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HORMONE BALANCE AND SHOOT GROWTH IN WHEAT (*Triticum durum* Desf.) PLANTS AS INFLUENCED BY SODIUM HUMATES OF THE GRANULATED ORGANIC FERTILIZER

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Abstract

Humic acids are formed in soil during decomposition of organic residues and are capable of increasing plant productivity. Plant growing widely utilizes application of the most soluble preparations on the base of sodium and potassium humates possessing hormone-like activity. Humic substances are capable of stimulating plant growth in very low concentrations that determines the necessity of targeting their uptake by plants. Such a possibility is provided by placing fertilizers in the direct vicinity of the seeds. The effect of incrustation of Nitrofosque granules with sodium humate (SH) on the crop yield of spring durum wheat has been shown previously. In the present experiments, the data on the changes in growth and hormone content of wheat plants (Bashkirskaya 27 cv.) treated with SH included into the granules of organic-mineral fertilizer (OMF) are reported for the first time. Granules of fertilizers without SH and those containing 1.25×10^{-2} %, 2.5×10^{-2} % and 5×10^{-2} % of humic preparation (of the granule mass) were placed at the distance of 2-3 cm from the 1-day seedlings planted into the soil. OMF was obtained from poultry waste (chicken manure) and dolomite and SH was extracted from brown coal. Plants that obtained neither humates nor OMF served as the control. Leaf length and transpiration (according to the decline in the mass of vessels with plants) were measured starting from the third day after sowing. Leaf samples for determination of hormones with enzyme-linked immunoassay were sampled on day 9 and leaf area and plant mass were measured on day 21 after sowing. OMF granules without SH stimulated leaf elongation resulting in their significantly longer length compared with the control (42, 156, 187 and 274 mm against 47, 167, 199 and 294 mm, $p \le 0.05$) detected in the first leaves 3, 6 and 8 days after sowing and 14 days after sowing in the second leaves. Meanwhile an increase in the OMF dosage did not increase their length significantly compared to the lower dose. Addition of humic substances to the OMF granules increased the promotive effect of the preparation on the leaf elongation most clearly manifested in the case of intermediate SH concentration ($2{,}5\times10^{-2}$ %), which significantly increased the leaf length during the time of registration (3, 6, 8, and 14 days after sowing) compared to the application of OMF without SH (6, 11, 13 mm increment of the first leaves and 9.4, 9, 22 mm of the second leaves, $p \le 0.05$). Unlike OFM without SH, which application did not change the shoot mass and leaf area, combination of OFM with intermediate SH concentration increased shoot mass (from 538 to 583 mg, $p \le 0{,}05$). Leaf area was significantly greater than in the control in the case of intermediate and maximal SH concentration (increments were 385 and 283 mm^2, $p \le 0.05$). Thus, addition of SH to OFM increased the effectiveness of the OFM action on growth characteristics. Accumulation of nitrogen in the shoots of the wheat plants

supplied only with OFM without SH did not differ from the control, while combination of OFM with SH resulted in accumulation of nitrogen at the level (by 8-15 %, $p \leq 0.05$) higher than in the control. Application of OFM without SH did not influence the content of studied hormones (auxins, cytokinins and abscisic acid — ABA), while addition of SH to the granules of fertilizers resulted in significantly higher concentration of abscisic acid (ABA) and cytokinins in the shoots, the 1.6-2.8 $ng \cdot g^{-1}$ increment (1.5-1.8 times) and 3.8-4.9 $ng \cdot g^{-1}$ increment (1.5-1.7 times) ($p \leq 0.05$). Concentration of indole acetic acid (IAA) with intermediate SH concentration was higher than in the control (40 against 15 $ng \cdot g^{-1}$, $p \leq 0.05$). The results obtained by us allow attributing increased effectiveness of fertilizers in the case of their supply with humates to their effect on the hormonal content. Increased content of hormones with promotive type of action is likely to enable activation of plant growth, while accumulation of ABA limits water losses by transpiration. The results of these laboratory experiments indicate perspectives of application of OMF containing sodium humates according to technology developed by us. Furthermore, the obtained data are of importance for revealing fundamental mechanisms, by which the action of humic compounds is manifested, in particular at the level of hormonal balance in plants.

Keywords: *Triticum durum,* organo-mineral fertilizer, sodium humate, auxins, cytokinins, abscisic acid, plant growth

Humic substances, mostly humic acids and fulvic acids synthesized in the soil during the decomposition of organic residues, can stimulate plant growth and yield [1]. Sodium and potassium humates manufactured commercially by alkaline extraction of caustobiolites (brown coal, peat, sapropel) are increasingly used in crop production. It is assumed that humates affect plant productivity both directly and indirectly. The indirect effect is associated with a modification of the soil structure in the rhizosphere and an increase in the availability of mineral elements for plants, while the direct effect lies in a change in the metabolism and development of plants [2, 3].

The mechanism underlying the effect of humic substances on physiological and biochemical processes in plants is not fully understood [4]. Humic substances of a relatively low molecular weight can form supramolecular associations consisting of hydrophilic and hydrophobic domains [5]. The hydrophobic components of humic substances resulted from destruction of plant residues capture the hydrophilic components of the soil and protect them from degradation [6). The compounds captured in this way can be further released due to changes in the structure of these associates under the influence of low-pH root exudates [4]. The direct effect of humic acids on the growth and development of plants is a manifestation of their hormone-like activity [7-9]. Auxins (mainly indoleacetic acid) [7, 10] and cytokinins (in the form of isopentenyladenine) [11] are identified in preparations of humic acids. Humic acids affect enzyme activity, gene expression, and proton pump activity in the same way as the plant hormone auxin [7-10]. There are reports that humic substances change the root architecture and metabolism due to such activity [12]. The hormone-like properties of humic preparations determine one of the advantages of organic fertilizers over chemical ones. However, the effects of humic acids on the levels of auxins and cytokinins in plants have not yet been studied.

The practical advantage of humic compounds is that they can stimulate plant growth in very low concentrations, from 20 mg / l^{-1} [7], which provides their cost-effectiveness. However, in case of low concentrations, the problem of targeting and entry into plants arises. The strip till technology and special seeders which allow fertilizers to be incorporated in the immediate vicinity of the seeds facilitate the problem.

Previously, we have shown the positive effect of sodium humate incorporated into nitrophoska granules on the yield of spring durum wheat [13]. This work is the first to report on the effect of different doses of sodium humate-based organic mineral fertilizers (OMF) on the growth and hormonal balance of wheat plants.

Our main purpose was to confirm or disprove the hypothesis that the effectiveness of the organomineral fertilizers combined with sodium humate is due to the sodium humate effect on the hormonal system of plants.

Materials and methods. Sodium humate (SH) was obtained according to a procedure similar to that described [14, 15]: 100 ml of 3% NaOH was added to 5 g of brown coal (Kumertau deposit, Republic of Bashkortstan) and stirred for 2 h at 60 °C, the undissolved precipitate (humin) was separated by centrifugation and rinsed by distilled water, 1% HCl was added to the resulting solution to pH = 2, the precipitate of humic acids was separated (a PE 6910 centrifuge, OOO EkrosKhim, Russia, 10 min, 4000 rpm/2325 g), rinsed by distilled water, dried in air, and dissolved in 1% NaOH (0.1 g per 10 ml).

The concentration of 0.01-0.001% solutions of humic acids in a 0.4% NaOH solution was measured by the optical density (OD) (Shimadzu UV 2600, Shimadzu, Japan, λ = 465 nm) as described [14, 15].

The organomineral fertilizers (OMF) were 60-65% fresh chicken manure and 54-33% dolomite (calcium and magnesium carbonates). SH was added to OMF at 1.25×10 2%, 2.5×10 2%, and 5×10 2% (SH1, SH2, and GN3, respectively) of the weight of the OMF granules. A portion of granules were not added with SH.

OMF granules, approximately 80 mg in weight with different contents of humic acids, were placed in pots filled with soil (about 0.82 kg, with a 2 cm layer of pebbles drainage) at a 5-6 cm depth from the soil surface at the rate of one or three granules per seed (80 or 240 mg). The seeds of durum spring wheat (*Triticum durum* Desf.) cv. Bashkirskaya 27 after 1-day germination on filter paper were placed at a depth of 3 cm from the soil surface (3 cm from the granules), 10 seeds per pot, 8 pots per treatment. Plants that did not receive additional OMF nutrition served as control. Plants were grown at PAR 400 μmol / m^{-2} (14 h daylight hours, 20/24 °C). The 60-80% soil moisture of the full moisture capacity was maintained with irrigation (2-3 times a week). From day 3 after planting the seedlings, the lengths of the 1st and 2nd leaves were measured. Twenty-one days after planting, the shoots were weighed, and the area of scanned leaf images were measured using the ImageJ software (National Institute of Health, USA; https://imagej.nih.gov).

The hormone concentration was determined on day 9 after planting. Phytohormones were extracted from homogenized shoots with 80% ethyl alcohol at 4 °C overnight. Purification and concentration of indoleacetic acid (IAA) and abscisic acid (ABA) from an aliquot of the aqueous residue after evaporation of the alcohol extract (from Petri dishes in a stream of air) was performed using ether extraction according to a modified scheme with a decrease in volume as described [16). IAA and ABA were twice extracted with diethyl ether from an aliquot of the aqueous residue acidified to pH 2-3 in a ratio of 1:3. Then they were returned to the aqueous phase (1% sodium bicarbonate solution, the ratio of the aqueous to organic phase is 1: 2), acidified again to pH 2-3, and, after 2-fold back-extraction with diethyl ether, methylated with diazomethane derived from nitrosomethylurea and added to the samples. The dried samples were dissolved in a small amount of 80% ethanol immediately before the immunoassay or in 100 μl of 80% ethyl alcohol before the enzyme-linked immunosorbent assay. Cytokinins from an aliquot of the aqueous residue (a sample previously purified by centrifugation) were concentrated in a C18 cartridge (Waters Corporation, USA) equilibrated with distilled water. The column with the sample was washed with 20 ml of distilled water. Cytokinins were eluted with 70% ethyl alcohol, then the alcohol was evaporated to dryness and, after dissolving the residue in 20 μl of 80% ethanol, the sample was analyzed by thin layer chromatography (TLC) in a silufol plate. TLC was

performed using a butanol:ammonia:water (6:1:2) solvent system as described [17]. After UV-detection (TCP-15.MC transilluminator, 4×8 W 312 nm lamps, Vilber Lourmat, France) of the zeatin, zeatin nucleotide and zeatin riboside markers, the zones were eluted with 0.1 M phosphate buffer (pH 7.2-7.4). To sediment silufol, the eluate was centrifuged for 10 min at 10000 rpm (Eppendorf MiniSpin, Eppendorf, US). The IAA, ABA and cytokinins were quantified by enzyme-linked immunosorbent assay (ELISA) with specific antibodies to hormones [16-18]. Antibodies to zeatin riboside were also used for the determination of zeatin and its nucleotide since they have cross-reactivity to these cytokinins.

ELISA test was performed in the wells of Castar polystyrene plates (Corning Incorparated, USA). The hormone-protein conjugate was immobilized on polystyrene. A 200 μl aliquots of the conjugate pre-diluted in 0.05 M immobilization buffer (9% NaCl) were poured into each well and kept at 4 °C for 18-20 h in a refrigerator or 2 h in a thermostat at 37 °C. The plates were washed thrice by physiological saline (pH 7.2-7.4) with 0.05% Tween 20 (PT solution). In all subsequent washings, the same solution was used. In some wells, 10-fold dilutions of hormone standards were poured to obtain a calibration curve. In the remaining wells, the aliquots of alcohol solutions of methylated hormones (IAA, ABA) or aliquots of phosphate buffer (pH 7.2-7.4) used to elute cytokinins from silufol were poured. Antisera to the corresponding hormone (100 μl per well) obtained as described [18, 19] and diluted with saline + 0.3% bovine serum albumin + 0.05% Tween 20 (PTB) were added to all wells. The plates were incubated at 37 °C for 1 h and rinsed with PT solution. To visualize the reaction of serum antibodies with the immobilized hormone conjugates, the peroxidase-conjugated bovine anti-rabbit antibodies were used. The secondary antibodies were diluted in PTB, 200 portions were poured into wells, incubated for 1 h at 37 °C, and rinsed by PT. The color reaction with the substrate ortho-phenylenediamine (0.4 mg/ml in 0.06 M phosphate buffer, pH 5.2, with 0.006% hydrogen peroxide) was stopped in 15-30 min using 4 N sulfuric acid. After measuring the optical density (photometer AIFR-01 UNIPLAN, ZAO PIKON, Russia; $\lambda = 492$ nm), the hormone concentration was calculated using the calibration curve.

On day 21, the total nitrogen concentration was measured according to Kjeldahl. Evapotranspiration of plants was evaluated throughout the experiment as pot weight losses.

Statistical processing was performed by standard methods using the MS Excel software. The means (*M*) and their standard errors (±SEM) were calculated. Differences were assessed by Student's *t*-test and considered statistically significant at $p \leq 0.05$. The number of replicates were 5 for nitrogen content assessment, 9 for hormones, 30 for water consumption, and 50 for assessment of plant weight, area and length of leaves.

Results. Mixing chicken manure with dolomite leads to formation of magnesium and calcium salts of uric and other organic acids contained in chicken manure, which reduces their solubility and a negative effect on plant growth when used in high concentrations. In addition, heating OMF to 100 °C due to friction during mixing and extrusion kills pathogenic microorganisms from chicken droppings.

As seen (Fig. 1), the OMF granules increased the length of the leaves by 5-17% compared to the control depending on the leaf age and the treatment option. In 3 days after sowing, the leaves were significantly longer than in the control ($p \leq 0.05$) only at a lower dose of OMF (one granule instead of three) and an intermediate concentration of humates (SH2) (see Fig. 1, A). As the plants grew, the effects of OMF and SH were also manifested at other doses and concentrations, i.e., the 1st leaves on day 8 and the 2nd leaves on day 14 were significantly

longer ($p \le 0.05$) than in the control for all treatments with OMF (with and without humate) (see Fig. 1). An increase in the dose of OMF did not lead to a significant increase in the length of leaves as compared to the minimum OMF dose, i.e., the increase in length compared to the control averaged 7.5 and 6.0% for one and three granules per seed, respectively.

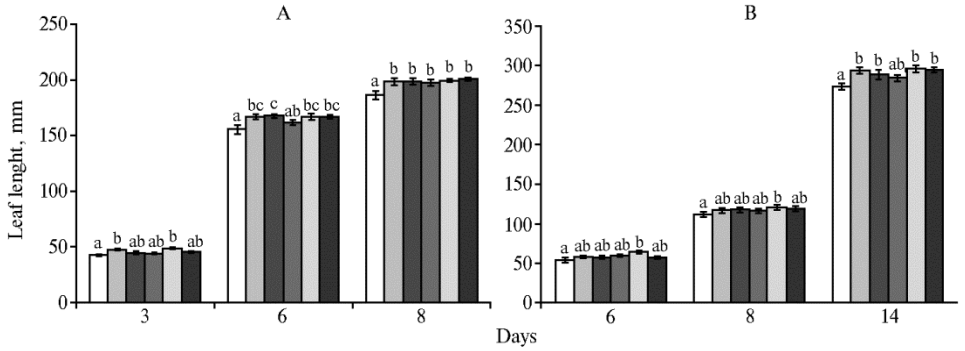

Fig. 1. The 1st (A) and 2nd (B) leaf length in spring durum wheat (*Triticum durum* Desf.) cv. Bashkirskaya 27 depending on the dosage pf organomineral fertilizers (OMF granules, mg per seed) **along and with different doses of sodium humate** (SH) (SH1 — $1{,}25\times10^{-2}$ %, SH2 — $2{,}5\times10^{-2}$ %, SH3 — 5×10^{-2} % of OMF granule weight). Bars from left to right are control, OMF 80 mg, OMF 240 mg, OMF 80 mg + SH1, OMF 80 mg + SH2, OMF 80 mg + SH3. Differences between the bars for which there is no coincidence in any letter designation are statistically significant at $p \le 0.05$ ($M\pm$SEM, $n = 50$, pot tests).

SH added to OMF granules increased their stimulating effect on the leaf elongation which was the clearest for SH2 (2.5×10 2% of the granule weight) as compared to the control. Upon this treatment, the average increase in leaf length over the entire observation period (3, 6, 8, and 14 days after planting) averaged 10% (differences as compared to the OMF effect without SH were statistically significant, $p \le 0.05$, paired *t*-test).

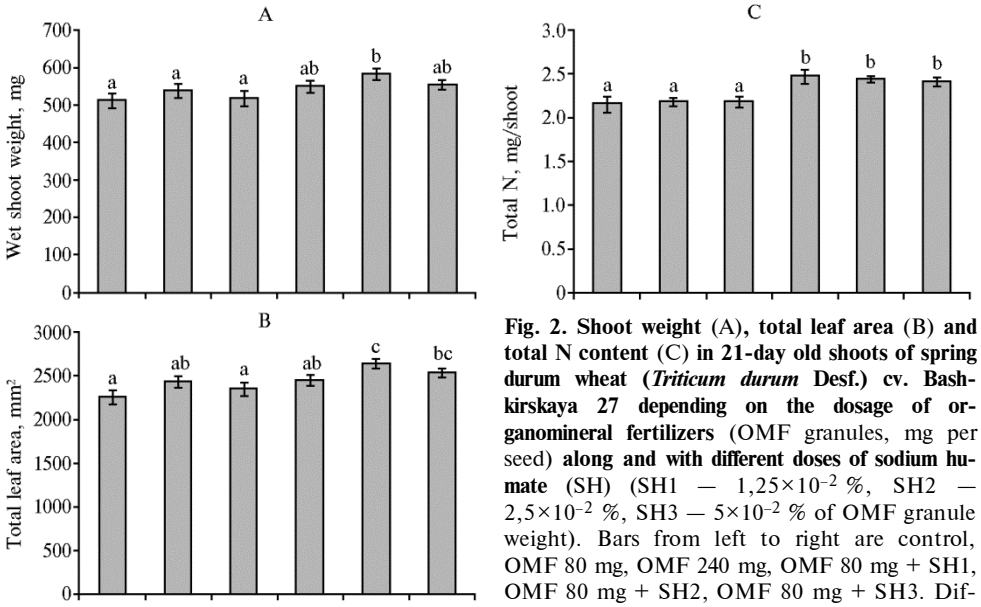

Fig. 2. Shoot weight (A)**, total leaf area** (B) **and total N content** (C) **in 21-day old shoots of spring durum wheat (*Triticum durum* Desf.) cv. Bashkirskaya 27 depending on the dosage of organomineral fertilizers** (OMF granules, mg per seed) **along and with different doses of sodium humate** (SH) (SH1 — $1{,}25\times10^{-2}$ %, SH2 — $2{,}5\times10^{-2}$ %, SH3 — 5×10^{-2} % of OMF granule weight). Bars from left to right are control, OMF 80 mg, OMF 240 mg, OMF 80 mg + SH1, OMF 80 mg + SH2, OMF 80 mg + SH3. Differences between the bars for which there is no coincidence in any letter designation are statistically significant at $p \le 0.05$ ($M\pm$SEM, for A and B $n = 50$, for C $n = 5$, pot tests).

Three weeks after planting, the OMF with SH2 led to a significant increase

in the fresh weight of the shoots compared to the control (Fig. 2, A). OMF without SH did not cause a significant increase in the shoot weight compared to the control. For the OMF supplemented with SH1 and SH3, the plants occupied an intermediate position between the control and the use of SH2, that is, their fresh weight did not differ statistically significantly from those in either the first or the second case. Thus, we revealed a tendency to increase the biomass of wheat plants under the influence of a OMF combination with SH, which was statistically significant at an intermediate SH concentration. For SH2 and SH3, the total leaf area was significantly greater than in the control (2637 and 2535 vs. 2252 mm^2, $p \le 0.05$), while OMF without SH and OMF plus the minimum SH1 concentration did not have a significant effect on the indicator (the differences from the control are not significant) (see Fig. 2, B).

The level of total nitrogen in shoots for OMF without humate did not differ from the control (see Fig. 2, C), being significantly higher ($p \le 0.05$) than in the control when OMF with SH were applied.

Evapotranspiration over the entire experiment did not differ between the control plants and those treated with fertilizers (1.6 g of water per plant per day, data not shown).

Upon application of OMF without SH, the concentration of hormones (auxins, cytokinins, and ABA) in wheat plants was at the control level (Fig. 3). The addition of sodium humate increased the content of hormones. To confirm the results of enzyme-linked immunosorbent assay, a comparison of ELISA and gas chromatography-mass spectrometry data was previously performed [17].

The total concentration of cytokinins was stably higher than in the control for all SH concentrations (see Fig. 3, A, $p \le 0.05$), and OMF without SH did not increase the cytokinin concentrations as compared to the control. The accumulation of auxins was pronounced at an intermediate concentration of humate, with the IAA level almost 3 times higher than in the control ($p \le 0.05$) (see Fig. 3, B). The ABA content was higher under the action of all tested concentrations of SH (differences from the control and the OMF without SH are significant at $p \le 0.05$) (see Fig. 3, C).

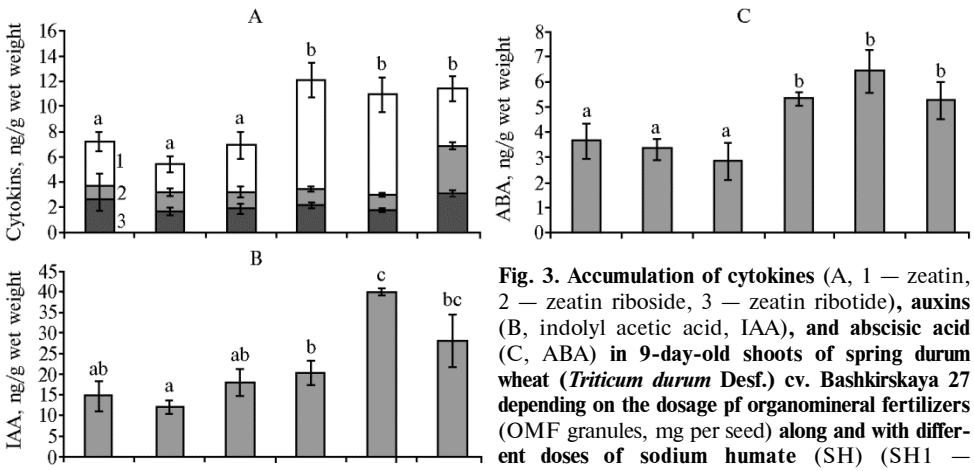

Fig. 3. Accumulation of cytokines (A, 1 — zeatin, 2 — zeatin riboside, 3 — zeatin ribotide)**, auxins** (B, indolyl acetic acid, IAA)**, and abscisic acid** (C, ABA) **in 9-day-old shoots of spring durum wheat (*Triticum durum* Desf.) cv. Bashkirskaya 27 depending on the dosage pf organomineral fertilizers** (OMF granules, mg per seed) **along and with different doses of sodium humate** (SH) (SH1 — $1{,}25\times10^{-2}$ %, SH2 — $2{,}5\times10^{-2}$ %, SH3 — 5×10^{-2} % of OMF granule weight). Bars from left to right are control, OMF 80 mg, OMF 240 mg, OMF 80 mg + SH1, OMF 80 mg + SH2, OMF 80 mg + SH3. Differences between the bars for which there is no coincidence in any letter designation are statistically significant at $p \le 0.05$ ($M\pm$SEM, $n = 9$, pot tests).

In general, the results on the length and area of leaves and the shoot weigh

showed that the humate added to organomineral fertilizers enhances their ability to stimulate the wheat plant growth. It was also revealed that OMF acts in low doses (one granule per seed), and an increase in the dosage to three granules does not enhance the positive effect.

An increase in nitrogen accumulation in shoots in response to application OMF with SH indicates an improvement in the ability of wheat plants to absorb mineral elements under the influence of humate, which corresponds to some reports [20]. Since the nitrogen has been repeatedly shown to stimulate the synthesis of cytokinins [21], their accumulation in the shoots of plants treated with SH can be associated with an increase in the nitrogen content. However, it is worth noting that the total nitrogen in shoots increased under the influence of SH to a lesser extent (to a maximum of 15%) than cytokinins the content which was 1.5 times higher than in the control, regardless of the SH concentrations. The lack of proportionality between the accumulation of nitrogen and cytokinins suggests the existence of additional mechanisms for stimulating the cytokinins accumulation in plants due to the action of sodium humates, e.g., their direct effect on plant hormones.

An alternative explanation may lie in the admixtures of hormone-like compounds in the SH preparation, which corresponds to the literature data [1]. Since physiologically active substances, including hormones, act in low concentrations, it is the presence of hormone-like substances that can explain the ability of small doses of SH to affect the growth and development of plants, as it was shown in our experiment. Auxin-like substances were found in humic preparations [4], which corresponds to our findings which revealed an increase in the content of auxins in plants under the influence of SH. It was also found that the use of a humic preparation increases the availability of nitrogen, phosphorus, and potassium [11].

The content of humic acids was 20-23% in fresh droppings and 15-17% in granulated OMF based on chicken manure and dolomite. However, the solubility of free humic acids is very low, while the solubility of their sodium salts is quite high. Therefore, the high efficiency of granular OMF containing readily soluble sodium humates on plant growth can be ensured by its high biodegradability.

Demin et al. [22] consider the direct penetration of humates into the cell unlikely, since this should be prevented by the formation of hydrogen bonds with the components of the cell walls, but it is possible for humic substances to enter the cell due to endocytosis and their further digestion in lysosomes. It is known that receptors for many hormones are located on the cell surface [23], which makes it possible to explain how hormone-like components contained in humates can affect plants by interacting with receptors on the cell surface without penetrating into the cell.

Our experiments do not allow us to conclude whether the increased levels of hormones are associated with their uptake from the SH preparation. The probability of this process is low, given the large sizes of humate molecules [24]. Nevertheless, the very fact of the accumulation of hormones in plants treated with the preparation we used clearly indicates the physiological activity of SH. It is important that in plants exposed to OMF without humate no increase in the hormone levels was recorded, that is, an increased content of hormones in plants is a characteristic response precisely to the SH in the preparations. The ability of cytokinins and auxins to stimulate shoot growth by activating cell division and elongation is well known [25]. This allows us to explain the activation of growth of wheat shoots under the influence of SH shown in our experiments by the accumulation of auxins and cytokinins.

The accumulation of ABA we found upon the introduction of granules

with sodium humate into the plant rhizosphere corresponds to some data [26]. The authors of the cited communication associate the ABA accumulation with the peculiarities of water exchange in plants treated with humate. We revealed a significantly greater accumulation of ABA under the influence of humates, i.e., almost 2-fold compared to the control, with the reliable differences from the control and OMF without SH ($p \leq 0.05$). As known, the water evaporation by leaves increases with an increase in total leaf area. In our experiment, the leaf area was significantly larger compared to the control at the intermediate and maximum doses of SH. However, we did not reveal a significant increase in transpiration losses (despite the large leaf area), which indicates a decrease in stomatal conductance. Since the ability of ABA to close stomata is well known, it is possible to explain the limitation of transpiration losses by an increase in the ABA content in plants under the influence of SH. We have previously shown that the activation of leaf growth by bacterial plant stimulants was accompanied by the accumulation of ABA which also limited evapotranspiration [18]. Thus, the change in the ABA accumulation under the influence of humate can be an adaptive response aimed at optimizing water relations when the humate acts on plants.

Thus, our findings show that sodium humate (SH) in the formulation of organic fertilizer (OMF) granules activates plant growth, which is most likely due to the ability of SH to influence the plant hormonal status. The accumulation of hormones possessing stimulating effects ensures the activation of plant growth, while the ABA accumulation normalizes water exchange. Our tests in which granulated fertilizers were placed at a distance of several centimeters from the seeds, simulating the strip till technology application, indicate the prospects of using SH-containing OMF in this technology.

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