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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.

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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 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, Tukh, 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 in100 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 3x 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% $CaCl_2$ **(**[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 version13.0. 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* (isolate11) 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	TCAAAACAAAAATAATCAAAACTTTTAACAATGGATCTCTTGGTTCTCGTA TCGATGAAGAACGCAGCGAAACGCGATATTTCTTGTGAATTGCAGAAGTG AATCATCAGTTTTTGAACGCACATTGCACTTTGGGGTATCCCCCAAAGTAT ACTTGTTTGAGCGTTGTTTCTCTCTTGGAATTGCATTGCTTTTCTAAAATTT CGAATCAAATTCGTTTGAAAAACAACACTATTCAACCTCAGATCAAGTAGG ATTACCCGCTGAACTTAAGCATATCAATAAGCGGA	100	*Candida pseudotropicalis*
9	TCAAAACAAAAATAATCAAAACTTTTAACAATGGATCTCTTGGTTCTCGT ATCGATGAAGAACGCAGCGAAACGCGATATTTCTTGTGAATTGCAGAAGT GAATCATCAGTTTTTGAACGCACATTGCACTTTGGGGTATCCCCCAAAGT ATACTTGTTTGAGCGTTGTTTCTCTCTTGGAATTGCATTGCTTTTCTAAAA TTTCGAATCAAATTCGTTTGAAAAACAACACTATTCAACCTCAGATCAAG TAGGATTACCCGCTGAACTTAAGCATATCAATAAGCGGA	98	*Candida salmanticensis*
11	TCAAAACAAAAATAATCAAAACTTTTAACAATGGATCTCTTGGTTCTCGT ATCGATGAAGAACGCAGCGAAACGCGATATTTCTTGTGAATTGCAGAAG TGAATCATCAGTTTTTGAACGCACATTGCACTTTGGGGTATCCCCCAAA GTATACTTGTTTGAGCGTTGTTTCTCTCTTGGAATTGCATTGCTTTTCTA AAATTTCGAATCAAATTCGTTTGAAAAACAACACTATTCAACCTCAGATC AAGTAGGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA	97	*Candida membranifaciens*
12	GCAAGAGCTCAGATTTGAAATCTCACCCTTCGTGGGGCCAATTTGTAAT TGGAAGTTGGAATCCCGGTTTCTACCTGTGGTCCATTTCCCTGGAACCA GGCCCCCCCCGAAGGTGAAAACCCCCGTGTGAGCACAATTCCCCCACCTA GGGCCTTCCGACCAGTCCAGTTGTTTGGGAATGCAACTCTTATTGGGTG GTAAATTCCATTCCATGGTAAATTTTGGCCAAAGAACGAACCCAAACCA ATACCGTGAAAGAAAGAAGAAAAGCACTTTGAAAAGAAAATGAAACGAC AAGTGGAATTGTTGAAAGGGAAGGGTATTGGGCTCGACCTGGGATTTTA TTTTCGTTGCCTCTCGGGGGCGGCGCTTTGGGTTTTTCCTGGGCCCCCC CCGTTTTTGTTGGGTGAGAAAGACCCTTGGAATGTGGCTCCCTCCGGAG	95	*Pichia guilliermondii*

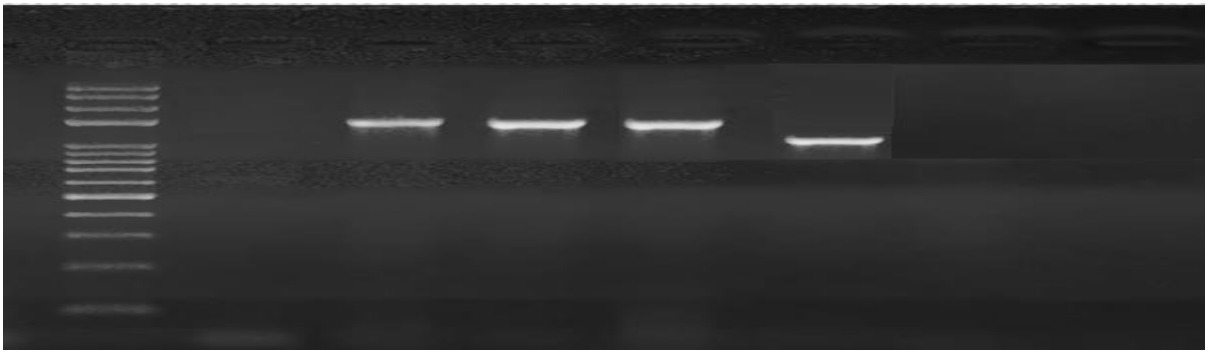

Fig (1): Molecular basis of identification of yeast isolates. PCR amplification of internal transcribed spacer sequences (ITS DNA) used as a molecular marker of yeast identity.

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	
	P. digitatum	***P. italicum****
Candida pseudotropicalis	65.4^{e}	76.5^{b}
Candida membranifaciens	69.6^{d}	–
Pichia guilliermondii	72^{c}	74.3^{d}
Candida salmanticensis	–	68.7^{e}
SBC	62.7^{g}	66.9^{f}
SBC+ *Candida pseudotropicalis*	83.1^{b}	76.1^{c}
SBC+ *Candida membranifaciens*	66.5^{f}	–
SBC+ *Pichia guilliermondii*	88.1^{a}	58.3^{g}
SBC+ *Candida salmanticensis*	–	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	
	P. digitatum	***P. italicum****
Candida pseudotropicalis	65.4^{d}	76.5^{c}
Candida membranifaciens	69.6^{c}	-
Pichia guilliermondii	72^{a}	74.3^{d}
Candida salmanticensis	–	68.7^{e}
SB	26^{g}	28.9^{g}
SB+ *Candida pseudotropicalis*	73^{b}	86.6^{a}
SB+ *Candida membranifaciens*	53.7^{f}	–
SB+ *Pichia guilliermondii*	58^{e}	86.8^{b}
SB+ *Candida salmanticensis*	–	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	
	P. digitatum	***P. italicum****
Candida pseudotropicalis	65.4^{e}	76.5^{a}
Candida membranifaciens	69.6^{d}	–
Pichia guilliermondii	72^{c}	74.3^{b}
Candida salmanticensis	–	68.7^{e}
$CaCl_2$	52.7^{g}	59.5^{f}
$CaCl_2$+ *Candida pseudotropicalis*	83.1^{a}	74.8^{c}
$CaCl_2$+ *Candida membranifaciens*	67^{f}	–
$CaCl_2$+ *Pichia guilliermondii*	74.8^{b}	73^{d}
$CaCl_2$+ *Candida salmanticensis*	–	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	
	P. digitatum	***P. italicum****
Candida pseudotropicalis	65.4^{d}	76.5^{a}
Candida membranifaciens	69.6^{c}	–
Pichia guilliermondii	72^{b}	74.3^{b}
Candida salmanticensis	–	68.7^{c}
SA	19^{g}	21.1^{g}
SA+ *Candida pseudotropicalis*	63.3^{e}	54.8^{d}
SA+ *Candida membranifaciens*	73.6^{a}	–
SA+ *Pichia guilliermondii*	48.3^{f}	39.3^{f}
SA+ *Candida salmanticensis*	–	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	
	P. digitatum	***P. italicum****
Candida pseudotropicalis	65.4^{e}	76.5^{a}
Candida membranifaciens	69.6^{d}	–
Pichia guilliermondii	72^{c}	74.3^{b}
Candida salmanticensis	–	68.7^{c}
Chitosan	28^{g}	34.7^{g}
Chitosan+ *Candida pseudotropicalis*	80.3^{b}	66^{d}
Chitosan+ *Candida membranifaciens*	60.4^{f}	–
Chitosan+ *Pichia guilliermondii*	87^{a}	46.6^{f}
Chitosan+ *Candida salmanticensis*	–	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 the 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 ≥ 40%, 18 strains were inhibited between 16 and 39%; and the remaining 8 strains showed ≤ 15% inhibition. *S. cerevisiae* (137) and *Kazachstania* (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 *Penecillium 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. membranifacien* 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. guilliermondi, C. pseudotropicalis,* are required in order to evaluate their potential against *P. digitatum* and *P. italicum* colonization in fruit wounds.

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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 المكافحة الحيوية بعد الحصاد. لوحظ أن ثمارالبرتقال تتدهور في كثير من الأحيان بعد الحصاد بسبب العفن الاخضر والعفن الازرق 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 لـعوامل المكافحة الحيوية عند إضافة 2% بيكربونات الصوديوم، 2% بنزوات الصوديوم، 2% كلوريد الكالسيوم، 0.2 %حمض السليسليك 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 digitatum* و *Penicillium italicum* وتم اختيارهما لاجراء الاختبارات المعملية. كذلك تم الحصول علي 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 المكافحة 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. membranifaciens* و *P. guilliermondii* و المركبات الطبيعة السابق ذكرها من فاعليتهم ضد فطر *P. italicum*. كما أظهرت الدراسة ان الجمع بين *C. membranifaciens* و حمض السليسليك أدي 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* .