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วิธีผสมผสานในการจัดจำแนกเชื้อ *Xanthomanas axonopodis* pv. *citri* ที่แยกจากมะนาว และมะกรูดในเขตจังหวัดมหาสารคาม

Polyphasic Characterization of *Xanthomanas axonopodis* pv. *citri* Isolated from Lemon and Leech Lime in Maha Sarakham Province

Aphidech Sangdee[1], Tananut Pratum[2] and Somkuon Wichachat[2]

Abstract

The objective of this study was undertaken to characterize *Xanthomonas axonopodis* pv. *citri* (Xac) by a polyphasic approach. Twenty-nine isolates of bacterial samples were isolated from infected leaf and fruit of lemon and leech lime. All isolates were used to pathogenicity test on lemon, leech lime and tangerine plant. The results showed 25 isolates showed typical symptoms and indicated that all bacterial isolates were *Xac*. Twelve isolates of *Xac* were selected and used to characterize the original host plant by biochemical and physiological characterization. The results showed, their biochemical and physiological properties were not related to the original host plant. Moreover, the results from protein pattern analysis, 16s rDNA gene analysis by ARDRA technique and REP-PCR fingerprinting analysis showed similarities agree with those biochemical and physiological results. After combining all data and analysis by using polyphasic method, the results showed these polyphasic characterization could classify all selected Xac isolates to original host plant.

Keywords: Bacterial canker disease, polyphasic characterization,

[1] Lecturer, Department of Biology, Faculty of Science, Mahasarakham University, Maha Sarakham 44150, Thailand
[2] Undergraduate student, Department of Biology, Faculty of Science, Mahasarakham University, Maha Sarakham 44150, Thailand

บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อจัดจำแนกเชื้อ *Xanthomonas axonopodis* pv. *citri* (*Xac*) โดยวิธีผสมผสาน โดยเก็บรวบรวมแบคทีเรียตัวอย่างจากใบและผลของมะนาวและมะกรูดที่แสดงอาการของโรคได้จำนวน 29 ไอโซเลต จากนั้นนำมาทดสอบความสามารถในการทำให้เกิดโรคบนพืชทดสอบ ได้แก่ มะนาว มะกรูด และส้มเขียวหวาน พบว่ามีแบคทีเรียตัวอย่างจำนวน 25 ไอโซเลต สามารถทำให้เกิดอาการเฉพาะของโรคแผลสะเก็ดบนพืชทดสอบได้จึงสรุปว่าแบคทีเรียทั้งหมดเป็นเชื้อ *Xac* จากนั้นคัดเลือกตัวแทนเชื้อ *Xac* จำนวน 12 ไอโซเลต เพื่อใช้ในการจัดจำแนกเชื้อตามชนิดของพืชอาศัยเริ่มต้น พบว่าการทดสอบคุณสมบัติทางชีวเคมีและ สรีรวิทยาไม่สามารถจำแนกตัวแทนเชื้อ *Xac* ทั้ง 12 ไอโซเลต ตามชนิดของพืชอาศัยเริ่มต้นได้ และเมื่อนำตัวแทนของเชื้อ *Xac* ทั้ง 12 ไอโซเลต มาวิเคราะห์รูปแบบโปรตีน, วิเคราะห์ 16s rDNA gene ด้วยเทคนิค ARDRA และวิเคราะห์ลายพิมพ์ดีเอ็นเอด้วยเทคนิค REP-PCR พบว่าผลการทดลองแต่ละวิธีให้ผลไปในทำนองเดียวกันกับการทดสอบคุณสมบัติทางชีวเคมีและสรีรวิทยา คือ แต่ละวิธีไม่สามารถจำแนกตัวแทนเชื้อ *Xac* ทั้ง 12 ไอโซเลต ตามชนิดของพืชอาศัยเริ่มต้นได้ แต่เมื่อนำข้อมูลจากการศึกษาทั้งหมดมารวมและวิเคราะห์ร่วมกันด้วยวิธีผสมผสาน พบว่าผลการวิเคราะห์ร่วมกันสามารถจำแนกตัวแทนของเชื้อ *Xac* ทั้ง 12 ไอโซเลต ตามชนิดของพืชอาศัยเริ่มต้นได้

คำสำคัญ: วิธีการจัดจำแนกแบบผสมผสาน โรคแผลสะเก็ดจากแบคทีเรีย

Introduction

Bacterial canker of citrus species, caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*), was initially observed in Florida and the adjacent states in 1933 (Schoulties et al., 1987). In the later years, pathogen spread and caused a serious disease in many commercial citrus varieties and some citrus species in many citrus producing areas in worldwide (Cubero and Graham, 2002). In Thailand, the pathogen was initially discovered in 1957 and its widespread to citrus and related species in many regions of Thailand and caused severe economically impact (Songkumarn et al., 2002)

The types of citrus bacterial canker have also been detected, diagnosed and characterized over the last 20 years using pathogenicity, biochemical, physiological, serological and molecular methods (Alvarez et al., 1991; Civerolo and Fan, 1982, Egel et al., 1991; Graham et al., 1990; Vauterin et al., 1995; Verniere et al., 1991). Classification of canker strains within the genus *Xanthomonas* has been discussed extensively because they have many host ranges such as sweet orange, sour orange (*Citrus aurantium*), citron (*C. medica*), trifoliate orange (*Poncirus trifoliate*) and lemon (*C. limon*) (Verniere et al., 1991; Sun et al., 2004). Moreover, canker disease symptom affected depending on type of citrus species, geographic locations and isolated-time (Songkumarn et al., 2002; Sun et al., 2004).

In the recent years, the various taxa of citrus canker, pathogenicity tests (Stall and Hodge, 1989), physiological analyses (Verniere et al., 1991), total protein profiles (Vauterin et al., 1991) and the polymerase chain reaction (PCR)-based assays (Cubero et al., 2001; 2002) were used as a taxonomic classification of the

citrus canker pathogen. Therefore, in this study many taxa method were combined as a polyphasic classification of the bacterial canker isolated from lemon and leech lime.

Materials and Methods

Bacterial isolation

Bacterial canker infected leaf and fruit of lemon and leech lime from Mahasarakham province were collected and isolated by crushing canker tissue in sterile water, streaking the macerate on nutrient agar (NA) plate, and picking up the single colonies 3 day after inoculation at 28 ° C. The cultures of isolated bacteria were grown and stored on NA slant and in 20% glycerol at -20 ° C.

Pathogenicity tests

Lemon (*Citrus limon*), leech lime (*C. hystrix* DC) and tangerine (*C. reticulate*) were inoculated with all of the isolated bacteria for their pathogenicity test. The bacterial suspension of all isolates was diluted to approximately 10^8 CFU/ml using sterile water. The inoculated plants were kept in the room an average temperature of 30 ° C and examined daily for canker symptoms for 1 month compare with water inoculation used as a negative control. Twelve isolates of pathogenic *Xac* consisted of 6 isolates (LL3-1, LL4-1, LL5-1, LF3-1, LF4-1 and LF5-1) from lemon leaf and fruit, and 6 isolates (KL2, KL3, KL5, KF1, KF3 and KF4) from leech lime leaf and fruit were selected for further studies based on the severity on test plants and original host plant.

Biochemical and physiological characteristics

The selected *Xac* isolates were cultivated on NA medium and then tested as biochemical and physiological tests consists of gram test, oxidation fermentation glucose metabolism, nitrate reduction, indole production, hydrolysis of starch and gelatin, cytochrome c oxidase activity and utilization of some carbon sources (glucose, lactose, mannital and fructose).

Total protein profile analysis by SDS-PAGE

All selected *Xac* were cultured on NA medium for 24 hours and washed their colony on the NA surface by using sterile water. The bacterial suspension were adjusted to the final concentration at OD_{600} = 1 and determined the protein pattern by using SDS-PAGE at 8% resolving gel. For protein analysis of each isolates, bands were scored as present (1) or absent (0) and the readings were entered in a computer file as a binary matrix. Similarity coefficients for all pairwise combinations were determined using Dice's coefficients and clustered by unweighted paired-group using arithmetic averages (UPGMA) by means of NTSYS program, version 2.10.

Genomic DNA preparation

All selected Xac isolates were grown on NA medium for 24 hours. Total genomic DNA from each isolates were prepared by using a modification of the phenol: chloroform procedure of Sambrook et al. (1989). DNA was precipitate at -20 °C for 2 hours with 3M sodium acetate and absolute ethanol. The DNA pellet was washed with 70% ethanol. After drying, the pellet was resuspended in 50 µl 1x TE buffer for the PCR-based assay.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The 16s rDNA gene of all selected *Xac* were performed by using FGPS6 (5'-GGAGAGTTAGATCTTGGCTCAG-3') and FGPS1509 (5'-AAGGAGGGGATCCAGCCG CA-3') primer (Nesme et al., 1995). PCR conditions were carried out in 50 µl volume containing 0.2 µM each primer, 5 µl 10X Taq DNA polymerase buffer (Promega, USA), 0.2 mM each dNTPs, 2 mM $MgCl_2$, 1 unit *Taq* DNA polymerase and 100 ng genomic DNA templates. The temperature cycling condition was performed as follows; initial denaturation at 94° C for 5 min and followed by 40 cycles of denaturatuion at 93° C for 1 min, annealing at 55° C for 1 min and extension at 72° C for 2 min 30 s. The final extension proceeded for 10 min at 72° C. The 16s PCR products were used as a template for restriction enzyme analysis with 2 digestive endonuclease (*Eco*R I and *Rsa* I). The restriction enzyme pattern were separated on 2% agarose gel in TBE buffer, stained with ethidium bromide and imaged. For restriction enzyme pattern analysis, the bands were scored and analyzed by using UPGMA method.

Repetitive-PCR fingerprinting

The amplification of all selected *Xac* were performed by using the primer set sequences corresponding to REP sequence, REP 1R-I (5'-IIIICGICGICATCIGGC-3') and REP2-I (5'-ICGICTTATCIGGCCTAC-3') primer (Versalovic et al., 1991). PCR conditions were carried out in 25 µl volume containing 0.4 M each primer, 2.5 µl 10X *Taq* DNA polymerase buffer (Promega, USA), 0.2 mM each dNTPs, 3.2 mM $MgCl_2$, 2 unit *Taq* DNA polymerase and 100 ng genomic DNA templates. The temperature cycling condition was performed as follows; initial denaturation at 94° C for 4 min and followed by 35 cycles of denaturatuion at 94° C for 1 min, annealing at 44° C for 1 min and extension at 65° C for 8 min. The final extension proceeded for 15 min at 65° C. The PCR-amplified products were separated on 1.5% agarose gel in TBE buffer, stained with ethidium bromide and imaged. For repetitive-PCR fingerprinting analysis, the bands were scored and analyzed by using UPGMA method.

Polyphasic characterization analysis

Polyphasic analysis, the biochemical and physiological data were converted to the

binary matrix as performed by the present of positive reaction were scored as present (1) and negative reaction were scored as absent (0). These binary matrixes were combined to the binary matrix of protein, restriction DNA pattern and repetitive-PCR fingerprinting analysis. The complete binary matrix was analyzed again by UPGMA method as NTSYS program, version 2.10.

Results

Bacterial isolation and their pathogenicity tests

Twenty-nine isolates of *Xac*-liked morphology were isolated form infected leaf and fruit of lemon and leech lime. All isolates were pathogenicity tested onto three kinds of test plant consisted of lemon, leech lime and tangerine. The results showed only six isolates (LL2-1, LL2-2, LL3-1, LL3-2, LL4-2 and LF2-1) could induce typical disease symptoms, i.e. small necrotic lesions in first, light green spots and become grayish white rupture and appear corky tissues surrounded by water-soaked tissues and a yellow halo on all test plant. Whereas, 18 isolates (LL4-1, LL5-1, LL5-2, LF1-1, LF1-2, LF2-2, LF3-1, LF3-2, LF4-1, LF4-2, LF5-1, LF5-2, KL2, KL3, KL5, KF1, KF3 and KF4) induced disease symptom on only one or two kinds of test plant and 5 isolates (KL1, KL4, KL6, KF2 and KF5) could not induce disease symptom on all test plants. Therefore, by using the evidence of Koch's postulate and colony morphology, all pathogenic bacteria defines as *Xanthomonas axonopodis* pv. *citri* (*Xac*) and then 12 isolates of these pathogenic bacteria were selected for further studies.

Biochemical and physiological characteristics

All selected Xac isolates were gram-negative rod, non-glucose utilized oxidatively by methyl red test. Starch and gelatin were hydrolysed. Cytochrome c oxidase was not detected. Indole was not produced and nitrates were not reduced. Whereas, the metabolic activities of 12 isolates with 4 carbon sources are shown different utilization depending on Xac isolate (Table 1).

Total protein profile analysis by SDS-PAGE

The protein profiles of total proteins extracted from all selected *Xac* showed almost isolates have similar protein profile except *Xac* isolate LL-3 showed extra band with molecular weight 13 kDa, 18 kDa, 20 kDa and 40.3 kDa (Fig. 1A). For UPGMA analysis, the protein profile of all selected *Xac* could divided into two group as A and B at 0.68 similarity index. Only LL-3 isolate was Group A while other isolates were classified as Group B (Fig. 1B).

Table 1 Metabolic activity of 12 isolates of *Xac* with 4 carbon sources

Isolate	Carbon Source			
	Glucose	Lactose	Mannital	Fructose
LL3-1	+	+	-	-
LL4-1	+	-	-	+
LL5-1	+	-	+	-
LF3-1	+	-	-	-
LF4-1	-	-	-	+
LF5-1	-	-	-	-
KL2	+	+	+	+
KL3	+	+	+	-
KL5	+	+	+	+
KF1	+	+	+	+
KF3	+	+	-	-
KF4	+	+	-	-

(-) = Non-utilized of carbon source
(+) = Utilized of carbon source

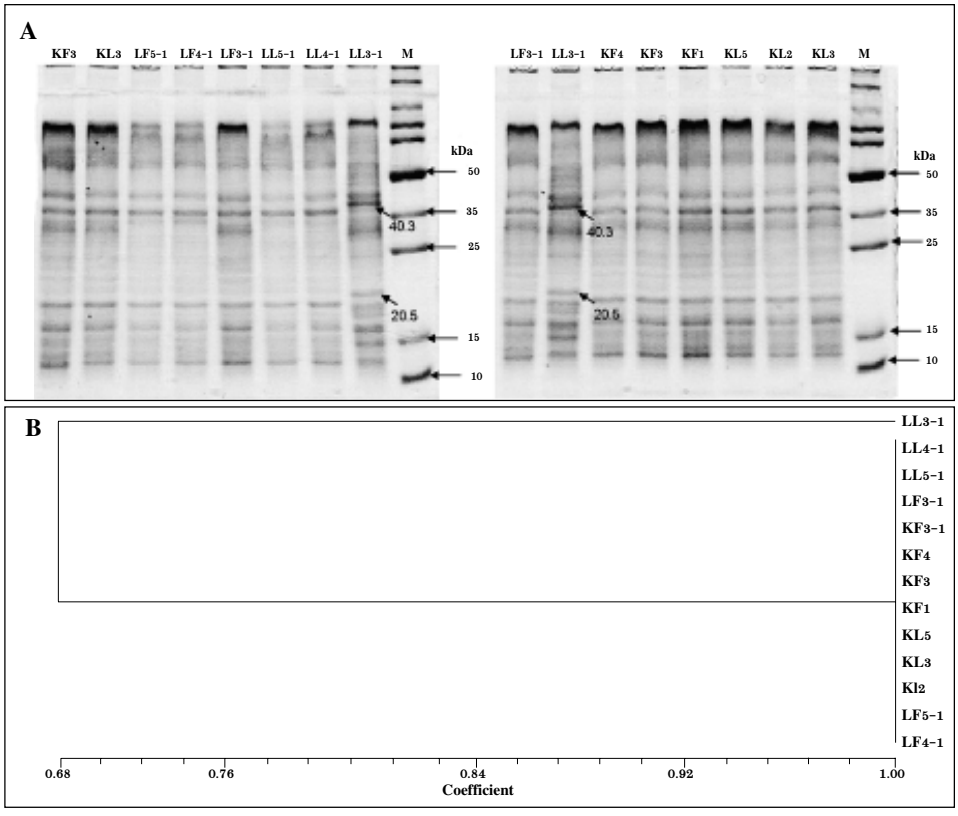

Fig. 1 Total protein profile of 12 selected Xac isolates were analyzed by SDS-PAGE at 8% resolving gel (A) and their protein profile were analyzed by UPGMA method, program NTSYSpc version 2.1 (B)

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Twelve selected *Xac* isolates were used to determine the DNA fingerprint by using ARDRA technique. The results showed that FGPS6 and FGPS1509 primer could amplify the 16s rDNA gene of all selected *Xac*, approximately 1500 bp. These 16s rDNA gene showed similar DNA pattern after digestion with *Eco*R I and *Rsa* I endonuclease (Fig. 2). Therefore, the selected *Xac* were not classified to the original host plant by using these enzyme.

Repetitive-PCR fingerprinting

The DNA fragments approximately 100-2000 bp were generated by using REP 1R-I and REP2-I primer (Fig. 3A). For UPGMA analysis, DNA fragments of 12 selected *Xac* isolates were divided into two major groups consisted of A and B at 0.50 similarity index. Only LL3-1 was a member of group A while the other isolates were classified as group B. Moreover, the selected *Xac* were not classified to the original host plant by using repetitive-PCR fingerprinting (Fig.3B) as same as protein profile from SDS-PAGE and restriction DNA pattern from ARDRA technique.

Polyphasic characterization analysis

After combined the unique characteristics of all selected *Xac* to the binary matrix and analyzed by the UPGMA method, these polyphasic characterizations had a power to divide the 12 selected *Xac* into two major groups as A and B at 0.68 similarity index. Only LL-3 isolate was classified as group A while the other isolates were classified as group B. Group B could be divided into two subgroup (B-1 and B-2) which were related to the original host plant. Group B-1 consisted of the 5 *Xac* isolates (LL4-1, LL5-1, LF3-1, LF4-1 and LF5-1) that originated from leaf and fruit of lemon. Whereas group B-2, had 6 isolates (KL2, KL3, KL5, KF1, KF3 and KF4) from leaf and fruit of leech lime (Fig. 4).

Conclusion and Discussion

According to the pathogenicity test, biolchemical and physiological properties of 12 selected plant pathogenic bacteria were classified as *Xanthomonas axonopodis* pv. *citri.* (Vudhivanich, 2003; Graham et al., 2004; Sun et al., 2004).

In this study, the classification of 12 *Xac* isolates that related to the original host plant could not be used only one classification technique. After, all data from many techniques were collected and analyzed by using polyphasic taxonomy, the results could be interpreted.

The polyphasic characterization was previously reported by many researchers. Sun et al. (2004) reported that combination of pathogenicity tests, ELISA, Fatty Acid (FAME) analysis, PCR-based assay, Pulsed-field gel electrophoresis and DNA reassociation was a

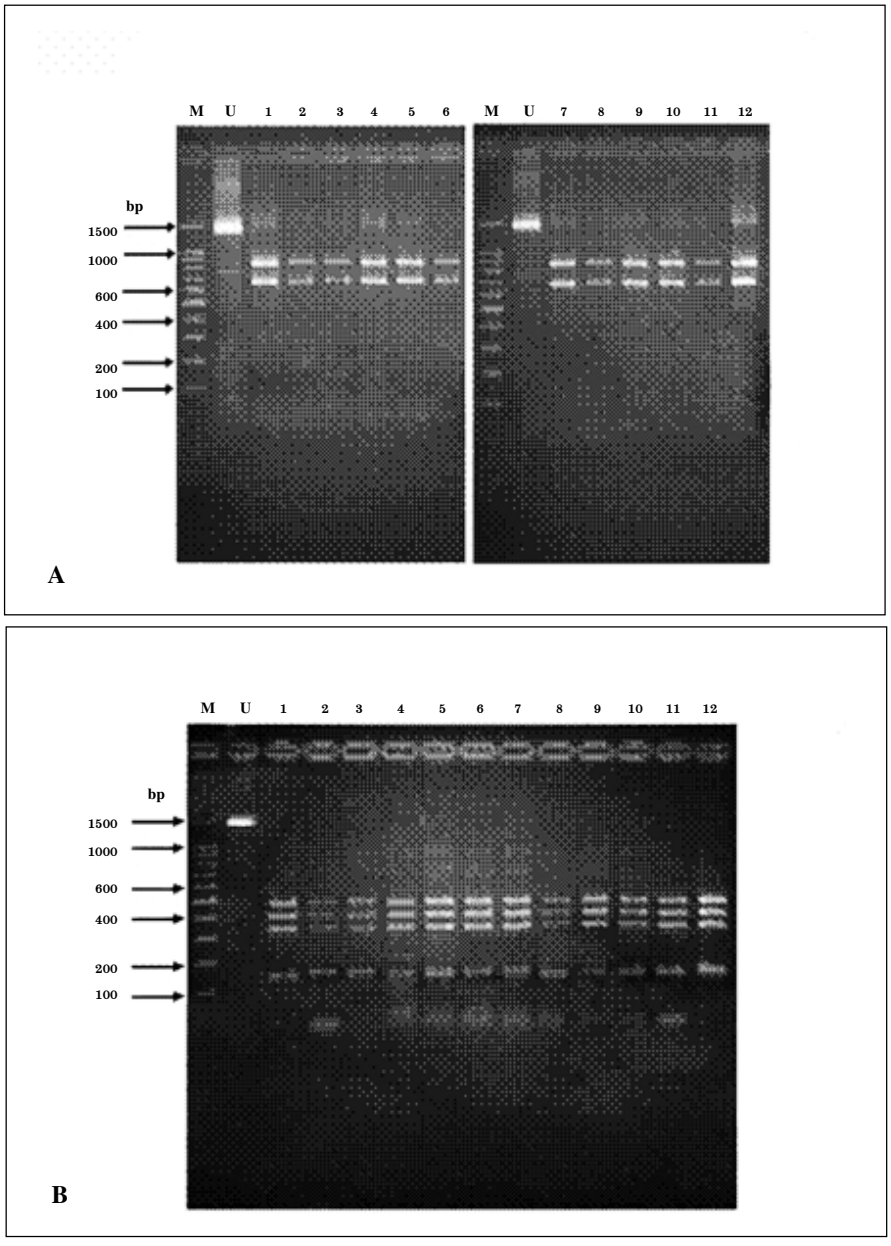

Fig. 2 **16s rDNA gene of 12 selected *Xac* isolates were analyzed by using restriction enzyme analysis with *Eco*R I and *Rsa* I and separated on 2% agarose gel electrophoresis**

Lane M = 100 bp standard marker Lane U = 16s rDNA-PCR product

Lane 1 = LL3-1	**Lane 7 = KL2**
Lane 2 = LL4-1	**Lane 8 = KL3**
Lane 3 = LL5-1	**Lane 9 = KL5**
Lane 4 = LF3-1	**Lane 10 = KF1**
Lane 5 = LF4-1	**Lane 11 = KF3**
Lane 6 = LF5-1	**Lane 12 = KF4**

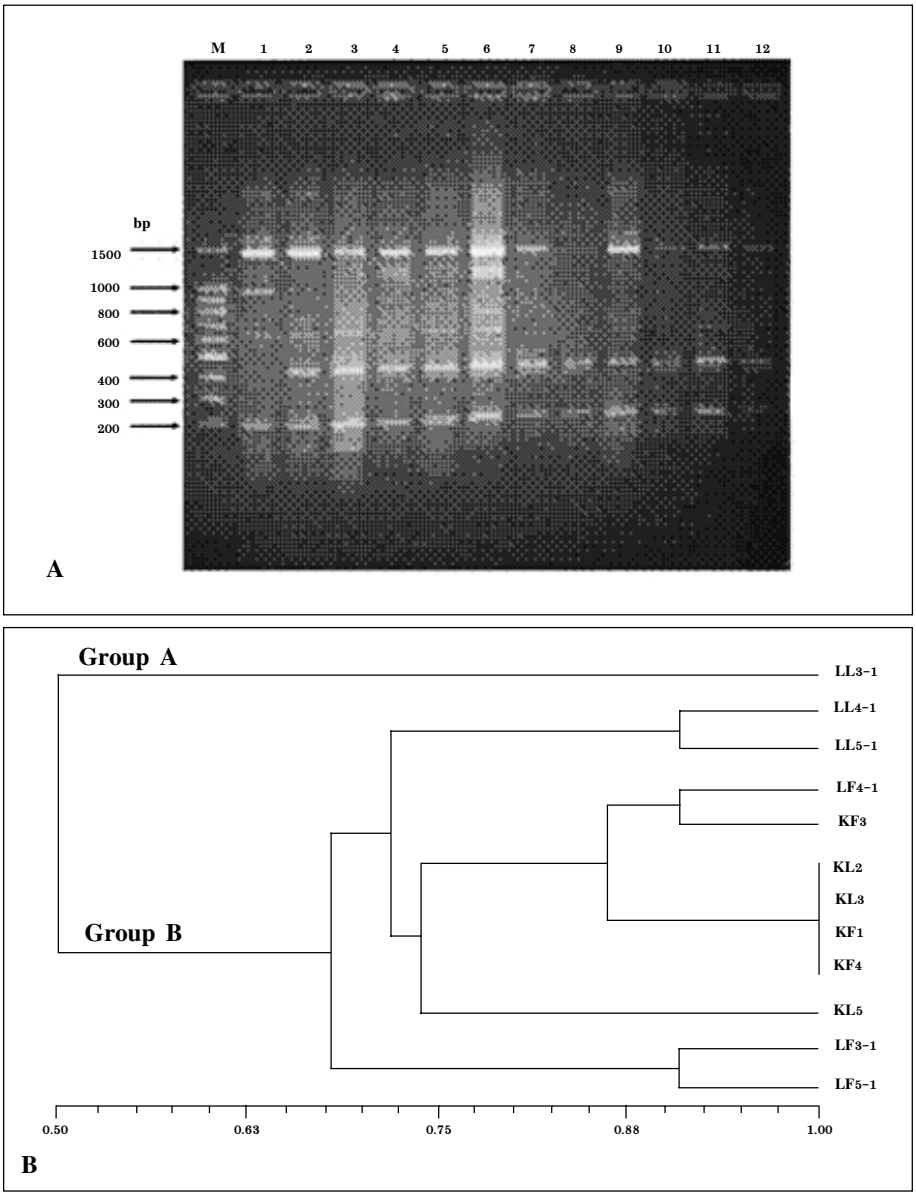

Fig. 3 **Repetitive-PCR fingerprinting performed with REP 1R-I and REP2-I primer and separated on 1.5 % agarose gel electrophoresis (A) and their dendrogram showed the relationships among 12 selected Xac isolates by UPGMA using the NTSYSpc version 2.1 (B).**

Lane M = 100 bp standard marker

Lane 1 = LL3-1
Lane 2 = LL4-1
Lane 3 = LL5-1
Lane 4 = LF3-1
Lane 5 = LF4-1
Lane 6 = LF5-1
Lane 7 = KL2
Lane 8 = KL3
Lane 9 = KL5
Lane 10 = KF1
Lane 11 = KF3
Lane 12 = KF4

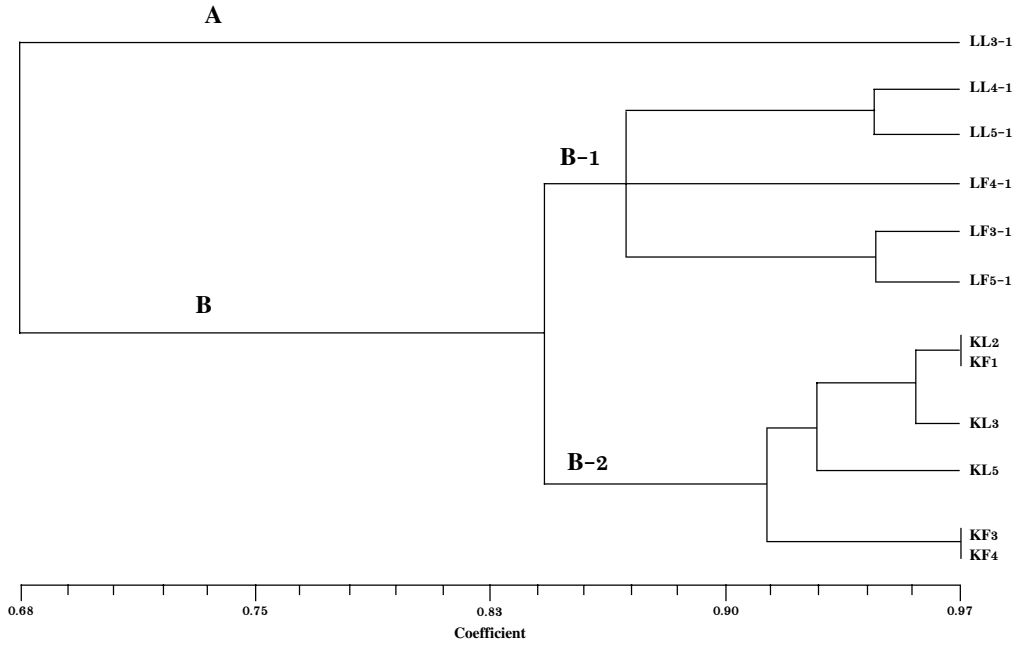

Fig. 4 The relationships dendrogram among 12 selected *Xac* isolates based on polyphasic characterization (biochemical and physiological properties, protein pattern analysis, 16s rDNA gene analysis and repetitive-PCR DNA fingerprinting). Similarities were calculated by using the Dice coefficient, and clustering was achieved by UPGMA using the NTSYSpc version 2.1.

powerful technique to characterize a new strain of citrus canker bacteria from Key/Mexican lime and alemow in South Florida. Moreover, The combination of pathogenicity test, biochemical and physiological characteristics, fatty acid methyl ester analysis, sequencing of the 16s rRNA gene, DNA-DNA hybridization and thermal stability of DNA reassociation as polyphasic characterization of xanthomonads isolated from onion, garlic and Welsh onion was reported by Roumagnac et al. (2004). From the research of Sun et al. (2004) and Roumagnac et al. (2004) were used the polyphasic characterization but the combination techniques were different.

Therefore, in this study we emphasize that the polyphasic characterization were used to be the powerful technique to classify the pathogen to relate with original host plant. And, these polyphasic characterization will be applied to use as a tool for determine the relationship between another pathogen and their related and between pathogen and geographic location.

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