GENETIC CHARACTERIZATION OF *Xylella fastidiosa* ISOLATED FROM CITRUS AND COFFEE PLANTS

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ABSTRACT: The Citrus Variegated Chlorosis and the Coffee Leaf Scorch are some of the many destructive diseases caused by *Xylella fastidiosa*, a gram-negative bacterium limited to the xylem of affected plants. As its genetic characterization is still not well established, different isolates of *X. fastidiosa* from citrus and coffee were evaluated through RAPD (Random Amplified Polymorphic DNA) technique to characterize and classify these isolates based on similarity coefficients. Sixteen isolates of *X. fastidiosa* were used on this trial, obtained from citrus, coffee and almond. The genetic polymorphism evaluation was performed using six arbitrary 10-base primer pairs. It was possible to establish a dendogram in which the isolates were classified into five groups (A, B, C, D and E). A prevalence of citrus isolates in groups A and D was observed. In groups B and C, there was a prevalence of coffee isolates meanwhile the group D consisted of the almond isolate, solely.
Key words: RAPD, gram-negative bacterium, genetic divergence

CARACTERIZAÇÃO GENÉTICA DE *Xylella fastidiosa* ISOLADA DE PLANTAS DE CITROS E CAFÉ

RESUMO: A Clorose Variegada dos Citros e a Requeima das Folhas do Cafeeiro são algumas das várias doenças destrutivas causadas pela *Xylella fastidiosa*, que é uma bactéria gram-negativa e limitada ao xilema de plantas afetadas. Como a sua caracterização genética ainda não está determinada, diferentes isolados da *X. fastidiosa* de citros e café foram avaliados pela técnica RAPD (Polimorfismo do DNA Amplificado ao Acaso) para caracterizar e classificar estes isolados com base em coeficientes de similaridade. Foram utilizados 16 isolados de *X. fastidiosa* provenientes de citros, café e amêndoa. A avaliação do polimorfismo genético foi realizada utilizando seis iniciadores randômicos de 10 pares de base. Foi possível estabelecer um dendograma no qual os isolados foram classificados em cinco grupos (A, B, C, D e E). Nos grupos A e D existe uma forte predominância de isolados de citros. Nos grupos B e C há predominância de isolados de café enquanto no grupo E ficou apenas o isolado de amêndoa.
Palavras-chave: RAPD, bactéria gram-negativa, divergência genética

INTRODUCTION

In the last 100 years, some fruit plants of economical importance mainly in North America were submitted to significative losses caused by *Xylella fastidiosa* (Wells et al., 1987), a xylem-limited gram-negative bacterium, which affects a large number of species, including mono and dicotyledon woody plants (Chagas et al., 1992).

In Brazil, this bacterium is the causal agent of the Citrus Variegated Chlorosis (CVC), Coffee Leaf Scorch (CLS) and Plum Leaf Scald (PLS) diseases. It was observed for the first time in the Sao Paulo and Minas Gerais states, in 1987 (Lee et al., 1993). At this moment, the CVC was found widespread over citrus orchards in São Paulo, Paraná (Leite & Jacomino, 1993), Minas Gerais (Mizubuti et al., 1994), Rio de Janeiro (Lee et al., 1991; Rossetti & De Negri, 1990), Goiás, Sergipe, Santa Catarina, Distrito Federal and Rio Grande do Sul states (Tubelis et al., 1993). The symptoms occur initially in a portion of the tree and then they spread very fast over the whole canopy. The branches located at the top of the tree die along time, the leaves fall and the fruit production is reduced one year after the tree has been infected (Lee et al., 1993).

The Coffee Leaf Scorch (CLS) was firstly reported in October of 1995 causing outbreaks in some regions of Sao Paulo state and south of Minas Gerais state (Paradela Filho et al., 1995). The causal agent was isolated and cultivated (Lima et al., 1996). At this mo-

ment, the CLS was found widespread over coffee areas on Paraná, São Paulo, Minas Gerais, Espírito Santo, Bahia, Rio de Janeiro and Rondonia states in Brazil (Matiello et al., 1998). Small and yellow and light green colored leaves, mainly on the top of coffee trees, short branch nodes, reduction of tree growth and death of lateral branches are symptoms observed for this disease (Matiello et al., 1998).

The genetic characterization of *X. fastidiosa* has not yet been established. Considering the above mentioned aspects, the objective of this study was to characterize the isolates of citrus and coffee using the RAPD (Random Amplified Poliforfism DNA) technique, and to build a dendogram for these isolates based on their similarity degree.

MATERIAL AND METHODS

Citrus and coffee plants used for isolation

Six year-old trees showing symptoms of CVC and CLS from North, South, East, and West of the Sao Paulo and Paraná states (Brazil), from Missiones (Argentina) and California (USA) were used in this trial. Symptomatic mature leaves of 'Pera' and 'Valencia' orange trees and 'Mundo Novo' coffee were removed to obtain extracts from the vessel tissues. In this way, 16 isolates were obtained (Table 1) from citrus and coffee which were used for all trials, from 1998 to 2000.

Growth medium

The periwinkle medium (PW) was used for bacterial growth, and consisted of soy peptone 4.0 g; hydrolyzed casein 1.0 g; K_2HPO_4, 1.2 g; KH_2PO_4, 1.0 g; $MgSO_4$ $7H_2O$, 0.4 g; agar, 12.0 g; hemine chloride (0.1%), 10.0 mL; phenol red (0.2%), 10.0 mL; distilled water, 920.0 mL; bovine serum albumin (BSA) (6.7%), 6.0 g/60.0 mL and glutamine (8.0%), 4.0 g/100.0 mL. The BSA and glutamine were diluted in water and then filtered (0.45 and 0.22 μm) before being added to the previously autoclaved medium.

Bacteria isolation technique

Leaves were placed on a slide glass and the petioles chopped. Then, petioles were sterilized using 70% ethanol during one min, washed three times in distilled water, exposed to 3% sodium hypochlorite during three min, and then washed three times in sterile distilled water (Lima et al., 1998).

For bacteria isolation, petioles were chopped into 2 mm sections, placed in a centrifuge tube (15 mL), adding 1 mL of PW liquid medium and centrifuged at 150 g during ten min at local temperature. Then, a fraction of 0.1 mL was removed from the supernatant and transferred to a glass tube containing 5.0 mL of PW medium.

***Xylella fastidiosa* culture**

Cultures were made on PW medium using agar (1.2%) or on liquid PW medium (pH 6.6) at 28°C. A dilution series was performed (1:100, 1:1000 and 1:10000) using bacteria cultured in liquid PW medium. Then, they were inoculated in solid PW medium to obtain isolated colonies. The colonies were removed and inoculated in PW liquid medium. This procedure was

Table 1 - Isolates of *X. fastidiosa* used on the trials.

Code	Location	Coordinates		Host	Cultivar
		Longitude	Latitude		
1	São Paulo, Cordeirópolis	47° 27' 25''	22° 28' 56''	Citrus	'Pera'
2	São Paulo, Jales	50° 32' 46''	20° 16' 09''	Citrus	'Pera'
3	São Paulo, Itapetininga	48° 03' 12''	23° 35' 31''	Citrus	'Pera'
4	São Paulo, São José Rio Preto	49° 22' 47''	20° 49' 12''	Citrus	'Pera'
5	São Paulo, Macaubal	49° 57' 51''	20° 48' 22''	Citrus	'Pera'
6	Missiones, Argentina	55° 40' 12''	27° 47' 23''	Citrus	'Valencia'
7	São Paulo, Matão	48° 21' 58''	21° 36' 13''	Coffee	'Mundo Novo'
8	Paraná, Londrina	51° 09' 47''	23° 18' 38''	Citrus	'Pera'
9	São Paulo, Taquaritinga	48° 30' 18''	21° 24' 23''	Citrus	'Pera'
10	São Paulo, Silvania	48° 19' 31''	21° 20' 14''	Coffee	'Mundo Novo'
11	São Paulo, Casa Branca	47° 05' 12''	21° 46' 27''	Coffee	'Mundo Novo'
12	São Paulo, Franca	47° 24' 04''	20° 32' 20''	Coffee	'Mundo Novo'
13	São Paulo, São Manuel	48° 34' 15''	22° 43' 53''	Coffee	'Mundo Novo'
14	São Paulo, Marília	49° 56' 46''	22° 12' 51''	Coffee	'Mundo Novo'
15	São Paulo, São José Rio Preto	49° 22' 47''	20° 49' 12''	Coffee	'Mundo Novo'
16	ATCC 35870 - California, USA	117° 17' 20''	34° 06' 30''	Almond	---

repeated three times in order to obtain a 3-time cloned isolate.

Extraction and amplification of DNA by the RAPD technique

Genomic DNA extraction of bacteria isolates was performed as described by Costa et al. (2000).

The isolates of *X. fastidiosa* were analyzed using the RAPD technique to know the level of genetic similarity among them. The genomic DNA was amplified through the reaction using six 10-base primers (Pharmacia Biotec). Primers and annealing temperatures were: 5'GGTGCGGGAA (34°C); 5'GTTTCGCTCC (32°C); 5'GTAGACCCGT (32°C); 5'AAGAGCCCGT (32°C); 5'AACGCGCAAC (32°C) and CCCGTCAGCA (34°C). The PCR conditions were: 94°C during 5 min, followed by 43 cycles at 94°C for 1 min, 35°C for 2 min, and 72°C for 2 min. After then, an additional step of 72°C was performed during 7 min. The band analyses were performed as conventionally by using 1% agarose gel electrophoresis (Figure 1).

Data analysis

Electrophoretic band patterns were compared to construct a binary matrix. The Jaccard - UPGMA (unweighted pair group method with arithmetic averages) method was used to calculate the genetic similarities using NTSYS-PC (Rohlf, 1989).

RESULTS AND DISCUSSION

Based on the dendogram (Figure 2), it was possible to classify the isolates into five groups: Group A (1, 4, 2 and 5), Group B (9, 12 and 14), Group C (7, 11, 10 and 13), Group D (3, 6, 8 and 15) and Group E (16). Groups A and D presented a prevalence of citrus isolates; groups B and C of coffee isolates, and group E consisted only of the almond isolate. In relation to the genetic distance, in groups with the prevalence of

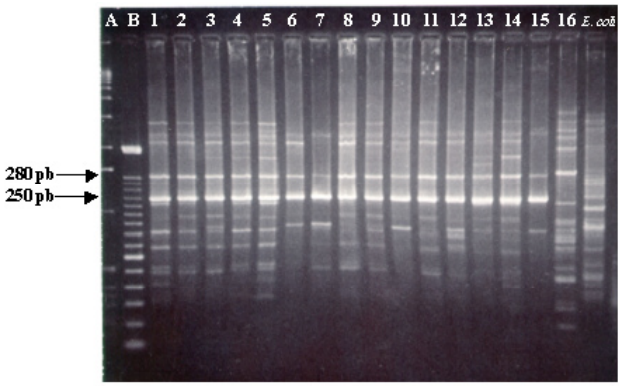

Figure 1 - RAPD of 16 isolates of *X. fastidiosa* using the primer 5 GGTGCGGGAA. *E. coli* profile was used as control. A and B are molecular standards.

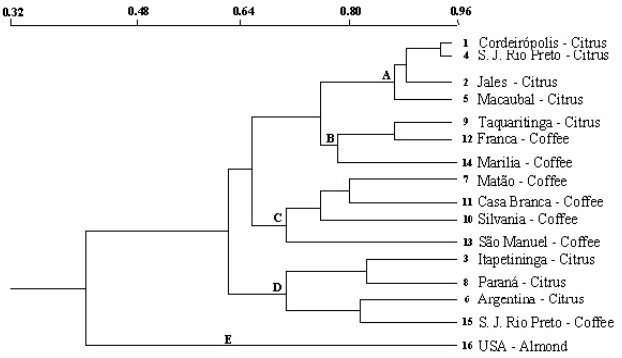

Figure 2 - Dendogram based on the genetic similarity among 16 *X. fastidiosa* isolates.

citrus isolates (A and D), this index ranged from 12 and 30%, approximately. Groups with prevalence of coffee isolates (B and C) it ranged from 22 and 30% and the almond isolate (E) showed a genetic distance of 58% in relation to the other groups. Rosato et al. (1999) analyzed 42 *X. fastidiosa* isolates of citrus, coffee, oleander and grapevine, and using eight distinct RAPD primers, classified the isolates of coffee and citrus into distinct groups.

Costa et al. (2000) classified citrus, coffee, plum and grapevine isolates into different groups using a distinct set of arbitrary primers in relation to those used in this research. These authors also observed a genetic divergence between isolates of citrus and coffee of 15%. Lacava et al. (2001) analyzed 16 bacteria isolates from citrus, two from coffee and one from grapevine, and classified them into distinct groups. The genetic divergence between citrus and coffee isolates was less than 30%. In another report, Qin et al. (2001) analyzed 78 isolates of *X. fastidiosa* from citrus, coffee, plum, grapevine, oak, almond and mulberry, classifying citrus and coffee isolates into different groups, with a genetic divergence of 20%. These results are very similar to those here observed.

Despite the results here shown and those observed in other reports that grouped the citrus and coffee *X. fastidiosa* isolates into distinct groups, today it is widely known that coffee derived isolates are able to infect and produce symptoms on citrus plants, indicating those genetic divergences are not sufficient to hinder the cross inoculation between *X. fastidiosa* isolates.

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