Effect of cadmium on polyphenol content in young barley plants (*Hordeum vulgare* L.)

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ABSTRACT

The effect of abiotic stress caused by cadmium on the total polyphenols content in root, shoots and leaf blades of barley was investigated in model experiments. Total polyphenols content was determined spectrophotometrically with Folin Ciocalteau's reagent, Cd content by atomic absorption spectrometry (AAS) in 28-day-old plants. Treatment of the barley plants with Cd (1.10^{-6} mol/l) in a nutrition solution caused the increase in the total polyphenols in all parts of the plant. The highest increase was found in the leaf blades (35.2%, 8340 mg/kg d.m.), a lesser increase found in the shoots (16.7%, 3590 mg/kg d.m.), and the lowest increase found in the roots (10.3%, 3650 mg/kg d.m.). The highest Cd increase in the treated plants was found in the roots (10 400%, 240 mg/kg d.m.), the lesser in their shoots (4990%, 16.3 mg/kg d.m.), and the lowest in the leaf blades (2580%, 5.78 mg/kg d.m.). There was found a statistically significant increase (at least $P < 0.05$) in the total polyphenols and Cd contents in all investigated parts of the plant. Statistically significant differences (at least $P < 0.05$) of the total polyphenols and Cd contents among barley roots, shoots, and leaf blades were found.

Keywords: barley; roots; shoots; leaf blades; polyphenols; Cd-stress effect

Polyphenols in barley

The outer layer coats of the plant seeds contain different polyphenolic compounds, e.g. flavonoids, phenolic acids, coumarins and anthocyanins. Their content and composition is strongly influenced by different factors (Weidner and Paprocka 1996, Weidner et al. 1996). *O*- and *C*-glycosides of flavones are characteristic for cereals, especially those derived from apigenin and luteolin. In rye and triticale there have been also described glycosides of quercetin. Flavonoids occurring in barley are given in Table 1.

Yu et al. (2001) identified in barley caryopses among cinnamic acid derivatives coumaric, caffeic, ferulic, and chlorogenic acids and among benzoic acid derivatives *p*-hydroxybenzoic, vanillic, and protocatechuic acids. Ferulic acid was the most abundant (Sancho et al. 2001). Markham and Mitchell (2003) found in young green barley leaf blades as the major flavonoid antioxidants saponarin, lutonarin and 2''-*O*-glucosylvitexin. The overview of phenolic acids in barley is given in Table 2.

The average content of the total polyphenols and especially those of catechol, resorcinol and phloroglucinol type compounds in barley caryopses differ from other cereals, e.g. triticale. The total

Table 1. Dominant polyphenols in barley (Lachman et al. 1998)

Polyphenol type	Compound
Flavones	apigenin 7-*O*-rhamnoglucoside
	isovitexin (6-*C*-glucosylapigenin)
	vicenin-1,2 (6,8-*C*-diglucosylapigenin)
	isoorientin (6-*C*-glucosylluteolin)
	2''-*O*-glycosylisovitexin
	lutonarin (isoorientin 7-*O*-glucoside)
	tricin
	glucotricin
	tricinin
Leucoanthocyanidins	procyanidin
	prodelphinidin
Catechins	gallocatechin
	(–)-epicatechin
Coumarins	umbelliferon
	esculetin
	scopoletin
	herniarin

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Table 2. Phenolic acids in barley (Lachman et al. 1998)

Acids derived from cinnamic acid	Acids derived from benzoic acid
Ćaffeic	vanillic
Ferulic	*m*-hydroxybenzoic
o-Coumaric	salicylic
p-Coumaric	*p*-hydroxybenzoic
Chlorogenic	syringic
	veratric
	protocatechuic
	gallic
	m-galloylgallic

Table 3. Composition of nutrient media-basic Knop's solution and modified Shive's solution with microelements used for barley cultivation

Knop's solution	
$Ca(NO_3)_2$ anhydrated	0.572 g/l
KNO_3	0.143 g/l
KCl	0.071 g/l
KH_2PO_4	0.132 g/l
$MgSO_4$ anhydrated	0.143 g/l
$FeCl_3.H_2O$	1 drop of 5% solution to 1 l of nutrient medium
Shive's solution	
H_3BO_3	1.43 mg/l
$MnCl_2.H_2O$	0.90 mg/l
$ZnSO_4.7\ H_2O$	1.00 mg/l
$CuSO_4.5\ H_2O$	0.08 mg/l

polyphenols and phloroglucinol type compounds content in barley caryopses varied from 857 to 1690 and from 95 to 448 mg/kg d.m., respectively, while corresponding values in triticale were 607–4400 mg/kg d.m., and 5.7–10.5 mg/kg d.m., respectively. It shows a considerable antioxidant activity in barley grains (Lee et al. 2003, Wu et al. 2003). The total polyphenols content in barley caryopses depends significantly on the variety and is influenced by the site, specific weather features of a year, and the conditions of ageing.

Effect of stresses in barley

Content and qualitative composition of polyphenolic complex has been affected by different stress factors (Weidner et al. 1996). Phenolic acids are regulating factors, which retard the processes of premature sprouting (Weidner and Paprocka 1996). Their changes in caryopses in the state of dormancy were determined both in free and bound forms (ester and glycosidic bounds) (Weidner et al. 1995). Regarding the fact that polyphenols are predominantly localized in the outer layers of caryopses, they protect the embryos against UV-irradiation.

Wu et al. (2003) investigated the effects of four Cd levels on lipid peroxidation and activities of antioxidant enzymes in barley plants during ontogenesis. This investigation showed that Cd-stress induced a concentration- and gene type-dependent oxidative stress response in barley leaf blades, characterised by an accumulation of malondialdehyde and the alteration pattern of antioxidative enzymes, regarding the genotypic difference in Cd tolerance.

The aim of this work was to determine the effects of the abiotic stress caused by cadmium on the polyphenol levels in barley plants.

MATERIAL AND METHODS

Cultivation of spring barley. The experiment was carried out under controlled conditions in a climate-controlled room at the Department of Plant Botany and Physiology of the Czech University of Agriculture in Prague. Spring barley variety Kompakt was selected as the experimental material. The caryopses were germinated in redistilled water at laboratory temperature (25°C) for 4 days. Then the seedlings were replanted into special boxes with a nutrient medium. Two variants of the cultivation experiment were carried out. The control variant without added cadmium and the experimental variant with the addition of Cd in the form of $CdCl_2.2\ H_2O$ into the nutrient medium in a concentration of 1.10^{-6} mol/l. Each variant was cultivated in 8 boxes. The number of cultivated plants varied from approximately 100 to 125 according to the dimensions of the boxes used. Plants were cultivated in conditioning plastic boxes for another 28 days after their replantation and the nutrient medium was exchanged one time per

Table 4. Assessment of obtained results by parallel analysis of standard and internal reference material

CRM/IRM	Cd_{found} (mg/kg d.m.)	$Cd_{certified/known}$ (mg/kg d.m.)
CRM 12-02-03	0.131 ± 0.044	0.136 ± 0.065
BIOMA 3	556 ± 17.8	573 ± 15.1

week. The young plants were grown at a stable light fluency rate (300 μmol photon/m^2/s) with the day period being 16 hours, at a temperature of 22°C and air humidity 60–80%. The nutrient mediums for both variants differed only in Cd occurrences. Basic nutrients were supplied in the form of Knop's nutrient solution diluted into a one half concentration (Table 3), which is useful for spring barley, and microelements were supplied in the form of Shive's solution diluted into one half concentration (Table 3). After cultivation, the plants were separated into roots, leaf blades and shoots and plant material was subsequently freeze-dried using a Lyovac GT 2 (Leybold-Heraeus, Germany) freeze-drier.

Determination of total polyphenol content. A modified method of Lachman et al. (1998) with Folin-Ciocalteau's phenol reagent was used. Freeze-dried and homogenised plant samples (approximately 0.3 g) were extracted in a Soxhlet apparatus with ethanol-water mixture (80:20 v/v) for 20 hours and the extract was quantitatively transferred into a volumetric flask and adjusted to 100 ml. After a dilution of 5 ml sample aliquot in 50 ml volumetric flask with 80% water-ethanolic solution to approximately 30 ml, 2.5 ml of Folin-Ciocalteau's phenol reagent (Fluka Chemie, AG) was added, agitated and mixed. Then was added 7.5 ml of 20% sodium carbonate solution, the volume was adjusted with distilled water to 50 ml and after thorough agitation it was left to stand at laboratory temperature for 2 hours for a quantitative formation of a blue colour. The same procedure was used for blank where instead of a sample solution; 5 ml of 80% ethanol was used. Then the solutions were centrifuged using a Janetzki T 30 centrifuge at 2000 cycles per minute for 12 minutes. Absorbance values were measured using a Heλios γ (Spectronic Unicam, GB) spectrophotometer against the blank at λ = 765 nm and expressed as mg of gallic acid in 1 kg dry matter of a sample. Relative standard deviation of this method was about 1.96% rel.

Determination of Cd content by a method of atomic absorption spectrometry (AAS). Freeze-dried samples (250–300 mg of leaf blades, 150–250 mg of shoots and 50–100 mg of roots) were decomposed according to the standard operational procedure described by Mader et al. (1998). They were charred on a hot plate in a temperature range of 350–500°C and then ashed in a muffle furnace at 350–500°C. Non-decomposed organic residues were oxidised with concentrated HNO_3 and decomposed at 500°C. The obtained white ash was dissolved in 1.5% HNO_3, the dissolving was accelerated by

Table 5. Increase of total polyphenols content caused by Cd treatment

Variant	Part of plant	Average total polyphenols content (mg/kg d.m.)	Increase (%)	Standard deviation
Control	roots	3310		522.096
	shoots	3080		400.595
	leaf blades	6170		645.374
Cd (1.10^{-6} mol/l)	roots	3650	10.3	579.527
	shoots	3590	16.7	725.123
	leaf blades	8340	35.2	606.717

Table 6. Increase of Cd content in barley in the experimental variants

Variant	Part of plant	Average Cd content (mg/kg d.m.)	Increase (%)	Standard deviation
Control	roots	2.29		4.0201
	shoots	0.32		0.2769
	leaf blades	0.22		0.1316
Cd (1.10^{-6} mol/l)	roots	240	10 400	106.3873
	shoots	16.3	4 990	4.5022
	leaf blades	5.78	2 580	1.3320

sonication. The Cd concentration in the digests of the control samples was determined using AAS with electrothermal atomisation (ET-AAS) with a standard deviation below 5% and detection limit 0.028 mg Cd/kg d.m. Cd concentration in the digests prepared from plant samples cultivated in the presence of cadmium chloride was determined in the acetylene-air flame (FAAS) using a Varian SpectrAA 110 spectrometer (standard deviation of measurement below 1%, detection limit < 2 mg/kg d.m). The determination of Cd concentration in the control samples was carried out in an argon atmosphere in a pyrolytic graphite tube with the platform using a spectrometer Varian SpectrAA 400 with graphite atomiser GTA-96 with compensation of non-selective absorption using deuterium corrector. The temperature of pyrolysis was 450°C and that of atomisation 1900°C and the injected volume was 20 µl. The analyte concentration was measured in the digests as well as in the calibration solutions in two replicates, and results were evaluated from a calibration curve constructed by a successive dilution of standard calibration ASTASOL solution with 1.5% HNO_3. All plant samples were analysed in three parallel determinations and the quality of the obtained results was assessed by a parallel analysis of standard reference material CRM 12-02-03 (alfalfa) and internal reference material with known Cd content BIOMA 3 (*Chlorella*) with the results given in Table 4.

Statistic evaluation. The results (mean values from three parallel determinations) were statistically evaluated with the Statgraphics programme by the analysis of variance with multiple grouping. More detailed evaluation was performed by the Scheffe's test.

RESULTS AND DISCUSSION

It is apparent from the data taken from Tables 5 and 6 that the treatment with Cd caused an increase of the total polyphenols content in comparison with the control variant. Regarding the plant parts, the increase of total polyphenol compounds after treatment with cadmium was as follows: leaf blades

Table 7. Statistical evaluation of the effect of investigated parameters on Cd and total polyphenols contents

Effect	Degrees of freedom	Sum of squares of deviations	Variance (average square)	*F*-test	*P* (level of significance)
Cd content (mg/kg d.m.)					
Variant	1	268 991	268 991.0	131.8454	0.0000
Part of plant	2	427 697	213 848.3	104.8173	0.0000
Replication	2	604	301.9	0.1480	0.8626
Variant × part of plant	2	412 828	206 414.2	101.1735	0.0000
Variant × replication	2	590	295.0	0.1446	0.8655
Part of plant × replication	4	1 494	373.6	0.1831	0.9468
Variant × part of plant × replication	4	1 447	361.8	0.1773	0.9497
Residual variance	126	257 065	2 040.2		
Total	143	1 370 717			
Total polyphenols content (mg/kg d.m.)					
Variant	1	3.661106E + 07	3.661106E + 07	100.002	0.0000
Part of plant	2	4.757074E + 08	2.378537E + 08	649.692	0.0000
Replication	2	2.543350E + 05	1.271675E + 05	0.347	0.7072
Variant × part of plant	2	2.448398E + 07	1.224199E + 07	33.439	0.0000
Variant × replication	2	7.932888E + 05	3.966444E + 05	1.083	0.3416
Part of plant × replication	4	1.883454E + 05	4.708636E + 04	0.129	0.9718
Variant × part of plant × replication	4	4.596943E + 05	1.149236E + 05	0.314	0.8682
Residual variance	126	4.612888E + 07	3.661022E + 05		
Total	143	5.846269E + 08			

Table 8. Scheffe's test applied on the experimental variants and parts of plant (Cd content)

Variant	Control	Cd (1.10^{-6} mol/l)					
Control		0.0000					
Cd (1.10^{-6} mol/l)	0.0000						
Part of plant	leaf blades	shoots	roots				
Leaf blades		0.8471	0.0000				
Shoots	0.8471		0.0000				
Roots	0.0000	0.0000					
Variant × part of plant		control			Cd (1.10^{-6} mol/l)		
		leaf blades	shoots	roots	leaf blades	shoots	roots
Control	leaf blades		1.0000	1.0000	0.9993	0.9093	0.0000
	shoots	1.0000		1.0000	0.9993	0.9116	0.0000
	roots	1.0000	1.0000		0.9999	0.9483	0.0000
Cd (1.10^{-6} mol/l)	leaf blades	0.9993	0.9993	0.9999		0.9852	0.0000
	shoots	0.9093	0.9116	0.9483	0.9852		0.0000
	roots	0.0000	0.0000	0.0000	0.0000	0.0000	

(35.2%) > shoots (16.7%) > roots (10.3%). The mean total polyphenols contents in the control and Cd treated variants were 6170 and 8340, 3070 and 3590, 3310 and 3650 mg/kg d.m. in leaf blades, shoots and roots, respectively. Incomparably higher increase was found in the Cd content in treated plants of barley. Cd mean contents in the control plants were 2.29, 0.32 and 0.22 mg/kg d.m., while in the treated plants 240, 16.3 and 5.78 mg/kg d.m. in roots, shoots and leaf blades, respectively. A relative increase of

Table 9. Scheffe's test applied on the experimental variants and parts of plant (total polyphenols content)

Variant	Control	Cd (1.10^{-6} mol/l)					
Control		0.0000					
Cd (1.10^{-6} mol/l)	0.0000						
Part of plant	leaf blades	shoots	roots				
Leaf blades		0.0000	0.0000				
Shoots	0.0000		0.4968				
Roots	0.0000	0.4968					
Variant × part of plant		control			Cd (1.10^{-6} mol/l)		
		leaf blades	shoots	roots	leaf blades	shoots	roots
Control	leaf blades		0.0000	0.0000	0.0000	0.0000	0.0000
	shoots	0.0000		0.8763	0.0000	0.1310	0.0627
	roots	0.0000	0.8763		0.0000	0.7626	0.5809
Cd (1.10^{-6} mol/l)	leaf blades	0.0000	0.0000	0.0000		0.0000	0.0000
	shoots	0.0000	0.1310	0.7626	0.0000		0.9998
	roots	0.0000	0.0627	0.5809	0.0000	0.9998	

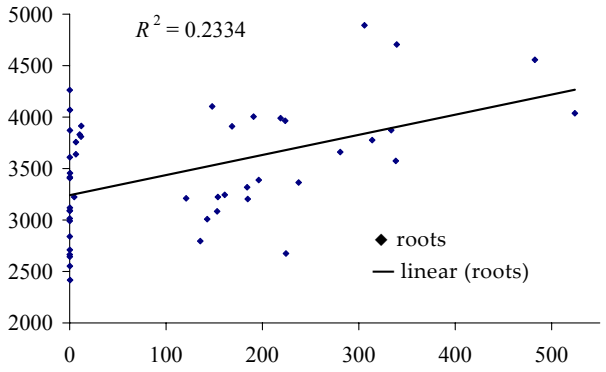

Figure 1. Relationship between total polyphenols and cadmium contents in roots of barley treated with Cd

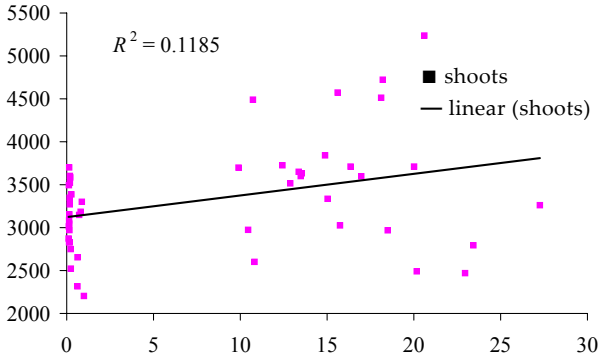

Figure 2. Relationship between total polyphenols and cadmium contents in shoots of barley treated with Cd

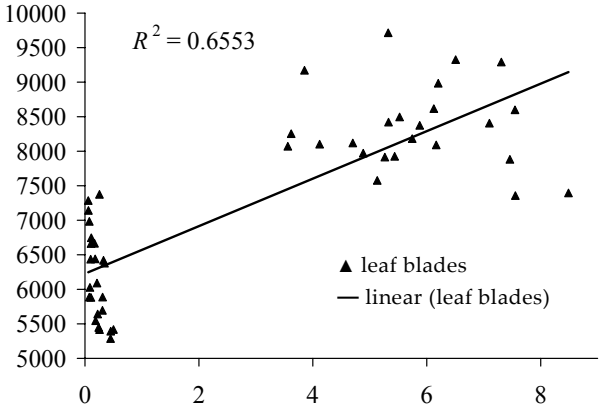

Figure 3. Relationship between total polyphenols and cadmium contents in leaf blades of barley treated with Cd

Cd content in the individual parts of the treated plants decreased in order roots (10 400%) > shoots (4990%) > leaf blades (2580%).

From the statistical evaluation (Tables 7–9) it can be concluded that the statistically significant differences in Cd and total polyphenols content were found in the both variants and that the treatment with Cd significantly affected the increase of the total polyphenols content. Statistically significant differences were found among the parts of plant and among variant × part of plant.

A relationship between the total polyphenol content and Cd content in all investigated barley organs was linear (Figures 1–3). The content of total polyphenols and Cd in barley roots, shoots, and leaf blades were reciprocal.

This investigation showed that Cd-stress (1.10^{-6} mol/l) caused an increase of both Cd and total polyphenols levels in barley plants and that the increase of the total polyphenols is a response to Cd-stress. This is the first report that is similar to other abiotic stress factors, UV and γ-irradiation, dryness, humidity or higher temperature (Lachman et al. 2001, Orsák et al. 2001, Hakala et al. 2002, Hideg et al. 2002). Also stress caused by Cd affects the level of polyphenol compounds as protective secondary metabolite compounds in plants. This is in accordance with the results obtained by Wu et al. (2003) that Cd-stress induced a concentration and gene type dependent oxidative stress response and changes in barley leaf blades characterised by the alteration pattern of antioxidative enzymes superoxide dismutase (SOD) and peroxidase (POD). The increase of total polyphenols content as a response to Cd-stress is similar, as Baisakhi et al. (2003) determined in the tolerant clone of *Chloris barbata* Sw. in protein-thiol level. As Chai et al. (2003) found in the French bean (*Phaseolus vulgaris*), the expression of stress-related genes as PvSR2 can enhance the Cd tolerance, esp. at higher external Cd concentrations. Evidence that phenolics play a key role in hardening responses to abiotic stressors support also results of Metwally et al. (2003), who found that salicylic acid alleviated Cd toxicity in barley plants and increased the level of tolerance toward high Cd concentrations. This supports the conclusion that salicylic acid is affecting mechanisms of Cd detoxification.

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ABSTRAKT

Vliv kadmia na obsah polyfenolů v mladých rostlinkách ječmene (*Hordeum vulgare* L.)

V modelových pokusech byl sledován vliv abiotického stresu způsobeného kadmiem na obsah celkových polyfenolů v kořenech, nadzemních částech a čepelích listů rostlin ječmene starých 28 dnů. Obsah celkových polyfenolů byl stanoven spektrofotometricky s fenolovým Folin-Ciocalteuovým činidlem, obsah kadmia atomovou absorpční spektrometrií (AAS). Přídavek kadmia (1.10^{-6} mol/l) do živného média způsobil nárůst celkových polyfenolů ve všech částech rostliny. Nejvyšší nárůst byl nalezen v čepelích listů (35,2 %, 8340 mg/kg sušiny), menší nárůst byl zaznamenán v nadzemní části (16,7 %, 3590 mg/kg sušiny) a nejmenší v kořenech (10,3 %, 3650 mg/kg sušiny). Největší nárůst obsahu kadmia v rostlinách pěstovaných na kultivačním médiu s přídavkem kadmia byl zjištěn v kořenech (10 400 %, 240 mg/kg sušiny), menší nárůst byl nalezen v nadzemní části (4990 %, 16,3 mg/kg sušiny) a nejnižší v čepelích listů (2580 %, 5,78 mg/kg sušiny). Ze statistického vyhodnocení analýzou rozptylu vícenásobného třídění vyplývá, že stres způsobený kadmiem měl za následek statisticky významný nárůst obsahu celkových polyfenolů i kadmia ve všech analyzovaných částech rostlin. Byly zjištěny statisticky významné rozdíly v obsahu celkových polyfenolů a kadmia v kořenech, nadzemních částech a čepelích listů ječmene.

Klíčová slova: ječmen; kořeny; nadzemní části; čepele listů; polyfenoly; vliv Cd-stresu

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