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Carbohydrate metabolism and fruit quality are affected in frost-exposed Valencia orange fruit

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Exposure of citrus fruit to frost often results in the development of freeze injury during their maturation in planta. This work was aimed to analyze changes in the biochemistry and enzymology of carbohydrate metabolism in freeze-injured orange fruit (*Citrus sinensis* var. Valencia late) and the involvement of oxidative stress in frost damage. The activities of pyrophosphate-dependent phosphofructokinase, phosphoenolpyruvate carboxylase and several fermentative enzymes increased in frost-exposed (FE) fruit, while NADP-malic enzyme and the mitochondrial isoform of NAD-malate dehydrogenase showed a reduction in their activities. Western blot analysis indicated a correlation between activity levels and protein content. Respiration rate in whole fruit was reduced by 40%, whereas the flavedo showed a more pronounced decline (53%). Volatile compound (i.e. ethanol and acetaldehyde) content was significantly higher in FE fruit than in control, as was that of L-malate (three-fold). Additionally, FE fruit showed a marked decrease in the maturity index (24%) because of a higher titratable acidity (39%). Evidence is presented that oxidative stress is involved in freeze-induced damage of orange fruit, where oxidative damage to lipids and proteins, and a greater electrolyte leakage in the flavedo were also observed. The results suggest that freezing temperatures provoke a notable metabolic switch in citrus fruit toward a fermentative stage, resulting in low-quality fruits.

Abbreviations – ADH, alcohol dehydrogenase; ADP, adenosine diphosphate; AlaAT, alanine aminotransferase; APX, ascorbate peroxidase; ATP-PFK, adenosine triphosphate–dependent phosphofructokinase; BSA, bovine serum albumin; CAT, catalase; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FE fruit, frost-exposed fruit; Fru-1,6-P_2, fructose-1,6-bisphosphate; Fru-2,6-P_2, fructose-2,6-bisphosphate; Fru-6-P, fructose-6-phosphate; FW, fresh weight; G6PDH, glucose-6-phosphate dehydrogenase; GAPC, glyceraldehyde-3-phosphate dehydrogenase; GAPN, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; GR, glutathione reductase; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid; LDH, lactate dehydrogenase; miETC, mitochondrial electron transport chain; NAD-MDH, NAD-malate dehydrogenase; NADP-ME, NADP-malic enzyme; PDC, pyruvate decarboxylase; PEG, polyethylene glycol; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PK, pyruvate kinase; PMSF, phenylmethyl sulfonyl fluoride; PPi-PFK, pyrophosphate-dependent phosphofructokinase; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; TEMED, tetramethyl ethylene diamine.

Introduction

Low temperature and frost constitute major risk factors for the production of citrus fruit. Although frost problems are negligible at lower latitudes, maximal quality is attained in a subtropical climate with moderately cold winter and with frost risk. Mature fruit display high resistance to frost because of the high content of sugars in the juice. Some recovery can occur when fruit are immature at the time of a frost event, but full quality is not completely achieved. In the case of late-ripening varieties, frosts can irreversibly arrest the evolution of the maturity index (ratio).

Several lines of evidence indicate that low temperatures alter cellular homeostasis by increasing the level of reactive oxygen species (ROS) at the transcript, protein and activity levels (Suzuki and Mittler 2006), which damage cellular components. In addition, the induction of oxidative stress by low temperatures would be the main factor that contributes to frost injury in different fruit (Kerdnaimongkol et al. 1997, Sala 1998). Moreover, it has been proposed that oxidative stress is involved in damage during cold storage of harvested citrus fruit (Sala 1998). Acquisition of freezing tolerance in plants is associated with the higher levels of ROS-scavenging enzymatic activities (Suzuki and Mittler 2006). Sala (1998) observed that chilling injury was reduced in cold-tolerant mandarin cultivars having a more efficient antioxidant system. There is also evidence that heat-induced chilling tolerance of citrus fruit is accompanied by an increase in the ROS-scavenging enzymes, catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) (Sala and Lafuente 1999). However, the involvement of oxidative stress in freeze injury of citrus fruit in planta has not been reported.

As sink organs, fruit heterotrophically utilize sucrose derived from photosynthetic cells (Iglesias and Podestá 2005). Thus, degradation of carbohydrates through glycolysis and respiration is a central process for the sustained provision of carbon and energy to the fruit. The respiration rate is one of the parameters that differentiate cold-sensitive from cold-resistant plants. Sensitive plants usually show lower respiration rates when exposed to cold, while a noticeable increase is observed when temperatures return to normal (Purvis 1990). Surprisingly, although several papers described the role of carbohydrates in response to cold (Echeverria and Valich 1988, Purvis 1990, Holland et al. 1999, Kaplan et al. 2006), few of them focused on the analysis of the relationship between the enzymology of carbohydrate metabolism (and its regulation) and the susceptibility to cold.

The main goal of this work was to analyze the biochemistry and enzymology of central metabolic pathways in orange fruit (*Citrus sinensis* var. Valencia late) when exposed to natural low temperatures next to the final maturation stage. Particularly, we examined the changes in respiration, the physiological parameters and fruit internal quality and the levels and activities of enzymes involved in the carbohydrate metabolism. Furthermore, the relationship between freeze injury and oxidative stress was evaluated by means of the analysis of levels of antioxidant enzymes and oxidative damage to cellular compounds.

Materials and methods

Plant material

''Valencia'' orange fruit (*C. sinensis* L. Osbeck) were harvested from trees located in selected lots of the INTA's Estación Experimental Agrícola Concordia, Entre Ríos, Argentina. For all experiments, fruit were harvested from the beginning of July until late September (the period of southern winter to early spring), and the studies were conducted during three consecutive seasons (from 2002 to 2004). Samples were processed at 48 h after the frost event. Sampled trees were located at spots within the same orchards at different terrain elevations providing high or low risk of frost. Immediately after a frost episode (considered as temperatures below −2°C with a duration of at least 4 h), exposure was visually confirmed and samples were collected. Visible characteristic symptoms were both external, as peel pitting (small pitted areas, discolored distributed over the fruit surface) and browning of the flavedo, and internal, as hesperidin crystals (white dots) above segment walls, soaked segment walls, zigzag deviation of segment walls and dried juice sacs. First, drying began in the peduncular zone, and subsequently, it reached the stylar end zone in severely damaged fruit. Frost juice sacs took a watery and pallid aspect. Fruit (approximately 25% of the total collected) exhibiting slight symptoms of frost injury were not included in these studies. Only severely damaged fruit were classified as frost-exposed (FE) fruit (35–40% of the total). Control fruit, coming from low-frost-risk places, were also inspected to ensure that no frost injury had occurred.

Temperature was registered hourly from 9 pm to 9 am. For biochemical analysis, fruit were peeled and classified as control or FE according to physical symptoms of frost injury, the segments were separated and segment walls were removed. The resultant tissue (the juice sacs) was frozen in liquid nitrogen and stored at −80°C until use. For all experiments, at least four individual fruit of each group were evaluated with duplicated assays of the same fruit. Fruit chosen for experiments were all of similar size and weight.

Total protein extraction

Fruit tissue (approximately 3–5 g) was powdered with liquid N_2 in a mortar and then homogenized with one-fifth volume of extraction buffer [1 *M* Tris, 40% (v/v) glycerol, 20 m*M* $MgCl_2$, 5 m*M* ethylenediaminetetraacetic acid (EDTA), 10 m*M* 2-mercaptoethanol] and 1 m*M* phenylmethly sulfonyl fluoride (PMSF). The final pH of the crude extract thus obtained was near 7. The homogenates were centrifuged for 15 min at 4°C in an Eppendorf microcentrifuge at maximum speed. The resulting supernatant was desalted in a Sephadex G-25 column previously equilibrated with an 1/10 dilution of extraction buffer (Penefsky 1977). The eluate was used as the source for enzyme activity measurements, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), non-denaturing electrophoresis and immunoblots.

Activity assays and kinetic studies

All enzymes with the exception of pyruvate kinase (PK) were assayed at 25°C in a MRX Microplate reader (Dynex Technologies, Chantilly, VA) following the oxidation of NAD(P)H or production of NADPH at 340 nm in a final volume of 0.2 ml. The assays were (1) optimized for pH and the concentration of cofactors/substrates, (2) corrected for NADH oxidase activity and (3) linear with respect to time and concentration of enzyme assayed. The enzymatic activities represent means of at least six determinations made in at least four individual fruit of each group. The experiments were repeated twice for the same fruit. The reaction mixtures for each assay were as follows.

Glyceraldehyde-3-phosphate dehydrogenase (GAPC, EC 1.2.1.12): 50 m*M* tricine-NaOH, pH 8.5, 4 m*M* NAD, 2 m*M* fructose-1,6-bisphosphate (Fru-1,6-P_2), 10 m*M* arsenate and 1 U aldolase. Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49): 100 m*M* Tris—HCl, pH 8, 0.2 m*M* NADP and 2 m*M* glucose-6-phosphate. Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN, EC 1.2.1.9): 50 m*M* tricine—NaOH, pH 8.5, 0.4 m*M* NADP, 2 m*M* Fru-1,6-P_2 and 1 U aldolase. NAD-malate dehydrogenase (NAD-MDH, EC 1.1.1.37): 50 m*M* imidazole, pH 6.9, 1 m*M* oxaloacetate and 0.15 m*M* NADH. Adenosine triphosphate-dependent phosphofructokinase (ATP-PFK, EC 2.7.1.11): 50 m*M* Tris–HCl, pH 7.5, 0.15 m*M* NADH 4 m*M* fructose-6-phosphate (Fru-6-P), 0.5 m*M* ATP, 5 m*M* $MgCl_2$, 5% (w/v) polyethylene glycol (PEG), 1 m*M* dithiothreitol (DTT), 0.2 U aldolase and 0.1 U glycerophosphate dehydrogenase triose phosphate isomerase. Pyrophosphate-dependent phosphofructokinase (PPi-PFK, EC 2.7.1.90): 50 mM Tris–HCl, pH 7.5, 0.15 m*M* NADH, 4 m*M* Fru-6-P, 0.5 m*M* PPi, 5 m*M* $MgCl_2$, 5% (w/v) PEG, 1 m*M* DTT, 1 μ*M* fructose-2,6-bisphosphate (Fru-2,6-P_2), 0.2 U aldolase and 0.1 U glycerophosphate dehydrogenase triose phosphate isomerase. Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31): 100 mM HEPES-NaOH, pH 7 or 8, 10% (v/v) glycerol, 5 m*M* $MgCl_2$, 10 m*M* HCO_3^-, 4 m*M* phosphoenolpyruvate (PEP), 0.15 m*M* NADH and 0.6 U MDH. K_m values for PEP were calculated by fitting to the Michaelis–Menten equation with a non-linear least-squares regression computer kinetics program (Brooks 1992). I_{50} values for malate (concentrations of inhibitor producing 50% inhibition of enzyme activity) were determined using the aforementioned computer kinetic program (Brooks 1992). All kinetics parameters are means of at least two determinations. NADP-malic enzyme (NADP-ME, EC 1.1.1.40): 50 m*M* Tris–HCl, pH 7.5, 0.5 m*M* NADP, 10 m*M* $MgCl_2$ and 10 m*M* L-malate. Lactate dehydrogenase (LDH, EC 1.1.1.27): 50 m*M* NaPi, pH 7.5, 10 m*M* pyruvate, 0.2 m*M* NADH and 1 m*M* methylpyrazole. Alcohol dehydrogenase (ADH, EC 1.1.1.1): 85 m*M* MES, pH 6.5, 5 mM acetaldehyde and 0.15 m*M* NADH. Pyruvate decarboxylase (PDC, EC 4.1.1.1): 85 m*M* MES, pH 6.5, 25 m*M* NaCl, 2 m*M* $MgCl_2$, 2 m*M* thiamine pyrophosphate, 2 m*M* DTT, 0.15 m*M* NADH, 50 m*M* oxamate, 3 U ADH and 10 m*M* pyruvate. The extracts were incubated for 30 min in the reaction medium in the absence of pyruvate and ADH for PDC activation before starting the reaction. Alanine aminotransferase (AlaAT, EC 2.6.1.2): 100 m*M* Tris–HCl, pH 8.0, 25 m*M* alanine, 10 m*M* α-ketoglutarate, 0.15 m*M* NADH and 0.2 U LDH. Glutathione reductase (GR, EC 1.8.1.7): 100 m*M* KPi, pH 7.8, 2 m*M* EDTA, 1 m*M* glutathione and 0.2 m*M* NADPH.

For the assay of PK activity (EC 2.7.1.40), the medium was previously bubbled with helium in order to remove the bicarbonate and avoid interference by endogenous PEPC and MDH. The enzymatic activity was corrected for interference by PEP-phosphatase activity by omitting adenosine diphosphate (ADP) from the reaction mixture. The activity measurement was performed in a Hitachi 150-20 spectrophotometer (Hitachi Corp., Tokyo, Japan) following the oxidation of NADH at 340 nm, and the reaction mixture contained 25 m*M* HEPES-NaOH, pH 7.2, 20 m*M* KCl, 10 m*M* $MgCl_2$, 2 m*M* PEP, 0.15 m*M* NADH, 5% (w/v) PEG, 1 m*M* ADP and 0.4 U LDH.

Fumarase (EC 4.2.1.2) activity was measured in a Hitachi 150-20 spectrophotometer following the production of fumarate at 240 nm, and the reaction mixture contained 100 m*M* KPi, pH 7.4, and 50 m*M* L-malate.

One unit of activity is defined as the amount of enzyme resulting in the consumption of 1 μmol substrate min^{-1}.

The determination of the activation constant (K_a) of PPi-PFK for Fru-2,6-P_2 was carried out at variable

concentrations of Fru-2,6-P_2 (2–50 n*M*), subsaturating concentration of Fru-6-P in a Unicam spectrophotometer (Pye-Unicam Ltd., Cambridge, England) following the oxidation of NADH at 340 nm. The reaction medium contained 100 m*M* Tris–HCl, pH 7.5, 0.15 m*M* NADH, 1.5 m*M* Fru-6-P, 0.5 m*M* PPi, 5 m*M* $MgCl_2$, 5% (w/v) PEG, 1 m*M* DTT, 1 U aldolase and 0.5 U glycerophosphate dehydrogenase-triose phosphate isomerase. The experiments were carried out four times with both control and FE fruit, and in each case, the assays were done twice for an individual fruit.

The differences in activity or kinetic parameters between two groups were assessed by the Student's *t*-test for paired samples.

Measurement of physiological, chemical and internal quality parameters

The respiratory activity of whole Valencia oranges and flavedo was measured by infrared gas analysis. Individual fruit or flavedo was placed in a 160-ml chamber through which air was pumped (at a flow rate of 300 ml min^{-1}). Increases in CO_2 in the chamber were monitored by passing the air stream through an infrared gas analyzer (IRGA, Qubit Systems Inc., Kingston, Canada), and from these data, the rate of CO_2 evolution was calculated. The average temperature was 23.4 ± 1.2°C, and the relative humidity was 83.2 ± 2.4%. The data presented are the results of measurements on six separate fruit ± SD. Two independent measurements were made of individual fruit.

Control and FE fruit were squeezed, and the resulting juices were used for the following determinations. Five fruit were used per replication (three replication samples).

The total soluble solid content in the juice was determined with a digital refractometer (expressed as °Brix), and the titratable acidity was measured by titrating with 0.1 N NaOH to pH 8.2. The results are expressed as percentage of anhydrous citric acid because this is the predominant organic acid in citrus. The maturity index was evaluated via the ratio total soluble solids : titratable acidity. The juice was extracted from individual fruit (at least 20 fruit per replication, three replication samples), and the juice content was expressed as percentage of fruit weight.

Ethanol and acetaldehyde content in juice were determined by gas chromatographic analysis of juice headspace using a gas chromatograph (Shimadzu Mod. GC17A; Shimadzu Corp., Kyoto, Japan) with a Supelco Omegawax 250 column (30-m × 0.25-mm internal diameter) (Supelco, Bellefonte, PA).

For extraction and determination of L-malate, the tissue (juice sacs) was homogenized with four volumes of perchloric acid (0.6 N). The homogenate was centrifuged at 6000 *g*, and the supernatant was neutralized with K_2CO_3. This solution was used for the determination of L-malate content by coupling the MDH reaction with glutamate-oxaloacetate transaminase. The reaction mixture contained 100 m*M* glycine, pH 10.0, 2 m*M* NAD, 50 m*M* glutamate, 30 U MDH, 20 U glutamate-oxaloacetate transaminase and variable amount of extracts. The blank was carried out without L-malate in the reaction mixture, and an L-malate standard was used to test the accuracy of the determination. The results were expressed as μmol L-malate g^{-1} fresh weight (FW). The experiments were carried out four times with both control and FE fruit, and in each case, the assays were done twice for an individual fruit.

For electrolyte leakage, two or three fruit per replication (three replication samples) were used. Peel was carefully removed, and 10-mm disks were cut with a cork borer. Samples of 10 disks per replicate were placed in a 200-ml glass bottle, washed twice with deionized water and then incubated in 100 ml of deionized water at 20°C. Conductivity of the incubation medium was measured with a conductivity meter after 3 h of incubation under constant shaking. After readings were taken, the flasks were autoclaved at 120°C for 20 min and cooled to 20°C, and the conductivity was measured again for total electrolytes. Data were expressed as (initial conductivity/total conductivity) × 100.

Weight loss was measured in 20 control and FE fruit.

Thiobarbituric acid reactive substances production and carbonyl assay in proteins

Crude extracts obtained as described above were incubated with equal volume of thiobarbituric acid [0.5% (w/v) in 20% (w/v) TCA] at 95°C for 45 min. After cooling, the samples were centrifuged at 10 000 *g* for 20 min. The supernatants were diluted in a 1:2 (v:v) ratio with water, and the absorbance was measured at 532 and 600 nm. The absorbance at 600 nm was subtracted from the absorbance at 532 nm, and the malondialdehyde extinction coefficient (155 mM^{-1} cm^{-1}) was used for calculations of the thiobarbituric acid reactive substances (TBARS) content.

Oxidation of proteins was analyzed by a spectrophotometric method that quantifies the carbonyl levels with 2,4-dinitrophenylhydrazine (DNPH). Crude extracts were obtained from control and FE fruit (approximately 5 g) by powdering with liquid N_2 in a mortar and then homogenizing with 1.25 ml of extraction buffer [1 *M* Tris, 40% (v/v) glycerol, 20 m*M* $MgCl_2$, 5 m*M* EDTA, 10 m*M* 2-mercaptoetanol, 5 μg ml^{-1} chymostatin, 1 m*M* 2,2′-dipyridyldisulfide, 5 μ*M* E-64 and 1 m*M* PMSF]. The

homogenates were centrifuged for 15 min at 4°C in an Eppendorf microcentrifuge at maximum speed. Extracted proteins were incubated with two volumes of 12 m*M* DNPH in 3 *M* HCl at room temperature for 1 h in the dark. Samples were vortexed every 15 min. Blanks were obtained by incubating extracted proteins with two volumes of 3 *M* HCl and then processed in the same manner as samples treated with DNPH. Next, proteins were precipitated by adding one-tenth volumes of 100% (w/v) TCA, and the samples were left for 1 h at 4°C. After centrifugation at maximum speed for 15 min, precipitated proteins were washed with 10% (w/v) TCA. Finally, pellets were washed three times with ethanol–ethyl acetate (1:1) (v/v) to remove the free DNPH and lipid contaminants. The final precipitates were dissolved in 6 *M* guanidine hydrochloride solution and left for 15 min at 37°C with agitation. Insoluble materials were removed by additional centrifugation. Carbonyl content in samples was calculated from the absorbance at 360 nm using HCl-treated samples as blanks and absorption coefficient of 22 000 M^{-1} cm^{-1}. The results were expressed in nanomoles of carbonyl per milligram total protein, considering that 15% of proteins are lost in the various washing steps (Levine et al. 1994). The experiments were carried out four times with both control and FE fruit, and in each case, the assays were done twice for an individual fruit.

Protein measurement

Protein concentration was determined according to the method described by Lowry et al. (1951) or by dye binding (Sedmak and Grossberg 1977) using a total protein commercial kit as standard.

Electrophoresis and immunoblotting

SDS-PAGE was performed according to the method of Laemmli (1970) using the Bio-Rad mini-gel apparatus (Bio-Rad, Hercules, CA). The final acrylamide concentration was 12, 10 or 8% (w/v) for the separating gel and 5% (w/v) for the stacking gel. The following standard proteins were included for the determination of the subunit molecular mass, phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozime (14.4 kDa), or alternatively myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), BSA (66 kDa) and ovalbumin (45 kDa). Immunoblotting was performed according to the method of Bollag and Edelstein (1991). Samples were run on a denaturing PAGE and transferred to nitrocellulose membrane. The following antibodies were used for detection: rabbit immune serum raised against maize recombinant photosynthetic NADP-ME (C.S. Andreo, Rosario, Argentina); rabbit immune serum raised against *Brassica napus* cytosolic PK (W.C. Plaxton, Kingston, Canada); affinity-purified anti *Amaranthus hypochondriacus* α subunit NAD-malic enzyme (NAD-ME) (J.O. Berry, Buffalo, USA); rabbit immune serum raised against barley LDH (A.D. Hanson, Florida, USA); affinity-purified antibodies against the cytosolic aldolase from germinated castor oil seeds endosperm (W.C. Plaxton, Kingston, Canada); and affinity-purified anti *Ananas comosus* cytosolic NAD-MDH, rabbit immune serum raised against potato tuber PPi-PFK and affinity-purified anti–*Amaranthus viridis* PEPC (authors lab).

Non-denaturing gel electrophoresis was performed according to the method of Davis (1964) using 6% (w/v) (for NADP-ME, LDH, ADH and CAT activities) or 8% (w/v) (for APX and SOD activities) for the separating gel and 5% (w/v) for the stacking gel. The gels were supplemented with 20% (v/v) glycerol and 10% (v/v) ethylene glycol. Gels were run at 12 mA and at 4°C until the dye front left the gel.

Enzymatic activities were detected by incubating the gels in the following reaction media until development of purple bands in the dark at 30°C.

NADP-ME: 100 m*M* Tris–HCl, pH 7.5, 20 m*M* $MgCl_2$, 20 m*M* L-malate, 1 m*M* NADP, 0.1 mg ml^{-1} nitroblue tetrazolium and 5 μg ml^{-1} phenazine methosulfate. LDH: 100 m*M* glycine, pH 9, 100 m*M* lactate, 2 m*M* NAD, 20 m*M* $MgCl_2$, 0.1 mg ml^{-1} nitroblue tetrazolium and 5 μg ml^{-1} phenazine methosulfate. ADH: 500 m*M* glycine, pH 9.0, 100 m*M* ethanol, 2 m*M* NAD, 0.1 mg ml^{-1} nitroblue tetrazolium and 5 μg ml^{-1} phenazine methosulfate.

Non-denaturing gels were also used to detect activities of the antioxidant enzymes SOD (EC 1.15.1.1), CAT (EC 1.11.1.6) and APX (1.11.1.11). For SOD isoforms, gels were equilibrated with 50 m*M* KPi, 1 m*M* EDTA, pH 7.8, for 30 min. Gels were then incubated in the same solution but supplemented with 120 μ*M* riboflavin, 28 m*M* tetramethyl ethylene diamine (TEMED) and 2.5 m*M* nitroblue tetrazolium for 20 min in the dark. Finally, gels were placed in distilled water and exposed in a light box for 15 min at room temperature. Observation of cyanide-sensitive or -insensitive isozymes was achieved by incubating gels in 50 m*M* KPi containing 3 m*M* KCN (inhibitor of Cu/Zn-SOD) or 5 m*M* H_2O_2 (inhibitor of Cu/Zn-SOD and Fe-SOD) for 30 min before staining for SOD activity. For CAT (EC 1.11.1.6) staining, the gels were washed three times with water for 10 min, incubated with 0.003% (v/v) H_2O_2 for 10 min, washed with water and submerged in a solution of 2% (w/v) $K_3Fe(CN)_6$ and 2% (w/v) $FeCl_3$. The reaction was continued for 2–3 min and stopped by a brief wash with water.

Non-denaturing PAGE for the detection of APX activity was achieved as described above, except that carrier buffer contained 2 m*M* ascorbate. The gels were prerun for 30 min to allow ascorbate to enter the gel prior to the application of samples. After electrophoretic separation, gels were equilibrated with 50 m*M* KPi, pH 7.0, containing 2 m*M* ascorbate for 30 min, followed by incubation in 50 m*M* KPi, pH 7.0, containing 4 m*M* ascorbate and 2 m*M* H_2O_2 for 20 min. The gels were washed with buffer for 1 min and submerged in a solution of 50 m*M* KPi, pH 7.8, containing 28 m*M* TEMED and 2.5 m*M* nitroblue tetrazolium with gentle agitation. The reaction was continued for 10–15 min and stopped by a brief wash with water.

Each isoform of the enzymes under study was quantified by densitometric analysis. The gels were scanned, and the area of the bands was obtained by integration using the Image-pro Plus program (Media Cybernetics Inc., Silver Spring, MD). Each densitometric analysis was determined in triplicate to minimize experimental error.

Results

Physiological and chemical parameters and internal quality in FE fruit

Table 1 summarizes the physiological and chemical parameters for control and FE oranges. The mean values for respiration rate of whole fruit decreased by 40% in FE fruit with respect to control, whereas this descent was more pronounced for flavedo (53%). Volatile compounds in juice, acetaldehyde and ethanol, increased in FE fruit 1.8 and 2.4-fold, respectively, and L-malate content showed also a marked increase in FE fruit (2.9-fold). Low temperatures also affected internal quality attributes. Titratable acidity increased in FE fruit (by around 39%), while soluble solids did not change. As a result, the maturity index was 1.3 times higher for control fruit. Finally, the percentage of juice was slightly lower in FE fruit (21%).

Table 1. Effect of freeze exposure on the physiological and chemical parameters and internal quality in *Citrus sinensis* oranges. The experiments were repeated at least four times in control and FE samples. * and **, data significantly different at $P < 0.01$ and 0.05, respectively; ns, data non-significantly different (paired Student's *t*-test).

Parameter	Control	Frost exposed	Percent of control
Respiration rate (nmol CO_2 min^{-1} g^{-1} fresh weight)			
Whole fruit	12.6 ± 1.1	7.6 ± 0.6	60*
Flavedo	117 ± 17	55.2 ± 5.9	47*
Volatile compounds			
Acetaldehyde (ppm)	4.0 ± 0.3	7.2 ± 0.9	179*
Ethanol (ppm)	152 ± 28	359 ± 97	236*
L-malate (μmol g^{-1} FW)	4.3 ± 0.9	12.3 ± 1.8	288*
Electrolyte leakage (%)	33.6 ± 3.9	44.3 ± 0.9	132*
Titratable acidity (%)	1.8 ± 0.1	2.5 ± 0.3	139*
Soluble solids (°Brix)	12.2 ± 1.1	12.6 ± 1.3	103 ns
Maturity index	6.6 ± 0.5	5.0 ± 0.4	76**
Percentage of juice	49.7 ± 5.1	39.3 ± 3.7	79**

Several studies report that freeze stress provokes an increase in the permeability of membranes, resulting in a higher leakage of ions and electrolytes (Mancuso et al. 2004). As shown in Table 1, electrolyte leakage was significantly higher in FE fruit (1.32-fold), clearly indicating damage in the membranes of the flavedo.

Total protein and enzymatic activities

SDS-PAGE of crude extracts from control and FE oranges did not show any remarkable qualitative change in the protein pattern (not shown). In contrast, total protein content of FE fruit on a FW basis showed a sharp decrease when compared with the control (Table 2). In addition, the final volume of crude extract obtained per gram of FW in FE oranges showed a significant decrease of 30%, probably as a result of the dehydration of juice sacs (Table 2).

Freeze exposure caused significant shifts in the activities of several enzymes (Table 2). NADP-ME activity showed a decrease of 30% as a function of protein, whereas a more significant decrease was observed when its activity was expressed as a function of the FW (50%, not shown). NAD-MDH lowered its activity significantly only as a function of the FW (30%, not shown), while its activity as a function of protein did not show any variation. PPi-PFK and PEPC activities increased markedly compared with control, while ATP-PFK and PK remained unchanged. The affinity of PPi-PFK by Fru-2, 6-P_2 was studied. In FE samples, PPi-PFK showed a high affinity toward its activator. The graphs obtained when PPi-PFK activity was analyzed against Fru-2,6-P_2 concentration were hyperbolic, with a K_a of 8.0 ± 1.7 n*M*. In contrast, in control samples, the activation kinetics did not reach saturation for Fru-2,6-P_2 even at high levels of this metabolite (Fig. 1).

In control fruit, PEPC activity determined at pH 7 was approximately 70% of that at pH 8, whereas decreasing the assay pH from 8 to 7 slightly decreased the enzyme's activity by 15% in FE fruit (not shown). In common with most plant PEPCs, the enzyme from citrus fruit displayed hyperbolic PEP saturation kinetics at pH 7 and 8, with K_m values of 110 ± 20 and 70 ± 10 μ*M*, respectively, in control fruit (not shown). A marked decrease (about 50%) in the enzyme's K_m (PEP) was observed in FE fruit at pH 7 and pH 8 (65 ± 4 and 34 ± 8 μ*M*, respectively). In

Table 2. Activity of enzymes in *Citrus sinensis* crude extracts of control and FE samples. The values represent means of at least six determinations carried out in at least two crude extracts of the same fruit. The experiments were repeated four times in control and FE samples. The enzymatic activities are expressed in terms of nmol mg protein^{-1} min^{-1}. sd, standard deviation; * and **significantly different at $P < 0.01$ and 0.05, respectively; ns, data non-significantly different (paired Student's *t*-test). PPi-PFK, pyrophosphate-dependent phosphofructokinase; PEPC, phosphoenolpyruvate carboxylase; NADP-ME, NADP-malic enzyme; LDH, lactate dehydrogenase; ADH, alcohol dehydrogenase; PDC, pyruvate decarboxylase; G6PDH, glucose-6-phosphate dehydrogenase; GAPC, glyceraldehyde-3-phosphate dehydrogenase; GAPN, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; NAD-MDH, NAD-malate dehydrogenase; PK, pyruvate kinase; ATP-PFK, adenosine triphosphate–dependent phosphofructokinase; AlaAT, alanine aminotransferase; FW, fresh weight.

Enzyme	Control	Frost exposed	Percent of control
PPi-PFK	80.0 ± 7.5	142.8 ± 23.8	180*
PEPC	204.0 ± 40.0	529.3 ± 40.9	260*
NADP-ME	231.8 ± 52.8	164.4 ± 37.6	71**
LDH	309.4 ± 61.8	1308 ± 147	420*
ADH	210.4 ± 41.4	985.0 ± 132.0	470*
PDC	371.8 ± 63.4	881.0 ± 157.3	240*
G6PDH	65 ± 11	81 ± 15	125**
GAPC	204 ± 50	247 ± 31	121 ns
GAPN	<0.3	<0.3	–
NAD-MDH	8093 ± 363	7121 ± 827	88 ns
PK	1097 ± 185	1088 ± 213	99 ns
ATP-PFK	484.3 ± 44	461.7 ± 40.7	95 ns
Fumarase	260.2 ± 28.2	156.1 ± 19.3	60*
AlaAT	989.3 ± 235.1	1096 ± 275	111 ns
Total protein content (mg g^{-1} FW)	0.49 ± 0.06	0.31 ± 0.04	63*
Volume of crude extract (ml g^{-1} FW)	0.81 ± 0.03	0.57 ± 0.04	70*

addition, PEPC from control and FE fruit showed different sensitivity toward malate. Thus, at subsaturating concentration of PEP, this metabolite yielded an extremely low I_{50} of 8.0 ± 2.0 μ*M* at pH 7 in control fruit, with a near-complete inhibition of PEPC activity (8% residual activity) occurring at a concentration of 2 m*M*. On the contrary, higher malate concentrations were needed to inhibit PEPC from FE fruit (I_{50} of 1.12 ± 0.15 m*M*, at subsaturating concentration of PEP) with 37% of residual activity at 2 m*M*.

AlaAT activity determined in the alanine → pyruvate direction was not affected in FE fruit compared with control samples. The same behavior was observed for GAPC, but G6PDH showed a moderate increase of 25% (Table 2). The activity of GAPN was extremely low both in control and in FE samples, suggesting a minor role for this enzyme in fruit metabolism at this stage of development.

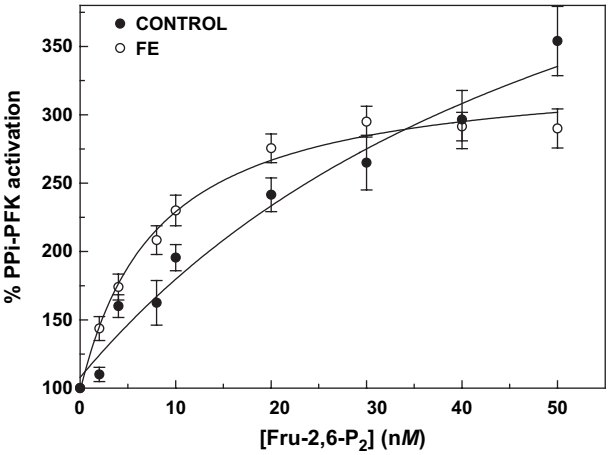

Fig. 1. Activation of pyrophosphate-dependent phosphofructokinase (PPi-PFK) from Valencia orange fruit by fructose-2,6-bisphosphate (Fru-2,6-P_2). The symbols represent the mean ± sd of four independent experiments. The assays were done twice on each individual fruit and three times for each concentration of Fru-2,6-P_2.

The greatest differences were found in the fermentative enzymes. Measured as a function of protein, PDC activity increased 2.4-fold, ADH activity 4.7-fold and LDH activity 4.2-fold (Table 2). Similar increases were observed when these activities were expressed as a function of FW (3.2, 4.6 and 3.6-fold, respectively, not shown).

FE fruit also showed a significant reduction in fumarase activity as a function of both protein (40%) and FW (58%, not shown).

The enzymes LDH, ADH and NADP-ME were also studied by non-denaturing PAGE followed by activity staining. As shown in Fig. 2, a single band with ADH activity was observed in control and FE fruit crude extracts, being 62% more intense in FE fruit. On the contrary, no band with LDH activity was detected in control samples, whereas a highly intense band was evidenced in FE fruit. Finally, one band with NADP-ME activity was observed in control and FE orange crude extracts, with 25% less intensity in the latter. Using the same approach, different bands with LDH and ADH activities corresponding to different isoforms have been reported in species such as maize, rice and barley (Rivoal et al. 1991, Christopher and Good 1996). Although a significant number of gels and crude extracts with variable protein loads were analyzed, a single band with activity was observed for LDH and ADH enzymes in Valencia oranges fruit.

Immunoblot analysis

In order to determine whether the changes in the enzyme activities were the result of variations in protein levels,

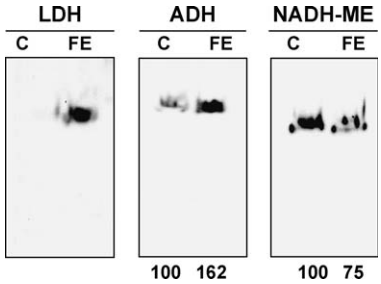

Fig. 2. Analysis of lactate dehydrogenase (LDH), alcohol dehydrogenase (ADH) and NADP-malic enzyme (NADP-ME) by non-denaturing polyacrylamide gel electrophoresis stained by enzymatic activity; C, crude extract from control Valencia orange fruit (20 μg); FE, crude extract from FE Valencia orange fruit (20 μg). The numbers below the figures indicate the relative intensities of reactive bands determined by densitometric analysis with respect to control samples (100%).

immunoblot analysis of control and FE fruit was conducted. Fig. 3 shows that PEPC and LDH were clearly induced in FE samples (1.4- and four-fold, respectively), whereas a decrease of 57% in the immunoreactive protein was observed for NADP-ME compared with the control. In the case of NAD-MDH, immunoblot analysis of Valencia orange crude extracts probed with anti-*Ananas comosus* cytosolic MDH antibodies did not show any variation in the immunoreactive protein between control and FE samples.

Fig. 3 shows an immunoblot analysis for the α subunit of NAD-ME, which reveals that it strongly decreased in FE

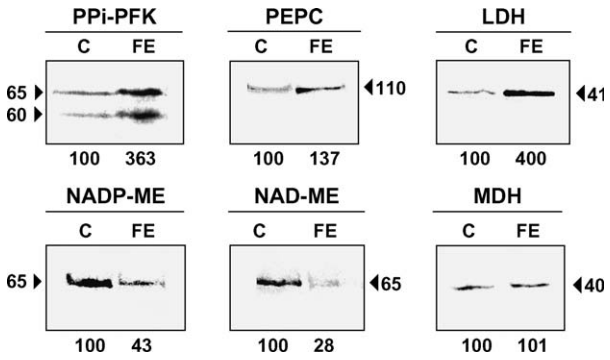

Fig. 3. Immunodetection of carbohydrate metabolism enzymes from Valencia orange fruit. C, crude extract from control Valencia orange fruit (20 μg); FE, crude extract from FE Valencia orange fruit (20 μg). The numbers at the sides of the figures represent molecular mass in kilodaltons. The numbers below the figures indicate the relative intensities of immunoreactive bands determined by densitometric analysis with respect to control samples (100%). For PPi-PFK, the numbers corresponded to the sum of the intensities of both bands. PPi-PFK, pyrophosphate-dependent phosphofructokinase; PEPC, phosphoenolpyruvate carboxylase; LDH, lactate dehydrogenase; NADP-ME, NADP-malic enzyme; MDH, malate dehydrogenase.

fruit (72%). Although its activity was not measured, the analysis of this enzyme is important as a mitochondrial marker. With respect to PPi-PFK, an increase in the level of both α (65 kDa) and β (60 kDa) subunits could be observed in FE fruit. The ratio α:β changed from 1.66 in control fruit to 1.00 in FE fruit. Cytosolic PK protein content remained constant, as observed in the activity assays (not shown). The cytosolic aldolase, which has been reported to be induced by different types of stress (Kelley and Freelin 1984), did not show any variation between control and FE fruit (not shown).

Enzymes involved in the antioxidant defense mechanisms

The enzymes APX, CAT and SOD were studied by non-denaturing PAGE followed by activity staining, while GR activity was measured spectrophotometrically. APX and CAT activities were very low in control fruit but were readily detected in FE fruit. Only one band was detected for both enzymes (Fig. 4). In addition, one SOD isoform was observed in fruit samples, and this enzyme's activity decreased by 60% in FE fruit (Fig. 4). To identify this isoform, gels were incubated with cyanide or peroxide before staining for SOD activity. As illustrated in Fig. 4, SOD activity was not inhibited by these compounds, indicating that the isoform detected is a Mn-SOD type. Non-denaturing gels of soluble and mitochondrial fractions stained by SOD activity showed one band with SOD activity only in the mitochondria (not shown), suggesting that the detected Mn-SOD isoform would be localized in this organelle. No SOD isoforms were identified as Cu/Zn-SOD or Fe-SOD in either sample. Cu/Zn-SOD has been detected in cultured cells and citrus leaves using a large amount of protein (Gueta-Dahan et al. 1997). However, because of the low protein content in orange crude extracts, the presence of this isoform in orange fruit cannot be disregarded. Finally, GR activity on a protein content basis was 25% lower in FE fruit compared with controls (0.19 ± 0.03 vs 0.16 ± 0.02 U mg^{-1}, respectively), while the decrease was more pronounced when the FW was taken in consideration, being 40% lower in FE fruit (0.08 ± 0.02 vs 0.05 ± 0.01 U g^{-1} FW, respectively).

Oxidative damage

Damage to membrane lipids by ROS action was assessed by measuring the TBARS content in citrus fruit after treatment with thiobarbituric acid. The level of lipid peroxidation showed an increase by 65% in FE fruit compared with controls (30.6 ± 2.5 vs 18.5 ± 2.3 nmol TBARS g^{-1} FW, respectively).

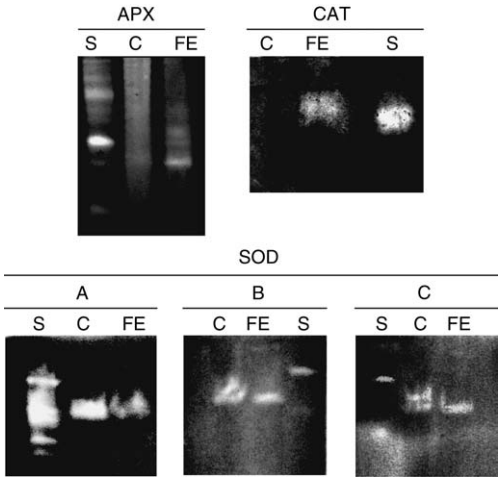

Fig. 4. Analysis of enzymes involved in antioxidant metabolism (APX, CAT and SOD) by non-denaturing polyacrylamide gel electrophoresis stained by enzymatic activity. APX: S, soybean 7-day–germinated cotyledon crude extract (50 μg); C, crude extract from control Valencia orange fruit (20 μg); FE, crude extract from FE Valencia orange fruit (20 μg). CAT: C, crude extract from control Valencia orange fruit (50 μg); FE, crude extract from FE Valencia orange fruit (50 μg); S, soybean 7-day-germinated cotyledon crude extract (50 μg). SOD: (A) Without addition of inhibitors of SOD isoforms. S, soybean 7-day-germinated cotyledon crude extract (50 μg); C, crude extract from control Valencia orange fruit (20 μg); FE, crude extract from FE Valencia orange fruit (20 μg). (B) With addition of 2 m*M* KCN. C, crude extract from control Valencia orange fruit (20 μg); FE, crude extract from FE Valencia orange fruit (20 μg); S, soybean 7-day-germinated cotyledon crude extract (50 μg). (C) With addition of 5 m*M* H_2O_2. S, soybean 7-day-germinated cotyledon crude extract (50 μg); C, crude extract from control Valencia orange fruit (20 μg); FE, crude extract from FE Valencia orange fruit (20 μg). APX, ascorbate peroxidase; CAT, catalase; SOD, superoxide dismutase.

The oxidative modification of proteins was determined by measuring the carbonyl content of fruit, which was 2.7-fold more elevated in FE fruit than in controls (7.4 ± 1.3 vs 2.8 ± 0.4 nmol mg^{-1} protein, respectively), indicating that a major oxidative damage to proteins had been induced by frost.

Discussion

Freezing temperatures registered in the late stages of maturation of citrus fruit provoke severe damage, which brings about low-quality fruit. Hence, the main objective of this work was to advance in the knowledge of the impact that this type of stress has in carbohydrate/respiratory metabolism.

Internal quality was notably affected in FE fruit. A higher acidity would be caused by the higher level of malate along with a possible reduction in the ability to metabolize citric acid. Finally, the percentage of juice was lower in FE fruit, reflecting dehydration underway in the juice sacs.

A remarkable reduction in total protein content was observed in FE fruit. This fact could be related to the action of proteases induced by cold that would degrade proteins oxidatively damaged or denatured by freeze stress added to a general reduction of protein synthesis because of an energetic deficit (Lafuente et al. 1991, Rozenzvieg et al. 2004).

FE fruit showed a marked alteration in some carbohydrate metabolism enzyme activities with respect to controls. The most dramatic change is the great increase in the activities of the fermentative enzymes PDC, ADH and LDH in FE fruit. The induction of both ADH activity and messenger ribonucleic acid levels by cold has been reported in cold-sensitive (rice and maize) (Christie et al. 1991) and cold-resistant (*Arabidopsis thaliana*) (Jarillo et al. 1993) plants exposed to low temperatures.

Activities of both PPi-PFK and PEPC, two important glycolytic enzymes in plants (Plaxton and Podestá 2006), increased in FE fruit, while those of ATP-PFK and PK, which catalyze reactions at the same level, were not affected. PPi-PFK and PEPC catalyze reactions that circumvent steps where ATP is used or produced and are induced under a number of conditions that lead to a drop in nucleotide levels, such as Pi deprivation (Plaxton 1996), anoxia (Mertens et al. 1990) or under cold stress in wheat (González et al. 2003). It is worth to emphasize the different sensitivity of PEPC from control and FE fruit toward its inhibitor, L-malate (with an I_{50} value in excess of two orders of magnitude higher in FE fruit) along with the different response of the enzyme's activity to pH changes. It is widely known that most plant PEPCs are subject to allosteric regulation, being activated by glucose-6-phosphate and inhibited by L-malate or aspartate (Izui et al. 2004). In addition, the enzyme is also regulated by reversible phosphorylation at the conserved serine located near the N terminus. The effects of phosphorylation of all plant PEPCs studied to date on its kinetics was to decrease the sensitivity of the enzyme to allosteric inhibition by L-malate (Chollet et al. 1996). Consequently, the striking difference observed in the PEPC's sensitivity to malate between control and FE fruit strongly suggests that there is a change in the enzyme's phosphorylation status by frost exposure of citrus fruit. Indeed, treatment of PEPC with alkaline phosphatase shows a marked increase in the sensitivity to malate in FE fruit, while no change is observed in control fruit (M.L. Falcone Ferreyra, V. Perotti, A. Iglesias and F.E. Podestá, unpublished results). It has been reported that during the development of tomato and grape berry, PEPC is less sensitive to low pH and malate inhibition, indicating that a phosphorylated form would control the accumulation of malic acid (Guillet et al. 2002). Accordingly, the lowered sensitivity of PEPC to inhibition by L-malate

added to the enzyme's induction can be rationalized in the context of an elevated fermentative metabolism because in combination with cytosolic NAD-MDH, it can also be useful to regenerate NAD^+, thus acting as an ancillary fermentation enzyme.

Alanine has the double advantage of acting as a biocompatible solute and serving as a storage form of pyruvate in the vacuole, controlling the supply of pyruvate to LDH and PDC and hence the flux to lactate and ethanol (Muench et al. 1998). AlaAT activity was not affected by freeze temperatures, which suggests that, in FE fruit, alanine may not be an important metabolic product of pyruvate under these stressful conditions.

Taking into account that the primary and secondary regulation of the glycolytic pathway are at the level of PEP and Fru-6-P utilization, respectively, an increase in PEPC activity would lower PEP and 3-phosphoglycerate levels (these metabolites are in equilibrium in vivo), both of which inhibit phosphofructokinase 2. Since PEP is also an inhibitor of PPi-PFK (Turner and Plaxton 2003), the net result would be an activation of the upper glycolysis by PPi-PFK activation. A higher PPi-dependent phosphorylation of Fru-6-P by the action of PPi-PFK with respect to ATP-PFK would be energetically beneficial in a stress situation where the energetic charge (ATP pool) would be limited, contrary to PPi levels that are remarkably insensitive to abiotic stresses (Plaxton and Podestá 2006).

Western blot analysis indicated that changes in the enzymatic activities are correlated with variations in the protein amounts for LDH, PPi-PFK, PEPC and NADP-ME (Fig. 3). With respect to the cytosolic NAD-MDH, no variation was observed in the immunoreactive protein content between control and FE samples. The drop in total NAD-MDH activity observed in FE fruit crude extracts could then be attributed to the mitochondrial isoform.

An examination of the subunit composition of PPi-PFK revealed a relative increase in the amount of the β subunit, which takes the ratio α/β from 1.66 to 1 upon freezing. Although the role of each subunit is not clearly defined, it has been proposed that the α subunit confers sensitivity to Fru-2,6-P_2, while β subunit is catalytic (Podestá and Plaxton 1994). The ratio α/β has been observed to change in response to stress or developmental cues (Botha and Botha 1991, Podestá and Plaxton 1994, Theodorou and Plaxton 1994). The response of PPi-PFK to Fru-2,6-P_2 differed in control and FE fruit. While the enzyme from control fruit was not saturated by this metabolite even at the millimolar range, in FE fruit, the K_a was 8 n*M*. This observation indicates that the increase in PPi-PFK activity in FE fruit would be because of not only a higher enzyme level (specially the β subunit) but also a higher affinity of the enzyme toward its activator, Fru-2,6-P_2. The parallel change in the ratio α/β and sensitivity toward the activator makes it tempting to establish a connection between both, but at this stage of our knowledge of PPi-PFK structure-to-function relationship, it is difficult to put forward a hypothesis without being too speculative. However, a possible explanation for this situation would be that at an α/β ratio of 1.66, part of the enzyme could exist as a less active, less sensitive form with a subunit stoichiometry different from 1:1, perhaps coexisting with forms having the 1:1 ratio. Of course, it cannot be disregarded that a new form of the enzyme could be expressed after frost exposure.

Associated with the fermentative trend observed in the enzyme content, a decrease in respiration of 40% was observed in whole FE fruit, while this reduction became more pronounced in the flavedo (53%). The volatile compound content was also significantly higher in FE fruit.

Although citrate is the main organic acid accumulated in Valencia orange fruit, the decrease observed in the NAD(P)-ME enzyme added to the induction of PEPC could generate an accumulation of malate in FE fruit. The enzymatic determination of this organic acid indicated an increase of three times in FE fruit in comparison with controls.

Electrolyte leakage is a useful parameter to estimate membrane integrity and evaluate damage by cold (Mancuso et al. 2004). FE fruit showed an increase of 32% in the electrolyte leakage, indicating that a major alteration in permeability had occurred (Table 1).

Several lines of evidence indicate that oxidative stress is involved in cold damage, and tolerance to low temperature has been related to a more efficient antioxidant system (Holland et al. 1999, Sala and Lafuente 1999, Suzuki and Mittler 2006). The analysis of ROS scavenger enzymes levels showed a strong increase in APX and CAT activity in FE fruit with respect to controls, while a SOD isoform, identified as Mn-SOD type, lowered. In addition, GR activity decreased in FE fruit both as a function of FW and milligram of protein. These results are also consistent with the establishment of oxidative stress in FE citrus fruit. The superoxide radical (O_2^-) is produced in the electron transport chains (in chloroplast and mitochondria) and for the action of enzymes such as NADPH and xanthine oxidases (plasmatic membrane and peroxisomes). SOD represents the first line of defense in the cell. Because biological membranes are impermeable to the superoxide radical, isoforms of SOD are present in different cellular compartments (Alscher et al. 2002). In heterotrophic tissues, such as fruit, the mitochondria are both the main source and target for oxidative stress induced by cold (Purvis et al. 1995, Bartoli et al. 2004). According to the results presented here, mitochondria of FE fruit would be less efficient to eliminate the superoxide radical generated by

the mitochondrial electron transport chain (miETC). On the other hand, higher CAT and APX activities would efficiently eliminate H_2O_2. Finally, GR is also involved in the detoxification of H_2O_2 in the ascorbate–glutathione cycle by means of the reduction of oxidized glutathione generated by the reaction catalyzed for dehydroascorbate reductase. The decrease observed in GR activity in FE fruit would lead to a limited or even impaired elimination of H_2O_2 in the cytosol and then to a reduction in reduced ascorbate and glutathione levels. Additionally, the overall level of glutathione could be lowered if ATP levels fall (Noctor and Foyer 1998). Furthermore, ascorbate levels could be also reduced because its synthesis is strictly linked to the miETC that is noteworthy impaired in FE fruit (Bartoli et al. 2000, Millar et al. 2003). Other factors responsible for a decreased level could be its direct destruction by O_2^- and derived species, and also its consumption for tocopherol regeneration (Iturbe-Ormaetxe et al. 1998).

NADP-ME are expressed in a tissue-specific manner as cytosolic or plastidic forms in C_3 plants (Gerrard Wheeler et al. 2005), but the localization of NADP-ME in citrus fruit is not known. Therefore, it cannot be assessed at this time which isozyme activities are been reduced. Although a lower capacity to produce NADPH by NADP-ME could be detrimental to the maintenance of the levels of reduced antioxidants, it could be compensated by a slightly higher G6PDH activity in FE fruit. On the other hand, it has been reported that GAPN could be involved in response to oxidative stress in wheat leaves, contributing to reductive power generation and also modulating levels of energy and reductants in the cytoplasm of the heterotrophic plant cell (Iglesias et al. 2000). In citrus fruit, GAPN activity was very low in control and FE samples, suggesting that this enzyme is not involved in the response to cold stress and would have a limited role in carbohydrate metabolism in citrus fruit.

An imbalance between the production of ROS and the level of ROS-detoxifying enzymes and reductants would lead to oxidative damage and impaired cellular functions. This fact was supported by the study of oxidative damage to lipids and proteins estimated by the TBARS level and carbonyl assay, respectively. Hence, FE fruit showed a notable increase in both TBARS and protein carbonyl content. The increased peroxidation of polyunsaturated fatty acids observed in FE fruit could be the source of unpleasant aroma compounds in damaged fruit.

The observations described above and the fact that FE fruit showed notable differences with respect to controls in mitochondrial marker enzymes such as a decrease in fumarase activity and NAD-ME immunoreactive protein point to a damage to the mitochondria, a hypothesis that needs further investigation to be substantiated.

Conclusions

Orange fruit exposed to natural frost exhibit notorious changes at the level of respiratory metabolism. The main features of frost-induced damage are (1) a surge in fermentative enzyme activities and the products of fermentation (volatiles and malate); (2) an increase in two glycolytic enzymes (PPi-PFK and PEPC) that catalyze bypasses to nucleotide-utilizing steps and (3) an increase in the ROS scavenging enzymes, CAT and APX, and a decrease in Mn-SOD and GR. Future studies focused in the proteomics (Renaut et al. 2006) and the investigation of other proteins and molecular mechanisms (Chinnusamy et al. 2006, Kaplan et al. 2006) involved in the response of citrus to frost exposure should be useful for a complete understanding of this extended, naturally occurring abiotic stress.

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