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ORIGINAL RESEARCH

Effects of humic acid application on physiological and biochemical characteristics of safflower cultivars under salinity and cadmium contamination

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Abstract

This study aimed to assess the impact of humic acid application on three safflower genotypes (AC-Sterling, Arack 2811, and C111) cultivated in cadmium contaminated soil with varying levels of salinity (0, 50, 100, and 150 mM NaCl). The experiment showed that the salinity decreased the K/Na ratio, photosynthesis rate, chlorophyll content, root volume as well as shoot and root dry weight, while it increased the cadmium concentration and the activities of catalase, peroxidase and ascorbate peroxidase. Of particular interest, the C111 genotype demonstrated the lowest decline in K/Na ratio and photosynthesis and exhibited the least increase in cadmium absorption. Furthermore, the salinity-induced increase in the antioxidant activity was most pronounced in catalase. The humic acid application led to increased activities of antioxidant enzymes, K/Na ratio, photosynthesis rate, chlorophyll content, root volume, shoot dry weight, and root dry weight. The humic acid application, however, resulted in decreased cadmium concentration in Arak 2811 while it increased cadmium concentration in AC-Sterling and C111. This indicates that the positive effects of humic acid on biomass were more pronounced in the C111 genotype under lower salinity levels and mostly manifested in root growth rather than shoot growth. The superiority of C111 in terms of the response to humic acid was more attributed to catalase and peroxidase activation compared to ascorbate peroxidase. Overall, the results showed a significant variation among safflower genotypes regarding the plant physiological attributes and the cadmium content in response to the salinity and the humic acid application.

Keywords Cadmium · Humic acid · Safflower · Salinity

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
Cd	Cadmium
Chl	Chlorophyll
EDTA	Ethylenediaminetetraacetic acid
HA	Humic acid
OM	Organic materials
PVP	Polyvinyl-pyrrolidone

Introduction

Soil salinity is one of the most serious abiotic stresses restricting crop growth and production (Parihar et al. 2015). While salinization can transpire across all climate zones, it is more common in arid regions (Bacilio et al. 2016). When exposed to heightened salt levels, plants undergo diverse physiological and biochemical reactions (Salem et al. 2014). Nonetheless, the tolerance to salt stress varies significantly among various plant species (Munns and Tester 2008), which could be categorized into tissue resistance and ion depletion (Aliyari Rad et al. 2021).

Soil physical properties may be affected by salinity, causing the deterioration of soil structure and reductions in soil hydraulic conductivity and infiltration rate. It also accelerates surface runoff and soil erosion (Edelstein et al. 2010) and affects soil microorganisms negatively (Yan et al. 2015). Furthermore, as the concentration of salt rises in the tissues, plants experience two main phases of stress: osmotic stress and specific ion (Na^+ and Cl^-) toxicity (Arzani and

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Ashraf 2016). These primary issues can trigger secondary stresses like oxidative stress and nutritional imbalances. Consequently, plants may suffer from impaired growth due to disruptions in metabolic and physiological functions. These disruptions include reduced nutrient and water uptake, membrane dysfunction, and disturbances in critical processes such as respiration, photosynthesis, and protein synthesis (Arzani et al. 2023).

Cadmium (Cd) is a toxic and highly mobile heavy metal that penetrates the environment from anthropogenic activities, particularly the use of phosphorous fertilizers containing Cd (Shafi et al. 2010; Khan et al. 2017). Moreover, using biosolids containing heavy metals such as Cd in order to improve saline soils quality in arid and semi-arid regions can also result in soil pollution (Mühling and Läuchli 2003). The toxicity of Cd has carcinogenic effects on various human organs like the kidneys, lungs, heart, and bones (Roberts 2014). Plants absorb Cd through the cortex tissue in their roots, which are then transported to aerial organs via the xylem transport system, alongside water and both macro- and micronutrients from the soil. Ultimately, these elements are stored in various parts of the plant, including leaves, fruits, and grains (Nayeri et al. 2023). Cadmium toxicity in plants leads to a range of detrimental effects, including chlorosis, necrosis, and wilting. It impairs photosynthesis, reduces chlorophyll content and decreases the transpiration rate. Additionally, cadmium exposure results in a decrease in xylem vessel density and leaf relative water content. Furthermore, the toxicity disrupts carbohydrate metabolism, inhibits stomatal opening, diminishes plasma membrane activity, and alters enzyme activities (Dias et al. 2013; Nayeri et al. 2023). To mitigate the harmful effects of heavy metals such as Cd, some plant species have evolved a range of defensive strategies. These adaptations include mechanisms for metal sequestration, compartmentalization within specific cell organelles, exclusion, and inactivation through the release of organic ligands (Nayeri et al. 2023). The extent to which plants uptake Cd depends on the soil Cd concentration, soil pH, soil mineralogy, soil organic matter, and the existence of other metals (Filipović et al. 2018). In addition, it has been shown that the Cd concentration in crops may be increased in saline soils fertilized by phosphorous fertilizers containing Cd (Huang et al. 2007). The combined effect of salinity and Cd stress on plants could cause membrane leakage and a rise in the production of oxygen radicals and H_2O_2 (Mühling and Läuchli 2003).

Soil organic materials (OM) play a crucial role in enhancing soil fertility via the chemical, physiological, and biological reactions in the soil (Khaled and Fawy 2011). Adding OM provides energy and nutrients for soil microorganisms to grow and function in poor soil conditions (Novair et al. 2024). One of the main soil OMs is humic acid (HA), which is extracted from plant and animal residues decomposition (Fan et al. 2014). Studies on different plants have shown that HA helps them directly and indirectly to grow better, and that the effects vary among plant types (Hemati et al. 2022). HA enriches plant growth indirectly by improving soil properties such as reducing soil compaction, water holding capacity, soil microbial activity, and availability of micro/macro elements. It also enriches the plant growth directly by increasing plant biomass in terms of the uptake of HA substances as well as transporting them to plant tissues (Ampong et al. 2022; Tahir et al. 2011; Hemati et al. 2022). Various studies indicate that the humification process leads to the formation of complex compounds between humic materials and mineral ions. This interaction enhances enzyme activity, resulting in increased respiration intensity, improved photosynthesis, and greater metabolism of nucleic acids (Hemati et al. 2022). In addition, the humic substances containing acidic electrolytes with carboxylic and phenolic hydroxyl (OH) functional groups may reduce the heavy metals toxicity by providing macromolecules that affect the bioavailability and solubility of heavy metals (Khan et al. 2017). Numerous studies have investigated the interactions between salinity stress and humic acid, however, there is a significant gap in the literature concerning the specific responses of safflower cultivars in a cadmium-contaminated soil. Therefore, the present experiment aimed to examine the effect of HA application on alleviating Cd-polluted soil and salinity stress in three safflower genotypes.

Materials and methods

Growth condition

Six seeds of three safflower genotypes, AC-sterling, Arak 111, and C111, were sown in 5-L plastic pots (20.5 cm diameter and 22.5 cm depth). These pots were filled with 5 kg of cadmium-contaminated soil derived from an area surrounding a steel plant in Isfahan, Iran. This soil was classified as a silty loam soil containing 0.99% organic matter, 131 mg kg^{-1} available K by 1 N acetate, 5.5 mg kg^{-1} total Cd, 0.85 available Cd by DTPA extraction method; having pH of 8.01 and EC of 0.51 dS^{-1} in saturated extract. Humic acid (from Biomega company) was applied to the soil before sowing at a rate of control and 1g kg^{-1} pot soil.

The emerged plants were initially watered with tap water (EC = 1.9 dS m^{-1}) until they were fully established when they had 4 to 6 true leaves. Three weeks after emergence, the seedlings were then irrigated with saline water at varying concentrations. Prior to full establishment, the plants were irrigated every 3 days. Once established, they were irrigated as needed (once or twice a week) until they

reached the time of applying salinity treatments at four-leaf stage. To minimize the risk of osmotic shock, the seedlings were gradually exposed to increasing levels of NaCl, beginning with a concentration of 25 mM until reaching final concentrations of either 50, 100, or 150 mM NaCl. The plants were irrigated after 50% of the available soil water had been depleted. Plants in all treatments received the same volume of irrigation water. The volume of irrigation water was calculated using the following formula:

$$V_{irrig} = \left(\theta_{FC} - \theta_{avg}\right) \times \rho_b \times V_{pot}.$$

Where V_{irrig} is the irrigation water volume (cm^3), θ_{FC} is the gravimetric water content (%) at field capacity, θ_{avg} the average moisture of soil layer in the root zone, ρ_b the apparent density of the soil, and V_{pot} the volume of the pot.

The pots were placed outdoors at the Isfahan University of Technology in Isfahan, Iran to expose them to natural environmental conditions.

Plants were harvested about 60 days after applying salinity treatment, at the beginning of flowering stage. Following the harvest of the shoots, the roots were carefully extracted from the soil and rinsed in a basin of water to remove any remaining soil particles. Subsequently, the roots and aerial organs were washed with distilled water then were dried separately at 65 °C for 72 h before measuring their dry weights.

Ion concentration analysis

The Na and K concentrations were measured using Hamada and El-Enany (1994) method. Each plant's powdered samples, weighing 0.2 g, were thoroughly burned in an electric oven. Subsequently, 10 mL of HCl 2 N was added to each sample while gently heated. The resultant solution was sieved through Whatman filter paper and the volume was then brought up to 100 mL using distilled water. The flame photometer (model Jenway PFP7 UK) was employed to measure the Na^+ and K^+ contents from 1:100 diluted root and shoot samples.

For the determination of Cd accumulation in plant tissues, the samples were extracted by using 4 mL of Merck HNO3, kept undisturbed for 24 h, heated in a laboratory oven, and determined by Perkin Elmer 3030 USA atomic absorption spectrophotometer.

Photosynthesis rate

The net photosynthetic rate was measured on three healthy leaves from each experimental unit using a calibrated portable gas exchange system (LCi, ADC Bioscientific Ltd., UK).

Measurement of photosynthetic pigments

Chlorophyll (Chl) contents of safflower genotypes leaves were determined following Lichtenthaler and Wellburn's method (1983). Leaf tissues chlorophyll was extracted with 10 mL 80% acetone and light absorbance was recorded at 645 and 663 nm by Hitachi U-1800 spectrophotometer for Chl *a* and Chl *b*, respectively.

Root volume

The root volume was measured using the water displacement method. First, the roots were submerged in a graduated cylinder filled with water. The water levels in the cylinder were recorded before and after submerging the roots into the cylinder. The volume of roots was calculated as follows:

root volume = volume of the water after submerging the roots into the cylinder − volume of the water before submerging the roots.

Determination of antioxidant enzymes activity

To determine the antioxidant enzyme activities (Peroxidase (POX), Catalase (CAT), and Ascorbate peroxidase (APX)), 0.1 g of frozen leaf samples were grounded with liquid nitrogen and homogenized with 1mL of 100mM K_2PO_4 containing (pH = 7) 0.1 mM Merck Ethylenediaminetetraacetic acid (EDTA) and 1% Merck polyvinyl-pyrrolidone (PVP) on ice. The solution was centrifuged by Hettich Benchtop centrifuge UNIVERSAL 320 at 1400 rpm for 30 min under 4 °C. The supernatant was used for the determination of antioxidant enzyme activities.

The CAT activity was measured using a spectrophotometer (Hitachi U-1800) at 240 nm, following the method described by Aebi (1984), using 3mL of a reaction solution containing 50 mM Na_2PO_4 (pH = 7), 10 mM H_2O_2 and 40 µL of the enzyme extract.

The APX activity was determined by the Nakano and Asada (1981) method using 1 mL of a reaction buffer containing 50 mM K_2PO_4 (pH = 7), 0.5 mM ascorbic acid, 0.1 mM EDTA, 1.25 mM H_2O_2, and 60 µL of the enzyme extract, at 290 nm in 1 min.

The determination of POX activity followed a procedure similar to that used for CAT, except that the extraction buffer for peroxidase contained an additional 3.35 µL of GUAIACOL per cuvette, in addition to 4.51 µL of H_2O_2 (30%). Absorbance was measured at 470 nm over a duration of 2 min (Change and Maehly 1955).

Statistical methods

The experiment was set up in a completely randomized design with a factorial arrangement of treatments (3 species, 2 HA levels, 4 NaCl levels, and 3 replications). Analysis of variance was carried out using SAS. Results were analyzed in terms of variance and LSD (at $p \leq 0.050$) values were calculated for cultivars, treatments, and their interactions.

Results

Cadmium concentration in shoot and roots

The effects of the interactions between salinity and genotype and HA and genotype were significant on the concentration of Cd in shoot and roots (Table 1). Salinity increased the concentration of Cd in shoot and roots in all tested genotypes (Table 2). At 50, 100, and 150 mM NaCl compared to non-saline control, Cd concentration in the shoot was increased by 27.8, 48.8 and 68.4% in AC-Sterling, 23.1, 39.0, and 67.6 in Arak 2811 and 12.0, 19.4, and 27.3 in C111 genotype, respectively.

The corresponding values for root Cd concentration were 16.2, 35.3, and 62.4 in AC-Sterling, 23.6, 44.7, and 67.1 in Arak 2811 and 12.9, 26.7, and 43.3 in C111 genotype.

The application of HA increased the concentration of Cd in shoot and root by 15.2 and 24.1% in AC-Sterling and by 10.1 and 11.4% in C111, respectively, but it decreased Cd concentration by 16 and 16.6% in Arak 2811 genotype (Table 3).

Table 1 Analysis of variance of shoot and root Cd concentration, Cd translocation, shoot and root K: Na concentration, photosynthesis rate and total chlorophyll concentration

Source of variation	df	Mean square						
		Shoot Cd concentration	Root Cd concentration	Cd translocation	Shoot K:Na ratio	Root K:Na ratio	Photosynthesis rate	Total chlorophyll concentration
Genotype (gen)	2	72.1^{**}	143^{**}	4378.1^{**}	86.9^{**}	1.58^{**}	18.4^{**}	0.062^{**}
Humic acid	1	4.21^{*}	6.36^{**}	305.5^{**}	164^{**}	27.1^{**}	82^{**}	0.4^{**}
NaCl	3	319^{**}	246^{**}	178.2^{**}	717^{**}	67.9^{**}	279^{**}	1.54^{**}
Gen × HA	2	100^{**}	93.9^{**}	200.4^{**}	10.0^{**}	1.33^{**}	0.727	0.009^{**}
Gen × NaCl	6	12.4^{**}	4.58^{**}	204.8^{**}	77.3^{**}	0.752^{**}	4.22^{**}	0.033^{**}
HA × NaCl	3	1.00	0.792	41.3	56.0^{**}	7.33^{**}	8.76^{**}	0.018^{**}
Gen × HA × NaCl	6	0.498	0.509	52.4^{*}	13.5^{**}	0.825^{**}	2.19^{*}	0.038
Error	48	0.81	0.574	11.38	0.798	0.096	0.699	0.0039

ns, *, and ** indicate non-significance at $P < .05$, significance at $P < .05$, and significance at $P < .001$, respectively

Table 2 Effect of different salinity levels on shoot, root and Shoot:Root Cd concentration, total chlorophyll concentration and antioxidant enzymes (CAT, APX and POX) of safflower genotypes

Experimental factor		Shoot Cd concentration (mg kg^{-1})	Root Cd concentration (mg kg^{-1})	Shoot:Root Cd concentration	Total chlorophyll concentration (mg g^{-1} FW)	Ascorbate peroxidase (unit mL^{-1})
NaCl	Genotype					
0	AC sterling	16.6^{h}	13.0^{h}	1.29^{cd}	0.975^{c}	1.7^{g}
	Arak 2811	18.2^{g}	16.1^{f}	1.13^{e}	1.19^{a}	1.7^{g}
	C111	22.7^{e}	16.1^{f}	1.41^{a}	1.11^{b}	2.54^{de}
50	AC sterling	21.2^{f}	15.1^{g}	1.41^{a}	0.762^{de}	1.91^{g}
	Arak 2811	22.4^{e}	19.9^{d}	1.13^{e}	0.698^{ef}	2.33^{ef}
	C111	25.4^{d}	18.2^{e}	$1.4a^{b}$	0.824^{d}	3.12^{c}
100	AC sterling	24.65^{d}	17.6^{e}	1.41^{a}	0.536^{gh}	2.17^{f}
	Arak 2811	25.3^{d}	23.3^{b}	1.09^{e}	0.558^{g}	2.68^{d}
	C111	27.1^{c}	20.4^{cd}	1.33^{bc}	0.684^{f}	3.58^{b}
150	AC sterling	$27.9b^{c}$	21.1^{c}	1.32^{cd}	0.33^{i}	3.39^{b}
	Arak 2811	30.5^{a}	26.9^{a}	1.13^{e}	0.48^{h}	3.02^{c}
	C111	28.9^{b}	23.1^{b}	1.25^{d}	0.378^{i}	4.36^{a}

Mean values in the same column followed by at least one common letter, are not significantly different at the 5% probability level based on the LSD test

Table 3 Effect of HA levels on shoot, root and Shoot:Root Cd concentration, total chlorophyll concentration, shoot dry matter and antioxidant enzymes (CAT, APX and POX) of safflower genotypes

Experimental factor		Shoot Cd concentration (mg kg^{-1})	Root Cd concentration (mg kg^{-1})	Shoot:Root Cd concentration	Total chlorophyll concentration (mg g^{-1} FW)	Shoot dry matter	Ascorbate peroxidase (unit mL^{-1})
HA	Genotype						
0	AC sterling	21.0^{e}	14.9^{e}	1.41^{a}	0.601^{d}	5.23^{c}	2.13^{d}
	Arak 2811	26.2^{b}	23.5^{a}	1.12^{c}	0.698^{c}	5.85^{b}	2.00^{d}
	C111	24.8^{c}	18.4^{d}	1.36^{ab}	0.604^{d}	5.32^{c}	2.81^{b}
1	AC sterling	24.2^{c}	18.5^{d}	1.31^{b}	0.700^{c}	5.93^{b}	2.46^{c}
	Arak 2811	22.0^{d}	19.6^{c}	1.12^{c}	0.762^{b}	6.67^{a}	2.87^{b}
	C111	27.3^{a}	20.5^{b}	1.34^{b}	0.894^{a}	6.81^{a}	3.99^{a}

Mean values in the same column followed by at least one common letter, are not significantly different at the 5% probability level based on the LSD test

Table 4 The three-way interaction of HA, NaCl and genotype on shoot and root K:Na ratio, photosynthesis rate, root volume, catalase, peroxidase and Cd translocation

Experimental factor			Shoot K/Na ratio	Root K/Na ratio	Photosynthesis rate	Root volume	Catalase	Peroxidase	Cd translocation
HA	NaCl	Cultivar							
0	0	AC-sterling	14.8^{b}	2.03^{g}	11.2de	9.17^{d}	0.176^{klm}	0.159^{k}	135.42^{def}
		Arak 2811	10.7^{c}	4.39^{c}	10.7e	11.7^{bc}	0.099^{m}	0.326^{hij}	115.82^{j}
		C111	5.6^{d}	3.87^{d}	12.7c	13.3^{b}	0.125l^{m}	0.259^{ijk}	142.34^{bc}
	50	AC-sterling	3.41^{e}	1.42^{hi}	8.18hi	2.5^{gh}	0.257^{ijk}	0.194^{k}	148^{a}
		Arak 2811	3.23e^{f}	2.02^{g}	6.95ij	6.17^{e}	0.199^{jkl}	0.349^{hij}	110.13^{klm}
		C111	2.04e-^{h}	1.84^{gh}	10.4ef	8.33^{d}	0.166^{lm}	0.330^{hij}	138.15^{cde}
	100	AC-sterling	1.22$^{g–j}$	0.906^{jkl}	5.37kl	2.17^{gh}	0.308^{ghi}	0.247^{jk}	147.74^{ab}
		Arak 2811	1.1$^{g–j}$	1.15^{ijk}	5.23kl	5.00^{e}	0.363^{fgh}	0.381^{hi}	106.48^{m}
		C111	1.33$^{g–j}$	0.958$^{i–l}$	6.94ij	5.00^{e}	0.203^{jkl}	0.402^{h}	138.46^{cde}
	150	AC-sterling	0.471^{ij}	0.45^{lm}	3.83m	1.00^{h}	0.530^{cd}	0.330^{hij}	133.32^{efg}
		Arak 2811	0.314^{j}	0.36^{m}	3.83m	4.5^{ef}	0.512^{de}	0.407^{h}	114.49^{jkl}
		C111	0.398^{j}	0.352^{m}	4.29lm	2.17^{gh}	0.391^{fg}	0.593^{g}	125.28^{hi}
1	0	AC-sterling	29.5^{a}	6.76^{a}	14.3b	15^{a}	0.202^{jkl}	0.344^{hij}	121.83^{i}
		Arak 2811	15.9^{b}	7.2^{a}	17.2a	15^{a}	0.184^{kl}	0.597^{g}	110l^{m}
		C111	9.81^{c}	5.51^{b}	15.5b	15^{a}	0.370^{fg}	1.35^{d}	139.93^{cd}
	50	AC-sterling	6.05^{d}	2.78^{ef}	10.1efg	5^{e}	0.391^{fg}	0.881^{f}	134.33^{ef}
		Arak 2811	6.22^{d}	3.23^{e}	9.00gh	12.5^{b}	0.283^{hij}	0.884^{f}	115.56^{jk}
		C111	5.34^{d}	2.65^{f}	12.2cd	10.^{cd}	0.436^{ef}	1.76^{c}	141.71^{c}
	100	AC-sterling	1.73$^{g–j}$	1.3^{ij}	7.03ij	3.25^{fg}	0.821^{b}	1.11^{e}	134.7^{def}
		Arak 2811	1.92$^{f–i}$	1.45^{hi}	5.82jk	9.33^{d}	0.574^{ed}	1.05^{e}	111.35^{jklm}
		C111	2.17^{efg}	1.32^{ij}	9.26fgh	5.5^{e}	0.608^{c}	2.05^{b}	128.19^{gh}
	150	AC-sterling	0.675^{hij}	0.905^{jkl}	4.87klm	1.33^{h}	1.30^{a}	1.42^{d}	131.24^{fg}
		Arak 2811	0.797$^{g–j}$	0.604^{lm}	4.86klm	4.67^{ef}	0.829^{b}	1.75^{c}	112.09^{jkl}
		C111	0.789$^{g–j}$	0.759^{klm}	5.09klm	4.5^{ef}	0.879^{b}	2.53^{a}	125.28^{hi}

Mean values in the same column followed by at least one common letter, are not significantly different at the 5% probability level based on the LSD test

The effects of the interactions between salinity and cultivar, as well as between HA and genotype, were significant on shoot/root Cd concentration (Table 1).

K/Na ratio in shoot and roots

The effect of the three-way interaction of salinity, cultivar, and HA and cultivar was significant on the K/Na ratio in

shoot and roots (Table 1). Salinity decreased the K/Na ratio in shoot and roots of HA applied and non-applied plants of all tested genotypes (Table 4). At 50, 100, and 150 mM NaCl compared to non-saline control, the K/Na ratio in shoots was decreased by 76.9, 91.7, and 96.8% in AC-Sterling, 69.9, 89.8, and 97.1% in Arak 2811 and 63.6, 76.3, and 92.9% in C111 genotype, respectively, under none HA applied control and by 79.5, 94.1, and 97.7% in AC-Sterling, 60.9, 87.9, and 95.0% in Arak 2811 and 45.6, 77.9, and 92.0% in C111, respectively, under HA application.

The corresponding reduction in the K/Na ratio in roots due to salinity were 30.0, 55.4, and 77.8% in AC-Sterling, 54.0, 73.8, and 91.8% in Arak 2811, and 52.5, 75.2, and 90.9% in C111 genotype, respectively, under none HA applied control and by 58.9, 80.8, and 86.6% in AC-Sterling, 55.1, 79.9, and 91.6% in Arak 2811, and 51.9, 76.0, and 82.0% in C111, respectively, under HA application.

The application of HA increased the K/Na ratio in shoots and roots of all tested genotypes under all salinity levels. At 0, 50, 100, and 150 mM NaCl the ratio of K/Na in shoot was increased by 99.3, 77.4, 41.8, and 43.3% in AC-Sterling, 48.6, 92.6, 74.5, and 154% in Arak 2811, and 75.2, 162, 63.2, and 98.2% in C111, respectively.

The corresponding values in the K/Na ratio in roots were 233, 95.8, 43.5, and 101 in AC-Sterling, 64.0, 59.9, 26.1, and 67.8% in Arak 2811, and 42.4, 44.0, 37.8, and 116% in C111, respectively.

Photosynthesis rate

The effect of the three-way interaction of salinity, cultivar, and HA was significant on the photosynthesis rate (Table 1). Salinity decreased the photosynthesis rate of both HA applied and non-applied plants of all tested genotypes (Table 4). At 50, 100, and 150 mM NaCl compared to non-saline control, the photosynthesis rate was decreased by 27.0, 52.1, and 65.8% in AC-Sterling, 35.1, 51.1, and 64.2% in Arak 2811, and 18.1, 45.4 and 66.2% in C111 genotype, respectively, under none HA applied control and by 29.4, 50.8, and 65.9% in AC-Sterling, 47.7, 66.2, and 71.7% in Arak 2811, and 21.3, 40.3 and 67.2% in C111, respectively, under HA application.

The application of HA increased the photosynthesis rate in shoot and roots of all tested genotypes under all salinity levels (Table 4). At 0, 50, 100, and 150 mM NaCl photosynthesis rate was increased due to HA application by 27.7, 23.5, 30.9, and 27.2% in AC-Sterling, 60.8, 29.5, 11.3, and 26.9% in Arak 2811 and 22.1, 17.3, 33.4, and 18.7% in C111, respectively.

Table 5 The effect of HA levels on different NaCl levels

Experimental factor		Total chlorophyll concentration (mg g^{-1} FW)	Shoot dry matter (g/pot)	Root dry matter (g/pot)
HA	NaCl			
0	0	0.977^{b}	7.52^{b}	1.18^{b}
	50	0.673^{d}	5.76^{d}	0.8^{d}
	100	0.538^{e}	4.8^{e}	0.58^{e}
	150	0.349^{g}	3.78^{f}	0.34^{g}
1	0	1.2^{a}	9.09^{a}	1.51^{a}
	50	0.849^{c}	6.96^{c}	1.01^{c}
	100	0.647^{d}	5.42^{d}	0.73^{d}
	150	0.442^{f}	4.4^{e}	0.47^{f}

Mean values in the same column followed by at least one common letter, are not significantly different at the 5% probability level based on the LSD test

Chlorophyll content

The effects of the interactions between salinity and cultivar, HA and cultivar as well as salinity and HA were significant on leaf chlorophyll content (Table 1). Salinity decreased the content of chlorophyll in all tested genotypes (Table 2). At 50, 100, and 150 mM NaCl salinity levels compared to non-saline control, the content of chlorophyll was decreased by 21.8, 45.0, and 66.2% in AC-Sterling, 41.3, 53.1, and 59.7% in Arak 2811, and 25.8, 38.4, and 65.9% in C111, respectively.

Regardless of the salinity level, the application of HA increased the concentration of chlorophyll by 16.5, 9.2, and 48% in AC-Sterling, Arak 2811, and C111 cultivars, respectively (Table 3). On the other hand, irrespective of cultivar, HA application increased chlorophyll content by 22.8, 26.2, 20.3, and 26.5%, respectively, under 0, 50, 100, and 150 mM NaCl salinity levels (Table 5).

Root volume

The effect of the three-way interaction of salinity, cultivar and HA was significant on root volume (Table 6). Salinity decreased root volume of both HA applied and non-applied plants of all tested genotypes (Table 4). At 50, 100, and 150 mM NaCl compared to non-saline control, root volume was decreased by 72.7, 76.3, and 89.1% in AC-Sterling, 47.1, 57.2, and 61.4% in Arak 2811 and 37.5, 65.5, and 83.7% in C111 genotype, respectively, under none HA applied control and by 66.7, 78.3, and 91.1% in AC-Sterling, 16.7, 37.8, and 68.9% in Arak 2811, and 33.3, 63.3, and 70.0% in C111, respectively, under HA application.

The application of HA increased the root volume of all tested genotypes under all salinity levels. At 0, 50, 100, and 150 mM NaCl root volume was increased due to HA

Table 6 Analysis of variance of root volume, shoot and root dry matter and antioxidant enzymes activity

Source of variation	df	Mean square					
		Root volume	Shoot dry matter	Root dry matter	Catalase	Peroxidase	Ascorbate peroxidase
Genotype	2	90.6**	2.95**	0.429**	0.096**	2.16**	8.73**
Humic acid	1	121**	18.2**	0.761**	1.57**	17.2**	11.3**
NaCl	3	336**	59.2**	2.9**	1.05**	1.39**	8.34**
Genotype × HA	2	4.70*	1.97*	0.022	0.066**	1.35**	1.10**
Genotype × NaCl	6	7.35**	0.421	0.021	0.036**	0.019**	0.295**
HA × NaCl	3	8.63**	0.978*	0.04	0.161**	685**	0.044
Genotype × HA × NaCl	6	5.78**	0.559	0.014	0.026**	0.019**	0.088
Error	48	1.069	0.244	0.012	0.003	0.006	0.048

ns, *, and ** indicate non-significance at $P < .05$, significance at $P < .05$, and significance at $P < .001$, respectively

application by 63.6, 100, 49.8, and 33.0% in AC-Sterling, 28.5, 103, 86.6, and 3.8% in Arak 2811, and 11.1, 20.0, 10.0, and 107% in C111, respectively.

The activities of antioxidant enzymes

The effect of the three-way interaction of salinity, cultivar and HA was significant on the activities of CAT and POX (Table 6). Salinity increased CAT activity in both HA applied and non-applied plants of all tested genotypes (Table 4). At 50, 100, and 150 mM NaCl compared to non-saline control, CAT activity was increased by 46.0, 75.0, and 201% in AC-Sterling, 101, 267, and 417% in Arak 2811, and 32.8, 62.4, and 213% in C111 genotype, respectively, under none HA applied control and by 93.6, 306 and 543% in AC-Sterling, 53.8, 212 and 351% in Arak 2811, and 17.8, 64.3, and 138% in C111, respectively, under HA application. The corresponding increases in POX activity due to salinity were by 22.0, 55.3, and 108% in AC-Sterling, 7.10, 16.9, and 24.8% in Arak 2811, and 27.4, 55.2, and 129% in C111 genotype, respectively, under none HA applied control and by 156, 223 and, 313% in AC-Sterling, 48.1, 75.9 and 193% in Arak 2811, and 30.4, 51.9, and 87.4% in C111, respectively, under HA application (Table 4).

The application of HA increased the activities of CAT and POX in all tested genotypes under all salinity levels. At 0, 50, 100, and 150 mM NaCl CAT activity was increased due to HA application by 14.8, 52.1, 167 and 145% in AC-Sterling, 85.9, 42.2, 58.1, and 61.9% in Arak 2811, and 196, 163, 200, and 125% in C111, respectively and POX activity was increased by 116, 354, 349, and 330% in AC-Sterling, 83.4, 153, 176, and 330% in Arak 2811, and 421, 433, 410, and 326% in C111, respectively.

The effects of the interactions between salinity and cultivar and HA and cultivar were significant on the activity of APX (Table 6). Salinity increased APX activity in all tested genotypes (Table 2). At 50, 100 and 150 mM NaCl compared to non-saline control, APX activity increased by 12.3, 27.7 and 99.4% in AC-Sterling, 37.1, 57.1, and 77.6% in Arak 2811, and 22.8, 40.9, and 71.7% in C111 genotype, respectively.

Regardless of salinity level, the application of HA increased the activity of APX on average by 15.5, 43.5, and 42.0% in AC-Sterling, Arak 2811, and C111.

Shoot and root weights

The effects of the interactions between salinity and HA and also cultivar and HA were significant on shoot weight and the effects of the interactions between salinity and HA were significant on root weight (Table 6). Salinity, regardless of cultivar, decreased shoot and root weights. The significant interactions between salinity and HA showed that at 50, 100, and 150 mM NaCl compared to non-saline control, shoot weight was decreased by 23.4, 36.2, and 49.7% under non-applied HA and by 23.4, 40.4, and 51.6% under HA application (Table 5). The corresponding reductions in root weight were 32.2, 50.8, and 71.2% under non-applied HA and 33.1, 51.7, and 68.9% under HA application.

Humic acid application, regardless of cultivar, increased shoot dry weight by 20.9, 20.8, 12.9, and 16.4%, while it increased the root dry weight by 28.0, 26.3, 25.9, and 38.2% under 0, 50, 100, and 150 mM NaCl salinity treatments.

The significant interactions between cultivar and HA showed that regardless of salinity level, the application of HA increased shoot weight on average by 13.4, 14.0, and 28.0% in AC-Sterling, Arak 2811, and C111 (Table 3).

Although the interactions between salinity and cultivar were not statistically significant on shoot weight, the reduction in shoot weight at 50, 100, and 150 mM NaCl compared to non-saline control were 23.5, 37.1, and 48.6% in AC-Sterling, 22.8, 40.6, and 53.0% in Arak 2811, and 21.7, 34.4, and 49.9% in C111.

Discussion

This study demonstrates that the concentration of Cd in the shoots and roots of all tested genotypes was elevated due to increased salinity. Cd phytoextraction increase by soil salinization has also been reported in tobacco and maize (López-Chuken et al. 2012), wheat (Abbas et al. 2018), bean (Rady 2011), castor, and mustard (Bauddh and Singh 2012). Studies have shown that soil salinity may increase the mobility and phyto-availability of heavy metals such as Cd by forming chloric complexes and even by altering root system functions (Ashrafi et al. 2015; Filipović et al. 2018). In fact, the increment of chloride ions in Cd-contaminated soils alters free Cd^{2+} to Cd-Cl complexes in soil solution, and as a result, thereby facilitating the transfer of Cd from the soil to the plant (Filipović et al. 2018).

However, the safflower genotypes exhibited variations in shoot Cd concentration and also their increased Cd concentration under saline conditions. AC-Sterling displayed a lower shoot Cd concentration (22.6 mg/kg DW) compared to Arak 2811 (24.1 mg) and C111 (26.0 mg). Interestingly, the extent of Cd accumulation in response to salinity was smaller in C111 (19.6%) in contrast to AC-Sterling (48.2%) and Arak 2811 (43.2%; an average of 50, 100, and 150 mM NaCl). The absorption and translocation of Cd in plants and the tolerance to Cd toxicity differ among plant species, and even different genotypes of the same species (Pourghasemian et al. 2013) depending on the genes and gene networks that involve in physiological and molecular mechanisms (Benavides et al. 2005). Studies on wheat (Lu et al. 2021), maize (Wu et al. 2023), cotton (Wu et al. 2004), rapeseed (Mwamba et al. 2020), sunflower (Tan et al. 2023), and safflower (Pourghasemian et al. 2013; Pourghasemian et al. 2019) demonstrates the variation in Cd uptake and tolerance among plant genotypes.

The interaction effect of salinity × cultivar was not statistically significant, indicating that the reduction in shoot weight followed similar trends across the three cultivars when exposed to varying NaCl concentrations. Additionally, C111 exhibited lower reductions in K/Na ratio (74.7%) and photosynthesis rate (43.1%) due to salinity when compared to AC-Sterling (89.5% and 48.5%, respectively) and Arak 2811 (83.4% and 56.0%). These findings may indicate that C111 is a more tolerant genotype when it exposes to salt stress. Studies indicate that the accumulation of salt in the soil causes plant growth reduction by osmotic stress, ion toxicity, and unbalancing mineral uptake and translocation (Hussain et al. 2016). In line with our findings, genotypic variation in salinity tolerance has been reported in safflower by other researchers (Yeilaghi et al. 2015; Thoday-Kennedy et al. 2021; Badran et al. 2022). For example, a study on 200 safflower genotypes showed at least four different genotypic salt tolerance classes for safflower in which the salt tolerance in safflower genotypes depended on Na^+ entry prevention to shoots and K^+ uptake preservation (Thoday-Kennedy et al. 2021).

In the current study, the impact of salinity stress on antioxidant enzymes was examined, revealing an elevation in their activities. Remarkably, among the enzymes studied, catalase (CAT) demonstrated the highest increase as compared to POX and APX. All biotic and abiotic stresses in plants alter the metabolism of reactive oxygen species (ROS), and as a result, to detoxify aggressive ROS, antioxidant enzymes activities such as superoxide dismutase, ascorbate peroxidase, and catalase would be increased (Munns and Tester 2008; Chaki et al. 2020). Despite the less negative salinity effects on physiological properties of the C111 genotype, it showed less increase in antioxidant enzymes activity under different saline conditions than the other two genotypes. It seems that this genotype may possess alternative mechanisms for tolerating salinity.

This study also illustrates that HA application improved nutritional status, growth attributes, and the activities of antioxidant enzymes in all tested genotypes. Several studies have shown that HA's hormone-like activity and chelating capacity affect plant functions and development (Ampong et al. 2022). Macro and micro nutrients, plant height, leaf area, dry matter, photosynthetic efficiency, protein content, antioxidant enzymes activity, and plant yield enhancement due to HA application have been observed in wheat, rice, maize, faba bean, pea and rapeseed (Abou-Aly and Mady 2009; García et al. 2012; Jannin et al. 2012; Osman and Rady 2012; Radwan et al. 2015; Lotfi et al. 2018; Dawood et al. 2019; Liu et al. 2019). Plant morphological and physiological properties are affected by HA due to its structural characteristics (Tavares et al. 2021).

The application of HA resulted in significantly greater increases in shoot weight in C111 (28%) compared to AC-Sterling (13.4%) and Arak 2811 (14%). Additionally, C111 showed a greater increase in chlorophyll content (48%) compared to AC-Sterling (16.5%) and Arak 2811 (9.2%) due to the application of HA. Furthermore, the increases in shoot weight were accompanied by higher increases in CAT and POX in C111 (159.3% and 384.8% respectively) compared to AC-Sterling (113.6% and 302.6%) and Arak 2811 (59.4% and 203.3%). These findings suggest that while all three antioxidant enzymes played a role in improving safflower biomass production through HA application, CAT and POX had a much greater influence. Besides, the stimulating activities of these two enzymes had an important role in the superiority of C111 in response to HA application in terms of biomass production. Some other studies illustrated the different effects of HA on wheat (Abbas et al. 2022), maize (Shahryari et al. 2011), and sorghum (Kandil et al. 2017) genotypes.

The positive effects of HA application were found to be even more pronounced on roots, with a significant increase of 29.6%, compared to a slightly lower increase of 17.8% on shoots. This may clarify the improvement of plant nutritional status under HA application. These results are in agreement with those of Rosa et al. (2018), which similarly observed a more noticeable impact of HA on wheat roots.

HA application had greater positive effects on biomass production under no-saline and low salinity treatments. Different results were obtained by (Jarošová et al. 2016), as in their study the application of HA had a greater positive effect on biomass production and growth attributes of barley under higher salinity levels. These incompatible results may be described by different HA levels and/or by different genotypes used in the two experiments. Nevertheless, in the current study, the larger effects of HA on roots under higher salinity levels were well-matched with the results reported by others.

Conclusion

Variation, in this study, existed among safflower genotypes in terms of Cd concentration and the response to salinity and HA application. Among the three genotypes, C111 stood out as superior in terms of having lower increases in Cd levels and less decrease in K/Na ratio and photosynthesis under salinity stress. Additionally, C111 exhibited the most positive response to HA application. The activities of antioxidant enzymes CAT, POX, and APX provided a significant role in differentiation responses of safflower genotypes to both salinity and HA application. Notably, HA application proved to be more effective in promoting biomass production under lower salinity levels.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors report there are no competing interests to declare.

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