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RESEARCH ARTICLE

Isolation, molecular identification and *in* vitro screening of *Talaromyces purpurogenus* for the first report as lemon soft- rot causal agent in Egypt.

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Abstract

lemon (*Citrus limon* L.) is one of the fruit crops that is most widely cultivated worldwide. Seven isolates of *Penicillium* sp were isolated from infected lemon fruits with soft rot pathogen collected from different lemon farm's grown under Qena governorate, Upper-Egypt. Pathogenicity test under laboratory conditions reveled that all isolates caused lemon soft rot with different degree. Isolate(Q-66) was the most aggressive one. Morphological identification appeared that the fungus related to *Talaromyces* species. Molecular analysis confirmed the fungus had 99.47% to 100% similarity of DNA with *Talaromyces purpureogenus*. Effect of *Trichoderma harzianum*, *Saccharomyces cerevisiae* and *Candida oleophila* for inhibit the soft rot pathogen growth linear *in* vitro was tested, the most significant reduction in the linear growth of the fungus was achieved by *Trichoderma harzianum* (88.3%) followed by *Saccharomyces cerevisiae* (80.4%), and *Candida oleophila* had (76%). For our knowledge this is the first report of *Talaromyces purpurogenus* fungus isolation in Egypt as lemon fruits soft rot causal agent.

Keywords: lemon soft rot; biocontrol; *Talaromyces purpurogenus*; *Trichoderma harzianum*; *Saccharomyces cerevisiae; Candida oleophila*.

1. Introduction

Lemon (*Citrus limon* L.) is the most prominent member of the Rutaceae families. People throughout the world used lemon as Vitamin C sources, cleaning and cooking (Shamsi *et al.*, 2016; Paciolla *et al.*, 2019). Through 2020 a bout of 21.4 million tonnes of lemons and limes were produced globally, 80% of all postharvest losses during storage period (Calavan *et al.*, 1989). The most harmful postharvest pathogens of citrus fruits are *Penicillium digitatum* and *Penicillium purpurogenum* (Steiner *et al.*, 1994; Ismail and Zhang, 2004;) *Talaromyces purpurogenus* fungus formerly known as *Penicillium purpurogenum* (Samson *et al.*, 2011). Among the most promising alternatives to synthetic fungicides is biological control (Wilson and Chalutz 1989). Citrus postharvest infections can be efficiently prevented by a variety of yeast antagonists. (Chalutz and Wilson, 1990; Arras, 1996; Taqarort *et al.*, 2008). Several commercial biological control formulations based on *Trichoderma harzianum*, *Bacillus subtilis*, and *Gliocladium virens* (Spadaro and Droby, 2016; Liu *et al.*, 2019). Struggle for nutrients and available space is thought to be the primary mechanism of action (Filonow, 1998; Spadaro *et al.*, 2004). Additional proposed mechanisms of action include antibiosis, increased resistance, and the production of lytic enzymes (Janisiewicz and Korsten, 2002). In order to improve the

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effectiveness of the antagonists and provide screening criteria for new isolates, a fuller understanding of their mechanism of action is essential. Therefore, the purpose of this research was to isolation, identification of the lemon soft rot pathogen which widespread during limon harvest season morphology and molecularly. The ability of *T. harzianum*, *Saccharomyces cerevisiae* and *Candida oleophila*, on suppressed the linear growth of *Talaromyces purpurogenus in* vitro was assessed as the first step in the pathogen biocontrol.

2. Materials and methods

2.1. Source of rotted lemon

Infected lemons fruits samples were collected from various lemon farms grown in Qena governorate Upper-Egypt during Season 2021-2022. The infected samples were preserved in sterile polypropylene containers under 4ºC. The isolation media used for isolation was Potato Dextran Agar (PDA).

2.2. Isolation of casual agents

A sterilized needle was used to remove a 2 mm-long rotting section of the lemon fruit peel. The tissue was aseptically put in Petri plates with (PDA) medium after being submerged in a 5% sodium hypochlorite solution for two minutes, rinsed in sterile distilled water (SDW) for 3 minutes and dried on filter papers in a laminar air flow cabinet. Petri dishes inoculated with the infected lemon peels were incubated for five days at 25Cº, and checked for the growth of fungi every day.

2.3. Pathogenicity test

With a few small adjustments, Amienyo and Ataga's (2006) technique was used to the pathogenicity test. Healthy lemon fruits were cleansed, washed, and sterilized for two minutes, then dried for twenty minutes. Next, a sterile cork borer was used to create cylindrical holes that were five millimeters in diameter in each lemon fruits. Each hole was filled with five mm-diameter discs of *Penicillium sp* that had been cultured on potato dextrose agar for five days before. In the control treatment, the holes injected with PDA discs had not been infused with the mycelial fungi and they were covered with sterile plastic sheets, incubated at 25 °C. The injected lemon fruits were observed every day for the soft rot symptoms and the degree of diseases incidence was determined by the scale from (1-5) as follows. Whereas 2 = 5–10 mm of rotting area (low virulent); 3 = 11–20 mm (moderately virulent); 4 = 21–30 mm (virulent); and 5 = ≥ 31 mm (high virulent); where 1, non-virulent symptoms are found. (Nash and Snyder, 1962).

2.4. Identification of the pathogen

The most pathogenic isolate was obtained through the sub-culturing of fungi derived from degraded lemon peel pieces and their incubation on sterile potato dextrose agar plates. Optical microscopy was used to identify the pure cultures of the fungus and determine its morphological characteristics before sending the pure culture for molecular identification.

2.5. DNA extraction and PCR assays

The Korean company Intron Biotechnology produced a kit called Patho-gene-spin DNA/RNA extraction that could be used to extract DNA. In order to perform the PCR, two universal fungal primers, ITS1 (forward) and ITS4 (reverse) were added to the reaction mixture. The chemical composition of the ITS1 was (5' - TCC GTA GGT GAA CCT GCG G - 3') and ITS4 was (5' - TCC TCC GCT TAT TGA TAT GC - 3'). The purified PCR product (amplicon) was confirmed again. Using size nucleotide marker (100 base pairs) and electrophoresis on 1% agarose gel, the purified PCR product (amplicon) was verified once again. After that, the reaction mixture was supplemented with dideoxynucleotides (dd NTPs) and these bands were eluted and sequenced. Every sample was sequenced in both sense and antisense orientations using ITS1 and

ITS4 primers (White *et al.,* 1990). Sequences were further analyzed via the National Centre for Biotechnology Information (NCBI) website's Basic Local Alignment Search Tool (BLAST). The sequences' phylogenetic analysis was performed using MegAlign (DNA Star) software version 5.

2.6. In vitro antagonism

Bioagents control *T. harzianum*, *Saccharomyces cerevisiae* and *Candida oleophila* were kindly provided from Department of Microbiology Faculty of Agriculture, South Valley University, Qena, Egypt. The antagonistic activities of the bioagents were assessed using the dual culture method. For *Candida oleophila and Saccharomyces cerevisiae* yeasts, fresh culture of each isolate were streaked in the side of a 9 cm Petri dish filled with PDA medium in a straight line and the soft rot pathogen was injected in other side on the same Petri dish. Regarding *T. harzianum,* 5 mm discs of the fresh culture of *T. harzianum* and pathogen were placed side by side on the same Petri dish, incubated for five days at a temperature of 25°C. Control plates were inoculated with the pathogen only. The percentage of inhibition was determined by (Korsten and De Jager 1995). R ─ R1/R × 100 = IPG (%) where R1 is the fungal growth distance from the point of inoculation to the colony margin in the treatment plates, IPG is the inhibition percentage growth, and mm is the distance (measured in mm) between the point of inoculation and the colony margin in the control plate.

2.7. Statistical analysis

The fully randomized experimental design included three copies of each treatment. After the experiment was run through at least two times. Comparison among means of treatments and appropriate control were made at P = 0.05 using a multiple range test(P>0.05 for the Duncan's test), (Gomez & Gomez, 1984) by SAS Statistical Software Package (v.9.2, 2008).

3. Results

3.1. Symptomology of lemon soft rot

lemon fruits collected from different lemon farms in Qena governorate, Egypt were severally suffered from soft-rot symptoms. First sign on infected fruits was appeared as dark soft spot-on lemon peel, these spots were covered with white mycelium of the pathogen after 3-5 days as illustrated in (Figure 1), which appears typically in pathogenicity test.

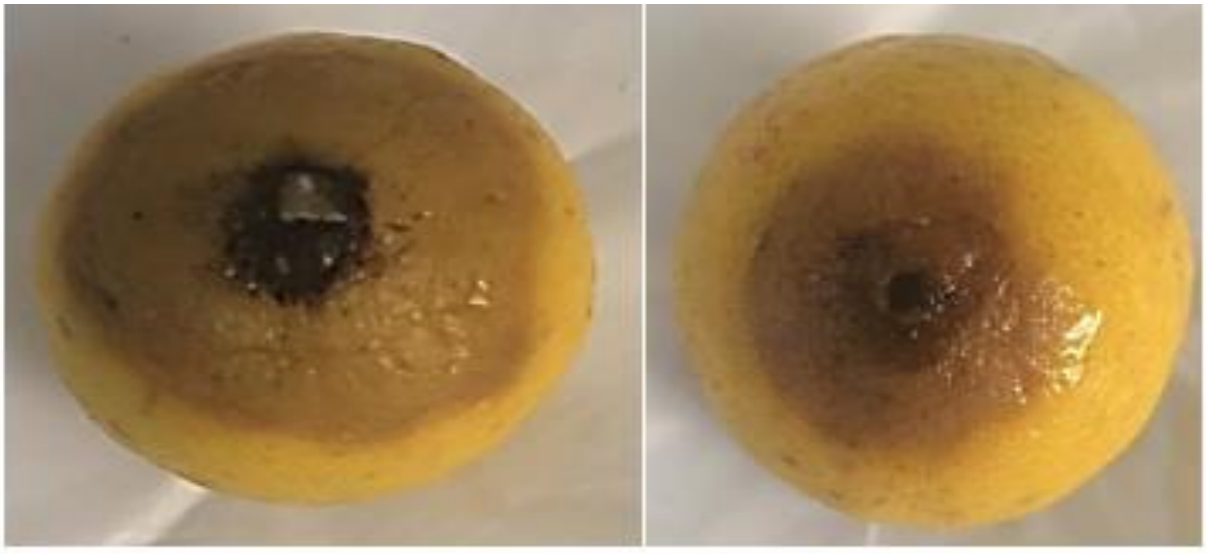

Figure 1. The natural symptoms on Lemon fruits collected from different farms grown under Qena governorate (Upper Egypt.)

3.2. Isolation of lemon soft rot pathogens

Seven different isolates of *Penicillium* sp were obtained from rotted lemon fruits.

3.3. Pathogenicity test

As illustrated in figure (2), all seven isolates were able to infect lemon fruits with different degree of soft rot under laboratory conditions. However, isolate (Q-66) was the virulent one caused 96% compared with others isolates which caused a weak symptoms in lemon fruits. Therefore isolate (Q-66) was subsequential used in all experiments. Under laboratory conditions, the infected lemon fruit areas first appear as a wetted area around the wound, followed by the production of a white mycelium of the pathogen fungus conidia, which in a short period of time causes the entire fruit to rot. At the final stage, the fungus' conidia were completely covering the infected fruits with black mycelium whereas, lemon fruits have no infection in control treatments. (Figure 3).

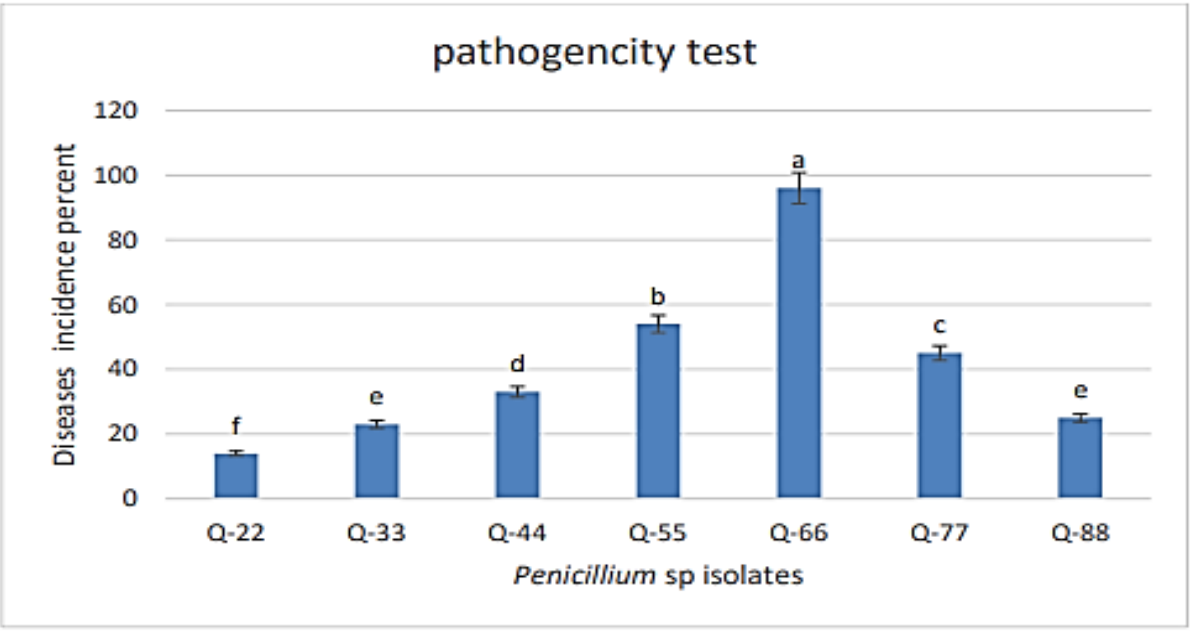

Figure 2. Pathogenicity test of different isolates of *penicillium* sp under laboratory conditions. Values in the column followed by the same letter are not significantly different according to Duncan's at P<0.05.

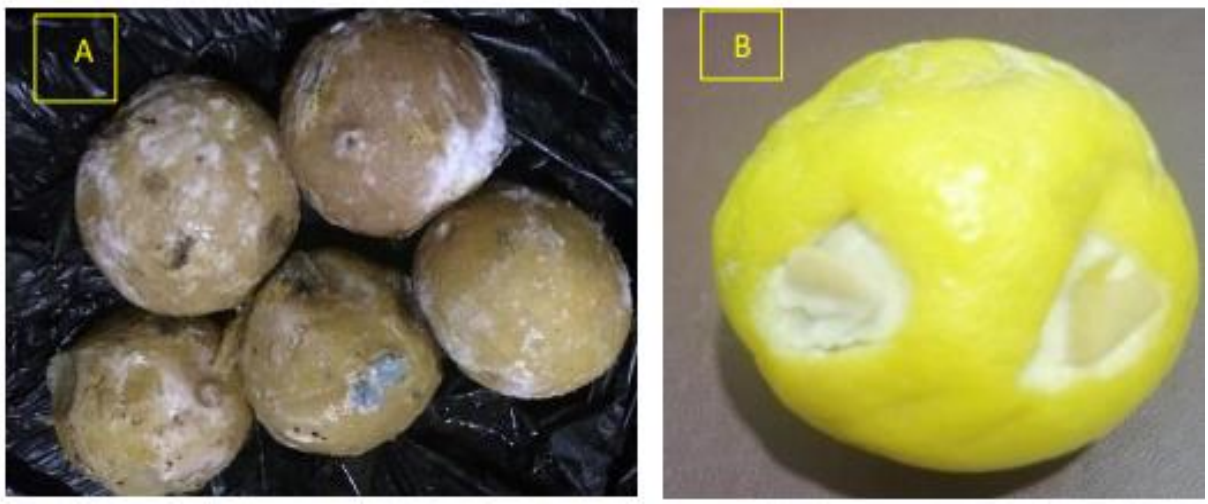

Figure 3. A: Soft rot symptoms appeared on inoculated lemon fruits with disc of *Talaromyces purpurogenus*, **B:** control lemon fruits inoculated with PDA discs has no pathogen.

3.4. Morphology of the pathogen

Talaromyces purpurogenus was isolated and identified as the fungus caused soft rots in lemon fruits. On potato dextrose agar, the pathogen's colony features were observed to generate a dark green colour with red pigmentation that spread as the fungus's mycelia grew (Figure 4. A). The pathogen's conidiophores are branching, glass-bearing-like globules, as seen by microscopic analysis (Figure 4. B).

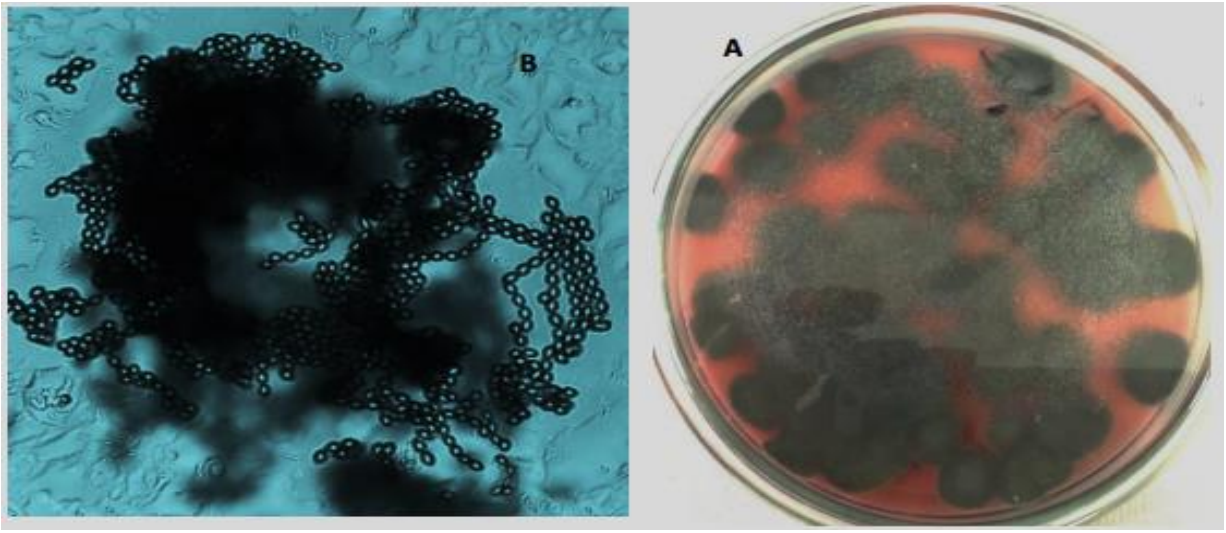

Figure 4. A: *Talaromyces purpurogenus* colony characteristics on potato dextrose agar.
B: Conidiophores of *Talaromyces purpurogenus* under optical microscopy.

3.5. Molecular identification of the pathogen

ITS sequences of the rDNA of the fungal strain (NM-3) isolated in this work were used to create a phylogenetic tree, which was then matched with closely similar sequences obtained from GenBank. Molecular tests revealed that the material had 99.47% - 100% identity with numerous strains of *Talaromyces purpurogenus* (Figure 5).

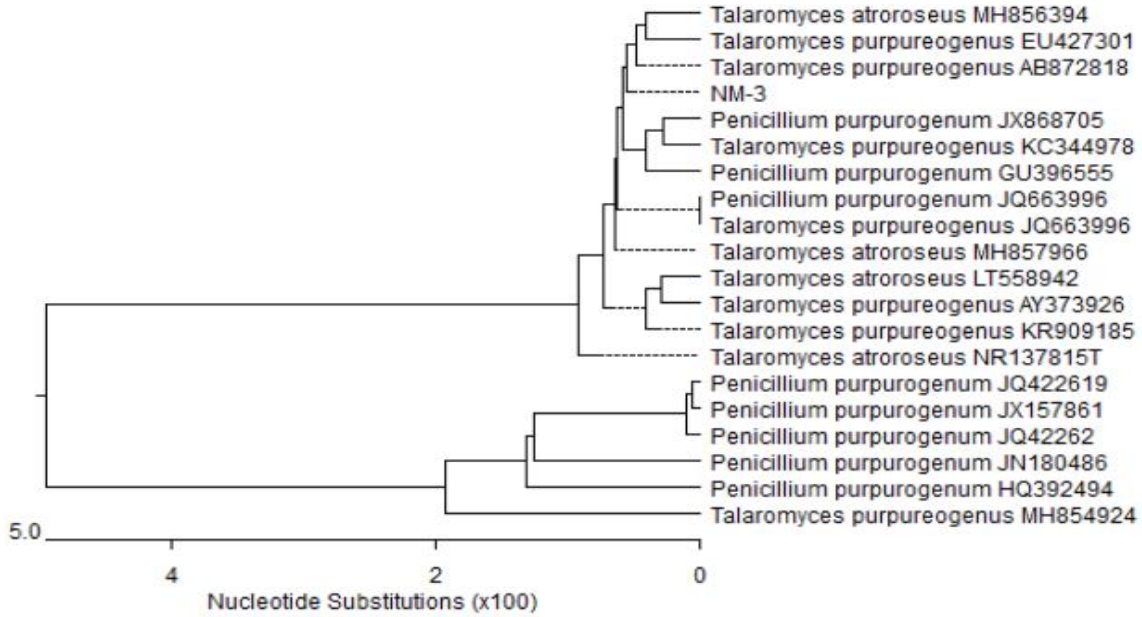

Figure 5. Phylogenetic tree based on ITS sequences of rDNA of the fungal strain isolated in the present study (NM-3) aligned with closely related sequences accessed from the GenBank. The sample showed 99.47% - 100% similarity with several strains of *Talaromyces purpurogenus.*

3.6. in vitro antagonistic

in vitro dual culture interactions between the bioagent and the fungus revealed that T. *harzianum* could limit the linear growth of the pathogen develop on the culture medium with 88.3% inhibition percent. However, yeasts, *Saccharomyces cerevisiae* was 80.4% and *Candida oleophila* was 76% inhibition percent. The results showed that *T. harzianum*, *Saccharomyces cerevisiae* and *Candida oleophila* bioagents had a substantial antagonistic effect on *T. purpurogenus* (Figure 6) with different degree.

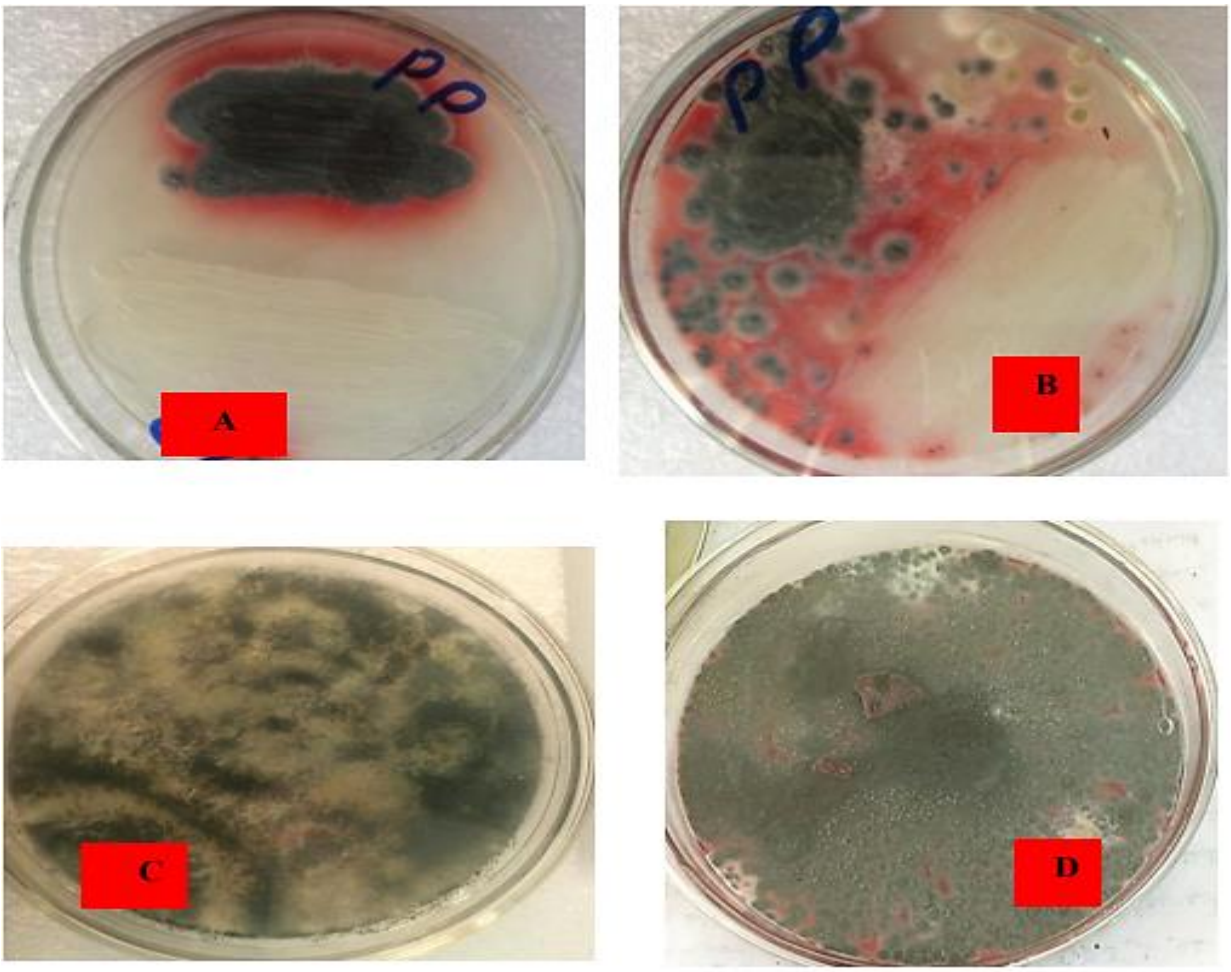

Figure 6. *In* vitro antagonistic within the pathogen of Lemon soft -rot and biocontrol agents. A. *Saccharomyces cerevisiae + T. purpurogenus*, B. *Candida oleophila+ T. purpurogenus,* C. *T. harzianum + T. purpurogenus, D.* control of *T. purpurogenus*

4. Discussion

Lemon is the most prominent member of the Rutaceae family's citrus fruit species, which have a global production of more than 98 million tons annually and have a substantial economic impact on the global fruit industry (United States Department of Agriculture, 2021). According to morphological and molecular identification in this investigation, the *T. purpurogenus* fungus was associated to soft rot in lemon fruits. The sexual *Penicillium* species were housed in the genus Talaromyces. (Benjamin, 1955). *Penicillium purpurogenus* has been identified as a fruit rot pathogen that affects Prunus armeniaca, Averrhoa bilimbi and strawberries (Gubler and

Converse, 1994), (Bhadwal and Sharma, 2011). White Yam rot and Pear blue mould (Sangoyomi *et al.,* 2004; Gwa and Abdulkadir, 2017; Stošić *et al.,* 2021). *Talaromyces* sp. caused sucrose loss in sugar beet piles (Strausbaugh and Dugan, 2017; Strausbaugh, 2018). *Talaromyces albobiverticillius* caused postharvest fruit rot on pomegranates (Mincuzzi *et al.,* 2020). According to (Ariza et al., 2002), fruits were infected by *Penicillium*.sp through wounds, after which the fungus's mycelium developed on the lesion; after a few days, the fruit began to rot completely; at last, the fruits were covered with the conidia of the *Penicillium*.sp that had infected them. Alternative methods include biological control using hostile microbes like fungi, bacteria, and yeast, as well as physical methods like heat or radiation, plant extracts, essential oils, chitosan, synthetic elicitors, and bio-fungicides were tested to control citrus post-harvest diseases (Palou *et al.,* 2016; Liu *et al.,* 2017; Palou, 2018; Papoutsis *et al.,* 2019). In vitro *T. harzianum* fungus could limited the growth of the pathogens on the culture medium. This may due to *Trichoderma* sp., have hyperparasites that interact directly to break down fungal cells or to exert antagonistic effects through antimicrobial chemicals, develop hyperparasiteism or directly attach to pathogen cells, and disrupt cellular functions. (Harman, 2004). Employ yeasts *Saccharomyces cerevisiae* and *Candida oleophila* considerably inhibited the pathogen's ability to develop linearly *in* vitro. The first step involves screening potential biological control agents (BCA) based on their *in* vitro antagonistic activity. (Paraveen *et al.,* 2016). Previous research looked on using yeasts to prevent post-harvest fungal (Abraham *et al.,* 2010; Platania *et al.,* 2012; Kupper *et al.,* 2013; Moretto *et al.,* 2014; Ferraz *et al.,* 2016; Liu *et al.,* 2017). Yeasts have desirable characteristics for pathogenic biocontrol since they rarely produce drugs or mycotoxins that could leave residues on fruits. (Droby *et al.,* 2002; Gamagae *et al.,* 2004; Zhang *et al.,* 2005). A number of antagonist yeasts, including *Candida oleophila* (Liou *et al.,* 2017; liou *et al.,* 2019), *Debaryomyces hansenii* (Hernandez-Montiel *et al.,* 2010; Cengiz and Füsun, 2018) *Candida oleophila* and *Debaryomyces hansenii* has been reported to be effectively controlled by Citrus fruit postharvest decay (Lahlali *et al.,* 2011; Hammami *et al.,* 2022). Yeasts have been utilised as biocontrol agents through a variety of processes, such as direct parasitism, the synthesis of volatile organic compounds, competition for nutrients or space with the fungal pathogen, and the secretion of lytic enzymes or antimicrobial molecules (VOCs). (Zhou *et al.,* 2014; Droby and Padaro, 2016; Chen *et al.,* 2020). Because they produce killer proteins that impede the growth of infections and deform fungal hyphae, certain strains of yeast have been referred to as "killing yeasts." (Comitini, *et al.,* 2009; Platania *et al.,* 2012; Aloui *et al.,* 2015; Ferraz *et al.,* 2016). Because synthetic fungicides have detrimental effects on humans and the environment, postharvest biological management of pathogenic fungus is now being evaluated as an effective fruit rot control strategy (Sharma *et al.,* 2009; Shahbazi *et al.,* 2014).

5. Conclusion

It could be concluded that a new emergency lemon soft rot pathogen isolated from different lemon fruits farms grown under Qena governorate in Upper - Egypt for the first time. The pathogen morphology was illustrated and molecularly analysis confirmed that the fungus had 99.47% to 100% similarity of DNA with various *Talaromyces purpurogenus*. Biological control trails using *Trichoderma harzianum, Saccharomyces cerevisiae* and *Candida oleophila* revealed that the bioagents have the potentiality to suppress the pathogen linear growth *In vitro*.

Authors' Contributions

All authors are contributed in this research

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Institutional Review Board Statement
All Institutional Review Board Statements are confirmed and approved.
Data Availability Statement
Data presented in this study are available on fair request from the respective author.
Ethics Approval and Consent to Participate
Not applicable
Consent for Publication
Not applicable.
Conflicts of Interest
The authors disclosed no conflict of interest.

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