

# Molecular Characterization of Brazilian Strains of *Xanthomonas campestris* pv. *viticola* by rep-PCR Fingerprinting

Loiselene C. Trindade<sup>1\*</sup>, Mirtes F. Lima<sup>2</sup> & Marisa A. S. V. Ferreira<sup>1</sup>

<sup>1</sup>Departamento de Fitopatologia, Universidade de Brasília, CEP 70910-900, Brasília, DF, fax (61) 272-1793, e-mail: marisavf@unb.br; <sup>2</sup>EMBRAPA- Semi Árido, Cx. Postal 23, CEP 56300-970, Petrolina, PE

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Corresponding author: Marisa A.S.Velloso Ferreira

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## ABSTRACT

Bacterial canker of grapevine (*Vitis vinifera*), caused by *Xanthomonas campestris* pv. *viticola* was first detected in Brazil in 1998, affecting grapevines in the São Francisco river basin, state of Pernambuco. The disease was also reported in Juazeiro, Bahia and later in Piauí and Ceará. Due to its limited geographical distribution and relatively recent detection in Brazil, very little is known about the pathogen's biology and diversity. Repetitive DNA based-PCR (rep-PCR) profiles were generated from purified bacterial DNA of 40 field strains of *X. campestris* pv. *viticola*, collected between 1998 and 2001 in the states of Pernambuco, Bahia and Piauí. Combined analysis of the PCR patterns obtained with primers REP, ERIC and BOX, showed a high degree of similarity among Brazilian strains and the Indian type strain NCPPB 2475. Similar genomic patterns with several diagnostic bands, present in all strains, could be detected. Fingerprints were distinct from those of strains representing other pathovars and from a yellow non-pathogenic isolate from grape leaves. The polymorphism observed among the Brazilian strains allowed their separation into five subgroups, although with no correlation with cultivar of origin, geographic location or year collected.

**Additional keywords:** diversity, grapevine bacterial canker, *Vitis vinifera*, repetitive DNA.

## RESUMO

### Caracterização molecular de isolados brasileiros de *Xanthomonas campestris* pv. *viticola* através de rep-PCR

O cancro bacteriano da videira (*Vitis vinifera*), causado por *Xanthomonas campestris* pv. *viticola*, foi primeiramente detectado no Brasil em 1998, em parreiras no município de Petrolina, Vale do São Francisco, Pernambuco. A doença foi também observada em Juazeiro, Bahia, e mais tarde nos estados de Piauí e Ceará. Devido à sua limitada distribuição geográfica e relativamente recente detecção no Brasil, pouco se conhece sobre a biologia e diversidade do patógeno. Padrões genômicos de rep-PCR foram gerados a partir do DNA purificado de 40 isolados de *X. campestris* pv. *viticola*, coletados entre 1998 e 2001, em diferentes localidades dos estados de Pernambuco, Bahia e Piauí. A análise combinada dos padrões obtidos com os "primers" REP, ERIC e BOX, mostraram alta similaridade entre os isolados brasileiros e o isolado tipo NCPPB 2475, originário da Índia. Os isolados brasileiros apresentaram padrões similares e algumas bandas diagnósticas, presentes em todos os isolados. Os padrões foram distintos dos isolados de outros patovares e de um isolado não-patogênico obtido de folhas de videira. O polimorfismo observado entre os isolados brasileiros permitiu a diferenciação de cinco subgrupos, sem nenhuma relação com cultivar de origem, local ou época de coleta.

**Palavras-chave adicionais:** diversidade, cancro bacteriano, *Vitis vinifera*, DNA repetitivo.

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## INTRODUCTION

In 1998, a new disease was reported affecting 'Red Globe' grapevines (*Vitis vinifera* L.) in Pernambuco and Bahia states. A gram-negative bacterium was isolated from all diseased plants and Koch's postulates were completed. The causal agent was identified through biochemical and pathogenicity tests as *Xanthomonas campestris* pv. *viticola* Nayudu (Dye) (Malavolta Jr. *et al.*, 1999a; Lima *et al.*, 1999). Later, the disease was also detected in the states of Piauí (Malavolta *et al.*, 1999b) and Ceará (Freire & Oliveira, 2001).

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This bacterium forms typical non-pigmented, smooth, round, mucoid, convex colonies with entire margins. Diagnostic features of tested Brazilian strains were: gram-negative, metabolism strictly aerobic, urease and oxidase negative, asparagine not utilized as the sole source of carbon and nitrogen, tolerance up to 2% NaCl and acid production from glucose, mannose, galactose, trehalose, cellobiose and fructose (Lima *et al.*, 1999). Colonies were visible after 48 to 72 h at 28 °C and 33 °C (Lima *et al.*, 1999), but no growth occurred at 41 °C (Malavolta Jr. *et al.*, 1999a). The hypersensitivity test was negative on tobacco (*Nicotiana tabacum* L.) leaves but positive on tomato (*Lycopersicon esculentum* Mill.) cv. "Santa Clara" (Malavolta Jr. *et al.*, 1999a). Symptoms on grapevine

plants could be detected from eight to 14 days after artificial inoculation depending on the method of inoculation used (Malavolta Jr. *et al.*, 1999a; Lima *et al.*, 1999).

*Xanthomonas campestris* pv. *viticola* was first described by Nayudu (1972) in India as *Pseudomonas viticola* Nayudu sp. nov., and its occurrence is apparently very restricted. Besides India, there are also reports of a "grapevine bacterial canker disease (GVBCD)" in Ukraine (Kochenko, 1993). Since the pathovar *viticola* has not been included in the reclassification work of Vauterin *et al.* (1995), its specific status may still be controversial. As with the pathovar *mangiferaeindicae* of the genus *Xanthomonas*, all strains could be referred to as members of the pathovar *viticola*. We chose here to use the name proposed by Dye (1978), considering that it has been used in all references to this disease found to date and until more information on their species classification is available.

Several molecular methods have been used to assess diversity and to detect plant pathogenic bacteria (Louws *et al.*, 1999). The presence of repetitive DNA sequences is common in the prokaryotic and eukaryotic genomes. In bacteria, the observed occurrence of three families of repetitive sequences, REP, ERIC and BOX elements (Stern *et al.*, 1984; Sharples & Lloyd, 1990; Koeuth *et al.*, 1995) led to the development of repetitive DNA PCR-based genomic fingerprinting protocols (referred as rep-PCR), in which primers were designed to amplify intervening DNA between two adjacent repetitive sequences (Louws *et al.*, 1999). The amplified fragments, then separated by electrophoresis on agarose gels, generate specific fingerprints (DeBruijn, 1992). Repetitive-PCR has been successfully used for identification and differentiation of strains and the assessment of genetic diversity of phytopathogenic bacteria such as *Xanthomonas* spp. and *Pseudomonas* spp. (Louws *et al.*, 1994, 1995; Opgenorth *et al.*, 1996; Weingart & Volksch, 1997; Restrepo *et al.*, 2000), *Clavibacter michiganensis* (Smith) Davis *et al.* (Louws *et al.*, 1998), and *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* (Horita & Tsuchiya, 2001).

Due to its relatively recent detection in Brazil, little is known about the biology and diversity of *X. campestris* pv. *viticola*. The objective of this study was to compare several field strains of *X. campestris* pv. *viticola*, collected from 1998 to 2001, using rep-PCR fingerprinting. The molecular characterization of these isolates could provide information not only on their diversity but also on their origin. Specific fingerprints may also be helpful in the identification process leading to a more rapid and effective diagnosis of grapevine bacterial canker.

## MATERIALS AND METHODS

### Bacterial strains: source and maintenance

Forty grapevine bacterial canker-derived strains previously identified as *X. campestris* pv. *viticola* were used in this study (Table 1). Reference strain NCPPB 2475 (originated from India), another 18 *Xanthomonas* sp. strains from various

host plants and one *Ralstonia solanacearum* strain were included for comparison purposes. One unidentified isolate, commonly isolated from grape leaves, was also included. This isolate grew on asparagine rich-medium, formed yellow, fast-growing and gram-negative colonies and did not induce a hypersensitive reaction on tobacco leaves. All isolates were cultivated on Kado's 523 medium (Kado & Heskett, 1970). For long-term maintenance, isolates were preserved on sterile distilled water at room temperature and frozen in 30% glycerol at  $-80^{\circ}\text{C}$ .

### Biochemical and pathogenic characterization

Initially all grapevine-derived isolates were identified and characterized according to conventional biochemical and physiological tests (Bradbury, 1986; Lelliott & Stead, 1987; Schaad *et al.*, 2001), except those obtained from other culture collections (IBSBF 1385, 1386 and 1369, and NCPPB 2475). The tests conducted were as described by Lima *et al.* (1999), except that the hypersensitivity test on tomato (cv. Santa Cruz), and growth on triphenyl tetrazolium chloride-containing medium (TTC) tests were included. All isolates were tested for pathogenicity by inoculation of petioles and leaves of grapevine cv. Catalunha after superficial wounding with a sterile needle.

### DNA isolation

Total bacterial DNA was extracted using a modified version of the CTAB method from Ausubel *et al.* (1992). Single colonies grown on Kado's 523 medium were transferred to 1.5 ml of 523 liquid medium and cultures were grown at  $28^{\circ}\text{C}$ , with shaking at 150 rpm. After 48 h the cultures were centrifuged at 4,600 g (8,000 rpm) for 5 min, the supernatant was removed and the pellets were resuspended in 520  $\mu\text{l}$  of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3  $\mu\text{l}$  of Proteinase K (20 mg/ml) were added. The mixture was incubated for 1 hour at  $37^{\circ}\text{C}$ , then 100  $\mu\text{l}$  of 5 M NaCl and 80  $\mu\text{l}$  of a 10% CTAB solution in 0.7 M NaCl were added and mixed. The suspension was incubated for 10 min at  $65^{\circ}\text{C}$  and kept on ice for 15 min. An equal volume of chloroform:isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7,200 g (10,000 rpm) for 20 min. The aqueous phase was transferred to a new tube, 0.6 volumes of isopropanol were added and DNA was precipitated at  $-20^{\circ}\text{C}$  for 16 h. DNA was collected by centrifugation at 7,200 g (10,000 rpm) for 10 min, washed with 500  $\mu\text{l}$  of 70% ethanol, air-dried at room temperature for approximately 3 h and finally resuspended in 50  $\mu\text{l}$  of TE buffer. The DNA was quantified on 0.8% agarose gels by comparison with DNA samples (phage  $\lambda$  DNA-*Hind* III fragments) of known concentration, diluted to working aliquots of 50 ng/ $\mu\text{l}$  and stored at  $-20^{\circ}\text{C}$  until needed.

### Repetitive elements-based PCR (rep-PCR)

Amplification reactions were conducted with three primer sets: (1) REP1R-I (5' IICGICGICATCIGGC 3') and REP2-I (5' ICGITTATCIGGCCTAC 3'); (2) ERIC1R (5' ATGTAAGCTCCTGGGGATTCA 3') and ERIC2 (5' AAGTAA GTGACTGGGGTGAGCG 3'), and (3) primer BOXA1R (5'

TABLE 1 - Species and strains used in this study

Strain	Host	Location and year collected
<i>Xanthomonas campestris</i> pv. <i>viticola</i>	<i>Vitis vinifera</i>	
NCPBP <sup>a</sup> 2475	cv. Anabi-e-Shahi	India, 1972
UnB <sup>b</sup> 1183, 1185, 1186, 1187, 1188, 1189, 1190, 1193, 1194, 1195, IBSBF <sup>c</sup> 1369	cv. Red Globe	Petrolina, PE, 1998
1191, 1192	cv. Itália	Petrolina, PE, 1998
IBSBF 1385	cv. Itália	Teresina, PI, 1998
IBSBF 1386	cv. Ribier	Teresina, PI, 1998
UnB 1201, 1202, 1203, 1206, 1207, 1208, 1209	cv. Itália	Petrolina, PE, 2000
UnB 1204	cv. Red Globe	Juazeiro, BA, 2000
UnB 1216, 1218	cv. Red Globe	Petrolina, PE, 2000
UnB 1205	cv. Itália	Sobradinho, BA, 2000
UnB1210	cv. IAC 572 (rootstock)	Petrolina, PE, 2000
UnB 1211	cv. Catalunha	Petrolina, PE, 2000
UnB 1212	cv. Itália	Petrolina, PE, 2001
UnB1213	cv. Red Globe	Petrolina, PE, 2001
UnB 1214, 1219, 1220, 1221	cv. Catalunha	Petrolina, PE, 2001
UnB 1222	cv. Perlette	Petrolina, PE, 2001
UnB 1223, 1224	cv. Red Globe	Juazeiro, BA, 2001
UnB 1225	cv. Festival	Petrolina, PE, 2001
UnB 1226	cv. Thompson Seedless	Petrolina, PE, 2001
UnB 1227	cv. Itália	Petrolina, PE, 2001
<i>X. axonopodis</i> pv. <i>glycines</i>		
UnB 306	soybean	Planaltina, DF, 1983
<i>X. axonopodis</i> pv. <i>manihotis</i>		
UnB 1126	cassava	Jataí, GO, 1996
UnB 1114	cassava	Mirador, PR, 1996
UnB 1111	cassava	Paranavaí, PR, 1996
UnB 1129	cassava	Rolândia, PR, 1996
<i>X. axonopodis</i> pv. <i>passiflorae</i>		
UnB 642	passionfruit	Planaltina, DF, 1986
<i>X. axonopodis</i> pv. <i>ricini</i>		
UnB 607 (white colonies)	castor	Ibiapina, CE, 1985
<i>X. campestris</i> pv. <i>campestris</i>		
UnB 159	cabbage	Vargem Bonita, DF, 1981
UnB 606	cabbage	Guaramiranga, CE, 1985
<i>X. campestris</i> pv. <i>carotae</i>		
UnB 107	carrot	Vargem Bonita, DF, 1981
<i>X. campestris</i> pv. <i>mangiferaeindicae</i>		
UnB 769 (yellow)	mango	Planaltina, DF, 1990
UnB 764 (white)	mango	Planaltina, DF, 1990
IBSBF 1230 (white)	mango	Brasil, 1996
IBSBF 1508 (white)	cashew	Brasil, 1999
<i>X. campestris</i> pv. <i>vesicatoria</i>		
UnB 604	tomato	Ibiapina, CE, 1985
UnB 605	tomato	CE, 1985
UnB 1174	pepper	DF, 1998
<i>X. melonis</i>		
UnB1080	cucumber	Vargem Bonita, DF, 1995
<i>Ralstonia solanacearum</i>		
UnB 486	potato	Lagoa Seca, PB, 1984
unidentified non-pathogenic isolate (NI)	<i>Vitis vinifera</i> cv. Catalunha	Petrolina, PE, 2001

<sup>a</sup> National Collection of Plant Pathogenic Bacteria, York, UK.

<sup>b</sup> Coleção de Bactérias Fitopatogênicas da Universidade de Brasília, Brasília, D.F.

<sup>c</sup> Instituto Biológico Setor de Bacteriologia Fitopatológica, Campinas, S.P.

CTCCGCAAGGCGACGCTGAC 3'), corresponding to REP, ERIC and BOX elements, respectively (Louws *et al.*, 1999). Primers were synthesized by Life Technologies do Brasil Ltda. Each reaction consisted of: 1.2 µl of 10X Taq buffer (500 mM KCl, 100 mM TRIS-HCl); 1 mg/ml BSA; 1.5 mM MgCl<sub>2</sub>; 0.2 mM each dNTP (GIBCO, BRL); 2 µM of each primer, 1.25 U of Taq DNA polymerase (GIBCO, BRL) and 50 ng of bacterial DNA, in

a final volume of 12 µl.

The PCR was carried out in a Gene Amp-PCR System 2400 thermal cycler (Applied Biosystems). With REP primers, REP1R-I and REP2-I, amplification began with a denaturation step at 95 °C for 7 min, followed by 35 cycles of 94 °C/1 min; 44 °C/8 min, and 65 °C for 8 min. With ERIC and BOX primers, cycling programmes started with denaturation at 95 °C for 7

min, followed by 30 cycles of: 94 °C/1 min; 1 min at 52 °C (ERIC) or 53 °C (BOX) for primer annealing, and 8 min at 65 °C for primer extension. After the extension step, the temperature was held at 65 °C for 15 min for all three primer sets. For each bacterial strain, reactions were repeated at least twice.

The PCR products were separated by electrophoresis on 1.5% agarose gels. Total volume of the reaction (12 µl) was analyzed. Electrophoresis was carried out at 80 V for 4 h in 0.5 X Tris-borate EDTA buffer. Gels were stained with ethidium bromide (0.5 µg/ml), and DNA migration patterns were analyzed visually. Sizes of fragments were determined by comparison with DNA molecular weight markers, 1 Kb and 100 bp DNA ladders (GIBCO, BRL).

Genomic fingerprint comparisons among bacterial strains were performed by measurement of band sizes. The presence or absence of each band was converted into binary data, scored as 1 for presence, and 0 for absence. Only the reproducible bands ranging from 200 bp to 3 kb were scored for each strain. Data generated with the three sets of primers were combined and analyzed using software MVSP 3.1 (Kovach, 1999). Genetic relationships among isolates were determined by Jaccard's similarity coefficients followed by UPGMA (unweighted pair-group method using arithmetic averages) clustering analysis. The reliability of the dendrograms was assessed by bootstrap analysis (Swofford & Olsen, 1990) with the software program WinBoot (Yap & Nelson, 1996) with 1,000 repeated samplings.

## RESULTS

All 37 field strains dated from 1998-2001, isolated from *V. vinifera* plants with bacterial canker symptoms showed biochemical properties of the genus *Xanthomonas* (Schaad *et al.*, 2001) and, apart from colony color, they could not be differentiated from a *X. campestris* pv. *campestris* (Pammel) Dowson strain (UnB 159) through biochemical and physiological tests. The pathogenicity assay on grape leaves and petioles along with the biochemical characteristics, confirmed the identity of all strains as the pathogen described by Nayudu (1972).

The DNA amplification from 41 *X. campestris* pv. *viticola* strains with primers REP, ERIC and BOX (rep-PCR) showed multiple fragments varying from 190 bp to 3054 bp. Initially, several tests with REP primers were carried out in order to assess the reproducibility of the method with purified DNA and whole cells collected directly from agar or liquid cultures grown for 24-48 h. The cells were resuspended in sterile water and 1 µl was used in the amplification reaction. The REP-PCR banding patterns were reproducible only when purified DNA was used. Only the major and more intense bands of 1 kb and 600 bp were visualized when using whole cells in the amplification reactions. With purified DNA, the same electrophoretic patterns were produced from independent amplification reactions and also from two independent DNA extractions of the same strain (data not shown).

With ERIC primers, 19 bands varying from 190-3054 bp

were visualized and scored (Figure 1). The majority of strains and the type strain NCPPB 2475 showed a similar pattern overall (Figure 1A), with several diagnostic bands. Polymorphic bands were also observed, and nine patterns could be differentiated. The genomic patterns of *X. campestris* pv. *viticola* were visually distinct from a non-pathogenic isolate (NI) and other xanthomonads (Figure 1B).

With primer BOX A1R a total of 12 bands varying from 210 bp to 2,036 bp were scored. An 800 bp-fragment was observed in the amplification patterns of 38 *X. campestris* pv. *viticola* strains. However, strains UnB 1212, 1222 and 1227 did not present that band. Visual analysis of the BOX fingerprinting patterns revealed five distinct patterns among the grape strains. Polymorphic bands were mostly detected in the region of 600-1000 bp. A high degree of similarity between Brazilian strains and the Indian strain was also observed (Figure 2).

The REP primers produced 16 scoreable bands ranging from 250 to 2900 bp. Seven distinct patterns were observed among *X. campestris* pv. *viticola* strains, with a 1,000 bp-band present in all strains. Polymorphism was observed between bands of 500 and 1000 bp.

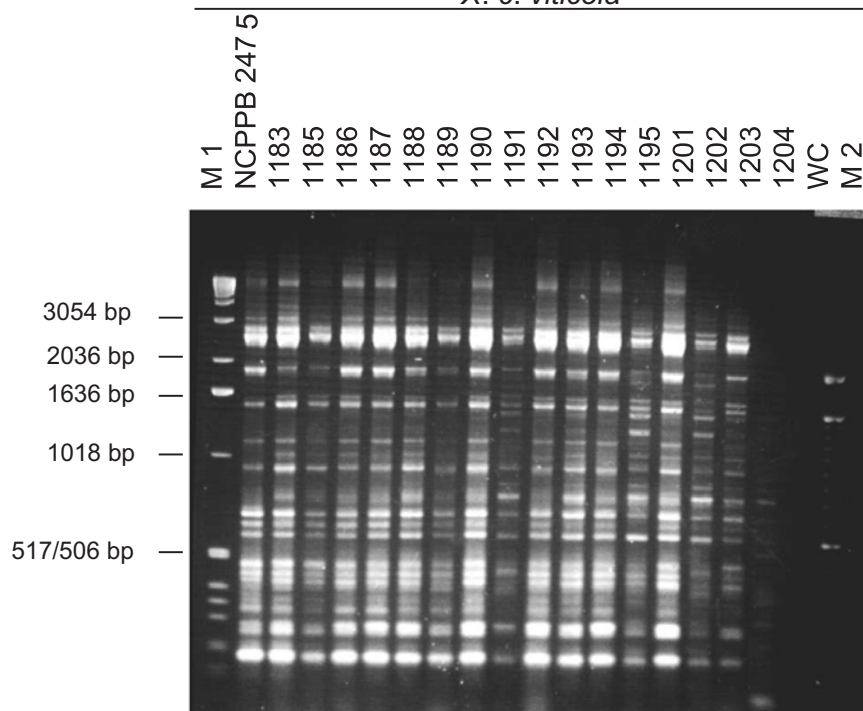
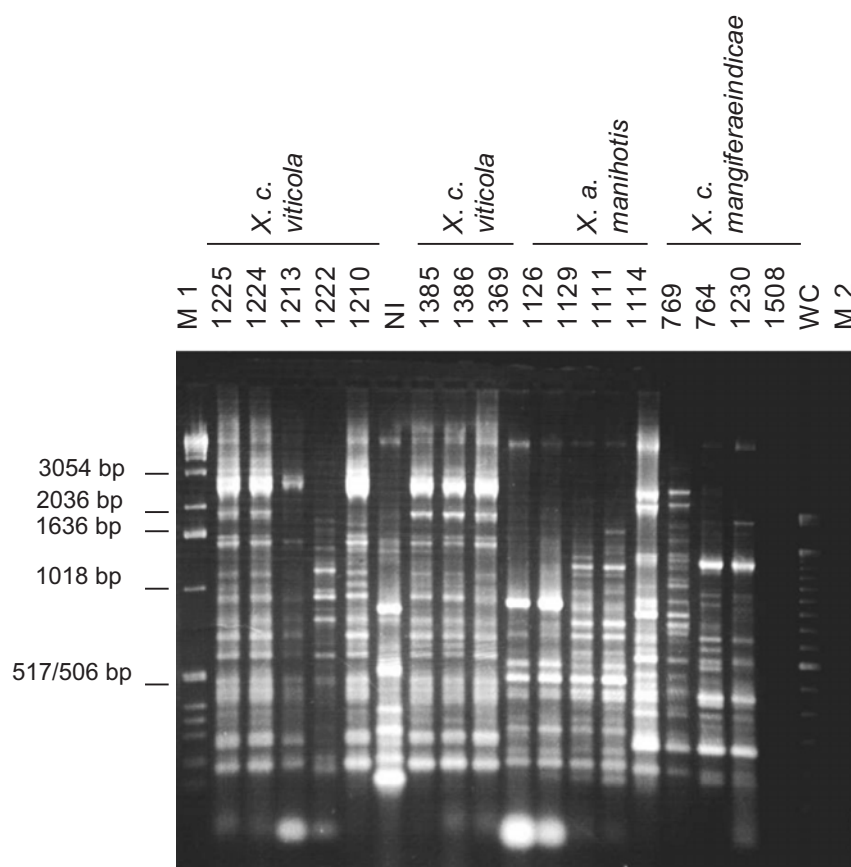
The PCR products of all 41 pathovar *viticola* strains, regardless of the geographic location, host cultivar or year collected, showed an overall homogeneous pattern sharing multiple bands (Figures 1 and 2). For all three primers used, the non-pathogenic yellow strain (NI) showed a distinct banding pattern from the *Xanthomonas* spp. strains.

Considering only the grapevine-derived strains and the combined data from the three primer sets, two distinct groups could be differentiated at 26% similarity: group 1, formed by the non-pathogenic strain (NI) only, and group 2, comprising all pathovar *viticola* strains (Figure 3). At 68% similarity, five subgroups were detected among the pathogenic strains. Thirty-two strains, representing 78% of the strains, formed one single group, which included the type strain (NCPPB 2475), 27 strains from Pernambuco, the two strains from Piauí (IBSBF 1385 and 1386) and two strains from Bahia (UnB 1224 and 1205). Reference strain NCPPB 2475 showed above 80% similarity to 29 grapevine strains (~73% of the tested strains).

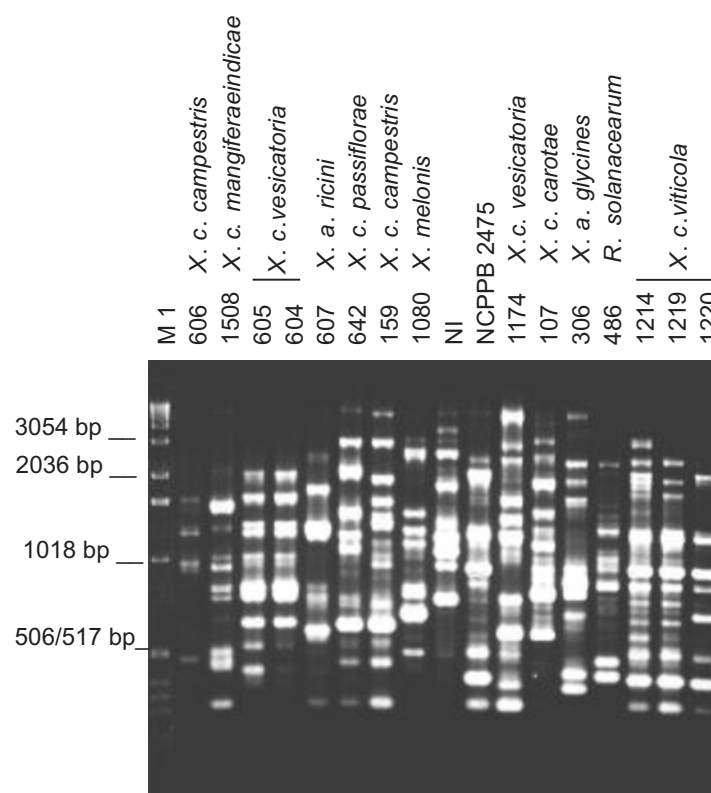
Dendrograms obtained with each individual set of primers showed an overall similar pattern of separation when compared to the combined analysis (data not shown). Of the three sets of primers tested, BOX was the most conserved, identifying a smaller number of subgroups among the strains.

Pathovar *viticola* strains representing all geographic locations and different rep-PCR groups (Figure 3) were selected and compared with other *Xanthomonas* sp. strains, associated with different hosts and one *R. solanacearum* strain. The combined analysis of the patterns generated by BOX, ERIC and REP-PCR showed the separation into two groups at 15% similarity (Figure 4). The first group was composed only of *Ralstonia solanacearum* (UnB 486), the second and largest group included all xanthomonads and the 12 selected *X. campestris* pv. *viticola* strains. Three mango (*Mangifera indica* L.) strains of *X. campestris* pv. *mangiferaeindicae* (Patel, Moniz & Kulkarni) Robbs *et al.*, UnB 764 (white), UnB



*X. c. viticola***A****B**

**FIG. 1** - Genomic fingerprints of *Xanthomonas* spp. generated from ERIC-PCR. (A) field strains of *X. campestris* pv. *viticola* collected in Brazil compared to type strain NCPPB 2475. (B) field strains of *X. campestris* pv. *viticola* collected in Brazil compared to other *Xanthomonas* spp. from different host species. NI: unidentified strain; WC: water control; M: molecular size standards 1 Kb DNA ladder (M1) and 100 bp-DNA ladder (M2). PCR products were separated by electrophoresis on 1.5% agarose gels.



**FIG 2** - Genomic fingerprints of *Xanthomonas* spp. from various host species and four strains of *X. campestris* pv. *viticola* generated from BOX-PCR. Lane M1: molecular size standard, 1 Kb- DNA ladder; NI: unidentified strain. PCR products were separated by electrophoresis on 1.5% agarose gel.

769 (yellow) and IBSBF 1230 (white), showed the highest similarity to *X. campestris* pv. *viticola*. Among the *X. campestris* pv. *mangiferaeindicae* strains, the strain IBSBF 1508 (white) from cashew was the most divergent. All other xanthomonads were grouped separately with similarity levels below 36% in relation to the grapevine strains.

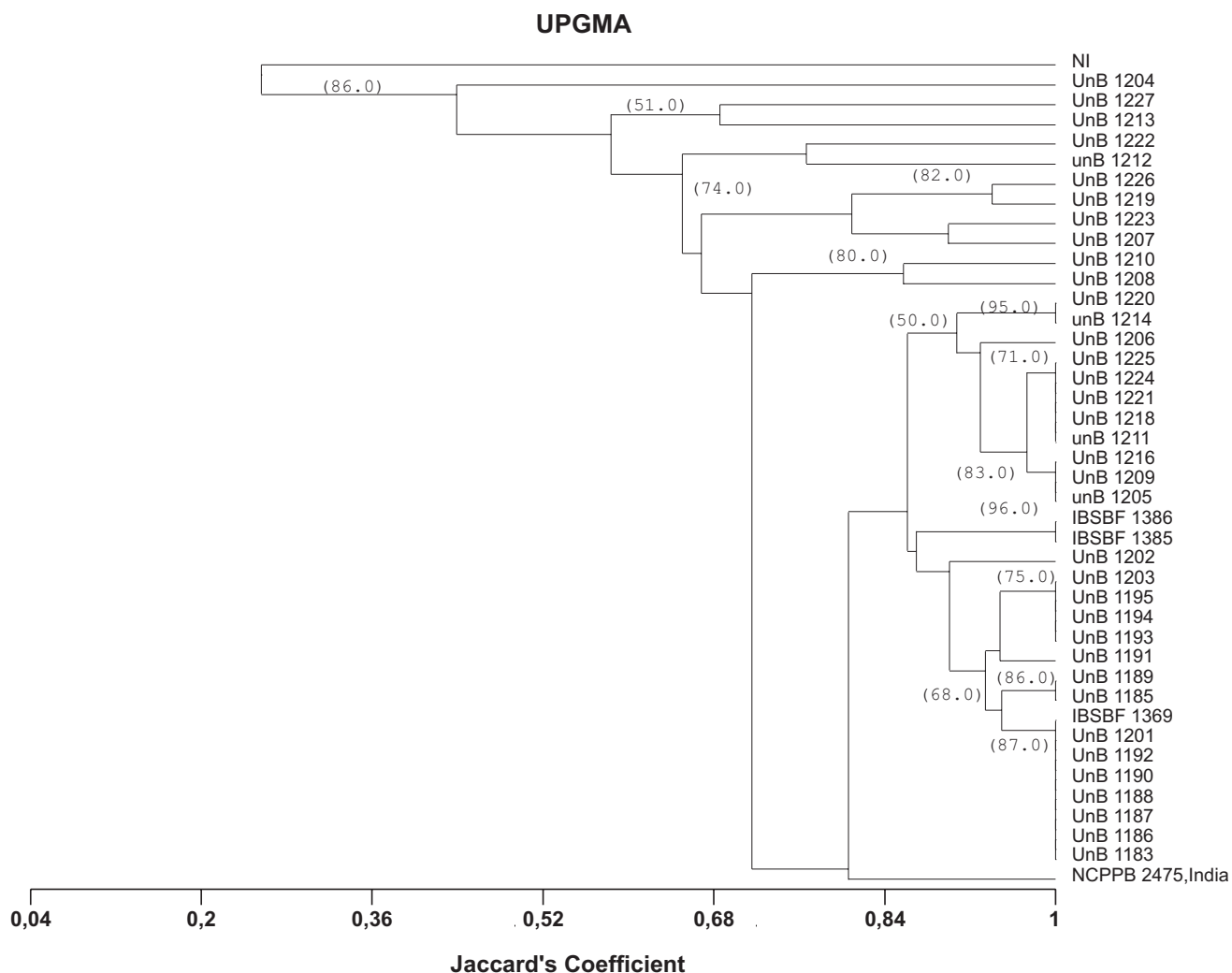
## DISCUSSION

Reproducible genomic fingerprints were produced for all field strains of *X. campestris* pv. *viticola*, collected in three different years (1998, 2000 and 2001), different locations and isolated from different grape cultivars. Although most bands were present in all tested strains, there were differences in the intensity of some amplified fragments as well as in the occurrence of several polymorphic bands. The rep-PCR fingerprints clearly differentiated the grapevine strains from the *R. solanacearum* strain as well as from the unidentified yellow strain and members of other pathovars, all of which produced unique profiles (Figures 1 and 2). In the genus *Xanthomonas*, rep-PCR has revealed both inter and intrapathovar variability (Louws *et al.*, 1994). For example, the studies of Louws *et al.* (1995) with *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye., confirmed the separation of this pathovar into two genetically distinct groups, thus confirming the taxonomic studies of Vauterin *et al.* (1995). On

the other hand, with primers REP and ERIC, a very high degree of homogeneity was found within a population of *Xanthomonas fragariae* Kennedy & King, pathogenic on strawberries (*Fragaria x ananassa* Duch.), collected during a four-year-period in California (Opgenorth *et al.*, 1996). Louws *et al.* (1994) suggested that intrapathovar diversity in *Xanthomonas* spp. could be grouped into two categories: (i) pathovars from which isolates had almost identical fingerprints or had overall unique profiles but shared multiple bands, and (ii) pathovars from which isolates could be divided into groups and did not share common banding patterns. The Brazilian strains of the pathovar *viticola* characterized in this study were found to belong to the first category.

The ERIC-PCR revealed more polymorphic bands among the strains than the other two sets of primers. Other studies have also shown differences in the ability of primers to reveal diversity in *Xanthomonas* spp. The REP element, for example, was the most conserved and the less efficient in revealing polymorphism in *X. campestris* pv. *vesicatoria* group B strains (Louws *et al.*, 1995). Within *Xanthomonas axonopodis* pv. *manihotis* (Bondar) Vauterin *et al.*, both REP and ERIC primers showed a high level of polymorphism among strains originating from different edaphoclimatic zones in Colombia (Restrepo *et al.*, 2000).

The great majority of field strains from Pernambuco, Piauí and Bahia, isolated from different cultivars over a three-

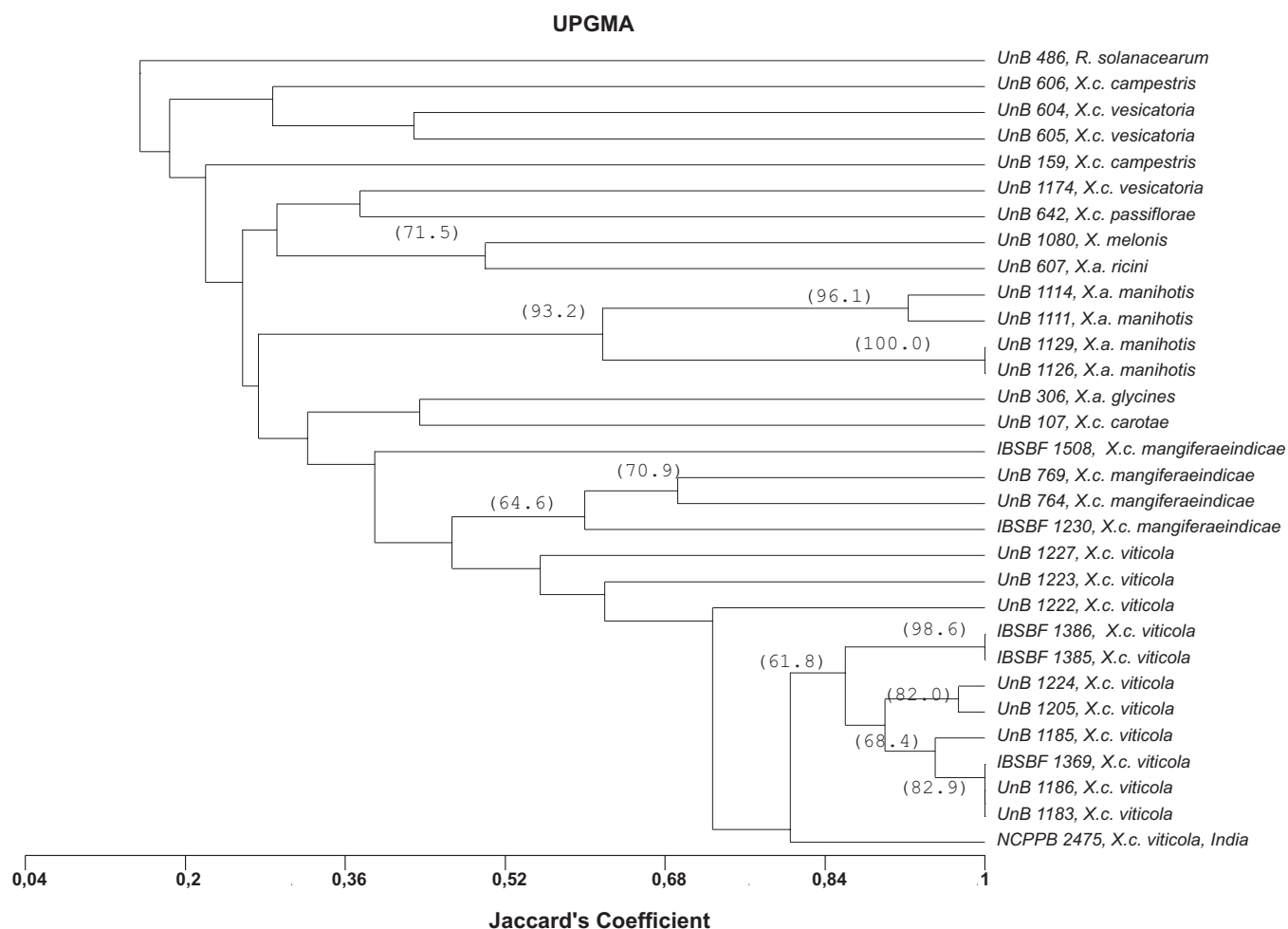


**FIG. 3** - Phenogram based on Jaccard's similarity coefficients among 40 *Xanthomonas campestris* pv. *viticola* strains collected in Brazil along with the type strain (NCPBP 2475) from India and a non-pathogenic yellow strain from grape (*Vitis* spp.) leaves (NI). Cluster analysis was performed using the UPGMA method. Values greater than 50% in 1,000 bootstrap samples are placed on the branches.

year period, showed similarity values above 80% in relation to the Indian strain. Considering their biochemical and physiological properties, the Brazilian and Indian strains have shown the same characteristics (Nayudu, 1972; Lima *et al.*, 1999; Araújo, 2001) except in the milk proteolysis test, which was negative for the strains from India studied by Chand & Kishun (1990). The high genetic similarity between Brazilian isolates and the Indian type strain supports the hypothesis that this bacterium may have originated in India, and that it was disseminated and introduced through contaminated planting material (Robbs & Rodrigues Neto, 1999). It is also possible that multiple introductions have occurred over time and/or that the bacterium may have been present in the country for a longer period, remaining undetected until the favorable climatic conditions of the summer of 1998 (Lima *et al.*, 1999). The development of irrigation projects in the São Francisco basin, including Bahia and Pernambuco started in the 1970's (Araújo, 2001). Introduction and transit of planting material

from other regions occurred and may have been responsible for the introduction of the bacterium. However, the lack of favorable environmental conditions may have prevented bacterial spread as the occurrence of an epidemic was not observed until 1998. Another hypothesis is that the bacteria may have been introduced by other potential hosts like mango and neem (*Azadirachta indica* A. Juss.). It is not known if the bacterium is naturally associated with these hosts in the area. Occurrence of symptomatic mango or neem plants has not been reported.

*Xanthomonas campestris* pv. *viticola* and *X. campestris* pv. *mangiferaeindicae* shared several bands and were found to be the most genetically similar. Both bacteria were originally described in India and are currently present in the northeast region of Brazil (Nayudu, 1972; Robbs *et al.*, 1978). Some grape-producing areas in the São Francisco basin also grow mango trees and use neem plants as wind-breakers. Both mango and neem have been reported as hosts of the pathovar *viticola*. It



**FIG 4** - Phenogram based on Jaccard's similarity coefficients showing relationships between the Brazilian strains of *Xanthomonas campestris* pv. *viticola*, the type strain from India (NCPBP 2475) and other bacterial species and genera. Cluster analysis was performed using the UPGMA method. Values greater than 50% in 1,000 bootstrap samples are placed on the branches.

should be considered that the use of neem for protecting vineyards may also represent a source of genetic variation since this species can be naturally infected thus providing more inoculum for potential disease outbreaks (Nayudu, 1972; Nascimento *et al.*, 2001).

The rep-PCR fingerprinting allowed us to identify *X. campestris* pv. *viticola* strains by the presence of several diagnostic bands present in all genomic fingerprints. Those bands were not present in the fingerprinting patterns of other pathovars, *R. solanacearum*, or the yellow strain associated with grape leaves. Intrapathovar variation was also detected and its significance should be further investigated with additional molecular markers. The lack of correlation between rep-PCR groupings and geographic origin of strains is an indication that this bacterium has spread among different vineyards in the Petrolina-Juazeiro area. Correlation between rep-PCR groupings and cultivar of origin or year of isolation were not evident either. The existing diversity in the pathovar *viticola* should be considered when adopting control measures based on selection of resistant material. Our study indicates

that rep-PCR fingerprinting can be considered an important tool for identifying and monitoring the diversity of this pathogen in the affected areas.

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#### LITERATURE CITED

- ARAÚJO, J.S.P. Perfil epidemiológico e subsídios para controle de *Xanthomonas campestris* pv. *viticola* (Nayudu) Dye, agente do cancro bacteriano da videira (*Vitis vinifera* L.) no Brasil. (Tese de Doutorado). Rio de Janeiro. Universidade Federal Rural do Rio de Janeiro. 2001.
- AUSUBEL, F.M., BRENT, R., KINGSTON, R.E., MOORE, D.D., SEIDMAN, J.G., SMITH, J.A. & STRUHL, K. (Eds). Short



- protocols in molecular biology. 2<sup>nd</sup> ed. London. Jonh Wiley & Sons Inc. 1992.
- BRADBURY, J.F. Guide to plant pathogenic bacteria Kew. C.A.B. International. 1986.
- CHAND, R. & KISHUM, R. Outbreak of grapevine bacterial canker disease in India. *Vitis* 29:183-188. 1990.
- DeBRUIJN, F.J. Use of repetitive (Repetitive Extragenic Palindromic and Enterobacterial Repetitive Intergenic Consensus) sequences and polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Applied and Environmental Microbiology* 58:2180-2187. 1992.
- DYE, D.W. Genus IX *Xanthomonas* Dowson 1939. In: Young, J.M., Dye, D.W., Bradbury, J.F., Panagopoulos, C.G. & Robbs, C.F. (Eds.). A proposed nomenclature and classification for pathogenic bacteria. *New Zealand Journal of Agricultural Research* 21:153-177. 1978.
- FREIRE, F.C.O. & OLIVEIRA, A.D.S. Ocorrência do cancro bacteriano da videira no Estado do Ceará. *Comunicado Técnico*, 62. Embrapa Agroindústria Tropical. 2001.
- HORITA, M. & TSUCHIYA, K. Genetic diversity of Japanese strains of *Ralstonia solanacearum*. *Phytopathology* 91:399-407. 2001
- KADO, C.I. & HESKETT, M. G. Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas* and *Xanthomonas*. *Phytopathology* 60:969-976. 1970.
- KOCHENKO, Z-I. Development of canker tumours on grape roots. *Zashchita-Rastenii-Moskva* 7:42-43. 1993.
- KOEUTH, T., VERSALOVIC, J. & LUPSKI, J. R. Differential subsequence conservation of interspersed repetitive *Streptococcus pneumoniae* BOX elements in diverse bacteria. *Genome Research* 5:408-418.1995.
- KOVACH, W.L. MVSP - Multivariate Statistical Package for Windows, Version 3.1 Pentraeth, Wales, UK. Kovach Computing Services. 1999.
- LELLIOTT, R.A & STEAD, D.E. Methods for diagnosis of bacterial diseases of plants. Oxford. Blackwell Science. 1987.
- LIMA, M.F., FERREIRA, M.A.S.V., MOREIRA, W.A. & DIANESE, J.C. Bacterial canker of grapevine in Brazil. *Fitopatologia Brasileira* 24:440-443. 1999.
- LOUWS, F.J., FULBRIGHT, D.W., STEPHENS, C.T. & DeBRUIJN, F.J. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology* 60:2286-2295. 1994.
- LOUWS, F.J., FULBRIGHT, D.W., STEPHENS, C.T. & DeBRUIJN, F.J. Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *vesicatoria*. *Phytopathology* 85:528-536. 1995.
- LOUWS, F.J., BELL, J., MEDINA-MORA, C.M., SMART, C.D., OPGENORTH, D., ISHIMARU, C.A., HAUSBECK, M. K., DeBRUIJN, F.J. & FULBRIGHT, D.W. rep-PCR- mediated genomic fingerprinting: a rapid and effective method to identify *Clavibacter michiganensis*. *Phytopathology* 88:862-868. 1998.
- LOUWS, F.J., RADEMAKER, J.L.W. & DeBRUIJN, F.J. The three Ds of PCR-based genomic analysis of phyto bacteria: diversity, detection and disease diagnosis. *Annual Review of Phytopathology* 37:81-125. 1999.
- MALAVOLTA JR., V.A., ALMEIDA, J.M.G., SUGIMORI, M.H., RIBEIRO, I.J.A., RODRIGUES NETO, J. & NOGUEIRA, E.M.C. Ocorrência de *Xanthomonas campestris* pv. *viticola* em videira no Brasil. *Summa Phytopathologica* 25:262-264. 1999a.
- MALAVOLTA JR., V.A., ALMEIDA, J.M.G., SUGIMORI, M.H., RIBEIRO, I.J.A., RODRIGUES NETO, J., PIRES, E.J.P. & NOGUEIRA, E.M.C. *Xanthomonas campestris* pv. *viticola* em videira no Estado do Piauí. *Summa Phytopathologica* 25:1999b (Abstract).
- NASCIMENTO, A.R.P., SILVA, V.A.V. MONTEIRO, L.C. AGUIAR, I.F. & CASTRO, G.S.S. *Azadirachta indica* – Hospedeiro alternativo de *Xanthomonas campestris* pv. *viticola* no submédio do Vale do São Francisco. *Fitopatologia Brasileira* 26:282. 2001 (Abstract).
- NAYUDU, M.V. *Pseudomonas viticola* sp. nov., incitant of a new bacterial disease of grapevine. *Phytopathologische Zeitschrift* 73:183-186. 1972.
- OPGENORTH, D.C., SMART, C.D., LOUWS, F.J., DeBRUIJN, F.J. & KIRKPATRICK, B.C. Identification of *Xanthomonas fragariae* field isolates by PCR genomic fingerprinting. *Plant Disease* 80:868-873. 1996.
- RESTREPO, S., VÉLEZ, C.M. & VERDIER, V. Measuring the genetic diversity of *Xanthomonas axonopodis* pv. *manihotis* within different fields in Colombia. *Phytopathology* 90:683-690. 2000.
- ROBBS, C.F., da PONTE, J.J. & SALES, M.G. Nota sobre *Xanthomonas mangiferaeindicae* no nordeste do Brasil. *Fitopatologia Brasileira* 3:215-218. 1978.
- ROBBS, C.F. & RODRIGUES NETO, J. Enfermidades causadas por bactérias em frutíferas tropicais no Brasil. *Summa Phytopathologica* 25:47-52. 1999.
- SCHAAD, N.W., JONES, J.B. & LACY, G.H. *Xanthomonas*. In: Schaad, N.W., Jones, J.B. & Chun, W. (Eds.) Laboratory guide for identification of plant pathogenic bacteria. 3<sup>rd</sup> ed. St. Paul. The American Phytopathological Society. 2001. pp. 175-200.
- STERN, M.J., AMES, G.F-L., SMITH, N.H., ROBINSON, E. C., & CHRISTOPHER, F.H. Repetitive extragenic palindromic sequences: a major component of the bacterial genome. *Cell* 37:1015-1026. 1984.
- SWOFFORD D.L. & OLSEN, G.J. Phylogeny reconstruction. In: Hillis, D.M. & Moritz, C. (Eds.). *Molecular systematics*. Sunderland, USA. Sinauer Associates. 1990. pp.411-501.
- SHARPLES, G. J. & LLOYD, R.G. A novel repeated DNA sequence located in the intergenic regions of bacterial chromosomes. *Nucleic Acids Research* 18:6503-6507.1990.
- VAUTERIN, L., HOSTE, B., KERSTERS, K. & SWINGS, J. Reclassification of *Xanthomonas*. *International Journal of Systematic Bacteriology* 45:472-489.1995.
- WEINGART, H. & VOLKSCH, B. Genetic fingerprinting of *Pseudomonas syringae* pathovars using ERIC-, REP-, and IS50-PCR. *Journal of Phytopathology* 145:339-345. 1997.
- YAP, I.V. & NELSON, R.J. WinBoot: a program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. Manila, Philippines. International Rice Research Institute. Discussion Paper Series no. 14. 1996.