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Mitigation of Cu stress by legume–*Rhizobium* symbiosis in white lupin and soybean plants

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ABSTRACT

The effect of *Bradyrhizobium*–legume symbiosis on plant growth, toxicological variables and Cu bioaccumulation was studied in white lupin and soybean plants treated with 1.6, 48, 96 and 192 μM Cu. In both species, those plants grown in the presence of root nodule-forming symbiotic *Bradyrhizobium* showed less root and shoot growth reduction, plus greater translocation of Cu to the shoot, than those grown without symbiotic *Bradyrhizobium*. The effective added concentrations of Cu that reduced shoot and root dry weight by 50% (EC_{50}), and the critical toxic concentration that caused a 10% reduction in plant growth ($CTC_{10\%}$), were higher in plants grown with symbiotic *Bradyrhizobium*, and were in general higher in the roots whether the plants were grown with or without these bacteria. The production of malondialdehyde and total thiols was stimulated by Cu excess in the shoots and roots of white lupin grown with or without symbiotic *Bradyrhizobium*, but mainly in those without the symbionts. In contrast, in soybean, the increases in malondialdehyde and total thiols associated with rising Cu concentration were a little higher (1.2–5.0 and 1.0–1.6 times respectively) in plants grown with symbiotic *Bradyrhizobium* than without. Finally, the organ most sensitive to Cu excess was generally the shoot, both in white lupin and soybean grown with or without symbiotic *Bradyrhizobium*. Further, *Bradyrhizobium*–legume symbiosis appears to increase the tolerance to Cu excess in both legumes, but mainly in white lupin; plant growth was less reduced and $CTC_{10\%}$ and EC_{50} values increased compared to plants grown without symbiotic *Bradyrhizobium*. *Bradyrhizobium* N_2 fixation in both legumes would therefore seem to increase the phytoremediation potential of these plants when growing on Cu-contaminated sites.

1. Introduction

Nitrogen (N) availability often limits plant growth. Most plants obtain their N from the soil, largely via fertilizers or mineralised indigenous organic matter. However, other plants, most notably legumes, can obtain N from atmospheric N_2 via the entry of their roots into an endosymbiotic association with N_2 fixing bacteria (Stougaard, 2000). This association is the main N-input process in natural ecosystems. It is also of great importance to agriculture since it provides a more effective, cheaper and cleaner way of improving soil fertility than the administration of either inorganic or organic fertilizers.

High soil concentrations of heavy metals have a detrimental effect on microbial activity, soil fertility and bacterial nitrogen fixation (McGrath et al., 1995; Giller et al., 2009), and can cause significant yield losses. Copper is an essential element for plant growth and development, playing an important role in the production of chlorophyll and certain enzymes, in protein and carbohydrate metabolism, and in symbiotic N_2 fixation. However, when it is present at leaf concentrations of 20 $\mu g\ g^{-1}$ dry weight (DW) it is highly toxic (Marschner, 1995). In general, Cu excess interferes with photosynthesis, pigment synthesis, plasma membrane permeability and other metabolic process, causing the strong inhibition of plant growth (Yruela, 2009). High Cu levels (>80 mg kg^{-1}) have been recorded in some natural soils, but Cu excess most frequently occurs in those polluted by Cu-rich pig and poultry slurries, fertilisers and fungicides, industrial and urban activities, metal mining and processing, and waste disposal (Kabata-Pendias and Pendias, 2001).

Contaminated soils are generally poor in nutrients and organic matter. The inclusion of N_2 fixing plants in stabilizing vegetation can, however, help in ecosystem development by increasing available soil N and by promoting plant cover (Frérot et al., 2006). The use of legumes for improving the fertility of soils contaminated with trace elements is therefore of great interest (Frérot et al., 2006). Soybean is the most economically important

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Abbreviations: BAF, bioaccumulation factor; $CTC_{10\%}$, critical toxic concentration of Cu causing a 10% reduction in plant growth; Cu, copper; DW, dry weight; EC_{50}, the effective concentration of Cu that reduces shoot or root dry weight by 50%; FW, fresh weight; MDA, malondialdehyde; N, nitrogen; ROS, reactive oxygen species; SB, symbiotic *Bradyrhizobium*; –SH, total thiols

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of all grain legumes. It is the main protein source in countries (e.g., China) with significant soil trace element (such as As and Cu) contamination, and is used as a model system for legume–*Rhizobium* investigations (Reichman, 2007). White lupin is a temperate grain legume of great agronomic potential due to its high seed protein content and positive effect on soil fertility. The ability of white lupin to survive in soils with low pH and nutrient availability, and the species′ intrinsic biomass production and relative tolerance to trace elements (measured via stress indicators) such as Cd, Hg and As (Zornoza et al., 2002; Esteban et al., 2008; Vázquez et al., 2009), suggest it to be a suitable legume for use in the remediation of contaminated soils.

Plant assays are required when assessing the ecotoxicity of different contaminants in soils. Efroymson et al. (1997) developed toxicological benchmarks in terrestrial plants for contaminants of potential concern. Endpoints such as shoot length, biomass production, percentage germination and root growth are frequently used in these assays (OECD, 2004; USEPA, 1996), and some studies have shown that excess heavy metals can influence these endpoints (An, 2006; Cao et al., 2007; Lee et al., 2008). The present study attempts to determine whether *Rhizobium*–legume symbiosis renders white lupin and soybean plants more or less tolerant to Cu excess. Differences in growth, Cu bioaccumulation and toxicological variables (metabolic and non-metabolic) were assessed in both legumes grown with or without root nodule-forming symbiotic *Bradyrhizobium* (SB) and/or Cu excess under controlled conditions, in order to test the hypothesis that the symbiotic association *Rhizobium*–legume reduces the toxic effect of Cu excess. The experiments were performed under hydroponic conditions, to control the bioavailability of the metal supplied. This system allows: (i) the effects of the Cu doses supplied to plants to be observed, (ii) the toxic Cu concentration for each species studied to be determined, and therefore, and (iii) the Cu tolerance of the different species to be compared.

2. Materials and methods

2.1. Growth conditions and Cu treatments

Seeds of white lupin (*Lupinus albus* L.) cv. Marta and soybean (*Glycine max* L.) cv. Williams were surface-sterilised in 10% v/v sodium hypochlorite for 15 min, rinsed thoroughly with deionised water and germinated on water-moistened filter paper in the dark at 28 °C for 3 days. These seedlings were then transferred to plastic Riviera pots (three seedlings to each pot) containing 2 L of perlite in the upper compartment. Each of the white lupin plants was inoculated twice (at sowing and 1 week later) with a 1 mL suspension of *Bradyrhizobium sp*. ISLU-16; each of the soybean plants received similar inoculations of *Bradyrhizobium japonicum* USDA-110. All bacteria were in the exponential growth phase (10^8–10^9 cells per mL).

The lower compartment of the Riviera pots containing plants grown with no SB was supplied with 0.75 L of nutrient solution; the composition of this solution was that reported by Zornoza et al. (2010). The lower compartment of the Riviera pots containing plants with SB was supplied with 0.75 L of N-free nutrient solution; the composition of this solution was that described by Sánchez-Pardo et al. (2012). All plants were grown in a controlled environment chamber under the following night/day conditions: temperature 20/25 °C, photoperiod 11/13 h, and relative humidity 60%/40%. The photon flux density during the light period was 520 μmol m^{-2} s^{-1}. Ten days after sowing, the plants were supplied with one of four Cu doses: 1.6, 48, 96 and 192 μM $CuSO_4 \cdot 5H_2O$. These high Cu doses were supplied to the plants since the perlite substrate retains approximately 50% of any heavy metals added (Vázquez and Carpena-Ruiz, 2005). Experiments were performed with four independent replicates (with three plants in each pot), following a randomised block design.

Plants were harvested 35 days after the initiation of the Cu treatments, separated into leaves, stems, roots and nodules, and each fraction weighed. They were then washed thoroughly with Tween 80 (0.1% v/v), and then three times with deionised water. One gram fresh weight (FW) of each fraction was frozen in liquid N_2 and stored at −76 °C for analytical determinations. The remaining plant material was dried at 80 °C for 3 days until a constant DW was achieved. It was then homogenised and the element contents determined.

2.2. Determination of Cu

The concentration of Cu in shoots (leaves plus stems) and roots was determined by digesting 200 mg DW of homogenised samples with a mixture of HNO_3:H_2O_2:H_2O (3:2:10, v-v:v) for 30 min at 125 °C under a pressure of 1.5 kPa (Lozano-Rodríguez et al., 1995). The Cu concentration was determined by atomic absorption spectrophotometry (Perkin-Elmer Analyst 800).

The bioaccumulation factor (BAF) for Cu in the white lupin and soybean plants grown under all conditions were calculated as the ratio between the Cu concentration in each plant organ per plant, and the total Cu concentration added per plant to the nutrient solution.

$$\mathrm{BAF} = \frac{\text{Cu concentration in plant organ } (\mu\text{g plant}^{-1})}{\text{Cu concentration in nutrient solution } (\mu\text{g plant}^{-1})}$$

2.3. Determination of malondialdehyde and total thiols

Frozen samples were homogenised to a fine powder in liquid N_2 using an ice-cooled mortar and pestle. Lipid peroxides were then determined as malondialdehyde (MDA, a cytotoxic product of lipid peroxidation normally considered as the major 2-thiobarbituric acid reacting compound) (Lozano-Rodríguez et al., 1997). Plant material (100 mg FW) was placed in 2.0 mL of TCA–TBA–HCl reagent (15% w/v TCA, 0.37% w/v TBA and 0.25 mM HCl), and the extract heated in a sand bath (90 °C, 30 min). After cooling, the flocculent precipitate was removed by centrifugation at 11,000*g* for 10 min. Absorbance of the supernatant was measured at 535 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. Total thiols (–SH) were assayed using 100 mg FW of plant material with 0.4 mL of NaOH (1 M) containing $NaBH_4$ (1 mg mL^{-1}) and 0.2 mL of deionised water. After centrifugation (11,000*g*, 10 min), 0.5 mL of supernatant was added to 0.5 mL of 5,5′-dithiobis (2-nitrobenzoic acid) dissolved in neutralising buffer (0.5 M potassium phosphate, pH 7.2), and absorbance measured at 410 nm (Jocelyn, 1987).

2.4. Toxicological variables and statistical analyses

Relative shoot and root growth rates were expressed as a percentage of the growth of plants (based on FW) compared to the corresponding control treatment (An, 2004a). The critical toxic concentration of Cu causing a 10% reduction in plant growth ($CTC_{10\%}$), and the effective added concentrations of Cu reducing shoot or root dry weight by 50% (EC_{50}), were calculated by regression analysis using SigmaPlot 9.0 software (SPSS Inc., Chicago, IL). EC_{50} values were calculated using a four-variable logistic curve, and $CTC_{10\%}$ values using a one-variable logarithmic curve.

The data presented are the means ± standard errors (S.E.) of four independent replicates. To ensure that the assumptions for statistical analysis were fulfilled, the equality of variances and the normality of the data were tested. Differences between means for each variable were tested for significance by one-way analysis of variance (ANOVA). Significant differences ($P < 0.05$) between treatments were sought using the least significant difference test or Duncan′s test as appropriate.

3. Results and discussion

3.1. Growth

Fig. 1 shows the relative growth rates of white lupin and soybean shoots and roots when grown with or without SB and exposed to different Cu doses. In both legumes, shoot growth with or without SB was generally adversely affected by Cu excess. With the 48 μM Cu dose, white lupin shoot growth was reduced by 35% and 45% when grown with and without SB respectively. In soybean these figures were 39% and 64%. The reduction was greater with increasing nutrient solution Cu concentration, especially in the plants grown without SB. As an exception to this trend, the white lupin plants treated with 192 μM Cu showed a milder reduction in shoot growth when grown without SB (59%) than with SB (71%). An increased translocation of Cu to the shoot might have occurred in plants grown with SB conditions, which might have affected the growth and development of this organ.

The relative growth rates of white lupin and soybean roots in plants grown without SB also fell with increasing Cu concentrations. However, the negative effect of Cu excess on root growth was less in plants grown with SB. Indeed, the plants grown with 48 μM Cu showed greater relative root growth (white lupin 12%,