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Release of phenols from *Lupinus albus* L. roots exposed to Cu and their possible role in Cu detoxification

Christoph Jung[1,4], Valérie Maeder[1], Felix Funk[1], Beat Frey[2], Hans Sticher[1] & Emmanuel Frossard[3]

[1]*Institute of Terrestrial Ecology, Swiss Federal Institute of Technology, Grabenstrasse 3, CH-8952 Schlieren, Switzerland.* [2]*Institute of Plant Sciences, Swiss Federal Institute of Technology, Eschikon 33, CH-8315 Lindau, Switzerland.* [3]*Swiss Federal Institute for Forest, Snow and Landscape Research, CH-8903 Birmensdorf, Switzerland.* [4]*Corresponding author**

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Abstract

The mechanisms enabling plants to tolerate high concentrations of available Cu in their rhizosphere are still poorly understood. To better understand the mechanisms involved, *Lupinus albus* L. (white lupin) was grown over 40 days in a hydroponic system compelling roots to develop under sterile conditions in the presence of a nutrient solution containing 0.5, 20 or 62 μM Cu. The following parameters were investigated in detail: low molecular weight phenols in nutrient solution (colorimetric assay), high molecular weight phenols in roots and in solution (HPLC-MS, HPLC-UV), pH, redox potential in solution (electrochemistry) and Cu distribution in the plant (AAS) as well as in apical root sections (EDX microanalysis). Finally, *in vitro* adsorption studies using voltammetry were conducted to evaluate the Cu adsorption behaviour of different phenolic compounds. When exposed to 62 μM Cu, biomass production of white lupin was strongly reduced. Plants grown in the presence of 20 μM Cu had a similar dry matter production compared to the control plants grown in a 0.5 μM Cu solution. However, an increased release of soluble and high molecular weight phenols into the solution was observed. The concentration of polyphenolic compounds in the roots (particularly isoflavonoids like genistein and genistein-(malonyl)-glucoside) was significantly higher for lupins grown in a 20 μM Cu solution compared to the control plants. As shown by an *in vitro* adsorption study, these phenolic compounds can bind Cu ions. In addition, plants exposed to 20 and 62 μM Cu cumulated high Cu amounts in root cell walls whereas only low amounts reached the symplasm. Therefore, it is proposed that the complexation of Cu^{2+} ions in the rhizosphere and in the roots apoplasm by phenolic compounds could alleviate Cu-mediated toxicity.

Abbreviations: Control – plants grown in nutrient solution containing 0.5 μM Cu^{2+}; 20Cu – plants grown in nutrient solution containing 20 μM Cu^{2+}; 62Cu – plants grown in nutrient solution containing 62 μM Cu^{2+}; HMWP – high molecular weight phenols; AAS – atomic absorption spectrometry; EDX – energy dispersive X-ray diffraction; DM – dry matter; ∅ – diameter; MS – mass spectrometry

Introduction

Plants can tolerate the presence of high concentration of toxic metals in soil, either through external mechanisms that limit the uptake of metals or through internal mechanisms which detoxify metals within the symplasm (Ernst et al., 1992; Marschner, 1995). The formation of non-toxic metal-ligands chelates in the rhizosphere involving, e.g., organic acids, phosphate or high molecular weight polysaccharides exuded from the root allowing plants to restrict metal uptake is a well documented mechanism (Horst, 1982;

* FAX No: +41-19-228-093. E-mail: christoph.jung@tecan.com

Ma et al., 2001; Ryan et al., 2001; Wenzl et al., 2001). The reaction of toxic metal species with ligands located on cell wall surfaces can also hinder the uptake of toxic metals. Ligands that have been described previously are, e.g., phosphate groups or phenols (Gaume et al., 2001; Heim et al., 1999; Horst, 1995). Finally, low molecular weight compounds such as organic acids, phytochelatins or metallothioneins contribute to detoxify metals within the plant (Hall, 2002; Ma et al., 2001; Marschner, 1995).

Little information is available on the mechanisms that allow plants to tolerate high concentrations of available Cu in their rhizosphere. Marked differences have been observed between plants in Cu tolerance: Minuartia hirsuta exhibited a root growth decrease of about 52% at 20 μM Cu in nutrient solution (Ouzounidou et al., 1994), whereas root growth decreased by 50% when tomato was exposed to only 4 μM Cu (Rahimi and Bussler, 1974). Differences in Cu tolerance might be based on ecological and physiological differentiation between plants from metal-enriched and non-contaminated habitats (Ernst et al., 1992). In tolerant populations of *Silene cucubalus*, the immobilization of Cu by ligands located in cell plasma membrane has been pointed out as a possible mechanism limiting Cu uptake by plants (De Vos et al., 1991). Further results of this study suggested that Cu tolerance was linkedto the ability of the plants to prevent membrane damage of root cells. Using EDX and EELS (electron energy loss spectroscopy) Neumann et al. (1995) detected Cu in the root vacuoles of idioblasts ('tannin cells') of heavy metal tolerant *Armeria maritime* and found Cu to be chelated by polyphenolic precipitates. Furthermore, a large part of the Cu in roots was localized in cell walls, in the cytoplasm, in the stroma of the plastids and in the nuclei where it was preferentially bound to proteins. Leopold et al. (1999) and De Vos et al. (1992) proposed that phytochelatins (thiol-rich peptides) play only a minor role in copper tolerance of *Silene vulgaris* and *Silene cucubalus*,respectively. In a recent study comparing tolerant and non-tolerant *Holcus lanatus* L., phytochelatin production has been found to correlate with increasing Cu and arsenate exposure (Hartley-Whitaker et al., 2001). However, based on the observed similar phytochelatin induction in tolerant and non-tolerant clones at Cu EC_{50} concentrations compared to the significantly different phytochelatin production at arsenate EC_{50} values the authors suggested that phytochelatins are involved in arsenic tolerance but not in copper tolerance.

Polyphenols, which provide by definition more than one phenolic hydroxyl groups, represent one of the most ubiquitous and numerous groups of plant metabolites. Due to their reducing properties as electron-donating agents, polyphenols can act as antioxidants by means of free-radical scavengers (Rice-Evans et al., 1997). Moreover, they have a high tendency to chelate heavy metals. In particular, the hydroxyl and carboxyl groups of polyphenolic compounds can strongly bind Cu and Fe (Martell and Smith, 1989). This chelating behaviour renders phenolic compounds strong candidate in preventing metal-catalysed free radical formation (Lopes et al., 1999). Herein polyphenols exert an additional antioxidant activity. A lot of phenolic compounds found in plant tissues have a higher tendency to form stable complexes with the most widespread toxic metals (Ni, Cu, Co, Mn, Al) than many organic acids (except citric acid; Martell and Smith, 1989). Therefore, it is reasonable to hypothesise that the formation of stable complexes between metals and phenols especially polyphenols can restrict metal uptake in plants and alleviate metal toxicity. Suresh and Subramanyam (1998) studied the role of polyphenolic compounds involved in Cu binding onto the cell walls of *Neurospora crassa*. Their ESR (electron spin resonance) and FTIR (Fourier transformation infrared) studies of the Cu–polyphenol complexes indicated Cu to be bound as Cu(I) in a octahedral geometry. Beside hydroxyl groups, nitrite groups were also identified. The authors proposed that both groups might participate in a binding mechanism and supposed that (poly)nitrophenols are the responsible ligands located in the cell wall. However, the possible release of phenolic compounds and the extracellular effect on Cu complexation had not been investigated in their study.

Flavonoids, a large group of low molecular weight polyphenolic substances, do also undergo chelate formation with Fe and Cu ions (Ramanathan et al., 1993; Wu et al., 1995). The generic chemical structure of isoflavonoids, a subgroup of flavonoids, is based on two aromatic and one pyrone ring. Due to various carbohydrate substitutions many isoflavonoids occur in plants as glucosides (Gagnon et al., 1992b; Shibuya et al., 1991). Both abiotic elicitors such as $CuCl_2$ and biotic elicitors such as yeast have been shown to induce isoflavonoid production in *Lupinus albus* L. (Bednarek et al., 2001; Gagnon and Ibrahim, 1997; Wojtaszek and Stobiecki, 1997). These observations suggest that isoflavones play a key role in plant defence against pathogens. The possible function of

isoflavones or other phenolic compounds in Cu detoxification of white lupin has not been investigated yet. This lack of information about a possible role of phenolic plant metabolites in metal detoxification prompted us to investigate the effect of high Cu availability on root exudation of phenols by white lupin in detail. White lupin has been considered as plant model since its polyphenolic compounds particularly isoflavonoids have been widely studied (Harborne, 1993; Harborne and Baxter, 1999; Tahara et al., 1989). Further objectives of the study were to analyse phenolic compounds in root tissues and to examine the adsorption of Cu by phenols.

Materials and methods

Materials

Seeds of *Lupinus albus* L. (white lupin, cultivar 'Weissblühende Tellerlupine) were obtained from Ufa AG (Switzerland). For pre-cultivation, agar plates ('Standard-I-Nähragar' Merck, Switzerland) and 250-ml Erlenmeyer flasks (autoclavable polycarbonate, Roth, Switzerland) were used. The following equipment was chosen to subsequently grow the plant under sterile conditions: Pyrex tubes (l, 30 cm ∅, 5 cm) with a little outlet tube (0.4 cm) at the top of the tube closed with sterile cotton, a grid (PTFE, 4-mm mesh, Angst & Pfister, Switzerland) for seed fixation, a layer of paraffin (melting point 46–48 °C, Merck) mixed with Vaseline (Manopharm, Germany) which covered the solution surface and enclosed the stem, a septum (∅, 3 cm, Merck) to close the bottom of the tube and two silicon tubes (∅, 4 mm, Merck): one for refilling with nutrient solution and sampling and the other one for aeration with filtered air (sterile filter, 0.22 μM, Millipore, Switzerland) (Figure 1).

Plant growth

Seeds were selected in a weight range of 350–450 mg. They were surface sterilised by being rinsed first in ethanol (70%) for 2 min and in $Ca(OCl)_2$ (30% solution) for 30 min. All materials used for plant growth were autoclaved at 1.5 bar/120 °C during 20 min to prevent microbial contamination. Seeds were germinated on nutrient agar. Roots of 7-day-old seedlings were inserted through a PTFE grid and then were transferred to 250-mL Erlenmeyer flasks to enable growth under sterile conditions during the next

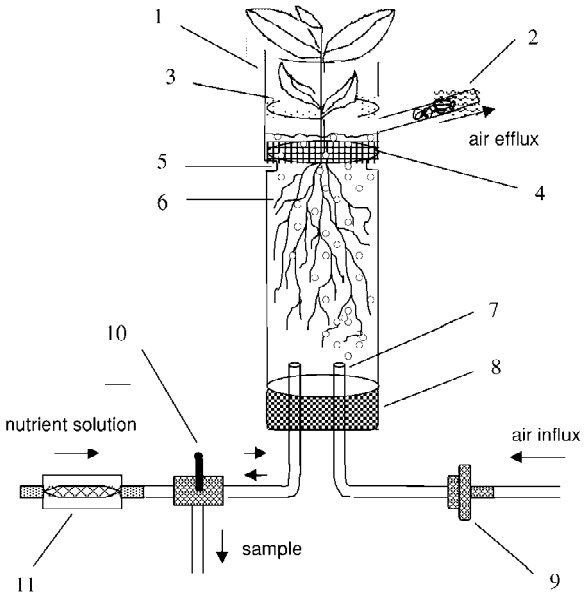

Figure 1. Glass tube (l=30 cm; ∅=5 cm) according to Tremblay-Boeuf et al. (1995); (2) air efflux tube (l=5 cm; ∅=0.4 cm) closed with sterile cotton wool and aluminum foil; (3) paraffin layer; (4) PTFE grid; (5) glass deformation for grid fixation; (6) Hoagland solution; (7) aeration; (8) septum (∅=3 cm); (9) air filter (0.22 μm); (10) PTFE tap; (11) sterile filter for solution (0.22 μm, 'Sterivex-GP', Millipore).

7 days (pre-cultivation period). Subsequently, cotyledons were cut off and each plant was fixed in an individual growth tube (according to Tremblay-Boeuf et al., 1995) where the roots were immersed in a permanently aerated Hoagland solution (Hoagland and Arnon, 1938) (Figure 1). The medium contained the following nutrient concentrations: KNO_3 (5 mM), $Ca(NO_3)_2 \cdot 4H_2O$ (5 mM), $MgSO_4 \cdot 7H_2O$ (2 mM), KH_2PO_4 (1 mM), FeNaEDTA (100 μM), KCl (50 μM), H_3BO_3 (24.3 μM), $MnSO_4 \cdot H_2O$ (2.3 μM), $ZnSO_4 \cdot 7H_2O$ (2 μM), $CuSO_4 \cdot 4H_2O$ (0.5 μM), $Na_2MoO_4 \cdot 2H_2O$ (0.5 μM). In order to keep the root system under sterile conditions it was separated from the shoot with the help of a paraffin/Vaseline layer covering the solution surface. Experiments were performed in a climate chamber using a photoperiod of 16 h, temperatures of 21/16 °C (day/night) and fluorescent lighting (Osram L36/77, L36/11-860, L36/31-830; 80 μmol m^{-2} s^{-1} PAR).

Cu ions were supplied as $CuSO_4 \cdot 4H_2O$ at concentrations of 0.5 (control), 20 and 62 μM.

All plants were grown for 40 days.

Cu content in the plants

After harvesting, leaves, stems and roots were dried at 40 °C and ground with an ultra-centrifugal mill (ZM-1, Retsch, Germany). One hundred mg of plant material were mixed with 5 ml HNO_3 (65%), 2 ml H_2O_2 (30%) and 2 ml H_2O and mineralised by microwave-digestion (EM-2, MLS GmbH, Switzerland). Cu was then measured using flame or furnace atomic absorption spectrometry (SpectrAA-400, Varian, Germany).

Metal determination in root cross-sections using X-ray microanalysis

Fresh root segments from the apical region of the main roots were taken from plants treated with 0.5 (control), 20 and 62 μM Cu. The samples were frozen at −196 °C in liquid nitrogen, fractured with a scalpel within a pre-chamber of a cryo-scanning electron microscope and immediately introduced in the microscope (Frey et al., 2000). The operation parameters used have been previously described (Heim et al., 1999). The SEM was equipped with a cryo unit and a Tracor Northern energy dispersive X-ray analysis system interfaced with a Voyager software package (Frey et al., 1996).

Isoflavones in the roots (HPLC-UV and -MS)

Phenols were extracted from 20 mg DM ground roots. Extraction and re-concentration of the extracts used for HPLC were performed as described by Münzenberger et al. (1995). Analytical HPLC was carried out with two LC systems. For quantitative analysis a two-pump system (LKB 2150, Pharmacia, Germany) with an UV/Vis detector (LKB 2151) using a Nucleosil 120-5 C18 column (Art. 720041, Macherey-Nagel, Switzerland) was applied. The data processing system comprised a Powerchrom interface and software (Hastings, UK). The elution system was a two-step linear gradient from solvent A (1.5% H_3PO_4 in H_2O) to 40% of solvent B (H_2O/CH_3OH/CH_3CN, 1:1:1, (v/v) in A in 60 min and then to solvent B in 40 min continuing for a further 30 min at solvent B. The flow rate was 1 ml min^{-1}, the injection volume 20 μL and the detection wavelength λ = 265 nm.

For structural analysis isoflavones were identified by mass spectrometry (MS) and UV absorption. Thereby, from all main peaks in the HPLC chromatogram MS and UV absorption spectra were obtained. The analytical set-up comprised a two-pump system (HP 1100 series), an ET 250/8/4 Nucleosil 7 C18 column (Art. 720018, Macherey-Nagel) with an UV/Vis detector (HP 1090 series DAD) and a quadrupole mass spectrometer (Finnigan TSQ-700, ionisation: APCI positive mode). The two-step linear gradient was the following: from 85% of solvent A (1.5% CF_3COOH in H_2O) and 15% of solvent B (H_2O/CH_3OH/CH_3CN, 1:1:1, (v/v) to 40% of solvent B in 30 min and then to solvent B (100%) in 30 min continuing for a further 20 min at solvent B. The flow rate was 1 ml min^{-1}, the injection volume 100 μL and the detection wavelength λ = 265 nm. The MS spectrum of genistein found in all root extracts could be verified with a control measurement of a purchasable genistein standard (Roth AG, Reinach, Switzerland). All other detected genistein derivates could not be purchased. Thus, root concentrations were only calculated for genistein and not for the genistein glucosides.

Solution sampling

Every 5 days a solution sample of 50 mL was taken from each growth tube and an aliquot of the medium was plated on nutrient agar to check sterility. After sampling, tubes were refilled with the corresponding nutrient solution to the initial solution level (350 mL). Due to the different nutrient and water consumption of the stressed and non-stressed plants, the volumes of fresh Hoagland solution, which had to be added to refill the tube, differed between 55 and 100 mL (V^{in}, see Equation (1)). After sampling, pH was measured immediately using a glass electrode (Art. 6.0204.100, Metrohm, Switzerland). Redox potential was determined with a Pt electrode (Metrohm, Art. 6.0431.100) and an Ag/AgCl reference electrode (Metrohm, Art. 6.0733.100).

Concentration of soluble phenols in the solution

The concentration of soluble phenols in the solution was measured with a colorimetrical assay (Swain and Hillis, 1959) using the Folins-Denis reagent (Fluka, Switzerland) using standards of phenol and caffeic acid for the calibration. The total amount of phenols released per unit DM root was then calculated for the entire period of plant growth using the Equation (1). This was necessary to take into account the variable water consumption observed in the different treatments and the variable amount of solution, which

was added to the tube after each sampling:

$$C[\text{Phenol}_{\text{cumulated}}] = C_0 + \sum_{k=1}^{n} C_k - C_{k-1} \left(\frac{V_T - V_{k-1}^{in}}{V_T} \right), \quad (1)$$

where $C[\text{Phenol}_{\text{cumulated}}]$ = cumulated phenol concentration (mmol/g DM); C_0 = phenol concentration of the first solution sample (mM); C_k = phenol concentration of sample k (mM); k = sample number (no unit); V_T = entire tube volume (350 ml); V^{in} = volume of solution being added after sampling to refill the growth tube (55–100 ml).

At the end the cumulated amount of phenols found in the solution was divided by the total dry matter of root to get cumulated phenol released per gram DM root.

High molecular weight phenols (HMWP) and qualitative phenol assays of methanolic HMWP extracts

At the end of the plant experiments the medium was centrifuged, the residue separated and freeze-dried. The substances (brown and yellowish coloured powders) were then suspended in 4 ml H_2O, acidified with 20 μL HNO_3 (5%), and purified twice by dialysis (MW1000) against water (nanopure). The remaining HMWP products weighed 2–14 mg (dry matter). Their total Cu content was measured and qualitative phenol assays of their methanol extracts were performed as follows. Between 2 and 6 mg of the dried HMWP products were dissolved in 5 ml methanol and placed in an ultra-sonic bath for 40 min. Afterwards, the solution was filtered (on a folded paper filter, Schleicher & Schuell, Switzerland) and the residue was rinsed twice with 1 ml methanol. The concentration of soluble phenol in the extracts was finally measured with the colorimetrical assay according to Swain and Hillis (see above).

Differential pulse cathodic sweep voltammetry

The influence of different organic compounds on the electrochemical behaviour of Cu in aqueous solution was investigated using differential pulse cathodic sweep voltammetry (DPV). The experimental set-up of DPV used in this study was described previously (Neubauer and Furrer, 1999). Caffeic acid (Fluka, Switzerland), genistein (Roth, Switzerland), HMWP (taken from the nutrient solution of a plant exposed to 20 μM Cu) and ethanol (p.a., Fluka, Switzerland) were used as ligands for Cu. The background electrolyte was $NaNO_3$ (0.1 M). The experiments with caffeic acid and genistein were carried out according to the following procedure: to 20 ml of a 100 μM $CuSO_4$solution the ligand was added sequentially in two steps to achieve ligand concentrations of 25 and 100 μM. After each step the pH was adjusted with NaOH and HNO_3 to values between 5.8 and 6.1. Subsequently three DPV measurements were done (for detailed measurement description please refer to Neubauer and Furrer, 1999). Three mg of the purified and freeze-dried HMWP were extracted with 10 ml ethanol. Two hundred, 400 and 800 μL of the filtered extract (0.45-μm filter) were added to 20 mL of a 50 μM $CuSO_4$ solution and the measurements were done as described above. In order to test the adsorption characteristics of the solvent, ethanol was measured likewise by adding 200, 400 and 800 μL of the pure solvent.

Statistical analysis

Comparisons of treatment effects on plant growth, Cu uptake and redox potential in solution, phenolic and genistein contents in roots between Cu treated plants (20 and 62 μM Cu, respectively) and control plants (0.5 μM Cu) were achieved with one-way analysis of variance (ANOVA). Comparisons of changes over time in pH values (Figure 5) and soluble phenol concentrations (Figure 6a) were carried out with repeated measures ANOVA. If the p value indicated significant differences between Cu treated plants and control plants ($p < 0.05$), *post hoc* pairwise comparisons were carried out using Bonferroni/Dunn adjustment of probabilities. Analysis were carried out using StatView 5.0 (SAS). The numbers of plant replicates in the growth experiments were $n = 4$.

Results and discussion

Plant growth

Forty days old *Lupinus albus* L. grown in the presence of 62 μM Cu exhibited a significant reduction of shoot and root weights of more than 70% compared to the control (Figure 2). However, a concentration of 20 μM Cu in the nutrient solution, which is 40 times higher compared to the control, did not result in a re-

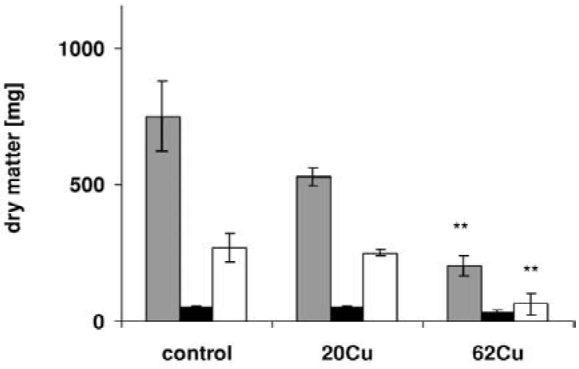

Figure 2. Growth responses of white lupin to different Cu supplies (62 and 20 μM Cu^{2+}, respectively) in leaves (▦), stems (■) and roots (□). Plant growth lasted 40 days for all treatments. Four replicates per plant treatment were used (all values are means ± SE, differences between treatment and control are significant at $p < 0.01$ (**); differences are related to leaves, stems and roots, respectively).

duction of root and shoot growth. During all applied Cu treatments, plants did not develop proteoid roots which are known to be developed by *Lupinus albus* L. under nutrient deficient conditions (Dinkelaker et al., 1989).

Cu uptake

After exposure to 20 and 62 μM Cu for 40 days plants accumulated considerable amounts of Cu in the roots while only a small fraction was translocated to aerial parts (Figure 3). This result clearly shows that roots are the main sites for Cu accumulation confirming results obtained in earlier studies (Hara and Sonoda, 1979). The Cu root-to-shoot ratio was highest for plants exposed to 62 μM Cu (ratio = 5.9) followed by those exposed to 20 μM Cu (ratio = 2.0) and those of the control (ratio = 0.8). In higher plants Cu is able to increase cell permeability through disruption of permeability barriers of cells (De Vos et al., 1989). We assume, that the exposure to 62 μM Cu may have affected the permeability barrier of white lupin root cells causing the influx of Cu ions from outer to inner parts of the cells. However, the EDX data in Figure 4 show a marked gradient in Cu concentration between the apoplasm and the symplasm in root epidermis and particularly in root cortex. As the Cu gradient in root epidermis is substantially smaller than in root cortex we presume a stronger influence of Cu on the permeability barrier of cells in the outer area of the roots (Figure 4).

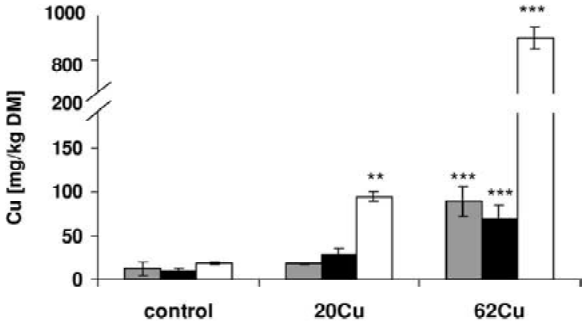

Figure 3. Effect of Cu excess on the Cu accumulation in leaves (▦), stems (■) and roots (□) of *Lupinus albus* L. Four replicates per plant treatment were used (all values are means ± SE, differences in Cu contents between treatment and control: significant at $p < 0.001$ (***); differences are related to leaves, stems and roots, respectively).

Table 1. Redox potential in the media at the beginning and at the end of the experiment related to different plant treatments. Values are means ± SE ($n = 4$), differences between initial and final value are significant at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***), respectively

Plant treatment	Initial value (mV) (after 7 days)	Final value (mV) (after 35 days)
20Cu	124 ± 10	209 ± 14***
62Cu	134 ± 4	168 ± 6*
Control	84 ± 5	187 ± 9***

pH and redox potential changes in the solution

At experimental start up, the pH of the nutrient solution was 5.0 ± 0.2. In the presence of plants exposed to 20 μM Cu or to 0.5 μM Cu (control plants) the pH remained close to the initial value until the 15th day, followed by a gradual decrease to pH 7.0 ± 0.2 after 36 days (Figure 5). No change in solution pH was observed during the entire experiment when plants were exposed to 62 μM Cu.

Redox potential of the solution significantly increased during 35 days when plants were grown in the presence of 0.5, 20 or 62 μM Cu (Table 1).

The different Cu treatments used in our nutrient solution experiments provoked different plant responses. A metal concentration of 62 μM Cu was toxic for the plant as indicated by the strong inhibition of plant growth. When plants were subjected to this high concentration, the pH of nutrient solutions did not change over the growth period presumably because NO_3^- uptake by plant roots was heavily impaired. In contrast, the physiologically active control plants still

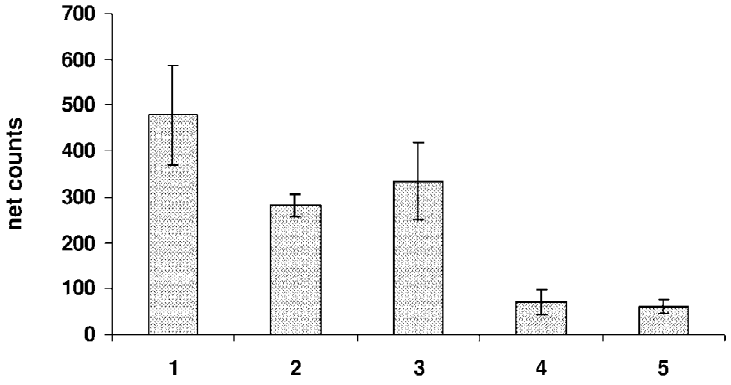

Figure 4. EDX results: X-Ray net counts of Cu in different sections of apical roots: (1) = apoplasm in rhizodermis; (2) = symplasm in rhizodermis; (3) = apoplasm in cortex; (4) = symplasm in cortex; (5) = central cylinder. Samples were taken from apical roots of a 62Cu plant after 40 days growth. Number of measurements per region are $n = 3$. Values are means ± SE.

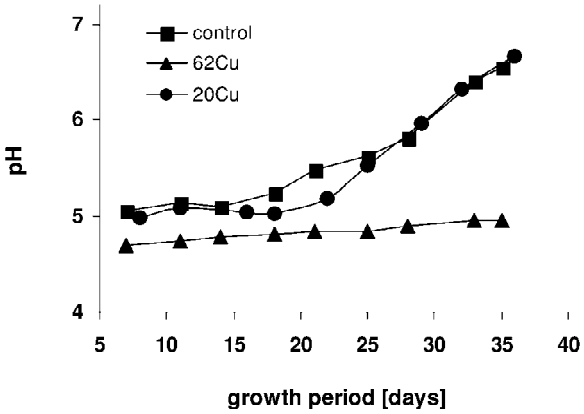

Figure 5. Influence of different plant treatments (▲, 62Cu; ●, 20Cu; ■, control) on the pH in the Hoagland solutions over the growth period. All values are means (means have been calculated from H_3O^+ concentrations). Differences between pH of 62Cu treated plants and pH of control plants are significant at $p < 0.001$ (***).

released OH^- and HCO_3^- as a consequence of the uptake of NO_3^-, that was the sole nitrogen source in the growth medium used in this study (Raven, 1986). The dry matter production and the changes in solution pH observed when plants were grown in the presence of 20 μM Cu were not different from control plants (exposed to 0.5 μM Cu) showing that white lupin can tolerate Cu concentrations as high as 20 μM but lower than 62 μM.

Release of soluble phenols to the nutrient solution

The concentration of soluble phenols in the solution remained constant throughout the experiment when plants were exposed to 0.5 μM Cu or to 62 μM Cu (Figure 6a). During the 20 μM Cu treatment, the concentration of soluble phenols in the solution proceeded similarly for a period of 25 days but then increased rapidly. The total amount of soluble phenols released per gram of root DM was significantly higher in the solution of 62 μM Cu treated plants compared to the total amount of soluble phenols released by the control plants (Figure 6b).

High molecular weight phenol (HMWP) concentration in solution and their Cu content

In the nutrient solutions of all plants yellowish and brown coloured precipitates were found particularly in apical regions of the roots. Qualitative assays of methanol extracts of these precipitates revealed the presence of phenols. On this account we named these precipitates high molecular weight phenols (HMWP). The yield of HMWP expressed in mg per g root or in mg per plant is presented in Figure 7. The net yield of HMWP expressed per g of DM root was highest for plants exposed to 62 μM Cu although the amount of HMWP obtained per plant was similar in all treatments.

The HMWP isolated from roots of plants grown in the presence of 20 and 62 μM Cu had a higher Cu concentration than HMWP isolated from the control (Table 2). Dialysed precipitates had either an equal or a higher Cu content than the non-dialysed precipitates. This suggests that the Cu ions were bound onto HMWP as could be assumed from a coordinative binding mechanism between Cu^{2+} and phenolic compounds.

The phenolic precipitates found in our study had formed a 1–2-mm thin layer around the apical roots.

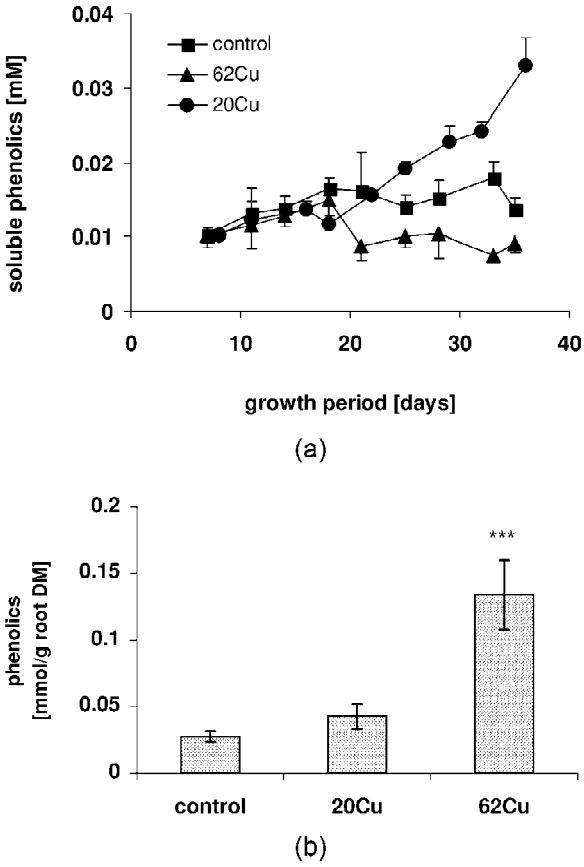

Figure 6. (a) Effect of Cu treatments (▲, 62Cu; ●, 20Cu; ●, control) on the soluble phenol concentration in the Hoagland solution over the growth period. All values are means ± SE. Differences between 62Cu and control as well as 20Cu and control are significant at $p < 0.01$ (**). (b) Soluble phenols released in the solution per gram root dry weight (cumulated amounts over a 35-day growth period). Values are means ± SE. Differences between 62Cu treatment and control: significant at $p < 0.001$ (***).

It could be proposed, that they act as a sink for Cu not in, but around the roots preventing Cu ions to reach the root cells. A similar effect is known for the mucilage, a gelatinous polysaccharide containing material that covers the root tips. These compounds bind heavy metals (Morel et al., 1987) and aluminium (Archambault et al., 1996) and have been proposed to be involved in Al tolerance in cowpea (Horst et al., 1982).

Genistein enrichment in the roots

In roots of all plants examined, genistein and five genistein glycosides (genistein-(malonyl)-glucoside, genistein-(acetyl)-glucoside, genistein-diglucoside and two genistein-glucosides) were found using HPLC-UV and HPLC-MS (Table 3). The genistein concentration was significantly higher in the roots of plants exposed to 20 μM Cu followed by those of the control plants (Table 4).

Table 2. Relationship between plant treatments and Cu contents in purified ($HMWP_p$) and non-purified high molecular weight phenols ($HMWP_{np}$). All values are means ± SE ($n = 3$ for $HMWP_p$, $n = 2$ for $HMWP_{np}$)

Cu [mg/kg DM]	62Cu	20Cu	Control
$HMWP_{np}$	1815 ± 813	612 ± 2	42 ± 4
$HMWP_p$	–[a]	609 ± 85	80 ± 4

[a]Due to insufficient amounts of isolated HMWP no data were available in one case.

All isoflavones were identified on the basis of their MS fragment patterns and their UV absorption spectra by comparison with corresponding data obtained from measurements of purchasable genistein. The dominant fragment at *m/z* 270 was found in all substances here presented. This fragment bases on the molecular ion of genistein. The characteristic fragment patterns in the MS spectra of the substances and λ_{max} values of 260 ± 1 nm (Table 3) are typical for genistein glucosides (Shibuya et al., 1991).

In this study, phenolic compounds were found in soluble as well as insoluble fractions in the nutrient solution and as isoflavones in the roots. A reasonable explanation for these results is root exudation of phenolic compounds. This view is in accordance with publications describing root excretion of organic compounds from white lupin roots (Dinkelaker et al., 1989; Huyghe, 1997). We consider the isoflavonoid metabolites found in our study to be part of the plant defence system (Gagnon and Ibrahim, 1997; Wojtaszek and Stobiecki, 1997). However, our results also suggest that white lupin releases phenolic compounds in its rhizosphere and increases the concentration of phenolic compounds in root tissues as a response to an increased Cu availability in the rhizosphere. In a further effort to elucidate the mechanisms involved, the ability of these phenolic compounds to chelate Cu was subsequently investigated using voltammetry.

Adsorption experiments with phenolic compounds using differential pulse cathodic sweep voltammetry (DPV)

With the help of DPV the influence of different polyphenols on the electrochemical behaviour of Cu in aqueous solution was studied. The addition of 100 μM of genistein to $Cu^{2+}{}_{aq}$ resulted in a decrease of

Table 3. Isoflavonoids found in root extracts of white lupin: retention time (t_R) of HPLC signals, MS (m/z) and UV (λ_{max}) absorption data

Proposed isoflavonoid	t_R (min)	Molecular ion (m/z (rel.int.%))	Main fragments (m/z (rel.int.%))	UV-λ_{max} (nm) (and according to Shibuya et al. (1991) (data in brackets))
Genistein	64.45	270.9 (100)	202.9 (40)	260
Genistein-(malonyl)-glucoside	52.6	518.9 (100)	270.9 (80) 474.9 (64)	259 (261.5)
Genistein-glucoside	49.05	432.9 (100)	270.9 (80) 202.9 (28)	260 (261)
Genistein-glucoside (isomer)	45.81	432.9 (100)	270.9 (80) 202.9 (28)	260 (261)
Genistein-diglucoside	32.51	595.5 (78)	270.9 (100) 202.9 (88) 432.9 (56)	260
Genistein-(acetyl)-glucoside	56.82	475.0 (100)	270.9 (68) 286.9 (44) 202.9 (24)	261

the $Cu^{2+}{}_{aq}$ signal (U = 0 V) by 80% (Figure 8). This peak decline indicates complexation of the metal by the ligand as shown by Neubauer and Furrer (1999) for another metal–ligand system but using the same method. As expected from its complex stability constant (Martell and Smith, 1989) the same experiment performed with caffeic acid resulted in a smaller but still significant reduction of the $Cu^{2+}{}_{aq}$ signal by 43%. Additionally, new signals appear at lower U values (from -0.15 to -0.3 V), most probably due to the formation of new Cu species. The addition of increasing volumes (200, 400, 800 μL) of an ethanol extract of the HMWP led also to a significant reduction of the $Cu^{2+}{}_{aq}$ signal by 40% for the highest ligand concentration (Figure 8). At the same time a broad peak at -0.2 V with a shoulder at -0.08 V arose indicating the formation of Cu complexes. The influence of the solvent was relatively small but not negligible. A peak reduction of 17% occurred after adding 800 μL of pure ethanol to the 50 μM Cu^{2+} solution.

The voltammetry study indicates that genistein – for which no stability constants with heavy metals are known so far – has a high affinity for Cu(II). The genistein concentration in the roots of plants exposed to 20 μM Cu (8.5 μmol genistein/g root DM) was almost 6-fold higher than the Cu concentration measured in the roots (1.5 μmol Cu/g root DM). Besides, the concentration of Cu found in root apoplasm of root epidermis and root cortex was nearly 2-fold higher than the concentration of Cu localised in symplasm. Previous works showed that genistein is located in the cell wall of white lupin (Ferrer et al., 1990; Gagnon et al., 1992a) and that β-glucosidase and isoflavone glucosides are coexistent in lupin cell walls (Pislewska et al., 2002). Hence, our results suggest that a substantial fraction of Cu had been immobilized onto genistein and its glucosides in the cell wall. Furthermore, the

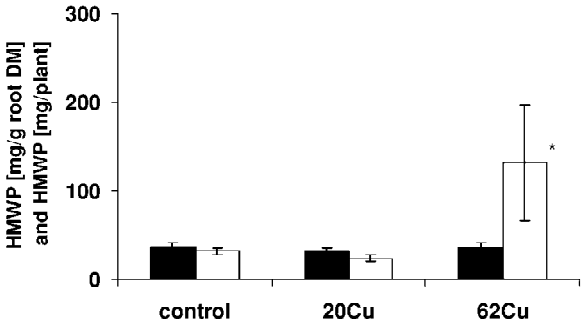

Figure 7. Exuded high molecular weight phenols (HMWP) in nutrient solution: relationship between absolute amounts in mg HMWP (■) and HMWP (mg) per g root DM (□). Values are means ± SE, $n = 3$. Differences in HMWP per g root DM between treatment and control: significant at $p < 0.05$ (*).

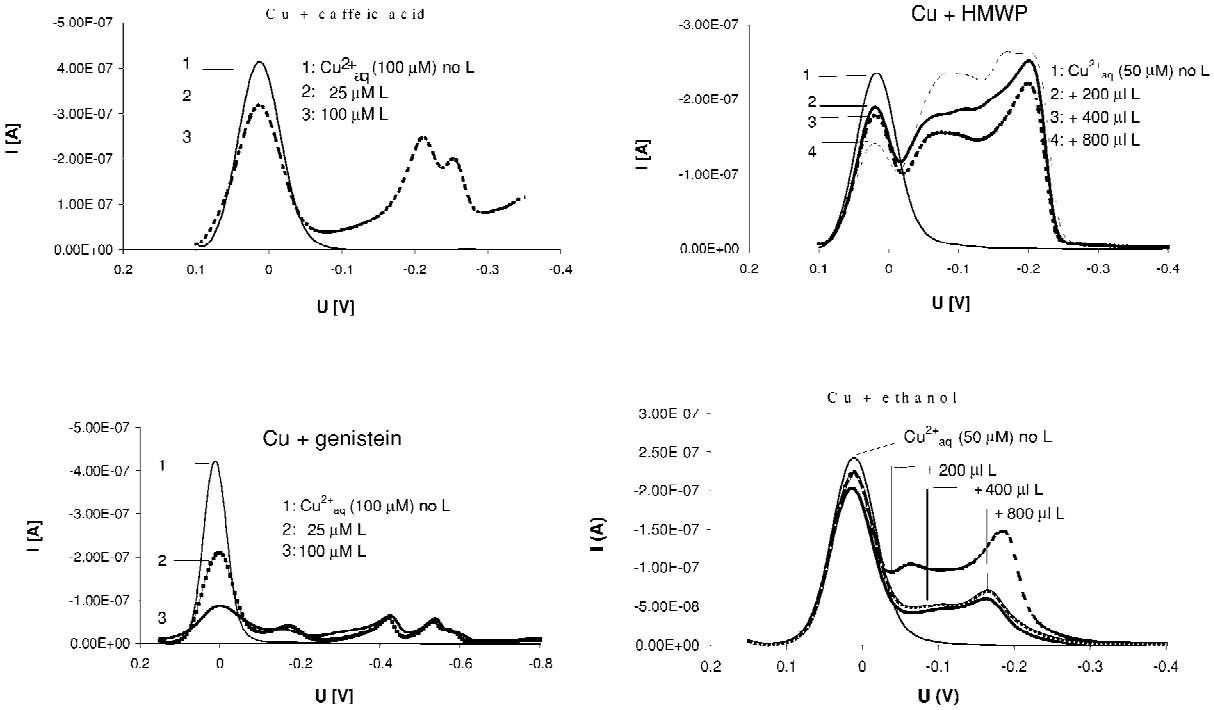

Figure 8. Influence of different phenolic compounds on the electrochemical behavior of Cu^{2+} in aqueous solution (pH 5.8–6.1) measured by DPV (differential pulse cathodic sweep voltammetry). In all voltammograms the fine solid line represents the DPV signal of Cu^{2+} without adding a ligand.

immobilization effect of the soluble phenols exuded from plants exposed to 20 μM Cu (15–33 μM phenols) could also be significant if the released compounds contained caffeic-like polyphenols (Figure 8).

Table 4. Effect of treatment with 20 μM Cu (20Cu) on genistein content in roots of white lupin measured by HPLC-UV. Values are means ± SE ($n = 4$), the difference between 20Cu and control is significant at $p < 0.01$ (**)

	20Cu	Control
Genistein (μmol/g root DM)	8.5 ± 1.6 **	1.5 ± 0.6

Conclusion

The reaction of *Lupinus albus* L. to increasing Cu concentrations (0.5, 20 and 62 μM Cu) was investigated in a hydroponic system where roots grew under sterile conditions. After exposure to a concentration of 20 μM Cu, plant dry matter production did not decrease in comparison to control plants grown with an optimal Cu nutrition (0.5 μM Cu). In contrast, plant roots treated with 62 μM Cu reduced plant biomass production significantly. During the 20 μM Cu treatment, the concentration of soluble (A) and high molecular weight (B) phenols increased in the root-bathing solution. We suppose that white lupin exuded these compounds indicating the plants were exposed to a physiological stress although plant dry matter production was not affected. Moreover, polyphenolic compounds, identified as isoflavones (C), were found in roots. They were presumably associated with structures of the cell walls as described in previous works (Ferrer et al., 1990; Gagnon et al., 1992a). As a further indication, using X-ray microanalysis, high Cu contents were observed in root cell walls (D), whereas only low contents were found in the symplasm. Finally, the studied phenolic compounds can bind Cu to a significant part as could be shown with voltammetry.

In conclusion, our observations suggest that phenolic compounds either exuded in the solution (A, B) or being located in the root cell walls (C) could have chelated Cu in the medium and in root cell walls (D). As a consequence, these effects could have restricted Cu toxicity to the plant.

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