PCR-RFLP ANALYSIS OF THE 16S RRNA AND ITS REGIONS IN BACTERIAL BLIGHT (*XANTHOMONAS AXONOPODIS* PV. *PUNICAE)* ACROSS POMEGRANATE FARMS IN KURDISTAN REGIONS-IRAQ.

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ABSTRACT

Bacterial blight caused by *Xanthomonas axonopodis pv. punicae* (Xap) is a major biotic diseases in pomegranate (*Punica granatum* L.). In the field survey which lasted 2 year in six different geographical locations, Xap strains from the severely infected plant material collected including DuhokCcenter, around Erbil, Zaxo, south of Duhok, Akre, and Amedi. *Xanthomonas* was detected from infected plant material and its identity was confirmed by morphological, Microbial and Molecular Characterization. To study its genetic variability and phylogenetic relationship two important loci were targeted namely ITS region of 16s rDNA, 16S rRNA and then a PCR-RFLP was performed for PCR product of 16s rRNA loci using *Hinf* I, *Hae* III, and *Rsa* I restriction enzymes. The PCR-RFLP showed the genetic similarity coefficient ranging from 1.00 to 3.73, and the dendrogram grouped all tested strains in 4 clusters. The result revealed that this disease is in blooming stage in the country which was thought that has not been recorded before in pomegranate. To the best of our knowledge this is the first record of Xap. In Kurdistan/ Iraq therefore, further studies are needed to be performed to manage this hazardous bacterium.

Keywords: survey , infected plants , phylogenetic genetic similarity ITS region, 16S rRNA .,

مجلة العلوم الزراعية العراقية -2023 :54(2):339-346 **سنجاري و جبرائيل**

*تحليل*PCR-RFLP للمواقع الجينيةITS of 16s rDNA, 16S rRNA *للكشف عن اللفحة البكتيرية Xanthomonas axonopodis pv. Punicae لمزارع الرمان في كوردستان/ العراق*

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المستخلص

تعتبر اللفحة البكترية المسببة من بكتريا *Xanthomonas axonopodis pv. punicae* (Xap) واحدة من اهم الامراض الحيوية التي تصاب بها الرمان. في هذه الدراسة تم جمع سلالات ال Xap من اجزاء مختلفة من نبات الرمان المصاب وذلك خلال سنتين من المسح الحقلي في ستة مناطق جغرافية مختلفة منها مركز دهوك- جنوب دهوك- اربيل – زاخو – عقرة و عمادية. تم تشخيص البكتريا والتاكد من وجودها عن طريق الصفات الظاهرية والصفات الميكروبية وايضا عن طريق التشخيص الوراثي الجزيئي. لغرض دراسة الاختلافات الوراثية والعلاقات التقارب الوراثي شملت هذه الدراسة على اختيار اهم موقعان وهما موقع ITS of 16s rDNA, 16S rRNA ولهذا الغرض تم هضم الجين الناتج من 16S rRNA باستخدام الانزيمات القاطعة التالية ا *Hinf* I, *HaeIII* and *Rsa* واظهرت نتائج الPCR-RFLP تشابه وراثي بين السلالات بنسبة (1.00-3.73) وايضا الشجرة الوراثية نتج عنها اربع مجاميع وراثية. من خلال النتائج التي تم الحصول عليها تمكنا ان نستنتج بان هذا المرض في بداية طور الانتشار في المنطقة والتي كان يعتقد سابقا بعدم تسجيلها في نبات الرمان.

الكلمات المفتاحية: الرمان . بكتريا اللفحة البكترية ITS region, 16S rRNA, PCR- RFLP

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INTRODUCTION

Pomegranate (*Punica granatum* L.) is an economically important crop with great nutritional values (15). Considering its importance many researchers focused on improving its productivity and quality (2, 9, 13,) Its farming was largely free of most pests and diseases (14). Diseases such as bacterial blight, anthracnose, and wilt are becoming more common as pomegranate cultivation extends. Pomegranate oily spot, also known as bacterial blight is one of the major diseases which is a dangerous and widely spread in many pomegranate-producing areas (26). Bacterial blight is caused by *Xanthomonas axonopodis* pv. *Punicae* characterized as yellow pigmented, Gram negative, and rod shaped (25). The disease affects all plant parts above ground, including flowers, leaves, twigs, stems, buds, and fruits. Early signs of the disease include water-soaked lesions on leaves, fruits, and stems, followed by corky, dark oily patches later in the infection. Damages caused by bacterial blight in pomegranate range from 30 to 100 percent thus, decreasing significantly yield, market value of the product and making it unsuitable for export. (4). The infection spreads in the garden readily via plant to plant, rain, wind-blown rainfall, the handling of the plant, contaminated equipment and insects. The disease spreads rapidly. After every watering, the occurrence of a disease increased under ideal circumstances of temperature and moisture (3). *Xanthomonas* is a large genus of pathogenic bacteria that collectively causes disease in many plant species. Morphological and physiological uniformity within *Xanthomonas* has hampered the establishment of a stable taxonomy task (11) Often, it was found difficult to detect *Xanthomonas* based only on microbial and biochemical characterization methods (18). Molecular techniques combined with traditional techniques offer an enormous potential to identify numerous plant pathogens rapidly. DNA-based markers have been used most extensively in diagnostic and genetic diversity research throughout the last two decades. For instance, investigations of bacterial plant pathogens, as an essential locus for identifying and determining phylogenetic distances, the 16S rRNA gene has been well established (1, 10). Though a considerable stretch of the 16S rRNA gene is conserved across bacterial genera, however a smaller variable region enables workers to estimate phylogenetic distances (21). The aim of the present study was to diagnose bacterial blight (*X. axonopodis)* across pomegranate growing farms in Kurdistan regions-Iraq using 16S rRNA, ITS region and PCR-RFLP markers.

MATERIAL AND METHODOLOGY

Field survey and sample collection

Field survey was conducted between 2019 and 2020 in several major pomegranate growing regions of Duhok center, south of Duhok, Erbil, Amedi, Zaxo and Akre. During the field survey pomegranate at the nursery stage and in orchard were inspected for incidence of bacterial blight which has not been recorded in these regions. For the purpose of this study, 80 plants were randomly selected. Infected plants were diagnosed based on standard bacterial blight symptoms, such as yellow water-soaked lesions in the early stages and corky, dark oily patches in the later stages of infection. The suspected plant parts were collected separately from the orchard, labeled and brought to the laboratory for the further studies. For this research the isolates were named as *X. axonopodis pv. Punicae* Xap1, Xap2, Xap3, Xap4, Xap5, Xap6, Xap7, Xap8 and Xap9 for bacterial isolate.

Pathogen isolation and characterization

To isolate bacteria from the infected leaves, small twigs and fruits of pomegranate were washed, air dried, cut into small sections with sterilized razor blades. Tissues were then disinfected with 0.1% HgCl2 for about half to one minute and washed twice with sterile distilled water to remove traces of HgCl2 (22). To allow the bacteria diffusion, approximately 4 mm diameter lesions were cut and macerated with sterilized blade in Petri dish containing few drops of sterile water. A loopful of suspension was then transferred onto sterilized LB nutrient agar (yeast extract 5 g, tryptone 10 g, NaCl 5g, agar 15 g and water 1 L, pH 7.0) and incubated at 28±2°C for 24-72 hrs. (26). After 2- 3 days, incubated plates were observed for the presence of typical pale yellow, glistening colonies. Pure cultures were then further sub-cultured and used for

pathological, biochemical studies. Purified strains were individually preserved in 40% glycerol stored for long time at -80°C for further studies.

Extraction of total genomic DNA from bacteria: Single colony of *X. axonopodis pv*. p*unicae,* bacteria were inoculated in LB broth and incubated at 28°C for 72 hours. Bacterial cells were harvested from10 ml of broth using centrifuging at 16099 Xg for 5 min. Cells were then treated with 1.8 mL lysis buffer (25 mM EDTA + 25 mM Tris base, pH 8 and 0.3 M sucrose) to this, 100 µl of 10 % SDS was added and vortexed vigorously and then samples were incubated for 1 hour at 37°C. The cell suspension was treated with 500 µl of 5 M Nacl and 500 µl of CTAB and all tubes were incubated at 65 °C for 10 min. After were cooled down at room temperature. Equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed thoroughly and centrifuged at 12,000 rpm for 10 min at room temperature. Supernatant was carefully transferred to fresh sterile centrifuge tubes and 0.6 volume of ice-cold Ethanol was added to precipitate DNA and incubated at -20 °C overnight. Tubes were then centrifuged at 16099 Xg for 10 min and the pellet was washed with 70 % ethanol. Tubes were air dried completely at room temperature and the DNA was dissolved in sterile nuclease free water and stored at 4 °C. The DNA samples were quantified using a Nanodrop spectrophotometer.

Direct detection from plant samples and genomic DNA extraction : The genomic DNA was extracted following the steps of (27), with few modifications. First, 3g of fresh and young pomegranate leaves were collected from around Duhok province, then ground in liquid nitrogen. The fine powder was dissolved in a pre-heated (60°C) 2x CTAB extraction buffer (2x CTAB, 5M NaCl, 1M Tris-HCl, 0.5 M EDTA), and incubate at 60°C in shaking water bath for 30 min. The mixture was extracted with an equal volume of chloroform/isoamyl alcohol (24:1 v/v). The mixture was then centrifuged (at 1792 Xg for 30 min). The aqueous phase was transferred into another tube and precipitated with 0.66 volume of isopropanol. Precipitated nucleic acids were then dissolved in TE-buffer and stored at -20°C until use. 1µl was used for PCR amplification. 5 µl of PCR product was loaded on agarose gel (1%) run in 1X TBE buffer at 80 volts for 40 min.

16S rRNA amplification

To amplify the 1500 bp gene of 16S rRNA region of *Xanthomonas axonopodis pv. Punicae*, the following pair of primers were used 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGT TACCTTGTTACGACTT-3') (20). A 10µl PCR reaction mixture was prepared containing 1 µl of DNA (25-50 ng), 1 µl (10 pmol) of each forward and reverse primers and 5 µl of (Go *Taq* G2 green) (Promega, USA) along with 2 µl of sterile dds water. An ABI Applied Biosystems PCR System 2720 Thermocycler was used with the following conditions, an initial denaturation step at 96°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10.

Molecular confirmation of *Xanthomonas* through Amplification of ITS region

ITS primers 799 F (5'-AACMGGATTAGATACCCKG-3'), 1193 R (5'-ACGTCATCCCCACCTTCC-3') (16) were used to amplify the 400 bp gene of ITS region. A 10µl PCR reaction mixture was prepared containing 1 µl of DNA (25-50 ng), 1 µl (10 pmol) of each forward and reverse primers and 5 µl of (Go *Taq* G2 green) (Promega, USA) along with 2 µl of DDs sterile water. PCR amplification was performed in an ABI Applied Biosystems PCR System 2720 thermo cycler using the conditions: an Initial denaturation step at 94ºCfor 2 min, 35 cycles consisting of 94ºC for 50 sec (denaturation), 55ºC for 30 sec(annealing), 72 ºC for 1 min (primer extension) and final extension of 72ºC for 10 min.

PCR-Restriction Fragment Length Polymorphism (RFLP): All *Xanthomonas* strains amplified product of the 16S rRNA obtained from direct extraction and bacterial cultures were further used for PCR- RFLP analyses. Enzymes that produced a smaller number and low-molecular-weight fragments were preferred. Using these criteria, the enzymes *Hin*fI, *Rsa*I and *Hae* III (Jena bioscience, Germany) were selected to digest the amplified 1500bp gene. Individual

digestion conditions were performed in a final volume of 50µl, as described by the manufacturer, 5µl of 10x universal buffer, 1µg of PCR product, 10 units of enzyme then the reaction was made up to 50µl with PCR grade water, the mixture then incubate at 37 ºC for 2 to 4 hours. Then the product of digested results was analyzed using 3% agarose gel electrophoresis.

Data analysis

The data were analyzed to form a dendrogram for each group of samples (direct and indirect pathogen extraction) by NTSYSpc 2.1 program.

RESULT AND DISCUSSION

The bacterial pathogen isolated from the infected plant parts like leaf and fruit, and cultured on LB Nutrient agar media was identified as *X. axonopodis pv. Punicae.* This was noticed through its morphological, microbiological and biochemical characteristics which showed yellow shiny mucoid colony, gram-negative rod-shaped bacteria, as shown in (Figure 1). The identification and characterization of this bacterium represents the first report in the region and was in agreement with other studies (6, 22).

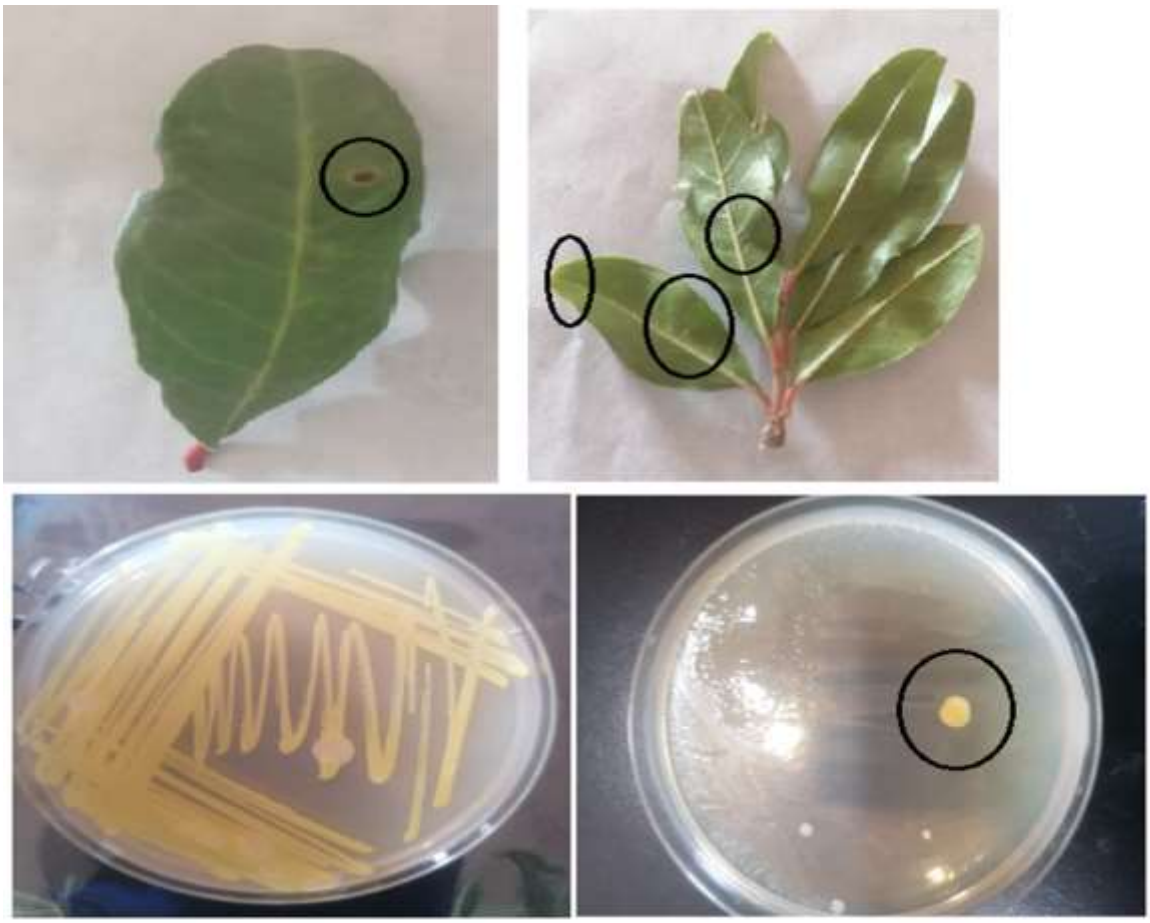

Figure 1. Pathogenicl variability of X.axanopodis pv. Punicae on suceptible pomegranate

Furthermore, the pathogen was confirmed as *X. axonopodis pv. Punicae* through molecular characterization using ITS region of 16s rDNA and 16s rRNA. The result of ITS region of 16s rDNA amplified with ITS marker yielded an amplicon size of 400 bp (Figure 2). Similarly, the 16s rRNA gene-based universal primers yielded an amplicon size of 1500bp in all isolates of *X. axonopodis pv. Punicae* (Figure 3). Based on the amplicons of 1.5kb and 400bp across all isolates the pathogen was confirmed as *X. axonopodis pv. Punicae*. (16, 20).

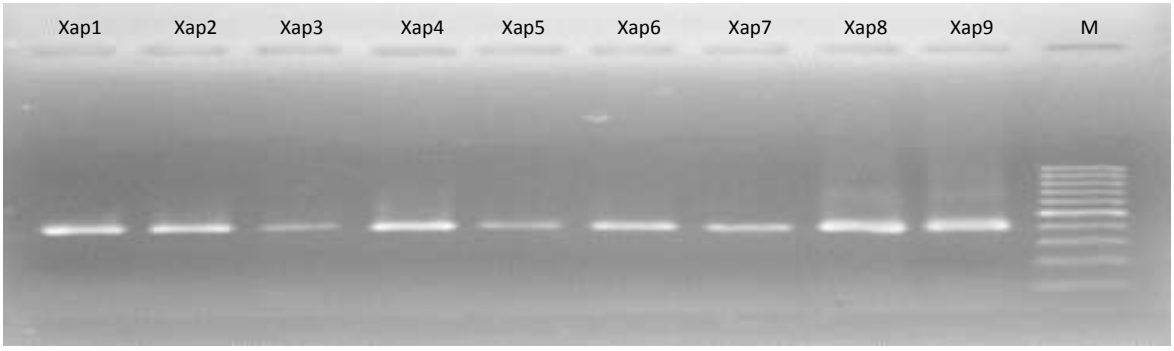

Figure 2. Molecular confirmation of X. *axopodia pv. Punicae by using the ITS primer*

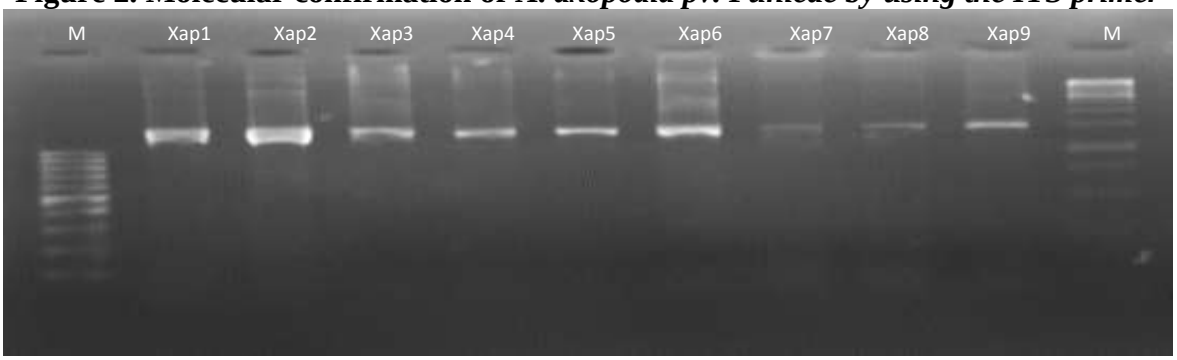

Figure 3. Molecular confirmation of X. *axopodia pv. Punicae by using the 16s rRNA primer*

Based on these molecular results, it was possible to develop and optimize a simplified protocol (less than 3 days) for direct detection of *X. axonopodis pv. Punicae* from the infected leaf and fruit tissues of pomegranate plants in the fields as well as from the artificially infected samples. This protocol involved DNA extraction from these samples and their subjection directly to the molecular investigations. The results of this protocol were successful in direct detection of the presence and/ or absence of bacteria in the tested samples and matched earlier molecular investigations. The results of these experiments on 80 apparently healthy leaf tissues showed that only 10 were found to be positive, indicating that the detection system could even work in asymptomatic (but suspected to be diseased) plant samples. However, the sampling of plant tissues to be tested for PCR detection need to be carried out very carefully as the blight bacterium, Xap is a mesophyll localized not systemic one. Application of this protocol may pave the way for a rapid and efficient management of the disease in pomegranate fields (19). The results of restriction enzymes digestion using *Hinf* I, *Hae* III and *Rsa* I of the 1.5 kb fragment obtained across all strains, and the amplified product of 16s rRNA, are shown in Figure 4. The PCR-RFLP profiles revealed different banding patterns, all strain produced relatively the same banding pattern except the Xap5 sample. The result of enzyme *Hinf* I well identified polymorphism between studied strains and showed different bands that ranged from 990bp to 200bp. The *Hae* III enzyme also showed high degree of polymorphism among all strains producing DNA banding pattern of 1200bp to 300bp. Meanwhile the most informative results were obtained using *Rsa* I enzyme which revealed clear and reproducible DNA bands ranging between 760bp to 300bp across all strains. The PCR-RFLP were previously reported in *Xanthomonas axopodia pv. Punicae* by isolation of bacterial plasmid (14). More recently, the PCR-RFLP was applied for 16s rRNA gene for identification of phytoplasma bacteria (8).

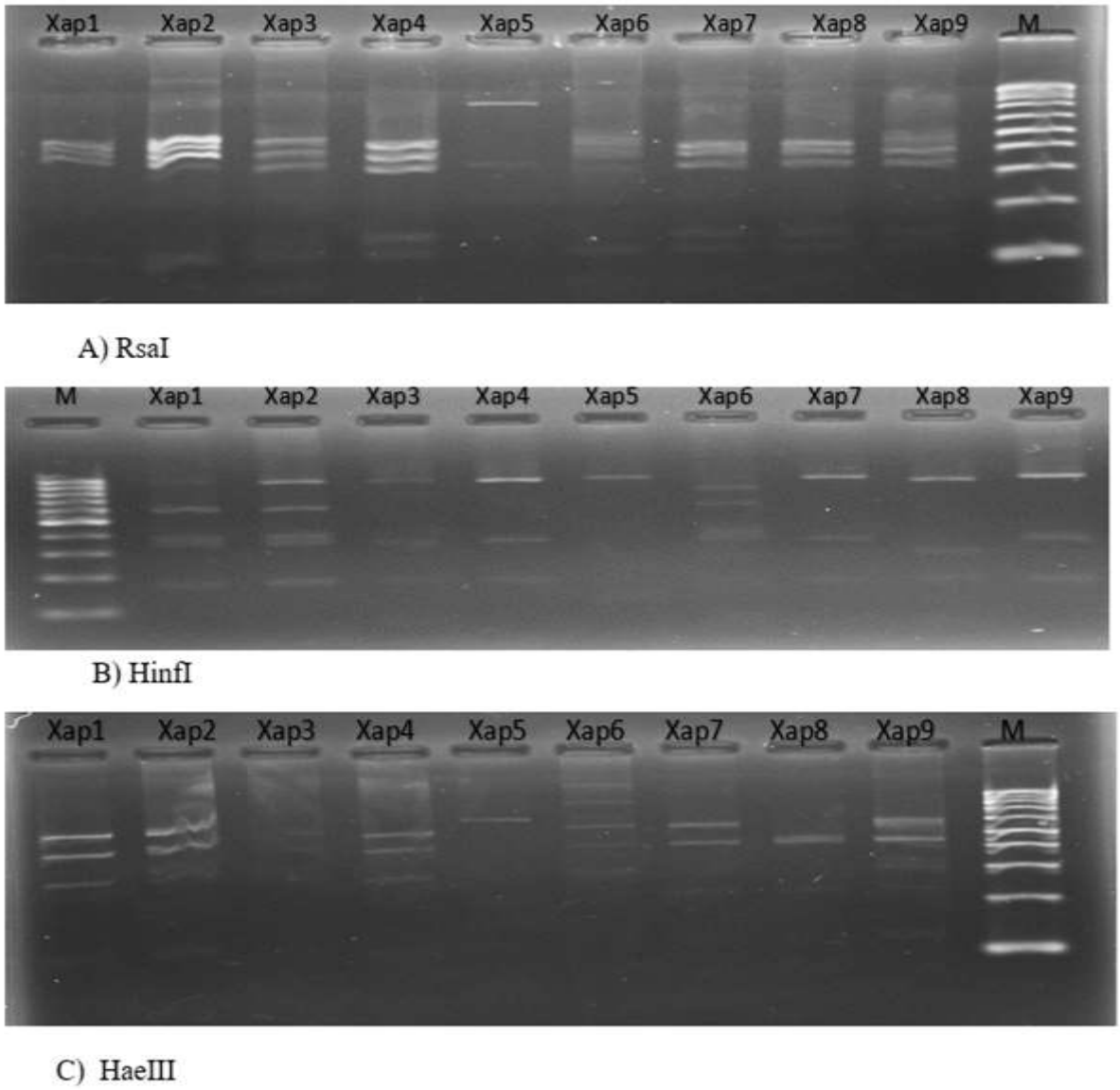

Figure 4. PCR-RFLP profiles of the 16s rRNA gene of the X. *axopodia pv. Punicae* obtained after digestion with the restriction enzymes (a) RsaI (b) HinfI and (c) HaeIII . M, marker of 100 bp (thermo fisher scientific).

The PCR-RFLP results obtained in this study were used to construct a dendrogram, which demonstrated the distinction between most of Xanthomonas isolate. The genetic similarity coefficient ranged from 1.00 to 3.73 similarity, the dendrogram revealed 4 clusters according to the statistical analysis performed using version 2.02 of NTSYS software (23). The most distinct cluster was cluster number 1 which involved most of the strains as Xap1 and Xap2 that showing 100% similarity. This cluster also contained Xap3, Xap4, Xap7, and Xap9 but in different ranges of similarity (1.34 between Xap4 and Xap9, then 2.19 to 3.73 among Xap7, Xap8, Xap6, and Xap5 respectively. All other three clusters involved one group as shown in (Fig.5). Similar results were obtained in the investigations which involved the direct detection of pathogen from leaves. The results presented here revealed the differentiation of Xap isolates by the analysis of the 16srRNA and ITS genes, which showed different levels of diversity. These results indicate that both loci can be successfully used as markers to distinguish different Xap strain spread in pomegranate orchid in Kurdistan region of Iraq.

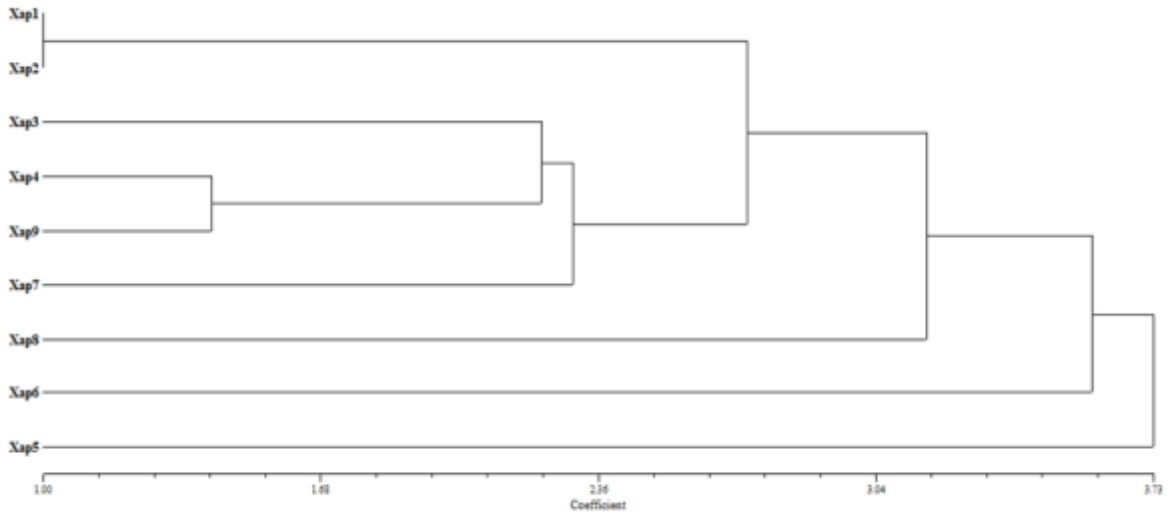

Figure 5. Dendrogram obtained for the different strain of Xap. based on the PCR-RFLP profile of the 16s rRNA gene

Further identification was also conferred by sequencing the bacterial stains showing an identity of 82% of partial sequence similarity to accession no. (CP030178.1) (26), from this result as mentioned before there were a few information available regarding the Xanthomonas species in general and Xap in specific. According to Sharma *et al*., 2012 the BLAST analysis of both 16S rRNA and complete rpoB gene sequences of *X. axonopodis pv. punicae* which revealed 99% identical to those of *Xanthomonas axonopodis pv. citri*, the main causative agent of citrus canker. Further phylogenomic analysis using highly conserved housekeeping genes described earlier for xanthomonads (5) confirmed its close relationship with *X. axonopodis pv. citri.* It is interesting *X. axonopodis pv. punicae* and *also X. axonopodis pv. citri* illness were first discovered in India, which also may be the area of origin of these bacterium (7, 12, 17) The comparison of the two data sets (PCR-RFLP and sequencing) must be made with caution, as sequence information of only a few Xanthomonas species are available in Gene Bank. Moreover, in the sequence analysis, the whole gene is being compared, whereas in PCR-RFLP, only a few restriction sites are analyzed. Future studies using the complete sequences of these gene may provide complementary information and may contribute to a more detailed understanding of the relationships between isolate of this complex species. Although the sequence analysis is promising, PCR-RFLP was able to distinguish most isolated strain and represents a fast and simple alternative to analyze the Xanthomonas rapidly. The present work gives a new insight into the genetic relatedness between the different Xanthomonas. Important gene with variable levels of diversity have been analyzed by the relatively simple technique of PCR-RFLP. This is the first studies on *X. axonopodis pv. Punicae* in Kurdistan area published to the best of our knowledge.

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REFERENCES

1. Adhikari T.B, C.M. Vera Cruz ,Q. Zhang , R.J.Nelson , D.Z. Skinner , T.W. Mew , and T.E. Leach. 1995 Genetic diversity of Xanthomonas oiyzae pv. oryzae in Asia. Appl Environ Microbiol 61:966–971.

2. Aljabary, A. M. A. O., A.A.Fatih and Z.M. Ahmad. 2022. Response postharvest quality of pomegranates to thermal treatments, immersing in black seed and flaxseed oils. Iraqi Journal of Agricultural Sciences – 2020:53(5):1048-1057. https://doi.org/10.36103/ijas.v53i5.1618

3. Anonymous 2008. Progress Report of Network Project on Mitigating The Bacterial Blight Disease Of Pomegranate in

Maharashtra, Karnataka and Andhra Pradesh. National Research Centre on Pomegranate (ICAR), Solapur-413 006, India
4. Anonymous 2006.. Diagnosis Of Bacterial Blight Of Pomegranate And Its Management-Training Manual, National Research Centre on Pomegranate, Solapur, 2006
5. Bogdanove AJ, et al. 2011. Two new complete genome sequences offer insight into host and tissue specificity of plant pathogenic Xanthomonas spp. J. Bacteriol. 193:5450–5464
6. Chand R,and R. Kishun, 1991. Studies on bacterial blight (Xanthomonas campestris pv. punicae) of pomegranate. Indian Phytopathology. 44(3):370-372
7. Fawcett HS, and AE Jenkins . 1933. Records of citrus canker from herbarium specimens of the genus *Citrus* in England and the United States. Phytopathology 23:820–824
8. GAO, R., WANG, J., ZHU, T., JIA, X., & LI, X. .2018. Identification and Molecular Characterization of a Phytoplasma Associated with Pomegranate Fasciation Disease. Horticultural Plant Journal, 4(1), 30–34. doi:10.1016/j.hpj.2018.01.001
9. Hamdan, A. Q. and F. F. Jomaa. 2020. Response of pomegranate " cv. wonderful" transplants to mineral nutrition and gibberrelic acid. Iraqi Journal of Agricultural Sciences –2020:51(1):339-346.
https://doi.org/10.36103/ijas.v51i1.933
10. Hauben L, L. Vauterin, J. Swings, and ERB Moore. 1997 Comparison of 16S ribosomal DNA sequences of all Xanthomonas species. Int J Syst Bacteriol 47:328–335
11 .Hayward, A. C. 1993. The hosts of Xanthomonas. In: Xanthomonas (Swings JG. and Civerolo EL eds). London: Chapman and Hall,; pp 1–119
12. Hingorani MK, and NJ.Singh. 1959. Xanthomonas punicae sp. now on Punica granatum. Indian J. Agric. Sci. 29:45–48
13. Jomaa, F. F., and A. A. Ali. 2016. Effect of foliar application of potassium zinc and gibberelic acid on leaves and peel content of mineral elements of pomegranate. Iraqi Journal of Agricultural Sciences –47(2): 533-542. https://doi.org/10.36103/ijas.v47i2.599
14. Kale, P., Chimote, V., Raghuwanshi, K., Kale, A., Jadhav, A., and S. Borkar. 2012. Microbial, biochemical, pathogenicity and molecular characterization of Xanthomonas axonopodis pv. punicae from pomegranate. Journal Pure Applied Microbiology, 6(4): 1699-1706
15. Lazeeza, S. O. 2021. Antioxidant activity of pomegranate, Iraqi journal of agricultural sciences, 52(1):196–203.
https://doi.org/10.36103/ijas.v52i1.1251
16. Lee JS, KS Ko and H.S. Jung . 2000. Phylogenetic analysis of Xylaria based on nuclear ribosomal ITS1-5.8S-ITS2 sequences. FEMS Microbiology Letters 2000; 187:89-93
17. Li W, Q. Song , RH. Brlansky and JS. Hartung. 2007. Genetic diversity of citrus bacterial canker pathogens preserved in herbarium specimens. Proc. Natl. Acad. Sci. U. S. A. 104:18427–18432
18. Lievens B, M. Brouwer, ACRC.Vanachter , CA Levesque, BPA. Cammue and BPHJ. Thomma. 2006.: Quantitative assessment of phytopathogenic fungi in various substrates using a DNA macroarray. Environ Microbiol 2005,**7** (11)**:**1698–710. 10.1111/j.1462-2920.2005.00816.x
19. Mondal, K. K., Rajendran, T., Phaneendra, C., Mani, C., Sharma, J., Shukla, R., and A. Kumar. 2012. The reliable and rapid polymerase chain reaction (PCR) diagnosis for Xanthomonas axonopodis pv. punicae in pomegranate. African Journal of Microbiology Research, 6(30): 5950-5956
20. Mondal, K. K., Verma, G., and C. Mani. 2013. Phylogenetic relatedness of Xanthomonas axonopodis pv. punicae, the causal agent of bacterial blight of pomegranate based on two loci, 16S rRNA and gyrB. Annals of Microbiology, 63(2): 801-804
21. Parkinson N, C. Cowie J. Heeney and D. Stead .2009. Phylogenetic structure of Xanthomonas determined by comparison of gyrB sequences. International journal of systematic and evolutionary microbiology. 2009; 59:264-274
22. Raghuwanshi K.S, B.A Hujare, V.P, Chimote and S.G. Borkar Characterization of Xanthomonas axonopodis pv. punicae isolates from western Maharashtra and their sensitivity to chemical treatments. The Bio scan. 2013; 8(3):845-850
23. Rohlf F. J. 1998 Numerical Taxonomy and Multivariate Analysis System, Version 2.0 (Exeter Software, New York), 1998

24. Schaad N . 1992. Laboratory Guide for Identification of Plant Pathogenic Bacteria. Second Ed. International Book Distributing Co, Lucknow, 1992, 165

25. Sharma, K., J. Sharma, . and V. Jadhav, 2015. In Recent Advances in Diagonosis and Management of plant Disease 119–126 (Springer 2015).

26. Sharma, V., S. Midha, M., Ranjan, A.K.., Pinnaka, and P.B. Patil, 2012. Genome Sequence of *Xanthomonas axonopodis* pv. punicae Strain LMG 859. Journal of Bacteriology, 194(9), 2395–2395. doi:10.1128/jb.00181-12

27. Weigand F., Baum and S. Udupa. 1993.. DNA Molecular Marker Techniques. Technical Manual No.20. International Center for Agricultural Research in the Dry Areas (ICARDA). Aleppo, Syria.