Plant and Soil **262:** 373–381, 2004.

Cadmium-induced senescence in nodules of soybean (*Glycine max* L.) plants

K.B. Balestrasse, S.M. Gallego & M.L. Tomaro[1]
Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, (1113) Buenos Aires, Argentina. [1]*Corresponding author**

Received 2 October 2003. Accepted in revised form 17 November 2003

Key words: antioxidant enzymes, cadmium, *Glycine max*, senescence, soybean

Abstract

The relationship between cadmium-induced oxidative stress and nodule senescence in soybean was investigated at two different concentrations of cadmium ions (50 and 200 μM), in solution culture. High cadmium concentration (200 μM) resulted in oxidative stress, which was indicated by an increase in thiobarbituric acid reactive substances content and a decrease in leghemoglobin levels. Consequently, nitrogenase activity was decreased, and increases in iron and ferritin levels were obtained. Senescent parameters such as ethylene production, increased levels of ammonium and an increase in protease activity were simultaneously observed. Glutamate dehydrogenase activity was also increased. Peroxidase activity decreased at the higher cadmium concentration while the lower cadmium treatment produced changes in peroxidase isoforms, compared to control nodules. Ultrastructural investigation of the nodules showed alterations with a reduction of both bacteroids number per symbiosome and the effective area for N_2-fixation. These results strongly suggest that, at least at the higher concentration, cadmium induces nodule senescence in soybean plants.

Abbreviations: EDTA – ethylenediaminetetraacetic acid (disodium salt); GDH – glutamate dehydrogenase; Lb – leghemoglobin; POD – peroxidases; PVP – polyvinylpyrrolidone; ROS – reactive oxygen species; TBA – thiobarbituric acid; TBARS – thiobarbituric acid reactive substances; TCA – trichloroacetic acid.

Introduction

Different kinds of environmental stresses (heat, drought, salinity, and heavy metals) have been shown to affect nodule formation and function in leguminous plants. Many of these environmental stresses are supposed to produce oxidative stress (Ferreira et al., 2002) and promotes ethylene production in plants (Morgan and Drew, 1997).

Nitrogen fixation is particularly sensitive to oxygen and reactive oxygen species (ROS) (superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$)). The rate of respiration, the high concentration of leghemoglobin (1–3 m*M*) and the very active iron metabolism in the nodule seems to be closely related to free radical reactions and oxidative stress (Robson and Postgate, 1980; Puppo et al., 1981; Davies and Puppo, 1992). There is compelling evidence that the biological damage attributed to superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) is dependent on the presence of iron such as heme-derived intracellular iron. Within most cells ferritin constitutes the major storage site for non-metabolized intracellular iron and therefore plays a critical role in regulating the availability of iron to catalyze such harmful reactions as the peroxidation of lipids and the Fenton reaction generating the highly reactive hydroxyl radical ($HO^\bullet$) (Lucas et al., 1998).

Hydrogen peroxide is involved not only in the cellular oxidative damage but also it is a signaling molecule in plants that mediates responses to abiotic and biotic stresses (Neill et al., 2002). Cellular H_2O_2 levels are determined by the rates of H_2O_2 production

*FAX No: 54-11-4508-3645. E-mail: ptomaro@ffyb.uba.ar

and metabolism via catalase and peroxidases. Peroxidases are ubiquitous hemoproteins that reduce H_2O_2 using different class of organic compound as electron donor. Plant growth, development and/or morphogenesis cause, or are caused by, consistent changes in peroxidase patterns which reflect alterations in the internal cellular milieu as well as the plant's interaction with the 'outside' environment (Siegel, 1993).

Soybean is an important crop in the world, offering high-quality protein and increasing the input of combined N into the soil. However, its yield may be adversely affected by heavy metals. Reduction of biomass production and nutritional quality has been observed in crops grown on soil contaminated with heavy metals. Heavy metals excess into the soils resulting from industrial and urban activities, sewage sludge and agrochemicals (Mhatre and Pankhurst, 1997).

Cadmium is a non-essential element that shows phytotoxicity even at low doses (Das et al., 1997; Sanita di Toppi and Gabbrielli, 1999). In roots and nodules of soybean plants high Cd causes oxidative damage and thus affects nitrogen fixation and assimilation (Balestrasse et al., 2001; Balestrasse et al., 2003). Detailed studies of cadmium effect on soybean nodules help to understand the mechanisms of metal toxicity. In the present work, we reported the relationship between cadmium-induced oxidative stress and the occurrence of senescence in soybean nodules subjected to two different concentrations of cadmium ions.

Materials and methods

Plant material and growing conditions

Seeds of soybean (*Glycine max* L.) were surface sterilized with 5% v/v sodium hypochlorite for 10 min and then washed with distilled water four times. The seeds were inoculated with 10^8 cell ml^{-1} of *Bradyrhizobium japonicum* (109, INTA Castellar) and were germinated in vermiculite for five days. After germination plants were removed from the pots, the root were carefully and gently washed and transferred to separated containers for hydroponics. Plants were grown in a controlled climate room at 24 ± 2 °C and 50% RH, with a photoperiod of 16 h. The hydroponics medium was Hoagland nutrient solution (Hoagland and Arnon, 1957). The medium was continuously aerated and replaced every three day. After four weeks plants were treated with nutrient solution devoid of cadmium (control) or containing 50 and 200 μM of $CdCl_2$. After 48 h of treatment, nodules were isolated and used for determinations. Three different experiments were performed, with five replicated measurements for each parameter assayed.

Thiobarbituric acid reactive substances (TBARS) determination

Lipid peroxidation was measured as the amount of TBARS determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Fresh control and treated nodules (0.3 g) were homogenized in 3 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at $3500 \times g$ for 20 min. To 1 ml of the aliquot of the supernatant, 1 ml of 20% TCA containing 0.5% (w/v) TBA and 100 μl 4% BHT in ethanol were added. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice. The contents were centrifuged at $10000 \times g$ for 15 min and the absorbance was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Leghemoglobin

Nodules (0.3 g) were homogenized in 3 ml of extraction medium containing 0.02% (w/v) potassium (K) ferricyanide and 0.1% sodium bicarbonate. Leghemoglobin (Lb) was estimated in the red supernatant (nodule cytosol) obtained after centrifugation of homogenates, using a fluorometric method as described by La Rue and Child (1979). Bovine hemoglobin was used as a standard.

Nitrogen fixation assay

Nitrogen fixation (nitrogenase: EC 1.18.6.1) was measured as acetylene reduction activity (Hardy et al., 1968). Nodules were enclosed in 100 ml bottles sealed with rubber stoppers containing C_2H_2 (10% v/v) in air. Gas samples (0.5 ml) were taken 60 min later and analyzed for ethylene in Konik 3000 HRGC chromatograph (Konik Inc., Tokyo, Japan) equipped with a hydrogen flame ionization detector (Hewlett Packard fused silica capillary HP-Plot Al_2O_3 column; oven temperature 120 °C; carrier gas: N_2 at a rate of 30 ml min^{-1})

Ferritin content determination

For ferritin assay the homogenate was prepared using 0.3 g of tissue in 3 ml of ice-cold 10 m*M* HEPES pH 7.9 solution containing 10 m*M* KCl and 0.5 m*M* dithiothreitol. Homogenates were centrifuged at 10000 × *g* for 5 min. Standard horse ferritin diluted in the range of 0.25–15 ng/50 μl Tris-Na (TS) and homogenates (diluted to approximately 50 μg protein/50 μl TS) were applied in triplicate onto nitrocellulose membranes (MSI, Westboro, MA) presoaked in TS using vacuum dot blot as described by Roskams and Connors (1994). Briefly, membranes were blocked for 1 h at 25 °C with 3% w/v Molico instant non-fat dry milk in TS, rinsed for 5 min three times with TS and incubated overnight at 4 °C with primary antibody: rabbit anti-horse ferritin. Membranes were rinsed and incubated with second antibody (peroxidase conjugated, goat anti-rabbit inmunoglobulins) for 1 h at 25 °C, rinsed again for 5 min three times with TS and developed with a solution containing α-chloro-naphthol in methanol and hydrogen peroxide. Blots were photographed in a Fotodyne equipment and analyzed with GelPro software.

Catalytic iron determination

Homogenates were prepared as described in ferritin content. After the precipitation of protein with 2.8 *M* HCl for 1 h, the supernatant (1 ml) was incubated with 20 μl of 2% hydroquinone and 20 μl of 1% o-phenanthroline and optical density at 505 nm was determined. A standard curve was generated based on the absorbance of standard solution of ferrous sulfate at pH 3.0.

Protease activity

Extracts for determination of protease activity were prepared from 0.2 g of plant material homogenized under ice-cold conditions in 2 ml of extraction buffer containing 50 m*M* phosphate buffer (pH 7.4), 1 m*M* EDTA and 1 g PVP at 4 °C. Protease activity was measured according to the method of Weckenmann and Martin (1984), using azocasein as substrate. Absorbance of the azo-dye released was measured at 340 nm and one unit of activity was defined as the activity producing an increase of 0.01 units of absorbance during 1 h incubation.

Ammonium determination

Plant material (0.5 g of nodule) was homogenized with 5 ml of 0.3 m*M* H_2SO_4, and centrifuged at 15000 × *g* for 15 min. Ammonium content was measured in the supernatants by the phenol-hypochlorite method (Weatherburn, 1967). A calibration curve with NH_4Cl was used as standard.

Ethylene determination

Nodules were placed in 10 ml gas-tight glass vessels and incubated at room temperature for 14 h. One ml sample of gas was removed and analyzed with a gas chromatograph (Konik 3000 HRGC, Japan) equipped with an alumina column and a flame ionization detector at 120 °C.

Glutamate dehydrogenase activity

Extracts for determination of glutamate dehydrogenase (EC 1.4.1.2.) activity were prepared from 0.3 g of nodules, homogenized in 3 ml of extraction buffer containing 100 m*M* MES-NaOH buffer (pH 6.8), 100 m*M* sucrose, 2% (v/v) 2-mercaptoethanol, and 15% (v/v) ethyleneglycol at 4 °C. The homogenates were centrifuged at 10000 × *g* for 20 min and the supernatant fraction was used for the assays. GDH was determined in the homogenates by measuring the decrease in absorption at 340 nm due to NADH oxidation, in a reaction medium containing 100 m*M* potassium phosphate buffer (pH 7.6), 0.1% 2-mercaptoethanol, 100 μ*M* NADH, 2.5 m*M* 2-oxoglutarate and 200 m*M* $(NH_4)_2SO_4$ (Groat and Vance, 1981).

Native PAGE, peroxidase activity staining and peroxidase activity

Extracts were prepared using 50 m*M* potassium phosphate (pH 7.4), 1 m*M* EDTA, 1 g PVP, 0.5% v/v Triton X-100. Equal amounts of protein from the different nodule extracts were subjected to discontinuous PAGE under non-denaturing, non-reducing conditions as described by Laemmli (1970), except that SDS was omitted. Electroforetic separation was performed at 4 °C for 3 h with a constant current of 30 mA per gel. Gels were stained for POD isozymes (Moore et al., 1978) and were photographed in a Fotodyne equipment and analyzed with GelPro software. For to quantify the levels of peroxidases, the activity was determined in

the homogenates by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol (extinction coefficient 26.6 mM^{-1} cm^{-1}) in a reaction contained extract, 50 mM K-phosphate buffer pH 7.0, 0.1 mM EDTA, 10 mM guaiacol and 10 mM H_2O_2.

Protein determination

Protein concentration was evaluated by the method of Bradford (1976), using bovine serum albumin as a standard.

Electron microscopy

Root nodules electron microscopy was performed in a Ziess Microscope (West Gernow). Samples were contrasted with uranyle acetate and lead citrate.

Statistics

Values in the text and tables indicate mean values ± S.E. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

Results

Oxidative stress generation and its effect on leghemoglobin content and nitrogen fixation

TBARS formation in plants exposed to adverse environmental conditions is a reliable indicator of free radical formation in the tissues. The TBARS content remained unaltered in nodules treated with 50 μM Cd(II) while 200 μM Cd(II) produced an increase close to 55% (Table 1). Leghemoglobin content and nitrogenase activity were measured as indicators of nodules effectiveness under oxidative stress conditions. Both parameters showed similar behavior at the two cadmium concentrations with a decrease in Lb and nitrogenase activity of 79% and 60%, respectively, for 200 μM Cd(II), and no changes for 50 μM cadmium, with respect to control values (Table 1).

Effect of cadmium stress on iron and ferritin content

Oxidative stress caused the breakdown of leghemoglobin. It could therefore be expected that if cadmium induced the formation of oxidant species it would also affect nodule free iron content. However, as shown in Figure 1, an opposite relation between ferritin and

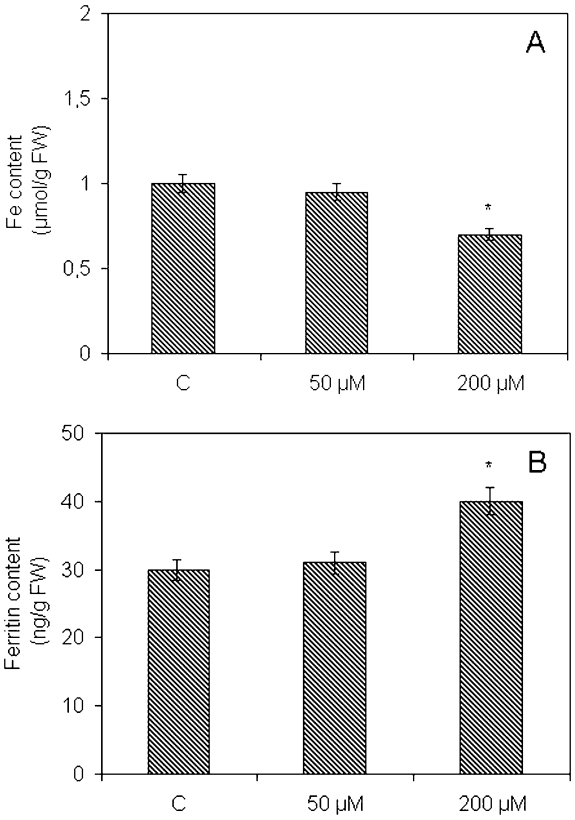

Figure 1. Effect of cadmium treatments on nodules iron (A) and ferritin (B) content. Values are the means of three different experiments with five replicated measurements, and bars indicate S.E. *Significant differences ($P < 0.05$) according to Tukey's multiple range test.

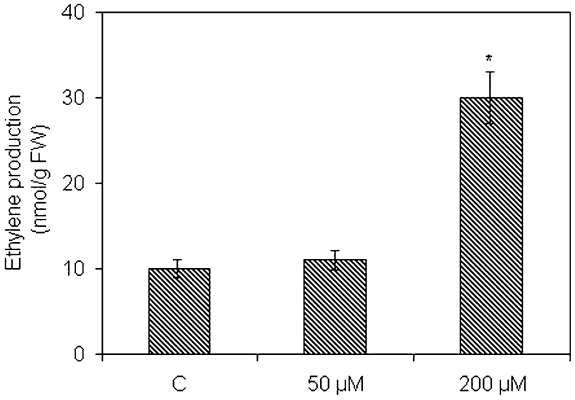

Figure 2. Effect of cadmium treatments on nodule ethylene content. Values are the mean of three different experiments with five replicated measurements, and bars indicate S.E. *Significant differences ($P < 0.05$) according to Tukey's multiple range test.

Table 1. Effect of cadmium ion treatments on nodule TBARS and leghemoglobin content and on nitrogenase activity. Data are the means ± S.E. of three different experiments with five replicated measurements. *Significant differences ($P < 0.05$) according to Tukey's multiple range test

Treatment	TBARS (nmol/g FW)	Leghemoglobin content (mg/g FW)	Nitrogenase activity (μmol ethylene/h/g FW)
Control	99.2 ± 9.1	2.10 ± 0.21	4.1 ± 0.2
50 μM	98.5 ± 9.2	1.95 ± 0.18	3.9 ± 0.2
200 μM	156.2 ± 10.3*	0.45 ± 0.03*	1.6 ± 0.1*

free catalytic iron levels was observed. An iron content decrease by about 30% (Figure 1A) and a 33% increase in ferritin levels (Figure 1B) was observed under 200 μM Cd(II) treatment, compared to controls. No changes in the two parameters were found for 50 μM cadmium, with respect to control values (Figure 1A and B).

Effect of cadmium treatments on appearance of senescence parameters

In order to assess the senescence parameters, ethylene production and ammonium content were evaluated. Nodules subjected to 200 μM Cd(II) shown a 2-fold increase in ethylene content, respect to controls (Figire 2). Likewise, ammonium levels were increased (190%) at the same cadmium concentration (Table 2). Two possible source of increased ammonium, GDH and protease activities were measured. While GDH activity was increased by 50 and 200 μM Cd(II) concentrations (35% and 200%, respectively, over the controls), protease activity was 50% increased only under the higher cadmium treatment (Table 2).

Effect of cadmium treatments on peroxidase activity and isoforms

As shown in Figure 3 peroxidase activity was decreased by about 40% at the higher cadmium concentration, while no change in the enzyme activity was found for 50 μM cadmium. Using the activity staining on native gels, control nodules showed four POD isoforms (r2, r6, r8 and r9) which were altered only by the lower cadmium concentration with disappearance of all isoforms present in control nodules, and the concomitant appearance of new isoforms (r1, r3, r4, r5 and r7) (Figure 4). Under the higher cadmium concentration the isoforms detected were similar to those found in control nodules (Figure 4).

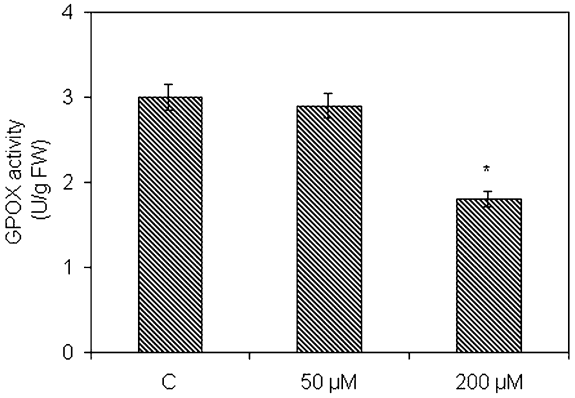

Figure 3. Effect of cadmium treatments on nodule peroxidase activity. Values are the mean of three different experiments with five replicated measurements, and bars indicate S.E. *Significant differences ($P < 0.05$) according to Tukey's multiple range test. One unit of POD forms 1 μmol of guaiacol oxidized per min under the assay conditions.

Table 2. Effect of cadmium ion treatments on nodule ammonium content, and on glutamate dehydrogenase and protease activities. Data are the means ± S.E. of three different experiments with five replicated measurements. *Significant differences ($P < 0.05$) according to Tukey's multiple range test. One unit of GDH oxidize 1 μmol of NADH per min under the assay conditions

Treatment (nmol/g FW)	NH_4^+ content (U/g FW)	GDH (U/g FW)	Protease
Control	40.2 ± 4.1	0.20 ± 0.01	0.10 ± 0.01
50 μM	39.5 ± 3.2	0.27 ± 0.02*	0.09 ± 0.01
200 μM	116.2 ± 10.3*	0.60 ± 0.01*	0.15 ± 0.01*

Nodule electronic microscopy

To complement the biochemical result, nodule ultrastructure was examined during cadmium-induced stress by electron microscopy. The control infected cells were densely packed with symbiosomes, enclos-

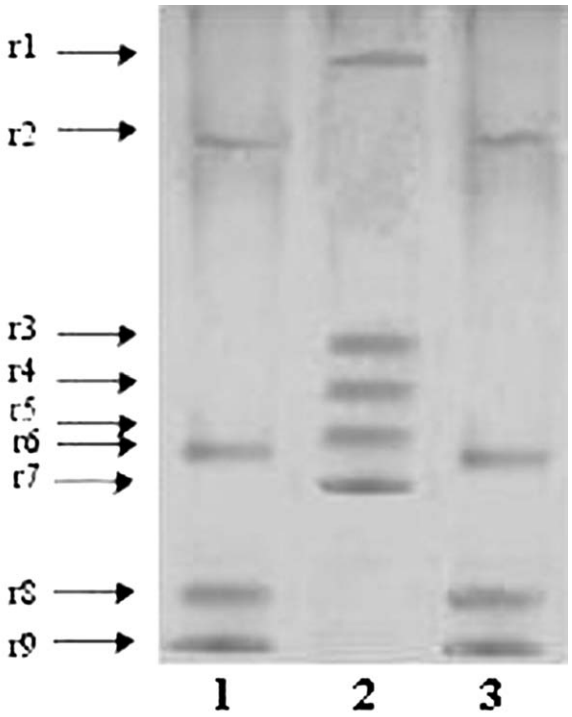

Figure 4. Effect of cadmium treatments on peroxidase isoforms. Proteins (4 μg protein per well) were separated by native-PAGE (10% polyacrylamide gels) and stained for peroxidase activity as described in Materials and methods. Lane 1: control, lane 2: 50 μM Cd(II), lane 3: 200 μM Cd(II).

ing 3–4 bacteroids (Figure 5A). The ultrastructure of the nodules treated with cadmium revealed a reduction of the bacteroids number per symbiosome. In nodules treated with 50 μM Cd this number decreased (Figure 5B), and at 200 μM Cd only a single bacteroid per symbiosome was observed (Figure 5C). The host cell cytosol appeared slightly dense to the electrons, and the intercellular space was largely extended.

Discussion

Cadmium causes oxidative damage and hence affects nitrogen fixation and assimilation in roots and nodules of soybean plants (Balestrasse et al., 2001; Balestrasse et al., 2003). In this study the effects of cadmium on senescence paramaters in nodule of soybean plants were examined. In addition, several senescence inductors were reported. As far as it is known, there is scanty information about the relationship between cadmium stress and senescence in symbiotic tissues. The cadmium concentrations used in the present work

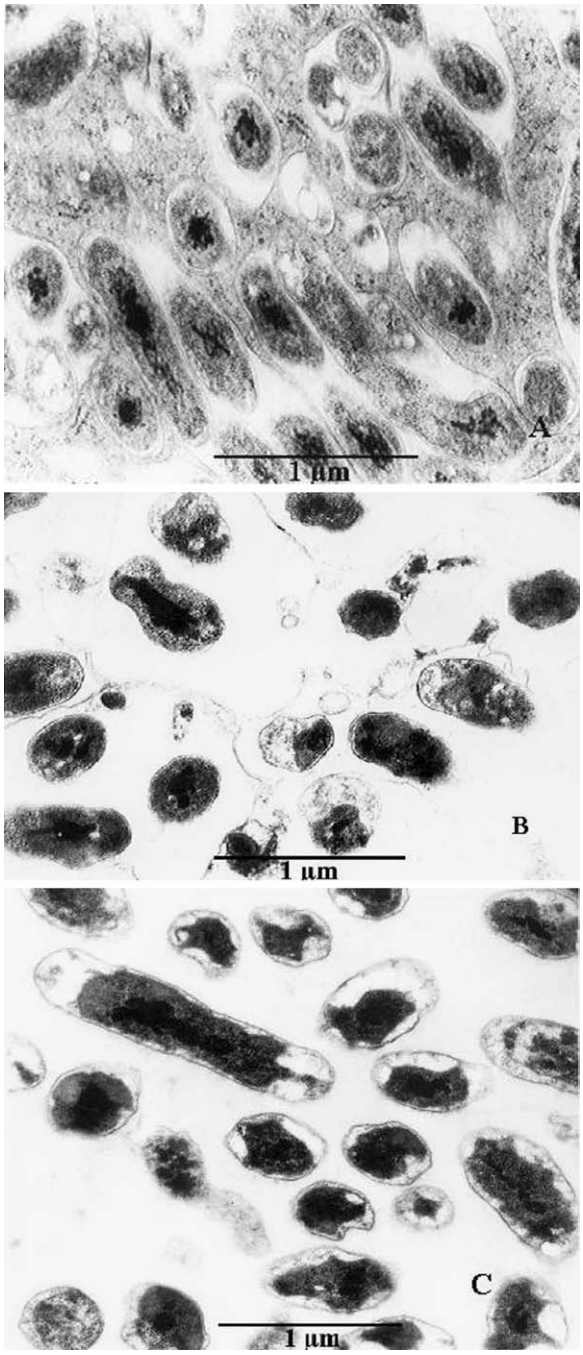

Figure 5. Electron micrographs showing the effect of cadmium treatments on soybean nodules. A, control infected cells; B, infected cells subjected to 50 μM Cd; C, infected cells treated with 200 μM Cd.

are similar to the levels observed in soils under different cadmium contamination conditions (Chaudhry et al., 1998; Kabata-Pendias, 2001; Schwartz et al., 2001). Besides, concentrations of 25 and 100 μM Cd (II) produced similar results to obtained using 50 μM cadmium concentration (data not shown).

Macromolecules, such as membrane lipids, proteins and nucleic acids, are broken down during senescence. Leaf senescence in particular may involve degradation of chlorophyll (Fang et al., 1998), nucleic acid (Buchanan-Wollaston, 1997), membranes (Trippi and Thimann, 1983) and protein (Lutts et al., 1996). The degradation of lipid and protein are probably the most significant breakdown process. In nodules treated with cadmium we observed increases in TBARS production and protease activity (Tables 1 and 2). In artificially senescing parsley leaves, protease activity increased considerably in parallel to the advance of senescence and enhanced by acceleration of senescence with ethylene (Jiang et al., 1999).

Ethylene has been shown to promote early plant senescence. The production of ethylene is tightly regulated by internal signals during development and in response to environmental stimuli from biotic (e.g., pathogen attack) and abiotic stresses. Among the environmental stresses, such as ozone, UV irradiation, and wounding, stimulation for ethylene synthesis has been reported to involve the generation of reactive oxygen species that cause damage to cellular organelles by lipid peroxidation (Surplus et al., 1998; Orozco-Cardenas and Ryan, 1999; Pellinen et al., 1999). In addition, ROS, in particular hydrogen peroxide, have been shown to function as signaling molecules (Levine et al., 1994). Our results indicate that, in nodules, cadmium stimulated oxidative stress generation and ethylene production. In *Phaseolus vulgaris*, Cd stimulated ethylene production and increased activity of guaiacol peroxidase (Sanita di Toppi and Gabbrielli, 1999). The stimulation of ethylene production in soybean nodules during the high Cd stress decreased peroxidase activity (Figure 3). Moreover, at the lower cadmium concentration changes in the isoforms pattern were observed (Figure 4), even though at the higher cadmium treatment this pattern was not altered. These results could be indicating that under moderate cadmium stress the nodules were able to counteract the cadmium-induced damage by induction of new POD isoforms absent in control nodules, and consequently the enzyme activity was similar to control values. At the higher cadmium concentration, POD isoforms pattern remains unaltered and therefore Cd-induced oxidative stress and senescence was observed in 200 μM Cd(II)-treated nodules.

Leghemoglobin content decreased with the higher cadmium treatment, and a consequent decrease in nitrogen fixation was obtained. A positive correlation exists between leghemoglobin content and nitrogenase activity (Dakora, 1995; Comba et al., 1998). Accordingly, in the present study both parameters vary in parallel (Table 1). The inhibition of acetylene reduction activity by 200 μM Cd(II) treatment could result from the effect of the heavy metal ions on bacteroid O_2 uptake, because bacteroid respiration provides the energy and reducing power that nitrogenase needs for efficient nitrogen fixation, similar to that which takes place under salt stress (Fernández-Pascual et al., 1996). Reactive oxygen species are involved in leghemoglobin breakdown. In dark-induced and natural nodule senescence (aging) in lupin (*Lupinus albus* L.) (indeterminate nodules) the elevated catalytic Fe content preceded the diminution of Lb and the increase in ferritin levels (Hernandez-Jimenez et al., 2002). In the present work, cadmium altered catalytic iron diminishing its concentration 48 h after the beginning of the experiment. Ferritin content showed an opposite behavior and therefore was increased. The ferritin accumulation may be interpreted as an antioxidant response (Lucas et al., 1998), thus indicating that Fe is not responsible for the oxidative damage generated by cadmium.

Another senescence indicators such as ammonium levels and GDH activity were increased (Table 2). Ammonium is released from plant tissues during physiological senescence (Loulakakis et al., 1994). Previous results obtained in our laboratory demonstrated that high cadmium concentrations (200 μM) induce oxidative stress in soybean nodules and roots (Balestrasse et al., 2001), and it is well known that oxidative stress induces senescence (Sandalio et al., 2001). Therefore, generation of oxidative stress could be responsible for the increased ammonium levels in soybean nodules treated with 200 μM Cd. Besides, the enzyme GDH catalyses a reversible enzymatic reaction involving the assimilation of ammonium into glutamate and the deamination of glutamate into 2-oxoglutarate and ammonium (Lancien et al., 2000). The induction of GDH activity observed in this study (especially by 200 μM cadmium), could be attributed to a higher amino acid catabolism, which resulted in increased ammonium concentration. Accordingly, Syntichaki et al. (1996) suggested a stress-related

function of GDH. Moreover, in maize, NH_4^+ induced the isomerization of GDH (Srisvastava and Singh, 1987), as well as lead, suggesting that this enzyme act as a sensor in monitoring environmentally-induced stress (Osuji et al., 1998).

The electron microscope investigation (Figure 5) revealed that cadmium treatment altered the ultrastructure of soybean nodules. Despite the increase in lipid peroxidation, disruption of symbiosome was not observed. However, the effective area for N_2-fixation in the nodule was reduced as the Cd concentration increased, as were the number of N_2-fixing cells inside the nodule. These results jointly with the significant decrease observed in nitrogenase activity, allow us to conclude that the number of bacteroids per symbiosome, and their N_2-fixing ability were both reduced by cadmium stress.

In conclusion, taking together, the present results clearly demonstrate that the high cadmium treatment caused oxidative stress, which in turn provoked the appearance of senescence indicators in nodules of soybean plants. Time course studies will be necessary in order to assess more precisely the role that Cd toxicity plays on nodule senescence.

Acknowledgements

This work was supported by grants from Universidad de Buenos Aires (Argentina) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (Argentina). M.L.T. is career investigator from CONICET.

References

Balestrasse K B, Benavides M P, Gallego S M and Tomaro M L 2003 Effect of cadmium stress on nitrogen metabolism in nodules and roots of soybean plants. Funct. Plant Biol. 30, 57–64.

Balestrasse K B, Gardey L, Gallego S M and Tomaro M L 2001 Response of antioxidant defence system in soybean nodules and roots subjected to cadmium stress. Aust. J. Plant Physiol. 28, 497–504.

Bradford M M 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Buchanan-Wollaston V 1997 The molecular biology of leaf senescense. J. Exp. Bot. 48, 181–199.

Chaudhry T M, Hayes W J, Khan A G and Khoo C S 1998 Phytoremediation-Focusing on accumulator plants that remediate metal-contaminated soils. Aust. J. Ecotoxicol. 4, 37–51.

Comba M E, Benavides M P and Tomaro M L 1998 Effect of salt stress on antioxidant defence system in soybean root nodules. Aust. J. Plant Physiol. 25, 665–671.

Dakora F D 1995 A functional relationship between leghaemoglobin and nitrogenase based on novel measurements of the two proteins in legume root nodules. Ann. Bot. 75, 49–54.

Das P, Samantaray S and Rout G R 1997 Studies on cadmium toxicity in plants: a review. Environ. Pollut. 98(1), 29–36.

Davies M J and Puppo A 1992 Direct detection of a globin-derived radical in leghemoglobin treated with peroxides, Biochem. J. 281, 197–201.

Fang Z, Bouwkamp J C and Solomos T 1998 Chlorophyllase activities and chlorophyll degradation during leaf senescence in non-yellowing mutant and wild type of *Phaseolus vulgaris* L. J. Exp. Bot. 49, 503–510.

Fernández-Pascual M, de Lorenzo C, de Felipe M R, Rajalakshmi S, Gordon A J, Thomas B J, and Minchin F R 1996 Possible reasons for relative salt stress tolerance in nodules of white lupin cv. Multolupa. J. Exp. Bot. 47, 1709–1716.

Ferreira R R, Fornazier R F, Vitoria A P, Lea P J and Azevedo R A 2002 Changes in antioxidant enzyme activities in soybean under cadmium stress. J. Plant Nutr. 25(2), 327–342.

Groat R G and Vance C P 1981 Root nodule enzymes of ammonia assimilation in alfalfa (*Medicago sativa* L.). Plant Physiol. 67, 1198–1203.

Hardy R W, Holstein R D, Jackson E K and Burns R C 1968 The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol. 43, 1185–1207.

Heath R L and Packer L 1968 Photoperoxidation in isolated chloroplasts. I. Kineties and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198.

Hernadez-Jimenez M J, Lucas M M and de Felipe M R 2002 Antioxidant and damage in senescing lupin nodules. Plant Physiol. Biochem. 40, 645–657.

Hoagland D R and Arnon D I 1957 California agricultural experimental station, Circular 347.

Jiang W B, Lers A, Lomaniec E and Aharoni N 1999 Senescence-related serine protease in parsley. Phytochemistry 50, 377–382.

Kabata-Pendias A 2001 Trace elements in soil and plants. 3rd. ed. CRC Press, Boca Raton, Florida, USA. 432 p.

La Rue T A and Child J J 1979 Sensitive fluorometric assay for leghemoglobin. Anal. Biochem. 92, 11–15.

Laemmli U K 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lancien M, Gadal P and Hodges M 2000 Enzyme redundancy and the importance of 2-oxoglutarate in higher plant ammonium assimilation. Plant Physiol. 123, 817–824.

Levine A, Tenhaken R, Dixon R and Lamb C 1994 H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79, 583–593.

Loulakakis K A, Roubelakis-Angelakis K A, and Kanellis A K 1994 Regulation of glutamate dehydrogenase anf glutamine synthetase in avocado fruit during development and ripening. Plant Physiol. 106, 217–222.

Lucas M M, Sype Van de G, Hérouart D, Hernández M J, Puppo A and de Felipe M R 1998 Immunolocalization of ferritin in determinate and indeterminate legume root nodules. Protoplasma 204, 61–70.

Lutts S, Kinet J M and Bouharmaont J 1996 NaCl-induced senescence in leaves of rice (*Oryza sativa* L) cultivars differing in salinity resistance. Ann. Bot. 78, 389–398.

Mhatre G N and Pankhurst C E 1997 Bioindicators to detect contamination of soils with special reference to heavy metals. *In* Biological Indicators of Soil Health and Sustainable Productivity. Eds. C E Pankhurst, B M Doube and V S R Gupta. pp. 349–369. CAB International, New York.

Moore R W, Welton A F and Aust S D 1978 Detection of hemoproteins in SDS-polyacrylamide gels. Meth. Enzymol. 52, 324–331.

Morgan P W and Drew M C 1997 Ethylene and plant responses to stress. Physiol. Plant. 100, 620–630.

Neill S, Desikan R and Hancock J 2002 Hydrogen peroxide signaling. Curr. Opin. Plant Biol. 5, 388–395.

Orozco-Cardenas M and Ryan C A 1999 Hydrogen peroxide is generated systemically in plant leaves by wounding and systemic via the octadecanoid pathway, Proc. Natl. Acad. Sci. USA 96, 6553–6557.

Osuji G O, Haby V A, Beyene A, Madu W C and Mangaroo A S 1998 The isomerization of glutamate dehydrogenase in response to lead toxicity in maize. Biol. Plant. 40(3), 389–398.

Pellinen R, Palva T and Kangasjärvi J 1999 Short communication: Subcellular localization of ozone-induced hydrogen peroxide production in birch (*Betula pendula*) leaf cells. Plant J. 20, 349–356.

Puppo A, Rigaud J and Job D 1981 Role of superoxide anion in leghemoglobine autoxidation. Plant Sci. Lett. 22, 353–360.

Robson R L and Postgate J R 1980 Oxygen and hydrogen in biological nitrogen fixation. Annu. Rev. Microbiol. 34, 183–207.

Roskams A J I and Connors J R 1994 Iron, transferring and ferritin in the rats brain during development and aging. J. Neurochem. 63, 709–716.

Sandalio L M, Dalurzo H C, Gomez M, Romero-Puertas M C and del Rio L A 2001 Cadmium-induced changes in the growth and oxidative metabolism of pea plants. J. Exp. Bot. 52, 2115–2126.

Sanita di Toppi L and Gabbrielli R 1999 Response to cadmium in higher plants. Environ. Exp. Bot. 41, 105–130.

Schwartz C, Gérard E, Perronnet K and Morel J L 2001 Measurement of *in situ* phytoextraction of zinc by spontaneous metallophytes growing on a former smelter site. Sci. Total Environm. 279, 215–221.

Siegel B Z 1993 Plant peroxidases-an organismic perspective. Plant Growth Reg. 12, 303–312.

Srisvastava H S and Singh R P 1987 Role and regulation of L-glutamate dehydrogenase during defence response in maize. Phytochemistry 26, 597–610.

Surplus S L, Jordan B R, Murphy A M, Carr, J P, Thomas B and Mackerness S A H 1998 Ultraviolet-B-induced responses in *Arabidopsis thaliana*: Role of salicylic acid and reactive oxygen species in the regulation of transcripts encoding photosynthetic and acidic pathogenesis-related proteins. Plant Cell Environ. 21, 685–694.

Syntichaki K M, Loulakakis K A and Roubelakisangelakis K A 1996 The amino-acid sequences similarity of plant glutamate dehydrogenase to the extremophilic archaeal enzyme conforms to its stress-related function. Gene 168(1), 87–92.

Trippi V and Thimann K V 1983 The exudation of solutes during senescence of oat leaves. Physiol. Plant. 58, 21–28.

Weatherburn M W 1967 Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. 39, 971–974.

Weckenmann D and Martin P 1984 Endopeptidase activity and nitrogen mobilisation in senescing leaves of *Nicotiana rustica* in light and dark. Physiol. Plant. 60, 333–340.

Section editor: C. Atkins