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AGAR GEL PHENOTYPING OF ROOT TRAITS AS RAPID AND SENSITIVE ASSAY OF WHEAT SEEDLINGS RESPONSE TO EDAPHIC FACTORS: ON EXAMPLE OF CADMIUM

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ABSTRACT. The influence of different concentrations of cadmium on root elongation, exudative activity of roots and seminal root angle of two wheat genotypes: common bread wheat – *Triticum aestivum* L. (cv. 'Favorytka') and emmer wheat – *Triticum dicoccum* Schrank. (cv. 'Holikovska') have been studied in the germination stage. Rapid changes in morphofunctional traits upon first three days of exposure to cadmium on early stages of growth have been studied on phenotyping plates with 25, 50 and 100 µM addition to agar-acid/base indicator medium. Significant inhibition of root elongation, exudative root activity and changes in seminal root angle were observed. Exposure to the highest Cd concentration led to a decrease in primal root length by 50%, decrease in root exudative activity by 88% and decrease in seminal root angle by 24 degrees in *T. aestivum* compared to a decrease by 12%, 83% and 17 degrees in *T. dicoccum*. Unlike root growth retardation, a decrease in exudative activity was observed on all three Cd concentrations. The root growth performance at starting stage of seedlings ontogenesis proposed as an express and sensitive phenotyping test method for determining plant response to edaphic stressors by cadmium toxicity example.

Introduction

Plant roots are involved in obtaining water and nutrients, rapid response to abiotic and biotic stressors in the soil and plant anchoring in the ground. Supervising plant root development and distribution is a fundamental part of insight of plant ontogenesis and evolution, permitting plants to respond to global climate changes and allowing plants to conquer in different ecological niches (Nibau *et al*., 2008).

In addition to measuring linear growth rates of primary and lateral roots, a lot of research is devoted to the root growth angle, which determines the direction of elongation of the roots and the area of distribution in the upper layers of the soil, affects the area in which the roots contact with water and substances dissolved in it (Nelson, Oliver, 2017). Several authors pointed to the significance of root growth angle as a potential trait to enhance crop yield because of the relation of root architectures to increased nutrition within the upper layers of the soil (Lynch, 2011). Thus, the route development and exudative activity of roots determine the ability of a plant to use nutrients that are unevenly distributed in the soil (López-Bucio *et al*., 2003; Uga *et al*., 2015).

In monocot species, the embryonic roots (primary and seminal) are especially important during early seedling establishment, but post-embryonic roots soon take over and are responsible for enhanced foraging (Koevoets *et al.*, 2016). Furthermore root efflux activity is important due to root exudates involving in the increase of bioavailavility and mobilization of nutrients. Organic acids are the most prevalent class of root exudates that have been extensively studied for their role in nutrient mobilization, pathogen resistance, heavy metals tolerance and other abiotic and biotic stresses (McGrail *et al.*, 2020). Heavy metals can inhibit the development of the root system of plants and limit the realization of the genetic potential of a variety and agroecosystems productivity (Rizvi *et al.*, 2020).

Therefore, this work aimed to investigate the roots morphofunctional response of two wheat genotypes (*Triticum aestivum* L. and *Triticum dicoccum* Schrank.) on germination stage under different concentrations of cadmium. The root growth performance at the initial stage of seedlings ontogenesis proposed as an express and sensitive test method for determining plant response to edaphic stressors by cadmium toxicity example.

Material and Methods

The objects of the study were two wheat genotypes – *Triticum aestivum* (cv. 'Favorytka') and *Triticum dicoccum* Schrank. (cv. 'Holikovska') on starting stage of development. Selected cultivars have passed preliminary screening for the level of resistance to changes in the osmotic potential of the growth medium (Smirnov *et al.*, 2020a; Smirnov *et al.*, 2020b). Seedlings were grown in vertical quadratic phenotyping plates 120×120 mm (Kartell Labware) in a growth chamber at 25 C without light for 3 days. The control variant of seedlings was grown on a 0.8% (w/v) agar layer prepared with distilled water (Manschadi *et al.*, 2008). Experimental seedlings were grown on a layer of agar with the addition of 25 µM, 50 µM and 100 µM of CdI_2 (pH 6.5).

Wheat seeds of both varieties were soaked in 10% (v/v) hydrogen peroxide and further in aseptic conditions within 6 hours were vernalized in distilled water at 4 °C to stimulate germination then transferred to a Petri dish on wet filter paper and placed in a thermostat at 25 °C for imbibition. Healthy seeds of both varieties were selected randomly. After 12 hours of imbibition, seeds were placed in an agar layer (50 mL of agar per phenotyping plate). For visual control of root exudation, acid-base indicator bromocresol green was used (Kosyan *et al.*, 2016).

Phenotyping of roots architecture and exudative activity (acidification of the medium) was carried out using morphological traits: lengths of primary and seminal roots, the angle between seminal roots (Waidmann *et al.*, 2020) and area of agar layer, which changed colour from blue to yellow under the influence of root exudates (Fig. 1 A, B). Root traits and area of agar layer with changed colour was measured using the ImageJ software, comparing with the scale bar 1 cm (Hohn, Bektas, 2020).

The experiment was repeated three times, with 4-fold biological repeats. The data were subjected to analysis of variance with subsequent Tukey's multiple range test (Two-way ANOVA) with GraphPad Prism 9.0 software. Data are expressed as means of replicates + standard deviation and were considered reliable at a significance level of $P < 0.05$.

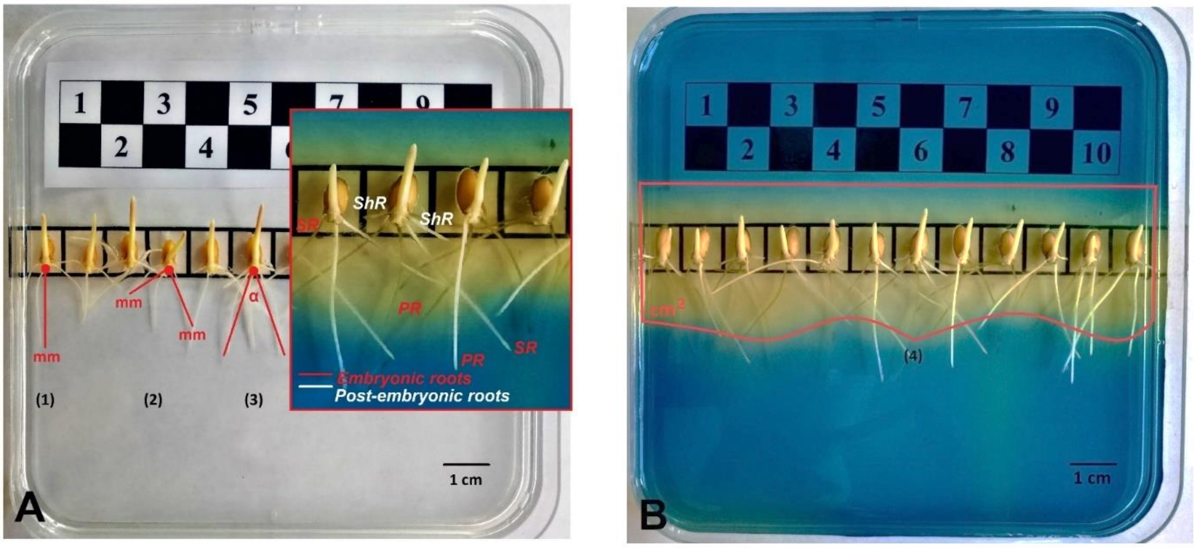

Figure 1. Phenotyping plates with germinated wheat seeds without an acid-base indicator (A) and with indicator addition (B); root traits: primary root length (1), seminal roots length (2), seminal roots angle (3) and root exudation activity as the area of agar gel colour change from blue to yellow (4). The inset to (A) schematically shows the PR – primary root, SR – seminal roots and ShR – shoot-born roots disposition.

Results

In response to cadmium toxicity, we observed growth inhibition of primal (PR) and seminal roots (SR), the degree of which differed between the two genotypes and depended on the concentration of Cd and the time of exposure. In addition, changes in seminal root angle (SRA) and exudative activity were noted (Tables 1–3).

Conditionally, it was possible to identify two opposite patterns of root growth. Reaction of *T. aestivum* cv. 'Favorytka' was distinguished by delay in germination and moderate root growth in the first 24 hours, followed by significant root length increase in the next two days – total increase in PR length on control samples was 398%, increase in SR was 458% of control variant. A high concentration of Cd (100 µM) caused a significant inhibition of the growth processes of PR, after 72 hours, the length of PR was 50% of the control level and moderate inhibition of the SR growth by 18%. Low and medium Cd concentrations (25 µM and 50 µM) did not have any significant effect on PR but had a pronounced stimulating effect on SR, which manifested itself as a 23–30% increase in SR length compared to the control variant.

Table 1. Root traits of wheat seedlings after 24 hours of germination on agar gel with different concentrations of cadmium

Variety	*Triticum aestivum* cv. 'Favorytka'				*Triticum dicoccum* cv. 'Holikovska'			
Variant, n = 36	Concentrations of cadmium, µM							
	0	25	50	100	0	25	50	100
Primary root length, mm	7.91 ± 1.46[a]*	6.18 ± 1.15[ab]	8.69 ± 1.22[a]	5.75 ± 0.91[b]	12.51 ± 1.74[a]	13.22 ± 2.46[a]	11.43 ± 2.22[a]	11.01 ± 1.91[a]
Seminal roots length, mm	5.41 ± 1.25[a]	4.81 ± 0.74[a]	7.65 ± 1.11[a]	6.22 ± 0.86[a]	11.73 ± 1.06[a]	11.78 ± 1.36[a]	14.47 ± 1.20[a]	12.38 ± 1.45[a]
Seminal root angle, degrees	61 ± 1[a]	64 ± 8[a]	63 ± 5[a]	48 ± 5[b]	88 ± 5[a]	85 ± 8[a]	62 ± 11[b]	68 ± 6[b]
Area of exudation activity, cm^2	76.25 ± 15.75	N/A	N/A	N/A	101.75 ± 2.02[a]	16.75 ± 2.75[b]	13.01 ± 1.50[b]	8.5 ± 1.50[c]

Data represented as mean values with standard deviation. Lower-case letters in the rows of each variety differ significantly by the Tukey's test (P < 0.05). N/A – not available

Table 2. Root traits of wheat seedlings after 48 hours of germination on agar gel with different concentrations of cadmium

Variety	*Triticum aestivum* cv. 'Favorytka'				*Triticum dicoccum* cv. 'Holikovska'			
Variant, n = 36	Concentrations of cadmium, µM							
	0	25	50	100	0	25	50	100
Primary root length, mm	16.47 ± 2.15[a]	17.75 ± 1.96[a]	16.93 ± 1.72[a]	11.07 ± 0.82[b]	19.51 ± 1.71[a]	21.61 ± 2.43[a]	19.51 ± 2.45[a]	17.87 ± 1.63[a]
Seminal roots length, mm	13.10 ± 1.02[a]	14.74 ± 1.37[a]	16.14 ± 1.38[a]	11.42 ± 0.78[b]	22.78 ± 1.18[a]	22.72 ± 1.55[a]	21.18 ± 1.43[a]	16.97 ± 1.18[a]
Seminal root angle, degrees	62 ± 3[a]	59 ± 4[a]	52 ± 2[b]	48 ± 3[b]	92 ± 2[a]	86 ± 4[a]	73 ± 4[c]	75 ± 2[c]
Area of exudation activity, cm^2	114.25 ± 4.7[a]	8.75 ± 2.05[b]	5.75 ± 1.9[b]	1.6 ± 1.02[c]	115.50 ± 2.86[a]	34.12 ± 4.32[b]	28.51 ± 2.81[b]	17.25 ± 2.67[c]

Data represented as mean values with standard deviation. Lower-case letters in the rows of each variety differ significantly by the Tukey's test (P < 0.05).

Table 3. Root traits of wheat seedlings after 72 hours of germination on agar gel with different concentrations of cadmium

Variety	*Triticum aestivum* cv. 'Favorytka'				*Triticum dicoccum* cv. 'Holikovska'			
	Concentrations of cadmium, µM							
Variant, n = 36	0	25	50	100	0	25	50	100
Primary root length, mm	31.15 ± 5.51[a]	29.36 ± 4.68[a]	30.52 ± 4.64[a]	15.59 ± 1.61[b]	26.21 ± 3.19[a]	32.37 ± 4.53[a]	29.24 ± 4.62[a]	23.05 ± 2.36[a]
Seminal roots length, mm	24.82 ± 2.28[a]	30.77 ± 2.81[b]	32.14 ± 3.31[b]	20.50 ± 1.38[c]	34.96 ± 2.05[a]	35.96 ± 2.51[a]	29.39 ± 1.97[a]	22.89 ± 1.48[b]
Seminal root angle, degrees	62 ± 3[a]	50 ± 6[b]	45 ± 2[b]	38 ± 4[c]	93 ± 4[a]	80 ± 6[ab]	73 ± 5[b]	76 ± 5[b]
Area of exudation activity, cm^2	132.0 ± 5.96[a]	105.50 ± 8.5[b]	54.25 ± 3.75[c]	15.2 ± 12.15[d]	130.5 ± 3.52[a]	101.01 ± 13.0[b]	42.02 ± 7.06[c]	21.25 ± 3.75[d]

Data represented as mean values with standard deviation. Lower-case letters in the rows of each variety differ significantly by the Tukey's test (P < 0.05).

T. dicoccum cv. 'Holikovska' was characterized by rapid growth in the first 24 hours followed by a moderate increase in root length – total increase in both PR and SR of control variant were more than 200%. High concentrations of Cd caused weak unreliable inhibition of PR growth by 12% and pronounced inhibition of SR growth by 35%. Low concentrations of Cd did not influence SR growth, but light unreliable stimulation of PR was observed.

For both cultivars, the first weak manifestations of growth retardation as a result of exposure to high concentrations of cadmium were noted at the time of two days; however, it became possible to unambiguously confirm their manifestations only after 72 hours. Inhibition of root growth processes is typically one of the first manifestations of cadmium toxicity (Ismael *et al*., 2019). The light stimulating effect of low cadmium concentrations was noted previously and is associated with a hyper compensatory response of the antioxidant system of plants (Carvalho *et al*., 20120.

Regarding the angle of root germination – seminal root angle trait, a general tendency was observed, expressed in its decrease as the metal concentration increased. It should be noted that although this parameter of the root system differs significantly between the two species, the degree of their reaction was close to each other. At 25 µM Cd on the third day, the difference between the control and experimental variants was 12 degrees for 'Favorytka' and 13 degrees for 'Holikovska', at concentrations of 100 µM – 24 and 17 degrees, respectively. At medium concentrations, SRA for 'Holikovska' was nearly identical to that at 100 µM, on the contrary, 'Favorytka' was closer to that at 25 µM, which could be attributed to the higher sensitivity of the seminal roots of 'Holikovska' to cadmium addition. In contrast to the root growth inhibition processes, a visible change in the root angle was noted already after 24 hours at high concentrations of cadmium for the 'Favorytka' and medium-high for 'Holikovska'. The response of SRA could be interlinked with disturbance of auxin homeostasis in roots, which is typically observed under Cd-stress (Uga *et al.*, 2015).

Evaluation of exudative activity showed that the control variant of *T. aestivum* had lower, by 25%, the extent of acidification of the medium in the first 24 h in comparison with *T. dicoccum*, however, there was no significant difference between control samples on the second and third day. Upon exposure to 25 µM of Cd, a sharp decrease in exudative activity was observed, up to the complete absence of any visible changes in the pH of the medium for *T. aestivum* and a decrease in the area of pH change by 83% for *T. dicoccum*. After 72 h samples with minimal cadmium concentrations did not reach the control values, the area of synthetic activity was 79% and 77% of the control, respectively. The subsequent increase in cadmium concentration led to a progressive decrease in the area of excretory activity, a time delay in recovery of excretory activity was noted for *T. aestivum* in comparison with *T. dicoccum*.

Discussion

Current data on the SRA in the early stages of development and the root architecture of adult plants indicate that cultivars with a small root angle tend to develop a compact deep-rooting system (Manschadi *et al*., 2008; Xie *et al*., 2017; Rich *et al*., 2020). Considering that the highest concentrations of bioavailable forms of cadmium are found in the first 20–40 cm of topsoil (Šichorová *et al*., 2004; Wang *et al*., 2019), due to oxidizing condition of topsoil, active use of phosphate-organic fertilizers, other similar agricultural practices and anthropogenic activities in general (Filipović *et al*., 2016; Wei *et al*., 2020), varieties with a small root angle can be considered as potential material for breeding low-cadmium accumulating wheat. In addition, differences in the root morphology of wheat varieties with high- and low-cadmium accumulation have previously been noted: Berkelaar and Hale (2000) reported that of the two durum wheat (*Triticum turgidum* L.) cultivars, which had less root surface area and fewer root tips tended to accumulate less Cd per gram of dry weight, a similar result was later acquired by Kubo *et al.* (2011) for two bread wheat cultivars.

Since the role of low-molecular-weight organic acids (LMWOA) in the mechanisms of extracellular protection against the toxic effects of metals is well known (Osmolovskaya *et al*., 2018), in particular, the excretion of malate by wheat in the response to aluminium (Delhaize *et al*., 1993; Sasaki *et al*., 2006), the relative excretory activity with Cd-stress has been evaluated. The relationship between the release of LMWOA's and resistance to cadmium through the exclusion mechanism was previously observed in a small number of plant species (Zhu *et al*., 2011; Guo *et al*., 2017), as well as in a transgenic species with a modified malate transporter (Ma *et al*., 2020).

In contrast to all other parameters described in the article, the difference in the parameter of exudative activity at concentrations of 0, 25, 50, and 100 µM Cd is very explicit. From a theoretical standpoint, the decrease in the area of pH change, which depends on the active excretion of organic acids by the root system, can be explained by one of the following assumptions or their combination: Cd-stress leads to inhibition of a group of membrane transporters, Cd-stress leads to inhibition of the synthesis of LMWOA's in the roots as such (Chaffai *et al*., 2006), there is active reabsorption of previously excreted acids in the form of Cd-OA conjugates. It is worth noting the available data indicating that when exogenous organic acids are added in medium, they are capable of forming soluble complexes with cadmium, which can be easily absorbed by plants without causing explicit phytotoxic effect (Nigam *et al*., 2000; Zhang *et al*., 2020; Lu *et al*., 2021).

Among other things, as can be seen from the above, as well as from the data given in the tables, such parameters as SRA and acidification of the medium, with all the simplicity of their measurement, exhibit a certain degree of specificity, thus, potentially, can be used to rapid-asset responsivity of plant organisms to low Cd-doses at a short period.

Conclusion

According to the received data on morphofunctional parameters in wheat seedlings upon three-day exposure to cadmium, genotype *Triticum aestivum* cv. 'Favorytka' was estimated to be more Cd-sensitive, as it exhibited much stronger inhibition in primal root growth, up to 50% compared to 12% in genotype *Triticum dicoccum* cv. 'Holikovska'. Significant inhibition in root grow was accompanied by a decrease in seminal root angle by 24 degrees in *T. aestivum*, compared to 17 degrees in *T. dicoccum*, and more prominent, up to complete absence, decrease in root exudative activity. However, a visible inhibition in root exudative activity was observed in both genotypes concerning all cadmium concentrations, and the degree of inhibition largely depended on the metal concentration.

The quality of the proposed phenotyping method, considering all its simplicity, was stated to be satisfactory, as it was found to be sensitive enough to demonstrate the difference in test-objects response depending on tested plant genotype and/or mental concentration. Although the proposed agar-gel phenotyping method has several downsides, such as the inability to model complex interaction between supposed stress-factor (in our case, Cd^{2+}) and soil particles, it still proposes an opportunity for non-invasive monitoring of early stages of root development and rapid, real-time assessment of its response to edaphic stressors. All while being cheap, cost-effective in terms of labour and time, and flexible to modifications.

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Conflict of interest

The author declares that there is no conflict of interest regarding the publication of this paper.

Author contributions

OS – author of the idea, guided the research and is the corresponding author.
TL – performed the phenotyping assays and calculation of the results.
MK – performed data statistical analysis.
LM, VS and NT – performed the literature data analysis and discussion of the results.
All authors read and approved the final manuscript.

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