The Impact of Short-Term Exposure to Pb and Cd on Flavonoid Composition and Seedling Growth of Common Buckwheat Cultivars

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Abstract

The aim of this study was to compare the tolerance of seedlings of three Polish buckwheat cultivars (Hruszowska, Kora, and Luba) for short-term exposure to Pb^{2+} and Cd^{2+}. Seedlings were grown under controlled conditions in Hoagland nutrient solution, with the addition of low/high Pb^{2+} or Cd^{2+} ions (0.01 and 1.00 mM, respectively). After 3 days of treated seedling growth, the levels of total anthocyanins and content of particular flavonoids were measured. The presence of low concentrations of both Pb^{2+} and Cd^{2+} resulted in a small stimulation of the growth of seedlings of all studied cultivars, while higher doses inhibit root growth and, to a much lesser extent, that of shoots. Cadmium (Cd^{2+}) ions were more harmful for growth of buckwheat seedlings than Pb^{2+} ions. More resistant to stress caused by the presence of high concentrations of Pb^{2+} and Cd^{2+} in the growth medium were seedlings of Hruszowska and Luba cultivars, compared to Kora seedlings. Cotyledons of more resistant cultivars (Hruszowska and Luba) contained much more flavonoids than cotyledons of Kora.

Keywords: lead, cadmium, common buckwheat, seedling growth, flavonoids

Introduction

While plants need many metals such as iron, magnesium, copper, or zinc, other metals such as lead or cadmium are highly toxic. Plants have evolved various mechanisms to deal with toxic metals in the soil [1]. For instance, specific proteins such as metallothioneins can effectively bind heavy metals [2, 3].

Lead is the most common heavy metal contaminant in the environment. It is accumulated in a dose-dependent manner in plants, which results in reduced growth, changes in chemical composition, enzyme activities, and lower uptake of many minerals. Lead can cause various physiological and biochemical dysfunctions during seed germination, plant growth, and many physiological and biochemical processes [4, 5]. Although lead transport from plant roots to shoots is usually limited, shoots usually can accumulate up to 1/6 of the level in roots [6, 7]. However, common buckwheat belongs to plants that accumulate high lead content in leaves without significant damage [8]. This indicates that buckwheat is recognized as a Pb hyperaccumulator. During 8 weeks of cultivation, buckwheat leaves accu-

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mulated ca. 2.5-times more Pb than the roots, and 4-times more than stems [8].

Among the heavy metals, cadmium (Cd) is one of the most toxic elements taken up by plants [9]. Cadmium is toxic to many plant species at low concentrations [10]. When Cd is present in the nutrient medium, it causes strong inhibition of growth and different metabolic processes [11]. At the root region Cd competes for absorption of several essential elements like calcium and magnesium, therefore causing their deficiency [12]. Cd can be accumulated to high concentrations in roots, whereas only minor amounts can be transported to the above-ground tissues in the form of metallo-organic complexes and reach tissues of aerial parts of the plants [13, 14]. Root elongation in plants was retarded rapidly within hours of Cd exposure, and this means that interactions with growth regulators may occur [15, 16].

Root growth is more sensitive to heavy metals than shoot growth [17]. This evidence correlates with the data that heavy metals accumulate predominantly in roots. In many plants Pb^{2+} inhibited growth more than Cd^{2+}, but in maize Cd^{2+} was found to exceed Pb^{2+} in its toxicity to roots [18, 19]. In general, Cd^{2+} and Pb^{2+} inhibits growth, but at low concentrations can promote the growth of root systems and shoots [20].

Previous studies showed that lead (Pb^{2+}) and cadmium (Cd^{2+}) ions, besides growth inhibition, can disturb many biochemical processes [21]. For instance, Cd^{2+} induced activity of chalcone synthase in soybeans [22]. This result suggests involvement of the phenylpropanoid pathway in the response of legume plants to metals [23, 24]. The gene encoding phenylalanine ammonia-lyase, a key enzyme in the phenylpropanoid pathway, was detected among Cd^{2+}-induced genes in *Brassica juncea* [25]. Several studies have found that heavy metals activated the phenylpropanoid pathway and increased lignin synthesis in many plant species [26-29]. Kováčik et al. [30] observed an increase of polyphenol oxidase activity in roots of *Matricaria chamomilla* by cadmium and suggested that the formation of polymerized phenols could be used to complex Cd ions.

Flavonoids are known to form complexes with heavy metals, and this could lead to an effective method of plant defensive responses to heavy metal stress [31-33]. It has been confirmed in numerous studies that flavonoids function as antioxidants mainly by chelating metal ions. Moreover, metal-flavonoid chelates are much more powerful free radical scavengers than the parent flavonoids, and play a prominent role in protecting cells from oxidative stress [34]. Flavonoids play an important role in limiting metal bioavailability and suppressing metal toxicity by forming complexes. Due to such properties, flavonoids appear to be a suitable antidote for heavy metal poisoning *in vivo* [35]. Among metal ions, easy-forming complexes with flavonoids are also Pb^{2+} and Cd^{2+} [35, 36].

Since many authors have found an induction of flavonoids upon heavy metal treatment [33, 37, 38], investigations on levels of endogenous flavonoids were done. After studies on micropropagated poplar (*Populus jacquemontiana*) plants, it was suggested that flavonoids are able to protect the plant tissues due to their Cd chelating properties [39]. In these studies Cd treatment caused enhanced accumulation of flavonoids in poplar seedlings. Evidence that flavonoids could be an additional factor in heavy metal tolerance in *Arabidopsis thaliana* also has been found [40].

The aim of the present study was to compare the sensitivity of seedlings of three Polish buckwheat cultivars (Hruszowska, Kora, and Luba) to the short-term exposure of Pb^{2+} and Cd^{2+}. Preliminary experiments evaluated the effects of different doses of Pb and Cd on the growth of buckwheat seedlings. The obtained data were the basis for the selection of the dose of 0.01 mM of each metal, which caused a slight stimulation of growth. For comparison, a concentration of 1.00 mM was used, which significantly inhibited seedling growth. In the work a possible relation between flavonoid level and Pb or Cd tolerance also was investigated in seedlings of common buckwheat cultivars. Among the studied cultivars the Hruszowska is the most widely cultivated in Poland, Kora is in second place, while Luba is only grown in experimental plots.

Material and Methods

Plant Material and Growth Conditions

Seedlings of buckwheat (*Fagopyrum esculentum* Moench) cv. Hruszowska, Luba, and Kora were used in this study. The germination process was carried out in darkness during four days, as described previously [41]. Afterward, the buckwheat seedlings were exposed for three days to low (0.01 mM) or high (1.00 mM) concentrations of $Pb(NO_3)_2$ or $CdCl_2$ dissolved in one fifth Hoagland solution, and grown in controlled-environment conditions with a 16/8 h 24/18ºC day/night scheme and light intensity of 100-120 $\mu mol \cdot m^{-2} \cdot s^{-1}$, which was provided by 400 W high-pressure sodium lamps [41].

Measurements of Plant Elongation

Before and after a 3-day period of exposure to Pb^{2+} or Cd^{2+}, shoot and main root length were measured in the seedlings. The differences between lengths before and after exposure were treated as a growth elongation. Mean results of lengths and their statistical evaluation were obtained from 60-80 seedlings.

Determination of Anthocyanins

Extraction and measurement of anthocyanins were carried out using the method described by Mancinelli [42] (slightly modified [43]). Briefly, hypocotyl or cotyledon tissues were extracted with acidified (1% HCl, w/v) methanol for 24 h at room temperature, in darkness with occasional shaking. Absorbance of the extracts was measured at $\lambda = 530$ nm (anthocyanins), and 657 nm (chlorophyll degradation products) using a Cecil spectrophotometer. The formula $A_{530} - 0.25 \cdot A_{657}$ was used to compensate for the absorp-

tion of chlorophyll degradation products at 530 nm. Anthocyanin content was calculated as cyanidin-3-gluco-side, whose molecular extinction coefficient equals 29,600. Analyses were carried out for three independent replicates for hypocotyls and cotyledons separately.

Determination of Flavonoids

Full details of flavonoid analysis were described earlier [41]. Briefly, freeze-dried samples of buckwheat tissues were extracted by sonication with a mixture of 60% methanol and 0.4% trifluoroacetic acid. The extraction was repeated five times. Pooled extracts were centrifuged and directly taken to HPLC analysis. A HPLC system equipped with a 250×2.0 mm i.d. Cadenza CD-C_{18} 3 µm column, and UV-Vis detector set at 350 nm. The flavonoids were eluted in a gradient system composed of solvents A (water/acetonitrile/formic acid, 89:6:5) and B (water/acetonitrile/formic acid, 15:80:5). Identification of flavonoids by comparing the retention times to the available standards was done. For calculating flavonoid content we used commercial standards, except for quercetin-galactosyl-rhamnoside, the contents of which were calculated based on the standard of the rutin. The quercetin-galactosyl-rhamnoside was identified as described earlier [41].

Statistics

In the case of anthocyanins a one-way analysis of variance complemented by the least significant difference was used with 5% level of probability (Newman-Keuls test). In the case of seedling elongation and flavonoid levels, statistical analyses were achieved with the t-test, and significant differences in relation to the control plants were indicated with *$p<0.05$ and **$p<0.01$.

Results and Discussion

Common buckwheat is recognized as a Pb hyperaccumulator [8]. It was found that buckwheat accumulates Pb in the shoots rather than in the roots. The mechanisms involved in Pb hyperaccumulation in buckwheat shoots remain unknown. Buckwheat is also able to accumulate a large amount of aluminum (Al) in the shoots [44]. In Al accumulation organic acids play an important role. There is a suggestion that the organic acids may also play a role in Pb tolerance and accumulation in buckwheat tissues [8].

During our studies low doses of Pb (0.01 mM) in nutrient solution have stimulated shoot and root growth of seedlings of all buckwheat cultivars, although the elongation was not statistically significant (Fig. 1). A similar phenomenon was also described for seedlings of *Pinus pinea* and *Pinus pinaster* [20]. A high Pb dose (1.00 mM) caused the elongation growth of roots and shoots to slow down. In the case of roots the growth inhibition was quite clear and statistically significant in comparison to roots of the control seedlings, at $p<0.01$. Buckwheat seedling root growth was more sensitive to Pb treatment compared to that of shoots.

The results confirm earlier findings that root growth is more sensitive to heavy metals than shoots [17]. Only growth elongation of shoots of Kora cv. was significantly lower at $p<0.01$. In seedlings of Hruszowska the elongation of shoots was significantly lower at $p<0.05$, and in the case of Luba the elongation decline was not significant, even at $p<0.05$. Reduced toxicity of heavy metals to the shoots due to the fact that plants have evolved mechanisms to limit the transport of metal ions to the aerial parts [6, 7]. It is well known that the heavy metal ions can bound to the cell walls in root cells. Lead ions exhibit the high affinity to the carboxyl and hydroxyl groups of the polysaccharides, which are the main components of cell walls [5]. For Pb, the process of binding to a cell wall is one of the major mechanisms of detoxification [45]. It seems that our results indicated much lower sensitivity buckwheat shoots than roots to lead ions, confirming these findings.

Buckwheat tissues are well known as a rich source of flavonoids, like glycosides of flavonols and flavones, as well as anthocyanins [41, 43]. Young seedlings of common buckwheat rapidly begin accumulating anthocyanins when exposed to light [46]. Accumulation of anthocyanins in young seedlings is stimulated by various environmental stresses [47]. Exposure to stress caused by excess Pb also resulted in an increased accumulation of anthocyanins in seedlings of all buckwheat cultivars (Fig. 2). Generally, the content of anthocyanins in the cotyledons and hypocotyl of
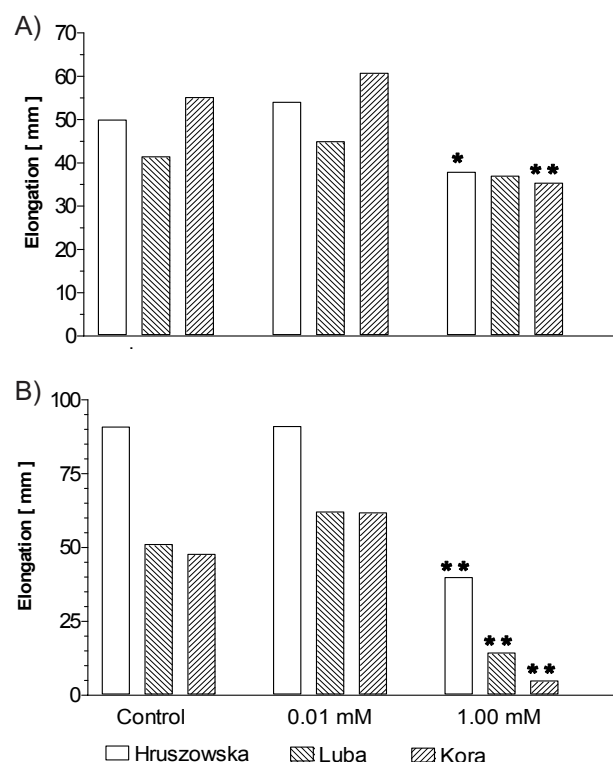

Fig. 1. Effect of 3-day treatment by low and high doses of Pb^{2+} in nutrient solution on elongation of shoots (A) and roots (B) in seedlings of three buckwheat cultivars. Means marked with one asterisk and two asterisks were considered statistically significant in comparison to control at $p<0.05$ and $p<0.01$, respectively. Lack of asterisk means no significant difference in comparison to control. Statistical calculations were carried out for each cultivar separately.

buckwheat was higher under high Pb doses (1.00 mM) rather than low (0.01 mM), and much higher than that in the control seedlings. Among the studied cultivars of buckwheat, Pb had the least impact on the content of anthocyanins in hypocotyls of Kora cv.

There is a well known relationship between exposure to heavy metals and accumulation of anthocyanins [48, 49]. In red maple (*Acer rubrum*) grown in soil with the addition of Pb^{2+} the amount of anthocyanins in the leaves increased nearly four-fold [50]. Increased synthesis of anthocyanins may be related to their role in reducing the toxicity of heavy metals on plants. According to Hale et al. [51], anthocyanins can protect cells from the damaging effects of heavy metals by the formation of stable complexes with them. After the absorption into plant tissues, heavy metals can be chelated in the cytosol, and finally allocated within the vacuole [52, 53]. The vacuole is one of the major cellular compartments involved in the maintenance of tolerance to the increased amount of heavy metals [52].

As regards the effects of Cd on plant development, it induces visible symptoms of phytotoxicity, such as reduced growth [54]. In present studies, unlike in the case of Pb, low Cd concentrations (0.01 mM) inhibited elongation of shoots and roots of Hruszowska and Kora seedlings (Fig. 3). Higher sensitivity of roots and shoots to cadmium toxicity, compared to lead, was noted earlier for wheat and other plants [18, 19]. Seedlings of Luba were more resistant
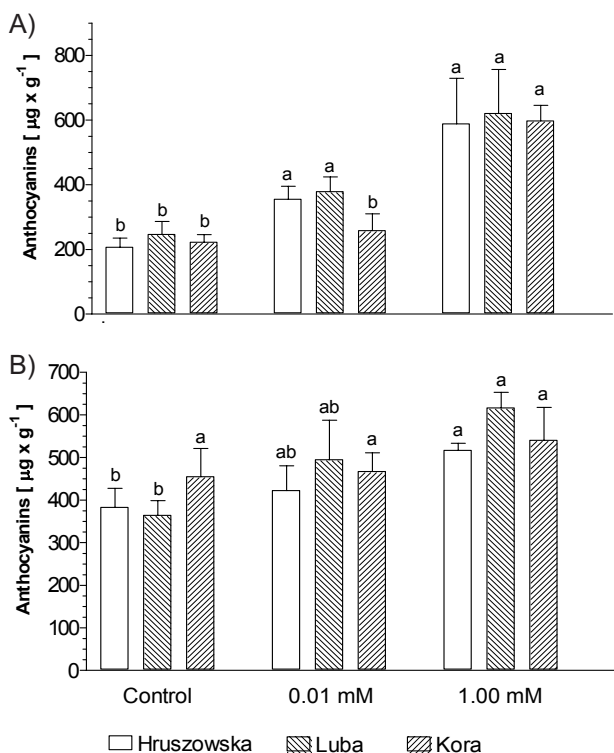

Fig. 2. Effect of 3-day treatment with low and high doses of Pb^{2+} in nutrient solution on anthocyanin content (on fresh weight basis) in seedling cotyledons (A) and hypocotyl (B) of three common buckwheat cultivars. Bars (means +SD) marked by the same letter(s) are not significantly different using Newman-Keuls test at 5% level of probability. Statistical calculations were carried out for each cultivar separately.
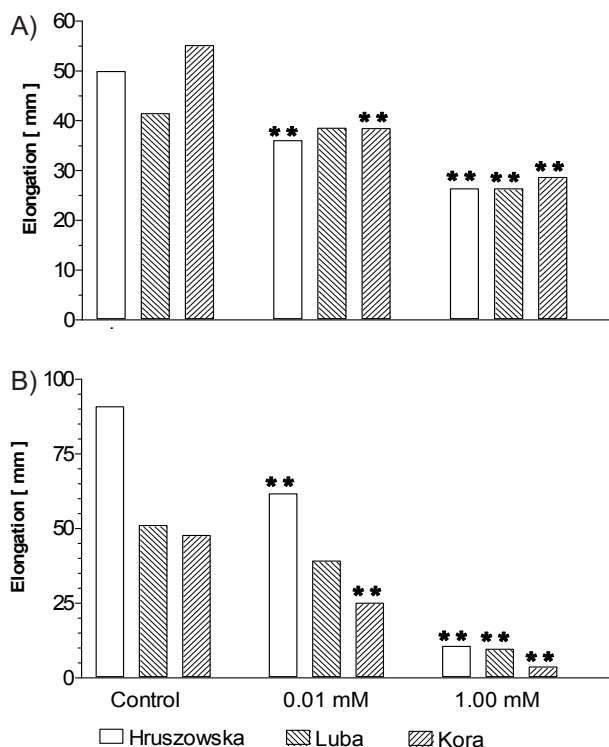

Fig. 3. Effect of 3-day treatment by low and high doses of Cd^{2+} in nutrient solution on elongation of shoots (A) and roots (B) in seedlings of three buckwheat cultivars. Means marked with two asterisks were considered statistically significant in comparison to control at p<0.01. Lack of asterisk means no significant difference in comparison to control. Statistical calculations were carried out for each cultivar separately.

to this concentration of Cd ions, although some, (not statistically significant), inhibition of growth occurred. Significantly greater inhibition of root and shoot growth was caused by a high dose Cd (1 mM). In the case of Kora, inhibition of root growth was almost complete, and in the case of all cultivars shoots reached 40-50% as compared to the control seedlings (Fig. 3). Buckwheat belongs to Pb-hyperaccumulator plants, and therefore has efficient mechanism of Pb-detoxification [8]. It is possible that this mechanism also plays a role in flavonoids.

The negative effect of cadmium on plant growth and development is well known [10]. Our results confirm strong inhibition of plant growth by Cd reviewed by Benavides et al. [11] and recently described for soybean [55]. Among tested buckwheat cultivars it has been found that Kora seedlings were most susceptible to the toxic effects caused by Cd^{2+}. Even a low dose of those ions (0.01 mM) caused growth inhibition of roots and shoots of the Kora seedlings, which was significantly lower compared to control at p<0.01 (Fig. 3). Also, growth of Hruszowska seedlings were significantly inhibited by both low and high concentrations of Cd^{2+}, although root elangation of the seedlings for the 1 mM concentration of Cd was almost three times higher than for the roots of the Kora (Fig. 3).

Similarly to Pb, the content of anthocyanins in the cotyledons and hypocotyl of buckwheat was higher under the high dose Cd (1.00 mM) than low (0.01 mM), and much

higher than that in the control seedlings. Exposure to excess Cd (1.00 mM) resulted in a much higher increased accumulation of anthocyanins in Hruszowska seedlings in comparison to Pb treatment (Figs. 2 and 4). In studied buckwheat cultivars, Cd had a smaller impact on the content of anthocyanins in tissues of Kora and Luba, than in Hruszowska.

In the reaction of leaf tissues to low concentrations of Cd^{2+} and Pb^{2+}, an increase in levels of all buckwheat flavonoids (glycosides luteolin, apigenin, and quercetin) was noted (Tables 1 and 2). A particularly large accumulation was observed in the case of rutin. Stimulation of flavonoid biosynthesis was noted in previous studies [33, 37, 38]. Recently, it also was found that activity of crucial enzyme in flavonoids biosynthesis and phenylammonia lyase (PAL), was increased in roots of soybean (*Glycine max*) and lupine (*Lupinus luteus*) seedlings treated with Cd^{2+} and Pb^{2+} [23]. Our results correspond with these findings.

When comparing the buckwheat cultivars, it may be noted that the high content of flavonoids seems to be associated with a higher resistance of the seedlings to Pb and Cd (Tables 1 and 2). Total content of flavonoids in cotyledons of Hruszowska and Luba was ca. 1.5-times higher than in Kora. The low level of flavonoids in Kora cotyledons was accompanied by substantial inhibition of root elongation. Shoot elongation of the Kora seedlings was also lower than in the case of Hruszowska and Luba. Thus,
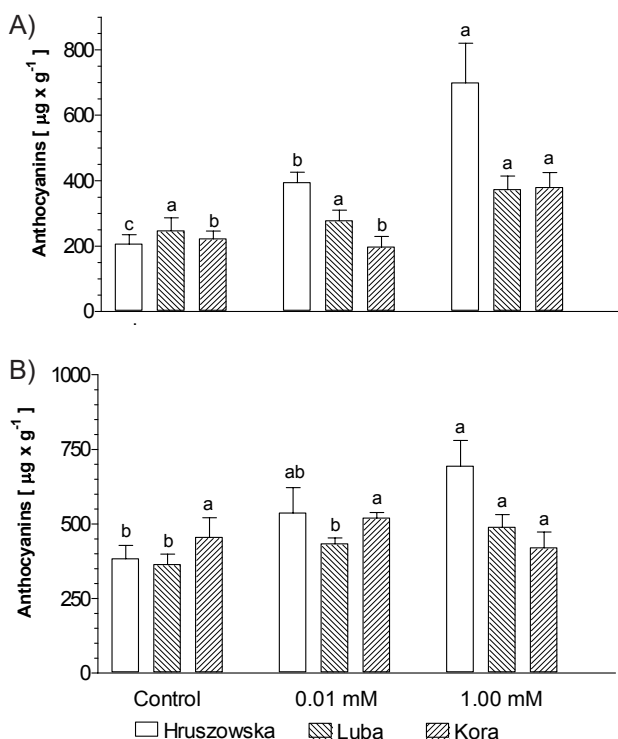

Fig. 4. Effect of 3-day treatment with low and high doses of Cd^{2+} in nutrient solution on anthocyanin contents (on fresh weight basis) in seedling cotyledons (A) and hypocotyl (B) of three common buckwheat cultivars. Bars (means +SD) marked by the same letter(s) are not significantly different using Newman-Keuls test at 5% level of probability. Statistical calculations were carried out for each cultivar separately.

Table 1. Effect of 3-day treatment by low and high doses of Pb^{2+} in nutrient solution on flavonoids levels (mg·g^{-1} DW) in cotyledons of buckwheat seedlings. The data were analyzed by one-way analysis of variance for each flavonoid separately. Means marked with one asterisk and two asterisks were considered statistically significant in comparison to control at $p<0.05$ and $p<0.01$, respectively. Lack of asterisk means no significant difference in comparison to control. NA – not analyzed.

Flavonoid	Control	0.01 mM	1.00 mM
Hruszowska			
Orientin	5.28	5.45	6.01
Iso-orientin	16.38	17.16	19.42*
Vitexin	4.89	5.88*	5.54
Iso-vitexin	10.66	11.57	12.35
Quercetin-galactosyl-rhamnoside	1.57	1.77	1.77
Rutin	7.46	9.91*	8.95*
Total	46.24	51.74*	54.04**
Luba			
Orientin	6.06	NA	5.88
Iso-orientin	19.16	NA	19.07
Vitexin	5.80	NA	4.89
Iso-vitexin	12.89	NA	10.96*
Quercetin-galactosyl-rhamnoside	2.02	NA	2.03
Rutin	9.58	NA	10.13
Total	55.51	NA	52.96
Kora			
Orientin	2.68	2.93	2.49
Iso-orientin	9.11	9.55	7.74*
Vitexin	3.50	3.71	3.18
Iso-vitexin	7.48	8.46	7.17
Quercetin-galactosyl-rhamnoside	0.95	0.89	1.44*
Rutin	5.72	9.05*	12.95**
Total	29.44	34.59*	34.97*

the detoxification mechanism in the buckwheat is probably related also to flavonoid levels. The enhanced content of flavonoids probably is responsible for binding of metal ions, and also for their deposition within the vacuole [24]. In a previous study the level of flavonols was increased in primary leaves of *Phaseolus coccineus* under heavy metal treatment, and the level depended on the metal and its concentration [26]. These results obtained in our study indicate that in reaction to Cd and Pb they can also involve other mechanisms, such as the accumulation of flavonoids,

Table 2. Effect of 3-day treatment by low and high doses of Cd^{2+} in nutrient solution on flavonoids levels (mg·g⁻¹ DW) in cotyledons of buckwheat seedlings. The data were analyzed by one-way analysis of variance for each flavonoid separately. Means marked with one asterisk were considered statistically significant in comparison to control at p<0.05. Lack of asterisk means no significant difference in comparison to control.

Flavonoid	Control	0.01 mM	1.00 mM
Hruszowska			
Orientin	5.28	6.14	3.91*
Iso-orientin	16.38	19.48*	12.79*
Vitexin	4.89	5.31	4.23*
Iso-vitexin	10.66	11.57	9.30*
Quercetin-galactosyl-rhamnoside	1.57	1.77	1.41
Rutin	7.46	10.10*	6.99
Total	46.24	54.37*	38.63*
Luba			
Orientin	6.06	6.44	6.06
Iso-orientin	19.16	21.95*	19.41
Vitexin	5.80	5.63	4.89
Iso-vitexin	12.89	12.27	10.75*
Quercetin-galactosyl-rhamnoside	2.02	1.81	1.96
Rutin	9.58	8.33*	8.26*
Total	55.51	56.34	51.33
Kora			
Orientin	2.68	2.99	3.26*
Iso-orientin	9.11	10.58*	10.27*
Vitexin	3.50	4.52*	4.54*
Iso-vitexin	7.48	9.66*	9.34*
Quercetin-galactosyl-rhamnoside	0.95	0.98	1.23
Rutin	5.72	10.17*	6.54
Total	29.44	38.90*	35.18*

which are metal chelators, and can also react with reactive oxygen species, converting the peroxyl radical into a highly stabilized radical [56].

Significant correlations were found between the levels of orientin and *iso*-orientin, as well as vitexin and *iso*-vitexin in the cotyledons of buckwheat (r=0.9944 and r=0.9742, respectively). Also, significant correlation was found between total content of both C-glucosides of luteolin and apigenin (r=0.9133). It probably means that biosynthesis of these flavonoids involves the same enzyme, or set of enzymes. No significant correlation was found between the content of both quercetin glycosides: rutin and quercetin-galactosyl-rhamnoside (r=0.2733).

Conclusions

Low concentrations of both Pb^{2+} and Cd^{2+} added to nutrient solution resulted in a small stimulation of the growth of seedlings of all studied cultivars of common buckwheat, while higher doses inhibit root growth, and to a much lesser extent, of shoots. Cd^{2+} ions were more harmful for growth of buckwheat seedlings than Pb^{2+} ions. More resistant to stress caused by the presence of high concentrations of Pb^{2+} and Cd^{2+} in the growth medium was seedlings of Hruszowska and Luba cultivars, in comparison to seedlings of Kora. Cotyledons of more resistant cultivars (Hruszowska and Luba) contained much more flavonoids than cotyledons of less resistant seedlings of Kora. It suggests that the detoxification mechanism of lead and cadmium in buckwheat seedlings probably involve flavonoids.

To the best of our knowledge, this is the first report uncovering the impact of genetic variability of flavonoid contents within seedlings of various buckweat genotypes on the ability to detoxify lead and cadmium ions. This hypothesis, however, requires further studies, which may explain the detailed mechanism of mentioned ion detoxification.

Acknowledgements

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