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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF POSTHARVEST FUNGAL PATHOGEN *(Penicillium chrysogenum*) IN POMEGRANATE FRUITS (*Punica granatum* L.) IN DUHOK PROVENCE- IRAQ

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ABSTRACT

The research aimed to identify and characterize six samples of *P. chrysogenum* isolated from pomegranate fruits in Duhok City – Iraq. The study used morphological, internal transcribed spacer (ITS) region anitsd PCR-RFLP markers. The morphological examination revealed consistent characteristics across all six samples, indicating their classification as *P. chrysogenum*. The macroscopic features showed dense, blue-green colonies with a velvety texture, revealing brush-like conidiophores and distinctive conidia structures. Molecular analysis of the ITS region showed an approximately 600bp band that confirmed the morphological identification, providing additional support for the presence of *P. chrysogenum* in the pomegranate samples. The obtained sequences using 28S rRNA partial gene of 1200bp showed a high degree of similarity with known *P. chrysogenum* reference sequences, further validating the identification. Furthermore, the enzymes Rsa I, Hae III, and Alu I were chosen to study the restriction site amplification of the affected ITS region of all examined P. chrysogenum. The results showed significant identical amplified bands among the studied isolates. This study may pave the way for the distribution of P. chrysogenum pathogens in Duhok City and highlights the importance of integrating molecular and morphological techniques for accurate identification.

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INTRODUCTION

The pomegranate (*Punica granatum* L.) tree remains famous for landscape greening because of its great decorative and economic value. Pomegranate fruit is abundant in carbs and other essential minerals and elements such as potassium, as well as multiple vitamins and flavonoids (Abdullah, 2010; Thomidis, 2014; Thannoun & Tayib, 2014 and Tufail *et al.*, 2023). Furthermore, pomegranate fruit involves a significant amount of pharmaceutical and therapeutically bio-active chemicals, which in turn could be included in the hypolipidemic and act as an essential antioxidant antibacterial in addition to vascular protective properties (Thannoun & Tayib, 2014 and Tufail *et al.*, 2023). Pomegranate diseases mainly caused largely through a range of bacteria and fungi, represent severs risks to the economic values, nutritional and postharvest losses. Several pathogens producing deterioration diseases among pomegranate has been identified via different studies

conducted in various states. *Zythia versoniana Sacc*. (Sun, 2008), *Botrytis cinerea* (Tedford, 2005), *Botryosphaeria dothidea* (Fu, 2007), *Pilidiella granati* (Mirabolfathy, 2012), *Neofusicoccum parvum*, and *Alternaria alternata* (Yao, 2017) are among the pathogenic organisms responsible for pomegranate rots. Several *Penicillium* species, such as *P. chrysogenum, P. digitatum, P. chrysogenum, P. italicum, P. crustosum, P. solitum, and P. verrucosum,* are known to cause blue mold decay, as reported by Palou *et al.,* 2010; Chen *et al*. in 2017; Papoutsis *et al*., 2019; Martín, 2020 and Duduk *et al*. in 2021.

Furthermore, different fungal pathogens were isolated from other fruits (Taha, 2018). Therefore, suitable fungicide must be administered in the cultivation area to combat such pathogens that harm the fruit as it matures. Moreover, postharvest fungicides might also be employed to suppress localized infections in fruit tissue, as well as avoid forming new infections throughout postharvest handling and storage (Strano *et al.,* 2022). The *Penicillium* genus is a commonly encountered group of fungi, readily isolated due to the widespread dispersion of their spores, as noted by (Myung *et al*. 2014). Identification of *Penicillium* species primarily relies on morphological characteristics, encompassing macroscopic and microscopic features that arise from the fungus's growth and sporulation patterns.

Molecular identification of fungi often relies on the DNA nuclear ribosome's internal transcribed spacer (ITS) region sequence (Schoch, 2012; Conti *et al.,* 2021). Individual fungi can be recognized and categorized rapidly and effectively by evaluating the information of the ITS region sequence. Furthermore, morphological and physiological variations might reveal species boundaries for DNA identification. These features may be utilized to identify a species and comprehend its biology (Maharachchikumbura *et al.,* 2021; Antil *et al.,* 2023). Therefore, the primary purpose of this study was to use morphological and molecular techniques to evaluate the prevalence of pomegranate-related post-harvest fungus. The study aimed to employ a combination of morphological, ITS region analysis, PCR-RFLP, partial 28S rRNA gene, and sequencing techniques to identify and characterize six *P.chrysogenum* accurately isolates collected from pomegranate fruits in Duhok City.

MATERIALS AND METHODS

Plant material

Approximately 40 pomegranate fruits with rot signs were gathered from the pomegranate store market (city center) and from Bebade / Sulav farm and brought to the laboratory for the next step of pathogen isolation.

Isolation of pathogen

Before cutting the epidermal tissues from the decaying part of the pomegranate, they were sanitized using 70% ethanol. These tissue sections, about 5 mm in length, were grown at 28 °C for two days on potato dextrose agar (PDA). The grown mycelia from the edges of the colonies were gathered and placed on fresh PDA plates to ensure purity. This process was repeated to harvest a single colony, and a single colony was isolated from each pathogen for subsequent identifications (Houbraken and Samson, 2011).

Pathogens Morphological Identification

Identification relied primarily on microscopic morphology. Microscopic observations were carried out using potato dextrose agar (PDA) for slide culture (Houbraken and Samson, 2011). Colony characteristics and diameters on potato dextrose agar media, Conidiophores, conidia, growth rate, pigmentation, color, surface texture, and phialide morphology were all measured on seven-day-old slide cultures (Frisvad and Samson, 2004); all these features are essential for species identification. The cultures obtained on PDA slants were kept in the refrigerator at five °C for further study.

DNA Extraction and PCR amplification

The Jena Bioscience plant and fungus DNA preparation kit (Jena Bioscience GmbH.07749 Jena Germany) was used to obtain the whole genomic DNA concentration of six isolated samples (177.8, 98.3, 245.2, 230.4, 278.6 and 125.7 nanogram respectively) from 20–30 mg single spore fungal culture samples.

Tables 1 and 2 show the primers used for amplification of the ITS region and the 28S rRNA partial gene, respectively.

Table (1): Primers used for amplification of ITS region.

Primer ITS region	Sequence	Tm (°C)	GC (%)	Product size
Forward ITS1	5'TCCTCCGCTTATTGATATGC3'	56.4	45.0	600bp
Reverse ITS4	5'TCCGTAGGTGAACCTGCGG3'	61.7	63.16	

For amplification of ITS region, a PCR reaction mixture of 20µl was prepared, which included 2µl of templet DNA (50 ng), 2µl of forward and reverse primer in concentration of (10 pmol each), and 10µl of Taq DNA Master (2x conc.) (Addbio, Korea). Additionally, 4µl of sterile water (DD water). An ABI Applied Biosystems PCR System 2720 thermo cycler was utilized to perform the PCR amplification, following optimized thermocycler conditions. These conditions involved a primary denaturation step for 5 minutes at 94 °C, following by 35 cycles of constant denaturation for 30 seconds at 94 °C, 58 °C annealing for 30 seconds, 72 °C extension for 30 seconds, last extension for 10 minutes at 72 °C.

Table (2): Primers used for 28S rRNA partial gene.

Primer 28S rRNA	Sequence	Tm (°C)	GC (%)	Product size
LUS Forward	5'- ACCCGCTGAACTTAAGC -3'	57.3	52.9	1200bp
LUS Reverse	5'- CGCCAGTTCTGCTTACC -3'	59.2	58.8	

The 28S rRNA partial gene was amplified using PCR in 50 µl of the reaction mixture that contained 25 µl of 2x Taq DNA Polymerase Master Mix (Addbio, Korea), 2 µl each of the LUS-forward and LUS reverse primers 17 µl of DNase-free

water, and four µl of DNA template. ABI Applied Biosystems PCR System 2720 thermal cycler was used to carry out the amplification. The PCR protocol included a 5-minute initial denaturation at 95 °C, 35 cycles of denaturation at 95 °C for one minute, primer annealing at 56 °C for one minute, an extension at 72 °C for one minute, and a final extra-extension step at 72 °C for ten minutes.

PCR-Restriction Fragment Length Polymorphism (PCR- RFLP)

The PCR products from the *Penicillium* ITS region was employed in PCR-RFLP analysis. Enzymes known for producing smaller and lower-molecular-weight fragments were selected. To be more precise, the amplified area was digested by the German enzymes HaeIII (GG/CC), RsaI (GT/AC), and AluI (AG/CT) from Jena Bioscience. The manufacturer's instructions were followed to prepare each digestive reaction in a final amount of 50µl: PCR-grade water was used to make up the remaining volume after adding 5µl of 10x universal buffer, 1µg of PCR product, and ten units of the enzyme. After that, the combination was incubated for two to four hours at 37°C. The resulting digested products were subsequently evaluated via 3% agarose gel electrophoresis.

Sequencing and Alignment of DNA

The 28S rRNA partial gene PCR result from the fungal sample was sequenced at the Korean company Macrogene using the ABI Prism Terminator Sequencing Kit (Applied Biosystem). Finch TV program software was used to modify 28S rRNA chromatograms and verify base calls. The Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) is used. The 28S rRNA gene sequence was aligned with other biological sequences to determine more similarities and/or differences with fungal species. The tool is accessible on the NCBI (National Center for Biotechnology Information) website.

RESULTS AND DISCUSSION

The Morphological Identification of Pathogens was accomplished at temperatures of 28 °C; *P. chrysogenum* mature colonies were spherical and proliferated, reaching a diameter of 5 cm in just seven days. The proliferation of colonies on PDA was observed in white with plentiful conidiogenesis after a week, giving the colonies a blue-green color. The colony edge was fully formed and thin, and the bottom was pale to cream-yellow (Figure 1, A and B). Conidia were hyaline, single-celled, ellipsoid-shaped conidia, whereas hyphae were septate, mono-verticillate, branching, septate conidiophores containing sporulating cells (phialides). Morphology of Conidiophores rise from superficial mycelium. Phialides and Conidia have a smooth texture, born in columns, globose to sub-globose. All isolates' conidia varied in size from 2.5- 3 x 2.5-3 µm, and conidiophore 8-9.5 x 2.5-3 (Figure 1).

The macroscopic and microscopic features of isolated pathogens were studied for morphological identification. The macroscopic properties, such as colony structure and color, corresponded with those described for *P. chrysogenum*. Microscopic examination revealed typical Penicillium species characteristics such as brush-like conidiophores and distinct conidia structures.

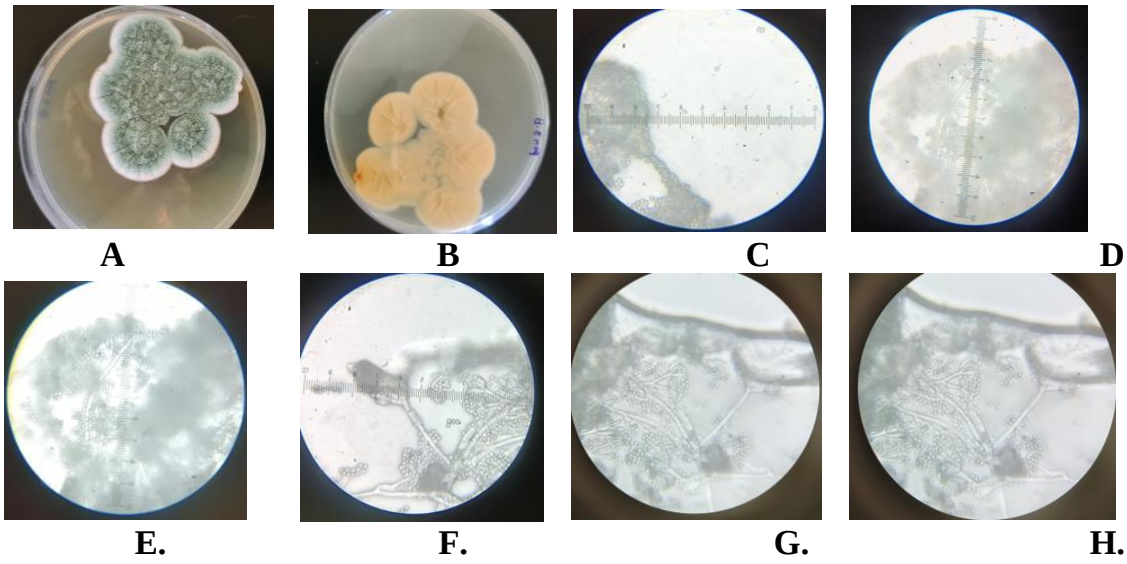

Figure (1): Morphological characteristics of *P. chrysogenum* grown on PDA for 7 days. (A) Colony surface on PDA (B) Colony reverse on PDA (C-H) conidia, phialide and structure.

The same morphological traits in all samples supported the conclusion that these samples were all *P. chrysogenum*. This confirms the finding of (Samson *et al.*, 1977). From the cultured isolation, six penicillium isolates were selected for DNA isolation and used for amplification of the ITS region that showed the clear and sharp band and was invariant in the length of about 600bp, as shown in (Figure 2).

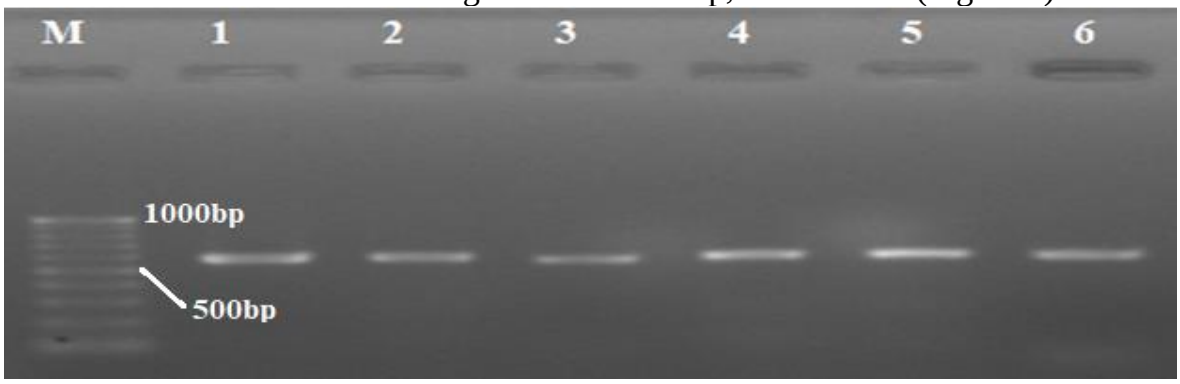

Figure (2): The 1.5% agarose gel of PCR amplification of ITS region. From left the first lane represent 1 kilo base pair ladder, lane no.1 to 6 represent penicillium *chrysogenum* isolated from pomegranate.

For the PCR-Restriction Fragment Length Polymorphism (PCR-RFLP), The digestion pattern for utilizing restriction enzyme shows a different range of bands in each isolate, as shown in (Figure 3). The RsaI enzyme showed a single band of about 590bp among isolates. On the other hand, the HaeIII restriction enzyme identified a clear single band of 350bp in all isolates. Meanwhile, the AluI enzyme also revealed a band of about 590bp.

Molecular identification utilizing the ITS region has proven a reliable approach for fungal species identification (Antil *et al.*, 2023). Furthermore, using PCR-RFLP provided an additional level of confirmation for species identification, as in the case of recognizing bacterial pathogens in pomegranate (Sinjare and Jubrael,

2023). Also, molecular markers such as RAPD could identify genetic variability (Ali Omarbly *et al.*, 2021).

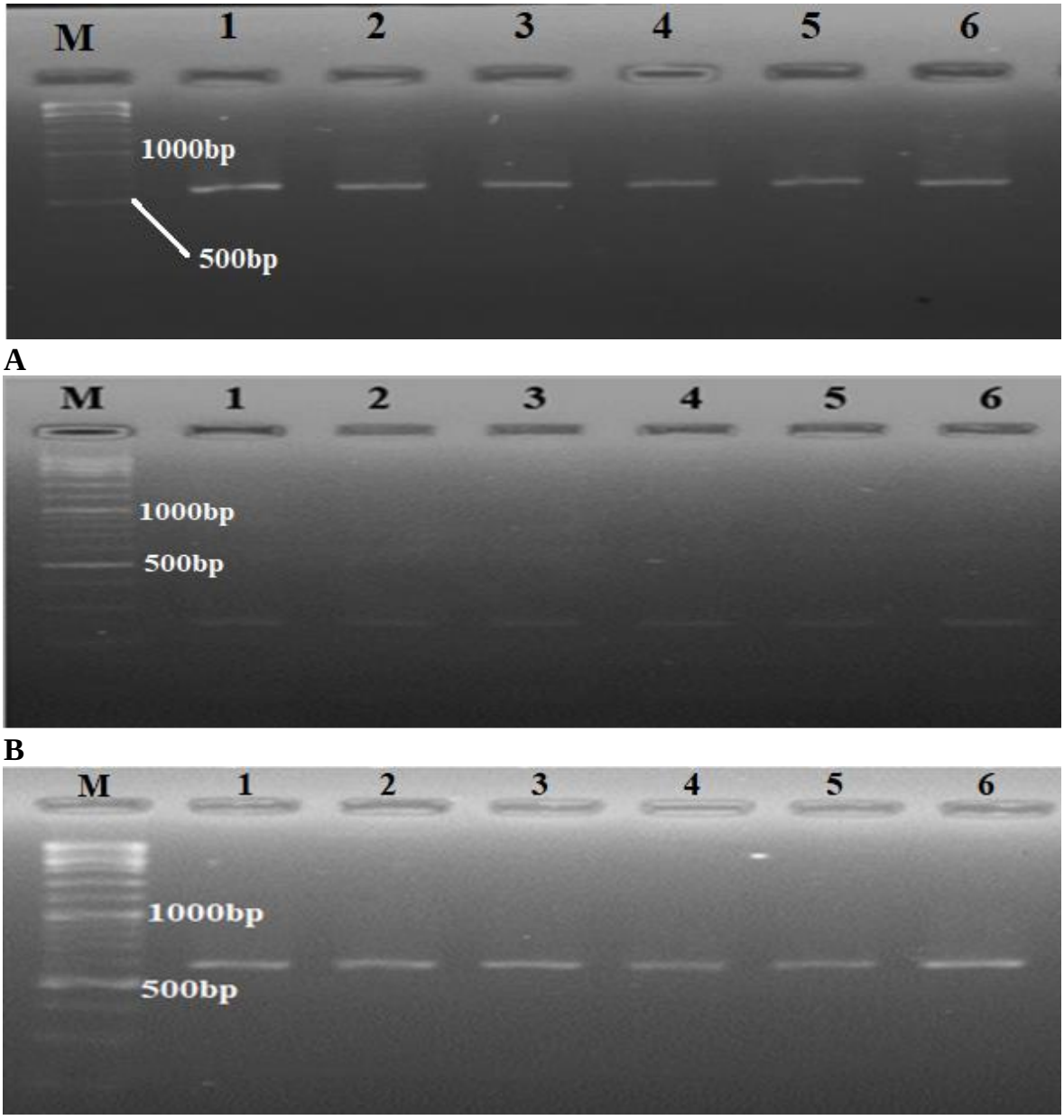

Figure (3): Represent 3% agarose gel for PCR- RFLP result digested with A) RsaI, B) HaeIII and C) AluI restriction enzyme. From left the first lane represent 1 kilo base pair ladder, lane no.1 to 6 represent penicillium *chrysogenum* isolated from pomegranate.

The PCR-amplified ITS region was digested using restriction enzymes, resulting in various fragment patterns based on alterations in the DNA sequences. The samples' identity was confirmed further using the ribosomal RNA sequences synthesized by Micro-Gene Company (South Korea), and primers specific to the 28S gene were designed. As predicted, a band of 1200 bp could be produced by the

primers. Using a 1.5% Agarose gel, the PCR product was electrophoresed and observed (Figure 4).

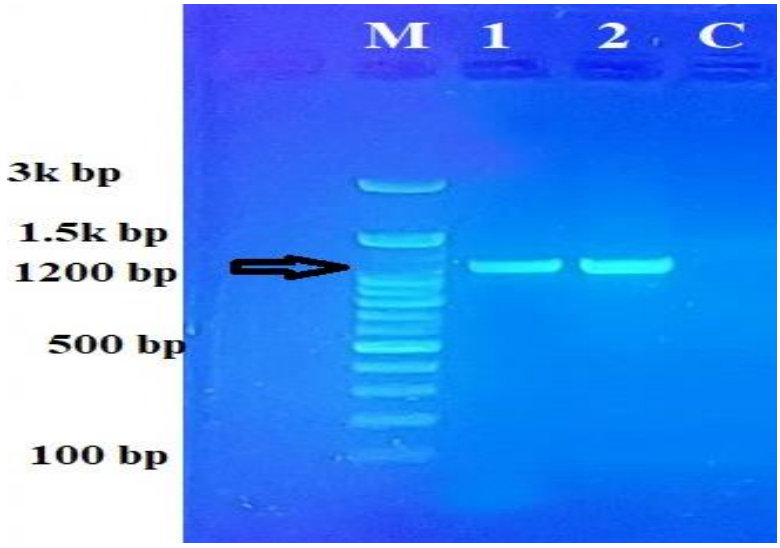

Figure (4): Partial 28S rRNA gene amplification from fungal materials using PCR: lane 1 has a ladder (3k bp–100 bp), lane 2 contains amplified gene bands, and lane C serves as a negative control.

The BLAST tool from Gen Bank (http://blast.ncbi.nlm.nih.gov/) was used. An amplicon size of 1200 bp of the 28S partial rRNA gene sequence was aligned. The amplified sequences were then compared to other species of *Penicillium* sequences that were saved. According to the BLAST findings in NCBI Genbank, the greatest identity number query sequence was 100% identity, given an accession number of OR226321, as shown in Table (3).

Table (3): Percentage distribution of *Penicillium chrysogenum* based on partial 28S rRNA according to nblast in Genbank of NCBI

Fungus Identified	Accession Numbers	Query Cover %	Identic Number %	Accession Number of BLAST Identification
Penicillium chrysogenum	OR226321	100	100	KY781803
		100	100	KX375781
		100	100	KX375766
		100	100	OQ787027
		100	100	KJ881371
		100	100	MW561583
		100	100	JQ434684
		100	100	JN938948
		100	100	JF922035
		100	100	MT226568
		100	100	MK713338

Furthermore, a species of *Penicillium chrysogenum* examined by the phylogenetic inference analysis based on the 28S rRNA nucleotide sequence was grouped along predicted lines. The phylogeny and sequence divergence similarity data was used, and it was possible to determine how closely related species within

different genera were to one another in biological sequences in the NCBI GenBank, as in Figure (5).

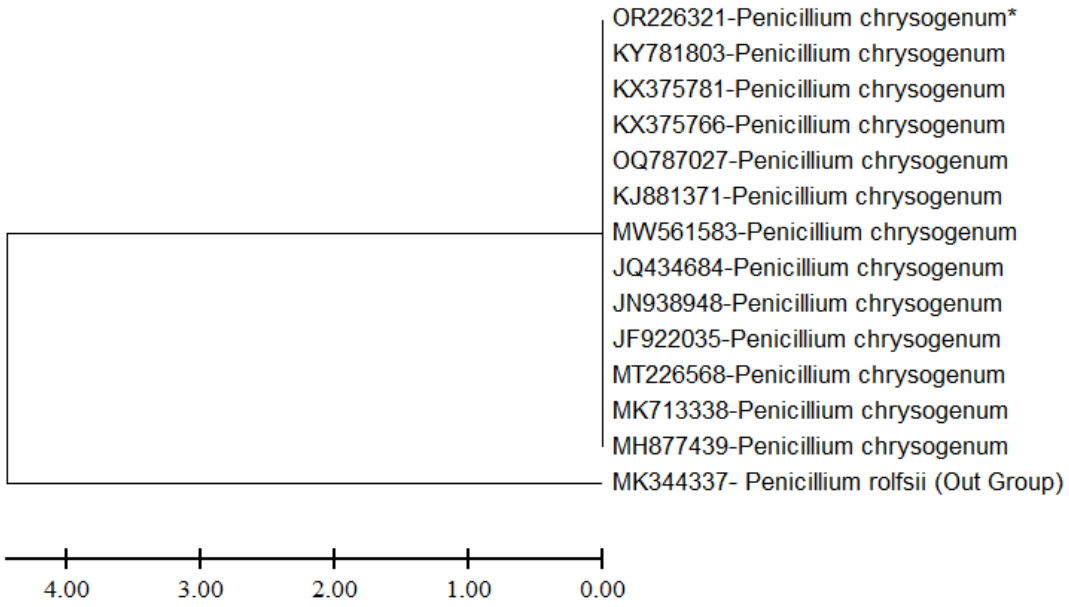

Figure (5): *Penicillium chrysogenum* sample's phylogenic tree from the Kurdistan area of Iraq (*). Using bootstrap analysis with 100 re-samplings and the Maximum Likelihood technique based on the Tamura-Nei model in MEGA11 software, the phylogenic tree was built. Concatenated partial 28S ribosomal rRNA gene partial DNA sequences of Iraqi isolate were utilized as input data compared with international *P. chrysogenum* isolates.

In this work, molecular approaches and morphological identification provide a complete methodology for precisely identifying *P. chrysogenum* isolates on pomegranate fruits. The findings obtained using both approaches were consistent, strengthening the trustworthiness of the identification method (Fierro *et al.*, 2022). This study's findings have implications for the managing pomegranate orchard postharvest loss caused by *P. chrysogenum*. Accurate pathogen identification is essential for executing successful disease management techniques. Knowing the presence of *P. chrysogenum* in Duhok City allows for the development of targeted control techniques, such as appropriate fungicide applications, improved storage conditions, and postharvest treatments.

CONCLUSIONS

This study identified and isolated six *Penicillium chrysogenum* isolates from rotted pomegranate fruits in Duhok city. From the morphological and molecular ITS region, PCR-RFLP identification was shown to be accurate and effective pathogen characterization results. The results of this research may help in the early identification of this pathogen in the region, allowing for the selection of an effective disease management strategy for pomegranate trees.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

There is no conflict of interest, the authors state.

التعريف الجزيئي لمسببات الامراض الفطرية بعد الحصاد للرمان (*Punica granatum L.*) في محافظة دهوك – العراق

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الخلاصة

تعد شجرة الرمان (*Punica granatum* L.) خيارًا شائعًا لتخضير المناظر الطبيعية بسبب قيمتها الزخرفية والغذائية الرائعة بالإضافة إلى قيمتها الاقتصادية. تمثل ثمار الرمان المصابة بالفطريات مخاطر جسيمة على القيمة الاقتصادية والخسائر الغذائية والتسويقية بعد الحصاد. هناك العديد من أنواع البنسليوم المسببة للأمراض الخطيرة المسؤولة عن تعفن الرمان. يعد *Penicillium chrysogenum* أحد مسببات الأمراض الفطرية المسؤولة عن التسبب في تعفن ما بعد الحصاد في مختلف الفواكه، بما في ذلك الرمان. يعد التحديد الدقيق والسريع لهذا العامل الممرض أمرًا حاسمًا لاستراتيجيات إدارة المرض الفعالة. يهدف هذا البحث إلى تحديد وتوصيف ست عينات من *P. chrysogenum* المعزولة من ثمار الرمان في مدينة دهوك – العراق باستخدام المؤشرات الشكلية والجزيئية ITS وPCR–RFLP. كشف الفحص المظهري (المورفولوجي) عن خصائص متسقة في جميع العينات الست، مما يشير إلى تصنيفها كـ *P. chrysogenum*. أظهرت السمات العيانية مستعمرات كثيفة، زرقاء مخضرة ذات ملمس مخملي، كما كشفت عن كونيديوفورات شبيهة بالفرشاة وتركيبة كونيديا مميزة. أظهر التحليل الجزيئي لمنطقة ITS ما يقرب من 600 نقطة أساس في النطاق الذي أكد التحديد المورفولوجي كما أظهرت التسلسلات التي تم الحصول عليها باستخدام الجين الجزئي 28S rRNA البالغ 1200 نقطة أساس درجة عالية من التشابه مع التسلسلات المرجعية المعروفة لهذا الفطر، مما يوفر دعمًا إضافيًا لصحة وجود *P. chrysogenum* في عينات الرمان. علاوة على ذلك، تم اختيار الإنزيمات القاطعة Rsa I وHae III وAlu I لدراسة تعدد الأشكال المستشهد بها لمنطقة ITS الناتجة لجميع العينات التي تم فحصها. أظهرت النتائج تعدد أشكال بين العزل المدروسة. قد تمهد هذه الدراسة الطريق لتوزيع مسببات الأمراض من *P. chrysogenum* في مدينة دهوك وتسلط الضوء على أهمية دمج التقنيات الجزيئية والشكلية (المظهرية) لتحديد دقيق.

الكلمات المفتاحية: تفاعل البلمرة المتسلسل–تعدد اطوال جزء الحصر، البنسيلين كريسوجينوم، الرمان، سلسلة Punica granatum L.) OR226321.

REFERENCES

Abdullah, G. (2010). Variation of morphological and pomological characters of some pomegranate genotypes in north-western regions of Syria. *Mesopotamia Journal of Agriculture*, 38, 53-62. https://doi.org/10.33899/magrj.2010.34166

Ali Omarbly, H. H., Khdr, D. M., Khailany, R. A., Ahmed, A. M., & Fateh Salai, S. A. (2021). Evaluation of genetic variability between three lines of chickens based on RAPD-PCR and 18s rRNA gene sequencing in Erbil (iraqi kurdistan region). *Mesopotamia Journal of Agriculture*, 49(3), 12-26. https://doi.org/10.33899/magrj.2021.129979.1129

Antil, S., Abraham, J. S., Sripoorna, S., Maurya, S., Dagar, J., Makhija, S., ... & Toteja, R. (2023). DNA barcoding, an effective tool for species identification: a review. *Molecular Biology Reports*, 50(1), 761-775. https://doi.org/10.1007/s11033-022-08015-7

Chen, L. S., Liu, Y. J., Xu, S. W., Chen, Y. L., Chen, M. J., & Zhou, L. J. (2017). First report of *Penicillium polonicum* causing blue mold on stored *Polygonatum cyrtonema* in China. *Plant Disease*, 101(12), 2149. https://doi.org/10.1094/PDIS-05-17-0756-PDN

Conti, A., Corte, L., Casagrande Pierantoni, D., Robert, V., & Cardinali, G. (2021). What is the best lens? Comparing the resolution power of genome-derived markers and standard barcodes. *Microorganisms*, 9(2), 299. https://doi.org/10.3390/microorganisms9020299

Fierro, F., Vaca, I., Castillo, N. I., García-Rico, R. O., & Chávez, R. (2022). *Penicillium chrysogenum*, a vintage model with a cutting-edge profile in biotechnology. *Microorganisms*, 10(3), 573. https://doi.org/10.3390/microorganisms10030573

Frisvad, J. C., & Samson, R. A. (2004). Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate *Penicillia* and their mycotoxins. *Studies in mycology*, 49(1), 1-174. https://www.studiesinmycology.org/sim/Sim49/Sim49Article1.pdf

Fu JuanNi, F. J., Liu XingHua, L. X., Cai FuDai, C. F., & Kou LiPing, K. L. (2007). Identification of pathogenic fungus causing a decay of stored pomegranate fruits using molecular biology technique. https://www.ahs.ac.cn/EN/abstract/abstract533.shtml

Houbraken, J. A. M. P., & Samson, R. (2011). Phylogeny of *Penicillium* and the segregation of *Trichocomaceae* into three families. *Studies in mycology*, 70(1), 1-51. https://pubmed.ncbi.nlm.nih.gov/22308045/

Martín, J. F. (2020). Insight into the genome of diverse *Penicillium chrysogenum* strains: specific genes, cluster duplications and DNA fragment translocations. *International Journal of Molecular Sciences*, 21(11), 3936. https://doi.org/10.3390/ijms21113936

Maharachchikumbura, S. S., Chen, Y., Ariyawansa, H. A., Hyde, K. D., Haelewaters, D., Perera, R. H., ... & Stadler, M. (2021). Integrative approaches for species delimitation in *Ascomycota*. *Fungal Diversity*, 109(1), 155-179. https://doi.org/10.1007/s13225-021-00486-6

Mirabolfathy, M., Groenewald, J. Z., & Crous, P. W. (2012). First report of *Pilidiella granati* causing dieback and fruit rot of pomegranate (*Punica*

granatum L.) in Iran. *Plant Disease*, 96(3), 461-461. https://doi.org/10.1094/PDIS-10-11-0887

Palou, L., Guardado, A., & Montesinos-Herrero, C. (2010). First report of *Penicillium* spp. and *Pilidiella granati* causing postharvest fruit rot of pomegranate in Spain. *New Disease Reports*, 22(21), 2044-0588. https://doi.org/10.5197/j.2044-0588.2010.022.021

Papoutsis, K., Mathioudakis, M. M., Hasperué, J. H., & Ziogas, V. (2019). Non-chemical treatments for preventing the postharvest fungal rotting of citrus caused by *Penicillium digitatum* (green mold) and *Penicillium italicum* (blue mold). *Trends in Food Science & Technology*, 86, 479-491. https://doi.org/10.1016/j.tifs.2019.02.053

Samson, R. A., Hadlok, R., & Stolk, A. C. (1977). A taxonomic study of the *Penicillium chrysogenum* series. Antonie van Leeuwenhoek, 43, 169-175. https://doi.org/10.1007/BF00395671

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., ... & White, M. M. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the national academy of Sciences*, 109(16), 6241-6246. https://doi.org/10.1073/pnas.1117018109

Sinjare, D. Y. K., & Jubrael, J. M. (2023). PCR-RFLP analysis of the 16S rRNA and its regions in bacterial blight (*Xanthomonas axonopodis pv. punicae*) across pomegranate farms in Kurdistan Regions-Iraq. *Iraqi Journal of Agricultural Sciences*, 54(2), 338-346. https://doi.org/10.36103/ijas.v54i2.1707 .

Strano, M. C., Altieri, G., Allegra, M., Di Renzo, G. C., Paterna, G., Matera, A., & Genovese, F. (2022). Postharvest technologies of fresh citrus fruit: Advances and recent developments for the loss reduction during handling and storage. Horticulturae, 8(7), 612. https://doi.org/10.3390/horticulturae8070612

Taha, H. (2018). Isolation and identification fungi from spoilage fruits and vegetables and study ability of it to produce enzymes lyase of cell wall. *Mesopotamia Journal of Agriculture*, 46(2), 266-277. https://magrj.mosuljournals.com/article_161467.html

Thannoun, A. M., & Tayib, M. M. (2014). Chemical composition and some characteristcs of pomegranate juice extacted by squeezing and centrifugation methods. *Mesopotamia Journal of Agriculture*, 42(1), 270-279. https://doi.org/10.33899/magrj.2018.161467

Thannoun, A. M., & Tayib, M. M. (2014). Effect of pomegranate juice consumption on lipid profile in healthy and hypercholesterolemic rats. *Mesopotamia Journal of Agriculture*, *42*(1), 280-292. https://magrj.mosuljournals.com/article_89360.html

Tedford, E. C., Adaskaveg, J. E., & Ott, A. J. (2005). Impact of Scholar (a new postharvest fungicide) on the California pomegranate industry. *Phytopathology News*, 39(2), 19. https://www.proquest.com/openview/4b9fbfde0df0f554ee8c387fd9aa3075/1?pq-origsite=gscholar&cbl=27382

Thomidis, T. (2014). Fruit rots of pomegranate (cv. Wonderful) in Greece. *Australasian Plant Pathology*, 43(5), 583-588. https://doi.org/10.1007/s13313-014-0300-0

Tufail, T., Ain, H. B. U., Shabbir, R., Hameed, M., Saeed, F., Afzaal, M., ... & Khan, A. A. (2023). Pomegranate. In Fruits and Their Roles in Nutraceuticals and Functional Foods (pp. 141-162). *CRC Press*.

Yao, X. (2017). Study on Postharvest Quality Deterioration and Control Technology of Pomegranate (Doctoral dissertation, Sichuan Agriculture University).