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Integration between Yeast and Some organic salts Treatments for controlling Tomato Fruits Decay

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Abstract- ***Saccaromyces cerevisiae, Candida tennis and /or some organic salts calcium chloride, sodium benzoate and potassium sorbate were evaluated in vitro for their activity against the fungal growth of Alternaria alternate and Rhizopus stolonifer the causal agents of tomato fruits decay. Results showed that S. cerevisiae has significantly the highest inhibitory effect on the growth of the pathogenic tested fungi followed the yeast C. tenuis. Also, data showed that all concentrations of tested salts could significantly inhibit the mycelia growth of decay fungi comparing with check control treatment. Potassium sorbate showed superior inhibitor effect against fungal growth followed by calcium chloride and sodium benzoate in respective order. Potassium sorbate at all tested concentrations have no significant inhibitory effect against the tested yeast isolates, while significant reduction in the populations of all yeast isolates was observed in sodium benzoate and calcium chloride treatments except at the lowest concentration of 0.5% of both salts. The synergistic effects of yeast and organic salts in combination against the fungal growth were tested. The data revealed that combination of yeast isolates either S. cerevisiae or C. tenuis and potassium sorbate showed the highest significant synergistic effect for inhibiting fungal growth followed by their combination with sodium benzoate and calcium chloride in respective order. Under storage conditions the applied formula containing combination between yeast and organic salts enhanced the efficacy of decay incidence of tomato fruits during storage better than each individual component. Application of S. cerviceae with calcium chloride or with potassium sorbate were the best treatment in reducing the percentage of disease incidence without affecting tomato fruit quality under storage conditions.***

Index Terms- **Saccaromyces cerevisiae- Candida tennis- Tomato -Fruit decay- calcium chloride- sodium benzoate- potassium sorbate.**

I. INTRODUCTION

Fresh –market tomatoes (*Lyopersicon esculentum*, Mill) are grown in most countries around the world. Tomato considered one of the most important vegetables crops for local consumption and exportation purpose. Tomato is susceptible to postharvest diseases caused by various pathogenic fungi. *Botrytis cinerea* [Pers., Ex. Fr.] (gray mould), *Rhizopus stolonifer* [Fhrenb., Fr. Will] (soft rot) and *Alternaria alternata* [(Fr.) Keissl.] (Black mould or Alternaria rot) are the most important decay pathogens of tomato causing postharvest losses at high frequency [1]. Postharvest diseases affect a wide variety of crops particularly in developing countries which lack sophisticated postharvest storage facilities [2]. Losses caused by postharvest diseases are greater than generally realized because the value of fresh fruits and vegetables increases several-fold while passing from the field to the consumer [3]. Postharvest losses are estimated to range from 10 to 30% per year despite the use of modern storage facilities and techniques [4]. Biological control has been advanced as an alternative to synthetic fungicides and considerable success in laboratory and pilot scale tests has been realized utilizing antagonistic microorganisms to control postharvest diseases. Several antagonistic yeasts and bacteria have been isolated and shown to have a broad spectrum of activity against a number of postharvest pathogens on a variety of fruit [5]. Recently, interest has been shown in combining microbial biocontrol agents with other chemical components to increase their activity against postharvest pathogens [6]. Organic salts and yeast often have a broad spectrum of activity, even though the mechanisms by which they inhibit microorganisms are not well understood. Therefore, research is needed to better understand not only the mechanisms through which pathogens can contaminate raw fruits and vegetables but also the procedures for killing or removing pathogens once they are present, either on the surface or in internal tissues, and the analytical methods for their detection. The objectives of this study were to evaluate the effectiveness of calcium chloride, sodium benzoate and potassium sorbate and/or antagonistic yeast isolates to inhibit the mycelia growth of *Rhizopus stolonifer* and *Alternaria alternata* under *in vitro* conditions. Also, pre storage approach of formulation of the bioagents and organic salts was evaluated for their ability to reduce the decay incidence of tomato fruits during storage.

II. MATERIALS AND METHODS

Pathogens and antagonistic microorganisms

The collected tomato fruits which showed decay symptoms were surface disinfested with 2.5% sodium hypochlorite for 3 min, then rinsed with sterilized water, and air-dried. The dried fruits were placed into desecrator for the visual appearance of fungal growth. Infected tomato tissue segments were transferred onto potato dextrose agar (PDA) plates and incubated at 25±2°C. Fungal colonies that appeared were sub-cultured and identified according to the key and description of [7,8,9]. The antagonistic yeasts were isolated from healthy tomato fruits. Fruits were placed into a 500ml beaker containing 200 ml sterilized distilled water. Beaker was placed on a rotary shaker at 100 r.p.m. for 10 minutes. A volume of 0.1 ml of suspended water was then spread on a nutrient yeast extract dextrose agar (NYDA) medium plates and incubated at 25±2°C for 24 hr. Developed several colonies

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were selected and purified in order to obtain single cell colony. Single cell colonies were picked up and maintained onto another slants and kept in a refrigerator for further studies. The purified isolates were identified according to the guide procedures described by [10,11].

Growth media

Potato dextrose agar (Difco Laboratories, Detroit, MI) and NYDB [8 g of nutrient medium (Difco Laboratories, Detroit, MI), 5 g of yeast extract, and 10 g of dextrose in 1 liter of water] were used for growing fungal and yeast isolates tested in the present work. Fungal and yeast cultures were maintained on PDA and NYD agar slant media at 5±2ºC as stock cultures until use. All isolates were activated by growing at the optimum growth conditions at the beginning of the present experiments.

Preparation of fungal spores and yeast cells Suspensions

Pathogenic fungal inocula were grown on PDA medium at 25±2ºC until an abundant heavy growth of conidia was evident. Conidia were harvested by scraping the surface of the colonies with a spatula, transferred to sterilized distilled water and filtered through nylon mesh. All spore suspensions were adjusted with sterile water to give a spore concentration of 106-107 spores per milliliter. Meanwhile, antagonistic yeast bio-agents were grown on NYDB medium and incubated in a rotary shaker at 200 rpm for 24 h at 28±2ºC. The yeast cells were harvested by centrifugation at 6,000 rpm for 10 min, washed twice with 0.05 M phosphate buffer at pH 7.0, and re- suspended in distilled water. The concentrations of yeast cells in the suspensions were adjusted to 3x 108 cells per milliliter. Concentrations of both yeast cells and fungal spores suspensions were adjusted with the aid of a haemacytometer slide.

In vitro growth inhibition of tested microorganisms and yeast

The inhibitory effect of the antagonistic yeast, Saccharomyces cerevisiae and Candida tenuis against the linear growth of tomato fruit decaying pathogenic fungi was evaluated using the modified dual culture technique [12]. Abundant fungal and yeast growth was first prepared. Ten mL of each individual yeast isolate at different cell concentration was grown for 48 h on NYDB broth medium and poured into flasks containing sterilized PDA medium. Before solidifying, each flask was rotated gently to ensure equal distribution of yeast growth, and then poured into 9-cm-diameter Petri dishes. Inoculated plates were incubated for 48 h at 28±1oC. For fungal growth, a 5-mm disk of each tested fungi was transferred to the centre of a PDA plate then incubated for ten days at 25±10C. In vitro antagonistic studies between biocontrol yeast and decay pathogenic fungi were performed on PDA medium in 9-cm-diameter Petri dishes. A 5-mm disk of each yeast growth culture was placed onto the PDA, 10mm from the edge of the Petri dish. Another disk of the same diameter of each decay pathogenic fungal growth culture was placed on the opposite side of the dish at the same distance. The control treatment was inoculated with a culture disk of either a pathogenic or antagonistic culture alone at the same conditions. Both experimental and control dishes were assigned to a completely randomized design, with five replicates per treatment. All inoculated Petri dishes were incubated at 25±1oC and the fungal growth diameter away from and towards the antagonist agent was measured after the pathogenic fungal growth in the control treatment had reached the edge of the Petri dish. This test was repeated three times and the inhibition was calculated as the percentage reduction in colony diameter growth compared with the control. The inhibitory effect of organic salts and/or antagonistic yeast isolates was evaluated *in vitro*. Organic salts, *i.e*. calcium chloride, sodium benzoate and potassium sorbate at concentrations of 0.25,0.5,1.0 and 1.5% against the linear growth of *Alternaria alternata* and *Rhizopus stolonifer* were tested. Different concentrations of organic salts added to conical flasks containing 100 ml of sterilized PDA medium before its solidification to obtain the proposed concentrations. The supplemented media were poured into Petri-dishes (9 cm) about 20 ml each. Control check treatment was PDA medium free of organic salts. Disks (5 mm-diameter) of each pathogenic fungi taken from seven days-old cultures were placed on the centre of Petri-dishes. All plates were incubated at 25±2ºC until the tested fungi reach full growth in check treatment. Reduction in mycelial growth was calculated as percentage of fungal growth diameter in treatment relatively to the growth diameter in control. The inhibitory effect of calcium chloride, sodium benzoate and potassium sorbate at the same previous concentrations on colony formed by antagonistic yeast isolates was assayed in NYPD broth using a modified method of [13]. Aliquots of 100 L of the yeast cell suspension ($3x10^8$) were transferred to glass tubes (180x16 mm) containing 5 mL sterilized distilled water, then the tested calcium chloride, sodium benzoate and potassium sorbate were added individually to each tube to achieve the proposed concentration. All tubes were left for 6 h, then shaking well using magnetic stirrer for 5 min. One ml of each test tube was dispensed into Petri dish and about 20 mL of semi-solidifying sterilized NYPD agar medium were poured into the inoculated plates and rotated gently to ensure equal distribution of the yeast inocula. Control check treatment was the yeast cell suspension free from tested organic salts. All plates were incubated for 72h and then examined. Percent of yeast isolates formed colonies was calculated comparing with their counts in check treatment. All treatments consisted of three replicates, and experiments were repeated three times. The efficacy of combined formula between organic salts and yeast isolates against the growth of decay pathogenic fungi was also evaluated. This test was carried out using Petri dishes containing PDA media supplemented with only one of the above mentioned organic salts concentrations (0.5%). Growth inhibition of pathogenic fungi affected by Yeast isolates in the presence of organic salts in the growth medium was evaluated following the dual culture technique (12). All the procedures of PDA supplementation with organic salts concentrations, plates inoculation with the isolates of yeast and fungal inocula,

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plates incubation and growth reduction measurement were carried out as stated before.

In vivo incidence of postharvest decay of tomato

The efficacy of combined formula between different organic salts at 0.25% and/or yeast isolates was evaluated against the decay incidence of tomato fruits storage conditions. Carnauba wax was used as the basic carrier solution for the mixture of organic salts and yeast isolates. Apparently healthy fruits of tomato (*Lycopersicon esculentum* L.) cv. Kasel Rock recently harvested collected from El-Ebour market the principle commercial market for vegetables and fruits at Cairo, Egypt. Tomato fruits surface disinfested with 2.5% sodium hypochlorite for 3 min, then rinsed with sterilized water and air-dried, then wounded using 1mm. diameter needle at one marked point and dipped for 3 min into the solution of tested mixtures individually, then picked up and left to air dried onto filter paper prior to use. After 1hr all treated fruits were inoculated individually by sprays with a fungal suspension ($1x10^6$ spore/ml). Check treatment was tomato fruits sprayed with sterilized distilled water. Thereafter, all treated fruits were air dried, placed into carton boxes (50 fruits per each), covered with plastic sheet to maintain a relative humidity at 100% and stored in cold room at 10 ± 2^oC for four weeks. Three boxes as replicates were used for each particular treatment as well as the control. At the end of storage period the decayed fruits were counted and then the percentage of disease incidence calculated in relative to control treatment.

Statistical analysis

Tukey test for multiple comparisons among means was utilized for analyzing the obtained results [14].

III. RESULTS AND DISCUSSION

Tomato decay Pathogens

The isolation trails of decayed tomato fruits revealed that the pathogenic fungi were *Rhizopes stolonifer* and *Alternaria alternata*. Meanwhile, the yeast *Candida tennis* was isolated from healthy tomato fruits. In this regards, several investigators isolated similar pathogens causing tomato fruits decay [1,2,3].

In vitro growth inhibition of tested decay Pathogens affected with antagonistic yeast and salts

The efficacy of yeast isolates was evaluated against the growth tomato decay fungi *in vitro*. Results in Table (1) showed that *S. cerevisiae* has significantly the highest inhibitory effect on the growth of the pathogenic tested fungi followed the yeast *C. tenuis*. The linear growth of *A. alternate* and *R. stolonifer* was recorded as 35.6, 22.5 and 66.6, 76.6 mm against the yeast, *S. cerevisiae* and *C. tenuis*, in respective order. Furthermore, reduction in the growth (Fig. 1) of *A. alternate* and *R. stolonifer* was recorded as 60.4 and 75.0%, respectively, when the yeast *S. cerevisiae* was inoculated in the growth medium. Meanwhile, *C. tenuis* showed the lowest inhibitory effect which recorded as 26.0 and 14.8%, respectively.

Table (1) Effect of yeast Saccharomyces cerevisiae and Candida tennis on the growth of pathogenic fungi cause Tomato fruit decay in vitro

Yeast	Fungal growth (mm)	
	A. alternate	R. stolonifer
S. cerevisiae	35.6 d	22.5 e
C. tennis	66.6 c	76.6 b
Control	90.0	90.0 a

Figures with the same letter are not significantly different (P≤ 0.05)

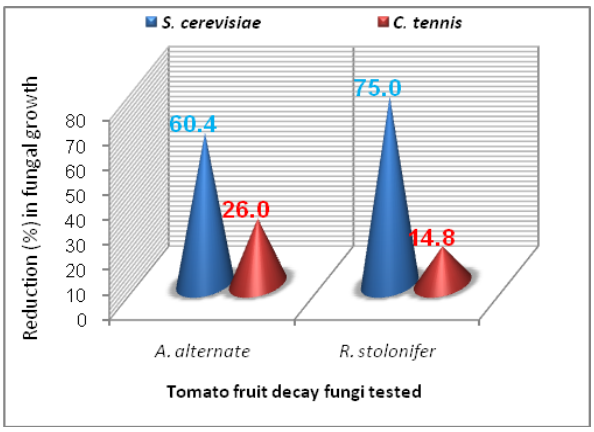

Fig. (1) Reduction in the growth of decay pathogenic fungi in response to antagonistic yeasts

Postharvest biological control is a relatively new approach and offers several advantages over conventional biological control [15,16]. Several biological control agents have been developed in recent years, and a few have actually been registered for use on fruit crops. Yeasts such as *Pichia guilliermondii* [17] and *Cryptoccocus laurentii*, yeast that occurs naturally on apple leaves, buds, and fruit [18] were the first to be applied for control of postharvest decay on fruit. Although there is no doubt that biocontrols are effective, they do not always give consistent results. This could be because biocontrol efficacy is so directly affected by the amount of pathogen inoculums present or antagonistic ability of the bio agent itself [19]. Furthermore, the individual inhibitory effect of tested salts against the linear growth of pathogenic fungi was shown in Table (2) and Fig. (2). Presented data showed that all concentrations of tested salts could significantly inhibit the mycelia growth of decay fungi comparing with check control treatment. Potassium sorbate showed superior inhibitor effect against fungal growth followed by calcium chloride and sodium benzoate in respective order. Illustrated data in Fig. (2) showed that the highest reduction in mycelia growth was recorded at the highest salt concentrations tested. Complete inhibition in all tested fungi was observed at concentration of 1.0 and 1.05% of potassium sorbate. Meanwhile, high reduction recorded in mycelia fungal growth was 63.3, 49.5% and 61.1, 63.0% for *A. alternate* and *R. stolonifer* at concentration of 1.0% of sodium benzoate and calcium chloride, respectively.

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Table (2) Effect of different concentrations of organic salts on the growth of tomato decay fungi *in vitro*

Tested salts	Concentration	Tomato decay fungi	
		A. alternata	R. stolonifer
Calcium chloride	0.25	90.0 a	90.0 a
	0.5	85.4 a	89.0 a
	1.0	60.5 b	60.0 b
	1.5	35.0 c	33.3 c
Sodium benzoate	0.25	90.0 a	90.0 a
	0.5	80.1 b	85.4 a
	1.0	66.6 c	77.8 a
	1.5	33.0 d	45.4 b
Potassium sorbate	0.25	52.7 b	42.3 b
	0.5	25.6 c	23.1 c
	1.0	0.0 d	0.0 d
	1.5	0.0 d	0.0 d
Control		90.0 a	90.0 a

Figures with the same letter are not significantly different (P≤ 0.05)

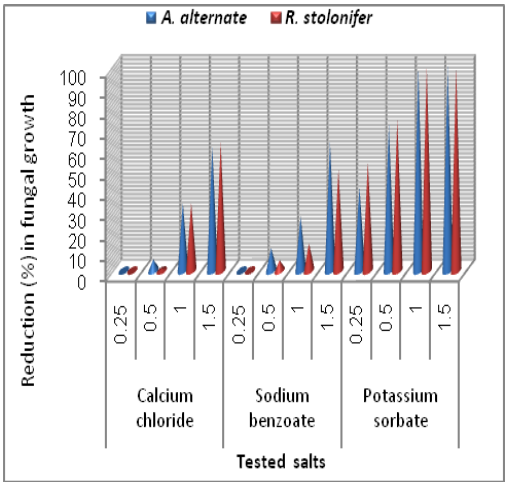

Fig. (2) Reduction in the growth of decay pathogenic fungi in response to different salts

Compatibility with chemicals used during handling is also important. Indications are that biological control agents must be combined with other disease control strategies if they are to provide acceptable control. Several proposed non- fungicidal approaches, including the use of biological control with antagonistic microorganisms, heat treatment, induction of resistance, natural fungicides and plant extracts and essential oils have been extensively studied. Unfortunately, none of them, when used alone, can provide satisfactory levels of decay control when compared with synthetic fungicides [20, 21, 22, 23]. Thus, an integrated disease control strategy has been investigated, in the present study, which is expected to provide high efficacy to bio control yeast agents. Calcium chloride, sodium benzoate and potassium sorbate were evaluated for their effect on viability of yeast isolates as well as the growth of pathogenic fungi. Furthermore, combinations salts and yeast isolates were also evaluated against the decay fungal growth. Data presented in Table (3) showed that all the potassium sorbate at all tested concentrations have no significant inhibitory effect against the tested yeast isolates, while significant reduction in the populations of all yeast isolates was observed in sodium benzoate and calcium chloride treatments except at the lowest concentration of 0.5% of both salts. This reduction was significantly different when compared with either potassium sorbate or control treatments.

Table (3) Counts of yeast colonies affected by different concentrations of salts in vitro

Tested salts	Con.	Number of colonies 10^6 (cfu/mL)	
		S. cerevisiae	C. tenuis
Calcium chloride	0.25	282 a	288 a
	0.5	268 bc	264 bc
	1.0	252 c	250 c
	1.5	230 c	224 c
Sodium benzoate	0.25	284 a	280 a
	0.5	270 b	278 b
	1.0	268 b	272 b
	1.5	266 b	270 b
Potassium sorbate	0.25	286 a	294 a
	0.5	286 a	288 a
	1.0	284 a	282 a
	1.5	280 a	280 a
Control		298 a	292 a

Figures with the same letter are not significantly different (P≤ 0.05)

Furthermore, the synergistic effects of yeast and organic salts in combination against the fungal growth were shown in Table (4). To achieve a suitable efficacy and avoid antagonistic effect that could be happened in the essential oil-yeast formula, melon and rose oils were neglected to be tested as combined factor with yeast isolates referring to their inhibitor effect on the viability of yeast isolates (Table, 3). Therefore**,** the combination between different salts at concentration of 0.5% and yeasts were tested. The data revealed that combination of yeast isolates either *S. cerevisiae* or *C. tenuis* and potassium sorbate showed the highest significant synergistic effect for inhibiting fungal growth followed by their combination with sodium benzoate and calcium chloride in respective order. Data also revealed that that the growth of *R. stolonifer* showed more sensitivity to applied treatment than *A. alternate*. Regarding to the presented data in Tables (1 and 2) it seems that the inhibiting effect on the growth of decay fungi referred mainly to the antagonistic yeast and enhancing with salt combination. In this regard, since no alternative to chemical control alone is as consistently effective as fungicides in reducing postharvest decay, promising alternatives of biological control with beneficial yeasts and organic salts treatments were tested to develop a strategy to provide satisfactory control of postharvest decay on tomatoes fruit in storage. In order to enhance bio control activity of antagonists against fungal pathogens, certain strategies, such as adding calcium salts, carbohydrates, amino acids and other nitrogen compounds to biocontrol treatments, were suggested [24,25].

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Table (4) Growth of decay fungi in response to different salts in combination with yeasts in vitro

Treatment	Fungal growth (mm)	
	A. alternata	R. stolonifer
S. cerevisiae + calcium chloride *	30.2 a	20.7 a
S. cerevisiae + sodium benzoate	27.2 c	18.2 c
S. cerevisiae + potassium sorbate	22.4 d	16.7 d
C. tenuis + calcium chloride	41.2 a	40.3 a
C. tenuis + sodium benzoate	36.4 b	37.3 b
C. tenuis + potassium sorbate	28.3 d	28.4 d

* All tested salts were used at concentration of 0.25%
Figures with the same letter are not significantly different (P≤ 0.05)

Table (5) Effect of organic salts alone or in combination with antagonistic yeasts on decay incidence of tomato fruits

Treatment	Decay incidence of tomato fruits (%)	
	Black rot A. alternata	Soft rot R. stolonifer
S. cerevisiae	41.1 b	39.2 b
C. tenuis	38.7 b	36.3 b
calcium chloride	31.2 bc	30.1 bc
sodium benzoate	27.4 c	25.3 c
potassium sorbate	28.8 c	26.7 c
S. cerevisiae + calcium chloride	21.3 d	19.3 d
S. cerevisiae + sodium benzoate	17.6 e	15.7 e
S. cerevisiae + potassium sorbate	11.8 f	11.3 f
C. tenuis + calcium chloride	14.2 f	13.3 f
C. tenuis + sodium benzoate	16.3 e	16.4 e
C. tenuis + potassium sorbate	13.7 f	12.2 f
Control	77.3 a	78.2 a

* All tested salts were used at concentration of 0.25%
Figures with the same letter are not significantly different (P≤ 0.05)

The results in the present work (Table 5) indicate that the applied formula containing combination between yeast and organic salts enhanced the efficacy of decay incidence of tomato fruits during storage better than each individual component. Results in Table (5) showed that application of carnauba wax containing *S. cerevisiae* or *C. tenuis* and potassium sorbate has superior effect for reducing the percentage of tomatoes fruits decay incidence caused by all the tested pathogenic fungi. It caused reduction recorded between 82.2 and 84.4% (Fig. 3) of all tested fruit decay incidence. *R. stolonifer* showed more sensitivity to most tested treatments than *A. Alternata*.

The potential of using carnauba wax supplemented with *S. cerevisiae* or *C. tenuis* and organic salts to control artificially-inoculated tomato fruits resulted in significant reduction in black rot and soft rot incidence. This combination had a suppressive effect on decay incidence of tomato fruits resulted in significant reduction compared with the other tested factors when applied individually. Many workers also successfully used different yeast isolates for controlling post harvest diseases during storage. They reported that treatment of fruit with yeast microbial agents was an efficient method for control of several postharvest decays [22,26,27, 28,29].

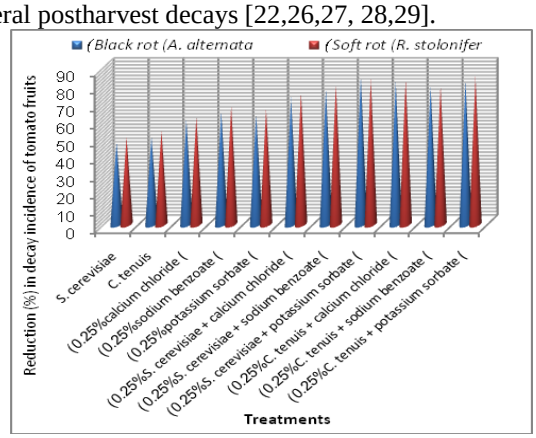

Fig. (3) Reduction in decay incidence of tomato fruits in response to organic salts alone or in combination with antagonistic yeasts

In this regard, many investigators reported that several inorganic and organic salts used in the food-processing industry have antimicrobial properties and could be useful as postharvest disease control treatment. These treatments are generally applied in combination with a surfactant or coating polymer [30,31]. Potassium sorbate and sodium benzoate have been used in commercial citrus packing-houses to control postharvest diseases of different fruits [32,33]. Therefore, alternative fungicide treatments have become an essentiality requirement for the management of postharvest diseases of many fruits. Application of good hygienic practice during production, transport and processing will certainly minimize the contamination of fruits and vegetables and reduce the mould infection. Several types of treatment are known to be partially effective in removing disease-causing organisms from the surface of fruits and vegetables or from contact surfaces during handling. Sodium benzoate is employed in a wide range of preservative applications because of its combination of bactericidal and bacteriostatic action with their properties of being nontoxic and tasteless. They are the most effective preservatives against mould. Several inorganic salts and organic lipophilic acids and their salts, some of which are used, in the food-processing industry, have antimicrobial properties and could be useful as postharvest treatment for decay control. The food preservatives potassium sorbate or sodium benzoate, have antifungal activities against postharvest decaying fungi [30,34]. Using potassium sorbate or sodium benzoate against postharvest diseases of tomato, apple, carrots and potato was reported by [31,32,33]. Also, it was reported that [35] the food preservatives potassium sorbate or sodium benzoate when applied to citrus fruits inoculated with *Penicillium digitatum* had similar fungicidal activity and are equivalent to the traditional treatment used as a postharvest fungicide for controlling citrus fruit decay.

The present study demonstrates that potassium sorbate and sodium benzoate in combination with yeast, *S. cerevisiae* or *C. tenuis* may have potential as environmentally friendly, nontoxic postharvest fungicides for tomato moulds control and could be suggested for

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commercial use in packing-houses in consideration to their wide consumption as safety food preservatives. However, several additional factors should be addressed to optimize disease control and integrate these postharvest treatments into the tomato production system. Protection of contamination of tomato fruits with pathogenic microorganisms should be the goal of everyone involved in both the preharvest and postharvest phases of delivering the product to the consumer. Reduction in the chances of contamination can be achieved, however, through appropriate agronomic practices, harvesting, processing, shipping, marketing and preparation.

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