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Involvement of glutathione and enzymatic defense system against cadmium toxicity in *Bradyrhizobium* sp. strains (peanut symbionts)

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Abstract In this study, the effects of cadmium (Cd) on cell morphology and antioxidant enzyme activities as well as the distribution of the metal in different cell compartments in *Bradyrhizobium* sp. strains were investigated. These strains were previously classified as sensitive (*Bradyrhizobium* sp. SEMIA 6144) and tolerant (*Bradyrhizobium* sp. NLH25) to Cd. Transmission electron micrographs showed large electron-translucent inclusions in the sensitive strain and electron-dense bodies in the tolerant strain, when exposed to Cd. Analysis of Cd distribution revealed that it was mainly bounded to cell wall in both strains. Antioxidant enzyme activities were significantly different in each strain. Only the tolerant strain was able to maintain a glutathione/oxidized glutathione (GSH/GSSG) ratio by an increase of GSH reductase (GR) and GSH peroxidase (GPX) enzyme activities. GSH S-transferase (GST) and catalase (CAT) activities were drastically inhibited in both strains while superoxide dismutase (SOD) showed a significant decrease only in the sensitive strain. In conclusion, our findings suggest that GSH content and its related enzymes are involved in the *Bradyrhizobium* sp. tolerance to Cd contributing to the cellular redox balance.

Keywords Antioxidant enzymes · *Bradyrhizobium* sp. · Cadmium · Glutathione · Tolerance

Abbreviations

CAT	Catalase
Cd	Cadmium
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
MDA	Malondialdehyde
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

Introduction

Cadmium (Cd) is a chemical element that belongs to the heavy metal group that does not have any biological function known and is highly toxic even at low concentration. This metal is introduced into agricultural soils through phosphate fertilizers, sewage sludge, and atmospheric fallout from industrial

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and urban activities (McGrath et al. 1995; Giller et al. 1998). In this way, Cd can not only affect soil microorganisms and plants that have a high economic value but also it can accumulate in organisms, transfer from one trophic level to the next and multiply its concentration along the food chain (De Acevedo 2003). One of the main effect of Cd on microorganisms is the alteration of their oxidative status, increasing the levels of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radicals (·OH) (Stochs and Bagchi 1995; Jamieson 1998; Sandalio et al. 2001). In addition, Cd may generate several celullar modifications: (1) substitution of metal–ligand binding, replacing one metal ion by another at the binding site of a specific biomolecule, thereby altering or destroying the biological function of the target molecule (Nieboer and Fletcher 1996); (2) generation of covalent and ionic reduction–oxidation (redox) reaction of metal ions with cellular thiols (R–SH), in particular glutathione (GSH) (Turner et al. 2001; Zannoni et al. 2007); (3) alteration of growth and cell morphology (Bååth et al. 1998; Lakzian et al. 2002; Khan and Scullion 2002), (4) oxidation of lipids, which results in the permeabilization of the plasma membrane (Howlett and Avery 1997).

Enzymatic and non enzymatic defense systems allow microorganisms to maintain their celullar redox status reducing deleterious effects of Cd (Silver and Phung 2005). GSH is the most abundant intracellular thiol that may complex heavy metals and is involved in ROS sequestration by its oxidation to oxidized glutathione (GSSG) (Sies 1990). This molecule keeps a strong reducing environment in the cell which plays an important role in different stressing conditions such as acidity, salinity, H_2O_2 and methylglyoxal (Sobrevals et al. 2006; Bianucci et al. 2008). On the other hand, several enzymes that are capable to remove ROS participate in the antioxidant defense. The enzymes superoxide dismutase (SOD) and catalase (CAT) are able to detoxify $O_2^{\cdot -}$ and H_2O_2, respectively (Cabiscol et al. 2000). GSH-related enzymes also play a critical rol in defense against ROS: (a) GSH peroxidase (GPX) is a H_2O_2 scavenger that use GSH as a reductant, (b) GSH reductase (GR) reduces GSSG using NADPH as a source of reducing power and maintaining the GSH/GSSG ratio in cells (Cabiscol et al. 2000), (c) GSH-S-transferase (GST) catalyzes the nucleophilic conjugation of GSH with several electrophilic substrates (Vuilleumier 1997).

Studies on the role of antioxidant defense system in soil microorganisms growing in Cd contaminated environment are scarce. In *Rhizobium leguminosarum* E20-8 (Cd tolerant strain), SOD, GPX, GR and CAT activities and GSH level were significantly increased when exposed to 1 mM $CdCl_2$ (Corticeiro et al. 2006). These authors postulated that the tolerance of this strain is not related to a higher efficiency in the oxygen scavengers but to changes in intracellular GSH/GGSG ratios. On the other hand, Pacheco et al. (2008) reported that the addition of 30 μg ml^{-1} Cd to the growth medium of *Escherichia coli gsh*A mutant (unable to produce GSH) reduced its growth and exacerbated the production of ROS. These data are confirming the importance of GSH in preventing Cd induced oxidative damage.

In a previous work, we classified the reference strain *Bradyrhizobium* sp. SEMIA6144 as sensitive to Cd and the native strains *Bradyrhizobium* sp. NLH25 and NOD31 as tolerant to this metal (Bianucci et al. 2011). Sensitive strain was capable to grow up to 10 μM Cd while tolerant isolates up to 30 μM. Furthermore, only the tolerant strains were able to increase the GSH synthesis. Both, sensitive and tolerant strains accumulated high amount of this metal when exposed to the higher concentration of Cd that allowed them to growth. *Bradyrhizobium* sp. is a Gram negative soil bacterium with high agronomic significance since it is able to establish a symbiotic nitrogen-fixing association with peanut plant (*Arachis hypogaea* L.), contributing in this way with the soil fertility (Fabra et al. 2010). Therefore, the aims of this work were to evaluate in *Bradyrhizobium* sp. strains whether Cd produces alteration in cell morphology and antioxidant enzyme activities as well as to determine the Cd distribution in different cellular compartments.

Materials and methods

Bradyrhizobium strains and growth conditions

Bradyrhizobium sp. SEMIA6144 was obtained from MIRCEN (Brazil) and *Bradyrhizobium* sp. NLH25 is a native isolate obtained from Córdoba. Both strains

are slow-growing soil bradyrhizobia, able to infect peanut with the same symbiotic effectiveness (Taurian et al. 2002). The tolerance assay was previously carried out measuring the number of viable cells (CFU ml^{-1}) each 24 h during bacterial growth at different Cd concentration (as Cl_2Cd 2.5 H_2O) in YEM broth (Vincent 1970) and incubated at 28°C on an orbital shaker at 150 rpm (supplementary data Fig. S1). According to this assay, strains were classified as sensitive (*Bradyrhizobium* sp. SEMIA6144) and tolerant (*Bradyrhizobium* sp. NLH25) (Bianucci et al. 2011). Since these strains tolerated different Cd levels, all the experiments were carried out with the maximum Cd concentration that allowed them to grow (10 μM Cd for *Bradyrhizobium* sp. SEMIA6144 and 30 μM Cd for *Bradyrhizobium* sp. NLH25).

Transmission electron microscopy

Bacterial samples were obtained from cultures in late exponential phase of growth by centrifugation for 10 min at 10,000×*g* at 4°C and washed twice with 0.85% sterile NaCl, as previously described (Bianucci et al. 2011). Samples were fixed by immersion in 2.5% glutaraldehyde in 0.1 M Sörensen buffer (pH 7.4) at 4°C for 2 h, and postfixed in 1% phosphate-buffered osmium tetroxide for 1 h. Then they were dehydrated in ascending concentrations of acetone (30–100%) and embedded in EMbed 812 resin. The resin was polymerized for 24 h at 60°C. Semithin sections (thickness, 0.5 mm) were stained with toluidine blue. Ultrathin sections (thickness, 70 nm) were double-stained with uranyl acetate and lead citrate (Jurado et al. 2007). Observations were performed with a transmission electron microscope (Elmiskop 101, Siemens).

Cadmium distribution

The quantity of Cd taken up by *Bradyrhizobium* sp. cells was measured in the late exponential phase of growth, by atomic absorption spectrometry using an inductively coupled plasma-atomic emission spectrometry (ICP-AES) according to Purchase et al. (1997). Distribution of metal was determined in different fractions obtained (loosely and tightly bound to cells and absorbed by cells).

Assessment of oxidative damage

The quantification of lipid peroxidation was estimated by determining MDA (malondialdehyde) reacting to TBA (thiobarbituric acid)-reactive substance following the method described by Steels et al. (1994) with modifications. Bacterial pellets were obtained from cultures in late exponential phase of growth by centrifugation for 10 min at 10,000 *g* at 4°C and washed twice with 0.85% sterile NaCl, as previously described (Bianucci et al. 2011). Samples were resuspended in 50 mM phosphate buffer at pH 7 and 28 μl of trichloroacetic acid (TCA), vortexed, sonicated (width: 80; time: 2 min, pulse, every 6 s) and then centrifugated at 2000×*g* at 4°C for 15 min. Finally, 100 μl of supernatant were resuspended in an assay mixture that contained 100 μl of 0.1 M EDTA and 600 μl of a solution of 1% (w/v) TBA and 50 mM NaOH. Samples were kept in boiling water for 15 min and, after cooling, the absorbance at 532 nm was measured.

Enzymatic assays

Bacterial pellets were obtained as described in assessment of oxidative damage. Pellets were resuspended in 2 ml of extraction buffer (50 mM phosphate potassium buffer, EDTA pH 7.5) and sonicated (width: 80; time: 2 min, pulse, every 6 s). The supernatants were used to determine enzymatic activities. Total protein content was assessed according to Bradford (1976) using bovine serum albumin as standard.

SOD (EC 1.15.1.1) activity was determined according to Beauchamp and Fridovich (1973) using nitro blue tetrazolium (NBT) in the presence of riboflavin. One ml of reaction mixture (0.54 μM EDTA, 75 μM NBT, 777 μM methionine and 50 mM phosphate buffer at pH 7.8), 4 μM riboflavin and 5 μg protein extract were placed under fluorescent light for 15 min. SOD specific activity was determined spectrophotometrically at 560 nm and expressed as units mg^{-1} protein. One unit of SOD activity is defined as the amount of enzyme required to inhibit in a 50% the reduction of NBT.

CAT (EC 1.11.1.6) activity was measured using the method described by Aebi (1984). The assay mixture contained 50 mM phosphate buffer at pH 7.4, 12.5 mM H_2O_2 and 100 μg protein extract. The

reaction was measured by the H_2O_2 decomposition at 240 nm. One unit of CAT is defined as the quantity of enzyme needed to degrade 1 μmol of H_2O_2 min^{-1}.

GPx (EC 1.11.1.9) activity was determined as described by Flohe and Gunzler (1984). The assay mixture in 1 ml contained 100 μl of protein extract, 0.1 M phosphate buffer at pH 7.4, 0.24 U GR and 10 mM GSH. Then 1.5 mM NADPH and 1.5 mM H_2O_2 were added. The reaction was measured following NADPH oxidation at 340 nm. One unit of GPx is defined as the quantity of enzyme needed to produce 1 μmol $NADP^+$ min^{-1}.

GR (EC 1.6.4.2) activity was determined as described by Sheadle and Bassham (1977). The assay mixture in 1 ml contained 300 μl protein extract, 0.5 mM GSSG and reaction buffer (50 mM Tris–HCl, 0.15 mM NADPH, 3 mM $MgCl_2$ at pH 7.5). A reaction without GSSG was performed in order to determine the non specific consumption of NADPH. The reduction of GSSG by GR was followed by the NADPH oxidation at 340 nm. One unit of GR is defined as the quantity of enzyme needed to produce 1 μmol $NADP^+$ min^{-1}.

GST (EC 2.5.1.18) activity was assayed by Habig et al. (1974) technique, measuring GSH conjugates with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay mixture in 1 ml contained 0.1 M phosphate buffer at pH 7, 0.25 mM CDNB, 4 mM GSH and 200 μg protein extract. The enzymatic activity was followed continuously at 340 nm. One unit of GST is defined as the quantity of enzyme needed to produce 1 μmol conjugated CDNB-GSH min^{-1}.

Statistical analysis

Differences among treatments were analyzed by two-way ANOVA, and $P < 0.05$ was considered significant according to Duncan′s test.

Results

Effect of cadmium on *Bradyrhizobium* sp. cell morphology

Transmission electron micrographs of metal-sensitive and tolerant *Bradyrhizobium* sp. strains showed differences in their cell morphology. In control conditions, *Bradyrhizobium* sp. SEMIA6144 showed a compact cellular structure with a low contrast area in cell wall and membrane and also the presence of electron-translucent inclusions in the cytoplasm with a size of about 0.19 μm (Fig. 1a). In Cd presence, these inclusions (0.78 μm) were larger than the control ones and a width increase of the space between cell wall and membrane was observed (Fig. 1b). Without Cd, *Bradyrhizobium* sp. NLH25 showed a well-defined cellular structure with electron-dense bodies in the cytoplasm with a size of about 0.27 μm and a cell wall slightly compacted (Fig. 1c). Similar electron-dense bodies (0.53 μm) were also apparent in cells exposed to Cd but they were greater and with a higher area of contrast than those found in control conditions. Moreover, a high electron-density zone was also found in the cell wall (Fig. 1d). No differences in cell size were found when both strains were exposed to Cd compared to control conditions (data not shown).

Cadmium distribution in *Bradyrhizobium* sp. strains

We have previously found that the total Cd accumulated was 1,278 and 7,669 μg g^{-1} dry weight by *Bradyrhizobium* sp. SEMIA6144 and *Bradyrhizobium* sp. NLH25, respectively. In order to determine the cellular metal distribution, Cd content was analysed in different fractions: loosely bound (extracellular polymers), tightly bound (firmly bound to cell wall) and absorbed (intracellularly bound). Table 1 showed that in both strains the highest amount of Cd was found in the cell wall followed by the intracellular fraction and, a little quantity bounded to extracellular polymers.

Parameters of oxidative stress in *Bradyrhizobium* sp.

In the tolerance assay, the addition of Cd allowed growth of *Bradyrhizobium* sp. SEMIA6144 and *Bradyrhizobium* sp. NLH25 up to 10 and 30 μM of metal, respectively (supplementary data Fig. S1). According to this tolerance assay, *Bradyrhizobium* sp. SEMIA6144 was classified as sensitive strain and *Bradyrhizobium* sp. NLH25 as a tolerant one. In order to study Cd tolerance, the effects of Cd on lipid peroxidation and antioxidant GSH-related enzymes were analyzed.

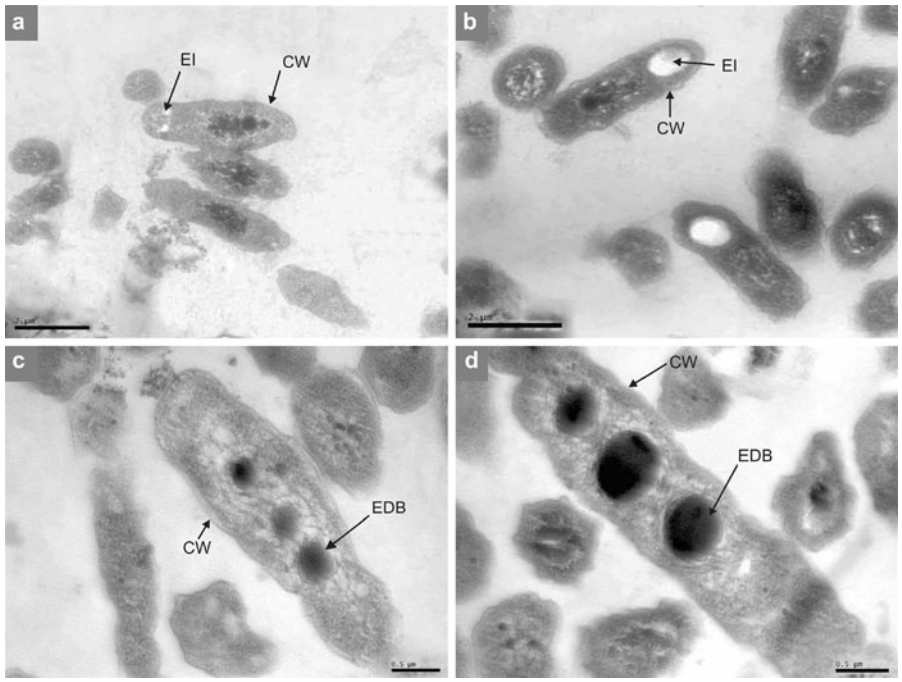

Fig. 1 Transmission electron micrographs of *Bradyrhizobium* sp. strains exposed to Cd. **a** *Bradyrhizobium* sp SEMIA6144 control. **b**. *Bradyrhizobium* sp SEMIA6144 growing with 10 μM Cd. **c**. *Bradyrhizobium* sp NLH25 control. **d**. *Bradyrhizobium* sp NLH25 growing with 30 μM Cd. Abbreviations: *CW* cell wall, *EDB* electron-dense bodies, *EI* electron-transparent inclusions. **a–b** magnification 16700×, *scale bar* represents 2 μm. **c–d** magnification 46460×, *scale bar* represents 0.5 μm

Table 1 Cadmium distribution in different cellular compartments of *Bradyrhizobium* sp

Bradyrhizobium sp. strains	Cd addition	Cd content (μg g^{-1} dry weight)		
	(μM)	Extracellular polymers	Cell wall	Intracellular
SEMIA6144	10	25 ± 0 (2%) a	1,110 ± 51 (87%) b	137 ± 18 (11%) c
NLH 25	30	686 ± 19 (9%) a	6,075 ± 177 (73%) b	784 ± 20 (13%) c

Data represent the mean ± SE ($n = 4$). *Different letters* in each row indicate significant differences ($P < 0.05$) according to the Duncan's test

Quantification of MDA content in *Bradyrhizobium* sp. SEMIA6144 showed a significant increase when exposed to Cd compared to control conditions. This behaviour was not observed in *Bradyrhizobium* sp. NLH25, whose MDA content remained unchanged (Fig. 2).

The determination of GSH-related enzymes (GPX, GR and GST) of *Bradyrhizobium* sp. strains showed different responses to Cd exposure (Fig. 3 a, b, c). When Cd was added, GPX activity was significantly increased in both strains meanwhile GR activity only increased in the tolerant one. GST and CAT was inhibited in both strains while SOD activity decrease only in *Bradyrhizobium* sp. SEMIA6144 (Fig. 4 a, b).

Discussion

The alteration of cell morphology is among the various effects caused by Cd (Bååth et al. 1998; Lakzian et al. 2002; Khan and Scullion 2002). By means of transmission electron microscopy it was observed the presence of electron-transparent inclusions or electron-dense bodies in *Bradyrhizobium* sp.

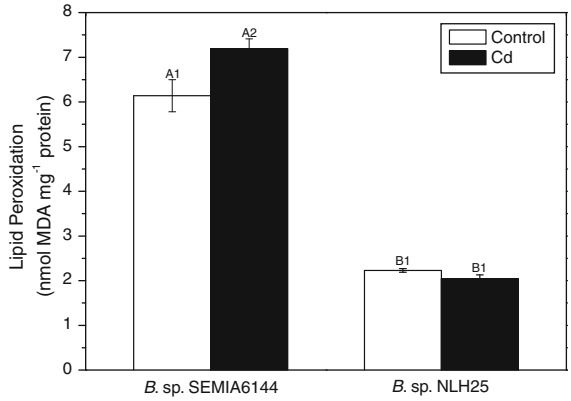

Fig. 2 MDA content in *Bradyrhizobium* sp. SEMIA6144 and NLH25 exposed to 10 and 30 μM Cd, respectively. Data represent the mean ± SE ($n = 4$). *Different letters* in each column indicate significant differences ($P < 0.05$) according to Duncan′s test

SEMIA6144 and *Bradyrhizobium* sp. NLH25, respectively. It is important to point out that an increase in the size of these inclusions was observed in both strains after growing in presence of Cd. It has been proposed for different rhizobial strains (*Rhizobium loti* Lu7, *Bradyrhizobium japonicum* USDA 110, *Rhizobium etli* CE3 and *Rhizobium trifolii* 0403) that the electron-transparent inclusions corresponded to polyhydroxybutyrate (PHB) while electro-dense bodies represent polyphosphate granules (Dazzo et al. 1984; Cevallos et al. 1996; Yang and Lin 1998). Literature about storage compounds in different bacterial strains under stress is scarce, but agrees with the general view that only one type of deposit is found for a given strain. In addition, the size of the inclusions augment in bacteria kept under several harmful environmental conditions (Stainer et al. 1992; Trautwein et al. 2008).

We have previously determined that *Bradyrhizobium* sp. strains accumulated Cd (Bianucci et al. 2011) and in this work the distribution of Cd-bound at different cellular fractions was analyzed. The fact that Cd was mainly found in the tightly bound fraction, corresponding to cell wall, suggests that retention in this compartment could be reducing intracellular Cd concentration being an important avoidance mechanism acting like a primary defense system. Lima et al. (2006) and Siñeriz Louis et al. (2009) demonstrated that the highest Cd concentration was found in the cell wall of *Rhizobium leguminosarum* biovar *viciae* and *Streptomyces* sp. F4, respectively. Other important

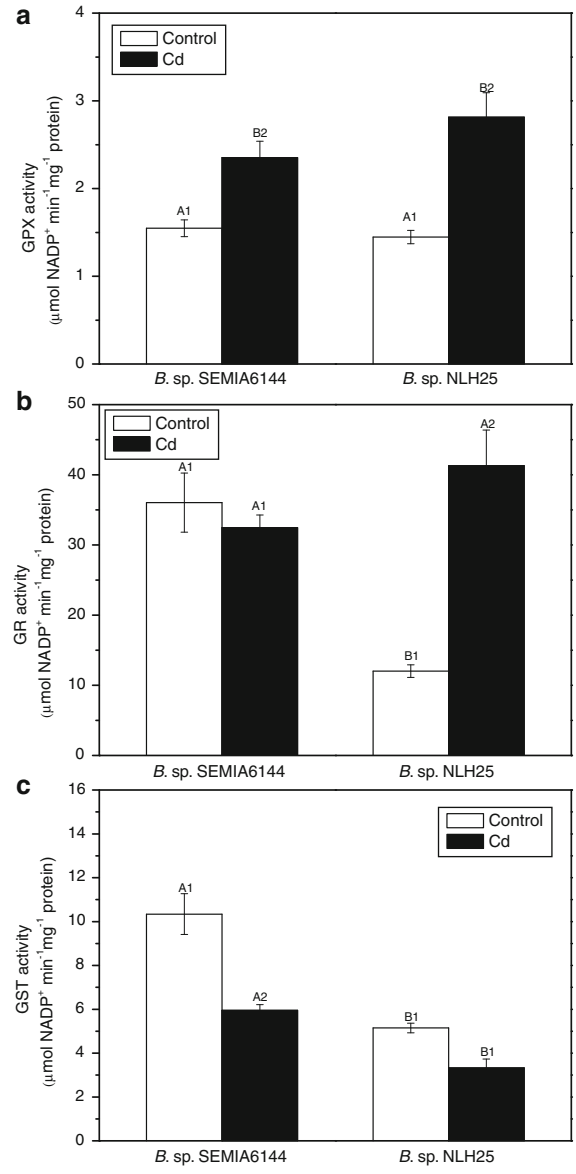

Fig. 3 Activities of GSH related enzymes in *Bradyrhizobium* sp. SEMIA6144 and NLH25 exposed to 10 and 30 μM Cd, respectively. **a** GPX. **b** GR. **c** GST. Data represent the mean ± SE ($n = 4$). Different letters in each column indicate significant differences ($P < 0.05$) according to Duncan′s test

defense mechanisms strategies to avoid Cd toxicity, specially found in bacteria that do not accumulate intracellularly this metal, could be based on efflux pumps or extracellular Cd sequestration (Cervantes and Gutierrez-Corona 1994; Purchase et al. 1997; Bruins et al. 2000). Results obtained in this work suggest that not only the cell wall is involved in *Bradyrhizobium* sp. NLH25 defense against the metal

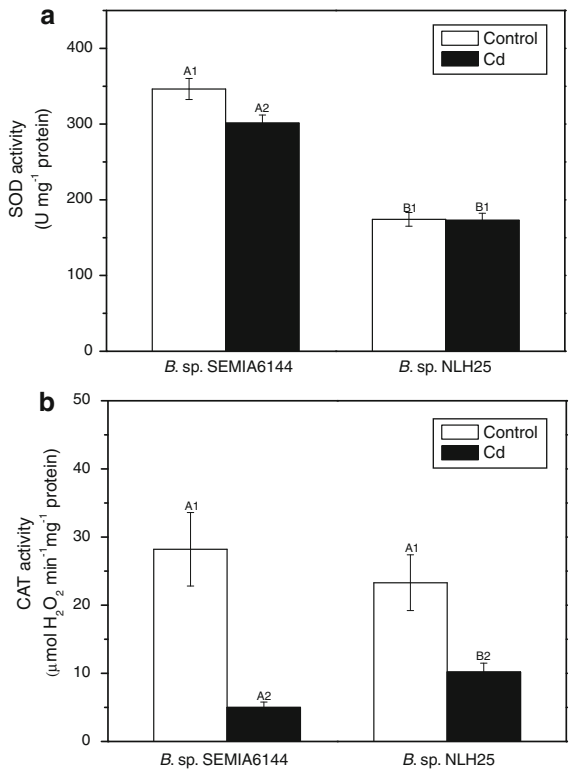

Fig. 4 Activities of SOD and CAT enzymes in *Bradyrhizobium* sp. SEMIA6144 and NLH25 exposed to 10 and 30 μM Cd, respectively. **a** SOD. **b** CAT. Data represent the mean ± SE ($n = 4$). Different letters in each column indicate significant differences ($P < 0.05$) according to Duncan′s test

but also intracellular mechanisms could be implicated in its tolerance, reducing Cd toxic effects. Some of the intracellular defense mechanisms are precipitation of metals as insoluble salt (Blake et al. 1993) or production of chelating agents with sulfur (-SH) groups that involve GSH and enzymes related to this molecule (Nies 1992). We demonstrated the important role of GSH in Cd tolerance in *Bradyrhizobium* sp. strains, in which a significantly increase of this thiol concentration was observed.

Lipids are major targets during oxidative stress. Free radicals can attack directly polyunsaturated fatty acids in membranes and initiate lipid peroxidation. A primary effect of lipid peroxidation is a decrease in membranes fluidity, which alters their properties and can disrupt membrane-bound proteins significantly. This effect acts as an amplifier, more radicals are formed, and polyunsaturated fatty acids are degraded to a variety of products (Cabiscol et al. 2000). In this research, only *Bradyrhizobium* sp. SEMIA6144 (sensitive strain) showed a significantly increase in lipid peroxides at 10 μM Cd. Similarly, Pacheco et al. (2008) showed that the degree of lipid peroxidation augmented significantly in *Escherichia coli* K-12 BW25113 when exposed to 30 μg ml^{-1} Cd.

The role of antioxidant enzymes in rhizobia exposed to heavy metals has not been well-studied yet. In order to evaluate whether CAT, SOD and GSH-related enzymes are involved in Cd tolerance, their specific activities were analyzed. GPX and GR have an important role in GSH/GSSG maintenance being crucial to keep cellular redox status. GPX and GR activities were enhanced in *Bradyrhizobium* sp. NLH25 while an increase in GPX activity was only observed in *Bradyrhizobium* sp. SEMIA6144 exposed to Cd. As only the tolerant strain has the ability to rise GSH levels and also to maintain GSH/GSSG ratio, it is possible that this behavior could be related with its tolerance to Cd, facing oxidative stress generated by this metal. Considering that no differences were observed in GPX activity between Cd sensitive and tolerant strains, this enzyme seems not be involved in tolerance to this metal.

GSTs constitute a protein superfamily which protects cells against oxidative stress, peroxidase and isomerase activities (Allocati et al. 2008). These enzymes may generate GSH conjugated with oxidative stress products as oxidized lipids, proteins and DNA. GSTs have been extensively studied in eukaryotic cells. In *Saccharomyces cerevisiae*, it was demonstrated that GSTs and GSH have an important role in defense against reactive oxygen species as well as in overall cellular detoxification of toxic oxidants such H_2O_2 and arsenic species (Todorova et al. 2007). Studies carried out in the unicellular protist *Euglena gracilis* showed that a moderate decrease in GST activity (<30%) could contribute to Cd accumulation by increasing the level of GSH (Cervantes et al. 2006). In bacteria have been reported that GST activity increased the antibiotic resistance (Piccolomini et al. 1989; Perito et al. 1996). This is one of the first reports about the effect of Cd on rhizobial GST activity. Our findings showed that GST activity was significantly decreased in both strains suggesting that Cd could interact negatively with the enzyme causing its inhibition. Furthermore, the reduced GST enzyme activity observed in *Bradyrhizobium* sp. strains could leave GSH available for the redox reactions and thus minimize the damage caused by the metal.

In this work, it was demonstrated that CAT activity was inhibited in both strains and SOD activity was reduced only in the sensitive strain exposed to this metal. Bučková et al. (2010) studied the effect of different environmental stresses on CAT and o-dianisidine-peroxidase enzyme activities in *Pseudomonas putida* and *Bacillus megaterium,* isolated from oil contaminated soils. These authors reported that CAT activity was drastically inhibited meanwhile a significant increase in o-dianisidine-peroxidase activity was observed. Grant et al. (1998) and Cyrne et al. (2003) reported that the decrease in CAT activity in *Saccharomyces cerevisiae* exposed to Cd could be due to the metal that inhibits the enzymatic activity. Moreover, they indicated that GSH could be related as an alternative or additional mechanism against H_2O_2. Thus, the increase in GPX activity observed in *Bradyrhizobium* sp. strains could be due to inhibition of CAT activity by Cd which allows ROS to detoxify, oxidizing GSH to maintain GSH/GSSG ratio.

In *Bradyrhizobium* sp. NLH25, Cd produced an inhibition of the GST activity while GR and GPX were significantly increased. Probably, the alteration in the cellular thiol redox balance could be due to the oxidative stress indirectly induced by Cd. Chrestensen et al. (2000) demonstrated that 10 μM Cd inhibited thioredoxins, thioredoxin reductase and GR activities in H9 and Jurkat cells. In addition, the inactivation of the thiol transferase pathway could lead to an increase in cellular lipid peroxidation products as a consequence of the low availability of the main electron donors for the thiol and GPX (Vido et al. 2001). Thus, the increase in GR and GPX activity observed in *Bradyrhizobium* sp. NLH25 confirms that the ability of this strain to maintain high levels of GSH, limiting ROS production, could be avoiding the oxidative damage to lipids.

In conclusion, one of the relevant findings of this work is that antioxidant enzyme activities were significantly different in each *Bradyrhizobium* sp. strains. *Bradyrhizobium* sp. cell wall constitutes a primary defense system against Cd toxicity, indicating that it is not a key component involved in tolerance. In contrast, GSH content and the activities of its related enzymes significantly contribute to *Bradyrhizobium* sp. NLH25 tolerance to Cd maintaining cellular redox balance. Further studies will be required to study GSH dynamics and GSH/GSSG redox pair quantification, together with a deeper characterisation of GR and GPX enzymes, under Cd stress.

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