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12 Cadmium in white lupin nodules: impact on nitrogen and carbon metabolism

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A B S T R A C T

The aims of this work were to investigate the microlocalisation of cadmium (Cd) in *Lupinus albus* L. cv. Multolupa nodules, and to determine its effects on carbon and nitrogen metabolism. Nodulated white lupin plants were grown in a growth chamber with or without Cd (150 μM). Energy-dispersive X-ray microanalysis showed the walls of the outer nodule cortex cells to be the main area of Cd retention, helping to reduce the harmful effect Cd might have on the amount of N_2 fixed by the bacteroids. Sucrose synthase activity declined by 33% in the nodules of the Cd-treated plants, and smaller reductions were recorded in glutamine synthetase, aspartate aminotransferase, alkaline invertase and NADP-dependent isocitrate dehydrogenase activities. The Cd treatment also sharply reduced nodule concentrations of malate, succinate and citrate, while that of starch doubled, but that of sucrose experienced no significant change. In summary, the present results show that white lupins accumulate significant amounts of Cd in their root nodules. However, the activity of some enzymes involved in ammonium assimilation did decline, promoting a reduction in the plant N content. The downregulation of sucrose synthase limits the availability of carbon to the bacteroids, which might interfere with their respiration. Carbon metabolism therefore plays a primary role in the impaired function of the white lupin root nodule caused by Cd, while N metabolism appears to have a more secondary involvement.

Keywords: Cadmium microlocalisation; carbohydrate metabolism; energy-dispersive X-ray microanalysis; *Lupinus albus* L.; nitrogen fixation

Introduction

Inorganic nitrogen (N) availability often limits plant growth. Most plants obtain their N from the soil, largely via fertilizers or mineralised indigenous organic matter. Other plants, most notably legumes, can also obtain N from atmospheric N_2 via the entry of the roots into an endosymbiotic association with nitrogen-fixing bacteria (Stougaard, 2000). This interaction gives rise to a new plant organ: the root nodule. These nodules provide optimal living conditions for nitrogen-fixing bacteria, which reduce N_2 to NH_4^+ via the action of nitrogenase (Nase) (Temple et al., 1998). Once NH_4^+ is formed it binds to glutamic acid (in the presence of ATP and via the action of glutamine synthetase [GS]), to form glutamine. Glutamine can then form two glutamic acid molecules in the presence of oxoglutarate and glutamate synthase (glutamine-2-oxoglutarate-amino-transferase; GOGAT). The glutamine synthetase/glutamate synthase (GS/GOGAT) system is of vital importance since the glutamine and glutamate produced act as N donors for the biosynthesis of major N-containing compounds such as amino acids, nucleotides and chlorophylls (Lea and Ireland, 1999). The symbiotic fixation of N_2 is dependent on the supply of carbohydrates by the host plant to the bacteroids in the root nodules. Photosynthates, predominantly sucrose, are translocated from the shoots to the root nodules, providing the bacteroids the energy and reductants required by Nase, and the carbon skeletons needed for the assimilation of NH_4^+. N_2 fixing bacteria-legume symbiosis is therefore based on the exchange of carbon and nitrogen between the symbiotic partners. Any circumstances that might negatively affect the functioning of the host plant might also have a harmful effect on the N_2 fixation process (Carpena et al., 2006).

Excessive amounts of heavy metals in soils lead to a reduction in soil microbe activity and soil fertility, causing significant yield losses (McGrath et al., 1995). Strong

evidence exists that soil microorganisms are more sensitive to heavy metals than animals or crop plants. In particular, N_2-fixing *Rhizobium* is highly sensitive to high concentrations of heavy metals (Giller et al., 1998; Broos et al., 2005). Cadmium (Cd), which has no biological function, is very toxic to both plants and animals (Sanità di Toppi and Gabbrielli, 1999); indeed, some authors refer to its toxicity as being 2-20 times greater than that of other heavy metals (Kabata-Pendias and Pendias, 2001). It enters agricultural soils via the application of sewage sludge and the extensive use of pesticides and phosphate fertilisers (Kabata-Pendias and Pendias, 2001), and is easily taken up by plant root systems. Many physiological disorders in plants are promoted by Cd-stress (Sanità di Toppi and Gabbrielli, 1999). In Cd-treated leguminous plants, structural alterations in leaves and roots have been reported (Vázquez et al., 1992; Vázquez et al., 2007), as well as in the root nodules of pea (Ausili et al., 2002), soybean (Chen et al., 2003) and white lupin (Carpena et al., 2003). Although the levels of Cd in agricultural soils are usually low, the metal has adverse effects on soil biological activity, reducing nodule formation and impairing nodule functioning in leguminous crops such as alfalfa (Porter and Sheridan, 1981), pea (Hernández et al., 1995), soybean (Balestrasse et al., 2001; Chen et al., 2003), white clover (Obbard and Jones, 1993) and white lupin (Zornoza et al., 2002).

White lupin (*Lupinus albus* L.) is a temperate grain legume of great agronomic potential given its high seed protein content and positive effect on soil fertility. This species enters into effective symbiosis with slow-growing *Bradyrhizobium* sp. (*Lupinus*). Lupin nodules are recognised as a special subclass of indeterminate nodules, known as lupinoid nodules (Fernández-Pascual et al., 2007). The ability of white lupin to survive in soils of low pH and low nutrient availability, and the species' intrinsic biomass production and relative tolerance to trace elements such as Cd, Hg

and As (Zornoza et al., 2002; Esteban et al., 2008; Vazquez et al., 2009), suggest it to be a suitable organism for use in the remediation of contaminated soils. While Cd excess is known to reduce the formation of root nodules in legumes, little is known about how the symbiotic union between legumes and N_2-fixing bacteria is affected by high Cd concentrations. To gain deeper insight into white lupin nodule metabolism under Cd stress, the microlocalisation of the heavy metal in the nodules and key nodule C and N metabolism variables were examined. So that sufficient quantities of Cd would accumulate and be detectable in nodules by low-temperature scanning electron microscopy (LTSEM), high doses of Cd (150 μM) were supplied to the plants.

Materials and Methods

Growth conditions and Cd treatments

White lupin cv. Multolupa seeds 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 28ºC for 3 days. The seedlings obtained were placed in plastic Riviera pots (four seedlings to each pot) containing 8 L of perlite in the upper compartment, and 2 L of nutrient solution in the lower compartment. The composition of the nitrogen-free nutrient solution was as follows: 0.50 mM $CaCl_2$, 0.50 mM KCl, 1.50 mM KH_2PO_4, 1.00 mM K_2SO_4, 1.00 mM $MgSO_4 \cdot 7H_2O$, 36 μM Fe-EDDHA, 33 μM $MnSO_4 \cdot H_2O$, 1.6 μM $ZnSO_4 \cdot 7H_2O$, 1.6 μM $CuSO_4 \cdot 5H_2O$, 46.2 μM H_3BO_3, 0.10 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.001 μM $CoCl_2 \cdot 6H_2O$, 0.001 μM $Ni(NO_3)_2 \cdot 6H_2O$ (pH 5.5 – 6.0). Deionised water was used for preparing all nutrient solutions and was added to replace transpiration losses every two days. The entire nutrient solutions were changed weekly. All plants were inoculated twice (once at sowing and again one week later) with a suspension of *Bradyrhizobium* sp. (*Lupinus*) strain ISLU-16 in the

exponential growth phase (2 mL of solution at a density of 10^8 cfu.mL^{-1}). The plants were grown in a growth chamber under the following conditions (night/day): T 20/25°C, photoperiod 11/13h, relative humidity 60/40%. The photon flux density during the light period was 520 µmol m^{-2} s^{-1}.Ten days after sowing, four replicates of two Cd treatments (0 and 150 µM as $CdSO_4$; the latter concentration was chosen to ensure Cd detection by energy-dispersive X-ray microanalysis [EDXMA; see below]) were established using a randomised block design. After 35 days of growth under these treatments, shoots and roots were separated and their fresh weight (FW) recorded. They were then washed thoroughly with tap water three times, and again with deionized water three times. Nodules from each root were collected, weighed and their number determined. Fresh nodule aliquots were immediately frozen in liquid N_2 and stored at -76ºC for analytical determinations. Other nodules and plant samples were oven-dried at 80ºC until a constant weight was reached; these dry weights (DWs) were then recorded. Dry samples were homogenised and used in element determinations.

Determination of Cd, N and stress indicators

The concentration of Cd in plant organs was determined by digesting 20 mg DW of homogenised nodule samples with a mixture of HNO_3: H_2O_2: H_2O (3:2:10, v:v:v) for 30 min at 125ºC under a pressure of 1.5 kPa. The Cd concentration was determined by atomic absorption spectrophotometry (Perkin-Elmer Analyst 800). Total shoot, root and nodule N concentrations were determined using the Dumas combustion method (Leco CHNS-932, St. Joseph, MI, USA) at the beginning and end of the Cd treatments. Fixed nitrogen was calculated as the total plant N content at harvest, minus the total N content at the start of the Cd treatments. The concentration of the stress indicators malondialdehyde (MDA) (a cytotoxic product of lipid

peroxidation normally considered the major 2-thiobarbituric acid-reacting compound) and total thiols (-SH) were examined in nodule samples homogenised to a fine powder in liquid N_2 using an ice-cooled mortar and pestle, and assayed as described elsewhere (Esteban et al., 2008).

Electron microscopy and energy-dispersive X-ray microanalysis

Small pieces (1 mm^2) of fresh nodules from Cd-treated and untreated nodules, selected at comparable stages of development, were mounted on a clamp holder with a special adhesive (Gurr®, OCT, BDH, Poole, UK). They were then cryofixed in slush nitrogen (−196ºC) and cryotransferred to a vacuum chamber at −180ºC to be fractured using a cooled, stainless steel spike. These samples were subjected to superficial etching under vacuum (−90ºC, 120 s, 2 kV) and gold coated for observation using a Zeiss DSM 960 digital scanning electron microscope (Oberkochen, Germany) at low temperature (LTSEM) and employing secondary and back-scattered electrons. EDXMA was performed in conjunction with low temperature scanning electron microscopy (LTSEM) using a Pentaflet apparatus (Pentaflet, Oxford, UK) at a resolution of 133 eV. Only smooth surfaces were contemplated for microanalysis, following the recommendations of Hess (1980). Semi-quantitative element analysis results were obtained using standard ZAF (atomic number, absorption and fluorescence) correction procedures employing Link Isis 3.2 software (Link Isis, Oxford, UK).

Extraction and enzyme assays

Frozen nodules (around 100 mg FW of liquid N_2-pulverised material) were homogenised with a buffer composed of 50 mM MOPS, 20% PVPP, 10 mM DTT, 10 mM 2-mercaptoethanol, 1 mM EDTA, 20 mM KCl and 5 mM $MgCl_2$, at pH 7 and 0-2ºC

169 (5 mL per g FW). The homogenate was centrifuged for 30 min at 20,000 *g* at 4ºC.
170 Samples (50 µL) of the supernatant were retained for the determination of
171 phosphoenol pyruvate carboxylase (PEPC; EC 4.1.1.31) activity (González et al.,
172 1995), the nodule protein fraction (Bradford, 1976), and leghaemoglobin (Lb) analysis.
173 The rest of the supernatant was desalted by low-speed centrifugation (180 *g*, 2 min)
174 through 5 mL columns of Bio Gel P6DG (BioRad, Hercules, CA, USA) equilibrated
175 with the following buffer: 250 mM MOPS, 25 mM $MgCl_2$ and 100 mM KCl, pH 7 at 0-
176 2ºC. The desalted extract was used to determine the following enzyme activities:
177 alkaline invertase (AI; EC 3.2.1.26), aspartate aminotransferase (AAT; EC 2.6.1.1),
178 glutamine synthetase (GS; EC 6.3.1.2) and sucrose synthase (SS; EC 2.4.1.13)
179 according to González et al. (1995), malate dehydrogenase (MDH; EC 1.1.1.37) and
180 uridine diphosphoglucose pyrophosphorylase (UDPGPP; EC 2.7.7.9) according to
181 Gordon and Kessler (1990), glutamate synthase (NADH-GOGAT; EC 1.4.1.14)
182 according to Groat and Vance (1981), and $NADP^+$-dependent isocitrate
183 dehydrogenase (ICDH; EC 1.1.1.42) according to Marino et al. (2007). All activities
184 were measured within the linear range at 30ºC, as described elsewhere (Marino et al.,
185 2006).

186 For the extraction of bacteroid proteins, the pellet remaining after removing the
187 host plant soluble proteins by centrifugation (see above) was washed three times in
188 0.5 mL extraction buffer, and centrifuged three times for 10 min at 14,000 *g*, on each
189 occasion discarding the supernatant. The pellet was then resuspended in bacteroid
190 extraction buffer (5 mL per g original nodule FW) (50 mM MOPS, 20% PVPP, 10 mM
191 DTT, 10 mM 2-mercaptoethanol, 1 mM EDTA, 20 mM KCl and 5 mM $MgCl_2$, pH 7 at 0-
192 2ºC), and the bacteroids broken open by sonication (3 x 30 s, 0ºC). After

centrifugation (20,000 *g*, 2ºC 30 min) the supernatant was retained for Western blotting and for bacteroid protein determination according to Bradford (1976).

Western immunoblotting

Samples of host plant soluble proteins and bacteroid protein extracts were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Runs were performed according to Laemmli (1970) using a 1 mm-thick 10% polyacrylamide (w/v) resolving gel and a 4.6% (w/v) stacking gel in a vertical electrophoresis cell (MiniProtean III, Bio-Rad, Hercules, CA, USA) at 150 V for 60 min. The gels were then electroblotted onto PVDF membranes for 75 min at 100 V in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA) and the blots blocked in 5% (w/v) skimmed milk in 20 mM Tris-buffered saline at 4ºC overnight. Anti-Lb was the primary antibody (1:5000, v/v) used against the host plant soluble protein extract. Anti-NifDK (1:2000, v/v) was used against the bacteroid soluble protein extract. Goat anti-rabbit lgG alkaline phosphatase (1:10.000, v/v Sigma-Aldrich, St. Louis, USA) was employed as the secondary antibody. Immunoreactive bands were visualized using the bCIP/NBT liquid substrate system (Sigma-Aldrich, St. Louis, USA).

Organic acid determination

Frozen nodule samples were homogenised in liquid N_2 using a pestle and mortar (around 100 mg FW), to which 1.5 mL of 10% (w/v) trichloroacetic acid (TCA) in water was then added. The homogenate was centrifuged for 10 min at 1750 *g* at 4ºC. The aqueous phase was washed five times with diethyl ether saturated with water. The ether was discarded and the aqueous phase purged with He for 2 min before filtering through a 0.45 µm syringe filter. Organic acids (malate, succinate,

citrate and α-ketoglutarate) levels were determined by ion chromatography in a DX-500 apparatus (Dionex, Salt Lake City, UT, USA) by gradient separation using a Dionex IonPac AS11 column as described elsewhere (Gálvez et al., 2005).

Sucrose and starch determination

Frozen nodules (around 100 mg FW of liquid N_2-pulverised material) were prepared for sucrose extraction in boiling 80% (v/v) ethanol. Ethanol-soluble extracts were dried in a Turbovap LV evaporator (Zymark Corp, Hopkinton, MA, USA). The soluble compounds were redissolved with 4 mL of distilled water, mixed, and centrifuged at 20,000 *g* for 10 min. The ethanol-insoluble residue remaining after the extraction of the soluble compounds was subjected to starch extraction as described by MacRae (1971), and the glucose produced determined by high-performance capillary electrophoresis in a Beckman Coulter PACE system 5500 apparatus (Beckman Instruments, Fullerton, CA, USA) as described by Marino et al. (2006). The sucrose of the ethanol-soluble fraction was determined in the same way.

Statistical analyses

The data shown 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 checked. Differences between means for each variable were tested for significance by one-way ANOVA. Means were compared using the least significant difference test ($P < 0.05$). Significant differences among the individual treatments are expressed by different letters (a, b, c). All calculations were performed using IBM SPSS 19.0 software.

Results

Effect of Cd treatment on plant and nodule weights, N status and total nodule Cd concentration

Table 1 shows the plant organ and nodule FWs, the mean nodule biomass, the total shoot, root and nodule N contents, and the Cd, MDA and total -SH concentrations in the nodules of plants grown with or without Cd for 35 d. Compared to the controls, the 150 μM Cd-treated plants showed clear reductions in shoot (32%), root (27%) and nodule (44%) FWs, whereas the average nodule weight almost doubled that of the controls. The shoot, root and nodule N content of the Cd-treated plants, expressed as DWs, showed significant declines - 34%, 20% and 35% respectively - compared to the controls. The roots of the Cd-treated plants had a dark brown colour, the main taproot was shorter, and the number of lateral roots smaller than in the untreated plants. The concentration of Cd in the nodules of the Cd–treated plants was almost five times that seen in the controls. The total –SH concentration in the nodules of the Cd-treated plants increased by 26%. The MDA concentration was not affected by the supply of Cd.

Cadmium localisation in nodule tissues

The microlocalisation of Cd at the tissue/cellular level was examined in the nodules of plants grown with and without Cd. Figure 1A provides an overview of the EDXMA results. Microprobe examinations were performed at the same sites in nodules of both untreated and Cd-treated plants. In the nodules of untreated plants, the Cd signal was below the EDXMA detection limit. In the nodules of the treated plants, Cd was localised in the cell walls of the nodule cortex cells, but it was not detected in those of the infected zone cells (Fig. 1B). The strongest Cd signal was

seen in the outer nodule cortex cell walls, falling towards the inner nodule cortex cell walls by 85%. Cd was also detected inside the nodule cortex cells and in the cells of the infected zone, showing a decreasing gradient from the outer to the inner nodule cortex (14%), and from the former to the inside of infected cells (47%).

Effect of Cd treatment on nitrogen and carbon metabolism enzyme activities, metabolites, leghaemoglobin, and Nase protein levels

Figures 2 and 3 show the activities of the N and C metabolism enzymes in the nodules of plants grown with and without Cd respectively. The 150 µM Cd treatment promoted a reduction in GS (*32*%) and AAT (22%) activity, but not in that of GOGAT compared to controls (Fig. 2). The activities of AI (27%), ICDH (22%) and SS (33%) declined in the nodules of the Cd-treated plants compared to the controls. The presence of Cd in the nutrient solution did not significantly modify the activities of PEPC, MDH or UDPGPP (Fig. 3A-B). No significant differences in Lb or Nase protein levels were seen between the treated and control plants (Fig. 4).

Table 2 shows the concentrations of organic acids, sucrose and starch in the nodules of plants grown with and without Cd. Malate, succinate and citrate concentrations were reduced by 35%, 22% and 39% respectively in the nodules of the Cd-treated plants compared to the controls. The starch content in the nodules of the Cd-treated plants was 2.3 times that seen in the controls. The levels of αKG and sucrose in the nodules did not change with Cd treatment.

Discussion

Early studies showed symbiotic N_2 fixation to be unaffected by even very high soil concentrations of heavy metals (Rother et al., 1983). However, a number of

290 authors later reported it to be affected even at relatively low soil concentrations
291 (Chaudri et al., 1993; Giller et al., 1998). The value of N_2 fixation as a marker of soil
292 heavy metal contamination – for which it has commonly been proposed (Brookes,
293 1995) – is therefore unsure. Cd is one of the most mobile trace elements in soils and
294 often accumulates in plants. In the present Cd-treated plants, the Cd concentration
295 reached 261.2 ± 14.7 nmol g^{-1} DW in the nodules after 35 days. The accumulation of
296 Cd reduced the growth of the plants, the number of nodules (by 70%), and their
297 biomass. Similar results have been reported for other Cd-treated plants such as alfalfa
298 (Ibekwe et al., 1996) and pea (Hernández et al., 1995). The inhibition of root
299 elongation and the reduction in the number and size of root hairs (essential for
300 nodulation) are responsible for the scant nodulation seen in plants treated with heavy
301 metals (Chaudhary et al., 2004). The altered root architecture and root growth seen in
302 the present Cd-stressed plants might be one of the reasons for poor nodule
303 occupancy.
304 The accumulation of Cd in the nodules depends on the ability of cell
305 compartments to bind Cd. The present results show the walls of the outer nodule
306 cortex cells to be the main area of Cd retention. The binding of Cd to cell walls in
307 variable proportions has been reported in other plant organs. For example, in earlier
308 EDXMA localisation analyses of 150 µM Cd-treated white lupin plants, Vázquez et al.
309 (2007) found root cell walls to be the main area of Cd binding. Cd-cell wall interactions
310 in flax plants have been attributed to changes in the homogalacturonan fraction of the
311 roots (Douchiche et al., 2010). In the present work Cd was detected inside the nodule
312 cortex cells and in those of the infected zone; the decreasing gradient of Cd
313 localisation from outer to inner nodular tissues prevented its accumulation and
314 therefore its harmful effect of Cd on the amount of N_2 fixed by the bacteroids.

315 In addition to the compartmentalisation of Cd, plant organs possess other
316 mechanisms of detoxifying Cd, e.g., phytochelatin synthesis (Sanitá di Toppi and
317 Gabbrielli, 1999). This may have also been operating in the nodules of the Cd-treated
318 plants. Thiol groups play an important role in cytoplasmic detoxification, but they are
319 also required to counteract the harmful effects of oxidative stress (Noctor and Foyer,
320 1998). The unchanged levels of MDA in the nodules of Cd-treated plants suggest that
321 non-oxidative stress is operating; although an increase in total -SH in the nodules of
322 the Cd-treated plants was seen. Contemplating a 2:1 stoichiometry for thiol-Cd
323 complexes, the Cd concentration in the nodules could be complexed by –SH groups
324 appearing in rising concentration in the Cd-treated plants, partly reducing the harmful
325 effect of Cd toxicity (Vazquez et al., 2009). Further, the enhanced production of –SH
326 groups in the Cd-treated plants might be related to an increased S concentration
327 under Cd stress (S rose from 90.6 to 143.7 μmol g^{-1} DW in the leaves and from 175.0
328 to 225.1 μmol g^{-1} DW in the roots). Cd-induced sulphate uptake increases in maize
329 roots for sustaining the high S demand during phytochelatin biosynthesis (Nocito et
330 al., 2002). Moreover, Cd and S were found co-localised in the vascular cylinder of the
331 present roots, as reported by Vazquez et al. (2007).
332 Reduced-GSH protects against Cd-induced oxidative damage, maintaining GS
333 and GOGAT activities close to those seen in the nodules of control soybean plants
334 (Balestrasse et al., 2006). In the present study, the supply of Cd reduced GS activity in
335 the nodules, but caused no change in GOGAT activity. The supply of high levels of Cd
336 to soybean nodules inhibits the GS/GOGAT cycle and hence affects nitrogen fixation
337 and assimilation, but low Cd levels do not (Balestrasse et al., 2005). In the present
338 work, Cd supply reduced the total N content of the treated plants and their nodules by
339 35%, suggesting alterations in processes related to N_2 fixation, such as bacteroid

carbon supply, oxygen availability and N feedback regulation. According to Marino et al. (2006), the regulation of biological nitrogen fixation (BNF) in legume-*Rhizobium* symbiosis may be dependent on these factors.

The main carbon source transported from the shoots to the nodules is sucrose, which is required for both N_2 fixation and NH_4^+ assimilation. Once in the nodule, sucrose may be hydrolysed by either SS or AI (Galvez et al., 2005). SS activity is essential for the maintenance of Nase activity, and it is reported that reduced SS activity greatly reduces N_2 fixation in a pea mutant (Gordon et al., 1999), promoting an eventual increase in the sucrose content. In the present study, the presence of Cd in the nodules inhibited SS and IA activities, leading to a slight (but not significant) increase in the sucrose content. The maintenance of normal sucrose levels in the nodules of Cd-treated plants may be due to the balance between the reduced demand for C skeletons for N_2 fixation leading to a lack of use of sucrose, and a possible decline in sucrose transport due to low photosynthetic rates (photosynthesis is highly sensitive to heavy metals, which inhibit the biosynthesis of chlorophyll and accessory pigments) (Sanitá di Toppi and Gabbrielli, 1999). However, the unchanged levels of sucrose do not rule out the involvement of SS in the functioning of the nodule. The supply of Cd also increased the starch content of the nodules of Cd-treated plants. A massive accumulation of starch granules was observed in the plastids and amyloplasts of the infected cells, as reported by Lucas et al. (1998) when investigating the effects of nodule senescence on white lupins. In contrast, other authors have detected reduced starch contents in lupin nodules grown under glyphosate stress (de Maria et al., 2006). According to these authors, SS plays an important role in the reversible conversion of sucrose into starch.

364 Hexoses, produced either by SS or AI, are hydrolysed through the glycolytic
365 pathway to form PEP, which is converted to malate in the PEPC/MDH cycle. This
366 malate can used be together with succinate as a source of carbon and energy for
367 bacteroid consumption (Cabrerizo et al., 2001). In the present study, the supply of
368 high Cd levels did not change the activity of PEPC and MDH in the plant nodules,
369 although reduced malate and succinate contents were seen. The reduction in organic
370 acids might be more related to the degradation of sucrose than the functioning of the
371 PEPC/MDH cycle, which is very stable under abiotic stress. A reduction in PEPC
372 activity, but no change in MDH activity, has been reported in lupin nodules treated with
373 glyphosate (de Maria et al., 2006). The present Cd treatment also reduced the
374 activities of AAT and ICDH, as well as the citrate concentration, and promoted a slight
375 reduction in the α-KG concentration. ICDH catalyses the conversion of isocitrate to α-
376 KG, which is necessary for the GS/GOGAT cycle and therefore for amino acid
377 synthesis (Gálvez et al., 2005).
378 An adequate oxygen flux is essential for the functioning of root nodules, and of
379 Nase in particular. Lb provides an adequate flux of O_2 to the bacteroids at very low
380 concentrations but Lb levels tend to decline when nodules are subject to abiotic
381 stresses (Fernández-Pascual et al., 2007). The direct interference of Cd in porphyrin
382 biosynthesis (Somashekaraiak et al., 1992) might contribute towards reduced Lb
383 levels, together with a reduction in O_2 diffusion. Balestrasse et al. (2001; 2004)
384 reported lower Lb levels in Cd-treated soybean plant nodules, especially in cells with
385 damaged bacteroids. However, in the present study, no alterations in Lb levels were
386 detected in the nodules of the Cd-treated plants, and no variation was seen in Nase
387 protein levels. This might indicate the existence of a positive correlation between the
388 Lb and Nase protein levels. Similar results have been reported for soybean nodules

389 treated with Cd and Al (Balestrasse et al., 2001; 2006). These authors observed a
390 reduction in Lb levels and Nase activity when their plants were thus treated.
391 In summary, the present results show that white lupin accumulates significant
392 amounts of Cd in its nodules. This Cd is mainly localised in the cell wall of cortex cells,
393 and only a small amount of Cd was detected inside of the infected zone cells. Nase
394 protein remained unchanged under Cd stress, but some the activities of enzymes
395 involved in NH_4^+ assimilation declined, promoting a reduction in the N content in the
396 nodules and other plant organs. The impairment of sucrose metabolism and the
397 downregulation of SS in the nodule might be responsible for the decline in N_2 fixation
398 in the presence of Cd via a limiting of the carbon flux for bacteroid respiration. Carbon
399 metabolism may therefore play a primary role in the impaired function of the white
400 lupin root nodule caused by Cd, while N metabolism appears to have a secondary
401 involvement.
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Table 1

Effect of Cd treatment on plant growth and nodule development, N status, total Cd concentration and stress indicators in nodules of 35 day-old plants. Data are means ± S.E. (*n* = 4). Means in the same row followed by the same letter do not differ significantly.

	Cd treatment (μM)	
	0	150
Shoots (g FW plant^{-1})	7.61 ± 0.29^{a}	5.17 ± 0.25^{b}
Roots (g FW plant^{-1})	3.28 ± 0.23^{a}	2.41 ± 0.15^{b}
Nodule biomass (mg FW plant^{-1})	206.25 ±19.48^{a}	115.25 ±10.18^{b}
Average nodule weight (mg FW per nodule)	2.57 ± 0.30^{a}	4.79 ± 0.12^{b}
Total shoot N content (μmol plant^{-1})	1945.51 ±91.48^{a}	1278.63 ±73.66^{b}
Total root N content (μmol plant^{-1})	268.19 ± 5.66^{a}	213.26 ± 2.92^{b}
Total nodule N content (μmol plant^{-1})	65.14 ± 5.68^{a}	42.42 ± 3.62^{b}
Total nodule Cd (nmol g^{-1} DW)	57.63 ± 4.55^{a}	292.19 ±15.67^{b}
MDA (nmol g^{-1} FW)	38.10 ± 1.94^{a}	37.30 ± 1.47^{a}
-SH (nmol g^{-1} FW)	344.06 ±23.82^{a}	434.78 ± 24.67^{b}

549 **Table 2**

550 Metabolite content in nodules of 35 day-old plants grown with or without Cd. Data are
551 means ± S.E. (*n* = 4). Means in the same row followed by the same letter do not differ
552 significantly.

553

	Cd treatment (µM)	
	0	150
Malate (mg kg^{-1} FW)	4963.32±92.58[a]	3210.80±204.04[b]
Succinate (mg kg^{-1} FW)	35.85 ± 2.81[a]	27.79 ± 2.11[b]
Citrate (mg kg^{-1} FW)	698.48±16.58[a]	428.06 ± 27.87[b]
αKG (mg kg^{-1} FW)	3.76 ± 1.45[a]	3.32 ± 2.19[a]
Sucrose (mg g^{-1} FW)	2.22 ± 0.18[a]	2.71 ± 0.15[a]
Starch (mg glucose g^{-1} FW)	0.42 ± 0.03[a]	0.96 ± 0.08[b]

554

555

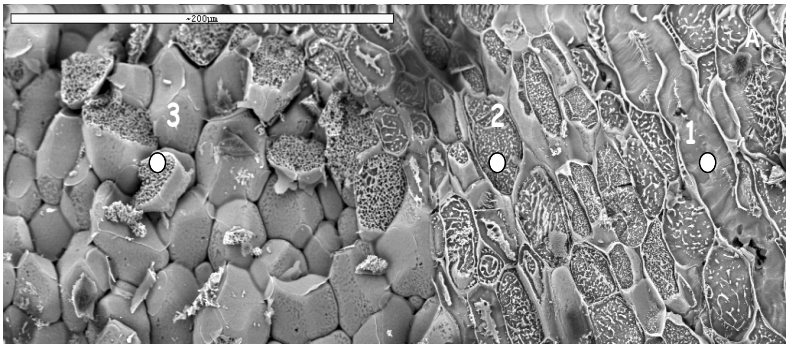
Legends of Figures:

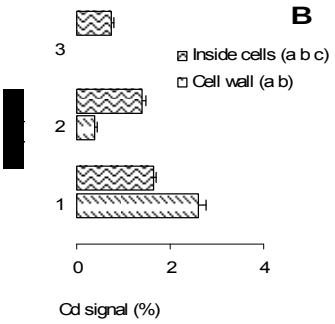
Fig.1. EDXMA localisation of Cd in transverse sections of the nodule of a Cd-treated plant: (A) Overview showing the nodule sample points (1 and 2: cortex; 3: infected zone) where Cd localisation was examined in freeze-fractured samples; (B) Cd values are expressed as percentages of the total signal. Data are means ± S.E. (*n* = 4).

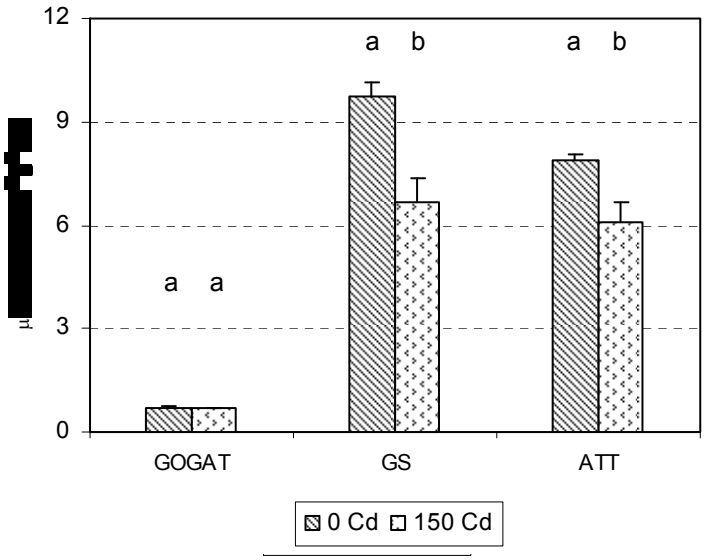
Fig. 2. Activities of nitrogen metabolism enzymes in the nodules of 35 day-old plants grown with and without Cd. Data are means ± S.E. (*n* = 4). Different letters above the bars indicate significant differences between treatments ($P < 0.05$).

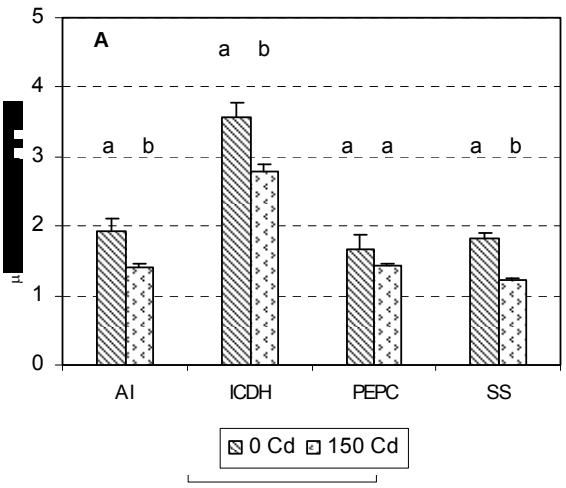
Fig. 3. Activities of carbon metabolism enzymes (A and B) in nodules of 35 day-old plants grown with or without Cd. Data are means ± S.E. (*n* = 4). Different letters above the bars indicate significant differences among treatments ($P < 0.05$).

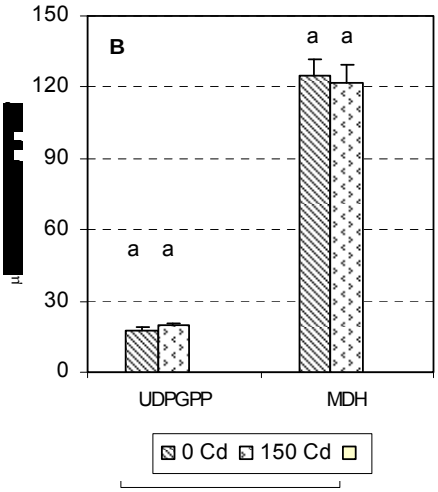
Fig. 4. Immunodetection of Lb and Nase protein levels in nodules of 35 day-old plants grown with or without Cd. Equal amounts of protein were loaded onto each track. Lanes C1 to C4 correspond to nodules of untreated plants, and Cd1 to Cd4 to those of treated plants.


~200nm
3
2
1


B
3
2
1
Inside cells (a b c)
Cell wall (a b)
0
2
4
Cd signal (%)


12
9
6
3
0
a b
a b
a a
GOGAT
GS
ATT
0 Cd
150 Cd


A
a b
a b
a a
a b
AI
ICDH
PEPC
SS
0 Cd
150 Cd


B
a a
a a
UDPGPP
MDH
0 Cd
150 Cd

575