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Efficiency index as the integral indicator of *Triticum aestivum* response to growth regulators

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Abstract

In the current study we investigated the effectiveness of synthetic Thidiazuron (TDZ), Cytodef (CTD), Epin-Extra® (24-EB) and natural Ribav-Extra® (RE) plant growth regulators (PGR) with a view to improving the heavy metal-tolerance in intact plants. To evaluate the effectiveness of PGR we chose the following parameters: the axial growth (as an integral indicator of the plant response to the environment), the superoxide generation (as an indicator of oxidative stress), the malondialdehyde (MDA) content (as an indicator of oxidative damage degree in plants), catalase (CAT) activity (as an indicator of the antioxidant defence systems activity) and the PGR capacity to change absorption and translocation of heavy metals in plants. The efficiency index was estimated on the basis of the results of winter wheat (*Triticum aestivum* L.) seedlings affected by 10 μM or 1 mM Zn^{2+}, Cu^{2+} and Ni^{2+} or Pb^{2+} heavy metals. An empirical scale of the efficiency of PGR was developed. The study found that the efficiency of the PGR investigated with respect to tolerance of heavy metals increased as follows: TDZ <CTD <RE <24-EB.

Key words: Cytodef, Epin-Extra®, heavy metals, oxidative stress, Ribav-Extra®, Thidiazuron.

Introduction

In recent decades, one of the research trends at the intersection of agriculture and environmental plant physiology was the study of the protective action of exogenous plant growth regulators (PGR) in plants exposed to extreme or unfavourable environmental factors. PGR can neutralize the negative impact of various stressors: chilling, drought, osmotic, heat, heavy metal stresses (Khan et al., 2012; Semenova, Lukatkin, 2015).

Soil contamination with various metals and their impacts on plant health and productivity are extensively reported (Anjum et al., 2015). Heavy metals cause various disorders in physiological and metabolic processes in plants. Toxic effects of heavy metals strongly depend on the metal, its concentration, the plant species and plant phenophase, as well as on environmental (edaphic, climatic, etc.) factors (Duchovskis et al., 2006; Башмаков, Лукаткин, 2002).

Synthetic and/or natural PGR, analogues of phytohormones are known to influence growth, seed germination, rhizogenesis, senescence, etc., and are also extensively reported to confer heavy metal stress tolerance in plants (Bashmakov et al., 2007; Lukatkin et al., 2007).

Usually, the stressful effects of the environment, as well as the effects of growth regulators, are estimated by the physiological or biochemical parameters of the plants or by the yield quantity and quality (Bashmakov et al., 2015; Anjum et al., 2016). Thus, under the stress effect of metals, the effectiveness of brassinosteroids was evaluated according to the following indices (for different species): in Indian mustard and common radish it was assessed by the activity of enzymes of radical scavenging system, plants growth and nickel (Ni) accumulation (Choudhary et al., 2011; Kanwar et al., 2013); in cucumber – by biomass accumulation, the content of chlorophyll and the activity of carbonic anhydrase (Fariduddin et al., 2013); in common radish – by the activity of 6 antioxidant defense system enzymes, lipid peroxidation (LPO) intensity and by proline content (Sharma et al., 2011); in wheat – by grain yield and heavy metals accumulation in aerial biomass (Kroutil et al., 2010).

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The efficacy of kinetin and other cytokinin-like substances was evaluated according to the following parameters: in pea seedlings by the rate of growth, soluble sugars and chlorophyll content, and on the rate of photosynthesis (Al-Hakimi, 2007); in zucchini – on the rate of cell division and growth (Stoynova-Bakalova, Petrov, 2010); in Mexican Palo Verde – by alterations in catalase and ascorbate peroxidase activities (Zhao et al., 2011); in maize cucumber – by seed germination and growth of axial organs (Lukatkin et al., 2007); in cucumber – by superoxide generation, LPO intensity, carotenoid content in leaves (Bashmakov et al., 2012).

There are also known protective properties of exogenous gibberellins in different plants, which were evaluated in cadmium stressed tomato by the activities of antioxidant enzymes and by the content of sugars (Khavari-Nejad et al., 2013); in pea – by the seed germination, by the accumulation of dry and fresh biomass, the length of axial organs and the activities of 8 key enzymes (Gangwar et al., 2011).

There are researches comparing several types of PGR that are close to the mode of action (Stoynova-Bakalova, Petrov, 2010; Choudhary et al., 2011), the effectiveness of several concentrations of the PGR (Gangwar et al., 2011), different exposure times (treatment times) with the PGR (Canakci, Dursun, 2011), the effectiveness of PGR in plants under several types of environmental stress (Fariduddin et al., 2013) or the effectiveness of PGR on several species/genotypes (Ali et al., 2015).

All these works use for a comparative evaluation a certain physiological or biochemical parameter, measured relative to the active heavy metal. However, to date, there are practically no studies in which any index has been developed that would allow a quantitative assessment of the effectiveness of growth regulators for any object, stressor, treatment method, etc. It should be assumed that such indices are absent or not used in the agricultural practice. Therefore, the aim of the current research is to develop the integral index of plant response to different growth regulators under stress conditions. The study summarises the experimental researches performed earlier by a uniform method, as well as new experimental data.

Materials and methods

Plant material. Commercial seeds of the winter wheat (*Triticum aestivum* L.) subspecies *lutescens* cultivar 'Mironovskaya 808'.

Reactants. Synthetic plant growth regulators (PGR) Thidiazuron (TDZ) – N-phenyl-N'-1,2,3-thiadiazol-5-ylurea, Cytodef (CTD) – N-(1,2,4-triazol-4-yl)-N"-phenylurea and Epin-Extra® (24-EB) contains 50 µM 24-epibrassinolide; natural plant growth regulator Ribav-Extra® (RE) is a product of the metabolism of mycorrhizal fungi isolated from ginseng (*Panax ginseng* C.A. Mey) roots. The sources of PGR were: TDZ – Schering (Germany), CTD – All-Russian Research Institute of Chemical Means of Plant Protection, 24-EB – NEST-M (Russia) and RE – "Selhozekoservis" (Russia). In Russia, these PGR are usually used in field conditions or greenhouses for seed treatment, spraying plants, or processing cuttings.

Experimental design. Wheat seeds were treated with 0.5% $KMnO_4$ for 5 min to surface sterilize then with 10 nM TDZ, 0.1 µM CDT, 10 ppm RE or 1 µM 24-EB for 4 to 8 h (control seeds were treated with distilled water), and then germinated in plastic pots (50 seeds per pot) in water (50 ml per pot) supplemented with 10 µM (low dose) or 1 mM (high dose) $Pb(NO_3)_2$, or $CuSO_4 \times 5H_2O$, or $NiSO_4 \times 7H_2O$ or $ZnSO_4 \times 7H_2O$, at temperature 22–24°C, photoperiod 16/8 h (day/night), PFD about 80 µmol m^{-2} s^{-1} for 7 days. In 7-day seedlings we measured heavy metals accumulation by atomic absorption spectrometry on a spectrometer Shimadzu AA-7000 (Japan) in shoots and roots (Bashmakov et al., 2015), growth and biochemical parameters in axial organs.

Superoxide anion generation. The estimation of $^{\bullet}O_2^-$ in leaf discs was based on the capacity of $^{\bullet}O_2^-$ to oxidize adrenaline to adrenochrome (Lukatkin, 2002 a). In brief, fresh discs (300 mg) were homogenized with 15 ml of distilled water. The solution was centrifuged for 15 min at 3000 rpm. To 3.0 ml of homogenate, 0.1 ml of adrenaline solution (0.01%) was added. The mixture was incubated for 45 min at ambient temperature with illuminance of about 200 µM m^{-2} s^{-1}. The optical density of the adrenochrome formed was measured immediately after incubation on a spectrophotometer UV-mini 1240 (Shimadzu, Japan) at 480 nm. The generation of $^{\bullet}O_2^-$ was calculated by molar extinction coefficient ε = 4020 M^{-1} cm^{-1} in µM g^{-1} min^{-1}.

Lipid peroxidation (LPO). The detection of LPO intensity in leaf discs was done using the method based on malondialdehyde storage (MDA) (Lukatkin, 2002 a). *T. aestivum* leaf discs (300 mg) were homogenized in 10 ml of isolation medium (with 0.1 M Tris-HCl buffer, 0.35 M NaCl, pH 7.6). To 3.0 ml of homogenate, 2.0 ml of thiobarbituric acid (prepared in 20% trichloroacetic acid) was added. The resultant solution was heated in a boiling water bath for 30 min and filtered. After stopping the reaction by keeping the tubes with solution on ice, the optical density was recorded on a spectrophotometer at 532 nm. The concentration of MDA was calculated by molar extinction coefficient ε = 1.56 × 10^5 M^{-1} cm^{-1} in µM g^{-1} of fresh leaf weight.

Catalase (CAT) activity. T. aestivum leaf discs (1.0 g) were homogenized with 10 ml of 50 mM phosphate buffer (pH 7.0). The homogenate was filtered and centrifuged for 10 min at 8000×*g*. To 25 ml of enzyme extract, 2.9 ml of phosphate buffer (pH 7.0) was added. Directly before the measurement, 90 µl of 3% hydrogen peroxide (H_2O_2) was added to the solution and decrease in the optical density during 1 min was measured on a spectrophotometer at 240 nm. The activity of CAT was calculated by molar extinction coefficient ε = 39.4 mM^{-1} cm^{-1} in mM g^{-1} min^{-1} (Lukatkin, 2002 b).

Calculation of efficiency index (IE) of the PGR. To evaluate the effectiveness of the PGR we developed a score index of the PGR efficiency for the best data interpreting. IE was calculated employing the formula:

$$IE = (\pm|P_1 - 100| \pm |P_2 - 100| \pm \ldots \pm |P_n - 100|) / n,$$

where P_1, P_2, etc. are the measured parameters (% relative to the untreated plants, which was taken as 100%); *n* is the amount of parameters considered in the IE calculation (7, for the experiment).

We took the difference with the sign "+" if the effect of the PGR was positive, and the sign "–" if the last one was negative. We recommend using the following empirically developed scale of the PGR efficiency: IE < 0 – the PGR is not effective, 0 < IE < 20 – the efficiency is very low, 21 < IE < 40 – the efficiency is low, 41 < IE < 60 – the efficiency is moderate, 61 < IE < 80 – the efficiency is above moderate, 81 < IE < 100 – the efficiency is high, and IE > 100 – the efficiency is very high.

Statistical analysis. All experiments were conducted in triplicate, and each experiment consisted of at least 150 seedlings. For all measurements averages and standard errors were calculated in *Microsoft Excel 2007*. Differences between means were assessed by the Duncan's test at $P = 0.05$.

Results and discussion

Heavy metals induced some changes in physiological and biochemical parameters associated with the metal accumulation. In most treatments of the experiment, the enhanced heavy metals accumulation was registered in different parts of plants, depending on metal concentration in the medium. Besides, high heavy metal doses lead to oxidative stress induction in tissues, which was registered by the increasing superoxide generation, LPO intensity, electrolyte leakage, alteration in activities of antioxidative enzymes. Eventually it resulted in a decrease in the growth of the plant's organs. However, when the plants were exposed to low doses (10 µM) of heavy metals, negative effects were poor or absent. Seed pretreatment with PGR contributed to a decrease in the observed negative heavy metals effects in wheat plants. In previous publications, the alterations in the physiological and biochemical parameters of plants under the action of the following PGR were observed: TDZ (Sazanova et al., 2012), CTD (Сазанова и др., 2012), 24-EB (Bashmakov et al., 2016) and RE (Грузнова и др., 2016).

To evaluate the effectiveness of PGR we chose the following parameters: the growth of axial organs (as an integral indicator of the plant response to the environment), the superoxide generation (as an indicator of oxidative stress), the concentration of MDA (as an indicator of oxidative damage degree in plants) and CAT (as an indicator of the antioxidant defence systems) activity. In addition, in agricultural practice it is very important to improve the heavy metals uptake and to reduce their accumulation in the yield. Therefore, when calculating the PGR efficiency, we also took into account the PGR capacity to change the heavy metals absorption in plants. We supplemented previous studies with some new research data to ensure that the measured parameters were the same for all the growth regulators (Table 1).

Analysing the data on the various PGR influence on the physiological and biochemical parameters of wheat plants exposed to heavy metals, ambivalent effects were obtained. Thus, in some cases PGR reduced oxidative stress (the level of superoxide generation decreased by 24-EB and especially RE) and increased the activity of antioxidant enzymes such as CAT (for example, TDZ). In other cases, the effect of PGR led to an accelerated repair of oxidative damage: 24-EB, RE (all cases except for 10 µM Pb^{2+}), TDZ in Cu-treated seedlings, or CTD in Pb- or Zn-treated seedlings, and to enhance in axial organs growth, for example, RE and 24-EB, except for 1 mM Cu^{2+}. And finally, in some cases, seed pretreatment with the PGR did not cause any positive changes in intact

Table 1. Effect of plant growth regulators (PGR) on the key parameters of the plant response to the heavy metals, % to the treatment without the regulator

Parameters	0 (water)	Cu		Ni		Pb		Zn	
		10 µM	1 mM	10 µM	1 mM	10 µM	1 mM	10 µM	1 mM
10 nM Thidiazuron (TDZ)									
Shoot length	128	115	81	178	115	107	134	108	126
Root length	224	169	96	89	174	182	189	203	152
$^{\bullet}O_2^-$ generation	166	386	411	329	199	602	276	24	364
LPO intensity	93	45	89	132	54	94	95	138	96
Catalase activity	139	162	162	237	227	107	160	168	107
Heavy metals in shoots	100	63	29	148	181	257	157	25	97
Heavy metals in roots	100	10	10	125	52	89	39	72	141
0.1 µM Cytodef (CTD)									
Shoot length	115	121	91	196	282	93	192	113	148
Root length	198	173	143	107	226	125	450	171	248
$^{\bullet}O_2^-$ generation	174	298	102	241	168	79	117	55	267
LPO intensity	88	215	244	100	206	53	42	75	72
Catalase activity	101	72	72	91	41	48	63	55	48
Heavy metals in shoots	91	132	92	1383	175	119	104	65	616
Heavy metals in roots	100	15	12	104	26	95	96	81	167
1 µM Epin-Extra® (24-EB)									
Shoot length	139	124	82	217	155	110	137	105	137
Root length	235	208	84	146	239	229	134	245	165
$^{\bullet}O_2^-$ generation	51	19	13	11	3	134	47	19	11
LPO intensity	46	64	7	26	48	15	15	92	16
Catalase activity	43	27	46	136	66	38	74	72	52
Heavy metals in shoots	100	189	68	87	49	67	65	73	76
Heavy metals in roots	92	5	15	61	108	137	90	88	74
10 ppm Ribav-Extra® (RE)									
Shoot length	172	112	59	199	399	150	179	147	152
Root length	298	184	184	191	683	220	278	221	263
$^{\bullet}O_2^-$ generation	46	36	42	69	28	72	67	75	69
LPO intensity	75	48	31	40	61	120	84	70	102
Catalas activity	74	103	93	85	79	103	89	90	96
Heavy metals in shoots	100	84	87	58	39	73	81	52	84
Heavy metals in roots	92	13	13	154	86	91	68	296	91

plants. Ambiguous effects were found when the PGR influenced on the absorption and translocation of heavy metals in plants was investigated. The PGR efficacy varied greatly depending on the metal, its concentration, the reactant and was often opposite in the roots and shoots. The most positive effects of seed-pretreatment with TDZ or CTD on heavy metals accumulation was found in *T. aestivum* seedlings exposed to Cu^{2+}. 24-EB was most effective against Zn-contaminated medium. The RE inhibited both the uptake and translocation of the metals in the majority of cases, except for 10 μM of Ni^{2+} and Zn^{2+}.

For the best data interpreting, we applied the score index of the PGR efficiency. All the PGR in the control (distilled water) had some stimulating effect, but their efficiency was different: IE was very low at cytokine-like TDZ and CTD, and moderate at RE and 24-EB. The IE of the PGR varied significantly when different concentrations of heavy metals were applied to wheat plants (Table 2).

Table 2. Modulation of efficiency index (IE) in *Triticum aestivum* seedlings exposed to different heavy metal treatment levels

	Distilled water	Cu^{2+}		Ni^{2+}		Pb^{2+}		Zn^{2+}	
		10 μM	1 mM	10 μM	1 mM	10 μM	1 mM	10 μM	1 mM
10 nM TDZ	8	−12	−32	−58	−18	−78	−15	26	−32
0.1 μM CTD	7	−19	2	−9	27	24	74	41	−11
1 μM 24-EB	48	47	45	50	62	36	44	44	53
10 ppm RE	56	45	40	42	157	30	53	12	39

Therefore, TDZ had low efficiency only at low dose of Zn^{2+}. Despite the detected positive effect of TDZ on individual parameters in wheat plants, the calculation of IE showed its ineffectiveness for wheat seedlings exposed to heavy metals.

CTD had very low efficiency at 1 mM Cu^{2+}, low – at 10 μM Pb^{2+} or 1 mM Ni^{2+}, moderate – at 10 μM Zn^{2+} and above moderate – at 1 mM Pb^{2+}. Nevertheless, CTD was ineffective against low concentrations of Cu^{2+} or Ni^{2+}, as well as a high concentration of Zn^{2+}.

Opposite to cytokinin-like PGR, RE was effective at all levels of metal treatment, but the IE values varied depend on the metal or the concentration. Therefore, RE had very low efficiency against the 10 μM Zn^{2+}. RE had the low efficiency at 10 μM Pb^{2+}, as well as 1 mM Cu^{2+} or Zn^{2+}. In other treatments of the experiment, the regulator had a moderate efficiency with the maximum at 1 mM Ni^{2+}.

Similar to RE, 24-EB had a beneficial effect on wheat plants against all the heavy metals studied. 24-EB had the moderate efficiency in seedlings exposed to almost all heavy metals treatment except for 10 μM Pb^{2+} (low efficiency) and 1 mM Ni^{2+} (efficiency above moderate). According to the data of the PGR effectiveness degree in wheat plants exposed to heavy metals, IE of all the growth regulators studied increased in the following: TDZ < CTD < RE < 24-EB.

Earlier, 24-EB has widely been reported to control plant growth and development by activating the cell cycle during seed germination, controlling progression of cell cycle (González-Garcia et al., 2011), inducing exaggerated growth in hydroponically grown plants (Arteca, Arteca, 2001), and also by controlling proliferation of leaf cells (Nakaya et al., 2002). In addition, 24-EB-mediated elevation in CAT activity and subsequent control of reactive oxygen species (ROS), such as $^{\bullet}O_2^-$, and reduction in heavy metals content observed herein can be convincing mechanism that eventually resulted in 24-EB-mediated improved growth traits in *T. aestivum* under different divalent metals.

Unfortunately, in the available references, we did not find an explanation for the protective effects of RE. However, as our studies have shown, RE is an auxin-like PGR, and perhaps its effects will be similar to those of auxins. There is increasing evidence that auxins play an important role in the protection and regulation of plant metabolism under stress conditions (Egamberdieva, 2009). The endogenous levels of auxins generally decrease following metal stress; thus, it is quite possible that exogenous application of auxins may complement the function of endogenous auxins, as reported in studies where indole-3-acetic acid (IAA) was exogenously applied (Srivastava et al., 2013). However, an exogenous supply of IAA improved growth of *Brassica juncea* under arsenic (As) stress by modulating expression of miR167, miR319 and miR854, suggests a protective role of IAA in enhancing metal tolerance.

In wheat seedlings, exogenous application of IAA is reported to increase growth of the root and shoot and to protect plants against stress (Egamberdieva, 2009). An active auxins pool has also been shown to be involved in reducing the toxicity of heavy metals. However, exogenous application of IAA may protect plants as well as enhance phytoextraction of heavy metals (Hadi et al., 2010).

Cytokinins have been shown to play an important role during plant acclimation to stress conditions and involve intense interactions and crosstalk with other hormones (Ha et al., 2012). Exogenous cytokinins application enhanced manganese (Mn) tolerance and the antioxidant defense system (Gangwar et al., 2010), reduced Cd-induced alterations (Al-Hakimi, 2007). Exogenous application of cytokinin-like PGR kinetin decreased toxic effects of cadmium (Cd) (Munzuroglu, Zengin, 2006), and thidiazuron had protective effect on cucumber seedlings exposed to heavy metals (Lukatkin et al., 2003). In addition, the presence of cytokinin in the rhizosphere could be an important factor affecting lead (Pb) toxicity (Vodnik et al., 1999). Thomas et al. (2005) observed that tobacco plants expressing the cytokinin-synthesizing gene (*Ipt*) showed enhanced tolerance against copper (Cu) stress as indicated by lower lipid peroxidation and increased expression of the metallothionein gene (*MT-L2*), despite accumulating a higher Cu concentration compared to nontransformed plants.

Conclusion

Based on the growth, membrane damage, oxidative stress and antioxidant activity parameters in *Triticum aestivum* exposed to stressors, a valid index of plant growth regulator (PGR) efficiency was developed, which allowed comparison of different PGR. Based on the index, it is possible to find a specific PGR that is most suitable for the cultivar exposed to a certain intensity of the stressor. However, in each case, careful analysis and selection of the parameters included in the efficiency index (IE) of the PGR calculation, as well as a preliminary determination of the stressor intensity, are necessary.

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Efektyvumo indeksas kaip *Triticum aestivum* reakcijos į augimo reguliatorius integralus rodiklis

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Santrauka

Siekiant pagerinti augalų sunkiųjų metalų toleranciją, tirtas sintetinių Thidiazurono (TDZ), Cytodefo (CTD) bei Epin-Extra® (24-EB) ir natūralaus Ribav-Extra® (RE) augalų augimo reguliatorių efektyvumas. Tikslui pasiekti buvo parinkti tokie parametrai: ašinis augimas kaip augalų reakcijos į aplinką integralus rodiklis), superoksido pokyčiai (oksidacinio streso rodiklis), malono dialdehido kiekis (oksidacinio pažeidimo laipsnio augaluose rodiklis), katalazės aktyvumas (antioksidacinės apsaugos sistemos aktyvumo rodiklis) ir augimo reguliatorių geba pakeisti sunkiųjų metalų absorbciją bei translokaciją augaluose. Efektyvumo indeksas apskaičiuotas remiantis žieminių kviečių daigų, paveiktų 10 µM arba 1 mM Zn^{2+}, Cu^{2+} ir Ni^{2+} ar Pb^{2+} sunkiaisiais metalais, tyrimo rezultatais. Taip pat buvo sukurta empirinė augalų augimo reguliatorių efektyvumo skalė. Nustatyta, kad tirtų augimo reguliatorių efektyvumas, atsižvelgiant į toleranciją sunkiesiems metalams, didėjo taip: TDZ < CTD < RE < 24-EB.

Reikšminiai žodžiai: Cytodef, Epin-Extra®, oksidacinis stresas, Ribav-Extra®, sunkieji metalai, Thidiazuron.