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Influence of cadmium on the symbiotic interaction established between peanut (*Arachis hypogaea* L.) and sensitive or tolerant bradyrhizobial strains

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ABSTRACT

Heavy metals in soil are known to affect rhizobia–legume interaction reducing not only rhizobia viability, but also nitrogen fixation. In this work, we have compared the response of the symbiotic interaction established between the peanut (*Arachis hypogaea* L.) and a sensitive (*Bradyrhizobium* sp. SEMIA6144) or a tolerant (*Bradyrhizobium* sp. NLH25) strain to Cd under exposure to this metal. The addition of 10 μM Cd reduced nodulation and nitrogen content in both symbiotic associations, being the interaction established with the sensitive strain more affected than that with the tolerant one. Plants inoculated with the sensitive strain accumulated more Cd than those inoculated with the tolerant strain. Nodules showed an increase in reactive oxygen species (ROS) production when exposed to Cd. The histological structure of the nodules exposed to Cd revealed a deposit of unknown material on the cortex and a significant reduction in the infection zone diameter in both strains, and a greater number of uninfected cells in those nodules occupied by the sensitive strain. In conclusion, Cd negatively impacts on peanut–bradyrhizobia interaction, irrespective of the tolerance of the strains to this metal. However, the inoculation of peanut with *Bradyrhizobium* sp. NLH25 results in a better symbiotic interaction suggesting that the tolerance observed in this strain could limit Cd accumulation by the plant.

1. Introduction

Living organisms require a low concentration of some heavy metals such as iron, cobalt, copper, manganese (Lane and Morel, 2000), which play a fundamental role in the cells as a part of their biomolecules. However, higher concentration of these metals can be toxic to the cells. In contrast, other heavy metals such as mercury, lead, cadmium, etc., do not have any vital function and are extremely toxic even at low concentrations. Cadmium (Cd) is one of the most harmful heavy metals known in nature (Ibekwe et al., 1995). Accumulation of Cd in soils is a result of several anthropogenic activities, such as the application of phosphate fertilizers and sewage sludge, and represents an important environmental risk (Gratão et al., 2005), which is exacerbated by the half life of this metal (15–30 years) (Henson and Chedrese, 2004; Maruthi et al., 2005). Cd could be accumulated by many organisms, being transferred from one component of the trophic chain to another and multiplying its concentration with time. This can cause serious problems to human safety especially in countries with intense agronomic activity.

Some of the consequences of higher concentrations of heavy metals in soils are associated not only with low rhizobia viability, but also with a low number of nodules and nitrogen fixation deficiency (Alexander et al., 1999; Broos et al., 2004; Younis, 2007). The effects of heavy metals on rhizobia–legume interactions could be direct, by alteration of nodule organogenesis, photosynthetic pigments (Bibi and Hussain, 2005; Wani et al., 2006) and Rubisco activity (Sheoran et al., 1990), or indirect affecting rhizobia viability due to an increase in reactive oxygen species (ROS) inducing

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Table 1
Growth under Cd stress. Shoot and root growth in peanut plants treated with 10 μM Cd, and infected with SEMIA6144 and NLH25 *Bradyrhizobium* strains.

Strains	Dry weight (g plant^{-1})				Length (cm)			
	Shoot		Root		Shoot		Root	
	Cd addition (μM)							
	0	10	0	10	0	10	0	10
SEMIA6144	0.99 ± 0.07 A1	0.42 ± 0.12 A2	0.20 ± 0.04 B1	0.17 ± 0.03 B1	18.55 ± 1.28 A1	11.94 ± 0.50 A2	28.06 ± 1.08 A1	30.00 ± 1.17 A1
NLH25	1.47 ± 0.20 A1	0.77 ± 0.10 A2	0.36 ± 0.03 A1	0.46 ± 0.07 A1	16.57 ± 0.47 A1	13.14 ± 0.61 A2	30.41 ± 0.75 A1	24.80 ± 0.71 B1

Data represent the mean ± SE ($n = 15$). Different letters in each column indicate significant differences between strains in the same treatment. Different numbers in each row indicate significant differences between treatment for each strain ($P < 0.05$) according to Duncan's test.

oxidative stress (Ortega-Villasante et al., 2005, 2007). It is known that Cd affects nitrogen assimilation by inducing oxidative damage on roots and nodules (Balestrasse et al., 2001, 2003). Furthermore, this metal alters nitrogen metabolism (Balestrasse et al., 2003, 2005) and accelerates nodule senescence in soybean plants (Balestrasse et al., 2004). Cd also reduces nodule organogenesis and functionality in a variety of leguminous plants such as pea (Hernández et al., 1995), soybean (Huang and Vanderhoef, 1974), alfalfa (Porter and Sheridan, 1981), bean (Vigue et al., 1981) and lupine (Carpena et al., 2003).

The peanut (*Arachis hypogaea* L.), also known as groundnut, is a leguminous plant of great agricultural and economic importance that can be used as a primary crop or as a source of several food products. Argentina, one of the major peanut producers in the world, concentrates about 93% of its production in the province of Córdoba (Cámara Argentina del maní, 2012). Traditionally, this legume is considered to be nodulated by slow-growing rhizobia of the genus *Bradyrhizobium* (Fabra et al., 2010). The symbiotic association between peanut and rhizobia is especially interesting because unlike other legume–rhizobia symbiosis such as *Sinorhizobium meliloti*–alfalfa and *Rhizobium tropici*–bean there are no cell-to-cell infection threads. Instead, infection through the epidermis involves intercellular penetration (crack entry), at the point of emergence of lateral roots. Therefore, several of the phenomena that are essential in the infection thread mode do not occur in the crack entry infection process. Rhizobia appear to enter the cortical cells through the structurally altered cell walls. Within cortical cells, rhizobia multiply rapidly, and the invaded cells divide repeatedly to form a determinate nodule (Boogerd and Van Rossum, 1997). *Bradyrhizobium* sp. is a Gram negative soil bacterium with high agronomic significance because it is able to establish a symbiotic nitrogen-fixing association with the peanut plant, and enhances soil fertility (Fabra et al., 2010a,b). In a previous study we classified two peanut microsymbionts as either sensitive (*Bradyrhizobium* sp. SEMIA6144) or tolerant (*Bradyrhizobium* sp. NLH25) to Cd (Bianucci et al., 2011). We also found that glutathione (GSH) and related enzymes (glutathione reductase and glutathione peroxidase) had a fundamental role in Cd detoxification in these strains (Bianucci et al., 2012a). In addition, we demonstrated that peanut plants exposed to Cd showed a significant reduction in growth and a high phytochelatins (PCs) induction (Bianucci et al., 2012b).

It was hypothesized that the tolerance of the bradyrhizobial strains to Cd could improve the symbiotic interaction established with peanut plants growing in contaminated soils. Data about the effect of Cd on bradyrhizobia–peanut interaction are scarce, and consequently we decided to analyze the impact of this metal on *Bradyrhizobium* sp.-peanut symbiosis, using a sensitive or tolerant strain to Cd, in a semi-hydroponic system through the determination of symbiotic parameters and stress indices.

2. Materials and methods

2.1. Bacterial strain and growth media

Bradyrhizobium sp. SEMIA6144 was obtained from MIRCEN (Brazil), and *Bradyrhizobium* sp. NLH25 is a native isolate obtained from Córdoba (Taurian et al., 2002). Both strains are slow-growing soil bradyrhizobia, able to infect peanut with the same symbiotic effectiveness (Taurian et al., 2002). Stock cultures were maintained on yeast extract mannitol–agar (YEMA) supplemented with Congo red (Vincent, 1970). The tolerance assay was carried out measuring the number of viable cells (CFU ml^{-1}) each 24 h during bacterial growth at different Cd concentration (as $Cl_2Cd\ 2.5{\cdot}H_2O$) in YEM broth (Vincent, 1970) and incubated at 28 °C on an orbital shaker at 150 rpm. According to this assay, strains were classified as sensitive (*Bradyrhizobium* sp. SEMIA6144) and tolerant (*Bradyrhizobium* sp. NLH25) (Bianucci et al., 2011). Cultures were grown in YEM medium and incubated at 28 °C on a gyratory shaker at 150 rpm for the length of the experiment. Growth was measured turbidimetrically at an absorbance of 620 nm, and the number of viable cells was determined by colony forming units (CFU) using by the drop-plate method on YEMA medium, using bacterial growth after 96 h incubation (Somasegaran and Hoben, 1994).

2.2. Nodulation assays

A. hypogaea L. cv. Granoleico (El Carmen S.A; General Deheza, Córdoba, Argentina) seeds were surface sterilized following the method described by Vincent (1970). Sterilized seeds were

Table 2
Symbiotic nitrogen fixation indexes under Cd stress. Number of nodules, nodule weight and nitrogen content in peanut plants treated with 10 μM Cd and infected with SEMIA6144 and NLH25 *Bradyrhizobium* strains.

Strains	Number of nodules (nodules plant^{-1})		Nodule dry weight (mg plant^{-1})		Nitrogen content (mg plant^{-1})	
	Cd addition (μM)					
	0	10	0	10	0	10
SEMIA6144	45.80 ± 6.62 A1	5.67 ± 0.82 B2	40.01 ± 0.07 A1	2.91 ± 0.02 B2	26.90 ± 0.17 B1	16.98 ± 0.21 B2
NLH25	46.58 ± 7.02 A1	21.00 ± 2.68 A2	30.02 ± 0.03 A1	9.40 ± 0.06 A2	41.10 ± 0.16 A1	24.20 ± 0.13 A2

Data represent the mean ± SE ($n = 15$). Different letters in each column indicate significant differences between strains for a same treatment. Different numbers in each row indicate significant differences between treatment for each strain ($P < 0.05$) according to Duncan's test.

Table 3
Concentration of Cd in peanut plants.

Strains	Cd concentration (μg g^{-1} dry weight)		
	Leaves	Roots	Nodules
SEMIA6144	39.51 ± 3.04 A3	242.33 ± 9.17 A1	65.57 ± 1.14 A2
NLH25	28.81 ± 1.59 B3	169.00 ± 12.42 B1	70.28 ± 4.70 A2

Data represent the mean ± SE ($n = 15$). Different letters in each column indicate significant differences between strains for the same organs. Different numbers in each row indicate significant differences between organs for each strain ($P < 0.05$) according to Duncan's test.

germinated at 28 °C in Petri dishes on a layer of Whatman N° 1 filter paper and moist cotton until the radicle reached 3–5 cm. The individual seedlings were placed aseptically in plastic containers on perlite inert substrate, a semi-hydroponic system in which root development resembled that of plants grown in soils (Vazquez and Carpena-Ruiz, 2005). The plants were watered once a week with Hoagland medium (Hoagland and Arnon, 1950) supplemented with Cd (0 and 10 μM). After 1 week the plants were inoculated with 3 ml *Bradyrhizobium* sp. SEMIA6144 or *Bradyrhizobium* sp. NLH25 strain (10^8 cfu ml^{-1}). Plants were grown in a greenhouse under controlled environmental conditions (light intensity 200 μmol m^{-2} s^{-1}, 16-h day/8-h night cycle, at a constant temperature of 28 °C and relative humidity of 50%) for 30 days. At harvest, plant lengths were measured and the shoots and roots were stored at −80 °C until analysis. The Nitrogen content of the peanut shoots was determined using the method described by Nelson and Sommers (1973).

2.3. Microscopy studies

For anatomical and histological studies, the root system was separated into main and lateral roots. From the main roots, portions of up to 5 mm in length were cut at approximately 1 cm from the of root tip. Roots and nodules were fixed in formol–acetic acid–ethanol–water at 30:5:50:15 v/v, and dehydrated in an ascending series of ethyl alcohol, xylol and finally embedded in histowax. Seriate sections, ranging from 8 to 10 mm thickness, were cut with a rotary microtome and stained with hematoxylin–safranin–fast green and mounted in distyrene, tricresyl phosphate and xylene (DPX) (Johansen, 1940; O'Brien and Mc Cully, 1981). The photomicrographs were taken using an Axiophot Carl Zeiss microscope (Germany).

2.4. Stress indices

Lipid peroxidation analysis was determined by estimating the malondyaldehyde (MDA) content as described by Heath and Packer (1968). Plant material (0.1 g) was homogenized in 20% (w/v) of trichloroacetic acid (TCA) and then mixed with 0.5% (w/v) of thiobarbituric acid (TBA). The extract was heated for 25 min in a cold bath (95 °C). After cooling, the samples were centrifuged for 6 min at 6200 g and the supernatant used to determine lipid peroxide content, measured at 535 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm (Spectrophotometer Spectronic® Genesys 2, USA).

Hydrogen peroxide (H_2O_2) content was measured spectrophotometrically after reaction with KI following the procedure described by Alexieva et al. (2001). The reaction mixture consisted of 0.1% (w/v) TCA root or leaf extract supernatant, 100 mM potassium phosphate buffer and 1M KI in fresh double-distilled water. The control probe consisted of 0.1% (w/v) TCA without root or leaf extract. The reaction was developed for 1 h in darkness and the absorbance measured at 390 nm. The amount of H_2O_2 was calculated using a standard calibration curve prepared with known H_2O_2 concentrations.

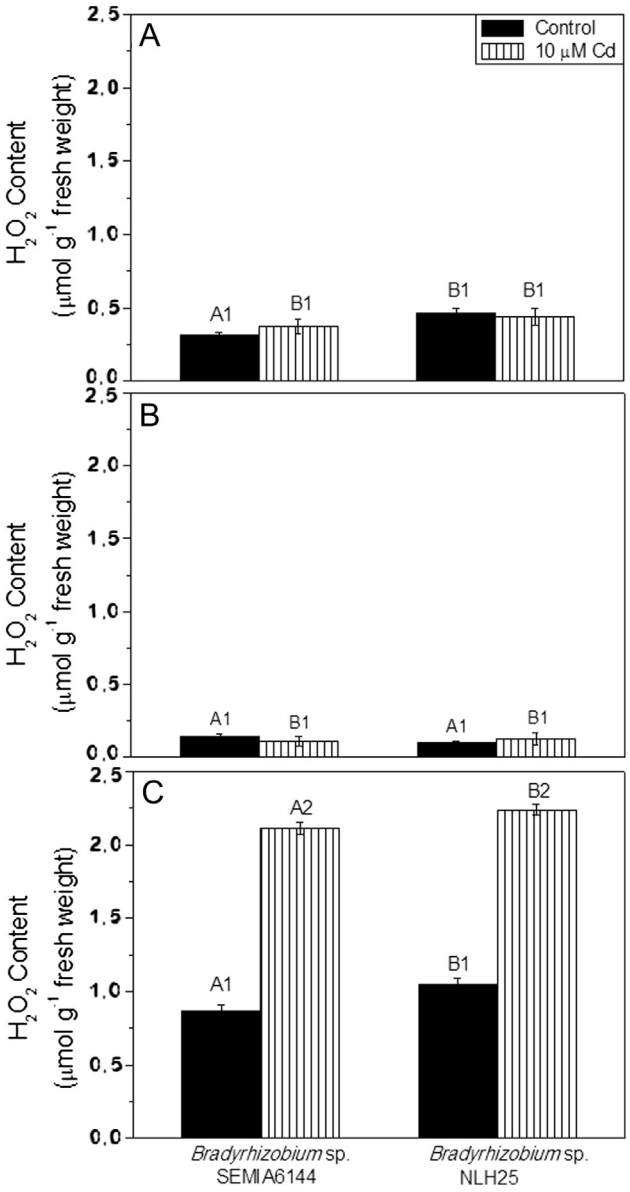

Fig. 1. Hydrogen peroxide content in peanut plants. A: leaves; B: roots; C: nodules. Data represent the mean ± SE ($n = 10$). Different letters in each column indicate significant differences between strains for a same treatment. Different numbers in each column indicate significant differences between treatment for each strain ($P < 0.05$) according to the Duncan's test.

For the detection of H_2O_2, nodules were detached and incubated with 1 mg ml^{-1} diaminobencidine (DAB)-HCl, pH: 3.8 for 8 h in darkness at room temperature. They were then boiled in ethanol, cut longitudinally and examined (Orozco-Cárdenas and Ryan, 1999).

In order to detect O_2^- production, the detached nodules were vacuum-infiltrated with 10 mM potassium phosphate buffer (pH 7.8) containing 10 mM NaN_3 and 0.1% (w/v) nitroblue tetrazolium (NBT) and incubated in darkness for one hour. They were then boiled in ethanol 96% (v/v) for 10 min cut longitudinally and photographed (Doke, 1983). They were examined and photographed using a stereoscopic microscope Stemi SV6, Carl Zeiss (Germany), with a digital Canon camera (China).

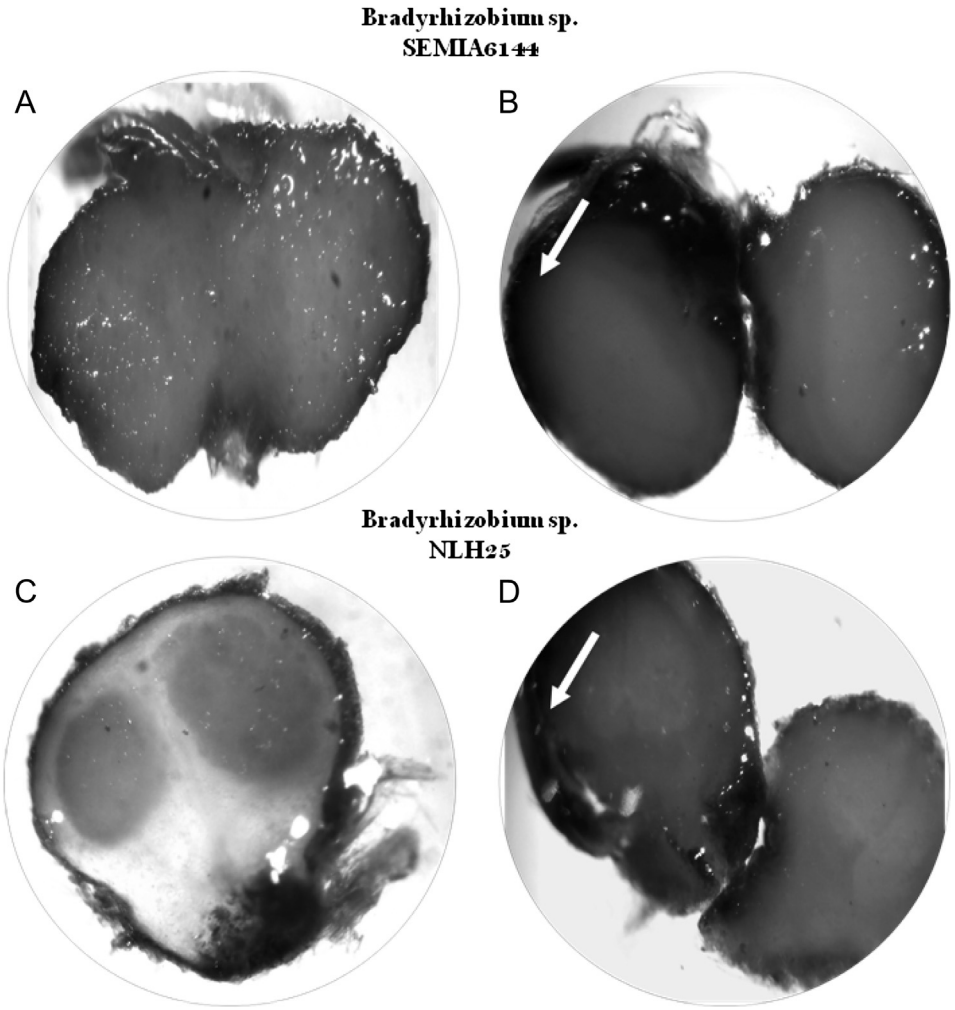

Fig. 2. Hydrogen peroxide production. A and C: Control, B and D: 10 μM Cd. The arrow indicates the brownish deposits resulting from the reaction between DAB and H_2O_2. Magnification 4.0X.

2.5. Cadmium determination in plants

The peanut leaves, roots and nodules that had been exposed to Cd were washed with distilled water and dried at 70 °C for 7 days. Samples were digested in an acid mixture H_2O:HNO_3:H_2O_2 (5:3:2) for 30 min in closed containers under pressure (Lozano-Rodríguez et al., 1997). Analysis of Cd in plant tissues was carried out using inductively coupled plasma mass spectrometry (ICP-MS), Elan 6000, Perkin–Elmer (San Jose, CA, USA).

2.6. Statistical analysis

The differences between treatments were analyzed by two-way analysis of variance, and a P value <0.05 was considered significant according to Duncan's test.

3. Results

3.1. Growth and nodulation of peanut plants exposed to Cd

Plants inoculated with sensitive or tolerant *Bradyrhizobium* sp. strains showed a significant reduction in shoot length and dry weight when exposed to 10 μM Cd when compared to the control plants. Peanut root length and dry weight were not affected by the concentration of this metal (Table 1).

The addition of Cd to the culture media decreased nodule number and dry weight, irrespective of the tolerance of inocula. However, a comparison between strains showed that this reduction was clearly more evident in the interaction established with the sensitive strain *Bradyrhizobium* sp. SEMIA6144 (Table 2).

3.2. Accumulation of Cd in plants inoculated with sensitive or tolerant strains

Cd was found mainly on the roots, followed by the nodules and finally the leaves, regardless of the tolerance of the inoculated strains. The Cd content of the roots and leaves of the plants inoculated with *Bradyrhizobium* sp. SEMIA6144 was significantly higher than that found on plants inoculated with the tolerant strain. Despite this, nodule Cd content did not show any significant change among the tested strains (Table 3). Interestingly, only 29 $\mu g\ g^{-1}$ was retained by the perlite inert substrate at the end of the experiment, which indicates that this substrate leaves Cd available to be absorbed by peanut plants.

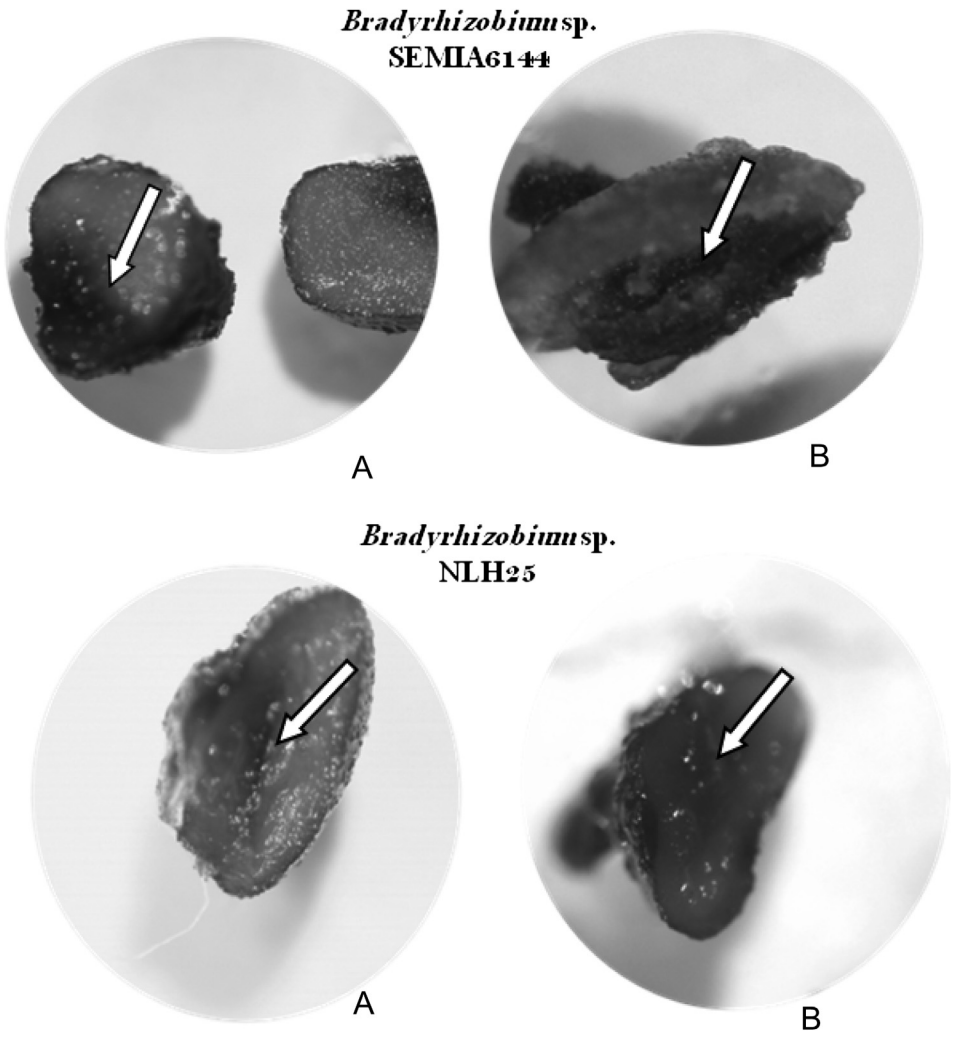

Fig. 3. Superoxide production in nodule. A: Control; B: 10 μM Cd. The arrow indicates formazan precipitated (blue). Magnification 3.2X.

3.3. Stress indicators on peanut plants

The hydrogen peroxide content of the roots and leaves was unchanged by the experiment. However, nodule hydrogen peroxide levels were elevated upon Cd treatment, regardless of the inocula (Fig. 1). Nodule H_2O_2 content was also evaluated by histological observations. H_2O_2 presence was detected through visualization of brownish deposits resulting from the reaction between H_2O_2 and the reactive DAB. Results showed that H_2O_2 deposits were observed only in nodules of inoculated plants exposed to Cd, mainly on nodule cortex zone (Fig. 2).

It was impossible to make a quantitative determination of O_2^- accumulation on nodules because of its rapid conversion to H_2O_2 by superoxide dismutase enzyme activity (Rubio et al., 2004, 2007) and, taking into consideration that a significant increase of H_2O_2 content was detected only in the nodules, we decided to evaluate nodule O_2^- production by histochemical analysis. The detection was determined using NBT reagent which reacts with O_2^- and produces a blue colored product, formazan. Results showed that the nodules treated with Cd and inoculated with a sensitive or a tolerant strain increased O_2^- production. The resulting formazan precipitate was observed mainly on nodule infection zone (Fig. 3). Root, leaf and nodule lipid peroxide content, quantified as MDA, was unchanged in the experiment, irrespective of the tolerance of the inoculated bradyrhizobial strains (Fig. 4).

3.4. Changes in anatomical and histological structure of peanut roots and nodules

In order to determine the effect of Cd on root structure, transverse cuts were made through the root. In the control and treated roots, the epidermis, cortex and vascular tissue could be identified. The epidermis consisted of cells arranged in compact form with a thin wall, the horizontal wall was wider than the vertical wall. Below it, the cortex and vascular tissue were found (Fig. 5A). At 10 μM Cd, the root tissue of inoculated plants with a sensitive or a tolerant strain, was affected by a deposit of an unknown material. In the symbiotic interaction established with the sensitive strain these deposits were found on the endodermis and epidermis (Fig. 5B) compared with plants inoculated with the tolerant strain, which were found only on the epidermis (Fig. 5C).

The anatomical structure of nodules of Cd-treated plants did not reveal any difference between these nodules and those of the control plants (Fig. 6A). However, the nodules of the treated plants showed histological modification of the cortex cells located outside the endodermis, with the deposition of an amorphous material of

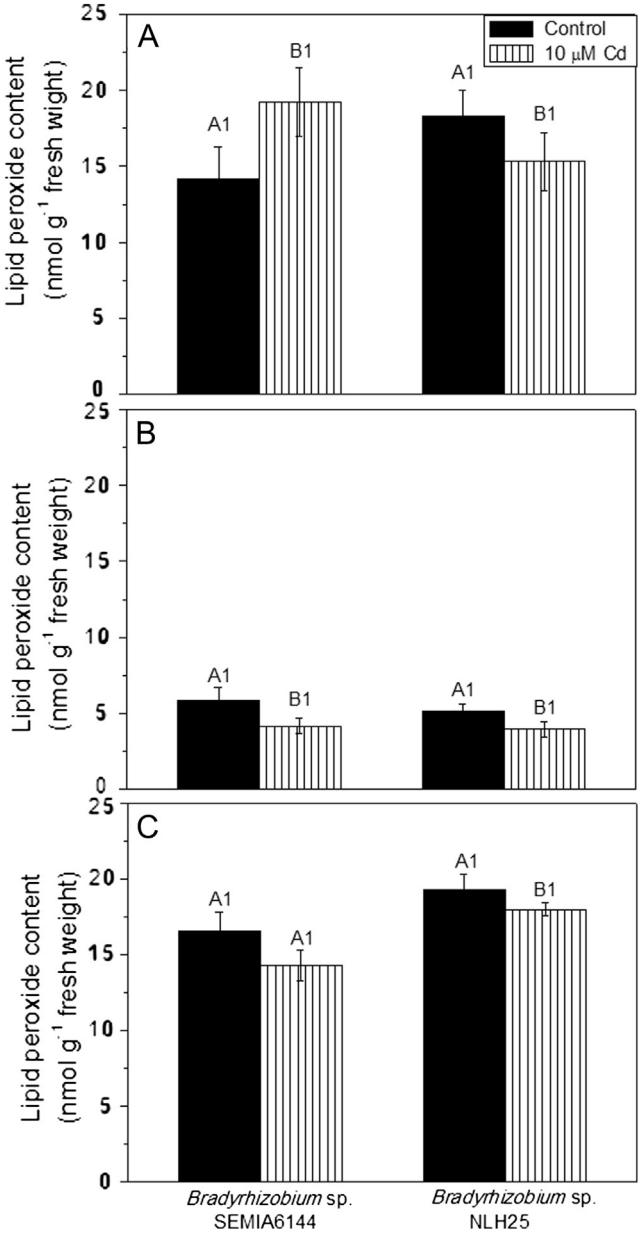

Fig. 4. Lipid peroxide content in peanut plants exposed to cadmium. A. leaves; B. roots; C. nodules. Data represent the mean ± SE (n = 10). Different letters in each column indicate significant differences between strains for a same treatment. Different numbers in each column indicate significant differences between treatment for each strain ($P < 0.05$) according to the Duncan's test.

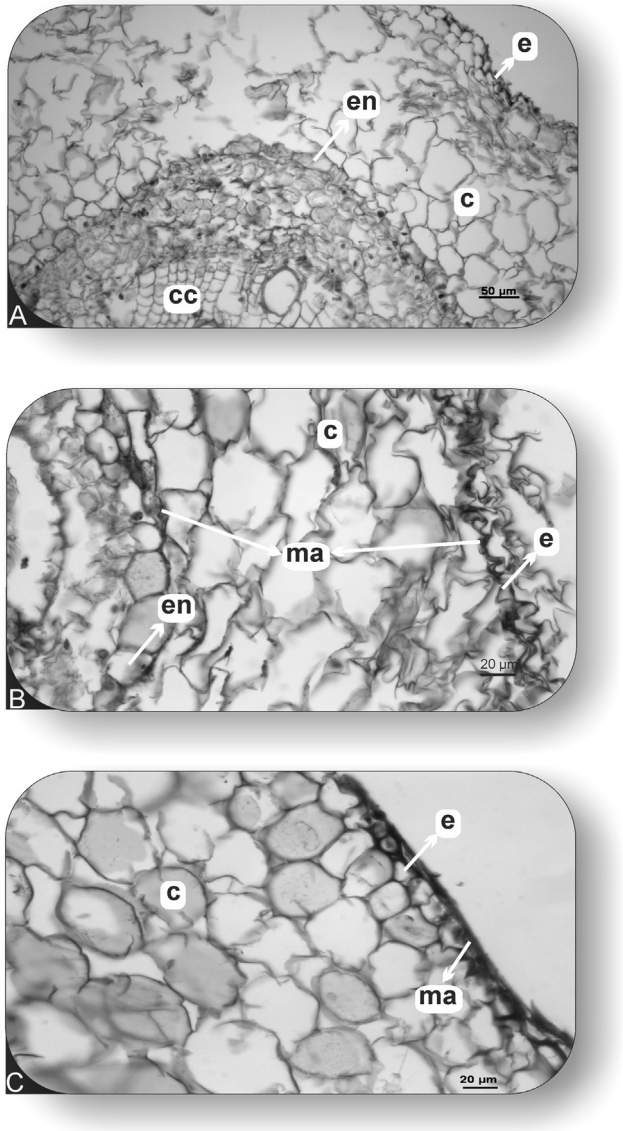

Fig. 5. Light micrographs of cross sections of principal peanut root exposed to Cd. A. control plants inoculated with *Bradyrhizobium* sp. SEMIA6144; B. peanut plant inoculated with *Bradyrhizobium* sp. SEMIA6144 exposed to Cd; C. peanut plant inoculated with *Bradyrhizobium* sp. NLH25 exposed to Cd. e, epidermis; en: endodermis; c: cortex; cc: central cylinder; ma: unknown material.

unknown composition (Fig. 6C). Moreover, those nodules occupied with the sensitive strain showed more uninfected cells than those occupied with the tolerant strain (Fig. 6D). Nodule diameter was not affected by Cd, however, a significant decrease in the diameter of the infection zone was observed, irrespective of the tolerance of the strain that occupied the nodule (Table 4).

4. Discussion

The experiments described in this study investigated the response of peanut plants inoculated with a sensitive or a tolerant bradyrhizobial strain to Cd. Treatment with Cd reduced peanut growth, which concurs with studies carried out on other inoculated legumes (Chen et al., 2002; Carpena et al., 2003), irrespective of the tolerance of the inoculated bradyrhizobial strain. There is some controversy about the effect of Cd on nodulation. A considerable increase in the number of nodules was found by Ibekwe et al. (1995) in their study of the symbiotic interaction established between *Sinorhizobium meliloti* –alfalfa/white or red clover. In *Mesorhizobium*-chickpea interaction, a reduction in the number of nodules of Cd treated plants was observed (Wani et al., 2007). These differences in the symbiotic properties of legumes exposed to Cd could be attributable, not only to the different substrate used, but also to Cd concentration, time of exposure and application of the metal (Sanitá di Toppi and Gabrielli, 1999). Adding Cd to the plant nutrient solution reduced nodule number and dry weight, regardless of the inoculated strain. However, this effect was more evident in peanut plants inoculated with the sensitive strain. Because *Bradyrhizobium* sp. SEMIA6144 could only tolerate up to 10 µM Cd the observation that the symbiotic interaction established with this strain was more affected than that established with the tolerant

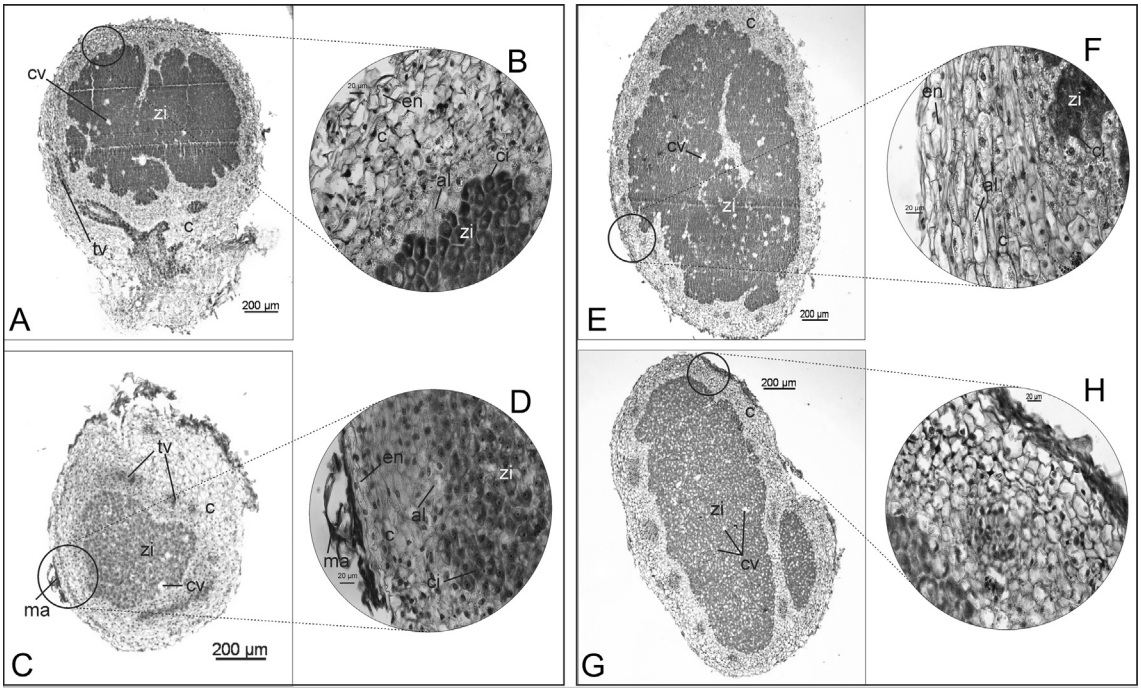

Fig. 6. Cd effects on histological structure of nodules occupied by a sensitive or tolerant strain. A, C (general view) of *Bradyrhizobium* sp. SEMIA6144 infected nodules in control and Cd treated conditions, respectively. B, D (detailed view) for the same conditions. E, G (general view) of *Bradyrhizobium* sp. NLH25 infected nodules in control and Cd treated conditions, respectively. F, H (detailed view) for the same conditions. al: starch, c: cortex, ci: infected cell, cv: non infected cell, en: endodermis, ma: unknown material, tv: vascular tissue, zi: infected zone.

strain, could be explained by a reduction in bradyrhizobial viability, caused by the metal at the time of infection.

The other symptoms caused by the addition of Cd associated with N_2 fixation are its effect on plant infection by bradyrhizobial strains, or its effect on nodule growth and development (Carpena et al., 2003). There are several reports that indicate a reduction in N_2 fixation in Cd treated legumes (Huang and Vanderhoef, 1974; Porter and Sheridan, 1981; Vigue et al., 1981; Hernández et al., 1995). In our work, Cd supply decreased total plant N content, regardless of the tolerance exhibited by the strain. Nevertheless, the inoculation with the tolerant strain resulted in a higher quantity of fixed nitrogen in both the control and treated conditions, than the interaction established with the sensitive strain. Because the plants only source of N was that derived from N_2 fixation by the nodules, the decrease observed here would indicate alterations in nodule function. The distinct response of peanut inoculation of each *Bradyrhizobium* sp. strain revealed that, even in closely related lineages, there were strain-specific variations influencing their behavior when exposed to metal.

With regard to Cd distribution and content: in studies carried out by Chan and Hale (2004) this metal was found mainly on the roots, followed by the stems, the leaves and then the fruit. However, the Cd content in lupin nodules was significantly greater than in roots. Our results reveal that the roots accumulated more Cd than leaves and nodules, which is similar to the findings of Lozano-Rodriguez et al. (1997) and Zornoza et al. (2002) using pea and lupin. The fact that the roots accumulated more Cd than the leaves demonstrates that there was a higher retention by this organ which could be translocated to aerial parts (Gussarsson, 1994; Guo et al., 1995; Zornoza et al., 2002). Conversely, those plants inoculated with the sensitive strain (*Bradyrhizobium* sp. SEMIA6144) accumulated more Cd than those inoculated with the tolerant strain. These results indicated that the metal absorption was highly restricted in peanut-tolerant strain interaction, limiting Cd accumulation in the different organs. Cd substrate content analysis, performed at the end of the experiment, indicated that a small quantity of Cd was retained in the perlite. It has been shown in previous studies that approximately 35% of the Cd present in a nutrient solution of 18 µM Cd was retained in this same substrate (Vázquez and Carpena-Ruiz, 2005). These authors argued that this system is appropriate for middle and long-term experiments, as this latter system more closely reproduces the natural soil conditions of Cd, humidity, root development, etc., in comparison with a hydroponic culture system.

It is well documented that root and leaf H_2O_2 content of non-inoculated plants exposed to Cd is significantly increased (Schützendübel et al., 2001; Schützendübel and Polle, 2002; Olmos et al., 2003; Romero-Puertas et al., 2004). However, the literature on the effect of Cd on O_2^- and H_2O_2 production on nodulated plants is scarce. The results obtained in this work reveal that the nodules of Cd treated plants had an increased ROS production, irrespective of the tolerance of the strains. Since Cd reduced not only the nodule number, but also the nitrogen content, of inoculated plants, it is possible to suggest that increasing ROS affects nodule efficiency in biological nitrogen fixation.

Table 4
Infection zone diameter and nodule diameter under Cd stress.

Strains	Infection zone diameter (µm)		Nodule diameter (µm)	
	Cd addition (µM)			
	0	10	0	10
SEMIA6144	788 ± 55 B1	520 ± 17 A2	1261 ± 92 A1	1149 ± 146 A1
NLH25	1179 ± 99 A1	653 ± 107 A2	1502 ± 130 A1	1110 ± 111 A1

Data represent the mean ± SE ($n = 15$). Different letters in each column indicate significant differences between strains for the same treatment. Different numbers in each row indicate significant differences between treatment for each strain ($P < 0.05$) according to Duncan's test.

The *Bradyrhizobium* sp. strains used in this research are known to accumulate large amounts of Cd when exposed to the metal in the culture media (Bianucci et al., 2011). Therefore, these strains could play a significant role in oxidative burst production on peanut nodules, possibly due to the metal content that they may have accumulated at the time of infection. The oxidative burst induces the oxidative stress promoted by Cd exposure and might cause lipid peroxidation (Hendry et al., 1992). In a previous work, we demonstrated that Cd addition did not modify root and leaf lipid peroxide content of non inoculated peanut plants (Bianucci et al., 2012b). In this work, inoculated peanut plants exposed to 10 μM Cd, did not affect leaf, root and nodule lipid peroxide content. In contrast, it was observed that the addition of 18 and 45 μM Cd did not alter root and leaf lipid peroxide content, but a significant increase in the number of nodules was observed (Carpena et al., 2002). However, in nodulated soybean plants exposed to 200 μM Cd an increase in the root and nodule lipid peroxide content was detected (Ballestrase et al., 2001). Our results differ from those reported by these authors as the lipid peroxide content of Cd-treated peanut plants remained unchanged in comparison with non-treated plants. Furthermore, although an increase of O_2^- and H_2O_2 content was observed in peanut nodules, the level of the increase may not be enough to cause lipid peroxidation. Besides, plant cells activate different antioxidant mechanisms in order to avoid Cd damage, such as phytochelatins production, compartmentalization in cellular organs (Sanitá di Toppi and Gabbrielli, 1999) and immobilization of Cd by cell pectin or carbohydrates (Benavides et al., 2005). Therefore, it is probable that the peanut presented a high peroxidation resistance, as was observed in lupin (Zornoza et al., 2002).

Regardless of the tolerance that *Bradyrizobium* sp. strains exhibited had to metal, Cd-treated plants revealed a deposit of an amorphous material on the dermal tissue. This result was consistent with that obtained in non-inoculated peanut plants (Bianucci et al., 2012b). Plants inoculated with the tolerant strain showed that this material was found mainly on the epidermis unlike that which occurred with plants inoculated with the sensitive strain, where the highest content of this material was found in the endodermis. It is known that the rhizodermis, exodermis and endodermis zones act as barriers to movement via apoplast of toxic elements such as Cd (Gierth et al., 1999; White, 2001; Enstone et al., 2003; Seregin et al., 2004; Seregin and Kozhevnikova, 2008). Considering the damage that was observed in the dermal cells of the peanut roots, it is suggested that the dermal tissue acts as a defence barrier, preventing the entry of Cd, and its translocation to the shoots.

5. Conclusions

Cd negatively impacts on peanut–bradyrhizobia interaction, resulting in a lower nodule number, oxidative burst production and histological modification. Despite the fact that Cd affects the establishment of the two bradyrizobial strains, regardless of the tolerance that they possess, inoculation of *Bradyrhizobium* sp. NLH25 results in a better symbiotic interaction. Moreover, we suggest that the tolerance observed in *Bradyrhizobium* sp. NLH25 could limit Cd accumulation by the plant. Further studies will be required to clarify the exact physiological mechanisms involved in this phenomenon.

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