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Differential Effects of Postharvest $CaCl_2$, Salicylic Acid and GABA Dips on Peel Gene Expression and Antioxidant Status of Thomson Navel Orange During Storage

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Abstract

Postharvest treatments can modulate molecular and biochemical responses in citrus fruit during storage. This study compared calcium chloride ($CaCl_2$, 2%), salicylic acid (SA, 2 mM), and γ-aminobutyric acid (GABA, 5 mM) dips with a water control in Thomson Navel sweet orange (*Citrus sinensis* (L.) Osbeck) stored at 7–10 °C for 90 days. Flavedo expression of *CsACS2*, *CsNCED,* and *CsPH8*, catalase (CAT) and peroxidase (POD) activities, total phenolic and flavonoid contents, and hydrogen peroxide (H_2O_2) levels were evaluated at 0, 30, 60, and 90 days. The treatments induced distinct temporal response patterns. $CaCl_2$ treatment was associated with a progressive decline in *CsACS2* expression relative to its day-0 baseline and increased CAT activity, which reached 246 U g^{-1} protein at day 90, compared with 95 U g^{-1} protein in the control. GABA treatment increased total phenolic content by 31–52% relative to the control across days 30–90. In contrast, SA-treated fruit exhibited the highest H_2O_2 levels at days 60 and 90, indicating a distinct late-storage redox response. Together, these findings reveal treatment-specific patterns of molecular and biochemical responses in Thomson Navel orange peel. $CaCl_2$ was primarily associated with altered *CsACS2* expression and enhanced CAT activity, whereas GABA consistently increased phenolic accumulation. These findings provide a basis for developing targeted postharvest strategies by identifying $CaCl_2$ and GABA as promising candidates for modulating peel physiological responses during cold storage.

Keywords *Citrus sinensis* · Postharvest storage · Calcium chloride · Salicylic acid · γ-Aminobutyric acid · Gene expression

1 Introduction

Postharvest diseases and physiological disorders limit the marketable life and storage potential of sweet orange (Citrus sinensis) (Strano et al. 2022). Identifying practical postharvest treatments is therefore important for citrus storage management. In Thomson Navel orange, postharvest storage is accompanied by changes in antioxidant capacity and secondary metabolism (Mohammadhosseini et al. 2013). However, the extent to which postharvest dips modify these responses in the peel requires further investigation.

Although citrus fruit are non-climacteric, storage conditions can alter hormone-associated transcription and redox metabolism. Monitoring selected transcripts alongside biochemical markers can help characterize these responses without assuming that changes in individual markers necessarily reflect changes in whole-fruit senescence. *CsACS2* encodes 1-aminocyclopropane-1-carboxylate synthase 2 (the prefix Cs denotes *C. sinensis*) and belongs to the ethylene-biosynthesis gene family of sweet orange (Sun et al. 2022*). CsNCED* encodes 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid (ABA) biosynthesis (Sun et al. 2012). In sweet orange, *CsNCED* expression increases during fruit maturation in parallel with ABA accumulation (Rodrigo et al. 2006). *CsPH8*, a P-type proton-pump gene associated with vacuolar acidification and citric acid accumulation in citrus fruit (Shi et al. 2019), was included as an exploratory marker because its response in the flavedo is less well established. Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), are involved in redox signaling and oxidative processes during storage (Huang et al. 2023). Catalase (CAT), peroxidase (POD), and phenolic and flavonoid compounds contribute to antioxidant responses.

$CaCl_2$, SA, and GABA have attracted interest as potential postharvest treatments because of their distinct physiological roles. Calcium chloride ($CaCl_2$) strengthens cell walls through interactions with pectic substances

(Zhang et al. 2019; Frempong et al. 2022). In navel orange, preharvest sprays of $CaCl_2$ and salicylic acid improved firmness and phenolic content during storage (Ramezanian et al. 2018), and $CaCl_2$ is also combined with biostimulants to improve the antioxidant status and quality of fruit, as in sweet cherry (Polat et al. 2026). Salicylic acid (SA) is a defense hormone that raises antioxidant capacity and induces resistance responses (Huang et al. 2023; Jiang et al. 2022; Gomathi et al. 2026). In Gonggan mandarin, SA combined with $CaCl_2$ changed disease resistance and defense-enzyme activity during storage (Zhang et al. 2022). γ-Aminobutyric acid (GABA) is a stress-signaling metabolite that affects ROS scavenging and organic acid metabolism through the GABA shunt (Nehela and Killiny 2023; Sheng et al. 2017b). In citrus, GABA altered citrate and amino acid metabolism and improved fruit quality and storage performance (Sheng et al. 2017a), and it extended the shelf life of Mexican lime (Assar et al. 2025). Preharvest GABA has also been associated with enhanced antioxidant responses in sweet cherry (Carrión-Antolí et al. 2024). Despite these reported effects, the three compounds may influence different components of the postharvest response system. $CaCl_2$ is associated with cell-wall stabilization, SA with defense-related signaling, and GABA with metabolic and redox regulation. Comparing their effects under the same cultivar and storage conditions can therefore help distinguish their respective molecular and biochemical response patterns.

Treatment responses can vary with concentration, cultivar, and storage conditions (Strano et al. 2022; Zhang et al. 2024). Although previous studies have examined these compounds individually in different fruit systems, their comparative effects on transcript profiles and redox-related biochemical traits in Thomson Navel orange flavedo during prolonged cold storage remain insufficiently characterized. In particular, evaluating these responses together over time may help reveal whether the treatments are associated with distinct patterns of hormone-related transcription, antioxidant activity, and phenolic accumulation. We therefore compared $CaCl_2$, SA, and GABA dips over 90 days, evaluating *CsACS2*, *CsNCED*, and *CsPH8* expression, CAT and POD activities, total phenolic and flavonoid contents, and H_2O_2 levels. This comparison aimed to characterize treatment-specific response patterns and identify promising candidates for postharvest management.

2 Materials and Methods

2.1 Plant Material, Treatments and Storage Conditions

Fully mature Thomson Navel orange fruits (*Citrus sinensis* (L.) Osbeck cv. 'Thomson Navel') were harvested from a commercial orchard in the Miandorood region, Sari County, Mazandaran Province, Iran. Fruits of uniform size and color and free from any visual defects or pathological symptoms were selected. The fruits were surface-disinfected by immersion in a 1% (v/v) sodium hypochlorite solution for 2 min, followed by thorough rinsing with distilled water and air-drying at ambient temperature (20 ± 2 °C).

The disinfected fruits were randomly divided into four treatment groups: distilled water (control), 2% (w/v) calcium chloride ($CaCl_2$), 2 mM salicylic acid (SA), and 5 mM γ-aminobutyric acid (GABA). The experiment was conducted as a factorial arrangement (four treatments × four storage times) in a completely randomized design. Each treatment consisted of three biological replicates of 60 fruits each. Fruits were immersed in the respective treatment solutions for 5 min and air-dried. The concentrations of $CaCl_2$, SA, and GABA were selected based on preliminary experiments and previous postharvest studies (e.g., 2 mM SA in Satsuma mandarin, Zhu et al. 2016; 1.5–3% $CaCl_2$ sprays on navel orange, Ramezanian et al. 2018).

The treated fruits were placed in plastic fruit baskets and stored in a storage room for 90 days; the temperature ranged approximately between 7 and 10 °C, and the relative humidity varied between 80% and 85%. Destructive sampling was conducted at day 0 (immediately after treatment), day 30, day 60, and day 90 of storage. At each sampling time, five fruits were randomly selected from each replicate, and the flavedo (peel) tissue was frozen in liquid nitrogen, ground into a fine powder, and stored at −80 °C for subsequent molecular and biochemical analyses. Analyses were restricted to flavedo tissue, the primary barrier against environmental stress and pathogen ingress.

2.2 Gene Expression Analysis by Quantitative Real-Time PCR (qPCR)

Total RNA was isolated from 100 mg of frozen peel tissue using a phenol-based extraction procedure followed by selective precipitation with lithium chloride (LiCl) according to a modified LiCl precipitation protocol (Verwoerd et al. 1989). RNA integrity was verified by agarose gel electrophoresis, whereas RNA purity and concentration were determined using a NanoDrop spectrophotometer. One microgram of DNase I-treated total RNA was reverse-transcribed into first-strand cDNA using an oligo(dT) primer and reverse transcriptase according to the manufacturer's instructions.

Quantitative real-time PCR (qPCR) was performed using a SYBR Green Master Mix on an Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each 20 µL reaction contained 1 µL of diluted cDNA, 0.5 µM of each gene-specific primer, and SYBR Green Master Mix. The thermal cycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 30 s.

A melting curve analysis was performed immediately after amplification to verify the specificity of the PCR products. The presence of a single peak in the melting curve confirmed specific amplification of the target genes without detectable non-specific products or primer-dimer formation. Primer sequences for the target genes (*CsACS2*, *CsNCED*, and *CsPH8*) and the reference gene (*Actin*) are listed in Table 1. *Actin* was used as the reference gene because it was stably expressed in our previous citrus postharvest studies. Gene expression analysis was conducted using three biological replicates, and each biological replicate was analyzed with two technical replicates.

Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), with the day-0 value of each treatment as the calibrator. Because each treatment is expressed relative to its own day 0, treatments were not compared directly at a given sampling time. Expression is plotted as $\log_2$ fold change ($-\Delta\Delta Ct$), so that zero means no change from day 0, positive values mean higher expression and negative values mean lower expression.

Table 1 Sequences of gene-specific primers for *CsACS2*, *CsNCED*, *CsPH8*, and reference gene *Actin* used in quantitative PCR

Gene Name	Primer Direction	Primer Sequence	Length (nt)	Tm (°C)	GC%
CsNCED	Forward	5′GGTGCCAACCCATTATTCGA3′	20	58.24	50
CsNCED	Reverse	5′GCCGTCACCGTCAAAGAAAT3′	20	59.13	50
Actin	Forward	5′CCAAGCAGCATGAAGATCAA3′	20	60.40	45
Actin	Reverse	5′ATCTGCTGGAAGGTGCTGAG3′	20	59.01	55
CsPH8	Forward	5′CCGTGAAGGAATTGATTTGG3′	20	54.94	45
CsPH8	Reverse	5′CCATGACAATGGATTCCACA3′	20	55.69	45
CsACS2	Forward	5′GGTGCCCCCAATATTAACAA3′	20	58.62	45
CsACS2	Reverse	5′AGAGCTCGGTCCCATATCAA3′	20	58.35	50

Tm, melting temperature; GC%, guanine–cytosine content; nt, nucleotides

2.3 Enzyme activity analysis

2.3.1 Enzyme extraction and protein quantification

Crude enzyme extracts were prepared from frozen flavedo (peel) powder stored at −80 °C (Sect. 2.1). Briefly, 0.5 g of powder was homogenized on ice in 5 mL of ice-cold 50 mM phosphate buffer (pH 7.0) containing 1% (w/v) polyvinylpyrrolidone (PVP), to bind phenolic compounds, and 1 mM ethylenediaminetetraacetic acid (EDTA), to chelate metal ions. The homogenate was centrifuged at 12,000 × g for 20 min at 4 °C, and the supernatant was kept on ice and used as the enzyme extract. Soluble protein content of the extracts was determined by the Bradford (1976) method using bovine serum albumin as the standard, and enzyme activities were expressed per gram of protein. Extractions were performed on three independent biological replicates per treatment and sampling day, each prepared from pooled flavedo tissue of five fruits, and each extract was assayed in two technical replicates.

2.3.2 Catalase activity assay

CAT (EC 1.11.1.6) activity was assayed according to Aebi (1984) by monitoring the decline in absorbance at 240 nm caused by H_2O_2 decomposition. The 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 and 50 µL of enzyme extract. Absorbance was recorded at 25 °C for 2 min using a UV–Vis spectrophotometer, against a blank without enzyme extract. Activity was calculated using an extinction coefficient of 39.4 mM^{-1} cm^{-1}. One unit (U) was defined as the amount of enzyme decomposing 1 µmol H_2O_2 per minute, and activity was expressed as U g^{-1} protein.

2.3.3 Peroxidase activity assay

POD (EC 1.11.1.7) activity was measured according to Chance and Maehly (1955) using guaiacol as the hydrogen donor. The 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.0), 20 mM guaiacol, 10 mM H_2O_2 and 50 µL of enzyme extract. The increase in absorbance at 470 nm due to guaiacol oxidation (tetraguaiacol formation; $\varepsilon = 26.6$ mM^{-1} cm^{-1}) was recorded at 25 °C for 3 min. One unit was defined as the amount of enzyme forming 1 µmol tetraguaiacol per minute, and activity was expressed as U g^{-1} protein.

2.4 Non-enzymatic antioxidants and hydrogen peroxide content

2.4.1 Total phenolic content

For phenolic and flavonoid determination, 0.5 g of frozen flavedo powder was extracted with 10 mL of 80% (v/v) methanol by shaking for 30 min in the dark, and the extract was centrifuged at 12,000 × g for 15 min at 4 °C. Total phenolics were determined using Folin-Ciocalteu reagent (Singleton et al. 1999). An aliquot (0.1 mL) of extract was mixed with 0.5 mL of Folin-Ciocalteu reagent (diluted 1:10, v/v) and, after 5 min, with 2 mL of 7.5% (w/v) Na_2CO_3. After incubation in the dark at room temperature for 60 min, absorbance was read at 765 nm. Concentrations were calculated from a gallic acid calibration curve and expressed as mg gallic acid equivalents (GAE) per g fresh weight (FW).

2.4.2 Total flavonoid content

Total flavonoids were measured in the same methanolic extract by the aluminum chloride colorimetric method (Zhishen et al. 1999). An aliquot (0.5 mL) of extract was mixed with 2 mL of distilled water and 0.15 mL of 5% (w/v) $NaNO_2$. After 5 min, 0.15 mL of 10% (w/v) $AlCl_3$ was added, followed after a further 6 min by 1 mL of 1 M NaOH. The volume was made up to 5 mL with distilled water, and absorbance was read at 510 nm immediately. Flavonoid content was calculated from a catechin calibration curve and expressed as mg catechin equivalents (CE) per g FW.

2.4.3 H_2O_2 Content

H_2O_2 was extracted by homogenizing 0.5 g of frozen flavedo powder in 5 mL of ice-cold 0.1% (w/v) trichloroacetic acid (TCA), followed by centrifugation at 12,000 × g for 15 min at 4 °C. The H_2O_2 content of the supernatant was measured spectrophotometrically after reaction with potassium iodide (Loreto and Velikova 2001): 0.5 mL of supernatant was mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI). After 60 min in the dark, absorbance was read at 390 nm against a blank in which TCA replaced the extract. H_2O_2 concentration was calculated from a standard curve of known H_2O_2 concentrations and expressed as μmol g^{-1} FW.

2.5 Statistical analyses

Data were analyzed with SPSS (version 21.0). Treatment, storage time and their interaction were tested by factorial analysis of variance (ANOVA) (Tables 2 and 3). Normality and homogeneity of variance were checked with the Shapiro–Wilk and Levene tests before ANOVA. Because the treatment × storage-time interaction was significant for all biochemical variables (Table 3), Duncan's multiple range test (DMRT) was applied to the interaction means at $P \leq 0.05$ instead of the main-effect means. DMRT is less conservative than Tukey's honestly significant difference test, so differences close to the threshold should be read with care. For the gene-expression data (Figs. 1–3), values at days 30, 60 and 90 were compared with the day-0 value of the same treatment (* $P \leq 0.05$; ** $P \leq 0.01$). The treatment × storage-time interaction was significant only for *CsACS2* (Table 2), so these comparisons are exploratory and were not corrected for multiple testing. Results are given as mean ± standard deviation (SD) of three biological replicates, and error bars in all figures show SD.

2.6 Use of generative AI tools

During preparation of this manuscript, the authors used ChatGPT (OpenAI) to translate author-written text into English and to improve language, grammar and readability, and Claude (Anthropic) to check the text, tables and

figures against the journal's formatting requirements, to suggest wording revisions and to propose the layout of the schematic summary in Fig. 9. The authors reviewed and edited all AI-assisted output and checked the content of Fig. 9 against their own data in Table 4. No AI tool was used to generate or modify research data. The authors take full responsibility for the accuracy and integrity of the final manuscript, including the experimental design, data analysis, interpretation and conclusions.

3 Results

3.1 Gene Expression

CsACS2, *CsNCED* and *CsPH8* followed different time courses in the four treatments (Figs. 1–3). Expression is given as $\log_2$ fold change relative to day 0 within each treatment, so each value shows how a treatment moved away from its own starting point, and the control shows the change caused by storage alone. By definition the day-0 mean is zero, and the day-0 error bars show only the scatter of the replicates around it. The treatment × storage-time interaction was significant for *CsACS2* ($P = 0.002$), storage time had a significant effect on *CsNCED* ($P = 0.007$), and no term was significant for *CsPH8* (Table 2); the comparisons with day 0 described below are therefore exploratory.

Table 2 Analysis of variance (ANOVA) examining the effects of treatment, storage duration, and their interaction on the expression levels of *CsACS2*, *CsNCED*, and *CsPH8* genes; values are mean squares (F and P values below)

Source of Variation	Degrees of Freedom	*CsACS2*	*CsNCED*	*CsPH8*
Treatment	3	85.483 $F = 2.61$; $P = 0.068$	271.718 $F = 2.78$; $P = 0.057$	438.950 $F = 1.76$; $P = 0.176$
Storage Time	3	85.087 $F = 2.60$; $P = 0.069$	470.289** $F = 4.80$; $P = 0.007$	619.822 $F = 2.48$; $P = 0.079$
Treatment × Time	9	126.379** $F = 3.86$; $P = 0.002$	144.404 $F = 1.48$; $P = 0.199$	453.529 $F = 1.81$; $P = 0.104$
Error	32	32.724	97.884	250.035

Values are mean squares with the corresponding *F* statistic and *P* value below. *, ** Significant at the 5% and 1% probability levels, respectively; values without asterisks are not significant

3.1.1 Ethylene- and ABA-Biosynthesis Genes

CsACS2 transcripts in $CaCl_2$-treated fruit declined progressively, with $\log_2$ fold changes of −5.73, −8.35 and −9.17 at days 30, 60 and 90, all significantly different from day 0 (Fig. 1). The control showed no significant change at any time (+1.82, −0.23 and −0.83), and neither did GABA (+2.48, −2.67 and −1.70). SA was unchanged at day 30 (+0.01), increased significantly at day 60 (+4.19) and returned to a non-significant value at day 90 (+1.41).

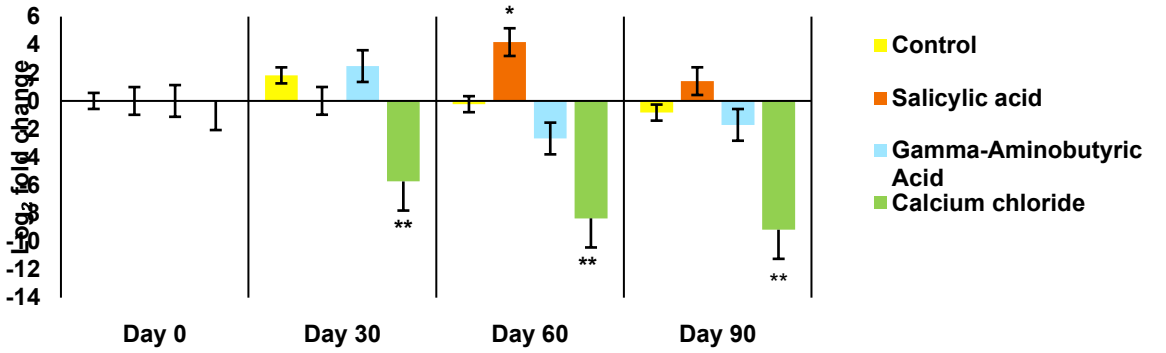


Fig. 1 Log_2 fold change in expression of *CsACS2* in Thomson Navel orange flavedo during storage. Expression was assessed immediately after treatment (day 0) and after 30, 60, and 90 days of storage in the control (distilled water), SA, GABA, and $CaCl_2$ treatments, and is shown relative to day 0 within each treatment. Values are means of three biological replicates; error bars show SD. Asterisks indicate significant differences relative to day 0 within each treatment (* $P \leq 0.05$; ** $P \leq 0.01$). $CaCl_2$, calcium chloride; GABA, γ-aminobutyric acid; SA, salicylic acid

CsNCED followed a different pattern (Fig. 2). In the control, transcripts rose significantly at day 30 (log_2 fold change +4.45; $P \leq 0.01$) and did not change significantly at days 60 and 90 (+0.24 and +3.17). In SA-treated fruit, *CsNCED* was significantly lower than at day 0 at day 30 (−4.83; $P \leq 0.01$) and at day 90 (−4.01), with a non-significant intermediate value at day 60 (−2.65). GABA-treated fruit showed non-significant changes at days 30 and 60 (+3.14 and −1.96) but the largest decrease at day 90 (−9.54). $CaCl_2$-treated fruit showed no significant change at any time (+1.27, +0.28 and +0.10).

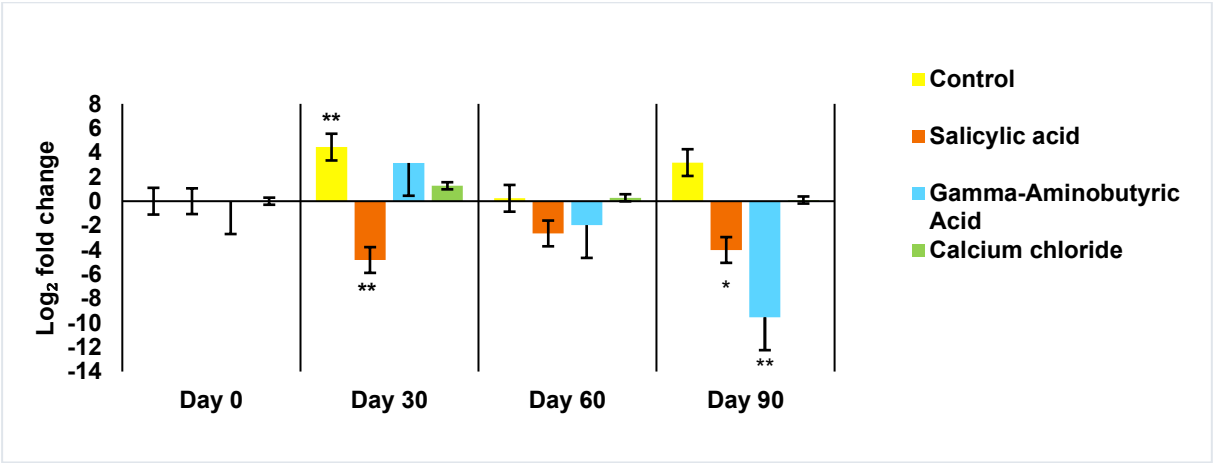


Fig. 2 Log_2 fold change in expression of *CsNCED* in Thomson Navel orange flavedo during storage. Expression was assessed immediately after treatment (day 0) and after 30, 60, and 90 days of storage in the control (distilled water), SA, GABA, and $CaCl_2$ treatments, and is shown relative to day 0 within each treatment. Values are means of three biological replicates; error bars show SD. Asterisks indicate significant differences relative to day 0 within each treatment (* $P \leq 0.05$; ** $P \leq 0.01$). $CaCl_2$, calcium chloride; GABA, γ-aminobutyric acid; SA, salicylic acid

3.1.2 Vacuolar Proton-Pump Gene

CsPH8 transcripts changed with both treatment and time (Fig. 3). At day 30, *CsPH8* rose significantly in GABA-treated fruit (log_2 fold change +4.30; $P \leq 0.05$) and fell significantly in $CaCl_2$-treated fruit (−3.34; $P \leq 0.05$), while the control (+1.71) and SA (+2.07) did not change significantly. At day 60 only $CaCl_2$ still differed from day 0 (−3.35; control −1.78, SA −0.92, GABA −2.69). By day 90, *CsPH8* was significantly lower than at

day 0 in all three treatments (−10.00 for GABA, −9.30 for $CaCl_2$ and −5.54 for SA), whereas the control showed a non-significant increase (+0.30).

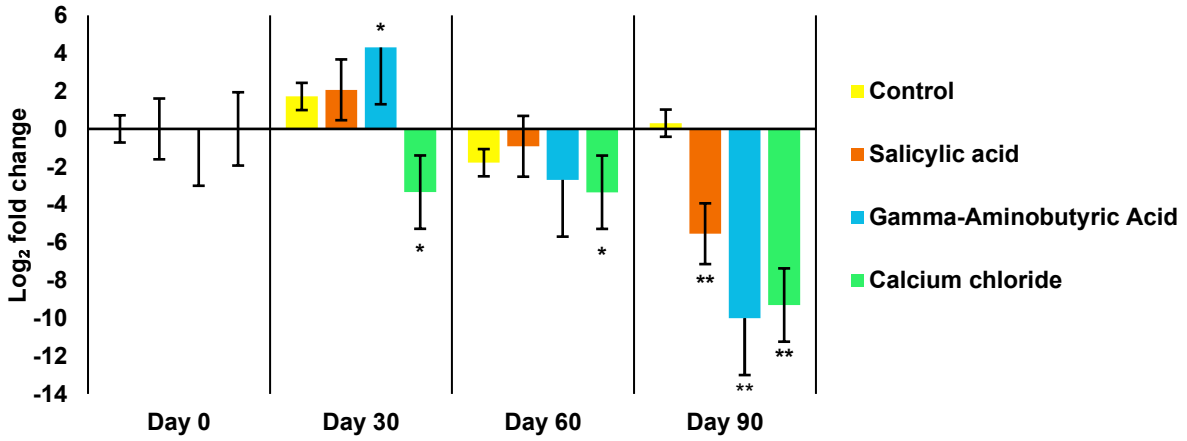


Fig. 3 Log_2 fold change in expression of *CsPH8* in Thomson Navel orange flavedo during storage. Expression was assessed immediately after treatment (day 0) and after 30, 60, and 90 days of storage in the control (distilled water), SA, GABA, and $CaCl_2$ treatments, and is shown relative to day 0 within each treatment. Values are means of three biological replicates; error bars show SD. Asterisks indicate significant differences relative to day 0 within each treatment (* $P \leq 0.05$; ** $P \leq 0.01$). $CaCl_2$, calcium chloride; GABA, γ-aminobutyric acid; SA, salicylic acid

3.2 Antioxidant Enzyme Activities

Treatment, storage time, and their interaction significantly affected all biochemical variables (Table 3). Differences between treated and control fruit at the same sampling time are reported only where significant (Duncan's test, $P \leq 0.05$).

Table 3 Analysis of variance (ANOVA) of the effects of treatment, storage time, and their interaction on biochemical parameters; values are mean squares (F and P values below)

Source of Variation	Degrees of Freedom	Flavonoid	Phenol	Hydrogen Peroxide	Peroxidase	Catalase
Treatment	3	1249.090** $F = 368.35$; $P < 0.001$	2379.099** $F = 31.54$; $P < 0.001$	0.135** $F = 45.00$; $P < 0.001$	244.247** $F = 8.83$; $P < 0.001$	8905.702** $F = 44.15$; $P < 0.001$
Storage Time	3	1094.530** $F = 322.77$; $P < 0.001$	3467.036** $F = 45.97$; $P < 0.001$	0.190** $F = 63.33$; $P < 0.001$	5540.983** $F = 200.43$; $P < 0.001$	92976.139** $F = 460.96$; $P < 0.001$
Treatment × Time	9	203.775** $F = 60.09$; $P < 0.001$	231.810** $F = 3.07$; $P = 0.009$	0.047** $F = 15.67$; $P < 0.001$	174.804** $F = 6.32$; $P < 0.001$	2475.865** $F = 12.28$; $P < 0.001$
Error	32	3.391	75.423	0.003	27.646	201.699

Values are mean squares with the corresponding F statistic and P value below. ** Significant at the 1% probability level ($P \leq 0.01$)

3.2.1 Catalase (CAT) Activity

CAT activity generally increased with storage in all groups, and treatments did not differ at day 0 or day 60 (Fig. 4). At day 30, only $CaCl_2$ increased CAT activity (107 vs. 74 U g^{-1} protein in the control). By day 90, all treatments significantly enhanced CAT activity: 190 U g^{-1} protein for both SA and GABA (2.0-fold) and 246 U g^{-1} protein for $CaCl_2$ (2.6-fold), compared with 95 U g^{-1} protein in the control.

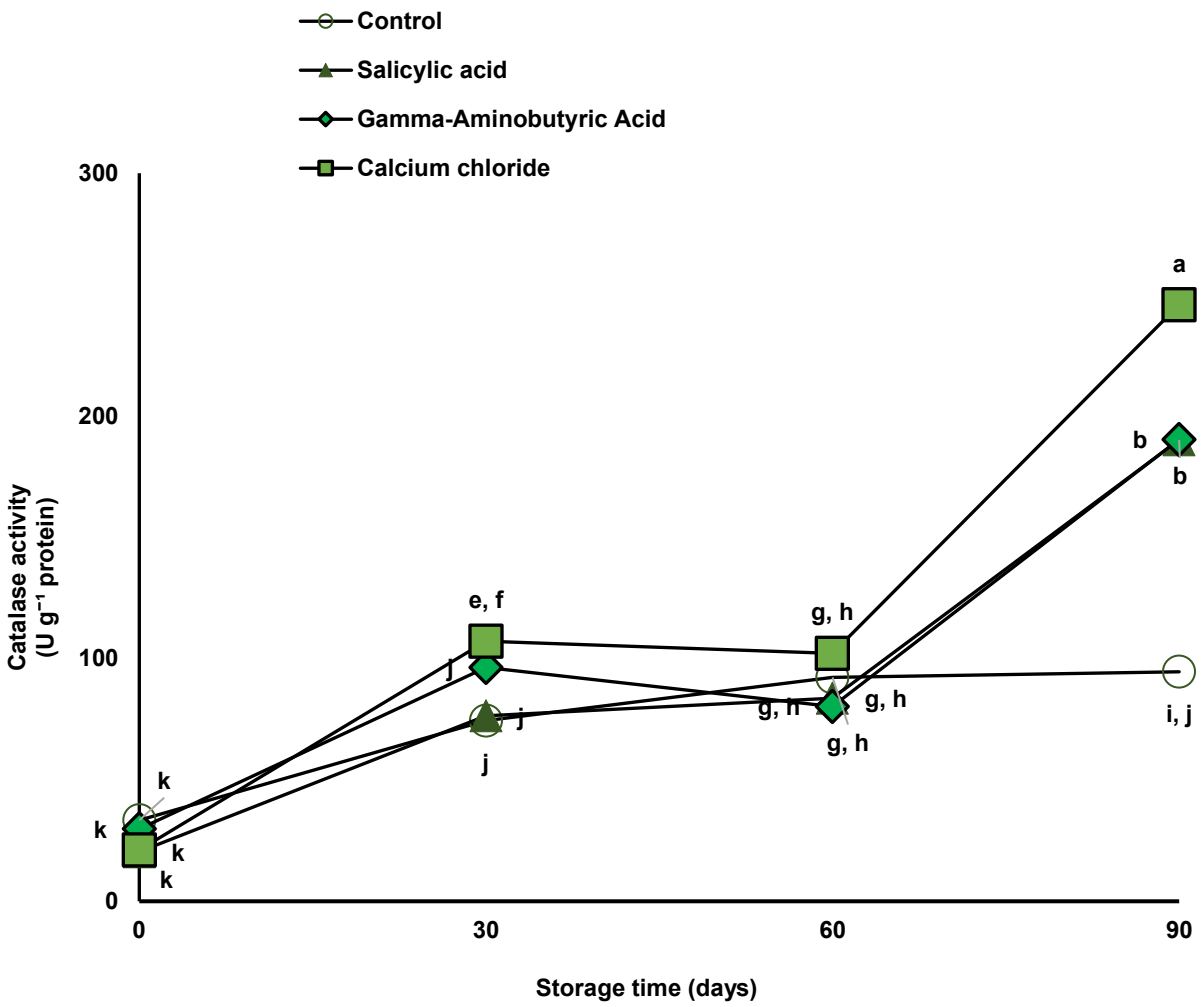


Fig. 4 Effects of postharvest $CaCl_2$, GABA, and SA treatments on catalase (CAT) activity in Thomson Navel orange flavedo during 90 days of storage. Means sharing the same letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$). Values are means of three biological replicates; error bars show SD. $CaCl_2$, calcium chloride; GABA, γ-aminobutyric acid; SA, salicylic acid

3.2.2 Peroxidase (POD) Activity

POD activity increased from less than 1 U g^{-1} protein at day 0 to 30–45 U g^{-1} protein at day 90 (Fig. 5) and did not differ among treatments at day 0. At day 30, GABA significantly increased POD activity (21.7 vs. 11.9 U g^{-1} protein in the control), whereas SA and $CaCl_2$ did not differ from the control. At day 60, POD activity in all treated fruit (11.3–28.0 U g^{-1} protein) was significantly lower than in the control (35.9 U g^{-1} protein). At day 90, only $CaCl_2$ differed from the control, with higher activity (44.6 vs. 29.8 U g^{-1} protein).

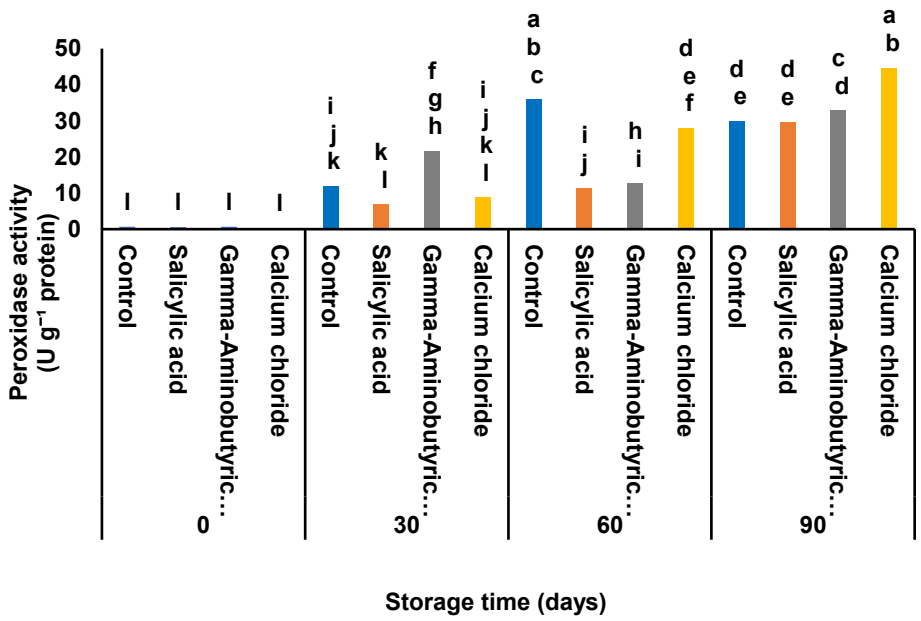


Fig. 5 Effects of postharvest $CaCl_2$, GABA, and SA treatments on peroxidase (POD) activity in Thomson Navel orange flavedo during 90 days of storage. Means sharing the same letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$). Values are means of three biological replicates; error bars show SD. $CaCl_2$, calcium chloride; GABA, γ-aminobutyric acid; SA, salicylic acid

3.3 Non-Enzymatic Antioxidants and Oxidative Status

Treatments produced time-dependent changes in total phenolic content, flavonoid content, and H_2O_2 accumulation (Figs. 6–8).

3.3.1 Total Phenolic Content

Total phenolic content did not differ significantly among treatments at day 0 (Fig. 6). GABA increased phenolic content at every later sampling time (+31%, +52%, and +49% relative to the control at days 30, 60, and 90). $CaCl_2$ increased phenolic content at days 60 and 90 (+36% and +29%), whereas SA did not differ from the control at any time.

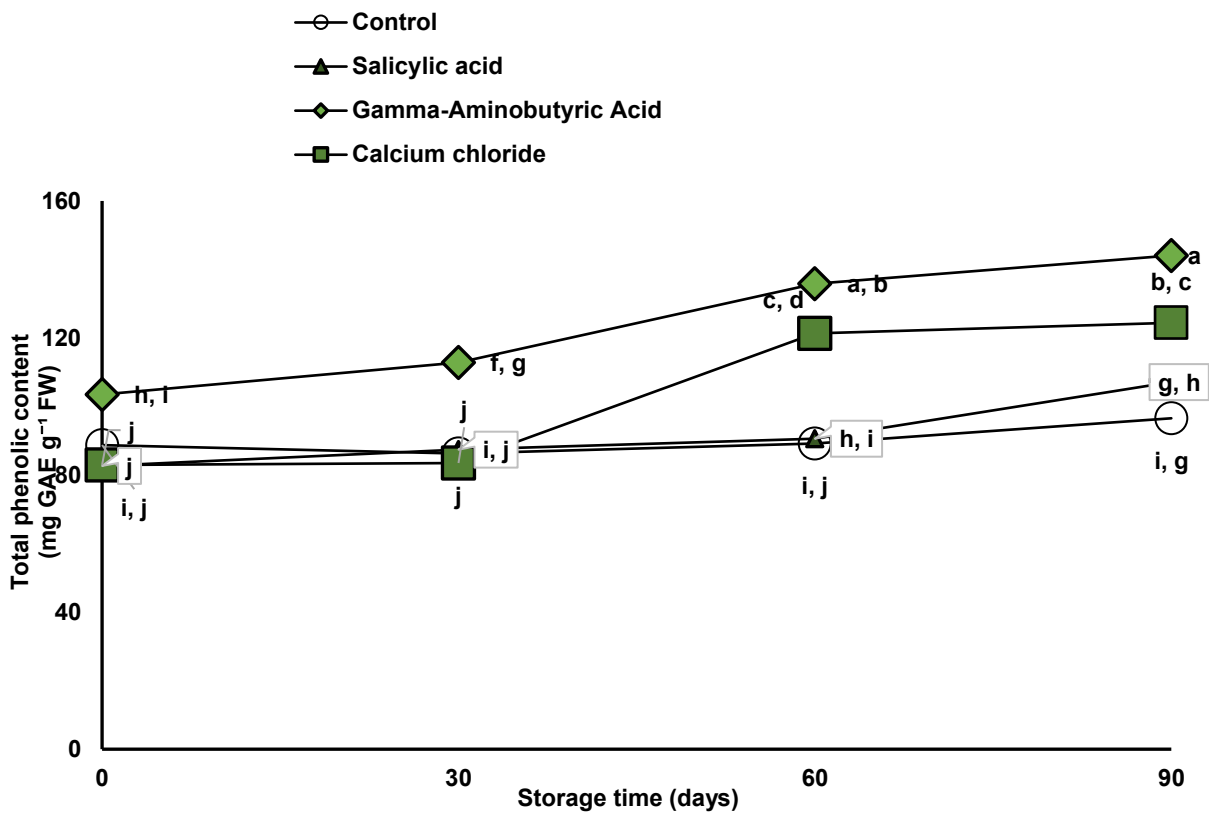


Fig. 6 Effects of postharvest $CaCl_2$, GABA, and SA treatments on total phenolic content in Thomson Navel orange flavedo during 90 days of storage. Means sharing the same letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$). Values are means of three biological replicates; error bars show SD. $CaCl_2$, calcium chloride; GABA, γ-aminobutyric acid; SA, salicylic acid; GAE, gallic acid equivalents; FW, fresh weight

3.3.2 Total Flavonoid Content

At day 0, GABA-treated fruit had 30% higher flavonoid content than the control, whereas SA and $CaCl_2$ did not differ from it (Fig. 7). At day 30, all treatments differed from the control: GABA (+23%) and $CaCl_2$ (+73%) were higher, whereas SA was lower (−19%). At day 60, only GABA differed from the control, with 36% lower flavonoid content. At day 90, SA (+58%) and $CaCl_2$ (+15%) were significantly higher than the control, whereas GABA remained lower (−34%).

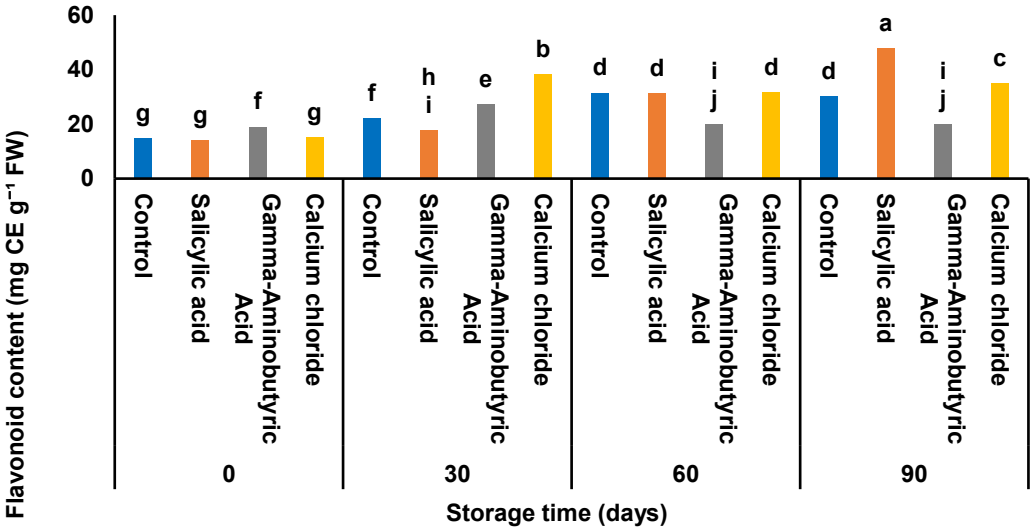


Fig. 7 Effects of postharvest $CaCl_2$, GABA, and SA treatments on total flavonoid content in Thomson Navel orange flavedo during 90 days of storage. Means sharing the same letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$). Values are means of three biological replicates; error bars show SD. $CaCl_2$, calcium chloride; GABA, γ-aminobutyric acid; SA, salicylic acid; CE, catechin equivalents; FW, fresh weight

3.3.3 Hydrogen Peroxide (H_2O_2) Accumulation

At day 0, H_2O_2 content was lowest in SA-treated fruit (82% lower than the control), with no difference for GABA or $CaCl_2$ (Fig. 8). At day 30, only $CaCl_2$ differed from the control (2.1-fold higher). By day 60, all treatments significantly increased H_2O_2 content relative to the control, with the highest increase in SA (5.2-fold), followed by $CaCl_2$ (3.2-fold) and GABA (1.6-fold). At day 90, SA and $CaCl_2$ remained significantly higher than the control (8.2-fold and 2.4-fold, respectively), whereas GABA did not differ from the control.

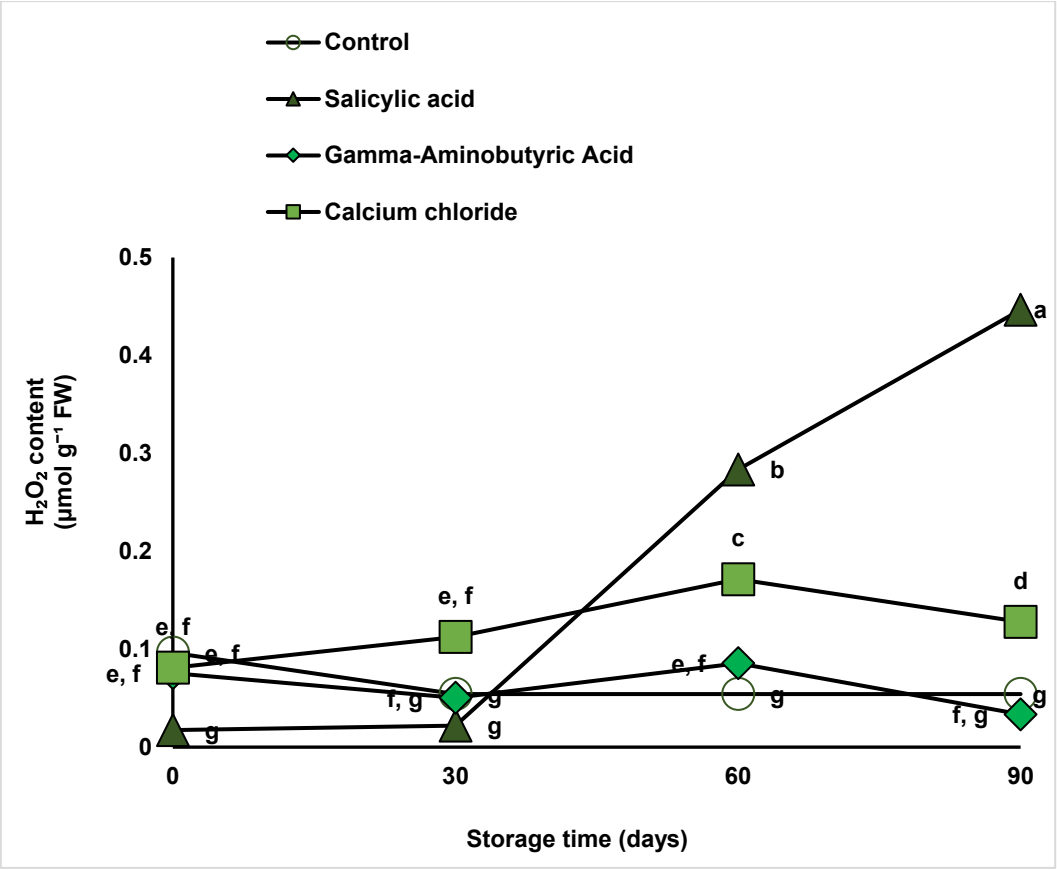


Fig. 8 Effects of postharvest $CaCl_2$, GABA, and SA treatments on hydrogen peroxide (H_2O_2) content in Thomson Navel orange flavedo during 90 days of storage. Means sharing the same letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$). Values are means of three biological replicates; error bars show SD. $CaCl_2$, calcium chloride; GABA, γ-aminobutyric acid; SA, salicylic acid; FW, fresh weight

4 Discussion

The three dips produced different transcript and redox profiles in the flavedo of Thomson Navel orange during cool storage (Table 4). $CaCl_2$ mainly lowered *CsACS2* transcripts and raised CAT activity. SA and GABA lowered *CsNCED* transcripts and differed from each other in their H_2O_2 and phenolic responses. Because expression was normalized to day 0 within each treatment, the gene-expression results describe how each treatment moved over storage, with the control as the reference for storage-related change.

$CaCl_2$ had the strongest effect on *CsACS2*. It lowered transcripts at every sampling time, to a $\log_2$ fold change of −9.17 at day 90 (a decline of several hundred-fold), whereas the control did not change significantly and SA caused a transient rise at day 60. *CsACS2* belongs to the ethylene-biosynthesis gene family of sweet orange (Sun et al. 2022), and calcium has been linked to phytohormone signaling in other fruit (Liu et al. 2024). A direct effect of calcium on ethylene-related genes in citrus peel is therefore plausible, but ethylene production was not measured here. CAT activity was also higher under $CaCl_2$, although the two responses may be unrelated.

CsNCED transcripts rose in the control at day 30 (+4.45), which fits a storage-related increase in ABA-biosynthetic capacity. SA lowered *CsNCED* at days 30 and 90 (−4.83 and −4.01) and GABA at day 90 (−9.54), while $CaCl_2$ had no significant effect. Lower transcript levels suggest reduced ABA-biosynthetic capacity, but ABA concentrations were not measured, so this remains to be checked.

CsPH8 transcripts rose in GABA-treated fruit at day 30 (+4.30) and were lower than at day 0 at day 90 in all three treatments (−10.00 for GABA, −9.30 for $CaCl_2$ and −5.54 for SA); $CaCl_2$ already lowered them at day 30 (−3.34), and the control showed no significant change. *CsPH8* is best known from the pulp, where it controls vacuolar acidification and citric acid accumulation (Shi et al. 2019), so these peel results do not show whether vacuolar pH, proton transport or organic acids changed (Sheng et al. 2017a, b).

At day 90, *CsNCED* and *CsPH8* transcripts fell together in GABA- and SA-treated fruit, most strongly under GABA (−9.54 and −10.00). Ma et al. (2024) linked an ABA-responsive transcription factor, CsABF3, to *CsPH8* through *CsAN1* and showed that this pathway drives citrate accumulation in citrus fruit under seasonal drought. The parallel decline of an ABA-biosynthesis gene and *CsPH8* seen here fits such an ABA–*CsPH8* link, but this is a hypothesis for the peel and was not tested. $CaCl_2$, in contrast, lowered *CsACS2* and *CsPH8* without changing *CsNCED*, so calcium may act on these genes by a route different from that of GABA and SA. In tomato, ABA promotes ethylene biosynthesis during ripening (Zhang et al. 2009); we saw no simple coupling between *CsNCED* and *CsACS2*, although the experiment was not designed to test it.

CAT activity did not differ among treatments early in storage, and the differences appeared later (Fig. 4). $CaCl_2$ raised CAT at days 30 and 90, whereas SA and GABA raised it only at day 90, when activity was 2.0–2.6 times that of the control. Increased antioxidant capacity after $CaCl_2$ or SA has also been reported in nectarine (Liu et al. 2024) and winter jujube (Sang et al. 2022), respectively. POD activity varied more (Fig. 5): GABA raised it at day 30, all treatments lowered it at day 60, when the control peaked, and only $CaCl_2$ raised it at day 90. CAT and POD therefore responded differently to each treatment and storage time rather than rising together.

H_2O_2 differed among treatments throughout storage (Fig. 8). SA-treated fruit had the lowest H_2O_2 at day 0 and the highest at days 60 and 90, so the SA effect changed sign over time. The SA-induced H_2O_2 increase agrees with the report on Satsuma mandarin (Zhu et al. 2016) but differs from pummelo, where SA reduced the storage-related rise in H_2O_2 (Huang et al. 2023); species, SA concentration or storage conditions may explain the difference. GABA raised H_2O_2 only at day 60. $CaCl_2$-treated fruit exceeded the control from day 30 onward but stayed below SA at days 60 and 90, which fits the improved stress tolerance reported for calcium in other fruit (Liu et al. 2024). H_2O_2 can act as a damage indicator or as a signal, and our data cannot separate the two.

GABA raised phenolic content at every sampling time after day 0 and $CaCl_2$ at days 60 and 90, whereas SA had no significant effect (Fig. 6); at day 90, phenolics were 49% (GABA) and 29% ($CaCl_2$) above the control. GABA is known to improve postharvest quality in citrus (Sheng et al. 2017a; Assar et al. 2025), and $CaCl_2$ enhanced phenylpropanoid metabolism in nectarine (Liu et al. 2024). Ramezanian et al. (2018) reported that preharvest $CaCl_2$ and SA sprays had the strongest effects on firmness and phenolic content of 'Washington Navel' oranges in storage; in our dips, $CaCl_2$ raised phenolics but SA did not. The lack of a phenolic response to SA also differs from custard apple, where preharvest SA at 1.0 mM raised phenolics and flavonoids (Gomathi et al. 2026), and the $CaCl_2$ response differs from sweet cherry, where preharvest $CaCl_2$ alone did not raise phenolics or DPPH activity (Polat et al. 2026). Species, application timing (preharvest spray versus postharvest dip) and concentration probably explain these differences. Flavonoid content also depended on treatment and time (Fig. 7): GABA caused an early rise followed by a decline, and SA and $CaCl_2$ raised flavonoids later in storage. Small differences were already present at day 0 (higher flavonoids under GABA, lower H_2O_2 under SA),

which may reflect baseline variation among replicate groups or an immediate effect of the treatment solutions; later effects should be read with these baselines in mind.

Table 4 Summary of significant treatment-associated changes in Thomson Navel orange flavedo during cool storage. Panel A: gene expression (relative to day 0 within each treatment). Panel B: biochemical variables (relative to the control at the same sampling time)

Panel A Gene expression (significant changes relative to day 0 within each treatment)

Gene	Control	$CaCl_2$	SA	GABA
CsACS2 (ethylene biosynthesis)	No significant change	Decrease at days 30, 60, and 90	Increase at day 60	No significant change
CsNCED (ABA biosynthesis)	Increase at day 30	No significant change	Decrease at days 30 and 90	Decrease at day 90
CsPH8 (vacuolar proton pump)	No significant change	Decrease at days 30, 60, and 90	Decrease at day 90	Increase at day 30; decrease at day 90

Panel B Biochemical variables (significant differences relative to the control at the same sampling time)

Parameter	$CaCl_2$	SA	GABA
CAT activity	Higher at days 30 and 90	Higher at day 90	Higher at day 90
POD activity	Lower at day 60; higher at day 90	Lower at day 60	Higher at day 30; lower at day 60
Total phenolic content	Higher at days 60 and 90	No significant change	Higher at days 30, 60, and 90
Total flavonoid content	Higher at days 30 and 90	Lower at day 30; higher at day 90	Higher at days 0 and 30; lower at days 60 and 90
H_2O_2 content	Higher at days 30, 60, and 90 (lower than SA at days 60 and 90)	Lower at day 0; higher at days 60 and 90	Higher at day 60

Figure 9 summarizes the measured transcript and biochemical responses of each treatment and the interpretations proposed from them.

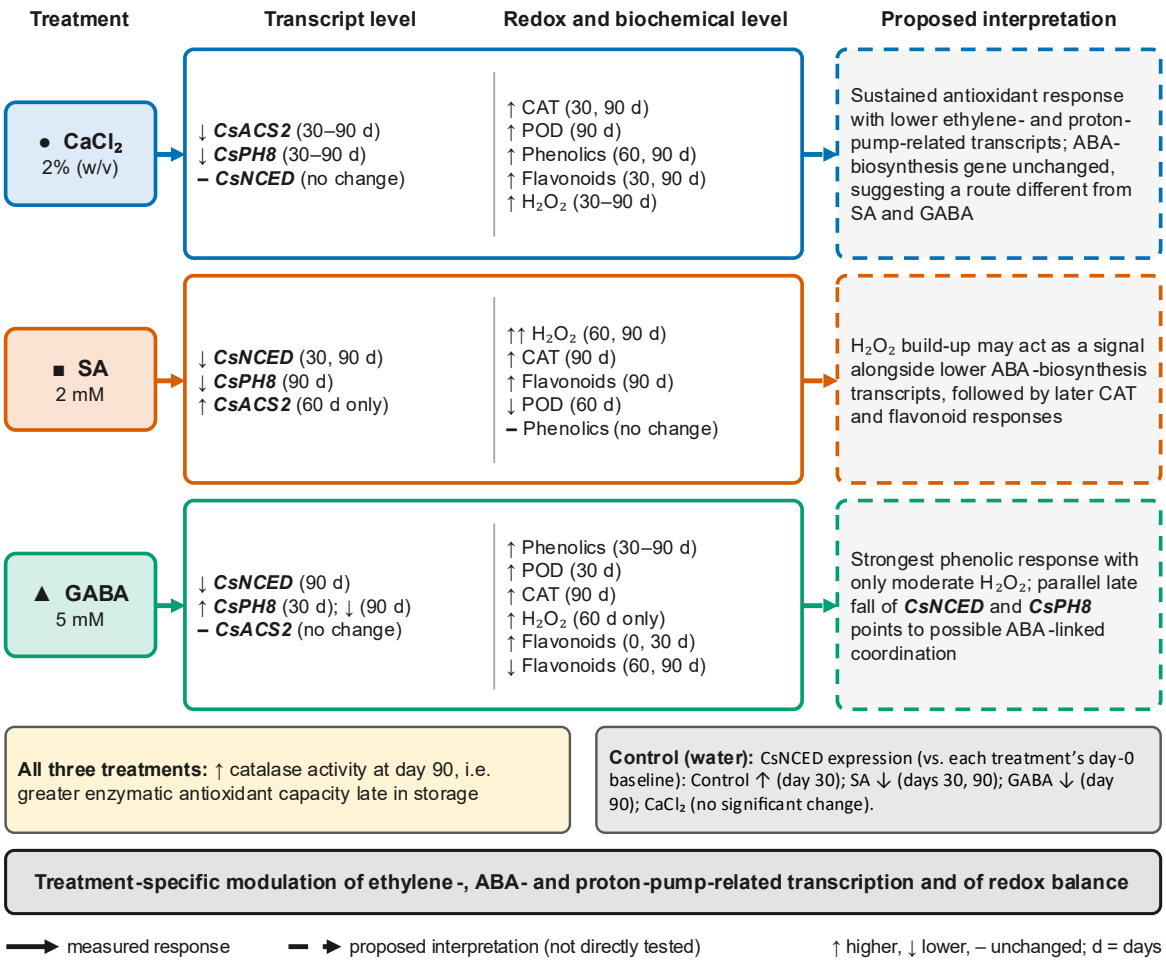


Fig. 9 Summary of the measured responses of Thomson Navel orange flavedo to postharvest dips of $CaCl_2$, SA and GABA during 90 days of cool storage, and the interpretations proposed from them. Solid arrows lead from each treatment to the responses measured in this study: transcript levels of *CsACS2*, *CsNCED* and *CsPH8* (relative to day 0 within each treatment) and redox and biochemical variables (relative to the control at the same sampling time). Dashed arrows lead to proposed interpretations that were not tested directly. ↑, higher; ↓, lower; –, no significant difference; numbers in brackets give the days (d) of storage at which the difference was significant ($P \leq 0.05$). The lower boxes show the response common to all treatments (higher CAT activity at day 90) and the storage-related rise of *CsNCED* in the control at day 30. $CaCl_2$, calcium chloride; SA, salicylic acid; GABA, γ-aminobutyric acid; CAT, catalase; POD, peroxidase; H_2O_2, hydrogen peroxide; ABA, abscisic acid

5 Conclusion

Postharvest dips of $CaCl_2$, SA and GABA produced different time courses of gene expression and redox-related markers in Thomson Navel flavedo during 90 days of cool storage. $CaCl_2$ lowered *CsACS2* transcripts and gave the strongest CAT response; SA and GABA lowered *CsNCED* transcripts at day 90; GABA gave the largest and most consistent increase in phenolics; and SA raised H_2O_2 late in storage. $CaCl_2$ and GABA are simple dips that now merit tests of weight loss, firmness, decay and sensory quality under commercial storage conditions. The transcript and antioxidant markers and sampling times reported here can guide those tests, together with direct measurements of ethylene, ABA and vacuolar pH.

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Competing interests

M. Biglarniya, H. Nosrati, A. Dehestani and P. Mehrabanjoubani declare that they have no competing interests.

Ethical standards

For this article no studies with human participants or animals were performed by any of the authors.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

Conceptualization: Ali Dehestani, Houshang Nosrati, Pooyan Mehrabanjoubani; Methodology: Maryam Biglarniya; Formal analysis and investigation: Maryam Biglarniya; Data curation: Maryam Biglarniya; Writing – original draft preparation: Maryam Biglarniya; Writing, review and editing: Ali Dehestani, Houshang Nosrati, Pooyan Mehrabanjoubani; Supervision: Houshang Nosrati, Ali Dehestani. All authors read and approved the final manuscript.

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