



Humic acids mitigate salt and drought stress in soybean (*Glycine max* (L.) Merr.) in vitro cultures

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Abstract

The aim of this study was to evaluate the protective effect of humic acids (HA) with different molecular weight fractions on the soybean Progres cultivar under drought and salinity stress in in vitro. HA were isolated from peat samples according to the International Humic Substances Society procedure. Three HA treatments were tested: HA<30 kDa, HA>30 kDa, and unfractionated HA (Mix). Sterilized soybean seeds were cultured on nutrient media supplemented with 100 mmol·dm⁻³ NaCl or 150 mmol·dm⁻³ Mannitol to simulate salinity and drought stress, respectively. HA fractions were added at 0.005 g C_{HA}·dm⁻³. No stress factors were used in the control samples. Biometric parameters (plant height, leaf number, root length, shoot and root biomass) and micromorphological traits (stomatal density and length) were measured. Micro- and macroelement contents in dry seedling matter were also analyzed. Soybean Progres exhibited greater sensitivity to salt than drought stress, shown by reduced biometric and micromorphological parameters and altered element contents. HA treatments demonstrated a protective role, which was dependent on the molecular fraction. The HA>30 kDa fraction and HA Mix provided the greatest protective and, at times, stimulatory effects, notably increasing stomatal density and biometric values under stress. HA, in the presence of salt and drought stress, did not specifically affect the uptake of the analysed micro- and macroelements by soybeans. The fractions HA<30 kDa and HA>30 kDa caused a decrease in the uptake of most analyzed elements. Unfractionated HA predominantly mitigated the effects of applied stresses. For HA Mix, the levels of micro- and macroelements in soybean seedlings were generally comparable to those in control plants.

Key message

This in vitro study demonstrates that humic acids, particularly those with a molecular weight greater than 30 kDa and unfractionated fractions, protect soybean Progres seedlings from drought and salinity stress by enhancing growth, stomatal traits, and nutrient balance.

Keywords Abiotic stress · NaCl · Mannitol · Humic acids · Soybean · Growth · Nutrients · Stomata

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Introduction

Traditional agriculture is at the forefront of climate change, being particularly affected by the increasing intensity and frequency of extreme events, such as floods, droughts, and rising temperatures (Canellas et al. 2024). Throughout their life cycle, plants are exposed to various stresses, including both biotic and abiotic factors. Salinity and drought are among the most significant abiotic stressors affecting plant growth and development. These stressors trigger morphological, biochemical, and molecular changes that severely limit crop production worldwide (Liu et al. 2017; Sahin et al. 2018; Bai et al. 2019; Singha et al. 2024; Akbari et al. 2024). Early responses to drought and salinity are similar, as both induce water stress that leads to reduced growth, decreased stomatal aperture, and nutrient deficiencies (such as K^+ and Ca^{2+}). However, long-term salt stress, in addition to causing dehydration, also leads to ionic stress, which contributes to leaf senescence and impaired photosynthesis (Feng et al. 2020; Ma et al. 2020; Cao et al. 2023).

Soybean (*Glycine max* (L.) Merr.) is one of the most essential leguminous crops, widely cultivated across diverse climates. It plays a pivotal role in global nutrition and food security, serving as a major source of both edible oil and plant-derived protein for humans and livestock. Globally, approximately 70% of plant-based proteins and 29% of edible oil are derived from soybeans (Hossain et al. 2024). In addition to its nutritional value, soybeans hold significant economic importance, providing raw materials for the animal feed and food industries. In recent years, soybeans have attracted increasing attention due to their high protein content, vitamin C, rich mineral composition, and reported anti-cancer properties (Cao et al. 2017; Feng et al. 2020). Moreover, soybean oil is considered a promising future bio-fuel source, with ongoing efforts to enhance soybean-derived biodiesel production (Santos et al. 2024). Under natural conditions, soybeans are exposed to various abiotic stress factors, including salinity and water deficiency. To achieve high yields, soybeans require an adequate water supply throughout their growth cycle (Buezo et al. 2019; Dong et al. 2019; Feng et al. 2020). Studies have demonstrated that both water deficiency and salinity stress negatively affect soybean growth and development (Matuszak-Slamani et al. 2017; Bai et al. 2019; Khattab et al. 2019; Matuszak-Slamani et al. 2022). Soybeans are moderately salt-tolerant and can be cultivated in light to moderately saline soils; however, their yields decline under salinity stress (Anwar-ul-Haq et al. 2023; Nasution et al. 2024).

To mitigate the negative impacts of abiotic stresses, various agronomic and biological strategies are employed. One example is the use of rhizosphere microorganisms, such as plant growth-promoting rhizobacteria, which enhance

nutrient uptake, reduce the toxic effects of salts, and support root development under challenging conditions (Khan et al. 2021; Kang et al. 2023; Handayani et al. 2024). Another approach involves appropriate soil management practices, such as mulching and balanced fertilization, which improve moisture conditions and alleviate stress caused by soil salinity (Jabeen et al. 2021). One of the most effective solutions is the application of biostimulants, which enhance plant physiological responses by strengthening their adaptive capabilities under stressful conditions. For instance, salicylic acid and algal extracts have been shown to significantly improve soybean drought tolerance by supporting antioxidant mechanisms (Ren et al. 2023).

Humic substances (HS) are among the most active components of soil organic matter. As polymeric substances with complex structures, they are formed through the condensation of biomass from microorganisms, plants, and animals. HS influence on the physical, chemical, and biological properties of soil by improving aggregate stability, buffering capacity, sorption of hydrophobic organic compounds, nutrient transport and bioavailability, as well as environmental metal complexation. In addition, they enhance the soil's ion exchange capacity and water retention (Ouni et al. 2014).

Humic substances (HS) are primarily composed of three fractions: humin, humic acids (HA), and fulvic acids. Humin are highly heterogeneous and recalcitrant fractions of soil organic matter that remain insoluble in both organic and inorganic solvents across the entire pH spectrum. Due to their insolubility and limited biological reactivity, humin receive less attention compared to other fractions: humic acids and fulvic acids (Ukalska-Jaruga et al. 2021). Humic acids (HA) and fulvic acids (FA) exhibit both chemical reactivity and resistance to microbial degradation, making them highly beneficial to soil and plant systems (Nardi et al. 2021; Ore et al. 2023; Canellas et al. 2024).

Humic acids (HA) are the major high-molecular-weight fraction of humic substances, formed during long-term decomposition of organic residues and organized into supramolecular aggregates rich in carboxyl and phenolic groups. Humic acids do not have a single, fixed molecular weight. Instead, they exist as complex, polydisperse mixtures of heterogeneous organic molecules (Nardi et al. 2021). Because of this, their molecular weight depends on the method used to measure them and the degree of aggregation. Humic acids are considered supramolecular associations held together by weak interactions (H-bonding, π - π stacking, hydrophobic interactions), so their 'molecular weight' reflects the size of aggregates (Piccolo and Drosos 2024). Humic acids are considered a potential strategy to mitigate the harmful effects of abiotic stress. They can be applied as plant growth stimulants and soil conditioners, and they can also reduce

irrigation frequency, improve water use efficiency, and mitigate the effects of drought stress on plants (Olk et al. 2018; Franzoni et al. 2022; Matuszak-Slamani et al. 2022; Rak-kammal et al. 2023; Canellas et al. 2024; Maffia et al. 2025). The effect of humic acids on plant growth depends on several factors, including their origin, concentration, molecular weight, application method, plant species, and developmental stage (Nardi et al. 2002, 2021).

Due to the complexity of HS structures and their certain fractions, it is important to identify which of them play a key role in plant growth and development under abiotic stress conditions, as the mechanisms of HS action are complex and require further research. Therefore, the aim of this study was to evaluate the protective effect of humic acids (HA) and their molecular fractions on the growth of soybean seedlings in *in vitro* cultures under laboratory-induced salinity and drought stress conditions.

Materials and methods

Extraction and fractionation of humic substances

The starting material for obtaining humic acids fractions was peat soils in the active accumulation stage of organic matter, located in Babia Góra National Park, Poland GPS: N 49°35'45"–E 19°30'21". Peat samples were collected from the subsurface layer up to a depth of 50 cm. The air-dried peat samples, with a moisture content not exceeding 12%, were homogenized after removing undecomposed plant residues. Humic acids were extracted using the method recommended by the International Humic Substances Society (Swift 1996). The first step of HA extraction included: decalcification of peat samples using HCl, extraction with $0.1 \text{ mol} \cdot \text{dm}^{-3}$ NaOH, precipitation of HA with $6 \text{ mol} \cdot \text{dm}^{-3}$

HCl, and purification of HA through deashing with an HCl/HF mixture, followed by washing with distilled water until no chlorides were detected. The purified, unfractionated humic acids was referred to as HA Mix. HA Mix preparation was frozen and lyophilized to achieve a constant mass.

The second step focused on the fractionation of HA Mix into molecular weight fractions by using an Amicon model 8400 apparatus equipped with Millipore filters with a 30 kDa molecular weight cut-off. The device comprises three main modules (Fig. 1). Module 1 supplies nitrogen under controlled pressure, which drives the separation of the polymer into distinct molecular fractions. Module 2 is a container equipped with a magnetic stirrer, where the solution to be fractionated is placed. Module 3 contains the Millipore membrane mount, and a nozzle connected to a hose that directs the filtered solution to an external collection container.

Millipore membranes can only be used with solutions within a pH range of 2 to 10. Therefore, appropriate amounts of humic acids were first dissolved in $0.1 \text{ mol} \cdot \text{dm}^{-3}$ NaOH and then diluted fivefold. The solution was then passed through the Amberlite H⁺120 ion-exchange resin to replace Na⁺ ions with H⁺ ions. The resulting acidic solution was transferred to the container (Module 2) equipped with a magnetic stirrer. Module 3, containing a Millipore filter with a 30 kDa cut-off, was mounted at the bottom. Module 1, supplying nitrogen, was positioned above Module 2 (the container holding the sample to be separated). The filtrate containing the fraction with a molecular weight below 30 kDa (HA < 30 kDa) was collected in a separate vessel and concentrated at a temperature not exceeding +40 °C. The concentrated solution was then frozen using a dry ice–alcohol mixture at –70 °C and lyophilized to constant weight. The supernatant containing the fraction with a molecular weight > 30 kDa (HA > 30 kDa) was similarly frozen using a dry ice–alcohol mixture at –70 °C and lyophilized to constant weight. The resulting HA preparation - HA > 30 kDa and HA < 30 kDa fractions, were dissolved in Michaelis buffer at pH 7.17. Chemical and spectral analyses of the molecular HA fractions and the unfractionated preparation were presented in our previous studies: Gawlik et al. (2016), Matuszak-Slamani et al. (2017, 2022).

In vitro cultures condition

Soybean seedlings of the Progres cultivar were analyzed under laboratory-induced stress conditions to evaluate the effects of salinity and drought, as well as the protective role of humic acids against these abiotic stresses. Soybean seeds of the Progres cultivar used in the *in vitro* studies were sterilized prior to the experiment. They were immersed in 70% ethanol for 10 s and then treated with 0.2% mercuric

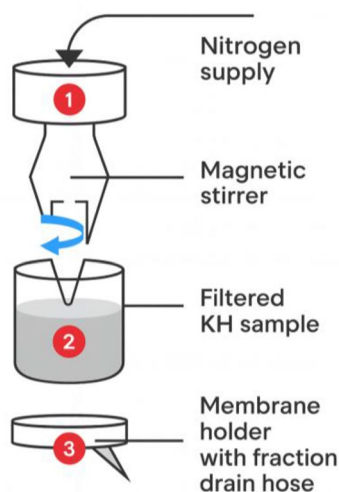


Fig. 1 Schematic of the Amicon 8400 device

chloride (HgCl_2) for 12 min. To prevent contamination, all subsequent procedures were carried out under sterile conditions in a laminar airflow chamber (Caetano-Anollés et al. 1990).

The Murashige and Skoog (1962) medium was used in the experiment. The pH of the medium was adjusted to 5.7 using $0.1 \text{ mol} \cdot \text{dm}^{-3}$ solutions of hydrochloric acid (HCl) and sodium hydroxide (NaOH). The media were supplemented with $8 \text{ g} \cdot \text{dm}^{-3}$ agar, $30 \text{ g} \cdot \text{dm}^{-3}$ sucrose, and $100 \text{ mg} \cdot \text{dm}^{-3}$ inositol. After the addition of the dry components, the media were heated to allow polymerization and solidification, poured into 900 cm^3 jars, and sterilized in an autoclave at 121°C for 20 min.

In the first stage of the *in vitro* study, the effects of salinity and drought on soybean growth and development were assessed.

For this purpose, sterilized soybean seeds were placed on a nutrient medium, to which $100 \text{ mmol} \cdot \text{dm}^{-3}$ NaCl and $150 \text{ mmol} \cdot \text{dm}^{-3}$ mannitol were added to simulate salinity and drought stress, respectively. The concentration of stress-inducing factors was selected based on previous studies (Kulpa et al. 2018). Simultaneously, the protective effect of humic acids (HA) on soybean growth and development under these abiotic stress conditions was investigated. Molecular fractions of HA were added to the nutrient medium containing the appropriate stress factor (NaCl or mannitol): humic acids with molecular weights less than 30 kDa ($\text{HA} < 30 \text{ kDa}$), greater than 30 kDa ($\text{HA} > 30 \text{ kDa}$), or unfractionated humic acids (HA Mix). For all tested molecular fractions, the concentration of HA in the nutrient

medium was identical, amounting to $0.005 \text{ g C}_{\text{HA}} \cdot \text{dm}^{-3}$. The control group consisted of sterilized soybean seeds of the Progres cultivar placed on Murashige and Skoog nutrient medium without stress factors.

Each experimental variant for each stress condition consisted of control (1) and four treatments: stress (2), stress + $\text{HA} < 30 \text{ kDa}$ (3), stress + $\text{HA} > 30 \text{ kDa}$ (4), and stress + HA Mix (5). All tested stresses and their combinations were applied simultaneously under controlled *in vitro* culture conditions. Ten jars were prepared for each combination, with ten seeds placed in each jar, resulting in a total of 100 seeds per combination (Fig. 2). The experiment was conducted in a phytotron at $25 \pm 1^\circ\text{C}$, under a light intensity of $40 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and a 16-hour photoperiod. After 21 days of soybean growth, biometric parameters, micromorphological features, and the content of micro- and macrolelements in the dry mass of soybean seedlings were measured.

Biometric measurements

Biometric measurements included plant height, number of leaves, root length, fresh weight, and the weight of the over-ground parts of soybean seedlings were conducted using an electronic caliper (Varel, accuracy $\pm 0.01 \text{ mm}$), an analytical balance (Radwag AS 82/220.R2 PLUS), and a moisture analyzer (Radwag MA 50.R).

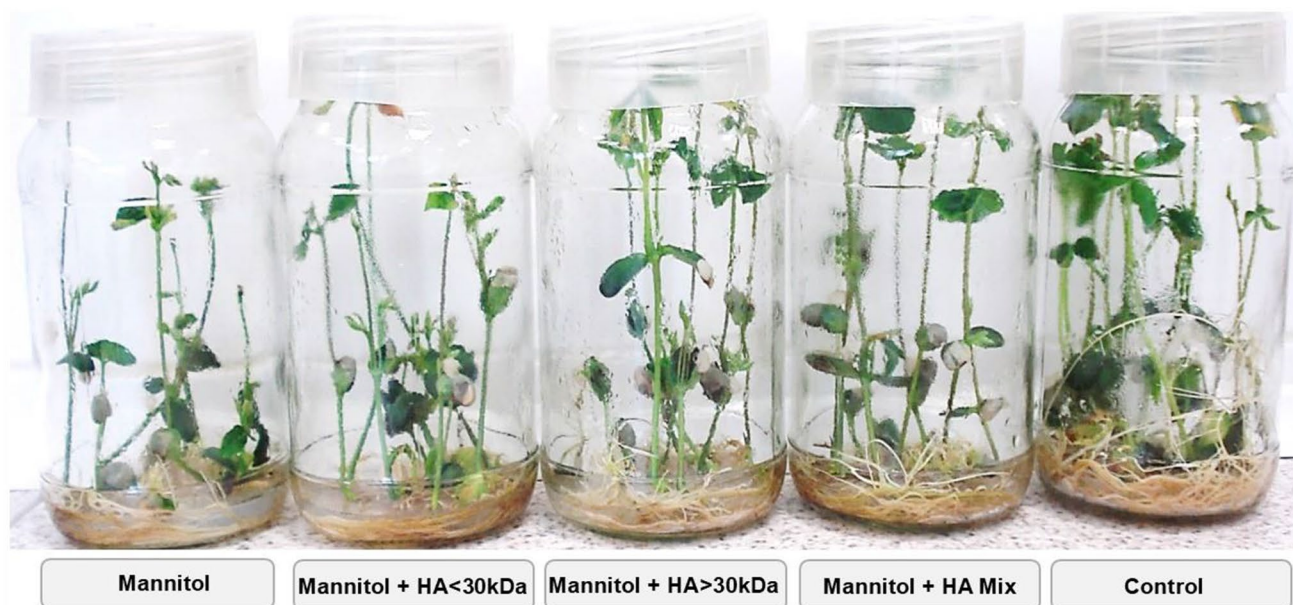


Fig. 2 Soybeans growing on medium with the addition of (from the left): mannitol; mannitol and $\text{HA} < 30 \text{ kDa}$; mannitol and $\text{HA} > 30 \text{ kDa}$, mannitol and unfractionated humic acids (HA Mix) and control medium

Micromorphological analysis

Leaves isolated from the middle part of soybean stems grown on media enriched with NaCl, mannitol, and humic acids were subjected to micromorphological analysis using a scanning electron microscope (SEM). The material was dried using a Critical Point Dryer (Quorum Technologies) and then coated with a gold layer using a Sputter Coater (Quorum Technologies). Observations were conducted with a Carl Zeiss EVO LS 10 microscope at an accelerating voltage of 15 kV. Determined stomatal length (μm) and stomatal density (the number of stomata per mm^2) on the surface of soybean leaf blades. Measurements were made on the abaxial (lower) surface of the soybean leaves.

Micro- and macroelement analysis

The content of five microelements (Cu, Mn, Zn, Fe, and Co) and four macroelements (Mg, Ca, K, and Na) in the dry matter of soybean seedlings of the Progres cultivar grown in vitro was determined. These elements were present in the basic medium (according to Murashige and Skoog 1962). The soybean seedlings were dried to constant weight at 105 °C, ground, and mineralized in a mixture of concentrated HNO_3 and HClO_4 acids in a 3:1 ratio. All determinations were performed in triplicate. Quantitative analysis was performed using the Solar S4 Atomic Absorption Spectrophotometer with an acetylene-air flame atomizer.

Statistical analysis

The experiment with plants in vitro cultures was conducted with 10 replications, each containing 10 plants (a total of 100 plants per experimental variant). Data for micro- and macrominerals are presented as the mean $\pm$ SD from three replicates. During the micromorphological analysis, 20 photos of leaf blades were taken for each combination, determining the stomatal density/ mm^2 . The length of 10 stomata randomly selected from each photo was determined, for a total of 200 for each combination. One-way analysis of variance (ANOVA) was used to compare mean values. The analysis formed the basis for identifying homogeneous groups using Tukey's test at a significance level of $p \leq 0.05$. Means that differed significantly were marked with different letters. The results are presented in graphs and tables separately for each stress factor (mannitol or NaCl). Statistical analysis was performed using Statistica 13.1 PL (StatSoft, Kraków, Poland).

Results and discussion

Research on the influence of humic acids on plant development has so far focused mainly on field, pot, or hydroponic experiments. In contrast, conducting research in vitro cultures allows for better standardization and more precise assessment of the effects of the studied factors on changes in plant morphological traits. In our study, we assessed the protective effects of humic acids and their molecular fractions on the growth of soybean seedlings in in vitro cultures under laboratory-induced salinity and drought stress conditions.

Growth in in vitro cultures

Salt stress, primarily induced by sodium chloride (NaCl), is one of the major abiotic factors affecting plant growth and development under both natural conditions and in vitro cultures (Parida and Das 2005). According to our experiment, the best growth parameters - longer shoots and roots, the highest shoot and root biomass, and the greatest number of leaves - were observed in soybean plants grown on control medium without stress factors (NaCl and mannitol). These differences were statistically significant for both stresses. Under salt stress, plant height decreased by 80%, root length by 65%, number of leaves by 79%, root weight by 51%, and above-ground biomass by 85% (Fig. 3). The results confirm that soybean is a salt-sensitive legume, and salinity impairs seed germination, seedling growth, and overall plant development (Rasheed et al. 2022). Under salt stress conditions, plants exhibit morphological changes such as reduced leaf size, delayed root growth, decreased biomass, and shortened shoot length. In in vitro cultures, salt stress can also hinder tissue regeneration processes, including somatic embryogenesis and rooting ability (Begum et al. 2022; Açıkbaş et al. 2023; Gobade et al. 2024). Salinity inhibits overall plant growth by reducing the osmotic potential of the growing medium, causing specific ion toxicity, inducing oxidative stress, and decreasing nutrient uptake (Parida and Das 2005; Liu et al. 2017; Atero-Calvo et al. 2024; Hossain et al. 2024).

The effect of mannitol on agricultural plant development has been widely studied, mainly due to its ability to regulate water management (Khatoon et al. 2018). Mannitol is commonly used as an osmotic stress agent, limiting water availability, disrupting metabolic processes, and reducing the rates of photosynthesis, growth, and protein synthesis in plants (Adrees et al. 2015; Saadaoui et al. 2023). Under drought stress, plant height decreased by 24%, root length by 55%, number of leaves by 36%, root weight by 50%, and above-ground biomass by 43% (Fig. 4). These results are consistent with our previous studies (Matuszak-Slamani

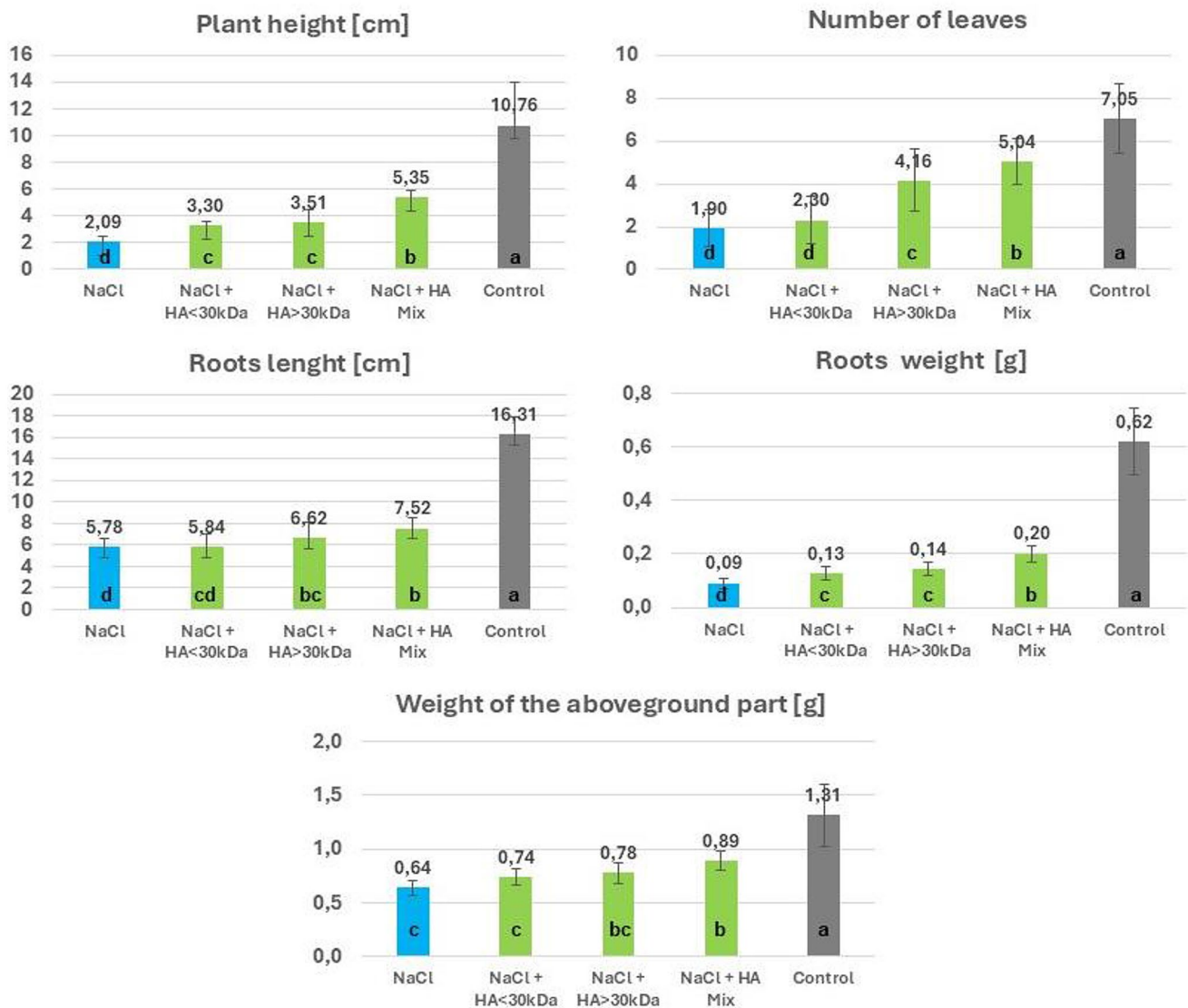


Fig. 3 Influence of humic acids on biometric parameters of overground parts of Progres soybean seedlings under salt stress conditions (Combinations: NaCl - salt stress; NaCl+HA < 30 kDa – salt stress+humic acids fraction < 30 kDa; NaCl+HA > 30 kDa – salt stress+humic acids

fraction < 30 kDa; NaCl+HA Mix – salt stress+unfractionated humic acids; C – control – medium). Values marked with the same letters do not differ significantly according to Tukey's test ($p \leq 0.05$)

et al. 2022) concerning soybean, as well as with studies by Ahmad et al. (2022) and Abu-Ria et al. (2024), which focused on the growth of sorghum and maize under drought stress conditions. The decrease in the analyzed biometric parameters is mainly attributed to drought-induced disruptions in water absorption, which leads to increased cell dehydration and inhibits cell division, expansion, and proliferation (Wahab et al. 2022).

Recent studies emphasize the key role of humic substances (HS), particularly humic acids (HA), in mitigating the adverse effects of abiotic stress and enhancing plant resilience (Nardi et al. 2021; Canellas et al. 2024; Nabi et al. 2025; Maffia et al. 2025). Our research demonstrated that

under salt stress, it is possible to enhance plant tolerance using humic acids. This is confirmed by the increased values of the analyzed parameters in soybean seedlings, particularly in the aboveground parts (Fig. 3).

Among the three HA combinations tested, the > 30 kDa fraction and the unfractionated preparation (Mix) had the most beneficial effects. Compared to the NaCl treatment alone, a significant increase was observed in plant height (58–156%), number of leaves (119–165%), and fresh mass of the overground parts (44–122%) (Fig. 3). For the root-related parameters, the protective effect of HA was less pronounced. Root mass and length increased by 16–39% and 14–30%, respectively, compared to plants subjected to salt

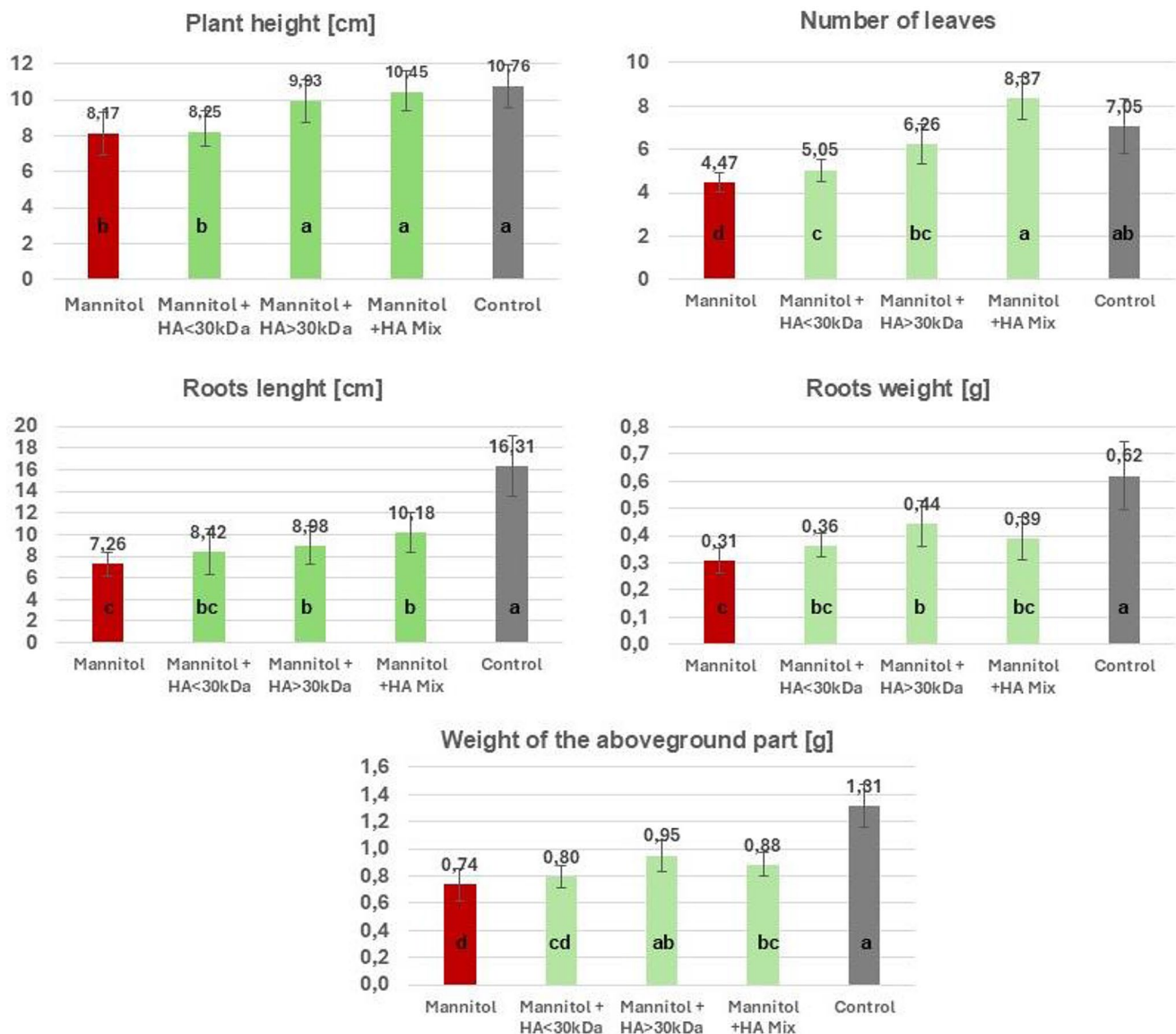


Fig. 4 Influence of humic acids on biometric parameters of over-ground parts of Progres soybean seedlings under drought stress conditions (Combinations: M (Mannitol) - drought stress, M+HA<30 kDa – drought stress+humic acids fraction<30 kDa; M+HA>30 kDa –

drought stress+humic acids fraction<30 kDa; M+HA Mix – drought stress+unfractionated humic acids; C – control – medium). Values marked with the same letters do not differ significantly according to Tukey's test ($p \leq 0.05$)

stress alone (Fig. 3). The obtained results indicate that the addition of humic acids activates many components of the ion homeostasis machinery in the presence of NaCl ions, thus highlighting their protective effect (Khaleida et al. 2017; Souza et al. 2021; Canellas et al. 2024; Nabi et al. 2025).

In the case of the second stress factor – drought, the protective effect of humic acids was less pronounced. However, similarly to salinity stress, their protective effect was most significant for the HA>30 kDa fraction and the HA Mix. Under drought conditions, the addition of humic acids led to an average increase of 20–40% in the analyzed biometric

parameters. An exception was observed for the HA Mix, where the number of leaves increased by 87% (Fig. 4).

Research shows that humic acids (HA) significantly alleviate drought-related stress in various plant species (sorghum, maize, rice, and wheat) by enhancing growth characteristics and biomass accumulation in leaves, stems, and roots (Ahmad et al. 2022; Mutlu et al. 2022; Abu-Ria et al. 2024). Humic acids exhibit pleiotropic effects, influencing plant growth, nutrient uptake, and stress tolerance (Nardi et al. 2021; Matuszak-Slamani et al. 2022; Abu-Ria et al. 2024; Maffia et al. 2025). Nardi et al. (2021) concluded that humic acids can stimulate chlorophyll synthesis and the

activity of enzymes responsible for regulating osmotic pressure in plant cells. Humic substances also support the reconstruction of cellular structures, resulting in improved plant growth under osmotic stress conditions.

Stress caused by drought and salinity leads to the production of reactive oxygen species (ROS) in plants, such as superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen, which are generated through the reduction of oxygen in cells (Nadarajah 2020). Excessive accumulation of ROS induces oxidative stress, resulting in damage to lipids, proteins, and DNA, disruption of membrane fluidity, loss of enzyme activity, protein cross-linking, inhibition of protein synthesis, and ultimately, cell death (Nadarajah 2020; Kumari et al. 2022). Plants counteract this through ROS-scavenging mechanisms, including both enzymatic and non-enzymatic antioxidants (Panda et al. 2024). Humic acids (HA), due to their phenolic and quinone groups, effectively neutralize free radicals, forming a protective barrier and enhancing the plant's innate defense mechanisms (Yang et al. 2021).

Micromorphological analysis

Stomata regulate the exchange of CO₂ and water vapor between the leaves and the atmosphere, serving as key indicators of a plant's resilience to environmental changes. Stomatal density directly influences photosynthesis and transpiration processes (Sultana et al. 2024). Example

photos of the leaf surface of control soybean plants grown in vitro cultures are shown in Fig. 5. The average stomatal length determined for control plants was 118.72 μm , and the average stomatal density was 296.85 mm^{-2} . It was found that the analyzed stresses (salinity and drought) had a negative effect on stomata.

Compared to the control of soybean plants, salinity stress significantly reduced stomatal length by 16%, while stomatal density decreased by 40%. In the case of drought, changes in the analyzed stomatal parameters also showed significant decreases, amounting to an 11% reduction in stomatal length and a 61% reduction in stomatal density (Figs. 6 and 7). Stomatal development adapts to environmental conditions throughout the plant growth cycle (Sultana et al. 2024), often by altering the density and size of stomata on the plant epidermis. Abiotic stresses, such as drought, decrease stomatal length and density (Han and Torii 2016; Lawson and Vialet-Chabrand 2019; Mohi-Ud-Din et al. 2025). Hughes et al. (2017) suggest that this is associated with the potential for reduced transpiration and improved water management by plants.

The use of humic acids in combination with salinity stress has a positive effect on stomata. The most beneficial effect was observed for the HA > 30 kDa fraction, where stomatal length increased significantly by 15%. Stomatal density increased by 144% compared to stress treatment and by 47% compared to control. For the other fractions, the

Fig. 5 Leaf surface of soybean cultured on control medium (a and b) and medium with NaCl (c) and mannitol (d) with visible stomata (a- magnification x 300; b- magnification x 1000)

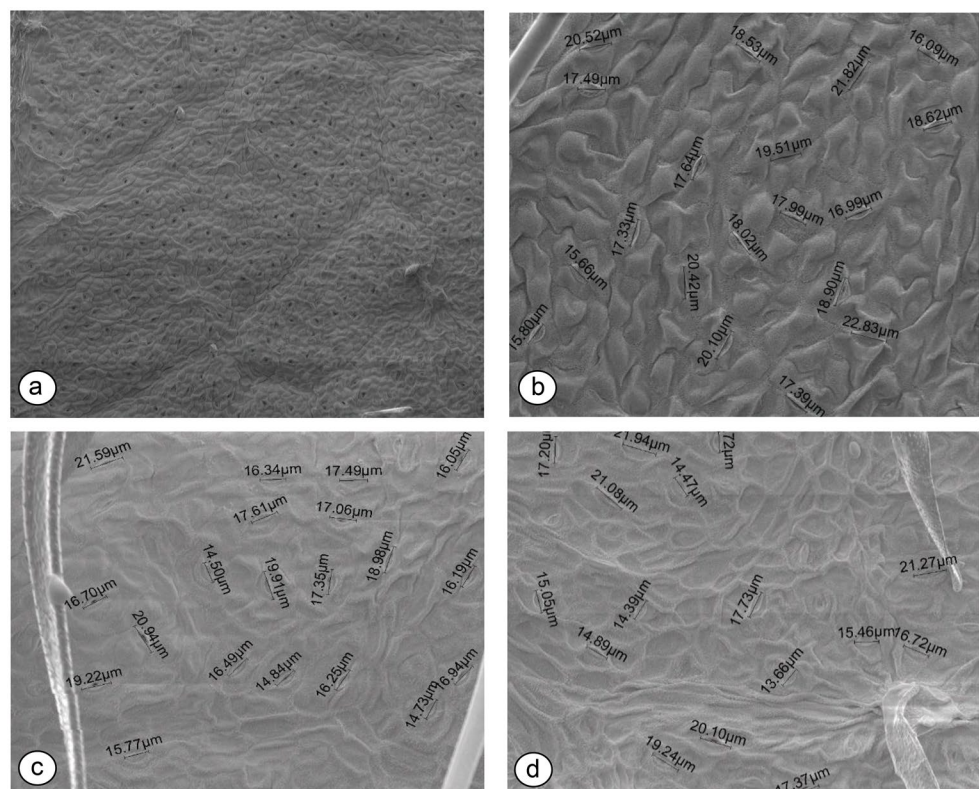


Fig. 6 The length and stomatal density on the surface of soybean leaf blades under salt stress conditions. Explanations as in Fig. 3

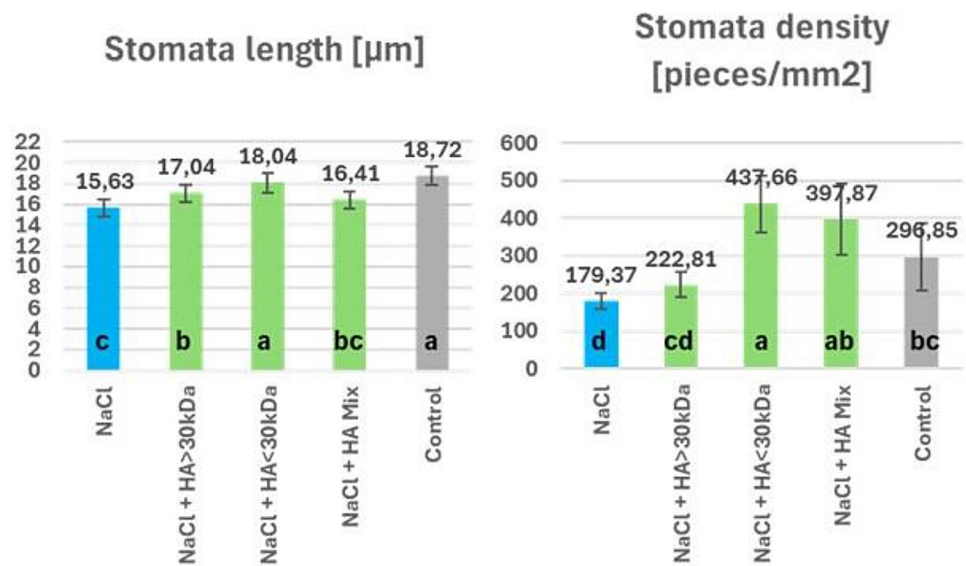
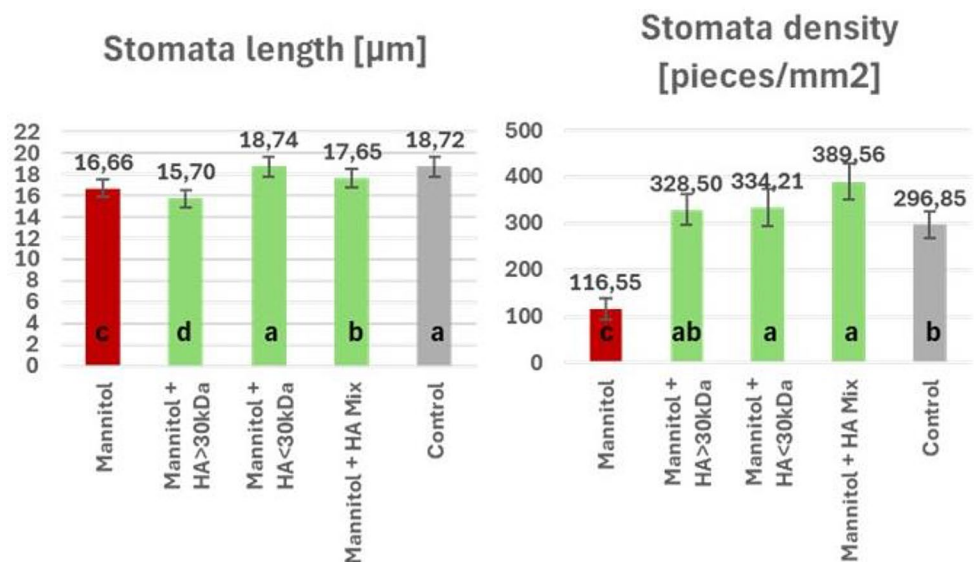


Fig. 7 The length and density of stomata on the surface of soybean leaf blades under drought stress conditions. Explanations as in Fig. 4



changes were smaller but comparable to the control values (Fig. 6).

In the case of drought stress, a significant increase in stomatal length ranging from 6% to 12% was observed only for two fractions: HA > 30 kDa and HA Mix, reaching values comparable to those of control plants. A significant effect of humic acids was also found in the case of stomatal density. This parameter increased markedly for all analyzed HA fractions, ranging from up to 182% (HA < 30 kDa) to 234% (HA Mix). The obtained values were also higher than those of the control plants from 11% (HA < 30 kDa, not statistically significant) to 31% (HA Mix, statistically significant) (Fig. 7).

Soybean is an amphistomatic species, with a higher stomatal density on the abaxial side than on the adaxial side. Stomata are distributed randomly across the leaf surface

(Amaliah et al. 2019; Sultana et al. 2024). Based on our results, it can be concluded that humic acids fractions, particularly HA > 30 kDa and HA Mix exhibit not only protective but also stimulatory effects, increasing stomatal density on soybean leaves exposed to stress conditions.

Micro- and macroelement analysis

The effect of humic acids (HA) on element uptake by plants depends on multiple factors, primarily the origin, type, dosage, and structural properties of HA, as well as the pH of the rooting medium, the concentration of exogenous elements, and the plant species (Nardi et al. 2017). The mechanism of element uptake by plant roots under natural conditions is complex, and the additional influence of humic substances and environmental stresses (such as drought and salinity)

makes it even more intricate and dependent on multiple factors.

The research conducted in this study on the effects of salt stress (NaCl), drought stress (mannitol), and humic acids fractions on the content of macro- and microelements in the leaves of the soybean cultivar Progres indicates differences in their accumulation. The impact of salt stress on the uptake of microelements from the medium by soybean seedlings of the Progres cultivar grown in *in vitro* cultures was observed (Table 1). According to Sahasakul et al. (2023), microelements are generally less susceptible to salt stress than macroelements. In our study, a decrease in the content of Mn, Zn, Fe, and Co was observed compared to the control, ranging from 15.41% (Mn) to 4.02% (Fe). A significant increase in Cu uptake by soybean seedlings was noted, exceeding 140%. On the other hand, Celik et al. (2011) and Katkat et al. (2009) reported that the foliar application of humic acids had a statistically significant positive effect on the uptake of Cu, Zn, Mn, Mg, and Fe by wheat.

The application of humic acid (HA) fractions in combination with NaCl modified microelement uptake by soybean seedlings of the Progres cultivar (Table 1). Salt stress alone increased Cu content relative to the control, whereas all HA treatments reduced Cu to control-like levels, indicating effective mitigation of NaCl-induced Cu accumulation. In contrast, Mn and Zn contents were reduced under salinity compared with the control, and all HA fractions maintained these elements at similarly lowered levels. Only the HA > 30 kDa fraction partially alleviated the negative effect of NaCl on Zn, increasing its content by 8.9% relative to NaCl alone and to a value close to the control.

The most pronounced HA effect was observed for Fe. While NaCl and HA < 30 kDa slightly reduced Fe content compared with the control, the HA > 30 kDa fraction in combination with NaCl almost doubled Fe content ($\approx 80\%$ increase vs. control), suggesting enhanced Fe availability, presumably via chelation and improved root uptake capacity to absorb microelements from the media (Zanin et al. 2019; Alvarez et al. 2023). The NaCl + HA Mix treatment restored Fe levels to a level comparable to those of control seedlings. Co content was increased only in the NaCl + HA < 30 kDa and NaCl + HA > 30 kDa combinations (by about 20% vs.

control, NaCl alone and NaCl + HA Mix), indicating fraction-specific stimulation of Co uptake.

Salt stress also affected the uptake of the analyzed macroelements. Among the four elements examined Na, K, Mg, and Ca, only Na showed increase in content compared to the control, with an approximately 350% rise in soybean seedlings. In contrast, the remaining macroelements K, Mg, and Ca experienced reductions in content by 19.3%, 27.5%, and 28.9%, respectively, confirming the harmful effects of salt stress on the uptake of essential nutrients by plants. Salt stress results in an ionic imbalance, leading to high cytosolic accumulation of Na^+ and Cl^- , which disrupts cellular homeostasis and interferes with essential metabolic processes in plants (Matuszak et al. 2009; Malekzadeh et al. 2024; Pour-Aboughadareh et al. 2024). Excessive sodium ions can displace other essential elements such as K, P, and Mg, which are crucial for enzyme activation and osmotic regulation, leading to impaired nutrient uptake and reduced plant growth (Atero-Calvo et al. 2024; Joshi et al. 2025). Furthermore, elevated chloride levels impair photosynthesis and cell membrane integrity, ultimately reducing plant vitality and productivity (Liu et al. 2017).

The effect of HA fractions applied in combination with NaCl on the macroelement content of soybean seedlings of the Progres cultivar was variable (Table 2). For Ca and Mg, the amounts measured in soybean seedlings were similar to those found in plants under salt stress (lower than the control). However, HA fractions mitigate the negative effects of stress on Na and K uptake. The ability of HA to adsorb exchangeable cations such as Mg, Ca, and K, preventing their leaching by percolating water, helps ensure the availability of these elements for plant uptake (Yang et al. 2021).

The measured K content in soybean seedlings treated with NaCl combined with humic acid molecular fractions < 30 kDa, > 30 kDa, and the HA Mix was comparable to the control level. In the case of Na, high content was observed in all tested plants subjected to salt stress, but this was significantly reduced by all analyzed humic acids molecular fractions. The greatest reduction in Na uptake by soybean seedlings was observed with the HA Mix, which decreased Na content by more than 30% (Table 2). A similar decrease in Na content in the shoots and roots of pepper with increasing doses of humic acids was reported by Çimrin et

Table 1 The content of microelements in soybean seedlings of progres cultivar under salt stress conditions

Combination	Microelements $\pm$ SD [mg·kg ⁻¹ DM]				
	Cu	Mn	Zn	Fe	Co
Control	16.57 $\pm$ 2.66 ^a	69.65 $\pm$ 1.58 ^a	61.68 $\pm$ 2.01 ^a	128.50 $\pm$ 2.22 ^a	0.434 $\pm$ 0.025 ^a
Salinity (NaCl)	40.02 $\pm$ 6.67 ^b	58.92 $\pm$ 1.06 ^b	54.57 $\pm$ 3.12 ^b	123.34 $\pm$ 0.82 ^b	0.408 $\pm$ 0.018 ^a
NaCl + HA < 30kDa	20.83 $\pm$ 2.82 ^a	53.66 $\pm$ 0.36 ^c	54.81 $\pm$ 0.82 ^b	120.13 $\pm$ 3.16 ^b	0.516 $\pm$ 0.011 ^b
NaCl + HA > 30kDa	14.93 $\pm$ 1.69 ^a	56.43 $\pm$ 0.85 ^{c, b}	59.43 $\pm$ 0.62 ^c	232.62 $\pm$ 3.73 ^c	0.510 $\pm$ 0.043 ^b
NaCl + HA Mix	15.71 $\pm$ 1.03 ^a	53.26 $\pm$ 1.41 ^c	52.75 $\pm$ 1.92 ^b	133.15 $\pm$ 2.99 ^a	0.416 $\pm$ 0.011 ^a

SD – standard deviation, *Values marked with the same letters do not differ significantly according to Tukey's test ($p \leq 0.05$)

Table 2 The content of macroelements in soybean seedlings of progres cultivar under salt stress conditions

Combination	Macroelements $\pm$ SD [$\text{mg}\cdot\text{kg}^{-1}$ d.m.]			
	Na	K	Mg	Ca
Control	3.35$\pm$0.52^a	17.79$\pm$0.97^a	2.769$\pm$0.191^a	1.772$\pm$0.203^a
NaCl	15.18 $\pm$ 0.79 ^b	14.36 $\pm$ 0.07 ^b	2.007 $\pm$ 0.093 ^b	1.260 $\pm$ 0.148 ^b
NaCl+HA<30kDa	12.42 $\pm$ 0.94 ^c	17.22 $\pm$ 0.16 ^a	2.127 $\pm$ 0.028 ^b	1.017 $\pm$ 0.093 ^b
NaCl+HA>30kDa	13.39 $\pm$ 0.21 ^c	17.79 $\pm$ 0.06 ^a	2.148 $\pm$ 0.067 ^b	1.292 $\pm$ 0.183 ^b
NaCl+HA Mix	11.03 $\pm$ 0.22 ^d	18.93 $\pm$ 0.48 ^a	2.124 $\pm$ 0.028 ^b	1.131 $\pm$ 0.167 ^b

SD – standard deviation, *values marked with the same letters do not differ significantly according to Tukey's test ($p\leq 0.05$)**Table 3** The content of microelements in soybean seedlings of progres cultivar under drought stress conditions

Combination	Microelements $\pm$ SD [$\text{mg}\cdot\text{kg}^{-1}$ d.m.]				
	Cu	Mn	Zn	Fe	Co
Control	16.57$\pm$2.66^{*a}	69.65$\pm$1.58^a	61.68$\pm$2.01^a	128.50$\pm$2.22^a	0.434$\pm$0.025^a
Mannitol (M)	14.34 $\pm$ 0.66 ^a	52.17 $\pm$ 1.15 ^b	57.87 $\pm$ 1.67 ^a	132.95 $\pm$ 5.54 ^a	0.383 $\pm$ 0.038 ^{a, b}
M+HA<30kDa	14.07 $\pm$ 1.00 ^a	56.08 $\pm$ 1.33 ^b	56.81 $\pm$ 0.50 ^a	116.10 $\pm$ 13.57 ^a	0.368 $\pm$ 0.030 ^b
M+HA>30kDa	13.37 $\pm$ 0.03 ^a	56.23 $\pm$ 0.05 ^b	45.90 $\pm$ 0.70 ^b	120.88 $\pm$ 0.86 ^a	0.456 $\pm$ 0.070 ^{a, c}
M+HA Mix	12.95 $\pm$ 2.35 ^a	62.94 $\pm$ 0.08 ^c	55.93 $\pm$ 2.71 ^a	121.39 $\pm$ 6.40 ^a	0.487 $\pm$ 0.008 ^c

SD – standard deviation, *Values marked with the same letters do not differ significantly according to Tukey's test ($p\leq 0.05$)**Table 4** The content of macroelements in soybean seedlings of progres cultivar under drought stress conditions

Combination	Macroelements $\pm$ SD [$\text{mg}\cdot\text{kg}^{-1}$ d.m.]			
	Na	K	Mg	Ca
Control	3.35$\pm$0.52^{*a}	17.79$\pm$0.97^a	2.77$\pm$0.19^a	1.772$\pm$0.20^a
Mannitol (M)	2.22 $\pm$ 0.40 ^b	16.69 $\pm$ 0.45 ^a	2.21 $\pm$ 0.02 ^b	0.907 $\pm$ 0.08 ^d
M+HA<30kDa	1.66 $\pm$ 0.22 ^b	18.05 $\pm$ 0.36 ^a	2.22 $\pm$ 0.04 ^b	1.129 $\pm$ 0.04 ^c
M+HA>30kDa	2.35 $\pm$ 0.10 ^b	17.53 $\pm$ 0.96 ^a	2.27 $\pm$ 0.01 ^b	1.171 $\pm$ 0.10 ^c
M+HA Mix	3.29 $\pm$ 0.20 ^a	17.59 $\pm$ 0.97 ^a	2.58 $\pm$ 0.07 ^a	1.502 $\pm$ 0.05 ^b

SD – standard deviation, *Values marked with the same letters do not differ significantly according to Tukey's test ($p\leq 0.05$)

al. (2010). Based on plant growth parameters and element content, they concluded that humic acids application could mitigate the harmful effects of salt stress on pepper plants.

Analysis of microelement and macroelements content in in vitro-grown Progres soybean seedlings under mannitol-induced drought stress indicates a relatively low sensitivity of this cultivar (Table 3). Drought did not significantly affect Cu, Zn, Fe or Co contents compared with the control, while Mn decreased by 25.6%, Hu and Schmidhalter (2005) stated that drought stress can significantly affect the uptake and translocation of mineral elements within plants. Under drought conditions, reduced water availability limits nutrient mass flow from the medium to the roots, thereby decreasing nutrient absorption (Nieves-Cordones et al. 2019).

Humic acid fractions applied under drought generally did not markedly modify microelement uptake. For Cu, Fe and most Zn treatments, contents in HA combinations did not differ significantly from either the control or drought alone. The only exception was M+HA>30 kDa, where Zn was significantly reduced (by 25.6% compared to the control and 20.6% compared to the drought-only treatment). For Mn, all HA treatments increased its content relative to drought alone, with a significant recovery observed only for the unfractionated HA Mix ($\approx 20\%$ increase). Co uptake was

fraction-dependent: HA<30 kDa further reduced Co compared with the control, whereas HA>30 kDa and especially the HA Mix increased Co content relative to drought, with a significant effect in the M+HA Mix combination.

Drought stress induced by mannitol addition also affected the uptake of macroelements by soybean seedlings of the Progres cultivar grown in vitro. Drought reduced the uptake of Na, Mg, and Ca by the test plants. No significant effect of mannitol on K content in soybean seedlings was observed - the K amounts were 17.79 $\pm$ 0.97 mg/kg in the control and 16.69 $\pm$ 0.45 mg/kg under drought conditions. Chen et al. (2011) reported contrasting findings to ours, observing that drought stress increased the content of several key macroelements, including K, Na, Ca, Mg, and Fe, in rice plants. This suggests that rice may activate specific physiological or biochemical mechanisms under drought conditions to enhance the uptake or retention of these essential nutrients, possibly as a strategy to maintain cellular homeostasis and metabolic functions.

Humic acids fractions affect the uptake of the analyzed macroelements by soybean seedlings from in vitro cultures under drought stress in different ways (Table 4). The HA fractions (HA<30 kDa and HA>30 kDa) did not cause any changes in the K content of soybean seedlings. Na and Mg

contents at levels was similar to those in plants subjected to drought stress. In contrast, for Ca, these fractions alleviated the effects of stress, resulting in a increase in Ca content by 25% and 30%, respectively, compared to drought alone. Unfractionated HA (HA Mix) showed the strongest alleviating effect, restoring Na and Mg to control values and markedly increasing Ca (by ~66% vs. mannitol), although its level remained slightly lower than in the control. Tokarz et al. (2020) and Solek-Podwika et al. (2023) reported that accumulation of inorganic ions (e.g., K^+ , Ca^{2+} , Na^+ , Mg^{2+} , and Cl^-) and dissolved organic substances (such as sucrose, polyols, glycine betaine, and proline) is a key osmotic adjustment mechanism, serving as one of the plant's defenses against drought stress.

Humic acids can influence plant nutrition directly by promoting nutrient uptake through interaction with specific nutrient master regulators and nonspecific targets, particularly at the plant cell membrane. Indirectly, HA affect the substrate and, under field conditions, the soil, altering its chemical, physical, and biological properties (Nardi 2017, Olivares et al. 2017). Humic acids-based biostimulants improve nutrient availability to plant roots, mainly by chelating metal ions and forming stable complexes with micronutrients like iron and phosphorus (Zanin et al. 2019; Alvarez et al. 2023). These interactions enhance iron uptake and increase nutrient use efficiency (Nardi et al. 2017, 2018). Additionally, humic substances facilitate iron and phosphate absorption by creating stable humic-metal complexes, boosting nutrient availability in the soil (Gerke 2021). The chelating properties of humic acids are key factors in improving element assimilation by crops (Zhang et al. 2013).

Conclusions

Plants are exposed to various environmental stresses during their growth and development under both natural and agricultural conditions. Stress is generally defined as an external factor that negatively affects plant growth and development, including crop quality and yield. Soybean Progres derived from in vitro cultures exhibits greater sensitivity to salt stress than to drought stress. This is evidenced by significantly lower values of the analyzed biometric parameters (i.e., fresh mass of shoots and roots, plant height, number of leaves, and root length), as well as micromorphological traits related to stomatal density and aperture. Additionally, significant changes were observed in the content of analyzed micro- and macroelements in the dry matter of soybean seedlings.

Humic acids (HA) play an important role in plant growth, yield, and resilience to abiotic stress. Our research confirms

their protective role in counteracting salinity and drought stress; however, this effect depends on their molecular fractions. Analysis of biometric and micromorphological parameters of soybean seedlings of the Progres variety derived from in vitro cultures indicates that the greatest protective and, in some cases even stimulatory effect (e.g., on stomatal density) was observed for the HA > 30 kDa fraction and unfractionated (Mix) humic acids. This is supported by their significantly higher values compared to plants grown under stress conditions.

Under NaCl stress, all HA fractions partially mitigated Na excess accumulation and macronutrient imbalances while differentially modulating micronutrient uptake. The < 30 kDa fraction limited Na accumulation and restored K to control levels, but its effect on micronutrients was moderate, primarily reducing NaCl-induced Cu excess and slightly increasing Co and Mn compared to salt alone. In contrast, the > 30 kDa fraction demonstrated a more pronounced regulatory effect on selected micronutrients: it mitigated the negative effect of salinity on Zn and strongly increased Fe uptake, which is consistent with the proposed chelating and Fe-mobilizing role of high-molecular-weight humic compounds. The unfractionated HA mix combined the beneficial characteristics of both fractions: it caused the strongest reduction in Na content under salinity, maintained K content at or above control values, normalized Fe to control levels, and increased Co content, indicating an overall improvement in ionic balance under salt stress.

Under mannitol-induced drought, Progres seedlings showed relatively low sensitivity to most trace elements, with Mn and Ca being the most affected. In this context, the effect of the HA fraction was again fraction-specific but generally weaker than under salinity. Fractions < 30 kDa and > 30 kDa did not significantly modify K, Na, or Mg content compared to drought alone, but partially mitigated Ca loss and increased Mn content compared to drought, while exerting divergent effects on Co content (decrease at < 30 kDa, increase at > 30 kDa). The HA mixture showed the broadest protective effect under drought conditions: it restored Na and Mg levels to the control level, significantly increased Ca levels, and significantly improved Mn and Co content.

These results indicate that humic substances, especially when used in the form of unfractionated mixtures, can support ionic regulation and microelement homeostasis under both salt stress and drought conditions, with high-molecular-weight fractions playing a key role in Fe and Zn nutrition, and low-molecular-weight fractions contributing to partial stabilization of Ca and Mn concentrations.

The obtained results confirm the protective role of humic acids, however, for the results of these studies to be applied in practice, they must be repeated under field conditions.

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Data availability Data will be made available on request.

Declarations

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