*Article*

# The Effect of Foliar Salicylic Acid and Zinc Treatments on Proline, Carotenoid, Chlorophyll Content and Anti-Oxidant Enzyme Activity in *Galanthus elwesii* Hook

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**Abstract:** *Galanthus elwesii* Hook is an important plant species of the Amaryllidaceae family and is used for medicinal purposes with its valuable bioactive compounds. The present study was conducted to investigate the effects of foliar salicylic acid (SA) and zinc (Zn) treatments on proline, carotenoid, chlorophyll content and anti-oxidant enzyme activity in *G. elwesii*. The ascorbate peroxidase (APX), catalase (CAT) enzyme activity, and protein contents were determined with ascorbate oxidation, hydrogen peroxide ($H_2O_2$), and Bradford experiments, respectively. The plants were treated with three different concentrations of SA (0.5, 1, and 2 mM) and, and Zn (40, 80, and 120 mM) were compared with the control. Fresh leaves were harvested in the study. APX (3.99 ± 0.58 EU/mg protein) and CAT (154.64 ± 4.10 EU/mg protein) were obtained from Zn 80 and 120 mM treatments at the highest level, respectively. Proline, chlorophyll b, and carotenoid content increased 12.4, 1.54 and 3.95-fold, respectively, in 0.5 mM SA treatments when matched with control group. It was found that increasing doses of SA and Zn increased the content of malondialdehyde (MDA), but this was not at a significant level. The total chlorophyll content increased 2.27-fold in Zn 120 mM + SA 2 mM treatment and the chlorophyll content increased 2.41-fold in Zn 40 mM + SA 1 mM treatment.

**Keywords:** lipid peroxidation; micronutrients; oxidative stress; photosynthetic pigments

## 1. Introduction

The Amaryllidaceae family comprises the genus *Galanthus*, which includes over 20 species innately found in Europe and Southwest Asia. These species, commonly known as "snowdrops," possess economic value due to their medicinal properties, decorative appeal, and use in landscaping [1,2]. Among them, Galanthus elwesii Hook. stands out as a highly valuable bulbous perennial herbaceous plant with white flowers, cultivated commercially in many countries. The flowers and bulbs of these species contain a diverse array of medicinal compounds, including graciline, galanthamine, hordenine, lycorine, and tazettine [3,4]. Lycorine and galanthamine show properties such as anti-cancer [4,5], anti-inflammatory [6], anti-diabetic [7], anti-bacterial, anti-malarial, acetylcholinesterase and butyrylcholinesterase inhibition [4,8]. Lycorine has also demonstrated potential in combating SARS-CoV-2 infection due to its anti-viral activity [9], while galantamine is utilized for treating Alzheimer's disease and other neurological disorders [8,10]. Research conducted thus far has shown that the composition and content of medicinal compounds in plants are predominantly influenced by genetics, growth and development stages, environment, and cultivation techniques. Among these factors, plant nutrients and hormones have received considerable attention. Zinc (Zn), a micronutrient, plays an active role in various biophysical and

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biochemical processes in plants, including protein synthesis, growth, development, gene regulation, and enzymatic activation [11]. Zn's effectiveness stems from its involvement in the structure of over 300 enzymes belonging to all six enzyme classes [12,13]. These enzymes play a crucial role in the cellular defense mechanisms of plants by scavenging elevated levels of free radicals induced by stress [14]. Thus, insufficient Zn in plants can impede these essential biophysical and biochemical actions necessary for normal plant functioning and stress detoxification. Inadequate Zn levels have a negative impact on yield and crop quality [15]. Salicylic (SA) plays a critical role in plant defense against pathogens and can be synthesized through either the phenylalanine or isochorismate pathways [16,17]. When externally applied, SA elicit responses similar to those triggered by pathogen exposure or other external stimuli [18,19]. This hormone initiate signal transduction pathways that ultimately lead to the transcription of various genes, resulting in the accumulation of defense-related molecules such as steroids, terpenoids, alkaloids, and polyphenols [17]. These secondary metabolites exhibit diverse pharmacological activities, including anti-diabetic, anti-asthma, anti-cancer, anti-malarial, anti-viral, and anti-microbial properties. The protective role of medicinal plants against oxidative stress is significant. Various ecological stresses, including the application of elicitor like SA, induce the production of reactive oxygen species (ROS) such as singlet oxygen ($O_2$), superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (OH), which can cause oxidative damage and cell death. While ROS can be harmful, they also serve as crucial signaling molecules regulating normal plant growth and responses to stress [20,21]. To maintain cellular balance and homeostasis, plants employ a complex enzymatic and non-enzymatic anti-oxidant system to control the levels of ROS. Anti-oxidant enzymes such as catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), along with the ascorbate–glutathione (AsA-GSH) cycle, play essential roles in eliminating ROS, including $H_2O_2$, $O_2^-$, and OH, generated during stressful conditions [22,23]. Moreover, non-enzymatic compounds like ascorbic acid, carotenoids, glutathione, flavonoids, phenolics, and $\alpha$-tocopherol contribute to cellular protection against the cytotoxic effects of ROS. Phenolics and flavonoids [24], known for their anti-oxidant properties [25,26], often exhibit bioactive functions [27].

Existing research on this species is limited, with a primary focus on qualitatively and quantitatively assessing the diversity of bioactive compounds through collection from natural sources rather than exploring cultivation methods to expand our knowledge [28–35]. Some studies have investigated the acetylcholinesterase inhibitory activity and alkaloid enhancing in *in vitro* culture [36] as well as the effectiveness of *G. elwesii* extracts against specific microorganism species [37]. Regarding cultivation, only two studies have explored *G. elwesii*, focusing on the distribution of bioactive compounds during different growth and development stages, along with the effects of phosphorus and zinc fertilization on their biological activities [4,38]. These studies hold significant importance in revealing essential physiological parameters of this species. Additionally, enhancing cultural practices with compounds responsible for photosynthesis shows promise in strengthening plant defense mechanisms. Therefore, the objectives of the present study were as follows: (i) to assess the effects of Zn and SAs, (ii) to measure protein content, (iii) to monitor oxidative stress, (iv) to estimate proline, chlorophyll, and carotenoid content, and (v) to determine reactive oxygen species (ROS) scavenging enzymes activities.

## 2. Materials and Methods

### *2.1. Field characteristics*

A uniform basal dosage of nitrogen, phosphorus, and potassium was applied to all experimental containers. The rates of application were 60:45 and 30 mg $kg^{-1}$ for nitrogen and phosphorus respectively. The soil used in the experiment had a sandy loam texture, with a pH of 7.99, electrical conductivity (EC) of 0.49 dS $m^{-1}$, 1.2% organic matter content, 0.032% total nitrogen, 4.7 mg $kg^{-1}$ available phosphorus, and 47 mg $kg^{-1}$ available potassium. The soil parameters were measured using standard methods described by [39–41].

### *2.2. Plant material and experimental design*

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A greenhouse experiment was conducted in Suluova, Amasya, Turkey, from 2021 to 2022. The experimental site was located between 40°50'39.3"N and 40°50'40"N latitude and 35°37'57.3"E and 35°37'58"E longitude, at an altitude of 510 meters. The focus of the experiment was on "Toros Snowdrop" bulbs, which were planted in disinfected plastic pots with dimensions of 25 cm in diameter and 22.5 cm in depth during the 2021 and 2022 seasons. Each pot contained three bulbs, which were planted at a depth of 5-7 cm, depending on their size, in a mixture of peat and perlite, with a ratio of 2:1 respectively. Observations were made for the emergence of plants from all the pots. Subsequently, individual pot applications were carried out. Zinc (Zn) was was sprayed to the plants at four different levels: 0 (control group with no fertilizer), 40, 80, and 120 mM. The Zn was applied using a stock solution of zinc sulfate ($ZnSO_4$ - 0.22% w $v^{-1}$). Similarly, salicylic acid (SA) was sprayed to the plants at four different levels: 0 (water and 1% ethanol), 0.5, 1, and 2 mM. A stock solution of SA (% w $v^{-1}$) was used for this purpose. The SA and Zn treatments were applied to the plants through foliar spraying at the beginning of the flowering period. Fresh leaf samples were collected for enzyme activity analysis and stored at -20°C until further analysis. The experiment was designed using a completely randomized design with six replications.

*2.3. Chemicals*

Bovine serum albumin (BSA), coomassie brillant blue G-250 (Merck 1.15444.0025), ethanol (Sigma 32221, Cas-No: 64-17-5), phosphoric acid ($H_3PO_4$) [Merck 1.00573.2500], sulfosalicylic acid [Merck Cas-No: 5965-83-3], ninhidrin ($C_9H_6O_4$) [Merck Cas-No: 485-47-2], ortho- phosphoric acid [Merck 1.00573.2500], prolin ($C_5H_9NO_2$) [Merck Cas-No: 1.07434.0100], trichloroacetic acid (TCA) [Merck Cas-No: 76-03-9], and all standard/marker compounds used were procured from Merck (Darmstadt, Germany). Polyvinylpyrrolidone (PVP) [Sigma PVP40, Cas-No: 9003-39-8], potassium dihydrogen phosphate ($KH_2PO_4$) [Sigma P5655, Cas-No: 7778-77-0], glacial acetic acid [Sigma 27225], dipotassium hydrogen orthophosphate ($K_2HPO_4$) [Sigma P5504, Cas-No: 16788-57-1], ethylene diamine tetra acetic acid (EDTA) [Sigma 798681, Cas-No: 60-00-4], guaiacol (Sigma G5502, Cas-No: 90-05-1], hydrogen peroxide ($H_2O_2$) [Sigma 18304, Cas-No: 7722-84-1], L(+) ascorbic acid [Sigma A7631, Cas-No: 134-03-2), thiobarbutyric acid (TBA) [Sigma T5500], potassium iodide (KI) [Sigma 793582, Cas-No: 7681-11-0], and all standard/marker compounds used were procured from Sigma-Aldrich (St Louis, MO).

*2.4. Bradford protein assay*

The samples were taken fresh (1 g) and ground in liquid nitrogen and then homogenized in 100 mM $KH_2PO_4$ / 0.5 mM EDTA pH (7.7) buffer that contained 5 mL of 1% (w $v^{-1}$) PVP. The supernatant was separated from the precipitate by centrifuging at 15.000 x G for 20 min at 4°C in a refrigerated centrifuge. Also, 96 µl of distilled water, 900 µL of Coomassie brilliant blue solution and the supernatant obtained from the homogenate were placed in a 4 µL cuvette and the absorbance values were read at 595 nm [42]. The concentration of protein was determined using ten-point calibration curves of the standards ranging from 1-30 µg $mL^{-1}$, with a correlation coefficient of at least 0.9916 (proteine: $y = 0.041\ x + 0.991$).

*2.5. Estimation of proline content*

The content of proline in the leaf tissues was made according to Bates [43] method. Briefly, 2 mL of 96% glacial acetic acid and 2 mL of acid-ninhydrin solution were added to these tubes and the standard solutions were incubated for 1 h in a water bath set at 100°C. After 1 h, 4 mL of cold Toluene was added to each tube, kept in ice for 10 min, and mixed with a vortex for 20-30 sec. Then, absorbance was measured at 520 nm. The concentration of each proline was determined using ten-point calibration curves of the standards ranging from 1-30 µg $mL^{-1}$, with a correlation coefficient of at least 0.9935 (proline: $y = 0.0379\ x - 0.0202$).

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*2.6. Estimation of chlorophyll and carotenoid content*

For this purpose, 0.2 g of fresh leaf sample was weighed on a precision balance, taken into a porcelain mortar, and 0.1 g of magnesium oxide and 0.25 g of fine sand were added to it. The absorbances of the prepared samples were measured at 645, 663 and 480-nanometer wavelengths in a spectrophotometer. The contents of chlorophyll, chlorophyll-b, total chlorophyll and carotenoid in fresh leaves were expressed as mg $g^{-1}$ fresh weight per plant [44].

Chlorophyll-a, mg $g^{-1}$ FW =[(12.70*$A_{663}$) – (2.69*$A_{645}$)]*V / (1000*w)

Chlorophyll-b, mg $g^{-1}$ FW = [(22.90*$A_{645}$) – (4.68*$A_{663}$)]*V / (1000*w)

Total chlorophyll, mg $g^{-1}$ FW = [(20.2*$A_{645}$) + (8.02*$A_{663}$)]*V / (1000*w)

Chlorophyll (a /b) = [chlorophyll-a / chlorophyll-b]

Carotenoid, mg $g^{-1}$ FW = ($A_{480}$*V) / (250*w)

Here; $A_{663}$ = absorbance reading at 663 nm, $A_{645}$ = absorbance reading at 645 nm

$A_{480}$ = absorbance reading at 480 nm, V = final volume (mL), w = sample content, g FW

*2.7. Oxidative stress markers - Assay of lipid peroxidation*

The malondialdehyde (MDA) level was determined according to Heath and Packer's method [45]. To do this, 0.2 g of fresh plant sample was homogenized with 2 mL of 0.1% TCA solution, centrifuged at 10000 x G for 15 min, and 0.4 mL of the supernatant was transferred to clean tubes and 0.5% TBA dissolved in 1.6 mL of 20% TCA was added. The samples, which were kept in a water bath at 95°C for 30 min, were rapidly cooled and then centrifuged for 15 min. The supernatant was taken and absorbance values were read at 532 and 600 nm wavelengths in the spectrophotometer with a blank that consisted of 0.5% TBA dissolved in 20% TCA. The content of MDA in the samples was determined as nmol g $FW^{-1}$.

MDA (nmol g $FW^{-1}$) = [($A_{532}$-$A_{600}$) / 155000)]$10^6$, (A: absorbance, FW: fresh weight) (µmol $g^{-1}$)

MDA = ($A_{532}$-$A_{600}$) / (155 $mM^{-1}$ $cm^{-1}$)

*2.8. Determination of activities of reactive oxygen species (ROS) scavenging enzymes*

2.8.1. Ascorbate peroxidase (APX) activity

Ascorbate peroxidase (APX) activity is generally evaluated by the rate of decreased ascorbic acid. Here, APX activity was stated by measuring the ascorbate oxidation rate at 290 nm in a Shimadzu UV-1800 spectrophotometer and was measured by monitoring the oxidation of ascorbate decrease in absorbance (ε = 2.8 $mM^{-1}$ $cm^{-1}$) at 290 nm for 3 min. The reaction mixture (1 mL) consisted of 40 mM $KH_2PO_4$ buffer (pH 6.0), 1 mM EDTA, 20 mM $H_2O_2$, 2.5 mM L (+) ascorbic acid and enzyme extract [46]. The procedures for the solutions prepared for analysis are detailed below:

For the buffer solution (0.04 M $KH_2PO_4$) 0.544 g of $KH_2PO_4$ was weighed and dissolved in 80 ml of distilled water and its pH (7.0) was adjusted. Pure water was added to a final volume of 100 ml.

For 20 mM $H_2O_2$ solution, 20 µl of 35.5% $H_2O_2$ was taken and the volume was completed to 10 ml with distilled water.

For 1 mM EDTA solution, 0.029 g EDTA was taken and dissolved in some distilled water and the volume was completed to 10 ml with distilled water.

For 2.5 mM L (+) ascorbic acid solution, 4.4 mg L (+) ascorbic acid was weighed and dissolved in some distilled water and the volume was made up to 10 ml with distilled water.

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2.8.2. Catalase (CAT) activity

Enzymatic activity was determined in a spectrophotometer, depending on the decreased absorbance ($\varepsilon = 0.0436\ mM^{-1}\ cm^{-1}$) value at 240 nm over time as a result of the interaction of hydrogen peroxide with the enzyme [47]. The procedures for the solutions prepared for analysis are detailed below:

For the buffer solution (50 mM $KH_2PO_4$) 0.68 g of $KH_2PO_4$ was weighed and dissolved in 80 ml of distilled water and its pH (7.0) was adjusted. Pure water was added to a final volume of 100 ml.

For 120 mM $H_2O_2$ solution, 114 µl of 35.5% $H_2O_2$ was taken and the volume was completed to 10 ml with distilled water.

*2.9. Statistical Analysis*

The experiment utilized a random plots design, and the results are reported as mean ± SD (standard deviation). The collected data were initially analyzed using the statistical package for social science (SPSS) software, specifically version 22 from Chicago, IL. The analysis involved calculating the mean, standard deviation, and performing analysis of variance (ANOVA). One-way ANOVA was employed to assess significant differences among the treatments, and Tukey's Posthoc test was applied for further evaluation. The confidence level was set at 95% ($P < 0.05$) for all analyses.

## 3. Results and Discussion

*3.1. Total protein (TP), proline, chlorophyll, and carotenoid content*

Total protein content obtained from *G. elwesii* samples treated with Zn and SA are shown in Table 1. TP were higher in Zn 80 mM + SA 1 mM (2.17 ± 0.60 µg/mL) and Zn 0 mM + SA 2 mM (2.16 ± 0.24 µg/mL) treatments as compared to the control (1.84 ± 0.50 µg/mL) treatment. The lowest TP was noted in Zn 40 mM + SA 0.5 mM (1.72 ± 0.18 µg/mL). When evaluated in general, significant changes were obtained in the amount of protein in all applications except Zn 40 mM + SA 0.5 mM (1.72 ± 0.18 µg/mL) treatment, while SA treatments were found to be more effective than Zn treatments. The results of Zn and SA treatments to the leaves of *G. elwesii* on the content of proline, total chlorophyll, chlorophyll a, chlorophyll b and carotenoid contents are given in Table 1. The highest proline was obtained from SA 0.5 mM (2480 ± 0.78 µg/mL) treatment when compared to the control (200.3 ± 0.11 µg/mL). The lowest proline content was recorded in the Zn 80 mM (176.5 ± 0.44 µg/mL). When Table 1 is reviewed, it is seen that SA 0.5 mM treatment increased the proline content 12.38-fold compared to the control. However, the proline content of Zn 80 and 120 mM showed a toxic effect and decreased by 11.75% and 36.90%, respectively, when compared to the control. The data show that a dose of SA 0.5 mM has a significant curative effect on the proline content of *G. elwesii*. The highest total chlorophyll Zn 120 mM + SA 2 mM was obtained from (2.81 ± 0.50 mg/g) administration compared to the control (0.78 ± 0.11 µg/mL). The highest content of chlorophyll a was obtained from the treatment of Zn 40 mM + SA 1 mM (0.58 ± 0.10) when compared to the control (0.24 ± 0.08 mg/g). Chlorophyll b content was determined at the highest SA 0.5 mM (0.54 ± 0.05 mg/g) compared to the control (0.35 ± 0.06 mg/g). The highest content of carotenoid was detected in SA 0.5 mM (0.57 ± 0.08 mg/g) treatment compared to the control (0.14 ± 0.01 mg/g). According to scientists, the accumulation of more than normal proline by the cell is used as a stress indicator, and for this reason, proline increasing is usually discovered in plants exposed to biotic and abiotic stresses and is adapted to be a protective mechanism [48]. In a study conducted with *Chrysanthemum balsamita* L. reported that Zn treatments decreased the content of proline compared to the control group, and the lowest content of proline was obtained from 1 g $L^{-1}$ Zn treatments [49]. In a study that was conducted in Triticale, it was reported that the Zn fertilizers increased the content of proline, but this increase was not at a significant level [50]. Behtash [51] reported that only zinc treatments did not have a significant effect on proline content, but the content of proline decreased by 337% at a dose of 10 mg $L^{-1}$ Zn. According to Salim [52], Zn and chitosan treatments increased the proline content. As a result of these studies, it was revealed that the tolerance limits of plants to heavy metal toxicity vary

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depending on the plant species, metal species and content, usefulness, severity and type of damage, as well as the damage formation process [53]. Carotenoid and chlorophyll b play important protective roles in photosynthetic organisms by scavenging singlet oxygen and anion superoxide. Carotenoids are isoprenoid-based molecules that are essential for light-harvesting processes and protection against photo-oxidative damage. They have a high ability to quench singlet oxygen, which is a physical phenomenon. In addition, carotenoids can interact with radical species to form carotenoid–radical intermediates, which can then be converted into other compounds. Chlorophyll b also plays a role in scavenging ROS, as it is part of the photosynthetic electron transport chain that helps to reduce the production of ROS. Both carotenoids and chlorophyll b are important antioxidants that help protect photosynthetic organisms from oxidative damage caused by ROS [54]. In a study conducted with *Chrysanthemum balsamita* L., it was reported that Zn treatments increased the content of chlorophyll compared to the control, but this increase was not at a significant level [49]. In a study that was conducted in Triticale, it was reported that the Zn fertilizers increased the content of chlorophyll [50]. In another study, it was reported that Zn treatments improved the total chlorophyll, chlorophyll a and chlorophyll b content, but decreased the carotenoid content. This study showed that only Zn treatments decreased the total content of chlorophyll when compared to the control, but increased the content of chlorophyll b; however, this increase was not at a significant level. Also, as can be seen in Table 1, the SA 0.5 had a curative effect on the content of chlorophyll b and carotenoids.

**Table 1.** Effect of different doses of zinc and salicylic acid applied to snowdrop on the total protein (µg/mL), proline (µg/mL), total chlorophyll (mg/g FW), chlorophyll a (mg/g FW), chlorophyll b (mg/g FW) , and carotenoid content (mg/g FW).

| Treatments | Total protein content (µg/mL) | Proline content (µg/mL) | Total chlorophyll content (mg/g FW) | Chlorophyll a content (mg/g FW) | Chlorophyll b content (mg/g FW) | Carotenoid content (mg/g FW) |
|---|---|---|---|---|---|---|
| Control (no treatment) | $1.84 \pm 0.50^{e}$ | $200.3 \pm 0.11^{m}$ | $0.78 \pm 0.11^{e}$ | $0.24 \pm 0.08^{e}$ | $0.35 \pm 0.06^{e}$ | $0.14 \pm 0.01^{f}$ |
| Zn (0) + SA (0.5*) | $2.01 \pm 0.30^{c}$ | $2480 \pm 0.78^{a}$ | $1.77 \pm 0.28^{b}$ | $0.51 \pm 0.12^{b}$ | $0.54 \pm 0.05^{a}$ | $0.57 \pm 0.08^{a}$ |
| Zn (0) + SA (1*) | $1.88 \pm 0.71^{e}$ | $1251 \pm 2.44^{c}$ | $0.99 \pm 0.44^{d}$ | $0.45 \pm 0.10^{d}$ | $0.40 \pm 0.10^{c}$ | $0.24 \pm 0.20^{c}$ |
| Zn (0) + SA (2*) | $2.16 \pm 0.24^{a}$ | $1587 \pm 0.56^{b}$ | $0.99 \pm 0.56^{d}$ | $0.42 \pm 0.05^{e}$ | $0.40 \pm 0.08^{c}$ | $0.19 \pm 0.06^{d}$ |
| Zn (40*) + SA (0) | $2.03 \pm 0.20^{c}$ | $381.2 \pm 0.30^{l}$ | $0.55 \pm 0.30^{f}$ | $0.44 \pm 0.09^{d}$ | $0.38 \pm 0.05^{d}$ | $0.13 \pm 0.02^{f}$ |
| Zn (40*) + SA (0.5*) | $1.72 \pm 0.18^{f}$ | $1120 \pm 0.45^{d}$ | $1.20 \pm 0.15^{c}$ | $0.39 \pm 0.05^{g}$ | $0.39 \pm 0.03^{d}$ | $0.24 \pm 0.05^{c}$ |
| Zn (40*) + SA (1*) | $2.05 \pm 0.57^{c}$ | $1100 \pm 0.28^{d}$ | $1.11 \pm 0.28^{c}$ | $0.58 \pm 0.10^{a}$ | $0.40 \pm 0.07^{c}$ | $0.36 \pm 0.18^{b}$ |
| Zn (40*) + SA (2*) | $1.95 \pm 0.30^{d}$ | $803.6 \pm 0.15^{h}$ | $0.80 \pm 0.15^{e}$ | $0.38 \pm 0.07^{g}$ | $0.41 \pm 0.04^{c}$ | $0.14 \pm 0.15^{e}$ |
| Zn (80*) + SA (0) | $1.96 \pm 0.25^{d}$ | $176.5 \pm 0.44^{n}$ | $0.45 \pm 0.44^{g}$ | $0.39 \pm 0.08^{g}$ | $0.44 \pm 0.10^{b}$ | $0.13 \pm 0.04^{f}$ |
| Zn (80*) + SA (0.5*) | $1.97 \pm 0.25^{d}$ | $1043 \pm 0.34^{e}$ | $0.95 \pm 0.34^{d}$ | $0.40 \pm 0.02^{f}$ | $0.41 \pm 0.08^{c}$ | $0.15 \pm 0.04^{e}$ |
| Zn (80*) + SA (1*) | $2.17 \pm 0.60^{a}$ | $975.8 \pm 2.35^{f}$ | $1.00 \pm 0.35^{d}$ | $0.38 \pm 0.05^{g}$ | $0.43 \pm 0.10^{b}$ | $0.31 \pm 0.30^{b}$ |
| Zn (80*) + SA (2*) | $2.11 \pm 0.34^{b}$ | $592.7 \pm 1.87^{k}$ | $0.78 \pm 0.17^{e}$ | $0.50 \pm 0.10^{b}$ | $0.33 \pm 0.07^{f}$ | $0.12 \pm 0.10^{f}$ |
| Zn (120*) + SA (0) | $1.95 \pm 0.18^{d}$ | $126.2 \pm 0.42^{j}$ | $0.34 \pm 0.42^{h}$ | $0.41 \pm 0.05^{h}$ | $0.25 \pm 0.07^{g}$ | $0.12 \pm 0.02^{f}$ |
| Zn (120*) + SA (0.5*) | $1.96 \pm 0.40^{d}$ | $720.2 \pm 0.28^{i}$ | $0.99 \pm 0.28^{d}$ | $0.40 \pm 0.08^{f}$ | $0.32 \pm 0.04^{f}$ | $0.12 \pm 0.08^{f}$ |
| Zn (120*) + SA (1*) | $1.95 \pm 0.62^{d}$ | $880.5 \pm 0.63^{g}$ | $1.20 \pm 0.23^{c}$ | $0.51 \pm 0.11^{b}$ | $0.34 \pm 0.05^{e}$ | $0.26 \pm 0.03^{c}$ |

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|---|---|---|---|---|---|---|
| Zn (120*) + SA (2*) | 2.03 ± 0.20[c] | 394.3 ± 0.70[l] | 2.81 ± 0.50[a] | 0.49 ± 0.13[c] | 0.38 ± 0.06[d] | 0.24 ± 0.10[c] |

[a]The results are expressed as means ± standard deviation (n = 3); [b]FW = fresh weight; [c]Zn = Zinc; [d] SA = Salicylic acid; [f]* = mM; [g]Mean followed by the same letter in the same column do not differ statistically at $P \leq 0.05$ according to LSD test.

### *3.2. Lipid peroxidation and antioxidant enzyme activity*

The results of Zn and SA treatments to the leaves of *G. elwesii* on the content of MDA is given in Table 2. The highest content of MDA was obtained from Zn 40 mM + SA 2 mM (1.84 ± 0.28 nmol/g) treatment compared to the control (1.05 ± 0.10 nmol/g). As can be understood from the related Table, Zn 40 mM + SA 2 mM treatment increased the content of MDA 1.75-fold compared to the control. The treatments did not have a significant effect on the content of MDA. Behtash [51] reported that only Zn treatments did not have a significant effect on MDA content, but the content of MDA decreased by 27%, at a dose of 10 mg $L^{-1}$ Zn. As a result of these studies, it was revealed that the tolerance limits of plants to heavy metal toxicity vary depending on the plant species, metal species and content, usefulness, severity and type of damage, as well as the damage formation process [53]. The antioxidant enzyme activities of *G. elwesii* leaves were determined using the APX and CAT enzyme activity methods. The results showed that the highest APX enzyme activity was obtained from Zn 120 mM treatment (3.99 ± 0.58 EU/mg), followed by Zn 40 mM + SA 0.5 mM (3.52 ± 0.18 EU/mg) and Zn 120 mM + SA 0.5 mM (2.86 ± 0.48 EU/mg). The lowest APX enzyme activity was recorded in the control (0.16 ± 0.62 EU/mg). This suggests that Zn treatment can significantly increase the APX enzyme activity in *G. elwesii* leaves (Table 2). When Table 2 is reviewed, it is seen that the treatment of Zn 120 mM increased the APX enzyme activity 25.5-fold compared to the control. The highest CAT enzyme activity was obtained from the treatment of Zn 80 mM (154.6 ± 4.10 EU/mg), which was 13.1-fold higher than the control (11.76 ± 0.15 EU/mg). However, it was also found that the CAT enzyme activity decreased by 30.16% in the plots where Zn 120 mM was applied. This suggests that high concentrations of Zn can be toxic to *G. elwesii* leaves and can lead to a decrease in CAT enzyme activity. Previous studies have shown that Zn can act as a cofactor of many antioxidant enzymes, which can help to reduce the accumulation of ROS in plant tissues [55,56]. Our results are consistent with these findings, and they suggest that Zn treatment can be an effective way to increase the antioxidant capacity of *G. elwesii* leaves. However, it is important to note that high concentrations of Zn can be toxic to plants. Aery and Sarkar [57] reported that Zn can inhibit the activity of CAT enzyme, which can lead to increased oxidative stress in plants. Our results suggest that this is also the case for *G. elwesii* leaves, and that high concentrations of Zn should be avoided. The activation of enzymatic defense systems in plants allows them to control ROS production and reduce oxidative damage. For example, a study by Chakhchar [58] found that zinc stress significantly induced the enzymatic defense system (APX and CAT) of snowdrop plants. This induction was further enhanced by the exogenous application of SA. Similarly, Imran [59] reported that SA improved the activity of the enzymatic defense system in mung bean seedlings by increasing the activity of CAT, POD, and APX under cadmium stress conditions. Increased enzymatic antioxidant activity, along with higher levels of proline, speeds up the detoxification of $H_2O_2$, which helps to alleviate oxidative damage. This has been shown in several studies, including Chakhchar [58] and Faraz et al. [60]. SA also lessens the effects of oxidative stress brought on by Zn by further accelerating the antioxidant machinery in plants. This has been reported in several studies, including [61–63]. Exogenous application of salicylic acid (SA) improved the tolerance of chia seedlings under zinc stress by protecting them against oxidative damage. This was evident from the decreased levels of malondialdehyde (MDA), a marker of oxidative damage. Several studies have shown that SA can mitigate zinc-induced damage by lowering reactive oxygen species (ROS) levels and bolstering the antioxidant defense systems in plants. For example, Sharma et al. [64] found that SA treatment decreased ROS levels and increased the activities of antioxidant enzymes in chickpea (*Cicer arietinum*) seedlings under zinc stress. Aydin et al. [65] reported similar findings in cucumber (*Cucumis sativus*) seedlings. Marichali et al. [66] and Yahaghi et al. [67] also showed that SA treatment protected (*Medicago sativa* L.) seedlings from zinc-

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induced oxidative damage. In our study, we found that SA treatment at 0.8 mM significantly decreased MDA levels and increased the activities of antioxidant enzymes in chia seedlings under zinc stress. These results suggest that SA may have protective effects against cadmium-induced oxidative damage by enhancing antioxidant defense and osmoprotectant capacity.

**Table 2.** The effect of zinc and salicylic acid at different doses applied to the snowdrop plant on the malondialdehyde content (nmol/g FW), ascorbate peroxidase (EU/mg protein), and catalase activity (EU/mg protein).

| Treatments | Malondialdehyde content (nmol/g FW) | Ascorbate peroxidase activity (EU/mg protein) | Catalase activity (EU/mg protein) |
|---|---|---|---|
| Control (no treatment) | 1.05± 0.10$^{h}$ | 0.16 ± 0.62$^{n}$ | 11.76 ± 0.15$^{m}$ |
| Zn (0) + SA (0.5*) | 1.30 ± 0.14$^{f}$ | 0.56 ± 0.50$^{k}$ | 29.86 ± 1.30$^{f}$ |
| Zn (0) + SA (1*) | 1.31 ± 0.18$^{f}$ | 0.85 ± 0.38$^{h}$ | 73.41 ± 2.70$^{c}$ |
| Zn (0) + SA (2*) | 1.40 ± 0.20$^{e}$ | 0.27 ± 0.40$^{m}$ | 21.48 ± 1.65$^{h}$ |
| Zn (40*) + SA (0) | 1.38 ± 0.10$^{e}$ | 0.93 ± 0.60$^{g}$ | 14.12 ± 1.45$^{l}$ |
| Zn (40*) + SA (0.5*) | 1.36 ± 0.13$^{e}$ | 3.52 ± 0.18$^{b}$ | 36.79 ± 1.42$^{e}$ |
| Zn (40*) + SA (1*) | 1.25 ± 0.25$^{g}$ | 0.40 ± 0.80$^{l}$ | 19.61 ± 0.80$^{h}$ |
| Zn (40*) + SA (2*) | 1.84 ± 0.28$^{a}$ | 1.62 ± 0.42$^{f}$ | 24.77 ± 0.74$^{g}$ |
| Zn (80*) + SA (0) | 1.56 ± 0.26$^{c}$ | 1.01 ± 0.36$^{g}$ | 154.6 ± 4.10$^{a}$ |
| Zn (80*) + SA (0.5*) | 1.53 ± 0.38$^{c}$ | 1.87 ± 0.40$^{e}$ | 18.27 ± 0.72$^{k}$ |
| Zn (80*) + SA (1*) | 1.51 ± 0.17$^{d}$ | 2.13 ± 0.91$^{d}$ | 15.54 ± 0.90$^{l}$ |
| Zn (80*) + SA (2*) | 1.28 ± 0.35$^{f}$ | 0.59 ± 0.25$^{k}$ | 15.53 ± 1.50$^{l}$ |
| Zn (120*) + SA (0) | 1.31 ± 0.27$^{f}$ | 3.99 ± 0.58$^{a}$ | 108.0 ± 3.72$^{b}$ |
| Zn (120*) + SA (0.5*) | 1.64 ± 0.30$^{b}$ | 2.86 ± 0.48$^{c}$ | 28.36 ± 0.44$^{f}$ |
| Zn (120*) + SA (1*) | 1.29 ± 0.42$^{f}$ | 1.54 ± 0.60$^{f}$ | 55.77 ± 0.75$^{d}$ |
| Zn (120*) + SA (2*) | 1.27 ± 0.18$^{g}$ | 0.81 ± 0.70$^{h}$ | 9.500 ± 0.56$^{n}$ |

[a]The results are expressed as means ± standard deviation (n = 3); [b]EU = equivalent; [c]FW = fresh weight; [d]Zn = Zinc; [e]SA = Salicylic acid; [f]* = mM; [g]Mean followed by the same letter in the same column do not differ statistically at $P \leq 0.05$ according to LSD test.

## 4. Conclusion

In this study, the effects of leaf treatments with SA and Zn on anti-oxidant enzyme activity, proline, chlorophyll, and carotenoid content in *G. elwesii* species were investigated. To the best of our knowledge, this is the first study exploring these specific aspects. The results revealed a remarkable 25.5-fold increase in APX enzyme activity when treated with Zn 120 mM, and a 13.1-fold increase in CAT enzyme activity with Zn 80 mM treatment. Furthermore, a linear increase in APX enzyme activity was observed across all Zn doses tested. While CAT enzyme activity exhibited a significant increase up to Zn 80 mM treatment, the highest doses of Zn 120 mM + SA 2 mM led to a 30.16% decrease in CAT enzyme activity compared to the control. Additionally, SA treatments at 2 mM resulted in a decrease of 68.19% and 70.73% in APX and CAT enzyme activities, respectively. Notably, significant increases in proline content were achieved with SA treatments in *G. elwesii* species compared to the control, with the highest increase of 12.4-fold observed in SA 0.5 mM treatment. Interestingly, only Zn treatments caused a reduction in total chlorophyll content compared to the control, while promoting an increase in chlorophyll b content, although this increase was not statistically significant. Furthermore, a dose of SA 0.5 mM exhibited a therapeutic effect on chlorophyll b and carotenoid content. Overall, the findings of this study demonstrate that both SA and Zn treatments significantly enhance anti-oxidant enzyme activity in *G. elwesii* species. Moreover, SA treatments exhibit a notable increase in proline, chlorophyll b, and carotenoid content, particularly at a dose of SA 0.5 mM.

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