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Biocontrol of the Growth of Some Fungal Isolates Causing Apple Fruit Rot in Local Markets

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ABSTRACT

During harvesting, transportation and storage, apples (*Malus domestica* Borkh) are exposed to scratches, which facilitate the process of fungi entering the healthy tissues of the fruit and rotting it and causing great economic losses. This study was aimed at finding alternatives (biological treatments) to preserve local apple fruits from infection with fungi and contamination with their toxins. Isolation and diagnosis of 92 fungal isolates belonged to six fungal genera *Alternaria* sp. 6/92 (6.52%), *A. alternata* 12/92 (13.04%), *Aspergillus flavus* 7/92 (7.60%), *A. niger* 5/92 (5.43%), *A. ochraceus* 6/92 (6.52%), *A. terreus* 5/92 (5.43%), *Penicillium* sp. 8/92 (8.69%), *P. expansum* 18/92 (19.56%), *P. italicum* 10/92 (10.86%), *Fusarium* sp. 6/92 (6.52%), *Mucor* sp. 4/92 (4.34%) and *Rhizopus stolonifer* 5/92 (5.43%). All filter concentrations of *T. harzianum* and *T. viride* had a significant inhibitory effect on the radial growth of *P. expansum*, *A. alternata* and *P. italicum* compared to the control treatment. The rates of colony diameters at the concentration of 15% ranged between (11.13-12.71) mm with inhibition percentage ranging between (85.87-87.63) for *T. harzianum* treatment and between (12.85-13.66 mm) with inhibition percentage ranging between (84.82-85.72) for *T. viride* treatment.

Key words: Apple fruit rot, *P. expansum*, biological treatments, *T. harzianum*, *T. viride*

INTRODUCTION

The apple (*Malus domestica* Borkh) belongs to the Rosaceae family, and it is one of the most common fruits all over the world. The fruits contain many carbohydrates, organic acids and mineral salts (such as potassium, sodium and calcium). It also contains some vitamins such as vitamins A, B and E, as well as fiber and carotene (Musacchi and Serra, 2018). Due to the high nutritional value of apples, it is considered a natural medium that helps in the growth of microorganisms, especially fungi (Amiri *et al*., 2021). During harvesting, transportation and storage, apples are exposed to scratches, which facilitate the process of fungi entering the healthy tissues of the fruit and rotting it and causing great economic losses. Warehouse diseases cause 20% losses in the global agricultural product due to partial or total spoilage of the fruits. In some hot and humid tropical countries, the percentage reaches 50 (El-Ramady *et al*., 2015; Blakeney, 2019).

The most essential fungi that contaminate vegetables and fruits after harvest, including apple fruits are *Aspergillus* spp., *Penicillium expansum*, *P. italicum*, *Botrytis cinerea*, *Alternaria alternata*, *Rhizopus stolonifer*, *Mucor piriformis*, *Geotrichum candidum*, *Phytophthora cactorum* and others (Khan *et al*., 2021). The effect of fungi to rot fruits, especially in apple, makes some fungal strains the capability to produce mycotoxins such as aflatoxin from *Aspergillus* spp. and patulin from *Penicillium* spp., which are the most potent toxins known to cause serious illnesses even at low concentrations. Further, the removing the infected parts of the fungus from food, as many people do, does not lead to the disposal of the mycotoxins formed in these foods (Dwiastuti *et al*., 2021). These toxins cause different types of cancers such as liver and kidney, as the fungi through these toxins transform the metabolic system of the cell from oxidized to fermented by the action of enzymes that protect it from being destroyed by oxygen. By taking advantage of the sugar present in the inactive body cavities as a source of energy, these abnormal cells are called cancer (Agriopoulou *et al*., 2020). The use of chemical pesticides, in general, is the main way to combat post-harvest diseases. Further, to increase the products free of pesticides and chemicals (because of their side effects on living organisms and the environment), and because of the presence of strains resistant to fungicides, there was a need to find alternative and safe methods that would be less risky to human health and environment (Tudi *et al.*, 2021).

One of the most alternative methods is the biological control method. The current studies have focused on this aspect in several parts of the world, resulting in the discovery of some important bacterial and fungal species that can be used in biological control (Leng *et al.*, 2022). The most important fungi that are used in biological control are the species of the fungus *Trichoderma* spp. because the types of this fungus can compete for food and space and parasitize with other microorganisms, as well as because of their ability to produce many degrading enzymes and antibiotics that affect the growth of fungi (Ferreira and Musumeci, 2021). Due to the difficulties facing our country in providing good storage means such as cold stores, as well as the high costs of using them, this study was aimed at finding alternatives to preserve local apple fruits from infection with fungi and contamination with their toxins.

MATERIALS AND METHODS

Samples of apple fruits were collected from local markets in different areas in the city of Al-Diwaniyah/Iraq, which showed rot and infection with fungi for the period from May to July 2022. Each parts of the fruit tissue were taken near the area of infection with a diameter of 0.5 cm and then these parts were superficially treated with 1% sodium hypochlorite solution for 3 min. Then washed with sterile distilled water, dried using sterile filter papers, and cultured in Petri dishes containing sterile PDA medium in addition to the antibiotic chloramphenicol at a concentration of 25 mg/l. Each piece was placed in the middle of the dish, then the dishes were incubated at 25°C for five days and once the incubation period finished, the fungal isolates were purified by taking a disc from each dish and cultured in a dish containing sterile PDA medium. This process was repeated several times until completely pure fungal isolates were obtained. It was identified based on taxonomic characteristics (Watanabe, 2018). The percentage of frequency was calculated as:

$$\text{Frequency \%} = \frac{\text{The number of isolates belonging to the same genus or species}}{\text{The total number of isolates}} \times 100$$

The antagonism fungi *T. harzianum* and *T. viride* were isolated from agricultural soil samples at the College of the Science, University of Al-Qadisiyah and identified after purification on the PDA medium based on taxonomic characteristics (Watanabe, 2018). Then the filtrates of two fungi were prepared by placing 50 ml of Potato Dextrose Broth (PDB) in 250 ml flasks and sterilized by autoclaving at 121°C for 20 min. Then the antibiotic chloramphenicol at a concentration of 25 mg/l was added. The flasks were inoculated by placing a 5 mm disc of antagonism fungi in each flask separately, and the flasks were placed in the incubator at 25°C for 14 days. Once the incubation period finished, the fungal cultures were filtered by filter papers under sterile conditions. The fungus filtrates were sterilized using (millipore filters 0.22 microns) and saved in the fridge at 4°C until use. To study the effect of *T. harzianum* and *T. viride* filtrates on the radial growth fungi, the PDA medium was prepared in 250 ml flasks and sterilized with an autoclave at 121°C for 20 min, after sterilization, the medium was cooled and the antibiotic chloramphenicol was added to it at a concentration of 25 mg/l. Three concentrations were prepared for the filtrates of *T. harzianum* and *T. viride*, which were 5, 10 and 15% separately, as well as the treatment of the fungicide Topaz at a concentration of 1 g/l. The control treatment contained the PDA medium without any addition. The flasks were shaken well and then the medium was poured into Petri dishes with a diameter of 90.00 mm with three replications for each treatment. The middle of each dish was inoculated with discs of the fungi under study (the most frequent) with a diameter of 5 mm from the culture at the age of seven days (one disc for each dish). After the mycelium in the control treatment reached the dish's edge, the percentage of inhibition was calculated as:

$$\text{Inhibition \%} = \frac{\text{Control colony diameter} - \text{Treatment colony diameter}}{\text{Control colony diameter}} \times 100$$

Petri dishes containing PDA sterile media were prepared with the antibiotic chloramphenicol at a concentration of 25 mg/l. Then the middle of the first half of the dish was inoculated with a disc diameter of 8 mm of *T. harzianum* at the age of seven days. The second half of the dish was inoculated with a disc of fungi under study (the most frequent) with the same diameter and age separately in three replications. The above steps were repeated using the *T. viride*. Now, the dishes were incubated at 25°C for seven days, and once the incubation period finished, the scale of Bell was adopted to calculate the area occupied by the antagonism fungus on one side and the pathogenic fungus on the other as:

Antagonism fungus covering the whole dish (1)
3/4 of the dish is covered in antagonism fungus (2)
1/2 of the dish is covered in antagonism fungus (3)
3/4 of the dish is covered in pathogen (4)
Pathogen covers the whole dish (5)

ANOVA was used for the statistical analysis, and significant differences between the means were determined using Duncan's polynomial test at 5%.

RESULTS AND DISCUSSION

The isolation and diagnosis of 92 fungal isolates belonging to 12 species of fungi for six fungal genera (*Alternaria* sp., *A. alternata*, *Aspergillus flavus*, *A. niger*, *A. ochraceus*, *A. terreus*, *Penicillium* sp., *P. expansum*, *P. italicum*, *Fusarium* sp., *Mucor* sp. and *Rhizopus stolonifer*) and the most frequent fungi was *P. expansum* in 18 isolates with a frequency of 19.56%, followed by *A. alternata* in 12 isolates with a frequency of 13.04%, then *P. italicum* with 10 isolates with a frequency of 10.86%, and the least frequent fungi was *Mucor* sp. in four isolates with a frequency of 4.34% (Table 1). Apple fruits become contaminated and infected with a variety of fungi after being stored for a long time, resulting in significant economic losses. Previous studies had revealed that fungi could be isolated from a range of fruits, which led to significant economic losses. El-Gali (2016) was able to isolate many fungi from different fruits, including apples in Libya, and found that *P. expansum* was one of the most isolated fungi. Abdullah *et al.* (2016) also mentioned that *P. expansum* was one of the most isolated fungi from apple fruits in Yemen. Embaby *et al.* (2020) were also able to isolate *A. alternata, A. niger, Fusarium* sp. and *P. expansum* from apple fruits in Egypt.

Table 1. Fungi isolated from apple fruits and their frequency

Fungi	No. of isolates	Frequency (%)
Alternaria sp.	6	6.52
A. alternata	12	13.04
Aspergillus flavus	7	7.60
A. niger	5	5.43
A. ochraceus	6	6.52
A. terreus	5	5.43
Penicillium sp.	8	8.69
P. expansum	18	19.56
P. italicum	10	10.86
Fusarium sp.	6	6.52
Mucor sp.	4	4.34
Rhizopus stolonifer	5	5.43
Total	92	

All concentrations of filtrates had a significant inhibitory effect on the growth of *P. expansum*, *A. alternata*, *P. italicum* on the PDA medium at a probability level of 5% compared to the control treatment (Tables 2, 3 and 4). The rate of colony diameters at the concentration of 15% was the best concentration, ranging from 11.13-12.71 mm with the inhibition percentage ranging between 85.87-87.63 for the treatment of *T. harzianum* and between 12.85-13.66 mm with an inhibition percentage between 84.82-85.72 for the treatment of *T. viride* compared to the control treatment in which the rate diameters of the colonies reached 90.00 mm. The results revealed that there were no significant differences between the effects of 15% concentration and the effect of the fungicide Topaz for all treatments and fungi under study, as the rates of colony diameters in the fungicide treatments ranged between 10.12-11.16 mm and the inhibition percentage ranged between 87.60-88.75. The use of some fungi in the biological control of plant pathogenic fungi is one of the safe and alternative methods of using fungicides. Katyayani *et al.* (2020) found that *Trichoderma* spp. were effective in inhibiting the growth of some fungi isolated from chickpea plant seeds. The antifungal activity of *T. harzianum* and *T. viride* filtrates was due to the ability of this species to produce lytic enzymes such as

Table 2. The effect of antagonism fungi filtrates on *P. expansum* radial growth

Concentration (%)	Fungus filters			
	T. harzianum		*T. viride*	
	Diameter of colonies (mm)	Percentage of inhibition	Diameter of colonies (mm)	Percentage of inhibition
5	22.15±0.21b	75.38	23.76±0.35b	73.60
10	16.75±0.23c	81.38	18.21±0.23c	79.76
15	11.13±0.12d	87.63	12.85±0.15d	85.72
Topaz (1 g/l)	10.12±0.17d	88.75	10.16±0.13d	88.71
Control	90.00a	0.00	90.00a	0.00

Same letters do not differ significantly among themselves for vertical comparisons.

Table 3. The effect of antagonism fungi filtrates on *A. alternata* radial growth

Concentration (%)	Fungus filters			
	T. harzianum		*T. viride*	
	Diameter of colonies (mm)	Percentage of inhibition	Diameter of colonies (mm)	Percentage of inhibition
5	23.56±0.33b	73.82	24.26±0.42b	73.04
10	17.77±0.46c	80.25	19.32±0.25c	78.53
15	12.43±0.24d	86.18	13.66±032d	84.82
Topaz (1 g/l)	11.12±0.25d	87.64	10.96±0.23d	87.82
Control	90.00a	0.00	90.00a	0.00

Same letters do not differ significantly among themselves for vertical comparisons.

Table 4. The effect of antagonism fungi filtrates on *P. italicum* radial growth

Concentration (%)	Fungus filters			
	T. harzianum		*T. viride*	
	Diameter of colonies (mm)	Percentage of inhibition	Diameter of colonies (mm)	Percentage of inhibition
5	25.14±0.42b	72.06	23.93±0.66b	73.41
10	18.67±0.28c	79.25	19.55±0.44c	78.27
15	12.71±0.27d	85.87	13.12±0.30d	85.42
Topaz (1 g/l)	11.16±0.21d	87.60	10.88±0.26d	87.91
Control	90.00a	0.00	90.00a	0.00

Same letters do not differ significantly among themselves for vertical comparisons.

chitinase, cellulase, 1,3-glucanase and polygalacturonase, which affect the building of the cell wall by degrading the most important substances in its structure, such as chitin, polysaccharide, in addition to its ability to produce many antibiotics such as alamethicin, alkylpyrone, diketopiperazine, isonitrile, trichodermin, harzianum A and harzianolide (Khan *et al.*, 2020; Sharma *et al.*, 2022).

The two antagonism fungi inhibited the development of all of the fungi under study (Table 5). These results confirmed the results of the effectiveness of the filtrates in the radial growth of fungi on the PDA medium, the antagonism value was 1 for *T. harzianum* against all fungi under study, while it was 1 for *T. viride* against *P. expansum*, *A. alternata* and 2 against *P. italicum* on the scale of Bell. The effectiveness of the antagonism fungi may be due to their ability to compete for food and

Table 5. Antagonism method

Antagonism fungi	Pathogenic fungi		
	P. expansum	*A. alternata*	*P. italicum*
T. harzianum	1	1	1
T. viride	1	1	2

parasitize on other microorganisms, as well as the production of many enzymes such as (Xylanase and chitinase) and toxic metabolic substances that affect the growth of fungi (Tyskiewicz *et al.*, 2022).

CONCLUSION

Fungal species belonging to the genera *Penicillium* spp. and *Alternaria* spp. were among the most prevalent fungi that caused post-harvest apple fruit rot, which resulted in large economic losses. The use of the antagonism fungi *T. harzianum* and *T. viride* had a significant effect on the growth of the fungi under study, with a concentration of 15% producing an effect similar to that of the fungicide Topaz. These alternatives were safe for the environment and therefore the possibility of using them in preserving fruits and vegetables after developing other experiments.

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