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Evaluation of the Native Killer Yeasts against the Postharvest

Phytopathogenic mould of Balady Orange Fruits

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Abstract:

Yeasts are some of the most important postharvest biocontrol agents (BCAs). Postharvest oranges frequently deteriorate due to green and blue moulds, leading to significant economic losses. The purposes of the present study were to isolate blue and green moulds from infected orange fruits, to assess the ability of killer yeasts isolated from healthy orange fruits and leaves from orange orchards to control blue and green moulds and to evaluate the additive effect of BCAs in combination with 2% sodium bicarbonate (SBC), 2%, sodium benzoate (SB), 2% calcium chloride, 0.2% salicylic acid (SA) or 0.5% chitosan. Among eight fungi isolated from orange fruits showing symptoms of green and blue mulds infection, two were identified as *P. digitatum* and *P. italicum* and selected for in vitro assays. Twenty eight yeast isolates were obtained from orange leaves and from the surface of fruits. All yeasts exhibited high killer activity. Twelve yeasts reduced 22.5–70% of *P. digitatum* growth while seven isolates reduced 21.1- 68.5% of *P. italicum* growth. The most potent yeast isolates were identified as *Candida pseudotropicalis*, *Candida salmanticensis*, *Candida membranifaciens* and *Pichia guilliermondii*. Combination of the BCAs, *C. pseudotropicalis*, *C. salmanticensis* and *P. guilliermondii* with SBC, CaCl₂ or chitosan increased their effectiveness against *P. digitatum*. While combination of *C. pseudotropicalis*, *C. membranifaciens* and *P. guilliermondii* with these natural compounds decreased their effectiveness against *P. italicum*. Combination of *C. membranifaciens* with SA increased its effectiveness against *P. digitatum*. Sodium benzoate has additive effect on *C. pseudotropicalis* against *P. digitatum* and *C. pseudotropicalis* and *P. guilliermondii* against *P. italicum*.

Keywords: Killer yeasts; Biocontrol agents; Phytopathogens; Orange fruits.

1. Introduction

Citrus is one of the most cultivated fruits in different countries of the world. The global citrus production was about 121 million tons, which represented 20.0% of the total fruit production

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in 2015 [1]. Citrus fruits play an important role in economic activity around the world. Citrus is also grown in various regions and can be grown in tropical and semi-tropical regions [2]. In Egypt, citrus is grown on an area of about 520,000 feddans, and it is the largest crop produced where orange represents about 65% of the total Egyptian citrus production [3].

Almost fresh fruit crops are perishable products with active metabolism and subject to extensive postharvest losses due to mechanical damage, physiological deterioration, water loss and microbiological decomposition that occur during harvesting, transportation and storage [4, 5]. Developing countries have the biggest crop losses in citrus accounts for 50% of the total production due to the lack in adequate crop protection measures [6].

About 20% of the harvested fruits undergo decay during the storage period before they reach the market for consumption [7]. Post-harvest diseases in long-term storage cause economic losses, which is one of the major problems of the world citrus industry [8, 9]. However, postharvest changes in fresh fruit cannot be stopped, but these can be slowed down within certain limits to increase its shelf-life [10].

Fungi are the causative agent of the most orange rot, due to the acidity of oranges, which is about 4-5 in healthy fruits [11]. The most common and serious diseases are caused by green and blue mould infection of *Penicillium digitatum* sacc, *Penicillium italicum* wehmer, respectively that affect citrus quality in Mediterranean climates [6, 12, 13].

The main pathogen present in citrus after harvesting, which causes 90% of the fruit loss, resulting in severe damage in commercial marketing is *Penicillium digitatum* [14]. Another common post-harvest disease of citrus fruits called blue mould rot is caused by *Penicillium italicum* [15].

Synthetic fungicides such as Thiabendazole (TBZ) and Imazalil (IMZ) are the most commonly used for control of post-harvest diseases in citrus because they are of low cost, easy to use and effective. However, resistant fungal strains arise at a high frequency, which reduces their effectiveness. Thus, their use has become highly restricted due to their high residual toxicity, carcinogenic effects, and environmental degradation [14]. Thus, with the increasing demand for methods with low environmental impact and lower risks to human health, biological control by using microbial agents is an alternative to synthetic fungicides as well as for post-harvest disease

management. Most biological control agents are isolated from the surfaces of fruits, giving them adaptive features, causing them to produce antifungals that have a better effect than synthetic fungicides [16, 17, 18, 19, 20].

Yeast has several properties, including the ability to survive in unfavorable environmental conditions, which makes it an ideal antagonist among microbial agents [17]. Killer yeast has the ability to produce toxins against susceptible yeasts, fungi and other filamentous bacteria [21, 22, 15].

The advantages of using killer yeasts as biocontrol agents are related to their adaptive traits, the ability to colonize and survive on fruit surfaces for long periods in diverse environmental conditions, the absence of the production of toxic substances, and the low cost-production of large amounts of yeast biomass. These advantages of killer yeast make it a better antagonist than other sources, because it is binding to a specific site, such as other yeasts, forming colonies in the wound that compete with the fungi for nutrients. They produce some enzymes and antimicrobial compounds, which can be soluble or volatile, and therefore they help in inhibiting the pathogen, by forming a biofilm on the inner surface of the wounds, which acts as a protective layer so that the fungus cannot develop the infection process [23].

Although microbial antagonists able to control postharvest diseases, application of antagonists alone is usually sufficient enough to achieve a consistently high level (> 95%) of disease control. Thus, the combination of biological control with chemical and physical control methods has been investigated and adopted in an integrated approach of postharvest disease management. This approach has the advantage of using the synergistic effects of each method and hence improves the overall performance and effectiveness of biocontrol agents [24, 25].

Antagonistic yeasts have still not been extensively applied and marketed, regardless of decades of research, [26]. up to date, only a few biocontrol yeasts (Nexy and Shemer) are commercially available for preventing postharvest green mould of citrus [27, 28]. Therefore, the search for new yeast antagonists with promising applications prospect is still occurring all over the world.

The objectives of this work were to isolate and identify native killer phenotype yeasts from the surface of leaves and orange fruits, and to evaluate their effectiveness against postharvest

phytopathogenic moulds of orange fruits, *penicillium digitatum* and *penicillium italicum* individually and/or in combination with natural compounds.

2. Materials and Methods

2.1. Plant material

The present study was carried out during season 2019, on Balady orange fruit (*Citrus sinensis* L.). Healthy fruits harvested at typical commercial level of maturity were collected from seven orange orchards in Qalyub, Shibin El Qanater, Tikh, Khanka, Agricultural Research Center, Sinnuris and Inshas in four Egyptian Governorates, Al Qalyubia, Giza, Al Fayoum and Al Sharqia. Fruits were washed with tap water and air dried, sorted to remove the mechanically injured and defected fruits. Fruits were used immediately after harvest, or held at 21 °C, 92–94% relative humidity for no longer than 2 days before use [9].

2.2. Isolation and identification of pathogenic fungi

Phytopathogenic moulds were isolated from infected orange fruits showing symptoms of green and blue moulds as described by [29] and cultured (Spores were suspended by adding a loopful from each surface infected orange fruit to 10 ml of sterile distilled water) on potato-dextrose agar plates (g/l: 200 potato extract, 20 dextrose and 20 agar, pH 5.6± 0.2) in triplicates and incubated for 7 days at 25°C and maintained at 4 °C. Fungal isolates were identified at the Regional Centre for Mycology and Biotechnology (Rcmb), Al Azhar University (Cairo / Egypt) [30, 31, 32].

2.3. Isolation of biocontrol agents

The yeast biological control agents were isolated from the surface of healthy orange fruits and leaves as described by [17]. Leaves or fruits' samples were suspended in 100 ml sterile saline (0.89% NaCl) and shaken vigorously for 30 min then, serial dilutions were made. An aliquot of 0.1 ml of each dilution was spread onto Yeast Extract Peptone Dextrose agar [33] (YEPD) plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar, pH 4.7) supplemented with 200 mg chloramphenicol L⁻¹ in triplicate and incubated at 28 °C for 48h. Each isolate was purified by subsequent streaking on the same medium with chloramphenicol.

Pure yeast cultures were preserved at – 80 °C in cryovials containing 1 ml of universal broth (1% glucose, 0.5 % peptone, 0.3% yeast extract, 0.3% malt extract, pH6) plus 20 % glycerol.

2.4. Assessment of killer yeasts activity

Killer activity was tested using the streak agar diffusion assay method [34]. Cells of sensitive yeast (*Saccharomyces cerevisiae*) were grown at 25 °C for 24 h on YEPD agar and suspended in sterile saline solution (0.85% NaCl) to obtain about 3×10^6 cells/ml. One millilitre of the suspension was thoroughly mixed with 20 ml of molten buffered YEPD medium adjusted to pH 4.5 with citrate-phosphate buffer and supplemented with 0.03% methylene blue and poured into a sterile Petri dish. A loopful of each yeast isolate was streaked on the cells of the sensitive yeast, and the plates were incubated at 25 °C for 48h. The production of killer factor and the death of sensitive cells were indicated by the presence of a growth inhibition zone and an adjacent blue zone.

2.5. *In vitro* efficacy of the killer yeasts in controlling phytopathogenic moulds

Agar well diffusion assay adapted from [35, 36, 37] with the aim to evaluate the antagonistic capacity of all of the isolated killer yeasts against the examined phytopathogenic moulds. Yeast suspensions were prepared by inoculating 25ml of YEPD in Erlenmeyer flasks with a loopful of cells and incubated at 25°C for 24h. The optical density of yeast cultures was adjusted to 0.3 at 620 nm Spectronic-21 with sterile distilled water. Mould spore suspensions were prepared from 5-day old colonies on potato dextrose agar (PDA) adjusted to 10^5 spores ml⁻¹ in sterile distilled water by a haemocytometer.

One ml of each mould suspension was added to 10 ml PDA around 45 °C and poured into a sterile Petri dish. After solidification the medium was perforated, leaving a 5 mm diameter well in the centre of the plate. Then, 100 µl of each yeast suspension was added into the well. A plate containing only the fungus was used as a control. All of the plates were incubated at 25 °C for 5 days. Finally, radial growth reduction was calculated in relation to growth of the control as follows:

$\%I = (C - T / C) \times 100$, where:

%I: The inhibition of radial mycelia growth,

C: Radial growth measurement in control and

T: Radial growth of the pathogen in the presence of yeast isolates. All treatments were applied in duplicates.

2.6. Identification of killer yeast isolates

The most potent killer yeast isolates were initially identified based on morphological characters and biochemical tests according to the manuals of [38, 39, 40] in addition to Integral system plus. Identification of the selected yeasts was confirmed by PCR-RFLP method, by amplifying and subsequent restriction digestion analysis of internal transcribed spacer region (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA) [41, 42]. Yeast isolates were grown for 48 h in YEPD broth at 25 °C and DNA extraction was performed using ABT DNA mini extraction kit (Applied Biotechnology Co. Ltd, Egypt). The contiguous ITS1-5.8S rDNA-ITS2 region was amplified with primers set ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The amplified PCR products were submitted to Solgent Co Ltd (South Korea) for DNA purification and sequencing. The resulted sequences were trimmed and assembled in Genius software (Biomatters). Consequently, the trimmed sequences were aligned and compared with the Gene Bank database (<http://www.ncbi.nlm.nih.gov/BLAST>) for molecular identification.

2.7. Enhancement of the biocontrol agents efficacy

The combination of biological control with various natural compounds was performed. Sodium bicarbonate, sodium benzoate, calcium chloride, salicylic acid and chitosan were used as an effective additive to improve the biocontrol performance of the antagonistic most potent yeast isolates. The effect of the potent yeast isolates combined with 2% sodium bicarbonate [43, 44], 2% Sodium benzoate [45,46], 2% CaCl₂ ([47, 48], 0.2% salicylic acid [49] or 0.5% Chitosan [50] on the growth of phytopathogenic moulds was examined, with some modifications. A hole of 5mm diameter was created in PDA plates seeded (107 spores/ 10 ml) with either blue or green moulds. To each hole of PDA plates, 100 µL of the following were added of the following: **Y:** killer yeast suspension (1×10⁸ cells/ml) alone, **Y+CA:** killer yeast suspension (1×10⁸ cells/ml) with the chemical additive, or **CA:** the chemical additive alone. Antifungal activity was monitored by determination of reduction % of fungal growth after 5 days of incubation at 25°C. All assays were carried out in triplicate.

2.8. Statistical analysis

All statistical analyses were performed using SPSS version 13.0. Data with a single variable (treatment) were analysed by one-way ANOVA, and mean separations were performed by Duncan's multiple range tests. Differences at $P < 0.05$ were considered significant. Data presented in this article were pooled across three independent repeated experiments [51].

3. Results

3.1. Isolation and identification of fungal isolates

Eight fungal isolates were obtained from orange fruits showing symptoms of green and blue moulds. Two fungal isolates were selected for *in vitro* assays. One of them was isolated from infected orange fruit showing symptoms of green mould from an orange orchard localized in Shibin El Qanater in Al Qalyubia governorate, Egypt while, the other one was isolated from infected orange fruit showing symptoms of blue mould from an orange orchard localized in Qalyub in Al Qalyubia governorate, Egypt. The isolates were identified as *Penicillium digitatum* (the causative agent of green mould) and *Penicillium italicum* (the causative agent of blue mould).

3.2. Isolation of yeasts

Twenty eight yeast isolates were obtained, of which 16 (57.2%) were isolated from orange leaves and 12 (42.8 %) from the surface of fruits. In vitro screening of the isolated yeast resulted in the selection of 4 isolates namely, No.3, 9, 11 and 12 as the most antagonistic ones against the two examined phytopathogens, *P. digitatum* and *P. italicum* which were isolated from orange leaves. Isolates No 3,9,11 were obtained from an orange orchard localized in Shibin El Qanater in Al Qalyubia governorate, Egypt while isolate No. 12 was obtained from an orange orchard localized in Qalyub, another location in the same governorate.

3.3. Assessment of killer yeast activity

All the twenty eight yeast isolates exhibited high killer activity against *S. cerevisiae* (sensitive strain) as they produced a blue inhibition ring or zone.

3.4. Efficacy of the killer yeasts in controlling *Penicillium digitatum* and *Penicillium italicum*.

Twelve out of the examined 28 isolates (42.9%), were able to inhibit mycelial growth of *P. digitatum* ranging from 22.5 -70% of reduction and seven (25%) out of the twenty eight yeast

isolates were able to inhibit mycelial growth of *P. italicum* ranging from 21.1- 68.5% of reduction. Among the yeast isolates, No. 3 and 12 exhibited antagonistic activity against *P. digitatum* and *P. italicum*. Isolate 3 reduced 41.1 and 62.2% of mycelial growth of *P. italicum* and *P. digitatum* respectively while isolate No. 12 reduced 70 and 39% of mycelial growth of *P. digitatum* and *P. italicum* respectively. Isolate No. 9 was the most potent reduced 68.5% of the mycelial growth of *P. italicum* and isolate No.11 reduced 54.5% of mycelial growth of *P. digitatum*. Thus, four yeast isolates were selected for further investigation (data not shown).

3.5. Identification of yeast isolates

Molecular identification results for the isolated strains are shown in **Table (1)** and **Fig. 1**. The most potent yeast isolates (3, 9, 11 and 12) for the control of orange blue and green moulds were identified as *Candida pseudotropicalis* (isolate 3), *Candida salmanticensis* (isolate 9), *Candida membranifaciens* (isolate 11) and *Pichia guilliermondii* (isolate 12).

Table (1): Identification of the yeast isolates based on partial sequencing of internally transcribed spacer (ITS) regions and 5.8S ribosomal DNA (rDNA)

Isolate no.	sequencing	ID %	Species
3	TCAAAACAAAAATAATCAAAACTTTTAAACAATGGATCTCTTGGTTCTCGTA TCGATGAAGAACGCGAGCGAAACGCGATATTTCTTGTAATTGCAGAAGT AATCATCAGTTTTTGAACGCACATTGCACCTTTGGGGTATCCCCCAAAGTAT ACTTGTTTGAGCGTTGTTTCTCTCTTGGAAATGCATTGCTTTTCTAAAAATTT CGAATCAAATTCGTTTGA AAAACAACACTATTCAACCTCAGATCAAGTAGG ATTACCCGCTGAACCTTAAGCATATCAATAAGCGGA	100	<i>Candida pseudotropicalis</i>
9	TCAAAACAAAAATAATCAAAACTTTTAAACAATGGATCTCTTGGTTCTCGT ATCGATGAAGAACGCGAGCGAAACGCGATATTTCTTGTAATTGCAGAAGT GAATCATCAGTTTTTGAACGCACATTGCACCTTTGGGGTATCCCCCAAAGT ATACTTGTTTGAGCGTTGTTTCTCTCTTGGAAATGCATTGCTTTTCTAAAA TTTCGAATCAAATTCGTTTGA AAAACAACACTATTCAACCTCAGATCAAG TAGGATTACCCGCTGAACCTTAAGCATATCAATAAGCGGA	98	<i>Candida salmanticensis</i>
11	TCAAAACAAAAATAATCAAAACTTTTAAACAATGGATCTCTTGGTTCTCGT ATCGATGAAGAACGCGAGCGAAACGCGATATTTCTTGTAATTGCAGAAG TGAATCATCAGTTTTTGAACGCACATTGCACCTTTGGGGTATCCCCCAA GTATACTTGTTTGAGCGTTGTTTCTCTCTTGGAAATGCATTGCTTTTCTA AAATTCGAATCAAATTCGTTTGA AAAACAACACTATTCAACCTCAGATC AAGTAGGATTACCCGCTGAACCTTAAGCATATCAATAAGCGGAGGA	97	<i>Candida membranifaciens</i>
12	GCAAGAGCTCAGATTGAAATCTCACCTTCGTTGGGGCCAAATTTGTAAT TGGAAGTTGGAATCCCGGTTTCTACCTGTGGTCCATTTCCTGGAACCA GGCCCCCCCCGAAGGTGAAAACCCCGTGTGAGCACAATTCCCCACCTA GGGCCTTCCGACCAGTCCAGTTGTTTGGGAATGCAACTCTTATTGGGTG GTAAATTCATTCCATGGTAAATTTTGGCCAAAGAAGCAACCCAAACCA ATACCGTGAAAGAAAGAAGAAAGCACTTTGAAAAGAAAATGAAACGAC AAGTGGAATTGTTGAAAGGGAAGGGTATTGGGCTCGACCTGGGATTTTA TTTTCGTTGCCTCTCGGGGGCGGCGCTTTGGGTTTTTCTGGGCCCCC CCGTTTTTGTGGGTGAGAAAGACCTTGGGAATGTGGCTCCTCCGGAG	95	<i>Pichia guilliermondii</i>

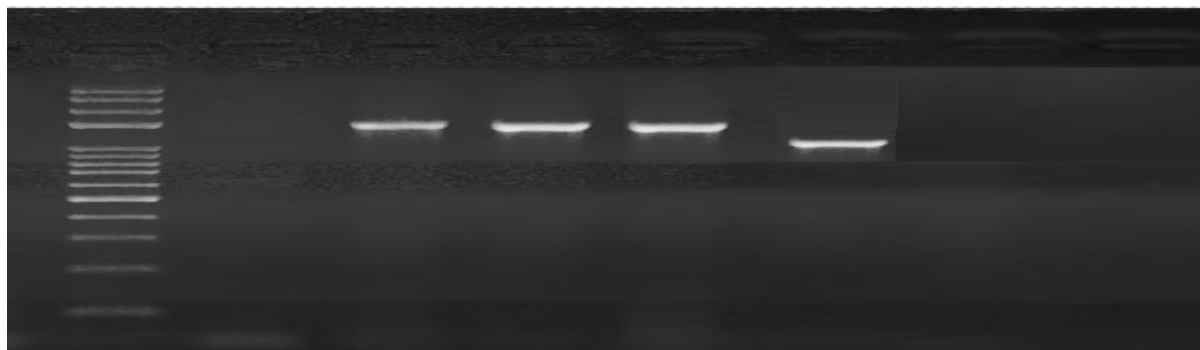


Fig (1): Molecular basis of identification of yeast isolates. PCR amplification of internal transcribed spacer sequences (ITS DNA) used as a molecular marker of yeast identity.

3.6. Enhancement of biocontrol efficacy

The obtained results (**Table 2**) showed that the antifungal activity of the killer yeast strains *Pichia guilliermondii* and *Candida pseudotropicalis* are more effective against *P. digitatum* that was enhanced in combination with 2 % sodium bicarbonate (88.1 and 83.1% reduction of fungal growth respectively) in comparison with the antagonists alone (72% and 65.4 reduction of fungal growth respectively) or with sodium bicarbonate alone (62.7% reduction of fungal growth). However, the antifungal activity of the killer yeast strain *Candia salmanticensis* was enhanced in combination with 2 % sodium bicarbonate (90.6% reduction of fungal growth) in comparison with the antagonist alone (68.7% reduction of fungal growth) or with 2 % sodium bicarbonate alone (66.9% reduction of fungal growth) (**Table 2**).

Table (2): Efficacy of the antagonistic killer yeast strains in combination with 2% sodium bicarbonate (SBC) in controlling *Penicillium digitatum* and *P. italicum* *in vitro*

Treatment	% growth reduction	
	<i>P. digitatum</i>	<i>P. italicum</i> *
<i>Candida pseudotropicalis</i>	65.4 ^e	76.5 ^b
<i>Candida membranifaciens</i>	69.6 ^d	–
<i>Pichia guilliermondii</i>	72 ^c	74.3 ^d
<i>Candida salmanticensis</i>	–	68.7 ^e
SBC	62.7 ^g	66.9 ^f
SBC+ <i>Candida pseudotropicalis</i>	83.1 ^b	76.1 ^c
SBC+ <i>Candida membranifaciens</i>	66.5 ^f	–
SBC+ <i>Pichia guilliermondii</i>	88.1 ^a	58.3 ^g
SBC+ <i>Candida salmanticensis</i>	–	90.6 ^a
f value = 38363.089***		
*f value = 25278.067***		

The obtained results in **Table (3)** showed that in exception to *C. pseudotropicalis* which showed high antifungal activity against *P. digitatum* in combination with 2 % sodium benzoate (73% reduction of fungal growth) in comparison with the antagonist alone (65.4 % reduction of fungal growth) or with sodium benzoate alone (26 % reduction of fungal growth). On the other hand, the antifungal activity of both of the killer yeast strains *P. guilliermondii* and *C. pseudotropicalis* against *P. italicum* was enhanced in combination with 2 % sodium benzoate (86.8 and 86.6% reduction of fungal growth respectively) in comparison with the antagonists alone (74.3 and 76.5% reduction of fungal growth respectively) or with sodium benzoate alone (28.9% reduction of fungal growth).

Table (3): Efficacy of the antagonistic killer yeast strains in combination with 2% sodium benzoate (SB) in controlling *Penicillium digitatum* and *P. italicum* *in vitro*

Treatment	% growth reduction	
	<i>P. digitatum</i>	<i>P. italicum</i> *
<i>Candida pseudotropicalis</i>	65.4 ^d	76.5 ^c
<i>Candida membranifaciens</i>	69.6 ^c	-
<i>Pichia guilliermondii</i>	72 ^a	74.3 ^d
<i>Candida salmanticensis</i>	-	68.7 ^e
SB	26 ^g	28.9 ^g
SB+ <i>Candida pseudotropicalis</i>	73 ^b	86.6 ^a
SB+ <i>Candida membranifaciens</i>	53.7 ^f	-
SB+ <i>Pichia guilliermondii</i>	58 ^e	86.8 ^b
SB+ <i>Candida salmanticensis</i>	-	60.2 ^f
f value = 81728.159***		
*f value = 109452.986***		

The obtained results (**Table 4**) showed that the antifungal activity of the killer yeast strains *C. pseudotropicalis* and *P. guilliermondii* against *P. digitatum* was enhanced in combination with 2 % calcium chloride (83.1 and 74.8% reduction of fungal growth respectively) in comparison with antagonists alone (65.4 and 72% reduction of fungal growth respectively) or with 2 % calcium chloride alone (52.7% reduction of fungal growth). However, the antifungal activity of the antagonistic yeast strains *C. pseudotropicalis*, *P. guilliermondii* and *C. salmanticensis* against *P. italicum* was reduced in combination with 2 % calcium chloride (74.8, 73 and 47.6% reduction of fungal growth respectively) in comparison with the antagonists alone (76.5, 74.3 and 68.7% reduction of fungal growth respectively) but it was better than with 2 % calcium chloride alone (59.5% reduction of fungal growth) (**Table 4**).

Table (4): Efficacy of the antagonistic killer yeast strains in combination with 2% calcium chloride in controlling *Penicillium digitatum* and *P. italicum* *in vitro*

Treatment	% growth reduction	
	<i>P. digitatum</i>	<i>P. italicum</i> *
<i>Candida pseudotropicalis</i>	65.4 ^e	76.5 ^a
<i>Candida membranifaciens</i>	69.6 ^d	–
<i>Pichia guilliermondii</i>	72 ^c	74.3 ^b
<i>Candida salmanticensis</i>	–	68.7 ^e
CaCl ₂	52.7 ^g	59.5 ^f
CaCl ₂ + <i>Candida pseudotropicalis</i>	83.1 ^a	74.8 ^c
CaCl ₂ + <i>Candida membranifaciens</i>	67 ^f	–
CaCl ₂ + <i>Pichia guilliermondii</i>	74.8 ^b	73 ^d
CaCl ₂ + <i>Candida salmanticensis</i>	–	47.6 ^g
f value = 27381.367***		
*f value = 26065.901***		

Candida membranifaciens showed much better antifungal activity against *P. digitatum* in combination with 0.2 mM salicylic acid (73.6% reduction of fungal growth) in comparison with the antagonist alone (69.6 % reduction of fungal growth) or with 0.2 mM salicylic acid alone (19% reduction of fungal growth) (**Table 5**). However, the antifungal activity of the tested killer yeast strains, *C. pseudotropicalis* , *P. guilliermondii* and *C. salmanticensis* against *P. italicum* (54.8 , 39.3 and 48.2 % reduction of fungal growth respectively) was reduced in combination with 0.2 mM salicylic acid in comparison with the antagonists alone but it was better than with 0.2 mM salicylic acid alone (21.1% reduction of fungal growth).

Table (5): Efficacy of the antagonistic killer yeast strains in combination with 0.2mM of salicylic acid (SA) in controlling *Penicillium digitatum* and *P. italicum* *in vitro*

Treatment	% growth reduction	
	<i>P. digitatum</i>	<i>P. italicum</i> *
<i>Candida pseudotropicalis</i>	65.4 ^d	76.5 ^a
<i>Candida membranifaciens</i>	69.6 ^c	–
<i>Pichia guilliermondii</i>	72 ^b	74.3 ^b
<i>Candida salmanticensis</i>	–	68.7 ^c
SA	19 ^g	21.1 ^g
SA+ <i>Candida pseudotropicalis</i>	63.3 ^e	54.8 ^d
SA+ <i>Candida membranifaciens</i>	73.6 ^a	–
SA+ <i>Pichia guilliermondii</i>	48.3 ^f	39.3 ^f
SA+ <i>Candida salmanticensis</i>	–	48.2 ^e
f value = 148990.313***		
*f value = 117358.5***		

Candida pseudotropicalis and *P. guilliermondii* showed much better antifungal activity against *P. digitatum* in combination with 0.5 % (w/v) chitosan (80.3 and 87% reduction of fungal growth respectively) in comparison with the antagonists alone (65.4 and 72 % reduction of fungal growth respectively) or with chitosan alone (28 % reduction of fungal growth). While the antifungal activity of the tested killer yeast strains against *P. italicum* was reduced in combination with 0.5 % (w/v) chitosan (66 and 46.6 % reduction of fungal growth respectively) in comparison with the antagonists alone (76.5 and 74.3 % reduction of fungal growth respectively) but it was better than with chitosan alone (34.7 % reduction of fungal growth).

It was noticed that both *P. guilliermondii* and *C. pseudotropicalis* were effective against both *P. digitatum* and *P. italicum* whether they were alone or combined with various natural compounds such as (sodium bicarbonate, sodium benzoate, calcium chloride, salicylic acid and chitosan).

In general and interestingly, *P. guilliermondii* and *C. pseudotropicalis* are very good antagonists when combined with natural compounds or alone.

Table (6): Efficacy of the antagonistic killer yeast strains in combination with 0.5% (w/v) of chitosan in controlling *Penicillium digitatum* *in vitro*

Treatment	% growth reduction	
	<i>P. digitatum</i>	<i>P. italicum</i> *
<i>Candida pseudotropicalis</i>	65.4 ^e	76.5 ^a
<i>Candida membranifaciens</i>	69.6 ^d	–
<i>Pichia guilliermondii</i>	72 ^c	74.3 ^b
<i>Candida salmanticensis</i>	–	68.7 ^c
Chitosan	28 ^g	34.7 ^g
Chitosan+ <i>Candida pseudotropicalis</i>	80.3 ^b	66 ^d
Chitosan+ <i>Candida membranifaciens</i>	60.4 ^f	–
Chitosan+ <i>Pichia guilliermondii</i>	87 ^a	46.6 ^f
Chitosan+ <i>Candida salmanticensis</i>	–	56.4 ^e
f value = 78571.851***		
*f value = 57344.167***		

4. Discussion

Postharvest diseases are among the most dangerous diseases of different fruits. Infection with these diseases sometimes occurs in the field during or before harvest, but development usually occurs during the post-harvest phase. Phytopathogenic fungi often produce toxins that are toxic to the host and other organisms living in the same environment [24]. In this study, eight fungal isolates were isolated from infected orange fruits that showed symptoms of green or blue rot. Two isolates were confirmed as *Penicillium digitatum* and *Penicillium italicum*. A previous report [52] referred to the sensitivity of oranges to infection by *P. digitatum* and *P. italicum*. These two fungi are the most common post-harvest pathogens responsible for heavy economic losses in

citrus crops [53]. The sensitivity of oranges to fungal decay was attributed to the high level of nutrients and sugars as well as low pH values [54].

Twenty eight yeast isolates were isolated from seven habitats of seven different orange orchards, of which 16 isolates (57.2%) were from orange leaves and 12 isolates (42.8%) from the surface of healthy fruits. These results are consistent with a previous study by [55] who reported that microorganisms naturally present on the fruit surface (such as yeast) account for most of the biological control agents used to control post-harvest disease. Yeasts in fruits and vegetables have been targeted as potential biological control agents for post-harvest diseases, due to their properties that enable them to improve their ability to colonize fruit surfaces. [27].

In addition, it has been generally found that fruits and vegetables provide a favorable environment for yeasts, especially killer yeast as about a quarter of yeast isolated from this source display a phenotype [56]. In a previous study, [57] reported that killer yeasts were recommended as biological control agents being a promising alternative to chemical fungicides due to their ability to control fungal diseases before and after harvesting and their low environmental impact. In the current study, all 28 yeast isolates showed high lethal activity against *S. cerevisiae*. This finding is supported by the study of [58] who showed that yeasts can produce so-called killer toxins, which are proteins or glycoproteins, which are toxic agents and can lead to the death of sensitive yeast isolates. More than 100 yeast species belonging to 20 genera were shown to have killing potential [59].

The protective efficacy of using killer yeasts and their toxins in the management of several economically harmful plant pathogens has been demonstrated in a variety of fruits, such as citrus, grapes, papaya, essential fruits, tomatoes and apples during pre- and postharvest [56]. According to a previous report [54], effective bio-agents are those isolated from the same site of their application. This might support the findings of the present study regarding the antifungal activity of yeasts isolated from healthy orange fruits and leaves against *P. digitatum* and *P. italicum* isolated from infected orange fruits. Some yeast has the ability to produce killer toxins and other antimicrobial agents that are lethal to filamentous fungi [60]. Fungal phytopathogen sensitivity to killer yeasts was first demonstrated in 1995[61]. Similar results were obtained with the killer yeast *Zygosaccharomyces bailii* in controlling *Fusarium oxysporum* and added that *Pichia*

membranifaciens inhibited *Botrytis cinerea* growth by killer toxin. [62]. Moreover, *P. expansum* infections could be controlled using killer yeasts *Pichia ohmeri* and *Candida guilliermondii* [63, 64]. According to [65] using killer yeasts as biological control agents of fungi causing postharvest diseases of fruits are being studied more recently. In a previous study, [9] isolated 437 native yeasts from fruits and leaves of citrus plants and from washing water of lemon peels, to study the killer activity against pathogens of citrus. Six yeast genera including *Pichia*, *Saccharomyces*, *Kazakhstania*, *Wickerhamomyces*, *Clavispora*, and *Candida* were identified in this study. Regarding the antifungal activity, 11 *P. italicum* strains showed growth inhibition of $\geq 40\%$, 18 strains were inhibited between 16 and 39%; and the remaining 8 strains showed $\leq 15\%$ inhibition. *S. cerevisiae* (137) and *Kazakhstania* (120) had protective characteristics against *P. italicum*. In addition, [66] isolated more than 400 yeasts from citrus plants, 8.5% of them exhibited killer activity. Two strains of *Pichia* sp., and one strain of *Wickerhamomyces* sp. reduced 93.6%, 82.5%, and 72.5% of *P. digitatum* growth respectively in *in vivo* assays. The present results are supported by the studies of [6, 67, 18] they stated that *Pichia guilliermondii* and *Pichia anomala* are usually used against postharvest green mould of citrus fruit and that *Pichia guilliermondii* has been widely demonstrated as an efficient biological control agent of pathogenic fungi, as blue and green moulds of citrus fruits.

It has been reported that the efficacy of most biocontrol agents is enhanced in combination with several natural compounds, either organic or inorganic salts as this provide wide spectrum, persistence and increase yeast concentration against pathogenic fungi [68]. In the present study the development of an integrated control strategy was carried out with an efficacy comparable to biocontrol agents by combining yeast strains with 2% sodium bicarbonate (SBC) salt to control the phytopathogenic fungi, *P. digitatum* and *P. italicum* *in vitro*. Presence of SBC in the cell suspension of the antagonists, *C. pseudotropicalis* or *P. guilliermondii* increased their effectiveness against *P. digitatum*. However, it did not increase their effectiveness against *P. italicum*. Combination of SBC with *C. salmanticensis* increased their effectiveness against *P. italicum*. Sodium carbonate, sodium bicarbonate, and ethanol are generally recognized as safe substances that reduced *P. digitatum* conidial germination [69]. In many studies, combining *Pantoea agglomerans* and a salt was very efficient in disease control [70]. [71] demonstrated that

bicarbonate salts have broad spectrum antimicrobial activity, and that they are effective against pathogens causing postharvest diseases. In addition, [72] reported that bicarbonate salts may inhibit pathogenic fungi causing postharvest diseases via reduction of fungal cell turgor pressure which leads to collapse and shrinkage of hyphae and spores, resulting in fungus inability to sporulate. [73, 74] revealed that applying 2% solution of SBC with the antagonist *K. marxianus* against *P. digitatum* on citrus fruit was markedly enhanced the antagonist's antifungal activity. However, [73] reported that sodium bicarbonate showed inhibitory effect on both pathogens and biocontrol agents.

In the current study, combination of a 2% solution of sodium benzoate (SB) with the yeast antagonist *C. pseudotropicalis* enhanced biocontrol of *P. digitatum*. On contrary, combination of SB with *C. membranifaciens* or *P. guilliermondii* did not increase their effectiveness against *P. digitatum*. Otherwise, applying SB with *C. pseudotropicalis*, *C. membranifaciens* or *P. guilliermondii* increased their effectiveness against *P. italicum*. However, combination of SBC with *C. salmanticensis* did not increase its effectiveness against *P. italicum*. The antimicrobial properties of sodium benzoate has been widely reported particularly, its efficacy as antifungal postharvest treatment on citrus, stone fruits or longan fruit [45].

The efficiency of the antagonist can be improved by either physical treatment or in combination with natural compounds as calcium salts [75]. The results of the current study revealed that CaCl_2 has an activity against both *P. digitatum* and *P. italicum* and improves biocontrol activity of *C. pseudotropicalis* and *P. guilliermondii* against *P. digitatum*. Earlier findings revealed that applying calcium salts with yeast antagonists' suspensions led to better control of *Penicellium expansum* and *Botrytis cinerea* on apple. In addition, it was assumed that applying calcium salts with the yeast antagonists increased their efficiency due to the osmotolerant nature of yeast. However, the addition of salt solutions with the lowest osmotic potential to apple wounds alone did protect the wounds against *Penicellium expansum* or *Botrytis cinerea* infections [76].

In addition, [69] showed that combination of the biological control agent *Candida sp.* with 2% solution of calcium chloride improved biocontrol of blue and gray moulds on apples, while calcium chloride alone failed to reduce decays. However, combining the antagonist *P.*

guilliermondi (US-7) with 68 mM CaCl_2 reduced the incidence of green mould decay of grapefruit by 97% while application of calcium chloride alone reduced decay by 43%. Previous studies reported that addition of CaCl_2 to *C. oleophila*, *C. guilliermondii*, *Cryptococcus laurentii* and *Pichia sp.* enhanced their biocontrol activities [77, 78, 79].

[80] studied the effect of Ca^{++} on the biological control activity of two *C. oleophila* strains against *Penicillium expansum* and found marked increase in their inhibitory activity. Reduction of the pectinolytic activity and inhibition of spore germination of *P. expansum* caused by calcium ions are the reasons that explain biocontrol improvement of this yeast.

[81, 49] reported that research proved that salicylic acid (SA) as a natural phenolic compound involved in transduction pathway, as a plant growth regulator and provides resistance to pathogenic microbes when applied at a non-hazardous concentration. The present study revealed that excluding *C. membranifaciens* the antifungal activity of the rest of antagonists did not enhance in combination with 0.2 mM salicylic acid. These results are in disagreement with research findings of [80]. However, it was found that SA showed little inhibition effect on *P. italicum* and *P. digitatum in vitro*, which was in accordance with the results of [49].

[82, 50] reported that application of natural polymers (as chitosan and its derivatives) with yeast could effectively improve their biocontrol activity. [83] revealed that chitosan inhibited fungal spores germination. However, [50] showed that high concentrations of chitosan are probably restraint for yeast growth. The obtained results in this study demonstrate that chitosan improved antifungal activity of *C. pseudotropicalis* and *P. guilliermondii* against *P. digitatum*. However, chitosan has no additive effect on the biological control agents in case of *P. italicum*. [84] showed that the *in vitro* growth of yeast was not influenced by chitosan lower optimal concentration, and that the application of chitosan with the antagonist *Cryptococcus laurentii* enhanced its antifungal activity against *P. italicum*. [85] reported that presence of 0.2% glycolchitosan with *Candida saitoana* suspensions was more efficient in controlling blue and gray moulds of apples and green mould of lemons and oranges than each treatment alone.

The difference in the efficacy of the killer yeast strains in combination with various natural compounds against *P. digitatum* and/ or *P. italicum* is due to the varieties of pH growth medium of all antagonists in controlling of *P. digitatum* and/ or *P. italicum in vitro* and most of

researchers applied the combination of killer yeast strains with various natural compounds and showed the antifungal activity against *P. digitatum* and/ or *P. italicum* on orange fruits (*in vivo*).

5. Conclusions

The studied killer yeasts as postharvest biocontrol agents against green and blue moulds are promising alternative to the use of synthetic fungicides, because of their less adverse effect on human health and the environment. *Pichia guilliermondii* and *C. pseudotropicalis* are very good antagonists when combined with natural compounds or alone. Hence, further studies of commercial formulations of the yeast strains *P. guilliermondii*, *C. pseudotropicalis*, are required in order to evaluate their potential against *P. digitatum* and *P. italicum* colonization in fruit wounds.

References

- [1] M. C. Strano, G. Altieri, N. Admane, F. Genovese, G. C. Di Renzo, Advance in citrus postharvest management: diseases, cold storage and quality evaluation. In: Citrus Pathology. In Tech, Harsimran Gill and Harsh Garg (2017).
- [2] G. F. Lambert, A. A. Lasserre, C. Azzaro-Pantel, M. A. Miranda-Ackerman, R. P. Vazquez, M. d. R. Salazar, Behaviour patterns related to the agricultural practices in the production of Persian lime (*Citrus latifoliatanaka*) in the seasonal orchard. Comput. Electron. Agric. 116: (2015) 162-172.
- [3] <https://www.freshplaza.com/article/9261344/egyptian-orange-export-volumes-surpass-those-of-spain-and-south-africa>.
- [4] L. Palou, J. Usall, J. A. Muñoz, J. L. Smilanick, I. Viñas, Hot water, sodium carbonate, and sodium bicarbonate for the control of postharvest green and blue moulds of clementine mandarins. Postharvest Biol. Technol. 24: (2002) 93–96.
- [5] R. Lahlali, M. N. Serrhini, D. Frie, M. H. Jijakli, *In vitro* effects of water activity, temperature, and solutes on the growth rate of *P. italicum* Wehmer and *P. Digitatum* Sacc. J. Appl. Microbiol. 101: (2006) 628–636.
- [6] R. Lahlali, Y. Hamadi, M. E. Guilli, M. H. Jijakli, Efficacy assessment of *Pichia guilliermondii* strain Z1, a new biocontrol agent, against citrus blue mould in Morocco under the influence of temperature and relative humidity. Biol. Control. 56: (2011a) 217–224.
- [7] N. Tao, N. Jia, H. Zhou, Anti-fungal activity of Citrus reticulata Blanco essential oil against *Penicillium italicum* and *Penicillium digitatum*. Food Chem. 153: (2014) 265–271.
- [8] C. Sun, D. Fu, H. Lu, J. Zhang, X. Zheng, T. Yu, Autoclaved yeast enhances the resistance against *Penicillium expansum* in postharvest pear fruit and its possible mechanisms of action. Biol. Control, 119: (2018) 51–58.

- [9] M. F. Perez, L. Contreras, N. M. Garnica, M. V. Fernández-Zenoff, M. E. Farías, M. Sepulveda, J. Ramallo, J. R. Dib, Native killer yeasts as biocontrol agents of postharvest fungal diseases in lemons. *PLoS One*, 11: (2016) 1-21.
- [10] A.A. Kader (ed.), *Postharvest Technology of Horticultural Crops*. Second edition, Univ. Calif., Div. of Agr. and Nat. Resources, Publ. 3311, (1992) pp. 296.
- [11] J. H. Costa, J. M. Bazioli, J. G. de Moraes Pontes, T. P. Fill, *Penicillium digitatum* infection mechanisms in citrus: what do we know so far? *Fungal Biol.* 123: (2019) 584–593.
- [12] M. N. Klein, K. C. Kupper, Biofilm production by *Aureobasidium pullulans* improves biocontrol against sour rot in citrus, *Food Microbiol.* 69: (2018) 1–10.
- [13] U.K. Bhatta, Alternative management Approaches of Citrus Diseases Caused by *Penicillium digitatum* (Green mold) and *Penicillium italicum* (Blue mold). *Front. Plant Sci.* 12: (2022) 833328. doi: 10.3389/fpls.2021.833328.
- [14] H. Singh, S. Yin-Chu, G. Al-Samarrai, M. Syarhabil, Potential of *Cerbera odollam* as a bio-fungicide for post-harvest pathogen *Penicillium digitatum*, *AIP Conf. Proc.* (2015).
- [15] A. M. Kanashiro, D. Y. Akiyama¹, K. C. Kupper, T. P. Fill, *Penicillium italicum*: An Underexplored Postharvest Pathogen. *Front. Microbiol.* 11: (2020) 1-17.
- [16] M. Wisniewski, S. Droby, J. Norelli, J. Liu, L. Schena, Alternative management technologies for postharvest disease control: the journey from simplicity to complexity. *Postharvest Biol. Technol.* 122: (2016) 3–10.
- [17] D. T. Cunha, L. P. Ferraz, P. P. Wehr, K.C. Kupper, Antifungal activity and action mechanisms of yeasts isolates from citrus against *Penicillium italicum*. *Int. J. Food Microbiol.* 276: (2018) 20-27.
- [18] H. Zhang, X. Zheng, F. Chengxin, Y. Xia, Postharvest biological control of gray mould rot of pear with *Cryptococcus laurentii*. *Postharvest Biol. Technol.* 35: (2020) 79–86.
- [19] B. G. Rowaa, M.A. Shima, A.A. Afaf, A.H. Maha, Application of Gum Arabic as Edible Coating for Improving Postharvest Quality of Potato Tubers. *Journal of Research in Science*. 38(1): (2021) 116- 141.
- [20] A.M. Hanahem, S.A. Wafaa, A.Y. Shymaa, E.M. Elham, M.H.H. Amr, Screening of Antifungal Activities of Five Algal Crude Extracts. *Journal of Research in Science*. 36: (2019).
- [21] R. S. Pimenta, F. L. Silva, J. F. M. Silva, P. B. Morais, D. T. Braga, C. A. Rosa, A. Corrêa, Biological control of *Penicillium italicum*, *P. digitatum* and *P. expansum* by the predacious yeast *Saccharomycopsis schoenii* on oranges. *Brazilian J. Microbiol.* 39: (2008) 85–90.
- [22] H. Aloui, F. Licciardello, K. Khwaldia, M. Hamdi, C. Restuccia, Physical properties and antifungal activity of bioactive films containing *Wickerhamomyces anomalus* killer yeast and their application for preservation of oranges and control of postharvest green mould caused by *Penicillium digitatum*. *Int J Food Microbiol.* 200: (2015) 22–30.

- [23] Y. Liu, S. Yao, L. Deng, J. Ming, K. Zeng, Different mechanisms of action of isolated epiphytic yeasts against *Penicillium digitatum* and *Penicillium italicum* on citrus fruit. *Postharvest Biol. Technol.* 152: (2019) 100–110.
- [24] A. S. Dukare, S. Paul, V. E. Nambi, R. K. Gupta, R. Singh, K. Sharma, R. K. Vishwakarma, Exploitation of microbial antagonists for the control of postharvest diseases of fruits: a review. *Crit. Rev. Food Sci. Nutr.* 59: (2019) 1498–1513.
- [25] G. Long, L. Yi, M. Xiaoxue, T. Rui, T. Boyun, Z. Zhiqin, Antifungal Activity of Polymethoxylated Flavonoids (PMFs)- Loaded Citral Nanoemulsion against *Penicillium italicum* by Causing Cell Membrane Damage. *J. Fungi* 8: (2022) 388. <https://doi.org/10.3390/jof8040388>.
- [26] S. Gross, L. Kunz, D. C. Müller, A. S. Kron, F. M. Freimoser, Characterization of antagonistic yeasts for biocontrol applications on apples or in soil by quantitative analyses of synthetic yeast communities. *Yeast*, 35: (2018) 559–566.
- [27] D. Spadaro, S. Droby, Development of biocontrol products for postharvest diseases of fruit: the importance of elucidating the mechanisms of action of yeast antagonists. *Trends Food Sci. Technol.* 47: (2016) 39–49.
- [28] K. Papoutsis, M. M. Mathioudakis, J. H. Hasperué, V. Ziogas, Nonchemical treatments for preventing the postharvest fungal rotting of citrus caused by *Penicillium digitatum* (green mould) and *Penicillium italicum* (blue mould). *Trends Food Sci. Technol.* 86: (2019) 479–491.
- [29] T. P. Canamas, I. Vinas, J. Usall, C. Casals, C. Solsona, N. Teixidó, Control of postharvest diseases on citrus fruit by preharvest application of the biocontrol agent *Pantoea agglomerans* CPA-2: Part I. Study of different formulation strategies to improve survival of cells in unfavorable environmental conditions. *Postharvest Biol. Technol.* 49: (2008) 86–95.
- [30] J. I. Pitt, The Genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. Academic Press Inc. Ltd, London UK (1979).
- [31] K. H. Domsch, W. Gams, T. H. Anderson, *Compendium of soil fungi*. Eching, Germany (1993).
- [32] R. A. Samson, E. S. Hoekstra, J. C. Frisvad, *Introduction to food and airborne fungi*. 7th (ed) Centraalbureau voor Schimmelcultures, Utrecht (2004).
- [33] C. Platania, C. Restuccia, S. Muccilli and G. Cirvilleri, Efficacy of killer yeasts in the biological control of *Penicillium digitatum* on tarocco orange fruits (*Citrus sinensis*). *Food Microbiol.* 30: (2012) 219–225.
- [34] C. P. Kurtzman, S. Droby, *Metschnikowia fructicola*, a new ascosporic yeast with potential for biocontrol of post-harvest fruit roots. *Syst. Appl. Microbiol.* 24: (2001) 395–399.
- [35] A. R. Coelho, G. M. A. Nóbrega, F. C. Pagnocca, F. L. Hoffmann, K. Harada, E. Y. Hirooka, Avaliação do potencial antagônico de leveduras, visando biocontrole de deterioração por *Penicillium expansum*. *Semin Cienc Agrar.* 32: (2011) 1879–1892.

- [36] G.E. Eman, A.H. Azhar, M.A. Sanaa, Y.I. Sahar, Antifungal activity of *Streptomyces canescens* MH7 isolated from mangrove sediment against some dermatophytes. Journal of Research in Science. 38(1): (2021) 36- 59.
- [37] L. Fayzi, L. Askarne, E.H. Boufous, O. Cherifi, K. Cherifi, Antioxidant and antifungal activity of some seaweeds against postharvest fungi pathogens. Asian J. Plant Sci 21: (2022) 328- 338.
- [38] N. J. W. Kreger Van Rij, The yeasts: A taxonomic study. Amsterdam, Elsevier Science Publishers BV (1987).
- [39] J.A. Barnett, R.W. Payne, D. Yarrow, Yeasts characteristics and identification. Cambridge University Press (2000).
- [40] C. P. Kurtzman, J. W. Fell, The yeasts: A taxonomic study, 4th ed. Elsevier Science Ltd. New York, (1998) 891- 947.
- [41] B. Esteve-Zarzoso, C. Belloch, F. Uruburu, A. Querol, Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol. 49: (1999) 329–337.
- [42] R. Mohammadi, H. Mirhendi, A. Rezaei-Matehkolaei, M. Ghahri, M. R. Shidfar, N. Jalalizand, K. Makimura, Molecular identification and distribution profile of *Candida* species isolated from Iranian patients. Med. Mycol. 51: (2013) 657–663.
- [43] H. M. Liu, J. H. Luo, P. Liu, B. Q. Wang, Y. J. Cheng, B. X. Deng, C. A. Long, Improvement of *Hanseniaspora uvarum* biocontrol activity against gray mould by the addition of ammonium molybdate and the possible mechanisms involved. J. Crop. Prot. 29: (2010) 277–282.
- [44] P. Geng, S. Chen, M. Hu, M. Rizwan-ul-Haq, K. Lai, F. Qu, Y. Zhang, Combination of *Kluyveromyces marxianus* and sodium bicarbonate for controlling green mould of citrus fruit. Int. J. Food Microbiol. 151: (2011) 190–194.
- [45] C. Montesinos-Herrero, A. Pedro, Moscoso-Ramirez, L. Palou, Evaluation of sodium benzoate and other food additives for the control of citrus postharvest green and blue moulds. Postharvest Biol. Technol. 115: (2016) 72-80.
- [46] M. El-Fawy, I. El-Sharkawy, S. Ahmed, Impact of pre and post-harvest treatments with chemicals preservatives on Botrytis gray rot disease and fruit quality of strawberry. Arch. Agri. Sci. j. 3: (2020) 178-194.
- [47] N. El-Mougy, M. Abdel-Kader, M. Aly, S. Lashin, Application of fungicides alternatives as seed treatment for controlling root rot of some vegetables in pot experiments. Adv. life sci. 2: (2012) 57-64.
- [48] V. H. Tournas, E. J. Katsoudas, Effect of CaCl₂ and various wild yeasts from plant origin on controlling *Penicillium expansum* postharvest decays in golden delicious apples. Microbiol. Insights, 12: (2019) 1–6.

- [49] J. Ahima, X. Zhang, Q. Yang, L. Zhao, M. Apaliya, H. Zhang, Biocontrol activity of *Rhodotorula mucilaginosa* combined with salicylic acid against *Penicillium digitatum* infection in oranges. *Biol. Control*, 135: (2019) 23-32.
- [50] F. Wang, J. Deng, J. Jiao, Y. Lu, L. Yang, Z. Shi, The combined effects of carboxymethyl chitosan and *Cryptococcus laurentii* treatment on postharvest blue mould caused by *Penicillium italicum* in grapefruit fruit. *Sci. Hortic.* 253: (2019) 35-41.
- [51] SPSS. , SPSS base 15.0 User's guide. SPSS inc., Chicago, USA (2012).
- [52] S. Droby, M. Wisniewski, D. Macarasin, C. Wilson, Twenty years of postharvest biocontrol research: is it time for a new paradigm? *Postharvest Biol. Technol.* 52: (2009) 137–145.
- [53] M. El-Otmani, A. Ait-Oubahou, L. Zacarías, Citrus spp.: Orange, Mandarin, tangerine, clementine, grapefruit, pomelo, lemon and lime. In E. M. Yahia (ed) *Postharvest biology and technology of tropical and subtropical fruits*, Woodhead Publishing. (2011) 437–516.
- [54] Q. Abdullah, A. Mahmoud, A. Al-harethi, Isolation and identification of fungal postharvest rot of some fruits in Yemen. *PSM Microbiol.* 1: (2016) 36-44.
- [55] S. Droby, M. Wisniewski, The fruit microbiome: a new frontier for postharvest biocontrol and postharvest biology. *Postharvest Biol. Technol.* 140: (2018) 107–112.
- [56] M. A. Díaz, M. M. Pereyra, E. Picón-Montenegro, F. Meinhardt, J. R. Dib, Killer yeasts for the biological control of postharvest fungal crop diseases. *Microorganisms*, 8(11): (2020) 1-14.
- [57] L. Parafati, A. Vitale, C. Restuccia, G. Cirvilleri, Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape. *Food Microbiol.* 47: (2015) 85–92.
- [58] M. J. Schmitt, F. Breinig, The viral killer system in yeast: From molecular biology to application. *FEMS Microbiol. Rev.* 26: (2002) 257–276.
- [59] P. Buzzini, A. Martini, Large-scale screening of selected *Candida maltosa*, *Debaryomyces hansenii* and *Pichia anomala* killer toxin activity against pathogenic yeasts. *Med. Mycol.* 39: (2001) 479-482.
- [60] L.P. Ferraz, D. T. Cunha, D. A.C. Silva, K.C. Kupper, Biocontrol ability and putative mode of action of yeasts against *Geotrichum citri-aurantii* in citrus fruit. *Microbiol. Res.* 188: (2016) 72–79.
- [61] G.M. Walker, A.H. Mcleod, V.J. Hodgson, Interactions between killer yeasts and pathogenic fungi. *FEMS Microbiol. Lett.* 127: (1995) 213-222.
- [62] F. Weiler, M. J. Schmitt, Zygoicin, a secreted antifungal toxin of the yeast *Zygosaccharomyces bailii* and its effect on sensitive fungal cells. *FEMS Yeast Res.* 3: (2003) 69-76.
- [63] A. Santos, A. Sanchez, D. Marquina, Yeasts as biological agents to control *Botrytis cinerea*. *Microbiol. Res.* 159: (2004) 331-338.
- [64] A.R. Coelho, Controle de *Penicillium expansum* / Biodegradação de Patulina: perfil cromatográfico de composto bioativo de leveduras killer visando aplicação póscolheita 130 f. Tese

(Doutorado em Ciência de Alimentos) Universidade Estadual de Londrina, Londrina. PR, Brazil (2005).

[65] M. F. Perez, A. S. Isas, A. Aladdin, H. A. El Enshasy, J. R. Dib, In: Zakaria Z A (ed) Killer yeasts as biocontrol agents of postharvest fungal diseases in lemons. Sustainable technologies for the management of agricultural wastes, applied environmental science and engineering for a sustainable future 71. Springer Nature Singapore Pte Ltd, (2018) 87–98.

[66] M. F. Perez, J. P. Ibarreche, A. S. Isas, M. Sepulveda, J. Ramallo, J. R. Dib, Antagonistic yeasts for the biological control of *Penicillium digitatum* on lemons stored under export conditions. Biol. Control, 115: (2017) 135–140.

[67] R. Lahlali, Y. Brostaux, M. H. Jijakli, Control of apple blue mould by the antagonistic yeast *Pichia anomala* strain K: screening of UV protectants for preharvest application. Plant Dis. 95: (2011b) 311–316.

[68] S. Panebianco, A. Vitale, G. Polizzi, F. Scala, G. Cirvilleri, Enhanced control of postharvest citrus fruit decay by means of the combined use of compatible biocontrol agents. Biol. Control, 84: (2015) 19–27.

[69] W. J. Janisiewicz, L. Korsten, Biological control of postharvest diseases of fruits. Annu. Rev. Phytopathol. 40: (2002) 411–441.

[70] R. Torres, C. Nunes, J. M. Garcia, M. Abadias, I. Vinas, T. Manso, M. Olmo, J. Usall, Application of *Pantoea agglomerans* CPA-2 in combination with heated sodium bicarbonate solutions to control the major postharvest diseases affecting citrus fruit at several Mediterranean locations. Eur. J. Plant Pathol. 118: (2007) 73–83.

[71] M. Mecteau, J. Arul, R. Tweddell, Effect of organic and inorganic salts on the growth and development of *Fusarium sambucinum*, a causal agent of potato dry rot. Mycol. Res. 106: (2002) 688–696.

[72] D.G. Alvindia, Sodium bicarbonate enhances efficacy of *Trichoderma harzianum* DGA01 in controlling crown rot of banana. J. Gen. Plant Pathol. 79: (2013) 136–144.

[73] O. A. Karabulut, U. Arslan, I. Kadir, K. Gul, Integrated control of postharvest diseases of sweet cherry with yeast antagonist and sodium bicarbonate applications within a hydrocooler. Postharvest Biol. Technol. 37: (2005) 135–141.

[74] W. J. Janisiewicz, R. A. Saftner, S. C. William, K. S. Yoder, Control of blue mould decay of apple during commercial controlled atmosphere storage with yeast antagonists and sodium bicarbonate. Postharvest Biol. Technol. 49: (2008) 374–378.

[75] G. Mandal, D. Singh, R. R. Sharma, Effect of hot water treatment and biocontrol agent (*Debaryomyces hansenii*) on shelf life of peach. Indian J. Hortic. 64: (2007) 25–28.

[76] R. J. McLaughlin, C. L. Wilson, E. Chalutz, W. F. Kurtzman, S. F. Osman, Characterization and reclassification of yeasts used for biological control of postharvest diseases of fruit and vegetables. Appl. Environ. Microbiol. 56: (1990) 3583–3586.

- [77] B. Scherm, G. Ortu, A. Muzzu, M. Budroni, G. Arras, Q. Migheli, Biocontrol activity of antagonistic yeasts against *Penicillium expansum* on apple. J. Plant Pathol. 85: (2003) 205–213.
- [78] H. Bastiaanse, L. de Bellaire, L. Lassois, C. Mission, M. Jijakli, Integrated control of crown rot of banana with *Candida oleophila* strain O, calcium chloride and modified atmosphere packaging. Biol. Control. 53: (2010) 100–107.
- [79] B. R. Gramisci, M. C. Lutz, C. A. Lopes, M. P. Sangorrín, Enhancing the efficacy of yeast biocontrol agents against postharvest pathogens through nutrient profiling and the use of other additives. Biol. Control, 121: (2018) 151–158.
- [80] M. Wisniewski, S. Droby, E. Chalutz, Y. Eilam, Effects of Ca^{2+} and Mg^{2+} on *Botrytis cinerea* and *Penicillium expansum* *in vitro* and on the biocontrol activity of *Candida oleophila*. Plant Pathol. 44: (1995) 1016–1024.
- [81] X. Qin, H. Xiao, C. Xue, Z. Yu, R. Yang, Z. Cai, L. Si, Biocontrol of gray mould in grapes with the yeast *Hanseniaspora uvarum* alone and in combination with salicylic acid or sodium bicarbonate. Postharvest Biol. Technol. 100: (2015) 160–167.
- [82] J. Liu, Y. Sui, M. Wisniewski, S. Droby, Y. Liu, Utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit. Int. J. Food Microbiol. 167: (2013) 153–160.
- [83] J. R. Gandra, C. S. Takiya, E. R. Oliveira, P. G. Paiva, R. H. T. Goes, E. R. S. Gandra, H. M. C. Araki, Nutrient digestion, microbial protein synthesis, and blood metabolites of Jersey heifers fed chitosan and whole raw soybeans. Rev. Bras. Zootec, 43: (2016) 130–137.
- [84] T. Yu, H. Y. Li, X. D. Zheng, Synergistic effect of chitosan and *Cryptococcus laurentii* on inhibition of *Penicillium expansum* infections. Int. J. Food Microbiol. 114: (2007) 261–266.
- [85] A. El-Ghaouth, J. L. Smilanick, G. E. Brown, A. Ippolito, M. Wisniewski, C. L. Wilson, Applications of *Candida saitoana* and glycolchitosan for the control of postharvest diseases of apple and citrus fruit under semi-commercial conditions. Plant Dis. 84: (2000) 243–248.

الملخص العربي

تقييم الخمائر القاتلة المحلية ضد مسببات الأمراض النباتية لثمار البرتقال ما بعد الحصاد

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الملخص العربي

تعد الخمائر من أهم عوامل مكافحة الحيوية بعد الحصاد. لوحظ أن ثمار البرتقال تتدهور في كثير من الأحيان بعد الحصاد بسبب العفن الأخضر والعفن الأزرق مما يؤدي الي خسائر اقتصادية هائلة .

تمثلت أهداف هذه الدراسة في عزل الفطريات المسببة للعفن الأزرق والأخضر من ثمار البرتقال المصابة وتقييم قدرة الخمائر القاتلة المعزولة من ثمار وأوراق البرتقال السليمة التي جُمعت من بساتين البرتقال للسيطرة على العفن الأزرق والأخضر وكذلك تقييم التأثير الإضافي لعوامل مكافحة الحيوية عند إضافة 2% بيكربونات الصوديوم، 2% بنزوات الصوديوم، 2% كلوريد الكالسيوم، 0.2% حمض السليسلوك و 0.5% كيتوزان. تم الحصول على ثماني عزلات فطرية تظهر أعراض العفن الأخضر والأزرق من ثمار برتقال مصابة، وقد تم تعريف عزلتان منهم على أنهما *Penicillium italicum* و *Penicillium digitatum* وتم اختيارهما لاجراء الاختبارات المعملية. كذلك تم الحصول على ثماني وعشرين عزلة خميرة من اوراق البرتقال ومن علي سطح الثمار. وقد اظهرت جميع الخمائر نشاطا قاتلا ملحوظا. فقد لوحظ أن اثنا عشر عزلة خميرة قد اختزلت من نمو فطر *P. digitatum* بنسبة 22.5- 70 % بينما اختزلت سبع عزلات من نمو فطر *P. italicum* بنسبة 21.1- 68.5%. وقد تم تعريف عزلات الخميرة الأكثر فاعلية على أنها *Candida pseudotropicalis* ، *Candida salmanticensis* ، *Candida membrtanifaciens* و *Pichia guilliermondii* . أظهرت الدراسة ان الجمع بين عوامل مكافحة الحيوية *C. pseudotropicalis* ، *C. salmanticensis* و *p. guilliermondii* و بيكربونات الصوديوم، كلوريد الكالسيوم، أو الكيتوزان أدى إلى زيادة فاعليتهم ضد فطر *P. digitatum* بينما قلل الجمع بين *C. pseudotropicalis* ، *C. membrtanifaciens* و *P. guilliermondii* و المركبات الطبيعية السابق ذكرها من فاعليتهم ضد فطر *P. italicum* . كما أظهرت الدراسة ان الجمع بين *C. membrtanifaciens* و حمض السليسلوك أدى الي زيادة فاعليتها ضد فطر *P. digitatum* . كذلك أظهرت الدراسة التأثير الإضافي لبنزوات الصوديوم على زيادة فاعلية *C. pseudotropicalis* ضد فطر *P. digitatum* ، *C. pseudotropicalis* و *P. guilliermondii* ضد فطر *P. italicum* .