Annals of Agricultural Science, Moshtohor (ASSJM)
https://assjm.journals.ekb.eg/
www.fagr.bu.edu.eg

ISSN:1110-0419
Original Article Vol. 62(4) (2024), 1 – 12 DOI: 10.21608/assjm.2025.327370.1320

Evaluation the Efficacy of Some Biotic and Abiotic Factors Against *Geotrichum citri-aurantii* and *Alternaria citri in vitro*

Aya M. Gafar; Abd El-Mageed M.H.; Eid Kh.E.; Eman O. Hassan
Plant Pathology Department, Faculty of Agriculture, Benha University, 13736, Egypt.
Corresponding author: khaled.alsayed@fagr.bu.edu.eg

Abstract

The Study aims to evaluate the effectiveness of various treatments against *Geotrichum citri-aurantii* and *Alternaria citri in vitro*. Samples were collected from three governorates: Beheira, Qalyubia, and Sharqiah. Qalyubia showing the highest frequency of *Geotrichum sp.* and *Fusarium sp.*, while *Alternaria sp.* was the most prevalent in all governorates. The molecular identification of *G. citri-aurantii* and *A. citri* offers valuable insights into the genetic makeup and diversity of fungal pathogens affecting Navel Orange fruits. By sequencing the ITS1 and 5.8S ribosomal RNA regions, the study successfully identified both species with high genetic similarity to known strains. Phylogenetic analyses further revealed genetic clustering, indicating some degree of diversity within these species. A range of biotic and abiotic treatments were tested, including bio-agents like *Trichoderma viride* and *Bacillus amyloliquefaciens*, essential oils *i.e.*, clove, thyme and ginger oils and chitosan and chitosan nanoparticles (NPs). Results showed that essential oils had varying inhibitory effects on fungal growth while chitosan and chitosan nanoparticles (NPs) were highly effective in reducing growth. Most notably, chitosan (NPs) showing highly effectiveness at lower concentrations. Additionally, *Trichoderma viride* was more effective than *B. amyloliquefaciens* in reducing the liner growth of *G. citri-aurantii* and *A. citri*.

Keywords: *G. citri-aurantii*; *A. citri*; *B. amyloliquefaciens*; Chitosan nanoparticles (NPs).

Introduction

The citrus industry, encompassing a diverse range of fruits within the genus Citrus of the Rutaceae family, plays a pivotal role in global agriculture. With an annual production of approximately 161.8 million tons cultivated across more than 10.2 million hectares, citrus fruits are among the most significant fruit crops worldwide **(Gonzatto and Santos, 2023)**. Oranges, in particular, dominate this sector, accounting for over 50% of global citrus production. Notably, Egypt stands out as one of the largest producers in the Arab world, contributing 3.7 million tons, which represents 8% of the global output. The economic implications of the citrus industry are profound, providing employment to millions across various sectors, including harvesting, transportation, and marketing. Beyond their economic value, citrus fruits are renowned for their nutritional benefits, serving as a rich source of vitamin C and acting as powerful natural antioxidants. Their high water content and nutrient profile bolster the body's immune system, making them a staple in healthy diets **(Abakpa and Adenaike, 2021; Ma *et al.*, 2020).** However, the citrus industry faces significant challenges, particularly concerning postharvest diseases primarily caused by fungal pathogens. These diseases can lead to substantial losses, prompting reliance on traditional control methods that often involve synthetic fungicides. While effective, these chemicals pose risks to human health and the environment **(Ismail and Zhang, 2004)**. The emergence of fungicide-resistant fungal strains further complicates disease management strategies **(Droby *et al.*, 2002)**. Recent research has shifted focus towards natural antimicrobial substances as viable alternatives to chemical fungicides. Essential oils, known for their antifungal properties, present a promising option due to their biodegradability and lower phytotoxicity **(Gatto *et al.*, 2011)**. Additionally, biocontrol agents such as Bacillus sp. and Trichoderma sp. have shown efficacy in managing postharvest diseases **(Mohammadi *et al.*, 2017; El-Katatny *et al.*, 2020)**. Among the most innovative strategies for controlling citrus rot is the use of chitosan and its nanoparticles. Chitosan nanoparticles have emerged as one of the most common antifungal coatings used to preserve fresh fruit post-harvest **(Li *et al.*, 2018)**. Their environmentally friendly properties, including biodegradability and safety for human health, make them an attractive option **(Shukla *et al.*, 2013).** Furthermore, chitosan has demonstrated extensive antifungal activities, reinforcing its potential as a key player in sustainable citrus disease management

(**Bautista-Baños *et al*., 2003)**. The study aims to investigate the effectiveness of various biotic and abiotic eco-friendly treatments, including some bioagents, essential oils and chitosan nanoparticles in the reduction of radial growth of G. citri-aurantii and A. citri, the causal pathogen of orange diseases in navel oranges fruits.

Materials and Methods

1. Survey of Navel Orange Fruit Diseases during the 2020/2021 Growing Seasons:

Navel oranges (*Citrus sinensis L.*) were procured from diverse governorates in Egypt, specifically Beheira, Qalyubia, and Sharqiah, throughout the 2020/2021 growing season. A total of one hundred fruits from each locale were meticulously examined for both disease incidence and severity.

1.1. Isolation and Purification of Causal Fungi from Infected Navel Orange Fruits:

Navel orange fruits were meticulously washed with sterilized water, diced into small pieces, and surface sterilized in 70% ethanol for one minute, followed by multiple washes with sterilized distilled water. The pieces were then dried using sterilized filter paper and transferred into Petri plates containing PDA media, incubated at 25±2°C for 7 days. Upon the emergence of fungal growth from the diseased specimens, these fungi were purified using the single spore technique as described by **Fang *et al*. (1983)** or through the hyphal tip transfer method outlined by **Hawker (1950)** and **Howard (1981)**. Stock cultures were preserved on PDA slants in a refrigerator at 5°C for subsequent studies, with sub culturing performed every 30 days. The purified fungi were processed in the Plant Pathology Laboratory, Plant Pathology Department, Faculty of Agriculture, Benha University. Identification of fungi from decayed fruits based on their morphological characteristics with the aid of light microscope according to **Gilman (1957), Ellis (1971)**, and **Barnett and Hunter (1972)** .Purified fungi were maintained on PDA slants under refrigeration at 5˚C as stock cultures for future investigations.

1.2. Frequency of Isolated Fungi from Navel Orange Fruits:

Infected Navel orange fruits were processed as previously described. The frequency of isolated fungi was recorded using the following equation provided by **Al-Masoodi *et al*., (2023)**:

$$\text{Frequency \%} = \frac{\text{Number of isolated fungi}}{\text{Total number of isolated fungi}} \times 100$$

2. Molecular Identification of *Alternaria sp.* and *Geotrichum sp.*:

The most virulent fungal isolates, *Alternaria sp*. (No.1) and *Geotrichum sp*. (No.1), were identified through polymerase chain reaction (PCR). The genomic Deoxyribonucleic acid (DNA) of these fungal isolates was extracted from pure cultures utilizing Fungi genomic DNA isolation kits (Cat. 27300), adhering strictly to the manufacturer's protocols. PCR amplification targeted a partial sequence of the internal transcribed spacer 1 gene and the 5.8S ribosomal RNA gene, employing universal primers (ITS) Internal Transcribed Spacer (ITS1F: 5' - TCCGTAGGTGAACCTGCGG - 3') and (ITS4R: 5' - TCCTCCGCTTATTGATATGC - 3'), which were validated using an in-silico PCR tool. The anticipated amplicon size was approximately 400-800 bp **(Fan *et al*., 2015)**.

A phylogenetic tree was constructed using CLC Sequence Viewer Version 6.3 based on UPGMA (unweighted pair group method for arithmetic analysis) **(Fan *et al*., 2015)**.

3. Isolation and Purification of Bioagent (*Bacillus* sp.):

The isolation and screening of biocontrol bacteria were conducted from the surface of healthy navel orange fruits. The fruits were rinsed in sterilized water to obtain a spore suspension of associated microorganisms. Subsequently, 0.1 mL of this spore suspension was distributed onto the surface of previously prepared potato dextrose agar plates. The inoculated plates were incubated at 25±2 °C for 4-7 days, during which the emerging colonies of microorganisms were isolated and sub-cultured to obtain single, clean isolates. Each isolated strain was placed in conical flasks containing 100 mL of sterile water. After oscillating for 10 minutes, the mixture was serially diluted to 10^{-6}. Then, 1 mL of the suspension at various concentrations was mixed with nutrient-agar (NA) media in Petri dishes, which were then inverted and incubated at 30 °C for 24 hours **(Hu *et al*., 2008)**. Single colonies on each plate were streaked onto fresh plates to verify purity. Following purification, the isolated bacterial strains were evaluated for their antagonistic capabilities against pathogens in vitro.

3.1. Molecular Identification of Bioagent (*Bacillus* sp.):

The total genomic DNA of the bacterial cells was extracted using the GeneJET™ Genomic DNA Purification Kit (Thermo Scientific®, Massachusetts, USA). The extracted DNA was used as a template for PCR amplification of the 16S rRNA gene using primer pairs 27F (50-AGAGTTTGATCCTGGCTCAG-30) and 1492R: 5" -GGTTACCTTGTTACGACTT-3" **(Badr *et al*., 2019)**. The 16S rRNA PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germany) and sequenced at Macrogen Inc., South Korea **(Weisburg *et al*., 1991).** Alignment of the 16S rRNA sequences was performed using the Clustal_W program. A phylogenetic tree was constructed using CLC Sequence Viewer Version 6.3 based on

UPGMA (unweighted pair group method for arithmetic analysis).**(Fan *et al*., 2015)**.

4. Evaluation of Various Biotic and Abiotic Factors on the the Linear Growth of *Alternaria citri* and *Geotrichum citri-aurantii*:

The virulent isolate of *Geotrichum citri-aurantii* (Isolate No.1) (accession no. PP124941), *Alternaria citri* (Isolate No.1) (accession no. PP124942), Which cause sour and black rot in navel orange fruits, respectively.

4.1. Evaluation of Various Biotic Factors on the the Linear Growth of *A. citri* and *G. citri-aurantii*:

4.1.1. Source of Antagonistic Organisms:

The isolate of *Bacillus amyloliquefaciens* (accession no. PQ135833.1) was procured from the surface of healthy navel orange fruits and identified by molecular biology additionally, the isolate of *Trichoderma viride* was graciously provided by the Plant Pathology Department at the Faculty of Agriculture, Benha University.

4.1.2. Effect of *B. amyloliquefaciens* on the Linear Growth of *A. citri* and *G. citri-aurantii*:

The antifungal efficacy of *Bacillus amyloliquefaciens* was meticulously evaluated against *A. citri* and *G. citri-aurantii* through the dual culture plate methodology. Discs measuring 5 mm, extracted from 7-day-old cultures of *A. citri* and 4-day-old cultures of *G. citri-aurantii*, were strategically positioned 1 cm from the periphery of each Potato Dextrose Agar (PDA) plate. Concurrently, a 1 ml loop of *B. amyloliquefaciens* was streaked from the opposite edge of the plate. Control plates were established, inoculated solely with the fungal discs. Each experimental condition was replicated thrice to ensure reliability. The plates were incubated at a controlled temperature of 25±2°C. The percentage reduction in mycelial growth was calculated using the previously mentioned formula **(Rashad *et al.*, 2020).**

4.1.3. Effect of *Trichoderma viride* on the Linear Growth of *A. citri* and *G. citri-aurantii:*

The antagonistic potential of the *Trichoderma viride* isolate against the linear growth of *A. citri* and *G. citri-aurantii* was scrutinized utilizing the dual culture technique within Petri dishes. Discs measuring 5 mm, containing fungal structures, were excised from the periphery of 7-day-old colonies of *A. citri* or *T. viride*, as well as from the edges of 4-day-old colonies of *G. citri-aurantii* cultivated on Potato Dextrose Agar (PDA). The pathogen and Trichoderma discs were strategically positioned on opposing sides of the Petri dishes, maintaining a fixed distance of 6 cm. In the control plates, only the pathogens were inoculated. The PDA plates were incubated at a temperature of 25±2°C for 7 days until the mycelial growth in the control plates reached the periphery of the Petri dishes. Three replicates were established for each treatment, alongside three control plates devoid of fungicide. The linear growth of the pathogens was meticulously measured once the fungal mycelium enveloped the control plates **(Rahman *et al.*, 2009)**. The reduction in mycelial growth was computed using the formula delineated by **Fokemma (1973)** as follows:

$$\text{Reduction \%} = \left[\frac{C-T}{C} X100\right]$$

Where, C = mean diameter in radial growth in control, T= mean diameter in radial growth in treatment.

4.2. Evaluation of Various Abiotic Factors on the the Linear Growth of *Alternaria citri* and *Geotrichum citri-aurantii*:

4.2.1. Commercial Plant Essential Oils:

A variety of essential oils, specifically Clove (*Syzygium aromaticum*), Thyme (*Thymus vulgaris*), and Ginger (*Zingiber officinale*), were procured from Al-Qus Company, renowned for cold oil extraction, located in Qena Governorate, Egypt.

4.2.2. Evaluation of Various Concentrations of Selected Commercial Plant Oils on the Linear Growth of *Alternaria citri* and *Geotrichum citri-aurantii*:

The antifungal potential of three commercial plant oils—Clove, Thyme, and Ginger—was scrutinized for their ability to inhibit the growth of *A. citri* and *G. citri-aurantii*. Specific volumes of each oil were incorporated into PDA media flasks to achieve the desired concentrations: Clove oil at (0.0%, 0.25%, 0.5%, and 1.0%), and both Thyme and Ginger at (0.0%, 3.0%, 6.0%, and 12.0%), supplemented with 0.1% Tween-80 **(Porcino *et al.*, 2023)**.

The treated and untreated (control) media were dispensed into three Petri dishes for each concentration. Following the solidification of the media, the Petri dishes were inoculated with a 5 mm disc from a 7-day-old culture of *A. citri* and a 4-day-old culture of *G. citri-aurantii*. The plates were then incubated at a controlled temperature of 25±2°C for 7 days. Three plates for each treatment served as replicates. The linear growth of the pathogens was measured once the fungal mycelium had completely covered the control plates, and the percentage reduction in mycelial growth was calculated using the previously mentioned formula.

4.3. Chitosan and Chitosan Nanoparticles:

Chitosan (CAS number: 9012-76-4) was sourced from Sigma-Aldrich, characterized by a molecular weight of 161.16 ($C_6H_{11}NO_4X_2$) and a degree of deacetylation of ≥80%.

4.3.1. Preparation of Chitosan Stock Solution:

A stock solution of chitosan was prepared by weighing 2 g of chitosan and dissolving it in 100 ml of distilled water, supplemented with 1 ml of acetic acid. The mixture was heated while being continuously agitated for 24 hours. The pH of the solution was subsequently adjusted to 5.6 by the

addition of 1 N sodium hydroxide (0.1 ml). This chitosan solution was then utilized to obtain various concentrations for *in vitro* studies **(Sánchez-Domínguez *et al*., 2011).**

4.3.2. Preparation of Chitosan Nanoparticles:

Chitosan nanoparticles were synthesized through ionotropic gelation with tripolyphosphate (TPP) anions. A 0.2% chitosan solution was prepared in a 1% acetic acid solution and agitated at room temperature for 1 hour at 1000 rpm. A TPP stock solution was created by dissolving 0.03 g of TPP in 11 ml of water. Chitosan nanoparticles formed spontaneously upon the dropwise addition of 1 ml of the TPP stock solution to the chitosan solution while stirring at 1000 rpm for 1 hour at room temperature. The resulting chitosan nanoparticles were sonicated for one hour to ensure their diminutive size **(Hassan *et al*., 2022)**.

4.3.3. Chitosan Nanoparticles (CHNPs) Transmission Electron Microscopy (TEM) Analysis:

For TEM analysis, a drop of the chitosan nanoparticle solution was placed on carbon-coated copper grids (CCG) and allowed to dry at room temperature to facilitate water evaporation. Electron micrographs were captured using a JEOL GEM-1010 transmission electron microscope at 70 kV, conducted at the research laboratories complex of the College of Agriculture, Cairo University **(Shoala *et al*., 2021).**

4.3.4. Evaluation of Different Concentrations of Chitosan and Chitosan Nanoparticles on the Linear Growth of *A. citri* and *G. citri-aurantii*:

Chitosan solutions were incorporated into PDA media before solidification to achieve the desired concentrations of chitosan (0.1%, 0.2%, 0.3%, 0.4%, and 0.5%) and chitosan nanoparticles (0.01%, 0.02%, 0.03%, 0.04%, and 0.05%). The prepared media were then poured into Petri plates. After solidification, the plates were inoculated with a 5 mm disc taken from the periphery of a 7-day-old culture of *A. citri* and a 7-day-old culture of *G. citri-aurantii*. The plates were subsequently incubated at a controlled temperature of 25±2°C. Three replicates were established for each treatment, along with three control plates that did not contain chitosan. The linear growth of the pathogens was measured once the fungal mycelium had completely covered the control plates, and the percentage reduction in mycelial growth was calculated using the previously mentioned formula.

5. Statistical analysis:

Statistical analysis of all the previously designed experiments have been carried out acccprding to the procedures (ANOVA) reported by **(Snedecor and Cochran 1989)**. Treatments means were compared by the least significant difference teast "L.S.D" at 5% level of probability.

Experimantal Results

1. Geographical variations in fungal frequencies:

The data collected from three governorates Beheira, Qalyubia, and Sharqiah. **Table (1)** illustrates the frequency distribution percentages of fungi isolated from diseased orange fruits collected from three governorates: Beheira, Qalyubia, and Sharqiah. The fungi identified include *Alternaria* sp, *Geotrichum* sp, *Penicillium* sp., *Fusarium* sp., and other fungi. The data reveal significant geographical variations in fungal frequencies across these regions. In *Qalyubia*, *Geotrichum* sp. and *Fusarium* sp. were notably prevalent, with frequencies of 25% each. *Alternaria citri* was the most widespread fungus across all governorates, with an average frequency of 27.3%. Specifically, Beheira and Sharqiah recorded identical frequencies for *Alternaria citri* at 28.5%, which was higher than the 25% observed in Qalyubia.

Table 1. Frequency of fungal diseases causing orange fruit rot in various governorates in the season 2020.

Governorates	Frequency of isolated fungus%				
	Alternaria sp.	*Geotrichum* sp.	*Penicillium* sp.	*Fusarium* sp.	Other fungi
Beheira	28.5 a*	14.2 b	28.5 a	14.2 b	14.6 a
Qalyubia	25.0 b	25.0 a	12.5 b	25.0 a	12.5 b
Sharqiah	28.5 a	14.2 b	28.5 a	14.2 b	14.6 a
Mean	27.3	17.8	23.2	17.8	13.9
LSD 0.05	0.44	0.21	0.36	0.26	0.39

*Values marked with different letters (a, b, c) indicate significant differences at the 0.05 significance level.

This significant difference underscores a greater prevalence of *Alternaria* sp. in Beheira and Sharqiah compared to Qalyubia. *Geotrichum* sp. exhibited a significantly higher frequency in Qalyubia (25%) than in Beheira and Sharqiah (14.2%), indicating a pronounced geographical disparity with a stronger presence in Qalyubia. Similarly, *Penicillium* sp. showed higher frequencies in Beheira and Sharqiah (28.5%) compared to Qalyubia (12.5%), with significant differences confirming the elevated presence in Beheira and Sharqiah. *Fusarium* sp. also demonstrated a higher frequency in Qalyubia (25%) relative to Beheira and Sharqiah (14.2%), highlighting a significant concentration in Qalyubia. The frequency of other fungi ranged from 12.5% to 14.6% across the three governorates, with no significant differences detected among them in this category. These findings emphasize the importance

of geographical factors in the distribution of fungal species affecting orange fruits.

2. Molecular identification of *Alternaria* sp. and *Geotrichum* sp .:

The molecular identification of the most virulent fungal isolates *Alternaria* sp. causing black rot of navel orange fruits and of *Geotrichum* sp. (Isolate 1) causing sour rot of navel orange fruit follows a standard molecular biology approach to characterize pathogenic strains affecting navel orange fruits.

2.1. Identification of *Geotrichum* sp. using molecular biology:

The molecular identification of *Geotrichum* sp. was achieved by sequencing the ITS1 and 5.8S ribosomal RNA gene regions, followed by comparison with known sequences in the NCBI database. The isolate PP124941 exhibited a 98.32% identity with the *Geotrichum citri-aurantii* strain GcaCC015 (EU131181) according to BLASTN and Jalview alignment results. This high sequence similarity strongly suggests that the fungal strain isolated in the study shares significant genetic overlap with the *Geotrichum citri-aurantii* reference strain. This high sequence similarity strongly suggests That the fungal strain isolated in the study shares significant genetic overlap with the *Geotrichum citri-aurantii* reference strain.

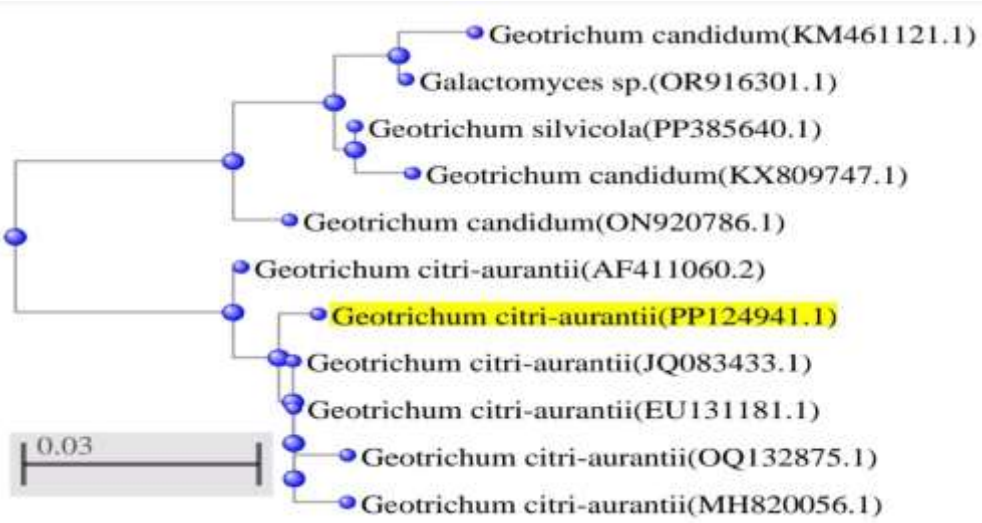

Fig. 1: The phylogenic tree showed convergence between our isolated (yellow color) and gene bank isolate. Our isolate showed in separated cluster that mean its diversity.

The phylogenetic tree for each isolate **(Fig.1)** confirmed the same identity ratio with the nearest sequences in the database. It can be inferred that isolate PP124941 has a close genetic relationship with *Geotrichum citri-aurantii* strain.

2.2. Identification of *Alternaria* sp. using molecular biology

The amplified PCR products of the ITS1 gene and 5.8S ribosomal RNA gene from *Alternaria* sp. (Isolate 1) was sequenced and deposited in the NCBI database under accession numbers PP124942. Similarly, *Alternaria citri* (PP124942) was identified by sequencing the ITS1 and 5.8S ribosomal RNA gene regions. The results revealed a 99.64% identity with *Alternaria alternata* (OP959991), which points to a high degree of similarity between the isolated strain and known *Alternaria* species. The phylogenetic tree **(Fig. 2)** again showed that the isolated strain forms a distinct cluster, indicating some level of genetic divergence from other isolates in the gene bank. In addition, the phylogenetic tree for each isolate confirmed the same identity ratio with the nearest sequences in the database. It can be inferred that isolate PP124942 has a close genetic relationship with *Alternaria citri* and *Alternaria alternate*.

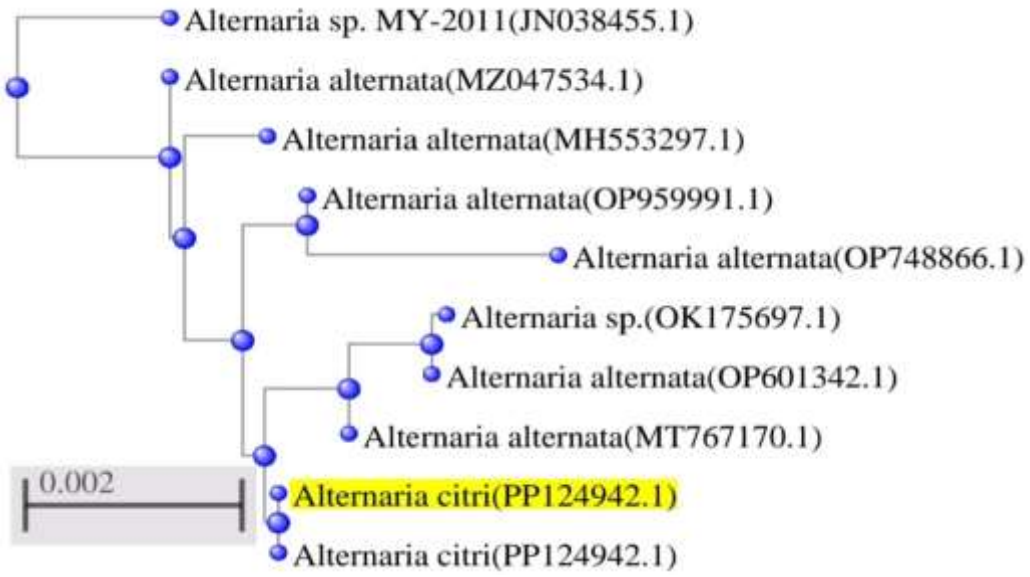

Fig. 2. The phylogenic tree showed convergence between our isolated (yellow color) and gene bank isolate. Our isolate showed in separated cluster that mean its diversity.

3. Molecular identification of *Bacillus* sp.:

An isolate of *Bacillus* sp. was obtained from the surface of healthy navel orange fruits. The isolate was identified through molecular characterization techniques, particularly sequence-based identification. The results presented that the isolate *Bacillus amyloliquefaciens* (Accession No. PQ135833.1) exhibits a close genetic relationship with the *Bacillus amyloliquefaciens* strain (Accession No. MK782759.1). As shown in **Fig. 3**, the phylogenetic analysis corroborated the high similarity between isolate PQ135833.1 and *Bacillus amyloliquefaciens* strain MK782759.1, confirming their close genetic relationship. The phylogenetic tree constructed from the sequence data demonstrated that the isolate shares significant identity with sequences from the database, further substantiating its molecular identification as *Bacillus amyloliquefaciens*.

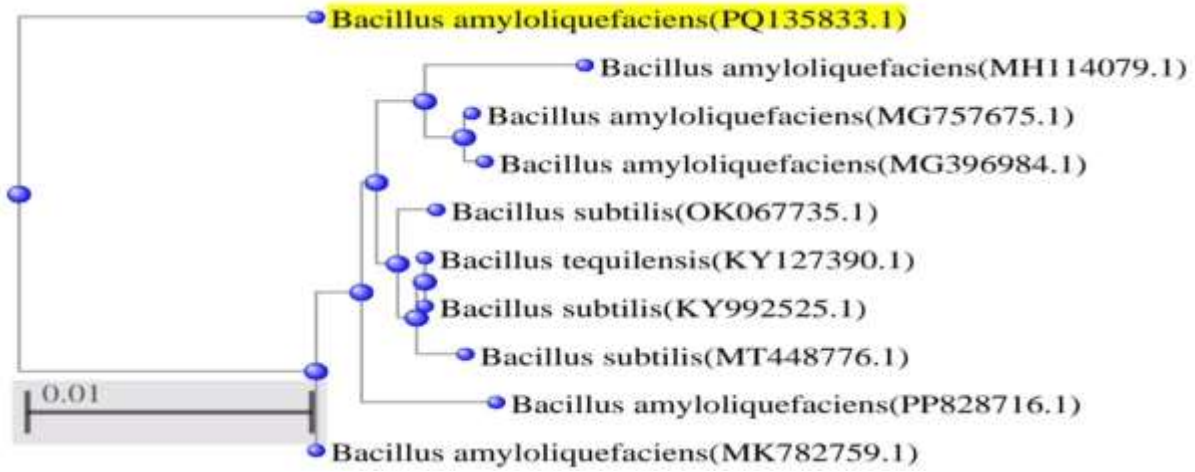

Fig. 3: The phylogenic tree showed convergence between our isolated (yellow color) and gene bank isolate. Our isolate showed in separated cluster that mean its diversity.

4. In vitro evaluation of some biotic and abiotic factors on linear growth and growth reduction % of *Alternaria citri* and *Geotrichum citri-aurantii*:

4. 1. Effect of *B. amyloliquefaciens* and *T. viride*. on linear growth and growth reduction % of *Alternaria citri* and *G. citri-aurantii*:

The study evaluated the antifungal properties of *B. amyloliquefaciens* and *T. viride* against *A. citri* and *G. citri aurantii*. The results, as presented in **Table (2)**, indicate that both bioagents are effective in suppressing the linear growth of these fungi. *Bacillus amyloliquefaciens* showed significant inhibitory effects, with a 66% reduction in the growth of *Alternaria citri* and a 55% reduction in the

growth of *Geotrichum citri aurantii*. However, *Trichoderma viride* consistently demonstrated higher effectiveness, achieving a 68% reduction in the growth of *Alternaria citri* and a 63% reduction in the growth of *Geotrichum citri aurantii*.

Table 2. Effect of *Bacillus amyloliquefaciens* and *Trichoderma viride*. on the linear growth and growth reduction percentages of *Alternaria citri* and *Geotrichum citri aurantii*.

Treatments	*Alternaria citri*		*Geotrichum citri aurantii*	
	linear growth (mm)	Reduction (%)	linear growth (mm)	**Reduction (%)**
Control	90 a	0.0 c	90 a	**0 c**
B. amyloliquefaciens	30 b	66 b	40 b	**55 b**
T. viride	28 c	68 a	33 c	**66 a**
LSD 0.05	2.62	1.31	2.62	**1.31**

*Values marked with different letters (a, b, c) indicate significant differences at the 0.05 significance level.

4.2. Effect of clove oil, Thyme oil and ginger oil with different concentrations on linear growth and growth reduction of *A. citri* and *G. citri –aurantii*:

The study examined the efficacy of clove oil, thyme oil, and ginger oil at various concentrations against the fungal pathogens *Alternaria citri* and *Geotrichum citri-aurantii*. The results in **Table (3)** indicated that clove oil was highly effective against both fungi, particularly at concentrations of 0.5% and 1%, resulting in a complete inhibition of growth**.** At a lower concentration of 0.25%, clove oil still achieved a substantial reduction in growth of 94%. Thyme oil, on the other hand, did not exhibit any significant inhibitory effect on the growth of either fungus at any of the tested concentrations. Ginger oil showed a moderate level of effectiveness against *Alternaria citri* at higher concentrations of 9% and 12%, with reduction percentages of 50% and 55%, respectively. However, at lower concentrations of 3% and 6%, ginger oil did not significantly impact the growth of *A. citri*.

Table 3. Effect of Various Concentrations of Clove Oil, Thyme Oil, and Ginger Oil on the Linear Growth and Reduction Percentage of Alternaria citri and Geotrichum citri-aurantii

Treatments	*Alternaria citri*		*Geotrichum citri-aurantii*	
	linear growth (mm)	Reduction (%)	linear growth (mm)	**Reduction (%)**
Control	90 a	0.0	90 a	**0 g**
Clove oil (0.25%)	5 d	94 b	50 e	**94 b**
Clove oil (0. 5%)	0 e	100 a	0 f	**100 a**
Clove oil (1%)	0 e	100 a	0 f	**100 a**
Thyme oil (1%)	90 a	0 e	90 a	**0 g**
Thyme oil (2%)	90 a	0 e	90 a	**0 g**
Thyme oil (3%)	90 a	0 e	90 a	**0 g**
Thyme oil (6%)	90 a	0 e	90 a	**0 g**
Thyme oil (12%)	90 a	0 e	90 a	**0 g**
Ginger oil (3%)	90 a	0 e	80 b	**11 f**
Ginger oil (6%)	90 a	0 e	70 c	**22 e**
Ginger oil (9%)	45 b	50 d	60 d	**33 d**
Ginger oil (12%)	40 c	55 c	50 e	**44 c**
LSD 0.05	1.3	0.74	1.47	**0.85**

*Values marked with different letters (a, b, c) indicate significant differences at the 0.05 significance level.

Against *G. citri-aurantii*, ginger oil demonstrated a gradual increase in effectiveness as the concentration increased, with the highest reduction of 44% observed at 12%. At lower concentrations of 9% and 6%, ginger oil showed moderate effectiveness with reduction percentages of 33% and 22%, respectively. These findings underscore the variability in the antifungal potency of essential oils, which is influenced by both the type of oil and its concentration.

4.3. Effect of chitosan and chitosan (NPs) with different concentrations on *Alternaria citri* and *Geotrichum citri aurantii in vitro* :

4.3.1 Characterization of Chitosan Nanoparticles (NPs)

Chitosan (CAS number: 9012-76-4) Sigma-Adrich. (Mw = 161.16 ; ≥ 80 % degree of deacetylation). Nanoparticles were prepared via iontropic chitosan gelation with tripolyphosphate(TPP) anions**.**

4.3.2. Transmission electron microscopy (TEM)

The morphological characteristics of Nanoparticles were investigated by transmission electron microscope (TEM) chitosan Nanoparticles were uniform and spherical with an average particle size of 15.3 -31.3 nm **(Fig 7)**.

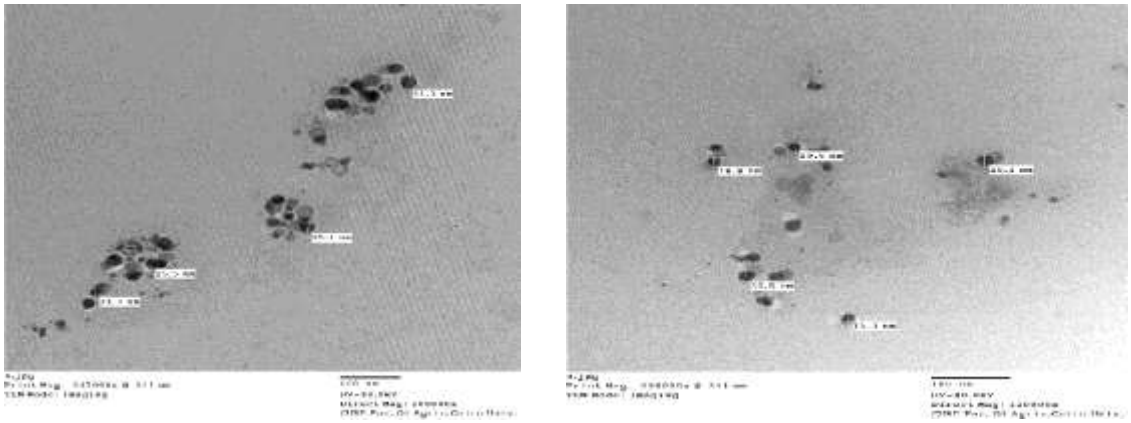

Fig. 4: Transmission electron microscope (TEM) image of chitosan Nanoparticles.

4.3.3. Antifungal activity of of chitosan and chitosan (NPs) with different concentrations on linear growth and growth reduction of *A. citri* and *G. citri aurantii*:

The study investigated the antifungal effects of chitosan and chitosan nanoparticles (NPs) against *A. citri* and *G. citri-aurantii*. The results, as presented in **Table (4)**, indicate that both chitosan and Chitosan-NPs are effective in suppressing the linear growth of these fungi, with the efficacy increasing in proportion to the concentration used.

Table 4. Effect of Various Concentrations of Chitosan and Chitosan (NPs) on the Linear Growth of *A. citri* and *G. citri aurantii*.

Treatments	*Alternaria citri*		*Geotrichum citri aurantii*	
	linear growth (mm)	Reduction (%)	linear growth (mm)	**Reduction (%)**
Control	90 a	0.0 d	90 a	**0 f**
Chitosan (0.1%)	50 c	94 b	80 b	**11 e**
Chitosan (0.2%)	22 e	75 e	20 d	**77 c**
Chitosan (0.4%)	10 f	88 d	10 e	**88 b**
Chitosan (0.5%)	0 g	100 a	0 f	**100 a**
Chitosan (NPs) at (0.01%)	70 b	92 c	90 a	**0 f**
Chitosan (NPs) at (0.02%)	45 d	55 f	40 c	**55 d**
Chitosan (NPs) at (0.03%)	10 f	88 d	0 f	**100 a**
Chitosan (NPs) at (0.04%)	0 g	100 a	0 f	**100 a**
Chitosan (NPs) at (0.05%)	0 g	100 a	0 f	**100 a**
LSD 0.05	1.41	1.20	1.35	**0.89**

*Values marked with different letters (a, b, c) indicate significant differences at the 0.05 significance level.

Chitosan at concentrations of 0.1% and 0.2% demonstrated a significant reduction in the linear growth of *G. citri-aurantii*, with reductions of 11% and 77%, respectively. For *A. citri*, the reductions were even more pronounced, with 94% and 75% at the same concentrations. A concentration of 0.4% chitosan resulted in an 88% reduction in growth for both fungi, while complete inhibition was achieved at 0.5% for both pathogens. Chitosan (NPs) appeared to be more potent at lower concentrations compared to chitosan. At a concentration of 0.01%, Chitosan-NPs showed a slight reduction in Geotrichum citri-aurantii but a significant reduction in *Alternaria citri*. At 0.02%, chitosan (NPs) exhibited moderate inhibition for both fungi. Complete inhibition of *G. citri-aurantii* was observed at 0.03% chitosan (NPs), with a significant reduction in *A. citri*. Notably, both fungi were completely inhibited by chitosan (NPs) at a concentration of 0.04%. These findings suggest that both chitosan and Chitosan (NPs) can be effective antifungal agents against *A. citri* and *G. citri-aurantii*, with chitosan (NPs) showing greater potency at lower concentrations. This has significant implications for the development of effective and environmentally friendly treatments for postharvest fungal diseases in citrus fruits.

Discussion

The citrus industry is significantly impacted by postharvest diseases, primarily caused by fungal pathogens such as *Penicillium digitatum*, *Geotrichum citri-aurantii*, and various species of *Alternaria* **(Talibi *et al.*, 2014)**. These pathogens not only affect the quality and shelf life of citrus fruits but also pose economic challenges for

producers. Understanding the geographical distribution and environmental factors influencing these pathogens is crucial for developing effective management strategies.

A survey conducted during the 2020 season across three Egyptian governorates—Qalyubia, Beheira, and Sharqiah—revealed valuable insights into the prevalence of these fungal pathogens. The findings indicated that *Alternaria citri* was the most widespread fungus among the sampled regions. This aligns with previous research that has identified *A. citri* as a significant pathogen in citrus fruits globally **(Timmer *et al.*, 2000)**. The uniformity in the frequency of *A. citri* across Beheira and Sharqiah, contrasted with the higher incidence in Qalyubia, suggests that environmental factors and agricultural practices may play a pivotal role in the distribution of these pathogens.

The increased severity of *A. citri* in Qalyubia can likely be attributed to specific environmental conditions that favor its growth, as noted by **Timmer *et al*. (2000)**. Additionally, the variability in disease metrics for *Geotrichum citri-aurantii*, *Fusarium* spp., and *Penicillium* spp. across the governorates underscores the importance of local conditions in shaping the epidemiology of these pathogens. Notably, the high frequency of *G. citri-aurantii* in Qalyubia compared to Beheira and Sharqiah suggests that specific regional conditions, such as humidity and air circulation, may favor this pathogen's proliferation **(Smilanick *et al*., 2005)**.

Molecular identification techniques, including sequencing of the ITS1 and 5.8S ribosomal RNA gene regions, have proven effective in characterizing these fungal pathogens. For instance, the isolate PP124941 exhibited a 98.32% identity with the *G. citri-aurantii* strain GcaCC015, indicating a strong genetic overlap with known reference strains. The use of ITS regions for fungal identification is well-established, providing robust taxonomic differentiation due to the variability within these regions among different fungal species **(Schoch *et al*., 2012)**. Phylogenetic analysis further supports these findings, revealing genetic relatedness and potential diversity within the species, which may have implications for pathogenicity and virulence **(Kurtzman and Robnett, 1997)**.

In addition to fungal pathogens, the study also identified *Bacillus amyloliquefaciens* from the surface of navel orange fruits, demonstrating a significant genetic similarity with existing strains. This bacterium is known for its biocontrol properties, playing a critical role in plant growth promotion and protection against phytopathogens **(Chen *et al*., 2009; Fan *et al*., 2011)**. The identification of *B. amyloliquefaciens* supports its adaptability to diverse environments, emphasizing its potential as a biocontrol agent in the citrus industry.

The antifungal efficacy of various biocontrol agents, including *Trichoderma viride* and *Bacillus amyloliquefaciens*, against *A. citri* and *G. citri-aurantii* was also investigated. The results indicated that *T. viride* exhibited superior efficacy compared to *B. amyloliquefaciens*, achieving more pronounced reductions in fungal proliferation. This finding is consistent with existing literature that highlights *Trichoderma* as an effective biocontrol agent due to its multifaceted modes of action, including mycoparasitism and competitive exclusion **(Harman *et al*., 2004)**.

Moreover, the study explored the antifungal properties of essential oils, revealing that clove oil achieved a 100% reduction in fungal growth, while thyme oil showed no significant inhibitory effects. The high efficacy of clove oil can be attributed to its active component, eugenol, known for its potent antimicrobial properties **(Pinto *et al*., 2009; Chaieb *et al*., 2007)**. Conversely, the ineffectiveness of thyme oil may be due to specific fungal strains tested or variations in experimental conditions.

The investigation into chitosan nanoparticles (NPs) revealed that they are more effective than conventional chitosan at lower concentrations, highlighting the potential of nanotechnology in developing potent antifungal treatments. Chitosan's mechanisms of action include cell membrane disruption and interference with nutrient uptake, which contribute to its antifungal efficacy **(Ziani *et al*., 2009; Badawy and Rabea, 2011)**.

In conclusion, the citrus industry faces significant challenges from postharvest diseases caused by fungal pathogens. The geographical distribution of these pathogens is influenced by environmental factors and agricultural practices, necessitating targeted management strategies. Molecular identification techniques and the exploration of biocontrol agents and natural antifungal compounds provide promising avenues for mitigating the impact of these diseases. Continued research in this area is essential for developing effective interventions that can enhance the resilience of citrus crops against postharvest diseases.

Reference

Abakpa, G. O. and Adenaike, O. (2021). Antioxidant compounds and health benefits of citrus fruits. *European journal of nutrition & food safety*, *13*(2): 65-74.

Al-Masoodi, I.H.; Al-Rubaye, A.F.M. and Hussein, H.J. (2023). Isolation and diagnosis of the fungi associated with maize seeds collected from local markets in Karbala, Iraq. Caspian *Journal of Environmental Sciences*, Vol. 21 No. 3 pp. 665-672

Badawy, F.M.I.; Sallam, M.A.N.; Ibrahim, A. and Asran, M.(2011). Efficacy of some essential oils on controlling green mold of orange and their effects on postharvest quality parameters. *Plant Pathol J.*, 10:168–174 p.

Badr, O. A. M.; El-Shawaf, I. I. S.; Khalil, M. H. A.; Refaat, M. H. and Ramadan, S. I. A. (2019). Molecular genetic diversity and conservation priorities of Egyptian rabbit breeds. *World Rabbit Science*, *27*(3): 135-141.

Barnett, H.L. and Hunter, B.B. (1972). Illustrated Genera of Imperfect Fungi. 3rd Edition, Burgess Publishing Co., Minneapolis, 241 p.

Bautista-Baños, S.;Hernández-López, M.; Bosquez-Molina, E. and Wilson, C. L. (2003). Effects of chitosan and plant extracts on growth of Colletotrichum gloeosporioides, anthracnose levels and quality of papaya fruit. *Crop protection*, *22*(9), 1087-1092.

Chaieb, K.; Hajlaoui, H.; Zmantar, T.; Kahla-Nakbi, A. B.; Rouabhia, M.; Mahdouani, K. and Bakhrouf, A. (2007). The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (Syzigium aromaticum L. Myrtaceae): A short review. *Phytotherapy Research*, 21(6): 501-506.

Chen, X. H.; Koumoutsi, A.; Scholz, R.; Schneider, K.; Vater, J.; Süssmuth, R. and Borriss, R. (2009). Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Nature Biotechnology*, 27(9): 1107-1114.

Droby, S., Vinokur, V., Weiss, B., Cohen, L., Daus, A., Goldschmidt, E. and Porat, R. (2002). Induction of resistance to Penicillium digitatum in grapefruit by the yeast biocontrol agent Candida oleophila. *Phytopathology*, 92: 393–399.

El-Katatny,M.H.; Ali,B.A.; Emam,A.S. and Yasen,M.G.(2020) . Biological effects of two Trichoderma harzianum isolates and mint oil on some postharvest fungal pathogens of sugarbeet. J. Adv. Biomed. & Pharm. Sci, 3: 65-79.

Ellis, M.B. (1971). Dematiaceous hyphomycetes. *Commw. Mycol. Inst. Kew. Surrey, England*.

Fan, B.; Chen, X. H. and Borriss, R. (2011). Bacillus amyloliquefaciens FZB42: a model for plant-associated growth-promoting bacteria. *Microbial Biotechnology*, 4(4): 459-467.

Fan, X.; Zhang, J.; Yang, L.; Wu, M.; Chen, W.and Li, G. (2015) Development of PCR-based assays for detecting and differentiating three species of Botrytis infecting broad bean. *Plant Dis*ease. 99(5):691–698 p.

Fang, Y.C.; McGraw, A.C.; Modjo, H. and Hendrix, J.W. (1983).A procedure for isolation of single-spore cultures of certain endomycorrhizal fungi.*New Phytologist.*, 95: 107-114 p .

Fokkema, N. J. (1973). The role of saprophytic fungi in antagonism against Drechslera sorokiniana (Helminthosporium sativum) on agar plates and on rye leaves with pollen. *Physiological Plant Pathology*, *3*(2): 195-205.

Gatto, M.A.; Ippolito, A.; Linsalata, V.; Cascarano, N.A.; Nigro, F.; Vanadia, S. and Di Venere, D. (2011). Activity of extracts from wild edible herbs against postharvest fungal diseases of fruit and vegetables. *Postharvest Biology and Technology* 61: 72–82.

Gilman, J.C. (1957). A Manual of Soil Fungi. Second Ed., the Iowa State College Press, Ames, Iowa, USA, 450 P.

Gonzatto, M. P. and Santos, J. S. (2023). Introductory chapter: world citrus production and research. In *Citrus Research-Horticultural and Human Health Aspects*. IntechOpen.

Harman, G. E.; Howell, C. R.; Viterbo, A.; Chet, I. and Lorito, M. (2004). Trichoderma species-opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology*, 2(1): 43-56.

Hassan, E.O.; Shoala, T.; Attia, A.M.F.; Badr, O.A.M.; Mahmoud, S.Y.M.; Farrag, E.S.H. and EL-Fiki, I.A.I.v (2022). Chitosan and Nano-Chitosan for Management of Harpophora maydis: Approaches for Investigating Antifungal Activity, Pathogenicity, Maize-Resistant Lines, and Molecular Diagnosis of Plant Infection. *Journal of Fungi*, *8*(5), 509.

Hawker, L.E. (1950). Physiology of Fungi.Univ.of London Press, LTD. Warwich Square, London.

Howard, R.J. (1981). Ultra structural analysis of hyphal tip cell growth in fungi: spitzenkorper, cytoskeleton and endomembranes after freeze-substitution. *J. Cell Sci.*, 48: 89-103 p.

Hu, Q.; Xu, J.; Song, P.; and Chen, W. (2008). Isolation and identification of a potential biocontrol agent Bacillus subtilis QM3 from Qinghai yak dung in China. *World J. Microbiol. Biotechnol*. 24: 2451-2458

Ismail, M. and Zhang, J. (2004). Post-harvest citrus diseases and their control. *Outlooks Pest Manage* 15: 29–35.

Kurtzman, C. P. and Robnett, C. J. (1997). Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. *Journal of Clinical Microbiology*, 35(5): 1216-1223.

Li, Y. N.; Ye, Q. Q.; Hou, W. F. and Zhang, G. Q. (2018). Development of antibacterial epsilon-polylysine/chitosan hybrid films and the effect on citrus. International Journal of Biological Macromolecules, 118, 2051–2056.

Ma, G.; Zhang, L.; Sugiura, M. and Kato, M. (2020). Citrus and health. In *The genus citrus* (pp. 495-511).

Mohammadi, P.; Tozlu, E.; KoTan, R. and Kotan, M. (2017). Potential of some bacteria for biological control of postharvest citrus green mould caused by Penicillium

digitatum. *Plant Protection Science*, *53*: 134–143.

Pinto, E.; Vale-Silva, L.; Cavaleiro, C. and Salgueiro, L. (2009). Antifungal activity of the clove essential oil from *Eugenia caryophyllata* (Syzigium aromaticum) on Candida, Aspergillus and dermatophyte species. *Journal of Medical Microbiology*, 58(11): 1454-1462.

Porcino, M.M.; Valdeir de S.; Oliveira,V.D.S; da Silva,H.F.; de Souza,M.D.S and do Nascimento,L.C. (2023). Essential oils in the management of Alternaria alternata f. sp. citri in 'Dancy' tangerine fruits**.** Rev. Caatinga, Mossoró, v. 36, n. 2: 291 – 299.

Rahman, M.; Begum, M. and Alam, M. (2009). Screening of Trichoderma isolates as a biological control agent against Ceratocystis paradoxa causing pineapple disease of sugarcane. *Mycobiology* 37: 277–285.

Rashad, Y.M.; Abbas, M.A.; Soliman, H.M.; Abdel-Fattah, G.G. and Abdel-Fattah, G.M. (2020). Synergy between endophytic Bacillus amyloliquefaciens GGA and arbuscular mycorrhizal fungi induces plant defense responses against white rot of garlic and improves host plant growth. *Phytopathologia Mediterranea* 59(1): 169-186.

Sánchez-Domínguez, D.; Ríos, M.Y.; Castillo-Ocampo, P. ; Zavala-Padilla, G. ; Ramos-García, M. and Bautista-Baños, S. (2011) . Cytological and biochemical changes induced by chitosan in the pathosystem Alternaria alternata–tomato**.** Pesticide Biochemistry and Physiology 99:250–255.

Shoala, T.; Gehan, A. and Basma,H.(2021). Effects of Salicylic Acid in The Normal and Nano Form against Selected Fungi That Infect Citrus Trees (Citrus sinensis). *Int. J. Sustain. Dev. Sci*. 4: 1–14.

Shukla, S. K.; Mishra, A. K.;Arotiba, O. A. and Mamba, B. B. (2013).Chitosan-based nanomaterials: A state-of-the-art review. *International journal of biological macromolecules*, *59*, 46-58.

Smilanick, J. L.; Mansour, M. F. and Gabler, F. M. (2005). Control of citrus postharvest green mold and sour rot by potassium sorbate combined with heat and fungicides. *Postharvest Biology and Technology*, 38(2): 105-114.

Snedecor, G. W. and Cochran, W. G. (1989). Statistical methods. Oxford and J.PH. Publishing Com. 8th edition

Talibi, I.; Boubaker, H.; Boudyach, E.H. and Ait Ben Aoumar, A.(2014). Alternative methods for the control of postharvest citrus diseases. J. Appl. Microbiol. 117: 1–17.

Timmer, L. W.; Solel, Z. and Gottwald, T. R. (2000). Alternaria brown spot of citrus: An overview of the disease and its management. *Plant Disease*, 84(4): 392-404.

Tripathi, P. and Dubey, N. (2003). Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. *Postharvest Biol Technol*. 32 : 235–245 p.

Weisburg, W. G.; Barns, S. M.; Pelletier, D. A. and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of.*

Ziani, K.; Oses, J.; Coma, V. and Maté, J. I. (2009). Effect of the presence of glycerol and chitosan on the mechanical and barrier properties of wheat gluten films. *Journal of Food Engineering*, 90(4); 570-578

تقييم فعالية بعض العوامل الحيوية واللاحيويه ضد *Geotrichum citri–aurantii* و *Alternaria citri* في المعمل

آية محسن جعفر؛ محمد هارون عبد المجيد ؛ خالد السيد عيد؛ إيمان عثمان حسن

**قسم أمراض النبات، كلية الزراعة، جامعة بنها، مصر

Corresponding author: khaled.alsayed@fagr.bu.edu.eg

تهدف الدراسة إلى تقييم فعالية العلاجات المختلفة ضد *Geotrichum citri–aurantii* و *Alternaria citri* في المختبر. تم جمع العينات من ثلاث محافظات: البحيرة والقليوبية والشرقية. أظهرت القليوبية أعلى معدل انتشار لـ *Geotrichum* sp. و *Fusarium* sp.، بينما كان *Alternaria* sp. هو الأكثر انتشارًا في جميع المحافظات. يوفر التعريف الجزيئي لـ *G. citri–aurantii* و *A. citri* رؤى قيمة حول التركيب الجيني وتنوع مسببات الأمراض الفطرية التي تؤثر على ثمار البرتقال السري. من خلال تسلسل مناطق ITS1 و S RNA5.8، حددت الدراسة بنجاح كلا النوعين مع تشابه وراثي كبير مع سلالات معروفة. كشفت التحليلات التطورية عن تكتل وراثي، مما يشير إلى درجة معينة من التنوع داخل هذه الأنواع. تم اختبار مجموعة من المعالجات الحيوية وغير الحيوية، بما في ذلك العوامل الحيوية مثل *Trichoderma viride* و*Bacillus amyloliquefaciens* والزيوت الأساسية مثل زيت القرنفل والزعتر والزنجبيل والشيتوزان وجسيمات الشيتوزان النانوية (NPs). أظهرت النتائج أن الزيوت الأساسية لها تأثيرات مثبطة متفاوتة على نمو الفطريات بينما كان الشيتوزان وجسيمات الشيتوزان النانوية (NPs) فعالة للغاية في الحد من النمو. والأكثر بروزًا، الشيتوزان (NPs) الذي أظهر فعالية عالية عند تركيزات أقل. بالإضافة إلى ذلك، كان *Trichoderma viride* أكثر فعالية من *B. amyloliquefaciens* في الحد من نمو بطانة *G. citri–aurantii* و*A. citri*.