BULG. J. PLANT PHYSIOL., 2003, 29(1–2), 44–54

CHILLING INJURY AND PEROXIDASE ACTIVITY CHANGES IN "FORTUNE" MANDARIN FRUIT DURING LOW TEMPERATURE STORAGE

F. El-hilali*[1], A. Ait-Oubahou[2], A. Remah[1], O. Akhayat[3]

[1]Department of Plant Protection, Institut Agronomique et Vétérinaire Hassan II, Complexe Horticole d'Agadir, B.P. 18/S, Agadir 80.000, Morocco

[2]Department of Horticulture, Institut Agronomique et Vétérinaire Hassan II, Complexe Horticole d'Agadir, B.P. 18/S, Agadir 80.000, Morocco

[3]Faculté des Sciences, Université Ibnou Zohr, Agadir

Received November 22, 2002

Summary. "Fortune" mandarin fruit kept at temperatures below 8 °C, showed severe peel pitting before even the transfer to ambient temperature. The severity of chilling injury increased by lowering temperature and prolonging storage duration. Preharvest spray of calcium nitrate and potassium nitrate 4 weeks prior to harvest improved the mineral content of fruit peel at harvest and contributed to a significant reduction of the peel disorders after storage at 4 °C and 8 °C. Rind firmness and juice acid content (A) were higher than control in treated fruit. Several other parameters such as rind thickness, juice content, total soluble solids (TSS) and TSS/A ratio, were not significantly affected by the treatments. Peroxidase (POD) activity increases continuously at 4 °C over the period of storage and the highest activity was observed in non-treated fruit. By contrast, at 8 °C POD activity increased during the first two weeks followed by a sharp decline for the rest of storage period. The increase in POD activity closely correlated with the importance of chilling disorders in the fruit peel.

Key words: chilling ingury, Ca, K, fortune mandarin frut, fruit quality, mineral content, peel disorders, POD activity.

* Corresponding author, e-mail: elhilali@iavcha.ac.ma

Introduction

Many fruits and vegetables from tropical and subtropical origin exhibit a physiological disfunction when exposed to non-freezing temperatures below 12 °C. Several physiological and biochemical alterations occur in these produce in response to chilling stress and often termed “chilling injury”. The indications of chilling symptoms vary with crop, temperature, physiological stage and storage length of exposure. Chilling disorders may appear already at low temperature storage for some crops but are usually enhanced or more visible when transferred to non-chilling temperatures. For citrus fruit, visible chilling injury symptoms include, excess weight loss, surface pitting and necrotic areas. Fortune mandarin fruit (*Citrus clementina* Hort. Ex. Tanaka x *Citrus reticulata* Blanco) is a late maturing variety known for its susceptibility to peel disorders at storage temperature below 10 °C (Martinez-Javega et al, 1992; Agusti and Almela, 1989). Peel pitting disorder in “Fortune” mandarin fruit as well as in other sensitive citrus species is characterized by sunken lesions and darkening of oil glands, which can affect large parts of the fruit. In addition, a number of metabolic and physiological processes are disrupted by chilling temperatures. Several mechanisms of chilling injury have been proposed. During the oxidative damage of the plant tissue as a response chilling temperatures, many free radicals are induced (Hariyadi and Parkin, 1991).

Plant resistance to different stresses is often expressed by an increase ih protective enzymes such as peroxidases and catalases. Peroxidase activity is expressed when plant tissue is subjected to stresses such as low temperature, pathogen infection, UV radiation, poisonous gases or heavy metals (Ashraf et al., 1994; Scalet et al., 1995) or during the senescence (Grover and Sinha, 1985). Peroxidaes have free radical scavenger properties under chilling conditions (Burris, 1960). Therefore, they play a major role in protecting the cell from hydrogen peroxide and thus reduce related stress damages (Edreva et al., 1993; Martinez-Tellez and Lafuente, 1993; Scalet et al., 1995) reported a positive correlation between phenylalanine ammonia-lyase (PAL) activity and the susceptibility of the tissue to chilling injury and little changes in polyphenol oxidase (PPO) and peroxidase (POD) in the peel of Fortune mandarin fruit stored for 25 days at 2.5 °C.

“Fortune” mandarin fruit shows positive effects of the calcium in reducing peel disorder incidence (Zaragoza et al., 1996; Ait-Oubahou et al., 2000). In avocado, Chaplin and Scott (1980) reported the reduction of chilling symptoms in fruits with high amount of calcium. The present paper summarizes some effects of preharvest application of Ca^{2+} and

K^{+} on “Fortune” fruit quality, chilling injury and POD activity in the peel of fruits kept at 4 °C and 8 °C for up to 4 weeks period.

Materials and Methods

A commercial orchard of "Fortune" mandarin was selected in the area of Marrakech. Eleven years old trees grafted on sour orange rootstock and planted at a spacing of 5 m×4 m were selected. The soil is a silty-loam with a pH of 7.6. Trees are drip irrigated and fertilizers are applied manually. During the winter or early spring, micronutrients are applied when needed. No disease symptom or mineral deficiency were apparent on selected plots. Soil and leaf analyses are done regularly to monitor the mineral nutrition status of the plantation. Different treatments containing calcium and potassium were sprayed on trees 4 weeks prior to harvest on January 19, 2001. For each treatment, 10 trees were selected in the same row and sprayed with an average volume of 8 to 10 liters of the prepared solution. The composition of tested treatments is given in Table 1.

After harvest, fruit were held at 4 °C and 8 °C for 2 and 4 weeks. Observations are performed on a sample of 10 fruits with four replicates at each observation date. Leaf sample is composed of about 20 mature leaves (6 to 7 months old) collected from different parts of the tree 1 day before application of treatments and at harvest. Studied parameters include firmness (g-force), juice content (%), total soluble solids (TSS) (°Brix) and juice acid content (A) in (%). Peel disorders were evaluated based on the presence and/or the absence following hedonic scale (0 – sound fruit; 1 – less than 10%; 2 – 10–20% pitting, 3 – 30–40% and 4 – more than 50% pitting) and a chilling index (CI) was calculated as follows:

$$CI = \frac{\Sigma \text{ (number of fruit with chilling x score of severity)}}{\text{total number of fruit}}$$

For enzyme studies, samples of fruit flavedo are collected at each sampling period. Horseradish peroxidase and anti-peroxidase were obtained from Sigma, France. Samples of fruit peel were collected and packed tightly in plastic film and stored at – 20 °C or below for further use. The extracts were prepared from three samples and two duplicates for measurements of peroxidase (POD) activity. Extraction of the per-

Table 1. Composition of treatments sprayed preharvest on trees 1 month prior to harvest.

Treatment	N (g/tree)	Ca^{2+} (g/tree)	K^+ (g/tree)
T0: Control	—	—	—
T1: 1% $Ca(NO_3)_2.4H_2O$	15.50	30.00	—
T2: 2% $Ca(NO_3)_2.4H_2O$	31.00	60.00	—
T3: 1.19% KNO_3	15.47	—	54.70
T4: 2.38% KNO_3	30.94	—	109.50
T5: 3.57% KNO_3	46.41	—	164.10

oxidase enzyme was performed according to a modified method described by Zheng et al. (1993). As the citrus peel contains pigments and essential oils, which may interfere with the measurement of enzyme activity, these compounds, were eliminated with cold acetone. Three grams (3 g) samples were crashed in liquid nitrogen, homogenized in cold acetone and filtered under a vacuum using cold acetone until a colorless powder was obtained. The powder was then dried under vacuum and stored at (–20 °C) or below for enzyme activity assay. Extraction procedure consists of taking 0.3 g of obtained white powder, grind for 10 min in 0.1 M sodium acetate buffrer, pH 7 until a homogenous paste was obtained. The mixture is centrifuged for 30 min at 15 000×g at 4°C. The supernatant was collected and used for peroxidase activity and protein content. Peroxidase activity was measured following the method described by Grzywnowicz et al. (1992). For POD activity, the guaiacol was used as an electron donor for oxidation. To 0.1 ml of supernatant, 0.09 ml of 30 mM H_2O_2, 50 µl of 1.5 mM of guaiacol and 2.75 ml of 0.1 M phosphate buffer, pH 7 was added. The mixture was shaken gently and the absorbance reading was done after 15 and 30 s at 470 nm. Enzyme activity was evaluated as follows: $A_{POD} = A_{470} \times (V.t)^{-1}$ where; A_{470} – absorbance at 470 nm; V – volume of the sample and t – time of the reaction. Total protein concentration was measured by dye binding (Bradford, 1976). Peroxidase specific activity is obtained by dividing units of POD by total soluble protein in the sample.

Enzyme-linked-immunosorbent assay (ELISA) test was performed following the modified method of Koenig (1981). Buffers used are; substrate buffer 10% (v/v) diethanolamine pH 9.8; coating buffer: 0.05 M sodium carbonate adjusted to pH 9.6 with 1.0 N HCl; rinsing buffer phosphate buffered saline tween (PBS-Tween) – 0.02 M PBS-Tween, pH 7.4 containing 0.15 M NaCl, 0.05% (v/v) Tween 20; extracting buffer – PBS-Tween containing 20 g/l (PVP) polyvinylpyrrolidone (MW 44 000), 2 g/l ovalbumin and 2 g/l sodium diethyldithiocarbamate, adjusted to pH 6.0 using 1.0 N HCl.

The samples were diluted 4 times and 200 µl were added into each well (2 wells per sample) and incubated over night at 4 °C. After washing with PBS-Tween, the plates were loaded with anti-peroxidase Horseradish from Sigma (P-7899) diluted 1 000 times, and then incubated at 37 °C for 4 hours. After washing with PBS-Tween, the anti-rabbit alkaline phosphatase (Sigma, A-3687) diluted 15 000 times and 200 µl were added to each well. The plate was incubated at 37 °C for 4 hours. After washing with PBS-Tween, the substrate (p-nitrophenyl phosphate: 1mg/ml) was added (200 µl per well) and the reaction was stopped after 30 min by adding 50 µl of 3 M NaOH. Photometric measurements at 410 nm were made using Dynatech ELISA reader, Model MR 250.

Leaf and peel mineral content were determined following conventional methods used in soil, plant and water analyses laboratories. All data were subjected to analysis of variance using statistical package COSTAT or STATITCF and mean separation was performed following Newman and Keuls range test at 5% level.

Results and Discussion

Preharvest sprays of trees with calcium nitrate and potassium nitrate 4 weeks prior to harvest and storage of fruit at 4 °C and 8 °C affected positively some parameters studied in our work.

Peel mineral content

Preharvest sprays of Ca and K increased Ca and K mineral content of the fruit peel at harvest (Fig. 1). All treatment showed a slight increase in their mineral content over the control. The highest concentration of Ca^{+} of 4.14% in the fruit peel was obtained from 2% $Ca(NO_3)_2.4H_2O$ treatment. Non-treated fruit have the lowest content with 2.78% Ca^{2+}. Potassium content in fruit rind of treated fruit increased slightly in all treatments over the control. However, no difference between various concentrations of potassium applied regarding potassium peel content.

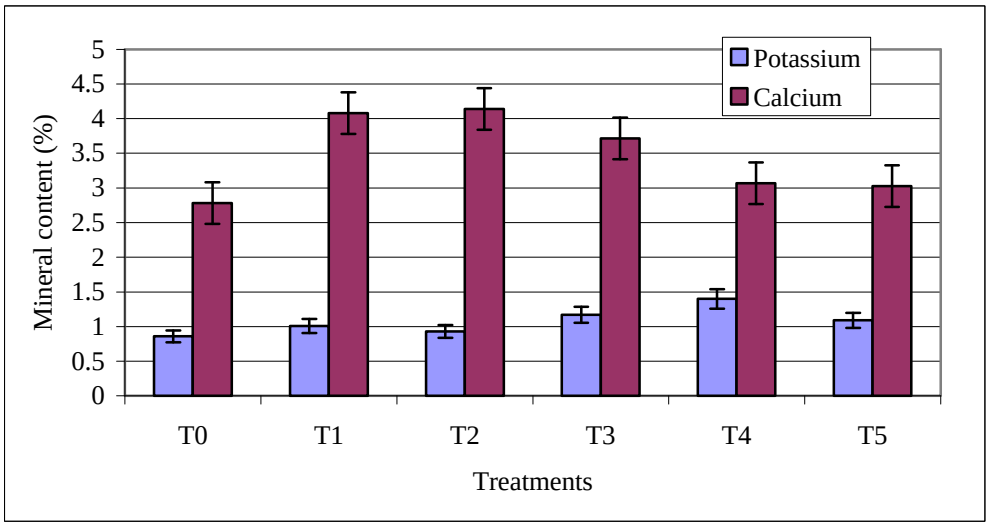

Fig. 1. Effect of preharvest foliar spray of fertilisers on calcium and potassium content (%) in the peel of "Fortune" mandarin fruit at harvest.

Peel pitting disorder

Temperature had a significant effect on the incidence of peel disorders (Table 2). More peel pitting were observed at 4 °C than at 8 °C. and the intensity of disorders in fruit rind increases with storage duration. The effect of temperature depends on storage duration and on applied elements. After 15 days at 4 °C, 30% of control fruits were affected in comparison to only 14 and 17% respectively for 1.19% KNO_3 and 2%

Table 2. Effect of different treatments on percent of "Fortune" fruit showing peel pitting and corresponding chilling index after 15 and 30 days of storage at 4 and 8°C.

Treatment	Storage duration			
	15 days		30 days	
	4°C	8°C	4°C	8°C
T0: Control	30b* (3.5)**	15a (2.1)	50bc (3.4)	17a (2.3)
T1: 1% $Ca(NO_3)_2.4H_2O$	24ab (1.1)	10a (1.3)	34abc (1.8)	17a (1.4)
T2: 2 % $Ca(NO_3)_2.4H_2O$	17a (0.8)	17ab (0.85)	23a (1.92)	13a (1.1)
T3: 1.9% KNO_3	14a (1.1)	5a (0.75)	14a (3.14)	7a (1.2)
T4: 2.38% KNO_3	24ab (1.2)	17ab (1.07)	30ab (2.65)	17a (1.6)
T5: 3.6% KNO_3	50c (2.85)	27b (1.1)	54c (3.42)	36b (1.8)

* Values within the column followed by the same letters are not significantly different from Newman and Keuls range test at 0.05 level.
** Values in parenthesis correspond to chilling index.

$Ca(NO_3)_2.4H_2O$. Fruit treated with 3.6% of KNO_3 have also showed a higher percentage of fruit with peel disorders both at 4 and 8°C. Treatments containing 1 to 2% of $Ca(NO_3)_2.4H_2O$ or 1.9% KNO_3 have the lowest percent of fruit with pitting in comparison to other treatments. Severe symptoms were observed in the control and in the fruit treated with 3.6% KNO_3 after both 15 and 30 days storage at 4°C. The severity of pitting varies between treatments with 1 to 2% of $Ca(NO_3)_2.4H_2O$ and 1.19 to 2.38% KNO_3 having the lowest values of chilling index of 1 to 2 in comparison to the control with values greater than 3 after 30 days storage at 4°C. Chilling index was lower for all treatments at 8°C than at 4°C.

Fruit internal quality

Flesh firmness of fruit treated with 1 and 2% of $Ca(NO_3)_2.4H_2O$ and 1.19 and 2.38% of KNO_3 was firmer than non-treated fruit. The control and fruit treated with 3.6% KNO_3 were less firm than other treatment (Table 3). Fruit stored at 8°C retained their firmness more than those stored at 4°C for the same period of storage. No quality parameters including acidity, firmness and total soluble solids have showed significant differences between treatments after 30 days storage at both 4°C and 8°C.

Peroxidase activity

At 4°C, POD specific activity increases continuously for all treatments during storage with non-treated fruit showing the greatest increase. After 30 days of storage, POD specific activity in the control was 2 to 9 fold higher than in other treatments. The smallest changes in POD specific activity was observed in fruit treated with 1%

Table 3. Effect of Ca^{2+} and K^{+} preharvest spray on the quality of "Fortune" mandarin fruit after 30 days storage at 4°C and 8°C.

	Measured parameters					
	Firmness (g-force)		Total soluble solids (°Brix)		Acid content (%)	
	4°C	8°C	4°C	8°C	4°C	8°C
T0	543ab*	527a	13.9ab	15c	1.65ab	1.85ab
T1	553 b	654c	13.9ab	15bc	1.88ab	1.74a
T2	599c	656c	13.9ab	14.8b	1.79ab	2.03b
T3	573bc	597b	13.5a	14.8b	1.72ab	1.64a
T4	544ab	586b	14.5c	13.9a	1.63a	1.67a
T5	509a	571b	14.5c	13.9a	1.67ab	1.54a

* Values followed by the same letters are not significantly different from Newman and Keuls range test at 0.05 level .

$Ca(NO_3)_2.4H_2O$, while most of the treatments showed a significant increase in activity (changes in % over the control at harvest) during storage (Fig. 2).

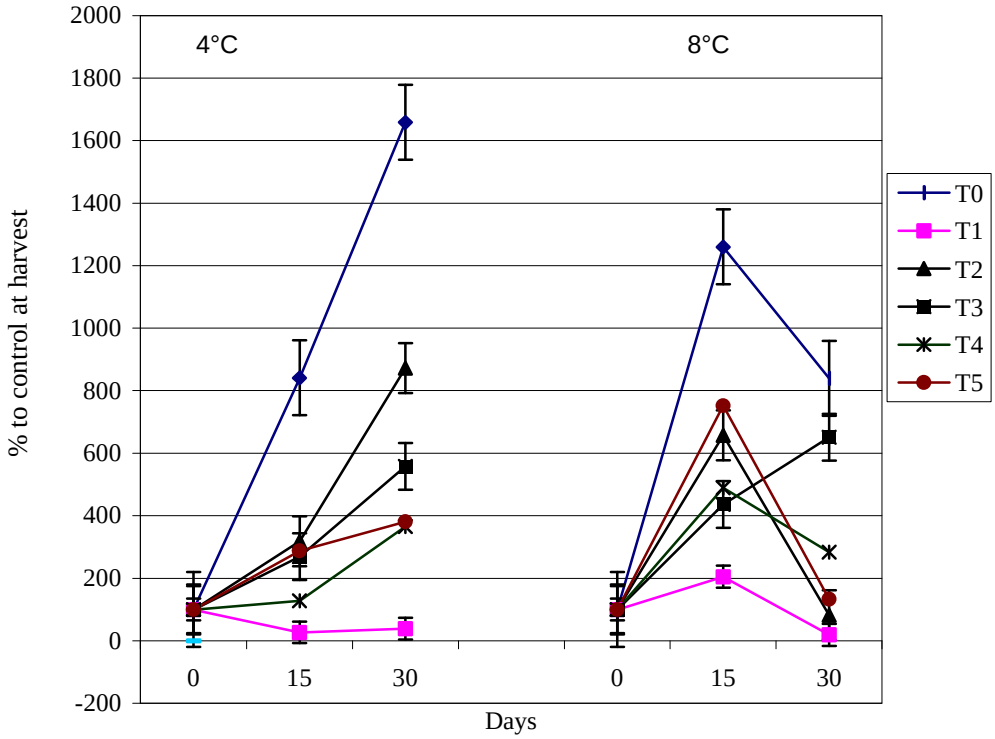

Fig. 2. POD specific activity changes (% to control at harvest) in "Fortune" mandarin fruit treated with different concentrations of calcium and potassium nitrates and stored at 4°C or 8°C for 30 days.

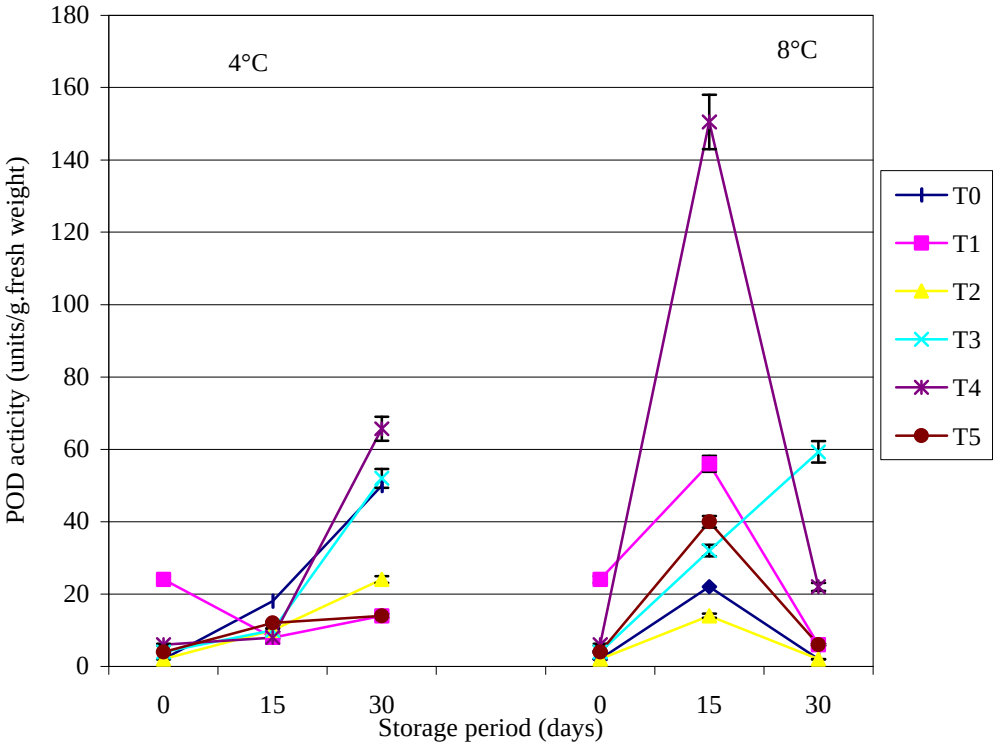

Fig. 3. Changes in POD activity in fruit pre-treated with Ca^{2+} and K^{+} and stored at 4°C and 8°C.

At 8°C, POD specific activity in the peel of fruit increased during the first two weeks of storage followed by a sharp decrease in the activity for the rest of storage. Non-treated fruit showed the greatest rate of POD specific activity as well as POD activity when compared to other treatments.

The increase in POD activity in non-treated fruit correlated with the chilling injury observed in these fruit. At 4°C, both POD activity and chilling index increased with storage period. In contrast, at 8°C most of the chilling disorders were expressed during the first two weeks of storage, which coincided with the highest increase in POD activity in the peel tissue (Fig. 3). These results support the relationship between chilling sensitivity of the fruit skin and the induction of peroxidase enzyme during storage. Setha et al. (2000) reported similar results on papaya stored at 5 and 15°C for up to 30 days. The authors reported that POD activity in fruits stored at 5°C was higher than that stored at 15°C and the activity at 5°C increased up to 15 days and then showed a rapid decrease until the end of storage, while papaya stored at 15°C showed very slight changes in POD activity throughout the storage period. In mango fruit stored at temperatures below 12.5°C, Zauberman et al. (1988) reported that chilling temperatures cause an increase of peroxidase activity. In their part, Aydin and Kadioglu (2001)

observed an increase in POD activity during fruit maturation and senescence. Edreva et al. (1993) reached the conclusion for the existence of close relationship between plant tissue damage and the activity of peroxidases in wheat exposed to low temperature. However, Martinez-Tellez and Lafuente (1993) did not find any significant changes in POD activity when “Fortune” mandarin fruit were stored at 2.5°C for several weeks. The results of our study indicated the enhancement of POD activity in the peel of fruit stored at low temperature for prolonged periods and that Ca^{2+} and K^{+} have an effect on chilling injury and enzyme activity. These findings were supported by enzyme -linked -immunosorbent assay (ELISA) test for total peroxidases (Fig. 4) which shows similar trend in changes of POD concentration in fruit tissue than for POD activity plotted in Fig. 2. In fact, the concentration of peroxidases has shown a continuous increase in storage at 4°C, while at 8°C, the concentration of peroxidases showed an increase during the first period and then a sharp decrease for the rest of storage period. These results also indicate a good relationship between the amount of peroxidases in the peel tissue and their activity during storage.

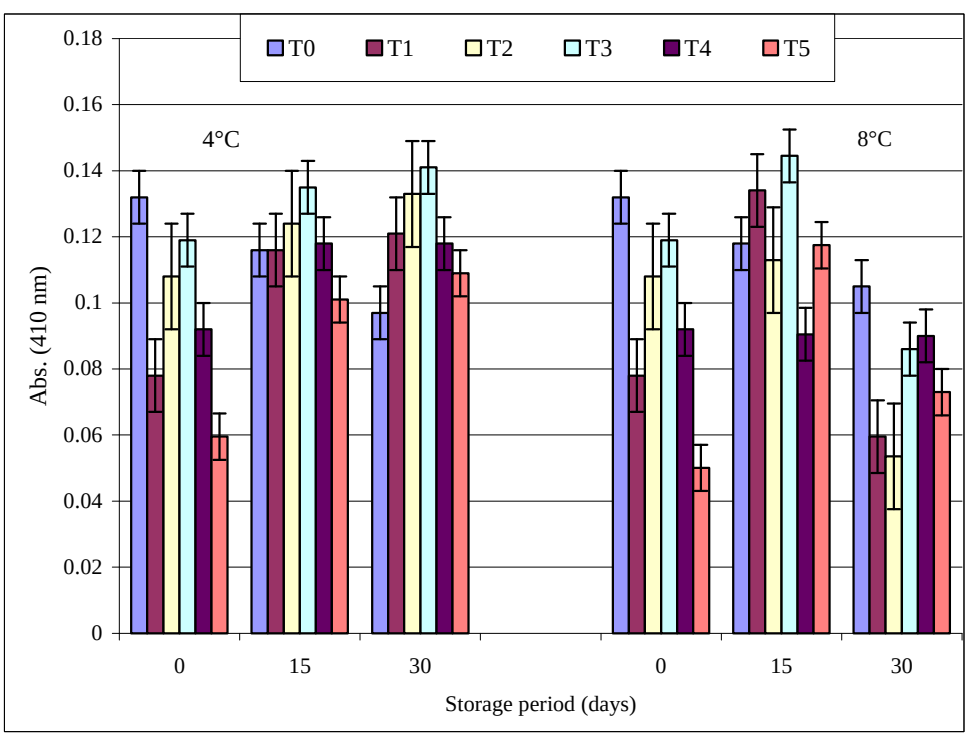

Fig. 4. Peroxidase concentration in the peel of “Fortune” mandarin fruit for different treatments during the storage at 4°C and 8°C following indirect ELISA test.

References

Ait-Oubahou, A., M. El-Otmani, R. Taraf, B. Nadori, D. Ezzoubir, M. Hanich, 2000. Effect of preharvest applications of Ca and K on the incidence of peel pitting of “Fortune” mandarin fruit at low temperature storage. Proc. Int. Soc. Citriculture 2000. Orlando Florida (In press)

Agusti, M., V. Almela, 1989. El cultivo de la mandarina Fortune en Espana. Problemas y perspectivas. Frutic. Prof., 25, 39–48.

Almela, V., M. Agusti, J. Pons, 1992. Rind spots in Fortune mandarin. Origin and Control. Physiol. Plant., 85, A65 (Abstract).

Ashraf, M. Y., A. R. Azmy, A. H. Khan, S. A. Ala, 1994. Effect of water stress on total phenols, peroxidase activity and chrolophyll content in wheat (*Triticum aestivum* L.). Acta Physiol. Plant., 16, 185–191.

Aydin, N., A. Kadioglu, 2001. Changes in the chemical composition, polyphenol oxidase and peroxidase activities during development and ripening of Medlar fruits (*Mespilus germanica* L.). Bulg. J. Plant Physiol., 27(3–4), 85–92.

Bradford, M, 1976 “A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding”, Anal. Biochem., 72, 248–254.

Burris, R. H. 1960. Hydroperoxidase (peroxidase and catalases). In: Encyclopedia of Plant Physiol., Ed. Ruhland W., 12. Berlin, Springer-Verlag, 365–400.

Chaplin, G. R., K. J. Scott., 1980. Association of calcium in chilling injury susceptibility of stored avocados. Hort Sci., 17, 238–239.

Edreva, A., G. Salcheva, D. Georgieva, 1993. Stress damage is related to peroxidase induction in wheat plants. In: Plant peroxidases, Biochemistry and Physiology, Eds. K. G. Welinder, S. K. Rasmussen, C. Penel, H. Greppin, University of Geneva, 401–404.

Grover, A., S. K. Sinha, 1985. Senescence of detached leaves in pigeon pea and chick pea: Regulation by developing pods. Physiol. Plant., 65, 503–507.

Grzywnowicz, K., Brzyska, M., Lobarzewski, J., Greppin, H., (1992). Changes in activity of soluble and immobilized cabbage peroxidase after treatment with seven different proteases. J. Mol. Catal,. 77, 365–376.

Hariyadi, P. K.L, Parkin,. 1991. Chilling-induced oxidative stress in cucumber fruits. Postharvest Biol. and technol., 1, 33–45.

Martinez-Javega, J. M., C. Saucedo, M. A. Del Rio, M. Mateos., 1992. Influence of storage temperature and coating on the keeping quality of “Fortune” mandarins. Proc. Int. Soc. Citriculture, 3, 1102–1103.

Martinez-Tellez, M. A., M. T. Lafuente.,1993. Chilling-induced changes in Phenylalanine ammonia–lyase, peroxidase, and polyphenol oxidase activities in citrus falvedo tissue. Acta Horticult., 343, 257–263.

Scalet M., R. Frederico, M. C. Guido, F. Manes. 1995. Peroxidase activity and polyamine changes in response to ozone and simulated acid rain in Aleppo pine needles. Environ. Exp. Bot., 35, 417–425.

Setha, S., S. Kanlayanarat,, V Srilaong, 2000. Changes in Polyamine Levels and Peroxidase Acrivity in “Khakdun” Papaya (*Carica papaya* L.) under Low Temperature Storage Conditions. In: Quality Assurance in Agricultural Produce, Ed. G. I. Johnson, Le Van To, N. D. Duc, M. C. Webb. ACCIAR Proceedings, 100, 593–598.

Zaragoza, S., R. Gazzola, I. Trenor, E. Alonso, E. Primo-Millo, V. Almela, M. Juan and M. Agusti, 1996. Control of Peel pitting of “Fortune” mandarin. Proc. Int. Soc. Citriculture, 1105–1109.

Zauberman, G., Y. Fuchs, I. Rot, A. Wexler, 1988. Chilling injury, peroxidase and cellulase activity in the peel of mango fruit at low temperature. Hort. Sci., 23, 732–733.

Zheng, X. H., Y. Ootani, M. Sawamura, 1993. Isozymic Analysis of peroxidase and Esterase in citrus Flavedo. Biosc. Biotech. Biochem., 57(10), 188–1802.