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Influence of Humic Acid on Morphophysiological Characteristics of *Oenothera speciosa* L. under Drought Conditions

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Abstract

Oenothera speciosa is a native flower of the central grasslands of the United States. In recent years, it has been widely cultivated in open fields in Iran due to its abundant flowering, high tolerance to environmental stresses, and ability to grow in various drained soils. This study investigates the effects of humic acid (HA) treatments at concentrations of 0, 16, and 32 mg.L^{-1} under different drought stress levels (90%, 70%, and 50% field capacity) on morphophysiological traits of *O. speciosa*. Compared to other concentrations, HA at 32 mg. L^{-1} under 90% field capacity significantly increased the average number of flowers per plant (99), anthocyanin content (53 mg. g^{-1}), and proline accumulation (7.57 µmoles. g^{-1}FW). Additionally, antioxidant activity inhibition was highest (37.10 µg. mL^{-1}) under 50% field capacity. As a natural substance, HA positively affects soil osmotic potential and nutrient availability under drought stress conditions. Therefore, it is recommended as a supplementary bio-fertilizer to enhance growth and development of ornamental plants in green spaces.

Keywords: Anthocyanin, Antioxidant activity, Environmental stress, Humic acid; Ornamental plant, Proline.

1. Introduction

Oenothera speciosa, commonly known as pink evening primrose, belongs to the *Oenothera* family, is a significant plant species valued for its medicinal, ecological, and ornamental properties. Due to its rich content of bioactive compounds such as flavonoids, antioxidants, and essential fatty acids particularly gamma-linolenic acid it has garnered attention in both traditional medicine and modern pharmacology for its anti-inflammatory effects, hormone regulation, and skin health benefits. Additionally, *O. speciosa* plays a vital role in biodiversity conservation and is used in ecological restoration and soil stabilization projects due to its drought resistance. Its attractive appearance and prolonged blooming period also make it a popular choice in landscape design and urban green spaces (Shawky et al., 2021).

O. speciosa is a perennial herb widely cultivated in green spaces across Iran. Flowering occurs from spring to autumn; flowers open in the afternoon and close in the morning (Munir et al., 2017). The most important characteristics of *O. speciosa* include typically four petals, a cup-shaped flower with pink or red veins, a height ranging from 30 to 150 cm, and propagation by stolons and seeds. *O. speciosa* has been studied and used in various herb gardens (Rowe et al., 2021). Its ability to thrive under different environmental conditions and stresses such as drought and poor soil nutrition makes it a suitable choice for designing public green spaces. However, nutrition has recently become a key factor for producers aiming to improve the growth and development of *O. speciosa* (Shawky et al., 2021).

Humus is a valuable material produced from the decomposition of plant residues and animal matter, a process carried out by soil microorganisms. Humic acid (HA) is an organic component of humus that improves soil physicochemical properties by enhancing cation exchange capacity (CEC), improving soil structure, and increasing nutrient availability. Essential nutrients in the soil, when present in an accessible form and location, help plants absorb and utilize them for growth. Moreover, HA acts as a natural fertilizer that regulates morphophysiological parameters because nutrient uptake is directly related to plant health. The above points indicate that HA can increase plant tolerance to both biotic and abiotic stresses (Allahkarami et al., 2025).

Drought stress is one of the most critical abiotic factors limiting plant growth and productivity, especially in arid and semi-arid regions. *O. speciosa*, a medicinal and ornamental plant, is sensitive to water deficit, which can adversely affect its physiological and morphological traits. Recent attention has focused on HA as a natural biostimulant capable of enhancing plant tolerance to environmental stresses by improving soil structure, nutrient availability, and root development. However, limited studies have investigated the role of HA in mitigating drought effects specifically in *O. speciosa* (Qiu et al., 2025). This study aims to fill this research gap by evaluating the potential of HA to improve drought resilience in pink evening primrose, thereby contributing to sustainable cultivation practices under water-limited condition.

2. Material and methods

2.1. Plant material and treatments

HA used in the current study consisted of 95% HA and 5% other nutrients and was obtained from Humic Growth Solution Company (USA). *O. speciosa* seedlings at the four-leaf stage were prepared from the research greenhouse of the Clinic Flower and Plant, Tehran Municipality (District 1). The experiment was in a research greenhouse during 2022–2023. Environmental conditions, including light intensity, relative humidity, and temperature, were controlled at 10,000–150,000 lux, 75–80%, and 15–30°C, respectively. The factors included foliar application of HA at 0 (control), 16, and 32 $mg.L^{-1}$, and drought stress at 90% (control), 70% (medium stress), and 50% (severe stress) field capacity (FC). The pot substrate consisted of 2 parts soil and 1-part cow dung; some of its properties are presented in Table 1.

Table 1
Analysis of soil and cow dung properties

	Texture	FC (%)	pH	EC ($dS.m^{-1}$)	TN ($g.kg^{-1}$)	TP ($g.kg^{-1}$)	TK ($g.kg^{-1}$)	TOC (%)
Soil	Silt loam	20	7.20	1.10	0.67	14.08	8.42	1.40
Cow dung	_	_	7.08	2.90	7.91	6.48	5.31	3000.16

FC: Field capacity; EC: Electrical conductivity; TN: Total nitrogen; TP: Total phosphorous; TK: Total potassium; TOC: Total organic carbon.

HA was sprayed at the beginning of flowering in the morning, twice a month until flower harvesting in October.

2.2. Number of Flowers

The average number of flowers per plant was counted in the early mornings, one month after the treatment started (Bohlouli et al., 2019).

2.3. Bud Diameter

Bud diameter was measured using a caliper, Model 16ER, China (Bohlouli et al., 2019).

2.4. Anthocyanin Content

Anthocyanin content was determined by pH-differential spectrophotometry; samples were measured at 510 and 700 nm using a spectrophotometer (Model 6850, UK), and results were calculated using the formula below (Nassour et al., 2020):

$$\text{Anthocyanin (mg. g}^{-1}) = \frac{(A \times 449.2 \times DF \times V \times 10^3)}{26900 \times 1 \times m}$$

A is the absorbance, DF is the dilution factor, V is the final volume (mL), and m is the petal weight (g).

2.5. Antioxidant Activity

Antioxidant activity was measured using the DPPH method, and the absorption spectrum was recorded at 517 nm. The inhibition percentage was calculated using the following formula (Smolinska-Kondla et al., 2022):

$$\text{Antioxidant activity (µg. mL}^{-1}) = \frac{(AB - AA)}{AB} \times 100$$

AA and AB are absorbance in the herbal extract and the blank, receptively.

2.6. Proline Content

To calculate proline content, toluene was used for the colorimetric assay, and absorbance was measured at a wavelength of 570 nm. The formula for evaluating proline content in flower tissue is provided below (Farhadi et al., 2024):

$$\text{Proline(µmoles.g}^{-1}\text{FW)} = \frac{[(\text{µg proline .mL}^{-1}) \times \text{mL toluene}) / 115.5 \text{ µg .µmole}^{-1}]}{[\frac{(\text{g sample})}{5}]}$$

2.7. Peroxidase enzyme

Peroxidase enzyme activity was determined using the method of Wu et al. (2022). The rate of oxidation was monitored at 460 nm, and the quenching factor for guaiacol peroxidase was considered as 26.6 $mµ^{-1}$ cm^{-1}.

2.8. Statistical analysis

This research was conducted in a completely randomized design with three replications under greenhouse conditions. Data analysis was performed using ANOVA, and mean comparisons were carried out using the Least Significant Difference (LSD) test.

3. Results and Discussion

3.1. Average number of flowers per plant

Tests of the main and interaction effects showed that all treatments used in the present study were statistically significant ($P \leq 0.01$) on the average number of flowers per plant (Table 2). Figure 1 (A), showed that the average number of flowers per plant was reduced at 50% FC, but increased with foliar application of 32 mg. L^{-1} HA, resulting in maximum average yields of 68 and 92 flowers per month, respectively. Statistical interaction results revealed that increasing HA had a significant effect on the number of flowers under drought stress. As presented in Figure 1 (B), application of the highest HA level improved the average number of flowers per plant even under severe stress.

Table 2.
Variance analysis (mean square) for some *O. speciosan* morpho-physiological traits.

S. V	df	Avereage number of flowers per plant	Diameter of bud	anthocyanin	Antioxidant activity	Proline	Peroxidase enzyme activity
Drought stress	2	31.80**	3.37**	8.03*	141.85**	1.11**	0.35**
HA	2	121.15**	6.18**	12.08**	38.41**	1.31*	1.18**
Drought stress × HA	4	11.44**	0.72ns	30.25*	251.67**	1.02*	0.40ns
Error	10	8.12	1.29	0.41	0.09	0.13	0.05
CV (%)		0.12	0.71	0.23	0.50	0.43	0.18

*, ** and ns significant at 0.05, 0.01 and not significant, respectively.

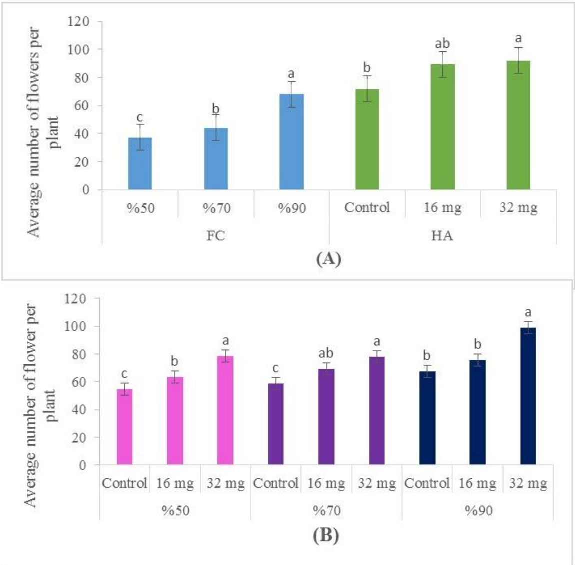

Figure 1.
Comparison of the main (A) and interaction (B) effects on the average number of flowers per plant under field capacity (FC) and humic acid (HA) in *O. speciosan*; ±SE.

Drought stress effects vary depending on the type and severity of stress, as well as the plant species and its tolerance threshold (Ding et al., 2025). Deficit irrigation, a water management technique commonly used for green spaces, induces a process known as "stress-induced flowering." While this serves as a survival mechanism, it typically results in a reduction of flower yield. Nutrient transport from the soil solution through the roots to the above-ground parts of the plant is essential for growth and development (Miri Seftejani et al., 2025). HA enhances soil fertility by improving the uptake of essential nutrients and increasing stress resistance. The findings of this study align with previous reports on marigold by Delvand et al. (2018) and Azama et al. (2022).

3.2. Diameter of bud

According to the analyzed results in Table 2, the main effects are statistically significant ($P \leq 0.01$); however, the interaction between treatments was not statistically significant. Figure 2, shows that there was no significant difference between the effects of 90% and 70% FC on bud diameter, with measurements of 28.51 mm and 28.11 mm, respectively. Additionally, it was observed that applying 32 mg.L^{-1} of HA significantly increased bud diameter compared to other treatments.

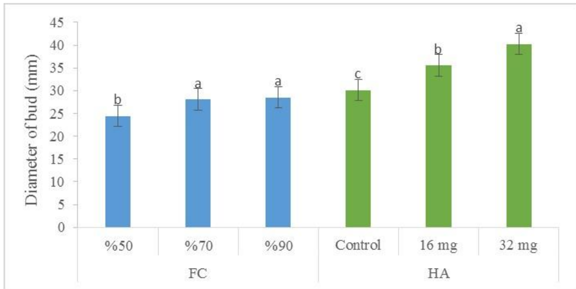

Figure 2.
Comparison of the main effects on bud diameter under field capacity (FC) and humic acid (HA) in *O. speciosan*; ±SE.

Due to the limitation of cell elongation under drought stress, flower size is reduced. Additionally, the acceleration of flowering to initiate reproductive growth results in a decreased bud diameter (Zarifi et al., 2025). HA can enhance flowering stages and petal development by improving water and nutrient availability to the plant. Macronutrients such as nitrogen and phosphorus are essential for promoting larger flowers and overall floral development (Al-Nafei and Al-Mohammad, 2021). These findings are consistent with the results reported by Bohlouli et al. (2019) and Dehestani-Ardakani et al. (2019) on evening primrose.

3.3. Anthocyanin content

The analysis of variance presented in Table 2 indicated significant differences between all treatments ($P \leq 0.05$ and $P \leq 0.01$). Both the main and interaction tests showed that anthocyanin content is directly influenced by drought stress and HA application, ranging from 32.14 to 60.52 mg. g^{-1} in the main effects (Figure 3. A), and from 40.32 to 53.00 mg. g^{-1} in the interaction effects (Figure 3. B).

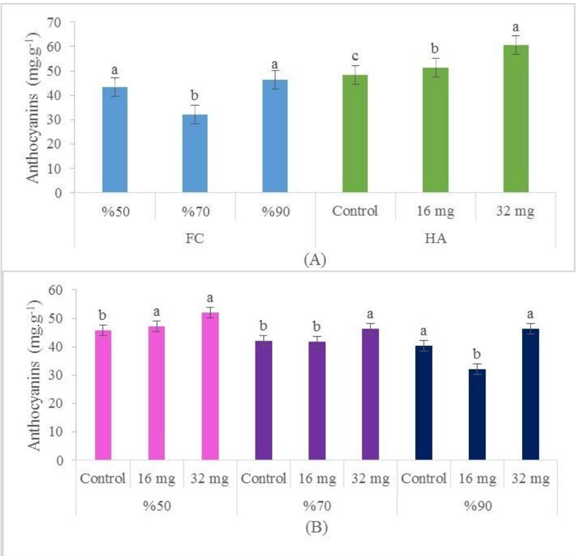

Figure 3.
Comparison of the main (A) and interaction (B) effects on anthocyanin content under field capacity (FC) and humic acid (HA) in *O. speciosan*; ±SE.

Anthocyanins are pigments with antioxidant functions (a type of flavonoid) in plants and are responsible for a range of flower colors including red, purple, pink, and blue. Therefore, the quality and synthesis of these compounds are important factors in ornamental plant studies. An increase in anthocyanin content is a common response mechanism to drought stress (Seth et al., 2025). HA has been shown to enhance anthocyanin content, likely by influencing the expression of genes involved in anthocyanin biosynthesis (Samia Ageeb and Heba Ibrahim, 2018).

Overall, anthocyanins play a crucial role not only in attracting pollinators but also in providing oxidative stress protection. However, this protective role can accelerate the process of flower senescence (Seth et al., 2025). The results of the current study are supported by findings from Taghizadeh et al. (2025) on rose.

3.4. Antioxidant activity

The ANOVA results in Table 2 show data significance at $P \leq 0.01$. As shown in Figure 4 (A), water deficit initially led to an increase in antioxidant activity, followed by a subsequent decrease. However, the application of HA promoted the production of antioxidant compounds. The interaction effects revealed the best results with the combination of 50% FC and 32 mg. L^{-1} of HA, achieving an antioxidant activity of 37.10 µg.ml^{-1} compared to other treatments and the control (Figure 4.B).

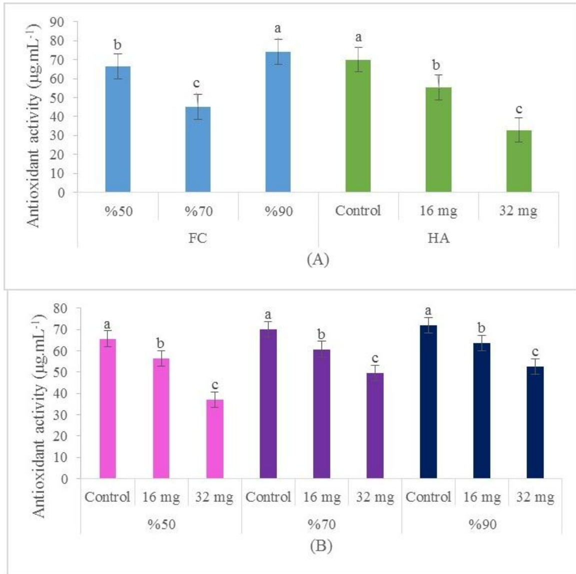

Figure 4.
Comparison of the main (A) and interaction (B) effects on antioxidant activity under field capacity (FC) and humic acid (HA) in *O. speciosan*; ±SE.

Drought conditions lead to an increased accumulation of reactive oxygen species (ROS) within plant cells. This excessive production of ROS creates an imbalance in cellular homeostasis, which can overwhelm the plant's natural antioxidant defense systems. When these defenses are insufficient, oxidative damage occurs to vital cellular components such as lipids, proteins, and DNA. Therefore, antioxidant activity plays a crucial role as a protective mechanism against free radicals generated by oxidative stress, helping to maintain cellular integrity and improve plant tolerance under drought conditions (Xu et al., 2025).

HA plays multiple roles as an antioxidant by scavenging reactive ROS, inhibiting their formation through metal ion chelation, and modulating the activity of key antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase. These mechanisms collectively contribute to reducing oxidative damage within plant cells under stress conditions (Mousavi et al., 2023). Moradi et al. (2024) demonstrated that rose plants treated with 1000 mg. L^{-1} HA exhibited significantly increased antioxidant activity, along with elevated levels of proline and total protein content, which are important indicators of improved stress tolerance and cellular protection.

3.5. Proline content

Based on Table 2, the mean square of proline was significant across all treatments ($P \leq 0.05$ and $P \leq 0.01$). Drought stress and elevated HA concentrations both contributed to enhanced proline accumulation in *O. speciosa* (Figure 5. A). However, the interaction effects presented in figure 5 (B) showed that the highest proline concentration (7.57 µmoles. g^{-1}FW) occurred under 90% FC without HA application.

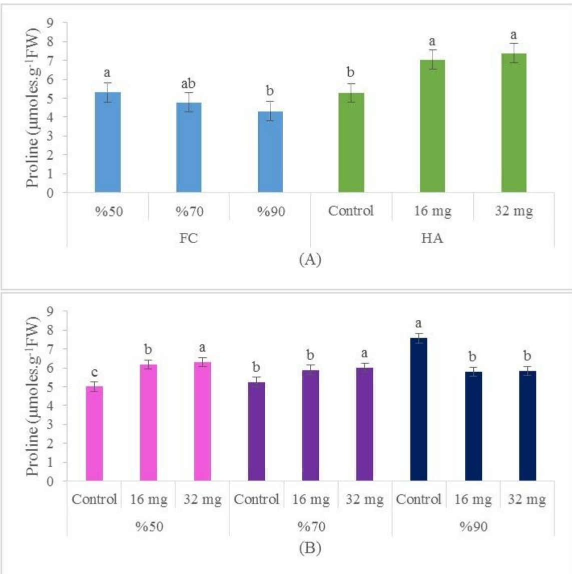

Figure 5.
Comparison of main (A) and interaction (B) effects of proline under field capacity (FC) and humic acid (HA) in *O. speciosan*; ±SE.

Proline, as an osmolyte molecule, helps plants enhance their tolerance to stress by maintaining cellular osmotic balance and protecting cells from environmental damage. HA, by improving nutrient uptake and increasing stress tolerance, indirectly promotes proline production (Xu et al., 2025; Roy et al., 2025). However, some studies have shown that while abiotic stresses such as drought or salinity typically induce proline accumulation as a protective response, the application of HA can mitigate this increase. This is likely because HA improves photosynthesis and protein synthesis, leading to better overall growth and reducing the plant's dependence on proline accumulation for stress management (Ghasemi et al., 2022).

3.6. Peroxidase enzyme activity

The data presented in Table 2 revealed that only the main effects on peroxidase enzyme activity were statistically significant ($P \leq 0.01$). As shown in Figure 6, peroxidase activity increased with the intensification of drought stress. Additionally, increasing the level of HA application influenced the enzyme activity, which ranged between 0.001 and 0.02 µmole.min^{-1}. g FW^{-1}.

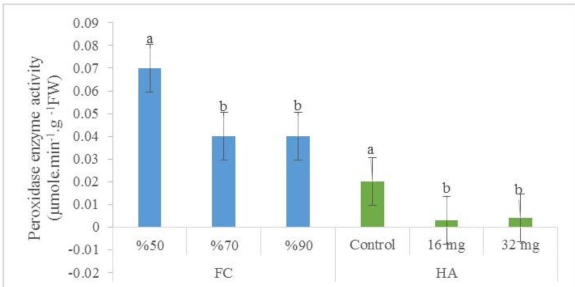

Figure 6. Comparison of the main effects on proline under field capacity (FC) and humic acid (HA) in *O. speciosan*; ±SE.

Drought stress has a positive impact on peroxidase enzyme activity in plants. Peroxidases are part of the plant's antioxidant defense system, and their activity increases to help scavenge reactive ROS produced under drought conditions (Osati et al., 2024). This increase in activity is a protective mechanism against the damaging effects of drought stress. Peroxidase enzyme activity can be inhibited by HA, which can interfere with peroxidase activity either by binding to the enzyme's substrate or by directly interacting with the enzyme itself (Sharif Soltani et al., 2023).

4. Conclusion

The market for organic products, including HA, within agriculture particularly in the ornamental plant industry is projected to expand significantly due to its efficacy in mitigating abiotic stresses. HA has been shown to enhance various morphophysiological characteristics of *O. speciosa*, including increased average flower number per plant, larger bud diameter, elevated anthocyanin concentrations, and improved antioxidant enzyme activity. These enhancements contribute to the plant's stress tolerance and overall aesthetic quality, which are critical factors in ornamental horticulture. Although numerous studies have investigated the effects of HA on soil fertility and plant growth parameters, most have been confined to controlled environments such as greenhouses or limited small-scale field trials. Laboratory and greenhouse experiments have demonstrated that HA facilitates nutrient chelation, thereby improving nutrient availability and uptake efficiency in plants, which in turn promotes growth and stress resilience. Given these findings, there is a strong impetus for the agricultural industry to commercialize HA applications on a larger scale, especially for enhancing crop performance under abiotic stress conditions.

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