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Postharvest Treatment of ‘Florida Prince’ Peaches with a Calcium Nanoparticle–Ascorbic Acid Mixture during Cold Storage and Its Effect on Antioxidant Enzyme Activities

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Abstract: Chilling injury (CI) is a physiological disorder resulting from low storage temperatures that affects the fruit quality and marketing of the ‘Florida Prince’ peach. In this study, the exogenous application of a mixture of calcium nanoparticles (CaNPs) and ascorbic acid was found to significantly alleviate the symptoms of CI in peaches during cold storage. Fruits were treated with CaNPs plus different concentrations of ascorbic acid (AA; 0, 3, 6, and 9 mM). Peaches were immersed in CaNP–AA for 15 min before being stored at 4 ± 1 °C and $95 \pm 1\%$ RH for 30 days. We observed that the 9 mM CaNP–AA treatment lowered the values for the CI index, ion leakage, and malondialdehyde (MDA) content and increased antioxidant enzyme activities (AEAs), such as for ascorbate oxidase (APX), catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR). Furthermore, the treatment reduced the accumulation of both H_2O_2 and $O_2^{\bullet-}$ and increased the level of DPPH reduction throughout the duration of cold storage. Our results suggest that 9 mM CaNP–AA treatment suppresses the incidence of CI in peach fruit throughout cold storage, possibly because 9 mM CaNP–AA is at least partly involved in enhancing the antioxidant system via its effect on antioxidant substances. The results indicate that applying the 9 mM CaNP–AA treatment afforded peaches with enhanced tolerance against cold storage stress.

Keywords: peach; cold storage; quality; calcium nanoparticles; antioxidant enzymes



Citation: A. A., L.; Ismail, H.; Kassem, H.S. Postharvest Treatment of ‘Florida Prince’ Peaches with a Calcium Nanoparticle–Ascorbic Acid Mixture during Cold Storage and Its Effect on Antioxidant Enzyme Activities. *Horticulturae* **2021**, *7*, 499. <https://doi.org/10.3390/horticulturae7110499>

Academic Editors: Maria Dulce Carlos Antunes, Custódia Maria Luís Gago and Adriana Guerreiro

Received: 17 September 2021
Accepted: 11 November 2021
Published: 15 November 2021

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1. Introduction

From the genus *Prunus*, the *Prunus persica* L. Batsch cv. ‘Florida Prince’ peach is the first early stone fruit crop to have been cultivated in Egypt. It displays high acclimation to local ecological conditions. A superior yield and fruit quality in comparison with others are recorded for this peach cultivar [1]. The area used for peach cultivation in Egypt is about 24,707 ha, corresponding to a total production of 360,723 tons [2]. Peaches decay easily during marketing processes, losing their value and quality after harvest. This decline occurs for several reasons, such as the rapid ripening of fruits, their sensitivity to mechanical injuries that occur throughout various handling processes, and their susceptibility to rapid infection from fungal diseases [3]. Therefore, for the reasons mentioned above, cold storage is an important way to reduce the rapid ripening of fruits and control the spread of diseases [4]. However, prolonged cold storage of peach fruits leads to biophysical changes in the cell walls that may later manifest as cold injury symptoms [5]. Symptoms of cold injury in peach fruits include the appearance of brown spots that vary in size and shape as well as the collapse of the interior tissue [6]. With an increase in storage duration, there is a tendency for greater development of chilling injury symptoms in fruit, reducing consumer acceptance [7].

Numerous studies conducted on CI symptoms have attempted to understand their prevalence and to mitigate their severity, frequently via treatments. Such studies tested the effect of cold storage of fruit [8], heat treatment of fruits to activate the action of antioxidants before cold storage [9], the use of Ultra-Violet (UV-C) [10] or salicylic acid to relieve the symptoms of cold injury [11], and the treatment of fruits with methyl jasmonate [12]. These studies aimed to reduce the occurrence of cold injury symptoms and control the quality of peaches during storage.

Recent research has examined the use of nano-calcium technology in the field of postharvest treatments for diminishing the phenomenon of CI symptoms during the cold storage of fruits [13]. Strengthening nano-calcium treatment by including antioxidants such as ascorbic acid (AA) plays an active role in countering the CI phenomenon. Generally, CI is directly correlated with the formation of reactive oxygen species (ROS) in fruit tissue throughout the storage period [14]. However, the AA present in plant cells reacts directly with ROS produced in cells [15], especially hydroxyl radicals [16]. Therefore, it increases the tolerance of fruit to the impact of the cold [17]. Furthermore, AA changes the oxidative state of alpha-tocopheroxyl radicals to normalize the action of plasma membranes on the plant cell membrane [18]. It also works to efficiently sustain antioxidant enzyme activity, which creates an equilibrium between the generation of ROS and antioxidants in the cell under chilling stress [19].

Calcium ions (Ca^{++}) are the main basis of pectin accumulation for supporting the cell wall and middle lamella to produce calcium pectate gel [20]. Calcium ions also balance the cell membrane and thus participate in firmness [21]. However, fruit firmness deteriorates considerably under the highest individually applied calcium chloride doses. Hence, using the authorized dose of calcium chloride (1%) is important because the application of high calcium chloride concentrations may cause stress on the tissues and thus a rapid increase in both the respiration rate and ethylene production [22]. Excessive respiration and ethylene production are associated with an increase in enzymatic breakdown, which leads to ripening and senescence [23]. Therefore, the tissues likely become softer than those of the untreated fruits. To the best of our knowledge, no prior studies have examined the role of calcium nanoparticles combined with ascorbic acid in protecting peach fruit during low-temperature storage. Therefore, this paper aims to assess the influence of calcium nanoparticles blended with ascorbic acid on CI increase in the 'Florida Prince' peach cultivar, and investigate the change model in fruit quality, chilling injury, and antioxidant enzyme potential throughout long periods of refrigeration.

2. Materials and Methods

2.1. Fruit Materials and Postharvest Treatments

The 'Florida Prince' peaches were from a commercial farm in Dakahlia province, Egypt (30.04° N, 31.25° E). Fruits were picked at commercial maturity (128 days after the full bloom stage) and included if they were free from peel defects and were uniform in size and shape. Fruits (600) were picked and delivered 2 h after harvesting. They were divided into two main batches. The first batch (300 fruits) was used for physical measurements, i.e., the chilling injury symptoms index, water loss, and fruit skin color (hue angle). This batch was divided into five lots of 60 fruits each for treatments, for which there were three replicates (e.g., 3 × 20 fruits). The second batch was used for the chemical analysis and had the same fruit distribution among the treatments as previously described.

2.2. Synthesis of Metal Calcium Nanoparticles (CaNPs) with Ascorbic Acid (AA)

Calcium nanoparticles (CaNPs) were prepared according to the procedure reported by Yugandhar and Savithamma [24]. A slight modification was made by adding AA at 3, 6, and 9 mM into a solution of CaCl (50 mM). Using distilled water, all weights of ascorbic acid were blended in a solution of calcium chloride at a concentration of 50 mM. The reaction mixture was spun on a checker at 5000 revolutions per minute for 1 h and then allowed to cool to room temperature for 2–3 days.

UV-vis spectroscopy was used to characterize the nanoparticles. The reduction of pure Ca^{++} particles and the subsequent topping of calcium nanoparticles were measured using ATI Unicomp UV-vis spectroscopic analysis vision software ver. 3.20 by comparing the UV-vis spectra of the response blend at various wavelengths. The combined metal nanoparticles' UV-vis spectra were measured between 240–440 nm. The investigation was successfully conducted at a temperature of 25 °C using quartz cuvettes with a 1 cm optical path (Figure 1).

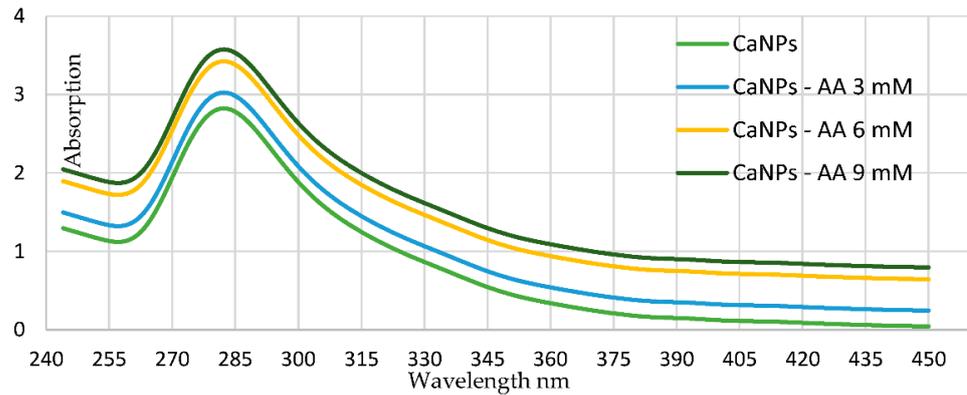


Figure 1. The UV-visible absorption spectra of the structure of calcium nanoparticles (CaNPs) mixed with various combinations of ascorbic acid (0, 3, 6, and 9 mM), displaying a peak at 282 nm.

The Zeta Potential test was used to determine the nanoparticles' (NPs) surface state and forecast the nanoparticle solution's long-term stability. The technique was used to define the CaNPs blended with ascorbic acid (AA) surface charge at the Central Laboratory, Electron Microscope Unit, Faculty of Agriculture, Mansoura University, Mansoura, Egypt, using Malvern Instruments Ltd. and Zeta Potential Ver. 2.3. The CaNP-AA mixture has an electrical charge on its surface, attracting a thin layer of ions with opposite directions to the surface. The Zeta Potential of nanoparticles provides information about their properties. Nanoparticles have two layers of ions that move as the solution diffuses. The electric potential at the end of the double layer is referred to as the Zeta Potential of the particles, and it varies between +100 and 100 mV. CaNPs containing AA were synthesized and had a Zeta Potential of -4.74 mV (higher stability). NPs with Zeta Potentials greater than or equal to $+25$ mV had a high degree of stability (Figure 2).

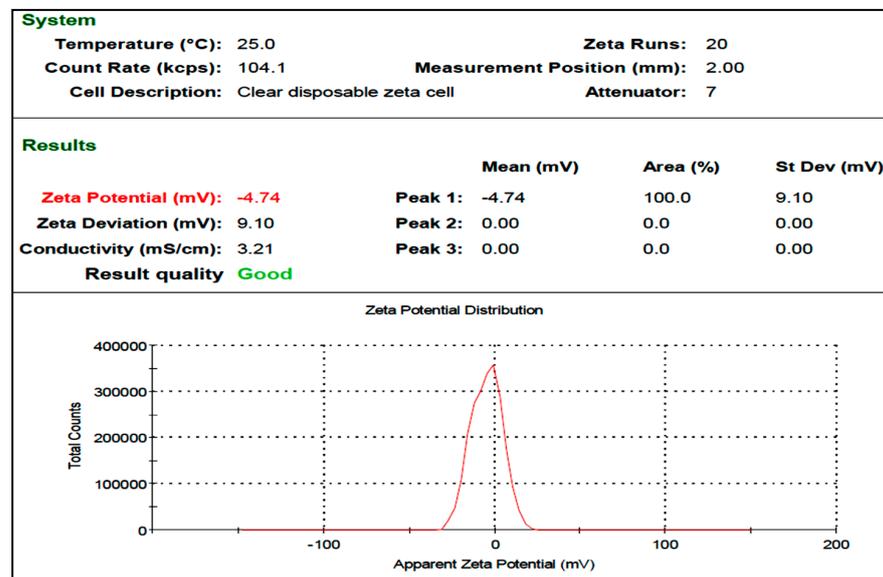


Figure 2. Zeta Potential determination technique for calcium nanoparticles with added ascorbic acid.

The nanoparticles' characterization was determined using transmission electron microscopy (JEOL TEM-2100) coupled to a CCD camera at a 200 kV acceleration voltage. Nanoparticles were defined according to size, shape, surface area, particle structure, and morphological characteristics. The integrated metal nanoparticles were created by suspending them on copper-coated carbon networks and allowing the dissolvable to dissolve gradually before chronicling the TEM images. TEM measurements were taken at Mansoura University's Central Laboratory, Electron Microscope Unit, Faculty of Agriculture, Mansoura, Egypt (Figure 3).

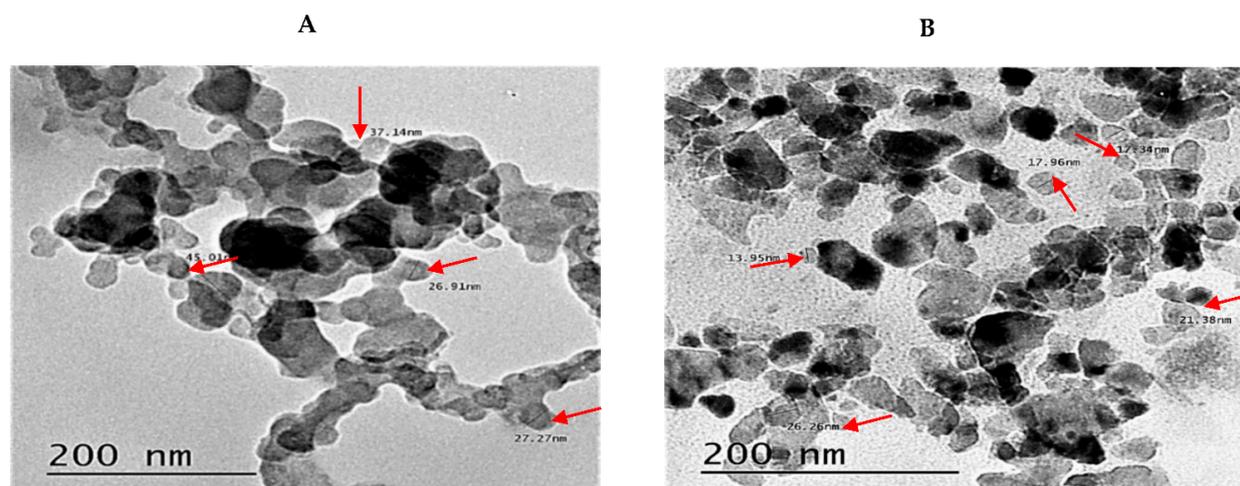


Figure 3. Transmission electron microscopy (TEM) images of the manufactured nanoparticles at 200 nm. The size of the CaNP particles (A) was between 27.27 and 45.01 nm. However, after mixing with ascorbic acid, the CaNPs reached about 13.95–21.38 nm in diameter (B). The particles were orbicular in appearance, and a few were tetragonal. The CaNPs particles tended to aggregate more in isolation than when mixed with ascorbic acid.

2.3. CaNP-AA Application Protocol

CaNPs were applied to the batches via five application approaches. The treatments were as follows: control, 0 mM CaNP-AA, 3 mM CaNP-AA, 6 mM CaNP-AA, and 9 mM CaNP-AA. Next, batches were soaked in the CaNP-AA treatments for 15 min at 4 °C, then placed into cold storage (4 ± 1 °C and air relative humidity, RH% 95 ± 1) for 30 days.

2.4. Chilling Injury Index, Water Loss%, and Fruit Skin Color

CI symptoms in peaches appear as brown shrunken areas/spots that increase in number and size as the duration of cold storage increases. The CI symptoms were inspected and scored on a scale from 0 (no injury) to 5 (very severe injury) based on necrotic spot area and browning intensity [25]. The CI index was computed according to the following formula:

$$CI - index = \sum_{k=5}^n \frac{(CI \text{ level}) * (\text{Number fruit at this level})}{\text{Total number of fruit}}$$

Water loss (WL%) was assessed by the following equation: $WL\% = (W_{t=0} - W_t) / W_{t=0} \times 100$; where $W_{t=0}$ is the initial weight of each fruit and W_t is their weight after five days [17]. However, the fruit skin color hue angle measurement was evaluated at intervals throughout the duration of storage by collecting images. To calculate the hue angle of peach, RGB signals were obtained using software ImageJ Ver. 1.43u (USA), according to Khojastehnazhand et al. [26].

2.5. Total Soluble Solid Content (SSC%), Total Acidity (TA%), and SSC/TA Ratio

The SSC% of peach juice was measured with a digital refractometer (PR32 ALAGO Co., Japan) at room temperature and was represented as a percentage. For TA%, peach

juice (20 mL) was used for titration with NaOH (0.1 N) [27]. The outcome was presented as a percentage according to the following formula:

$$TA\% = \frac{[0.1 \text{ M NaOH} * \text{vol. of NaOH (in liter)} * 192.43]}{wt \text{ of sample}} * 100$$

where 192.43 g/mol is the molecular weight of citric acid.

The SSC/TA ratio was computed to judge peach maturity [25].

2.6. Fruit Pigments and Fruit Firmness (N)

The total anthocyanin in the fruit material was extracted using methanol mixed with 1% hydrochloric acid. After grinding in liquid N₂, the samples were incubated at room temperature overnight. The extracts were then centrifuged at 16,000× g for 16 min and their absorbance at 530 and 657 nm was measured using a spectrophotometer set at 421 nm [28].

The freeze-dried materials were first pulverized in a ball mill to extract the carotene, then 5 mL of N,N-dimethylformamide (DMF) was added to 0.8 g of this powder. To ensure a thorough extraction of carotene, the sample powder was immersed in DMF for 16 min at 4 °C [29], and then stored at 4 °C for 16 h. Finally, 1 mL of the suspension was centrifuged at 16,000× g for 5 min at 4 °C to remove all particles, and the supernatant solution was measured using a spectrophotometer [30].

Fruit firmness measurements were taken using a Zwick Universal Testing Machine equipped with a 60° conical probe 6.35 mm in diameter. The apparatus determined the force needed by the mechanical probe to penetrate 8 mm into the tissue of fruits at a speed of 3 mm s⁻¹ [31].

2.7. Antioxidant Enzyme Activities (AEAs)

To determine catalase (CAT) activity, fruit pulp (2 g) was homogenized with 20 mL of a solution of 100 mM potassium phosphate (KH₂PO₄). The mixture was centrifuged (30,000× g) twice for 25 min at 4 °C. The clear extraction quantity was utilized for observations of the CAT activity in a final volume of 5 mL that contained 1 mL of the catalase extract (400–800 mg protein). A unit of CAT activity was defined as the amount of the compound that could oxidize 1 mM H₂O₂ min⁻¹ at 25 °C [32].

Ascorbate peroxidase (APX) was isolated from 2 g of fruit pulp tissue ground with 20 mL of 50 mM potassium phosphate (KH₂PO₄). Additionally, EDTA, ascorbic acid (AA, 1 mM), and polyvinylpyrrolidone (PVPP, 1%) were added at 5 °C. The materials were mixed and centrifuged twice at 35,000× g for 30 min at 4 °C, and the clarified supernatant was utilized to monitor the APX activity in a final quantity of 3 mL. This included 150–300 mL of the clear fraction (40/240 mg protein). A unit of APX was characterized as the amount of the compound that oxidized 1 mM of ascorbate min⁻¹ at 25 °C [33].

Glutathione reductase (GR) was separated from 1 g of peach pulp tissue ground in 25 mL of a 100 mM KH₂PO₄ buffer (pH 7.4) containing 0.6 mM EDTA at 5 °C. The mixture was centrifuged twice at 25,000× g for 25 min at 5 °C. An aliquot of the clear supernatant was applied to observe the GR activity in a final volume of 4 mL [34]. It contained 100 mL of the accelerator extract (40–80 mg protein). Each GR unit was defined as the amount of accelerator that oxidized 1 mM of NADPH min⁻¹. Moreover, the activity was counted on the standard curve as reported in [35].

Superoxide dismutase (SOD) was isolated from 1 g of peach pulp tissue ground in 10 mL of 50 mM KH₂PO₄ with the addition of 1.33 mM of diethylenetriamine penta-acetic acid (DTPA, C₁₄H₂₃N₃O₁₀) at 4 °C, then the mixture was centrifuged twice at 30,000× g for 30 min at 4 °C. The clear supernatant was used to detect SOD activity [36] in a final volume of 4 mL, which contained 70–80 mL of the mixture concentrate (24–56 mg of protein). A unit of SOD was described as the quantity of the substances that produced a half-maximal decrease. All the macromolecules were prepared and examined for calculating the catalyst activity [37].

2.8. Lipid Peroxidation, Malondialdehyde (MDA) Accumulation, and Ion Leakage%

Peach pulp (3 g) was ground and combined with 30 mL of metaphosphoric acid (HPO_3 , 5%) and 500 μL of butylated hydroxytoluene (2%) in ethanol; then, the mixture was homogenized. 1,1,3,3-Tetra-ethoxy-propane (Sigma-Aldrich, St. Louis, MO, USA) was used to vary the amount of TBARS from 0 to 20 mM relative to 0–1 mM malondialdehyde (MDA) as a calibration standard to evaluate MDA accumulation product for the peach samples. The stoichiometry of MDA was calculated throughout the acid-heating step of the assay [38].

The ion leakage (IL%) of all peach samples was measured initially (M_0) by using an INE-DDSJ-318 conductivity meter. Later, after 3 h, all samples were heated at 100 °C in a water bath for 30 min to measure the total leakage after the samples reached room temperature (M_1) [39]. The percentage of IL% was calculated by the following formula:

$$\text{IL\%} = M_0 \text{ reading after 3 h} - M_1 \text{ (reading after heating)} / M_0 \times 100$$

2.9. Ethylene and Respiration Assessment

Ethylene concentrations and CO_2 respiration were determined at 5-day intervals in five peaches. For all experimental treatments, fruits were placed and sealed in 1000 mL glass jars with a 1-h gap between each interval of cold storage duration (in days). Gas chromatography techniques were used to extract gas samples from the headspace atmosphere surrounding the fruit and analyze them for ethylene and carbon dioxide (GC). Ethylene concentrations were determined using a GC-6000 Vega Series from Carlo Erba Ins., Milano, Italy, while CO_2 concentrations were determined using a GC PBI-Dansensor Checkmate-9900 from Denmark [40].

2.10. H_2O_2 and $\text{O}_2^{\bullet-}$ Production Rate and DPPH Reduction

One gram of fruit tissue was added to 3 mL of a KH_2PO_4 buffer 50 mM (pH 7.8) under cooling at 4 °C. The reagent was combined with polyvinylpyrrolidone (PVP 1% *w/v*) and immediately centrifuged at 10,000 rpm at 4 °C for 15 min. The $\text{O}_2^{\bullet-}$ production rate was determined by observing the development of NO_2 from hydroxylamine with the introduction of $\text{O}_2^{\bullet-}$ [41]. A linear curve with NO_2 was utilized to establish the $\text{O}_2^{\bullet-}$ formation rate from the response of $\text{O}_2^{\bullet-}$ with hydroxylamine. $\text{O}_2^{\bullet-}$ production was determined as $\text{nmol min}^{-1} \text{g}^{-1} \text{FW}$.

In the H_2O_2 assay, 1 g of the fruit pulp sample was added to 6 mL of 100% $(\text{CH}_3)_2\text{CO}$ and immediately centrifuged at $10,000 \times g$ for 15 min at 4 °C, then 1 mL of the clarified supernatant was combined with 0.1 mL of 5% $\text{Ti}(\text{SO}_4)_2$ and 0.2 mL of a NH_4OH solution. The hydrogen peroxide sample was accelerated, and the residue was reduced by adding 4 mL of 2 M H_2SO_4 after centrifugation at $10,000 \times g$ for 20 min, then the absorbance was quantified on a photometer at 415 nm. The H_2O_2 content was calculated from a standard curve and the fixation rate was shown as $\mu\text{mol g}^{-1} \text{FW}$ [42].

The inhibitory activity of DPPH was examined in the peach pulp sample and the dismutation of radical activity technique was applied. The scavenging results of flavedo samples of DPPH radicals were given as percentages. In brief, a 2 mL sample of peach extract (with methanol) was combined with 2 mL of 0.16 mM DPPH methanolic solvent. Afterward, samples were shaken for 1 min and stored for 30 min at room temperature in dark conditions. Subsequently, samples were evaluated on a photometer at a wavelength absorbance of 517 nm. The final amount of the DPPH radical was assumed by applying the formula of [43].

2.11. Statistical Analysis

The experiment was conducted during two growth seasons (2018–2019) using the Co-Stat software package (Ver. 6.303; 789 Lighthouse Ave PMB 320, Monterey, CA 93940, USA). The chilling injury, water loss, and peach skin color profile were analyzed in a randomized complete block design for the effect of fruit color maturity stages (applied to the same fruit

throughout the storage period). A factorial analysis displayed the main comparisons and the interaction effect of storage time and CaNP-AA under refrigeration. The appropriate significant differences among CaNP-AA treatments were tested by utilizing Duncan's multiple range test at the 5% level.

3. Results

3.1. Synthesis of Metal Calcium Nanoparticles (CaNPs)

We observed that the formation of calcium nanoparticles (CaNPs) blended with ascorbic acid at different concentrations was synthesized, and we confirmed treatments at a peak at 282 nm by UV-visible absorption spectra (Figure 1). Another technique to prove the formation of nanoparticles was using the Zeta Potential test for calcium nanoparticles with added ascorbic acid (Figure 2). To be certain, we used Transmission Electron Microscopy (TEM) images at 200 nm. The size of the CaNP particles (Figure 3A) was between 27.27 and 45.01 nm. However, after mixing with ascorbic acid, the CaNPs reached about 13.95–26.26 nm in diameter (Figure 3B).

3.2. CI Index, Water Loss%, and Peach Skin Color

Figure 4 shows the changes in the CI index, water loss, and peach skin color (h°) as physical features of peach quality throughout a cold storage period of several weeks. CaNP-AA treatments had a significant influence when studied as a factor. Regarding the various CaNP-AA applications, a significantly greater increase in CI incidence and water loss occurred, while peach skin color declined, in the untreated fruit compared with fruit treated throughout the 30 days. Nevertheless, fruit subjected to the 9 mM CaNP-AA treatment presented significantly lower CI incidence and water loss and the highest preservation of peach skin color (h°) compared with other CaNP-AA treatments throughout the storage period. Moreover, the 9 mM CaNP-AA treatment minimized CI incidence over the full storage period (30 days). This began on the 20th day (1.01), then increased slightly up until the end of storage (1.19). The outcomes indicated a reduction in the rate of water loss (19.57%) and a slight decline in the skin color of the fruit ($h^\circ = 75.31$) on the 30th day of the cold storage period. The change in physical parameters could be due to the cold temperature stress effects, which enhanced the formation of reactive oxygen species (ROS).

3.3. Total Soluble Solids (SSC%), Total Acidity (TA%), and SSC/TA Ratio

Figure 5 depicts the differentiation in chemical quality, i.e., SSC%, TA%, and SSC/TA ratio, as a function of storage duration in days for 'Florida Prince' peach. Evidently, SSC% increased significantly in the control fruit during the storage period. However, the CaNP-AA at 9 mM treatments presented a different trend. We observed the lowest changes in SSC% compared to untreated and treated fruit. The chemical quality elements revealed a significant interaction ($p < 0.001$) when storage time (days) and CaNP-AA treatments were examined.

The chemical quality results for fruit treated with the control treatment showed a gradual increase in SSC% and the SSC/TA ratio throughout the 30 days. Nevertheless, reductions in total TA% throughout storage duration in fruit treated with 9 mM CaNP-AA were seen. This produced the lowest changes in both SSC and the SSC/TA ratio throughout the cold storage period compared with other treatments and the initial values at harvest time, with 11.82% SSC and an SSC/TA ratio of 12.23%. However, a stable TA value (0.960%) was maintained on the 30th day of the storage period compared with the initial value (1.016%) at harvest time.

3.4. Fruit Pigments (Anthocyanin and Carotene) and Fruit Firmness (N)

Figure 6 displays the variation in fruit pigment (i.e., anthocyanin (peel; $\text{mg } 100 \text{ g}^{-1}$ FW) and carotene (pulp; $\mu\text{g } 100 \text{ g}^{-1}$ FW)) and firmness (N) as a function of storage duration in days for 'Florida Prince' peach. Evidently, both pigments decreased significantly in all CaNP-AA mixtures and control fruit throughout the storage period, as did fruit firmness. The 9 mM CaNP-AA treatment significantly reduced the fruit pigment degradation and

firmness throughout 30 days compared with the initial value at harvest time. Fruit pigments on the 30th day were $14.06 \text{ mg } 100 \text{ g}^{-1} \text{ FW}$ and $1.94 \text{ } \mu\text{g } 100 \text{ g}^{-1} \text{ FW}$ compared with the initial values of $14.67 \text{ mg } 100 \text{ g}^{-1} \text{ FW}$ and $2.00 \text{ } \mu\text{g } 100 \text{ g}^{-1} \text{ FW}$, respectively. Furthermore, fruit firmness was recorded at 61.87 N , compared with an initial value of 63.26 N .

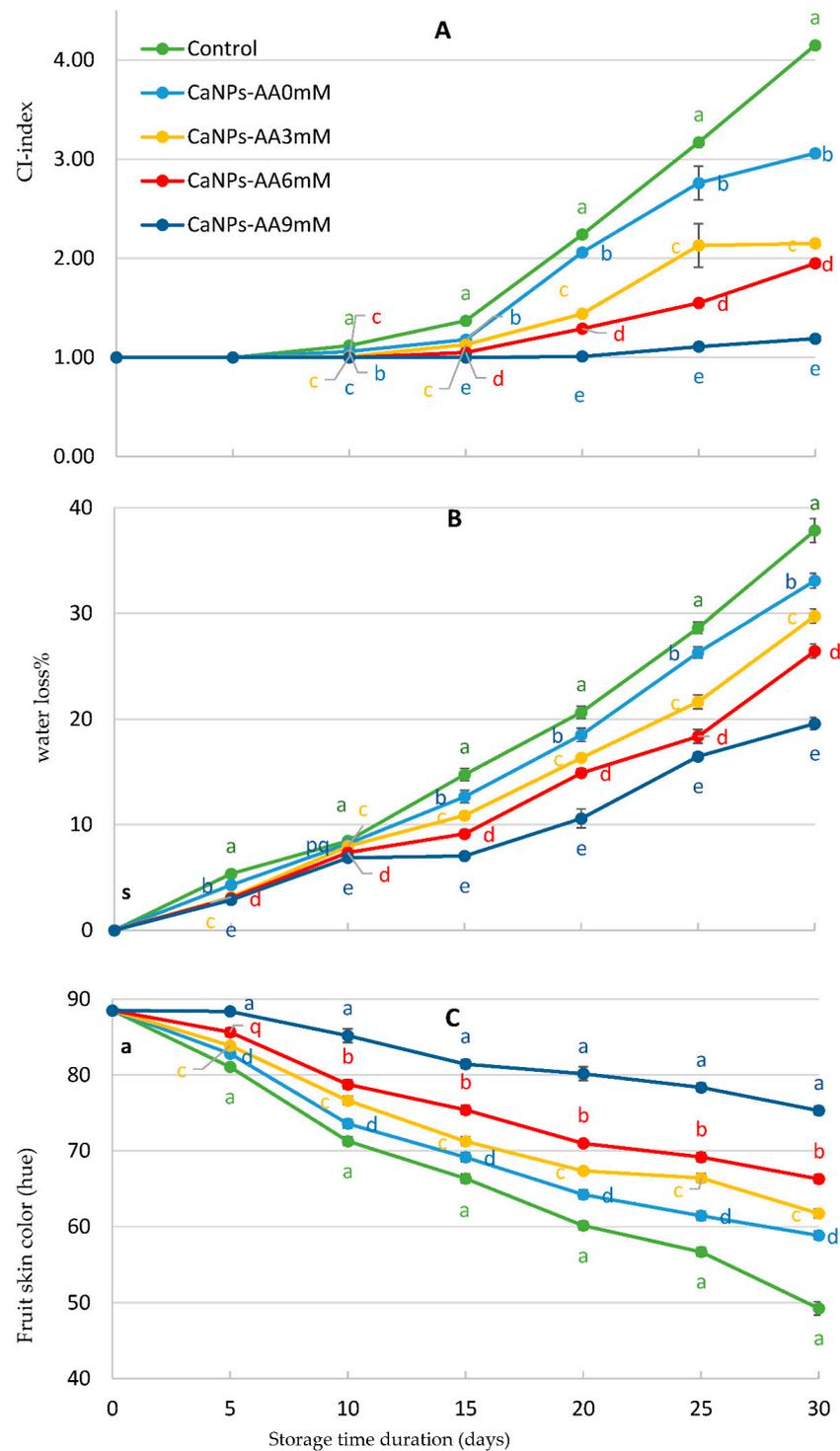


Figure 4. The chilling injury index (A), water loss percentage (B), and fruit skin color (hue) (C) of ‘Florida Prince’ peaches immersed in CaNPs mixed with ascorbic acid at different concentrations (0, 3, 6, and 9 mM) and stored at a low temperature ($4 \text{ } ^\circ\text{C}$ and $95\% \text{ RH}$) for 30 days. Error bars represent standard errors and different letters indicate significant differences at $p \leq 0.05$ among treatments for each storage period.

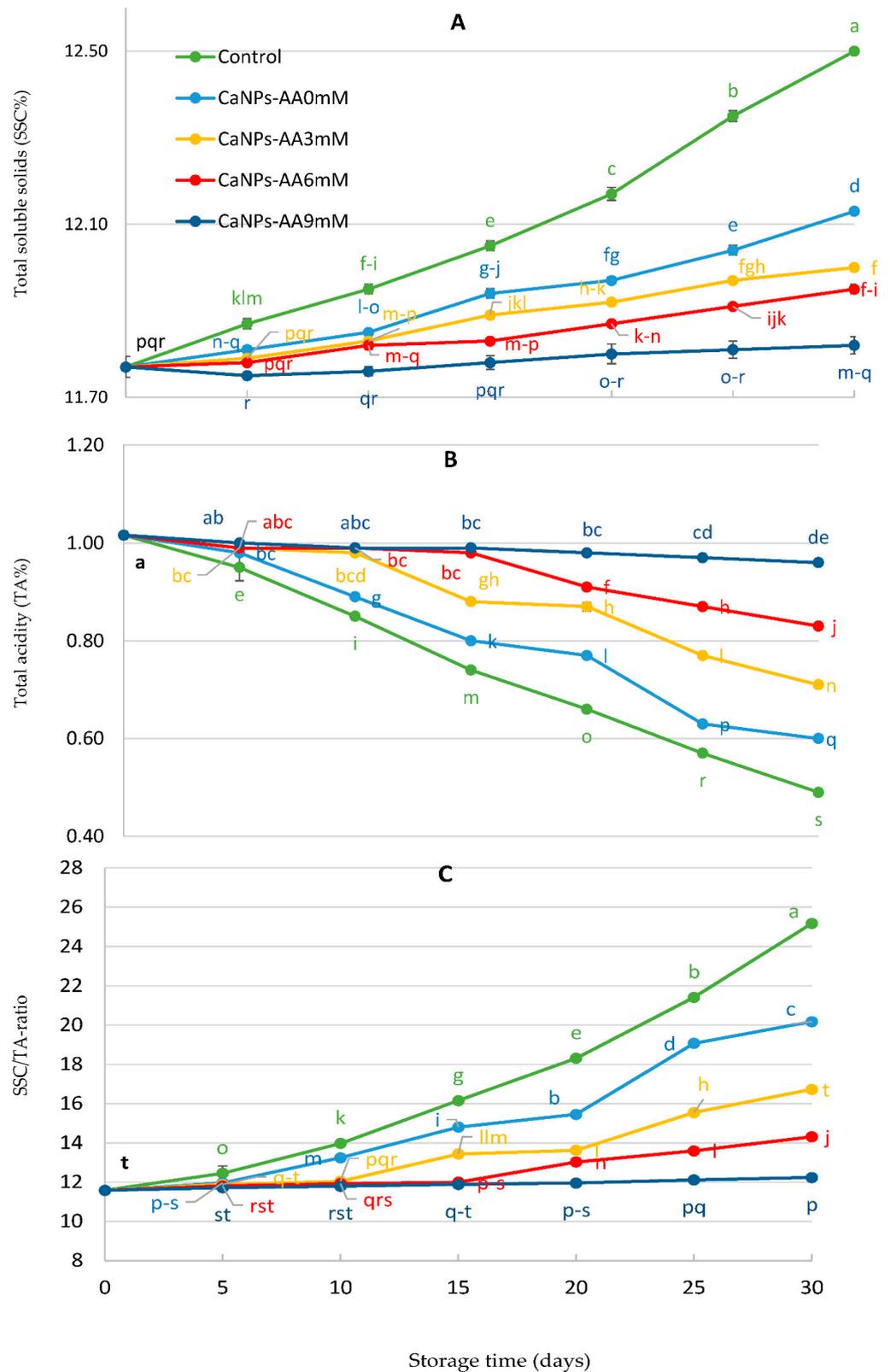


Figure 5. The total soluble solid content (SSC%) (A), total acidity percentage (TA%) (B), and SSC/TA ratio (C) of ‘Florida Prince’ peaches immersed in CaNPs mixed with ascorbic acid at different concentrations (0, 3, 6, and 9 mM) and stored at a low temperature (4 °C and 95% RH) for 30 days. Error bars represent standard errors and different letters indicate significant differences at $p \leq 0.05$ among treatments for each storage period.

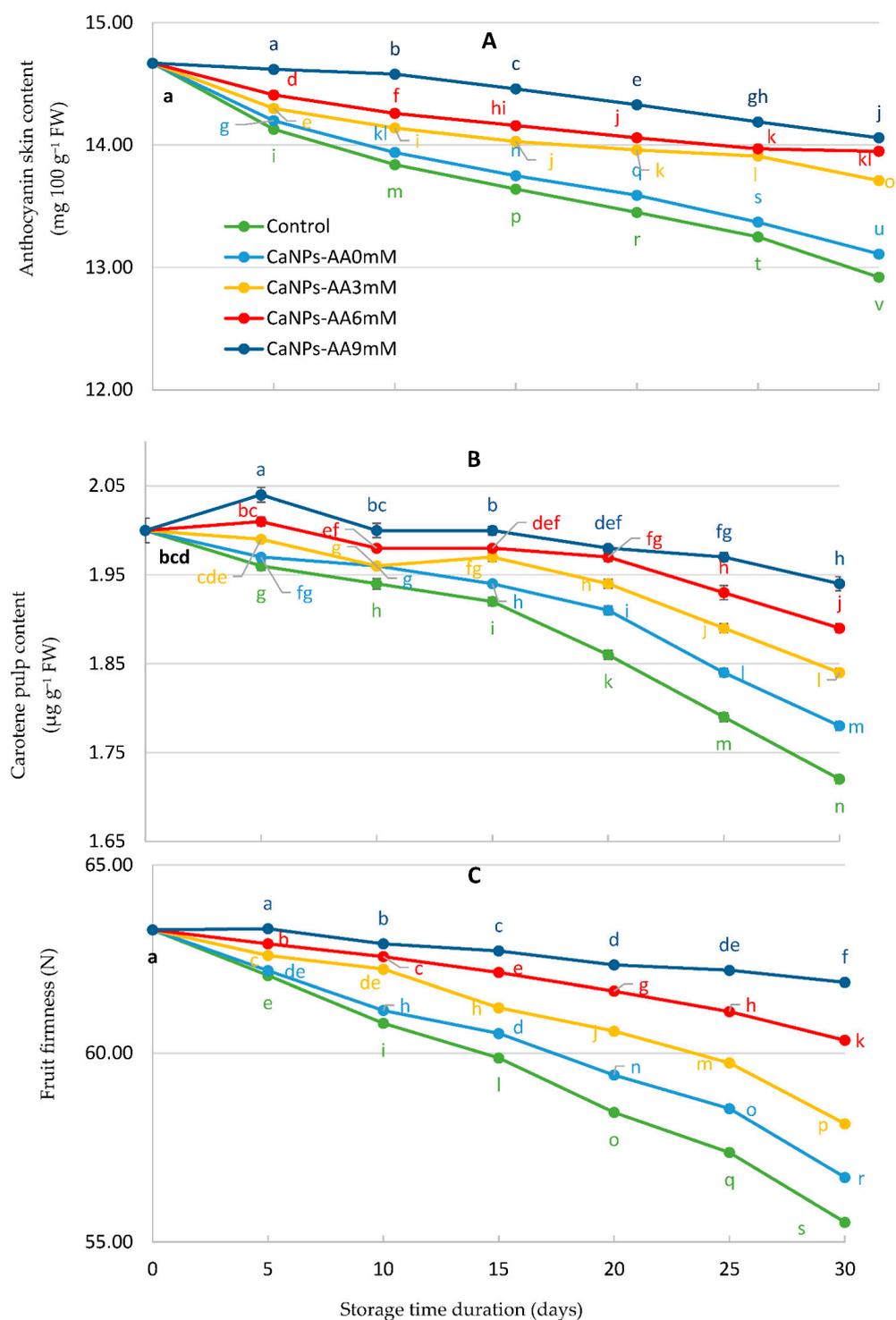


Figure 6. Fruit pigments (i.e., anthocyanin (A) and carotene (B)) and fruit firmness (C) of ‘Florida Prince’ peaches immersed in CaNPs mixed with ascorbic acid at different concentrations (0, 3, 6, and 9 mM) and stored at a low temperature (4 °C and 95% RH) for 30 days. Error bars represent standard errors and different letters indicate significant differences at $p \leq 0.05$ among treatments for each storage period.

3.5. Antioxidant Enzyme Activity (AEA)

Figure 7 displays the antioxidant enzyme activities (AEAs) as a function of storage time in weeks. Apparently, the AEAs displayed a significant interaction at 5% when the CaNP-AA treatments and storage time duration (days) were considered as an experimental factor.

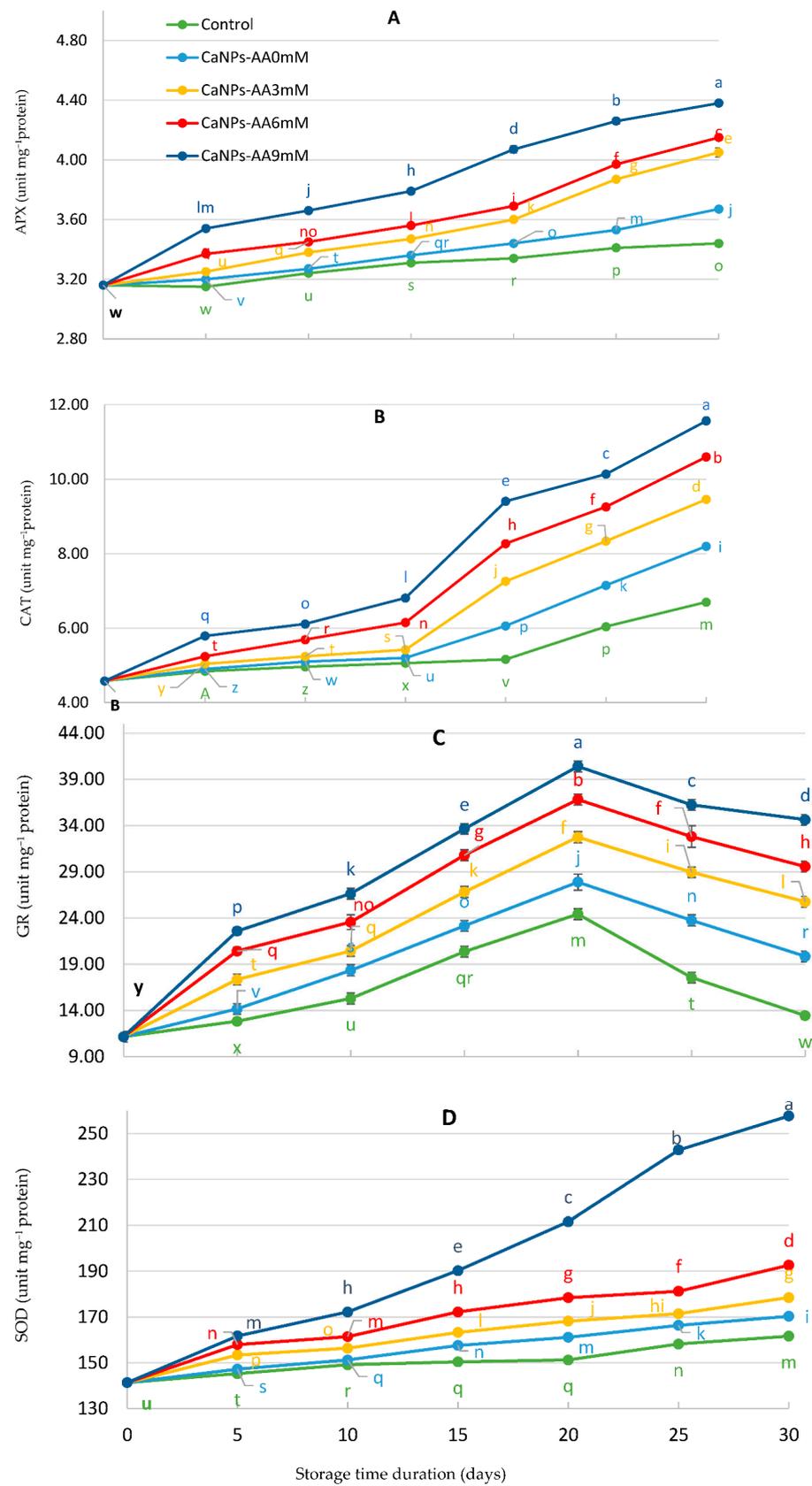


Figure 7. The AEAs, such as APX (A), CAT (B), GR (C), and SOD (D), of ‘Florida Prince’ peaches immersed in CaNPs mixed with ascorbic acid at different concentrations (0, 3, 6, and 9 mM) and stored at a low temperature (4 °C and 95% RH) for 30 days. Error bars represent standard errors and different letters indicate significant differences at $p \leq 0.05$ among treatments for each storage period.

The AEAs continued to increase differently until the end of the storage period. The changes in overall levels of AEAs for all treatments increased slightly throughout the first 20 days, then they became different up to the end of the cold storage period for all treatments.

However, the GR activity increased up to the 20th day and then declined in all treatments until the end of the experiment. Obviously, exogenous treatment with 9 mM CaNP-AA increased the AEAs in peach. The activity increased (APX, 4.38; CAT, 11.57; SOD 257.68) until the 30th day, but GR (40.40) increased up to the 20th day and then declined (34.63 units g⁻¹ protein) until the end of the experiment.

3.6. Estimation of Malondialdehyde (MDA), Ion Leakage (IL%), Ethylene Production, and Respiration Rate

In peach pulp, MDA accumulation and IL% significantly ($p > 0.01$) and dramatically increased throughout the duration of storage, dependent on the CaNP-AA treatment (Figure 8). A significant interaction among storage factors, storage duration (weeks), and CaNP-AA treatment was considered as an experimental factor. MDA content and IL% clearly increased in the CaNP-AA treatments compared with the initial values. Furthermore, differences among the CaNP-AA treatments appeared on the 10th day and became more pronounced during the storage duration until the end of the experiment. Certainly, the lowest values of MDA and IL% were identified in peaches treated with 9 mM CaNP-AA (MDA = 0.24 and IL = 23.37%) on the 30th day of the storage period compared with other treatments. However, the control treatment exhibited the highest accumulation of MDA (0.44) and IL (44.85%) in the same time interval. Moreover, the ethylene evolution and respiration rate in peach increased gradually and independently in the CaNP-AA treatments up to the maximum peak on the 10th and 5th days of storage. Both then declined until the end of storage time. However, it can be seen in Figure 5 that respiration showed an increase in CO₂ rate until the end of storage. The increases in CO₂ were independent and based on the CaNP-AA treatments. However, lower ethylene production and CO₂ over the 30 days was observed in the control.

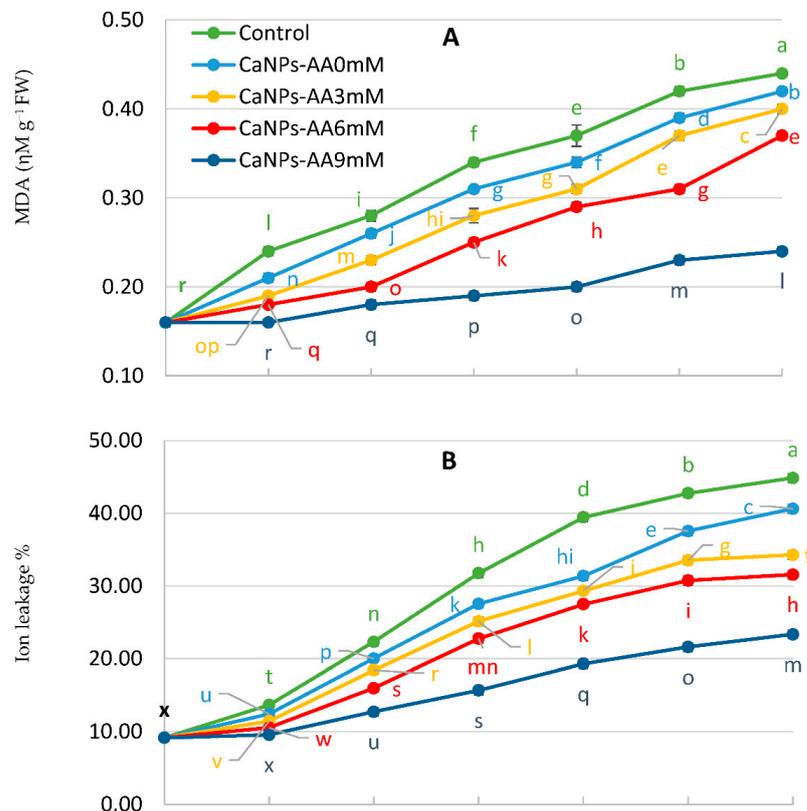


Figure 8. Cont.

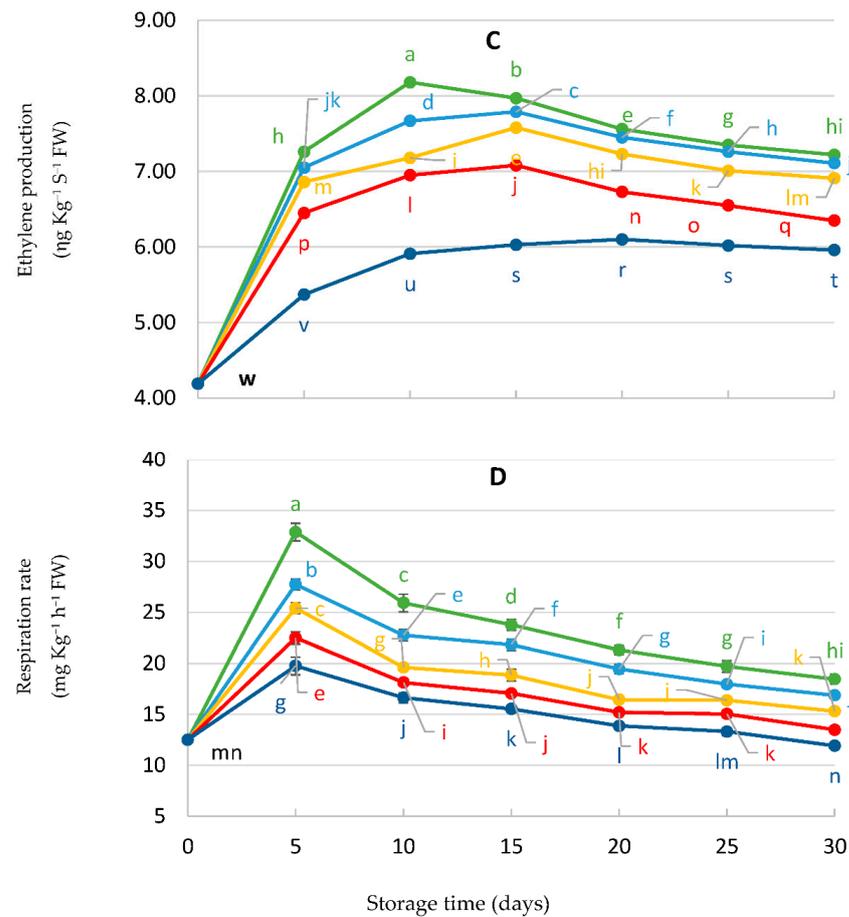


Figure 8. MDA (A), IL% (B), ethylene (C), and respiration rate (D) of ‘Florida Prince’ peaches immersed in CaNPs mixed with ascorbic acid at different concentrations (0, 3, 6, and 9 mM) and stored at a low temperature (4 °C and 95% RH) for 30 days. Error bars represent standard errors and different letters indicate significant differences at $p \leq 0.05$ among treatments for each storage period.

3.7. H_2O_2 and $O_2^{\bullet-}$ Production and DPPH Reduction

Differences in H_2O_2 and $O_2^{\bullet-}$ generation rates and antioxidant performance (utilizing the DPPH technique) could be seen as a function of time in days. The parameters produced a significant effect ($p < 0.003$) when the storage time and CaNP-AA applications were used as experimental factors (Figure 9).

H_2O_2 and $O_2^{\bullet-}$ generation in peach pulp increased continuously from the time of fruit collection up to the 30th day of the experiment. Consequently, increases and differences were independently associated with CaNP-AA applications on the 30th day of storage time. The 9 mM CaNP-AA treatment produced the lowest H_2O_2 ($0.12 \text{ mM min}^{-1} \text{ g}^{-1} \text{ FW}$) and $O_2^{\bullet-}$ ($0.34 \text{ mM g}^{-1} \text{ FW}$) amounts throughout the storage period compared with different CaNP-AA treatments on the 30th day. The assessment of AEAs by utilizing the DPPH decrease demonstrated that their activity improved spontaneously and slowly in all CaNP-AA treatments throughout the storage period. In any case, the control displayed the greatest reduction in DPPH (41.86%) on the 30th day of the trial.

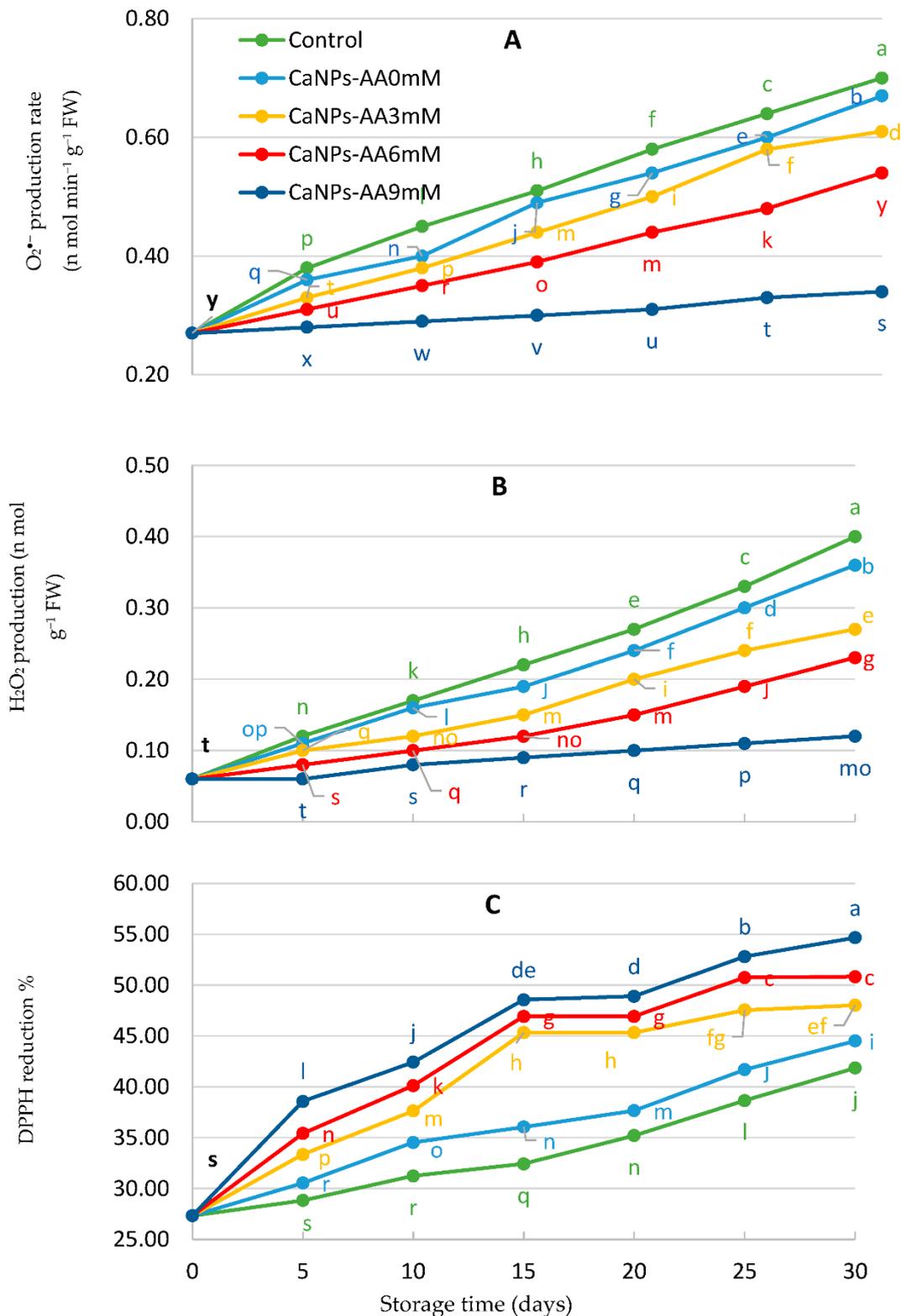


Figure 9. H₂O₂ (A), O₂^{•-} (B) production rates, and DPPH reduction (C) in ‘Florida Prince’ peaches immersed in CaNPs mixed with ascorbic acid at different concentrations (0, 3, 6, and 9 mM) and stored at a low temperature (4 °C and 95% RH) for 30 days. Error bars represent standard errors and different letters indicate significant differences at $p \leq 0.05$ among treatments for each storage period.

4. Discussion

This study examined the effectiveness of CaNP–AA treatment for reducing chilling injury in peaches during cold storage. Chilling injury has been recognized as a physiological disorder caused by low temperatures [44]. A variation was observed during 30 days of cold storage stress in peach fruit. Usually, low temperatures generate reactive oxygen species (ROS) during long-term cold storage [43]. The most abundant ROS produced is the hydroxyl radical ($\cdot\text{OH}$), which reacts with lipids and proteins of the plasma cell membrane [45]. Over a long period of cold storage, malondialdehyde (MDA) and protein carbonyl groups (PCG) terminate the lipid and protein of the cell membrane [46]. Consequently, cell membrane structure and function are lost [13] and cell death occurs [15]. With cell death, CI symptoms appear [45] via an equilibrium between ROS formation and antioxidant system activity under low temperatures [15,19]. Our results confirmed that the most severe symptoms of CI were observed in untreated fruit compared with CaNP–AA treatments. We observed that the 9 mM CaNP–AA treatment minimized CI symptoms and water loss. These results were due to the efficacy of AA at 9 mM for quenching ROS formation and preventing cell damage [15,47]. The reduction in CI incidence was reflected in fruit color (hue angle) (Figure 4). This may demonstrate that the presence of AA in combination with CaNP application preserved the water content during cold storage [48]. The results are consistent with other previous studies on peaches [49,50], which confirmed the positive influence of CaNP–AA treatment on minimizing chilling injury symptoms and improving fruit quality during cold storage.

In the present study, 9 mM CaNP–AA produced fewer changes in SSC and TA than other treatments (Figure 5). The SSC and TA remained at the same level as the initial values, which could be due to the role of AA as an antioxidant that decreased the oxidation reaction [51]. The SSC/TA ratio is an indicator of the good taste and flavor of peach fruit [52].

The changes in both SSC% and TA% in stressed peach fruit during cold storage after CaNP–AA treatment can be attributed to the conversion of organic acids to sugar [53]. Moreover, the highest SSC% seen throughout the storage period may have occurred as a consequence of decreasing AA and acidity under prolonged storage [17]. Furthermore, it could be associated with increases in starch enzyme activities that change organic acids to sugar, as described previously [54]. Similar results were observed in sweet orange fruit [55], lime [56], and pawpaw [57] stored at low temperatures.

The results revealed that fruit treated with 9 mM CaNP–AA maintained better fruit pigmentation and fruit firmness than the control (Figure 6). Based on the fruit quality data, it is clear that with the 9 mM L^{-1} CaNP–AA treatment, no adverse effects were observed in the fruit. However, higher AA doses (9 mM L^{-1}) were more effective at reducing the decline in peach quality. Thus, the 9 mM CaNP–AA application maintained the fruit pigments and firmness of peach fruit under cold storage. These findings may be linked to the physiological roles of calcium and AA in the CaNP–AA mixture. Calcium is essential for maintaining cell wall stability and integrity, as well as determining fruit quality [58]. Calcium has been demonstrated to be effective at preserving the quality of fruit [58], increasing antioxidant capacity [59], preventing softening [58], alleviating chilling injury [60], controlling postharvest decay [61], and delaying the fruit ripening process [62]. Previously, calcium lactate had better textural and sensory properties on peaches than calcium chloride and calcium propionate [58]. Supplementing CaNPs with AA provided additional benefits, such as quenching ROS [63], maintaining fruit quality throughout cold storage [64], delaying fruit ripening, and reducing microbial infection [65]. These results are in line with the findings of Campos-Vargas et al. [66] on ‘O’Henry’ peaches.

In our study, APX, CAT, GR, and SOD activities were more strongly activated in 9 mM CaNP–AA-treated fruit. According to several reports, APX donates an electron to free radicals and converts H_2O_2 to oxygen and water, thereby mitigating oxidative damage [67]. The activity of the APX enzyme is directly proportional to the amount of AA [68]. Thus, the increased activity in 9 mM CaNP–AA-treated peaches could be a result of stable AA

concentrations in the fruit. Additionally, findings have shown that both CAT and SOD are essential enzymes for reducing ROS damage in litchi fruit [69]. Reduced CAT and SOD activity are typically associated with increased fruit senescence [70]. The increased CAT and SOD activity may be attributed to the fruit treated with 9 mM CaNP-AA producing fewer $O_2^{\bullet-}$ and H_2O_2 radicals. Their activities were enhanced with CaNP-AA treatments based on the concentration of AA. Certain differences in AEAs among the CaNP-AA applications can be revealed. The presence of AA in combination with CaNP mixture at a high concentration (9 mM) improved AEAs during cold storage. The CaNPs also increased AA to quench ROS formation throughout storage [71]. However, the AA works indirectly as a quencher of ROS [27]. Moreover, AA might be forced to balance the production of ROS and AEAs [19]. Hence, AA can be used as a stabilizer of the network of AEAs [15]. The results are in line with other studies conducted on peach [49,72], persimmon fruit [73], and banana [44].

Calcium (Ca^{2+}) is an essential mineral for fruit and plays an important role in forming cell walls and membranes [74]. A second messenger in plant signal transduction, Ca^{2+} is implicated in stress responses [75]. Furthermore, different calcium-binding proteins perceive transitory increases in cytosolic Ca^{2+} under cold stress and trigger varied physiological responses [76]. Calcium in the form of $CaCl_2$ treatment reduced peel browning caused by freezing injury in pear fruit by inhibiting membrane lipid peroxidation and increasing SOD activity and expression [77]. It reduced chilling injury symptoms in winter jujube fruit [78]. By modulating SOD, POD, CAT, and the AsA-GSH cycle, $CaCl_2$ increased chilling tolerance in green peppers [79]. A previous study on loquat fruit found that $CaCl_2$ treatment increased cold tolerance by regulating energy metabolism and accumulating osmotic substances [72,80].

In this work, MDA accumulation was the most prevalent side effect of lipid peroxidation, a process that can cause cellular membrane damage [81]. Elevated ROS levels can lead to more lipid peroxidation, leading to damaged membranes and decreased storage capacity [82]. Due to the reduction in oxidative damage and senescence, 9 mM CaNP-AA-treated peaches showed reduced MDA and IL% concentrations. CaNPs were paired with AA to minimize ethylene and respiration. In this context, further investigations are required to determine how these two methods achieved such positive results.

The results were obtained due to the decline in oxygen-consuming respiration under cold storage, which diminishes both H_2O_2 and $O_2^{\bullet-}$ production [83]. Furthermore, the increase in AEAs (Figure 4) may diminish both the production of H_2O_2 and $O_2^{\bullet-}$ correlated with improved SOD activity, as indicated by Lo'ay and El-Khateeb [17]. The relationship between both CAT and APX in the network, besides other antioxidants [15], could be strongly connected to the quenching of both H_2O_2 and $O_2^{\bullet-}$ or the minimization of the effects of both [41].

5. Conclusions

CI significantly impairs the storage of peach fruits, as measured by fruit loss over the course of a cold storage period. The 9 mM CaNP-AA treatment clearly ameliorated CI in peach fruit by improving AEAs and minimizing ion leakage. Thus, treatment with AA at 9 mM L^{-1} together with CaNPs suppressed the incidence of CI during cold stress, particularly in terms of ROS. It also activated antioxidant enzymes, which was reflected by the low MDA content throughout the cold storage period. Our results suggest that a mixture of AA with CaNPs could be used as a tool adjuvant to maintain fruit quality traits under cold storage.

Author Contributions: Conceptualization, L.A.A.; data curation and validation, L.A.A.; methodology, L.A.A. and H.S.K.; formal analysis, L.A.A. and H.S.K.; writing—original draft preparation, L.A.A. and H.S.K.; writing—review and editing, H.I. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Researchers Supporting Project Number (RSP-2021/403), King Saud University, Riyadh, Saudi Arabia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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