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EFFECT OF EXOGENOUS TREATMENT WITH ABSCISIC ACID ON PHYTOHORMONES ACCUMULATION IN WINTER WHEAT SEEDLINGS UNDER ZINC STRESS

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Abstract. Among heavy metals (HM), zinc is essential for plant growth and development. It plays an important role in metabolism, is critical for the action of Zn-dependent enzymes involved in physiological processes. However, at elevated concentrations, like other heavy metals, it negatively affects the plant metabolism In this work, we investigated whether exogenous treatment with abscisic acid (ABA) alters endogenous phytohormones balance of zinc-stressed winter wheat seedlings. It was shown, that under zinc stress in the presence of exogenous abscisic acid (ABA) in three-day-old seedlings of *Triticum aestivum* L. cv. Podolyanka changed the character of accumulation and balance between hormones stimulators of growth processes and hormones involved in the formation of protective mechanisms. Zinc at a concentration of 228 mg/l inhibited the growth of the root system. Under these conditions, a decrease in the content of endogenous indole-3-acetic acid (IAA), zeatin and ABA and an increase in gibberellic acid (GA_3), isopentenyladenosine (iPA) and salicylic acid (SA) took place. After adding to incubation medium10^{-6} M ABA, the growth of the seedling roots intensified and the negative effect of zinc was mitigated. The level of stressful hormones ABA and SA became higher. The strategy of wheat seedlings adaptation to zinc stress in the presence of exogenous ABA was aimed at activation of root growth. Changes in the phytohormones balance initiate protective mechanisms and further adaptation of plants to high concentration of zinc, and the treatment of grains with exogenous ABA can be used to enhance stress resistance.

Abbreviations:
IAA – indole-3-acetic acid
ABA – abcisic acid
CK – cytokinin
GA_3 – gibberellic acid
HM – heavy metal
HPLC-MS – high performance liquid chromatography-mass-spectrometry
iP – isopentenyladenine
iPA – isopentenyladenosine
SA – salicylic acid
t-Z – *trans*-zeatin
t-ZG – *trans*-zeatin-*O*-glucoside
t-ZR — *trans*-zeatin riboside

1. Introduction

Heavy metals, as constituents of various chemical compounds, are essential natural components of the earth's crust. As essential microelements, they affect plant growth, development and metabolism. However, at levels exceeding a certain threshold, HMs act as pollutants and pose an environmental threat. Excessive concentrations of HMs inhibit plant growth, biomass accumulation, adversely affect photosynthetic activity (Chandra & Kang, 2016; Mathur *et al.*, 2016), mineral nutrition (Vernay *et al.*, 2007), water exchange (Mukhopadhyay & Mondal, 2015). Zinc is one of the essential microelements necessary for the normal life of plants. Its effect is manifested in the growth promotion, the initiation of flowers and formation of grains, activation of reparative processes (Alloway, 2008). It functions as a cofactor of enzymes, is involved in the synthesis of tryptophan, a precursor of indole-3-acetic acid (Alloway, 2008), is a component of the zinc finger proteins associated with signal transduction within the cell (Figueiredo *et al.*, 2012), is implicated in protecting cell membranes (Cakmak, 2000). At high concentrations zinc is toxic to plants. In wheat, its excess inhibited shoot growth and reduced the rate of photosynthesis (Kaznina & Titov, 2017), in tomato plants it caused a decrease in protein content in roots and shoots (Kösesakal & Ünal, 2012). In our studies, exogenous ABA was shown to mitigate the negative effects of high zinc concentrations on the grain germination and the growth of winter wheat seedlings (Vasyuk *et al.,* 2019). Zinc ions Zn $^{2+}$, ZnON $^{+}$, ZnCl $^{+}$ are freely adsorbed by soil colloids. The concentration of this metal in the soil is 17-160 mcg/g, and in plant leaves it is in the range of 20-100 mcg/g of dry weight (Kots & Peterson, 2005). In areas contaminated by industrial ferrous and non-ferrous metallurgical emissions, zinc content reaches 1200 mg/kg (Svitowyi *et al.*, 2014).

Plant growth and development is regulated by a complex hormonal system, the interaction between components of which is synergistic or antagonistic. Hormonal substances occur in plant tissues at low concentrations and function at sites of synthesis, or are transported to other organs and tissues (Li *et al.*, 2016; Santner *et al.*, 2009). Due to their complex and balanced interaction, plants maitain hormonal homeostasis in various organs at all stages of ontogeny (Kohli *et al.*, 2013; Ravindran *et al.*, 2017; Stamm *et al.*, 2012; Verma *et al.*, 2015).

In recent years, the involvement of phytohormones in the induction and integration of plant defense responses under HM impact has been actively investigated (Bücker-Neto *et al.*, 2017; Rajewska *et al.*, 2016; Sah *et al.*, 2016). Phytohormone priming positively affects nitrogen metabolism, cell division, transpiration, and enzymatic activity under conditions of metalic stress (Sytar *et al.*, 2019). Thus, treatment with exogenous abscisic (Pantin *et al.*, 2013) and salicylic acids (Metwally *et al.*, 2003) at toxic concentrations of HM activated the signaling cascades and simulated phytoprotective effects. IAA, combined with selenium, reduced stress in conditions of arsenic compounds contamination (Pandey & Gupta, 2015). Due to the activation of defense mechanisms and inhibition of growth and development (Bartels & Sunkar, 2005; Danquah *et al.*, 2014, Voytenko & Kosakivska, 2016), ABA is considered vital for the plant because of biotic and abiotic stressors effects. Therefore, our aim was to determine the effects of exogenous treatment with ABA on the phytohormones accumulation and balance in *Triticum aestivum* L. seedlings affected by high concentrations of zinc and to investigate the possible use of the hormone to enhance stress resistance.

2. Materials and methods

Plant material and growth conditions

The study was conducted on the winter wheat (*Triticum aestivum* L.) cv. Podolyanka. The cultivar belongs to the group of strong wheats, the variety is lutescence, high-yielding, intensive type, frost- and drought-resistance, tolerant of powdery mildew, rust and fusariosis. Wheat grains were obtained from the collection of the Institute of Plant Physiology and Genetics, NAS of Ukraine. Dry calibrated grains were sterilized in 80% ethyl alcohol solution, washed with distilled water and laid out in Petri dishes (50 pcs) on distilled water-moistened filter paper (control) and filter paper moistened with solutions of zinc sulfate, 10^{-6} M ABA solution and a mixture of zinc sulfate solutions and ABA. The plants were grown in the dark at $+ 24°$ C. The zinc solution was prepared from aqueous zinc sulfate ($ZnSO_4 \times 7H_2O$) at a rate of 228 mg of pure zinc per liter. Our previous studies showed that such zinc concentration inhibited the germination of grains and growth of wheat seedlings (Vasyuk et al., 2019), whereas priming in a 10^{-6} M ABA solution stimulated these processes (Kosakivska et al., 2019). To determine phytohormones, there were selected etiolated wheat seedlings with grains, which were grown on zinc and ABA solutions for three days (Fig. 1).
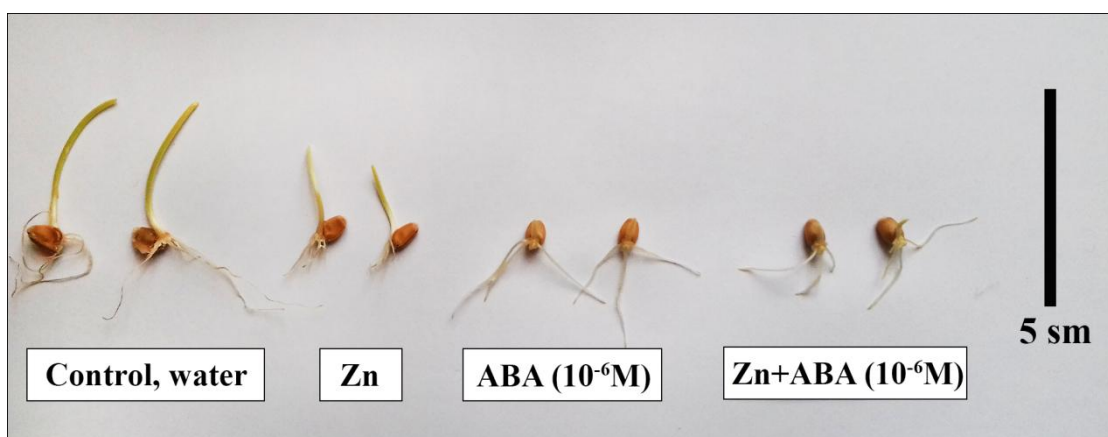

Fig. 1. The three-day-old *Triticum aestivum* L. cv. Podolyanka seedlings cultivated on zinc and ABA solutions

Morphological analysis

The effect of zinc and ABA on seed germination and seedling growth was investigated on the third day of cultivation. The following parameters were determined: length and fresh weight of the most developed root, height and fresh weight of shoot, fresh and dry weight of seedling. The dry weight was measured after drying at $105°$ C to constant weight. Biological replication within each variant of the experiment was 10 plants in three time analytical replication.

Extraction and assay of phytohormones

Material samples (2 g) were triturated in liquid nitrogen and homogenized in 10 ml of extraction solution (ethanol, water, formic acid in a ratio of 15: 4: 1) and extracted for 24 hours. The extracts were centrifuged for 30 min. at 15000 rpm at $+ 4°$ C on a Janetski K-24 centrifuge (Germany). The supernatants were drained, and 5 ml of the extraction solution was added to the precipitate and kept for another 30 minutes, then

re-centrifuged. The combined supernatants were evaporated to 5 ml using a Type 350 P vacuum evaporator (Poland). Further isolation of phytohormones was performed by the method (Dobrev *et al.,* 2012) on two solid phase SPE C18 columns, Sep-Pak Plus, Waters and SPE Oasis MCX, 6 cc / 150 mg, Waters. Column C18 was used to remove lipophilic substances, proteins and pigments. The SPE Oasis MCX column was used to sorbs IAA, ABA, GA$_3$ and SA. Elution of IAA, ABA and GA$_3$ was performed with 100% methanol, SA using a solution containing 60 ml of 100% methanol and 2.5 ml of 26% ammonia brought to 100 ml with bidistilate. The resulting eluents were evaporated to dryness on a vacuum rotary evaporator at a temperature not exceeding + 40° C. The dry residue was reconstituted to a volume of 200 μm with 45% methanol.

Analytically, phytohormones were determined applying the HPLC-MS on an Agilent 1200 LC liquid chromatograph with a G 1315 V diode-matrix detector (USA) in tandem with an Agilent G6120A single-quadrupole mass spectrometer. For chromatographic isolation, an Agilent ZORBAX Eclipse Plus C18 column with a lipophilic-modified sorbent was used, the particle size of which was 5 μm (reverse phase chromatography). Chromatographic isolation of the sample components with a volume of 20 μl by a solvent system (methanol, ultra-pure water, acetic acid in a volume ratio of 45: 54,9: 0,1) was followed by IAA and ABA detection in the UV absorption region at an analytical wavelength of 280 and 254 nm. To determine the SA content, samples with a volume of 20 μl were isolated using a solvent system (methanol, ultra-pure water, acetic acid in a volume ratio of 45: 54,9: 0,1) and SA in the UV absorption at an analytical wavelength 302 nm was detected. After isolation of the samples using the solvent system (acetonitrile, ultra-pure water, acetic acid - 30: 69,9: 0,1), GA$_3$ was detected through the mass detector signal. Samples with SA were isolated using a solvent system (methanol, water, acetic acid - 35: 64.5: 0.5), and detection was performed at 269 nm. The mobile phase velocity of the solvents during detection of IAA and ABA was 0.7 ml / min, SA - 0.8 ml / min, GA$_3$ and CTC - 0.5 ml / min. Non-labeled IAA, ABA, SA, GA$_3$, *trans*-zeatin glucoside (*t*-ZG), *trans*-zeatin (*t*-Z), *trans*-zeatinriboside (*t*-ZR), isopentenyladenine (iP) and isopentenyladenosine (iPA) manufactured by Sigma-Aldrich (USA), were used as standards for the construction of the calibration tables.

The control of the analytes content in the samples was carried out using a combined mass spectrometer (electrospray and chemical ionization at atmospheric pressure) with a negative polarity of the ionization of the analyte molecules during the analysis of IAA, ABA, SA, GA$_3$ and positive polarity during CK analysis. For the quantitative analysis of GA$_3$, the signal of the mass detector MSD SIM (setting 50% of the scan time of the detector indicator m / z 345) was used.

Statistical analysis

The experiments were performed in three biological and three analytical replicates. Analysis and calculation of phytohormone content were performed using Agilent Open LAB CDS Chem Station Edition software (rev. C.01.09). The quantitative values obtained were statistically processed using Microsoft Excel 2016. The significance of the difference was assessed by Student's t test using a 5% significance level (P ≤ 0.05; 0.01 and 0.001).

3.   Results and discussion

Morphometric study

The seed germination, root system formation and tillering are the decisive stages of ontogenesis, which affect the productivity of crops and wheat yield. Germination of seeds occurs under conditions of moisture and oxygen supply, corresponding temperature and light regimes (Cardoso *et al*., 2015), the presence of microelements (Khan *et al*., 2009; Mirshekari *et al*., 2012) and is regulated by the phytohormonal system (Han & Yang, 2015; Vishal & Kumar, 2018). Morphometric parameters of growth under stress factors are integral characteristics of the plant physiological state. On the first day of seed germination, enzymes are activated; the intensity of respiration increases, hydrolysis of the reserve substances used by the embryo for germination takes place. On the 2-3rd day embryo roots emerge, which provide the inflow of moisture and minerals to the seedling. Under favorable conditions when germinating in the soil for 3-4 days, the shoot goes to the surface. During this period seedlings going from heterotrophic to autotrophic nutrition.

In the model we created, all solutions inhibited the growth of seedlings. Exogenous ABA slowed shoot deevelopment, and zinc sulfate reduced root growth (table 1). Their combined application mitigated the individual inhibitory effects of these substances, but growth rates remained to be less than those of controls. It should be noted that biomass of seedlings, grown on zinc sulfate solution, were higher. Analysis of whole seedlings biomass indicated that it increase was due to shoots development. When incubated with ABA+zinc, seedlings biomass was less than control (table 1).

Table 1. Morphometrical study of three-day-old *Triticum aestivum* L. cv. Podolyanka seedlings grown on ABA and zinc solutions. Fresh weight – FW; dry weight – DW

Parameters influence	Control, water	10^{-6} M ABA	Zn (228 mg/l)	Zn (228 mg/l) + 10^{-6} M ABA
Leaves, mm	36,3±1,8	**7,5±0,3**	24,1±1,2	7,9±0,3
Leaves, FW mg	38±1,9	**5±0,3**	23±1,2	4±0,2
Roots, mm	63,4±3,2	27,9±1,4	**18,0±0,8**	24,1±1,2
Roots, FW mg.	27±1,4	19±1,0	**13±0,7**	18±0,9
Seedlings, FW mg	145,2±7,3	90,4±4,5	104,1±5,2	98,4±4,9
Seedlings, DW %	30,3±1,5	43,5±2,2	37,8±1,9	45,7±2,4

As it has been reported by Leung, Girandat (1998) in the first hours of seeds incubation on ABA solutions, the hormone does not inhibit the synthesis of hydrolases and does not affect the cleavage of the reserve substances involved in the initiation of growth processes. Thus, we can assume that in our experimant ABA, in accordance with its physiological function, inhibited the early development of seedlings shoots.

Effect of zinc and ABA on IAA accumulation

Auxins are involved in the regulation of the seed germination, division, elongation and differentiation of cells, photo- and gravitropisms, apical dominance, embryo-, organo- and morphogenesis, development of the root system (Cheng & Zhao, 2007; Ludwig-Muller, 2011; Mano & Nemoto, 2012; Teale *et al*., 2006). As a result of HM effect, auxin homeostasis is disturbed, and that adversely affects plant growth and development (Hu *et al.* 2013). Zinc has been reported to be involved in IAA metabolism, and excess metal influences the hormone content (Yamaji *et al.,* 2013).

However, with an excessive zinc concentration and treatment with exogenous ABA, a decrease in IAA level in the roots and leaves of *Populus anescanscens* was observed (Shi *et al*., 2015). At the same time, under zinc stress there was revealed a rise in IAA content in *Quercus suber* leaves (Disante *et al*., 2014), whereas no changes in hormone concentrations were observed in the roots and shoots of *Arabidopsis thaliana* (Sofo *et al*., 2013). We showed that incubation on ABA and zinc sulfate solutions caused respectively a two-fold and three-fold fall in endogenous IAA content in three-day-old wheat seedlings, whereas after the combined use of phytohormone and HM – 2.6 times. Thus, the addition of the hormone slightly mitigated the negative effect of zinc on the accumulation of IAA (Fig. 2 a).

Effect of zinc and ABA on ABA accumulation

Abscisic acid plays a key role in the formation of plant defense mechanisms (Vishwakarma *et al.,* 2017), regulation of ripening and seed germination (Finkelstein *et al.*, 2008), influences the architecture of the root system (Harris, 2015). HM was found to substantially increase hormone content in plant tissues of barley (Hollenbach *et al*., 1997), and exogenous ABA, reducing the level of transpiration, restricted the supply of zinc to the aboveground organs of rice seedlings (Zhao *et al*., 2009). Excessive zinc concentrations slowed germination of cucumber, chickpea and bean seeds and led to an increase in endogenous ABA (Atici *et al*., 2005; Wang *et al*., 2014; Zengin, 2006). However, it has been reported that zinc does not affect or reduce the hormone content of the Arabidopsis seedlings (Sofo *et al*., 2013). The decrease of ABA content in the roots and a rise in poplar leaves was observed after prolongation of zinc stress in conditions of seedlings exposure to exogenous ABA (Shi *et al.,* 2015). Our study showed that when grown on ABA solution, the content of endogenous ABA in three-day-old winter wheat seedlings increased 10.5 times. During the action of zinc, it remained at the control level, whereas the combined use of ABA and zinc caused a 4-fold rise in the hormone content (Fig. 2 b). Incubation with ABA solution mitigated the negative effect of zinc on wheat root growth (Fig. 1, table).

Effect of zinc and ABA on GA_3 accumulation

Gibberellins are activators of processes that occur during seed germination, they stimulate linear growth of axial organs, increase the leaf surface (Gantait *et al*., 2015). GA_3 content is known to rise during seed germination (Debeaujon & Koornneef, 2000; Atıcı *et al*., 2003). Gibberellins content in plant tissues is significantly affected by environmental conditions. Thus, the high concentration of zinc caused a fall in GA_3 content in germinating chickpea seeds (Atıcı *et al*., 2005) and Arabidopsis roots, that occurred through the hypersynthesis of DELLA proteins – repressors of the hormone signaling pathway (Sofo *et al*., 2013; Khan *et al*., 2015). Excess zinc led to a decrease in the concentration of GA_3 in the roots and leaves of poplar regardless of the exposure duration (Shi *et al*., 2015). At the same time, low concentrations of zinc initiated the accumulation of endogenous GA_3, whereas high concentrations acted as a stress factor (Atıcı *et al*., 2005). We demonstrated that incubation on zinc solution enlarged the content of endogenous GA_3 in wheat seedlings, whereas the amount of hormone decreased almost fourfold when there were applied ABA solutions and a mixture of ABA and zinc sulfate (Fig. 2 c). Zinc had a positive effect on the accumulation of GA_3, but an enlargement of the three-day-old wheat seedlings shoots was not observed (Fig. 1, table).

Effect of zinc and ABA on cytokinins accumulation

Cytokinins control cell division, stimulate the formation and activity of shoot meristems, form tissue attractive capacity, repress leaf senescence, inhibit root growth and branching, are involved in regulating seed germination, response to stress (Vedenicheva & Kosakivska, 2016; 2017; Veselov *et al*., 2017; Wybouw & De Rybel, 2019). The class of cytokinins includes adenine derivatives; the compounds are similar in structure but different in biological activity and functions. Hormone molecules with some variations in the structure of the side chain are likely to mediate different biological signals: it has been established that *t*-Z and iPA are involved in the transmission of long-range signals in the acropetal and basipetal directions, respectively (Kudo *et al*., 2012). During the germination of chickpea seeds under zinc stress, a gradual increase in Z content and a decrease in ZR were detected. Z is suggested to play a dominant role in overcoming such stress and can be synthesized *de novo* (Atıcı *et al.* 2005). In our study, the cytokinins ZG, ZR, and iP were found in trace amounts (less than 0.5 ng/g of crude substance) in three-day-old winter wheat seedlings. Only Z and iPA were identified in significant quantities. Exogenous ABA caused a threefold rise in Z content, whereas zinc partially suppressed the hormone accumulation, and the lowest level of Z was recorded under combined action of exogenous ABA and HM. The iPA accumulation pattern had the opposite direction, namely, as result of exogenous ABA effect the hormonal content diminished while zinc effect led to its increase. Growing on a mixture of ABA and HM inhibited iPA accumulation (Fig. 2 d).
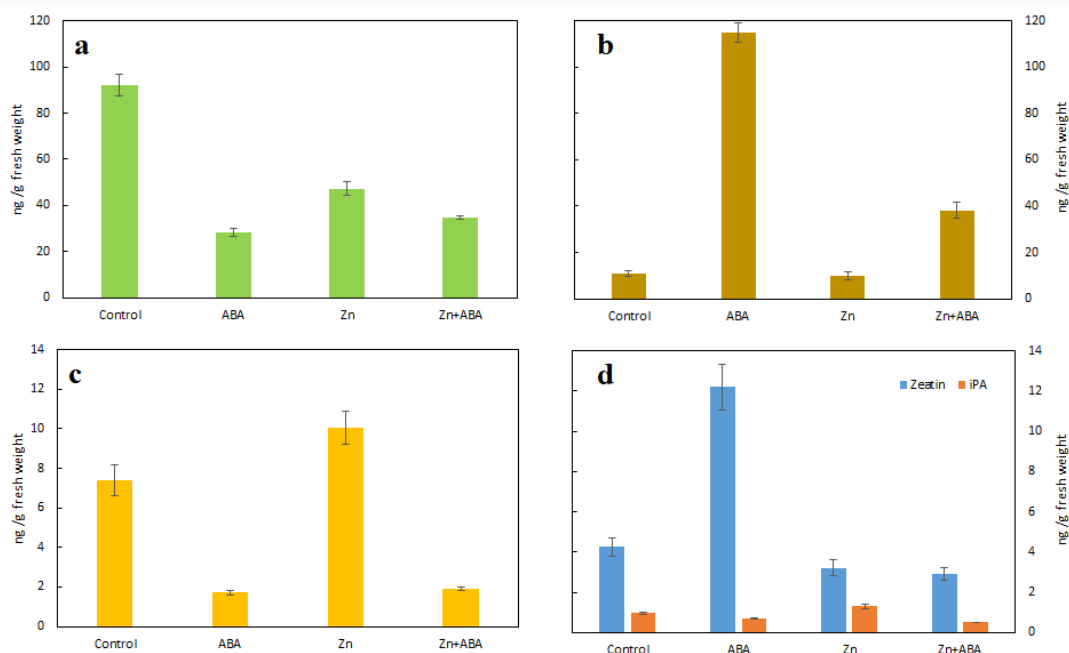

Fig. 2. Phytohormones content in three-day-old *Triticum aestivum* L. cv. Podolyanka seedlings grown on ABA and zinc solutions (a – IAA; b – ABA; c – GA$_3$; d – cytokinins)

Effect of zinc and ABA on SA accumulation

Salicylic acid plays a key role in the formation of the resistance of germinating seeds and adult plants under various biotic stressors (Vicente & Plasencia, 2011), and thus, the hormone is positioned as an effective remedy (Dempsey & Klessig, 2017). HM action was reported to enhance exogenous SA antioxidant protection, to mitigate lipid

peroxidation, and to increase photosynthetic activity (Noriega *et al*., 2012; Sytar *et al*., 2019). An increase in endogenous SA content was observed in *Thlaspi praecox* leaves due to HM hyperaccumulation (Lugany *et al*., 2013). Some rise in SA biosynthesis during HM impact in roots and poplar leaves was observed (Shi *et al*., 2015). We established that when incubated on a solution of zinc sulfate, three-day-old wheat seedlings showed a two-fold increase in SA content, and it increased seven times with exogenous ABA. A ten-fold rise in SA content was observed with the combined action of zinc sulfate and ABA solutions (Fig. 3).
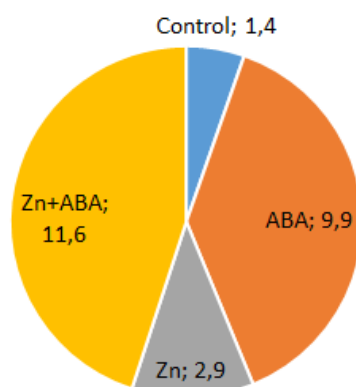

Fig. 3. The content of salicylic acid in three-day-old *Triticum aestivum* L. cv. Podolyanka seedlings grown on ABA and zinc solutions (ng/g fresh weight)

Effect of zinc and ABA on the endogenous phytohormones balance

Under control growing conditions, endogenous IAA in three-day-old seedlings of winter wheat dominated, its content reached 92 ng/g fresh weight. The ratio of total content of hormones stimulator IAA, GA_3, and CK to total contents of stress hormones ABA and SA under control conditions was 9:1, for ABA actions – 0.3:1, zinc – 4.8:1, zinc + ABA – 1.3:1 (Fig. 4).
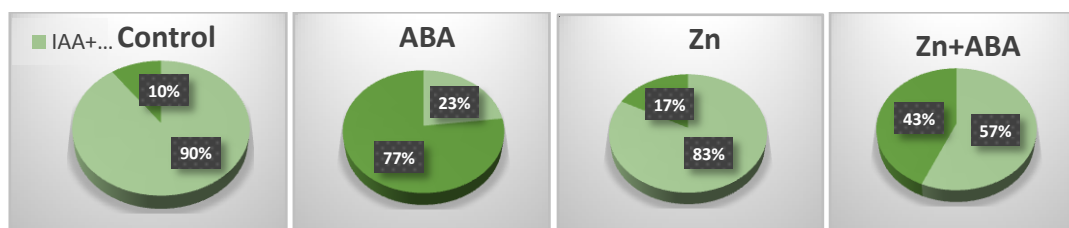

Fig. 4. Ratio between IAA+GA_3+CK content and ABA+SA content in three-day-old *Triticum aestivum* L. cv. Podolyanka seedlings grown on ABA and zinc solutions (%).

The pattern of changes in endogenous phytohormones content showed that zinc and exogenous ABA acted as abiotic stressors. Changes in morphometric parameters observed in our study indirectly indicate the involvement of stimulating hormones IAA, Z, iPA and GA_3 in the regulation of shoots growth under zinc stress. Some rise in SA content occurred in all experimental variants, testifying the hormone involvement to the formation of defense mechanisms.

4.   Conclusion

Our study showed that high zinc concentrations caused the changes in hormones accumulation and balance in winter wheat seedlings at the juvenile stage of their development. Zinc at a concentration of 228 mg/l inhibited the growth of the root, which was the first to be adversely affected by the excessive concentration of HM. Under these conditions, a fall in endogenous IAA, zeatin and ABA content and a rise in GA$_3$, iPA and SA were observed. Seedlings incubation on 10^{-6} M ABA solution induced the growth of root system. A combined action of the hormone and zinc resulted in the leveling of HM inhibitory effect, and the content of stress hormones SA and ABA increased. Our results are consistent with those of other researchers regarding the involvement of ABA in triggering SA and IAA signaling cascades capable of reducing the toxic effect of HM (Noriega et al., 2012; Trinh et al., 2014). Therefore, the strategy of wheat seedlings adaptation to zinc loading under the action of exogenous ABA was aimed at inhibiting shoot growth and activating root growth. Changes in the balance of phytohormones are able to initiate protective mechanisms and further adaptation of plants to the effects of excessive HM concentrations, and the priming of grains with exogenous ABA can be used to improve stress resistance.

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