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Activation of phenylpropanoid pathway in legume plants exposed to heavy metals. Part II. Profiling of isoflavonoids and their glycoconjugates induced in roots of lupine (*Lupinus luteus*) seedlings treated with cadmium and lead

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We examined changes in profiles of isoflavonoids in roots of lupine (*Lupinus luteus* L. cv. Juno) seedlings in response to treatment with two heavy metals: cadmium (at 10 mg/l) and lead (at 150 mg/l). Overall, 21 flavonoid conjugates were identified in root extracts, some of them with up to six positional isomers. The total amount of all isoflavonoids increased by about 15% in cadmium-treated plants and by 46% in lead-treated ones. Heavy metals markedly increased the content of two compounds: 2′-hydroxygenistein glucoside and 2′-hydroxygenistein 7-*O*-glucoside malonylated. Possible functions of the identified isoflavonoids in yellow lupine exposed to heavy metal stress are discussed.

Keywords: cadmium, isoflavonoid, lead, lupine, phenylpropanoid pathway

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INTRODUCTION

Heavy metals, such as cadmium and lead, are environmental pollutants, that affect various physiological processes in plants. The plant response to different abiotic and biotic stress factors is frequently correlated with an enhancement of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) activity, a key enzyme of the phenylpropanoid pathway. Several products of this pathway such as coumarins, lignin, flavones, isoflavones, flavonols and anthocyanins are involved in plant defense against various environmental factors (Dixon & Paiva, 1995; Dixon *et al.,* 2002; Winkel-Shirley, 2002; Jung *et al*., 2003; Treutter, 2005; Taiz & Zeiger, 2006). Heavy metals activate the phenylpropanoid pathway and increase lignin synthesis in many plant species (Diaz *et al.,* 2001; Jouili & Ferjani, 2003; Lin *et al.,* 2005; Yang *et al*., 2007; Kovacik & Klejdus, 2008). However, not all plants respond to heavy metals in the same manner. Recently we have found that while the treatment of soybean (*Glycine max*) seedlings with cadmium (Cd^{2+}) or lead (Pb^{2+}) trigged an increase of *PAL* mRNA level, PAL activity and lignin content, the same treatment of yellow lupine (*Lupinus luteus)* seedlings stimulated of PAL activity but decreased the lignin content (Pawlak-Sprada *et al*., 2011). This suggest that the activation of phenylpropanoid pathway by Cd^{2+} or Pb^{2+} in lupine roots enhanced the synthesis of secondary metabolites other than lignin.

The aim of this study was to determine whether Cd^{2+} and Pb^{2+} affect the profile of flavonoids in the root of yellow lupine seedlings. The flavonoid compounds were analyzed by liquid chromatography-mass spectrometry (LC/ESI/MS/MS) (Bednarek *et al.,* 2003; Kachlicki *et al*., 2005; 2008; Muth *et al.,* 2008) that allowed differentiation and quantification of flavonoids (flavones and isoflavones) and their glycosides.

MATERIALS AND METHODS

Plant material. Seeds of yellow lupine (*Lupinus luteus* L. cv. Juno) were surface sterilized with 75% ethanol for 5 min, followed by 1% sodium hypochloride for 10 min, washed in water and germinated for 48 h on water-moistened filter paper in Petri dishes. The seedlings were then transferred to dishes containing 5 ml of either distilled water (control), aqueous solution of $CdCl_2$ containing 10 mg/l of Cd^{2+} (which corresponds to 89 μM $CdCl_2$) or aqueous solution of $Pb(NO_3)_2$ containing 150 mg/l of Pb^{2+} (which corresponds to 724 μM $Pb(NO_3)_2$). The seedlings were incubated in the dark at 22°C for 48 h.

Analysis of isoflavonoids. Isolation of phenolic compounds from plant tissue. Prior to LC profiling of isoflavone glucosides, samples of frozen plant material (0.1 g fresh weight of roots) were homogenized in 80% methanol (10 ml/g fresh weight of tissue) in a mortar with pestle and then sonicated for 30 min. The suspension was filtered through a Buchner funnel and concentrated under vacuum at 40°C. The samples were purified and concentrated by solid phase extraction (SPE) on cartridges containing a cation exchanger and RP C-18 silica gel (Alltech, Carnforth, England) used in tandem, according to the method of Stobiecki *et al.* (1997).

Identification and quantification of isoflavonoids and their glycoconjugates. Identification was done on the basis of high resolution collision induced dissociation (CID) MS/MS spectra (*m/z* values registered with accuracy better than 5 ppm) and their retention times registered for consecutive compounds analyzed with a liquid chromatography tandem mass

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Abbreviations: CID, collision induced dissociation; LC/ESI/MS/MS, liquid chromatography electrospray ionisation tandem mass spectrometry; LC, liquid chromatography; *m/z*, mass-to-charge ratio; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PAL, phenylalanine ammonia-lyase

spectrometry (LC/ESI/MS/MS), consisted of LC system model 1200 SL containing binary pump SL, diode array detector G1315C Starlight and automatic injector G1367C SL WP (Agilent Technologies, Waldbronn, Germany) connected to a micrOTOF-Q spectrometer (Bruker, Bremen, Germany). The analyses were carried out using a Zorbax Eclipse XDB-C18 column (2.1mm × 100 mm, grain diameter 1.8 μm) from Agilent. Chromatographic runs were performed at a 0.5 ml/min flow rate using mixtures of two solvents: A (99.5% H_2O, 0.5% formic acid, v/v) and B (99.5% acetonitrile, 0.5% formic acid, v/v) with a split of the column effluent 3:2 so 0.2 ml/min was delivered to the ESI ion source. The elution steps were as follows: 0–8 min linear gradient from 5 to 30% of B, 8–10 min linear gradient to 95% B, 10–12 min isocratic at 95% B. After return to the initial conditions column equilibration was achieved after 3 min.

The micrOTOF-Q mass spectrometer was run with ESI at ±4.5 kV, nebulisation with nitrogen at 1.6 bar and dry gas flow of 8.0 L/min at 220 °C. The system was calibrated externally using a calibration mixture containing sodium formate clusters. Additional internal calibration was performed for every run by injection of the calibration mixture using the divert valve during the LC separation. All calculations were done with the HPC quadratic algorithm. Such a calibration gave at least 5 p.p.m. accuracy of m/z value annotation. MS/MS spectra were acquired with the frequency of one scan per second for ions chosen on the basis of preliminary experiments. The collision energy depended on the molecular masses of the compounds and was set between 10 and 25 eV. The instrument operated at a resolution higher than 10 000 (FWHM, full width at half maximum) under the program micrOTOF Control version 2.3 and data was analysed using the DataAnalysis version 4 package delivered by Bruker.

Identification of 21 isoflavone glycoconjugates was done on the basis of high resolution MS and CID MS/MS spectra.

Quantification of identified compounds was performed on the basis of peak areas registered in single ion chromatograms of protonated molecules $[M+H]^+$ of consecutive compounds eluted from the LC column. Peak areas were compared with a calibration curve prepared with genistein 7-*O*-β-D-glucoside at concentrations between 50 ng/ml and 2 μg/ml. The concentrations of all recognized isoflavonoid glycoconjugates were expressed as equivalents of genistein 7-*O*-β-D-glucoside.

Statistics. All results are presented as means ±S.D. (standard deviation) obtained from six independent replicates derived from tree independent biological experiments.

RESULTS AND DISCUSSION

Methods based on liquid chromatography mass spectrometry that allow the identification and quantification

Table 1. Compounds identified in *Lupinus luteus* roots treated with heavy metals

Peak #	Rt (min)	Compound identified	$[M+H]^+$ *m/z* (calculated)[b]	$[M+H]^+$ *m/z* (registered)[b]
1	1.4	Genistein 8-*C*-7-*O*-diglucoside	595.1663	595.1667
2	1.9	Genistein 4′,7-*O*-diglucoside	595.1663	595.1671
3	3.2	Genistein 4′,7-*O*-diglucoside malonylated I[a]	681.1667	681.1693
4	3.4	2′-Hydroxygenistein glucoside	449.1084	449.1089
5	3.5	Genistein 4′,7-*O*-diglucoside malonylated II[a]	681.1667	681.1672
6	3.8	Genistein 8-*C*-glucoside	433.1135	433.1135
7	3.8	Genistein 4′,7-*O*-diglucoside malonylated III[a]	681.1667	681.1669
8	4.0	Genistein 4′,7-*O*-diglucoside malonylated IV[a]	681.1667	681.1682
9	4.0	Genistein 8-*C*-glucoside malonylated I[a]	519.1139	519.1147
10	4.3	2′-Hydroxygenistein 7-*O*-glucoside malonylated I[a]	535.1088	535.1085
11	4.3	Genistein 4′,7-*O*-diglucoside dimalonylated I[a]	767.1671	767.1683
12	4.5	Genistein 8-*C*-glucoside malonylated II[a]	519.1139	519.1151
13	4.5	Genistein 7-*O*-glucoside	433.1135	433.1131
14	4.6	2′-Hydroxygenistein 7-*O*-glucoside malonylated II[a]	535.1088	535.1078
15	4.7	Genistein 4′,7-*O*-diglucoside dimalonylated II[a]	767.1671	767.1665
16	4.8	2′-Hydroxygenistein 7-*O*-glucoside malonylated III[a]	535.1088	535.1079
17	5.0	Genistein 4′ or 7-*O*-glucoside malonylated I[a]	519.1139	519.1132
18	5.2	Genistein 4′-*O*-glucoside	433.1135	433.1130
19	5.5	Genistein 4′ or 7-*O*-glucoside malonylated II[a]	519.1139	519.1138
20	5.7	Genistein 4′ or 7-*O*-glucoside malonylated III[a]	519.1139	519.1137
21	6.2	Genistein 4′ or 7-*O*-glucoside malonylated IV[a]	519.1139	519.1134

$[M+H]^+$, protonated molecule. [a]Substitution of glucose moiety on an aglycone and position of sugar malonylation position is not defined on the basis of registered mass spectra. [b]Mass to charge ratio (*m/z*) is calculated to the fourth decimal point for defined elemental composition, these values correspond within 5 p.p.m. to exact mass of $[M+H]^+$ ions registered for respective compounds (**1–21**).

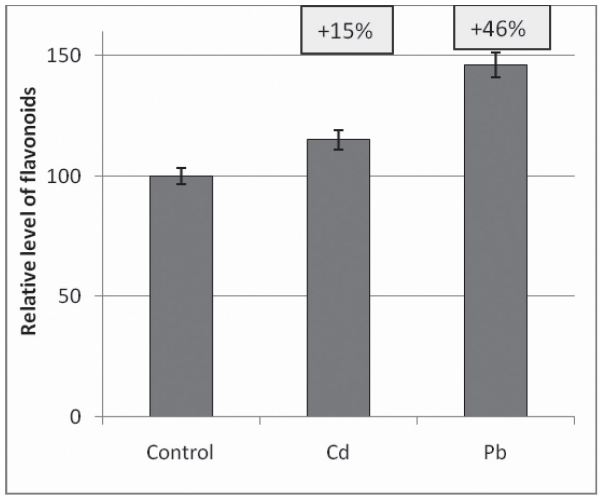

Figure 1. Total flavonoids in roots of *L. luteus* under heavy metal stress
Values are means ± S.D. of three independent experiments.

of various flavonoids derived from plant material have been described (Fossen & Andersen, 2006; Stobiecki & Kachlicki, 2006). In the present study 21 different compounds were identified in yellow lupine root extracts (Table 1). In the case of genistein and 2'-hydroxygenistein glycoconjugates, especially malonylated glycosides, various positional isomers were found. Ultrahigh pressure liquid chromatography has earlier been shown to resolve isomers of malonylated isoflavonoid glycosides from *Lupinus angustifolius* plants (Muth *et al*., 2008). However, in the present study, application of ultra performance liquid chromatography (UPLC) permitted to separate the isomers, but it was not possible to define the position of malonylation on the basis of registered mass spectra. All identified compounds are isoflavone mono- or diglucosides and 15 of them are isomers of the isoflavone glycoconjugates malonylated at different positions of the sugar moiety. A similar composition of isoflavonoids has been detected in roots of two other lupine species: *L. albus* and *L. angustifolius* (Bednarek *et al.,* 2003; Kachlicki *et al.,* 2005) and most of them were also identified in leaves of *L. angustifolius* (Bednarek *et al.,* 2003; Muth *et al.,* 2009). The most abundant compounds in yellow lupine roots of control plants are: genistein 4'- or 7-*O*-glucoside and 8-*C*-glucoside (**6, 13, 18**) (Fig. 3a–c) and some of their malonylated derivatives (**17, 19, 20, 21**) (Fig. 3d). The qualitative composition of isoflavone glycoconjugates derived from control, metal-untreated roots, was the same as that of isoflavonoids isolated from lupine roots treated with cadmium (Cd^{2+}) or lead (Pb^{2+}). The total amount of the isoflavonoids increased by about 15% in Cd^{2+}-treated plants and by about 46% in Pb^{2+}-treated plants (Fig. 1). Most of the identified isoflavone glycoconjugates showed some increase upon the treatment (Figs. 2–4). The largest increase was observed for two compounds: 2'-hydroxygenistein glucoside (**4**) and its malonylated isomers (**10**, **14**, **16**) (Fig. 2a and 2b). The amount of 2'-hydroxygenistein glucoside (**4**) in roots of Cd^{2+}-treated seedlings was 36 times higher and in Pb^{2+}-treated seedlings 219 times higher than in the control plants (Fig. 2a). Also the level of malonylated derivatives of 2'-hydroxygenistein 7-*O*-glucoside increased substantially upon treatment with Cd^{2+} or Pb^{2+}: 21-fold and 85-fold, respectively (Fig. 2b). Smaller increases were found for genistein diglucosides (Fig. 4a) and their malonylated (Fig. 4b) and dimalonylated forms (Fig. 4c) and smaller still for genistein monoglucosides (Fig. 3a–c) and theirs malonylated forms (Fig. 3d). The only compound whose level was decreased in the presence of either metal was genistein 4'-*O*-glucoside (Fig. 3c). These data suggest that two types of genistein modifications are im-

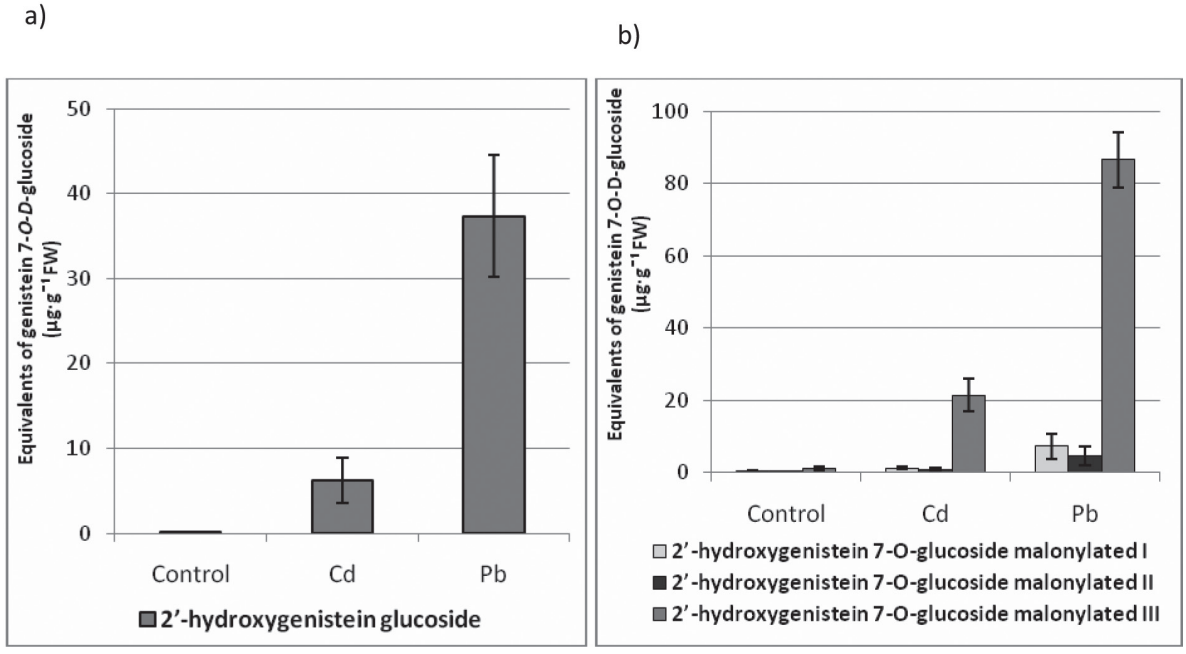

Figure 2. Concentrations of 2′-hydroxygenistein glucoside (a) and 2′-hydroxygenistein 7-*O*-glucoside malonylated (b) in *L. luteus* roots treated with Cd^{2+} or Pb^{2+}
Concentrations of metabolites were calculated as equivalents of genistein 7-*O*-D-glucoside used for preparation of calibration curve. Values are means ± S.D. of three independent experiments. FW, fresh weight.

portant in the plant response to heavy metals: hydroxylation and the level of glycosylation. Genistein diglucosides were more strongly induced by heavy metals than the respective monoglucosides. The position of glucose moiety attachment to genistein seems to play a role as well. A stimulatory effect of heavy metals was observed for of 7-*O* and 8-*C* glucosides of genistein (Fig. 3a and 3b), whereas the amount of 4'-*O* glucoside of genistein was actually decreased in the heavy metals-treated plants (Fig. 3c).

Ample data indicate changes of flavonoid composition in plants exposed to various biotic interactions, both pathogenic and symbiotic (Kosslak *et al.,* 1987; Grandmaison & Ibrahim, 1995; Morkunas *et al.,* 2005; Muth *et al*., 2009). Stimulation of genistein and its derivatives was mostly observed in lupine roots colonized by symbiotic bacteria, whereas prenylated genistein and 2'-hydroxygenistein derivatives (wighteone, luteone) were synthesized during a defense reaction (Bednarek *et al*., 2003; Łuczkiewicz, 2008; Muth *et al.,* 2009). An increase of various flavonoids and isoflavononids has also been documented in different legume plant species in response to infection or elicitation (Treutter, 2005; Naoumkina *et al.,* 2007; Subramanian *et al*., 2007; Wasson *et al*., 2009). Increased synthesis of isoflavones was also observed in legumes due to interactions with various abiotic factors, such as light conditions (Bednarek *et al*., 2003), toxic compounds (potassium cyanide and sodium chloride) (Kneer *et al.,* 1999) as well as heavy metals (Jung *et al.,* 2003; Mithöfer *et al*., 2004; Michalak, 2006). Treatment of soybean with Hg^{2+} increased the level of glyceollins

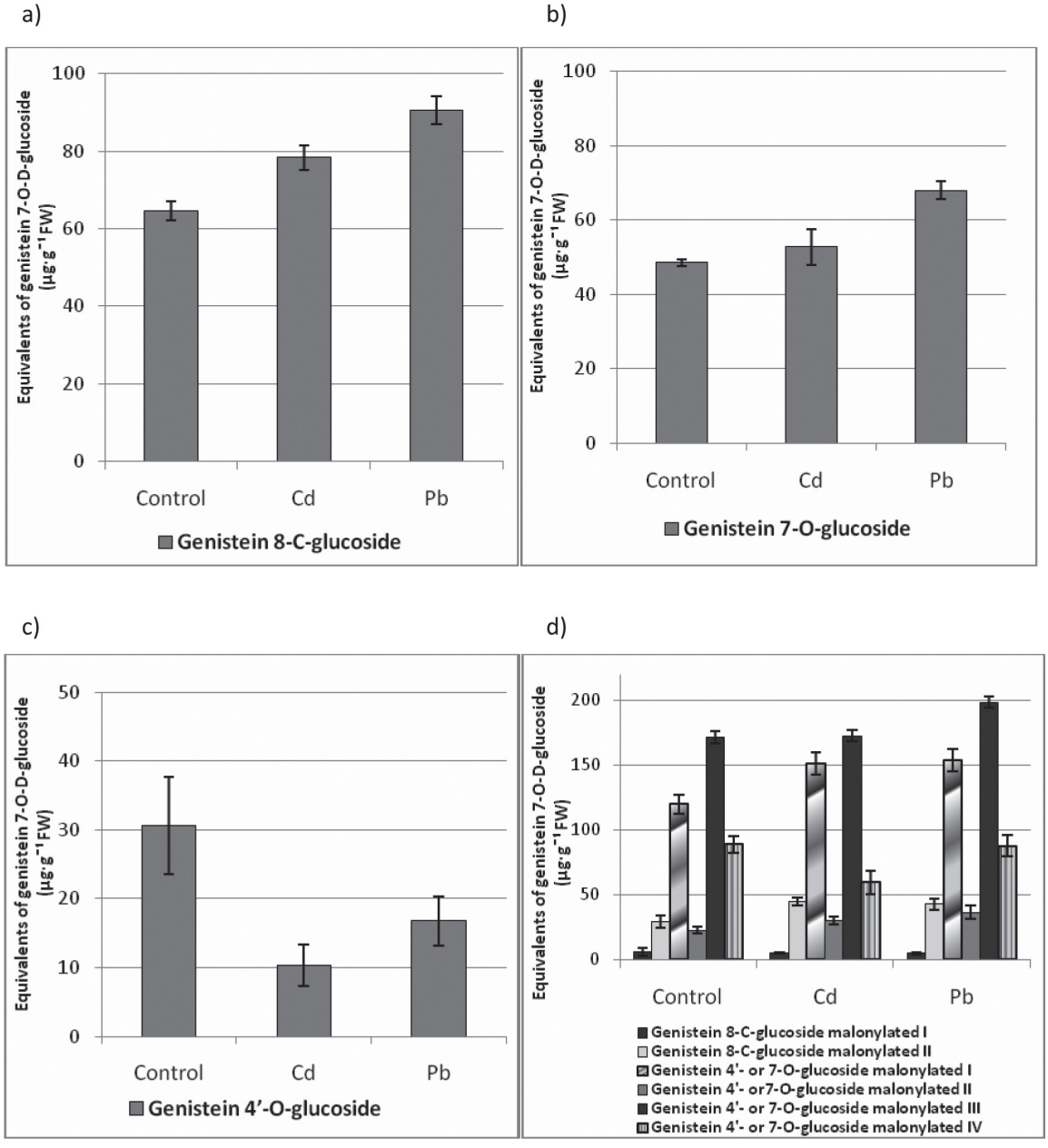

Figure 3. Concentrations of genistein 8-*C*-glucoside (a), genistein 7-*O*-glucoside (b), genistein 4'-*O*-glucoside (c) and genistein 8-*C*- and 4'- or 7-*O*-glucoside malonylated (d) in *L. luteus* roots treated with Cd^{2+} or Pb^{2+}
Concentrations of metabolites were calculated as equivalents of genistein 7-*O*-D-glucoside used for preparation of calibration curve. Values are means ± S.D. of three independent experiments. FW, fresh weight.

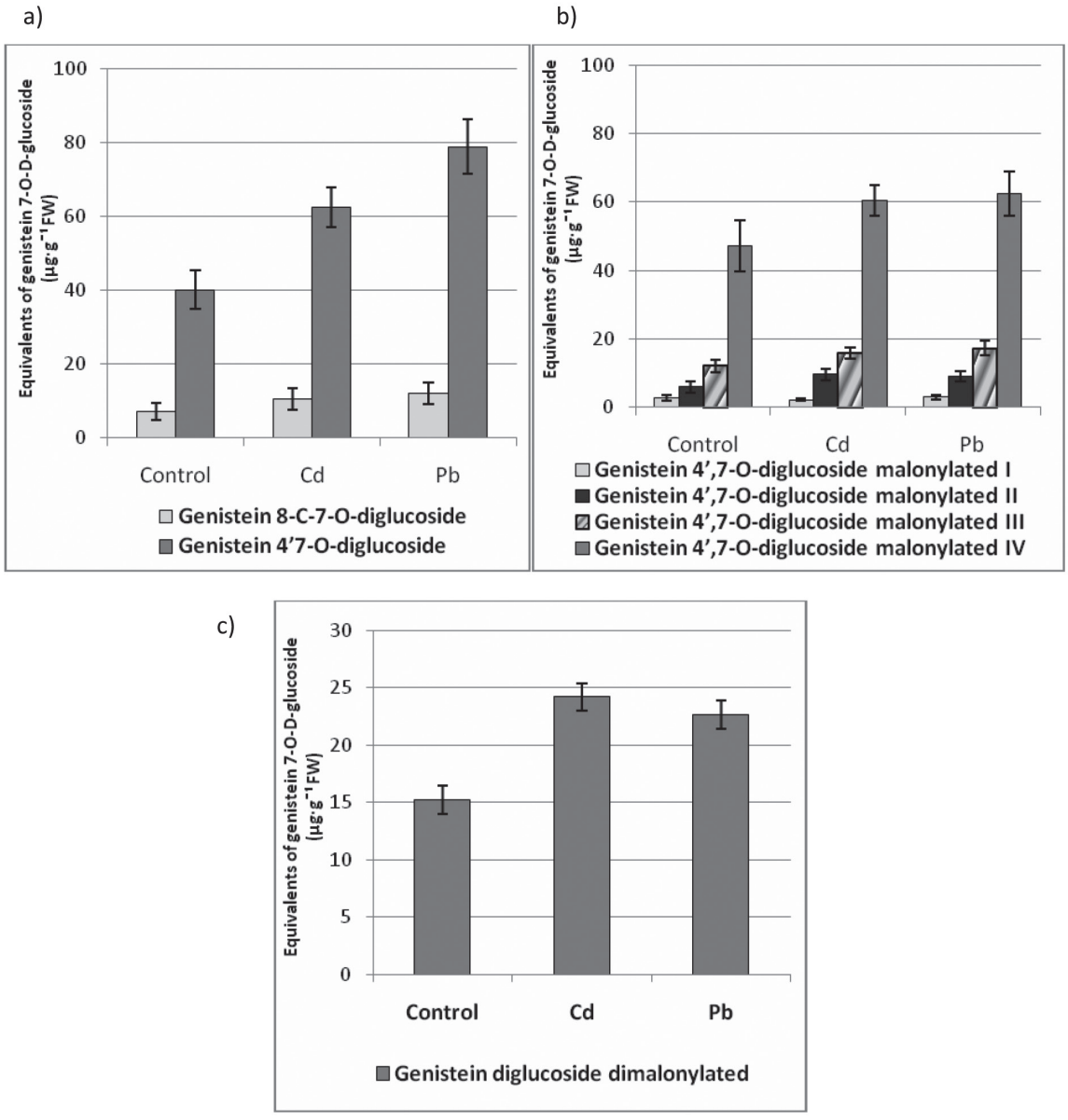

Figure 4. Concentrations of genistein 8-*C*-7-*O*- and 4',7-*O*-diglucoside (a), genistein 4',7-*O*-diglucoside malonylated (b), genistein diglucoside dimalonylated (c) in *L. luteus* roots treated with Cd^{2+} or Pb^{2+}
Concentrations of metabolites were calculated as equivalents of genistein 7-*O*-D-glucoside used for preparation of calibration curve. Values are means ± S.D. of three independent experiments. FW, fresh weight.

(Mithöfer *et al.,* 2004), while in Cu^{2+}-treated white lupine (*L. albus*) cotyledons elevated levels of genistein and 2'-hydroxygenistein were observed (Gagnon & Ibrahim, 1997; Wojtaszek & Stobiecki, 1997). Stimulation of genistein synthesis and secretion was also observed in yellow lupine (*L. luteus)* roots treated with various elicitors, such as soluble chitosan, salicylic acid and potassium cyanide (Kneer *et al.,* 1999). However, the effects of toxic heavy metals, such as cadmium or lead, on isoflavonoid composition in legume plants have not been analyzed so far. Our data indicate that the main compounds which are induced in the root of yellow lupine treated with Cd^{2+} or Pb^{2+} are 2'-hydroxygenistein glucoside and 2'-hydroxygenistein 7-*O*-glucoside malonylated (Fig. 2a, 2b). We did not observe stimulation of free aglycones of genistein and 2'-hydroxygenistein or their prenylated derivates (wighteone, luteone). It is generally accepted that glycosylation increases the solubility and stability of flavonoids and isoflavonoids (Modolo *et al.,* 2007). According to Zhao and Dixon (2009) glycosylation is essential for vacuolar uptake whereas malonylation of the sugar group may facilitate transport of the glycoconjugates through membranes. Flavonoids have been suggested to act as antioxidants, protecting cells from oxidative stress (Rice-Evans *et al.*, 1996; Winkel-Shirley, 2002; Skórzyńska-Polit *et al.*, 2004; Michalak, 2006; Keiling & Ludwig-Müller, 2009). Heavy metals increase the level of reactive oxygen species (ROS) which can inflict a serious damage on plant cells (Rucińska *et al.,* 1999; Moller *et al.*, 2007; Sharma & Dietz, 2009). The ability of flavonoids to chelate metal ions appears to contribute to their antioxidant activity *in vitro* (Cheng & Breen, 2000; Mira *et al.*, 2002). It has been documented that a flavonoid complex with a transition metal (Fe^{3+}, Fe^{2+}, Cu^{2+}) exhibits a superoxide dismutase activity (Kostyuk *et al.,* 2004; 2007). However, the antioxidant function claimed for flavonoids in plants

is still a matter of debate (Hernandez *et al.,* 2009). The data presented here may suggest additional functions played by isoflavonoid glycoconjugates in plants exposed to heavy metals. The reaction of yellow lupine root cells to Cd^{2+} and Pb^{2+} involves an increase of glycosylated and malonyladed forms of the isoflavonoid 2'-hydroxygenistein (Fig. 2a and 2b). Such forms of isoflavonoids may be responsible not only for binding of metal ions, but also for deposition and maintaining of isoflavonoid-metal complexes within the vacuole. Glycosylation and malonylation of flavonoids has recently been attributed to their targeting to the vacuoles (Zhao & Dixon, 2009).

Based on the obtained data we can also explain the decrease in lignin content in heavy metal-treated roots of yellow lupine (Pawlak-Sprada *et al.*, 2011). It was shown that genistein from *L. albus* is a potent inhibitor of the peroxidase-catalyzed oxidation of coniferyl alcohol, the first step in lignin biosynthesis (Ferrer & Barcelo, 1994). A similar inhibitory effect on lignin synthesis can be assumed for by 2'-hydroxygenistein glucoside and 2'-hydroxygenistein 7-*O*-glucoside malonylated, which are strongly stimulated in *Lupinus luteus* roots treated with Cd^{2+}or Pb^{2+}.

Although many facts concerning the participation of flavonoids in plant stress response are known the exact function of these metabolites in the plant reaction to toxic heavy metals is still a matter of debate.

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