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Original Research

Comparison between Traditional and PCR Methods for Identifying *Fusarium oxysporum f. sp. Melonis*, the Causal Agent of Watermelon *Citrullus lanatus* Wilt

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ABSTRACT:

Fungal diseases have become a major threat to field crops, particularly watermelons, due to the escalating prevalence of *Fusarium oxysporum*. This soil-borne pathogen has been identified as a primary cause of significant yield losses in watermelon cultivation across various regions of eastern Libya, including Tobruk, Al Marj, and Tukrah. The study aimed to investigate the prevalence of *F. oxysporum f. sp. melonis*, the causal agent of Fusarium wilt in watermelons, and to establish a reliable and efficient diagnostic protocol.Visual symptoms such as leaf chlorosis, wilting, and root rot were observed in watermelon plants across the study sites. Fungal isolates were obtained from symptomatic tissues and subjected to morphological, microscopic, and molecular analyses. Morphological and microscopic characterization provided preliminary evidence of *Fusarium* infection; however, molecular identification using polymerase chain reaction (PCR) with specific primers confirmed the presence of *F. oxysporum f. sp. melonis* with a high degree of accuracy (99%).The study highlights the utility of PCR-based diagnostics in the

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rapid and accurate detection of *F. oxysporum f. sp. melonis* in watermelons. This molecular approach offers significant advantages over traditional methods in terms of sensitivity, specificity, and time efficiency. The findings underscore the importance of implementing integrated disease management strategies, including the use of resistant cultivars, crop rotation, and appropriate cultural practices, to mitigate the impact of Fusarium wilt on watermelon production in eastern Libya.

KEYWORDS: *Fusarium oxysporum f. sp. Melonis* ,Watermelon Wilt , Citrullus lanatus , Traditional Methods /PCR Technology , Identification.

INTRODUCTION

Watermelon (*Citrullus lanatus*), a highly sought-after summer cucurbit, is cultivated extensively due to its desirable taste, appearance, and nutritional value. Given its market appeal, it is a significant commodity both domestically and internationally. China leads the world in watermelon cultivation in terms of both area and production (FAO, 2016), while Egypt tops the list in the Arab world (Arab Organization for Agricultural Development, 2015).

Fusarium oxysporum is a fungal pathogen that infects watermelon plants, causing distinct symptoms at various growth stages. Seed infection can lead to seed rot and reduced seedling emergence. Young seedlings may wilt and die, while older plants exhibit wilting and eventual death. Fruit set is also affected, resulting in smaller, unmarketable fruits. Root examination of infected plants in the study sites revealed yellowing and death of leaves, as well as lesions at the points of fungal penetration. Disease severity is exacerbated by high temperatures and extended periods of light.

This pathogen poses a significant threat to watermelon production as it can persist in the soil for up to 15 years through resistant structures (Kaur et al., 2012) or in alternative hosts like weeds without displaying symptoms (Negreiros et al., 2019). The fungus thrives in water-stressed and arid environments (Cohen et al., 2014), and its severity increases with each successive cropping cycle due to the accumulation of inoculum (Ambrósio et al., 2015; Lodha & Mawar, 2020). Consequently, early detection of the fungus in production areas is crucial for implementing effective control measures.

Traditionally, *F. oxysporum* has been identified through morphological and microscopic examination of colonies and spores (Biswas et al., 2014). However, this method lacks specificity due to the significant morphological diversity among fungal species (Iqbal, U., & Mukhtar, 2014) and is time-consuming and laborious (Biswas et al., 2014). In this context, PCR has emerged as a rapid and reliable technique for detecting plant pathogens (Martinelli et al., 2015). Species-specific primers developed by Babu et al. (2007) have facilitated the detection and identification of *F. oxysporum* and other pathogens in various crops worldwide.

The conventional methodology involves isolating the fungus from soil or infected plant tissues onto culture media, followed by DNA extraction, purification, and detection using PCR (Babu et al., 2013). Direct PCR, on the other hand, amplifies DNA directly from samples without prior isolation, reducing time and costs and allowing for the rapid analysis of a large number of samples (Ben-Amar et al., 2017). Direct PCR has been successfully employed to detect plant pathogenic bacteria (Fujikawa et al., 2013), fungi (Ben Amar et al.,

2012), and viruses (Biswas et al., 2014) directly from plant tissues.

This study aimed to detect and identify *F. oxysporum* in watermelon-growing areas using molecular methods for rapid identification and comparing the results with traditional methods. Plant tissue samples were collected from three different regions: Al-Qa'arah, east of Tobruk; Butrabeh coast in Tukra; and Al-Zawawi farms in Al-Marg. DNA was extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) method, and amplification was performed using direct PCR. The identity of the samples was confirmed through DNA sequencing. This study represents the first molecular diagnosis of *F. oxysporum*-associated watermelon disease in these regions. The methodology presented here can be applied for the reliable and simple diagnosis of pathogens in other crops.

MATERIALS AND METHODS

1.Sample Collection

Plant samples, including leaves and roots, were collected from three watermelon-producing regions in eastern Libya: Al-Qa'arah area in Tobruk, Butrabeh coast in Tukra, and Al-Zawawi farms in Al-Marj. These regions exhibit a Mediterranean climate according to the Köppen climate classification. Healthy leaves were collected as negative controls. Samples were placed in plastic bags and transported to the laboratory for sterilization (in 10% sodium hypochlorite solution for one minute, followed by rinsing with distilled water for two minutes), and then stored at -20°C (Watanabe, 2010).

2.Isolation of Fungi from Plant Tissues

To isolate the target fungus from plant tissues, leaves and roots collected from plants were first washed under tap water and thoroughly dried. These samples were then subjected to surface sterilization, followed by immersion of tissue pieces for 30 seconds in a 6-14% sodium hypochlorite solution, rinsing with distilled water, and drying on sterile filter paper. The dried tissue sections were aseptically cut into smaller pieces and inoculated onto 9 mm Petri dishes containing Potato Dextrose Agar (PDA) medium. Inoculations were performed in 3 replicates for each sample, resulting in 3 Petri dishes per sample. Petri dishes were then wrapped in Parafilm and incubated at room temperature for 10 days (Galleglly and Hong, 2008).

3.Morphological Identification of Fungal Isolates

Pure cultures of the target fungus, previously isolated at room temperature and cultivated on Potato Dextrose Agar (PDA), were obtained. These cultures were then incubated on fresh PDA plates at 25°C for 10 days. To facilitate morphological examination, additional cultures were established on Corn Mail Agar (CMA) and incubated under the same conditions. Fungal colonies on PDA plates were observed under a stereomicroscope and compared to reference images in fungal atlases (Akılı et al., 2010).

4. Microscopic Identification of Fungal Spores

A microscopic slide was prepared by placing a small sample of fungus from a CMA culture plate onto a clean glass slide. A drop of water and a stain were added to enhance spore visibility. The slide was then covered with a cover slip and examined under a microscope. The shape, size, color, and arrangement of the spores were observed and recorded. Microscopic images were captured for further analysis. The recorded observations were compared to fungal atlases and specialized databases to identify the spore species. Additional information about different spore

morphologies was obtained from scientific literature (Kim, Ji Yeun, et.al 2011).

5. Molecular Definition of Fungi

5.1 Before Beginning

Extracting deoxyribonucleic acid (DNA) from fungi and conducting polymerase chain reaction (PCR) on fresh fungal tissue requires precise steps.

This is to ensure the quality of the extracted DNA and the success of the analysis (Ben-Amar et al. 2017, Bellstedt et al. 2010).

Preparation of materials HiBind® DNA Mini Kit, a water bath set at 65°C, a centrifuge with a minimum speed of 10,000 rpm, 1.5 ml or 2 ml centrifuge tubes, 100% ethanol, a vortex mixer, a micropipette, and liquid nitrogen (Figure 1).

Figure 1: Equipment to Extract DNA.

5.2. Fungal DNA Extraction Protocol

1. Sample Preparation

Weigh approximately 100 mg of fungal tissue and transfer it to a 2 ml microcentrifuge tube. Grind the tissue in liquid nitrogen using a mortar and pestle.

2. Cell Lysis

Add 400 µl of lysis buffer SFG1 (containing 1% SDS, 50 mM Tris-HCl, pH 8.0) and 4 µl of RNase A to the ground tissue. Vortex the mixture vigorously for 1 minute to ensure complete mixing. Incubate the mixture at 65°C for 10 minutes with occasional gentle shaking to lyse the cells and release the DNA.

3. Protein Precipitation

Add 140 µl of buffer SFG2. Centrifuge the mixture at 10,000 rpm for 10 minutes to pellet the cell debris and proteins.

4. DNA Binding

Transfer the supernatant to a new 2 ml microcentrifuge tube. Add 1.5 volumes of binding buffer SFG3 (e.g., for 500 µl of extract, add 750 µl of SFG3). Vortex the mixture thoroughly. Transfer the mixture to a spin column and centrifuge at 10,000 rpm for 2 minutes.

5. Washing

Discard the flow-through and place the spin column in a new collection tube. Add 650 µl of wash buffer SPW and centrifuge at 10,000 rpm for 1 minute. Repeat the wash step once more.

6. DNA Elution

Centrifuge the spin column at maximum speed for 2 minutes to dry the membrane. Add 100 µl

of elution buffer SPW preheated to 65°C directly to the membrane. Incubate at room temperature for 3-5 minutes.Centrifuge at 10,000 rpm for 1 minute to collect the eluted DNA.

7. DNA Quantification and Quality Assessment

Measure the absorbance of the eluted DNA at 260 nm and 280 nm to determine the concentration and purity. Store the DNA at -20°C.

8. PCR Amplification and Sequencing

Amplify the ITS region of the ribosomal RNA using primers ITS1 and ITS4. Sequence the PCR products using an ABI 3730XL sequencer.

The sequence data were analyzed to identify the fungal species. (White et al, 1990)

5.3.Performing a PCR reaction on the pure products of fungal isolates

PCR amplification of pure fungal isolates using ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5' TCCTCCGCTTATTGATATGC3') primers (Babu et al., 2007).

5.4. Fungal diagnosis

The comparison of nucleotide sequences following DNA sequencing was performed (Figure 2).

The resulting DNA sequences were initially processed using various computer programs and then compared to other sequences in the NCBI GenBank (National Center for Biotechnology Information) using BLAST nucleotide algorithm (Mauchamedov et al., 1994).

Figure.2. ABI 3730XL

RESULTS AND DISCUSSION

The fungus *F. oxysporum* was observed in all three sampling sites. It was also observed in all root samples collected, but was not apparent in the leaves except in the Al Marj city site. Additionally, many different types of fungi were observed, both saprophytes and pathogens, which could be the subject of future studies.

1.Morphological and microscopic definition (traditional methods)

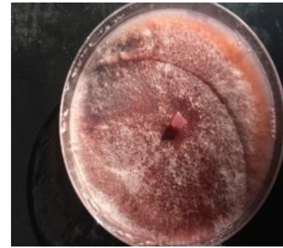
1.1. To identify and confirm the presence of the fungus *F. oxysporum*, symptoms were observed on the cultivated plants. The plants exhibited various signs of infection, all indicative of a possible *F. oxysporum* infestation. Upon examination of the leaves, yellowing and wilting of both new and old leaves were noted in some plants. A general weakening of the leaves, as evidenced by their reduced number and size, was also observed. Examination of the roots revealed rotting, which would hinder the absorption of water and nutrients from the soil. A cross-section of the root showed a darkening of the vascular bundles, a characteristic symptom of *F. oxysporum* infection. This darkening leads to the constriction and blockage of the vascular vessels. When examining the sampling sites during fruit maturation, it was observed that the fruits of the symptomatic plants were smaller in size compared to those of asymptomatic

plants of the same age. The fruits also exhibited deformities such as sunken or raised spots. In some cases, both internal and external fruit rot was observed (Figure 3).

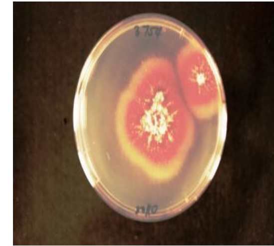
Figure.3 Internal and External Fruit Rot.

1.2. Through monitoring the growth of fungi in Petri dishes containing PDA medium, which were isolated pure from the study areas and compared to images of the target fungus *F. oxysporium* growth in previous studies (Figure.4.2. Snyder, et.al 1940) , a match was observed in the growth patterns of the fungi. This indicates the possible presence of the fungus.

Additionally, the possibility of the presence of other fungi, which may be saprophytic or parasitic, was identified. The fungus was observed to grow rapidly, forming large colonies. Initially, the colonies were white or pink in color, but over time, the color became darker, turning purple or reddish-brown. Concentric rings of different colors were also observed on the Petri dish, with a reddish-pink color being prominent (Figure.4.1).



(Figure.4.1) *F. oxysporium* in the Petri Dish.



(Figure.4.2) *F. oxysporium.* (Snyder, et.al 1940)

1.3. Microscopic examination using a microscope was conducted after preparing a slide using cotton blue lactophenol stain. Thin, branched fungal hyphae were observed (Figure 5.2), along with the characteristic crescent-shaped conidia of the fungus *F. oxysporium*. These included small, transparent, and slightly curved microconidia, as well as larger, more curved macroconidia with thick walls and septa (Figure 5.1). Additionally, small, round bodies resembling chlamydospores were observed. These thick-walled structures were often found singly or in pairs in the middle or at the tips of the fungal mycelium.

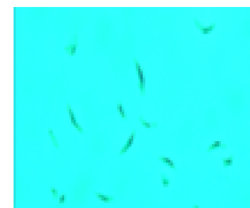


Figure 5.1 Fungal Chlamydospores

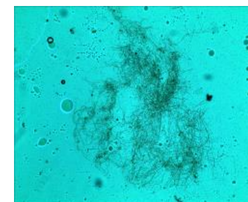
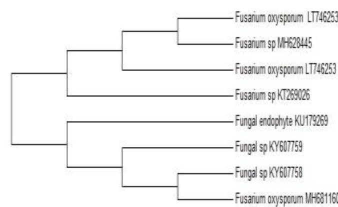


Figure 5.2 Fungal Hyphae.

2. Molecular identification of the fungus using PCR technology

A polymerase chain reaction (PCR) was performed at the Razi Laboratory in Benghazi, amplifying the internal transcribed spacer (ITS) region of the ribosomal RNA of *F. oxysporum* isolates obtained from three different locations. Using specific primers, PCR products of 550, 180, and 600 base pairs were obtained, corresponding to the expected sizes of ITS-1, ITS-5 sequences, respectively (Figure 6.1). The resulting sequences were compared to the NCBI GenBank database using the BLASTn tool (Figure 6.2). The analysis revealed a 99% identity with reference sequences of *F. oxysporum*, indicating that the three isolates obtained from the study sites were indeed *Fusarium oxysporum f. sp. Melonis*, the causal agent of watermelon Fusarium wilt (Figure 7).

(Figure 6.1)



(Figure 6.2)

(Figure 6.1) PCR products of mushroom tissue samples after amplification by electrophoresis using primers for samples taken from the roots; 1. From Tobruk watermelon root/ 2. From Tokra watermelon root/ 3. From Marj watermelon root/ 4. Control. **(Figure 6.2)** Comparison tree between F. oxysprum and other fungi in GenBank.

GTGAACCTGCGGAGGGATCATTACCGAGTTTAC
AACTCCCAAACCCCTGTGAACATACCACTTGTT
GCCTCGGCGGATCAGCCCGCTCCCGGTAAAACG
GGACGGCCCGCCAGAGGACCCCTAAACTCTGTT
TCTATATGTAACTTCTGAGTAAAACCATAAATA
AATCAAAACTTTCAACAACGGATCTCTTGGTTCT
GGCATCGATGAAGAACGCAGCAAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGAATCATC
GAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAA
CCCTCAAGCACAGCTTGGTGTTGGGACTCGCGT
TAATTCGCGTTCCTCAAATTGATTGGCGGTCACG
TCGAGCTTCCATAGCGTAGTAGTAAAACCCTCG
TTACTGGTAATCGTCGCGGCCACGCCGTTAAAC
CCCAACTTCTGAATGTTGACCTCGGATCAGGTA
GGAATACCCGCTGAACTTAAGCATATGAATAAC

Total Number of Base : 530 / **Similarity Score:**972 / **Array Matching Ratio:**99% / **Similarity Ratio:**99%

(Figure .7 Fungus DNA sequences) .

CONCLUSION

In a 2022 study of watermelon crops in eastern Libya, we identified *Fusarium oxysporum* as a major causative agent of vascular wilt and plant death. The study, conducted across three sites with high watermelon production, highlighted the significant impact of fungal diseases on these crops.

The study aimed to identify the primary causative agent of these diseases using traditional methods (morphological and microscopic diagnosis) followed by molecular diagnosis of the pathogen. The diagnostic methods were compared to determine the most accurate and rapid method for identifying and characterizing the pathogen. The study also aimed to promote the use of PCR technology, which has proven to be highly accurate, rapid, and sensitive in identifying plant pathogens of various types.

Visual diagnosis of plants at the study sites revealed scattered symptoms of vascular wilt disease in some plants, manifested by yellowing of both young and old leaves, and the death of some. At the end of the season, examinations showed that the fruits were relatively small. Upon inspection of the roots, they were observed to be rotten and dead. A cross-section of the roots of infected plants revealed black discoloration in the conductive vessels of the roots, suggesting a possible infection with the fungus *F. oxysporum* .

By isolating the infected parts of leaves and roots from diseased plants and obtaining pure cultures of the target fungus, the plates

inoculated with parts of diseased plants were examined after the fungus grew to a suitable degree. After the fungus had completed its growth, it was observed that the mycelium turned brownish-red, which may indicate the presence or infection by the fungus *F. oxysporum* .

As part of the fungal detection steps, a microscopic examination of the fungal hyphae was conducted. Crescent-shaped fungal spores, which are typically indicative of the fungus F. oxysporum, were observed. However, it was difficult to determine whether these spores were septate or aseptate. Chlamydospores were also observed on the fungal mycelium in the slide, both in the middle of the hyphae and at the tips of other hyphae. Despite the examination providing a strong probability of infection with F. oxysporum, it was challenging to accurately identify the fungal strain.

Despite extensive examinations conducted on infected plants throughout the disease season, it was not possible to definitively confirm the causal race of the pathogen. Traditional methods enabled the identification of the fungal genus and species only, with a relatively high but uncertain probability. This necessitated the use of PCR analysis, which conclusively demonstrated that the pathogen causing the infection was *F. oxysporum* belonging to the fungal race *Melonis*, the causal agent of Fusarium wilt in watermelon *C. lanatus* .

The primary goal of this study was to demonstrate the effectiveness of PCR technology in identifying the sex, species, and strain of plant pathogenic fungi directly from plant tissues. PCR offers significant time savings, high accuracy (up to 90%), and applicability to a wide range of pathogens, including fungi, bacteria, and viruses. Compared to traditional methods, PCR is more accurate, faster, and less costly

The direct PCR method described here presents a highly efficient and economical approach for diagnosing *F. oxysporum* infections. By rapidly identifying this pathogen in large sample sets, it enables timely and targeted disease management strategies. This versatile technique can be tailored for diverse crops and pathogens worldwide, promoting sustainable agriculture .

The main conclusions of the experimental work should be presented. The contribution of the work to the scientific community and its economic implications should be emphasized.

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الملخص

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تنتشر الامراض الفطرية بكثرة في المحاصيل الحقلية بشكل كبير نتيجة لتغير الظروف البيئة التي تحدث في الفترة الحالية ، مما يسبب خسائر كبيرة في تلك المحاصيل ، ومن ضمن هذه الفطريات فطريات ساكنات التربة يصعب الكشف عليها وتعريفها لأجراء عمليات الوقاية والمكافحة لها في الوقت المناسب ، ويصيب الفطر محاصيل البطيخ في مناطق كثيرة من شرق ليبيا مسبب خسائر اقتصادية كبيرة للمزارعين ، وقد اجريت الدراسة خلال موسم زراعة البطيخ بالعام 2022 في ثلاث مناطق من شرق ليبيا وهي طبرق والمرج وتوكرة ، وذلك من خلال الكشف الظاهري للنباتات التى أظهرت أعراض الإصابة من خلال أصفرار الأوراق وذبول النباتات وتعفن الجذور ، وأجريت خلال الدراسة عمليات العزل اللازمة للفطر من النباتات التي ظهرت عليها الأعراض ، وقامت الدراسة بالكشف على الفطر المعزول من خلال الكشف المورفولوجي الظاهري والمجهري ثم تم أجراء استخدام تقنية PCR ، وقد كانت نتائج الكشف الظاهري للنباتات وللفطر المعزولة تظهر احتمال كبير الإصابة بالفطر دون التأكيد أن سلالة الفطر هي التي تصيب البطيخ أو سلالة أخرى ، ولكن عند أجراء PCR وجد ان الفطر الذي تم الكشف عنه هو *Fusarium oxysporum f. sp. Melonis* السلالة التي تسبب الذبول الوعائي لمحاصيل البطيخ بنسبة دقة وصلت الى 99% ، كما استغرقت مدة الكشف وأجراء التحليل الجزئيى للفطر وقت أقل وأسرع مقارنة بالطرق التقليدية المعروفة ، وقد هدفت الدراسة هنا الى التشجيع على استخدام التقنية الحديثة في تحديد المسببات المرضية والكشف عنها وتوضيح خطوات العمل لذلك واتخاذ اجراءات المكافحة وطرق الوقاية اللازمة لحماية المحاصيل الزراعية وتقليل الخسائر.

الكلمات المفتاحية: *Fusarium oxysporum f. sp. Melonis* ، ذبول البطيخ ، *Citrullus lanatus* ، الطرق التقليدية، التقنية الحديثة، PCR.