Effect of Antagonistic Yeast Treatment on Extension of Wounded Fruit Shelf- Life and Avoid Damage of Rough Harvest

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ABSTRACT

Fruit surface wounding caused by rough harvesting or insect attack provides many entries to mold organisms and cause loss of fruits. Forty five yeast isolates were classified to five genera according to their morphological characteristics and they screened for antagonistic activity. Twelve isolates represented to *Candida* spp. (five); *Pichia* spp. (three) ; *Kluyveromyces* spp. (three); *Saccharomyces* spp.(one) were identified and tested against Penicillium digitatum and *Rhizopus stolonifer*. The largest inhibition (%) of the tested fungi was recorded by *Candida sake* isolates. Yeast application significantly decreased fruit decay severity on fruits of mandarin (*Citrus reticulata* Blanco) by 15% or strawberry (Fragaria × ananassa) by 24% than untreated ones. The fungicidal effect was increased with increases of yeast concentrations. However, there is no treatment gave completely inhibition to these mold fungi. The best yeast concentration was 1×106 cfu /ml. The combination of the highly concentration of *C. sake* (1×106cfu /ml) and 1% CaCl2 enhanced the antagonistic effect of the yeast, where they exhibited completely inhibition to *P. digitatum* on mandarin fruits and *R. stolonifer* on strawberry fruits after 6 days post infection.

Key Words: *Candida sake*, Fruit shelf- life, *Penicillium digitatum*, *Rhizopus stolonifera*.

INTRODUCTION

Postharvest diseases play an important role in reducing the quantity and quality of fruits specially the highly perishable ones such as mandarin and strawberry (Darrow and Wallace, 1966). The Egyptian growers normally use chemical fungicides to reduce mold problem (Embaby *et al.*, 2016). There is global concern about the residues of chemical pesticides on fruit surfaces regarding health of human and the environment (Aktar *et al.*, 2009). Persistent attempts to find alternatives to pesticides are still underway since many fungi have become resistant to commercial pesticides (Horuz and Kinay, 2010). Citrus was the most important fruit crops in Egypt, mandarin (*Citrus reticulata* Blanco) represent about 16.4% of the total Citrus land area and about 14.6% of the total Citrus production (Anon., 2015). Injuries on mandarin fruits, especially during rough harvesting, cause entries to wound fungi such as *Penicillium italicum* and *P. digitatum*, the causal agents of blue and green mould, respectively. The decays caused approximately 60-80% of all citrus postharvest losses (Sallam *et al.*, 2012). Also, strawberry fruits are highly perishable and cannot be stored for longer duration (Tahir *et al.*, 2018). The strawberry fruit rots in Egypt (soft rot caused by *Rhizopus stolonifer* and grey mold caused by *Botrytis cinerea*) are the most important diseases attach these fruits, but soft rot is the most dangerous factor under high temperature (El-Mougy *et al.*, 2008).

Using of antagonistic microorganisms as biocontrol agents against postharvest mold fungi is an alternative way to the use of commercially pesticides (Sharma *et al.*, 2009). Application of yeasts such as *Candida* spp. as biocontrol agents acts as a promise method against different pathogens. Since the biocontrol agents are less effective than the pesticides, they should be activated and more effective with some additives such as adding calcium salts to biocontrol treatments (El-Ghaouth *et al.*, 2000; Karabulut and Baykal, 2003).

Therefore, this investigation was undertaken to study the postharvest behavior of mandarin and strawberry fruits after treating with the antagonistic yeast, *Candida sake* during their shelf-life time, and to identify the ap-

propriate concentration of calcium chloride as a simultaneous application.

MATERIALS AND METHODS

Fruit materials:

Healthy looking fruits of mandarin (*Citrus reticulata* Blanco) cv. Baladi and strawberry (Fragaria × ananassa) cv. Vienna were collected from an orchard and a field, respectively at Badrashin, Giza Governorate, Egypt during 2017, and brought in sterile polyethylene plastic bags to Laboratory of Plant Pathology, National Research Center, Dokki, Egypt. They were in the same size, with stalk, without wounds and washed with tap water. The surfaces were sterilized by dipping in sodium hypochlorite 1% for 2 min., then rinsed three times in sterile distilled water and air-dried.

Fungal mold agents:

Native strains of *P. digitatum* and *R. stolonifer* were obtained from Microbiological Resources Center (Cairo MIRCEN) for their high level of aggressiveness on fruits of mandarin (Smilanick *et al.*, 2008) or strawberry (Kwon *et al.*, 2009), respectively. The fungal cultures were maintained on PDA at 5 °C.

Isolation of yeasts:

Some yeast isolates from surface of orange fruits were isolated according to the protocol described by (Fiss *et al.*, 2003). Fruit samples were washed by tap water and rinsed three times in distilled water. They were then cut, squeezed and collected in the sterile test tubes. Mixed samples were dilute serially and 0.1 ml of diluted fruit juice was plated on yeast extract peptone-dextrose agar medium (YPD) supplemented with antibiotics (chlora-mphenicol at 25 µg / mL) and incubated at 30ºC for 48 hours. Yeast colony was sub-cultured until the purified cultures were maintained and kept at 4ºC.

Identification and characterization of yeast species:

Morphology of the yeasts and their appearance on YPD agar was examined based on their cultural characteristics (Colony shapes, size, pigment, elevation, edge and surface appearance). Primary identification of the yeast species was carried out by using the conventional methods described by (Kurtzman *et al.*, 2011) while the final identification was carried out only to the highly antagonistic strain according to the morphological, physiological and biochemical characters of the yeast which are used in yeast taxonomy (Yarrow, 1998 and Madhavan *et al.*, 2011).

Inoculum preparation of the isolated yeasts:

The isolated yeasts were grown at 24°C for 48 hrs on nutrient yeast broth (20.0g peptone, 20.0g glucose; and 10.0g yeast extract) with shaking. The culture was centrifuged at 400 g. for 10 min. and pellets were re-suspended in distilled sterilized water and centrifuged again. The resulting pellets were dispersed in sterilized distilled water. The concentration of isolate was adjusted to 1×10^6 colony forming units (cfu) ml^{-1} using a hemacytometer slide. The concentrations of the highly effective yeast were adjusted to 1×10^4, 1×10^5 and 1×10^6cfu/ml.

Antagonistic activity in vitro of the isolated yeasts on two fungal mold agents:

Dual culture was used to test the antagonism between the isolated yeasts, and both of *P. digitatum* or *R. stolonifer*. Twenty five µl of each concentration of the yeast was placed into well at the center of Petri dishes that containing yeast extract malt agar medium and then plates were incubated at 25˚C for 48 hrs. Surface media was mulched by 50 µl of *P. digitatum* or *R. stolonifer* spore suspension. Two replicates were prepared for each concentration. All the dishes were incubated at 28˚C for 3 and 6 days and the experiment was carried out twice. To compare the inhibitory effects of different concentrations of *C. sake* (Strain CAP1), the growth inhibition percentage was determined according to the

scale adopted by Bell *et al.*,1982 where:

100% inhibition = the yeast completely grows over the pathogen and covered the entire medium surface.

75% inhibition = the yeast grows at least 2/3 of the medium surface.

50% inhibition = each of the yeast and the pathogen grows approximately ½ of the medium surface and nether organism appeared to dominant the other.

25% inhibition = the pathogen grows at least 2/3 of the medium surface.

0% inhibition = the pathogen completely overgrows the yeast and occupied the entire medium surface.

Calcium chloride preparations:

Different concentrations of calcium chloride were prepared, viz. 0.50, 0.75 and 1%.

Fungicidal activity of the highly effective yeast isolate on wounded fruit mold severity:

The fruits were soaked in $CaCl_2$ solutions (0.50, 0.75 and 1%) for 15 min. and soaked again in the highly effective yeast solution for another 15 min. The test fruit surfaces were artificially wounded (1 mm wide and 2 mm deep) with sterile needles in the middle, each mandarin fruits was inoculated with 20 µl of *P. digitatum* (10^6 spores ml^{-1}) while each strawberry fruit was inoculated with R. stolonifer with the same concentration. The control fruits treatments were inoculated with distilled sterilized water only. A total of 100 treated fruits from each of mandarin or strawberry were kept on 10 open trays. Each tray (10 fruits) was considered as one treatment. Each treatment was replicated two times. After the treatments, the fruit trays were left overnight in an open shelf at room temperature (22°C). Then, the trays packs in plastic boxes containing moistened filter paper, covered with cling-films, and stored at 25˚C. After 3 and 6 days, the fruits were removed and decay severity%, were determined according to (Coco *et al.*, 2002) as follows:

None = 0% of fruit surface was injured ; Minor = slight to 10% of fruit surface was injured ; Moderate = 11 to 30% of fruit surface was injured and Severe = More than 30 of fruit surface was injured.

Effect of combination treatment of the highly effective yeast and calcium chloride on wounded fruit mold severity:

To test the effect of $CaCl_2$ in combination with the highly effective yeast (*Candida sake*) on development of fruit molds, each wounded and inoculated fruit was treated with 20 µl of the best concentration of yeast suspended in the best concentration of $CaCl_2$ solution. The fruits of control treatments were treated with the test $CaCl_2$ or yeast concentration only. After 3 and 6 days, the fruits were removed and radii of rotted symptoms at site of inoculation were measured as previously described.

Statistical analysis:

All trials were carried out on the basis of completely randomized designs. For statistical analysis, data were subjected to the analysis of variance. Statistical comparisons among means were performed using Duncan's multiple range tests.

RESULTS

Identification and characterization of some yeast species:

The isolated yeasts (45 isolates) were classified to five groups according to their morphological characteristics. The frequency of yeasts on culture media recorded as 15 spp. of *Candida*, 10 spp. of *Saccharomyces*, 8 spp. of *Pichia* and 7 spp. of *Kluyveramyces*, while five species were identified as *Schizosaccharomyces* spp. (Table 1).

Table (1): Morphological characteristics of the isolated yeasts.

Groups of yeast	Pigmentation	Colony morphology	Cell size	Frequency
Candida spp.	Brown	Mucoid, circular	Medium	15
Kluyveromyces spp.	Creamy	Flat, fury	Medium	7
Pichia spp.	White	Raised, circular, smooth	Medium	8
Saccharomyces spp.	White	Raised, circular, smooth	Large	10
Schizosaccharomyces spp.	White	Dry, flat, rough	Large	5

Antagonistic activity in vitro of the isolated yeasts on two fungal mold agents:

In our study, 45 test yeast isolates were screened for antagonistic activity. The data showed that 5 isolates of *Candida* spp.; 3 isolates of *Pichia* spp.; 3 isolates of *Kluyveromyces* spp.; 1 isolate of *Saccharomyces* spp. and no isolates of *Schizosaccharomyces* spp. are antagonistic to the two test pathogenic fungi, *P. digitatum* and *R. stolonifer*. The largest inhibition% of the test fungi was recorded in (Table 2) by *Candida* spp.

Table (2): Antagonistic activity in vitro of the isolated yeasts on *P. digitatum* and *R. stolonifer* growth.

Groups of yeast	Antagonistic isolates	*P. digitatum* inhibition%*	*R. stolonifer* inhibition%*
Candida spp.	5	75	100
Kluyveromyces spp.	3	50	75
Pichia spp.	3	50	50
Saccharomyces spp.	1	25	0
Schizosaccharomyces spp.	0	0	0
LSD at 5%	-	5.1	7.2

*100% inhibition = The yeast completely grows over the pathogen; 75% inhibition = The yeast grows at least 2/3 of the medium surface; 50% inhibition = Each of the yeast and the pathogen grows approximately ½ of the medium surface and 25% inhibition = The pathogen grows at least 2/3 of the medium surface.

Fungicidal activity of the highly effective yeast isolate on fruit mold severity:

The highly effective yeast isolates were identified as *Candida sake* (Saito and Oda) Van Uden and H. Buckley. Yeast application (Table 3) significantly decreased fruit mold severity on mandarin (15% decrease) or strawberry fruits (24% decrease) than untreated ones. The fungicidal effect was increased with increase of yeast concentrations. However, there is no treatment gave completely inhibition to these mold fungi. The effective concentration was 1×10^6cfu/ml.

Table (3): Fungicidal activity of the yeast *C.sake* at three concentrations on mold severity of the test fruit after 3 and 6 days.

C. sake yeast cons. (cfu /ml)	Mandarin mold severity% after:		Strawberry mold severity% after:	
	3 days	6 days	3 days	6 days
1×10^4	7	10	12	17
1×10^5	5	7	8	13
1×10^6	3	5	6	8
Inoculated, not treated fruits	12	20	22	32
Healthy looking fruits	None	2	None	4
LSD at 5%	1.9	2.3	2.1	3.0

None = 0% of fruit surface was injured; Minor = slight to 10% of fruit surface was injured; Moderate = 11 to 30% of fruit surface was injured and Severe = More than 30 of fruit surface was injured.

Effect of the combination of the highly effective yeast and calcium chloride on fruit mold severity:

Data in (Table 4) stated that the combination of the highly concentration of *C. sake* was (1×10^6 cfu/ml) and three concentrations of $CaCl_2$ enhanced the antagonistic effect of the yeast. The effective treatment was *C. sake* 1×10^6 + $CaCl_2$1%, where it exhibited completely inhibition to *P. digitatum* on mandarin fruits and *R. stolonifer* on strawberry fruits after 6 days of the artificially infection.

Table (4): Effect of combination of *C. sake* (1×10^6 cfu /ml) with three concentrations of $CaCl_2$ on mold severity of the test fruit after 3 & 6 days.

Combination treatment	Mandarin mold severity after:		Strawberry mold severity after:	
	3 days	6 days	3 days	6 days
C. sake 1×10^6+$CaCl_2$0.50%	5	7	8	10
C. sake 1×10^6+$CaCl_2$0.75%	3	5	6	8
C. sake 1×10^6+$CaCl_2$1%	None	None	None	None
Inoculated, not treated fruits	12	20	22	32
Healthy looking fruits	None	2	None	4

None = 0% of fruit surface was injured; Minor = slight to 10% of fruit surface was injured; Moderate = 11 to 30% of fruit surface was injured and Severe = More than 30 of fruit surface was injured.

DISCUSSION

The general strategy of biological control is to use one living organism to control another. Among these antagonistic organisms, natural yeasts have been efficacious as biological control agents. Yeasts generally have simple nutritional requirements, can grow rapidly on inexpensive substrates and are able to colonize dry surfaces for long periods of time. The author used healthy citrus fruit surface as a source to isolate of epiphytic yeasts. The largest obtained number of antagonistic yeasts (5 isolates) and the highly effective against strawberry soft rot (75% inhibition) and mandarin green mold (100% inhibition) were belonged to isolates of *Candida* yeast followed by *Kluyveromyces* and Pichia isolates, whereas *Schizosaccharomyces* ones were without antagonistic effect against these pathogens. These results are in agreement with those reported by Mercier and Wilson (1995), Arras (1996) and El -Ghaouth *et al.* (2003) who used many species of *Candida* for controlling fruit molds. Several routes have been proposed to explain the action mechanism of biological control agents (Lo, 1998).Various observations suggest that induction of host resistance, competition for space and nutrients between yeasts and pathogens, parasitism and also resistance to oxidative stress are likely to be the main mechanisms of yeast action (El-Ghaouth *et al.*, 2003 and Gholamnejad *et al.*, 2010). Also, Demirci (2011) found that *Candida famata* enhanced the accumulation of phytoalexins, scoparone and scopoletin in citrus wound tissues.

The combination of the highly concentration of *C. sake* (1×10^6 cfu/ml) + $CaCl_2$1%, exhibited completely inhibition to *P. digitatum* on mandarin fruits and *R. stolonifer* on strawberry fruits after 6 days of the artificially infection. In this respect, Geng *et al.* (2011) combined between *Kluyveromyces marxianus* and sodium bicarbonate for controlling green mold of citrus fruit.

For explanation the action of calcium, Hussain *et al.* (2012) reported that calcium is readily enters the apoplast and is bound in exchangeable from to cell wall and exterior surface of plasma membrane. It serves as a detoxifying agent. Calcium in cell walls serves as a binding agent in the calcium pectates form. Calcium has received considerable attention due to it can delay ripening and senescence, reduce the physiological disorders and reduce respiration, extend shelf life

Application of a combination of calcium chloride along with antagonistic yeasts could be used as an integrated management practice against some postharvest fruit or vegetable diseases.

ERFERENCES

Aktar, M., Sengupta, D., and Chowdhury, A. 2009. Impact of pesticides use in agriculture: Their benefits and hazards. Interdiscip. Toxicol. 2(1): 1–12.

Anonymous. 2015. Analytical economic study of Egyptian citrus exports. Agricultural Department of Economics, National Research Centre, Dokki, Giza, Egypt, 5 pp.

Arras, G. 1996. Mode of action of an isolate of *Candida famata* in biological control of *Penicillium* digitatum in orange fruits. Postharvest Biology and Technology. 8: 191-198.

Bell, D., Wells, H., and Markhan, C. 1982. *In vitro* antagonism of *Trichoderma* species against six fungal pathogens. Phytopathology. 72: 379-382.

Coco, V., Grimaldi, V., Campisano, A., and Catara, A. 2002. Assessment of orange fruit colonization by biocontrol yeasts. Plant Protection Science. 38 (Special Issue 2): 638-641.

Darrow, G., and Wallace, H. 1966. The Strawberry, History, Breeding, and Physiology. Holt, Rinehart and Winston, New York.

Demirci, F., 2011. Effects of *Pseudomonas fluorescens* and *Candida famata* on blue mould of citrus caused by *Penicillium italicum*. Agric. J. of Crop Sci. 5(3):344-349.

El-Ghaouth, A., Smilanick, J., Wisniewski, M., and Wilson, C. L. 2000. Improved control of apple and citrus fruit decay with combination of *Candida saitoana* and 2-deoxy-D-glucose. Plant Disease. 84:249-253.

El- Ghaouth, A., Wilson, C., and Wisniewski, M. 2003. Control of postharvest decay of apple fruit with *Candida saitoana* and induction of defense responses. Phytopathology. 93:344-348.

El-Mougy, N., El-Gamal, N., and Abdalla, M. 2008. The use of fungicide alternatives for controlling postharvest decay of strawberry and orange fruits. J. of Plant Protection Research. 48(3):385-395.

Embaby, E., Abdel-Galil, M., and Aiat, N. 2016. Control strawberry fruit decay caused by some mold fungi. International J. of Pharm. Tech. Research. 9(10): 184-193.

Fiss, M., Barchhausen, O., Gherbawy, Y., Kollar, A., Hamamoto, M., and Auling, G. 2003. Characterization of epiphytic yeasts of apple as potential biocontrol against apple scab (*Venturia inaequalis*). J. Plant Diseases and Protection. 110(6): 513-523.

Geng, P., Chen, S., Hu, M., Rizwan-ul-Haq, M., Lai, K., Qu, F., and Zhang, Y. 2011. Combination of *Kluyveromyces marxianus* and sodium bicarbonate for controlling green mold of citrus fruit. Int. J. Food Microbiol. 151: 190-194.

Gholamnejad, J., Etebarian, H., and Sahebani, N. 2010. Biological control of apple blue mold with *Candida* membranifaciens and *Rhodotorula mucilaginosa*. African J. of Food Science. 4(1): 1-7.

Horuz, S., and Kinay, P. 2010. The effects of some new postharvest fungicides and combination of hot water with sodium bicarbonate against *Geotrichum citri-aurantii* on citrus. Acta Horticulturae. 877(877):1551-1557.

Hussain, P., Meena, R., Dar, M., and Wani, A. 2012. Effect of post-harvest calcium chloride dip treatment and gamma irradiation on storage quality and shelf-life extension of Red delicious apple. J. Food Sci. Technol. 49(4): 415-426.

Karabulut, O., and Baykal, N., 2003. Biological control of postharvest diseases of peaches and nectarines by yeasts. J. Phytopath. 155:130-134.

Kurtzman, C., Fell, J., and Boekhout, T. 2011. The Yeasts, a Taxonomic Study. 5th Edition, Elsevier Science, New York, 2354 pp.

Kwon, J., Yoon, H., Nam, M., and Park, C. 2009. Occurrence of strawberry soft rot by *Rhizopus stolonifer* on distribution after harvest. Korean J. of Medical Mycology. 37(1): 117-119 ·

Lo, S. 1998. General mechanisms of microbial biocontrol agents. Plant Pathology Bulletin. 7: 155-166.

Madhavan, P., Jamal, F., Chong, P., and Ng, K. 2011. Identification of local clinical *Candida* isolates using CHROM agar *Candida* as a primary identification method for various *Candida* species. Trop. Biomed. 28: 269-274.

Mercier, J., and Wilson, C. 1995. Effect of wound moisture on the biocontrol by *Candida* oleophila of gray mold rot of apple. Postharvest Biology and Technology. 6(1/2): 9-15.

Sallam,N., Badawy, I., and Ibrahim, A. 2012. Biocontrol of green mold of orange using some yeasts strains and their effects on postharvest quality parameters. International J. of Plant Pathology. 3: 14-24.

Sharma, R., Singh, D., and Singh, R. 2009. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. Biol. Control. 50:205-221.

Smilanick, J., Mansour, M., Gabler, F., and Sorenson, D. 2008. Control of citrus postharvest green mold and sour rot by potassium sorbate combined with heat and fungicides. Postharvest Biology and Technology. 47: 226-238.

Tahir, H., Xiaobo, Z., Jiyong, S., Mahunu, G., Zhai, X., and Mariod, A. 2018. Quality and postharvest-shelf life of cold-stored strawberry fruit as affected by gum Arabic (Acacia senegal) edible coating. J. Food Biochem. e 12527.

Yarrow, D. 1998. Methods for the isolation, maintenance and identification of yeasts. Yeast. 14:77-100.

التأثير التضادي للمعاملة بالخميرة بهدف إطالة صلاحية الثمار للتداول وتجنب ضرر الحصاد الخشن

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الملخص

تجريح سطح الثمار نتيجة الحصاد الخشن أو هجوم الحشرات يوفر مداخل لمسببات الأعفان وبالتالي يسبب خسارة هذه الثمار، مما يستوجب تقليل نسبة حدوث العفن بالمبيدات الحيوية الآمنة دون اللجوء إلى المبيدات الكيميائية.

تم عزل 45 عزلة من الخمائر من سطح ثمار موالح محلية، ثم توزيعها على 5 أجناس حسب خصائصها الشكلية، وتم اختبار قدرتها على التضاد معمليًّا، أظهرت خمس عزلات من الكانديدا وثلاثًا من البتشيا وثلاثة من الكليفيروميسس وعزلة من السكارواميسس قدرتها على التضاد مع فطري عفن الموالح الأخضر (بنسليوم دجتاتم) وعفن الثمار الطري (ريزوباس ستلونيفر)، وتم اختيار أكثر العزلات شراسة وتعريفها كعزلة من كانديدا ساك.

تم خلال عام 2017م إجراء تجارب التضاد الحيوي بالعزلة الشرسة على ثمار الفراولة واليوسفي التي تم جمعها من مزارع نموذجية في قرية البدرشين محافظة الجيزة، نجحت تركيزات الخميرة المذكورة في خفض شدة العفن، وكان أعلى التركيزات (106cfu/ml ×1) هو أشدها تأثيراً، إلا أنه لم ينجح وحده في القضاء الكامل على العفن، لكن النتائج تحسنت عند معاملة الثمار معاملة مشتركة من الخميرة وتركيزات كلوريد الكالسيوم خاصة التركيز 1%.

الكلمات المفتاحية: بينيسيليوم ديجيتاتوم، تداول ثمار، ريزوبوس ستولونيفر، كانديدا ساكي.