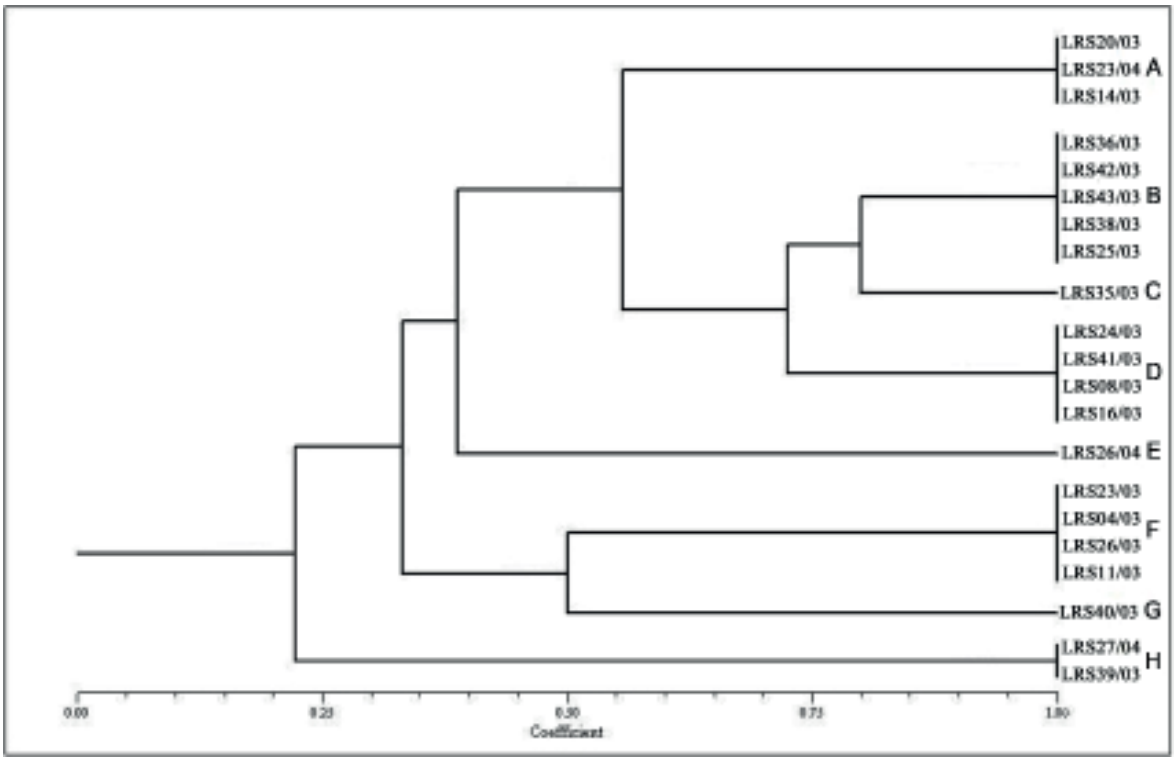

Figure 2. Dendrogram of genetic similarity of 21 isolates of *Alternaria alternata* by application of the Jaccard coefficient and grouping method UPGMA.

Table 2. Enzymatic activity of *Alternaria alternata* isolates.

Isolates	Enzymatic Index[1]			
	Amylase	Cellulase	Pectinase	Poligalacturonase
LRS04/03	1.16 b[2]	1.04 b	1.13 c	1.03 d
LRS08/03	1.17 b	1.08 b	1.19 b	1.10 c
LRS11/03	1.18 b	1.14 a	1.12 c	1.17 b
LRS14/03	1.16 b	1.02 c	1.01 d	1.09 c
LRS16/03	1.27 b	1.17 a	1.16 b	1.05 d
LRS20/03	1.45 a	1.00 c	1.00 d	1.06 d
LRS23/03	1.15 b	1.07 b	1.08 c	1.05 d
LRS24/03	1.15 b	1.10 a	1.17 b	1.03 d
LRS25/03	1.15 b	1.02 c	1.02 d	1.08 c
LRS26/03	1.41 a	1.14 a	1.06 d	1.11 c
LRS35/03	1.23 b	1.07 b	1.14 c	1.01 d
LRS36/03	1.43 a	1.07 b	1.13 c	1.08 c
LRS38/03	1.08b	1.12 a	1.11 c	1.06 d
LRS39/03	1.23 b	1.02 c	1.18 b	1.06 d
LRS40/03	1.31 a	1.15 a	1.22 b	1.15 b
LRS41/03	1.35 a	1.05 b	1.11 c	1.09 c
LRS42/03	1.14 b	1.00 c	1.00 d	1.05 d
LRS43/03	1.22 b	1.10 a	1.07 c	1.12 c
LRS23/04	1.17 b	1.10 a	1.03 d	1.00 d
LRS26/04	1.41 a	1.07 b	1.06 d	1.02 d
LRS27/04	1.43 a	1.07 b	1.39 a	1.29 a

[1]Enzymatic index (EI) = halo diameter/colony diameter.
[2]Means followed by the same letter in the column do not differ statistically by the Scott-Knott (p≤0.05) test.

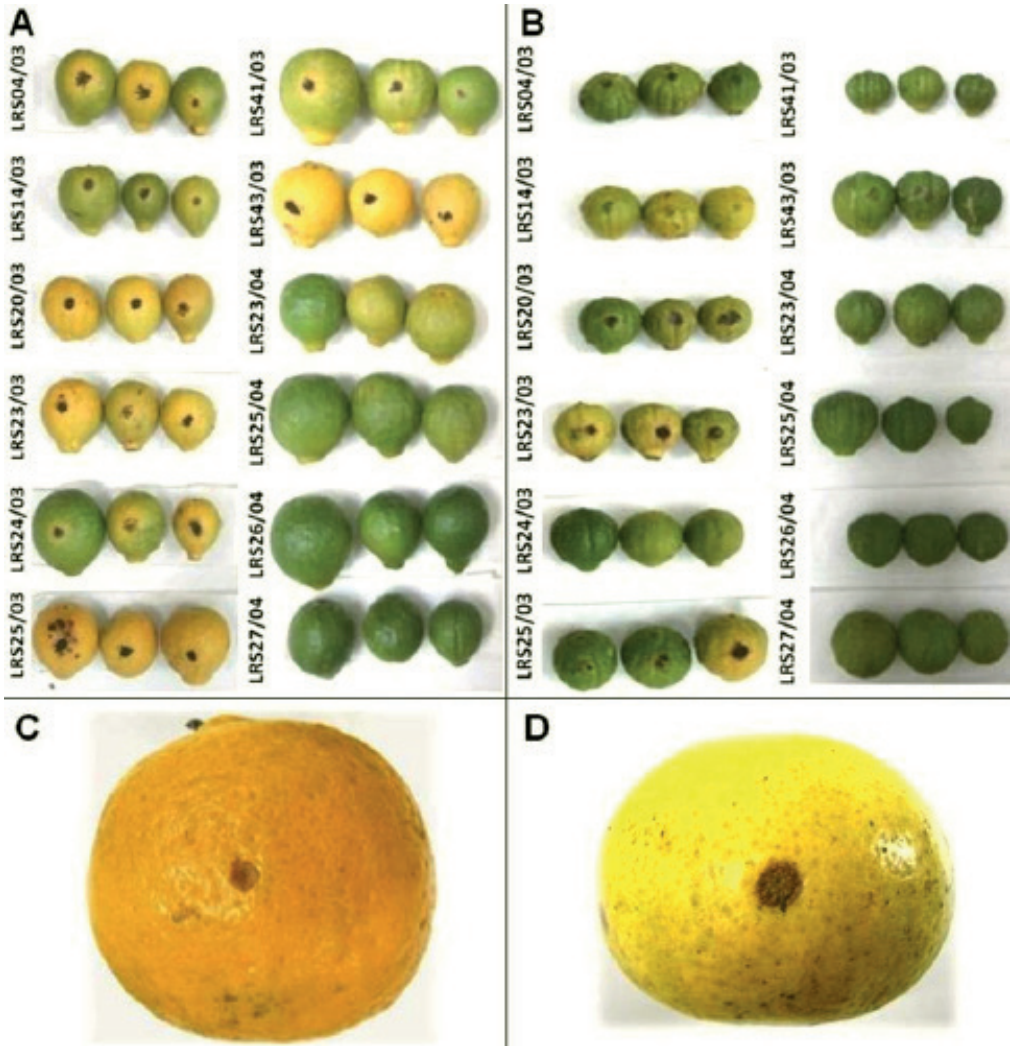

Figure 3. (a) Tangerine 'Ponkan' fruits - F3 phenological stage, (b) 'Murcott' tangor fruits - F3 phenological stage, (c) Tangerine 'Ponkan' fruit – F8 phenological stage - inoculated with the isolate LRS25/03, (d) and 'Murcott' tangor fruit – F8 phenological stage - inoculated with the isolate LRS25/03.

Table 3. Incidence of alternaria brown spot symptoms in 'Murcott' tangor fruit and 'Ponkan' tangerine inoculated at different phenological development stages with *Alternaria alternata* isolates, tangerine pathotype (TP) and 'Rough' lemon (RLP).

Isolates[1]	'Murcott'				'Ponkan'			
	Phenological stage[2]							
	F3	F4	F5	F8	F3	F4	F5	F8
	Incidence (fruits with symptoms: total of fruits assessed)							
LRS04/03 (TP)	5:5	0:5	0:5	3:5	5:5	2:5	0:5	5:5
LRS14/03 (TP)	5:5	0:5	1:5	4:5	5:5	4:5	0:5	1:5
LRS20/03 (TP)	5:5	3:5	4:5	0:5	5:5	5:5	2:5	0:5
LRS23/03 (TP)	5:5	4:5	0:5	3:5	5:5	3:5	0:5	2:5
LRS24/03 (TP)	0:5	0:5	0:5	4:5	5:5	0:5	0:5	1:5
LRS25/03 (TP)	5:5	0:5	0:5	5:5	5:5	0:5	1:5	5:5
LRS41/03 (TP)	3:5	4:4	0:5	3:5	5:5	4:5	0:5	3:5
LRS43/03 (TP)	5:5	5:5	5:5	5:5	5:5	4:5	3:5	3:5
LRS23/04 (PRL)	0:5	0:5	0:5	0:5	0:5	0:5	0:5	0:5
LRS25/04 (PRL)	0:5	0:3	0:5	0:5	0:5	0:5	0:5	0:5
LRS26/04 (PRL)	0:5	0:5	0:5	0:5	0:5	0:5	0:3	0:5
LRS27/04 (PRL)	0:5	0:5	0:5	0:5	0:5	0:5	0:5	0:5

[1]TP: Tangerine pathotype, PRL: 'Rough' lemon pathotype. [2]F3, F4, F5 and F8 phenological stages.

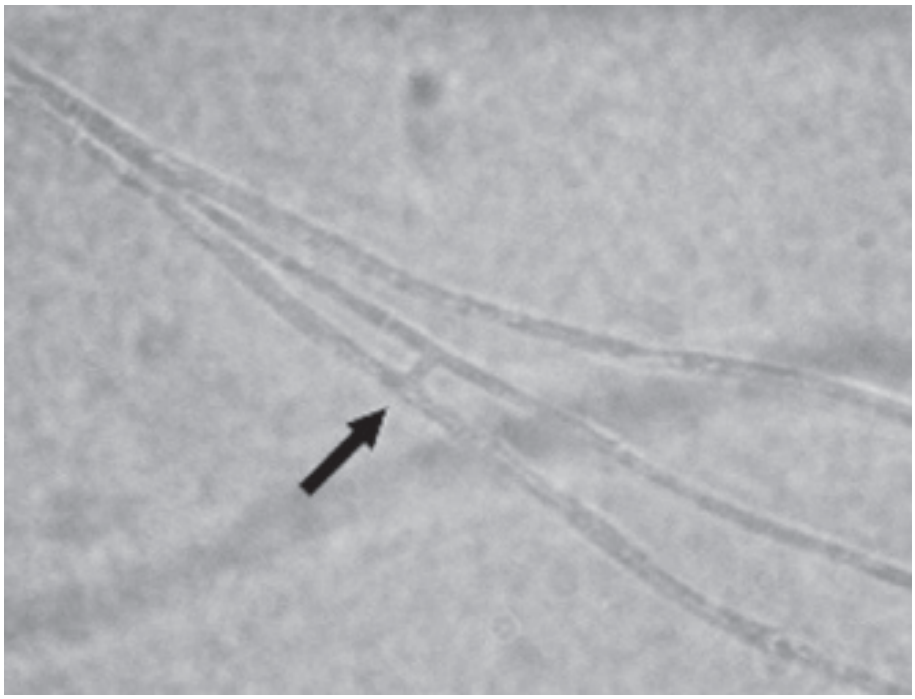

Figure 4. Micrography of the hyphal anastomosis (arrow) between isolates LRS14/03 (isolated from the 'Murcott' tangor fruit) and LRS04/03 (isolated from the 'South African' tangerine leaf) of the *A. alternata* tangerine pathotype.

CONCLUSIONS

Polymorphism for the esterase enzyme has low variability among isolates from the same pathotype. However, differences among isolates suggest a specialization of each pathotype to the conditions they were submitted. No correlation was found among isolates with host site of origin.

It was impossible to correlate the production of lipase, protease and amylase enzymes with *A. alternata* pathogenicity. This finding suggests that a minimum production of these enzymes is necessary for infection to occur.

Cellulolytic, pectinolytic and poligalacturonase activity is sufficient for the distinct grouping of isolates even under low quantity. Like other fungi, *A. alternata* have the ability to perform hyphal anastomosis among the isolates, indicating potential development of heterokaryons.

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