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Effect of Humic Acids on Soybean Seedling Growth under Polyethylene-Glycol-6000-Induced Drought Stress

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Abstract: Humic substances (HS) are the most important natural biostimulant of plants. However, the relationship between their structure and biological activity in plants is still not well recognized. The objective of this paper was to assess the influence of molecular fractions of humic acids (HA) (HA < 30 kDa and HA > 30 kDa) on reducing negative effects of drought stress in soybean (*Glycine max* (L.) Merr.) seedlings of Progres and Nawiko cultivars. Drought stress was induced in laboratory conditions by the addition of polyethylene glycol 6000 (PEG 6000) to make a water potential of −0.5 MPa. HA were extracted according to the International Humic Substances Society procedure, and then were separated into two molecular fractions by membrane filtration. The following physiological and biometric parameters were determined: chlorophyll content, photosynthesis activity, electrical conductivity, fresh and dry mass of overground and roots, and plant length. The enzyme activity and ion contents were also measured. Differences in response to drought stress with the addition of HA < 30 kDa and HA > 30 kDa or not to the Hoagland's solution were observed among studied cultivars. Drought stress caused a decrease in the most physiological parameters and increase in peroxidase activity in the case of both studied cultivars. However, the results of biometric measurements showed that the Progres cultivar appears to have better tolerance to drought stress. The significant influence of water deficit on most macroelement content in dry matter leaves of both studied cultivars was not observed, while its effect on microelement uptake by soybean plants was concluded. In the case of the Progres cultivar, the results showed a significant decrease in microelement content in the dry matter of leaves, whereas in the leaves of Nawiko cultivar there was a significant increase. The influence of HA > 30 kDa and HA < 30 KDa fractions on physiological features of both studied cultivars was varied. HA > 30 kDa fraction better up-regulated the antioxidant defense system. Unfortunately, no effect of either HA fraction on the macro- and micronutrients uptake system of both studied cultivars was observed.

Keywords: humic acid fractions; drought; hydroponics; nutrient uptake; plant growth; soybean (*Glycine max* (L.) Merr.)

1. Introduction

Soybean (*Glycine max (L.) Merr.*) is the most important leguminous crop species grown in 95 countries of the world. In 2019, the soybean cultivation area was 120.5 million ha, while the average yield was 2.77 tha^{-1} [1,2]. The soybean is indispensable for people and animals, due to its chemical composition. Its seeds contain many proteins, fats, large volumes of unsaturated fatty acids, as well as vitamins and minerals [1,3,4]. Soybean oil is considered a future fuel source, and efforts are made to increase soybean-derived diesel production [5]. The capability of binding atmospheric nitrogen is a valuable feature of the legume, including soybean, and the process itself is beneficial for this plant (cultivation of soybean plants does not require large doses of nitrogen) as well as succeeding crops [6,7].

Soybean is exposed to multiple stresses during their growth. One of major abiotic stresses that have a negative impact on their growth and development is drought [8,9]. Tolerance to the drought stress varies between species, strains, growth phases, drought duration and intensity, and other conditions [10,11]. Soybean is considered sensitive to several abiotic stresses as compared to other legumes and crop [12–14], especially at particular phases of its life cycle [15]. Drought stress can cause soybean yield reductions of up to 40% per year [16]. Soybean shows different responses to drought depending on the different plant growth stages [17] and different genotypes [18]. Generally, soybean yield is decreased by drought [19]. Soybean seed quality is also altered by drought [20,21]. Sheteiwy et al. [22] demonstrated that the contents of soluble sugars, lipids, pro-teins, and oils in soybean decreased under drought stress.

Plants show various physiological and biochemical reactions at the cellular and entire organism levels to drought-related stress. This makes drought stress a complex phenomenon [23,24]. Drought stress inhibits sprouting, growth, and development, and interrupts appropriate physiological processes. Additionally, this stress leads to inhibition of photosynthesis, composition changes, and the activity of hormones. The same applies to permeability of cell membranes and production of reactive forms of oxygen [23–26]. Usually, plants accommodate to the stress using various adaptive mechanisms. However, climate changes and contemporary agricultural systems adversely affect the quality and yield of crops. To increase productivity and mitigate consequences of the abiotic stress, including drought stress, we can use various strategies, such as mass screening and breeding, growth of tolerant strains, exogenous application of hormones and osmoprotectants, nanoparticles, and humic acids [13,27–29].

Biostimulants such as humic acids (HA) play an important role in the growth of plants, yield, and resilience to abiotic stress. Nevertheless, this phenomenon demonstrates the complex nature [13,30–34]. The biological activity of HA finely relies on their dosage, origin, molecular size, degree of hydrophobicity and aromaticity, and spatial distribution of hydrophilic and hydrophobic domains [35]. HA have both indirect and direct influence on plant growth. Indirect influence includes improvements in soil properties, such as aggregation, aeration, permeability, water-holding capacity, and micronutrient transport and availability, whereas direct influence includes improvement in the overall plant biomass [30,36,37]. HA influence a number of processes in plants, e.g., enzyme activity, protein metabolism, photosynthesis, respiration, abstraction of water and nutrients, hormone fluxes, cell membrane permeability, and reactive oxygen species [31,38,39]. According to the literature, it is not possible to unambiguously conclude whether the influence of HA on plants is related to the molecular mass [30,36] or the structural characteristic of those molecules [40]. Despite different opinions regarding the influence of HA involving mechanisms on plants, most authors are in agreement about the positive effects of HA on plants and the link between HA and increased metabolic efficiency [39].

Due to the complex nature of drought stress and the interaction with other abiotic stresses, it is important to examine thoroughly the physiological and molecular aspects of plant resilience to drought. Moreover, the biological activity of HA to stimulate plant growth is becoming important, especially in the context of sustainable agriculture. Therefore, the aim of the study was to assess the influence of molecular fractions of HA on reducing negative effects of drought stress in soybean of Progres and Nawiko cultivars.

2. Materials and Methods

2.1. Determination of HA Fractions and Their Spectral Characteristic

Humic acids were isolated from peat samples (collected from Babiogórski National Park, Poland: GPS: N 49°35′45″–E 19°30′21″) according to the International Humic Substances Society procedure [41]. The extraction was performed in the following steps: (1) decalcitation of peat samples with HCl, (2) triple extraction with NaOH, (3) HA precipitation by HCl, (4) HA purification by HCl/HF, (5) HA reprecipitation by HCl (6), washing out by redistilled water until the negative Cl^- test with silver nitrate $AgNO_3$, and (7) HA freeze-drying.

HAs were separated into two molecular fractions (HA > 30 kDa and HA < 30 kDa) with application of Amicon 8400 cell and Millipore filters with cut-off point 30 kDa.

For HA molecular fractions, the following measurements were performed: spectra of UV-VIS (Specord M-42 spectrophotometer) and scanning electron microscope images (FEI Quanta 2000). Spectrophotometric UV-Visible measurements were performed using SPECORD UV-VIS M-42, a computer-aided dual-beam spectrophotometer with the START software by Carl Zeiss Jena. All solutions subjected to photometric analysis were characterized by the same carbon concentration: 10 $mgC \cdot dm^{-3}$ in a solution of phosphate buffer of pH = 7.0. SEM images; the best results were produced by samples overlaid with gold.

2.2. Plant Growth Conditions and PEG 6000 Treatment Procedures

The studies were carried out on soybean seedlings of (*Glycine max (L.) Merr.)* Progres and Nawiko cultivars. Soybean seeds were washed thrice with distilled water. The 100 soybean seeds of each cultivar were germinated on trays with roasted silica sand. The substrate moisture was roughly 50% of water weight-holding capacity. After 4–5 days (BBCH 10 growth phase acc. to [42]), soybean seedlings were moved to germination apparatus of $0.08 \times 0.012 \times 0.036$ m (height $\times$ width $\times$ length) and volume of 2.5 dm^3. The experiment was conducted in water cultures with Hoagland's nutrient solution at pH 5.6. Soybean seedlings were grown under controlled temperature and lighting (PPFD 350 μmol (photons) m^{-2} s^{-1}, 25 °C, photoperiod 16 h/8 h (day/night). The solution was aerated.

After 7 days of growth, soybean seedlings were randomly divided into 4 groups (10 plants in each variant):

1. Control (C) (only Hoagland's solution);
2. 0.5 MPa (DS) + Hoagland's solution;
3. 0.5 MPa (DS) + HA < 30 kDa + Hoagland's solution;
4. 0.5 MPa (DS) + HA > 30 kDa + Hoagland's solution.

Two replications were made for each combination of the experiment. The concentration of HA fractions was 0.005 gC_{HA} dm^{-3}. Polyethylene glycol 6000 (PEG 6000) was added into the nutrient solution to make the water potential of –0.5 MPa [43], which was simulated by drought stress treatments (DS). In such conditions, soybean seedlings were grown for another 7 days.

2.3. Physiological Measurements

Physiological measurements on soybean plants were carried out after 7 days of growth under induced drought stress, with and without HA fractions.

2.3.1. Relative Chlorophyll Content

The relative chlorophyll concentration was determined using a non-destructive method. The SPAD value of the leaf was determined using a chlorophyll meter SPAD-502 (Minolta CO. Ltd., Osaka, Japan). Results are given in SPAD, the value of which is proportional to the content of chlorophyll in the examined leaf area (6 mm^2) [44]. SPAD measurements were carried out on 10 randomly selected plants from each experimental variant

2.3.2. Photosynthesis Activity

The photosynthesis rate was determined using TPS-2 (PP System, Amesbury, MA, USA). During the measurement, leaves were illuminated at 400 $\mu mol\,m^{-2} \cdot s^{-1}$ using the LED light system.

2.3.3. The Relative Electrolyte Leakage

Leaf membrane damage was determined by recording of electrolyte leakage (EL) as described by Dexter et al. [45] with modifications. To assess damage to the cell membrane structure and the loss of controlled and selective permeability of water solutions, the study used a modified conductometric method. Both in control and drought-exposed groups with and without the HA fractions, the leaves' tissues were cut with cork borer into 15 mm discs. The discs were rinsed in redistilled water and placed in test tubes. Each test tube was filled with 7 cm^3 of redistilled water of up to 1.6 $\mu S \cdot cm^{-1}$. Pieces of leaves were completely immersed in water (of room temperature −20 °C). After 4 h at shaker, electric conductivity of solutions was measured (W_1) using a conductometer CPC–551 (Elmetron, Poland). After measurement, the solution was poured back to the test tube with a piece of a plant. Then, to cause extreme damage, pieces of plants immersed in water were placed in a freezer (−30 °C, 24 h). After defrosting and reaching the room temperature hours after they were taken out of the freezer (2 h of which was in shaker), conductivity was measured again (W_2). Permeability of cytoplasmic membranes was measured to assess the impact of electrolytes from plant tissues based on the changes of conductivity of solutions. The electrolyte leakage (EL) was calculated as a relative value of electrical conductivity (EC):

$$EC = [(W_1 - W_0)/(W_2 - W_0)] \cdot 100\% \quad (1)$$

where:

W_0—conductivity of redistilled water ($W_0 < 1.6\ \mu S \cdot cm^{-1}$);

W_1—mean value of electrical conductivity of leakage from leaves' tissues;

W_2—mean value of electrical conductivity of leakage from broken leaves' tissues (freezer −30 °C, 24 h).

Values were expressed in the percentage of maximum leakage of electrolytes from leaves' tissues (EC).

2.4. Biometric Measurements

The length of plants was measured (cm). Fresh and dry matter of the overground parts and roots of soybean seedlings of the Nawiko and Progres cultivars were also performed. To determine dry matter, samples were divided into roots and overground parts and oven dried at 105 °C for 12 h and then weighed. Overground parts and roots dry matter were expressed as $g \cdot plant^{-1}$.

2.5. Enzyme Measurements

Activity of peroxidase was determined using a spectrophotometric method according to Chance and Machly [46]. The method is based on the pyrogallol oxidation to purpurogallin with the presence of hydrogen peroxide. The amount of purpurogallin was photometrically measured at 430 nm wavelength. The measurements were carried out in a 2 cm^3 of reactive mixture. The mixture consisted of 0.1 cm^3 of plant extract, diluted with: 0.9 cm^3 0.05 $mol \cdot L^{-1}$ of actetate buffer, 0.5 cm^3 0.06 $mol \cdot L^{-1}$ hydrogen peroxide and 0.5 cm^3 0.02 $mol \cdot L^{-1}$ of pirogallol. The control sample was made without H_2O_2 to eliminate polyphenol oxidase activity, which also has oxidation properties. The samples were incubated for 4 min at 30 °C. The activity of peroxidase was calculated by subtracting absorbance of control samples from absorbance of test samples. The peroxidase activity was expressed in mmol purpurogallin g^{-1} fresh weight min^{-1}. The concentration of enzyme in the solution was expressed in units per 1 cm^3.

The activity of catalase was also determined using spectrophotometric method according to Lück [47]. The method is based on a direct measurement of the decrease in absorbance of a sample at $\lambda = 240$ nm, caused by the decomposition of H_2O_2 into molecular oxygen and water in the presence of the enzyme. The catalase activity was calculated acc. to the following formula:

$$\text{Catalase activity} = \Delta A \cdot V_0 / \bar{E} \cdot l \cdot a \cdot V_1 \cdot \Delta t \quad (2)$$

where:

ΔA—change of absorbance in time;
V_0—total extract volume [cm^3];
l—optical path length [cm];
a—fresh plant mass [g];
V_1—volume of enzyme extract used for determination [cm^3];
Δt—reaction time [min.];
$\bar{E}$—mean value of molar absorbance coefficient of H_2O_2.
The concentration of the enzyme in a solution was expressed in units per 1 cm^3.

2.6. Ion Measurements

Dry matter of Progres and Nawiko cultivar leaves was mineralized in the mixture of concentrated acids, e.g., HNO_3 and $HClO_4$ (3:1). Mineralization was carried out for 1.0 ± 0.01 g in triplicate. After mineralization, the content of selected macro- and microelements—Na, K, Ca, Mg and Cu, Zn, Mn, Fe, and Co—was measured by using the method of atomic absorption and emission spectrometry (SOLAAR Spectrometer AA Thermo Elemental Series). The quantitative analysis of the analyzed metals was based on Merck's standards and standard curves for which the correlation coefficient was at least 0.995.

Based on the standard curves, the detection limit and the limit of quantification were calculated for each metal. All determinations were performed in triplicate, for which the coefficient of variation was calculated. When the coefficient of variation exceeded 10%, the analysis was repeated.

2.7. Statistical Analyses

Statistical analysis was performed using Statistica 12.0. Prior to the analysis, the data for normal distribution (Shapiro–Wilk's test) and homogeneity of variances (Levene's test) were checked. One-way analysis of variance (ANOVA) was used to determine the variant experiment on the studied features within one cultivar. The analysis was the basis to separate homogenous groups while using the Tukey's test at a significance level of $\alpha = 0.05$.

3. Results and Discussion

3.1. Analysis of HA Fractions' Properties

The elemental analysis of studied HA molecular fractions was presented and described previously [48].

Many different spectroscopic techniques have been used to study the HA structure, but UV–Visible spectroscopy is a non-destructive and simple method, which allows determining the presence of various aliphatic and aromatic groups important in terms of the ability to sorb macro- and micro-components and their soil–plant migration. The absorption curves of HA < 30 kDa and HA > 30 kDa fractions are shown in Figure 1. The curves are similar in shape: monotonic and non-characteristic. According to Stevenson [49] and Kumada [50], this may signify that the same groups of chromophores are responsible for the absorption of electromagnetic radiation, although their quantity varies and their effects may overlap. These groups include those directly related to the aromatic ring of the HA nucleus ($\lambda_{max.}$ = 280 nm), as well as those which constitute bridges linking parts of their structure.

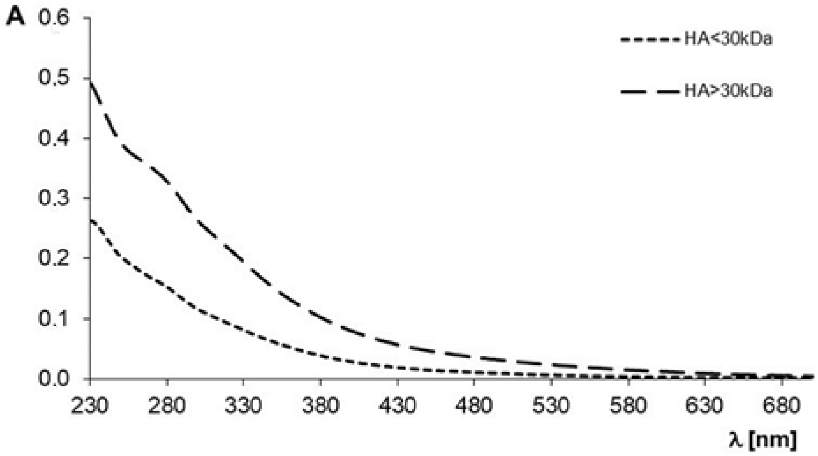

Figure 1. Absorption spectra of HA < 30 kDa and HA > 30 kDa fractions.

The differences in the spatial structure of HA < kDa and HA > kDa fractions are shown in Figure 2. The HA < kDa fraction does not form a distinguished spatial structure, whereas in the HA > kDa fraction a polymeric structure is visible.

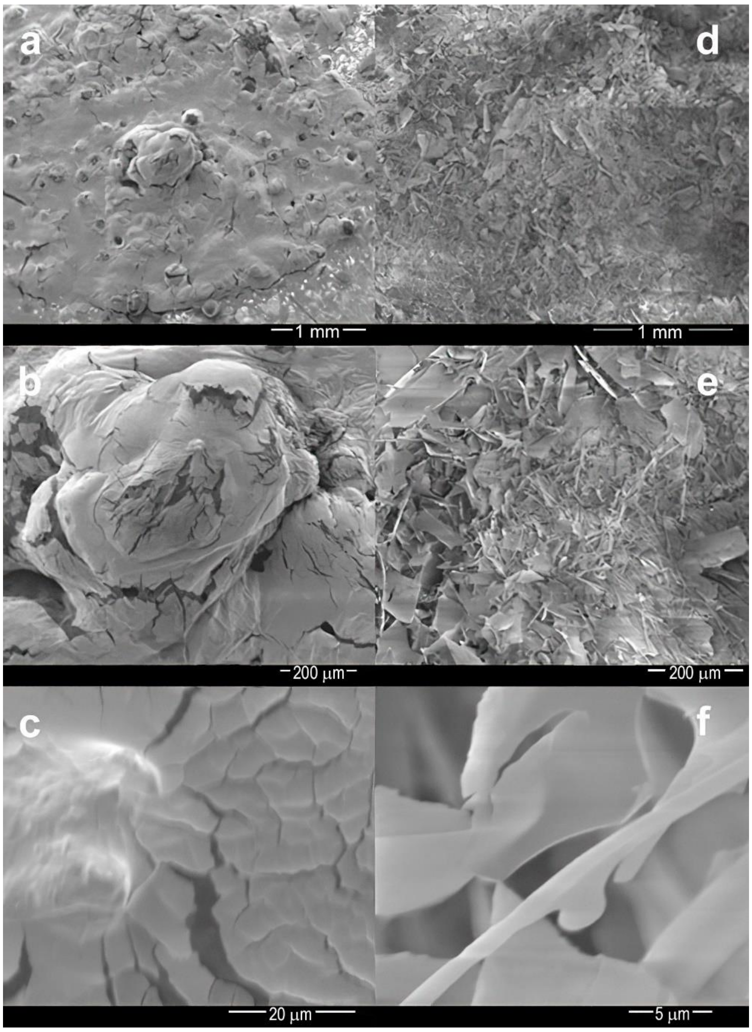

Figure 2. The scanning electron microscope images of spatial structure of HA < 30 kDa in magnifications: (**a**) 74×, (**b**) 300×, (**c**) 5000×; and HA > 30 kDa in magnifications: (**d**) 110×, (**e**) 400×, (**f**) 13,000×.

3.2. Physiological Measurments

The influence of HA fractions on chlorophyll content in leaves of Nawiko and Progres soybean seedlings growing under drought stress is presented in Figure 3.

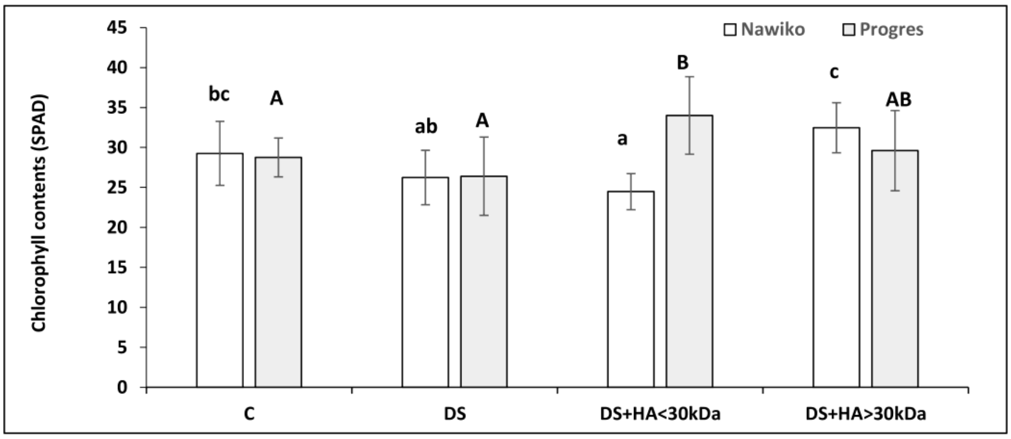

Figure 3. Influence of HA fractions on chlorophyll content in leaves of Nawiko and Progres soybean seedlings growing under drought stress. C-control; DS-drought stress: growing in Hoagland's solution with PEG 6000; DS + HA <30 kDa; DS + HA >30 kDa (average values ± SD). Average values marked with the same letters do not differ at the significance level $p = 0.05$; Tukey's test; a, b, c-for Nawiko; A, B, C-for Progres.

Our results showed that soybean seedlings growing under drought stress exhibited slightly lower content of chlorophyll than the control plants, but these differences were not statistically significant (Figure 3). Therefore, the reduction in chlorophyll production has been considered as a typical symptom of oxidative stress or may be the result of pigment photo-oxidation and chlorophyll degradation in plants [51]. Similar to our observations, many studies have reported a decrease in chlorophyll contents caused by drought stress in numerous plant species [21,52–55]. Moreover, it was found that damage of photosynthetic pigments by drought stress leads to the degradation of the thylakoid membrane and damage of photosynthetic apparatus [56–58] which leads to the direct susceptibility of chlorophyll to degradation. Bai et al. [59] assume that higher content of chlorophyll causes greater plant resistance to drought stress, wherein chlorophyll b fades faster than chlorophyll a [60,61].

An increase in chlorophyll content was observed in the case of the Progres cultivar growing under drought stress with HA < 30 kDa and HA > 30 kDa fractions, compared to the plants growing only under drought stress and the control plants. The HA < 30 kDa fraction caused an increase in chlorophyll content significantly by 30% compared to drought stress and by 18% compared to the control plants. Although, the HA > 30 kDa fraction caused the tendency to increase chlorophyll content in leaves of the Progres cultivar. In the case of the Nawiko cultivar, the HA fraction above 30 kDa significantly increased (by 24%) the content of chlorophyll compared to the drought stress. The application of humic substances may increase the content of chlorophyll and thus influence photosynthesis. However, the increase in chlorophyll content does not always result in higher yield of photosynthesis [30]. Nasiri et al. [62] observed that foliar application of HA significantly increased the chlorophyll content of canola genotypes. Lotfi et al. [63] concluded that the chlorophyll content of rapeseed plants decreased as a result of water stress, whereas the application of HA positively affected the chlorophyll content. According to Tehranifar and Ameri [64], the increase in the chlorophyll content due to HA may be related to the enhancement in CO_2 assimilation and photosynthetic rate, which is probably due to the increased rubisco enzyme activity. A comparison of the influence of HA fraction on photosynthesis rate of Nawiko and Progres soybean seedlings growing under drought stress is presented in Figure 4.

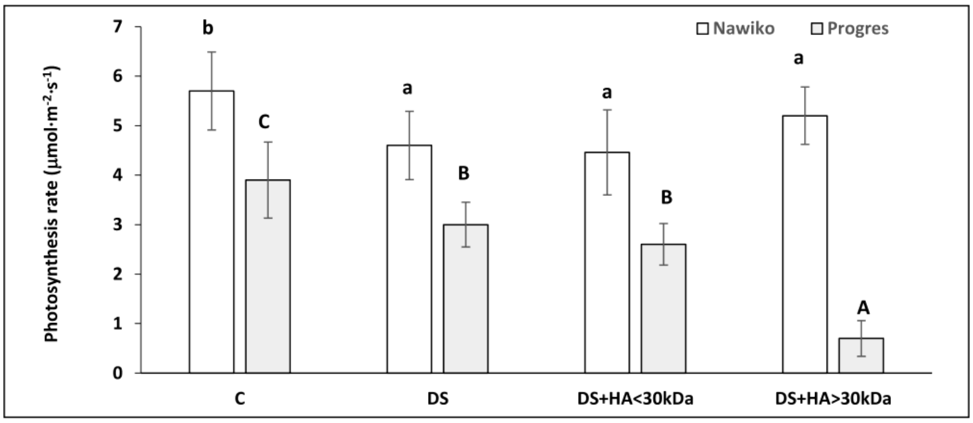

Figure 4. Influence of HA fractions on photosynthesis rate of Nawiko and Progres soybean seedlings growing under drought stress. C-control; DS-drought stress: growing in Hoagland's solution with PEG 6000; DS + HA < 30 kDa; DS + HA > 30 kDa (average values ± SD). Average values marked with the same letters do not differ at the significance level $p = 0.05$; Tukey's test; a, b, c-for Nawiko; A, B, C-for Progres.

Soybean seedlings exposed to drought stress significantly reduced photosynthesis rate by about 20% compared to the control plants (Figure 4). Similar results were obtained by de Souza et al. [65]. During drought stress, the stomatal and non-stomatal limitations have a negative impact on the rate of photosynthesis [57,66–68]. The decrease in the net photosynthesis rate, and consequently the decrease in assimilation, reduces the growth and yield due to the drop in stomatal conductance and leaf water potential [26,57,69].

According to the study, both cultivars showed lower photosynthesis rate due to the drought stress with HA fractions compared to the control plants. In the case of the Nawiko cultivar, growing under drought stress with HA < 30 kDa photosynthesis rate decreased by 20%, whereas with HA > 30 kDa by 9% compared to the control plants. HA fractions did not have a significant impact on the photosynthesis rate compared to drought stress alone in the case of the Nawiko cultivar. In the case of the Progres cultivar, a larger decrease in photosynthesis rate than in Nawiko was observed. It was 23% lower under drought stress and 33% lower with HA < 30 kDa compared to the control plants. HA > 30 kDa reduced the assimilation rate by about 82% compared to the control plants and by 76% compared to drought stress alone. According to Lotfi et al. [70] the application of HA improved plants' net photosynthesis under water stress via increasing the rate of gas exchange and electron transport flux in plants.

The assessment of damage to cell membrane structure and the loss of controlled and selective permeability of water solutions were made using a modified conductometric method (Figure 5).

The value of electric conductivity of the solution, and consequently the leakage of electrolytes (calculated by Formula 2), was smaller in particular variants of the experiment for both soybean cultivars, compared to the control plants, except the DS + HA > 30 kDa variant for Progres cultivar. However, it was not a statistically significant difference. No significant impact was found regarding HA fractions on the scope of damage to the cell membrane compared to drought stress. A statistically significant decrease of 50% in the leakage of electrolytes and the relative electrical conductivity of the solution, compared to control plants, was found in the case of the Nawiko cultivar growing under drought stress with HA > 30 kDa. This suggests that HA > 30 kDa reduces the permeability of membranes. In normal conditions, the cell membrane is selectively permeable. Different stress factors cause damage to the cell membrane. While examining morphological and physiological responses of seven different soybean cultivars to drought stress, Rao and Chaitanya [57] discovered an increase in electrolytic leakage due to drought stress. The larger the damage, the larger the degree to which part of the cell content flows outside, causing an increase in electric conductivity [71].

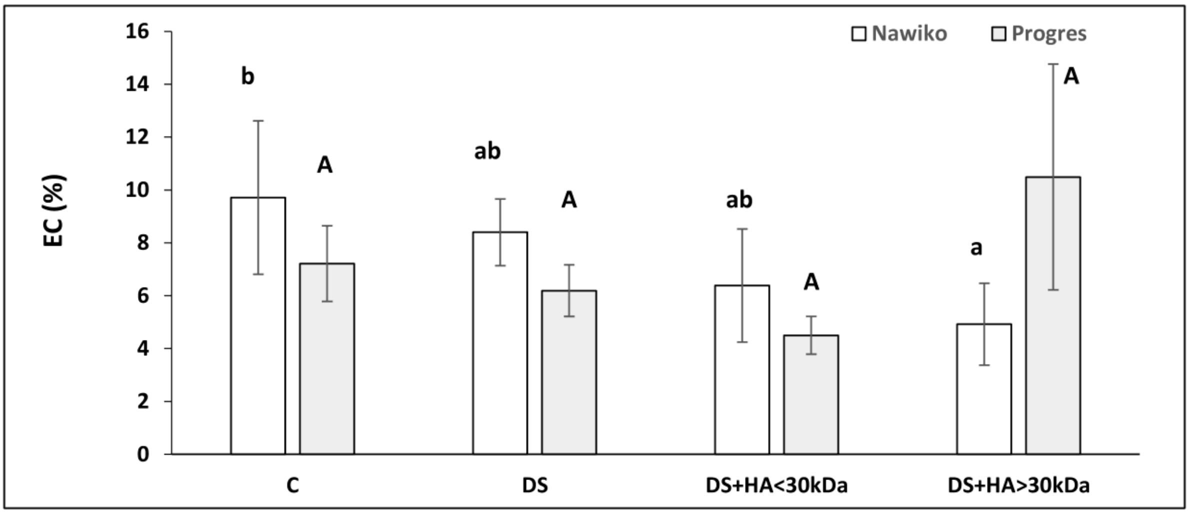

Figure 5. Influence of HA fractions on electric conductivity (EC) of Nawiko and Progres soybean seedlings growing under drought stress. C-control; DS-drought stress: growing in Hoagland's solution with PEG 6000; DS + HA <30 kDa; DS + HA >30 kDa (average values ± SD). Average values marked with the same letters do not differ at the significance level $p = 0.05$; Tukey's test; a, b, c-for Nawiko; A, B, C-for Progres.

3.3. Biometric Measurements

The influence of HA < 30 kDa and HA > 30 kDa fractions on fresh and dry matter of overground parts and roots of Nawiko and Progres soybean seedlings is presented in Figure 6.

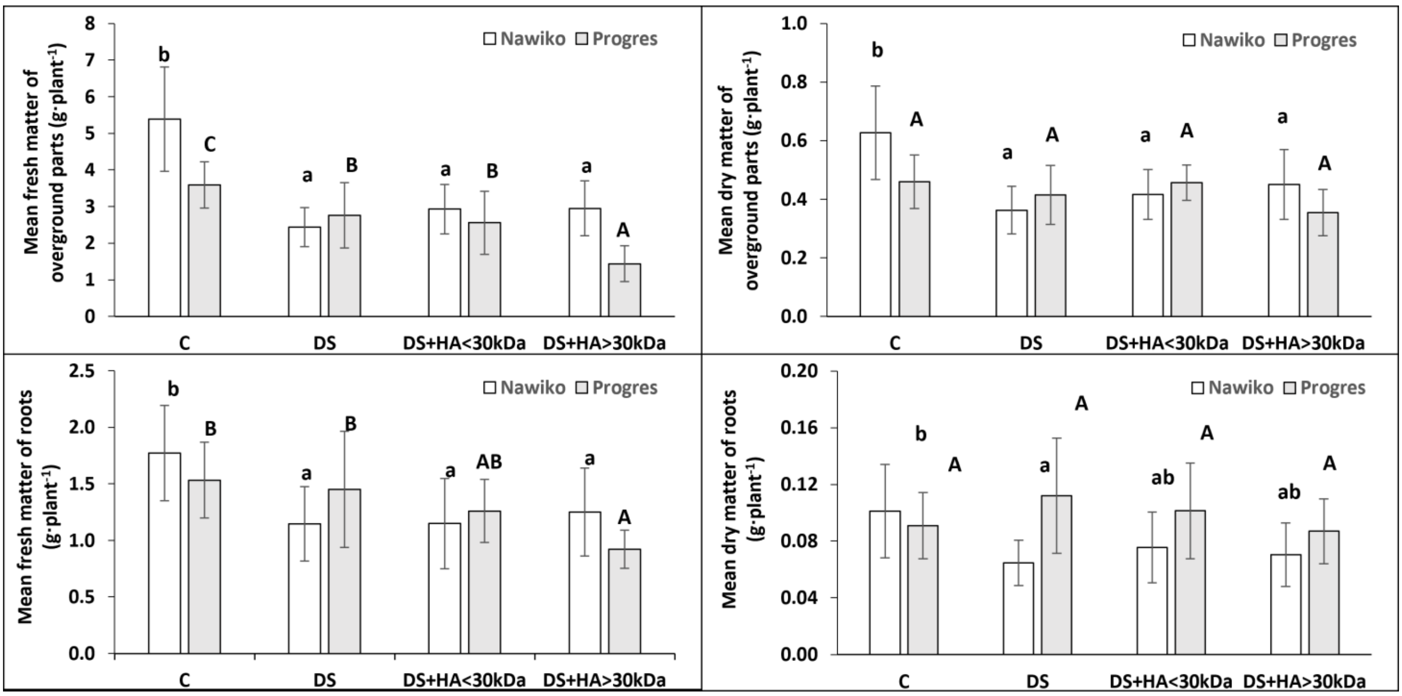

Figure 6. Influence of HA fractions on biometric parameters of Nawiko and Progres soybean seedlings growing under drought stress. C-control; DS-drought stress: growing in Hoagland's solution with PEG 6000; DS + HA < 30 kDa; DS + HA > 30 kDa (average values ± SD). Average values marked with the same letters do not differ at the significance level $p = 0.05$; Tukey's test; a, b, c-for Nawiko; A, B, C-for Progres.

In this study, the growth rate of cultivar soybean Nawiko was inhibited under drought stress conditions. The fresh and dry matter of the overground parts and roots was significantly lower (by about 40%) compared to the control plants (Figure 6). Rao et al. [25] also observed a decrease in leaf and root fresh and dry mass in soybean seedlings under drought stress. Biomass is one of the major parameters reflecting growth and development of plants exposed to stress factors. Poor access to water results in a number of modifications of cell membranes, changes which have a negative impact on the total biomass of the plant [23]. Sheteiwy et al. [22] reported the exposure to drought stress resulted in a significant reduction in plant height, fresh weight, pods/plants, and weight of 100 seeds compared with the well-watered conditions. Reduction in biomass under drought was reported in the case of various plants [72–75]. Bai et al. [76] concluded that soybean seedlings exposed to single drought stress had less biomass, since they needed to invoke additional energy to synthesize osmolytes for osmotic adjustment, which also affected and hindered biomass accumulation. Plants need to consume more energy for accumulating inorganic ions and organic osmolytes, and accordingly the energy available for plant growth is relatively reduced. This results in reduced biomass accumulation and crop production of plants under drought stress [76]. The Progres cultivar appears to show better tolerance to drought stress; there were no significant differences in the dry matter of the overground fresh and dry matter of roots (Figure 6). Only the fresh matter of overground parts of seedlings growing under drought stress was significantly decreased by 23% compared to the control plants. The dry matter of roots was 20% higher compared to the control plants, but this was not a statistically significant difference. The roots (root growth, size, proliferation, density, and distribution) are considered the key organ for plant adaptation to drought [77]. Increased matter of roots was a strategy to increase the water uptake from soil under water deficit conditions [78].

The influence of HA < 30 kDa and HA > 30 kDa fractions on fresh and dry matter of overground parts and roots of Nawiko and Progres soybean seedlings was varied. The fresh matter of the overground part of seedlings under drought stress with HA > 30 kDa was significantly reduced (by 48%) in relation to those under drought stress only in the case of the Progres cultivar. As with the overground part, the fresh matter of roots was reduced (by 36%) under drought stress with HA fractions.

It was observed that addition of HA fraction had a positive influence on growth rate of Nawiko cultivar under drought stress. Generally, HA < 30 kDa and HA > 30 kDa fractions increased fresh and dry matter of overground parts by 20% compared to drought stress, but it was not a statistically significant difference. Similarly, HA fractions had a positive influence on the dry matter of roots by increasing it by 17% (HA > 30 kDa) and 33% (HA < 30 kDa), and also fresh matter by 8% (HA > 30 kDa) compared to drought stress alone. The increase in root growth is one of the major effects of HA [79,80].

Under drought stress, the length of both cultivars' soybean seedlings was reduced significantly compared to the control plants (Figure 7).

The reduction in plant length could be attributed to the decline in the cell enlargement and more leaf senescence in the plant under water stress [51,81]. Drought stress inhibited increases in the soybean plant height and leaf area in the study by Dong et al. [21]. This inhibition became more significant as the level, duration, and frequency of the drought stress increased. HA < 30 kDa and HA > 30 kDa stimulated the increase in the length of Nawiko and Progres cultivars. In the case of the Nawiko cultivar, it was by 18% (HA < 30 kDa) and 30% (HA > kDa), whereas for the Progres cultivar, it was by 27% and 3%, respectively, compared to seedlings growing under drought stress only.

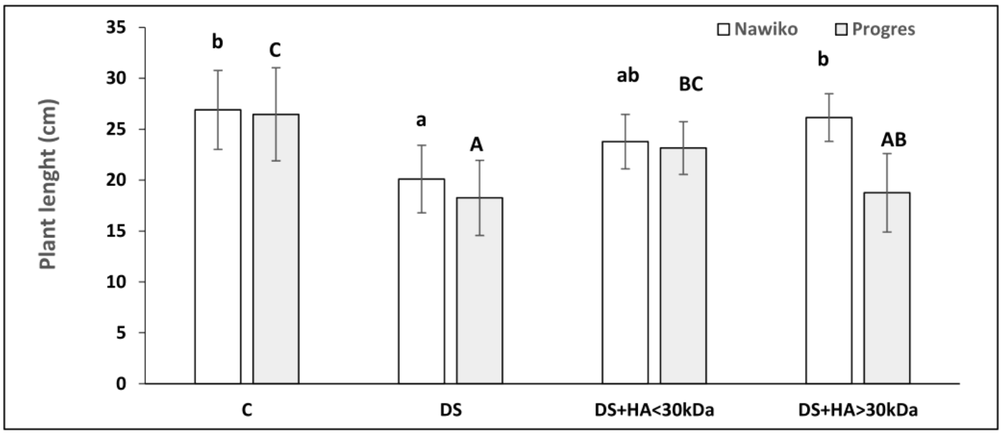

Figure 7. Influence of HA fractions on plant length of Nawiko and Progres soybean seedlings growing under drought stress. C—control; DS—drought stress: growing in Hoagland solution with PEG 6000; DS + HA < 30 kDa; DS + HA > 30 kDa (average values ± SD). Average values marked with the same letters do not differ at the significance level $p = 0.05$; Tukey's test; a, b, c—for Nawiko; A, B, C—for Progres.

3.4. Results of Enzyme Activities

The research into the response of plants to abiotic and biotic stress factors has clearly shown that in each case these processes are related to uncontrolled increases in the level of reactive oxygen species (ROS). To minimize the effects of oxidative stress, plants put in place strategies to balance the ROS production and the enzyme activities. Several studies have shown that higher levels of enzyme activities may contribute to better drought tolerance. This indicates an increased ability to protect against oxidative damage [82–84].

Drought stress induced increases in peroxidase activity in both cultivars compared to control plants. Increased catalase (calculated by Formula 3) was observed in Nawiko seedlings only (Figure 8). The HA < 30 kDa fraction reduced peroxidase and catalase activities in the case of the Nawiko cultivar compared to plants growing under drought stress only. The opposite trend was observed with HA > 30 kDa. The HA < 30 kD did not have an influence on catalase activity in the case of the Progres cultivar under drought stress. However, it reduced peroxidase activity. The HA > 30 kDa reduced catalase and increased peroxidase activities. Increased production of antioxidants has been observed in plants treated with HA [85].

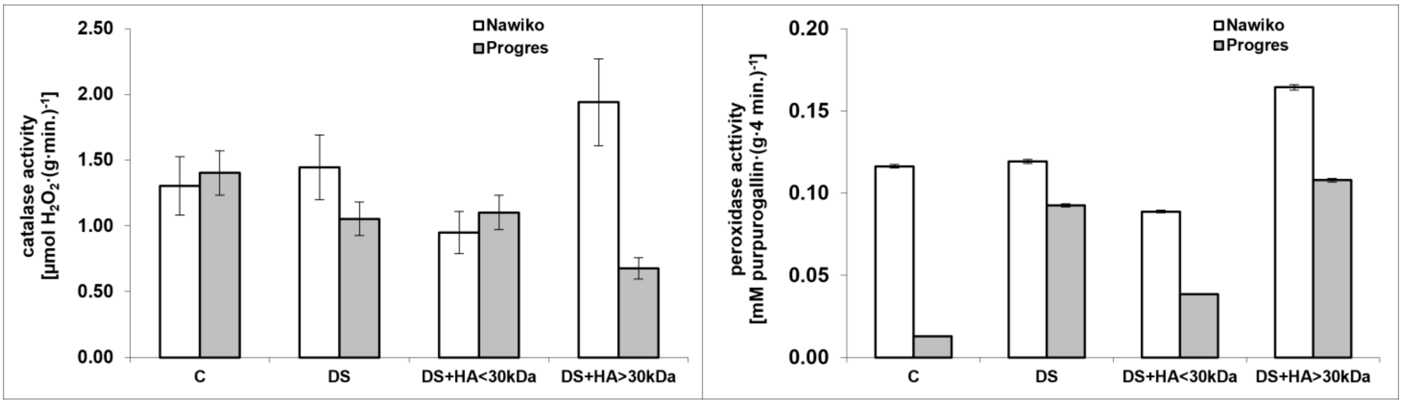

Figure 8. Influence of HA fractions on catalase and peroxidase activity of Nawiko and Progres soybean seedlings growing under drought stress. C-control; DS-drought stress: growing in Hoagland solution with PEG 6000; DS + HA < 30 kDa; DS + HA > 30 kDa ((average values ± SD). SD did not exceed 17% for catalase activity and 5% for peroxidase activity.

3.5. Ion Measurements

The study on the influence of drought stress and HA fractions on the content of macro- and microelements in leaves of Nawiko and Progres soybean seedlings showed a variable capability to accumulate macro- and microelements in the solution (Tables 1 and 2).

Table 1. Influence of drought on the content of selected macroelements in soybean leaves.

Cultivar	Combination	Macroelements g·kg^{-1} (d.m.)			
		Na ± SD	K ± SD	Mg ± SD	Ca ± SD
Progres	Control	1.24 ± 0.08 ^{*a,A}	38.57 ± 1.24 ^{*a,A}	3.63 ± 0.04 ^{*a,A}	9.14 ± 0.46 ^{*a,A}
	DS	1.30 ± 0.04 ^{a}	36.17 ± 0.75 ^{a}	3.71 ± 0.07 ^{a}	10.40 ± 0.44 ^{b}
	DS + HA < 30 kDa	1.81 ± 0.03 ^{b}	37.38 ± 1.29 ^{a}	3.53 ± 0.05 ^{a}	9.97 ± 0.06 ^{b}
	DS + HA > 30 kDa	1.61 ± 0.17 ^{b}	32.21 ± 0.27 ^{b}	2.99 ± 0.06 ^{b}	8.10 ± 0.55 ^{a}
Nawiko	Control	2.48 ± 0.30 ^{*a,B}	22.09 ± 0.01 ^{*a,B}	3.50 ± 0.01 ^{*a,A}	8.76 ± 0.31 ^{*a,A}
	DS	2.16 ± 0.19 ^{a}	21.89 ± 0.13 ^{a}	3.66 ± 0.01 ^{a}	7.97 ± 0.21 ^{b}
	DS + HA < 30 kDa	1.14 ± 0.17 ^{b}	21.67 ± 0.30 ^{a}	3.58 ± 0.07 ^{a}	7.84 ± 0.11 ^{b}
	DS + HA > 30 kDa	2.56 ± 0.28 ^{a}	25.93 ± 0.67 ^{b}	3.59 ± 0.04 ^{a}	9.01 ± 1.46 ^{a,b}

SD—standard deviation * values marked with the same letters do not differ significantly according to Tuckey's test at the significance level $p \leq 0.05$; lowercase—drought stress, capital letter—cultivar.

Table 2. Influence of drought on the content of selected microelements in soybean leaves.

Cultivar	Combination	Microelements mg·kg^{-1} (d.m.)				
		Cu ± SD	Mn ± SD	Zn ± SD	Fe ± SD	Co ± SD
Progres	Control	16.16 ± 0.12 ^{*a,A}	150.45 ± 3.27 ^{*a,A}	134.75 ± 2.75 ^{*a,A}	317.85 ± 5.80 ^{*a,A}	0.218 ± 0.014 ^{*a,A}
	DS	13.24 ± 0.15 ^{b}	119.53 ± 2.20 ^{b}	113.58 ± 3.38 ^{b}	117.34 ± 0.48 ^{b}	0.399 ± 0.023 ^{bc}
	DS + HA < 30 kDa	12.19 ± 0.63 ^{b}	116.70 ± 6.35 ^{b}	124.21 ± 0.84 ^{c}	85.43 ± 1.17 ^{c}	0.347 ± 0.039 ^{b}
	DS + HA > 30 kDa	10.78 ± 0.14 ^{c}	117.63 ± 6.43 ^{b}	103.47 ± 4.15 ^{d}	126.90 ± 3.80 ^{d}	0.422 ± 0.007 ^{c}
Nawiko	Control	12.25 ± 0.31 ^{*a,B}	67.94 ± 2.29 ^{*a,B}	34.72 ± 1.67 ^{*a,B}	164.34 ± 5.07 ^{*a,B}	0.363 ± 0.038 ^{*a,B}
	DS	11.79 ± 0.04 ^{a}	98.72 ± 1.44 ^{b}	42.49 ± 5.28 ^{b}	166.67 ± 2.93 ^{a}	0.463 ± 0.011 ^{b}
	DS + HA < 30 kDa	8.86 ± 0.46 ^{b}	104.43 ± 0.91 ^{b,c}	66.60 ± 3.83 ^{c}	196.84 ± 4.85 ^{b}	0.571 ± 0.058 ^{c}
	DS + HA > 30 kDa	9.91 ± 0.33 ^{c}	110.29 ± 2.20 ^{c}	67.20 ± 1.20 ^{c}	185.52 ± 3.13 ^{b}	0.726 ± 0.090 ^{c}

SD—standard deviation * values marked with the same letters do not differ significantly according to Tukey's test at the significance level $p \leq 0.05$; lowercase—drought stress, capital letter—cultivar.

Plants growing in natural conditions show variable content of trace elements in their dry matter. It varies between species and cultivars, as well as conditions of vegetation [86,87]. The mechanism that plants use to absorb elements is rather complex and depends on a number of factors, e.g., cation exchange through cell membranes, intracellular transport, and ions and substances secreted by roots and microorganisms [87]. The presence of stress factors makes the process even more complex.

In the case of macroelements, differences in the content of monovalent elements were found (Table 1). Leaf dry matter of the Progres cultivar was characterized by an increased content of K to 3.86%, which is 40% higher than in Nawiko. In contrast, in the case of Progres leaves' dry matter, the Na content was reduced to 0.12%, twice less than in Nawiko. No significant differences regarding the content of Ca and Mg in leaves of soybean cultivars were found. Both cultivars showed higher content of Ca than Mg. The content of Ca in the dry matter of leaves varied from 0.88% to 0.91%, whereas Mg varied from 0.35 to 0.36% (Table 1).

Larger differences in the case of microelement content in leaves' dry matter of both cultivars were found. The Progres cultivar had better capability to accumulate the microelements (Table 2). Its total content in the dry matter of leaves was two times higher compared to Nawiko. This was due to a significantly higher content of Cu, Mn, Zn, and Fe. Only Co showed significantly higher accumulation by 34% in leaves of the Nawiko cultivar. However, its contribution to the dry matter of plants was the lowest.

Progres and Nawiko plants showed different reactions to drought stress induced by polyethylene glycol. Hu and Schmidhalter [87] stated that the uptake and translocation of mineral nutrients within the plant could be affected by drought stress. In the case of macroelements, both cultivars did not show a significant impact of drought on the content of Na, K, and Mg in the dry matter of leaves. In the case of Ca, the soybean cultivars showed different reactions to the deficit of water. Progres leaves recorded a statistically significant increase in Ca (by 14%), whereas Nawiko leaves showed a significant decrease of 10% (Table 1).

It was concluded that the water deficit has an influence on the uptake of microelements present in the solution by soybean plants, and this was also determined by their cultivar (Table 2).

In the case of Progres, the study showed a significant decrease in microelement content in the dry matter of leaves (by 70%) compared to control plants. This was due to a reduced accumulation of Cu, Mn, Zn, and Fe. The largest change was recorded for iron (by 63%) whereas the smallest for Zn (by 15%). It was also found that drought stress increases uptake of Co. The content of Co was 80% higher compared to control plants.

In the case of the Naviko cultivar, the deficit of water did not have a significant influence on the content of Cu and Fe, whereas its presence significantly increased accumulation of Mn, Zn, and Co. Compared to control plants, the increase in the content of Mg in the dry matter of leaves was the largest (by 45%), and the smallest in the case of Zn (by 22%). Due to these changes, the total presence of microelements in the dry matter of Nawiko soybean leaves increased by about 13%.

HA fractions with polyethylene glycol did not have a direct impact on the content of micro- and macroelements in the dry matter of soybean leaves. The impact depended on HA fractions and soybean cultivar (Tables 1 and 2).

The HA < 30 kDa fraction did not have a significant impact on the content of K and Mg in the soybean cultivars, since their content in the dry matter of leaves was not different compared to control plants and plants growing under drought stress only. The study concluded that the deficit of water determined the accumulation of Ca and Mn in leaves of both soybean cultivars and Cu, Zn, and Co in leaves of the Progres cultivar. In the case of the combination DS + HA < 30 kDa, their content in the dry matter of leaves was at the same level of DS (Tables 1 and 2).

In the case of the HA < 30 kDa fraction, the study recorded a reduced accumulation of Na and Cu for the Nawiko cultivar and Fe for the Progres cultivar compared to the DS and control plants. When combined with the drought stress, the fine-particle HA fraction stimulated an increased accumulation of Zn, Fe, and Co in soybean leaves of Nawiko and accumulation of Na soybean leaves of Progres. The increase was statistically significant both in the combination with DS and the control plants.

Larger differences in the content of macro- and microelements in soybean leaves of the soybean cultivars were obtained for the combination of DS + HA > 30 kDa. It reduced the uptake of K, Mg, Zn, and Cu by Progres and Cu by Nawiko. The changes were statistically significant compared with the DS only and the control plants.

It was concluded that the presence of the HA > 30 kDa fraction may reduce drought stress and increase the content of K, Mn, Zn, Fe, and Co in Nawiko soybean leaves and Co in Progres soybean leaves compared to DS and control plants. In the case of combined DS + HA > 30 kDa, the content of Ca in dry matter of both cultivars' leaves and Na in dry matter of Nawiko leaves was at the level of control plants. The content of Fe in the dry matter of Progres leaves significantly increased compared to DS by 8%. However, its contribution to the dry matter of leaves was still about 60% lower than in control plants.

In the case of the HA > 30 kD fraction, the drought stress factor determined the level of Mn in Progres soybean leaves, since its contribution to the dry matter of leaves was at the same level as for DS.

While comparing the impact of HA on the uptake of nutrients in the presence of drought stress, it was concluded that HA > 30 kDa reduces the uptake of K, Mg, Ca, Cu and Zn by the Progres cultivar, whereas it increases the uptake of Na, K, and Cu by the Nawiko cultivar, as well as Fe and Co by the Progres cultivar. Differences in the size of HA

(<30 kDa, >30 kDa) do not have a statistically significant influence on the uptake of Mg, Ca, Zn, Fe, Co, and Mn by Nawiko and Ca and Mn by Progres.

4. Conclusions

This research will ensure a better understanding of the relationship between HA molecular structure activity and plant stress response. Most studies confirm the beneficial effect of HS on plant growth by mitigating the negative effects of abiotic stresses, but the physiological mechanism has not been well known. Our research indicates that the studied soybean cultivars respond differently to drought stress alone and with the addition of different molecular HA fractions to the nutrient solution.

Drought stress caused a decrease in most physiological parameters and an increase in peroxidase activity in the case of both studied cultivars. However, the results of biometric measurements showed that the Progres cultivar appears to have better tolerance to drought stress. The significant influence of water deficit on most of the macroelement content in the dry matter leaves of both studied cultivars was not observed while its effect on microelements uptake by soybean plants was concluded. In the case of the Progres cultivar, the results showed a significant decrease in microelement content in the dry matter of leaves, whereas in the leaves of Nawiko cultivar a significant increase.

The influence of HA > 30 kDa and HA < 30 KDa fractions on physiological features of both studied cultivars was varied. In the case of the Nawiko cultivar growing under drought stress with HA addition, an increase in values of morphological features (fresh and dry matter of aboveground parts and roots, and plant length) was observed compared to drought stress only; however, the difference was not statistically significant. Both fractions also had a positive effect on membrane permeability; however, it was also not a statistically significant difference. The addition of the HA > 30 kDa fraction caused a statistically significant increase in chlorophyll compared to drought stress only. In the case of the Progres cultivar, the statistically significant decrease in fresh matter of aboveground parts and roots compared to drought stress only was found only for HA >30 kDa. A significant increase in length and chlorophyll content was found for HA < 30 kDa compared to drought stress only.

The HA > 30 kDa fraction better up-regulated the antioxidant defense system. Unfortunately, no effect of either HA fraction on the macro- and micronutrients uptake system of both studied cultivars was observed.

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