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Effect of Salicylic Acid Pretreatment and Humic Acid Soil Application on Physiological and Biochemical Traits of Saffron (*Crocus sativus* L.)

Running Title: Enhancing Saffron Traits with Salicylic and Humic Acid

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ABSTRACT

Studying compounds like Safranal is essential to understanding saffron's aroma, medicinal properties, and overall quality. This study aimed to evaluate the impact of salicylic acid (SA) and humic acid (HA) applications on the physiological and biochemical responses of saffron (*Crocus sativus* L.) under stress conditions, water deficit, and low soil organic matter, which are common challenges in Iranian agricultural soils. The experiment was conducted over two years (2018–2019) in Marvast, Yazd Province, using a split-plot design with three replications. Treatments included three levels of SA (0, 1, and 2 mM) and four levels of HA (0, 5, 10, and 15 kg ha^{-1}). The results demonstrated that both SA and HA significantly improved plant nutrition and stress tolerance, with combined applications being more effective than individual treatments. Notable increases were observed in key traits: 64% in relative water content, 83% in Proline, and 330% in catalase with 1 mM SA + 15 kg HA; 127% in chlorophyll with 2 mM SA + 10 kg HA; 49% in carotenoids, 77% in sugars, 220% in superoxide dismutase, 84% in Picrocrocin, and 100% in Safranal with 2 mM SA + 15 kg HA; 240% in ascorbate peroxidase and 500% in glutathione peroxidase with 1 mM SA + 10 kg HA; 370% in protein with 2 mM SA + 5 kg HA; 158% in polyphenol oxidase with 1 mM SA; and 45% in crocin with 2 mM SA + 15 kg HA. These findings highlight the synergistic role of SA and HA in enhancing saffron's resilience and productivity, offering promising strategies for saffron cultivation in arid and semi-arid regions of Iran.
Keywords: Humic acid, Saffron, Salicylic acid, Secondary compounds

INTRODUCTION

Studying bioactive compounds in saffron, such as Safranal, Picrocrocin, and crocin, is essential for understanding its medicinal properties, aroma, and overall quality. These compounds play a key role in saffron's therapeutic characteristics and commercial value, making their analysis critical for scientific research and industrial applications [1]. The principal organoleptic properties of saffron—color, aroma, and taste—are primarily determined by three bioactive constituents: Safranal as the volatile compound responsible for aroma, Picrocrocin as the bitter glycoside contributing to taste, and crocin as the carotenoid pigment conferring color [2]. In terms of treatment, Safranal and crocin in saffron have the same effect as fluoxetine and prevent the reabsorption of dopamine, norepinephrine, and serotonin, therefore having an antidepressant effect [3].

According to comprehensive analyses of saffron production in Iran, the main limiting factors are drought and climate change, particularly in arid and semi-arid regions. Despite saffron's inherent tolerance to water scarcity, reduced rainfall and rising temperatures significantly decrease yield. In these regions, saffron cultivation is of major importance in two aspects: first, its relative resilience to drought compared with other crops, and second, its crucial role in sustaining the livelihoods of local farmers [4]. From an economic perspective, saffron (*Crocus sativus* L.) cultivation offers substantial profitability for farmers due to its high market value and minimal input requirements. Agriculturally, saffron's growth cycle begins in the cooler months, which significantly reduces its irrigation demand. The plant's morphological traits—such as narrow leaves and underground corms—contribute to low transpiration rates and minimal nutrient needs. These characteristics make saffron particularly suitable for cultivation in arid and semi-arid regions, where water scarcity is a major constraint. Recent studies confirm the increasing interest among farmers in expanding saffron production in such climates, driven by both economic incentives and ecological compatibility [5].

Although saffron (*Crocus sativus* L.) is well-suited for integration into cropping systems of arid and semi-arid regions due to its low water and nutrient requirements, recent evidence highlights a concerning trend: climate change has significantly impacted its productivity. Rising temperatures, erratic precipitation patterns, and increased frequency of extreme weather events have led to a noticeable decline in saffron yield per unit area. These climatic stresses disrupt key physiological stages such as flowering induction and corm development, ultimately reducing both the quantity and quality of harvests. Studies conducted across diverse agro-climatic zones confirm that saffron cultivation is increasingly vulnerable to climate variability, necessitating adaptive management strategies to sustain its economic viability [6]. Simultaneously, implementing appropriate agricultural practices—such as optimized irrigation scheduling, balanced fertilization, and the application of organic compounds—can significantly enhance saffron (*Crocus sativus* L.) yield. These interventions improve soil fertility, stimulate corm development, and support the physiological processes essential for flowering and stigma production. Integrated nutrient management, particularly the use of organic and inorganic fertilizers in precise combinations, has been shown to increase both the quantitative and qualitative traits of saffron. Moreover, organic treatments contribute to improved soil structure and microbial activity, further supporting sustainable saffron cultivation in diverse agro-climatic conditions [7].

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Among nature-compatible organic fertilizers, humic acid. stands out for its multifaceted benefits to soil and plant health. humic acid enhances the biological, chemical, and physical properties of soil by improving nutrient availability, water retention, and microbial activity—all without causing environmental harm. Its hormonal-like compounds stimulate plant metabolism, leading to improved growth, flowering, and stress tolerance. In saffron (*Crocus sativus* L.), the application of humic acid. has shown significant positive effects on both qualitative and quantitative traits, including increased stigma yield, flower number, and corm development. These improvements make humic acid a valuable input for sustainable saffron cultivation, especially in resource-limited conditions [8]. In a study, Ahmadi and Aminifard (2017) showed that the use of humic acid increased the content of chlorophyll compared to the control, so that the highest content of chlorophyll (41410 mg/g fresh weight) was related to the treatment of 10 kg/ha of humic acid It seems that humic acid improves the growth of saffron by exerting direct effects (including the induction of hormonal responses, increasing the concentration of chlorophyll, and increasing the rate of respiration) and indirectly (influencing the biochemical structures of the soil) [9].

In addition to humic acid, salicylic acid—a phenolic compound—plays a pivotal role in regulating plant physiological processes. Its influence extends to enhancing chlorophyll and carotenoid content, modulating stomatal behavior, and activating key enzymes involved in stress response and metabolic pathways. The effects of salicylic acid are highly dependent on the plant species and its developmental stage, with optimal concentrations promoting growth, flowering, and the production of secondary metabolites [10]. In saffron (*Crocus sativus L.*), salicylic acid has been shown to stimulate crocin and phenolic exudation, improve antioxidant activity, and influence enzyme dynamics, thereby contributing to improved yield and quality under controlled conditions [11]. According to physiological and biochemical findings, several studies have investigated the effect of salicylic acid on the properties of saffron. For instance, the highest levels of crocin, Picrocrocin, and Safranal were obtained at a lower concentration of salicylic acid (1 mM), showing a statistically significant difference compared with the higher concentration (2 mM) [12]. Investigating the effects of salicylic acid (SA) and humic acid (HA) on saffron yield under arid and semi-arid conditions may enhance yield stability. While their individual effects on saffron traits have been studied, the combined impact on key characteristics remains unexplored. This study aimed to evaluate the synergistic effects of SA and HA on the physiological and biochemical traits of saffron.

MATERIAL AND METHODS

Plant Material and Experimental Design

The corms of high-quality ecotype Torbet Heydarieh were studied in the form of a split-plot experiment as a completely randomized block design with 3 replications for two years (2018 and 2019) in the research farm of the Payame Noor University, Marvast Branch, Yazd Province

Agricultural Treatments and Operations

In May 2018, the experimental field was prepared through plowing and leveling. Well-decomposed manure was then incorporated into the soil at a rate of 70 tons per hectare to enhance soil fertility, structure, and microbial activity. The field was subsequently harrowed to ensure uniform distribution and optimal soil conditions for saffron planting.

. After the land was leveled, Main plots measuring 3 × 8 meters (24 m²) and secondary plots measuring 2 × 3 meters (6 m²) were established. Each sub-plot measured 3 meters in length and contained six planting rows, with 25 cm between rows. The distance between blocks was 300 cm, and the distance between plots was 50 cm. Coarse tubers weighing 8–12 g were manually planted on November 7, 2018. Tubers were spaced 10 cm apart within rows, placed at a depth of 15 cm, resulting in a planting density of 50 tubers per square meter.

The experimental study involved two factors: salicylic acid at three levels (0, 1, and 2 mM) and humic acid. at four levels (0, 5, 10, and 15 kg/ha). humic acid was applied in the form of Humex commercial powder containing 80% humic acid during the first irrigation, which was carried out on November 1, 2018, when the mean temperature was approximately 2 °C, according to data from the Yazd Province Meteorological Organization. For salicylic acid treatment, the tubers were divided into three groups: two groups were pre-treated with 1 M and 2 M salicylic acid solutions, while the third group served as a control and was treated with water only. The tubers were pre-treated separately for 8–10 hours under dark conditions. The first irrigation coincided with planting, followed by four additional irrigation stages according to the crop's water requirements.

Measurement of Physiological and Biochemical Traits

Relative water content (RWC): Five leaf samples with an approximate area of 1 cm^2 were prepared, and their fresh weight was determined immediately. Next, the leaf pieces were floated in the petri dishes with lids in distilled water for 2-3 hours. After this period, the leaf pieces were removed from the distilled water, their surface was gently wiped with a dry paper towel, and their weight was determined quickly. After that, the samples were placed in an oven at 80°C for 24 hours, and their dry weight was recorded [13]. Based on the following formula, the relative water content was calculated, where WD is dry weight, WF is wet weight, and WT is leaf turgor weight.

$(\%)\ RWC = [(WF - WD) / (WT - WD)] \times 100$

Proline content was determined following Bates *et al.* (1973) (26) using spectrophotometry at 515 nm. Briefly, 0.3 g of leaf tissue was homogenized in 10 ml of 3% sulfosalicylic acid, and the extract was filtered through Whatman No. 2 paper. Two milliliters of the filtrate were reacted with 2 ml glacial acetic acid and 2 ml ninhydrin, incubated in a boiling water bath for 1 h, cooled, and extracted with 4 ml toluene. The upper red-colored phase was used for absorbance measurement.

Superoxide dismutase (SOD) activity was assayed according to Beyer and Fridovich (1987) (14) in a reaction mixture containing 0.025% Triton X-100, 100 mM phosphate buffer (pH 7.6), 12 mM methionine, and 75 μM nitroblue tetrazolium. Absorbance was read at 560 nm. Catalase (CAT) activity was measured as described by Torabi et al. (2016) (15) at 25°C in a mixture of 100 μl enzyme extract, 5 μl 3.41 M H2O2, and 3 ml of 50 mM potassium phosphate buffer (pH 7).

Ascorbate peroxidase (APX) activity was determined according to Ranieri *et al.* (2003) (16) at 290 nm, using 10 μl protein extract, 1 mM H2O2, 5 mM ascorbate, and 50 mM potassium phosphate buffer (pH 7.8).

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Glutathione peroxidase (GPX) activity was assayed following Caverzan *et al.* (2016) (17) at 25°C in a mixture containing 50 µl protein extract, 15 mM H_2O_2, 10 mM guaiacol, and 50 mM phosphate buffer (pH 7). Absorbance was recorded at 470 nm.
Polyphenol oxidase (PPO) activity was determined according to the method of Caverzan *et al.* (2016) (17).
Extraction of total protein and determination of antioxidant enzyme activity:
Total protein was extracted by mixing 0.25 g of leaf powder with 2.5 ml of extraction buffer (100 mM phosphate buffer, pH 7.6) and vortexing. Samples were centrifuged at 13,000 × g for 15 min at 4°C, and the resulting supernatant (the clear liquid above the sediment) was collected for enzyme assays.
. Protein concentration was determined according to the Bradford method [25].
Catalase (CAT) activity was measured as described by Torabi *et al*. (2016) [15] at 25°C. The reaction mixture consisted of 100 µL enzyme extract, 5 µL of 3.41 M H_2O_2, and 3 mL of 50 mM potassium phosphate buffer (pH 7).
Ascorbate peroxidase (APX) activity was determined following Ranieri *et al*. (2003) [16] at 290 nm, using 10 µl protein extract, 1 mM H_2O_2, 5 mM ascorbate, and 50 mM potassium phosphate buffer (pH 7.8).
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Polyphenol oxidase (PPO) activity was determined based on the descriptive method of Caverzan *et al.* (2016) [17].

Chlorophyll and Carotenoid Content

Leaf tissue (0.1 g) was homogenized in a porcelain mortar with 3 mL of 80% (v/v) acetone, and the resulting extract was brought to a final volume of 15 mL with the same solvent. Spectrophotometric measurements were performed using 80% acetone as the reference blank. Absorbance values were recorded at 645, 663, 480, and 510 nm, and the concentrations of chlorophyll a, chlorophyll b, and total carotenoids were calculated based on the spectrophotometric equations established by Arnon (1949) [19].

Soluble Sugars Content

Fresh leaf tissue (0.5 g) was homogenized and extracted with 10 mL of 96% (v/v) ethanol. The extracts were shaken at 360 rpm for 15 minutes and centrifuged at 3500 rpm for 15 minutes. Subsequently, 3 mL of anthrone reagent was added to the supernatant, and the solution was boiled for 10 minutes in a water bath. After cooling to room temperature, absorbance was measured at 630 nm. The soluble sugar content was quantified according to the anthrone method as described by Irigoyen et al. (1992).

Quantitative Analysis of Saffron Constituents

Saffron stigmas collected at the flowering stage were immediately transported to the laboratory and cryogenically ground into a fine powder using liquid nitrogen. The quantification of key saffron constituents was performed following the Iranian National Standard Method (28). Briefly, 500 mg of powdered stigmas was dissolved in distilled water to obtain a 1000 mL solution. The solution was stirred in the dark for 20 min using a magnetic stirrer to ensure complete homogenization. Absorbance measurements were recorded at 257 nm for Picrocrocin, 330 nm for Safranal, and 440 nm for crocin using an optical spectrophotometer. The concentrations of the compounds were subsequently calculated from the absorbance values. In the calculation, X represents the compound concentration, A denotes the absorbance at the specified wavelength, and M corresponds to the dry weight of the stigma (mg) (28).
$X = [(A * 100)/M] * [(100/100-H)]$

Data Analysis Method

Analysis of variance (ANOVA) was performed using SAS 9.4 statistical software, and the means were compared using Duncan's multiple range test at a 5% significance level.

RESULTS AND DISCUSSION

Analysis of variance revealed that the main effects of humic acid and salicylic acid were highly significant for most physiological and biochemical responses of saffron, including relative water content (RWC), *Proline* accumulation, photosynthetic pigment concentrations, soluble sugars, protein content, and antioxidant enzyme activities. Furthermore, a significant interaction between humic acid and salicylic acid was observed for the majority of traits, indicating a synergistic effect of their combined application in enhancing stress-related responses. In contrast, the three-way interaction of year × humic acid × salicylic acid did not exhibit statistical significance for key physiological parameters, such as RWC, Proline content, total chlorophyll (TC), chlorophyll a (TCa), and chlorophyll b (TCb). Consequently, the two-way interaction of humic acid × salicylic acid was used to interpret treatment effects and conduct mean comparisons for these variables.
Furthermore, humic acid. was found to enhance cell membrane permeability, stimulate antioxidant enzyme activity, and increase the synthesis of alpha-tocopherol, which plays a critical role in tolerance to biotic and abiotic stresses. It also improved nutrient uptake through the roots, thereby contributing to higher relative water content in saffron. salicylic acid, due to its phenolic nature, influenced chlorophyll and carotenoid levels, stomatal regulation, enzyme activity, and osmotic adjustment. Its application led to increased Proline accumulation through reduced incorporation into protein synthesis, decreased Proline oxidase activity, enhanced biosynthesis from glutamate, and protein degradation. Proline, as a compatible osmolyte, played a key role in osmotic regulation, maintaining cell turgor, and protecting molecular structures under stress conditions. Additionally, carotenoids, as tetraterpenoid pigments, act as antioxidants by scavenging reactive oxygen species, thereby protecting saffron plants against oxidative stress.
Overall, the combined use of humic acid and salicylic acid effectively improved saffron performance and quality under water-deficit conditions.

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Table 1 The results of analysis of variance of physiological and biochemical traits of saffron ecotype Torbat Heydarieh under humic acid and salicylic acid treatment

S.O. V	Df.	M.S. RWC	Pro	TC	TCa	TCb	Car	Sugar	Protein	CAT	APX	GPX	Saf	Pic
Y.	1	39.38^{ns}	14.29^{ns}	0.6^{ns}	0.002^{ns}	0.002^{ns}	0.116**	8.77**	0.42^{ns}	3.23*	1.65**	1.003**	87.59	9**
Y.×Rep	4	16.03^{ns}	32.74^{ns}	0.3^{ns}	0.003**	0.007^{ns}	0.002^{ns}	0.13**	0.004^{ns}	0.011^{ns}	0.013^{ns}	0.103^{ns}	61.11^{ns}	90.005^{ns}
HA	2	490.96**	225.2**	0.217**	0.2**	0.056**	0.108**	12.32**	6.66**	6.688**	6.41**	9.44**	1560.6**	97.53*
Y.× HA	3	250.82**	106.01**	0.124**	0.002^{ns}	0.007^{ns}	0.058**	0.32^{ns}	1.24^{ns}	0.77^{ns}	0.25^{ns}	0.797^{ns}	447.65**	876.64**
Error	8	5.61	14.03	0.005	0.009	0.003	0.003	0.19	0.006*	0.005	36526*	0.034**	29.3	67.39
SA	3	329.08**	457.2**	0.118**	0.059**	0.034**	0.029**	4.05**	1.69**	0.924**	3.48**	1.36**	393.29**	364.75**
Y.× SA.	3	17.87	192.51**	0.004^{ns}	0.0012^{ns}	0.003^{ns}	0.017**	1.82^{ns}	0.36**	0.849^{ns}	7.28**	0.63**	33.43	403.12**
HA × SA.	6	203.82**	150.45**	0.025**	0.035**	0.0043**	0.015**	2.85**	0.67**	0.419**	0.75**	1.67**	71.12**	186.74**
Y×HA.×SA.	6	170^{ns}	73.86^{ns}	0.016^{ns}	0.01**	0.013^{ns}	0.015**	0.58**	0.056**	0.258**	0.92**	0.517**	94.55**	109.58**
Error	36	30.96	16.98	0.004	0.003	0.0049	0.0036	0.22	0.004	0.019	0.049	0.074	26.26	38.54
C.V.	-	12.06	9.63	28.16	19.74	27.52	10.16	11.26	16.03	11.55	11.7	19.05	13.11	10.9

*and ** are significant at the probability level of five percent and one percent, respectively.

Abbreviation: Relative water content (RWC); Proline (Pro); Total Chlorophyll (TC); Chl a (TCa); Chl b (TCb); Carotenoid (Car); Sugar; Protein; Catalase (CAT); Ascorbate Peroxidase (APX); Glutathione Peroxidase (GPX); Safranal (Saf); Picrocrocin (Pic); Crocin (Cro).

Proline Content

The results of a two-year experiment showed that the Proline content increased significantly with the application of humic acid treatments in saffron. The results indicated that the application of 15 kg of humic acid enhanced Proline accumulation by 50%, 55.8%, and 33.27%, respectively, relative to the control.

The process of changes of Proline due to the use of salicylic acid in humic acid. levels were a quadratic process, with an increase in the level of salicylic acid, up to 1 mM, the amount of Proline increased, and then decreased with an increase in salicylic acid Based on these results, the highest average content of this osmolyte was obtained by applying the combined treatment of 1 mM salicylic acid and 15 kg/ha of humic acid., with an average of 61.795 mmol/g of fresh weight (Fig. 1).

Proline plays various roles in stress conditions, including maintaining protein and membrane stability, supporting subcellular structure and function, and scavenging ROS [20]. Proline is a key non-enzymatic antioxidant that accumulates in plants under abiotic stress, particularly drought, contributing to enhanced stress tolerance. In saffron (*Crocus sativus* L.), increased Proline levels were observed following the application of salicylic acid (SA) and humic acid. (HA) can be attributed to several physiological mechanisms: reduced incorporation of Proline into protein synthesis, inhibition of Proline oxidase activity, upregulated biosynthesis from glutamate, and proteolysis leading to elevated free amino acid pools. As a compatible osmolyte, Proline plays a vital role in osmotic adjustment, maintaining cell turgor, stabilizing proteins and membranes, and protecting cellular structures against dehydration and oxidative damage. These effects collectively enhance saffron's resilience under drought conditions and support its sustainable cultivation in arid conditions [20]. Under water-deficient conditions, the accumulation of compatible solutes such as Proline is a critical adaptive strategy employed by plants to regulate cellular osmotic pressure and preserve turgor. Proline functions as an osmoprotectant, enabling cells to retain water and maintain structural integrity under drought stress. Beyond its role in osmotic adjustment, elevated Proline levels contribute to the stabilization of proteins and cellular structures and play a key role in detoxifying reactive oxygen species (ROS). This dual functionality—osmotic regulation and oxidative stress mitigation—positions Proline as a central molecule in plant resilience. In saffron (*Crocus sativus* L.), increased Proline accumulation following the application of salicylic acid and humic acid. has been linked to enhanced drought tolerance, improved antioxidant capacity, and protection of cellular components under stress conditions [20].

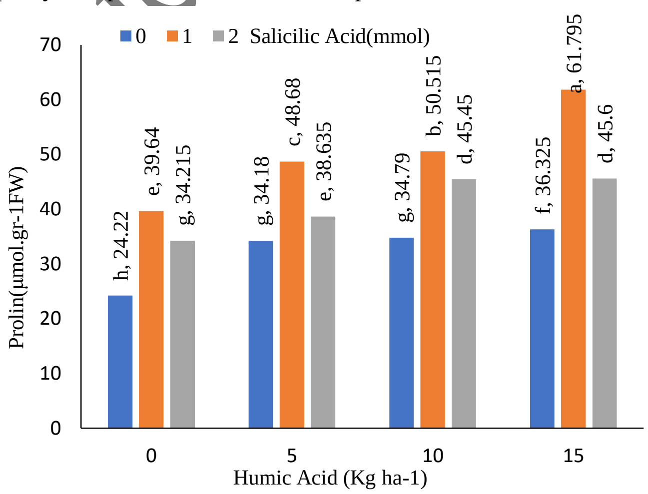

Fig. 1 The average content of Proline due to the interaction of Salicylic and humic acid.
Means with at least one common letter do not have statistically significant differences (Duncan 5%)

Relative Water Content (RWC)

In each of the levels of salicylic acid, the relative content of saffron leaf juice increased with the increase in the level of humic acid. At each level of humic acid, the highest relative content of leaf water was obtained by using one mMol of salicylic acid. As the level of

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salicylic acid increased, the relative content of leaf water decreased. The highest percentage of leaf water content was obtained by using 15 kg/ha of humic acid along with 1 mMol of salicylic acid (Fig. 2).

The higher relative water content may be due to the acceleration of the response to environmental conditions and the closure of the stomata due to the application of salicylic acid [13]. Humic acid enhances saffron growth through both direct and indirect mechanisms. Indirectly, it improves soil biochemical properties, which can positively influence nutrient availability and root activity. Direct effects primarily include the stimulation of vegetative growth and enhancement of yield-related traits. Although some studies suggest that humic acid may also modulate physiological processes such as chlorophyll content and metabolic activity, these effects require further species-specific investigation [9]. In addition to its role in improving soil structure and nutrient availability, humic acid. exerts several biochemical and physiological effects that enhance plant resilience. humic acid. increases cell membrane permeability, facilitating more efficient nutrient uptake and signaling. It also stimulates the activity of antioxidant enzymes such as superoxide dismutase and catalase, which are crucial for mitigating oxidative stress. Notably, humic acid. promotes the biosynthesis of alpha-tocopherol (vitamin E), a lipid-soluble antioxidant that stabilizes cellular membranes and contributes to tolerance against these synergistic effects. Positioning humic acid as a valuable biostimulant in sustainable saffron production [27].

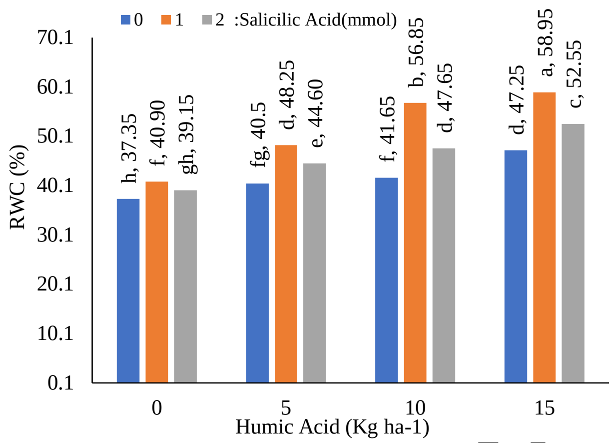

Fig. 2 The average of RWC due to the interaction of Salicylic and humic acid.
Means with at least one common letter do not have statistically significant differences (Duncan 5%)

The highest total chlorophyll content was observed with the application of 2 mM salicylic acid and 15 kg/ha humic acid, representing an increase of nearly threefold compared to the control. This enhancement was also reflected in both chlorophyll a and chlorophyll b, indicating a general improvement in leaf pigment accumulation. The highest amount of chlorophylls a and b was obtained from the combined treatment of 15 kg of humic acid. and 2 mmol of salicylic acid. The use of 10 kg of humic acid. and the same amount of salicylic acid did not have a statistically significant difference with this treatment. In addition to the positive effect of these two compounds on the content of leaf chlorophylls, our findings indicated the significant effect of the separate treatment of humic acid. and salicylic acid and their mutual effect on the content of carotenoids in saffron, so that the maximum content of carotenoids is obtained with the simultaneous application of 2 ml of Molar salicylic acid and 15 kg/ha of humic acid. were obtained, which was associated with an increase of about 2.5 times compared to the control without both agents (Fig. 3).

Similar to our findings, Ansariyan *et al.* (2019) suggested that salicylic acid inhibits the degradation of chlorophyll oxidase, similar to inhibiting the activity of chlorophyll oxidase enzymes, thereby increasing chlorophyll and total photosynthesis [12]. salicylic acid prevents the destruction of chlorophyll molecules by preventing the activity of ACC oxidase enzyme, controlling the function of Abscisic acid, and preventing the production of Ethylene hormone, and thus can increase the content of chlorophyll [15]. In agreement with what was observed in this study, Ahmadi and Amini Fard (2017) showed that the consumption of humic acid. increased the content of chlorophyll compared to the control, so that the highest amount of chlorophyll (41410 mg/g fresh weight) was related to treatment 15 kg/ha was humic acid. They stated that organic fertilizers, such as humic acid. increase the availability of nutrients, especially nitrogen (an important compound for photosynthetic cells), and increase chlorophyll [9]. Also, meeting nutritional needs increases soil microscopic organisms and, as a result, increases the absorption of trace elements such as Manganese, Iron, and Magnesium, which play a role in chlorophyll synthesis [22].

Carotenoids are tetraterpenoid organic pigments synthesized in the plastids of plants and other photosynthetic organisms, where they play essential roles in light harvesting and photoprotection. Located primarily in chloroplasts and chromoplasts, carotenoids contribute to the structural integrity of photosystems and serve as precursors for key phytohormones such as abscisic acid and strigolactones. One of their most critical functions is their antioxidant activity: carotenoids effectively scavenge reactive oxygen species (ROS), thereby mitigating oxidative damage to cellular components under stress conditions. This protective role is especially vital during environmental challenges such as high light intensity, drought, and temperature extremes, making carotenoids indispensable for plant survival and stress adaptation [29, 30]. In line with our observations, Ansariyan *et al*. (2020) showed that the use of salicylic acid increases the content of carotenoids. The concentration of 1 mM compared to 2 mM of salicylic acid had a greater effect on the content of carotenoids, so that in the concentration of 1 mM of salicylic acid, the content of carotenoids reached 1.2 mg/g of the fresh weight of the sample [13]. By increasing the antioxidant capacity of non-enzymatic agents such as Carotenoids, salicylic acid causes a decrease in lipid peroxidation and the amount of Hydrogen Peroxide, more protection of cell membranes and photosynthetic organelles, and pigments [23]. Although the content of

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carotenoids after humic acid. treatment was associated with a slight increase in our study, Ahmadi and Aminifard (2017) showed that the consumption of humic acid. did not change the content of carotenoids compared to the control [28].

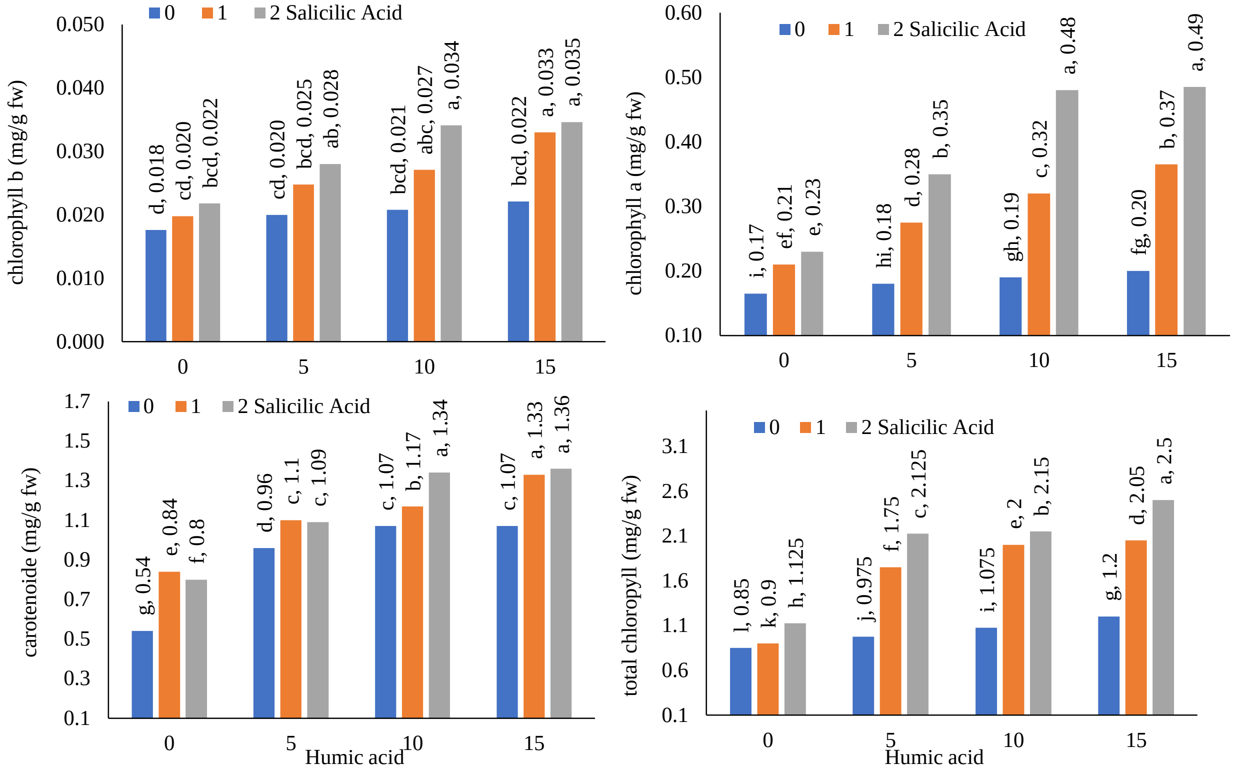

Fig. 3 The average of Total Chlorophyll (TC), Chl a (TCa), Chl b (TCb), Carotenoid (Car), due to the interaction of Salicylic and humic acid. Means with at least one common letter do not have statistically significant differences (Duncan 5%)

Soluble Sugars

The sugar content increased with the application of separate treatments of salicylic acid and humic acid. This increase was more significant in their simultaneous treatment. For the treatment of 2 mM salicylic acid and 15 kg/ha of humic acid, the sugar content increased up to 90% in the first year and about 71% in the second year (Table 2). The results indicated that the soluble sugar content was higher in the first year compared to the second year of the experiment, although this difference was not statistically significant in the control treatment without salicylic acid. The results also showed that increasing the level of humic acid. in each of the levels of salicylic acid, there was no cause for a statistically significant increase in the soluble sugar content in both years. The highest soluble sugar content was obtained from the treatment with the maximum Salicylic and humic acid. There was no statistically significant difference in the soluble sugar content measured in these two treatments in the first and second years of the experiment. Soluble sugars play a crucial role in various physiological and biochemical processes. Via enhanced intracellular accumulation, they prevent cell damage in adverse environmental conditions and protect proteins and plasma membranes from damage. Similar to our findings, Ansariyan *et al.* (2020) demonstrated that the use of salicylic acid increases Carbohydrates, with higher salicylic acid concentrations leading to a higher increase in this ratio. For example, at a salicylic acid concentration of 2 mM, the carbohydrate content reached 5.2 mg/g of sample fresh weight. The increase in sugar content may be attributed to the Proline and glycine betaine synthesized when saffron is exposed to unfavorable conditions in its dry and semi-arid cultivation areas, as compatible osmolytes, resulting in an improvement in water relations. In line with this, our findings showed that the changes in Proline level are proportional to the content of sugars [13].

Soluble Proteins

The average total protein amount in the second year at each humic acid and salicylic acid level was lower than in the first year. The total protein content in saffron significantly increased following the application of humic acid and salicylic acid treatments. The highest total protein content, averaging 0.258 mg/g of dry weight, was recorded from the interaction of the highest levels of salicylic acid and humic acid. with the treatment of 2 mM salicylic acid and 1 kg/ha of humic acid. in the first year (Table 2).

Researchers studying plant responses to adverse environmental conditions have found that cellular and metabolic processes change under these conditions. These changes require the accumulation of soluble and apoplastic proteins, and their synthesis changes depending on temperature. Examples of these changes include the accumulation of proteins involved in carbohydrate metabolism, increased activity of antioxidant enzymes such as catalase, and the synthesis of heat shock proteins in response to adverse environmental conditions. Therefore, investigating changes in total protein content in different treatments and genotypes of plants is crucial for researching plant tolerance to adverse environmental conditions.

Our findings are consistent with those of Ansariyan et al. (2020), who showed that the use of salicylic acid increases the content of soluble proteins. Additionally, our research revealed the positive effect of humic acid on the content of soluble proteins. Interestingly, the results showed that the simultaneous use of salicylic acid and humic acid. could yield more protein in saffron tissues. Previous studies have shown that the reduction of leaf-soluble protein during stress acts as a limiting factor for photosynthesis, leading to decreased plant performance.

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Therefore, it can be concluded that the treatment of humic acid. and salicylic acid preserves or increases the soluble proteins, which is generally an important challenge in agricultural areas, especially in arid and semi-arid regions [13].

Table 2 The average content of sugar and soluble proteins of saffron leaves under the influence of the interaction of Salicylic and humic acid. in the studied years.

S.A (m.M)	humic acid (Kg ha-1)	Soluble Sugar (Mg. g^{-1} FW)				Soluble protein (Mg. g^{-1} FW)			
		2018		2019		2018		2019	
0	0	3.27	hij	2.85	j	0.028	k	0.05	ij
	5	3.37	hij	3.1	ij	0.046	jk	0.054	hij
	10	3.49	hij	3.13	ij	0.053	hij	0.062	ghij
	15	3.59	g-j	3.25	hij	0.054	hij	0.071	fgh
1	0	3.75	e-i	3.32	hij	0.066	ghi	0.074	fg
	5	4.28	efg	3.42	hij	0.086	f	0.086	f
	10	4.36	efg	3.66	fghi	0.12	e	0.087	f
	15	4.49	de	3.98	e-h	0.125	e	0.125	e
2	0	5.38	bc	4.2	e-h	0.146	cd	0.138	de
	5	5.8	abc	4.37	ef	0.192	b	0.157	c
	10	6.35	ab	5.15	cd	0.242	a	0.179	b
	15	6.8	a	5.58	abc	0.258	a	0.192	b

Means with at least one common letter do not have statistically significant differences (Duncan 5%)

Antioxidant Enzymes

Our research demonstrated that the activity of Catalase, Ascorbic peroxidase, and Glutathione peroxidase increased significantly when a combined treatment was applied to saffron, compared to when single treatments were used. The treatments that resulted in the highest activity values of antioxidant enzymes were as follows: a 2.8- and 5.2-fold increase in catalase activity with the treatment of 2 mM salicylic acid and 15 kg/ha of humic acid., compared to the control treatment without Humic and salicylic acid; 4.36 and 3.9 times increase in APX activity with the treatment of 2 mM salicylic acid and 15 kg/ha of humic acid., compared to the control without humic acid. and salicylic acid in the first and second years, respectively; and a 1.68 and 1.45% increase in GPX activity with the treatment of 2 mM salicylic acid and 15 kg/ha of humic acid. compared to the control without humic acid. and salicylic acid in the first and second years, respectively (refer to Table 4 for details). These results are consistent with previous studies. Aslezaeem *et al.* (2018) demonstrated that the highest activity of catalase and GPX enzymes was achieved at a high concentration of salicylic acid (1 mM) [25]. Similarly, Torabi *et al*. (2016) found that the activity of catalase, APX, and GPX enzymes decreased at higher concentrations of salicylic acid (1 mM), but increased at lower concentrations (0.5 mM) [15]. Furthermore, a recent study identified the synergistic effect of salicylic acid and humic acid. on the activity of these enzymes for the first time. The study revealed that the pattern of changes in catalase, APX, and GPX activity under the influence of the examined treatments was the same, confirming the coordination of cell components in response to the plant environment.

Table 3 The average activity level of some saffron antioxidant enzymes under the influence of Salicylic and humic acid. interaction in the years under review

S.A m.M	humic acid Kg ha-1)	Cat				Apx				Gpx			
		Unit mg-1protein											
		2018		2019		2018		2019		2018		2019	
0	0	0.56	j	0.50	jk	0.80	k	0.91	hijk	1.16	i	1.37	hi
	5	0.58	ij	0.59	g-j	0.88	k	1.32	jk	1.18	ghi	1.43	gh
	10	0.66	f-j	0.75	ghij	0.97	ijk	1.37	ghi	1.24	fghi	1.44	fghi
	15	0.77	e-j	0.76	e-i	1.25	hijk	1.61	gh	1.35	fgh	1.44	fg
1	0	0.78	e-i	0.81	d-g	1.48	ghij	1.71	efg	1.50	ef	1.47	f
	5	0.80	e-i	0.94	defg	1.52	fg	1.74	def	1.52	ef	1.49	cde
	10	0.81	e-h	0.94	de	1.76	fg	1.90	de	1.73	def	1.49	cde
	15	0.81	def	1.60	b	1.83	def	1.93	cde	1.79	bc	1.62	cde
2	0	0.99	cd	1.76	b	2.40	cd	2.73	b	1.84	b	1.64	cde
	5	1.17	c	2.44	a	2.73	c	2.98	b	1.86	a	1.78	cde
	10	1.23	b	2.60	a	3.43	a	3.43	a	1.93	a	1.88	cd
	15	1.61	b	2.64	a	3.49	a	3.63	a	1.95	a	1.98	a

Means with at least one common letter do not have statistically significant differences (Duncan 5%)

Secondary Metabolites

Based on the comparison of means, the highest Safranal content was obtained with the simultaneous application of 2 mM salicylic acid and 15 kg/ha humic acid in both experimental years (2018 and 2019), showing an increase of approximately 93% and 95%, respectively, compared to the control (Fig. 4). Similarly, the maximum Picrocrocin content under these treatments increased by approximately 50% in 2018 and 100% in 2019 relative to the control (Fig. 4).

In justifying the increase of Picrocrocin and Safranal in saffron with salicylic acid and humic acid. treatments, it can be noted that these treatments lead to corms with more weight, which in turn expands the area of root activity in absorbing nutrients from the soil. And the development of saffron growth will be effective. At the beginning of autumn and before the appearance of the leaves, the growth and development of saffron plants largely depend on the reserves stored in the corms. Larger corms, containing higher nutrient reserves and greater energy potential, support enhanced development of both root and aerial organs [22]. Therefore, according to this issue, one of the

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reasons for the increase in these compounds due to the treatment of humic acid. and salicylic acid can be attributed to the increase in photosynthesis, the production of more compounds resulting from this process, and the subsequent growth and development of saffron. Since the effective compounds of saffron are made up of glycosides, it is logical that by increasing the chlorophyll content and subsequently increasing the photosynthetic power, the synthesis of sugars increases, which in turn leads to an increase in the biosynthesis of Picrocrocin and Safranal.

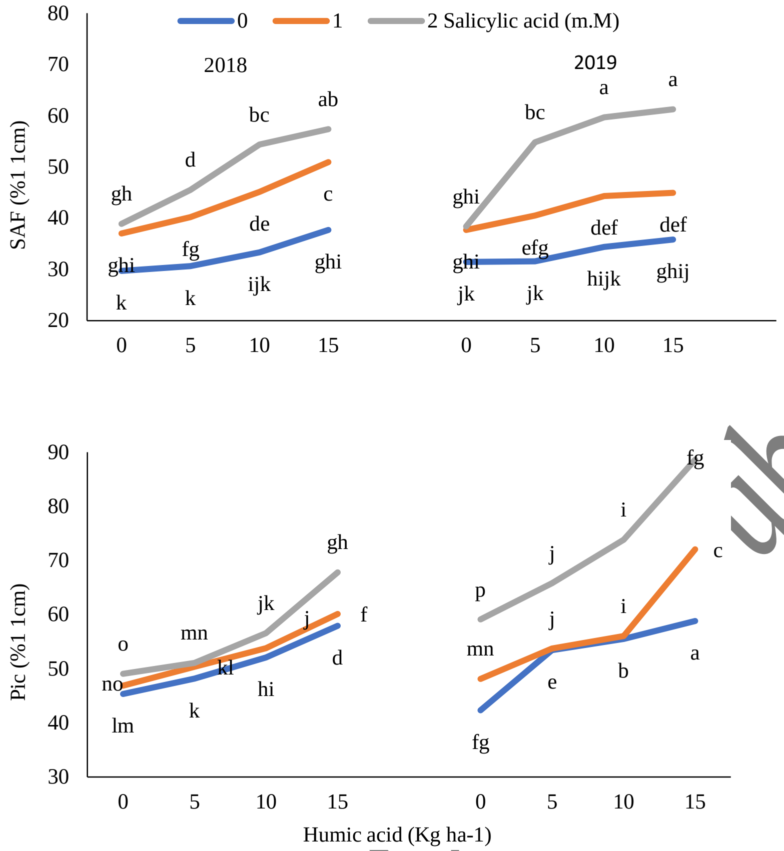

Fig. 4 The average concentration of Picrocrocin and Safranal under the influence of the interaction of Salicylic and humic acid. in the studied years. Means with at least one common letter do not have statistically significant differences (Duncan 5%)

Relationships Between Traits

The correlation analysis revealed that Safranal was positively and significantly associated with Protein, Soluble Sugars, Chlorophyll a and Chlorophyll b, as well as Carotenoids, suggesting that the accumulation of primary metabolites and pigments may directly contribute to the biosynthesis and stability of secondary metabolites in saffron. In contrast, Picrocrocin showed no significant correlation with most traits and even weak negative correlations with Carotenoids and Glutathione Peroxidase, which may indicate that its accumulation is regulated by distinct biochemical pathways independent of pigment metabolism or antioxidant enzyme activity. Furthermore, the strong positive correlation between Catalase activity and Proline implies a coordinated role of Osmo protectants and antioxidant enzymes in maintaining cellular homeostasis under stress conditions, which has been highlighted in previous studies on stress physiology of saffron and other medicinal plants. Similarly, the strong correlations of Soluble Sugars with Chlorophyll a, Chlorophyll b, Carotenoids, and Proteins emphasize the central role of carbohydrates in supporting both primary metabolism and secondary metabolite formation, thereby contributing to the overall biochemical quality of saffron. These findings are consistent with earlier reports that identified close linkages between photosynthetic pigments, antioxidant systems, and secondary metabolite accumulation in *Crocus sativus* L.

Table **4** Correlation between traits in saffron treated with humic acid. and salicylic acid

Variables	RWC	Pro	TC	TCa	TCb	Car	Sugar	Proein	CAT	APX	GPX	Saf	Pic
RWC	1												
Pro	0.47 *	1											
TC	0.69 **	0.28	1										
TCa	0.53 **	0.39	0.78 **	1									
TCb	0.38	0.28	0.59 **	0.66 **	1								
Car	0.48 *	0.25	0.58 **	0.63 **	0.41 *	1							
Sugar	0.28	0.03	0.66 **	0.71 **	0.51 *	0.77 **	1						
Proein	0.26	0.26	0.56 **	0.73 **	0.62 **	0.65 **	0.76 **	1					
CAT	0.43 *	0.72 **	0.34	0.28	0.42 *	-0.02	0.02	0.16	1				
APX	0.28	0.26	0.47	0.46 *	0.64 **	0.26	0.29	0.51 *	0.44 *	1			
GPX	0.3	0.3	0.29	0.29	0.3	0.56 **	0.31	0.33	0.23	0.22	1		
Saf	0.35	0.57 **	0.41	0.63 **	0.56 **	0.64 **	0.59 **	0.71 **	0.44 *	0.36	0.48 *	1	

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Pic	0.08	0.08	0.49	0.37	0.24	-0.05	0.19	0.25	0.15	0.13	-0.08	-0.01	1

*and ** are significant at the probability level of five and one percent, respectively.

Abbreviation: Relative water content (RWC); Proline (Pro); Total Chlorophyll (TC); Chl a (TCa); Chl b (TCb); Carotenoid (Car); Sugar; Protein; Catalase (CAT); Ascorbate Peroxidase (APX); Glutathione peroxidase (GPX); Safranal (Saf); Picrocrocin (Pic)

CONCLUSION

The results of this study revealed that the application of humic acid. and salicylic acid significantly increased protein content. This effect is likely due to the induction of ROS-related signaling pathways and the enhancement of antioxidant enzyme activity. The increase in the content of soluble sugars can also be because the Proline induced with the help of these treatments, as a compatible osmolyte, leads to the improvement of the water relations of saffron, which was visible in the measurement of the relative water content. The positive effect of the treatments on the pigments is probably due to the increase in the availability of nutrients, especially nitrogen (as an important component of photosynthetic pigments), which causes an increase in chlorophyll. In addition to the separate effects of the two compounds of humic acid. and salicylic acid on the physiological and biochemical traits of saffron, we witnessed the synergistic effect of these two compounds on most of the mentioned traits, which can be useful in developing agronomic strategies to increase the yield of saffron. However, the mechanisms involved in this synergism are not yet known and need further study.

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