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Biocontrol of Green Mold of Orange using Some Yeasts Strains and their Effects on Postharvest Quality Parameters

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ABSTRACT

Three isolates of yeasts, *Candida sake* (Cs), *Pichia gluilliermondii* (Pg) and *Pichia membranifaciens* (Pm) were evaluated for their activities in reducing postharvest green mold decay of orange fruits caused by *Penicillium digitatum*. *In vitro* experiments, all tested isolates inhibit the growth of *Penicillium digitatum*. *In vivo* experiments, treating fruits 15 days before harvest and after harvest or only after harvest (natural and artificial infection of fruits) by three isolates of yeasts significantly reduced the disease severity in natural and artificial infection of fruits compared with untreated fruits (control). *Pichia gluilliermondii* (Pg) was the best yeast than others isolates in controlling the disease in artificial infection. All treatments significantly decreased fruit weight loss percentage (%) for nine weeks comparing with control. Results also indicated that all isolates significantly reduced the undesirable fruits percentage during cooling storage period (5°C) compared with control. Prolonging cooling storage at 5°C for nine weeks significantly increased total soluble solids percentage of all treatments and control. Total acidity slightly decreased after cold storage period, while post-harvest treatments with the *Pichia gluilliermondii* caused significant decrease in total acid. Vitamin C was gradually decreased as storage period prolonged, for both control and tested treatments. No constant effects between all treatments and controls, except post harvest treatment fruit with *Pichia gluilliermondii* which caused high decrease in ascorbic acid content compared with controls.

Key words: *Penicillium digitatum,* biocontrol, *Pichia, Candida,* Vitamin C

INTRODUCTION

Green mold of citrus caused by *Penicillium digitatum* (Pers. Fr.) Sacc. is the most economically important postharvest diseases and the limiting factor for storage life of citrus worldwide (Soylu *et al*., 2005; Latifa *et al*., 2011; Badawy *et al*., 2011). Traditionally, plant diseases controlled by applying synthetic fungicides. By contrast, fungicide treatments of postharvest fungi may have several side effects, including the development of resistant strains, environmental contamination and harmful to human health (Rosslenbroich and Stuebler, 2000). Biological control has been developed as an alternative method to us the fungicides for many plant pathogenic fungi (Wilson *et al*., 1993). There are many studies demonstrating postharvest disease control of different fruit species by using bacteria, yeast and other microorganisms as biological agents which protect fruits and vegetables against postharvest pathogens (Janisiewicz and Koresten, 2002; Cota *et al*., 2008). Currently, two commercial products, Aspire (based on *Candida oleophila* Montrocher) and

Yield Plus (based on *Cryptococcus albidus* (Saito) Skinner) have been registered for controlling such diseases in the United States or South Africa (Janisiewicz and Koresten, 2002; Fravel, 2005). The antagonist yeast, *Rhodotorula glutinis* (Fresenins) Harrison has been proposed for the postharvest biological control of blue mold decay of pears (Zhang *et al*., 2005), green mold decay of oranges (Zheng *et al*., 2005), gray mold decay of strawberry (Zhang *et al*., 2007a) and gray mold decay of apple (Sansone *et al*., 2005), as well as sweet cheery (Tian *et al*., 2004). Yan *et al*. (2008) reported that use of yeast strain of *Pichia gluilliermondii* was effective in controlling tomato fruit caused by *Rhizopus nigricans* under storage conditions.

The objective of this study was to determine the postharvest control of green mould decay of orange by using *Candida sake* (Cs), *Pichia gluilliermondii* (Pg) and *Pichia membranifaciens* (Pm) *in vitro* and *in vivo*. Also, physical (weight loss% and Fruit decay%) and chemical characteristics (Total Soluble Solids (TSS%), Titratable acidity and Vitamin C contents) of treated fruits were also determined biweekly.

MATERIALS AND METHODS

Pathogen inoculums: *Penicillium digitatum* was isolated from infected orange fruits and cultured on potato dextrose agar (PDA: extract of boiled potatoes, 200 mL; dextrose, 20 g; agar, 20 g and distilled water, 800 mL) at 25°C for 7 days. Spore suspensions were prepared by removing the spores from the sporulating edges of 7 days old culture with a bacteriological loop and suspending them in sterile distilled water. Spore concentrations were determined with a hemacytometer. The spore concentrations of *Penicillium digitatum* were adjusted to 5×10^4 spores mL^{-1}.

Antagonists: The yeast antagonist *Candida sake* (Cs), *Pichia gluilliermondii* (Pg) and *Pichia membranifaciens* (Pm) used in this study were isolated and identified previously by authors. They were identified based on their morphological and physiological characteristics (Hashem, 2005).

The yeasts were cultivated in 250 mL erlenmeyer flasks with 50 mL of nutrient yeast dextrose broth (NYDB: nutrient broth 8 g L^{-1}; yeast extract 5 g L^{-1}, dextrose 10 g L^{-1}) on a rotary shaker at 200 rpm for 24 h at 28°C. The medium was centrifuged at 2500×g for 10 min. The yeast cells were resuspended in sterile distilled water and adjusted to concentrations of 10^8 CFU mL^{-1} for *Candida sake*, *Pichia gluilliermondii* and *Pichia membranifaciens*, respectively by means of a hemacytometer.

Biological control studies

***In vitro*:** Dual culture was carried out according to Gholamnejad *et al*. (2010). Yeast strains were streaked on half of plates. After incubation at 20°C in the dark for 48 h, a plug (10 mm diameter) cut from the leading edge of a 7 day old culture of *P. digitatum* on PDA medium was placed on the other half of the plate. Potato dextrose agar inoculated with the pathogen alone served as the control. Plates were incubated at 20°C for 7 day then the colony diameters were measured.

All tests were carried out in four replicates. The percent of the growth inhibition was calculated using the formula n = (a-b)/a×100, where n is the percent growth inhibition, a is the colony area of *P. digitatum* growth inhibition and b is the colony area of treated , as described previously by Etebarian *et al*. (2005).

***In vivo*:** Orange trees (*Citrus sinensisl*) Balady cultivar forty years old, in good physical condition, free of insects, damage and diseases are selected and used as plant material of this investigation.

Three trees for each treatment were used as a replicates (three branches were used of each tree). Trees were spraying 15 days before harvested (pre harvest treatments) time with 5 L of yeasts suspension per tree afternoon.

Orange fruits (Baladi cultivar) were harvested at commercial maturity. Fruits were used immediately after harvested, surface washed with tap water and then air dried. Fruits randomly divided into 2 equal groups; first group was wounded in three points around the entire equatorial region of each orange in depth of 5.0 mm with a 1.25 mm diameter needle at the equator of each fruit to prepare for inoculation. The second group, fruits were used without wound. The 7 treatments were carried out as following:

- Preceding harvest spraying with *Candida sake*+post harvest spraying with *Candida sake* isolate
- Post-harvest spraying with *Candida sake* isolate
- Preceding harvest spraying with *Pichia gluilliermondii*+postharvest spraying with *Pichia gluilliermondii* isolate
- Post harvest spraying with *Pichia gluilliermondii* yeast isolate
- Preceding harvest spraying with *Pichia membranifaciens*+post harvest spraying with *Pichia membranifaciens* isolate
- Post-harvest spraying with *Pichia membranifaciens* isolate
- Infected control (spraying with *P. digitatum*)
- Healthy control (spraying with tap water)

This protocol was repeated in a separate trial and conducted for two seasons (2009 and 2010). All treatments were inoculated by dipping in suspension of *Penicillium digitatum* (5×10^4 spores mL^{-1}). Fruits were air dried, then put into 440×300×100 mm plastic trays, retained high humidity (about 85-90%) and stored at 5°C. Infection incidence was observed periodically up to 30 days in the first group and 60 days in second group percentage of disease severity was calculated by dividing the weight of infected area by weight of orange. There were three replicate trials of 5 fruits per replicate.

Representative samples of five fruits per replicate were taken biweekly storage period until the percentage of decay reached 50%. Physical and chemical fruit properties were estimated biweekly.

Physical characteristics

Fruit weight loss (%): This character was determined by weighing 5 fruits in each replicate. Percentage of weight loss was calculated by determination the progressive reduction in fruit weight during storage period relative to the original fresh weight at the beginning of storage.

Undesirable fruits%: Caculated by:

$$\text{Undesirable fruit (\%)} = \frac{\text{No. of undesirable fruits}}{\text{Total No. of fruit}} \times 100$$

Chemical characteristics

Total Soluble Solids (TSS%): Total Soluble Solids (TSS) were determined by measuring the refractive index of the same juice with a hand refractometer and the results were expressed as

percentages (g/100 g fruit weight) (Larrigaudiere *et al.*, 2002). TSS% in fruit juice was determined by using a hand refractometer.

Titratable acidity: :Total acidity in fruit juice was determined by titrating fruit juice against 0.1 N NaoH with phenol phethalin as an indicator and calculated as gram citric acid/100 mL fruit juice.

Vitamin C contents: The 2, 6-dichloroindophenol titrimetric method (AOAC, 1995) was used to determine the ascorbic acid content of pressed fruit juice. Results were expressed as milligrams of ascorbic acid/100 g sample (Ozden and Bayindirli, 2002).

Ascorbic acid content was determined by using 2.6 dichlorophenol. Indophenol as described by AOAC (1995).

Statistical analysis: All data obtained throughout the study were tabulated and statistically analyzed, according to methods described by Snedecor and Cochran (1990) and using L.C.D test to recognize the significance of the differences among various treatments means.

RESULTS AND DISCUSSION

Biological control studies

***In vitro*:** An antagonistic effect was observed on PDA plates which had *Penicillium digitatum* followed by *Candida sake* (Cs), *Pichia gluilliermondii* and *Pichia membranifaciens*. The three antagonists tested yeasts could inhibit significantly the *P. digitatum* mycelia growth of in dual culture. However, there were significant differences among yeast strains. Growth inhibition of *P. digitatum* by strain (Pm) was significantly greater than those by of other strains, whereas strain (Pg) had less effect on the growth of the pathogen (Table 1).

In vivo

Effect of yeasts on artificially inoculated infection: In 2009 and 2010 seasons (Table 2), treated fruits with three isolates of yeasts (Cs, Pg and Pm) were effective in controlling green mold of fruits caused by *P. digitatum* at 5°C compared with control. There was no significant difference found between the two methods of applying yeasts on fruits. However, spraying fruits with yeasts,15 days before harvest and after harvest exhibited higher decrease in disease severity than spraying yeasts one time after harvest. Results also indicated that, *Pichia gluilliermondii* (Pg) showed higher decrease in disease severity of green mold of orange fruits than other tested yeast strains.

Effect of yeasts on naturally infection: Data presented in Table 3 showed that all treatments with tested yeasts to control green mold of fruits stored at 5°C disease severity was significantly

Table 1: Percentage of *P. digitatium* growth inhibition caused by Cs, Pg and Pm yeasts strains *in vitro*

Yeasts	Inhibition (%)
Candeda sake (Cs)	20.1[b]
Pichia gluilliermondii (Pg)	25.2[a]
Pichia membranifaciens. (Pm)	15.2[c]

Values in the column followed by different letters indicate significant differences among treatments according to lease significant differences test (p = 0.05)

Table 2: Effect of applying three isolates of yeasts at different times of harvest on developing citrus mold disease of citrus wounded fruits inoculated by *P. digitatum*

	2009 season			2010 season		
Antagonistic yeasts	Before and after sparing (%)	After sparing (%)	Mean	Before and after sparing (%)	After sparing (%)	Mean
Cs	27.37^{cd}	32.17^{bc}	29.77^{b}	30.33^{bc}	32.67^{bc}	30.5^{b}
Pg	23.47^{d}	26.67^{cd}	25.07^{c}	22.0^{c}	27.5^{bc}	24.75^{c}
Pm	32^{bc}	36.67^{b}	34.33^{b}	33.7^{bc}	38.67^{b}	36.18^{b}
Control	64.67^{a}	69.33^{a}	66.95^{a}	69.33^{a}	67.33^{a}	68.33^{a}
Mean	36.85^{a}	41.21^{a}		38.84^{a}	42.04^{a}	

Cs: *Candida sake*, Pg: *Pichia gluilliermondii*, Pm: *Pichia membranifaciens*, Values in the column followed by different letters indicate significant differences among treatments according to lease significant differences test (p = 0.05)

Table 3: Effect of applying three isolates of yeasts (15 days before harvest and after harvest or after harvest to natural infection of unwounded citrus fruits *P. digitatum*

	2009 season			2010 season		
Antagonistic yeasts	Before and after sparing (%)	After sparing (%)	Mean	Before and after sparing (%)	After sparing (%)	Mean
Ca	14.2^{bc}	19.67^{bc}	16.93^{b}	15.27^{b}	21.33^{c}	18.3^{b}
Pg	11.0^{c}	15.6^{bc}	13.3^{b}	9.67^{c}	14.87^{b}	12.27^{b}
Pm	18.53^{bc}	24.0^{b}	21.27^{b}	18.27^{b}	25.67^{c}	21.97^{b}
Control	52.27^{a}	53.67^{a}	52.97^{a}	52.33^{a}	58.0^{a}	55.17^{a}
Mean	24.0^{a}	28.23^{a}		23.88^{a}	33.96^{a}	

Values in the column followed by different letters indicate significant differences among treatments according to lease significant differences test (p = 0.05)

lower than non treated fruits (control). Results also demonstrated that no significant differences were found between yeasts isolates in controlling green mold disease. Also, there is no significant differences were observed between the two tested methods of yeasts applications on fruits. Spraying fruits 15 days before harvest and after harvest were better than spraying only after harvest.

Results reported herein are in accordance and confirm those reported by several researches. They reported that, yeasts are one of most common biological control agents against postharvest of certain disease (Janisiewicz and Koresten, 2002; Cota *et al*., 2008). The ability of the yeasts to rapidly colonize the wounded or surface of fruits may indicate their ability in biocontrol or by nutrient competition and/or site exclusion. Spraying antagonistic yeasts before picking fruits may protect the wounds that from during picking and transport, prevent the invasion of pathogenic fungi and prevent the incidence of fruit rot during storage. Also, the results showed that applying *Pichia gluilliermondii* (Pg) for controlling green mold of orange fruits in artificial or by *P. digitatum* was the best than other yeasts such results are in agreement with those reported by Zhang *et al*. (2007b) and Zhao *et al*. (2009).

Physical characteristics

Fruit weight loss%: Data in Fig. 1 show that fruit weigh less percentage increased by extending cold storage period, during the two investigated season. These results could be due to the loss in moisture content. This agrees with the finding of Elshiekh and Abo-Goukh (2008), they found that weight loss percentage progressively increased during storage at 18±1 for 3 months.

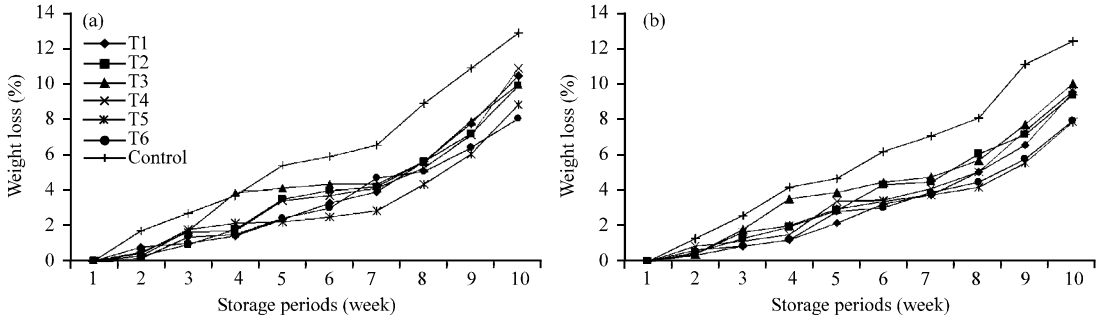

Fig. 1(a-b): Effect of *Candida sake, Pichia gluilliermondii* and *Pichia membranifaciens* applications on Fruit weight loss% of Balady Oranges, during cold storage for nine weeks in (a) 2009 and (b) 2010 seasons. T1: Preceding harvest spraying with isolate *Candida sake* (Cs)+post harvest spraying with *Candida sake* (Cs). T2: Post-harvest spraying with isolate *Candida sake* (Cs). T3: Preceding harvest spraying with isolate *Pichia gluilliermondii* (Pg)+postharvest spraying with *Pichia gluilliermondii* (Pg). T4: Post harvest spraying with isolate *Pichia gluilliermondii* (Pg). T5: Preceding harvest spraying with isolate *Pichia membranifaciens* (Pm)+post harvest spraying with *Pichia membranifaciens* ((Pm). T6: Post-harvest spraying with isolate *Pichia membranifaciens* ((Pm). T7: Control (spraying with Tap water)

Generally, results indicate that all treated fruits by three isolates of yeasts significantly decreased fruit weight loss percentage (%) during cooling storage for nine weeks, comparing with control. Treated fruits with *Pichia gluilliermondii* (Pg) in (pre+post) harvest caused significant decrease in fruit weight loss percentage by (47.19, 44.74%), respectively in 2009 and 2010 seasons compared with controls. Post harvest treatment with *Pichia gluilliermondii* has the best result, it caused significant decrease in fruit weight loss percentage by (44.87, 47.30%), respectively in the two seasons compared with controls.

The positive effects of yeast isolates on decreasing fruit decay might be attributed to making a thin film of yeast surrounding the fruit peel, meanwhile induced a modification of microclimatic of fruits. From such results we can notice that there was no significant difference between times of application within the same yeast treatments during the two seasons.

Undesirable fruits percentage: Data in Fig. 2 illustrates that undesirable fruit percentage increased by extending cooling storage period. These finding are in agreement with that reported by Shehata (1998).

Generally, results indicated that all treatments significantly reduced the undesirable fruits percentage during cooling storage for nine weeks, compared with controls during the two experimental seasons. Also there were significant differences between the effect of *Pichia gluilliermondii* and the other two yeasts during the two seasons. Where post-harvest treatment with *Pichia gluilliermondii* (Pg) has the best result, it caused significant decrease in undesirable fruits percentage by (78.71, 61.07%), respectively, in 2009 and 2010 seasons, compared with controls. While (pre+post) harvest treatment with *Pichia gluilliermondii* (Pg) caused significant decrease in undesirable fruit percentage by (52.48, 50.07%), respectively in 2009, 2010 seasons compared with controls. There was no significant between times of application. These results are in agreement with Hong *et al.* (1998), Mari and Guixxardi (1998) and Zhang *et al.* (2007b).

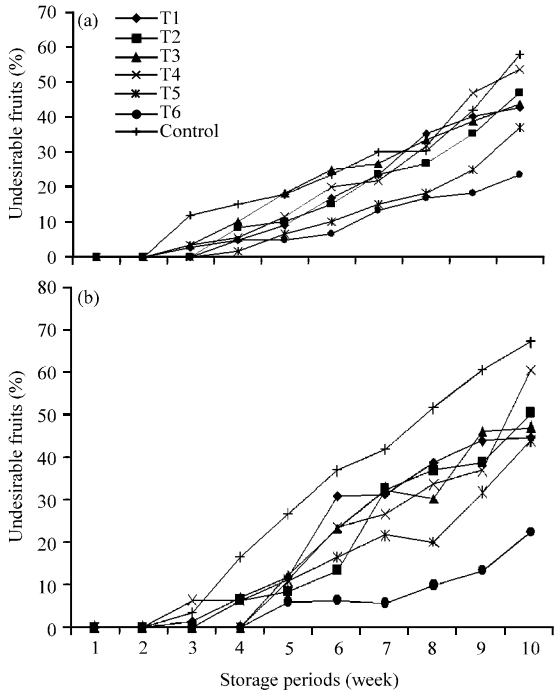

Fig. 2(a-b): Effect of *Candida sake*, *Pichia gluilliermondii* and *Pichia membranifaciens* applications in controlling undesirable fruits percentage of "balady" oranges, during cold storage for nine weeks, in a (a) 2009 and (b) 2010 seasons. Whereas T1-T6 As described in footnote of Fig. 1

This finding may be due to the competition for nutrients and space involved along with direct parasitism as for the yeast strains antagonist of *P. digitatum* in citrus fruit, in addition to the ability of yeast cells to grow very quickly and thus to remove nutrients and space from pathogen, also they may be able to produce hydrolytic enzymes capable of attacking the pathogens cell walls and extracellular polymers that appear to have antifungal activity. It is possible to hypothesize the induction of resistance in the host through the accumulation of phytoalexins like scoparone and scopoletin in citrus fruits.

Chemical characteristics

Total Soluble Solids (TSS%): From Fig. 3 it is clear to notice that prolonging cooling storage at 5°C for nine weeks caused an increase of total soluble solids percentage of control by (9.82, 1.05%) respectively in 2009 and 2010.

These results may be due to the loss in moisture content which lead to the concentration of total soluble solids. These finding are in agreement with Salih and Abdalla (1982), Attia (1995) and Marcilla *et al.* (2006).

From figure we can notice that there were no significant differences between the effect of both *Candida sake* and *Pichia membranifaciens* compared with controls, also there were no significant differences between times of applications. The two experimental seasons 2009, 2010 took similar trend, where treatment with *Candida sake* or *Pichia membranifaciens* either (pre+post) harvest treatments or post-harvest treatments significantly reduced TSS%, compared with controls, during cold storage.

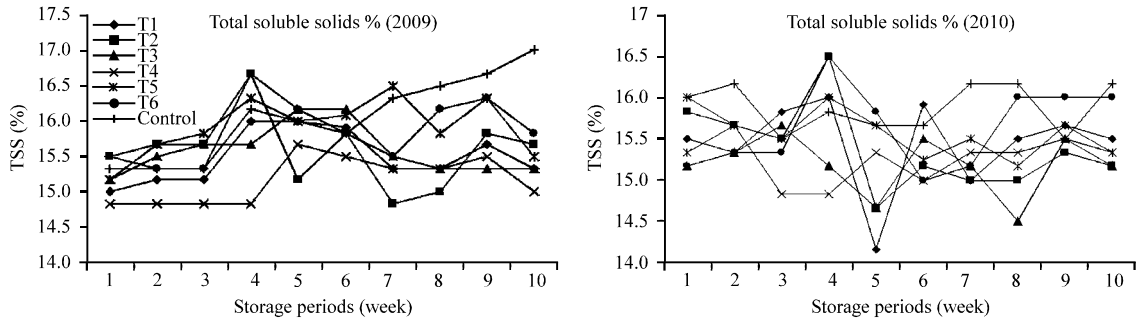

Fig. 3: Effect of *Candida sake*, *Pichia gluilliermondii* and *Pichia membranifaciens* applications on Total Soluble Solids percentage (TSS%) of balady Oranges, during cold storage for nine weeks, in (2009) and (2010) seasons. Whereas T1-T6 As described in footnote of Fig. 1

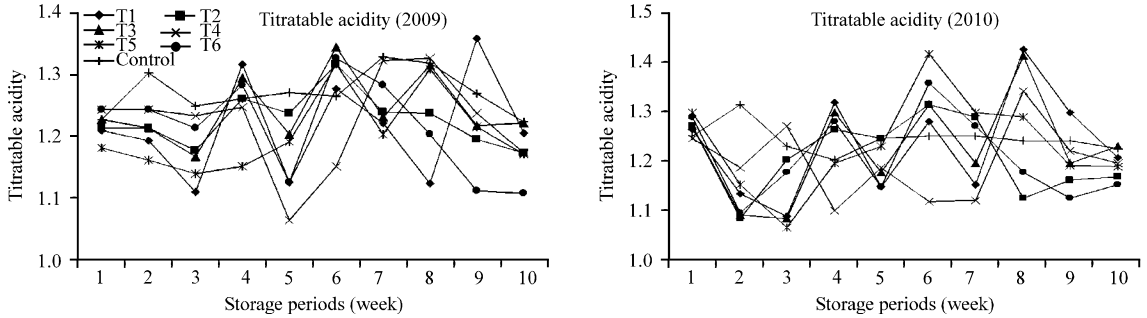

Fig. 4: Effect of *Candida sake*, *Pichia gluilliermondii* and *Pichia membranifaciens* applications on Titratable acidity, of balady Oranges, during cold storage for nine weeks, in (2009) and (2010) seasons. Whereas T1-T6 As described in footnote of Fig. 1

Moreover (pre+post) harvest treatments with *Pichia gluilliermondii* caused in no significant decrease in TSS% by (0.75%) compared with control in 2009. While it caused significant decrease in TSS% by (2.39%) compared with control in growing season 2010. This decrement may be due to the transformation in part of TSS to acids. While post harvest treatments with the same yeast caused in slight decrease in TSS% by (1.12, 1.64%) compared with controls in 2009 and 2010, respectively.

These finding are in agreement with Marcilla *et al.* (2006). They reported that only small differences were found between (SSC) of orange fruits just after harvest and after two months of cold storage at 5°C.

Titratable acidity: From Fig. 4 it was clear to notice that there were no significant differences between the effect of all treatments and controls, on Total Acidity (TA). Also the two experimental seasons 2009, 2010 took similar trend. There were no differences between times of applications. These results are agreement with Marcilla *et al.* (2006).

Vitamin C contents: During cold storage for nine weeks it is clear from Fig. 5 that Vitamin C was gradually decreased as storage prolonged, for both control and all treatments. This agree with Trifiro *et al.* (1995) who describe maximum decrease of 8% in ascorbic acid in fresh juices of blood

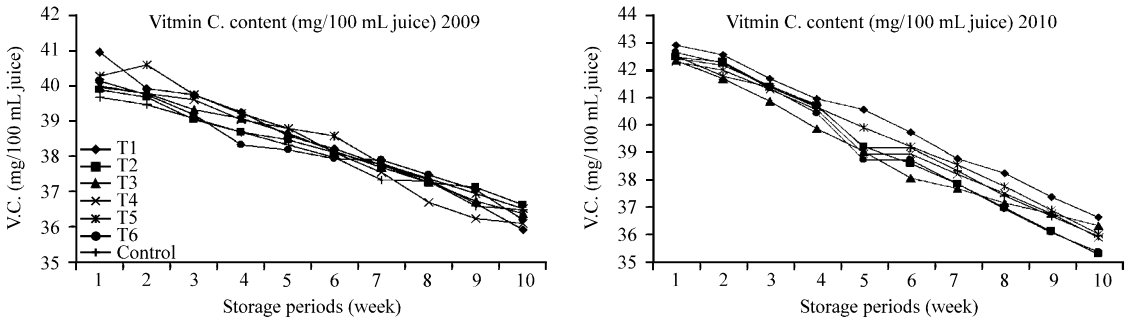

Fig. 5: Effect of *Candida sake*, *Pichia gluilliermondii* and *Pichia membranifaciens* applications on Vitamin C content (mg/100 mL juice) of balady Oranges, during cold storage for nine weeks, in (2009) and (2010) seasons. Whereas T1-T6 As described in footnote of Fig. 1

Oranges stored at 3°C for 30 days, Kabasakalis *et al.* (2000) and Del Caro *et al.* (2004) reported that cold storage of fresh Orange juice for 31 days lost 7 to 13% of its ascorbic acid content. Also they reported that segments of Minneola and salustiana Orange cultivars showed a significant decrease in ascorbic acid after 12 days of storage at 4°C. Ajibola *et al.* (2009), found a significant decrease in ascorbic acid after 4 weeks of cold storage at 4±1°C.

Data illustrated in such table reported that there was no significant between all treatments and control expert post harvest treatment with *Pichia gluilliermondii*. In postharvest treated fruits with *Pichia gluilliermondii* lost (9.65, 17.0%) of its ascorbic acid content after nine weeks of cold storage in 2009 and 2010, respectively.

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