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# EFFECTS OF ZINC-NANO OXIDE, SALICYLIC ACID, AND SODIUM NITROPRUSSIDE ON PHYSIOLOGICAL PROPERTIES, ANTIOXIDANT ENZYME ACTIVITIES, AND SECONDARY METABOLITES OF *Viola odorata* UNDER DROUGHT STRESS

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## ABSTRACT

One of the most important abiotic stresses and limiting factors (closing pores, lack of $CO_2$ entry, reduced photosynthesis, and reduced yield) of plant products around the world is water-deficit stress. This study aimed to examine the water deficit stress and foliar application with anti-stress compounds (ASC) on characteristics of *Viola odorata*. The study was carried out as a factorial experiment based on a randomized complete design. The factors consisted of water deficit and the foliar application of ASC at six levels [zinc-nano oxide (ZnO, 1000 and 1500 mg $l^{-1}$), salicylic acid (SA, 200 and 300 mg $l^{-1}$), and sodium nitroprusside (SNP, 200 and 300 μM)], and the control. The water deficit reduced the leaf water potential, cell membrane stability, and the shoot and root fresh weight but increased electrolyte leakage and soluble sugar accumulation. However, foliar applications, particularly SA and SNP, positively affected the measured parameters. The activities of superoxide dismutase and guaiacol peroxidase at all three field capacity levels were higher in the plants treated with SA and SNP than in the control and plants treated with ZnO. In sum, using 200 mg $l^{-1}$ of SA as a foliar application, in addition to improvement of the growth and developmental conditions of the aromatic violet plant, moderated the adverse effects of water deficit stress and increased the plant resistance to water deficit stress. Based on the results, the application of SA, SNP, and ZnO reduced electrolyte leakage and enhanced the plant's resistance to water deficit by increasing the compatible osmolyte accumulation and antioxidant enzyme activity.

**Key words:** compatible osmolytes, salicylic acid, Violaceae, zinc

## INTRODUCTION

*Viola odorata* from the Violaceae family is one of the most dominant ornamental plants native to cold regions and is popular for many reasons, including the ability to flower throughout the year and variety in color, leaf shape, and petals. It originated from humid and shady areas of Europe and Asia. *Viola odorata*, a perennial, herbaceous plant, has no distinct stems and may attain a height of 3 to 12 cm. Its stems are creeping surfaces that form roots at the end. This plant grows in wet and shady places such as plains, forests, or low slopes and even at high altitudes, especially under the shade of trees. This plant is very important

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due to its planting in green space and its medicinal effects. Used parts of *Viola odorata* are fragrant flowers, leaves, roots, seeds, and even all parts of this plant [Tavakoli Saberi and Sedaghat 2005]. There is limited information concerning the effect of drought stress on the quantitative and qualitative characteristics of *Viola odorata*. To achieve the medicinal benefits of this plant, it is indispensable to study the response of this plant to environmental stresses at large-scale and commercial production conditions outside its natural habitat.

The amount of plant available water is one of the climatic factors affecting plant yield and distribution. Water deficit stress is considered one of the most important abiotic stresses and a limiting factor for crop productivity around the globe. This stress has drastic adverse effects on plant growth and development, which leads to disruption of physiological processes, changes in carbohydrate metabolism, and alterations in protein structure and enzyme activity [Hajiboland and Amirazad 2010]. The application of plant growth regulators and proper methods of supplying mineral nutrition to plants can be an effective strategy to prevent the destructive effects of drought stress and to provide the basis for plant adaptation. Plant growth regulators are used to improve crop yields; hence, successful application of these substances to overcome adverse effects of stress on various plants has been reported [Metwally et al. 2003].

Drought stress results in the generation of reactive oxygen species (ROS), which have negative consequences at a cellular level: damage of membrane, DNA, lipids, and amino acids and limiting the activities of several enzymes [ElSayed et al. 2019, Maurino and Flügge 2008, Abedi and Pakniyat 2010]. Excessive ROS formation can cause oxidative stress, which damages plants by oxidizing photosynthetic pigments, membrane lipids, proteins, and nucleic acids [Abedi and Pakniyat 2010]. To keep the levels of active oxygen species under control, plants have non-enzymatic and enzymatic antioxidant systems to protect cells from oxidative damage [Mittler 2002]. To minimize these damaging effects, plants activate antioxidative guard systems involving both enzymatic and non-enzymatic antioxidants. The enzymatic antioxidant system that regulates ROS and redox homeostasis includes superoxide dismutase (SOD) [ElSayed et al. 2019; Alscher and Erturk 2002], glutathione peroxidases (GPXs), peroxiredoxins (Prxs), ascorbate peroxidase (APX), and catalase (CAT) [ElSayed et al. 2019].

Zinc-nano oxide (ZnO), salicylic acid (or ortho-hydroxy benzoic acid; SA), and sodium nitroprusside (SNP) belong to anti-stress compounds (ASC). The application of nano-fertilizers is a technique to increase plant resistance to environmental stresses. Zinc acts as a co-factor for antioxidant enzymes and increases the plant's resistance to drought stress through enhancing enzymatic activity [El Fouly et al. 2011]. Also, Zn plays a chief role in nitrogen metabolism and stimulates plant growth [Maurya and Kumar 2014]. It exerts a positive effect on protein formation and metabolism in plants, acting as a functional, structural, or regulatory co-factor for a large number of enzymes [Alloway and Alloway 2008, Nie et al. 2018]. Zinc is necessary for the activity of the enzyme RNA polymerase, which is involved in protein synthesis [Nie et al. 2018].

The role of zinc in cell division and protein synthesis has been known for a long time, but a new class of zinc-dependent protein molecules (zinc metalloproteins) has been identified in DNA replication and transcription, thus regulating gene expression [Vallee and Falchuk 1993, Coleman 1992]. Zinc is required for binding of specific genes with tetrahedral bonds that result in transcription. By this means, the polypeptide chain forms a loop of usually 11 to 13 amino acid residues, which bind the specific DNA sequences. Zinc is, therefore, directly involved in the translation step of gene expression of DNA elements in these DNA-binding metalloproteins.

Amino acids accumulate in zinc-deficient plants as protein content decreases [Tsui 1984]. Protein synthesis resumes when zinc is resupplied because zinc is a structural component of the ribosomes and responsible for their structural integrity. Ribosomes disintegrate in the absence of zinc, but reconstitution reoccurs with the resupply of zinc. According to Nie et al. [2018], whether the enhanced protein synthesis with Zn is related to the amino acid accumulation involved in N metabolism in plants requires further study.

Salicylic acid is an endogenous plant growth regulator that originated from natural phenolic compounds and is involved in the regulation of physiological and biochemical processes in case of biotic and abiotic stresses. It is considered a strategy to inhibit the destructive effects of environmental stresses and

to increase some growth parameters. The application of SA on "Sports Grass" has improved the quantity and quality of grass, increased vegetative growth and relative water content, and plant resistance to water deficit stress. Salicylic acid protects plants from oxidative damage by increasing the activity of antioxidant enzymes [Metwally et al. 2003].

Sodium nitroprusside is one of the compounds recently examined to reduce the effects of stresses on plants. This compound is involved in plant growth regulation, message transmission, and responses to biotic and abiotic stresses. Positive effects of SNP application, such as reduction of ion leakage, stimulation of auxin production, increase of growth, improvement of water relations, reduction of water loss, and reinforcement of antioxidation system, have been reported in plants under the water deficit stress [Gorgini Shabankareh and Khorasaninejad 2017]. Sodium nitroprusside is a nitric oxide (NO) donor. Nitric oxide is involved in stomatal closure induced by a phytohormone – abscisic acid (ABA). This hormone promotes NO synthesis in guard cells, while the application of NO-scavengers prevents ABA-induced stomatal closure [van Meeteren 2020].

This study aimed to investigate the effect of different levels of water deficit stress with foliar application of ASC (ZnO, SA, and SNP) on morphological characteristics, activity of antioxidant enzymes, and secondary metabolites in *Viola odorata.*

## MATERIAL AND METHODS

### The plant material and application of treatments

The *Viola odorata* seedlings were collected at the 2–4 leaf stage from Makidi Valley in Kaleybar, East Azerbaijan province (38°52'N, 47°02'E, 1144 m, from sea level), Iran on March 28, 2018. They were then planted in pots containing soil with a loamy sand texture at the research farm of the Agricultural and Natural Resources Research and Education Center of East Azerbaijan province. All agronomic and irrigation needs of the plant until the establishment in the cultivation bed were met. Two weeks later, the ASC was applied in two steps with a one-week interval. The drought stress was applied one week after the second step of the foliar application based on the substrate's field capacity (FC) using the weight method. The drought stress was continued until the end of the experiment (October 10, 2018), when the leaves turned yellow and flowering was terminated. The plants were six months old and were used for further experiments.

### Experimental design and treatments

In order to investigate the effect of ZnO, SA, and SNP on the drought tolerance of *V. odorata*, a factorial experiment was conducted with two factors based on a randomized complete block design with three replications. The first factor was assigned to the drought stress (irrigation to supply 85%, 65%, or 55% of FC), and the second factor was dedicated to the foliar application of ASC at six levels [SA (200 and 300 mg $l^{-1}$), ZnO (1000 and 1500 mg $l^{-1}$) and SNP (200 and 300 μM)] and the control. To do so, the control plants were sprayed with distilled water.

The experiment was performed outdoors with a total of 21 treatments, three replications, 63 plots, and four pots per plot and a total of 252 pots. Foliar application for all treatments at the rate of 50 ml for each pot was done in two stages at a distance of one week from each other, two weeks after the plants were placed in the culture medium, and 50 ml of distilled water was used for foliar application for control plants.

Deficit stress began one week after the second stage of FC-based foliar application by weight and continued until the leaves turned yellow, which coincided with the end of the experiment [Sadeghian et al. 2013]. The plants were in an open environment exposed to temperature and light changes. The conditions were the same for all plants, and the treatments and changes had the same effects and never interfered with the effects of the studied treatments and deficit stress was 55% of FC as severe stress in this experiment.

### Shoot and root fresh weight

At the end of the experiment, in each replication, one plant was uprooted and cleaned from the pots. Then, it was cut from the crown to determine the shoot and root fresh weight (FW) separately using a 0.001 g digital scale.

### Soluble sugars

To measure the soluble sugars, an alcoholic extract was prepared following Sanchez et al. [1998] method. So, 0.5 g of the fresh leaf tissue was extracted using 5 ml of 95% ethanol. Then, the superna-

tant of the solution was separated and centrifuged at 3500 rpm for 10 min. Next, 0.1 ml of the resulting alcoholic extract was mixed with 3 ml of newly prepared solution of 0.15 g of anthrone in 100 ml of 72% sulfuric acid and placed in a hot water bathroom for 10 min until a colorful substance appeared. After cooling the solution, its soluble sugars were measured at 665 nm with a Shimadzu UV-120-02 spectrophotometer and expressed as mg $g^{-1}$ FW.

### Cell membrane stability

To measure the stability of the cell membrane, 20 leaf disks were prepared from each experimental unit. Five samples of disks (the control) were in 10 ml of distilled water, and five other samples (drought treatments) were placed in 10 ml of 6000 PEG solution (40%). After 24 h, the solution was discarded, and 10 ml of distilled water was added to both groups of samples. Again, after 24 h, the electrical conductivity (EC) of samples immersed in water was determined using a Metrohm (644, Switzerland) EC meter (µS $cm^{-1}$). The samples were then placed in an autoclave for 15 min, and their EC was reread. The damage percentage caused by the water deficit stress was identified according to the relationship between damage percentage and cell membrane stability. In this regard, $C_1$ and $C_2$ were the EC of the control samples in the first and second readings, while $T_1$ and $T_2$ were the EC of PEG-treated samples in the first and second readings (µS $cm^{-1}$) [Blum and Ebercon 1981].

$$\text{Percentage of cell membrane damage} = \left(1 - \frac{1 - \left(\frac{T1}{T2}\right)}{1 - (C1 - C2)}\right) \times 100$$

### Electrolyte leakage

Lutts et al. [1996] methodology was used to measure the leaf electrolyte leakage (EL); healthy leaves were collected from each replication and placed in screw-cap test tubes containing 20 ml of distilled water. They were then shaken at 25°C for 24 h, and the (EC) of the solution was measured ($EC_1$). In the next step, the samples were autoclaved at 120°C for 20 min, and their EC was re-measured ($EC_2$). Finally, the following equation was used to find out the EL as a percentage:

$$\text{Electrolyte leakage} = \frac{EC_1}{EC_2} \times 100$$

### Leaf water potential

Leaf water potential (LWP) was measured between 12:00 and 14:00 (peak atmospheric temperature) on the stems of fully-developed leaves with a pressure chamber (Model 1900 Sky Instrument, England) [Shackel et al. 1997].

### Superoxide dismutase (SOD) enzyme

The SOD activity was determined using del Río et al. [2018] method. The reaction compound was made from 300 µl of 0.01 mM methionine, 100 µl of 1 mM nitroblue tetrazolium (NBT), 200 µl of 0.01 M EDTA, and 2350 µl of 50 mM potassium phosphate buffer. Then, 2.95 ml of the solution, along with 50 µl of 0.2 mM riboflavin and 50 µl of the enzymatic extract, were mixed in a test tube and exposed to radiation for 15 min. To determine SOD activity, the absorbance of the solution was read by a spectrophotometer (Unico-2150, China) at 560 nm at 23 ±2°C upon specific intervals with a spectrophotometer for 2 min. The activity was expressed as unit $mg^{-1}$ FW protein $min^{-1}$.

### Guaiacol peroxidase (GPX) enzyme

The reaction medium for measuring GPX activity was composed of 25 mM potassium phosphate buffer (pH = 8.6), 40 mM hydrogen peroxide, and 20 mM guaiacol. The reaction was triggered by adding 100 µl of the enzymatic extract to the final volume of 3 ml. The absorption increase was recorded by the formation of tetraguaiacol at 470 nm for three min. Then, the enzyme activity was expressed as the absorption variations as unit $mg^{-1}$ FW protein $min^{-1}$ [Dazy et al. 2008].

### Secondary metabolites

Essential oil of *Viola odorata* flowers or leaves was extracted by hydro-distillation. At first, 30 g of flowers or leaves were ground and then subjected to a Clevenger-type collector apparatus for 3 h. Then, essential oil was isolated following the method described in the Russian Pharmacopoeias. All experiments were

carried out in triplicate, and the obtained results were expressed based on the flowers' dry weight.

Shimadzu SCL-POA reversed-phase high-performance liquid chromatography (HPLC) system was used to assay the compositions of phenolic compounds. The applied system consisted of an LC-10ADVP pump equipped with an SPD-10AVP Diode Array (UV) detector. The column type was a Kintex 5u RP C18 µ, 4.6 mm internal diameter × 250 mm. The mobile phase was composed of (A) 0.05% formic acid (HCOOH) and (B) 0.05% formic acid-acetonitrile ($CH_3CN$) (50 : 50 v/v). Gradient elution was performed as follows: 0 min, 95 : 5; 10 min, 90 : 10; 40 min, 60 : 40; 55 min, 45 : 55; 60 min, 20 : 80; and 65 min, 0 : 100. The mobile phase was filtered under the vacuum through a 0.45 µm membrane filter before usage. The flow rate was 1.5 ml/min, and the UV absorbance was measured at 260–380 nm. The operating temperature was the room temperature. Identification of phenolic compounds was achieved by comparing retention times of standards, UV spectra, and calculation of UV absorbance ratios after the co-injection of samples and standards. To prepare the standard solution, 5 mg of coumaric acid, quercetin, and gallic acid were dissolved in 50 ml of methanol. All analyses were performed in triplicate [Pallag et al. 2016].

### Data analysis

Data were analyzed in the $SAS_{9.2}$ statistical software package, and the graphs were drawn in Excel. Also, the means of the data were compared using the least significant difference (LSD) test at the $P < 0.05$ probability level.

## RESULTS

### Morphological traits

Results showed that the interactive effect between water deficit stress and ASCs (ZnO, SA, and SNP) had a significant influence on shoot and root FW ($P < 0.01$) (Tab. 1). Shoot and root FW in the Viola odorata plant was reduced under the application of water deficit stress. Application of SA and SNP at three FC levels was more effective than ZnO in increasing shoot and root FW. The highest shoot and root FW were observed after the application of 200 mg $l^{-1}$ SA + 85% FC and 300 mg $l^{-1}$ SA + 65% FC, respectively. The lowest value was obtained in the control plants (Tab. 3).

### Physiological traits

The results showed that the interactive effect between the water deficit stress and ASCs (ZnO, SA,

**Table 1.** The analysis of variance for the effect of water deficit and ASC foliar application

| Source of variables | df | Mean squares (MS) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | SFW | RFW | SSU | CMS | EL | LWP | SOD | GPX |
| WD | 2 | 46.4** | 4.21** | 0.303ns | 42.8$^{ns}$ | 121* | 8157** | 0.107* | 0.0015* |
| ASC | 6 | 17.6** | 7.54** | 0.857** | 418** | 237.** | 8566** | 0.241** | 0.0027** |
| WD × ASC | 12 | 38.54** | 5.91** | 0.382* | 112* | 657** | 3980** | 0.183** | 0.0013** |
| Error | 40 | 3.1073454 | 0.679 | 0.1904762 | 676** | 42.85714 | 172.8373 | 0.0476191 | 0.0004762 |
| CV (%) | – | 8.13 | 13.6 | 20.7 | 42.8 | 24.1 | 7.26 | 23.4 | 17.7 |

*, **, $^{ns}$ significant at $P < 0.05$, $P < 0.01$ and insignificant, respectively
WD – water stress; ASC – anti-stress compounds; SFW – shoot fresh weight; RFW – root fresh weight; SSU – soluble sugars; CMS – cell membrane stability; LWP – leaf water potential; EL – electrolyte leakage; SOD – superoxide dismutase activity; GPX – guaiacol peroxidase activity; CV – coefficient of variation

**Table 2**. The analysis of variance for the effect of water deficit and ASC foliar application

| Source of variables | df | Mean squares (MS) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Butyl-2 ethyl hexyl phthalate | Phytol | Violin | Hexadecanoic acid | 3-Hexenyl acetate | Leaf oil | Flower oil |
| WD | 2 | 2.210$^{ns}$ | 1.90** | 2.210** | 45.7** | 10.9** | 0.049** | 0.366 ** |
| ASC | 6 | 4.28** | 0.895** | 4.28** | 4.16** | 0.762** | 0.0606** | 0.0087$^{ns}$ |
| WD × ASC | 12 | 1.92* | 0.289* | 1.92* | 3.51** | 1.89** | 0.0265** | 0.022** |
| Error | 40 | 0.86 | 0.14 | 0.866 | 0.23 | 0.18 | 0.008 | 0.005 |
| CV (%) | | 5.57 | 22.02 | 13.9 | 10.52 | 4.63 | 12.88 | 17.1 |

$^{*}$, $^{**}$, $^{ns}$ significant at $P < 0.05$, $P < 0.01$ and insignificant, respectively
WD – water stress; ASC – anti-stress compounds; CV – coefficient of variation

and SNP) had a significant impact on soluble sugar content ($P < 0.05$) and cell membrane stability, EL and LWP ($P < 0.01$) (Tab. 1). The amount of soluble sugar increased along with increasing the water deficit stress in all treatments (except 1000 mg $l^{-1}$ ZnO treatment at 85% FC), while 200 mg $l^{-1}$ SA treatment at 55% FC resulted in the highest soluble sugar content (Tab. 3). The results showed that along with increasing the water deficit stress, cell membrane stability decreased and EL increased. The outcomes also showed the positive effect of foliar application at three levels of FC on cell membrane stability and EL reduction compared to the control treatment. The highest stability of the cell membrane was obtained in the treatment of 300 mg $l^{-1}$ SA at 85% FC, while the most effective treatment in reducing EL was 200 mg $l^{-1}$ SA at 85% FC. The lowest cell membrane stability was obtained in the control treatment at 55% FC (Tab. 3). According to the findings, the LWP decreased with increasing the water deficit stress. The highest level of LWP was found in foliar spraying of the plants with SA at three FC levels. The highest and lowest values of LWP were obtained in the treatment of 200 mg $l^{-1}$ SA and the control treatment at 55% FC (Tab. 3).

### Superoxide dismutase (SOD) and guaiacol peroxidase (GPX)

The results demonstrated that the interactive effect of the water deficit stress and ASCs (ZnO, SA, and SNP) was significant on the activity of SOD and GPX enzymes ($P < 0.01$) (Tab. 1). The enzyme activity increased under the water deficit stress. The highest activity of SOD and GPX enzymes was found in the treatment of 200 mg $l^{-1}$ SA at 55% FC. The lowest activity of SOD and GPX enzymes was obtained in 1000 mg $l^{-1}$ ZnO treatment and the control treatment in 85% FC, respectively (Tab. 3).

### Secondary metabolites

The interactive effect of the water deficit stress and ASCs was significant on *Viola odorata* secondary metabolites such as butyl-2-ethylhexyl phthalate, phytol, violin ($P < 0.05$), hexadecanoic acid, 3-hexenyl acetate (Fig. 1), and the leaf oil and flower oil ($P < 0.01$) – Table 2. The content of secondary metabolites increased under the water deficit stress. The highest and lowest levels of the secondary metabolites were observed in the control treatment at 55% FC and the treatment of 200 mg $l^{-1}$ SA at 85% FC (Tab. 4). The highest and lowest percentages of hexadecanoic acid were obtained in the treatment of 1000 mg $l^{-1}$ ZnO at 55% FC and the treatment of 200 mg $l^{-1}$ SA at 85% FC (Tab. 4).

## DISCUSSION

### Morphological traits

Water deficit reduces turgor pressure, inhibits cell growth and development, disrupts photosynthesis and nutrient uptake, and finally, reduces the growth and weight of different parts of the plant [Albergaria et al. 2020]. In the present study, the application of water deficit stress by irrigation at a rate of 65% and 55% of FC reduced the plant weight; nonetheless, the foliar application of ASCs alleviated the loss of plant and root we-

**Table 3.** Means' comparison for the interactive effect of water stress × ASC foliar application

| Water deficit (%) | ASCs | Shoot fresh weight (g) | Root fresh weight (g) | Soluble sugars (mg $g^{-1}$ FW) | Cell membrane stability (%) | Electrolyte leakage (%) | Leaf water potential (Pa) | SOD (unit $mg^{-1}$ FW protein $min^{-1}$) | GPX (unit $mg^{-1}$ FW protein $min^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| 85% FC | Control | 19.9 f–i | 5.40 e–k | 1.59 fg | 57.5 ef | 38.80 bc | 133 jkl | 0.796 bc | 0.081 i |
| | 1000 mg $l^{-1}$ ZnO | 20.1 e–h | 5.24 f–k | 1.49 g | 77.9 ab | 28.60 def | 148 g–j | 0.704 c | 0.096 g–i |
| | 1500 mg $l^{-1}$ ZnO | 21.1 e–h | 5.93 d–h | 1.78 e–g | 71.9 b–d | 22.80 fg | 162 fgh | 0.804 bc | 0.109 e–i |
| | 200 mg $l^{-1}$ SA | 31.7 a | 7.94 bc | 2.53 a–d | 80.3 ab | 14.80 g | 200 de | 1.101 b | 0.156 ab |
| | 300 mg $l^{-1}$ SA | 25.0 b–d | 8.46 ab | 2.41 a–e | 83.7 a | 19.00 fg | 240 b | 0.974 bc | 0.134 a–f |
| | 200 µM SNP | 22.4 c–f | 6.70 c–e | 2.27 a–f | 83.1 a | 18.90 fg | 182 ef | 0.853 bc | 0.138 a–f |
| | 300 µM SNP | 22.9 c–e | 6.31 d–f | 2.03 b–g | 77.4 a–c | 21.00 fg | 178 ef | 0.826 bc | 0.119 c–h |
| 65% FC | Control | 17.7 i | 5.35 e–k | 1.64 fg | 54.3 f | 45.90 b | 127 jk | 0.763 bc | 0.083 hi |
| | 1000 mg $l^{-1}$ ZnO | 18.8 hi | 5.68 e–j | 1.69 fg | 66.3 de | 32.50 c–e | 147 h–j | 0.796 bc | 0.103 f–i |
| | 1500 mg $l^{-1}$ ZnO | 19.8 f–i | 6.07 d–g | 1.94 c–g | 67.0 c–e | 27.80 d–f | 167 f–h | 0.845 bc | 0.113 e–i |
| | 200 mg $l^{-1}$ SA | 26.8 b | 8.22 ab | 2.69 ab | 78.4 ab | 16.40 g | 215 cd | 1.053 bc | 0.153 a–c |
| | 300 mg $l^{-1}$ SA | 22.1 efg | 9.23 a | 2.55 a–c | 80.1 ab | 19.00 fg | 248 b | 0.983 bc | 0.136 a–f |
| | 200 µM SNP | 19.9 f–i | 7.17 b–d | 2.28 a–f | 80.9 ab | 19.90 fg | 177 f | 0.939 bc | 0.141 a–e |
| | 300 µM SNP | 22.2 d–g | 6.22 d–g | 2.19 a–g | 78.0 ab | 22.40 e–g | 180 ef | 0.872 bc | 0.129 b–g |
| 55% FC | Control | 17.7 i | 4.11 k | 1.61 f g | 33.4 g | 66.70 a | 123 k | 0.783 bc | 0.093 g–i |
| | 1000 mg $l^{-1}$ ZnO | 19.3 g–i | 4.37 jk | 1.73 e –g | 61.1 ef | 42.51 bc | 150 g–i | 0.802 bc | 0.105 e–i |
| | 1500 mg $l^{-1}$ ZnO | 20.0 f–i | 4.48 i–k | 1.82 d–g | 58.3 ef | 33.20 cd | 160 f–h | 0.828 bc | 0.117 c–i |
| | 200 mg $l^{-1}$ SA | 25.1 bc | 4.86 g–k | 2.84 a | 81.1 ab | 16.89 g | 297 a | 1.940 a | 0.168 a |
| | 300 mg $l^{-1}$ SA | 22.3 c–f | 5.84 d–i | 2.57 a–c | 81.9 ab | 18.10 fg | 230 bc | 1.033 bc | 0.139 a–f |
| | 200 µM SNP | 20.9 e–h | 4.87 g–k | 2.30 a–f | 78.3 ab | 21.96 fg | 170 fg | 0.926 bc | 0.149 a–d |
| | 300 µM SNP | 19.3 g–i | 4.64 h–k | 2.310 a–f | 72.2 b–d | 21.40 fg | 165 f–h | 0.905 bc | 0.127 b–g |

*In each column, the means with similar letters are not significantly different ($P < 0.05$) using the LSD test

**Table 4.** Means' comparison for the interactive effect of water stress × ASC foliar application

| Water deficit | ASCs | Butyl-2 ethylhexyl phthalate (%) | Phytol (%) | Violin (%) | Hexadecanoic acid (%) | 3-Hexenyl acetate (%) | Leaf oil (%) | Flower oil (%) |
|---|---|---|---|---|---|---|---|---|
| 85% FC | Control | 17.42 ab | 2.21 bc | 7.40 a–c | 7.36 ab | 10.50 ab | 0.60 d–f | 0.64 ab |
| | 1000 mg $l^{-1}$ ZnO | 16.70 a–e | 1.91 b–f | 7.41 ab | 5.14 d | 9.65 d–e | 0.741 b–d | 0.50 c–e |
| | 1500 mg $l^{-1}$ ZnO | 17.40 a–c | 1.78 c–g | 7.36 a–c | 4.91de | 9.30 d–g | 0.74 b–d | 0.48 c–e |
| | 200 mg $l^{-1}$ SA | 14.46 g | 1.10 h | 4.46 g | 2.48 i | 8.08 j | 0.44 g | 0.23 i |
| | 300 mg $l^{-1}$ SA | 15.86 c–g | 1.39 d–h | 5.45 e–g | 4.62 d–f | 8.21 ij | 0.87 ab | 0.32 g–i |
| | 200 µM SNP | 16.73 a–e | 1.54 d–h | 6.82 a–e | 3.11 hi | 8.40 h–j | 0.67 c–f | 0.35 f–h |
| | 300 µM SNP | 17.42 ab | 1.55 d–h | 6.09 b–f | 3.97 fg | 8.91 f–i | 0.74 b–d | 0.35 f–h |
| 65% FC | Control | 17.36 a–c | 2.45 ab | 7.42 ab | 6.61 bc | 10.19 bc | 0.87 ab | 0.60 a–c |
| | 1000 mg $l^{-1}$ ZnO | 17.40 a–c | 2.00 b–d | 7.42 ab | 6.55 c | 9.93 b–d | 0.74 b–d | 0.55 b–d |
| | 1500 mg $l^{-1}$ ZnO | 17.40 ab | 1.84 b–f | 7.40 ab | 5.41 d | 9.23 d–g | 0.74 b–d | 0.44 d–f |
| | 200 mg $l^{-1}$ SA | 15.746 d–g | 1.19 gh | 4.81f g | 2.82 hi | 7.95 j | 0.54 fg | 0.23 i |
| | 300 mg $l^{-1}$ SA | 14.81 fg | 1.28 f–h | 5.74 d–g | 3.08 hi | 8.22 ij | 0.62 d–f | 0.32 g–i |
| | 200 µM SNP | 16.42 a–e | 1.49 d–h | 7.06 a–d | 3.05 hi | 8.60 g–i | 0.67 c–f | 0.34 f–i |
| | 300 µM SNP | 17.06 a–d | 1.71 c–h | 6.42 a–e | 4.29 ef | 9.043 e–h | 0.74 b–d | 0.41 e–g |
| 55% FC | Control | 17.68 a | 2.87 a | 7.68 a | 6.82 bc | 11.10 a | 0.93 a | 0.69 a |
| | 1000 mg $l^{-1}$ ZnO | 17.41 ab | 2.33 b–c | 7.40 a–c | 7.68 a | 10.33 bc | 0.79 a–c | 0.54 b–d |
| | 1500 mg $l^{-1}$ ZnO | 17.40 ab | 1.98 b–e | 7.42 ab | 4.91 de | 9.66 b–d | 0.74 b–d | 0.43 d–g |
| | 200 mg $l^{-1}$ SA | 16.09 b–f | 1.190 gh | 5.86 c–g | 2.90 hi | 8.00 j | 0.57 e–g | 0.26 hi |
| | 300 mg $l^{-1}$ SA | 15.45 e–g | 1.35 e–h | 6.70 a–e | 3.10 hi | 8.43 h–j | 0.64 c–f | 0.32 g–i |
| | 200 µM SNP | 16.820 a–e | 1.55 d–h | 7.40 ab | 3.30 gh | 8.98 e–g | 0.70 c–e | 0.35 f–h |
| | 300 µM SNP | 17.42 ab | 1.79 c–g | 6.73 a–e | 4.88 de | 9.40 d–f | 0.74 b–d | 0.41 e–g |

*In each column, means with similar letters are not significantly different ($P < 0.05$) using the LSD test

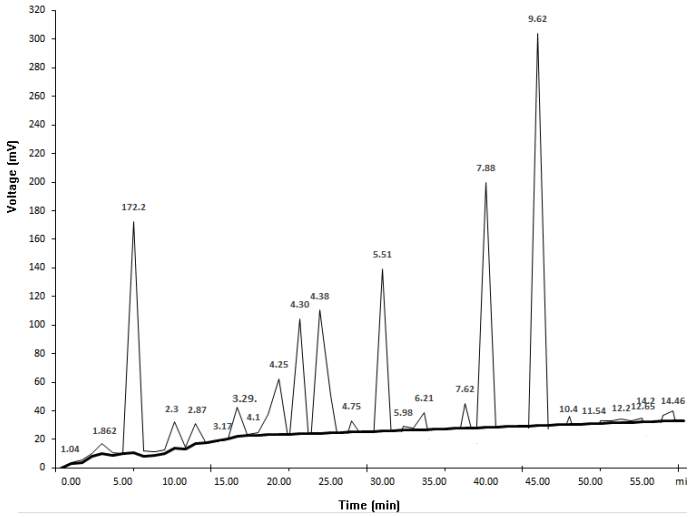


**Fig. 1.** The HPLC chromatogram of *Viola odorata* volatiles constitutes of flowers: butyl-2-ethylhexyl phthalate (17.261), phytol (9.13), violin (2.443), hexadecanoic acid (14.74), 3-hexenyl acetate (11.89)

ight due to the water deficit by improving plant tolerance. Among ASCs, SA levels were more effective than other treatments in improving plant growth under both water deficit and no water deficit conditions, which is consistent with the results of Damalas [2019]. Some researchers believe that SA increases plant growth by influencing the photosynthesis process and enzyme activity as well as accelerating the mobilization of assimilates from the source to the sink [Damalas 2019].

The SNP, as a nitric oxide-releasing compound, enhances the plant's tolerance to drought stress. It inhibits leaf water wastage under drought conditions by accelerating stomatal closure and contributes to maintaining and increasing the plant weight via preserving cell turgor [Mohasseli and Sadeghi 2018]. In the present study, the foliar application of SNP on the sweet violets increased the shoot FW compared to the control. It can be due to the impact of SNP on plant tolerance improvement against stress, increasing the photosynthesis rate and water use efficiency, and increasing photosynthetic availability to sustain vegetative growth and subsequently increase the FW [Zangani et al. 2018].

It was revealed that, along with increasing the water deficit, the leaf soluble sugar content increased in all treatments, except the SA and SNP treatments. Sugars are compatible with osmolytes in osmotic adjustment, and their increased accumulation in the leaf mesophyll cells under stressful conditions contributes to preserving cell turgor, proteins, and membrane integrity [Ashraf and Foolad 2007]. In a similar study, Bijanzadeh et al. [2019] concluded that SA application under stressful conditions increases soluble sugar content in the plants via influencing the polysaccharide -hydrolyzing enzymes, and the accumulation of these sugars increases the osmotic pressure and enhances the plant's potential to take up water and nutrients from the soil. In another similar study focused on the impact of SA on the cumin plants exposed to water deficit stress, it was revealed that exogenous application of 0.5 mM SA increases plant biomass by increasing the accumulation of chlorophyll and soluble sugars and photosynthesis rate [Rebey et al. 2012]. Soluble sugars are highly sensitive to environmental stresses, which act on the supply of carbohydrates from source organs to sink ones [Rosa et al. 2009]. Starch is emerging as a critical molecule in mediating plant responses to abiotic stresses, such as drought. Under stress conditions, plants generally remobilize starch to provide simple sugars (glucose and fructose) and energy at times when photosynthesis may be potentially restricted. The released sugar metabolites support plant growth under stress and function as osmoprotectants to mitigate the negative effect of the stress [Dien et al. 2019, Krasensky and Jonak 2012].

### Physiological traits

Salicylic acid, as an osmotic regulator, considerably affects most metabolic reactions of plants. It reduces damages caused by ROSs and increases the stability of cell membranes by increasing the activity of antioxidant enzymes and improving the amount of polyamines in plants [Acosta-Motos et al. 2017]. Previous research proved that SA increases the stability of cell membranes under water deficit. The stability of the membrane was induced by SA foliar application, which was better than other treatments because SA was able to reduce the destructive effects of water deficiency on the cell membrane. Salicylic acid stabilizes cell membranes by preventing lipid peroxidation and increases the plant tolerance to water deficit stress [Ibrahim et al. 2016]. Consistent with this, SA application was shown to enhance antioxidant enzyme activity in different plant species subjected to various abiotic stresses [Nahrjoo and Sedaghathoor 2018, Yusuf et al. 2008, Sabzmeydani et al. 2020].

The profound impacts of the water deficit are the inhibition of cell wall evolution, the reduction of cell membrane stability, and an increase in EL. The superoxide enzymatic decomposition by antioxidant enzymes and scavenging free radicals with sugars, proline, and anions under stressful conditions would hinder any damage to the membrane and preserve its structure [Xiong et al. 2012]. In our study, the treatments with higher soluble sugar content exhibited lower EL, reflecting the positive effect of the foliar application on sweet *Viola odorata* EL reduction. The membrane is the first part of the cell that is injured by the drought stress. Several researchers argue that the role of SA in preserving membrane integrity and reducing EL under drought stress is related to its impact on increasing the antioxidants and polyamines synthesis, H2O2 content decrease, and lipids peroxidation prevention [Fotouhi Ghazvini et al. 2011]. Furthermore, SNP inhibits the occurrence of membrane oxidative stress and damage by enhancing the antioxidant system potential of the plant and removing ROS [Farooq et al. 2009].

The LWP is an essential indicator for determining the plant water status; thus, it is used to detect the occurrence of drought stress. During the environmental stresses, RWC (relative water content), LWP, and osmotic potential would notably diminish. In general, plants reduce LWP by increasing the compatible osmolytes such as sugars and proline. Moreover, through osmotic regulation, plants permit more water to be absorbed from the soil [Ashraf and Foolad 2007]. In our study, the foliar application of SA, SNP, and ZnO increased LWP compared to the control treatment at all three FC levels. Thus, these applications have assisted in preserving the leaf water by modulating the effects of stress and osmotic regulation. Yadollahi et al. [2017] reported that the application of SNP improves plant water status by removing ROSs, which is in agreement with the results of the present study.

### Activity of antioxidant enzymes and secondary metabolites

One of the most critical physiological mechanisms of plant tolerance against stress and adverse environmental conditions is increasing the activity of antioxidant enzymes to eliminate or reduce ROS function [Gill and Tuteja 2017]. In sum, the destructive effects of stress begin with an increase in ROS levels. Superoxide dismutase forms the defensive first line against ROS-induced damages. Plant adaptation to environmental stresses is generally associated with an increase in SOD activity. In the current study, the foliar application increased SOD and GPX enzyme activity at all levels. The highest activity of both enzymes was observed in the treatment of 200 mg $l^{-1}$ SA at 55% FC (Tab. 1). The SA and SNP reduced the damages caused by the drought stress by increasing the plant's antioxidant capacity. In plants treated with anti-stress and growth-promoting compounds, increasing the accumulation of soluble sugars and proline leads to increased oxidative enzyme activity (GPX, SOD, and CAT) [Chavoushi et al. 2019].

In many cases, water restriction increases the content of secondary metabolites such as alkaloids and essential oils, and any deficiency that restricts growth more than photosynthesis increases the production of secondary metabolites [Gorgini Shabankareh and Fakheri 2015]. In some medicinal plants, like balm (*Melissa officinalis* L.), water deficit stress increases the effective ingredients and volatile oils [Aliabadi Farahani et al. 2009]. In general, medicinal plants produce essential oils under drought-stress conditions in order to prevent the cells from oxidation [Kazemi et al. 2017]. For example, an increase in the cumin essential oil percentage due to drought stress was reported [Pirzad et al. 2015].

When subjected to water deficit, plants initiate a cascade of signals that activate homeostatic factors.

These signals increase certain organic and inorganic compounds in the cytosol to modify the osmotic pressure and thus maintain turgor and water gradient at the cellular level [Okunlola et al. 2016]. Among known secondary metabolites affected by a water deficit are capsaicinoids, phenolic compounds, and flavonoids. Furthermore, at different phenological stages, the effects vary both between species and genotypes of the same species [Ruiz-Lau et al. 2011, Phimchan et al. 2012].

Another compound that is involved in the transmission of messages and response to biotic and abiotic stresses is SNP. In an experiment conducted on savory, the results showed that the highest percentage of essential oil (1.58%) was obtained from the combined treatment of 40% of field capacity and 100 mM SNP [Gorgini Shabankareh and Fakheri 2015]. According to the results of this experiment, El-Tohamy et al. [2009] reported that foliar application of onion with ZnO increased the essential oil percentage, which is consistent with the results obtained in this study where foliar application with ZnO increased the performance of secondary metabolites in the aromatic *Viola odorata* plant (Tab. 4).

## CONCLUSIONS

Deficit stress caused a decrease in shoot and root FW, decreased cell membrane stability and LWP, and increased soluble sugar, EL, and antioxidant enzyme activity in *Viola odorata*. However, foliar spraying with ASC, especially 200 mg $l^{-1}$ SA, moderated the deficit stress effects and improved the quantitative and qualitative traits of *V. odorata*. The alleviating effect of ASC and SA on water-stressed plants was associated with the improvement of physiological processes through increasing the plant's tolerance and maintaining water retention in plant tissues. Findings proved that ASC and SA can regulate *V. odorata* plant growth in water stress conditions. According to our results, with the intensification of drought stress, most of the secondary metabolites, including phytol, violin, and hexadecanoic acid, increase significantly in *Viol*a. Due to SA's low price and availability, it may be applied to plant cultivation in dry and waterlogged areas, making the plant resistant to water deficit stress.

## COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants as objects of research.

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