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(Original Article)

Isolation and Molecular Characterization of Yeast Killer Isolates from Natural Habitats

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Abstract

Several yeast isolates can secrete antifungal components. These include toxic proteins or enzymes, cyclic dipeptides, surfactants, or volatile small molecules, all of which act against fungi. Although the term 'killer yeast' traditionally refers to those that secrete protein toxins, some literature uses the term more broadly for any yeast with antifungal activity. This study aims to obtain highly potent killer yeast isolates to be used in biological control against several other microorganisms. 30 yeast isolates were collected from three distinct sources (soil, juice, and fruit) from seven different locations: Assiut town, Bosra, Sahel Selim, Elbadari, AlGhanium, Abnob, and Manfalout, all located at Assiut goernorate, Egypt. The isolates were characterized and tested for their killing activity. Five isolates out of 30 showed killing activity. ITS gene sequence determination method was used to identify the strongest isolate molecularly, and the results showed that isolate no. 9 was 99% related to *Meyerozyma carbibica*. The potential of the tested isolates to eliminate various pathogens was demonstrated by their antagonistic against several plant pathogenic microorganisms. The results showed the ability of the tested isolates to kill different pathogens. Therefore, these isolates can be used to biologically control various plant pathogens.

Keywords: *Antagonism, Killer Yeast, Meyerozyma carbibica, Mycocins*

Introduction

Yeasts are single-celled, eukaryotic microorganisms that belong to the fungus kingdom. Yeasts are found in many different environments throughout nature, and they are frequently isolated from materials that are high in sugar. They are present in soil, as well as on the leaves, flowers, and fruits of plants. Yeast biomass has a low natural salt and fat content and is a rich natural source of protein, comprising amino acids, enzymes, peptides, carbs, and B vitamins (Jach*, et al.,* 2022). Yeasts play important roles in industry, the environment, and medical science (Walker, 2009).

The “killer” phenomenon was discovered first by Bevan and Makower (1963). It was observed in yeast cells of certain strains of *Saccharomyces cerevisiae*. Three phenotypes are described namely, killer (K), sensitive (S), and

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neutral (N). Killer strains that kill sensitive strains, while neutral strains do not produce toxin, and are immune to toxins secreted by others. The discovery of a fourth yeast phenotype termed "killer/sensitive" was reported, this phenotype is sensitive to "killer factor" but produces a new "killer factor" which kills sensitive cells. The killer strain is immune to the effect of its own toxin. They have been isolated from environmental, clinical, industrial, and agricultural sources (Golubev, 1998). Thereafter, it was detected that killer yeasts secrete a proteineous mycocins (killer toxins) lead to killing the sensitive strains (Woods and Bevan, 1968; Bussey, 1972; and Golubev, 1998). Killer activity has been reported in almost 100 yeast species belonging to 20 or more genera (Bajaj and Singh, 2017).

Killer phenomena have generated a lot of interest because of their potential applications, which include safeguarding industrial fermentation against infecting yeasts (Martini and Rosini, 1989). In addition of being used as antimitotic drugs, killer yeasts have been employed in the bio-typing of pathogenic yeasts. Killer yeast may have potential for application as bio preservatives, biocontrol agents and as new therapeutic molecules especially against multidrug resistance pathogens (Georgescu *et al*., 2024).

The genetic studies of different types of killer phenotypes showed that this character may be due to dsRNA viruses, or linear dsDNA plasmids, or chromosomal genes. The killer toxins interact with the receptors on the cell wall or cell membrane of yeasts cause killing the microorganisms (Marquina, *et al.,* 2002; and Alturki *et al*., 2019).

Killing habitat is characterized by high sugar concentration and low pH but there is still much to learn about the killer toxin production and the probability that a killer toxin produced by yeast may kill certain susceptible yeast (Crabtree *et al.,* 2023).

Killer factors had no effect on producing killer strains which were immune. This suggests that the poisons don't have a site of action, or inactivating substances. On the other hand, when the killer strain kills another, the secreted toxins must be different because the specific immunity system of the killed strain immune against its own toxin but not against the other (Buzzini and Martini, 2000).

The killing trait is not limited to yeasts only but is found in other microorganisms such as bacteria, and termed bacteriocins (Konisky, 1982).

Throughout the years, yeasts have been identified using morphological, biochemical, and physiological traits. Barnett and Hemsworth, (1990), have been described around 1000 species of yeast. Of the 62000 yeast species and 0.065% of yeast genera, only 0.22% and 0.065%, respectively, have been isolated and characterized.

To characterize yeasts, both morphological and molecular techniques have been used frequently. Molecular characterization tends to be preferred for precise and accurate identification of yeasts due to its higher resolution and ability to resolve closely related species or strains. Morphological characterization remains valuable for preliminary identification and in environments where molecular

resources are unavailable. Combining both approaches can often provide a comprehensive understanding of yeast species (James and Nair*,* 2012).

This study aimed to collect different yeast isolates from different natural sources, phenotypic and molecular characterization of the isolates, detection of the presence of killer and sensitive isolates, study the antagonism between killer yeast and some others pathogenic microbes.

Materials and Methods

1-Sampling area and sample collection

Several fruit, vegetables, juices, and soil samples collected from different areas in Assiut governorate, Egypt (Assiut, Bosra, Elbadari, El Ghanaym, Manfalut, Abnob, and Sahil-salim) were used to isolate different yeast isolates.

2-Isolation and purification of yeast

Yeasts were isolated from the collected samples using dilution plate method according to the methods of Kurtzman *et al.* (2011) as follows:

Isolation was performed on yeast extract peptone dextrose (YEPD) agar plates containing: 10g yeast extract; 20g peptone; 20g glucose, and 20g agar-agar dissolved in one liter of distilled water. The media were supplemented with 100 µg/ml streptomycin solution to avoid bacterial growth. The medium was autoclaved at 121ºC for 20 minutes. Each sample (1 g), and 50 ml of sterile distilled water were added to a conical flask. A mechanical shaker was used to shake the suspension for 30 minutes. 0.1 ml of the suspension was plated on agar media, thereafter, the plates were incubated at 30ºC for 48-72 hours. After incubation, different individual yeast colonies were picked up according to their morphological characteristics (colony shape, color, and cell shape) and microscopic examination. The selected yeast colonies were purified by single colony isolation after triple re-streaking on the YPD agar plates and were selected for further investigations.

3-Determination of killer activity

The killer activity was investigated by 2 different methods; firstly, using the antagonism method, then the results were confirmed using Methylene blue (MB) agar method.

In the antagonism method, yeast isolates were spread on laboratory YEPD solid culture media and were kept drying. Then the potential killer isolates were streaked above the sensitive isolate. The two different isolates were grown together at 30ºC for 48 h. A killer effect was recorded when the zone of inhibition around the tested isolates appeared on. The test was performed in triplicate.

The killing activity was confirmed using Methylene blue (MB) agar diffusion assay according to (Kurtzman, *et al.,* 2011) as the following:

On a Petri dish with 20 mL of YEPD agar medium buffered to pH 5.0 (0.1 M citric acid-phosphate) and containing 0.003% methylene blue, 1.0 mL of yeast isolate broth culture was plated. The plates were incubated for 48 h at 30ºC. A zone

of blue-colored zone of inhibition surrounded by the cells indicated killer activity (Qiu, *et al*., 2022).

4-Identification by gene sequencing

The strongest yeast killer isolate was identified molecularly using ITS gene sequencing as following: Firstly, DNA was isolated by CTAB Protocol (Saghai-Maroof *et al.,* 1984), by the centrifugation of 1 ml of overnight liquid culture for 10 minutes at 7,500 rpm to collect the cell. Then the pellet was resuspended in 0.2 ml of PBS buffer. 0.4 ml of CTAB extraction buffer and 40 µl of β-mercabtoethanol were added then the mixture was mixed gently. The tube was incubated for 30 minutes in a water bath at 60 ºC. Following cooling, an equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed. After centrifuging this mixture at maximum speed for 5-10 minutes, the aqueous supernatant was transferred to a fresh tube. Equal volume of cold, 100% ethanol were added, and the mixture was chilled for 30 minutes at -4°C, centrifuged for 5 min. at 1300 rpm to pellet the DNA. 70% ethanol was used for washing, and then the mixture was centrifuged for five minutes. After one hour of room temperature drying, the pellets were dissolved in a warm distilled H_2O.

The isolated DNA sample were sent to Gene Analysis Unit (Macrogene Inc., Seuol, Korea) for the sequencing of the ITS gene with Primers; ITS1 (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), which target the fungal internal transcribed spacer 1 (ITS1) region of the rRNA gene. The obtained gene sequence was compared to complete sequences found in the GenBank database to identify the isolated fungus using a BLAST search (NCBI). The phylogenetic tree based on the sequences of DNA was created using MEGA11 software.

5-Antagonism against some plant pathogenic bacteria

The plant pathogenic bacteria, *Erwinia carotovora, Pseudomonas aeruginosa* and *Streptomyces Scabies,* were used as indicator bacteria. 10 µL of yeast killer of overnight liquid culture was spotted onto a YEPD solid media with a bacterial cell layer of fresh pure cultures of pathogens. The plates were incubated at 30ºC for 24 h and the width of the inhibition zones was measured. The control was sterile water spotted on the pathogen-containing plates (Mannazzu, *et al* . 2019).

Results and Discussion

1-Sampling and Yeast isolation

In this study, 30 yeast isolates were collected from different natural sources in the Assiut regions, these isolates were morphologically characterized (Table 2).

Colony surface (rough or smooth), margin (undulating and entire), elevation (convex and flat), and color (red, cream, dark cream and white) of the 30 yeast isolates were detected and the results as shown in Table (2). The results showed different shapes and colors of colonies that were different from each other (Figure 1).

Table 2. Morphological charachterization of the different isolates and their sources.

No	Strain code	Isolation source	Location	Surface	Margin	Elevation	Colors
1	A	Juice	Assiut	Rough	Entire	Convex	White
2	A1	Fruit	Assiut	Rough	Entire	Convex	White
3	B	Soil	El Badary	Rough	Entire	Convex	White
4	B1	Soil	El Badary	Rough	Entire	Convex	White
5	C	Fruit	Bosra	Smooth	Entire	Convex	Dark cream
6	C1	Soil	Bosra	Smooth	Entire	Convex	Dark cream
7	D	Fruit	Bosra	Rough	Undulating	Convex	White
8	D1	Fruit	Bosra	Rough	Undulating	Convex	White
9	E	Fruit	Bosra	Smooth	Entire	Convex	Dark cream
10	E1	Juice	El Ghanaym	Rough	Undulating	Convex	White
11	F	Soil	El Ghanaym	Smooth	Entire	Convex	Dark cream
12	F1	Juice	El Ghanaym	Smooth	Entire	Convex	Dark cream
13	G	Juice	ELGhanaym	Rough	Undulating	Convex	White
14	G1	Soil	Assiut	Rough	Entire	Flat	White
15	H	Soil	Assiut	Rough	Entire	Flat	White
16	H1	Soil	Assiut	Smooth	Entire	Convex	Cream
17	I	Soil	Assiut	Smooth	Entire	Convex	Cream
18	I1	Soil	Assiut	Rough	Undulating	Convex	Dark cream
19	J	Soil	Assiut	Rough	Undulating	Convex	Dark cream
20	J1	Soil	Assiut	Rough	Undulating	Convex	Dark cream
21	K	Soil	Sahel Selim	Rough	Undulating	Convex	Dark cream
22	K1	Soil	Sahel Selim	Rough	Entire	Convex	Dark cream
23	L	Soil	Sahel Selim	Smooth	Entire	Convex	Cream
24	L1	Soil	Manfalut	Smooth	Entire	Convex	Bright red
25	M	Soil	Manfalut	Rough	Undulating	Convex	Cream
26	M1	Soil	Manfalut	Rough	Undulating	Convex	Cream
27	N	Soil	Manfalut	Rough	Entire	Convex	Dark cream
28	N1	Soil	Abnob	Rough	Entire	Convex	Dark cream
29	O	Soil	Abnob	Rough	Entire	Convex	Dark cream
30	O1	Soil	Abnob	Smooth	Entire	Convex	Bright red

According to the results in Table (2), the different yeast isolates were grouped in four classes, namely, white (10 isolates), dark cream (13 isolates), cream (5 isolates), and red (2 isolates), and the dominant one were - dark cream-colored colonies (43.3%).

Figure 1. Different morphological characteristics of some wild yeast isolates.

2-Killer activity for different isolates

The toxicity of the isolates was tested against each other. The toxicity was recorded after 48 hours. The results (Table 3) showed that, among the thirty yeast isolates collected from the different sources only five killer yeast isolates were founded (isolate no. 1, 9, 10, 16, and 21), while the other 25 isolates do not have killing activity. The killer isolates caused killing in varying proportions, so there are isolates that completely kill other sensitive yeast isolate and other killer yeast isolates cause partial killing.

Figure 2. Inhibitory effect of a yeast killer isolate against a sensitive yeast isolates.

Table 3. The toxicity of the isolates against each other

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
2	++																												
3	+	+																											
4	+	-	-																										
5	++	-	+	-																									
6	+	-	-	+	+																								
7	+	-	-	-	-	-																							
8	++	-	+	-	+	-	-																						
9	+	-	-	-	+	+	-	+																					
10	+	-	-	-	+	+	-	-	+																				
11	++	-	-	-	+	-	-	-	++	+																			
12	++	-	-	-	-	-	-	-	++	-	-																		
13	+	+	+	-	-	-	-	-	-	-	-	-																	
14	+	-	-	-	-	-	-	-	-	-	-	-	-																
15	++	-	-	-	-	-	-	-	-	-	+	-	-	-															
16	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-														
17	++	-	+	-	-	-	-	-	++	+	-	-	-	-	-	++													
18	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-												
19	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-											
20	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-										
21	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	+	-	-	-	-									
22	++	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	-	-	-	++								
23	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-							
24	++	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	++	-	-						
25	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	++	-	-	-	-	++	-	-	-					
26	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-				
27	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-			
28	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	++	-	-	-	-	-	-		
29	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	++	-	-	-	-	-	-	-	
30	++	-	-	+	+	-	-	-	++	+	-	-	-	-	-	++	-	-	-	-	++	-	-	-	-	-	-	-	-

Complete killing (++), Partial killing (+), and No killing activity (-).

From the previous table, we observed that the 5 killer isolates caused complete killing to some isolates, partial killing to other isolates, and no killing activity against some isolates. Therefore, we concluded that the killing activity would also depend on other factors, and an immunity system to the toxins would be present in some isolates.

The killing activities of the 5 killer isolates were confirmed using Methylene blue (MB) agar method (Figure 3).

The results showed that isolate no. 21 and no. 1 produced the highest activity of killer toxin. The other three killer yeast isolates caused killing, but not as the same degree as the other killer yeast strains.

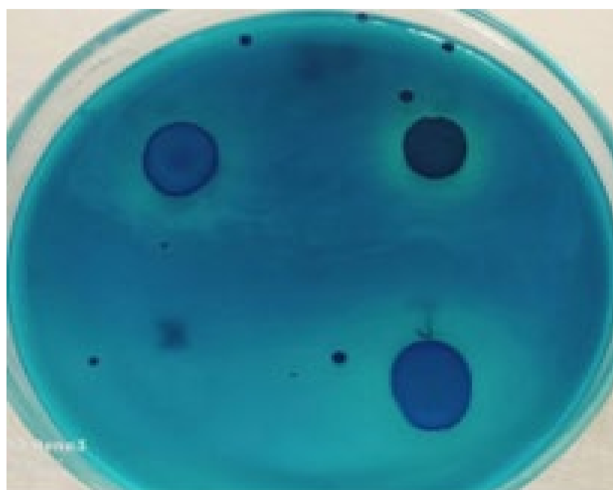

Figure 4. Toxicity yeast killer isolate confirmed by Metlylene blue (MB) agar method.

Identification of the best yeast killer isolate by ITS gene sequencing

The identification of killer isolate by gene sequencing was done for isolate no.9 which is one of the best killer yeast isolates. The sequencing of the isolate was done by the Gene Analysis Unit (Macrogene Inc., Seuol, Korea). The partial gene sequence of isolate no. 9 was in 99% similarity to *Meyerozyma carbibica* available in Gene bank database (Figure 4). *M. caribbica* is a yeast species that has shown potential in various biocontrol applications (Qiu *et al*., 2022), but there's not much information available about it being a producer of toxins.

From our results, *M. carbibica* could be considered as a killer yeast, having a broad range of antimicrobial activity against bacteria.

```
NR_149348.1 Meyerozyma caribbica CBS 9966 ITS region; from TYPE material
ength=642

Score = 1026 bits (555),  Expect = 0.0
Identities = 563/566 (99%), Gaps = 3/566 (1%)
Strand=Plus/Plus

Query  1    GTAGGTGAAACCTGCGGAAGGATCATTACAGTATTCTTTTGCCAGCGCTTAACTGCGCGG  60
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  74   GTAGGTG-AACCTGCGGAAGGATCATTACAGTATTCTTTTGCCAGCGCTTAACTGCGCGG  132

Query  61   CGAAAAACCTTACACACAGTGTCTTTTTGATACAGAACTCTTGCTTTGGTTTGGCCTAGA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  133  CGAAAAACCTTACACACAGTGTCTTTTTGATACAGAACTCTTGCTTTGGTTTGGCCTAGA  192

Query  121  GATAGGTTGGGCCAGAGGTTTAACAAAACACAATTTAATTATTTTTATTGATAGTCAAAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  193  GATAGGTTGGGCCAGAGGTTTAACAAAACACAATTTAATTATTTTTATTGATAGTCAAAT  252

Query  181  TTTGAATTAATCTTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  253  TTTGAATTAATCTTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAA  312

Query  241  CGCAGCGAAATGCGATAAGTAATATGAATTGCAGATTTTCGTGAATCATCGAATCTTTGA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  313  CGCAGCGAAATGCGATAAGTAATATGAATTGCAGATTTTCGTGAATCATCGAATCTTTGA  372

Query  301  ACGCACATTGCGCCCTCTGGTATTCCAGAGGGCATGCCTGTTTGAGCGTCATTTCTCTCT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  361  CAAACCCCCGGGTTTGGTATTGAGTGATACTCTTAGTCGAACTAGGCGTTTGCTTGAAAA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  421  GTATTGGCATGGGTAGTACTGGATAGTGCTGTCGACCTCTCAATGTATTAGGTTTATCCA  480
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  553  ACTCGTTGAATGGTGTGGCGGGATATTTCTGGTATTGTTGGCCCGGCCTTACAACAACCA  612

Query  541  AACAAGTTTGACCTCAAATCAGGTAG  566
            |||||| ||||||||||||||| |||
Sbjct  613  AACAAG-TTGACCTCAAATCAG-TAG  636
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Figure 4. Sequence alignment of the isolate no. 9 (Query) against the partial ITS gene sequence data of *M. carbibica* in GenBank.

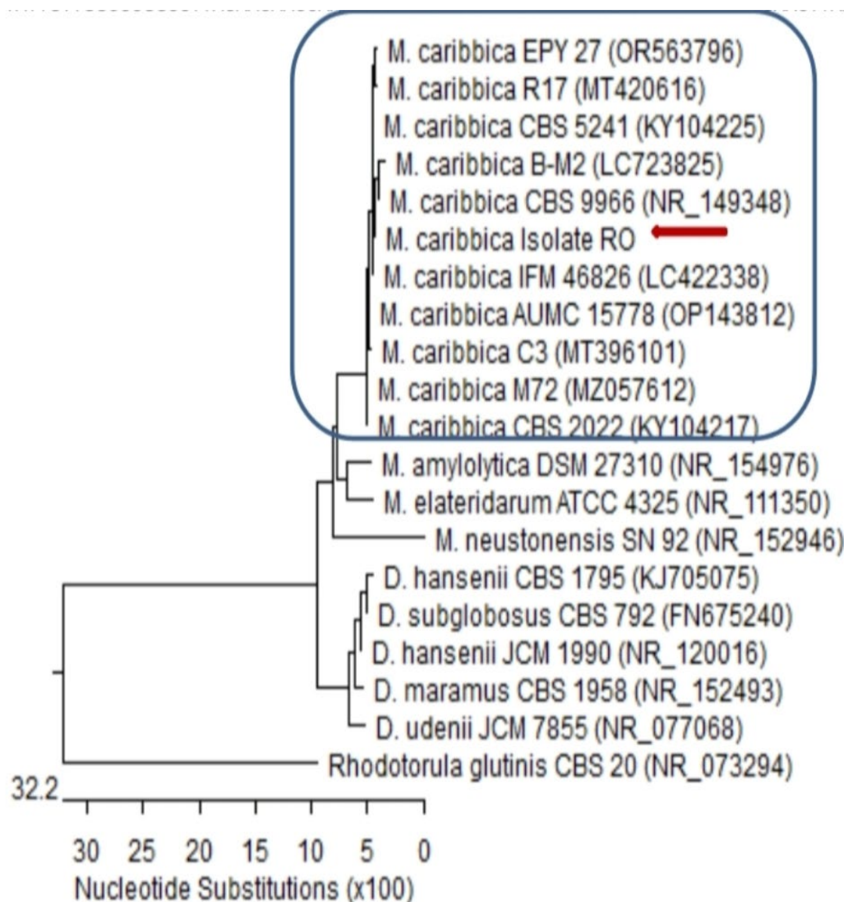

Figure 5. The phylogenetic tree based on ITS sequences of *M. caribbica* isolate no.9, arrowed, aligned with the ITS sequences obtained from the GenBank for closely related strains.

The Phylogenetic tree results (Figure 5) showed that isolate no.9 had 99.47% - 99.83% identity and 98% - 100% coverage with several strains of the same species including *M. caribbica* CBS 9966 with GenBank accession no. 149348.

4-Killer yeasts as biocontrol agents

The uses of yeasts for biological control against various pathogens relay on antagonistic action mechanisms. Killer yeasts have a great potential to act as biocontrol agents, since they produce toxins which do not harm humans or the fruit (Ocampo-Suarez *et al*., 2017) but can overcome different pathogenic bacteria. Three Killer isolates (isolate no. 9, isolate no. 1, and isolate no. 21) were screened for their potential applications as living biocontrol agents against some plant pathogenic bacteria such as *S. scabies, E. carotovora,* and *P. aeruginosa*

Isolate no. 9 and isolate no.1 were able to inhibit only the bacterial growth of both *S. scabies and E. carotovora*, but *P. aeruginosa* showed resistance to the toxins produced by these isolates. On the other hand, isolate no. 21 was able to inhibit the bacterial growth of all the 3 tested pathogenic bacteria including *P. aeruginosa* but with different levels (Figure 6).

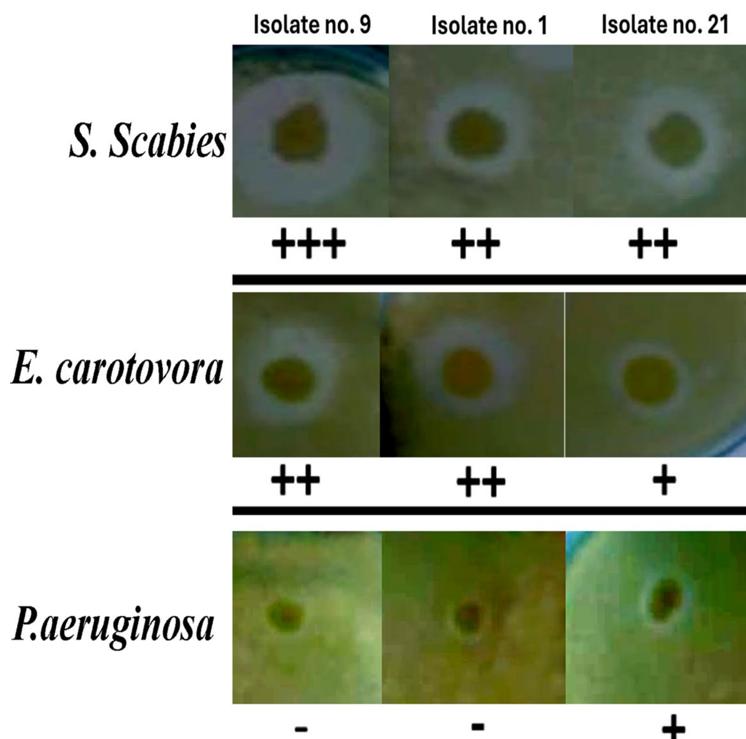

Figure 6. Screening of some yeast isolates as a bioagent against different pathogenic bacteria.

Conclusion

This study was conducted in the Microbial Genetics and Biotechnology Laboratories, Department of Genetics, Faculty of agriculture, Assiut University, Assiut, Egypt in the period from 2019 to 2024. The aim of the study was to obtain killer yeast isolates with high effectiveness against some other microbes to be used in biological control. 30 yeast isolates from 7 different locations: Assiut Town, Bosra, Sahel Selim, Elbadari, AlGhanium, Abnob, and Manfalout from three different sources (soil, juice, fruit) were isolated, characterized and screened for their killing activity. The results showed the presence of 5 yeast killer isolates. The molecular identification of the strongest isolate was done through the ITS gene sequence determination technique, which confirmed that isolate No. 9 is 99% similar to *Meyerozyma carbibica*. The antagonism between the isolated killer yeasts and some other plant pathogenic microbes showed the ability of the tested isolates to kill different pathogens. So, these isolates can be used to biologically control various plant pathogens.

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العزل والتوصيف الجزيئي لعزلات الخميرة القاتلة من المصادر الطبيعية

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الملخص

تم اجراء هذه الدراسة في معامل الوراثة الميكروبية والتكنولوجيا الحيوية بقسم الوراثة بكلية الزراعة جامعه اسيوط وكان الهدف من هذه الدراسة هو الحصول على عزلات خميرة قاتله ذات فعالية عالية للاستخدام في المكافحة الحيوية ضد الفطريات والبكتيريا المسببة للأمراض. وقد تم عزل 30 عزله من الخميرة من مواقع ومصادر مختلفة وتم توصيف هذه العزلات مورفولوجيا ومجهريا. كما تم الكشف عن عزلات خميرة ذات القدرة على القتل وذلك بطريقتين مختلفتين: باختبار التضاد بين العزلات وبعضها البعض وباستخدام صبغه الميثيلين الأزرق. أظهرت النتائج وجود 5 عزلات من الخميرة القاتلة. تم التعرف الجزيئي للعزلة الأقوى من خلال تقنية تحديد تتابعات جين الـ ITS والذي أكد ان العزلة رقم 9 متشابهة بنسبة 99% مع الخميرة *Meyerozyma carbibica*.

كما تم اختبار خاصيه التضاد بين الخمائر القاتلة المعزولة وبعض الميكروبات الأخرى المسببة للأمراض النباتية مثل:*P. aeruginosa* ، *E. caortovora*، *S. scabies*. واظهرت النتائج قدرة العزلات المختبرة على قتل المسببات المرضية المختلفة.

الكلمات المفتاحية: *خاصيه التضاد، الخمائر القاتلة، الميوكسين، Meyerozyma carbibica*