**Journal of Animal & Plant Sciences, 35(2): 2025, Page: 445-457**
**ISSN (print): 1018-7081; ISSN (online): 2309-8694**
https://doi.org/10.36899/JAPS.2025.2.0037

# ANTHOCYANIN ACCUMULATION AND GENE EXPRESSION ANALYSIS IN BLACK CARROT CALLI UNDER SALT AND SALICYLIC ACID STRESSES

B. Odabaşı[1], S. Şebikbay[1], A. Üner[2], G. Yıldırım-Baştemur[2], M. Pekmez[2], S. Perçin-Özkorucuklu[2] and Ö. Çakır[2*]

[1]Institute of Graduate Studies in Science, Program of Molecular Biotechnology and Genetics, Istanbul University, Istanbul, Turkiye
[2]Faculty of Science, Department of Molecular Biology and Genetics, Istanbul University, Istanbul, Turkiye
Corresponding author's email: ozgurckr@istanbul.edu.tr

## ABSTRACT

Black carrot (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.), is an important crop plant from the Apiaceae family, rich in anthocyanins, which contributes to its antioxidant capacity. Anthocyanins are secondary metabolites give purple to red color to plants, aid to adapt plants to severe environmental conditions. The main purpose of this study is to investigate the effects of NaCl and salicylic acid (SA) stress conditions on anthocyanin accumulation and antioxidant activity of total anthocyanin extract from black carrot callus cultures. In this study, one month old calli elicited with 50, 100, 150, 200 mM NaCl and 50, 100, 200, 400 µM SA. qRT-PCR analyses were performed to examine gene expression in the phenylpropanoid pathway. The anthocyanin profiles of the extraction obtained from stressed purple carrot calli were determined through HPLC-DAD while antioxidant capacities were evaluated with DPPH and CUPRAC assays. Results indicated that SA application generally increased antioxidant capacity, with the highest activity observed particularly in calli treated with 150 mM NaCl on day 15. NaCl elicitation was more effective in inducing anthocyanin biosynthesis compared with SA. HPLC analysis demonstrated a notable increasement of anthocyanin accumulation in NaCl treated samples, dramatically on the 10th day with 200 mM NaCl. This study indicated that elicitation with NaCl and SA affected anthocyanin accumulation and antioxidant capacity in black carrot calli. Thus, it is suggested that optimized elicitation conditions can be used to provide an alternative approach to increase the amount of anthocyanins to utilize its valuable health-promoting properties. By exploring the molecular mechanisms of anthocyanin biosynthesis under NaCl and SA stress, future studies could shed light on understanding of plant defense, potentially contributing to the development of more resilient varieties.

**Keywords**: Anthocyanin, Antioxidant Activity, *Daucus carota* ssp., Elicitation, Gene Expression, HPLC

This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Published first online March 01, 2025 Published final April 28, 2025

## INTRODUCTION

Carrots *(Daucus carota* L.), a member of Apiaceae family are the most popular vegetables among the crops. Cultivated carrots can be subdivided into two main groups, Western carrot *(D. carota* ssp. *sativus)* and eastern or anthocyanin carrots *(D. carota* ssp. *sativus* var. *atrorubens* Alef.*)* (Que *et al*., 2019; Nath *et al*., 2022; Polat *et al.*, 2022). Black carrot has a high moisture content of 88%, contains 1% protein, 2.5% fiber and 0.14% fat as well as several micro elements and vitamins including magnesium (9 mg), calcium (34 mg), potassium (240 mg), phosphorus (25 mg), zinc (0.2 mg) and copper (0.02 mg); riboflavin (0.02 mg), thiamine (0.04 mg), niacin (0.2 mg), and carotene (5.33 mg) per 100 g (Blando *et al*., 2021; Pandey *et al*., 2021).

Plant secondary metabolites; phenolics, terpenes, steroids, alkaloids, and flavonoids are not essential for plant living but crucially important for plant defense to environmental stresses and adaptation. Black carrots have a remarkably flavonoid content compared to orange carrots which play a crucial role in several biochemical and pharmacological processes. These flavonoids have antioxidant, anti-inflammatory, anti-atherosclerotic, antiplatelet aggregation, antitumor, antimicrobial, and anti-allergic activities (Rao *et al*., 2007).

Anthocyanins, water-soluble pigments derived from the amino acid phenylalanine, are subclass of flavonoids (Bai *et al*., 2019). Anthocyanins are synthesized from the flavonoid branch of the phenylpropanoid pathway. Structural and regulatory genes that take part in anthocyanin biosynthesis pathway is well studied (Ram *et al*., 2013). Phenylpropanoid pathway starts with PAL (phenylalanine ammonia lyase), followed by; C4H (cinnamate 4-hydroxylase) and 4CL (4-coumarate-CoA ligase). Flavonoid branch of the pathway continues as early genes *CHS* (chalcone

synthase), *CHI* (chalcone isomerase), *F3H* (flavanone 3-hydroxylase) and late genes *DFR* (dihydroflavonol 4-reductase), *LDOX* (leucocyanidin oxygenase), *UFGT* (UDP-glucose:flavonoid 3-O-glucosyltransferase) (Chaves-Silva *et al*., 2018; Fang *et al*., 2019). LDOX catalyses the reaction that converts leucoanthocyanidins to anthocyanidins. Given that PAL is the first enzyme in the phenylpropanoid pathway and LDOX plays a pivotal role in anthocyanin production, the examination of gene expression for these two enzymes is of utmost importance in research focused on anthocyanin accumulation (Khusnutdinov *et al*., 2021; Li *et al*., 2021; Sharma *et al*., 2022). Previous studies showed that anthocyanins have biological functions that are beneficial for human health (Li *et al*., 2017). Anthocyanins have important features for plants as it is in human health. In plants, anthocyanin accumulation rises under a range of abiotic stresses, such as heavy metal exposure, salinity, drought, UV radiation, nutrient deficiency, and extreme temperature (Li *et al*., 2018).

Salinity is one of the main abiotic stress factors that affect agricultural production all over the world and have serious effects on plant growth and development (Farooq *et al*., 2015; Liang *et al*., 2018) Under salinity, various crops have been observed to accumulate higher anthocyanin level, help plants to improve their tolerance to the challenging conditions (Mansour *et al*., 2023). Researchers showed that salt treatment, leads to accumulation of anthocyanins and proline, which facilitates better and faster adaptive response to salt stress in the colored wheat genotype (Mbarki *et al.,* 2018). In another study, salt stress triggers anthocyanins accumulation in *D. carota* cell culture, resulting from alterations in the expression of genes responsible for anthocyanin biosynthesis and storage (Saad *et al.,* 2021).

Salicylic acid (2-hydroxybenzoic acid) is also known as a signaling molecule or elicitor to protect plants against harsh environmental conditions derived from biotic and abiotic stresses (Hussain *et al*., 2009; Rivas-San Vicente and Plasencia, 2011). As an example, increase in anthocyanin levels observed in SA treated callus cultures of *D. carota* (Sudha and Ravishankar, 2003). In a study conducted on grapes, SA was applied at four different concentrations using the spray method and the anthocyanin content was reported to be much higher than in untreated berries. In particular, 200 µM SA application was reported to have almost twice the accumulation of malvidin-3-O-β glucoside, an anthocyanin derivative, in berries compared to the control (Oraei *et al*., 2019). When all these properties are considered, anthocyanins have become a research hotspot recently by scientists (Zheng *et al*., 2020).

Elicitation is one of the most efficient strategies for enhancing secondary metabolite biosynthesis, especially for anthocyanins. Physical, chemical or biological elicitors mimic the effect of environmental stresses which leads to activation of plant defense mechanisms and production of secondary metabolites (Saw *et al*., 2010; Fazili *et al*., 2022; Selwal *et al*., 2023).

It is of great importance to accurately determine the chemical profiles and concentrations of anthocyanins with antioxidant properties (Singh *et al*., 2020). In such cases, advanced techniques such as high-performance liquid chromatography (HPLC) are required to accurately and reproducibly detect the molecular diversity and low concentrations of anthocyanins (Valls *et al.*, 2009).

The callus stage is efficient for the studies focusing on enhancing the accumulation of secondary metabolites such as anthocyanins (Hosseini *et al*., 2020). To the best of our knowledge, there has been no study investigating anthocyanin gene expressions, quantity and antioxidant activity of black carrot callus anthocyanins under NaCl and SA treatment. This study aimed to explore how NaCl and SA influence anthocyanin accumulation in black carrot calli, with the goal of promoting higher anthocyanin accumulation during the callus stage.

## MATERIALS AND METHODS

**Plant Material and Sterilization:** Black carrot (*D. carota* ssp. *sativus* var. *atrorubens* Alef.) seeds were supplied from Atatürk Horticultural Central Research Institute in Yalova, Türkiye and kept at 4 °C in fridge. Surface sterilization performed according to Shiyab *et al*. (2019) with slight changes. Seeds were washed with 2-3 drops of liquid soap under tap water for 40 mins. Then they were sterilized with 70% ethanol (v/v) for 15 sec, 60% sodium hypochlorite (v/v) for 20 mins, and 70% ethanol (v/v) for 1 min respectively. Subsequently, seeds were rinsed with distilled water for 15 mins thrice and dried on sterile blotting paper.

**Germination and Callus Tissue Induction:** This study was designed with three biological replicates, where callus cultures were derived from plants grown from seeds for a month at three different time points. Sterilized seeds were transferred to hormone-free Murashige and Skoog (MS) basal medium containing 3% (w/v) sucrose, 0.9% (w/v) agar and pH adjusted to 5.6-5.8 with NaOH. Petri dishes were maintained in phytotron under 16/8 h photoperiod, light intensity of 2000–2500 Lux and 24±2°C temperature. The process of germination, seedling development and callus tissue induction were conducted following the procedure applied by Karataş *et al.*, (2014). Briefly, seeds were germinated in the dark for 7-10 days, then they were transferred to glass tubes containing hormone-free MS media for a month for development. Afterwards for callus tissue induction, purple hypocotyl parts of developed seedlings have been used as explant. Approximately 1 cm cutted and wounded hypocotyl tissues were planted on MS medium

supplemented with auxin (2 mg/L 2,4-D (2,4-Dichlorophenoxyacetic acid)) and cytokinin (0,2 mg/L BAP (6-Benzylaminopurine)) phytohormones. Tissues were subcultured monthly, undergoing a step-by-step progression from tissue to callus formation for 3 months. By the end of this period, purple, friable calli were successfully developed. These calli were subsequently exposed to elicitor treatments, including NaCl and SA, for further analysis.

**SA and NaCl Elicitation:** Subcultured purple and friable calli was used for elicitor treatments. 50, 100, 200, 400 of µM SA and 50, 100, 150, 200 mM of NaCl were applied individually to 0.2 g initial biomass of calli. The specified concentrations of NaCl were measured and added to the callus induction MS media before autoclave. The main stock solution was prepared as 50 mM SA. Measured SA was diluted with 50 mL solvent (EtOH 1:1 distilled water) and sterilized before addition to the culture medium with 0.22 µM pore-sized micro filter and added to medium after autoclave at stated concentrations (Ram *et al*., 2013). Following the elicitor treatment, samples gathered on 5, 10, 15 and 20th days. Fresh weight (FW) of the collected samples were weighted to determine the growth index. Then they were immediately frozen with liquid nitrogen and stored at -80°C freezer for further analysis

**Anthocyanin Extraction:** Anthocyanins from freezed callus tissue were extracted according to Gras *et al*. (2015) for HPLC and antioxidant activity analyses. 0.2 g of tissue was disintegrated in 4 mL solvent (75% distilled water, 20% MetOH, 5% formic acid) using an ultrasonicator 5 times for 20 seconds. After centrifugation, supernatant was vapoured by evaporator, 2 mL of MetOH was added to the pellets and dissolved in an ultrasonic bath, respectively. Finally, obtained solvents were kept at 4°C in the fridge.

**HPLC Instrument and Conditions:** High-performance liquid chromatography (HPLC) was used to determine the profiles and concentration of anthocyanin pigments in the black carrot callus extracts. The HPLC system (Shimadzu system) consisted of a LC- 10AD VP pump, a CTO-10AS column oven, a DGU-20A degasser and a SPD-M20A diode array detector (DAD). The HPLC method used in this study was modified based on the protocol proposed by Baştemur *et al*. (2024). As the stationary phase, a SUPELCOSIL LC-18 column measuring 25 cm x 4.6 mm was utilized, employing a gradient program. The mobile phase consisted of deionized water with 0.5% orthophosphoric acid (A) and methanol (B) with the gradient elution procedure: 35% B (3 min), 35% to 45% B (12 min), 45% to 35% B (2 min), at 3 min returning to initial conditions and total run time was 20 min. The flow rate of mobile phase was kept constant at 1.0 mL/min and the column temperature was at 25°C. The DAD detector was configured to a wavelength of 520 nm, and the injection volume for each sample and standard was 20 µL. The standard stock solution cyanidin 3-O-glucoside and cyanidin chloride were prepared in methanol and stored at 4°C. All analyses were performed in triplicates.

**Antioxidant Activity Assays**

**DPPH Assay:** DPPH assay is a commonly used method to measure the ability of compounds to serve as free radical scavengers or hydrogen donors. The antioxidant activity of foods is especially evaluated by using this method. Deep purple coloured DPPH reagent (2,2-Diphenyl-1-picrylhydrazyl (DPPH$^\bullet$) is used for DPPH assay due to its losing color property when reacted with antioxidants. Color altering is determined by UV-VIS Spectrophotometer at 515-520 nm. In this study, DPPH scavenging activity of salicylic acid and NaCl treated black carrot callus samples were measured based on the (Kang *et al*., 2022) protocol with slight changes (since the extracts were prepared in methanol, the stock DPPH solution was also prepared in methanol). For control and blank, ascorbic acid and methanol were used, respectively. Concentrations of samples between 0.1-3.0 mg $mL^{-1}$ and ascorbic acid between 0.001-0.1 mg $mL^{-1}$ were analyzed. Analyses were performed as 3 biological and 2 technical repeats. Methanolic 0.2 mM, 160 µL DPPH radical solution and 40 µL sample solution were mixed in 96-well microplates. Mixtures were measured with UV-VIS spectrophotometer at 520 nm after incubating for 10 min. in dark conditions in a shaker (Kang *et al*., 2022).

**CUPRAC Assay:** The CUPRAC assay is based on the reduction of $Cu^{2+}$ into $Cu^{+}$ by the action of the non-enzymatic antioxidants (for ex: Glutathione etc.) in the sample that will be analyzed. In this study, total antioxidant capacity of SA and NaCl treated black carrot callus samples were analyzed with the Cupric Ion Reducing Antioxidant Capacity (CUPRAC) method. Principle of the Cuprac method is based on reduction of Cu (II)-Nc complex to Cu (I)-Nc chelate under antioxidant presence (Apak *et al.* 2004). 250 µL of 10 mM $CuCl_2.2H_2O$, 250 µL of 7.5 mM Nc (in ethanol), 250 µL of 1.0 M $NH_4$-Ac buffer solution at pH 7, 25 µL sample and 250 µL distilled water added sequentially in a 24-well microplate. After that, the solution was kept at dark for 1 hour before measurement at 450 nm. Molar absorptivity (ε) of Trolox was calculated using the linear calibration curve of trolox that was created marking absorbance versus concentrations (25-1000 µg $mL^{-1}$) (Apak *et al*., 2004).

**qRT-PCR Analysis:** For qRT-PCR (Quantitative real-time polymerase chain reaction) analyses, total RNA was isolated from the frozen black carrot calli by the phenol:chloroform method using a phenol solution with a

similar mechanism of action to TRIzol reagent. Isolated RNAs were dissolved in DEPC (Diethyl Pyrocarbonate)-treated water. RNA samples were run in 1.2% agarose gel electrophoresis to analyze for quality and total RNA concentration was analyzed by using NanoDrop 2000 (Thermo Scientific), the purity of RNA was determined by absorbance ratio (A260/A280 and A260/A230), values approximately 2 are deemed as pure. cDNA was synthesized by using the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific, 4368814) in a total volume of 20 µl according to manufacturer's instructions.

Anthocyanin biosynthesis genes, *PAL3* and *LDOX2* were analyzed with a CFX96 Touch Real-Time PCR System (BioRad), using SYBR Green Real-Time PCR Master Mix (Hibrigen, Türkiye) All reactions were carried out in a final volume of 25 µL containing 1 µL cDNA, 0.5 µl of forward and reverse primer listed in Table 1 (for *PAL3* and *LDOX2*, as previously reported in Yildiz *et al., 2013*), 12.5 µL SYBR Green mix, 10.5 µL Nuclease- free water. The qRT-PCR reaction was performed with two technical replicates for each three biological replicates. The reaction performed was as follows: 50°C for 1 min, followed by 40 cycles of 94°C for 30 sec, annealing at 56 °C for 30 sec and 72 °C for 30 sec. Housekeeping gene ACTIN was used as reference. The relative expression ratio of each gene was calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

**Table 1. Forward and reverse primer sequences that used in qRT-PCR analysis.**

| Gene | Forward Primer Sequence | Reverse Primer Sequence | Product Length | GenBank Accession Number |
|---|---|---|---|---|
| *Actin* | 5'- TATGAGCAAGAGTTGGAGACT -3' | 5'- CATAGATGGCTGGAAGAGGAC -3' | 132 bp | MG931007 |
| *PAL3* | 5'- GAACTTGAGCACTTCCATC -3' | 5'- GCACTCCTTAATCCTGTTAG -3' | 139 bp | AB089813 |
| *LDOX2* | 5'- CATTATCTTTCGGACTGGGACTTG -3' | 5'- GGTTGAGGACACTTCGGGTAG -3' | 112 bp | AF184274 |

**Statistical analysis:** In order to ensure reliability and reproducibility of the results, all experiments were conducted with three independent replicates. The data were statistically analyzed using a two-way ANOVA to examine the comparison among control and treated groups and it followed by Tukey's test using GraphPad Prism (version 8.4.3) software for means comarison. A p-value of ≤ 0.05 was set as the threshold for statistical significance, indicating a meaningful difference in the results.

## RESULTS

**Callus Growth:** The maximum growth measured in callus tissues exposed to salt stress was recorded as 1.21 ± 0.24 g on the day-20 of 50 mM treatment which representing a 6.04 times increase compared to the initial mass. The maximum growth of the control group was 1.14 ± 0.16 g on the day-20, but the increase in the 50 mM NaCl compared to control groups was statistically non-significant. Growth in 150 mM and 200 mM NaCl treated calli were greatly inhibited and these conditions caused lethal effect on the day-20 (Fig. 1).

Salicylic acid had no stimulating effect on callus growth under any conditions. The maximum growth among the treated groups was 0.72 ± 0.19 g on the day-20, 50 µM SA samples. The control group on the same day has a mass of 1.14 ± 0.16 grams. After 20 days, SA concentrations and the increasing growth ratios were found to be inversely proportional. Potentially, 400 µM SA treatment could have led to death of callus tissues (Fig. 2).

### Gene Expression Analysis

**Effect of NaCl on Anthocyanin Biosynthesis Genes:** Inhibitory effect of salt stress on the relative expression of the *LDOX2* gene was observed in this study from the downregulations compared to *ACTIN* (Fig. 4). Based on the expression of *ACTIN* as the housekeeping gene, the lowest expression of *LDOX2* was seen in the group treated with 50 mM NaCl for 15 days.

Conversely, treatment with 200 mM of NaCl for 5 days led to a 28-fold increase in the relative expression of *PAL3*. However, there was no upregulation observed in the following days, rather *PAL3* gene was found to be downregulated. Treatment of 100 mM NaCl for 10 and 20 days, and 150 mM NaCl for 15 days, all led to slight upregulations in the *PAL3* gene, though these changes were not statistically significant (Fig. 3).

**Effect of SA on Anthocyanin Biosynthesis Genes:** Overall, SA typically negatively affected the transcription of the *LDOX2* gene and causes downregulation (Fig. 5), and only 100 µM SA treatment for 5 days slightly increased the gene expression. The dramatic decrease in expression observed in 400 µM SA treated samples on the day-20 is thought to be related to death of the tissue, as indicated in the growth index graph (Fig. 2).Thus, in all treated calli, expressions of *PAL3* and *LDOX2* genes on days 10, 15, and 20 were significantly downregulated compared to the control, as determined by statistical analysis.

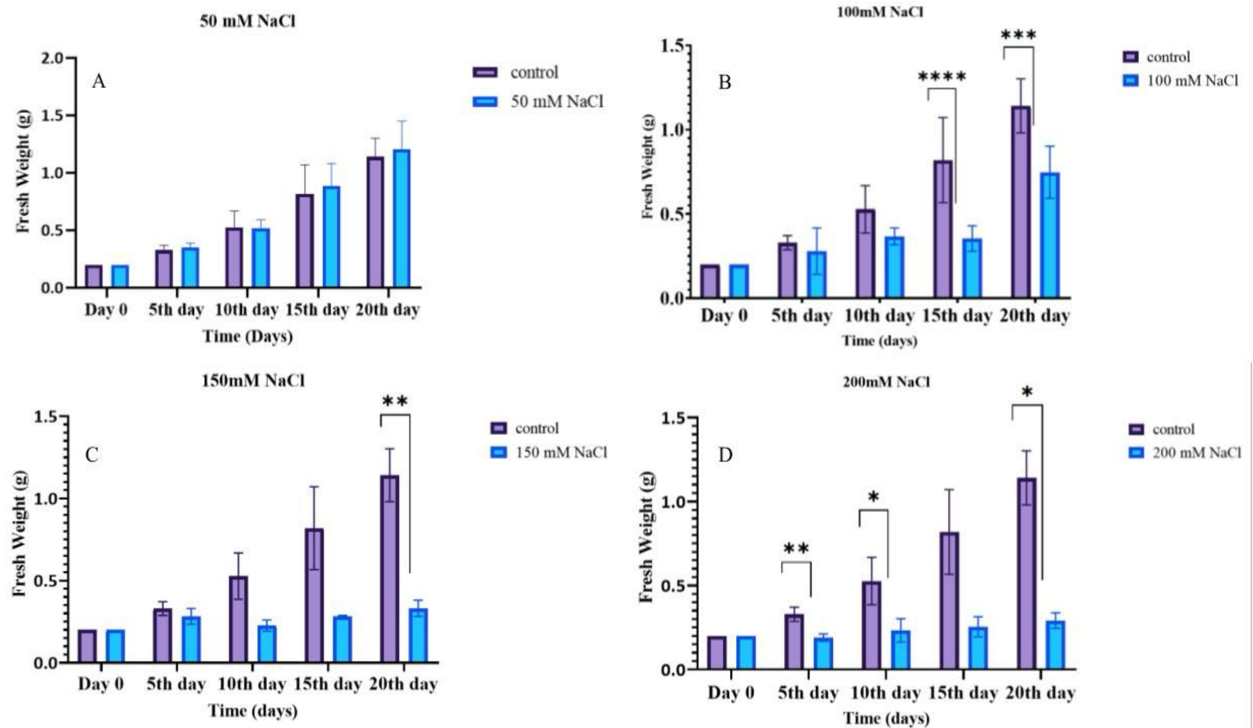


**Figure 1*:* Effects of 50, 100, 150, 200 mM NaCl elicitation on *Daucus carota* ssp. callus growth rates after 5, 10, 15, 20 days. Day 0 represents 0.2 g initially measured callus. A 50 mM NaCl B 100 mM NaCl C 150 mM NaCl D 200 mM NaCl conditions. p<0.05 significance level (Two-way ANOVA followed by Sidak's multiple comparisons test).**

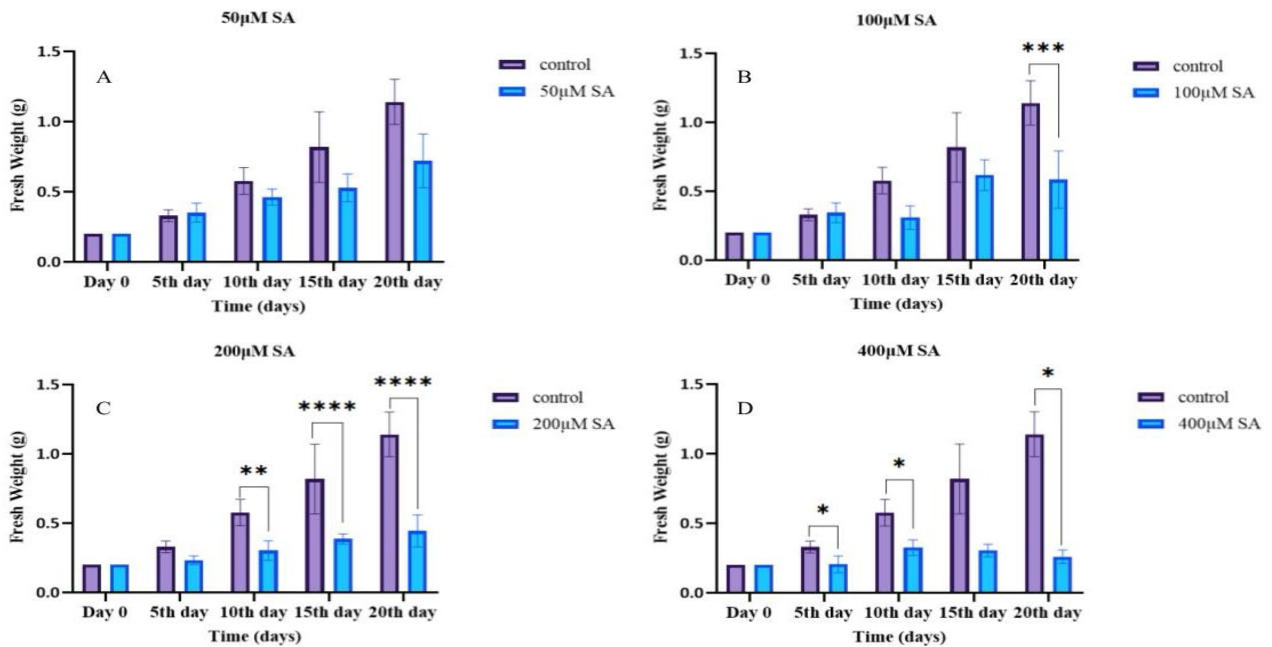


**Figure 2: Effects of 50, 100, 200, 400 µM SA elicitation on *Daucus carota* ssp. callus growth rates after 5, 10, 15, 20 days. Day 0 represents 0.2 g initially measured callus. A 50 µM SA B 100 µM SA C 150 µM SA D 200 µM SA conditions. p<0.05 significance level (Two-way ANOVA followed by Sidak's multiple comparisons test).**

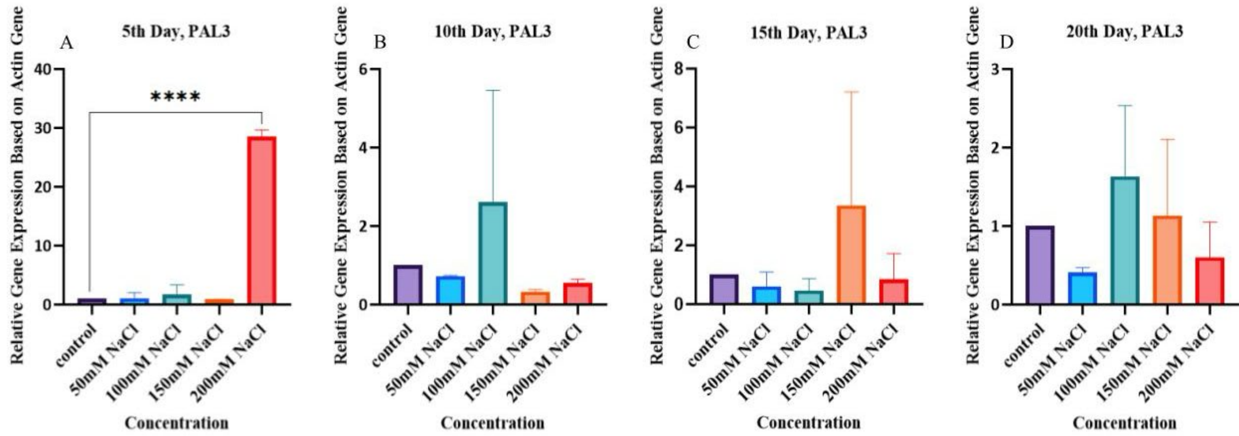


**Figure 3: Relative expression analysis of *PAL3* gene under NaCl elicitation. A on 5th day B on 10th day C on 15th day D on 20th day. p<0.05 significance level (One-way ANOVA followed by Tukey's multiple comparisons test).**

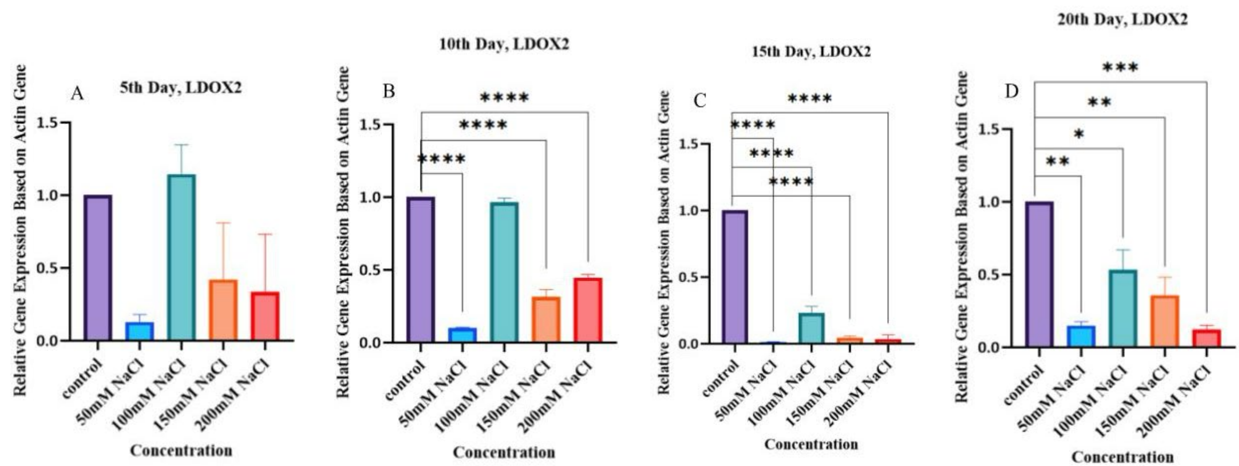


**Figure 4: Relative expression analysis of *LDOX2* gene under NaCl elicitation. A on 5th day B on 10th day C on 15th day D on 20th day. p<0.05 significance level (One-way ANOVA followed by Tukey's multiple comparisons test).**

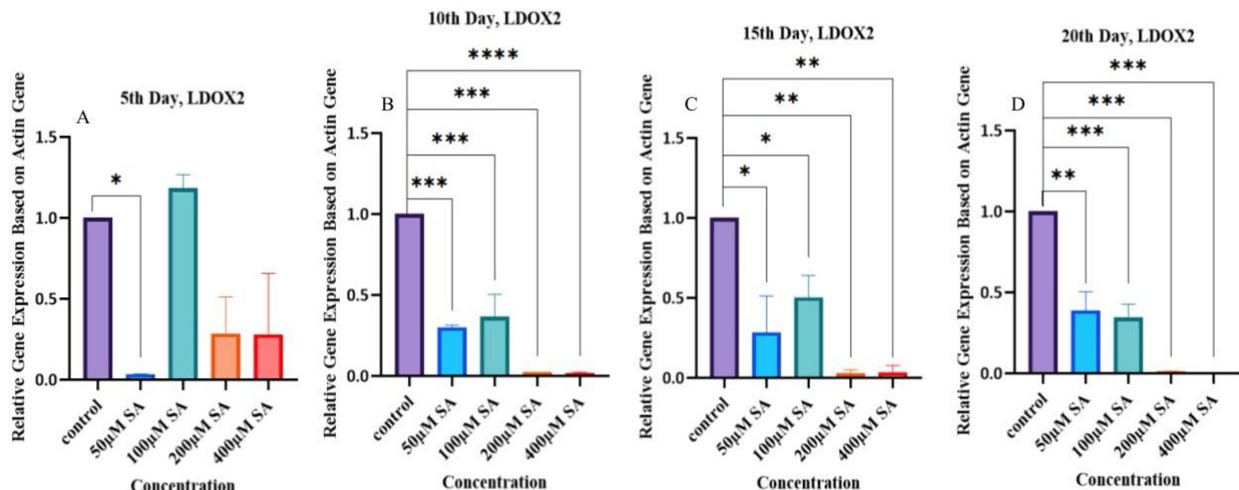


**Figure 5*:* Relative expression analysis of *LDOX2* gene under SA elicitation. A on 5th day B on 10th day C on 15th day D on 20th day. P<0.05 significance level (One-way ANOVA followed by Tukey's multiple comparisons test).**

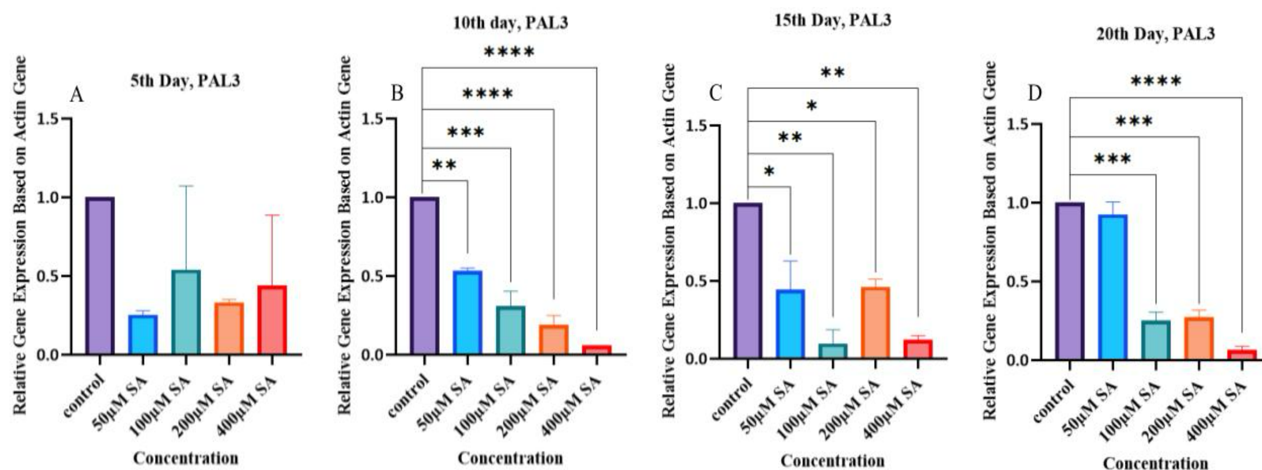


**Figure 6: Relative expression analysis of *PAL3* gene under SA elicitation. A on 5th day B on 10th day C on 15th day D on 20th day. P<0.05 significance level (One-way ANOVA followed by Tukey's multiple comparisons test).**

**Antioxidant Activity:** The antioxidant capacity of NaCl and salicylic acid treated purple carrot callus samples were determined using DPPH radical scavenging and CUPRAC assays (Results are shown in (Fig. 7, Fig. 8, Fig. 9 and Fig. 10). Different concentrations of callus extracts ranging between 0.1 and 3 mg $mL^{-1}$, along with different concentrations of ascorbic acid as the reference ranging between 0.001 and 0.1 mg $mL^{-1}$ were tested for DPPH activity assay. To determine the Cupric reducing activity, a concentration of 3 mg $mL^{-1}$ was used. With respect to Cupric reducing activity of the NaCl treated samples, there was no significant change for 5- and 10-day treatments. For 15-day NaCl treatment, while all concentrations caused an increase, the 150 mM concentration caused a statistically significant increase compared to control. This increasing trend continues similarly in the 20-day treatment. The increase in the treatments of 50 and 100 mM NaCl especially showed statistical significance compared to control. SA treatments seemed much more effective in terms of antioxidant activity compared to NaCl treatments. Relatively higher concentrations of SA treatments (200 and 400 µM ) for all durations caused a statistically significant decline for Cupric reducing activity, whereas 50 µM SA seemed to have caused an increase. Comparison of DPPH radical scavenging activity for NaCl treated samples showed that, only 15-day 150 mM treatment resulted in significantly higher activity. Whereas all, 10-day long SA treatments significantly increased the DPPH activity compared to control. Results indicated that 50 µM SA treatment for 20 days had the highest values of DPPH scavenging activity and total antioxidant capacity among all samples.

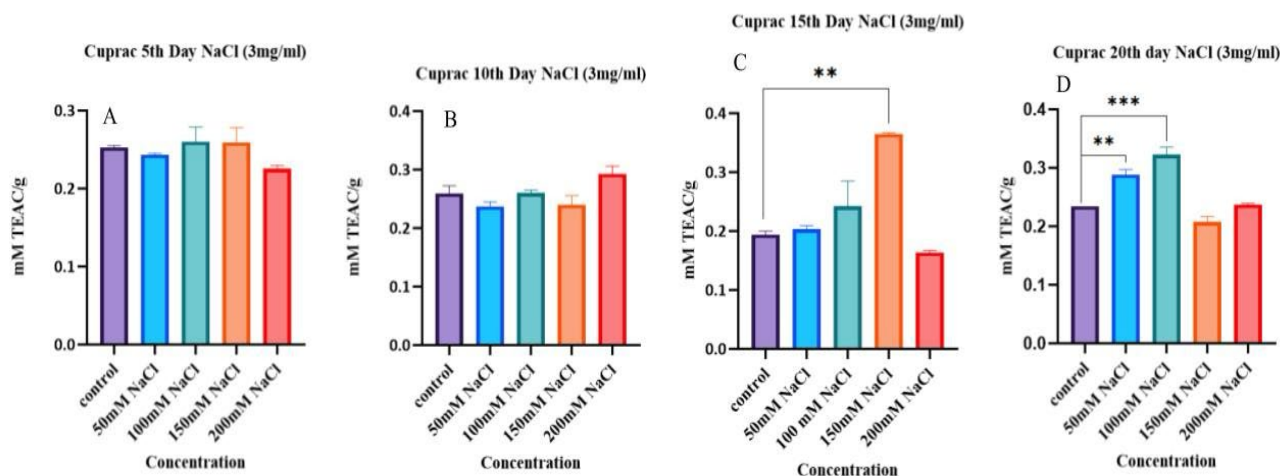


**Figure 7: Cuprac assay of NaCl treated callus. A on 5th day B on 10th day C on 15th day D on 20th day. P<0.05 significance level (one-way ANOVA followed by Dunnett's multiple comparisons test).**

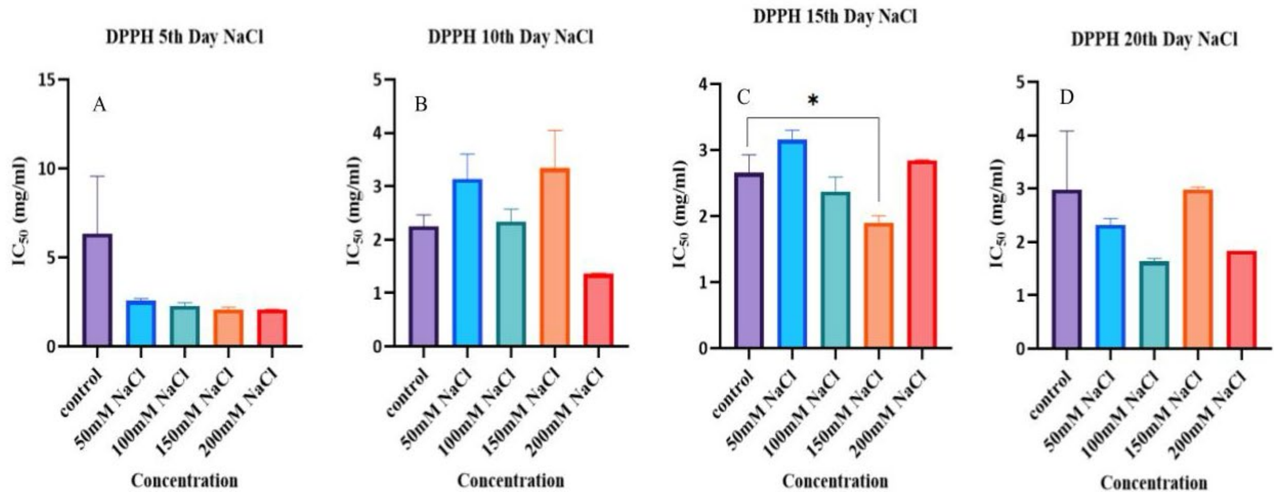


**Figure 8: DPPH assay of NaCl treated callus. A on 5th day B on 10th day C on 15th day D on 20th day. P<0.05 significance level (one-way ANOVA followed by Dunnett's multiple comparisons test).**

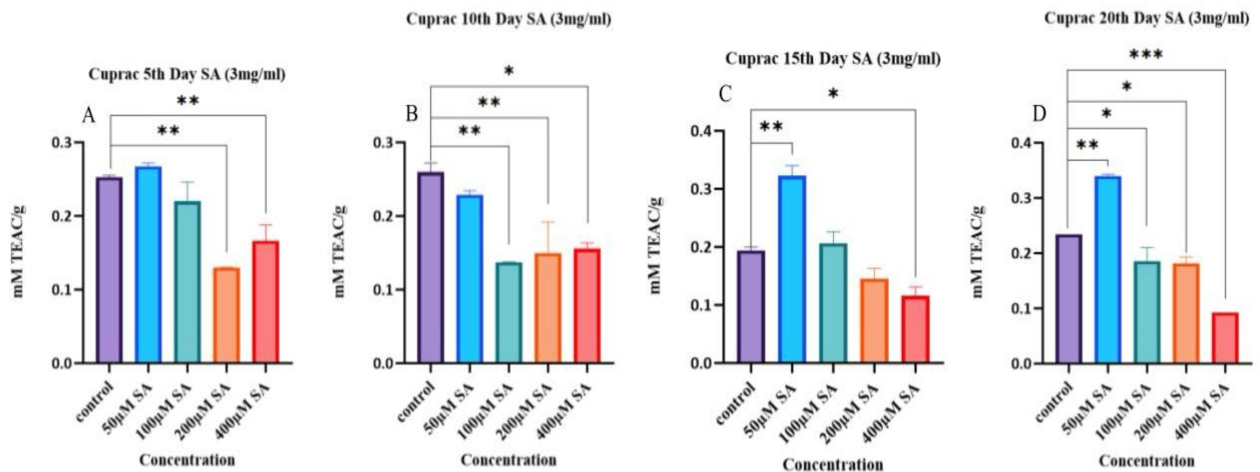


**Figure 9: Cuprac assay of SA treated callus. A on 5th day B on 10th day C on 15th day D on 20th day. P<0.05 significance level (one-way ANOVA followed by Dunnett's multiple comparisons test).**

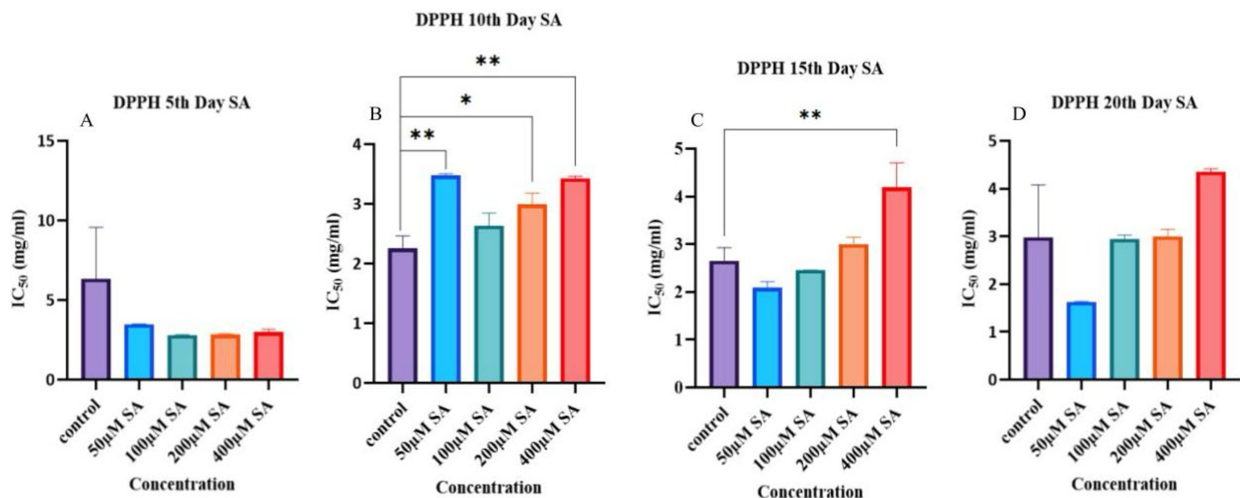


**Figure 10: DPPH assay of SA treated callus. A on 5th day B on 10th day C on 15th day D on 20th day. P<0.05 significance level (one-way ANOVA followed by Dunnett's multiple comparisons test).**

**HPLC analysis of anthocyanins:** The anthocyanin profile of purple carrot extracts was established using High-Performance Liquid Chromatography (HPLC). Cyanidin 3-O-glucoside and cyanidin chloride were analyzed to assess the effects of salt and SA stress on the anthocyanin biosynthesis pathway to correlate these changes with gene expression of *PAL3* and *LDOX2*. The peaks in the extracts were identified by comparison with the standard chromatogram, and the quantitative analysis of both pigments was completed within in a short period, specifically within 18 minutes. As a result of the analysis, on the 10th day of 200 mM NaCl treatment, the amounts of cyanidin 3-O-glucoside and cyanidin chloride pigments were determined to be 2.06 ±0.001 µg/mg and 0.66±0.09 µg/mg, respectively. In the SA treatment, cyanidin 3-O-glucoside was found to be 1.91±0.007 µg/mg on the 20th day (50 µM), while cyanidin chloride was measured at 0.56±0.01 µg/mg on the 5th day (100 µM) (Table 2). In conclusion, a comparison of the two treatments revealed that the NaCl treatments resulted in higher anthocyanin pigment levels than the SA treatments.

**Table 2. Pigment content of purple carrot extracts. (µg/mg).**

| Extract | CyanidinCl ± SD | Cyanidin 3-O-glucoside ± SD |
|---|---|---|
| Control 5th day | 0.125±0.006 | 1.310±0.003 |
| Control 10th day | 0.197±0.152 | 1.309±0.001 |
| Control 15th day | N.D | 0.640±0.009 |
| Control 20th day | N.D | 5.536±0.058 |
| 50 mM NaCl 5th day | 0.294±0.054 | 1.201±0.006 |
| 50 mM NaCl 10th day | N.D | 0.508±0.001 |
| 50 mM NaCl 15th day | 0.174±0.046 | 0.911±0.001 |
| 50 mM NaCl 20th day | N.D | 1.191±0.001 |
| 100 mM NaCl 5th day | 0.195±0.041 | 1.118±0.014 |
| 100 mM NaCl 10th day | 0.352±0.018 | 1.560±0.016 |
| 100 mM NaCl 15th day | 0.168±0.070 | 1.180±0.008 |
| 100 mM NaCl 20th day | 0.158±0.011 | 2.055±0.005 |
| 150 mM NaCl 5th day | 0.053±0.043 | 0.748±0.002 |
| 150 mM NaCl 10th day | N.D | 0.363±0.004 |
| 150 mM NaCl 15th day | N.D | 0.437±0.002 |
| 150 mM NaCl 20th day | N.D | 0.435±0.001 |
| 200 mM NaCl 5th day | 0.242±0.005 | 0.834±0.002 |
| 200 mM NaCl 10th day | 0.663± 0.088 | 2.061±0.001 |
| 200 mM NaCl 15th day | N.D | 0.316±0.002 |
| 200 mM NaCl 20th day | N.D | 0.864±0.006 |
| 50 µM SA 5th day | 0.447±0.180 | 1.544±0.021 |
| 50 µM SA 10th da | N.D | 0.496±0.003 |
| 50 µM SA 15th day | 0.035± 0.038 | 1.401±0.015 |
| 50 µM SA 20th day | 0.203± 0.072 | 1.911±0.007 |
| 100 µM SA 5th day | 0.515±0.012 | 0.917± 0,008 |
| 100 µM SA 10th da | 0.322±0.009 | 0.791±0.007 |
| 100 µM SA 15th day | 0.074±0.033 | 0.818±0.008 |
| 100 µM SA 20th day | 0.067± 0.003 | 0.860±0.011 |
| 200 µM SA 5th day | 0.214±0.016 | 0.764±0.002 |
| 200 µM SA 10th da | N.D | 0.499±0.002 |
| 200 µM SA 15th day | N.D | 0.325±0.002 |
| 200 µM SA 20th day | N.D | 0.266±0.002 |
| 400 µM SA 5th day | N.D | 0.415±0.001 |
| 400 µM SA 10th da | N.D | 0.304±0.002 |
| 400 µM SA 15th day | N.D | 0.192±0.002 |
| 400 µM SA 20th day | N.D | 0.151±0.003 |

N.D: Not detected.

## DISCUSSION

Secondary metabolite production in plants is heavily influenced by various biotic and abiotic stress factors. The objective of this study was to explore the role of SA and NaCl in enhancing anthocyanin accumulation in black carrot calli. Studies investigating the effects of different treatments on various plans in

terms of anthocyanin biosynthesis reported varying outcomes. Zheng *et al*. (2020), investigated how nitrogen deficiency, phosphorus deficiency, and the combined deficiency of both elements affected anthocyanin biosynthesis in grape skin callus, determining cyanidin 3-O-glucoside levels for all treatments to be in the range of 1.11±0.0071 mg/kg to 10.72±0.064 mg/kg. Saad *et al*. (2018) evaluated the optimization of nutrient intake and various physical parameters to achieve higher biomass yield and enriched anthocyanin production in suspension cultures of *D. carota* ssp. Study reported the highest anthocyanin content (9.30±0.25 mg/100 g FW) to be was observed in callus grown in the medium supplemented with 20.0 mM $NH_4NO_3$: 37.6 mM $KNO_3$, and the lowest in the medium containing 40.0 mM $NH_4NO_3$: 18.8 mM $KNO_3$ (2.74±0.27 mg/100 g FW). Sun *et al*. (2017) investigated the effects of methyl jasmonate, abscisic acid, and their combination on anthocyanin metabolism in callus material derived from red apple leaves. Cyanidin 3-O-galactose content in callus treated with $10^{-4}$ mol/L methyl jasmonate was found to be 1.3 times higher compared to the control (130.096 µg/g).

Salicylic acid and NaCl are known as signaling molecules with significant roles in plant defense mechanisms and plant stress tolerance (Rivas-San Vicente and Plasencia, 2011; Hawrylak-Nowak *et al.,* 2021). Previously, many studies reported SA and NaCl as positive regulators of the secondary metabolite pathways such as anthocyanin biosynthesis pathway. As examples of in vitro studies carried out in this context; in *Rosa hybrida* L. and *D. carota* callus cultures (Sudha and Ravishankar, 2003), anthocyanin production was higher than control conditions after 200 µM SA treatment (Ram *et al*., 2013). El-Beltagi *et al*., (2022) demonstrated that 50 mg $L^{-1}$ SA treated *Oryza sativa L.* cell suspensions showed increased anthocyanin production and antioxidant activity. Likewise, in *Vitis vinifera* cell suspension cultures, Wang *et al*. (2017) stated that anthocyanin accumulation and related gene expressions reached maximum level with 100 µM SA treatment after 2 days of incubation, and later on the anthocyanin accumulation decreased.

It can be proposed that the antioxidant activity might increase due to the increased Cyanidin3-O-glucoside levels for the related samples. For the total antioxidant activity, it can be deduced that 150 mM NaCl treatment for 15 days has the highest capacity. Previously in black carrot crude extracts, Smeriglio *et al*., (2018) quantified 25 polyphenols and 78.06% was composed of anthocyanins. According to their study when the concentrations of Cyanidin 3-O-glucoside and cyanidin were compared, cyanidin concentration was much higher than cyanidin 3-O-glucoside. In our study, however, the cyanidin 3-O-glucoside content was much higher than aforementioned report. This variation can be explained by the elicitation effect of the different agents used in our study. Resultingly, both SA and NaCl conditions are believed to be valuable and useful for the elucidation of anthocyanin accumulation causes, identification of polyphenolic profiles and biological activities of black carrot. Also, the accumulation of cyanidin 3-O-glucoside seems to be beneficial, due to the antioxidant, anti-inflammatory, and antitumor properties of this natural anthocyanin molecule (Hao *et al*., 2021; Qi *et al*., 2023).

When the effects of different concentrations and durations of NaCl and SA treatments on anthocyanin accumulation, antioxidant activity of the total anthocyanin extract, and the expression of genes involved in the anthocyanin biosynthesis pathway were considered, the observed inconsistencies between the results are likely related to multiple factors. Presence of all antioxidants in the extracts, including non-anthocyanin phenolics, interfered with the DPPH and Cuprac results. Besides, not all molecules were detected in HPLC-DAD, thus mismatches between these may have been observed. Here, we focused on the expression of a limited subset of genes within the phenylpropanoid pathway. However, further investigation is needed, as it is known that the expressions of many other genes within this pathway contributes to the biosynthesis of other compounds with antioxidant activity.

This study gives valuable insights on the impacts of NaCl and salicylic acid on anthocyanin accumulation and antioxidant capacity in black carrot calli; however, there are some limitations needing to be addressed. Firstly, due to the narrow concentration range of the treatments and the long interval between measurement days, the plants' responses to these stresses could not be thoroughly assessed. Another drawback is the genetic instability due to soma clonal variations possibly occuring in the calli leading to differences between cultures and impacting plant metabolism and stress responses. Future studies should focus on improving elicitation conditions and using different elicitors for large-scale production of anthocyanins. Moreover, molecular mechanisms in the plants' response to stress can be elucidated in more detail, allowing for the production of more resistant plant varieties through gene editing strategies.

**Conclusion:** HPLC analysis revealed cyanidin 3-O-glucoside as the major anthocyanin pigment in purple carrot extracts. NaCl treatment increased the amount of this compound, ranging from 0.316±0.002 µg/mg to 2.061±0.001 µg/mg, while salicylic acid treatment resulted in levels ranging from 0.151±0.003 µg/mg to 1.911±0.007 µg/mg. Additionally, 150 mM NaCl treatment for 15 days upregulated *PAL3* gene and led to the highest observed antioxidant activity. These findings point to the enhancing effect of NaCl treatment on cyanidin 3-O-glucoside accumulation, which could be

linked to increased antioxidant capacity detected in black carrot callus.

**Funding:** This research is financially supported by Istanbul University Scientific Research Project Coordination Unit with project no FLO-2021-37286 and FLO-2022-39546.

**Conflicts of Interest:** The authors declare that there is no conflict of interest to disclose.

**Competing Interests:** The authors have no relevant financial or non-financial interests to disclose.

**Author Contributions:** All authors contributed equally to the conceptualization, methodology, data collection, analysis, and writing of the manuscript.

**Acknowledgements:** We sincerely thank Elif Pulat for her invaluable assistance in editing and refining this manuscript. Her contributions greatly enhanced its clarity and quality.

**Data Availability:** Authors declare that the data could be available upon request.

## REFERENCES

Apak, R., K. Güçlü, M. Özyürek and S.E. Karademir (2004). Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. J. Agric. Food. Chem. 52(26): 7970–7981. https://doi.org/10.1021/jf048741x

Bai, S., R. Tao, Y. Tang, L. Yin, Y. Ma, J. Ni, X., Yan, Q. Yang, Z. Wu, Y. Zeng and Y. Teng (2019). BBX16, a B-box protein, positively regulates light-induced anthocyanin accumulation by activating *MYB10* in red pear. Plant Biotechnol. J. 17(10): 1985-1997. https://doi.org/10.1111/pbi.13114

Baştemur, G.Y., R. Akpınar, E. Kır and S.P. Özkorucuklu (2024). Development and validation of the HPLC-DAD method for the simultaneous determination of anthraquinones in *Rumex crispus* L. and *Rumex acetosella* L. plants and evaluation of their antioxidant capacities. J. Anal. Chem. 79(2): 180-190. https://doi.org/10.1134/S1061934824020035

Blando, F., S. Marchello, G. Maiorano, M. Durante, A. Signore, M.N. Laus, M. Soccio and G. Mita (2021). Bioactive compounds and antioxidant capacity in anthocyanin-rich carrots: a comparison between the black carrot and the Apulian landrace “Polignano” carrot. Plants. 10(3): 564. https://doi.org/10.3390/plants10030564

Chaves-Silva, S., A.L. Dos Santos, A. Chalfun-Júnior, J. Zhao, L.E. Peres and V.A. Benedito (2018). Understanding the genetic regulation of anthocyanin biosynthesis in plants–tools for breeding purple varieties of fruits and vegetables. Phytochem. 153: 11-27. https://doi.org/10.1016/j.phytochem.2018.05.013

El-Beltagi, H.S., H.I. Mohamed, M.I. Aldaej, J.M. Al-Khayri, A.A. Rezk, M.Q. Al-Mssallem, M.N. Sattar and K.M. Ramadan (2022). Production and antioxidant activity of secondary metabolites in Hassawi rice (*Oryza sativa* L.) cell suspension under salicylic acid, yeast extract, and pectin elicitation. In Vitro Cellular and Developmental Biology-Plant. 58(4): 615-629. https://doi.org/10.1007/s11627-022-10264-x

Fang, H., Y. Dong, X. Yue, J. Hu, S. Jiang, H. Xu, Y. Wang, M. Su, J. Zhang, Z. Zhang, N. Wang and X. Chen (2019). The B-box zinc finger protein MdBBX20 integrates anthocyanin accumulation in response to ultraviolet radiation and low temperature. Plant, Cell and Environment. 42(7): 2090-2104. https://doi.org/10.1111/pce.13552

Farooq, M., M. Hussain, A. Wakeel and K.H.M. Siddique (2015). Salt stress in maize: effects, resistance mechanisms, and management. A review. Agron. Sustain. Develop. 35: 461–481. https://doi.org/10.1007/s13593-015-0287-0

Fazili, M.A., I. Bashir, M. Ahmad, U. Yaqoob and S.N. Geelani. (2022). *In vitro* strategies for the enhancement of secondary metabolite production in plants: a review. Bull. Natl. Res. Cent. 46(1): 35. https://doi.org/10.1186/s42269-022-00717-z

Gras, C.C., R. Carle and R.M. Schweiggert (2015). Determination of anthocyanins from black carrots by UHPLC-PDA after ultrasound-assisted extraction. J. Food Compos. Anal. 44: 170–177. https://doi.org/10.1016/j.jfca.2015.08.011

Hao, X., R. Guan, H. Huang, K. Yang, L. Wang and Y. Wu (2021). Anti-inflammatory activity of cyanidin-3-O-glucoside and cyanidin-3-O-glucoside liposomes in THP-1 macrophages. Food Sci. Nutr. 9(12): 6480–6491. https://doi.org/10.1002/fsn3.2554

Hawrylak-Nowak, B., S. Dresler, M. Stasińska-Jakubas, M. Wójciak, I. Sowa and R. Matraszek-Gawron (2021). Nacl-induced elicitation alters physiology and increases accumulation of phenolic compounds in *Melissa officinalis* L. Int. J. Mol. Sci. 22(13): 6844. https://doi.org/10.3390/ijms22136844

Hosseini, N.S., Z. Ghasimi Hagh and H. Khoshghalb (2020). Morphological, antioxidant enzyme activity and secondary metabolites accumulation in response of polyethylene glycol-induced osmotic stress in embryo-derived plantlets and callus cultures of *Salvia leriifolia*. Plant Cell Tissue Organ Cult. 140: 143–155. https://doi.org/10.1007/s11240-019-01718-z

Hussain, M., M.A. Malik, M. Farooq, M.B. Khan, M. Akram and M. F. Saleem (2009). Exogenous glycinebetaine and salicylic acid application improves water relations, allometry and quality of hybrid sunflower under water deficit conditions. J. Agron. Crop Sci. 195: 98-109. https://doi.org/10.1111/j.1439-037X.2008.00354.x

Kang, M. H., Y.J. Ji, Y.M. Han, G.Y. Jang, D.H. Kim, J.H. Lee, G.S. Kim, S.J. Choi and H.D. Kim (2022). *Sasa borealis* ethanol extract protects PC12 neuronal cells against oxidative stress. Appl. Sci. (Switzerland). 12(12). https://doi.org/10.3390/app12126155

Karataş, İ., M. Elmastaş, and R. Karataş (2014). Black Carrot (Daucus carota ssp. sativus var. atrorubens Aleph) effect of some practices on anthocyanin production in callus culture. Gaziosmanpaşa J. Scientific Research. (9): 62-73.

Khusnutdinov, E., A. Sukhareva, M. Panfilova and E. Mikhaylova. (2021). Anthocyanin biosynthesis genes as model genes for genome editing in plants. Int. J. Mol. Sci. *22*(16): 8752. https://doi.org/10.3390/ijms22168752

Liang, W., X. Ma, P. Wan and L. Liu (2018). Plant salt-tolerance mechanism: a review. Biochem. Biophys. Res. Commun. 495(1): 286–291. https://doi.org/10.1016/J.BBRC.2017.11.043

Li, D., P. Wang, Y. Luo, M. Zhao and F. Chen (2017). Health benefits of anthocyanins and molecular mechanisms: update from recent decade. Crit. Rev. Food Sci. Nutr. 57(8): 1729–1741. https://doi.org/10.1080/10408398.2015.1030064

Li, N., X. Wang, B. Ma, Z. Wu, L. Zheng, Z. Qi and Y. Wang (2021). A leucoanthocyanidin dioxygenase gene (*RtLDOX2*) from the feral forage plant *Reaumuria trigyna* promotes the accumulation of flavonoids and improves tolerance to abiotic stresses. J. Plant Res. 134(5): 1121-1138. https://doi.org/10.1007/s10265-021-01315-2

Li, X., X. Lv, X. Wang, L. Wang, M. Zhang and M. Ren (2018). Effects of abiotic stress on anthocyanin accumulation and grain weight in purple wheat. Crop Pasture Sci. 69(12): 1208-1214. https://doi.org/10.1071/CP18341

Livak K.J. and T.D. Schmittgen (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 25(4): 402–408. https://doi.org/10.1006/meth.2001.1262

Mansour, M.M.F. (2023). Anthocyanins: biotechnological targets for enhancing crop tolerance to salinity stress. Sci. Hortic. 319: 112182. https://doi.org/10.1016/j.scienta.2023.112182

Mbarki, S., O. Sytar, M. Zivcak, C. Abdelly, A. Cerda and M. Brestic (2018). Anthocyanins of coloured wheat genotypes in specific response to salstress. Molecules. 23(7): 1518. https://doi.org/10.3390/molecules23071518

Nath, P., A. Dukare, S. Kumar, S. Kale and P. Kannaujia (2022). Black carrot (*Daucus carota* subsp. *sativus*) anthocyanin-infused potato chips: Effect on bioactive composition, color attributes, cooking quality, and microbial stability. J. Food Process. Preserv, 46(1): e16180. https://doi.org/10.1111/jfpp.16180

Oraei, M., S. Panahirad, F. Zaare-Nahandi, and G. Gohari (2019). Pre-véraison treatment of salicylic acid to enhance anthocyanin content of grape (*Vitis vinifera* L.) berries. J. Sci. Food Agric. 99(13): 5946-5952. https://doi.org/10.1002/jsfa.9869

Pandey, P., K. Grover, T.S. Dhillon, A. Kaur and M. Javed (2021). Evaluation of polyphenols enriched dairy products developed by incorporating black carrot (*Daucus carota* L.) concentrate. Heliyon. *7*(5): e06880. https://doi.org/10.1016/j.heliyon.2021.e06880.

Polat, S., G. Guclu, H. Kelebek, M. Keskin and S. Selli (2022). Comparative elucidation of colour, volatile and phenolic profiles of black carrot (*Daucus carota* L.) pomace and powders prepared by five different drying methods. Food Chem. 369: 130941. https://doi.org/10.1016/j.foodchem.2021.130941

Qi, Q., M. Chu, X. Yu, Y. Xie, Y. Li, Y. Du, X. Liu, Z. Zhang, J. Shi and N. Yan (2023). Anthocyanins and proanthocyanidins: chemical structures, food sources, bioactivities, and product development. Food Rev. Int. 39(7): 4581-4609. https://doi.org/10.1080/87559129.2022.2029479

Que, F., X.L. Hou, G.L. Wang, Z.S. Xu, G.F. Tan, T. Li, Y.H. Wang, A. Khadr and A.S. Xiong (2019). Advances in research on the carrot, an important root vegetable in the Apiaceae family. Hortic. Res. 6: 69. https://doi.org/10.1038/s41438-019-0150-6

Ram, M., K.V. Prasad, S.K. Singh, B.S. Hada and S. Kumar (2013). Influence of salicylic acid and methyl jasmonate elicitation on anthocyanin production in callus cultures of *Rosa hybrida* L.

Plant Cell Tissue Organ Cult. 113(3): 459–467. https://doi.org/10.1007/s11240-013-0287-1

Rao, A.V. and L.G. Rao (2007). Carotenoids and human health. Pharmacol. Res. 55(3): 207-216. https://doi.org/10.1016/j.phrs.2007.01.012

Rivas-San Vicente, M. and J. Plasencia (2011). Salicylic acid beyond defence: its role in plant growth and development. J. Exp. Bot. 62(10): 3321-3338. https://doi.org/10.1093/jxb/err031

Saad, K.R., G. Kumar, P. Giridhar and N.P. Shetty (2018). Differential expression of anthocyanin biosynthesis genes in *Daucus carota* callus culture in response to ammonium and potassium nitrate ratio in the culture medium. 3 Biotech. 8: 1-11. https://doi.org/10.1007/s13205-018-1447-0

Saad, K.R., G. Kumar, S.N. Mudliar, P. Giridhar and N.P. Shetty (2021). Salt stress-induced anthocyanin biosynthesis genes and *MATE* transporter involved in anthocyanin accumulation in *Daucus carota* cell culture. ACS omega. 6(38): 24502-24514. https://doi.org/10.1021/acsomega.1c02941

Saw, N.M.M.T., H. Riedel, O. Kutuk, K. Ravichandr and I. Smetanska (2010). Effect of Elicitors and Precursors on the synthesis of Anthocyanin in grape *Vitis vinifera* cell cultures. Energy Res. J. 1(2): 189–192. https://doi.org/10.3844/erjsp.2010.189.192

Sharma, H., N. Chawla and A.S. Dhatt (2022). Role of phenylalanine/tyrosine ammonia lyase and anthocyanidin synthase enzymes for anthocyanin biosynthesis in developing *Solanum melongena* L. genotypes. Physiol. Plant. 174(5): e13756. https://doi.org/10.1111/ppl.13756

Selwal, N., F. Rahayu, A. Herwati, E. Latifah, Supriyono, C. Suhara, I.B.K. Suastika, W.M. Mahayu and A.K. Wani (2023). Enhancing secondary metabolite production in plants: exploring traditional and modern strategies. J. Agric. Food Res. 14: 100702. https://doi.org/10.1016/j.jafr.2023.100702

Shiyab, S., B. Al-Qarallah and M. Akash (2019). Influence of salinity on growth and organic compounds content of carrot (*Daucus carota* L.). Acta Scientiarum Polonorum. Hortorum Cultus. 18(6): 85-96. 10.24326/asphc.2019.6.9

Smeriglio, A., M. Denaro, D. Barreca, V. D'Angelo, M.P. Germanò and D. Trombetta (2018). Polyphenolic profile and biological activities of black carrot crude extract (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.). Fitoterapia. 124: 49–57. https://doi.org/10.1016/j.fitote.2017.10.006

Singh, M.C., C. Kelso, W.E. Price and Y. Probst (2020). Validated liquid chromatography separation methods for identification and quantification of anthocyanins in fruit and vegetables: a systematic review. Food Res. Int. 138: 109754. https://doi.org/10.1016/j.foodres.2020.109754

Sudha, G. and G.A. Ravishankar (2003). Elicitation of anthocyanin production in callus cultures of *Daucus carota* and the involvement of methyl jasmonate and salicylic acid. Acta Physiol Plant., 25: 249–256. https://doi.org/10.1007/s11738-003-0005-4

Sun, J., Y. Wang, X. Chen, X. Gong, N. Wang, L. Ma, Y. Qiu, Y. Wang and S. Feng (2017). Effects of methyl jasmonate and abscisic acid on anthocyanin biosynthesis in callus cultures of red-fleshed apple (*Malus sieversii* f. *niedzwetzkyana*). Plant Cell Tissue Organ Cult. 130(2): 227–237. https://doi.org/10.1007/s11240-017-1217-4

Valls, J., S. Millán, M.P. Martí, E. Borràs and L. Arola (2009). Advanced separation methods of food anthocyanins, isoflavones and flavanols. J. Chromatogr. A. 1216(45): 7143–7172. https://doi.org/10.1016/j.chroma.2009.07.030

Wang, H., W. Wang, W. Huang and H. Xu (2017). Effect of salicylic acid on the gene transcript and protein accumulation of flavonoid biosynthesis-related enzymes in *Vitis vinifera* cell suspension cultures. Hort Sci. 52(12): 1772–1779. https://doi.org/10.21273/HORTSCI12515-17

Yildiz, M., D.K. Willis, P.F. Cavagnaro, M. Iorizzo, K. Abak and P.W. Simon (2013). Expression and mapping of anthocyanin biosynthesis genes in carrot. TAG Theor. Appl. Genet. Theoretische und angewandte Genetik, 126(7): 1689–1702. https://doi.org/10.1007/s00122-013-2084-y.

Zheng, H. Z., H. Wei, S.H. Guo, X. Yang, M.X. Feng, X.Q. Jin, Y.L. Fang, Z.W. Zhang, , T.F. Xu and J.F. Meng (2020). Nitrogen and phosphorus co-starvation inhibits anthocyanin synthesis in the callus of grape berry skin. Plant Cell Tissue Organ Cult. 142(2): 313–325. https://doi.org/10.1007/s11240-020-01864-9.