Universidade de Aveiro Departamento de Biologia
Ano 2012

Sofia Caçoilo Corticeiro

Estudo da complexação de cádmio por glutationa em *Rhizobium leguminosarum*

Study of the Cd complexation mechanism by glutathione in *Rhizobium leguminosarum*

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Etelvina Maria de Almeida Paula Figueira, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e da Doutora Odete Abreu Beirão da Cruz e Silva, Professora Auxiliar da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro

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palavras-chave

Rhizobium leguminosarum, cádmio, glutationa, complexos, periplasma, glutationa-s-transferases.

resumo

A associação simbiótica de plantas leguminosas com bactérias do género *Rhizobium* é o maior e mais eficiente contribuinte de azoto fixado biologicamente (Somasegaran e Hoben, 1994; Zahran, 1999). No entanto, o constante aumento da poluição em solos agrícolas, nomeadamente a contaminação por metais devido à aplicação de fertilizantes e de lamas, está a tornar-se um problema ambiental cada vez mais preocupante (Alloway, 1995a; Giller et al., 1998; Permina et al., 2006; Thorsen et al., 2009; Wani et al., 2008), influenciando de forma negativa a persistência destas bactérias nos solos agrícolas, assim como a sua eficácia de nodulação (Broos et al., 2005; Wani et al., 2008;. Zhengwei et al., 2005). Desta forma, o estudo dos mecanismos de tolerância de *Rhizobium* a metais tornou-se uma área de investigação de elevada importância. Com o trabalho apresentado nesta tese pretendeu-se perceber melhor a tolerância *Rhizobium leguminosarum* ao cádmio (Cd), dando particular atenção a um mecanismo de tolerância previamente descrito em *R. leguminosarum* (Lima et al., 2006): a complexação intracelular de Cd pelo tripéptido glutationa (GSH). Assim, o principal objectivo deste trabalho foi perceber melhor qual a importância deste mecanismo nos níveis de tolerância de rizóbio ao Cd. Como já tinha sido descrito em trabalhos anteriores (Figueira et al., 2005; Lima et al., 2006), foi possível verificar que a estirpe mais tolerante ao metal apresenta níveis mais elevados de Cd e GSH intracelulares. Demonstrou-se ainda que a tolerância ao Cd está dependente da maior eficiência no mecanismo de complexação observada na estirpe tolerante, logo durante as primeiras 12 h de crescimento. Gomes et al. (2002) verificou que a acumulação de complexos GSH-Cd no citoplasma inibe a entrada de metal na célula. Como neste trabalho se observou um aumento nos níveis de Cd intracelular na estirpe tolerante ao longo do tempo, surgiu a hipótese dos complexos serem excretados para o espaço periplasmático. Os elevados níveis de GSH e de Cd determinados no espaço periplasmático corroboraram esta hipótese. Neste trabalho demonstrou-se ainda que a eficácia do mecanismo de complexação, depende da actividade enzimática de uma isoforma específica de GST, que apresentou um elevado acréscimo de actividade na presença do metal. Desta forma, os resultados desta tese indicam que, a maior tolerância de *R. leguminosarum* ao Cd, depende da capacidade das estirpes para induzir a síntese de GSH na presença de Cd e, simultaneamente aumentar a actividade enzimática da GST específica, optimizando assim o mecanismo de complexação de Cd intracelular.

keywords

Rhizobium leguminosarum, cadmium, glutathione, complexes, periplasm, glutathione-s-transferase.

abstract

The symbiotic association of leguminous plants and rhizobia also has a crucial impact on the nitrogen cycle: estimates are that rhizobial symbioses, with a number greater than 100 important agronomical legumes, contribute nearly half to the annual quantity of nitrogen fixed biologically entering soil ecosystems (Somasegaran and Hoben, 1994; Zahran, 1999). Nevertheless, the permanent increased of metal pollution in agriculture soils, due to the current use of fertilizers and industrial and domestic sludge, is becoming one of the most troublesome environmental problems (Alloway, 1995a; Giller et al., 1998; Permina et al., 2006; Thorsen et al., 2009; Wani et al., 2008), has a negative impact in rhizobia persistence in agricultural soils and its ability to form nitrogen-fixing nodules (Broos et al., 2005; Wani et al., 2008; Zhengwei et al., 2005). So, the study of the tolerance mechanisms of soil bacteria to metals arises as a research area with great importance. In this thesis it was intended to draw attention to the tolerance to Cd of *Rhizobium leguminosarum*, a specific soil bacteria that establish symbiotic associations with legumes.

The present study focused on a particular strategy that *Rhizobium leguminosarum* possess to tolerate Cd: the intracellular chelation of Cd by the tripetide GSH. Thus the elucidation of the role of GSH as a metal chelating agent in bacteria tolerance to Cd was considered to be the main aim of this work. Higher intracellular Cd and GSH levels were observed in the tolerant strain as it had already been demonstrated by others authors (Figueira et al., 2005; Lima et al., 2006). It was also demonstrated that *Rhizobium leguminosarum* tolerance to Cd was dependent on the higher efficiency of the chelation mechanism exhibited by the tolerant strain. This mechanism was activated at the beginning of the lag phase (12 h of growth). As the accumulation of GSH-Cd complexes may inhibit intracellular Cd uptake (Gomes et al., 2002), which was not observed in the tolerant strain, the GSH-Cd complexes were suggested to be transported into the periplasmic space. The results point out this hypothesis as the most viable as high levels of GSH and Cd were found in the periplasmic space but only a small percentage of GSH-Cd complexes were quantified. It was also established that the chelation mechanism occurred in the cytoplasm, and its efficiency appeared to be dependent on the enzymatic activity of a specific GST isoform. So, the ability of the tolerant strain to induce GSH synthesis under Cd exposure and, simultaneously, to increment the activity of a specific GST was point out as the main reasons behind the differences the tolerance to Cd observed between the two strains.

Index

Abbreviations

- SH	Sulfhydryl group
°C	Celsius degrees
ADP	Adenosine diphosphate
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
Ca	Calcium
Cd	Cadmium
CDNB	1-chloro-2,4-dinitrobenzene
CF	Cytoplasmic fraction
Cr	Chromate
Cu	Copper
CydDC	ABC binding transporter
DCNB	2,4-dichloro-1-nitrobenzene
DNA	Deoxyribonucleic acid
DNPH	2,4-dinitrophenyl hydrazine
DTE	1,4-Dithioerythritol
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	DL-Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ETHA	Ethacrynic acid
g	Gravitational acceleration (when referring to centrifugation)
GPx	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutathione synthethase
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione-s-transferase
h	Hour
H_2O_2	Hidrogen peroxide
HCl	Hydrochloric acid

HEPES	N-2-hydroxythylpiperazine – N'-2-ethanesulphonate
ICP-MS	Inductively coupled plasma mass spectrometry
IM	Inner membrane
KCl	Potassium chloride
LPS	Lipopolysaccharides
M	molar
mBBr	Monobromobimane
MDA	Malondialdehyde
MDH	Malate dehydrogenase
Mg	Magnesium
min	Minute
ml	Milliliter
N	Nitrogen
NaCl	Sodium chloride
NADH	β-Nicotinamide adenine dinucleotide
NADPH	β-Nicotinamide adenine dinucleotide phosphate
$NADPH_2$	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
OD	Optical density
OM	Outer membrane
P	Phosphor
PEP	Phospho(enol)pyruvate
PF	Periplasmic fraction
ROS	Reactive oxygen species
RP-HPLC	Reverse phase high pressure liquid chromatography
Rpm	Rotations per minute
s	Second
SE	Standard error of the means
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl) aminomethane
v/w	Volume/ weight
Ycf1p	Yeast cadmium factor protein
YEM	Yeast-extract-mannitol broth
Zn	Zinc
γ-GCS	γ-glutamylcysteine synthetase

Chapter 1

General Introduction

1.1 Introduction

1.1.1 Nitrogen fixation by Rhizobia

The 6 billion people on earth consume an average of nearly 11 g of N per person per day (Fink et al., 1999). Plant sources satisfy up to 80% of dietary needs in much of the tropics and sub-tropics. With the earth's population increasing and being expected to reach 9.4 billion in 2050 (Nature News, 2009), world food production rates will need to be increased by at least 50% (Murchie et al., 2009). In this way unprecedented increases in crop production will be needed if the current levels of dietary proteins and caloric intake are to be maintained. In many developing countries, the effective management of nitrogen in the environment is essential for agricultural sustainability (Rehman and Nautiyal, 2002). Rhizobia are Gram-negative soil bacteria, present in soils as free living bacteria or in mutualistic symbiosis with leguminous plants (Figure 1.1) resulting in nitrogen-fixing root nodules (Figure 1.2) (Atlas and Bartha, 1997; Muglia et al., 2007; Somasegaran and Hoben, 1994). Since plants do not assimilate the chemical form of atmospheric nitrogen (Abbas and Kamel, 2004; Atlas and Bartha, 1997; Figueira, 2000), rhizobia are essential in agriculture and crop production, particularly in nitrate-poor soils (Odee et al., 2002;

Viprey et al., 2000). Biological nitrogen fixation in legumes is often used to improve infertile agricultural soils (Rehman and Nautiyal, 2002). The symbiotic association of leguminous plants and rhizobia also has a crucial impact on the nitrogen cycle: estimates are that rhizobial symbioses, with a number greater than 100 important agronomical legumes, contribute nearly half to the annual quantity of nitrogen fixed biologically entering soil ecosystems (Somasegaran and Hoben, 1994; Zahran, 1999).

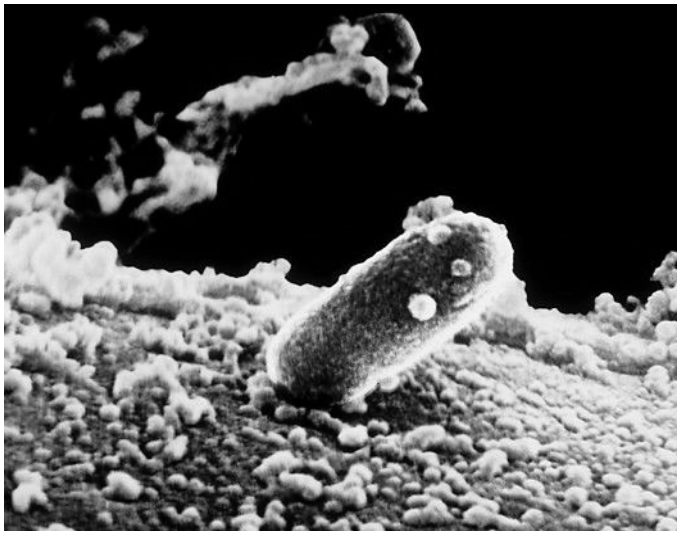

Figure 1.1 – Scanning electron micrograph (SEM) of a nitrogen-fixing bacterium, *Rhizobium leguminosarum*, on the root hair of a pea plant, *Pisum sativum*. (in Burgess, 2010).

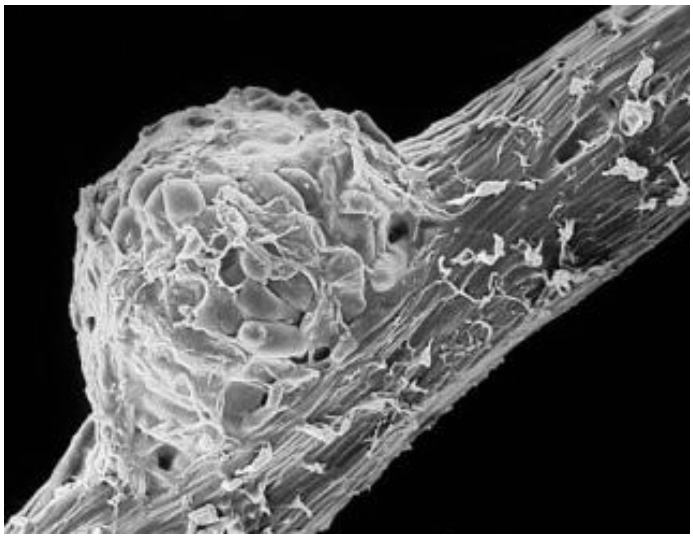

Figure 1.2 – Scanning electron micrograph (SEM) of a root nodule on *Pisum sativum* caused by the nitrogen-fixing soil bacteria *Rhizobium leguminosarum*. (in Burgess, 2010).

The survival and symbiotic efficiency of rhizobial populations are influenced by several factors: the presence of the host plant, nutrient availability, soil characteristics, climatic factors and the level of exposure to abiotic stresses (Muglia et al., 2007). One of the abiotic stresses highly affecting symbiotic efficiency and rhizobia in agriculture soils is metal contamination (Broos et al., 2005; Wani et al., 2008; Zhengwei et al., 2005). Nevertheless, the application of legume-rhizobia symbiosis in metal contaminated soils may also improve agriculture soils by diminishing soil contamination through metal extraction (Zhengwei et al., 2005).

1.1.2. Metal contamination

Many agricultural and industrial practices, as well as other anthropogenic activities, have been adversely affecting the environment, increasing the occurrence of several toxic compounds, such as metals, in the ecosystems (Pazirandeh and Mauro, 2000; Trajanovska et al., 1997). In the last decades, an increase of heavy metal contamination of water and soil has been considered as one of the most current troublesome environmental problems (Alloway, 1995a; Giller et al., 1998; Permina et al., 2006; Thorsen et al., 2009; Wani et al., 2008). These elements are ubiquitous and persistent pollutants that are introduced into the environment mainly through anthropogenic activities, such as smelters, power station industry, mining and the application of metal-containing pesticides, fertilizers, herbicides and sludges (Alloway, 1995a; Carrasco et al., 2005; Giller et al., 1989; McGrath et al., 1995; Robinson et al., 2001; Saxena et al., 1999). Atmospheric deposition of industrial dust, mining operations, incineration processes, burning of fossil fuels (Alloway and Steinnes, 1999) and military activities also contribute to the increased concentration of metals in soils (Giller et al., 1989; McGrath et al., 1995; Pazirandeh et al., 1998; Robinson et al., 2001). Agricultural soils often present deficiencies in nutrients that affect plant growth and development, hence requiring the addition of fertilizers and sludges (Alloway, 1995a). According to Alloway (1995b), phosphate fertilizers are widely regarded as being the most ubiquitous source of cadmium (Cd) contamination in agricultural soils (< 500 mg. kg^{-1}), since relatively high concentrations of this metal

can be found in phosphorites, used to manufacture these fertilizers. Sewage sludge application to land is also very common, resulting in an improvement of the soil physical and chemical characteristics (Abd-Alla et al., 1999; Giller et al., 1989; Obbard, 2001), since sludges are a rich source of organic matter and inorganic nutrients, such as N, P, Ca and Mg (Chander and Brookes, 1993). However, there has been a growing concern over the use of sludges due to their content in non essential metals and other potential toxic compounds from both industrial and domestic sources (Horswell et al., 2003; Obbard, 2001; Purchase and Miles, 2001), thus contributing to increase soil contamination. Metals become irreversibly immobilized in soil components being a cause of toxicity to microorganisms, plants, animals and in particular to humans (McGrath and Lane, 1989). Metals can accumulate in biological systems and ultimately be introduced into food web via different mechanisms (Giller et al., 1998). Among non essential metals, Cd is generally considered one of the most toxic elements, exhibiting highly adverse effects on soil biological activity and plant metabolism, even at low concentration (Carpena et al., 2003). Metal contamination in soils inhibits nodules formation and reduces the symbiotic efficiency on the formed nodules (Carpena et al., 2003; Chaudri, 1993; Chaudri et al., 2000; Giller et al., 1998; Hirsch et al. 1993; McGrath et al., 1995; McGrath et al., 1988), consequently affecting plant growth and crop production (Zhengwei et al., 2005).

1.1.3. Cadmium

Cd is a highly toxic metal, presence in the environment essentially due to human activities. Due to its severe toxicity, Cd is a serious threat to soil bacterial communities, as well as to plants, animals and other organisms, through the transfer of metal ions along the food chain, constituting a serious problem of public health (Ibekwe et al., 1995). According to the Agency for Toxic Substances and Disease Registry in 2009, Cd was considered to be one of the most toxic elements in the world. Cd is emitted to soil, water, and air as a consequence of the practice of several activities such as air emissions, waste and sewage disposal, industrial and mining processes, and application of phosphate fertilizers and sludges (Alloway and Steinnes,

1999; Obbard, 2001; Zhu et al., 1999). Unlike other metals that are considered essential nutrients in trace amounts (Gadd et al., 1990), Cd has no physiological function in terrestrial organisms, being considered toxic even at low concentrations (Adamis et al., 2004; Pan et al., 2009). Among the non-essential metals, Cd poses a most concerning threat due to its high mobility and bioavailability, being one of the reasons why metal contamination has been the concern of numerous scientific investigations. Cd has a much higher affinity for sulfur than for oxygen (Outten and O'Halloran, 2001), so its toxicity will be the result mainly of the affinity of Cd for sulfur generated during the biosynthesis of cysteine and of iron-sulfur centers; binding to thiol groups; and the replacement of other transition-metal cations from such sulfur-rich complex compounds (Helbig, 2008). Cd may enter living cells via transporters for the uptake of essential cations such as Ca, F and Zn (Clemens, 2006). Some of the toxic effects attributed to Cd in the intracellular environment are related to its ability to induce oxidative stress, by increasing cellular ROS (reactive oxygen species) levels (Sandalio et al, 2001; Winterbourn, 1982), which are extremely harmful to cellular components such as proteins, DNA and lipids (Romero-Puertas et al., 1999; Sandalio et al., 2001; Wolff et al., 1986,) and consequently to the cellular metabolism. This metal may also replace zinc and iron ions present in metalloenzymes, causing both the inactivation of the enzymes and the increase in free iron ions, which consequently increase the levels of oxidative stress (Stochs and Bagchi, 1995; Vido et al., 2001). Nevertheless, the major toxicity of Cd comes from its high affinity towards sulfhydryl groups (SH), inactivating important metabolic enzymes and consequently interfering with the cell metabolism (Bruins et al., 2000; Nies, 1992), potential resulting in cell death (Tamás et al., 2006). To cope with Cd toxicity, living organisms have developed different strategies and mechanisms (Prévéral et al., 2009), in order to reduce the concentration of cytosolic free Cd and hence its potential toxicity (Leverrier et al., 2007).

1.1.4. Cadmium tolerance mechanisms

Despite the toxic effects of Cd, some microorganisms had develop diverse metal tolerance mechanisms (Roane and Pepper, 1999), such as energy-dependent efflux of the metal (Grass et al., 2000; Munson et al., 2000; Nies et al., 1998; Peitzsch et al., 1998; Purchase et al., 1997; Saltikov and Olson, 2002), precipitation as insoluble salts (Blake et al., 1993), immobilization of the metal within the cell wall (Cervantes and Gutierrez-Corona, 1994) and production of chelating agents (Silver and Phung, 1996; Lima et al., 2006). The search for other strategies that enhance Cd tolerance in bacteria will help us to understand how bacteria cope with metal stress. Under this context, Silver and Misra (1988) referred the importance of reevaluating the role of thiol in bacterial cell grown under Cd exposure.

1.1.5. Glutathione and its cellular functions

Glutathione (GSH) (Figure 1.3) is a well-known thiol-containing tripeptide and a ubiquitous molecule with several roles in the cell metabolism (Meister, 1995; Noctor and Foyer, 1998).

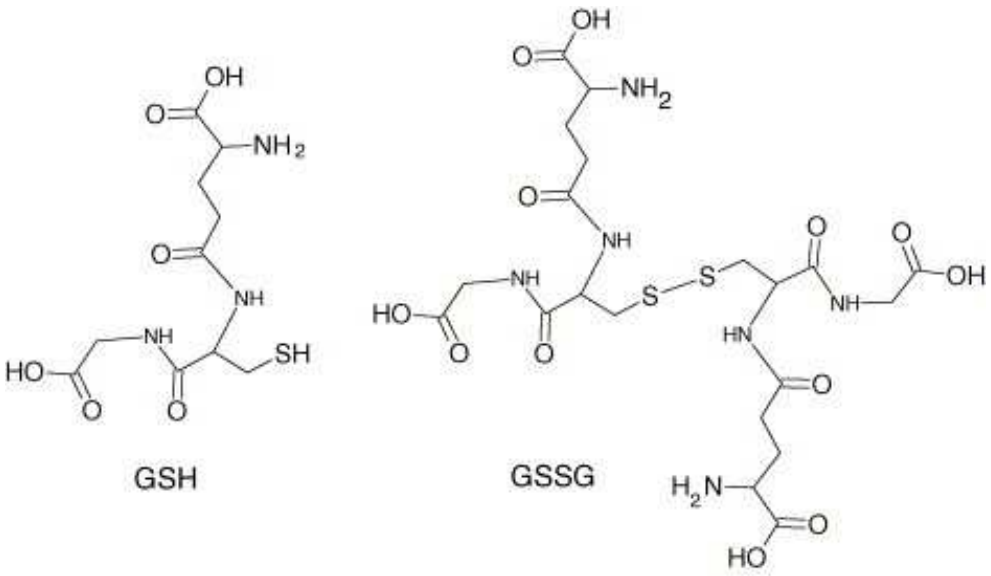

Figure 1.3. Chemical forms of reduced glutathione (GSH) and oxidized glutathione (GSSG) (in Sakhi et al., 2006).

The tripeptide GSH is involved in the metabolism of sulfur-containing amino acids; in the biosynthesis of DNA; in the detoxification of toxic xenobiotics; in the scavenging of ROS and in the regulation of the redox environment of the cell by reducing disulfide proteins and other thiol containing molecules (Ashida et al., 2005; Griffith and Mulcahy 1999; Meister and Anderson, 1983).

In bacteria GSH was also reported to be important in metal, acid, osmotic and oxidative stress induced by peroxides, such as hydrogen peroxide (H_2O_2) or alkyl hydroperoxides (Corticeiro et al., 2006; Figueira et al., 2005; Ferguson and Booth, 1998; Lima et al., 2006; Masip et at al., 2006; Muglia et al, 2007; Riccillo et al., 2000; Vergauwen et al., 2003) and in protection against toxins like methylglyoxal, chlorine compounds like hypochlorous acid and monochloroamine (Chesney et al., 1996; Ferguson and Booth, 1998; Masip et al., 2006; Saby et al., 1999). Moreover, GSH was involved in the regulation of intracellular potassium levels (Ferguson and Booth, 1998) and in preventing the formation of aberrant protein disulfides in the cytoplasm (Masip et al., 2006) and in the regulation of cell cycle (Meister, 1992). In *Rhizobium*, this tripeptide is one of the biomolecules with higher influence on tolerance of metals in free-living bacteria (Figueira et al., 2005, Lima et al., 2006) and plays an important role in the nodulation and fixation processes (Frendo et al., 2005; Harrison et al., 2005; Helbig et al., 2008). GSH main functions are due to its sulfhydryl group (-SH), the electronic cloud surrounding the nucleus of an atom of sulfur is highly polarized, making GSH a highly reactive molecule (Adamis et al. 2004; Josephy et al., 1997). The abundance of GSH in aerobic organisms combined with their chemical properties, reinforces the importance of this tripeptide as a protective and detoxifying agent in many taxonomically diverse organisms such as bacteria, fungi, plants and animals (Josephy et al., 1997, Masip et al., 2006), being the most abundant low molecular weight thiol found in nature (Ferguson and Booth, 1998; Vergauwen et al., 2003).

1.1.5.1. Glutathione biosynthesis and recycling pathway

In gram negative bacteria GSH biosynthesis is achieved by two steps and catalyzed by the products of *gshA* and *gshB* (Griffith and Mulcalhy, 1999; Janowiak, and Griffith, 2005; Masip et al., 2006; Meister, 1974; Meister and Anderson, 1983). The first step is catalyzed by *gshA* product, the cytosolic ATP-dependent enzyme γ-glutamylcysteine synthetase (γ-GCS) that catalyzes the addition of cysteine and glutamate to produce γ-glutamylcysteine (Figure 1.3) (Meister, 1974).

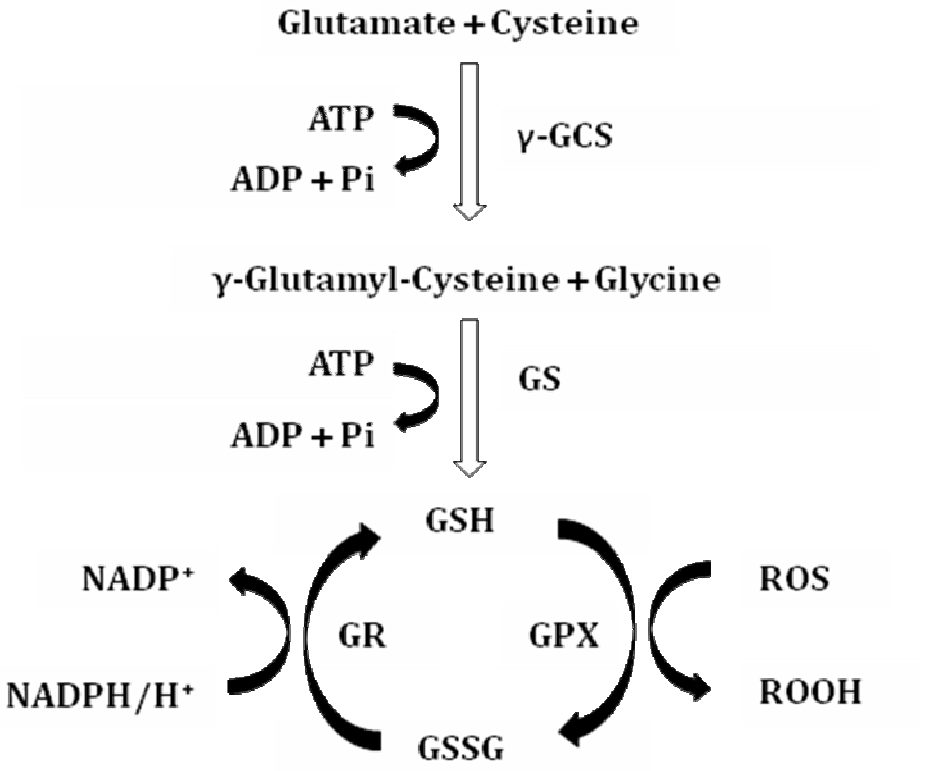

Figure 1.4. The schematic of GSH redox cycle shows the relationship between antioxidant enzymes and GSH. GSH is synthesized from the amino acids glutamate and cysteine by the action of γ-GCS, the rate-limiting enzyme, followed glycine addition by GS. Both reactions are ATP-limited. GSH undergoes the GPx coupled reaction, thereby detoxifying reactive oxygen species (ROS). During this reaction, GSH is oxidized to generate GSSG, which is recycled back to GSH by the action of GR at the expense of $NADPH_2$ (adapted from Haddad, 2004)

This reaction requires coupled ATP hydrolysis and Mg to form an amide bond between the c-carboxyl group of glutamate and the amino group of cysteine (Huang et al., 1993). The second step is the addiction of glycine to the dipeptide γ-

glutamylcysteine to form GSH (Figure 1.3), a reaction catalyzed by *gshB* product, the cytosolic ATP-dependent enzyme glutathione synthetase (GS) and also requires coupled ATP hydrolysis and Mg (Meister, 1974). γ-GSC enzyme is feedback inhibited by GSH, which binds to the glutamate binding site on the active site and at another position that interacts with the thiol group of GSH (Huang et al., 1988), being the rate-limiting step in GSH synthesis (Kelly et al., 2002).

GSH provides reducing equivalents for the glutathione peroxidase (GPx) catalyzed reduction of lipid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water. In the GPx catalyzed reaction, the formation of a disulfide bond between two GSH molecules gives rise to oxidized glutathione (GSSG). The enzyme glutathione reductase (GR) recycles GSSG to GSH with the simultaneous oxidation of nicotinamide adenine dinucleotide phosphate ($NADPH_2$) (Haddad, 2004).

1.1.5.2. GSH as a metal chelator in *Rhizobium leguminosarum*

In previous results it was demonstrated that GSH was dramatically increased in *Rhizobium* tolerant strains after Cd exposure, suggesting its importance in metal stress coping (Figueira et al., 2005). It was also demonstrated that the exposition to Cd induced oxidative stress in *Rhizobium leguminosarum* cells (Corticeiro et al., 2006), however our findings suggested that the higher Cd tolerance presented by some *Rhizobium* strains was not related to a higher efficiency of the antioxidant stress mechanism, but to higher levels of intracellular GSH (Figueira et al., 2005). One important aspect of Cd ions is their ability to covalently bind to sulfhydril groups. Although this is partially the cause for its high toxicity, this feature is also used by several organisms to reduce its toxicity to the cell, through sequestration with metal-detoxifying ligands, which converts it into a less harmful form. Some works have shown that isolate GSH molecules can also sequester Cd in yeast cells (Adamis et al., 2004) and in *Rhizobium leguminosarum* (Lima et al., 2006). GSH-Cd complexes had been isolated, demonstrating that intracellular complexation in this species exists and it was proven to be an important intracellular Cd tolerance mechanism in *Rhizobium leguminosarum* (Lima et al., 2006). Although this mechanism was found to be present

in both tolerant and sensitive strains, different strains showed efficiency differences in the GSH-metal complexation, that are not understood. It is known however, that the efficiency of this mechanism correlates with variations in the tolerance of different strains to Cd. Understanding the complex formation between metal and GSH in *Rhizobium* is crucial to elucidate this molecular mechanism and to understand at what extent it confers protection against metal stress. The GSH-metal formation may be spontaneous, but can also be mediated or both. A strong candidate for mediating GSH-metal complexation are glutathione-S-transferases (GSTs), which are detoxifying enzymes present in all aerobic organisms (Vuillemier and Pagni, 2002). These enzymes catalyze the nucleophilic attack of the sulphur atom of GSH on the electrophilic group of other molecules. GSTs have been extensively studied in several species, including mammals, in which multiples isoforms are present. However much less information is available on the biological functions of bacterial GSTs (Allocatti et al., 2009), been mainly related with growth and with the degradation of aromatic compounds (Vuillemier and Pagni, 2002). Although the bind of metal ions to GSH was proven to be mediated by GSTs in yeasts (Adamis et al., 2004) it was not described in bacteria.

The fate of the complexes within the cell is another aspect that remains to be elucidated. It was already demonstrated the existence of a vacuolar membrane protein, the yeast cadmium factor protein (Ycf1p), which is involved in metal and drug detoxification in *Saccharomyces cerevisiae* (Li et al., 1997). It has been referred as an ATP-dependent pump able to transport organic GSH conjugates, GSH-metal complexes and GSH. In bacterial cells there are no vacuoles, however in gram-negative bacteria, as *Rhizobium*, there is the periplasmic space where some polypeptide products can be sequestered (Herrmann et al., 2009; Marco, 2009). Recently it was described the transport of GSH by CydDC, also an ABC-type transporter, into the periplasmic space in *E. coli* (Pittman et al., 2005). So it is crucial to understand if the complexes or other GSH-conjugates are formed or transported into the periplasmic space, or if the GSH-Cd complexes are formed or accumulated in the cytoplasm. Each option entails different consequences for the cell. The complexation of metal ions in the periplasmic space reduce the amount of free ions in the cytoplasm, where their toxic effects are more detrimental.

1.1.6 General aims of this study

The main aim of this thesis is to highlight the GSH-Cd chelation mechanism in *Rhizobium*. By increasing the knowledge on this intracellular Cd detoxification mechanism, it will be possible to understand the tolerance differences of bacteria to Cd and to develop strategies to improve bacterial tolerance to this metal. This aim will be accomplished by:

A. The evaluation of the oxidative stress level experienced by *Rhizobium leguminosarum* strains at 50 % and 70 % growth inhibition, achieved by the quantification of lipid peroxidation and protein oxidation (Chapter 2).

B. The study of GSH-Cd chelation mechanism at 50% and 70 % growth inhibitions in *Rhizobium leguminosarum* strains (Chapter 2), through the monitor of Cd and GSH levels as well as the formation of GSH-Cd complexes at 0h, 12h, 24h, 48h and 72h.

C. The purification of the periplasmic space and the determination of its importance in Cd tolerance (Chapter 3), by determining Cd and GSH subcellular distribution, and localization of the GSH-Cd.

D. To analyze the influence of GSTs in the GSH-Cd complexes formation and their regulation under Cd exposure (Chapter 4), through the purification of GST isoforms present in bacterial cells in the absence and in the presence of Cd exposure.

Chapter 2

GSH-Cd chelation mechanism in *Rhizobium leguminosarum*

2.1. Introduction

2.1.1. Cd toxic effects

The permanent increased of metal pollution in agriculture soils, due to the current use of fertilizers and industrial and domestic sludge, is becoming one of the most troublesome environmental problems (Alloway, 1995a; Giller et al., 1998; Permina et al., 2006; Thorsen et al., 2009; Wani et al., 2008), which negatively affects rhizobia persistence in agricultural soils and its ability to form nitrogen-fixing nodules (Broos et al., 2005; Wani et al., 2008; Zhengwei et al., 2005).

Cd deserves a particular attention, due to its high mobility and bioavailability in agriculture soils. This metal often causes toxicity to both eukaryotic and prokaryotic cells even at low concentrations (Prévéral et al., 2009; Zhengwei et al., 2005), adversely influencing the survival and biodiversity of soil microbial communities (Roane and Pepper, 1999). Cd enters the cells through transports usually used for the uptake of essential cations such as calcium, iron and zinc (Clemens, 2006; Nies, 1992; Outtenand O'Halloran, 2001) and is a non-redox active metal, indirectly inducing oxidative stress by displacement of redox-active metals, by the depletion of endogenous radical scavengers, such as GSH, (Penninckx, 2002; Masip, 2006), or by affecting the activity of antioxidant enzymes. Nevertheless, the depletion of GSH is considered to be the main cause for the generation of ROS

(reactive oxygen species) and, consequently oxidative stress (Almazan et al., 2000; Avery, 2001; Ercal et al., 2001; Liu et al., 2005; Rikans and Yamano, 2000; Stohs et al., 2001; Wolf and Baynes, 2007). ROS formation due to metal exposure enhanced lipid oxidation, consequently increasing membrane permeability (Gadd, 1993; Howlett and Avery, 1997). Nevertheless, the major toxicity of Cd comes from its high affinity towards sulfhydryl groups (SH), inactivating important metabolic enzymes and consequently interfering with the cellular metabolism (Bruins et al., 2000; Helbig et al., 2008; Nies, 1992).

2.1.2. Glutathione

GSH is a vital antioxidant and detoxifier, with important cellular functions in both prokariotic and eukaryotic organisms (Masip et al., 2006), being involved in the metabolism of sulfur-containing amino acids; in the biosynthesis of DNA and in the regulation of the redox environment of the cell by reducing disulfide proteins (Meister and Anderson 1983, Griffith and Mulcahy, 1999, Ashida et al., 2005). In bacteria GSH was also reported to be important in metal, acid, osmotic and oxidative stress (Corticeiro et al., 2006; Figueira et al., 2005; Ferguson and Booth, 1998; Lima et al., 2006; Masip et at al., 2006; Muglia et al, 2007; Riccillo et al., 2000; Vergauwen et al., 2003) and in protection against toxins like methylglyoxal and chlorine compounds (Chesney et al., 1996; Ferguson and Booth, 1998; Masip et al., 2006; Saby et al., 1999). GSH is synthesized in most organisms by the sequential action of γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase (GS) (Equation 2) (Griffith and Mulcalhy, 1999; Meister and Anderson, 1983).

$$\text{L-glutamate + L-cysteine + ATP} \rightarrow \text{L- γ- glutamyl-L-cysteine + ADP + } P_i \quad \text{(Equation 1)}$$

$$\text{L-γ-glutamyl-L-cysteine + glycine + ATP} \rightarrow \text{GSH + ADP + } P_i \quad \text{(Equation 2)}$$

γ–GCS is the rate-limiting step in GSH synthesis and feedback inhibited by GSH (Huang et al., 1988; Kelly et al., 2002), for ensuring a constant level of GSH in the cell cytoplasm (Masip et al., 2006).

2.1.3. GSH-Cd complex formation

In previous results it was demonstrated that GSH was dramatically increased in *Rhizobium* tolerant strains after Cd exposure, suggesting its importance in metal stress coping (Figueira et al., 2005). It was also demonstrated that the exposition to Cd induced oxidative stress in *Rhizobium leguminosarum* cells (Corticeiro et al., 2006), however our findings suggested that the higher Cd tolerance presented by some *Rhizobium* strains was not related to a higher efficiency of the antioxidant stress mechanism, but to higher levels of intracellular GSH (Figueira et al., 2005).

Cd ions bind covalently to sulfhydril groups. Although this is partially the cause for its high toxicity, this feature is also used by several organisms to render the metal less harmful to the cell, through sequestration with metal ligands. Lima et al. (2006) demonstrated that GSH was related with bacterial tolerance to Cd due metal chelation. GSH-Cd complexes were isolated, demonstrating the existence of intracellular Cd chelation mechanism in *Rhizobium leguminosarum* and suggesting that the efficiency of this mechanism could justify the tolerance differences observed among different strains (Lima et al., 2006). Although this mechanism was found to be present in both tolerant and sensitive strains, different strains showed differences in the formation of GSH-Cd complexes which, remained to be clarified. Understanding the process of complex formation between metals and GSH in *Rhizobium* appears to be crucial to understand the extent of its protection against metal toxicity.

2.1.4. Aims of the chapter:

The intracellular GSH-Cd complexation mechanism has been reported in *Rhizobium leguminosarum* (Lima et al., 2006) when exposed to severe levels of stress (growth inhibitions of 80%). Although this mechanism had been described to such level of stress, the efficiency of the complexation mechanism at lower levels of metal

exposure remains unknown. Thus, the study of the GSH-Cd chelation mechanism at different degrees of metal exposure will highlight the real importance of this intracellular Cd tolerance mechanism. So, in this chapter this information was obtained by:

A. Screening the Cd tolerance of two *Rhizobium leguminosarum* strains;
B. Evaluating the cellular damage induced by different degrees of Cd exposure;
C. Determining the enzymatic activity of γ-GCS under different levels of metal stress;
D. Investigating the GSH-Cd chelation mechanism during *R. leguminosarum* growth;
E. Determining the importance of the intracellular metal chelation mechanism in *Rhizobium leguminosarum* growing at different levels of Cd stress.

2.2. Material and methods

2.2.1. *Rhizobium* strains and growth conditions

Two *Rhizobium leguminosarum* biovar *viciae* strains, E20-8 and NII-1, formerly isolated from *Pisum sativum,* grown in soils from Estação Nacional de melhoramento de Plantas em Elvas, and previously used in other studies (Figueira, 2000; Figueira et al., 2005; Corticeiro et al., 2006; Lima et al., 2006), were screened for their Cd tolerance by growing in yeast extract – mannitol (YEM) medium (Somasegaran and Hoben, 1994) supplemented with increasing Cd concentrations. Cells were incubated at 26°C, in an orbital shaker at 200 rpm, during 72 h. To estimate growth, optical density was measured at 600 nm.

2.2.2. Lipid peroxidation

The level of lipid peroxidation in *Rhizobium* cells was determined in terms of malondialdehyde (MDA) content, after reaction with thiobarbituric acid (TBA), as described by Steels et al., (1994) with some modifications. One of the final products of lipid peroxidation is MDA which can react with TBA *in vitro* to form a chromogenic adduct that can be measured spectrophotometrically, with maximum absorption between 532 and 535 nm. Briefly, *Rhizobium* cells were collected, washed in ddH2O and centrifuged for 15 min at 10000 *g* and 4°C. The pellet was resuspended in 500 µl of 50 mM potassium phosfate buffer (pH 7.0) and disrupted by sonication for 15 s, at 0.7 cycles. s^{-1}. To each sample, 56 µl of 100 % TCA was added and the mixture was vortexed at a maximum speed for 1 min. Extracts were then centrifuged for 15 min at 10000 *g* and 4°C. The reaction mixture containing 150 µl of supernatant, 100 µl of 100 mM EDTA and 450 µl of a solution with TBA 1% (w/v), 50 mM NaOH 0.025% BHT (w/v) were kept in boiling water for 15 min and cooled on ice for 15 min. The

resulting mixture was centrifuged at 8000 *g* for 5 min and absorbance of the supernatant was measured at 532 nm. The measurements were corrected for non specific absorbance by subtracting the absorbance at 600 nm. The amount of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1}.

2.2.3. Protein oxidation

Rhizobium cells were collected, washed in ddH2O and centrifuged for 12 min at 10000 *g* and 4°C. The pellet was resuspended in 500 µl of 10 mM phosphate buffer (pH 7.4) and 1mM EDTA and disrupted by sonication for 15 s, at 0.7 cycles. s^{-1}. Cell extracts were centrifuged during 10 min at 10000 *g* and 4°C. This method (Levine et al., 1990) was based on the reaction of carbonyls resulting from free radical modification of proteins and 2,4-dinitrophenyl hydrazine (DNPH). Briefly, 5% of streptomycin was added to the sample and incubated for 20 min at room temperature. The resulting mixture was centrifuged at 4000 *g* for 5 min. 800 µl of 10 mM DNPH in 2.5 mM HCl was added to 250 µl of sample extract, and the mixture was incubated in the dark for 1 hour. The sample was then precipitated with 1 ml of 20% (w/v) TCA, centrifuged at 10000 *g* for 10 min at 4°C and the supernatant discarded. The procedure was repeated with the addition of 1 ml of 10% (w/v) TCA. After being washed twice with 1 ml ethanol: ethyl acetate (1:1), the pellet was dissolved in 500µl of 20 mM sodium phosphate buffer (pH 2 - 3) containing 6 M guanidinium hydrochoride. The final mixture was incubated at 37°C during 15 min and centrifuged at 10000 *g* during 5 min at 4°C. Carbonyl concentration was calculated from the difference in absorbance recorded at 370 nm for DNPH-treated and HCl-treated (blank) samples and expressed in nmol of DNPH by mg protein. Protein carbonyl content estimated by using the molar absorption coefficient of 22 mM^{-1} cm^{-1} for DNPH derivatives.

2.2.4. Total thiol quantification

Total thiol quantification was performed according to Ellman (1959) with some modifications. *Rhizobium* cells were collected, washed in ddH2O and centrifuged for 12 min at 10000 *g* and 4°C. The pellet was resuspended in 600 µl of 1 M HCl and disrupted by sonication for 15 s, at 0.7 cycles. s^{-1}. Cell extracts were centrifuged during 10 min at 10000 *g* and 4°C. The sample was neutralized with 0.1 M NaOH, after the addition of 800 µl of 100 mM Tris–HCl buffer (pH 8.6) and 1 mM EDTA. After incubation for 15 min at room temperature, 25 µl of 10 mM DTNB (Ellman's reagent) prepared in 10 mM phosphate buffer (pH 7.5), was added. Derivatization was performed in the dark, for 30 min at 26°C. The intensity of the yellow colour formation due to 2-nitro-5-mercaptobenzoate was measured at 412 nm, in a spectrophotometer (Beckman Model DU-68). Total thiols were quantified using GSH as standard.

2.2.5. γ-Glutamylcysteine synthetase (γ-GCS) enzymatic assay

γ-GCS activity was determined by following spectrophotometrically the ADP formation using the pyruvate kinase-lactate dehydrogenase-coupled assay (Seelig and Meister, 1985). *Rhizobium* cells were collected, washed in ddH2O and centrifuged for 12 min at 10000 *g* and 4°C. The pellet was resuspended in 500 µl of 600 mM Tris HCl buffer (pH 8.2), and disrupted by sonication for 15 s, at 0.7 cycles. s^{-1}. Cell extracts were centrifuged during 10 min at 10000 *g* and 4°C. The reaction mixture, equilibrated at 37 °C, contained 250 µl of 600 mM Tris HCl buffer (pH 8.2), 100 µl of 250 mM L-glutamate, 100 µl of 250 mM L-aminobutyrate, 50 µl of 100 mM ATP, 50 µl of 100 mM (PEP) phospho(enol)pyruvate, 60 µl of 500 mM $MgCl_2$, 100 µl of 1 M KCl, 25 µl of 8 mM EDTA, 70 µl of 6 mM NADH, 35 µl of 200 U. ml^{-1} pyruvate kinase, 60 µl of 200 U. ml^{-1} lactate dehydrogenase, and 100 µl of cell extract, which was added last to start the reaction. The oxidation of NADH was monitored at 340 nm (ϵ = 6.2 mM^{-1}) and was assumed to equal the rate of ADP formation. 1 U of enzyme was determined as the amount of enzyme used to form 1 µM produt. h^{-1}.

2.2.6. Analysis of Cd complexes

2.2.6.1. Cd extraction

Rhizobium cells were harvested by centrifugation, for 10 min at 10000 *g* at room temperature. Cell pellets were suspended in ddH2O, immersed in an ultrasonic water bath for 5 min to release the loosely bound Cd and centrifuged for 10 min at 10000 *g* and at room temperature. The supernatant was collected for metal quantification. For the intracellular Cd extractions, the procedure was followed as in Lima et al. (2006): the pellet was resuspended in 1ml of 100 mM HEPES (pH 8.6), 1mM phenylmethylsulfonyl fluoride and 0.2% (v/v) Tween 20 and cellular disruption was achieved through ultrasonication with a low repeating duty cycle of 0.3 cycles. s^{-1} in an ice bath for 10 min. Extracts were centrifuged at 14000 *g*, for 10 min at 4 °C and the supernatant collected. The remaining cell debris was again resuspended in 500 µl extraction buffer, and the procedure was repeated three more times, to assure the full extraction of intracellular Cd. The extracted supernatants were pooled and contained the intracellular, cytosolic Cd, which provided the material for the peptide–Cd complex characterisation. The Cd firmly bound to the cell walls was obtained by resuspending the remaining pellets with 1 ml of 400 mM HNO_3. All extracts were sub-sampled for metal quantification and immediately analyzed.

2.2.6.2. Size exclusion chromatography

Buffer Cd-extracts provided the material for the analysis of metal–peptide complexes, which were separated by gel filtration (Lima et al., 2006). Intracellular Cd extracts were eluted in a Sephacril S-100 column (25 cm×0.5 cm i.d.). The gel bed was equilibrated with degassed elution buffer, 10 mM HEPES (pH 8.0) and 300 mM KCl. Elution was achieved with an injection of 1ml of sample, at a flow rate of 0.4 ml. min^{-1}, at room temperature. The absorbance was detected at 254 nm and fractions were collected every 2 min intervals. All fractions were sub-sampled for Cd quantification and those corresponding to peptide–Cd complexes were pooled and frozen for metal and peptide analysis.

2.2.6.3. Metal quantification

Wall bound Cd, total intracellular Cd and Cd collected from eluted fractions were quantified by ICP-MS.

2.2.6.4. HPLC analysis of the complexes

In order to characterize the peptide nature of the Cd peaks, fractions were analyzed for their thiol content by HPLC, as described by Lima et al. (2006). Selected fractions were collected and complexes were dissociated by acidification with 0.4M HNO3. Thiols present in the Cd–peptide complex were analyzed by HPLC with pre-column derivatisation with monobromobimane (mBBr). Samples (100 µl) were neutralized with 100 mM NaOH, after the addition of 200 µl of 0.1 M Tris–HCl buffer (pH 8.0), 1 mM EDTA and 25 µl of 2 mM DTE. After incubation for 1 h at room temperature, 50 µl of 20mM mBBr (Calbiochem) was added. Derivatization was performed in the dark, for 40 min at 35°C. The reaction was stopped by the addition of 5 % (v/v) acetic acid up to a total volume of 1.5 ml. Samples were stored at 4°C before RP-HPLC analysis. GSH and cysteine content was determined using GSH and cysteine as standard.

2.2.7. Protein quantification

Soluble protein concentration was measured following the method described by Bradford (1976) using bovine serum albumin (BSA) as standard. The assay was based on the binding of the dye Coomassie Blue G250 to protein, forming a stable dye–albumin complex, which can be quantified spectrophotometrically at 595 nm.

2.2.8. Statistical analysis

For both strains, data from all the biochemical parameters were submitted to hypothesis testing using permutation multivariate analysis of variance with the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008), following the calculation

of Euclidean distance matrices among samples. A one-way hierarchical design, with the Cd exposure concentration as the main fixed factor, was followed in this analysis. The pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance. When the main test revealed statistical significant differences ($p<0.05$), pairwise comparisons were performed. The t-statistic in the pair-wise comparisons were evaluated in terms of significance among different conditions and species. Values lower than 0.05 were considered as significantly different.

The null hypotheses tested were: a) for each strain and for each biochemical parameter: no significant differences exist between Cd exposure concentrations; b) for each Cd exposure concentration and for each biochemical parameter: no differences exist between strains.

The results obtained were represented as superscript letters (for each strain, Cd concentrations) or asterisks (for each Cd concentration, between strains) and the significance level for each comparison.

2.3. Results

2.3.1. Susceptibility of *Rhizobium leguminosarum* strains to Cd

To establish the tolerance levels of *Rhizobium* strains E20-8 and NII-1 to Cd, bacterial cells were incubated in YEM containing different metal concentrations, from 0 to 1 mM Cd. Although Cd concentrations used in this study were reported to be higher than those observed in most contaminated ecosystems (Pan et al., 2009; Wagner, 1993), the growth of microorganisms at extreme stress levels was reported as crucial to trigger the tolerance mechanisms relevant for *Rhizobium* survival under Cd contaminated conditions (Figueira et al., 2005).

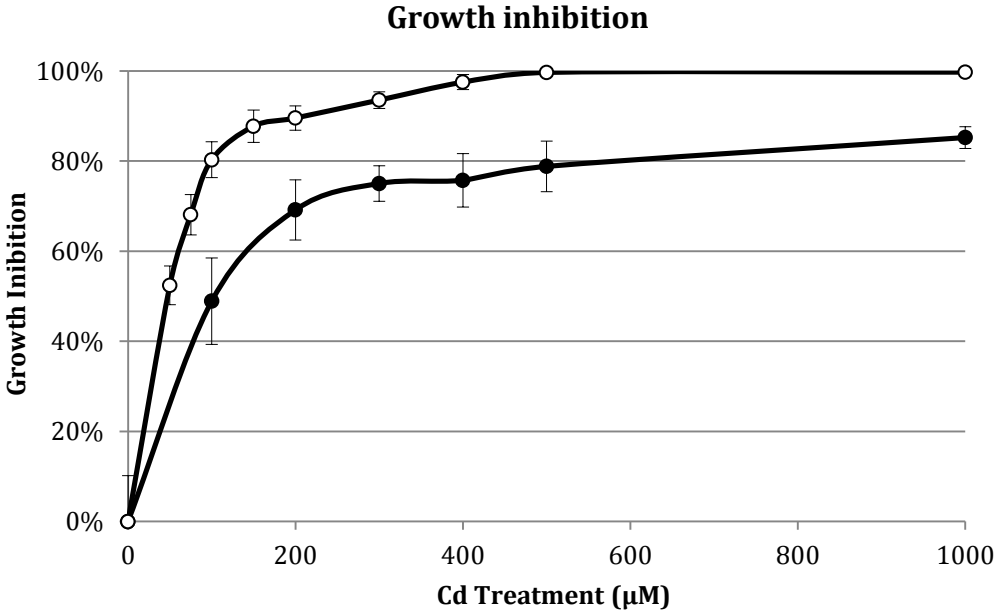

Figure 2.1 – Cd tolerance of *Rhizobium leguminosarum* strain E20-8 (full circles) and strain NII-1 (open circles) exposed to increasing Cd concentrations during a 72h growth period. Results are expressed as the percentage of growth inhibition induced by metal exposure. Data are the means of three replicate experiments.

Cd tolerance of *Rhizobium* strains E20-8 and NII-1 was achieved by determining the growth inhibition levels induced by metal exposure during a 72h growth period (Figure 2.1). The results demonstrated that E20-8 *Rhizobium* strain was able to grow under all Cd concentrations used in this study, presenting growth inhibitions of 50% and 70% of the control at 100 and 200 μM Cd, respectively. The highest metal concentration tested, 1000 μM Cd, lead to a growth inhibition of 80% when compared to growth under control conditions. On the other hand, NII-1 *Rhizobium* strain was not able to survive at Cd concentrations higher than 400 μM Cd, exhibiting at this concentration, a reduction of 98% when compared to the growth observed under control conditions. Growth inhibitions of 50% and 70% were detected at Cd concentrations of 25 and 50 μM Cd, respectively. Taking into consideration these results, from now on, E20-8 will be referred as tolerant and NII-1 as sensitive to Cd. In order to be able to compare the stress level of each strain as well as the mechanisms induced by metal exposure, the subsequent studies will be performed at growth inhibitions of 50% and 70% of control for both strains, which implies that they will be grown at different Cd concentrations. The growth inhibition was chosen as a parameter to compare both strains, as it may be an indication that, although the Cd concentrations were different, metal exposure was inducing an equivalent stress level. Cd concentrations selected to perform the following analysis are summarized in table 2.1.

Table 2.1. Cd concentrations inducing 50% and 70% growth inhibitions in *Rhizobium leguminosarum* tolerant (E20-8) and sensitive (NII-1) strains.

	Growth inhibition	
	50 %	**70%**
Tolerant strain (E20-8)	100 μM	200 μM
Sensitive strain (NII-1)	25 μM	50 μM

2.3.2. Cellular damage

The analysis of the cellular damage experienced by tolerant (E20-8) and sensitive (NII-1) strains under Cd exposure was performed to demonstrate that similar growth inhibitions were related to similar oxidative stress degrees in both *Rhizobium leguminosarum* strains. Cellular damage provided information concerning the toxic effect of Cd in *Rhizobium leguminosarum* cells, particularly the estimation of lipid peroxidation and protein oxidation levels. Both assays were also performed in *Rhizobium* cells growing under control conditions (absence of Cd) determining lipid peroxidation and protein oxidation levels under non stress conditions.

2.3.2.1. Lipid peroxidation

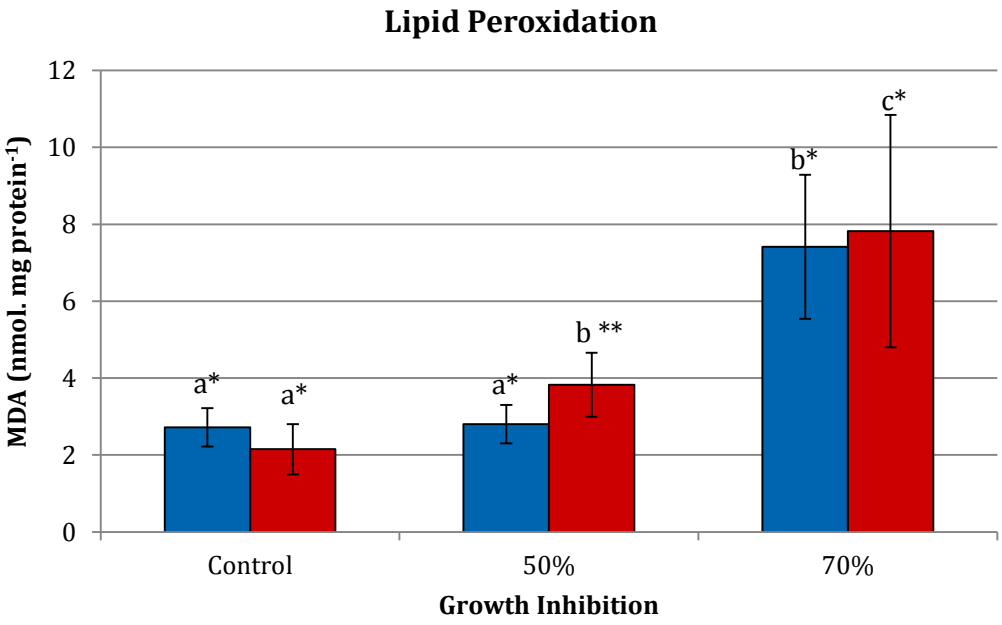

Figure 2.2 – Lipid peroxidation levels of *Rhizobium leguminosarum* tolerant (blue bars) and sensitive (red bars) strains under control and Cd exposure. Data are the means of three replicate experiments, with standard errors. Different letters represent different significant differences ($p < 0.05$) between treatments and asterisks represent different significant differences ($p < 0.05$) between strains in the same treatment.

Lipid peroxidation, resulting of free radical attack to membrane lipids (Heath and Packer, 1968), was determined in both *Rhizobium* strains, under control

conditions and under Cd exposure (at 50% and 70% growth inhibition). Results are presented in Figure 2.2. In the tolerant strain, E20-8, a 50% growth inhibition did not significantly ($p > 0.05$) affect lipid peroxidation level, compared to control. However under a higher degree of metal stress, 70% growth reduction, a significant ($p < 0.001$) increased in lipid peroxidation was observed from 2.72 nmol. mg protein^{-1} under control conditions to 7.41 nmol. mg protein^{-1}. In the sensitive strain, lipid peroxidation levels were significantly enhanced by Cd exposure at 50% ($p<0.001$) and 70% ($p<0.05$) growth inhibition, from 2.15 nmol. mg protein^{-1} to 3.83 and 7.82 nmol. mg protein^{-1}, respectively. Lipid peroxidation levels were not significantly ($p>0.05$) different between strains at 70% growth inhibition.

2.3.2.2. Protein oxidation

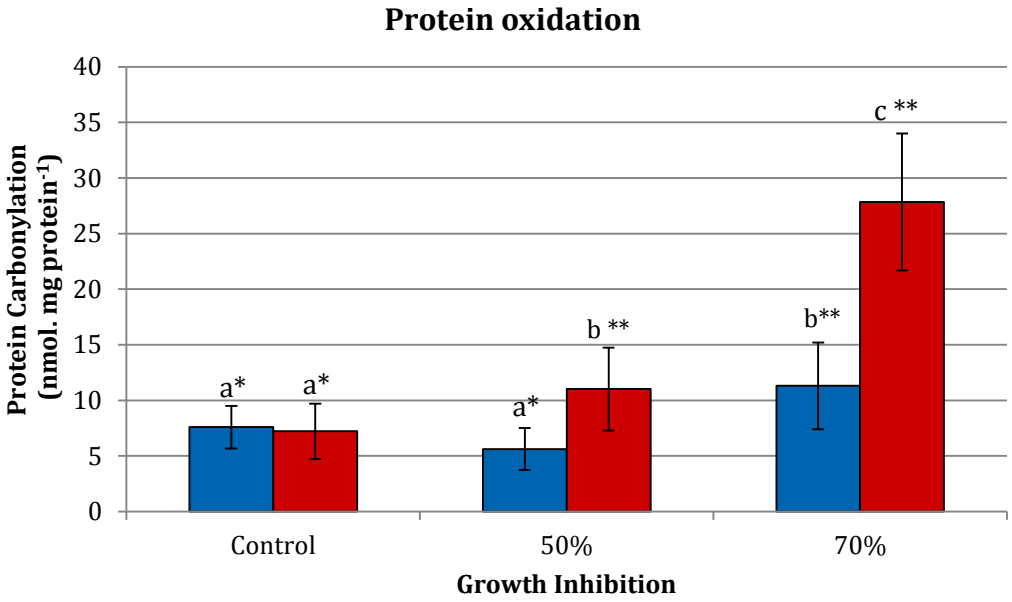

Figure 2.3. – Protein oxidation levels of *Rhizobium leguminosarum* E20-8 (blue bars) and NII-1 (red bars) strains under Cd exposure. Data are the means of three replicate experiments, with standard errors. Different letters represent different significant differences ($p < 0.05$) between treatments and asterisks represent different significant differences ($p < 0.05$) between strains in the same treatment.

Protein oxidation levels were measured in *Rhizobium* strains, under control conditions and under Cd exposure (at 50% and 70% growth inhibition). Results are

expressed in figure 2.3. Similar to lipid peroxidation results, Cd exposure inducing a 50% growth inhibition was not sufficient to affect significantly ($p>0.05$) protein oxidation levels in *Rhizobium* tolerant strain. Nevertheless, under a severe stress exposure, 70% growth reduction, protein oxidation was significantly ($p< 0.05$) enhanced from 7.59 nmol. mg protein^{-1} to 11.31 nmol. mg protein^{-1}. Sensitive strain presented higher protein oxidation values under both Cd exposures when compared to the control. Cells exposed to 25 µM Cd, 50% growth inhibition, presented a protein oxidation value of 11.02 nmol. mg protein^{-1}, while in cells expose to 50 µM Cd, 70% growth inhibition, the levels of protein carbonylation were 27.81 nmol. mg protein^{-1}. Comparing both strains, under control conditions the protein oxidation levels were not significantly ($p>0.05$) different. However, Cd exposure leaded to higher protein carbonylation levels at both, 50% ($p< 0.05$) and 70% ($p< 0.001$) growth inhibition.

2.3.3. Total thiol content in *Rhizobium leguminosarum*

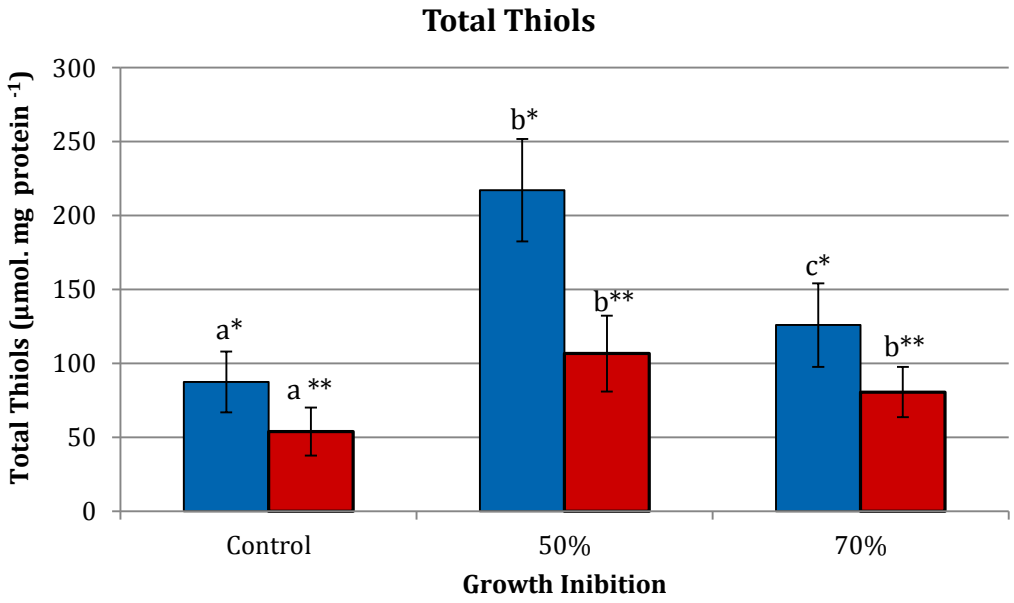

Figure 2.4 – Total thiol levels of *Rhizobium leguminosarum* tolerant (blue bars) and sensitive (red bars) strains growing under Cd exposure. Data are the means of three replicate experiments, with standard errors. Different letters represent different significant differences ($p < 0.05$) between treatments and asterisks represent different significant differences ($p < 0.05$) between strains in the same treatment.

GSH is considered to be crucial to *Rhizobium leguminosarum* metal tolerance, having important functions in the antioxidant machinery and in metal detoxification (Masip et al., 2006; Lima et al., 2006). Taking this into consideration, total thiol levels were quantified in *Rhizobium* cells growing in the absence of metal and under Cd exposure. Results are presented in figure 2.4.

Total thiol levels of *Rhizobium* tolerant strain were enhanced due to Cd stress ($p < 0.001$). At 100 μM Cd, 50% growth inhibition, total thiols were 217.2 nmol. mg protein^{-1}, almost 2.5 higher than the value measured in the control. In *Rhizobium* cells exposed to a higher metal concentration, 70% growth reduction, the thiol level was 125.9 nmol. mg protein^{-1}. It is important to notice that, in the tolerant strain, cells with a growth inhibition of 70% presented a lower total thiol content ($p < 0.001$) than cells exposed to Cd concentrations inducing a 50% growth reduction, nevertheless the thiol level remained higher ($p < 0.001$) than controls.

In *R. leguminosarum* sensitive strain, total thiol levels were also enhanced ($p < 0.05$) by Cd exposure, being the highest value registered at a growth inhibition of 50%, 106.6 μmol. mg protein^{-1}, twice the value measured in control conditions. Comparing both strains, results demonstrated marked differences between the tolerant and the sensitive strain at all conditions: under control conditions, *R. leguminosarum* tolerant strain had higher thiol content than the sensitive strain ($p<0.05$); under Cd exposure both strains demonstrated the ability to increment intracellular thiol pool, although the tolerant strain presented higher ($p < 0.001$) ability to increase total thiol levels, than the sensitive strain, NII-1.

2.3.4. γ-Glutamylcysteine synthetase in *Rhizobium leguminosarum*

GSH is synthesized in a highly conserved two-step ATP-dependent biosynthesis pathway by two specific enzymes: γ-Glutamylcysteine ligase (γ-GCS) and Glutathione synthetase (GS), being the first the key step of GSH synthesis. So in order to understand if GSH synthesis was affected by Cd exposure, γ-GCS activity was determined in *R. leguminosarum* strains under control conditions and under metal stress. Results indicate that γ-GCS activity was significantly ($p<0.001$) enhanced by Cd

exposure in *R. leguminosarum* tolerant strain, being observed an increment of 3.6 and 2.7– fold at 50% and 70 % growth inhibition, respectively (figure 2.5). In the sensitive strain, γ-GCS activity was also significantly ($p<0.05$) affected by Cd exposure, nevertheless the increment under Cd stress was only 1.5 – fold, under both levels of metal stress.

The comparison of γ-GCS enzyme activity of both strains showed that, although the enzymatic activity of E20-8 tolerant strain was significantly ($p<0.05$) higher in the absence of metal stress, the increment detected under metal exposure was also much higher ($p<0.001$) than in the sensitive strain.

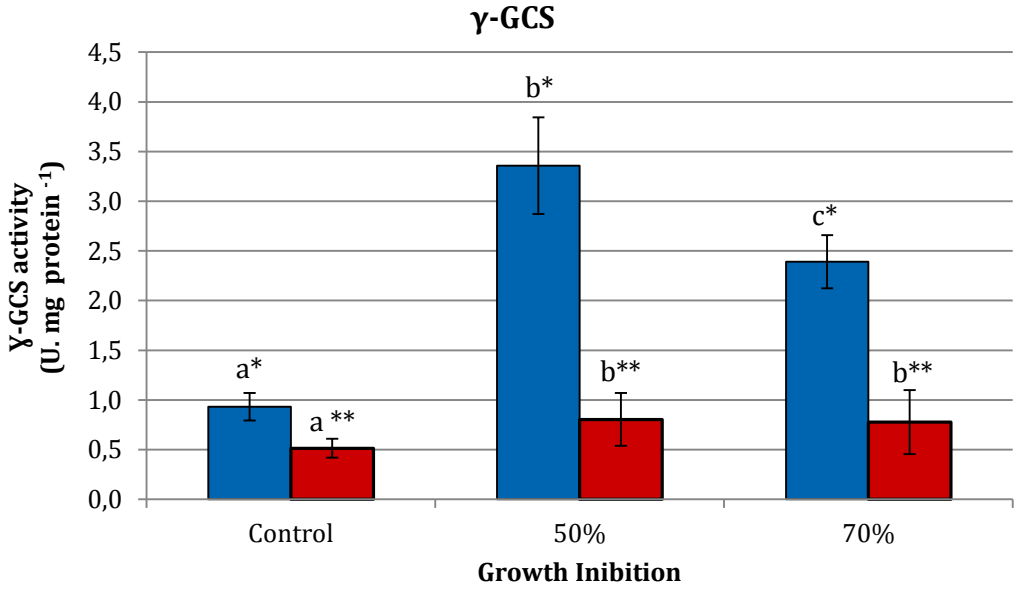

Figure 2.5 – γ-Glutamylcysteine ligase (γ-GCS) activity of *Rhizobium leguminosarum* tolerant (blue bars) and sensitive (red bars) strains growing under Cd exposure. Data are the means of three replicate experiments, with standard errors. Different letters represent different significant differences ($p < 0.05$) between treatments and asterisks represent different significant differences ($p < 0.05$) between strains in the same treatment.

2.3.5. Cd distribution within *Rhizobium leguminosarum* cells

Different growth inhibitions due to Cd exposure revealed significant alterations in cellular damage and in intracellular thiol content. To better understand the cellular alterations detected and to possibly explain the tolerance differences

exhibited by E20-8 and NII-1 *Rhizobium* strains, it was important to analyze the intracellular Cd levels as well as the efficiency of the GSH-Cd chelation mechanism during the growth period of both *Rhizobium leguminosarum* strains.

The first approach was to determine the intracellular Cd content in *Rhizobium* cells, knowing that not all the metal present into the growing medium is usually assimilated by bacterial cells and that, mostly of the cellular Cd was expected to be retained in the cell wall (Bianucci et al., 2012; Lima et al., 2006; Pereira et al., 2006). Cd partitioning into wall bound Cd and intracellular Cd was achieved by separating cells into two distinct fractions: the cell walls and the intracellular fraction. The intracellular Cd was obtained through sequential extractions with an alkaline buffer and the metal bound to cell walls, was separated by acid-extraction of the cellular debris resulting from the intracellular Cd extractions. The partitioning results through the sub-cellular fractions are presented in Table 2.2.

Table 2.2 - Cd partitioning in *Rhizobium leguminosarum* strains E20-8 (tolerant) and NII-1 (sensitive) exposed Cd. Data are the means of three replicate experiments with standard errors. Different letters represent different significant differences ($p < 0.05$) between treatments and asterisks represent different significant differences ($p < 0.05$) between strains in the same treatment.

Strain	Cd treatment (µM)	Growth inhibition	Cell wall bound Cd (µmol. mg protein^{-1})	Intracellular Cd (µmol. mg protein^{-1})	Intracellular Cd (%)
E20-8	**100**	**50%**	242.76 ± 64.62 (a*)	18.79 ± 7.26 (b*)	6.9
	200	**70%**	277.87 ± 85.75 (a*)	92.81 ± 29.85 (c*)	18.8
NII-1	**25**	**50%**	127.04 ± 10.17 (a* *)	10.55 ± 1.05 (b* *)	20
	50	**70%**	110.22 ± 10.61 (a* *)	6.36 ± 1.15 (b**)	9.3

Cd distribution differed between cellular fractions, being mostly bound to the cell walls. The concentration of Cd bound to the cell wall was clearly higher in the tolerant strain, E20-8, than in the sensitive strain. In both strains wall bound Cd was not significantly ($p>0.05$) affected by the level of Cd exposure. Under a growth inhibition of 50%, the tolerant strain accumulated 18.79 µmol Cd. mg protein $^{-1}$, intracellular. This concentration increased almost 5-fold to 92.8 µmol Cd. mg protein $^{-}$

^{1} at 70% growth inhibition. The sensitive strain accumulated 10.55 μmol Cd. mg protein $^{-1}$ intracellular at 50% growth inhibition and the concentration decreased to 6.36 μmol Cd. mg protein $^{-1}$, at 70% growth inhibition. These results clearly show that, despite being more tolerant to Cd, the E20-8 strain accumulated more Cd intracellular than the sensitive strain.

2.3.6. Intracellular Cd and GSH content

Although the majority of Cd was wall bound, it was crucial to investigate intracellular Cd tolerance mechanisms as the metal present in the cytoplasm is considered highly toxic to the bacterial cells, by disturbing cellular metabolism and consequently affecting *Rhizobium* survival. Thus, intracellular Cd and GSH levels were monitored during the 72 h of bacterial growth, to determine the profile of cellular Cd uptake and the GSH levels in both *Rhizobium leguminosarum* strains.

2.3.6.1 Intracellular Cd accumulation in *Rhizobium* cells

Intracellular Cd levels were monitored during a 72 h growth period (in the stationary phase) and Cd levels quantified, at 0h, 12h, 24 h, 48 h and 72 h (figure 2.6).

At 50% growth inhibition, the tolerant strain E20-8 presented intracellular metal levels significantly ($p<0.05$) increased during the first 12 h of growth, followed by significant reduction ($p<0.05$) after 24 h of stress, to increase once more ($p< 0.05$), reaching the highest level of Cd accumulation at 72 h of metal exposure. At 70% growth inhibition Cd accumulation was initially (12 h) low, followed by an increment until 48 h, and after that Cd accumulation stabilized until 72 h of growth.

In the sensitive strain, NII-1, the profile of Cd uptake during the growth period was dependent of the degree of metal exposure. At a 50% growth inhibition, 3.59 μmol. mg protein^{-1} of intracellular Cd (34%) was uptake during the first 12h of growth, a level which was not significantly different ($p> 0.05$) from the quantifications performed after 24h and 48h of metal exposure; the highest amount

of Cd uptake was observed at 72h, reaching an intracellular Cd concentration of 10.55 µmol Cd. mg protein^{-1}.

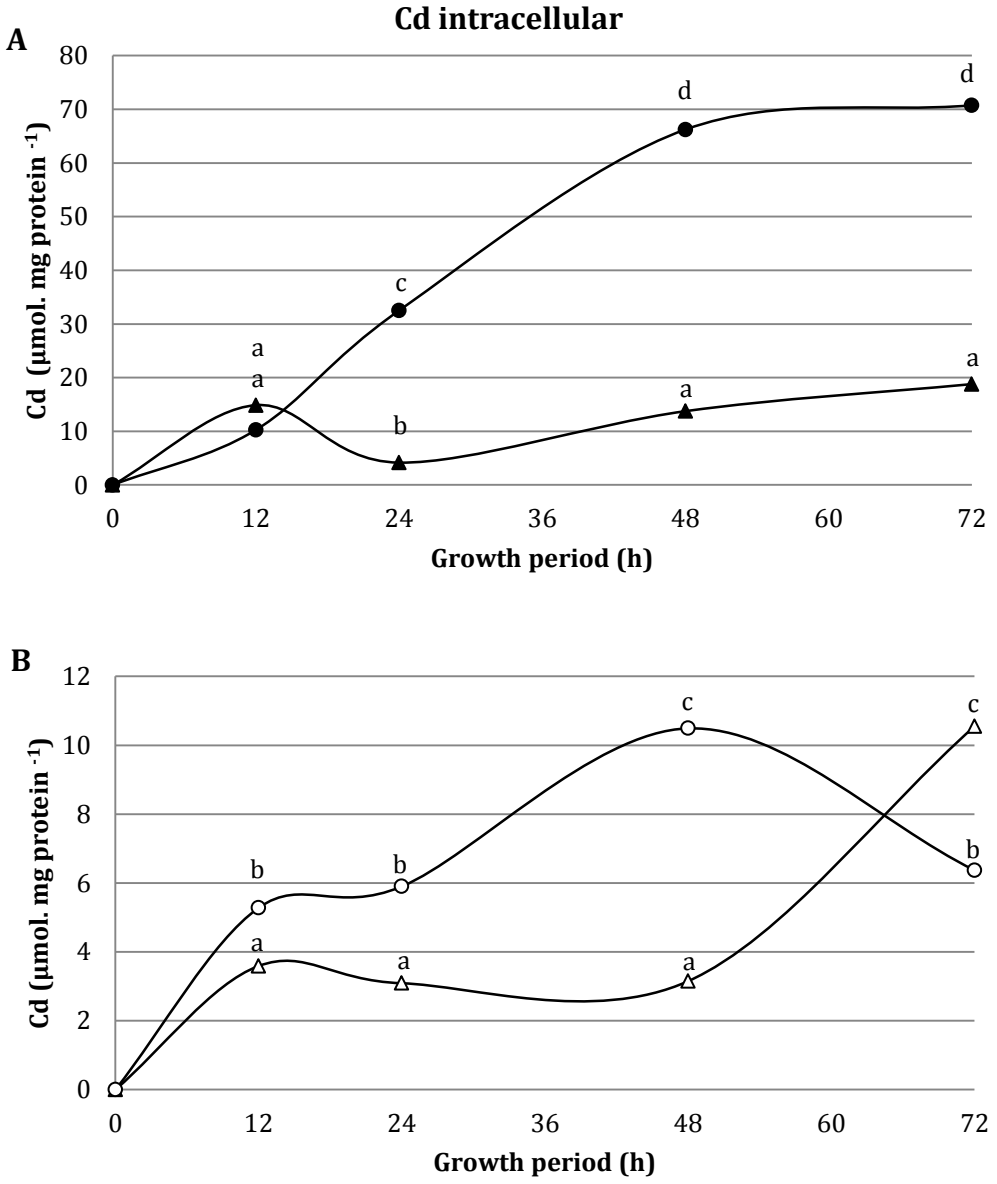

Figure 2.6 – Total intracellular Cd levels during growth period of *Rhizobium leguminosarum* strains, tolerant (A), under a 50% (Full triangles) and 70% (Full circles) of growth inhibition, and sensitive (B) under a 50% (Open triangles) and 70% (Open circles) of growth inhibition. Intracellular Cd was quantified at 0h, 12h, 24h, 48h and 72h of the growth period. Data are the means of three replicate experiments. Standard errors are less than 10%. Different letters represent different significant differences ($p < 0.05$).

In the sensitive strain, at 70% growth inhibition, 5.28 μmol Cd. mg protein^{-1} (50%) entered the cells during the first 12 h of exposure, and increased steadily again between 24 h and 48 h of growth, reaching the highest value, 10.5 μmol Cd. mg protein^{-1}. At 72 h, the metal present in the intracellular was 6.4 μmol. mg protein^{-1}. It is important to underline that, at 70% growth inhibition the intracellular Cd concentration at 72h was lower than the concentration observed at 50% growth inhibition. The results also demonstrated that intracellular Cd levels of *Rhizobium* tolerant strain were always higher than the levels quantified in the sensitive strain, under the same degree of metal stress, indicating that E20-8 had to cope with higher intracellular metal concentrations during the entire growth period.

2.3.6.2. Intracellular GSH content of *Rhizobium* cells

The analysis of the thiol pool of bacterial cells demonstrated that GSH was the main thiol in *Rhizobium leguminosarum* strains, being also quantified very small amounts of cysteine (results not shown). Taking this into consideration total thiol levels will be hereafter referred only as GSH. Intracellular GSH levels during the 72 h of *Rhizobium* growth (0 h, 12 h, 24 h, 48 h and 72 h) under control conditions and under Cd exposures are present in figure 2.7.

In the absence of metal stress, intracellular GSH levels of the tolerant strain increased to 51.86 μmol. mg protein^{-1} (61%) at the first 12 h of growth, reaching 78,12 μmol. mg protein^{-1} GSH (92%) after 24 h. The maximum value of intracellular GSH observed in E20-8 under control conditions was 84. 35 μmol. mg protein^{-1}, at 72 h of growth. At a Cd exposure inducing a 50% growth inhibition, the tolerant strain demonstrated the ability to increment GSH levels during the 72 h of growth: thiol levels incremented to 76.71 μmol. mg protein^{-1} (37%) during the first 12 h of growth, after 48 h reached 187.81 μmol. mg protein^{-1} (92%). The highest intracellular GSH level observed in the tolerant strain was 204. 74 μmol. mg protein^{-1} determined at 72 h. GSH profile in the tolerant strain, at 70% growth inhibition, was different from other treatments. Intracellular GSH concentrations reached 87.82 μmol. mg protein^{-1} (72%) during the first 24 h of growth, but at 48 h decreased to 70.55 μmol. mg

protein^{-1}, 58% of the highest GSH concentration determined (120.63 µmol. mg protein^{-1}) at 72 h.

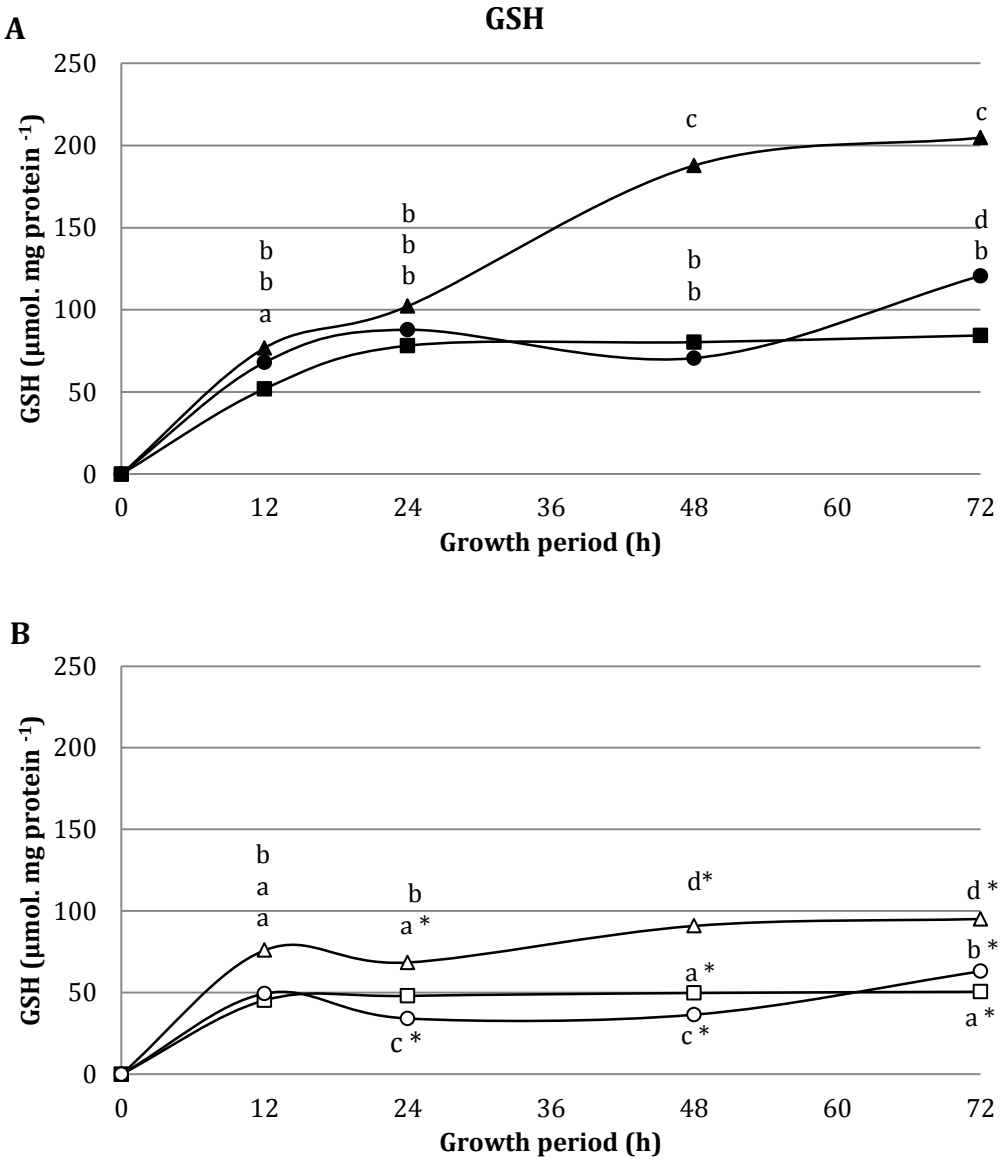

Figure 2.7 – Intracellular GSH levels during growth period of *Rhizobium leguminosarum* strains, tolerant (A), under control conditions (Full squares), and under a 50% (Full triangles) and 70% (Full circles) of growth inhibition, and sensitive (B) under control conditions (Open squares), and under 50% (Open triangles) and 70% (Open circles) of growth inhibition. Intracellular Cd was quantified at 0h, 12h, 24h, 48h and 72h of the growth period. Data are the means of three replicate experiments. Standard errors are less than 10 %. Different letters represent different significant differences ($p < 0.05$) between treatments and asterisks represent different significant differences ($p < 0.05$) between strains in the same treatment.

When the sensitive strain grew under control conditions, GSH content reached 45.27 μmol. mg protein^{-1}, 89 % of the total intracellular GSH at 12 h. After 24 h the tripeptide level increased to 48.00 μmol. mg protein^{-1} (95%), continuing to be enhanced to 50.52 μmol. mg protein^{-1} at 72 h. At 50% growth inhibition, the tripeptide levels increased to 8.77 μmol. mg protein^{-1} (80%) during the first 12 h of growth. The value observed at 24 h was lower (72%), recovering in the subsequent 24 h, reaching 96% of the intracellular GSH level (95.12 μmol. mg protein^{-1}) registered at 72 h. At 70% growth inhibition, after the first 12 h of growth GSH was 8.19 μmol. mg protein^{-1} (78%), of the highest value recorded. After 24 h of growth it was observed a decrease to 5.29 μmol. mg protein^{-1} (54%), only recovered at the end of the growth (72 h), which presented the highest GSH value observed in NII-1 to this treatment, 63.05 μmol. mg protein^{-1}. Although the intracellular GSH levels were higher in *Rhizobium* tolerant strain in control conditions. The highest difference between the two strains were in the presence of Cd, tolerant strain demonstrated a higher ability to increase the thiol levels under metal stress, particularly at 50% growth inhibition, than sensitive strain. Nevertheless both strains presented higher levels of intracellular GSH under Cd exposure, being that increment more evident under a lower degree of metal stress.

2.3.6.3. Intracellular Cd complexation

Rhizobium leguminosarum strains possess tolerance mechanisms to cope with metal toxicity. The formation of GSH-Cd complexes was already described in *Rhizobium leguminosarum* (Lima et al., 2006) as an important intracellular Cd detoxification mechanism dependent on GSH. Lima et al. (2006) also suggested that the efficiency of the complexation mechanism could justify differences in Cd tolerance, detected among *Rhizobium* strains. Thus in this chapter, it was investigated the GSH-Cd complexation mechanism during the *Rhizobium leguminosarum* growth (figure 2.8 – 2.11). The analysis of GSH-Cd complexes was performed in *Rhizobium leguminosarum* cells exposed to Cd stress (figures 2.8 and 2.9) at 12 h, 24 h, 48 h and 72 h of the growth period. The results were obtained by undertaking three distinct

analyses: the separation of the intracellular peptide content through size exclusion chromatography and the quantification, in each separated protein peak, of Cd by ICP-MS and GSH by RP-HPLC.

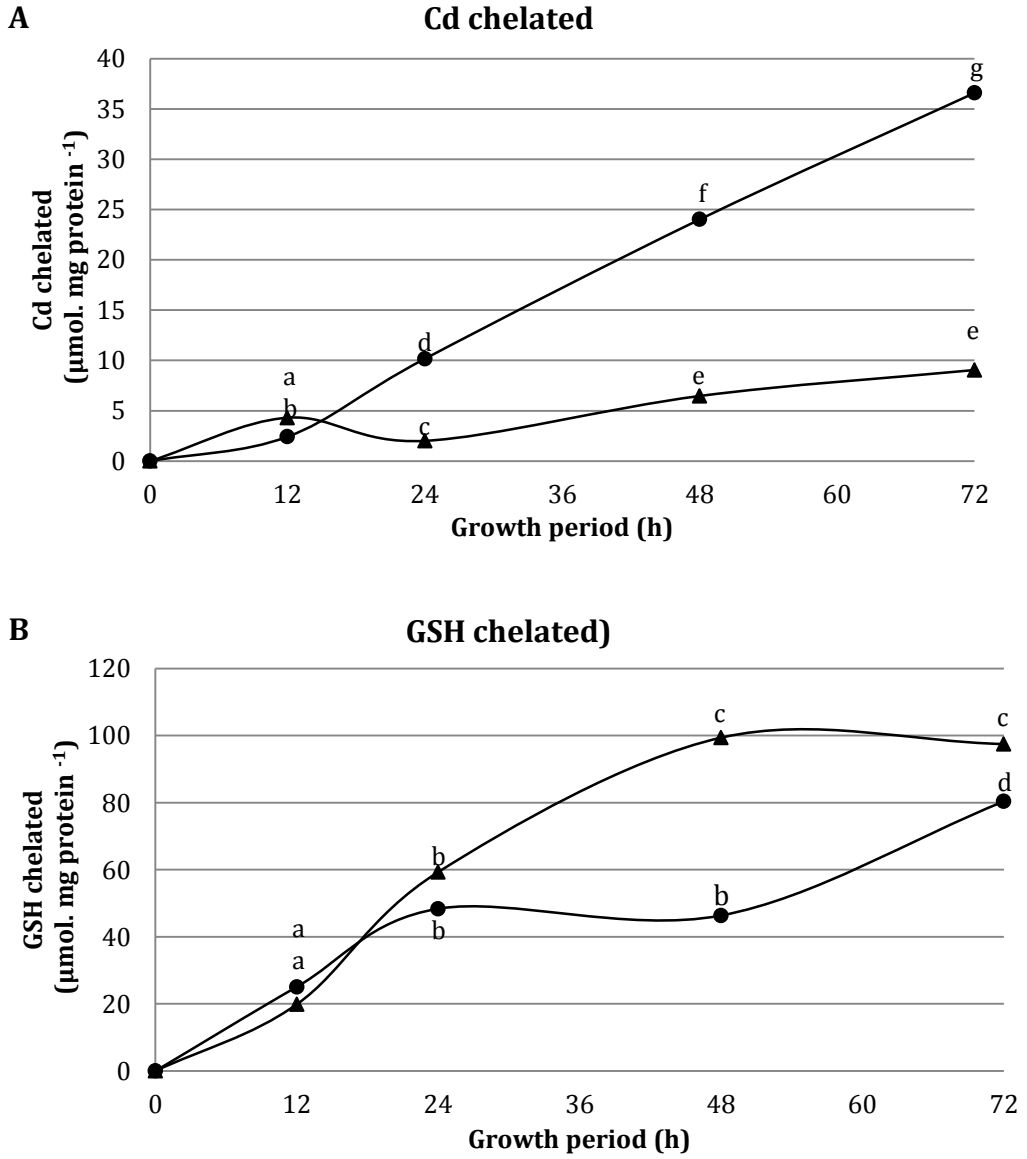

Figure 2.8. Cd (A) and GSH (B) content in the separated protein peak determined in *Rhizobium leguminosarum* tolerant strain under a 50% (Full triangles) and 70% (Full circles) of growth inhibition. Intracellular Cd and GSH levels were quantified at 0h, 12h, 24h, 48h and 72h of the growth period. Data are the means of three replicate experiments with standard errors. Different letters represent different significant differences ($p < 0.05$).

Results demonstrated the presence of Cd in GSH peak, indicating the existence of the GSH-Cd complexation mechanism in both, tolerant and sensitive *Rhizobium*

strains, growing under metal exposure. Complex formation was first detected after 12 h of growth, as it was possible to quantify a significant level of intracellular Cd chelated to GSH. The highest intracellular metal concentration observed in GSH peak was detected after 72 h of growth (figure 2.8A and 2.9A), when the level of GSH available to form the GSH-Cd complexes also was the maximum (figure 2.8B and 2.9B).

In the tolerant strain, at a 50% growth inhibition, Cd quantified in GSH peak during the first 12 h of stress was 4.31 μmol Cd. mg protein^{-1}, after 24 h the metal GSH chelated decreased ($p<0.05$), recovering to 9.05 μmol Cd. mg protein^{-1} after 72 h of growth ($p<0.05$) (figure 2.8A). At 70% growth inhibition, the concentration of Cd chelated during the first 12 h was lower ($p<0.05$), 2.42 μmol Cd. mg protein^{-1}, but was continuously increased ($p<0.05$) during the 72 h of metal exposure, when 36.59 μmol. mg protein^{-1} Cd was observed in GSH protein peak (figure 2.8B). GSH concentrations present in protein peak and were also dependent on the level of stress and growth phase. In *Rhizobium* tolerant strain, during the first 12 h of growth, GSH chelated to Cd was 19.88 μmol. mg protein^{-1}; the highest GSH complexed with Cd was quantified after 48 h of metal exposure, 99.40 μmol. protein^{-1}, and was not significantly different ($p>0.05$) from the GSH concentration observed after 72 h of growth. At 70% growth inhibition, GSH detected in the peptide peak was 25.09 μmol. protein^{-1} after 12 h of growth, and was enhanced, reaching 80.39 μmol. protein^{-1} at 72 h.

Similar profiles were registered to NII-1 sensitive strain, although Cd chelated to GSH chelated was much lower than in the tolerant strain (figures 2.9A e 2.9B). At 50% growth inhibition 0.32 μmol Cd. mg protein^{-1} were quantified during the first 12 h and after 72 h the Cd chelated to GSH was 1.53 μmol Cd. mg protein^{-1}; at 70% growth inhibition 0.21 μmol Cd. mg protein^{-1} of metal were quantified after 12 h of exposure and the highest value of metal chelated to GSH was 1.69 μmol. mg protein^{-1} at 72 h of growth. The GSH levels complexed to Cd in *Rhizobium* sensitive strain are represented in figure 2.9.B. At 50% growth inhibition, the level of GSH quantified after 12 h of exposure increased to 8.77 μmol. protein^{-1} until the end of growth period, when GSH concentration was 13.11 μmol. protein^{-1}. At 70% growth inhibition, GSH concentrations during the first 12 h and after 72 h of growth were not significant

different (p> 0.05) from the values quantify at 50% growth inhibition to the same growth phases, nevertheless it was possibly to detected a decreased in GSH chelated to Cd at 24 h and 48 h of stress.

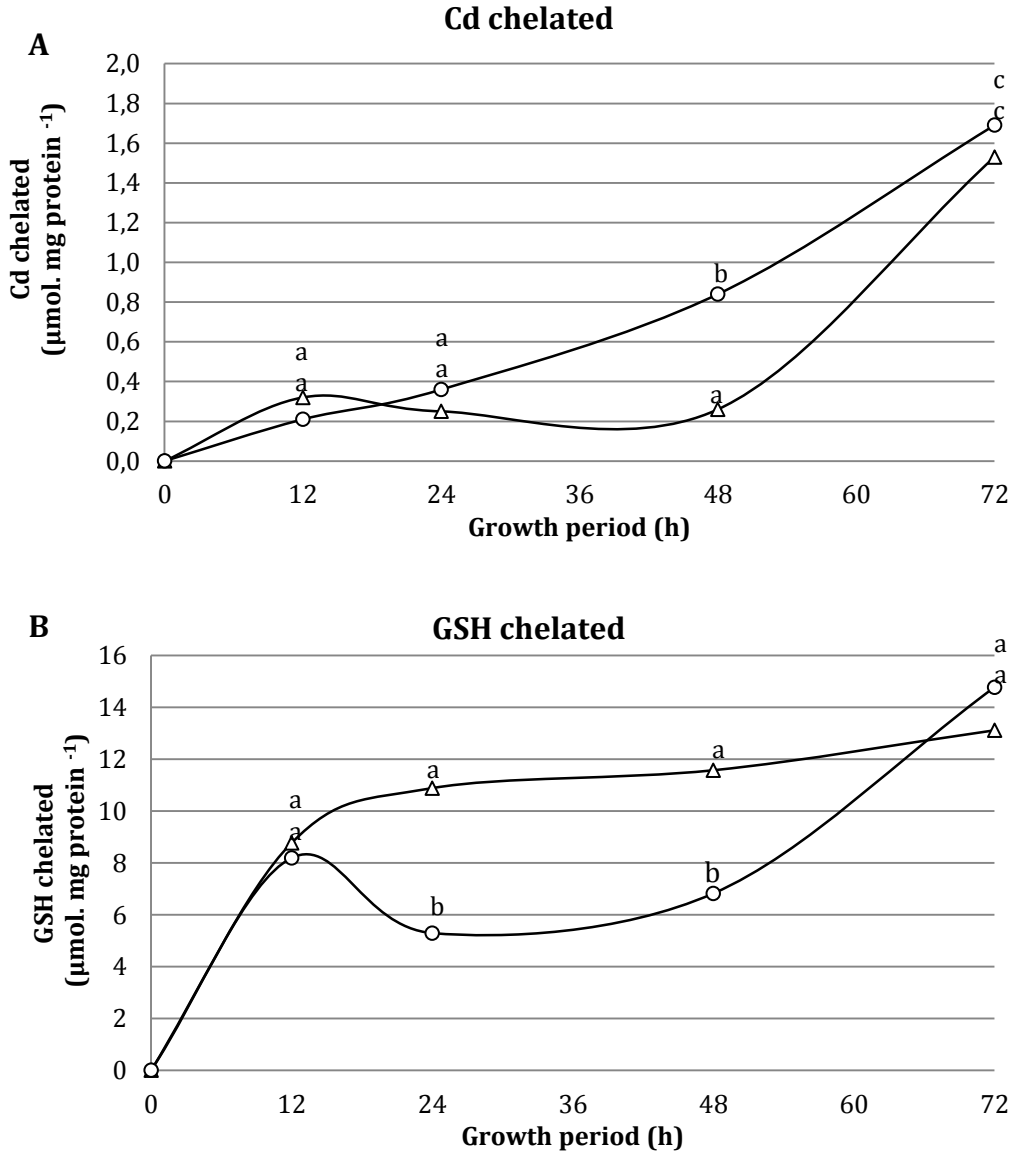

Figure 2.9 - Cd (A) and GSH (B) content in the separated protein peak in *Rhizobium leguminosarum* sensitive strain under a 50% (Open triangles) and 70% (Open circles) of growth inhibition. Intracellular Cd and GSH levels were quantified at 0h, 12h, 24h, 48h and 72h of the growth period. Data are the means of three replicate experiments with standard errors. Different letters represent different significant differences ($p < 0.05$).

Taking into account the levels of total and complexed intracellular Cd and GSH, (figures 2.6 – 2.9), differences between strains emerged. In order to highlight those

differences, the relative amount of Cd and GSH involved in the chelation process was obtained and expressed as percentage of total intracellular Cd and as percentage of total intracellular GSH (figures 2.10 – 2.11).

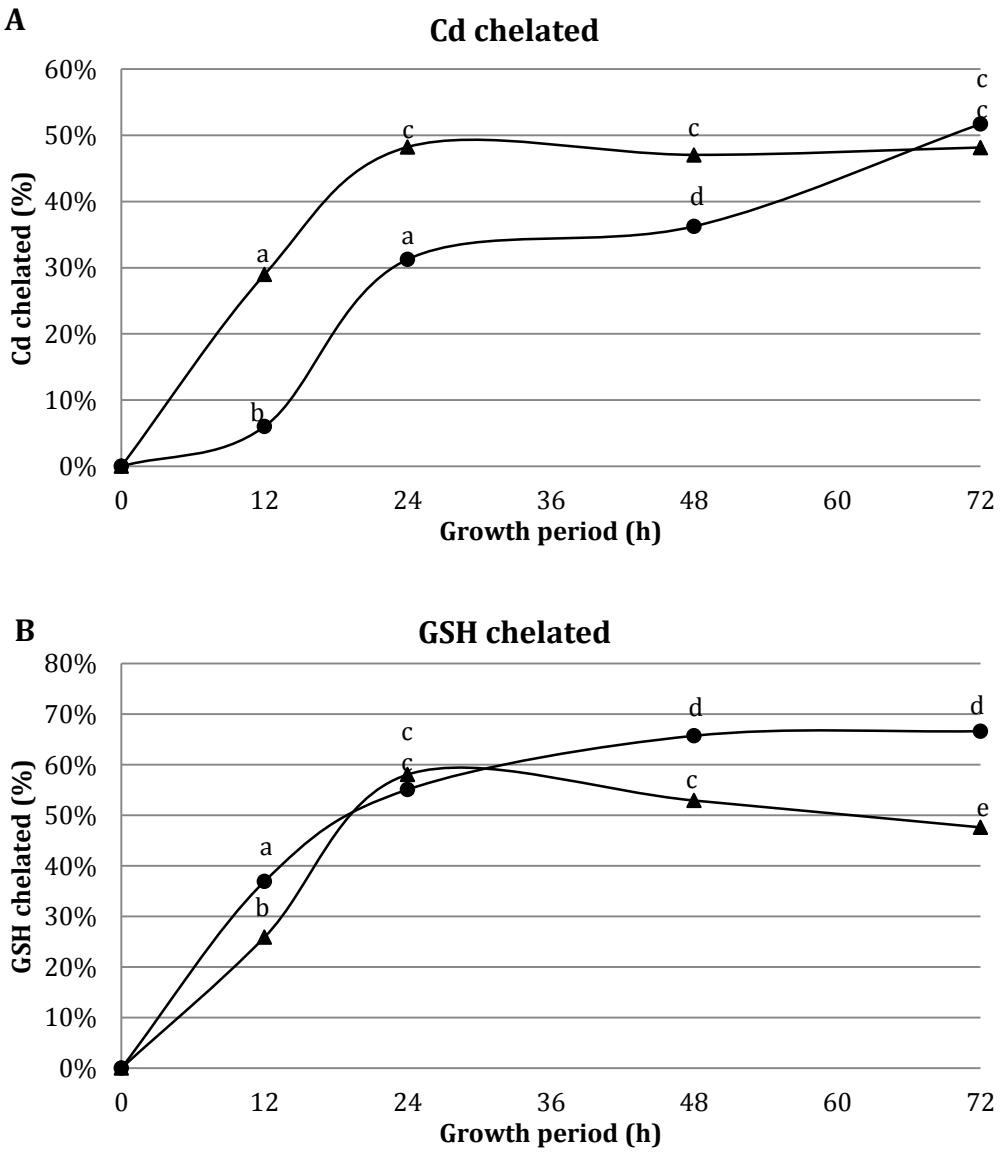

Figure 2.10 – Cd (A) and GSH (B) chelated in the separated protein peak as percentage of the total intracellular Cd and GSH, respectively, quantified during growth period of *Rhizobium leguminosarum* tolerant strain under a 50% (Full triangles) and 70% (Full circles) of growth inhibition. Intracellular Cd and GSH levels were obtained at 0h, 12h, 24h, 48h and 72h of the growth period. Data are the means of three replicate experiments with standard errors. Different letters represent different significant differences ($p < 0.05$).

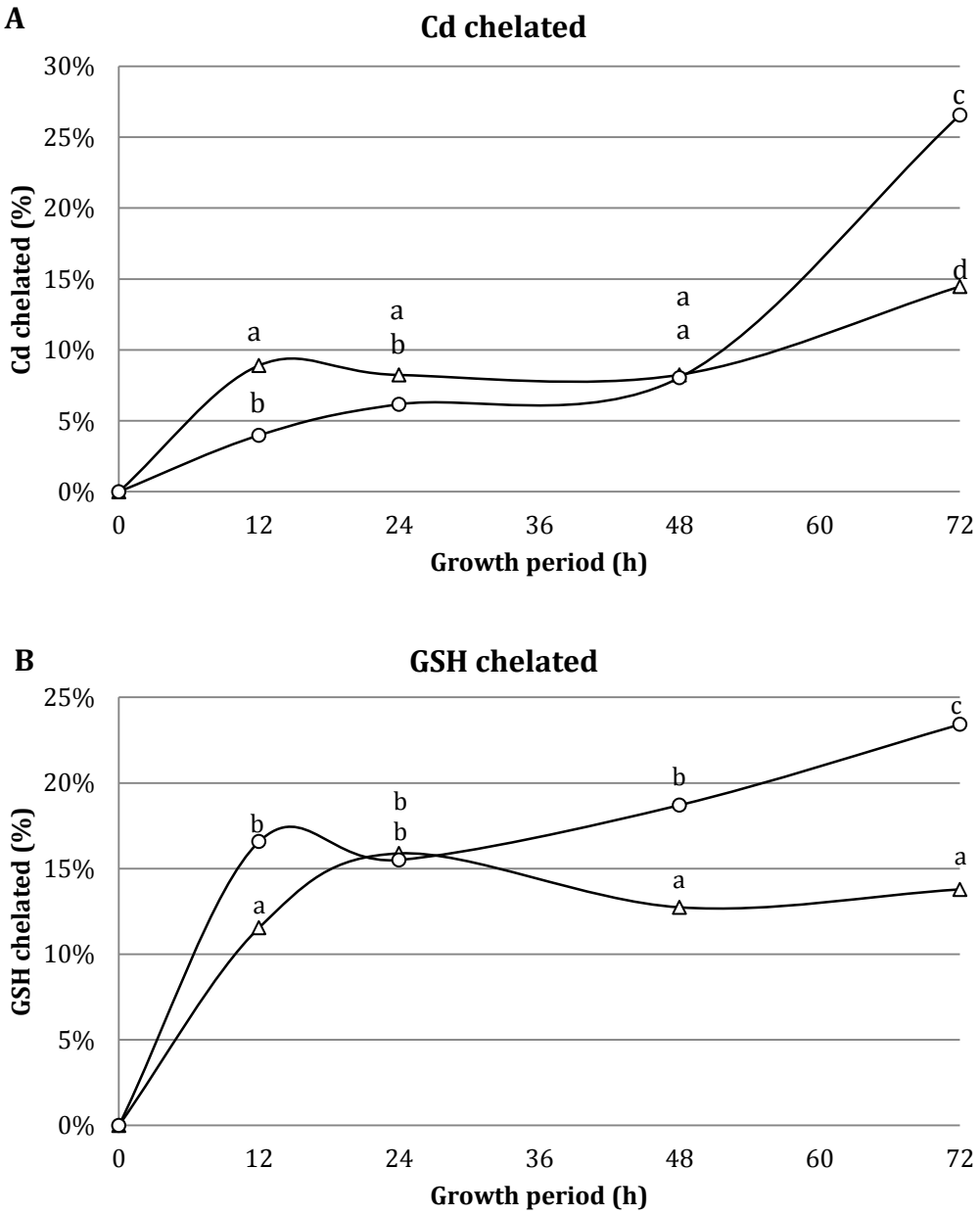

Figure 2.11- Cd (A) and GSH (B) chelated in the separated protein peak as percentage of the total intracellular Cd and GSH, respectively, quantified during growth period of *Rhizobium leguminosarum* sensitive strain under a 50% (Open triangles) and 70% (Open circles) of growth inhibition. Intracellular Cd and GSH levels were obtained at 0h, 12h, 24h, 48h and 72h of the growth period. Data are the means of three replicate experiments with standard errors. Different letters represent different significant differences ($p < 0.05$).

In *Rhizobium leguminosarum* tolerant strain (figure 2.10.), at a 50% growth inhibition, 48% of the total intracellular Cd was chelated during the first 24 h of

growth and maintained ($p > 0.05$) after 72 h of metal exposure. At 70% growth inhibition, the efficiency of metal chelation was initially (first 12 h of growth) modest, only 6% of the total intracellular Cd was chelated. Nevertheless it increased steadily the following 12 h, reaching 32% at 24 h and increased until the end of the growth period (72 h) reaching 52 % of total intracellular Cd.

The GSH-Cd complexation mechanism should also be evaluated by the ability to increase GSH levels and the percentage of thiol used in metal chelation should also be considered as an important indicator of the efficiency of this intracellular Cd detoxification mechanism. Thus, the percentage of GSH used in metal chelation was also analyzed during metal exposure. In *Rhizobium* tolerant strain (figure 2.10.B), at 50% growth inhibition, the percentage of GSH use to chelate Cd increased during the first 24 h, when 58.1% of GSH was chelated to Cd, but decreased with time, and at 72 h only 47.6% of GSH was found to be metal chelated. At 70% growth inhibition, during the first 48 h of growth the tolerant strain was able to increase the percentage of GSH used to chelate Cd, 65.7% reaching at this time a "plateau".

In the sensitive strain, the percentage of intracellular Cd chelated to GSH was 9% to 14% at a growth inhibition of 50%, and 4 to 27% at 70% growth inhibition (figure 2.11.A). NII-1 showed a low GSH ability to chelate Cd. At 50% growth inhibition, the percentage of GSH bound to Cd was never higher ($p > 0.05$) than 15.9% of the total intracellular GSH level. At 70 % growth inhibition, presented similar percentages to 50% growth inhibition of GSH chelated to Cd in the first 24 h. Increasing at 48 h and at 72 h, when the percentage was the highest recorded (23.4%).

2.4 Discussion

2.4.1 *Rhizobium* tolerance to Cd

In previous reports (Corticeiro et al., 2006; Lima et al., 2006), it was demonstrated the importance of intracellular GSH in Cd detoxification at extreme Cd stress levels, (80% growth inhibition) in *Rhizobium leguminosarum*. At that time the question if intracellular Cd chelation by GSH is only triggered as an ultimate effort to survive or if it is a cellular response to decrease intracellular Cd ions toxicity, induced at lower metal stress levels was raised. Thus, the study of the GSH-Cd chelation mechanism at different degrees of Cd stress during *Rhizobium* growth was believed to be crucial to better understand the role of the tripeptide GSH in Cd tolerance.

Previous results had already described E20-8 and NII-1 as Cd tolerant and Cd sensitive, respectively (Figueira et al., 2005; Lima et al., 2006). The screening of Cd tolerance performed in this thesis was important to show that tolerance differences between these *Rhizobium leguminosarum* strains were maintained, even though these strains had been preserved for several years in laboratory conditions. In this chapter results demonstrated that *R. leguminosarum* strain E20-8 was able to grow at 1000 μM Cd, while NII-1 strain did not survive to Cd concentrations higher than 400 μM Cd. E20-8, the tolerant strain, presented a growth inhibition of 50% at 100 μM Cd and of 70% at 200 μM Cd, and NII-1, the sensitive strain, presented a growth inhibition of 50 % at 25 μM Cd and of 70% at 50 μM Cd (figure 2.1). Cd concentrations inducing growth inhibition of 50% and 70% were chosen to perform the subsequent analysis, in order to investigate the importance of the GSH-Cd chelation mechanism in stress levels inferior to those reported by Lima et al., (2006), as different degrees of metal stress could trigger distinct cellular responses (Hu et al., 2005; Roane and Pepper, 1999). Roanne and Pepper (1999) observed that some bacterial Cd-resistant populations increase resistance under higher levels of metal stress, and therefore suggested a possible change in resistance mechanism, depending on the level of Cd exposure. A similar conclusion was obtained in *Pseudomonas putida* exposed to

different Copper (Cu) concentrations (Rensing et al., 2002). So, the GSH-Cd complexation mechanism found in *R. leguminosarum* under 80% growth inhibition (Lima et al., 2006) could not be so important in bacterial tolerance under low levels of metal stress.

2.4.2 Cd, cellular damage and the importance of GSH

Cd had been described as a non redox-active metal, although indirectly inducing oxidative stress (Penninckx, 2002), due to the formation of reactive oxygen species (ROS). ROS damage biologically relevant macromolecules, such as nucleic acids, membrane lipids and proteins (Mostertz and Hecker, 2003), leading to cellular damage. Lipids are considered to be major targets during oxidative stress (Bianucci et al., 2012). 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 (Cabiscol et al., 2000). Thus, the determination lipid peroxidation was crucial to assess the level of cellular damage experienced by bacterial strains. Cellular damages, lipid peroxidation (figure 2.2) and protein oxidation (figure 2.3), in *Rhizobium* tolerant strain were not affected in a Cd exposition inducing 50% growth inhibition, probably due to the higher levels of total thiols. Only the higher stress level (70% growth inhibition) affected lipid peroxidation, inducing an increment in MDA levels. The sensitive strain presented higher lipid peroxidation and protein oxidation levels than the control, under both metal exposures. Similarly it was observed a significantly increase in lipid peroxides in *Bradyrhizobium sp.* (Bianucci et al., 2012) and in *E. coli* when exposed to Cd (Helbig et al., 2008; Pacheco et al., 2008). Due to the rapid turnover of proteins, protein oxidation is considered to contribute less prominently to total cellular damage (Mayo, 2003). A severe Cd exposure, leading to a growth inhibition of 70%, induced a higher degree of cellular damage in both *Rhizobium* strains, possibly as a consequence of the lower levels of total thiols. The depletion of reduced GSH and protein-bound sulfhydryl groups also resulted in the production of ROS, consequently, it enhanced

cellular damage, such as lipid peroxidation and protein oxidation (Stohs and Bagchi, 1995). Corticeiro et al., (2006) demonstrated that the oxidative status in tolerant *Rhizobium* strains was better maintained due to the ability to improve GSH:GSSG ratio, than NII-1 *Rhizobium* sensitive strain. So, as it was reported by Corticeiro et al. (2006), these results demonstrated that the *R. leguminosarum* tolerant strain was able to better tolerate Cd stress. The aptitude to better cope with metal stress was believed to be dependent on its ability to improve or maintain the intracellular thiol pool (Figueira et al., 2005; Lima et al., 2006). Cd exposure lead to an increase in total intracellular thiol levels in both strains (figure 2.4), primarily at 50% growth inhibition. Nevertheless the increment was more evident in E20-8 tolerant *Rhizobium* strain, suggesting that Cd tolerance differences between the two strains were related to the ability to increase thiol levels under metal stress. As GSH is the main thiol present in *R. leguminosarum* strains, it was possibly to assume that the cellular damage exhibited by these strains under Cd exposure was inversely related to GSH levels, since higher levels of this tripeptide had a protective effect on bacterial cells growing under metal stress, as it was observed in other bacteria strain under Cd exposure (Bianucci et al., 2012; Figueira et al., 2005; Lima et al., 2006; Mendoza-Cózatl et al., 2005).

The study of the enzymatic activity of γ-GCS was considered to be important parameter to understand the higher thiol pool observed particularly in *R. leguminosarum* tolerant strain under Cd stress (figure 2.5). In this thesis only the activity of γ-GCS was determined as this enzyme is the rate-limiting in GSH synthesis (Huang et al., 1988; Kelly et al., 2002). In *R. leguminosarum* strains, parallel to the levels of total thiols, the activity of γ-GCS was enhanced under Cd exposure and much more pronounced in the tolerant strain, particularly at 50% growth inhibition. Cd has also been reported to induce the activity of γ-GCS, and consequently GSH pool, in *E. coli* (Chen et al., 2009) yeasts (Mendoza-Cózatl et al., 2005) and plants (Mendoza-Cózatl et al., 2005; Zhu et al., 1999). Since γ-GCS activity is feedback inhibited by GSH (Huang et al., 1988), the higher activity of this enzyme detected under Cd stress may be explain by the depletion of this tripeptide, possible in metal chelation, as GSH is

consumed in the detoxification of heavy metals, leading to the increment of its synthesis (Coblenz and Wolf, 1994).

2.4.3 Cd cellular distribution in *Rhizobium leguminosarum*

Cd distribution within *Rhizobium* cells demonstrated that, in both strains, the majority of Cd was retained in the cell wall, being intracellular Cd only a small fraction of the total Cd (table 2.2). These results confirm bacterial cell wall as the primary defense mechanism against metal, acting like a primary defense, which avoids the entrance of Cd into the cell. Similar results were obtained in *Rhizobium leguminosarum* (Lima et al., 2006) and in *Pseudomonas,* where 90% of the initial Cd was bound to the surface of the cells (Pabst et al., 2010). Several authors demonstrated that the extracellular barrier of LPS was an important mechanism of Cd resistance in *A. caulinodans* (Zhengwei et al., 2005). In *Klebsiella aerogenes*, the extracellular capsule prevented the entry of up to 1 nM of Cd (Mergeay, 1991). Pereira et al. (2006) demonstrated that Cd sequestration by exopolysaccharides was an important avoidance mechanism in *R. leguminosarum* strains isolated from metal-contaminated soils. Moreover, Cd exposure was proven to increase bacterial production of extracellular polymeric substances (EPS) (Guibaud et al., 2005; Henriques et al., 2007), providing protection to the cell by restricting its contact with the outer membrane layer (Ueshima et al., 2008). In this chapter, both, *Rhizobium* strains presented similar percentages of Cd retention in the cell wall, suggesting that there are no differences in Cd binding process to the cell wall among strains. Intracellular Cd was much lower than the metal bound to the cell wall. Nevertheless intracellular Cd is considered to be much more toxic, due to interfering with metabolism (Lima et al., 2006). At 50% growth inhibition, *Rhizobium* tolerant strain accumulated 18,79 μmol .mg protein $^{-1}$ Cd intracellular, while NII-1 sensitive strain presented 10.55 μmol .mg protein $^{-1}$. At 70% growth inhibition, tolerant *Rhizobium* strain E20-8 accumulated 92.81 μmol .mg protein $^{-1}$ Cd intracellular, while in the sensitive strain only 6.36 μmol .mg protein $^{-1}$ was quantified. Results from this chapter demonstrated that the tolerant *Rhizobium* strain presented higher levels of intracellular Cd than sensitive strain as was described by other authors (Figueira et

al., 2005; Lima et al., 2006). *Rhizobium leguminosarum* tolerant strain presented higher intracellular Cd levels in both treatments but, according to the analysis of cellular damage parameters, intracellular metal toxicity was not higher when compared to the sensitive strain, NII-1. Thus, the tolerance difference of these strains rely, at least in part, on the efficiency of intracellular mechanisms. Lima et al. (2006) had already established that tolerance differences detected among *R. leguminosarum* strains subjected to severe levels of metal stress, could be explained by the efficiency of the GSH-Cd chelation mechanism. Some Cd resistant strains increased their resistance under higher levels of metal stress (Roane and Pepper, 1999), suggesting a possible change in the resistance mechanism according to the degree of metal stress induced in bacterial strains (Hu et al., 2005; Roane and Pepper, 1999). Thus, GSH-Cd complexation may be an important intracellular Cd tolerance mechanism only triggered by extreme levels of Cd stress, as an ultimate cellular effort to survive, or may be an important strategy to improve *Rhizobium leguminosarum* strains at different levels of Cd.

2.4.4. Intracellular Cd and GSH levels during *Rhizobium* growth

The analysis of intracellular Cd levels during *Rhizobium* growth demonstrated that after 24 h of growth, intracellular Cd levels in E20-8 tolerant strain decreased at 50% growth inhibition (figure 2.6A), suggesting the activation of an efflux mechanism as a consequence of Cd exposure, not observed in *R. leguminosarum* sensitive strain (figure 2.6B). The stress response strategy of lowering intracellular Cd levels was also reported in several bacteria such as *Caulobacter crescentus*, (Hu et al., 2005), *Alcaligenes eutrophus* (Nies et al., 1992) and *E. coli* (Nies, 2003) due to the up-regulation of efflux pumps. Zhengwei et al. (2005) reported that *Azorhizobium caulinodans* sensitive strains presented higher Cd uptake than tolerant strains, being able to accumulate 3 to 15 times more Cd in intracellular than metal tolerant strains (Bruins et al., 2000), suggesting that Cd tolerance was related to the ability to maintain Cd intracellular levels low in tolerant strains. Nevertheless, the high Cd concentrations tolerated by *Rhizobium* E20-8 strain demonstrated that such efflux mechanism was not efficient to avoid metal accumulation in the tolerant strain at

longer periods of metal exposure, leading to intracellular Cd levels much higher than in the sensitive strain. Cd increment after 24 h of growth was concomitant with the increment of intracellular GSH levels, suggesting that intracellular Cd ions induced GSH synthesis in *Rhizobium* tolerant strain. In *E. coli*, GSH overproduction resulted in a higher uptake of Cd (Wawrzynska et al., 2005). As it was also reported in *E. coli* by Vlamis-Garlikas (2008), GSH levels in *Rhizobium* tolerant strain were enhanced even throughout the stationary phase of growth.

2.4.5. The importance of GSH in intracellular Cd detoxification

The chromatographic analysis demonstrated that GSH-Cd chelation mechanism was present in both *Rhizobium leguminosarum* strains, being already observed during the first 12 h of metal exposure (figure 2.8 and 2.9). The results present in this chapter, proved that GSH chelation mechanism was activated by the entrance of Cd into the cell and that it was not dependent on the degree of metal stress. Tolerance differences among sensitive and tolerant *Rhizobium* strains were suggested (Lima et al., 2006) to be dependent, not only on the ability to induced GSH synthesis, but also on the efficiency of the chelation mechanism, as Cd sequestration is dependent on GSH synthesis and on a rapid formation of GSH conjugates with metal ions (Adamis et al., 2004), but the overprodution of GSH in *E. coli* resulted in a higher intracellular Cd accumulation but not altered its metal tolerance (Wawrzyńska et al., 2005). Results obtained in this work show a higher efficiency of metal chelation observed under the severe level of metal stress (figure 2.10 and 2.11). During the first 12 h of growth, the GSH-Cd chelation mechanism presented a similar efficiency between strains, exception made to the tolerant strain under a 50% growth inhibition where 29% of the intracellular metal was already GSH chelated. Nevertheless, after 72 h the tolerant strain, presented 48% and 67% of intracellular metal chelated to GSH at 50% and 70% growth inhibition, respectively, while the highest percentage of metal chelated by NII-1 was only 27%. These results demonstrated the importance of GSH-Cd complexation in the detoxification of intracellular Cd in *R. leguminosarum*. This mechanism was demonstrated to be activated by metal exposure, during the first

12 h of metal stress, and not to be dependent on the degree of metal stress as it was observed in both strains. It was also proven that the tolerance differences detected among *Rhizobium* strains can be explain at least in part by the ability to increase GSH synthesis and to form GSH-Cd complexes. GSH may not be a vital metabolite for bacteria, as demonstrated by the survival of GSH mutants of *E. coli* (Chesney et al., 1996), nevertheless it was already proven that its presence is crucial to cope with several types of abiotic stresses (Bianucci et al., 2012; Chesney et al., 1996; Fergunson et al., 1998; Figueira et al., 2005; Lima et al., 2006 ; Pabst et al., 2010; Riccillo et al., 2000).

The accumulation of GSH-Cd complexes may not be a successful strategy in metal contaminated environments, as may be deleterious to the cellular metabolism. In *Saccharomyces cerevisiae* it was demonstrated that the accumulation of GSH-Cd complexes in the cytoplasm inhibited Cd uptake, nevertheless intracellular Cd levels in tolerant strain were continuously enhanced during the growth period, suggesting that GSH-Cd complexes may be excreted. In *S. cerevisiae* the formed complexes are transported into the vacuoles (Li et al., 1997), but as bacterial cell do not have such cellular organelles, the fate of GSH-Cd complexes are uncertain. In *E. coli* it was reported a periplasmic transporter mediated by GSH conjugates implicated in metylglyoxal and N-ethylamide detoxification (Ferguson et al., 1995). A similar transporter may be active in *R. leguminosarum*. Thus, as *R. leguminosarum is* a gram-negative bacteria, the periplasmic space of the tolerant and sensitive strains will be isolated and the role of this compartment in the intracellular Cd detoxification mechanism will be evaluated (Chapter 3). As the tolerance of E20-8 was suggested to be related to the efficiency of the GSH-Cd chelation mechanism, the reasons behind the higher efficiency of the GSH-Cd chelation mechanism will also be investigated. Adamis et al. (2004) demonstrated that Glutathione-S-Transferases (GST), a multifamily of detoxifying enzymes, were crucial to GSH-Cd complexes formation in *S. cerevisiae*. Thus, in Chapter 4, the GSTs of *Rhizobium leguminosarum* strains will be investigated in order to determine the importance of this family of enzymes in the efficiency of the GSH-Cd chelation mechanism and, therefore in the tolerance of E20-8 strain.

Chapter 3

The importance of the periplasmic space in Cd tolerance

3.1 Introduction

3.1.1. Gram-negative bacteria

Gram-negative bacteria, like *Rhizobium leguminosarum*, do not have the complex compartmentalization and organelles of eukaryotic cells but are divided in four compartments: the cytoplasm, the inner membrane, the periplasm and the outer membrane (Salton, 1963; Stock et al., 1977). The outer membrane, which separates the external environment from the periplasm, is composed by phospholipids, lipopolysaccharides, β-barrel proteins and lipoprotein (Beveridge, 1999; Tokuda, 2009), and the inner, membrane is composed of phospholipids in both the inner membrane and the outer membrane constituted gram-negative cell envelope (Beveridge, 1999; Nikaido, 2003; Salton, 1963; Tokuda, 2009). The cell envelope provides a boundary between the environment and the cytoplasm of the cell, and this boundary is a main line of defense protecting the cell from stressful environments (Beveridge, 1999; Ruiz et al., 2006). The periplasm is an aqueous compartment enclose by the two membranes of gram-negative cell envelope, which contains a layer of peptidoglycan and a high variety of macromolecules (Beveridge, 1999; Nikaido, 2003; Ruiz et al., 2006; Tokuda, 2009).

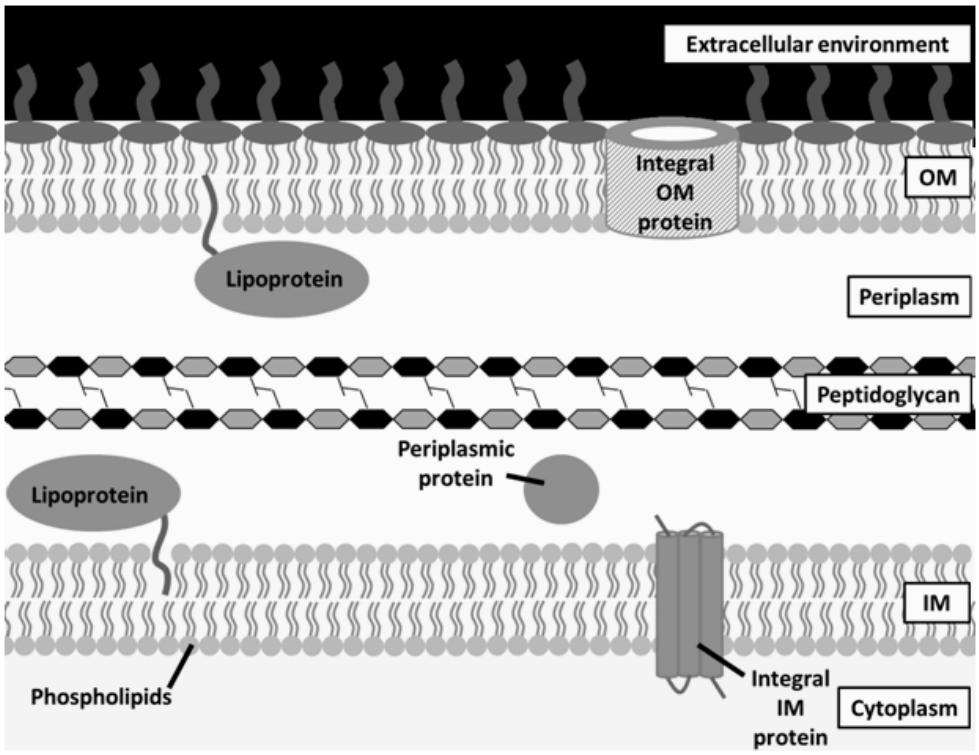

Figure 3.1 –The cell envelope of Gram-negative bacteria includes an outer membrane (OM), an inner membrane (IM), a layer of peptidoglycan, and the aqueous periplasm. The OM is an asymmetric bilayer composed of lipopolysaccharides (LPS) in the outer leaflet and phospholipids in the inner leaflet. The IM is a symmetric bilayer composed of phospholipids in both the inner leaflet and the outer leaflet. Lipoproteins in the IM and OM are anchored to the membranes through lipid tails while the proteins are in the periplasm. Soluble periplasmic proteins are in the periplasm and are not anchored to either membrane. Integral OM proteins are usually β-barrel proteins and act as channels between the periplasm and the extracellular environment. Integral IM proteins span the entire IM and have various functions. (in Poole et al., 2012).

3.1.2. The GSSG:GSH subcellular ratio

All cells must maintain intracellular compartments at appropriate reduction potentials for metabolism (Krebs, 1967). In the bacterial cytoplasm, thiol groups are maintained in a reduced state by thioredoxins and other reducing agents, whereas in the periplasm, cysteine residues are rapidly oxidized as soon as newly synthesized proteins are translocated across the inner membrane (Herrmann et al., 2009; Marco, 2009; Smirnova and Oktyabrsky, 2005; Pittman et al., 2005). GSH reduces disulfide bonds in proteins including those that may form on exposure to oxidative stress, neutralizes free radicals, and is involved in the detoxification of xenobiotics and in metal chelation (Adamis et al., 2004; Lima et al., 2006). Cellular GSH homeostasis has

long been considered as a key element of signaling cascades, transducing information on environmental constraints to their respective targets (May et al., 1998). Due to the central cysteine, GSH can undergo reversible oxidation and reduction, and can thus be present in either the reduced form or oxidized GSH disulfide (GSSG) (Haddad, 2004), and the oxidation of GSH pool is often expressed in terms of the GSH:GSSG ratio (Kirlin et al., 1999). GSH:GSSG ratio reflects the dynamic balance between GSH synthesis, decomposition, transport, oxidation, and reduction, so it depends on cellular state and compartment; and environmental conditions, such as metal stress (Corticeiro et al., 2006; Pittman et al., 2005; Smirnova and Oktyabrsky, 2005). In *E. coli* cells, under non stress conditions, 99.5% GSH exists in the reduced form, while GSSG is 0.33% of the total intracellular GSH, being the ratio GSH:GSSG between 300 and 600 (Dalle-Donne, 2009**;** Smirnova and Oktyabrsky, 2005).

3.1.4. The importance of periplasmic space in metal tolerance

GSH is involved in an important intracellular Cd detoxification mechanism found in *Rhizobium leguminosarum*: the chelation of the metal by GSH (Lima et al., 2006; Chapter 2). The efficiency of this chelation mechanism appears to be crucial to explain the Cd tolerance levels presented by some *Rhizobium* strains. Nevertheless, the accumulation of GSH-Cd complexes in the cytoplasm may possible interfere with the cellular metabolism, affecting cell survival (Adamis et al., 2009; Gomes et al., 2002). Furthermore, the accumulation of GSH-Cd complexes in the cytoplasm would inhibit Cd absorption (Gomes et al., 2002). However, as it was demonstrated in Chapter 2, Cd chelation did not inhibit metal uptake in *R. leguminosarum* tolerant strain. In *Saccharomyces cerevisiae*, the GSH–Cd complex was proven to be transported into the vacuole through the Ycf1 protein (Li et al., 1997). Thus, in bacteria, the fate of the complexes may be the exclusion out of the cell into the environment or the transport and accumulation of the complexes into the periplasmic space. In *E. coli* GSH was reported to be excreted or leaked out into the periplasm (Pittman et al., 2005), however there are no information regarding the destination of GSH-Cd complexes in bacteria.

3.1.5. Aims of the chapter

The results of the Chapter 2 suggested the non accumulation of the GSH-Cd complexes in the cytoplasm, raising the hypothesis of their transport into the periplasmic space of *R. leguminosarum.* In order to study the periplasmic space as the potential fate of the GSH-Cd complexes and its metabolic implications, the aims of this chapter were:

A. To isolate the periplasmic space from the cytosolic fraction;
B. To determine the subcellular GSH:GSSG ratios;
C. To study Cd and GSH distribution between the periplasm and the cytoplasm;
D. To analyze the possible GSH-Cd complex formation in the periplasmic space or its transport into that compartment.

3.2. Material and methods

3.2.1. Bacterial strains and growth conditions

Rhizobium leguminosarum strains E20-8 and NII-1 were grown in YEM (Somasegaran and Hoben, 1994) supplemented with Cd (0, 100 and 200 μM Cd to the tolerant strain and 0, 50 and 75 μM Cd to the sensitive strain). Cells were incubated at 26°C, in an orbital shaker, during 72 h at 200 rpm. To estimate growth, optical density was measured at 600 nm.

3.2.2. Purification of periplasmic and cytoplasmic fractions

The isolation and identification of the periplasmic and cytoplasmic fractions was accomplished by following the method described by De Maagd and Lugtenberg (1986) with some modifications. All the procedure was carried out at 0 to 4°C. *Rhizobium* cells were grown as described previously and harvested by centrifugation during 10 min at 10000 *g* and 4°C. The pellet was suspended in 5 ml of 30 mM Tris (pH 8.0) and 20% (w/v) sucrose. 2 $mg.ml^{-1}$ of lysozyme was added and incubated at room temperature for 45 min. The cells were pelleted by centrifugation at during 10 min at 10000 *g* and 4°C. Part of the supernatant fluid, the periplasm, was kept apart for measuring the activities of malate dehydrogenase (Kitto, 1969) and alkaline phosphatase (Malamy et al., 1964), markers for the cytoplasmic and periplasmic fractions, respectively. To isolate the cytoplasmic proteins, spheroplasts were lysed in 500 μl of 30 mM Tris-HCl (pH 8.0), by sonication during 15 s at 0.5 cycles. s^{-1}. Subsequently, unbroken cells were pelleted, being a small fraction kept apart for measuring malate dehydrogenase and alkaline phosphatase activities. The supernatant fluid, containing cytoplasmic fraction, was further diluted, and KCl was added to a final concentration of 200 mM. Cell envelopes were removed by centrifugation at 262000 *g* for 2 h at 4°C.

3.2.3. Alkaline Phosphatase activity

Alkaline phosphatase (AP) activity was assayed in 3 ml of 100 mM Tris-HCl pH 9.8, containing 30 µl of 65 mM p-nitrophenyl phosphate, 100 µl of 10 mM $MgCl_2$ and 100 µl of cell extract. The reaction mixture was incubated al 37 °C for 15 min. The reaction was stopped by adding 500 µl of 1 M NaOH and the p-nitrophenol produced was measured at 405 nm, using a control, lacking the enzyme, as blank, using a UV/VIS spectrophotometer (Beckman Model DU-68). A unit of ALP activity was defined as the amount of enzyme catalyzing the liberation of 1 µmol p-nitrophenol min^{-1} under standard conditions. p-nitrophenol was used as standard (Malamy et al., 1964).

3.2.4. Malate dehydrogenase activity

Malate dehydrogenase was assayed by measuring the decrease rate absorbance at 340 nm during the conversion of oxaloacetate to malate with NADH as the cofactor. Reaction mixture of 825 µl contained 60 mM HEPES (pH 7.2), 50 µl of 6 mM NADH, and 100 µl of cell extract. Before starting the reaction by the addition of 25 µl of 10 mM oxaloacetate solution, the endogenous NADH oxidase activity was measured (Kitto, 1969) using a UV/VIS spectrophotometer (Beckman Model DU-68).

3.2.5. Reduced and oxidized GSH quantification

GSH and GSSG were quantified according to the DTNB-GSSG reductase recycling assay (Anderson, 1985). In this reaction GSH is oxidized by DTNB to give GSSG with stoichiometric formation of TNB. GSSG is reduced to GSH by the action of the highly specific GR and NADPH. The reaction mixture, containing 300 µl of 150 mM Potassium phosphate buffer (pH 7.5) and 100 mM EDTA, 50 µl of 10 mM DTNB, 5 µl of 100 U. ml^{-1} GR, 90 µl of cell extract and 50 µl of NADPH, was incubated at 30°C during 15 min. TNB formation was measured at 412 nm using a UV/VIS spectrophotometer (Beckman Model DU-68) and was proportional to the sum of GSH

and GSSG present. GSSG was determined by the same method, with previously incubation of 90 µl of cell extract with 2 µl of 2-vinylpyridine during 60 min. GSH content was calculated as the difference between the two forms. GSSG and GSH were used as standard.

3.2.6. Total thiol quantification

Total thiol quantification was performed as previously described in Chapter 2, in periplasmic and cytoplasmic fractions (Ellman, 1959).

3.2.7. Analysis of the complexes

The analysis of the complexes was determined as previously described in Chapter 2, in the periplasmic and cytoplasmic fractions (Lima et al., 2006).

3.2.8. Cd quantification

Cd was quantified in both periplasmic and cytoplasmic fractions by ICP-MS, as previously described in Chapter 2.

3.2.9. Protein quantification

Protein quantification was performed in both periplasmic and cytoplasmic fractions as described in Chapter 2 (Bradford, 1976).

3.2.1. Statistical analysis

For both strains, data all the biochemical parameters in the periplasmic and cytoplasmic fractions were submitted to hypothesis testing using permutation

multivariate analysis of variance with the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008), following the calculation of Euclidean distance matrices among samples. A one-way hierarchical design, with the Cd exposure concentration as the main fixed factor, was followed in this analysis. The pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance. When the main test revealed statistical significant differences ($p<0.05$), pairwise comparisons were performed. The t-statistic in the pair-wise comparisons was evaluated in terms of significance among different conditions and strains. Values lower than 0.05 were considered as significantly different.

The null hypotheses tested were: a) for each strain, for each cellular fraction and for each biochemical parameter: no significant differences exist between Cd exposure concentrations; b) for each strain, for each biochemical parameter: no significant differences exist between cellular fractions; c) for each Cd exposure concentration, for each cellular fraction and for each biochemical parameter: no differences exist between strains.

3.3. Results

3.3.1. Purification of periplasmic and cytoplasmic fractions

Separation of the periplasmic and cytoplasmic fractions was achieved by employing the experimental methodology described by De Maagd and Lugtenberg (1986) with same alterations. *R. leguminosarum* cells grown under control conditions and under Cd stress were first treated with lysozyme to obtain the periplasmic fraction, and then disrupted by ultrasonication to obtain the cytoplasmic fraction.

3.3.1.1. Quantification of alkaline phosphatase (AP) activity

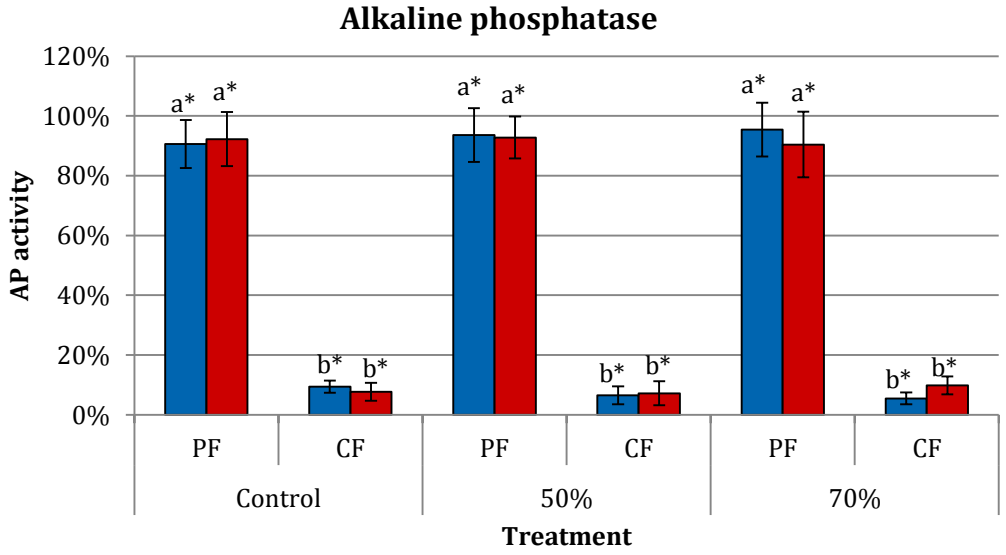

Figure 3.2. – Alkaline phosphatase activity, expressed in percentage, obtained from the periplasmic (PF) and cytoplasmic fractions (CF) of *Rhizobium leguminosarum* tolerant (blue bars) and sensitive (red bars) strains growing under control conditions and under Cd exposure inducing 50% and 70% growth inhibition. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between treatments and asterisks represent significant differences ($p < 0.05$) between strains in the same treatment.

The purification of the sub-cellular fraction was validated by performing the quantification of alkaline phosphatase activity, an enzyme specific to the periplasmic

space. AP activity of the periplasmic space was significantly ($p<0.001$) different from the enzymatic activity observed in the cytoplasmic fraction. The purification procedure with the concentration of 2 mg. ml^{-1} of lysozyme allowed the detection of more than 90% of the total alkaline phosphatase activity in the periplasmic fraction (figure 3.1). These results were not dependent on *Rhizobium leguminosarumn* strains, or on the level of Cd exposure, as no significant ($p>0.05$) differences were detected. In the cytoplasmic fraction, the enzymatic activity quantified was never higher than 10% of the total AP activity, demonstrating that the purification was achieved with a residual level of contamination between fractions.

3.3.1.2. Quantification of malate dehydrogenase (MDH) activity

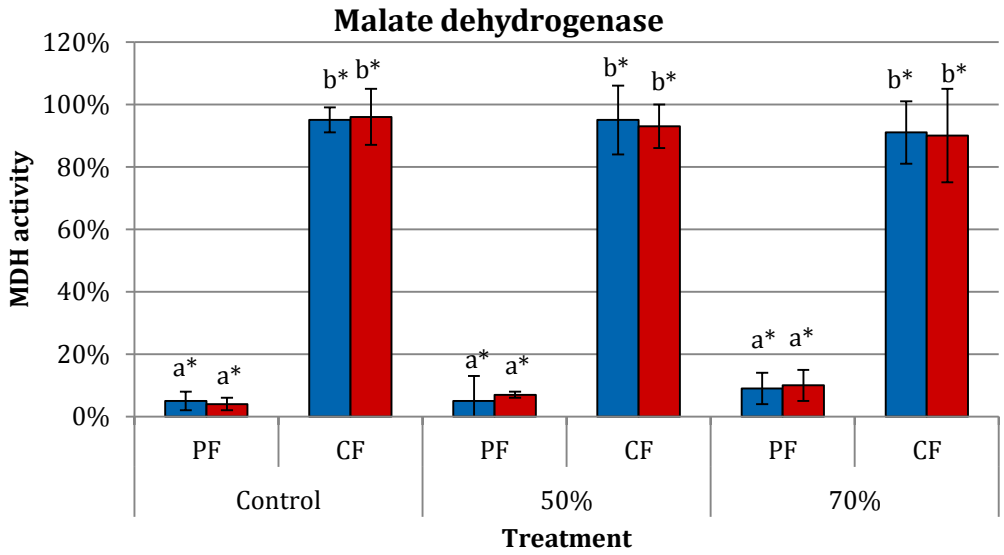

Figure 3.3. – Malate dehydrogenase activity, expressed in percentage, obtained from the periplasmic (PF) and cytoplasmic fractions (CF) of *Rhizobium leguminosarum* tolerant (blue bars) and sensitive (red bars) strains growing under control conditions and under Cd exposure inducing 50% and 70% growth inhibition. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between treatments and asterisks represent significant differences ($p < 0.05$) between strains in the same treatment.

The activity of the cytoplasmic enzyme malate dehydrogenase was determined to ensure that the cytoplasmic content did not contaminate the periplasmic fraction. Analogous to, the results obtained to AP activity, MDH activity was significantly

($p<0.001$) different between the periplasmic fraction and the cytoplasmic fraction, nevertheless no significant ($p>0.05$) differences were detected between strains or treatments. Less than 10% of MDH activity was detected in the periplasmic fraction, while more than 90% MDH activity was found in the cytoplasm (figure 3.2). So, these results indicate that the chosen methodology was efficient to purify periplasmic fraction, leaving cytoplasmic membrane intact. The cytoplasmic fraction could be obtained after lysis of the cells by ultrasonication, with subsequent removal of the cell envelopes by centrifugation. So, the concentration of 2 mg. ml^{-1} of lysozyme allowed the lysis of the cell wall with the release of the periplasmic content without disrupting the inner membrane, and thus avoiding contamination of the periplasmic fraction with cytoplasmic content.

3.3.1.3. Proteins recovered from the periplasm and cytoplasm fractions

As it was already established, the chosen purification procedure efficiently separated periplasm from cytoplasm content. Protein quantification was performed in each sub-cellular fraction and the results are presented in figure 3.4. Results indicated that, in each bacterial strain and growth condition, protein concentration was significantly ($p<0.001$) lower in the periplasmic fraction than in the cytoplasm. In *Rhizobium* tolerant strain, the periplasmic protein concentration (0.058 mg. ml^{-1}) quantified under control conditions, was not significantly ($p>0.05$) affected by Cd exposure. Nevertheless, at 70% growth inhibition, the protein concentration in the cytoplasm was significantly ($p<0.001$) affected by the metal exposure decreasing from 0.349 mg. ml^{-1} to 0.138 mg. ml^{-1}.

In NII-1 sensitive strain protein content was significant ($p<0.05$) affected by Cd exposure in both subcellular fractions. The protein content in the periplasmic fractions were significantly ($p<0.05$) reduced due to meta, but were not significant ($p>0.05$) affected by the degree of stress. In the cytoplasmic fraction it was observed a significant ($p<0.001$) decrease in protein concentration as the level of Cd exposure has been enhanced, reaching 0.152 mg. ml^{-1} at 70% growth inhibition.

Under control conditions and at 70% growth inhibition, periplasmic and cytoplasmic protein levels of both *R. leguminosarum* strains were not significantly

(p>0.05) different. Although under Cd concentrations inducing 50% growth inhibition, NII-1 sensitive strain presented significantly (p<0.05) lower peptide content in both fractions, being the highest difference observed in the cytoplasm where protein concentration in the sensitive strain was 2-fold lower.

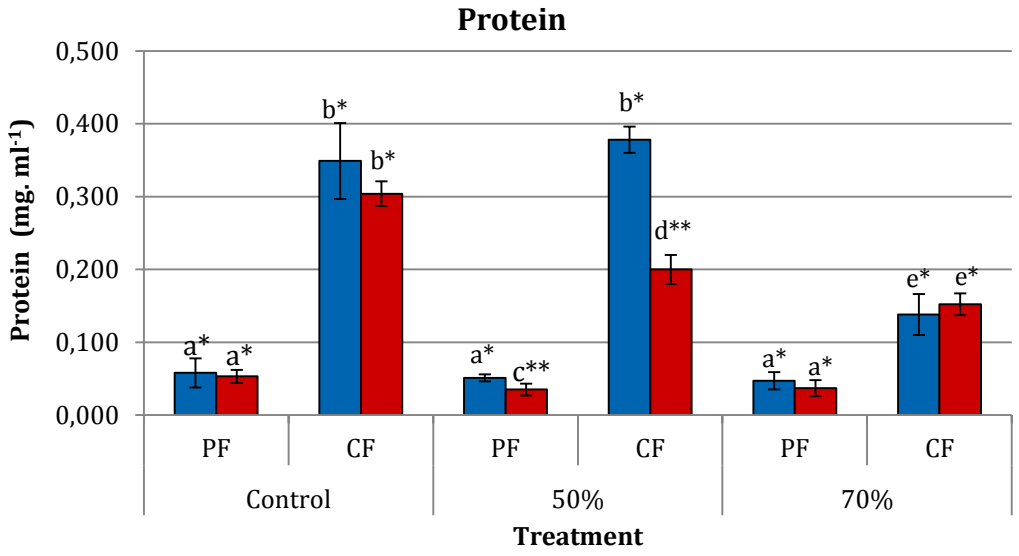

Figure 3.4. – Protein from the periplasmic (PF) and cytoplasmic fractions (CF) of *Rhizobium leguminosarum* tolerant (blue bars) and sensitive (red bars) strains growing under control conditions and under Cd exposure inducing 50% and 70% growth inhibition. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between treatments and asterisks represent significant differences ($p < 0.05$) between strains in the same treatment.

The ratio between the periplasmic and cytoplasmic proteins was significantly (p<0.05) different among treatments, but was similar between the two *Rhizobium leguminosarum* strain. The percentage of periplasmic proteins was only significantly (p<0.05) higher than the control at 70% growth inhibition, increasing from 14% to 26% of the total protein content and decreasing from 86% to 74% in the cytoplasm. Similar results were observed in NII-1 (sensitive strain) at 70% growth inhibition, the concentration of periplasmic proteins was enhanced to 20% of the total protein content. Furthermore, it was not possible to detect significant differences (p>0.05) among *Rhizobium* strains under control conditions or under Cd exposure. So, despite the differences in protein quantifications observed in both strains, the relation between the periplasmic and the cytoplasm peptide content was very similar in tolerant and sensitive strains.

3.3.2. GSH and GSSG levels in the periplasm and cytoplasm

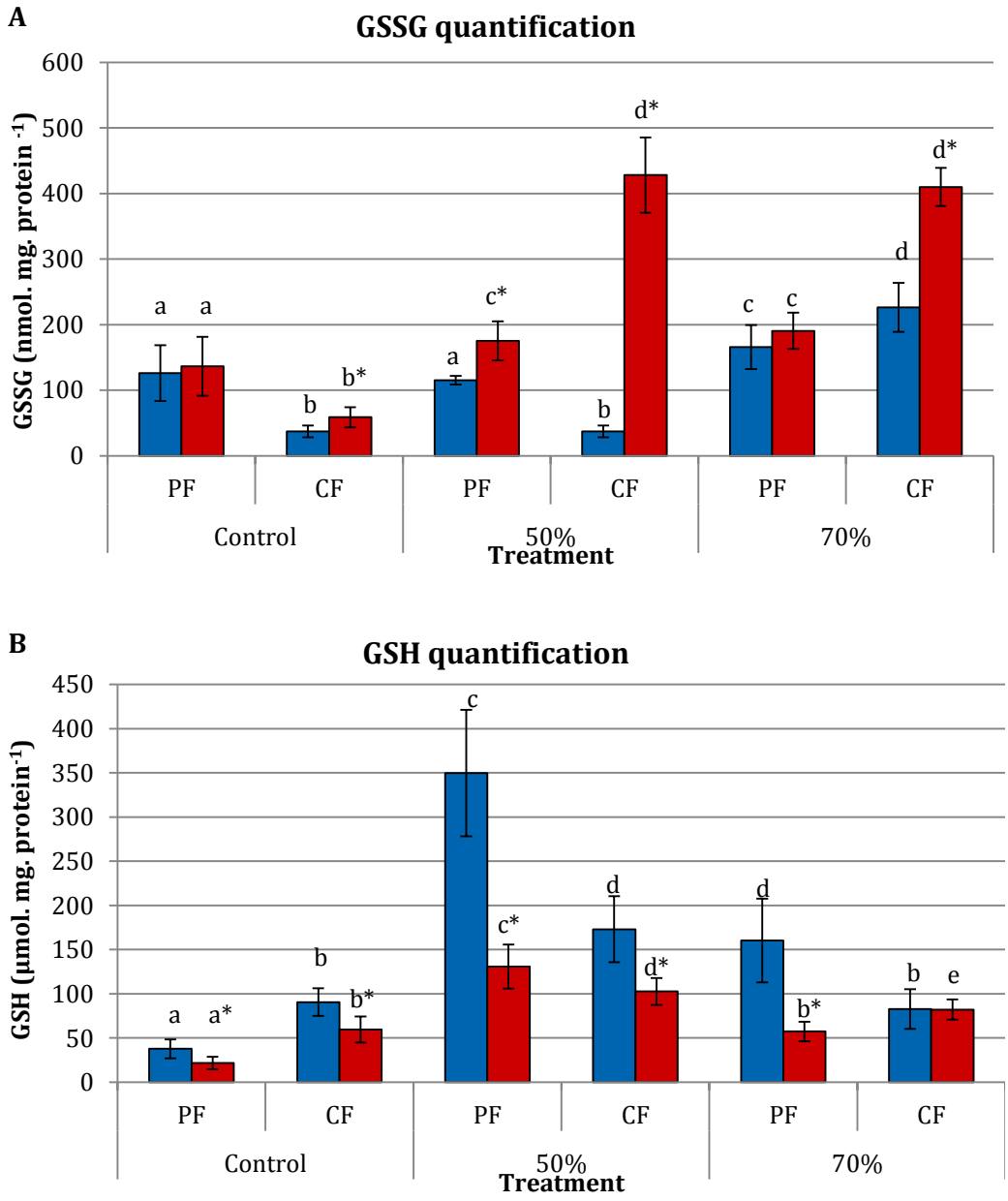

Figure 3.5. – GSSG (A) and GSH (B) in the periplasmic (PF) and in the cytoplasmic (CF) fractions of *Rhizobium leguminosarum* tolerant (blue bars) and sensitive (red bars) cells growing under control conditions and under Cd exposure inducing 50% and 70% growth inhibition. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between treatments and asterisks represent significant differences ($p < 0.05$) between strains in the same treatment.

In the tolerant strain GSSG levels were significantly ($p<0.001$) higher in the periplasm space, than in the cytoplasm, in control and 50% growth inhibition (figure 3.5.A). However, 70% growth inhibition induced a significant ($p<0.001$) increment in

GSSG content in both fractions, much evident in the cytoplasmic fraction. At this level of stress, GSSG concentration was significantly ($p<0.001$) higher in the cytoplasm than in the periplasm. In the sensitive strain, most of the GSSG was located in the periplasm, in control, as it was observed in the tolerant strain, but under Cd exposure, GSSG levels increased 7-fold in the cytoplasm compared to control conditions surpassing periplasmic concentrations. No significant ($p>0.05$) differences were detected in periplasmic GSSG levels between Cd treatments. For the three conditions tested, the tolerant strain presented significantly lower ($p<0.001$) GSSG values in the cytoplasm than the sensitive strain.

Under control conditions, both strains presented significantly ($p<0.05$) higher GSH levels in the cytoplasm than in the periplasm (figure 3.5.B). Under Cd exposure it was observed a significant ($p<0.001$) increase of GSH in the periplasm, at both levels of metal concentration. The highest level of periplasmic GSH was observed at 50% growth inhibition, 349.66 μmol. mg protein^{-1}. In the cytoplasm GSH increased at that level of metal stress, but at 70% growth inhibition was similar to control. GSH values obtained at 50% growth inhibition were the highest quantified in subcellular fractions of NII-1, 130.55 μmol. mg protein^{-1} in the periplasm space and, 102.75 μmol. mg protein^{-1} in the cytoplasm. The results also showed that, under metal stress, GSH subcellular levels of the sensitive strain were significantly ($p<0.001$) lower that the ones observed in the tolerant strain, being the major difference in the periplasmic fraction under a 50% growth inhibition.

GSH:GSSG ratio was estimated in the subcellular fractions of *Rhizobium leguminosarum* and the results are expressed in table 3.1. Under control conditions both *Rhizobium* strains presented a significantly ($p<0.05$) higher GSH:GSSG ratio in the cytoplasm, than in the periplasmic space (6 to 8 fold). Under Cd exposure, tolerant and sensitive strains showed marked differences. In the tolerant strain, only at 70% growth inhibition, it was determined a GSH: GSSG ratio significantly ($p<0.05$) higher in the periplasmic fraction than in the cytoplasm. In the sensitive strain, Cd exposure induced a significant ($p<0.05$) increment in GSH: GSSG ratio in the periplasmic fraction, 3.1-fold at 50% growth inhibition and 1.5-fold under 70% growth inhibition. Tolerant strain always presented significantly ($p<0.05$) higher GSH:GSSG ratio in both subcellular fractions than the sensitive strain, being the highest GSH:GSSG ratio

detected in the cytoplasmic fraction of this strain when exposed to a Cd concentration inducing 50 % growth inhibition.

Table 3.1. GSH:GSSG ratios in periplasmic and cytoplasmic fractions in *Rhizobium leguminosarum* strains tolerant and sensitive under control conditions and under Cd exposure inducing 50% and 70% growth inhibition, after 72 h of growth. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between treatments and asterisks represent significant differences ($p < 0.05$) between strains in the same treatment.

		Fraction	GSH: GSSG ratio
Tolerant (E20-8)	**Control**	Periplam	299 ± 53 a*
		Citoplasm	2430 ± 276 b*
	50%	Periplam	3037 ± 591 c*
		Citoplasm	4640 ± 564 d*
	70%	Periplam	971 ± 107 e*
		Citoplasm	366 ± 77 a*
Sensitive (NII-1)	**Control**	Periplam	158 ± 48 a **
		Citoplasm	1013 ± 151 b **
	50%	Periplam	745 ± 99 c **
		Citoplasm	240 ± 36 a **
	70%	Periplam	301 ± 25 d **
		Citoplasm	198 ± 61 a **

3.3.4. Cd distribution in *R. leguminosarum* cells

The protocol used to separate periplasmic from cytoplasmic fraction also allowed to determine the subcellular localization of the metal within *Rhizobium leguminosarum* cells: wall bound Cd and intracellular Cd values were previously presented in Chapter 2. Nevertheless intracellular Cd comprises the metal in the periplasmic space as well as in the cytoplasm. The metal distribution between subcellular fractions is presented in figure 3.6.

In the tolerant strain, at 50% growth inhibition, the Cd concentration found in the periplasmic space was significantly higher ($p<0.001$) from the level in the cytoplasmic fraction. However, at 70% growth inhibition, Cd levels in the cytoplasm were significantly ($p<0.001$) enhanced to 79.61 μmol. mg protein^{-1}, while Cd concentration in the periplasm was 35.12 μmol. mg protein^{-1}. Although the Cd

concentrations determined in the periplasmic space of both Cd exposures were similar, at 50% growth inhibition, it corresponded to 24% of the total intracellular Cd content, whereas at 70% growth inhibition it only corresponded to 12% of the total intracellular Cd.

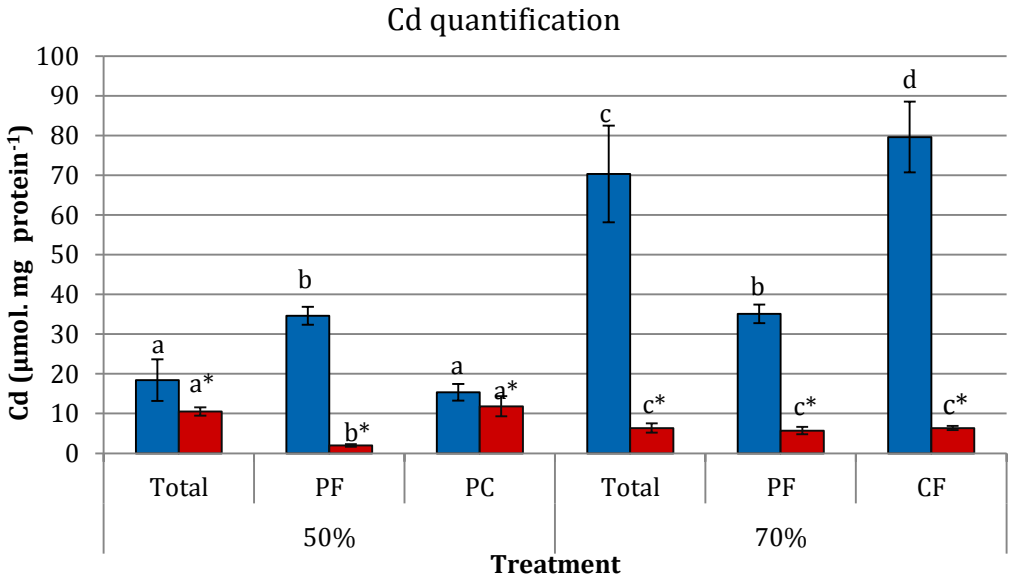

Figure 3.6. – Cd in the periplasmic (PF) and in the cytoplasmic (CF) fractions of *Rhizobium leguminosarum* tolerant (blue bars) and sensitive (red bars) cells growing under Cd exposure inducing 50% and 70% growth inhibition. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between treatments and asterisks represent significant differences ($p < 0.05$) between strains in the same treatment.

In NII-1 sensitive strain it was observed a different subcellular distribution of Cd: at 50% growth inhibition, a significant ($p<0.001$) higher Cd concentration was found in the cytoplasm, 11.85 µmol.mg protein^{-1}. At 70% growth inhibition there were no significant ($p>0.05$) differences between both fractions. However, under the lowest level of metal stress, only 3% of the total intracellular Cd was found in the periplasmic fraction, whereas at 70% growth inhibition it reduced to 20% of total intracellular Cd. So, at 50% growth inhibition, the tolerant strain presented a significantly ($p<0.001$) higher Cd concentration in the periplasmic fraction than in the cytoplasm, a trend that was inverted at 70% growth inhibition. The concentration of Cd in the sensitive strain was always higher on the cytoplasm at 50% growth inhibition, being similar at 70% growth inhibition.

3.3.5. Distribution of GSH-Cd complexes in *R. leguminosarum* cells

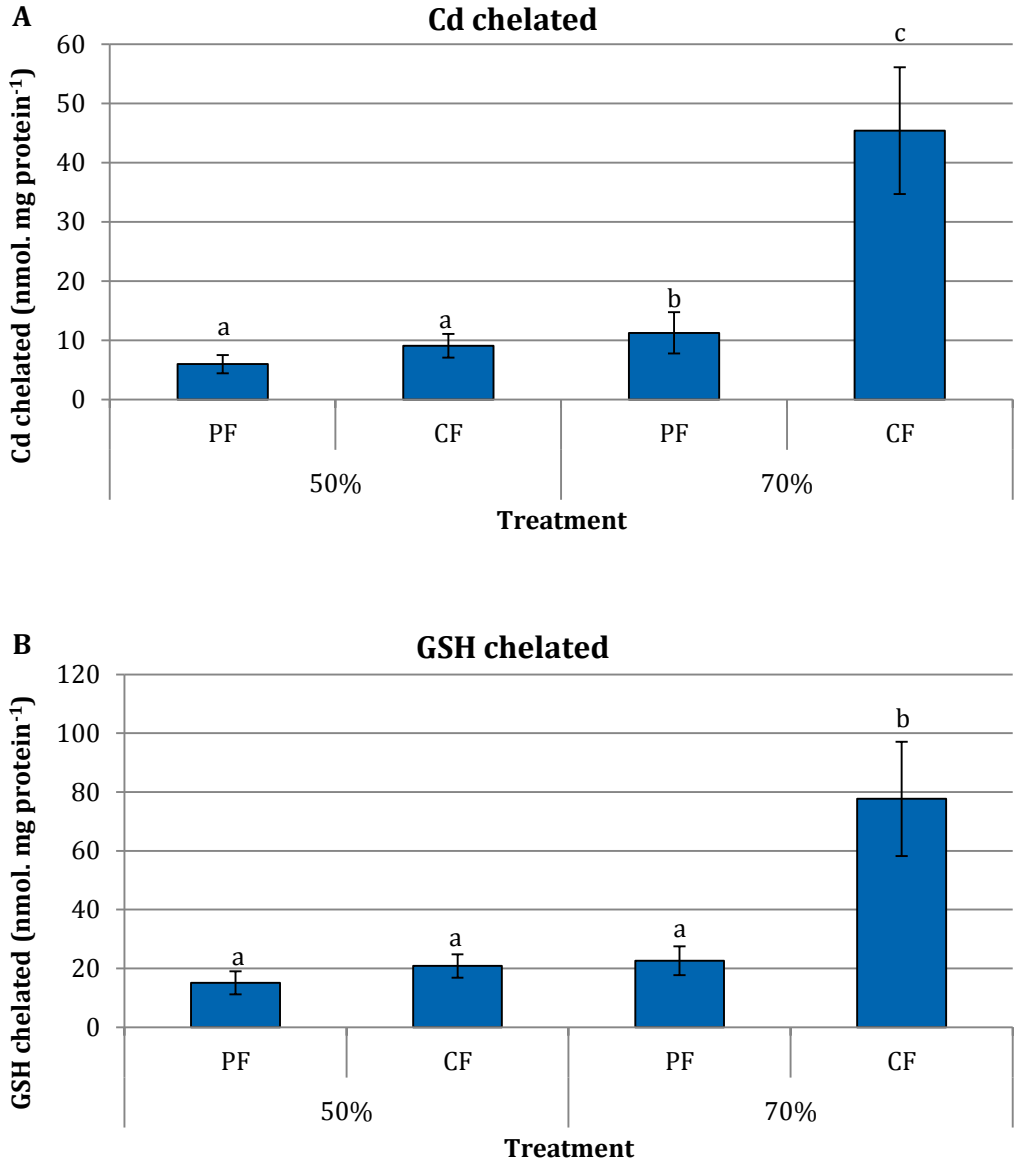

Figure 3.7. Cd (A) and GSH (B) content in the separated protein peak obtained in periplasmic (PF) and cytoplasmic (CF) fractions in *Rhizobium leguminosarum* tolerant strain, E20-8, under Cd exposure inducing 50% and 70% growth inhibition. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between treatments.

Buffer extracts, containing periplasmic and cytosolic Cd fractions, provided the source of peptide–Cd complexes and were separated through gel filtration, as it was reported in Chapter 2. Cd bound to GSH in both periplasmic and cytoplasmic fractions are expressed in figure 3.7. The analysis of the GSH-Cd distribution demonstrated that, in *Rhizobium leguminosarum* strains, most of Cd complexes were localized in the

cytoplasm. In the tolerant strain Cd chelated in the cytoplasm was 1.5-fold higher than in periplasmic fraction at 50 % growth inhibition and 4-fold at 70 % growth inhibition, reaching 9.07 µmol. mg protein^{-1} and 45.37 µmol. mg protein^{-1}, respectively (figure 3.7.A). In the tolerant strain higher levels of GSH chelated were found in the cytoplasm under both levels of metal stress, being 1.4-fold and 3.4 fold higher, respectively than in the periplasmic space (figure 3.7.B).

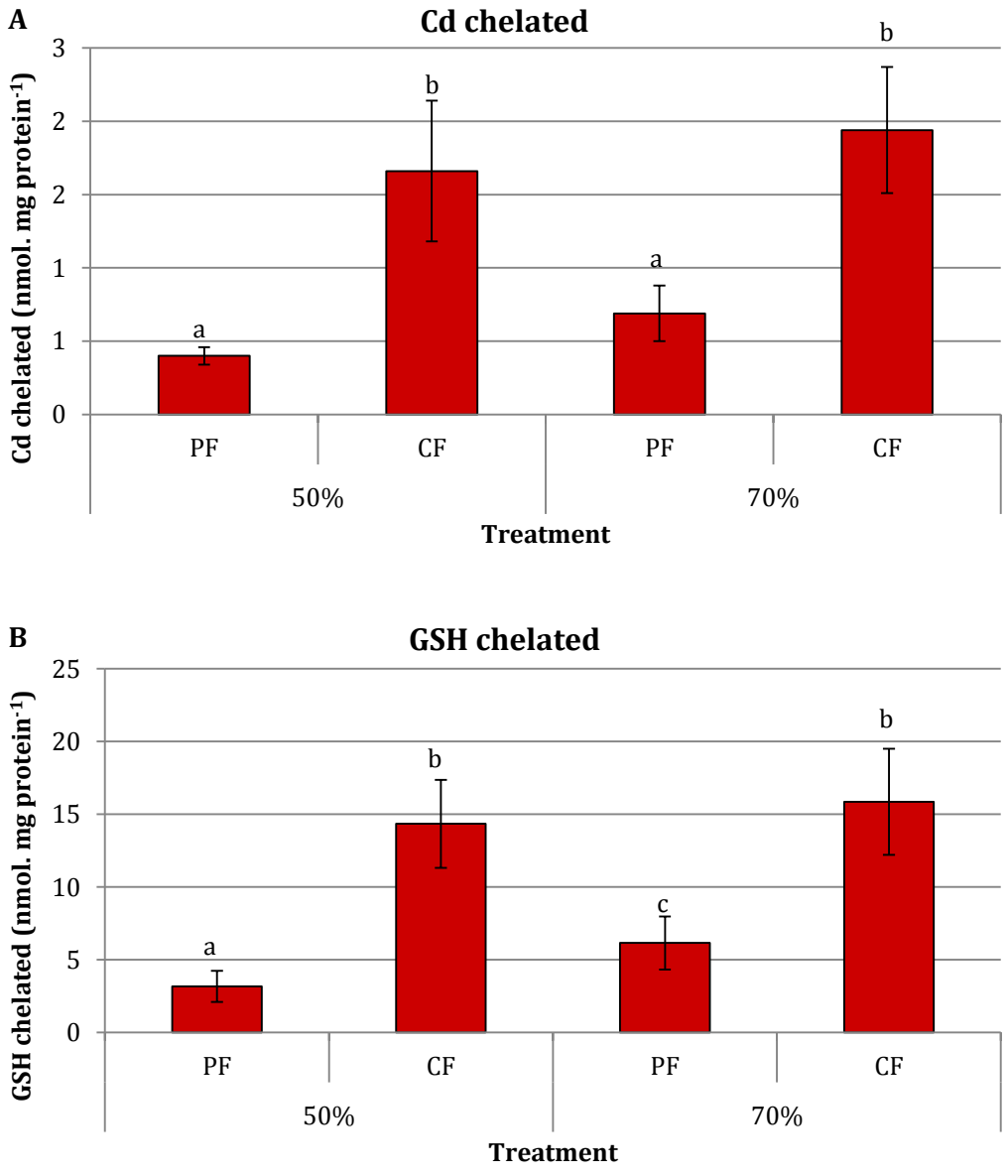

Figure 3.8. Cd (A) and GSH (B) content in the separated protein peak obtained in periplasmic (PF) and cytoplasmic (CF) fractions in *Rhizobium leguminosarum* sensitive strain, NII-1, under Cd exposure inducing 50% and 70% growth inhibition. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between treatments.

The same profile was observed in the sensitive strain, under the lower level of metal exposure, Cd chelated in the cytoplasm was 4.2-fold higher than in the periplasmic space and at 70% growth inhibition it was 2.8-fold higher (figure 3.8.A). The level of GSH chelated in the sensitive strain was also higher in the cytoplasmic fraction, 14.34 µmol. mg protein^{-1} at 50% growth inhibition and, 15.85 µmol. mg protein^{-1} at 70% growth inhibition (figure 3.8.B).

The efficiency of the GSH-Cd chelation mechanism was also found to be different according to its subcellular localization (figure 3.9). In the tolerant strain more than 50 % of the Cd present in the cytoplasm was found to be chelated, 59 % under the lower level of metal stress and, 57 % under the severe Cd exposure. In the periplasm fraction the efficiency was much lower, but dependent on the degree of metal stress, reaching 32 % of Cd chelated at 70 % growth inhibition.

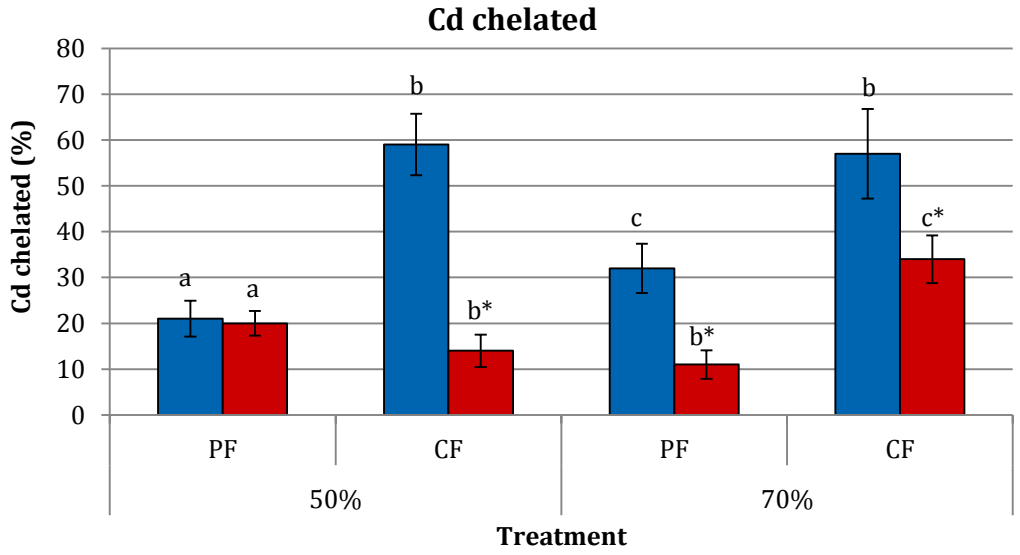

Figure 3.9. The percentage of Cd chelated in periplasmic (PF) and cytoplasmic fractions in *Rhizobium leguminosarum* tolerant (blue bars) and sensitive (red bars) strains under Cd exposure inducing 50% and 70% growth inhibition. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between treatments and asterisks represent significant differences ($p < 0.05$) between strains in the same treatment.

At 50% growth inhibition, the efficiency of the GSH-Cd chelating mechanism in tolerant strain was achieved using only 4% of the GSH quantified in the periplasm and 12% of the tripeptide present in the cytoplasm. At 70% growth inhibition, 14% of the GSH from the periplasm fraction was found to be chelated to Cd, but in the

cytoplasm 89% of GSH was involved in the chelation mechanism. In the sensitive strain the highest percentage of GSH involved in Cd chelation was 20% in the cytoplasmic fraction, under the severe level of metal stress. Overall *R.leguminosarum* tolerant strain demonstrated significantly ($p<0.001$) higher levels of Cd chelated to GSH than the sensitive strain.

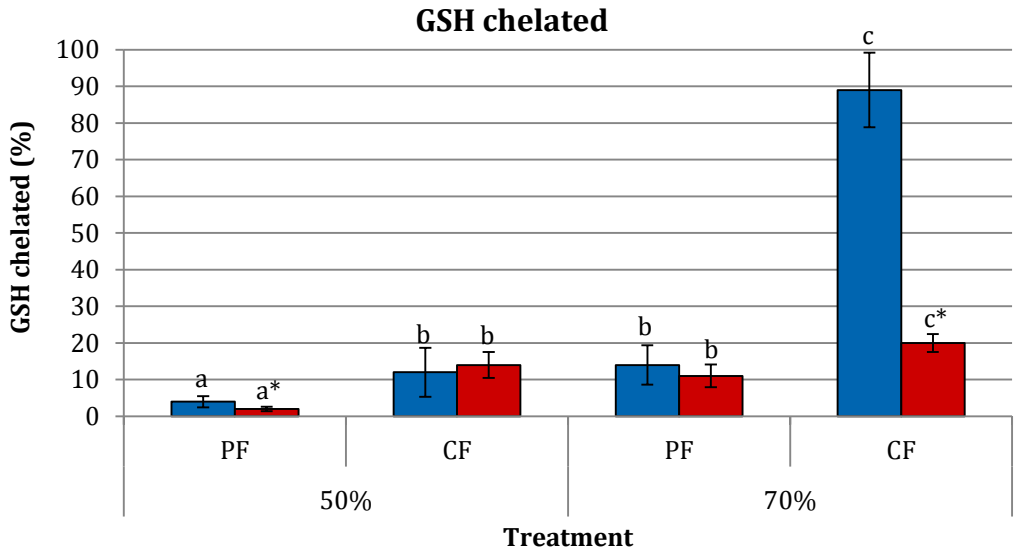

Figure 3.10. The percentage of GSH chelated in periplasmic (PF) and cytoplasmic (CF) fractions in *Rhizobium leguminosarum* tolerant (blue bars) and sensitive (red bars) strains under Cd exposure inducing 50% and 70% growth inhibition. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between treatments and asterisks represent significant differences ($p < 0.05$) between strains in the same treatment.

3.4. Discussion

3.4.1. Subcellular fractionation: periplasmic and cytoplasmic fractions

In order to investigate the subcellular localization of GSH-Cd complexes in *R. leguminosarum*, bacterial cells were sub fractionated into periplasmic and cytoplasmic fractions, according to the method describe by (De Maagd and Lugtenberg, 1986) with some adaptations. The efficient separation of cellular fractions was demonstrated by determining the activities of alkaline phosphatase (AP) and malate dehydrogenase (MDH) enzymes, markers for the periplasmic and cytoplasmic fractions, respectively (De Maagd and Lugtenberg, 1986; Molenaar et al., 2000). The efficiency of the procedure had already been demonstrated in *R. leguminosarum* (De Maagd and Lugtenberg, 1986), and in several other bacteria such as *Agrobacterium tumefaciens* (Wu et al., 2008) and *E. coli* (Arrecubieta et al., 2001). Results demonstrated that this protocol was efficient in releasing periplasmic proteins, without disturbing cytoplasmic membrane, in *R. leguminosarum* strains under control conditions and Cd exposure. In the periplasmic fraction more than 90% of the total AP activity was quantified, while only less than 10% of the total MDH activity was detected. In the cytoplasmic fraction more than 90% of the total MDH activity was quantify, demonstrating the efficient disrupt of the inner membrane and consequently release of cytoplasmic proteins.

3.4.2. The GSH: GSSG ratio in subcellular fractions

Within bacterial cells, more than 99% of GSH exists in the thiol-reduced form (GSH). The remaining amounts undergo thiol oxidation to form GSH disulfide (GSSG) and mixed disulfides with target proteins (Dalle-Donne, 2009). The GSH:GSSG redox ratio can directly influence disulfide bonding in proteins, functioning as an indicator of cellular oxidative stress level (Wang and Ballatori, 1998). In *R. leguminosarum* it

was demonstrated that the majority of GSH was in the reduce form in both subcellular fractions, nevertheless important biological levels of GSSG were observed in the periplasmic space of *Rhizobium* strains in the absence of metal stress, as it was shown by Eser et al. (2009) in *E. coli*. Such GSSG levels corroborated the oxidative environment in the periplasm, even under non stress conditions. In *R. leguminosarum* tolerant strain the GSSG increment induced by Cd exposure was only detected at 70% growth inhibition. In NII-1 sensitive strain, GSSG increase was more pronounced, particularly in the cytoplasm, independently of the degrees of metal stress, evidencing the higher susceptibility of *Rhizobium* sensitive strain to oxidative stress. Moreover GSSG in the cytoplasm may be highly toxic to cellular metabolism because it may easily react with free sulfhydryl groups (Smirnova and Oktyabrsky, 2005). The high GSH:GSSG ratio observed in both cellular fractions of E20-8 under the lowest degree of Cd stress, showed that *R. leguminosarum* tolerant strain was able to cope with metal stress and consequently avoid oxidative stress effects by increasing GSH levels in both subcellular fractions. In gram-negative bacteria, GSH was reported to be synthesized in the cytoplasm and subsequently exported into the periplasm space (Suzuki et al., 1986). This statement was confirmed by the results expressed in this chapter, since in *R. leguminosarum* it was not detected activity of γ-GCS in the periplasmic space (results not shown), corroborating that periplasmic GSH was previously synthesized in the bacterial cytoplasm. In the GSH-dependent detoxification of N-ethylmaleimide, GSH was reported to be recycled in the cytoplasm (MacLaggan et al., 2000). However in other detoxifying pathways GSH was leaked out into the periplasm space (CrowyChanel et al., 2001) through an ABC-type transporter (Pittman et al., 2005) or was excreted to the extracellular medium, with the following reentry into the cell (Smirnova and Oktyabrsky, 2005). The results of this chapter suggested that GSH was exported into the periplasmic space therefore protecting cellular metabolism from the metal toxicity. In yeast, GSH can be decomposed by gamma-glutamyl transferase (γ-GT) and Lap4 (an aminopeptidase) inside the vacuole, restoring glutamate, cysteine and glycine in the cytoplasm, which can be used for new GSH synthesis through GSH synthesis enzymes (Adamis et al., 2007; Adamis et al., 2009). Thus, GSH would be recycled for continuous protection against metals, xenobiotics and oxidative stress. In gram-negative bacteria this tripeptide may be

cleaved in the periplasmic space and resynthesized in the cytoplasm, as the peptide bond between glutamate and cysteine can only be cleave by the periplasmic enzyme γ-glutamyl transpeptidase (Smirnova and Oktyabrsky, 2005), conserving the low levels of GSH in the periplasm space (Vlamis-Gardikas et al., 2008). Once more, as in *R. leguminosarum* Cd exposure enhanced GSH levels in both subcellular fractions, even at 70% growth inhibition, high levels of GSH were synthesized and transported into the periplasmic space, possibly as GSH-Cd complexes.

3.4.3. Cd distribution in *Rhizobium* subcellular compartments

Intracellular Cd ions are assumed to be highly toxic to the cellular machinery. Consequently, the high intracellular Cd concentrations found in *R. leguminosarum* tolerant strain may be considered dangerous to cell metabolism, even with the demonstrated efficiency of the chelation mechanism in this strain. In *Pseudomonas putida*, Cd was reported to be storage in the periplasm limiting metal toxicity in the cytoplasm (Pabst et al., 2010). In this work, the purification and analyzes of the periplasmic space content was believed to be crucial to highlight the intracellular Cd detoxification pathway in *R. leguminosarum.* Pabst et al. (2010) suggested that, during metal uptake, Cd was initially retained in the periplasmic space before entering the cytoplasm as 4.3% of the total Cd was found in the periplasmic space and only 0.25% of the metal was found in the cytoplasm (Pabst et al., 2010). On the other hand, Rensing et al. (2002) suggested that, in gram-negative bacteria, Cd was first accumulated in the cytoplasm and consequently exported into the periplasm by a P-Type ATPase. The high levels of Cd present in the cytoplasm of *R. leguminosarum* strains suggest that Cd was uptaken into the cytoplasm and exported to the periplasm to avoid the toxicity due to metal accumulation. In *R. leguminosarum* tolerant strain, under a 50% growth inhibition, the higher levels of Cd quantified in the periplasmic space, when compared to the levels in the cytoplasm, could suggest that Cd was first uptaken into the periplasmic space. Nevertheless the results obtained at the extreme level of stress show that the high levels of metal in the cytoplasm may only be due to the direct entrance of the metal, as upon saturation of the periplasmic space, Cd ions

can be exported extracellular, as observed by Rensing et al. (2002) by the CZC system exporting Cd out of *E. coli* cells. In *P. aeruginosa* it was determined that an outer membrane purine (OprE purine) was involved in the efflux of chromate from the periplasm to outside the cell, thus avoiding accumulation of chromate (Cr) in the cytoplasm (Rivera et al., 2008). In *E. coli* one of the pathways described for copper (Cu) resistance was the active efflux of the metal from the cytoplasm into the periplasmic space, carried out by ATPases located in the internal membrane (Outten and O'Halloran, 2001; Rensing et al., 2000).

3.4.4. GSH-Cd complexes in *R. leguminosarum* subcellular fractions

The higher levels of Cd in the cytoplasm and the cytoplasmic synthesis of GSH, indicated that the GSH-Cd complexes were formed in the cytoplasm. Although the possible accumulation of the GSH-Cd complexes in the cytoplasm was reported to inhibit Cd uptake (Gomes et al., 2002), it may have a negative impact on the cellular metabolism. So, even with the demonstrated efficiency of the chelation mechanism in *R. leguminosarum* tolerant strain (Chapter 2), the accumulation of GSH-Cd complexes in the cytoplasm may not emerge as an improved survival strategy in a permanent contaminated environment. The increment in intracellular Cd levels found in the tolerant strain, parallel to the efficiency in GSH-Cd chelation and suggested that the complexes were excreted from the cytoplasm. Indeed, *R. leguminosarum* tolerant strain presented GSH-Cd complexes in the periplasmic space, suggesting that the GSH-Cd complexes were excreted to the periplasmic space. In gram-negative bacteria, as *Rhizobium*, GSH-Cd complexes may be exported into the periplasmic space, to increase cell tolerance to Cd. In *S. cerevisiae*, the formed GSH-Cd complexes are transported into the vacuole through the yeast cadmium factor protein (Ycf1) (Li et al., 1997), to avoid its accumulation in the cytoplasm and Pittman et al. (2005) suggested that in *E. coli*, GSH transport by CydDC, an ABC-type transporter, was similar to that of Ycf1 described by Li et al. (1997).

Chapter 4

The role of GSTs in *R. leguminosarum* tolerance to cadmium

4.1 Introduction

4.1.1 Glutathione-S-transferases

Glutathione-S-transferases (GST; EC 2.5.1.18) are a family of multi-functional dimeric proteins implicated in a variety of stress conditions, particularly in the cellular detoxification of xenobiotic (Allocati et al., 2009; Favaloro et al., 2000; Hayes et al., 2005) such as drugs and pesticides (Kanai et al., 2006). The common feature of these enzymes is to catalyze the conjugation of the sulphur atom of GST with a large variety of hydrophobic toxic compounds of both endogenous and exogenous origin (Allocati et al., 2009; Armstrong, 1991). Furthermore, GSTs are involved in other cellular functions, such as peroxidase and isomerase activity (Hayes and McLellan, 1991; Hayes and Pulford, 1995; Mannervik and Danielson, 1988). GSTs, using GSH as co-enzyme and/ or as substrate, are also involved in several degradation reactions and have the ability to bind to a range of lipophilic compounds (Favaloro et al., 2000). GSTs are widely distributed in nature, been found in both prokaryotes and eukaryotes. Presently it is possible to distinguish, at least, four major families of these proteins: cytosolic GSTs, mitochondrial GSTs, microsomal GSTs and bacterial

fosfomycin-resistance proteins (Allocatti et al., 2009; Armstrong, 2000; Hayes et al., 2005). The cytosolic GSTs are divided into numerous divergent classes, according to their chemical, physical and structural properties (Hayes et al., 2005; Sheehan et al., 2001), and represent the most biologically relevant and well studied group of GSTs (Allocati et al., 2009); the mitochondrial GSTs, also known as kappa class GSTs, are soluble enzymes that have been characterized in eukaryotes (Robinson et al., 2004); the microsomal GSTs include membrane-bound transferases known as membrane-associated proteins that are involved in ecosanoid and GSH metabolism (Jakobsson et al., 1999); and the fosfomycin-resistance proteins that are exclusive to bacteria (Allocati et al., 2009).

Cytosolic GSTs, referred only as GST in this thesis, are divided into different classes, mainly α, μ, π, θ, σ, ζ and ω in mammals, φ and τ in plants, δ in insects and β, θ, χ and ζ in bacteria, according to their specific characteristics (Allocatti et al., 2009; Vuilleumier and Pagni, 2002). GSTs are accepted to belong to the same class, generally, when proteins present a sequence similarity higher than 40% in their primary structure (Armstrong, 1997). GSTs of different classes share less than 25% sequence identity. The identity increases only if the N-terminal region is considered, since this region comprises part of the active site, with residues that interact with GSH, and it is evolutionarily conserved (Hayes et al., 2005; Sheehan et al., 2001). In addition to amino acid sequence identity, immunological properties, kinetic features as well as similarity of the crystal structures provide additional supporting data (Allocatti et al., 2009; Vuilleumier and Pagni, 2002). Despite the low inter-class sequence identitiy, crystallographic analyzes demonstrated that the overall protein fold is conserved among GSTs classes (Dixon et al., 2002). GSTs are dimeric proteins homo or heterodimers of subunits with apparent molecular masses in the range of 23–27 kDa (Yang et al., 2004), with each subunit divided in a two-domain structure: the N-terminal thioredoxin-like domain and the C-terminal α-helices domain (Kanai et al., 2006). The active site of these enzymes consists of two binding sites: the G-site, where GSH binds; and the H-site, where the hydrophobic electrophiles bind (Armstrong, 1997; Sheehan et al., 2001).

4.1.2. Bacterial GSTs

Four different classes of GSTs have been identified in bacteria: β, θ, χ and ζ (Di Ilio et al., 1988; Rossjohn et al., 1998; Sheehan et al., 2001; Wiktelius and Stenberg, 2007). β class GSTs is characterized by the presence of a cysteine residue at the GSH site (Rossjohn et al., 1998), by the ability to efficiently conjugate with the most GST common substrate, CDNB, and binding to GSH-affinity matrix. The first β class GST was characterized from *Proteus mirabilis* (PmGST) (Di Ilio et al., 1988), but GSTs from this class had already been purified and characterized from several other bacteria, such as *E. coli.*

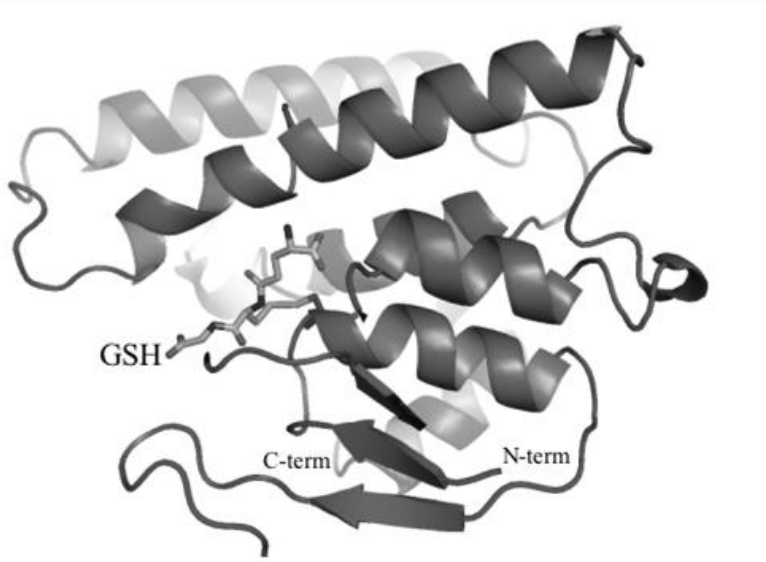

Figure 4.1- Beta class GST structure from *Proteus mirabilis* (in Rossjohn et al., 1998)

4.1.3. Putative GSTs in the genomes

Bacterial genomes encoding multiple GST genes with extensively different sequences have been investigated in the last years. Nevertheless, most of the gene products do not present functional features or have unknown functions (Vuilleumier and Pagni, 2002). In the *E. coli* genome, in addition to the beta class GST and to Stringent starvation protein A, a RNA polymerase, six other GST homologues have been identified (Rife et al., 2003). These proteins had GST and GSH dependent

peroxidase activities and were involved in the defense against oxidative stress (Kanai et al., 2006). In *P. mirabilis* as well as in *Proteus vulgaris* three and four different GSTs were identified, respectively (Allocatti et al., 2009). According to Rife et al. (2003) there are eight possible GSTs in *E. coli* but only two of the eight gene products have functional characteristics. There is no information in *R. leguminosarum* GSTs. According to the genome sequencing there are 19 putative GSTs in the chromosome plus four in the plasmids (Young et al., 2006).

4.1.4. The role of GSTs in Cd tolerance

A general mechanism for Cd detoxification is the chelation of the metal by GSH (Adamis et al., 2009). In *R. leguminosarum* the GSH-Cd complex formation is an important Cd detoxifying mechanism present in both, tolerant and sensitive strains (Chapter 2), but showing a higher efficiency of Cd chelation to GSH in the tolerant strain, suggesting that the sensitivity of NII-1 may be justified by the low efficiency of the complexation mechanism in this strain. The tolerant strain was able to complex 52% of the intracellular Cd with 67% of the intracellular GSH, while sensitive strain only sequestrated 27% of intracellular Cd and used only 23% of total GSH. So, apparently intracellular GSH content do not explain *per se* the GSH-Cd complexation differences between strains. GSTs are detoxifying enzymes that catalyze the nucleophilic attack of the sulfur atom of GSH on the electrophilic group of the substrate, both from endobiotic and xenobiotic origin. In *Saccharomyces cerevisiae* it was demonstrated that this family of enzymes have distinct roles in response to Cd stress: one is involved in the formation of GSH-Cd complexes, while the other appears to be involved in the regulation of GSH homeostasis (Adamis et al., 2004).

4.1.5. Aims of the chapter:

GSTs may be involved in the GSH-Cd complex formation in *Rhizobium leguminosarum* and its activity under metal stress may explain the chelation efficiency differences detected among strains and, consequently, their tolerance

differences under metal stress. However there are no reports regarding the study of GSTs in rhizobia under metal stress. So, in order to understand the role of this family of enzymes in the formation of GSH-Cd complexes, the aims of this chapter are:

A. To determine GST activity of *Rhizobium* strains in the absence and in the presence of Cd inducing 50% growth inhibition.
B. To purify GST isoforms under control conditions and under Cd exposure.
C. To understand the role of GSTs on GSH-Cd complex formation, through quantification of GST activity using Cd as substrate.

4.2 Material and methods

4.2.1 Bacterial strains and growth conditions

Rhizobium leguminosarum strains E20-8 (tolerant) and NII-1 (sensitive) were grown in YEM (Somasegaran and Hoben, 1994) supplemented with Cd (0 and 100μM Cd to the tolerant strain and 0 and 50 μM Cd to the sensitive strain). Cells were incubated at 26°C, in an orbital shaker, during 72 h at 200 rpm. To estimate growth, optical density was measured at 600 nm.

4.2.2 Isolation of GSTs

Rhizobium GSTs were purified according to the method of Di Ilio et al. (1988). Briefly, cells were washed twice with 10 mM potassium phosphate buffer pH 7.0 containing 1mM EDTA and 1mM DTT and disrupted by sonication during 20 s at 0.7 cycles. s^{-1}). The homogenated material was centrifuged at 105.000 *g* for 1 h and the supernatant was loaded onto a GSH-Sepharose affinity column equilibrated with 10 mM potassium phosphate buffer pH 7.0 with 1 mM EDTA. The column was washed using 15ml of the same buffer with 0.5M KCl to rinse away non-specific proteins; non-retained effluent was collected. The GSTs were then eluted in 1 ml fractions with 50 mM Tris–HCl buffer pH 9.6 with 5mM GSH. Fractions were tested for activity towards CDNB and assayed for protein using the Bradford Assay. Retained and non-retained fractions were then frozen at −80°C for further analysis and the procedure was repeated three times.

4.2.3 Purification of GSTs isoforms

Fractions presenting GST activity were pooled and submitted to anion exchange chromatography. The sample was eluted with a linear salt gradient (0 to

750 mM NaCl). Detection was performed at 280 nm. Fractions were tested for activity towards CDNB and assayed for protein using the Bradford Assay (Bradford, 1976). Retained fractions were then frozen at −80◦C for further analysis and the procedure was repeated three times.

4.2.4 Enzyme activity

GST activity was determined spectrophotometrically with CDNB, ETHA and DCNB and Cd by the method of Habig and Jakobi (1981). The activity towards CDNB was determined at 340 nm by adding 200 µl of 50 mM phosphate potassium buffer (6.8), 100 µl of 5 mM GSH, 100 µl of cell extract and finally 100 µl of 5 mM CDNB to start the reaction. The activity towards ETHA was determined at 270 nm by adding 250 µl of 50 mM phosphate potassium buffer (6.8), 100 µl of 5 mM GSH, 100 µl of cell extract and finally 50 µl of 2 mM ETHA to start the reaction. The activity towards DCNB was determined at 344 nm by adding 50 µl of 50 mM phosphate potassium buffer (6.8), 250 µl of 5 mM GSH, 100 µl of cell extract and finally 100 µl of 5 mM DCNB to start the reaction. The activity towards Cd was determined at 254 nm by adding 210 µl of 50 mM phosphate potassium buffer (6.8), 90 µl of 5 mM GSH, 100 µl of cell extract and finally 100 µl of 4 mM Cd to start the reaction. GSTs activity was obtained based on the extinction coefficients of 9.6 mM. cm^{-1} (CDNB), 5.0 mM cm^{-1} (ETHA) and 10.0 mM cm^{-1} (DCNB). All the assays were performed at 25 °C, using a UV/VIS spectrophotometer (Beckman Model DU-68) and activity was calculated using protein concentrations determined via the Bradford assay, with BSA used as standard.

4.2.5 Protein quantification

Protein quantification was performed by the Bradford method as previously described in Chapter 2 (Bradford, 1976).

4.2.6 Statistical analysis

For both strains, data from GST activity and protein peak isolated were submitted to hypothesis testing using permutation multivariate analysis of variance with the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008), following the calculation of Euclidean distance matrices among samples. A one-way hierarchical design, with the Cd exposure concentration as the main fixed factor, was followed in this analysis. The pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance. When the main test revealed statistical significant differences ($p<0.05$), pairwise comparisons were performed. The t-statistic in the pair-wise comparisons was evaluated in terms of significance among different conditions and strains. Values lower than 0.05 were considered as significantly different.

The null hypotheses tested were: a) for each strain, for each substrate and for total GST activity: no significant differences exist between Cd exposure concentration; b) for each strain and for each Cd exposure concentration: no significant differences exist between GSTs isoforms; c) for each strain and for each GST isoform: no significant differences exist between Cd exposure concentration; d) for each Cd exposure concentration and for total GST activity: no differences exist between strains; e) for each Cd exposure concentration and for each GSTs isoform: no differences exist between strains.

4.3 Results

4.3.1 GST activity in *Rhizobium leguminosarum* cells

GST activity was assessed in *Rhizobium leguminosarum* strains, under control conditions and under Cd exposure, in order to understand the role of this family of enzymes in *Rhizobium* metal tolerance. Cd concentration inducing 50% and 70% growth inhibitions in both *Rhizobium* tolerant and sensitive strains were chosen to perform several analysis (Chapters 2 and 3), however in this chapter all analysis were performed at 50% growth inhibition, due to the amount of cells needed to perform all the methodologies. The enzymatic activity of GSTs was obtained towards three substrates presenting different chemical properties: CDNB, ETHA and DCNB, in tolerant and sensitive (figure 4.2.) strains grown under control conditions and under Cd exposure. Under control conditions, the GST activity of the tolerant strain towards CDNB was not significantly ($p>0.05$) different from the activity detected towards ETHA, but was significantly higher ($p<0.001$) than the GST activity quantified towards DCNB. Results also demonstrated that GST activity towards the different substrates was significantly ($p<0.001$) affected by metal exposure. GST activity towards CDNB and DCNB was significantly enhanced ($p<0.001$) by Cd exposure, 4 and 3.5 fold, respectively. GST activity towards ETHA decreased 2 fold in the presence of Cd. In the sensitive strain GST activity towards the three different substrates was similar in cells growing in the absence or in the presence of Cd. The highest GST activity was quantified towards ETHA in both treatments, being the only significant difference ($p<0.05$) detected due to metal exposure. Comparing GST activity between both strains it was evident that, under control conditions, tolerant strain presented a significant ($p<0.001$) higher GST activity towards CDNB, but no significant ($p>0.05$) differences were obtain towards ETHA and DCNB. Under Cd exposure, differences in GST activity towards CDNB was even higher between both strain, since GST activity was highly enhanced in the tolerant strain, while in the sensitive strain GST activity

was negatively affected by the metal. Cd decreased GST activity towards ETHA in both strains, although its effect was more pronounced in the tolerant strain. For DCNB, strains showed different Cd activity, being higher in the tolerant strain than in the sensitive strain.

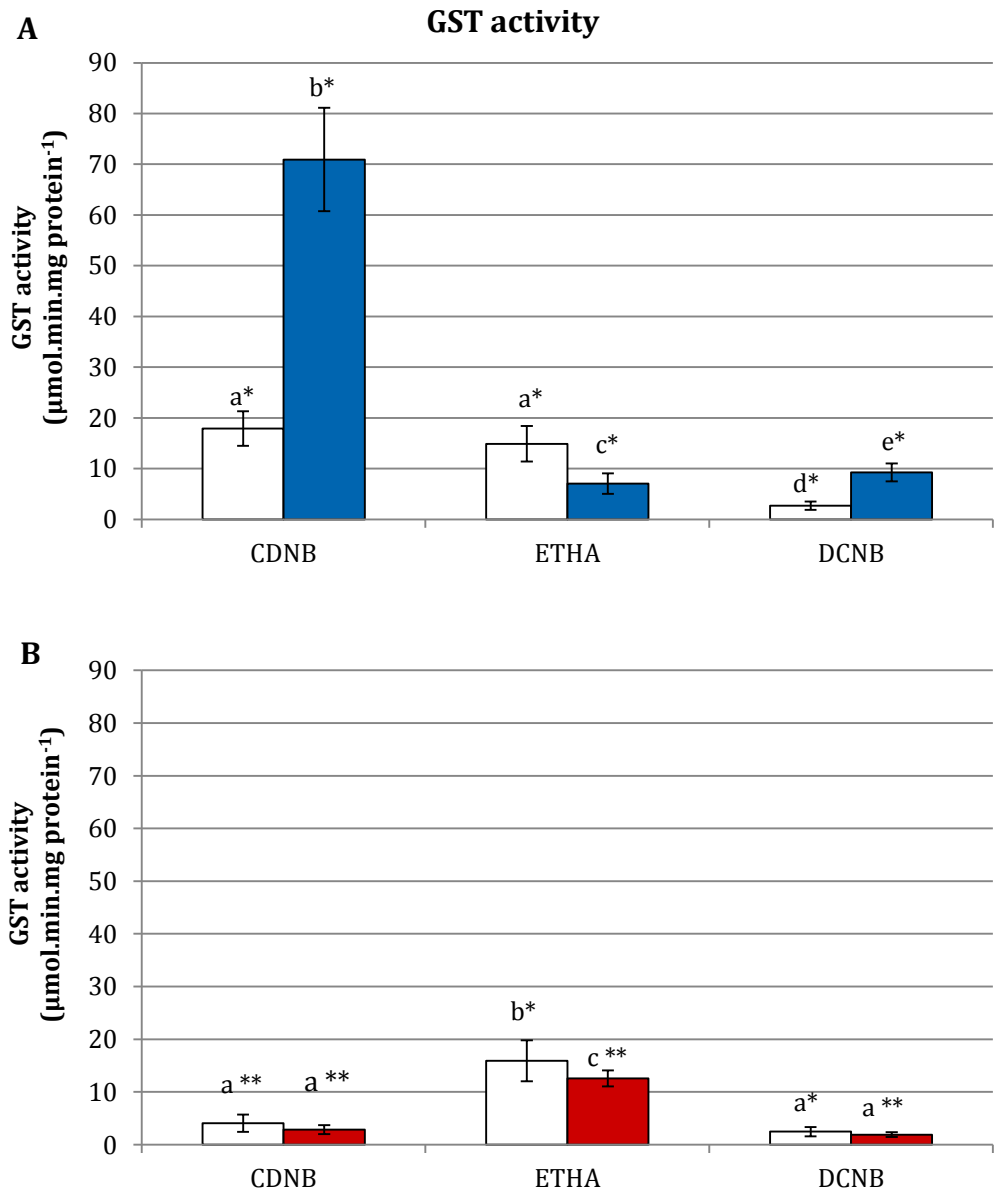

Fig. 4.2 – GST activity in *Rhizobium leguminosarum* tolerant (A) and sensitive (B) strains, grown under control conditions (open bars) and under Cd exposure inducing 50% growth inhibition (full bars), towards different specific substrates: CDNB, ETHA and DCNB. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between treatments and asterisks represent significant differences ($p < 0.05$) between strains in the same treatment.

4.3.2. GST activity in the periplasmic and cytoplasmic fractions

The GST isolation procedure was performed in the periplasmic fraction and in the cytoplasmic fraction in order to understand GST localization within the cell. Despite the effort it was not possible to isolated different GST isoforms from the periplasmic fraction. GST activity from the isolated fractions is expressed in figure 4.3.

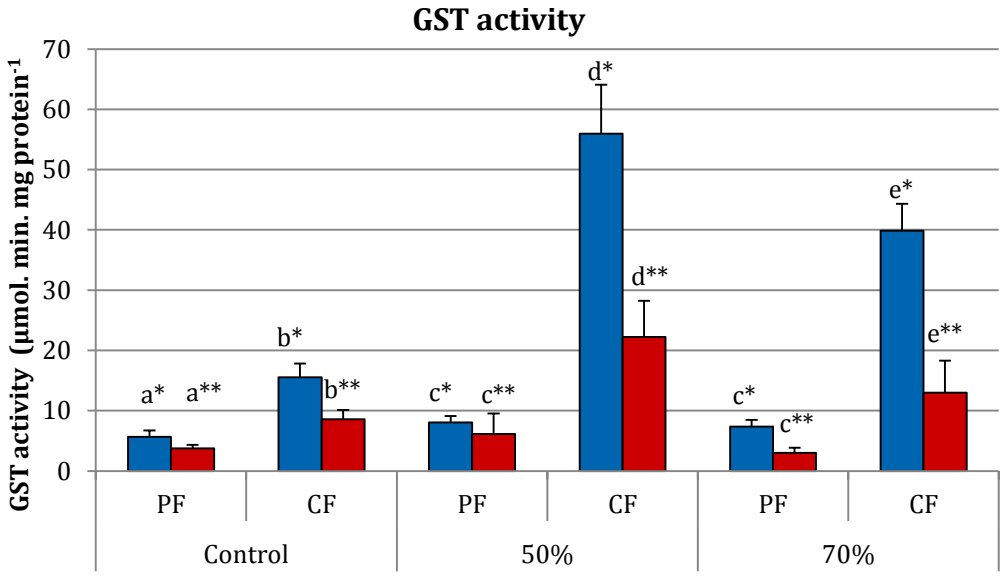

Figure 4.3. – GST activity towards CDNB recovered from the periplasmic (PF) and cytoplasmic fractions (CF) of *Rhizobium leguminosarum* tolerant (blue bars) and sensitive (red bars) strains growing under control conditions and under Cd exposure inducing 50% and 70% growth inhibition. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between treatments and asterisks represent significant differences ($p < 0.05$) between strains in the same treatment.

The GST activity levels detected in periplasmic fraction were very low, when compared to the ones obtained in the cytoplasm, indicating that GST activity was mainly located in the cytoplasm.

4.3.3. Isolation and purification of *Rhizobium* GSTs

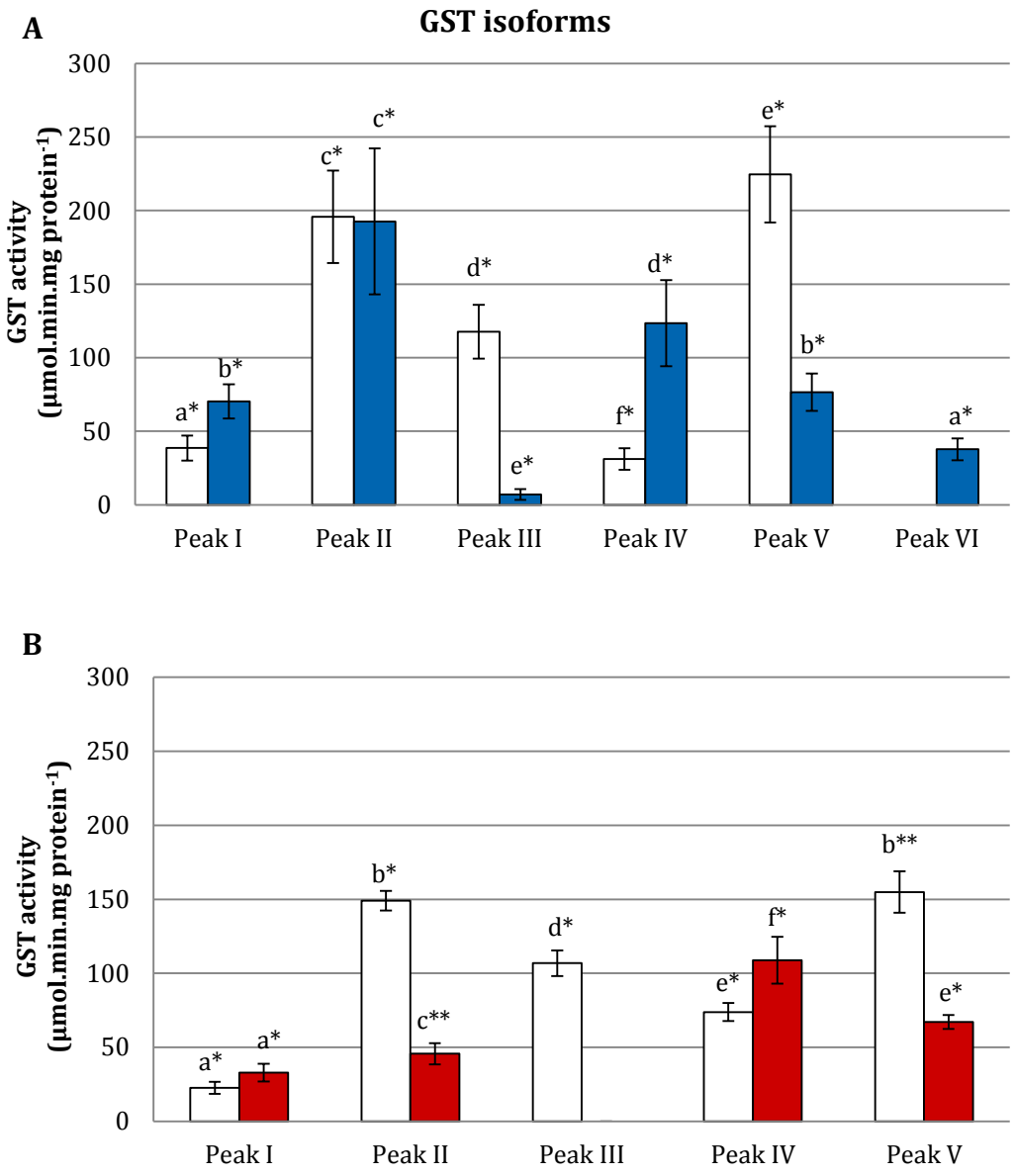

Figure 4.4 – GST activity of protein fractions from anion exchange chromatography in *Rhizobium leguminosarum* tolerant (A) and sensitive (B) strains grown under control conditions (open bars) and under Cd exposure inducing 50% growth inhibition (full bars), towards CDNB. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between treatments and asterisks represent significant differences ($p < 0.05$) between strains in the same treatment.

Putative GST isoforms were isolated from *Rhizobium leguminosarum* cells grown under control conditions or exposed to 50% growth inhibition Cd concentrations. The purification procedure was achieved through two distinct steps: 1) separation of GST from the other bacterial proteins by GSH affinity chromatography; and 2) isolation of the different GST isoforms by anion exchange chromatography. After the first step affinity chromatography performed on a GSH-Sepharose column, eluded fractions containing GST activity were loaded into an anion exchange chromatography column. GST activity towards CDNB was quantified in each eluted fraction and after the entire purification procedure. The obtained results are presented in figure 4.4.

When the tolerant strain was grown under control conditions, the purification procedure resulted in the separation of five protein peaks with GST activity towards CDNB, corresponding to five GST isoforms. Peaks II and V presented the higher GST activity towards the universal CDNB substrate. The lowest GST activity towards CDNB was observed in the isolated protein reported as peak IV. Under Cd exposure it was possible to separated six protein peaks with GST activity towards CDNB, suggesting the presence of an additional GST isoform induced by metal stress. The presence of Cd also altered the activity of some GST isoforms, being the higher GST activity quantified in peaks I and IV and significant ($p<0.001$) lower GST activity was detected in peak III. Cd exposure did not significantly ($p>0.05$) affect GST activity of peak II.

In the sensitive strain, the methodology used allowed the isolation of five protein peaks with GST activity towards CDNB in control conditions and only four in the presence of Cd. Peaks II and V presented the higher GST activity values towards CDNB in control conditions. Cd exposure significantly ($p<0.05$) affected GST activity, exception made to peak I. The enzymatic activity detected in peak IV was significantly ($p<0.05$) higher, but in peaks II and V the activity decreased and in peak III no activity was detected towards CDNB.

Both strains presented similar GST profiles under non stress conditions: it was possible to separate five distinct protein peaks presenting comparatively analogous enzymatic activities. However, when exposed to Cd, differences in the purified protein peaks were evident: a new peak the tolerant strain, peak VI, not detect in the sensitive

strain; the non-detection of GST activity in peak III of the sensitive strain. In peak II, the enzymatic activity decreased in the sensitive strain but not in the tolerant strain.

4.3.4. The role of GSTs in *Rhizobium leguminosarum* Cd tolerance

The role of the purified GSTs in GSH-Cd complexes formation was studied by quantifying the complexes resulting from GSH-conjugation activity of the purified enzymes from the tolerant strain (Figure 4.5) in the presence of Cd and GSH. In the sensitive strain no activity towards Cd was detected.

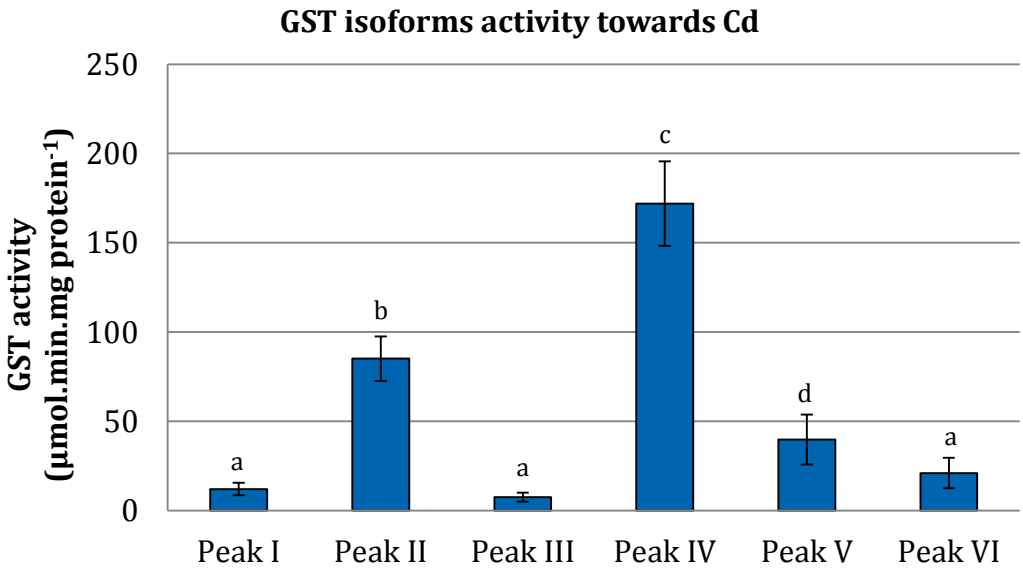

Fig. 4.5. – GSH-conjugation activity of the protein peaks purified from tolerant strain grown under Cd exposure inducing 50% growth inhibition. Data are the means of three replicate experiments, with standard errors. Different letters represent significant differences ($p < 0.05$) between values.

According to the results obtained, all proteins were able to successfully conjugate GSH towards Cd, leading to the formation of GSH-Cd complexes. However, the protein from peak IV was the one presenting a higher GSH-conjugation efficiency in the chelation between the metal and the tripeptide. These results suggest that, although all GST were able to catalyze the binding between Cd and GSH, the enzyme

isolated in peak IV presented a higher specificity to that reaction not being the most reactive enzyme towards the universal GST substrate, CDNB.

4.4 Discussion

4.4.1 GSTs catalytic ability with different substrates

GSTs have been extensively studied in several species, including mammals, in which multiple isoforms, composed of two subunits, are present. GST fold is highly conserved from bacteria to mammals (Federici et al., 2007). However, much less information is available on the presence, structure and biological functions of bacterial GSTs. GST activity in *Rhizobium* cells was determined towards different substrates, since the catalytic ability towards selected substrates is considered to be an important parameter to distinguish, among different GST isoforms (Dainelli et al., 2002). Some GST isoforms may have low specific activity towards the universal substrate CDNB, but an extraordinary higher activity towards other substrates (Alkafaf, 1997). The GST activity, determined in *R. leguminosarum* cells towards three different substrates, revealed marked differences between the two *Rhizobium* strains (figure 4.2) . The tolerant strain (E20-8) demonstrated the ability to increase GST activity towards the specific substrate CDNB as a consequence to Cd exposure, while under the same level of metal stress, the GST activity of the sensitive strain (NII-1) was negatively affected. It had already been reported that bacterial GSTs have no specific relevant activity towards CDNB (Zablotowicz et al., 1995), as bacterial GSTs were first described not as detoxifying enzymes but rather as being involved in primary metabolism (Wiktelius and Stendberg, 2007). On the other hand Favaroloro et al. (1998) demonstrated that OaGST from *Proteus mirabilis* presented high activity towards that specific substrate. Even though the present work demonstrated that the metal tolerant strain present higher GST activity towards CDNB when exposed to Cd. Zablotowicz et al. (1995) had already suggested a high affinity of gram-negative bacteria GST towards CDNB, even though the affinity was considerable lower than in eukaryotic organisms.

In this work the level of GST activity under control conditions was similar to those found in other bacteria (Emtiaze et al., 2009; Favarolo et al., 1998; Federici et al., 2007; Kanai et al., 2006) but lower than the values obtained to other organisms (Adewale et al., 2006; Donham et al., 2005; Dainelli et al., 2002). Nevertheless under Cd stress GST activity levels in the tolerant strain were highly increased, indicating that GSTs must have an important role in the tolerance to Cd. There are several studies regarding the identification and characterization of bacterial GSTs. Nevertheless there are no evidences supporting the role of GSTs in bacterial tolerance to Cd stress. In *Saccharomyces cerevisiae* it was reported two important GSTs in Cd stress: Gtt1 involved in the regulation of GSH homeostasis and Gtt2 involved in the formation of GSH-Cd complexes (Adamis et al., 2004). The increment observed in the activity of GSTs towards CDNB, in the *Rhizobium* tolerant strain exposed to Cd, suggested that bacterial GSTs may be involved in the formation of GSH-Cd complexes. In plants and animals it was demonstrated that some GSTs may be inducible by the exposure to some xenobiotics, and even though some reports confirmed that gram-negative bacteria may use GST as a potential detoxification mechanism (Favarolo et al., 1998; Favarolo et al., 2000; Zablotowicz et al., 1995), there were no evidences regarding the presence of inducible GSTs in bacteria.

4.4.2 GST isoforms under Cd stress

It was already demonstrated in *S. cerevisiae* (Adamis et al., 2004) that GSTs appear to be crucial to the efficient formation of GSH-Cd complexes and consequently to increase Cd tolerance. The results presented in this chapter confirm that in *Rhizobium leguminosarum* GST may have a similar role. GSTs are a multigene family of enzymes (Allocati et al., 2009) with possible different roles in cellular detoxification against harmful xenobiotics (Adamis et al., 2004; Oakley, 2005). Thus, it was important to identify the GST isoform directly involved in the formation of GSH-Cd complexes. To accomplish this goal different GST isoforms, present in *Rhizobium leguminosarum*, were isolated in the absence and in the presence of metal (figure 4.4). Both *Rhizobium* strains presented five GST like proteins, presenting different GST activities towards CDNB. Similar results were obtained in other bacteria

species. In *Proteus mirabilis* were identify three GSTs (Di Ilio et al., 1988), while in *Proteus vulgaris* were identify four (Hong et al., 2003) and in *E. coli*, apart from beta class GST (Nishida et al., 1998), there are reports of at least six GST homologous (Rife et al., 2003). Although Cd exposure inhibited the activity of some GSTs, results indicated the induction of a new GST isoform in the tolerant strain. The isolated GST isoforms may not be the only GSTs present in *Rhizobium leguminosarum*, as the analysis of *R. leguminosarum* genome revealed 19 putative GST (Young et al., 2006), that were not expressed under the experimental conditions or could not be purified by the procedure used.

Bianucci et al. (2012) demonstrated that, in *Bradyrhizobium*, GST activity was inhibited by Cd exposure. Nevertheless there are no reports regarding the purification of GST isoforms under Cd stress in bacteria. The results of this thesis indicated that Cd exposure had a different influence in GSTs isoforms, as the activity of some isoforms was inhibited and of others was enhanced. Furthermore, the increment in the activity of some GST isoforms was related with the higher metal tolerance exhibited by the tolerant *Rhizobium* strain. The isoform isolated in peak IV had a particularly affinity towards Cd in the presence of GSH (figure 4.5), suggesting its crucial importance in the chelation mechanism. Moreover, the lower GST activity detected in the periplasmic fraction and the difficulty to isolate GST isoforms from that fraction, suggested that GSTs were mainly present in the cytoplasm (figure 4.2). Taking this into consideration, the results from this thesis suggest that GSH-Cd complexes were formed in the cytoplasm of *Rhizobium leguminosarum* cells, and that this complexation mechanism was mainly mediated by a specific GST isoform (peak IV).

Chapter 5

Final remarks

Given the pressure caused by the global population increase, over the past 50 years there has been a profound transformation of agricultural practices. The widespread use of pesticides, chemical fertilizers and household or industrial sludges, increased soil contamination with toxic substances that interfere with soil microflora. Agricultural soils often present deficiencies in nutrients that affect plant growth and development, hence requiring the addition of fertilizers and sludges (Alloway, 1995a), which frequently present high levels of metals such as Cd. The study of soil bacteria tolerance to contaminants arises as a research area with great importance and short time to produce results. Throughout the chapters of the thesis it was intended to draw attention to the influence of Cd on the tolerance of *Rhizobium leguminosarum,* a bacterial species with high importance to agricultural soils, due to the establishment of endosymbiosis with legumes high economic and dietary value.

The study focused on a particular strategy that *Rhizobium leguminosarum* possess to tolerate Cd, and possible other metals. Mechanisms of tolerance to Cd as metal efflux systems mediated by active transport (Nies, 1999; Saltykov and Olson, 2002), precipitation of metal ions as insoluble salts (Blake et al. 1993 ; Wang et al., 2000), change in the permeability of membranes (Levine and Marzluf, 1989), immobilization of metals in the cell wall (Cervantes and Gutierrez-Corona, 1994) and production of chelating agents (Silver and Phung, 1996) have been described for

several bacterial species. However the role of GSH, a biomolecule present in the majority of species, on the chelation of metals was poorly studied. Thus the elucidation of the GSH role as a metal chelating agent in bacteria tolerance to Cd was the main aim of this work.

In Chapter 2 it was investigated the GSH-Cd chelation mechanism. Higher intracellular Cd levels were observed in the tolerant strain as it had already been demonstrated by others authors (Figueira et al., 2005; Lima et al., 2006). It was also demonstrated that *Rhizobium leguminosarum* tolerance to Cd was dependent on the higher efficiency of the chelation mechanism exhibited by the tolerant strain. This mechanism was activated at the beginning of the lag phase (12 h of growth). As the accumulation of GSH-Cd complexes may inhibit intracellular Cd uptake (Gomes et al., 2002), which was not observed in the tolerant strain, the GSH-Cd complexes were suggested to be transported into the periplasmic space (Chapter 3). The results point out this hypothesis as the most viable as high levels of GSH and Cd were found in the periplasmic space but only a small percentage of GSH-Cd complexes were quantified. Furthermore, as GSH synthesis appeared to occur in the cytoplasm, GSH content in the periplasm had to be transported from the cytoplasm. It was also established that the chelation mechanism occurred in the cytoplasm, as its efficiency was dependent on the enzymatic activity of a specific GST isoform, present in that cytoplasm (Chapter 4). The ability of the tolerant strain to induced specific GST activity was probably the most important reason to explain differences in Cd tolerance between the two strains. Thus, results of this thesis suggested that, Cd was uptaken by bacterial cells directly into the cytoplasm, where it was GSH chelated, a reaction catalyzed by a specific GST isoform. The formed complexes were then excreted into the periplasmic space, where complexes dissociated and metal ions were accumulated or excreted. Figure 5.1 represents the Cd tolerance mechanism, suggested by the results obtained in this thesis, that may explain the higher tolerance demonstrated by the tolerant *Rhizobium leguminosarum* strain, E20-8.

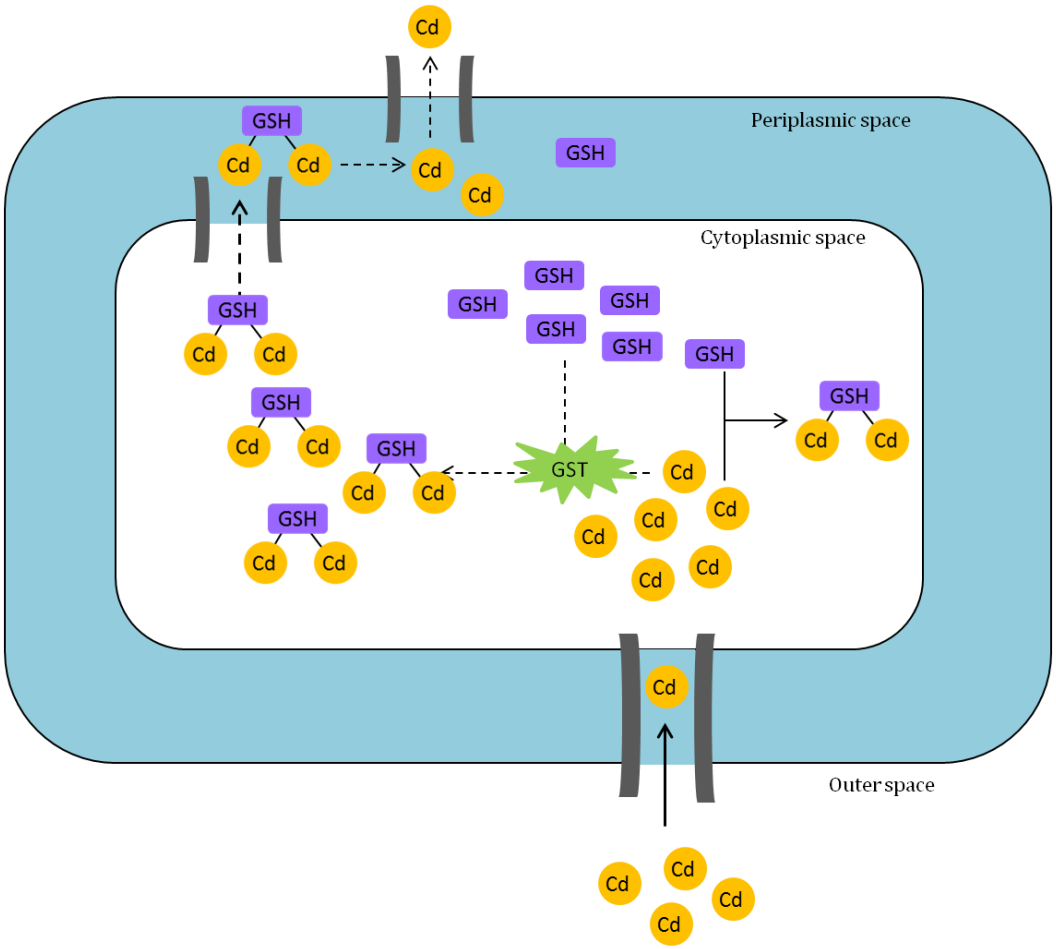

Figure 5.1. – The putative Cd tolerance mechanism that present in *Rhizobium leguminosarum* tolerant strain. Cd enters bacterial cells directly into the cytoplasm, where it is chelated by GSH, a reaction catalyzed by a specific GST isoforms. This mechanism leads to the increment of γ-GCS activity and consequently, to a higher of GSH synthesis. The formed GSH-Cd complexes are then excreted into the periplasmic space, where complexes are dissociated and metal ions are accumulated or excreted.

Chapter 6

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