Universidade de Aveiro Departamento de Biologia
2019

Carina Flávia Ribeiro Sá

Airborne exposure of *Rhizobium* to volatile organic compounds: biochemical effects on cells challenged or not by cadmium

Exposição aérea de *Rhizobium* a compostos orgânicos voláteis: efeitos bioquímicos em células expostas ou não a cádmio

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Etelvina Maria de Almeida Paula Figueira, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e coorientação científica da Doutora Adília da Conceição Marques de Oliveira Pires, Investigadora Auxiliar do Departamento de Biologia & CESAM, Universidade de Aveiro.

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agradecimentos

À minha orientadora, Doutora Etelvina Maria de Almeida Paula Figueira, pela oportunidade de realizar este trabalho, pelo constante apoio, disponibilidade, por me ajudar a ver como as peças se encaixam quando nada parecia fazer sentido.
À minha co-orientadora, Doutora Adília da Conceição Marques de Oliveira Pires, pela paciência e apoio, por perante situações difíceis nunca perder a calma e ver soluções onde eu só via problemas, por explicar e relembrar todos os passos da bioquímica e da análise estatística.
Ao Paulo Cardoso que me ensinou a base do trabalho de laboratório, incutindo-me "picuinhices" que melhoraram as minhas capacidades no laboratório e ao qual serei para sempre grata; e ainda pela sua disponibilidade e análise crítica ao ler os meus textos, possibilitando uma leitura mais correta e focada do meu trabalho.
À Diana Matos pela companhia durante as longas horas de trabalho, apoio e paciência perante as minhas dúvidas. Ao Ricardo Pinto pela sua ajuda e bom-humor quando o trabalho era muito e o tempo era pouco. A todos os membros do Rhizolab (Catarina, Beatriz, Tiago, João, Daniel, Miguel, Ricardo, Diana, Paulo) pelas reuniões e momentos descontraídos.
A todo o pessoal do laboratório Ângela, Luísa, Lucia, Francesca, Anthony, Madalena, Simão, Ruis, Carla, Silvana, Matilde, Marta, Henrique, Marcelo, pela ajuda e boa disposição e alegria, proporcionando um ambiente de trabalho descontraído, animado mesmo nos dias de maior stress.
Aos meus amigos pelo apoio e momentos de descontração
À minha família que me apoiou em todos os momentos não permitindo o meu isolamento total do resto do mundo durante a elaboração desta tese. Em especial à minha mãe por me ouvir e me dar os conselhos que só uma mãe sabe dar.

palavras-chave

Rizóbio, cádmio, compostos orgânicos voláteis, monoterpenos, compostos voláteis de enxofre, stresse oxidativo.

resumo

Os rizóbios são bactérias do solo que promove o crescimento de leguminosas. O crescimento e a persistência destas bactérias pode ser limitado por diversos fatores, como por exemplo o cádmio (Cd). O aumento das concentrações de Cd no solo desencadeia diferentes mecanismos nas células de forma a tolerar o stress. A produção de compostos orgânicos voláteis (VOCs), resultantes do mecanismo secundário das bactérias, pode ser um dos mecanismos a que estas bactérias recorrem como forma de tolerar situações de stress. Nesta tese foram estudados os efeitos de cinco monoterpenos (α-pineno, limoneno, eucaliptol, linalol e mentol) e quatro compostos de enxofre (dimetil sulfeto (DMS), dimetil dissulfeto (DMDS), dimetil trissulfeto (DMTS) e metil tioacetato (MTA)) no crescimento, estado oxidativo e mecanismos antioxidantes em *Rhizobium leguminosarum* na estirpe E20-8, com o intuito de testar a hipótese de que a exposição aérea a estes VOCs poderiam influenciar o crescimento e tolerância de rizóbio ao cádmio.

Os resultados obtidos durante este estudo permitiram verificar efeitos diversos de cada um dos compostos em *Rhizobium* E20-8 que também divergiam com o stresse (exposição ou não a Cd). Deste modo foi possível provar que os efeitos divergem entre VOCs da mesma família química. Na ausência de Cd os monoterpenos testados apresentam atividade antibacteriana (linalol, limoneno, mentol), ou propriedades antioxidantes (α-pineno e eucaliptol). Na presença de Cd apenas o limoneno (1 e 100 mM) e o eucaliptol (100 nM) foram capazes de induzir o crescimento das células. De um modo geral, os compostos de enxofre testados desencadeiam mecanismos antioxidantes semelhantes. Apesar dos compostos voláteis de enxofre não afetarem o crescimento, são capazes de reduzir o stress oxidativo das células, tendo um efeito protetor a nível das membranas (DMDS e DMTS) e deste modo minimizando a toxicidade do Cd.

As baixas concentrações de voláteis testadas permitem-nos prognosticar que estes efeitos poderão ocorrer no ecossistema do solo, influenciando o crescimento e a tolerância das células bacterianas quer estas se encontrem ou não em stresse. Este estudo contribui para compreender melhor o efeito dos compostos voláteis nas interações dos organismos que habitam o solo. O contributo é especialmente importante na influência que estes compostos possam ter em contextos de stresse ambiental.

keywords

Rhizobium, cadmium, volatile organic compounds, monoterpenes, volatile sulfur compounds, oxidative stress

abstract

Rhizobia are soil bacteria that promote the growth of legume plants. The growth and persistence of these bacteria may be limited by several factors, such as cadmium (Cd). Increasing concentrations of Cd in soil triggers different mechanisms in bacterial cells in order to tolerate stress. The production of volatile organic compounds (VOCs), resulting from the secondary metabolism of bacteria, may be a way to overcome stress situations. In this thesis the effects of five monoterpenes (α-pinene, limonene, eucalyptol, linalool and menthol) and four sulfur compounds (dimethyl sulfide (DMS), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and methyl thioacetate (MTA)) on growth, oxidative status and antioxidant mechanisms of *Rhizobium leguminosarum* E20-8 were studied, to test the hypothesis that these VOCs could influence rhizobial growth and tolerance to cadmium.

The results obtained during this study showed that different compounds have different effects on *Rhizobium* E20-8, not being related to the chemical family to which they belong. The tested monoterpenes displayed antibacterial activity (linalool, limonene, menthol), or antioxidant properties (α-pinene and eucalyptol) in the absence of Cd. In the presence of Cd only limonene (1 and 100 mM) and eucalyptol (100 nM) were able to induce cell growth. The tested sulfur compounds generally triggered similar antioxidant mechanisms in *Rhizobium*. Although volatile sulfur compounds did not affect growth, they were able to reduce the oxidative stress of cells, having a membrane-protective effect (DMDS and DMTS) and thus minimizing Cd toxicity.

The low concentrations of volatiles tested allow us to predict that these effects may occur in the soil ecosystem, influencing the growth and tolerance of bacterial cells whether they are under stress or not. This study contributes to better understand the effect of volatile compounds on the interactions of soil-dwelling organisms. The contribution is especially important on the influence that these compounds may have in environmental stress contexts.

Index

Index of Figures XIX

Index of Tables XXI

List of Abbreviations XXIII

Chapter 1 – State of the art 1

Abstract 1

1. Introduction 1

1.1. *Rhizobium* 2

1.2. Cadmium 3

1.2.1. Cd contamination 3

1.2.2. Cd tolerance mechanisms in bacteria 5

1.3. Volatile organic compounds 6

1.3.2. VOCs in the soil 8

1.4. General objectives 10

Chapter 2 – Airborne exposure of *Rhizobium leguminosarum* strain E20-8 to volatile monoterpenes: effects on cells challenged by cadmium 11

Abstract 11

2.1. Introduction 12

2.2. Results 15

2.2.1. Sole exposure to Cd 15

2.2.2. Effects of α-pinene on *Rhizobium* grown in the presence and absence of Cd 15

2.2.3. Effects of limonene on *Rhizobium* grown in the presence and absence of Cd 17

2.2.4. Effects of linalool on *Rhizobium* grown in the presence and absence of Cd 18

2.2.5. Effects of eucalyptol on *Rhizobium* grown in the presence and absence of Cd 19

2.2.6. Effects of menthol on *Rhizobium* grown in the presence and absence of Cd 20

2.3. Discussion 23

2.4. Concluding remarks 28

Chapter 3 – Effects of volatile sulfur compounds on growth and oxidative stress of *Rhizobium leguminosarum* strain E20-8 challenged by cadmium 29

Abstract 29

3.1. Introduction 30

3.2. Results 32

3.2.1. Sole exposure to Cd 32

3.2.2. Growth 32

3.2.3. Oxidative damage 33

3.2.4. Antioxidant enzymes 34

3.2.5. Metabolic response 36

3.2.6. Principal Coordinates (PCO) 36

3.3. Discussion 38

3.4. Concluding remarks 41

Chapter 4 – Final remarks and future work 43

Chapter 5 – Experimental procedures 45

5.1. Growth of E20-8 45

5.2. Tolerance to cadmium 45

5.2.1. Growth medium preparation 45

5.2.2. Inoculation 45

5.2.3. Growth determination 46

5.2.4. Calculation of IC_{50} 46

5.3. Influence of the VOCs in the growth of E20-8 in the presence of Cd 46

5.3.1. Experimental conditions 46

5.4. Influence of VOCs in E20-8 biochemistry at 0 and 100 µM Cd 47
5.4.1 Extraction 47
5.4.2. Protein content 48
5.4.3. Lipid peroxidation 48
5.4.4. Protein carbonylation 48
5.4.5. Superoxide dismutase activity 48
5.4.6. Glutathione peroxidase activity 49
5.4.7. Glutathione-S-transferases activity 49
5.3.4. Statistical analyses 49
Chapter 6 – Bibliography 51
Annex 73
Supplemental files 75
Chapter 2 - Supplemental files 75
Chapter 3 - Supplemental files 80

Index of Figures

Figure 1 – Representation of the cadmium (Cd) biogeochemical cycle and the natural and anthropogenic release of Cd into the environment. 3
Figure 2 – Scheme of volatile organic compound (VOC) emissions by soil organisms and their interactions. .. 6
Figure 3 – Main metabolic pathways for the production of microbial volatiles. 9
Figure 4 - Growth, antioxidant and biotransformation activity and damage in Rhizobium cells exposed to Cd and α-pinene. 16
Figure 5 – Growth, antioxidant and biotransformation activity and damage in Rhizobium cells exposed to Cd and limonene. 17
Figure 6 – Growth, antioxidant and biotransformation activity and damage in Rhizobium cells exposed to Cd and linalool. 19
Figure 7 – Growth, antioxidant and biotransformation activity and damage in Rhizobium cells exposed to Cd and eucalyptol. 20
Figure 8 – Growth, antioxidant and biotransformation activity and damage in Rhizobium cells exposed to Cd and menthol. 21
Figure 9 – Growth of Rhizobium cells exposed to Cd and sulfur compounds. 33
Figure 10 – Damage in Rhizobium cells exposed to sulfur compounds in the absence or presence of Cd. 34
Figure 11 – Antioxidant activity in Rhizobium cells exposed to sulfur compounds in the absence or presence of Cd. 35
Figure 12 – Biotransformation activity and damage in Rhizobium cells exposed to sulfur compounds in the absence or presence of Cd. 36
Figure 13 – Principal Coordinates with centroids ordination (PCO) of the biochemical determinants for each of the sulfur compounds. 37
Figure 14 – Growth of Rhizobium strain E20-8 in the absence (A to F) and presence (G to L) of Cd when exposed to α-pinene concentration 0 nM (A, G), 1 nM (B, H), 100 nM (C, I), 10 µM (D, J), 1 mM (E, K), 100 mM (F, L), during 96 hours. 47

Index of Tables

Supplementary Table S1 – Growth, protein, antioxidant and biotransformation (SOD, GPX, GSTs) activity and damage (LPO) in Rhizobium cells exposed to Cd and α-pinene. *75*

Supplementary Table S2 – Growth, protein, antioxidant and biotransformation (SOD, GPX, GSTs) activity and damage (LPO) in Rhizobium cells exposed to Cd and limonene. *76*

Supplementary Table S3 – Growth, protein, antioxidant and biotransformation (SOD, GPX, GSTs) activity and damage (LPO) in Rhizobium cells exposed to Cd and linalool. *77*

Supplementary Table S4 – Growth, protein, antioxidant and biotransformation (SOD, GPX, GSTs) activity and damage (LPO) in Rhizobium cells exposed to Cd and eucalyptol. *78*

Supplementary Table S5 – Growth, protein, antioxidant and biotransformation (SOD, GPX, GSTs) activity and damage (LPO) in Rhizobium cells exposed to Cd and menthol. *79*

Supplementary Table S6 – Growth, in Rhizobium cells exposed to Cd and MTA; DMS; DMDS; DMTS. *80*

Supplementary Table S7 – Lipid peroxidation (LPO), in Rhizobium cells exposed to Cd and MTA; DMS; DMDS; DMTS. *81*

Supplementary Table S8 – Protein carboxylation (PC), in Rhizobium cells exposed to Cd and MTA; DMS; DMDS; DMTS. *82*

Supplementary Table S9 – Superoxide dismutase (SOD), in Rhizobium cells exposed to Cd and MTA; DMS; DMDS; DMTS. *83*

Supplementary Table S10 – Glutathione s-transferases (GSTs), in Rhizobium cells exposed to Cd and MTA; DMS; DMDS; DMTS. *84*

Supplementary Table S11 – Glutathione peroxidase (GPx), in Rhizobium cells exposed to Cd and MTA; DMS; DMDS; DMTS. *85*

Supplementary Table S12 – Protein, in Rhizobium cells exposed to Cd and MTA; DMS; DMDS; DMTS. *86*

List of Abbreviations

AHL – *N*-acyl homoserine lactone

BSA – bovine serum albumin

CDNB – 1-chloro 2,4 dinitrobenzene

CAS – chemical abstracts service

CG – carbonyl groups

DNA – deoxyribonucleic acid

DMS – dimethyl sulfide

DMDS – dimethyl disulfide

DMTS – dimethyl trisulfide

DNPH – 2,4-dinitrophenylhydrazine

DTPA – diethylenetriaminepentaacetic acid

IC_{50} – half maximal inhibitory concentration

GPx – glutathione peroxidase

GSH – Glutathione

GSTs – glutathione s-transferases

g – gravitational force

H_2S – hydrogen sulfide

LPO – lipid peroxidation

MDA – malondialdehyde

MTA – methyl thioacetate

mU – miliunit of enzyme activity

MVOCs – microbial volatile organic compounds

NBT – nitroblue tetrazolium

NH_4 – ammonium

PC – protein carbonylation

PCO – principal coordinates

PROT – protein

QQ – quorum quenching

QS – quorum sensing

ROS – reactive oxygen species

rRNA – ribosomal ribonucleic acid

SOD – superoxide dismutase

Tris-HCl – tris(hydroxymethyl)aminomethane hydrochloride

TBA – 2-Thiobarbituric acid

TBARS – thiobarbituric acid reactive substances

U – unit of enzyme activity

VOCs – volatile organic compounds

VSCs – volatile sulfur compounds

YMA – Yeast mannitol agar

Chapter 1 – State of the art

Abstract

Rhizobium is an important genus of soil bacteria that enables better plant growth and development. However, the presence and number of these bacteria in the soil depends on many factors, both biotic and abiotic. The presence of cadmium (Cd) in the soil is a limiting factor for *Rhizobium* growth. Anthropogenic activities have led to increased concentrations of Cd in certain locations. In order to fight / survive stress, bacteria resort to different mechanisms. The production of secondary metabolites, specifically volatile organic compounds (VOCs), is known to be involved in the communication mechanisms of microorganisms and can interfere with growth. The following chapter aims to gather information on these three topics (*Rhizobium*, cadmium stress and VOCs) in order to understand the possible implications of VOCs production on the survival of microorganisms under stress (Cd).

1. Introduction

Soil surrounds all the superficial layer of the earth's crust, consisting of minerals, organic matter and living organisms, creating the conditions for autotrophs colonization, such as plants, that are the base of trophic webs. The complex soil matrix allows for the existence of several microhabitats with distinct physicochemical gradients and discontinuous environmental conditions (Torsvik and Øvreås, 2002). Microorganisms capable to adapt in these microhabitats can be found either in the surface (0 to 25 cm deep) or subsurface of the soil. However, a larger number of studies are carried out on the microorganisms that inhabit the surface of the soil since in this layer a higher number and diversity of microorganisms can be found (Fierer et al., 2003; Konopka and Turco, 1991). The surface microorganisms assist in soil support and plant development as they play an important role in soil formation, biogeochemistry, contaminant degradation, and the maintenance of groundwater quality (Fierer et al., 2003; Hiebert and Bennett, 1992; Konopka and Turco, 1991). Thus, soil

microorganisms and their interactions with each other or with the remaining of the soil biota (Torsvik and Øvreås, 2002) are important for the development of the communities and food webs present in an ecosystem.

1.1. *Rhizobium*

Rhizobium is a genus of gram-negative, aerobic bacteria that inhabits the superficial layer of the soil and rhizosphere, obtaining energy from different carbon sources (Deshwal and Abhishek, 2014; Sethi et al., 2019). In the rhizosphere, they interact with plant roots and when in symbiosis with legumes, atmospheric nitrogen can be fixed and used by plants, which otherwise would be unavailable (Masson-Boivin and Sachs, 2018). Nitrogen is essential for plant development, but frequently is present at low concentrations in the soil. Thus, biological nitrogen fixation provides optimal nitrogen nutrition even in nitrogen deficient soils, leading to better plant development (Mabrouk et al., 2018; Mabrouk and Belhadj, 2010; Sadowsky, 2005; Zahran, 1999). Therefore, *Rhizobium* strains have a great agricultural and economic interest for improving crop yield (Rehman and Nautiyal, 2002) and reducing the use of inorganic nitrogen fertilizers (Coleto et al., 2014). For these reasons, most studies on rhizobia aimed to study this symbiosis and nitrogen fixation, and little attention has been paid to the role of rhizobia in the soil in its free-living state.

Rhizobia can use different carbon sources and can be classified into fast and slow growers, depending of the carbon source that they are able to metabolize (Stowers, 1985). Free-living rhizobia are also capable of utilizing sources of nitrogen from the surrounding environment such as ammonia (Brown and Dilworth, 1975). A study by Denison and Kiers (2011) verified the ability of *Rhizobium* to form biofilms with other species, allowing its persistence for long periods of time. *Rhizobium*'s production and accumulation of poly-β-hydroxybutyrate and extracellular polysaccharides are used by *Rhizobium* when it is in the free-living state, which probably allows its survival when other carbon sources are not available (Patel and Gerson, 1974; Pauling et al., 2001; Stowers, 1985), evidencing the metabolic plasticity of these soil bacteria and the ability to persist in soils.

1.2. Cadmium

Cadmium (Cd) is a toxic metal that exists in rocks, sediment, soil and dust (Pinot et al., 2000; Thornton, 1992). Cadmium is found in small concentrations in nature, typically between 1.8 and 53 µM in soil (Helmke, 1999; Kabata-Pendias, 2010). Volcanic activity, weathering of rocks, and soil erosion are some of the natural forms of emission of this toxic metal (Adamu and Nganje, 2010) (Fig. 1). However, the increase of Cd concentrations in soil were found to be related to increased anthropogenic activities such as industrial production, mining and intensive agriculture (Foy et al., 1978; Khan et al., 2017; Thornton, 1992; Ursínyová and Hladíková, 2000) (Fig. 1). Air, effluent, sludge, or solid waste (Pinot et al., 2000; Thornton, 1992) resulting from industrial activity as well as overuse of phosphate fertilizers (Khan et al., 2017) led to changes in soil composition, and concentrations higher than 2669 µM Cd could be found in extremely contaminated sites (Itoh and Yumura, 1979).

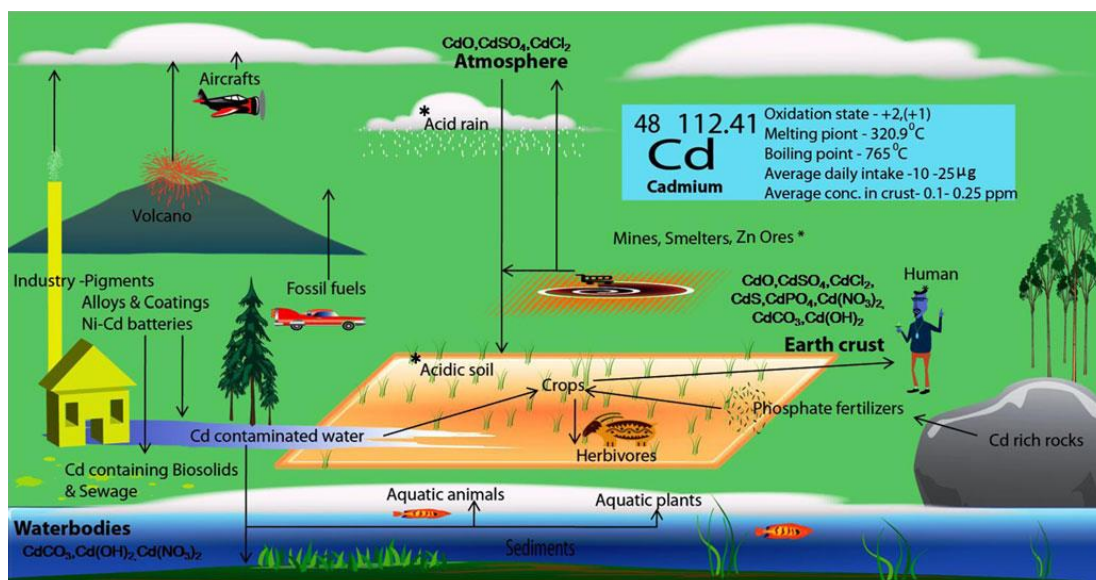


Figure 1 – Representation of the cadmium (Cd) biogeochemical cycle and the natural and anthropogenic release of Cd into the environment. (Image from Sebastian and Prasad (2014)).

1.2.1. Cd contamination

Changes in soil composition such as nutrient availability and physicochemical properties will influence the organisms that inhabit it (plants, insects, nematodes and microorganisms). Soils are the main accumulator of metals released into the environment by anthropogenic activities and unlike organic compounds they do not suffer degradation by chemical or

microbial action, meaning that after its introduction into the soil, its total concentration does not change significantly over time (Wuana and Okieimen, 2011). Soil composition and physical and chemical characteristics (e. g. pH, electric conductivity, oxygen concentration), will influence the chemical form of metals in the soil and their availability. Soil enzymes are deeply affected by metal ion toxicity and prolonged soil exposure to metals leads to a decrease in soil enzymatic activity (Maliszewska-Kordybach and Smreczak, 2003; Pereira et al., 2007; Tyler et al., 1989; Wang et al., 2007; Wyszkowska and Wyszkowski, 2003) due to the formation of catalytically active groups or protein denaturation (Das et al., 1997; Gill et al., 2013; Leita et al., 1995). The high toxicity and solubility in water of Cd makes it an extremely important pollutant (Beesley and Marmiroli, 2011; DalCorso et al., 2010; Lockwood, 1976; Pinto et al., 2004).

The use of Cd-contaminated waters or phosphate fertilizers in rice fields leads to the discarding of large amounts of contaminated food, as well as the emergence of disease in people who daily eat contaminated rice (20-40 µg Cd) (Sebastian and Prasad, 2014). This demonstrates that humans are affected by the presence of Cd in the ecosystem and that solutions to minimize this problem are needed (Liu et al., 2018).

Due to the important role played by microbial communities in soil maintenance their characterization (biomass, diversity and activity) are often used as indicators of soil quality (Hamman et al., 2007; J. Li et al., 2015; Q. Li et al., 2015; Rubin et al., 2013). The persistence and toxicity of metals in the soil are one of the factors that most influence microorganisms in soils contaminated by metals (Alkorta et al., 2004). In the presence of Cd the ecological functions performed by microbial communities are negatively affected (Harichová et al., 2012; Hurdebise et al., 2015). Microorganisms are susceptible to metals and are generators of biodiversity and density alteration (He et al., 2005; Lu et al., 2013; Roane and Pepper, 1999; Vodyanitskii and Plekhanova, 2014; Wuana and Okieimen, 2011). A recent study by Lou et al. (2019) reported that an increase in Cd concentrations influenced in different ways the microorganisms community of the soil, leading to the increase in the number of bacteria in certain phyla (*Chloroflexi*, *Actinobacteria*, *Firmicutes*) or the decrease (*Proteobacteria*, *Verrucomicrobia*, *Nitrospirae*) or no alteration in others (*Acidobacteria*, *Thaumarchacota*). These results allow to deduce that prolonged exposure to high Cd concentrations would lead to changes in the microbial population density therein as well as in diversity (Luo et al., 2019).

As previously reported, microorganisms present different tolerances mechanisms to metals: exclusion by permeability barrier; intra- and extra-cellular sequestration; active transport efflux pumps; enzymatic detoxification; and reduction in the sensitivity of cellular targets to metal ions (Bruins et al., 2000; Gadd and Griffiths, 1977; Olaniran et al., 2013). For these reasons the identification and study of Cd tolerant strains has been carried out to determine the impact of Cd on microorganisms and to find strategies that can minimize this impact or be used to decontaminate polluted sites (Lin et al., 2016; Lu et al., 2013; Olaniran et al., 2013; Siripornadulsil and Siripornadulsil, 2013).

The study conducted by Pramanik et al. (2018) observed that *Enterobacter aerogenes* K6, besides exhibiting plant growth promotion, presented resistance to Cd and was able to improve the germination rates of rice seedlings. Kotoky et al. (2019) observed a similar effect with *Serratia marcescens*. This rhizobacteria was able to promote plant growth and resist high concentration of Cd, demonstrating that the resistance mechanism identified was related with glutathione S-transferase mechanism for detoxification of Cd (Kotoky et al., 2019) corroborating the results of Corticeiro et al. (2013).

1.2.2. Cd tolerance mechanisms in bacteria

The concentration of toxic metals in the soil is not the only factor that influences microorganisms tolerance, since nutrient availability, temperature, water availability, pH, electric conductivity, oxygen concentration, root interaction and other microorganisms also contribute to the level of tolerance displayed by microorganisms (Sadowsky, 2005; Stotzky and Pramer, 1972; Zahran, 1999). In order to withstand metal-induced stress conditions microorganisms developed different strategies. The decrease of cell activity (becoming dormant) allow them to survive for a longer period of time, since Cd accumulating is slower and toxic Cd concentrations are more difficult to reach (Garbeva et al., 2014a, 2011; Garbeva and de Boer, 2009; Hibbing et al., 2010; Laskaris et al., 2010). Several detoxification mechanisms also increase microorganisms tolerance to Cd: sequestration and precipitation of Cd ions using different inorganic and organic compounds both extra and intracellularly are mechanisms avoiding interference with cell metabolism and thus decreasing metal toxicity (Abbas et al., 2018; Aiking et al., 1982; Bramhachari and Nagaraju, 2017; Gadd and Griffiths, 1977; Herman et al., 1995; Jezequel and Lebeau, 2008; Mota et al., 2015; Pistocchi

et al., 2000). For example, Lima et al. (2006) verified that in a Cd tolerant strain of *Rhizobium* the glutathione (GSH) chelated 75% of the intracellular metal. The bacterial sulfate compounds (diallyl disulfide, dimethyl disulfide and diallyl sulfide) led to the decrease of soluble Cd, due to the S-Cd complexation outside the cell (Essa et al., 2006; Kamyabi et al., 2018). A recent study reported that in presence of Cd *Rhizobium* cells changed their volatile profile (Cardoso et al., 2017). These authors also observed an increase in some VOCs in the presence of stress, which can be involved in the tolerance of bacteria to Cd stress.

1.3. Volatile organic compounds

Volatile compounds are present in our daily lives, been used for a long time as aroma and flavor of some fermented foods such as cheese and wine (Deetae et al., 2007; Goniak and Noble, 1987; Kreitman et al., 2019; Lamberet et al., 1997; Landaud et al., 2008; Law and Sharpe, 1978). Volatile compounds can be produced by different organisms such as plants, animals and microorganisms, having an important role in the interactions between organisms (Fig. 2). VOCs produced by bacteria may act as an attractant or repellent to other organisms (Schulz and Dickschat, 2007).

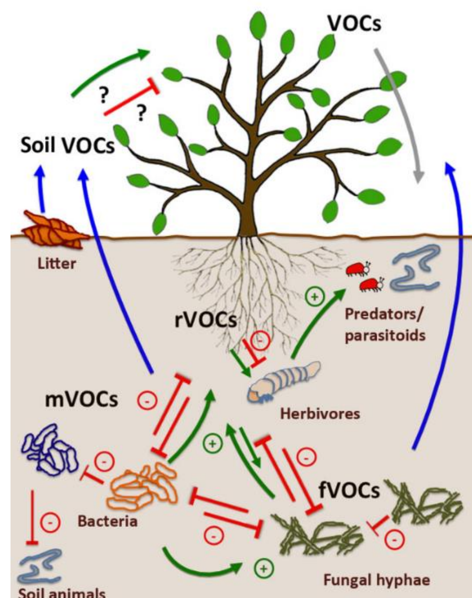


Figure 2 – Scheme of volatile organic compound (VOC) emissions by soil organisms and their interactions. VOCs (blue arrows) emitted by bacteria (mVOCs), fungi (fVOCs) and roots (rVOCs). The direct negative effects (red arrows) and the positive effects (green arrows) of the VOCs are represented (Image from Peñuelas et al. (2014)).

Currently 2000 microbial volatile (organic and inorganic) compounds have been estimated (Lemfack et al., 2018). However, due to the reduced number of species studied compared to the estimated ones (Locey and Lennon, 2016), the diversity of volatile compounds that occur on nature could be much higher. The volatiles compounds are considered secondary metabolites, being originated from intermediates of primary metabolism and their functions are not fully understood (Schmidt et al., 2015; Schulz and Dickschat, 2007). Unlike primary metabolites, they are not essential for cell development, but they seem to have a defence and communication functionality (Schulz and Dickschat, 2007).

A study performed by Ahmad et al (2014) found that volatiles produced by plants influenced quorum sensing (QS) in bacteria, observing that few compounds stimulated QS, but the vast majority inhibited QS interactions, and demonstrating a potential antimicrobial activity of these compounds (Bodini et al., 2009; Szabó et al., 2010). Chernin et al. (2011) verified that the volatile organic compounds produced by two rhizospheric strains interfered with the network mediated by *N*-acyl homoserine lactone (AHL) inhibiting QS communication in various strains of bacteria. It was also observed that dimethyl disulfide (DMDS) was able to produce the same effect on AHL-producing bacteria (Chernin et al., 2011). This communications mechanisms (Huang et al., 2016; Winson et al., 1998) are some of the responses of the microorganisms to their habitat changes, allowing a collective response of microorganisms to climate change (Dong and Zhang, 2005). Since VOCs produced by bacteria or plants can interfere with these mechanisms, it is possible to deduce that the presence or absence of these compounds is an important defence mechanism of organisms under stress conditions, acting as signalling entities.

The slow growth of bacteria compared to fungi may have led to the development of volatiles with antifungal activity (Fernando et al., 2005). Chaves-López et al. (2015) found that *Bacillus* strains were able to inhibit the growth of a set of fungi, however they had different degrees of inhibition. Studies have found that VOCs produced by bacteria and yeast help control sapstain and mould in wood by limiting their growth (Bruce et al., 2003; Payne et al., 2000). Volatiles that are likely to be involved in growth inhibition are dimethyl disulfide, dimethyl trisulfide and in particular ketones (Bruce et al., 2004). Inhibition of hyphae growth and germination of fungal spores by bacteria through the use of volatile compounds has long been known (Herrington et al., 1987). However, due to the renewed

interest in the study of these compounds it was possible to verify that exposure to bacterial volatile compounds leads to morphological, enzymatic activity and gene expression alterations in fungi (Effmert et al., 2012; Garbeva et al., 2011; Kai et al., 2009; Vespermann et al., 2007; Wheatley, 2002). The volatiles emitted by the rhizospheric strains *Pseudomonas fluorescens* and *Serratia plymuthica* are able to inhibit the growth of the bacterial phytopathogens *Agrobacterium tumefaciens* and *A. vitis*, allowing better development of tomato infected plants (Dandurishvili et al., 2011). An analysis of the released compounds evidenced DMDS has the primarily responsible for the observed inhibition (Dandurishvili et al., 2011)

Ryu et al. (2003) found that VOCs produced by *Bacillus subtilis* and *B. amyloliquefaciens*, in particular 2,3- butanediol and acetoin, promoted plant growth (*Arabidopsis thaliana*) and Cardoso et al. (2018a) also found that rhizospheric strains of different bacterial genus were able to promote plant (*A. thaliana*) growth. Responses related to stress and hormonal regulation could explain the growth promotion in *A. thaliana* exposed to volatile compounds from *B. subtilis* (Zhang et al., 2007). However, the biological functions and the mode of action of most of the VOCs emitted by bacteria remain unclear (Farag et al., 2006)

1.3.2. VOCs in the soil

Although the existence and importance of volatile organic compounds produced by bacteria has long been recognized (Stotzky et al., 1976), only recently the study of these compounds has arose greater interest with the view to improve agricultural production by fighting pests and improving crop growth with lower amounts of agrochemicals (Brilli et al., 2019; Kim et al., 2013; Schmidt et al., 2015). The ability of microbial volatile organic compounds to diffuse in the soil (Audrain et al., 2015; Effmert et al., 2012; Schmidt et al., 2015) is due to their low molecular mass, low boiling point and high vapour pressure (Chung et al., 2016; Schmidt et al., 2015; Vespermann et al., 2007). Their high volatility makes diffusion the main dispersion mechanism of these compounds in the complex soil matrix (Effmert et al., 2012; Schmidt et al., 2015). The biodegradation of these compounds is less likely to occur in comparison with the ones soluble in water (Koske and Gemma, 1992). This feature allows them to be considered a good at-a-distance communication system between microorganisms

(Chernin et al., 2011; Effmert et al., 2012; Garbeva et al., 2014a, 2014b; Kai et al., 2009), influencing not only growth but also antibiotic production and gene expression of soil bacteria (Audrain et al., 2015; Garbeva et al., 2014b; Schmidt et al., 2015). Given the diversity of microbial volatile organic compounds (MVOCs), their interaction in the environment in which they are released and the target organism, different effects may be observed (Chung et al., 2015).

Several studies have found antioxidant properties of VOCs in animal and plant cells (Edris, 2007; Farag et al., 2013). However, little is known about their influence on bacteria and especially on bacteria inhabiting the soil (Bitas et al., 2013).

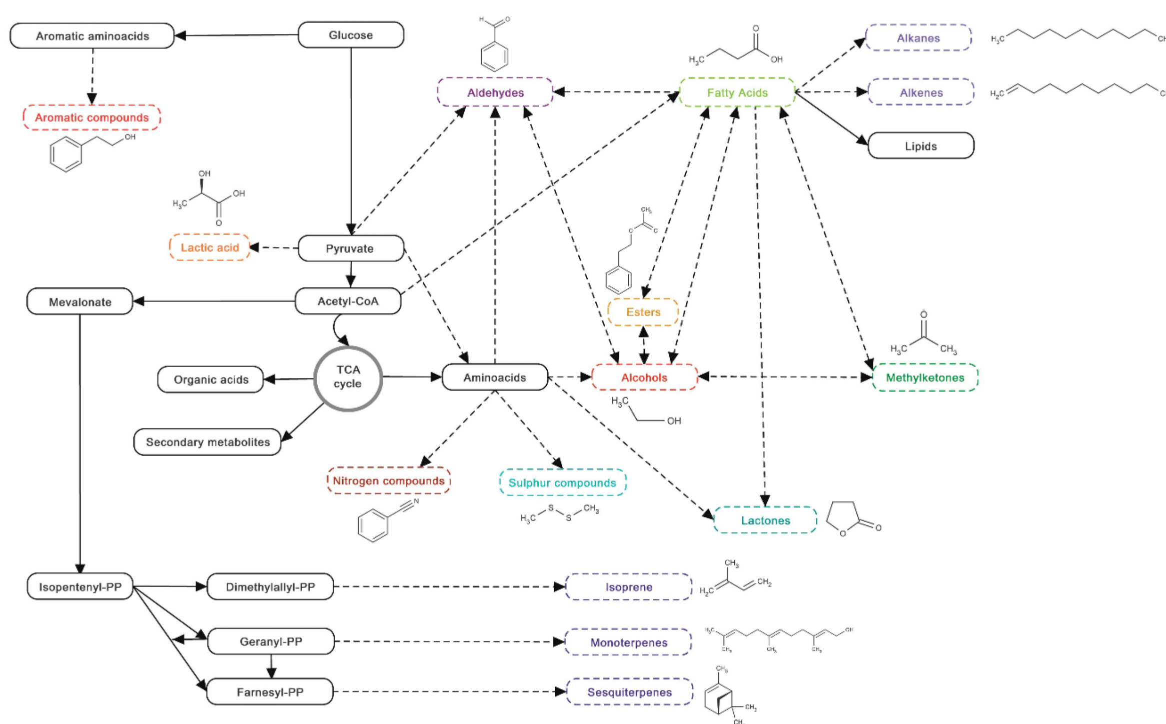


Figure 3 – Main metabolic pathways for the production of microbial volatiles. The colored dashed rectangles represent the volatiles of different chemical classes (Image from the Schmidt et al. (2015)).

Piechulla and Degenhardt (2014) found that the majority of volatiles released by bacteria are alkenes, alcohols, ketones, terpenes, benzenoids, pyrazines, acids, esters and sulfur compounds (Fig. 3). Some terpenes produced by plants are known to have antimicrobial and anti-herpetic activity (Chaves-López et al., 2015; Chen et al., 2004; Langenheim, 1994; Song and Ryu, 2013) and to have antioxidant properties (Gonzalez-

Burgos and Gomez-Serranillos, 2012; Ng et al., 2000; Zengin and Baysal, 2014). However, little is known about the effects of terpenes produced by bacteria. The synthesis of terpenes is widely distributed in bacteria, so the effects of this compounds must be similar to those produced by plants (Chen et al., 2004; Yamada et al., 2015).

The volatile sulfur compounds (VSCs) have anti-yeast (Kim et al., 2004), antifungal (Fernando et al., 2005), nematicidal (Tada et al., 1988) and insecticidal activity (Gautier et al., 2008) and could be used in agriculture as biocontrol agent. Some VSCs were reported to reduce bacterial stress (Mi et al., 2016), others to have little antibacterial effect (Kim et al., 2004). Similarly to terpenes, antioxidant and detoxifying properties of sulfur compounds have been verified (Thomson and Ali, 2003; Wu et al., 2002).

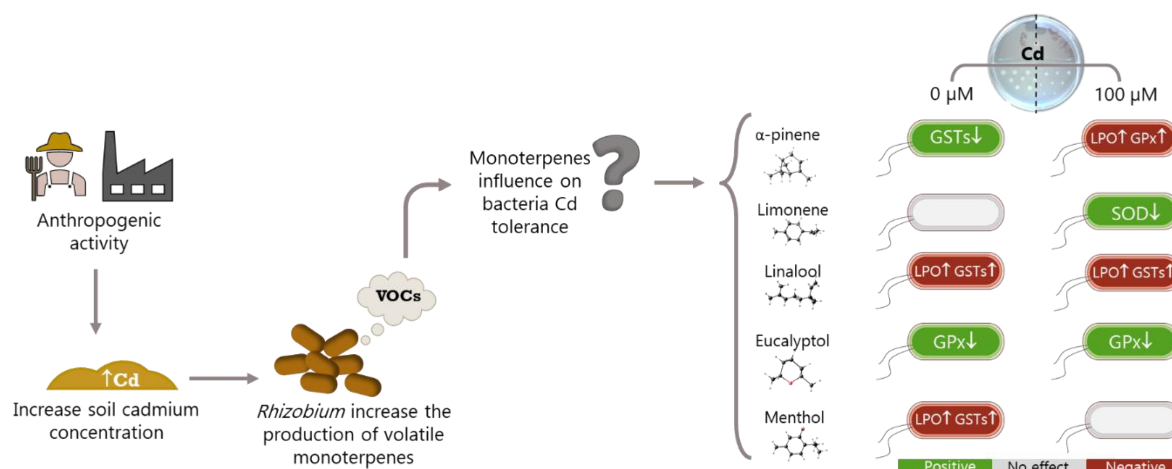
1.4. General objectives

This thesis aimed to identify the influence of VOCs on growth and metabolism of soil bacteria, in order to understand how these compounds interfere with cell metabolism in two cell physiological conditions: no Cd (control) and addition of 100 µM Cd (stress). With this approach it is intended to increase the knowledge about the role, importance and mode of action of volatile compounds in bacteria and to clarify interactions between organisms producing (influencers) and receiving (influenced) these compounds.

The effect of monoterpenes in *Rhizobium* strain E20-8 exposed or not to Cd, will be addressed in chapter 2. The effect of volatile sulfur compounds in *Rhizobium* strain E20-8 exposed or not to Cd, will be addressed in chapter 3.

Chapters 2 correspond to an article, submitted to Journal of Hazardous Materials, that is currently under reversion. Chapter 3 is a paper, still in progress, that will be submitted to a journal in the near future.

Chapter 2 – Airborne exposure of *Rhizobium leguminosarum* strain E20-8 to volatile monoterpenes: effects on cells challenged by cadmium



Abstract

Volatile organic compounds (VOCs) are produced by plants, fungi, bacteria and animals. These compounds are metabolites originated mainly in catabolic reactions and can be involved in biological processes. In this study, the airborne effects of five monoterpenes (α-pinene, limonene, eucalyptol, linalool, and menthol) on the growth and oxidative status of the rhizobial strain *Rhizobium leguminosarum* E20-8 were studied, testing the hypothesis that these VOCs could influence *Rhizobium* growth and tolerance to cadmium. The tested monoterpenes were reported to have diverse effects, such as antibacterial activity (linalool, limonene, α-pinene, eucalyptol), modulation of antioxidant response or antioxidant properties (α-pinene and menthol). Our results showed that non-stressed cells of *Rhizobium* E20-8 have different responses (growth, cell damage and biochemistry) to monoterpenes, with α-pinene and eucalyptol increasing colonies growth. In stressed cells the majority of monoterpenes failed to minimize the detrimental effects of Cd and increased damage, decreased growth and altered cell biochemistry were observed. However, limonene (1 and 100 mM) and eucalyptol (100 nM) were able to increase the growth of Cd-stressed cells. Our study evidences the influence at-a-distance that organisms able to produce monoterpenes may have on the growth and tolerance of bacterial cells challenged by different environmental conditions.

2.1. Introduction

Rhizobium is a bacteria genus that inhabits the superficial layer of soil and have the ability to fix atmospheric nitrogen when in symbiosis with legumes (Uma et al., 2013). For this reason, *Rhizobium* strains are focus of great agricultural and economic interest for promoting plant growth, increasing productivity (Rehman and Nautiyal, 2002) and reducing inorganic nitrogen fertilization (Coleto et al., 2014). *Rhizobium* also exists as a free-living form and is known for its ability to create biofilms with other species, allowing its persistence for long periods of time, yet with little information regarding its role in the soil (Denison and Kiers, 2011).

Microorganisms can communicate with each other by quorum sensing (QS) (Winson et al., 1998) or quorum quenching (Huang et al., 2016) allowing a collective response to environmental changes (Dong and Zhang, 2005). Microorganisms are also capable of producing and releasing a wide range of volatile organic compounds (Audrain et al., 2015; Schulz and Dickschat, 2007), which can diffuse through the complex soil matrix (Audrain et al., 2015). Microbial volatile organic compounds (MVOCs) are important in the interactions of microbial communities at-a-distance (Audrain et al., 2015; Schmidt et al., 2015). Depending on the volatile compound produced, the interactions with the environment might have a positive or a negative effect in the growth and physiology of the target organisms (Chung et al., 2015). To date, approximately 2000 microbial volatile compounds were found in almost 1000 species studied (Lemfack et al., 2018). Most MVOCs are alkanes, alcohols, ketones, aldehydes, volatile sulfur compounds, aromatic compounds and terpenoids (Schmidt et al., 2015; Schulz and Dickschat, 2007). However, due to the small number of species studied, compared to the 1 trillion (10^{12}) microbial species estimated to exist (Locey and Lennon, 2016), the list of MVOCs could be highly underestimated.

Volatile organic compounds are involved in several ecological roles in the soil, namely in the communication between organisms (plant-plant, plant-insect or plant-microbe) (Asensio et al., 2008). For example, plant volatiles can influence the microorganism's communication (QS) (Ahmad et al., 2014), demonstrating a volatile interaction between these different two life kingdoms. VOCs can also play a role in the communication between bacteria, influencing their motility and drug resistance (Kim et al., 2013). Previous studies (Edris, 2007; Farag et al., 2013) have reported antioxidant properties

of VOCs in animal and plant cells, however the influence of these compounds in soil bacteria is scarce (Bitas et al., 2013).

Monoterpenes may play a role in nutrient cycling and in the rhizosphere community, some can be used as substrates for bacterial growth (Asensio et al., 2008), and they might be also involved in microbial interactions (Schmidt et al., 2015). Plant monoterpenes can be synthetized and accumulated in roots and rhizomes of numerous plant species (Bos et al., 2002; Kovacevic et al., 2002) and were reported to induce the production of violacein (which has antibiotic activity) and pyocyanin (Ahmad et al., 2014). Bacteria are also capable of producing terpenoid compounds and are considered an unexplored source of new natural products (Yamada et al., 2015). The concentrations of monoterpenes in the environment depend on the number, proximity and the presence of terpene releasing organisms, and so a high variability is expected. Although little information exists regarding the concentration of monoterpenes in the soil, the concentration for α-pinene and limonene were reported to be between 2.2-1500 µg/g and 4.0-920 µg/g, depending on the type of soil (Asensio et al., 2008; McCreary et al., 1983; Wilt et al., 1988). Monoterpenes were reported to have antimicrobial and antiherbivore activities, providing a belowground protection to the plants capable of accumulating them (Chaves-López et al., 2015; Chen et al., 2004; Song and Ryu, 2013). Monoterpenes released by microorganisms might also have identical properties (Chen et al., 2004).

Soil microorganisms are influenced by a panoply of conditions including nutrient availability, physical-chemical conditions, interaction with plant roots and other organisms, and toxic compounds (Sadowsky, 2005; Zahran, 1999). Among the many factors that influence soil microbial communities toxic elements are one of the most detrimental, due to their high toxicity and persistence (Wuana and Okieimen, 2011). Cadmium (Cd) is a toxic metal that occurs in nature at low concentrations as a component of rocks, sediments, soils and dusts (Pinot et al., 2000). However, anthropogenic activities such as industrial production of batteries, plastics, alloys and synthetic materials lead to an increase of natural levels of Cd in the soil (Ursínyová and Hladíková, 2000) by releasing Cd into the environment through atmospheric emissions, effluents, sludges, or solid waste (Pinot et al., 2000). In agricultural soils the main route of Cd contamination is the application of phosphate fertilizers (Khan et al., 2017). Usually, in soil, 1.8–53 µM of Cd can be found (Helmke, 1999). However, the concentration can surpass 2669 µM in extremely

contaminated sites (Itoh and Yumura, 1979), with impacts to microbial communities (Lu et al., 2013).

A study performed by Cardoso et al. (2017) reported that *Rhizobium* cells stressed by exposure to Cd changed their volatile profile, and suggested this alteration to be linked to an increase of catabolic pathways or to the induction of tolerance mechanisms. Since MVOCs can be used as a means of communication between organisms, the higher production of some compounds, when bacteria are under stress, could be a way to signal neighboring cells of an eminent environmental stress situation and allow them to prepare in advance. To test this hypothesis, in this work *Rhizobium* cells were subjected to airborne exposure to different concentrations of α-pinene, limonene, eucalyptol, linalool and menthol. Growth and biochemical status were assessed in the presence and absence of Cd.

2.2. Results

Each figure represents the growth (A) and the biochemical endpoints (B and C) obtained from the airborne exposure of *Rhizobium* colonies to six concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM), of five monoterpenes (Fig. 4 - α-pinene, Fig. 5 - limonene, Fig. 6 - linalool, Fig. 7 - eucalyptol and Fig. 8 - menthol) for two Cd conditions (0 and 100 µM). No differences on colony macroscopic characteristics (shape, color) besides colony size were noticed when comparing colonies exposed and not exposed to the monoterpenes.

The experimental procedures of this chapter can be found in chapter 5, pages 49 to 54.

2.2.1. Sole exposure to Cd

When exposed to Cd a 55% reduction in growth of E20-8 compared to control condition, is observed (Fig. 4A, Fig. 5A., Fig. 6A, Fig. 7A and Fig. 8A). Cd also induced alterations in all the biochemical parameters determined, mostly increases (Fig. 4B, Fig. 5B, Fig. 6B, Fig. 7B and Fig. 8B). Result showed a considerable increase of the protein content and antioxidant activity (SOD and GPx). Regarding LPO and GSTs a small increase was observed.

2.2.2. Effects of α-pinene on *Rhizobium* grown in the presence and absence of Cd

Through the analysis of E20-8 growth, it was possible to observe that in the absence of Cd α-pinene increased growth (Fig. 4A). Maximum growth was observed in the presence of 1 mM of α-pinene, being this concentration the only significantly different from control. α-pinene decreased protein comparatively to control, especially at higher concentrations (less 25%) (Fig. 4B; supplementary table S1). The activity of SOD and GSTs also decreased. No variation was observed in the levels of lipid peroxidation (LPO) and GPx activity up to 1 mM, however at 100 mM a 4-fold significant increase was observed for GPx (Fig. 4B; supplementary table S1).

In the presence of Cd, α-pinene did not alleviate the growth decrease imposed by Cd (Fig. 4A). In the combined exposure to Cd and α-pinene protein and LPO were increased by

α-pinene especially at higher concentrations (Fig. 4B and Supplementary Table S1). α-pinene did not influence GSTs activity and GPx activity only increased at 100 mM α-pinene. On the other hand, a decrease in SOD activity was observed. (Fig. 4B and Supplementary Table S1).

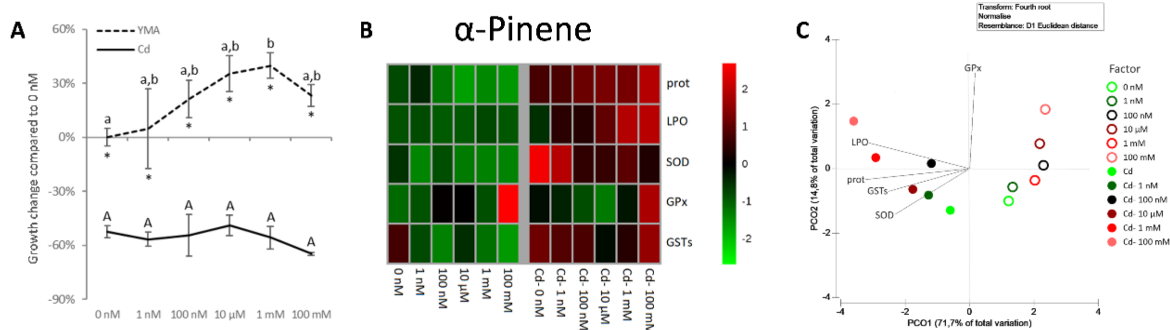


Figure 4 - Growth, antioxidant and biotransformation activity and damage in *Rhizobium* cells exposed to Cd and α-pinene. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 α-pinene concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) in a total of 12 conditions. (A) growth variation relatively to control (no Cd no compounds). Cells were exposed only to the α-pinene and not to Cd (dashed line); to the α-pinene and Cd (full line). Values are means of 3-6 replicates ± standard errors. Different lowercase letters indicate significant differences among α-pinene concentrations in YMA (0 µM Cd) condition; uppercase letters indicate significant differences among α-pinene concentrations in Cd condition, and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. (B) Heatmap of the biochemical determinants for each condition: lipid peroxidation (LPO); protein content (prot); glutathione peroxidase (GPx) activity; glutathione s-transferases (GSTs) activity; and superoxide dismutase (SOD) activity. For mean values, standard errors and statistical significance see Supplementary Table S1. (C) Principal Coordinates with centroids ordination (PCO) of the biochemical determinants for each condition: cells exposed to α-pinene and not to Cd (open circles); α-pinene and Cd (closed circles); detailed color scheme in the figure. Pearson correlation vectors were imposed: LPO; prot; SOD; GSTs and GPx activity ($r \geq 0.70$).

The Principal Components Ordination (PCO) of α-pinene data (Fig. 4C) showed that PCO1 was responsible for 71.1% of the differences separating bacteria exposed and not exposed to Cd based on their different biochemical behavior. PCO2 was responsible for 14.8% of the variation showing that GPx activity was the main mechanism of distinction between α-pinene concentrations (Fig. 4C).

2.2.3. Effects of limonene on *Rhizobium* grown in the presence and absence of Cd

The growth of colonies exposed to limonene was not significantly different from control although a decrease trend was observed (Fig. 5A). Limonene did not change protein content but increased LPO levels and enzymes activity (SOD, GSTs and GPx) (Fig. 5B and Supplementary Table S2).

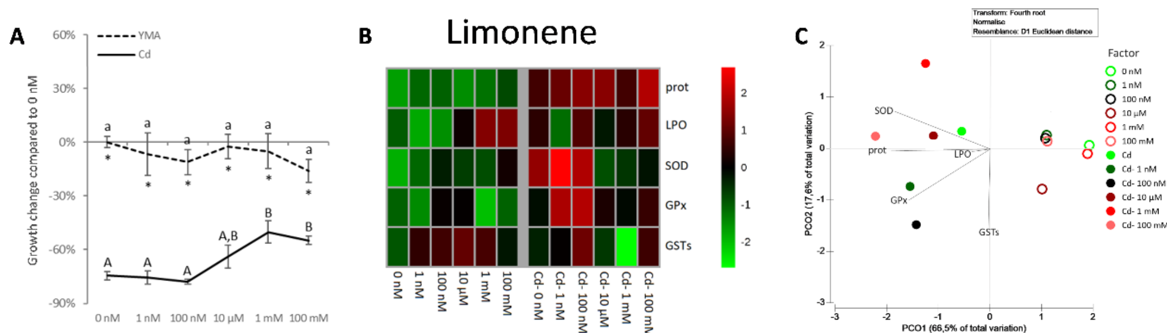


Figure 5 – Growth, antioxidant and biotransformation activity and damage in *Rhizobium* cells exposed to Cd and limonene. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 limonene concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) in a total of 12 conditions. (A) growth variation relatively to control (no Cd no compounds). Cells were exposed only to the limonene and not to Cd (dashed line); to the limonene and Cd (full line). Values are means of 3-6 replicates ± standard errors. Different lowercase letters indicate significant differences among limonene concentrations in YMA (0 µM Cd) condition; uppercase letters indicate significant differences among limonene concentrations in Cd condition, and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. (B) Heatmap of the biochemical determinants for each condition: lipid peroxidation (LPO); protein content (prot); glutathione peroxidase (GPx) activity; glutathione s-transferases (GSTs) activity; and superoxide dismutase (SOD) activity. For mean values, standard errors and statistical significance see Supplementary Table S2. (C) Principal Coordinates with centroids ordination (PCO) of the biochemical determinants for each condition: cells exposed to limonene and not to Cd (open circles); limonene and Cd (closed circles); detailed color scheme in the figure. Pearson correlation vectors were imposed: LPO; prot; SOD; GSTs and GPx activity ($r \geq 0.70$).

In the presence of Cd, limonene concentrations higher than 10 µM increased colonies growth, with the two highest concentrations of the compound (1 mM, 100 mM) being significantly different from sole exposure to Cd (Fig. 5A). LPO was not changed by limonene compared to Cd alone (Fig. 5B; Supplementary Table S2). However, protein levels

and SOD and GSTs activity increased significantly for some concentrations of limonene comparatively to sole exposure to Cd (Fig. 5B; Supplementary Table S2). GPx activity was increased by the lowest limonene concentrations (1 nM, 100 nM), but remained similar at the higher concentrations (10 µM, 1 mM and 100 mM).

PCO analysis demonstrated that the abscissa axis was responsible for 66.5% of the differences separating the bacteria exposed and not exposed to Cd based on their different biochemical features. The ordinate axis was responsible for 17.6% of the differences, pointing that GSTs activity was the main mechanism of distinction between the concentrations of limonene in the presence of Cd (Fig. 5C).

2.2.4. Effects of linalool on *Rhizobium* grown in the presence and absence of Cd

Linalool appears to decrease colony growth both in the presence and absence of Cd, especially at the highest concentrations, where a significant decrease was observed (Fig. 6A). Sole expose to linalool did not influence proteins content and SOD activity, increased LPO and GSTs activity and decreased GPx activity in some concentrations (1 nM and 10 µM) (Fig. 6B and Supplementary Table S3).

The combined exposure to Cd and linalool led to increases of protein, GPx and GSTs activity comparatively to Cd (significant only at milimolar range of the compound) (Fig. 6B and Supplementary Table S3). Furthermore, linalool decreased LPO at lower concentrations (1 nM, 100 nM) and increased at higher concentrations (10 µM, 1 mM and 100 mM) compared to sole exposure to Cd (Fig. 6B).

PCO analysis showed that the abscissa axis was responsible for 78% of the differences separating bacteria exposed and not exposed to Cd based on their different biochemical traits (Fig. 6C). The ordinate axis was responsible for 12.7% of the differences showing that exposure to 100 mM of linalool further increased the damage inflicted by Cd on membranes (LPO), which cells tried to restrain by increasing GSTs activity.

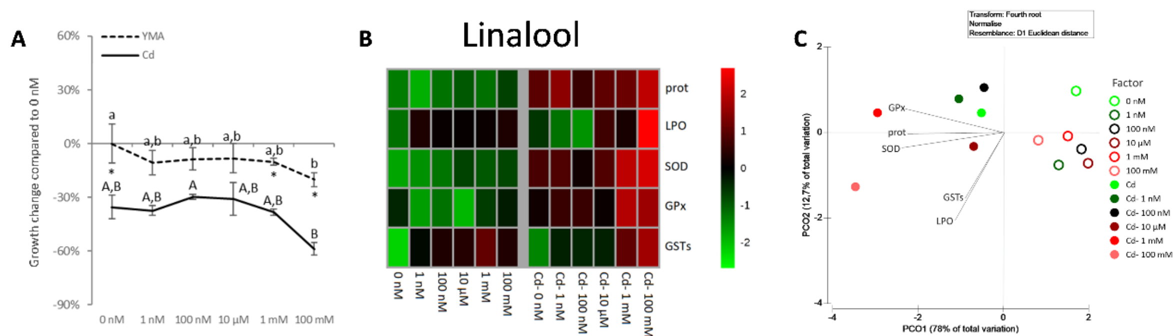


Figure 6 – Growth, antioxidant and biotransformation activity and damage in *Rhizobium* cells exposed to Cd and linalool. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 linalool concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) in a total of 12 conditions. (A) growth variation relatively to control (no Cd no compounds). Cells were exposed only to the linalool and not to Cd (dashed line); to the linalool and Cd (full line). Values are means of 3-6 replicates ± standard errors. Different lowercase letters indicate significant differences among linalool concentrations in YMA (0 µM Cd) condition; uppercase letters indicate significant differences among linalool concentrations in Cd condition, and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. (B) Heatmap of the biochemical determinants for each condition: lipid peroxidation (LPO); protein content (prot); glutathione peroxidase (GPx) activity; glutathione s-transferases (GSTs) activity; and superoxide dismutase (SOD) activity. For mean values, standard errors and statistical significance see Supplementary Table S3. (C) Principal Coordinates with centroids ordination (PCO) of the biochemical determinants for each condition: cells exposed to linalool and not to Cd (open circles); linalool and Cd (closed circles); detailed color scheme in the figure. Pearson correlation vectors were imposed: LPO; prot; SOD; GSTs and GPx activity (r ≥ 0.80).

2.2.5. Effects of eucalyptol on *Rhizobium* grown in the presence and absence of Cd

Eucalyptol induced a dose response increase trend in colony growth both in the presence and absence of Cd although most values were not significantly different from control (Fig. 7A).

Eucalyptol decreased protein (only significantly at 1 mM), LPO (100 mM) and enzymes activity for one (GPx) or more concentrations (SOD, GSTs) of the compound (Fig. 7B and Supplementary Table S4). In the presence of Cd, eucalyptol decreased protein, SOD and GPx activity, but increased LPO levels and GSTs activity at higher concentrations (1 mM and 100 mM).

PCO analysis (Fig. 7C) demonstrates that the abscissa axis was responsible for 75,4%

of the differences, separating the bacteria exposed and not exposed to Cd based on their different biochemical characteristics. The ordinate axis was responsible for 12.6% of the differences, showing that GPx activity was negatively influenced by eucalyptol both in presence and absent of Cd.

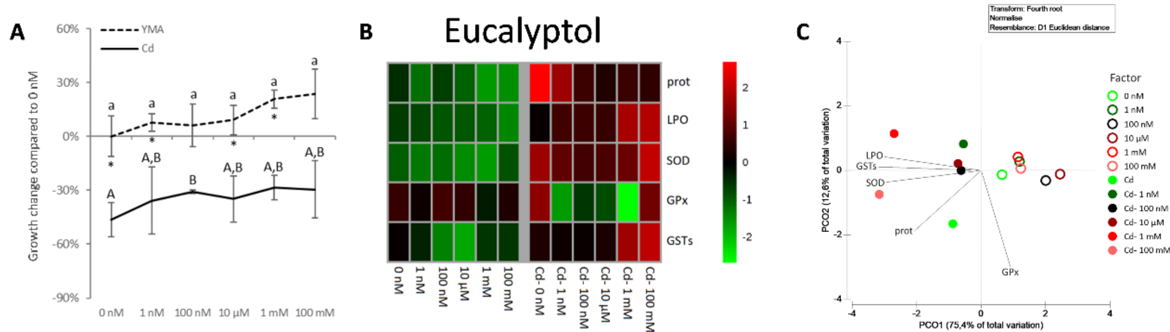


Figure 7 – Growth, antioxidant and biotransformation activity and damage in *Rhizobium* cells exposed to Cd and eucalyptol. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 eucalyptol concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) in a total of 12 conditions. (A) growth variation relatively to control (no Cd no compounds). Cells were exposed only to the eucalyptol and not to Cd (dashed line); to the eucalyptol and Cd (full line). Values are means of 3-6 replicates ± standard errors. Different lowercase letters indicate significant differences among eucalyptol concentrations in YMA (0 µM Cd) condition; uppercase letters indicate significant differences among eucalyptol concentrations in Cd condition, and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. (B) Heatmap of the biochemical determinants for each condition: lipid peroxidation (LPO); protein content (prot); glutathione peroxidase (GPx) activity; glutathione s-transferases (GSTs) activity; and superoxide dismutase (SOD) activity. For mean values, standard errors and statistical significance see Supplementary Table S4. (C) Principal Coordinates with centroids ordination (PCO) of the biochemical determinants for each condition: cells exposed to eucalyptol and not to Cd (open circles); eucalyptol and Cd (closed circles); detailed color scheme in the figure. Pearson correlation vectors were imposed: LPO; prot; SOD; GSTs and GPx activity ($r \geq 0.70$).

2.2.6. Effects of menthol on *Rhizobium* grown in the presence and absence of Cd

Growth of bacterial cells exposed to increasing concentrations of menthol decreased (significantly at 100 mM). In Cd challenged cells menthol did not have a defined trend in colony growth (Fig. 8A).

The increase in menthol concentrations led to protein increase (Fig. 8B and Supplementary Table S5). LPO decreased significantly at 100 nM and 10 µM. SOD activity increased at higher concentrations. GPx activity decreased at 1 mM and GSTs decreased at lower concentrations (1 nM to 10 µM) and increased at higher concentrations (1 mM and 100 mM).

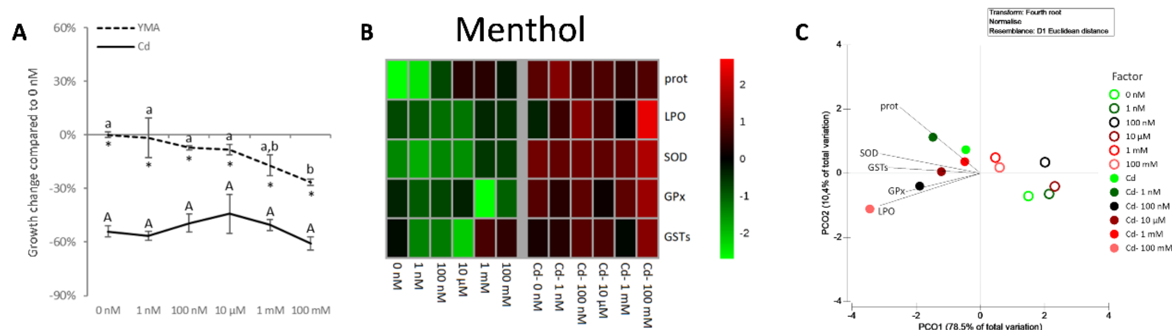


Figure 8 – Growth, antioxidant and biotransformation activity and damage in *Rhizobium* cells exposed to Cd and menthol. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 menthol concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) in a total of 12 conditions. (A) growth variation relatively to control (no Cd no compounds). Cells were exposed only to the menthol and not to Cd (dashed line); to the menthol and Cd (full line). Values are means of 3-6 replicates ± standard errors. Different lowercase letters indicate significant differences among menthol concentrations in YMA (0 µM Cd) condition; uppercase letters indicate significant differences among menthol concentrations in Cd condition, and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. (B) Heatmap of the biochemical determinants for each condition: lipid peroxidation (LPO); protein content (prot); glutathione peroxidase (GPx) activity; glutathione s-transferases (GSTs) activity; and superoxide dismutase (SOD) activity. For mean values, standard errors and statistical significance see Supplementary Table S5. (C) Principal Coordinates with centroids ordination (PCO) of the biochemical determinants for each condition: cells exposed to menthol and not to Cd (open circles); menthol and Cd (closed circles); detailed color scheme in the figure. Pearson correlation vectors were imposed: LPO; prot; SOD; GSTs and GPx activity ($r \geq 0.70$).

The presence of menthol in Cd stressed cells led to variation in protein content (both increases and decreases), increases in LPO levels and GSTs activity and no significant influence on SOD and GPx activity (Fig. 8B and Supplementary Table S5).

PCO analysis (Fig. 8C) showed that the abscissa axis was responsible for 78.5% of

the differences separating the bacteria exposed and not exposed to Cd based on their different biochemical features. It was also possible to observe that SOD and GSTs activity and protein content are the main mechanisms that discriminate the influence of menthol (ordinate axis).

2.3. Discussion

Previous studies demonstrated that Cd decreases the diversity and number of microorganisms found in contaminated soils (Luo et al., 2019; Wu et al., 2018), evidencing the detrimental effect of this toxic element on microorganisms growth and survival. In this study, Cd induced differences in the growth and biochemistry of *Rhizobium,* with increased damage (LPO) and metabolic alterations (proteins), including antioxidant (SOD) and biotransformation (GSTs) responses. This is in agreement with previous studies reporting Cd to trigger the antioxidant response in *Rhizobium* (Corticeiro et al., 2006; Figueira et al., 2005). We also observed that monoterpenes influenced colony growth and biochemistry and changed cells response to Cd stress, with each compound evidencing distinct influences and the same compound inducing different responses at different conditions (presence and absence of Cd). In this way, compounds belonging to the same chemical family (monoterpenes) had different effects on bacteria. In fact, terpenes and terpenoids can have different functional groups, rendering different terpenoid molecules with diverse bioactivities. Monoterpenes might contain an aldehyde, alcohol, ketone, ester and ether functional groups. Bioactivity of monoterpenoids depends on the nature and position of functional groups and molecular configuration (Tripathi and Mishra, 2016). Monoterpene hydrocarbons are antioxidants, however oxygenated monoterpenes are more powerful antioxidants (Zengin and Baysal, 2014). Regarding antibacterial activity, terpenoids that contain alcohols possess higher activity than the corresponding carbonyl compounds, and the number of double bonds and the acyclic or cyclic nature of the structure do not appear to have a big effect on antibacterial activity, with the exception of aromatic compounds, which can evidence higher inhibitory activity (Zengin and Baysal, 2014). Eucalyptol and linalool have been reported as inhibitory of bacterial growth (Zengin and Baysal, 2014). These authors also tested α-pinene, but concentrations up to 2% did not inhibit bacterial growth; nevertheless α-pinene was reported in the literature as inhibitory of bacterial growth (Ait-Ouazzou et al., 2011). It is also important to note that, as opposed to inhibition studies which used relatively high concentrations and tested direct contact (Zengin and Baysal, 2014), in our study low concentrations (down to nanomolar range) were used, and due to the volatile nature of the interaction, the changes that were elicited were likely due to small concentrations reaching the cells. Thus, it is not far-fetched to assume that similar

interactions exist in the soil. α-pinene, limonene, linalool, eucalyptol, and menthol are all produced by plants and thus have a natural presence in the ecosystems. Moreover, terpenoid compounds are also produced by bacteria (Cardoso et al., 2017; Schulz and Dickschat, 2007; Yamada et al., 2015).

In our study, α-pinene increased colony growth. Previous studies have found that α-pinene can be used as a carbon source by microorganisms (Griffiths et al., 1987). It is known that LPO is one of the main effects on cells under oxidative damage (El-Nekeety et al., 2011). Since cells exposed to different concentrations of α-pinene had similar LPO values compared to the control, this suggests that α-pinene is not detrimental to the membrane lipids of E20-8. An earlier study (Türkez and Aydın, 2016) showed that this compound may be beneficial for cells due to its antioxidant properties. In our study, the antioxidant nature of α-pinene could be proven by the decrease in SOD and GSTs activities without increasing LPO levels and by growth promotion. This antioxidant effect disappeared in Cd stressed cells, since membrane damage (LPO) increased compared to sole exposure to Cd, however the higher increase in proteins evidence the metabolic effort of cells to trigger mechanisms to fight Cd induced stress and α-pinene joint toxicity, such as the increase of GPx activity. Previous studies reported that a high level of glutathione (GSH) allows cells to better manage the oxidative stress created by Cd (Corticeiro et al., 2006; Figueira et al., 2005). Glutathione peroxidase (GPx) plays a key role in cellular antioxidant activity by catalyzing the reduction of hydroperoxides using GSH as a reducing agent (Galiazzo et al., 1987). In accordance with these studies it is possible to observe that α-pinene induced GPx activity in a concentration-dependent manner, either in the presence and absence of Cd, evidencing this enzyme as the main mode of α-pinene to modulate the cell antioxidant response both in the presence and absence of Cd.

Limonene is produced by many plant species (Jongedijk et al., 2016) and has also been detected in the headspace of microorganisms (Cardoso et al., 2017; Jongedijk et al., 2016). Our results evidence a dual effect of limonene in cells exposed or not exposed to Cd. In the absence of Cd, limonene did not influence cell growth, but in Cd stressed cells increased growth compared to sole exposure to Cd at higher concentrations. Although limonene may be used by some bacteria as a carbon source (Griffiths et al., 1987), it is mainly known for its antimicrobial activity (Ahmad et al., 2014; Espina et al., 2013). In the absence of Cd, limonene showed pro-oxidant activity, increasing membrane damage (LPO) and

triggering antioxidant and biotransformation responses. The ability of cyclic hydrocarbons, including limonene, to interact with the microbial plasma membrane leads to a disruption in the integrity of the membrane (Sikkema et al., 1994), thus justifying the observed LPO values. When cells are in the presence of Cd a positive influence of limonene on cell growth is observed, especially at higher concentrations. The increase of antioxidant enzymes in *Escherichia coli* exposed to metals showed that cells were under oxidative stress, despite protection mechanisms were triggered (El-rab and Abskharon, 2013). In our study, the increases of antioxidant/biotransformation enzymes activity (GSTs) by limonene in the presence of Cd compared to sole Cd exposure, can be considered a stimulation of the antioxidant and biotransformation action of cells towards Cd toxicity. The higher growth observed could be linked to the increase in GSTs activity, since these enzymes are known to be part of the cell detoxification process and the formation of Cd-GS complexes (Cardoso et al., 2018b; Lima et al., 2006) that decreases free Cd ions concentration in cells thus reducing their toxicity (Cardoso et al., 2018b). PCO brings GSTs activity as the main mechanism triggered by limonene to fight Cd stress. Thus, increasing limonene production by microorganisms or exposing microorganisms to limonene applied directly to soil or by limonene root exuding plant species may benefit *Rhizobium* when exposed to Cd.

Coriander essential oil was described as having antioxidant and antigenotoxic activity towards bacteria (*E. coli*) (Mitić-Ćulafić et al., 2009) and its main constituent is linalool (Duarte et al., 2016). In our study linalool had a negative effect on cell growth both in the presence and absence of Cd stress. Thus, the antibacterial effect of this compound (Duarte et al., 2016; Fisher and Phillips, 2006) is also observed and may might be due to the biochemical response of the cell to linalool, evidencing an increase in oxidative stress levels. Van Bogelen et al. (1987) observed that in response to Cd, *E. coli* cells increased the synthesis of specific proteins (proteins induced by cadmium) in order to combat Cd stress. Enzymes capable of removing oxygen radicals and their products are important actions of cellular antioxidant defense, and Cd-susceptible strains (with lower growth) were reported to have higher SOD and GPx activity (Birben et al., 2012), evidencing that the most efficient mechanisms are those avoiding the buildup of oxidative stress (Corticeiro et al., 2006). Oxidative damage (LPO) increased probably because antioxidant activity was not activated (SOD and GPx), and E20-8 cells decreased growth, even though GSTs activity was increased. In our study, PCO analysis evidence GSTs and LPO as the main endpoints that

distinguish linalool concentrations in the presence of Cd.

Previous studies have found that eucalyptol antioxidant properties are concentration dependent, inducing oxidative damage in membranes and DNA at high concentrations. (Özkan and Erdoğan, 2013). Taking into account the pro-oxidant effects described, it would be expected that eucalyptol would induce cellular damage and antioxidant response, along the increase of the concentrations. However, the decrease in oxidative damage (LPO) together with the decrease in the antioxidant and biotransformation activity (SOD, GSTs, GPx) observed in our study is more related with an antioxidant role and may support the increase in growth observed. The biochemical data are in agreement with a study by Mitić-Ćulafić et al. (2009), where reduction of lipid peroxidation and antioxidant and antigenotoxic capacity were reported in *E. coli* cells exposed to eucalyptol. However, eucalyptol did not reduce the oxidative stress imposed by Cd in E20-8. The increase in oxidative damage (LPO) and the decrease in GPx and SOD activity corroborate that under Cd stress the effect of eucalyptol shifts from anti- to pro- oxidant effect. However, the biotransformation response (GSTs) was activated, protecting cells from the toxic compounds derived from lipid hydroperoxides and catalyzing the formation of Cd-GS complexes, turning cells less vulnerable to Cd, and supporting higher growth than when only exposed to Cd.

Menthol is widely used in food, cosmetic and pharmaceutical industries (Schelz et al., 2006; Yu et al., 2007). This compound is not common in the VOCs matrix released by bacteria (Karami et al., 2017), and little is known about the individual effect of this compound on microorganisms. Menthol is one of the constituents of essential oils of several plant species, which were described to have antibacterial, antiviral and antioxidant properties (Kaya and Duran, 2018; McKay and Blumberg, 2006). Our results showed that menthol inhibits bacterial proliferation, demonstrating its antibacterial activity in the absence of Cd. At low concentrations menthol decreased membrane damage (LPO) and GSTs activity; at higher concentrations SOD and GSTs activities increased and GPx activity decreased, evidencing a shift from anti- to pro-oxidant activity as concentrations increase. In the presence of Cd menthol exhibited a pro-oxidant activity for all concentrations tested towards membranes, but little influence on cytoplasm biochemistry and on growth. GSTs activity was the only parameter increased by menthol in Cd exposed cells. GSTs were reported to increase the formation of Cd-GSH complexes, minimizing metal toxicity (Cardoso et al., 2018a), which can explain the maintenance of growth similar to sole exposure to Cd, without

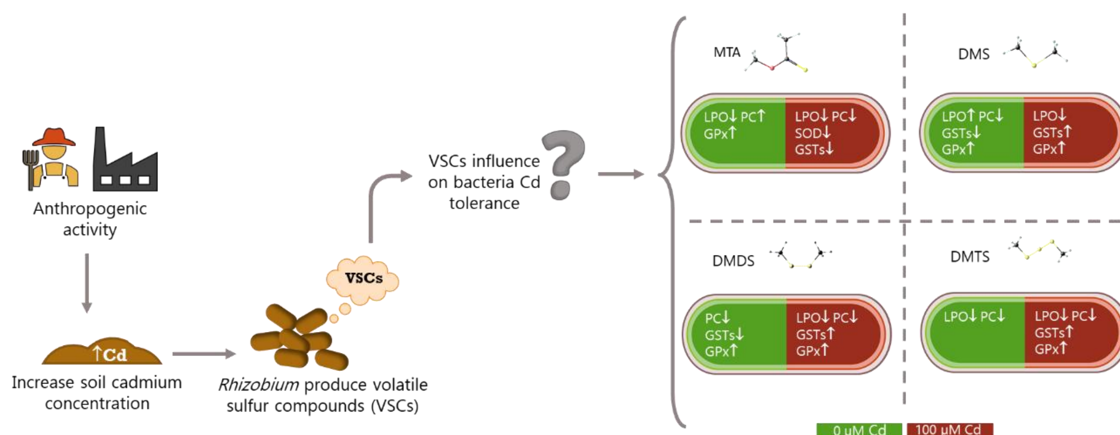
the activation of the antioxidant enzymes. Thus, menthol had little influence on Cd-induced cytosolic oxidative stress. Through the analysis of the PCO it is possible to observe that protein, LPO and GPx are the main endpoints to distinguish the effects of menthol concentrations on cells in the presence and absence of Cd, demonstrating the biochemical effects of menthol behind its antimicrobial activity.

2.4. Concluding remarks

Airborne exposure of *Rhizobium* colonies to monoterpenes evidenced differences among compounds, both in Cd exposed and not exposed cells. In the absence of Cd eucalyptol and α-pinene increased growth due to a low influence on cell biochemistry. Limonene did not influence growth, probably due to higher allocation of energy to combat oxidative stress, living less energy available for growth. Linalool and menthol evidenced antimicrobial activity. Linalool affected both membranes and cytosol, but menthol toxicity was not related to membrane damage, but to higher toxicity in the cytoplasm. Most monoterpenes further increased the oxidative stress of cells generated by Cd, specifically in membranes. The influence of these compounds on growth was linked to the ability of cells to activate the metabolism (higher protein level) and to trigger the antioxidant (SOD and GPx activity) and biotransformation (GSTs activity) response. Eucalyptol was the only monoterpene extending its protective effect to E20-8 cells challenged by Cd, improving growth significantly when present at 100 nM, although limonene was also capable of promoting growth significantly in the presence of Cd conditions when applied at 1 and 100 mM.

Our study thus evidences the influence at a distance that organisms (plants or microorganisms), capable of producing volatile compounds (such as monoterpenes), may have on the growth and tolerance of bacterial cells living in different environmental conditions and sheds some light on the communication and interaction among communities that coexist spatially and temporally.

Chapter 3 – Effects of volatile sulfur compounds on growth and oxidative stress of *Rhizobium leguminosarum* strain E20-8 challenged by cadmium



Abstract

Volatile sulfur compounds have been reported to be produced by many bacterial species. These compounds can be used as a biocontrol strategy in agriculture since some of these compounds negatively influence fungal species, nematodes and insects. Moreover, dimethyl disulfide released by bacteria has also been reported to promote plant growth. Some of these compounds have also been hypothesized to play a role in bacterial response to Cd induced stress. This study aimed to assess the potential effects of four volatile sulfur compounds (VSCs) (dimethyl sulfide - DMS, dimethyl disulfide - DMDS, dimethyl trisulfide - DMTS and methyl thioacetate - MTA) on the growth and oxidative state of *Rhizobium* sp. strain E20-8 via airborne exposure, in order to test the hypothesis that these volatile compounds could influence growth and cadmium tolerance of the bacteria. Our results show that, overall, the tested compounds triggered similar antioxidant mechanisms in *Rhizobium*. Although growth was not very affected, VSCs can minimize Cd toxicity towards cells by reducing oxidative stress. The protective effect at the membrane level by DMDS and DMTS particularly demonstrates the antioxidant effect of these volatile compounds. Due to the volatile nature of these compounds, the low concentrations tested, and considering that they are released by bacteria and other organisms such as plants, it is possible that these effects also occur in the soil ecosystem.

3.1. Introduction

Rhizobium is a soil gram-negative bacterium that has a great economic and agricultural interest due to its ability to fix atmospheric nitrogen when in symbiosis with legumes (Mabrouk et al., 2018; Mabrouk and Belhadj, 2010), leading to an increase in agricultural production (Rehman and Nautiyal, 2002) and the decrease in the use of nitrogen-based inorganic fertilizers (Coleto et al., 2014).

The use of phosphorus fertilizers, which may contain traces of Cd, is the main reason for the increase of cadmium (Cd) concentrations in agricultural fields (Khan et al., 2017). Cd is a naturally occurring toxic metal whose concentrations in the environment have increased due to anthropogenic activities (Thornton, 1992; Ursínyová and Hladíková, 2000). Due to their persistence and high toxicity, metals are harmful to microbial communities (He et al., 2005; Wuana and Okieimen, 2011). In this way high concentrations of Cd will have impacts on the microbial communities (Lu et al., 2013; Roane and Pepper, 1999).

The survival of microorganisms under stress conditions depends on tolerance mechanisms, such as specific pathways originating secondary metabolites (Garbeva et al., 2014a, 2011; Garbeva and de Boer, 2009; Hibbing et al., 2010; Laskaris et al., 2010). As a result of the secondary metabolism, microorganisms are capable of producing and releasing a variety of volatile organic compounds (VOCs) (Audrain et al., 2015; Schulz and Dickschat, 2007). Microorganisms have different ways of alerting the changes that occur in their environment (Dong and Zhang, 2005). The easy diffusion of microbial VOCs (MVOCs) by the complex soil matrix (Effmert et al., 2012; Schmidt et al., 2015) allows them to be considered a good system of communication at a distance between microorganisms (Chernin et al., 2011; Effmert et al., 2012; Garbeva et al., 2014b, 2014a; Kai et al., 2009), influencing growth, antibiotic production and gene expression of soil bacteria (Audrain et al., 2015; Garbeva et al., 2014b; Schmidt et al., 2015).

Volatile sulfur compounds (VSCs) are chemical compounds that are released in large amounts from the ocean to the atmosphere (Andreae, 1990), having been the topic of countless studies. The most important pathway for the formation of dimethyl sulfide in the marine and estuarine ecosystems involves the cleavage of 3-dimethylsulfoniopropionate and play an important role in acid precipitation, cloud formation, climate regulation (Nriagu et al., 1987; Schulz and Dickschat, 2007), and the global sulfur cycle (Lomans et al., 2002).

VSCs are responsible for the aroma and flavor of some fermented foods such as cheese and wine (Deetae et al., 2007; Kreitman et al., 2019; Landaud et al., 2008). In these cases the VSCs originate from the secondary metabolism of bacteria and can be derived from amino acid (cysteine and methionine) catabolism, originating for example methanethiol (Scully et al., 1997), dimethyl sulfide (DMS) (Yoshimura et al., 2000), dimethyl disulfide (DMDS) (Fukamachi et al., 2005) and dimethyl trisulfide (DMTS) (Chu et al., 1997; Schulz and Dickschat, 2007; Sreekumar et al., 2009). The methyl tioacetate (MTA) synthesis, in microorganisms, use as precursors the acetyl CoA or methanethiol (Arfi et al., 2002; Schulz and Dickschat, 2007). VSCs can have anti-yeast properties (Kim et al., 2004), as well as antifungal (Fernando et al., 2005), nematicidal (Tada et al., 1988) and insecticide (Gautier et al., 2008) activities, allowing their use as biocontrol in agriculture and thus decrease the application of more persistent agrochemicals (Fernando et al., 2005). Despite their use as pest control in agricultural soils, information about the influence of these compounds on soil bacteria is scarce (Bitas et al., 2013). Moreover, other authors did not observe a strong antibacterial of these compounds (Kim et al., 2004), and in some cases a bacterial stress relief was observed , due to antioxidant and detoxifying properties (Mi et al., 2016; Thomson and Ali, 2003; Wu et al., 2002).

A recent study showed a change in the VOCs released by *Rhizobium* cells in the presence of Cd (Cardoso et al., 2017). These authors also verified an increase in the peak areas of some VSCs (DMS, DMDS, DMTS) and the decrease of others (MTA) in the presence of stress, which could be involved in the antioxidant response, and thus could constitute a possible mechanism of cellular tolerance to Cd. VOCs might also be used as an environmental stress alert allowing cells to communicate their current state and prepare neighboring cells for eminent stress. In order to evaluate the effects that the increase of VSCs may have on bacterial cells the sulfur compounds MTA, DMS, DMDS and DMTS were used to test the hypothesis that their presence (through airborne exposure) affects the growth and biochemical status of *Rhizobium* cells exposed or non-exposed to Cd.

3.2. Results

Results were evaluated by colony weight (supplementary tables). Graphs were built with values relative to the control (no Cd and no VSCs).

The experimental procedures of this chapter can be found in chapter 5, pages 49 to 54.

3.2.1. Sole exposure to Cd

When exposed to Cd a 60% reduction in growth of E20-8 compared to control condition was observed (Fig. 9). Cd also induced alterations in all the biochemical parameters determined, increasing for the majority of the conditions (Fig. 10 to 12).

3.2.2. Growth

Through the analysis of E20-8 growth, it was possible to observe that in the absence of Cd methyl thioacetate (MTA) increased growth at low concentrations (1 nM and 100 nM) (Fig. 9). Maximum growth was observed in the presence of 100 nM. However, the two highest concentrations show a decrease in growth, not significantly different from control but significantly different from 100 nM, 10 µM. For the remaining compounds (DMS, DMDS, DMTS) little variation was observed in the growth of E20-8, with no significant increase or decrease being observed relatively to control. In the presence of Cd, the two highest concentrations of MTA lead to a significant increase in growth relative to control. An increase in growth at 1 mM concentration was also observed for DMTS, yet it was not statistically significant. The compounds DMS and DMDS did not alleviate the growth decrease imposed by Cd (Fig. 9).

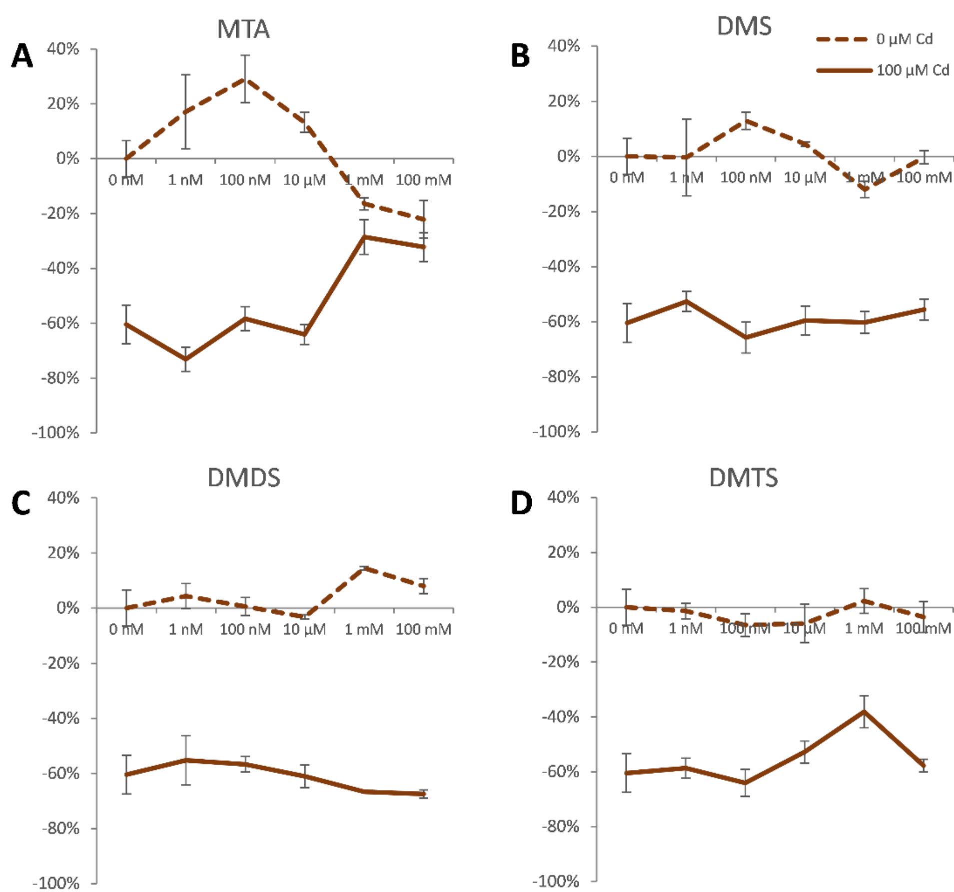


Figure 9 – Growth of *Rhizobium* cells exposed to Cd and sulfur compounds. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 concentrations (0nM, 1nM, 100nM, 10µM, 1mM and 100mM) of each VSC in a total of 12 conditions. Growth variation relatively to control (no Cd no compounds). (A) Cells were exposed only to MTA and not to Cd (dashed line); MTA and Cd (full line). (B) Cells were exposed only to DMS and not to Cd (dashed line); DMS and Cd (full line (C) Cells were exposed only to DMDS and not to Cd (dashed line); DMDS and Cd (full line). (D) Cells were exposed only to DMTS and not to Cd (dashed line); DMTS and Cd (full line). Values are means of 3-6 replicates ± standard errors. Different lowercase letters indicate significant differences among each VSC concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among each VSC concentrations in Cd condition, and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. For mean values, standard errors and statistical significance see Supplementary Table S6.

3.2.3. Oxidative damage

In the absence of Cd the presence of MTA and DMTS led to a decrease in LPO, while DMS and DMDS led to an increase (Fig. 10A). MTA was the only compound that led to increase of PC, in the absence of Cd (Fig. 10C).

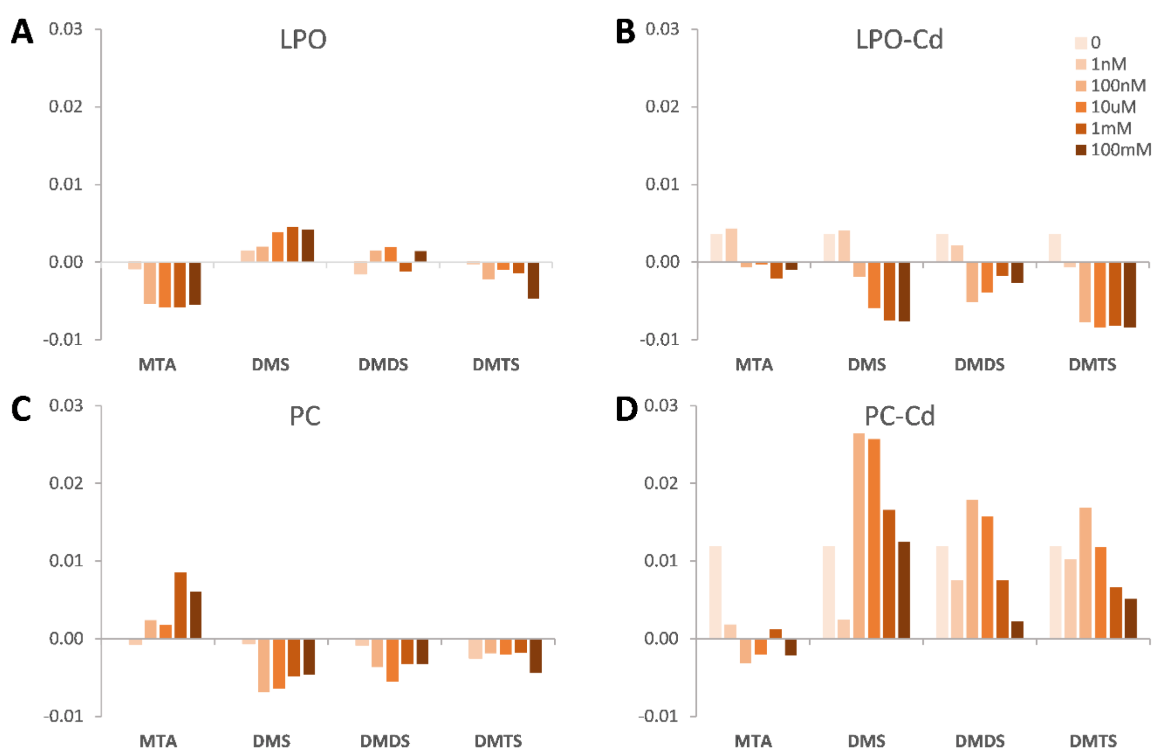


Figure 10 – Damage in *Rhizobium* cells exposed to sulfur compounds in the absence or presence of Cd. Cells were simultaneously exposed to 6 concentrations (0nM, 1nM, 100nM, 10µM, 1mM and 100mM) of the sulfur compounds (MTA, DMS, DMDS, DMTS) in a total of 24 conditions. Biochemical results variation relatively to control (no Cd, no compounds). (A) lipid peroxidation in the absence of Cd (LPO); (B) lipid peroxidation in the presence of Cd (LPO-Cd); (C) protein carboxylation in the absence of Cd (PC); (D) protein carboxylation in the presence of Cd (PC-Cd). For mean values, standard errors and statistical significance see Supplementary Table S7 and S8.

In the presence of Cd all compounds led to a decrease in LPO at concentrations of 100 nM and higher. This decrease was more pronounced in DMS and DMTS, which have values significantly different from the control (Fig. 10B). However, an increase of PC in the presence of compounds was observed (Fig. 10D), with the exception of MTA (PC decreased significantly).

3.2.4. Antioxidant enzymes

In the absence of stress, the increment of SOD and GPx was observed in the presence of the compounds (Fig. 11A; E). Significant increase occurs in the activity of GPx for DMDS and

MTA compounds, while the GSTs activity decreases in the presence of the compounds (Fig. 11C; E).

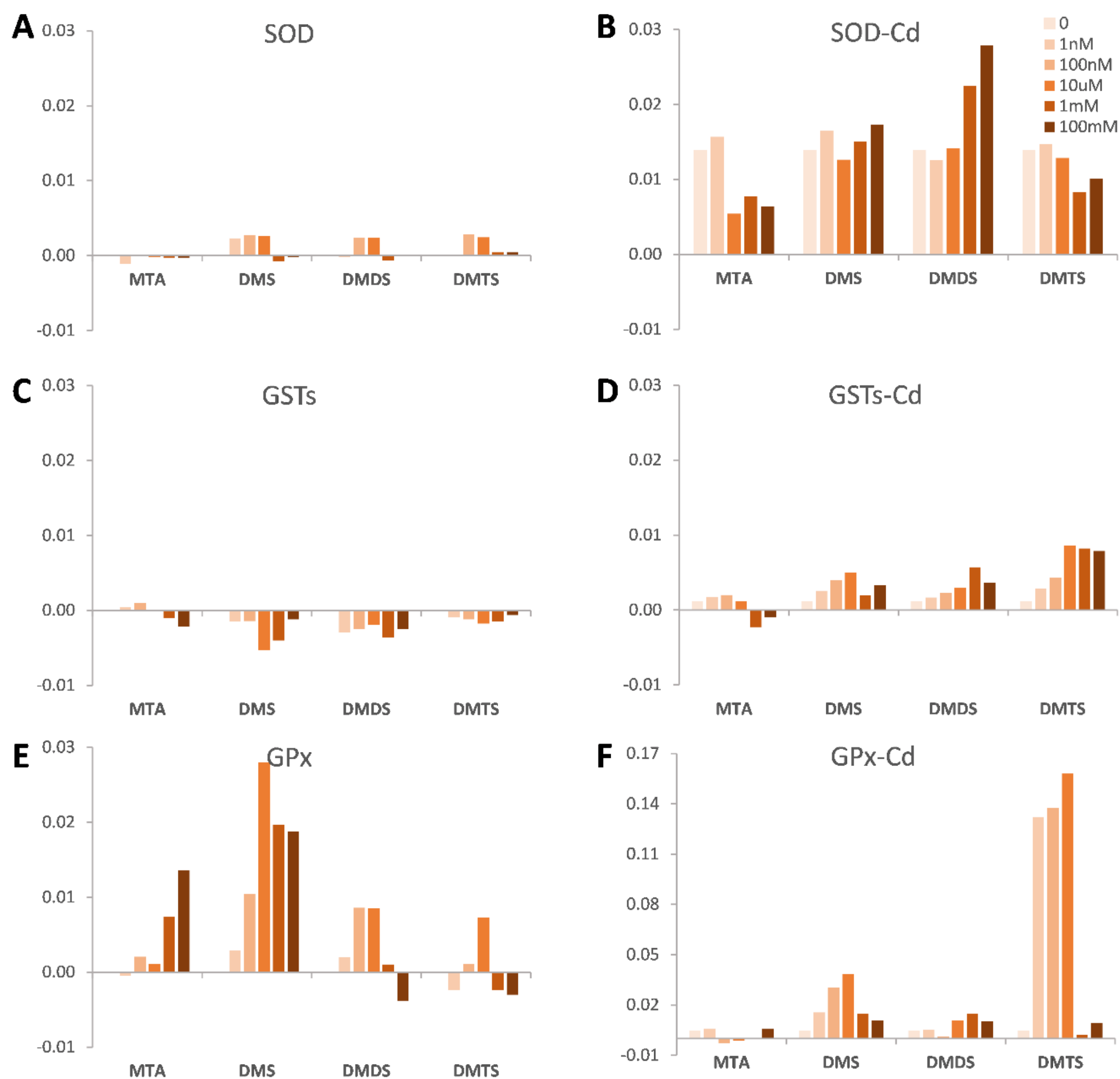


Figure 11 – Antioxidant activity in *Rhizobium* cells exposed to sulfur compounds in the absence or presence of Cd. Cells were simultaneously exposed to 6 concentrations (0nM, 1nM, 100nM, 10µM, 1mM and 100mM) of the sulfur compounds (MTA, DMS, DMDS, DMTS) in a total of 24 conditions. Biochemical results variation relatively to control. (A) superoxide dismutase activity in the absence of Cd (SOD); (B) superoxide dismutase activity in the presence of Cd (SOD-Cd); (C) glutathione s-transferases activity in the absence of Cd (GSTs); (D) glutathione s-transferases activity in the presence of Cd (GSTs-Cd); (E) glutathione peroxidase activity in the absence of Cd (GPx); and (F) glutathione peroxidase acivity in the presence of Cd (GPx-Cd). For mean values, standard errors and statistical significance see Supplementary Table S9-S11.

In the presence of Cd most of the compounds led to an increase of SOD, GSTs and GPx (Fig. 11B; D; F), being observed a significant increase in the SOD for the DMDS and in the GPx for the DMTS compound.

3.2.5. Metabolic response

In the absence of Cd, a decrease in the levels of protein in the presence of all the compounds under study (MTA, DMS, DMDS, DMTS) was observed, however this decrease was not significantly different from control (Fig. 12A). In the presence of Cd, higher concentrations of MTA and DMTS led to a decrease and a significant decrease, respectively, of the protein levels, while DMS and DMDS led to an increase of protein level (Fig. 12B).

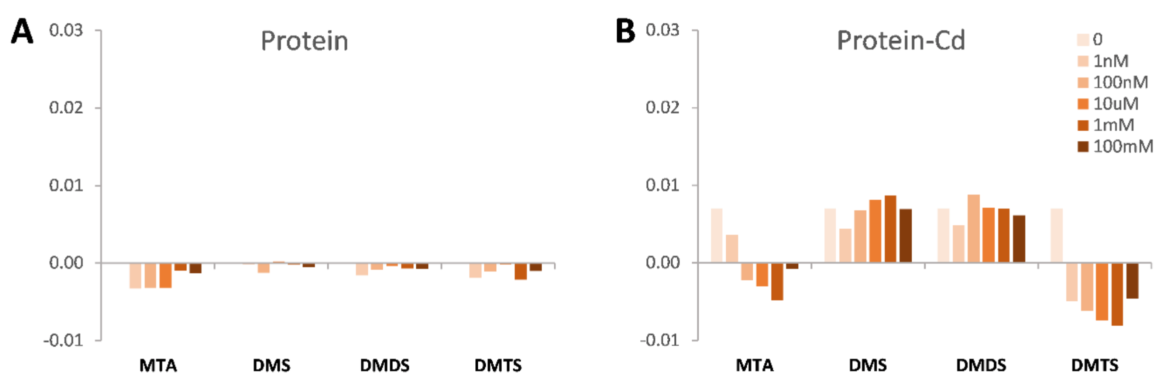


Figure 12 – Biotransformation activity and damage in *Rhizobium* cells exposed to sulfur compounds in the absence or presence of Cd. Cells were simultaneously exposed to 6 concentrations (0nM, 1nM, 100nM, 10µM, 1mM and 100mM) of the sulfur compounds (MTA, DMS, DMDS, DMTS) in a total of 24 conditions. Biochemical results variation relatively to control. (A) protein content in the absence of Cd (Protein); (B) protein content in the presence of Cd (Protein-Cd). For mean values, standard errors and statistical significance see Supplementary Table S12.

3.2.6. Principal Coordinates (PCO)

PCO analysis of results in the absence of Cd (Fig. 13A) shows that the abscissa axis is responsible for 71.7% of the differences separating the compounds based on their different biochemical features. It is possible to observe that MTA and DMTS led to an increase of

cells PC and GSTs while the remaining compounds (DMS; DMDS) influenced more the membrane (LPO), protein level and SOD and GPx activity.

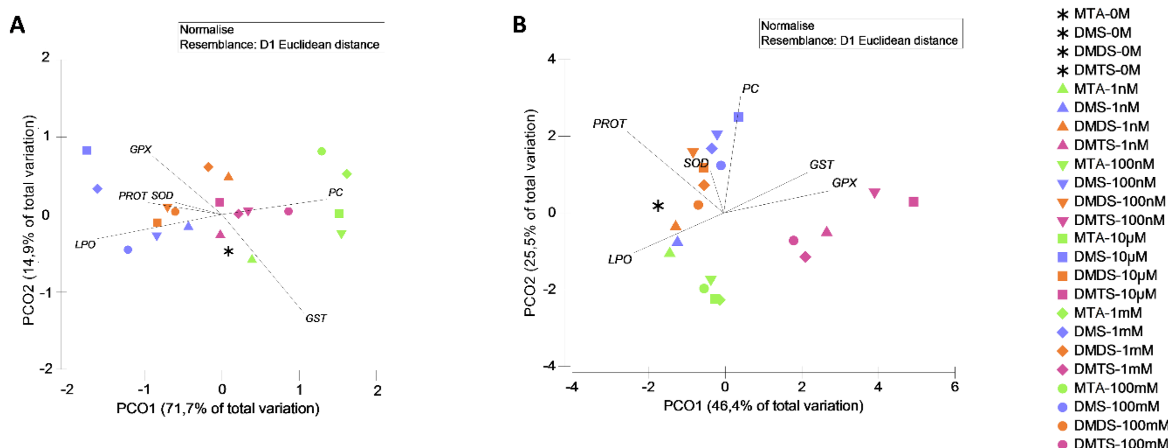


Figure 13 – Principal Coordinates with centroids ordination (PCO) of the biochemical determinants for each of the sulfur compounds. (A) Cells exposed to MTA, DMS, DMDS, DMTS and not to Cd; Pearson correlation vectors were imposed: lipid peroxidation (LPO); protein carboxylation (PC), superoxide dismutase activity (SOD); glutathione S-transferases activity (GSTs); glutathione peroxidase activity (GPx) and protein content (PROT) ($r \geqslant 0.30$). (B) Cells exposed to MTA, DMS, DMDS, DMTS and Cd; detailed color scheme in the figure. Pearson correlation vectors were imposed: LPO; PROT; SOD; GSTs and GPx activity ($r \geqslant 0.30$).

PCO analysis in the presence of Cd (Fig. 13B) showed that the abscissa axis was responsible for 46.4% of the differences separating the compounds based on their different biochemical features. However, cells exposed to Cd and higher concentrations of MTA (10 µM, 1 mM, 100 mM) have similar biochemical features to the control, with less oxidative mechanisms being activated. It was also possible to observe that the levels of LPO decreased with the growth of the number of sulfur atoms. When exposed to Cd the biochemical behavior of cells changed with the presence of the compound, with DMS and DMDS affecting SOD activity and PC while the main mechanisms that discriminate the influence of DMTS were the activity of GSTs and GPx.

3.3. Discussion

The presence of Cd in the soil decreased the diversity and number of microorganisms found in uncontaminated soils (Luo et al., 2019; Wu et al., 2018), demonstrating that this element influences growth and survival of soil microorganisms. Previous studies reported that in the presence of Cd *Rhizobium* displayed antioxidant responses (Corticeiro et al., 2006; Figueira et al., 2005). The present study is in line with those studies, with changes in *Rhizobium* growth and biochemical response also being observed as a consequence of exposure to Cd. However, other effects of Cd on bacterial cells remain to be elucidated. The ability to grow in metal contaminated environments that some bacteria present (Aiking et al., 1982; Bruins et al., 2000; Rajbanshi, 2009) may be related to different strategies. One mechanism could be the production of hydrogen sulfide leading to the formation of insoluble metal-sulfide complexes (Essa et al., 2012; Gadd and Griffiths, 1977). VSCs produced by bacteria were also capable of precipitating different heavy metals (Essa et al., 2012), and thus increase bacterial tolerance to metals (Gadd and Griffiths, 1977; Ramírez-Díaz et al., 2008).

The synthesis of the four VSCs studied was reported to be common in bacteria (Ali et al., 2015; Boden et al., 2011; Kelly et al., 2006; Lefebvre et al., 2007; Schulz and Dickschat, 2007). The metabolism of L-methionine, which increases by the degradation of oxidized proteins originates methanethiol (Helinck et al., 2000). Methanethiol is toxic to cells (Finkelstein and Benevenga, 1986), but can be converted to the less toxic compound methyl thioacetate (MTA) (Helinck et al., 2000). Methanethiol can also lead to DMS, DMDS and DMTS synthesis (Schulz and Dickschat, 2007). From the four VSCs studied, MTA had the lowest toxicity. DMS, DMDS and DMTS are toxic to a wide range of organisms, including fungi and bacteria (Bending and Lincoln, 1999) and thus display broad-spectrum antibiosis against fungal and bacterial pathogens (Ali et al., 2015), being DMDS even used as a soil fumigant (Ajwa et al., 2010). In the study by Cardoso et al. (2017) it was found that along an increase of Cd concentrations, the MTA peak area decreased while the remaining compounds analyzed (DMS, DMDS, DMTS) increased. Being DMS, DMDS and DMTS toxic why do Cd-exposed *Rhizobium* cells decrease MTA concentration and increase the levels of the other three VSCs? Cardoso et al. (2017) proposed this variation of VSCs concentration as a detoxification mechanism of methanethiol, leading to a decrease of reactive oxygen species (ROS) concentration and consequently Cd stressed cells with lower

lipid peroxidation and protein carbonylation, which however was not proven.

The aim of this paper was not only to prove the possible antioxidant role that these VSCs may have as a mechanism to reduce Cd damage in *Rhizobium* cells, but to further unveil the role that these compounds may play in Cd tolerance. According to Schulz and Dickschat (2007), methanethiol can react with hydrogen sulfide (H_2S) forming dimethyl sulfide, disulfide and trisulfide, a reaction involving hydroxyl radicals and ascorbate (Chin and Lindsay, 1994). Moreover, Guan et al. (2017) reported DMS to scavenge ROS via its thiomethyl groups. Accordingly, DMDS and DMTS may also exhibit the same activity as they also have thiomethyl groups, evidencing ROS scavenging as a cause of VSCs formation and an effect of VSCs antioxidant properties (Carrión et al., 2015).

Exposure of non-stressed *Rhizobium* cells to these compounds allowed us to assess their influence on cell metabolism (soluble protein levels), their toxicity on cell structures such as membranes (LPO) and proteins (PC), as well as their ability to induce mechanisms of cellular hemostasis maintenance, such as the activity of antioxidant and biotransformation enzymes (GSTs, GPx, SOD). The results showed that the four VSCs had little influence on cell metabolism and homeostasis with few significant changes compared to control (cells not exposed to VSCs), demonstrating a small influence of these compounds on cell growth and contradicting the reported toxic effects (Ajwa et al., 2010; Ali et al., 2015; Bending and Lincoln, 1999).

In the presence of Cd, the influence of VSCs on cell metabolism is higher. Results showed that MTA was able to combat the toxicity caused by Cd and approximated the levels of the endpoints determined to values close to cells growing under control (no Cd and no airborne exposure to VSCs). The antioxidant effect of MTA in Cd stressed cells was supported by the decrease in antioxidant (SOD, GPx) and biotransformation (GSTs) activities, which proved to be effective since lower damage (LPO and PC) and protein content were observed compared to sole exposure to Cd. The other three VSCs showed a more powerful antioxidant effect, leading to LPO levels even lower than control, decreasing PC (DMDS and DMTS) relatively to sole exposure to Cd, and inducing more effectively the activity of antioxidant and biotransformation enzymes such as GPx and GSTs. The consumption of hydroxyl radicals during the formation of DMS, DMDS and DMTS and their own scavenging activity as mentioned earlier may also have contributed to the lower oxidative effects, and in combination with the stimulation of antioxidant enzymes activity

may explain why cells switch from producing MTA from methanethiol to DMS, DMDS and DMTS.

The increase in antioxidant enzymes in cells when exposed to a stress such as Cd indicates that cells are sensitive to the original stress inducing oxidative stress and damage (El-rab and Abskharon, 2013). Thus, the most efficient mechanisms are those that prevent the build-up of oxidative stress (Corticeiro et al., 2006). According to Kamyabi et al. (2018) and Essa et al. (2012) DMDS forms complexes with Cd, being Cd-S bonds the main interactions in the complexes (Kamyabi et al., 2018). The reduction of free Cd ions in the cytoplasm, will decrease Cd displacement of essential metals in many metalloenzymes, inactivation of enzymes that act via sulfhydryl groups (Lehninger, 1970; Santos et al., 2016; Torres et al., 2000) and generation of ROS. This lower interference with cellular metabolism may explain the lower cellular damage (LPO and PC) observed in cells stressed by Cd and simultaneously exposed to DMDS. The identical effects observed for DMS may come from a similar mechanism, as Kamyabi et al. (2018) detected the formation of Cd complexes with VSCs with only one S atom, such as DMS, although with lower capacity to chelate metal ions, which was explained by the bonding efficiency being proportional to the number of S ions present. Admitting that this assumption is correct, then DMTS (with three S atoms) would complex more efficiently Cd ions than DMS and DMDS, and therefore would have a higher protective effect from the damage caused by Cd in cells, which is actually supported by the results of the present study.

Previous studies showed that one of the main mechanisms of Cd tolerance in the *Rhizobium* strain used in this study (E20-8) was the use of most of the cells GSH to form Cd-bisglutathionate complexes (Cardoso et al., 2018b; Lima et al., 2006). The formation of Cd complexes with VSCs may decrease GSH expenditure in controlling Cd toxicity. Being GSH the major redox scavenging molecule in bacterial cells (Kabil and Banerjee, 2010; Riccillo et al., 2000), the lower use of GSH in Cd complexation may allow for higher GSH concentrations in cells, leading to lower oxidative stress and higher activity of enzymes that depend on GSH concentration to maintain full activity, such as GPx and GSTs. PCO analysis evidence GSTs and GPx activity as the biochemical endpoints that were most correlated with DMTS in Cd exposed cells. In fact, while induction of the activity of these two enzymes is observed in Cd-stressed cells exposed to DMS, DMDS, and DMTS, the increase in activity of these two enzymes is much more influenced by DMTS than by the other two VSCs.

3.4. Concluding remarks

The present study showed that in the absence of stress, VSCs had little influence on cell metabolism and homeostasis, evidencing the small influence of these compounds on cell growth. In the presence of Cd stress, although growth was not very affected, VSCs minimized Cd toxicity towards cells by reducing oxidative stress. The transformation of methanethiol into DMDS and especially DMTS, that occurs in cells exposed to Cd, created conditions for the reduction of free Cd ions in the cytosol, which protects cells from the damage caused by Cd. The decrease in ROS concentration caused by the lower concentration of free Cd ions in cells, by the formation of VSCs and by the antioxidant activity of these VSCs contributed to protect membranes and proteins. Higher activity of GSTs and GPx reduced more rapidly the products derived from lipid-based hydroperoxide metabolism, further reducing LPO. The sum of all these processes may decrease the cellular damage caused by Cd and produce an unexpected effect of lower LPO levels compared to control (cells not exposed to Cd or VSCs). Airborne exposure to VSCs did not appear to affect soil bacteria in the concentration range (nM to mM) used in this study, reduces and even appeared to have beneficial effects when bacteria are in metal-induced stress conditions.

Chapter 4 – Final remarks and future work

The increase of soil toxicity through anthropogenic activities, specifically the increase of Cd, leads to a degradation of the soil and its biodiversity. The study of different survival and warning mechanisms by microorganisms, such as VOCs, may lead to a better understanding of the functioning of soil microbial communities and the changes they are subjected to. The elucidation of these mechanisms allows to understand and anticipate the dynamics and changes that occur in these communities both in undisturbed and stressed conditions and may provide tools to minimize the impact of stressors and to restore soil functionality. In this study we verified that each compound had a different influence on *Rhizobium* both in the presence and absence of Cd. Clear positive effects were observed for α-pinene, eucalyptol, MTA and DMTS in the absence of Cd. In the presence of Cd positive effects were observed in the presence of limonene, eucalyptol, DMDS and DMTS. However, the modes of action differed. Most of monoterpenes tested increased membrane damage, whereas sulfur compounds were able to protect membranes from Cd toxicity. The decrease in stress by the cells is related to the uptake of free ions of Cd by sulfur compounds. The positive influence of monoterpenes was attributed to activation of the metabolism and the antioxidant response. VSCs were reported to act in different ways, complexing Cd ions, scavenging ROS and increasing the activity of GSH-dependent enzymes. Altogether, the results obtained in this thesis allowed to identify not only new strategies to combat stress, but also to evidence the interconnection between different processes occurring in a cell and that contribute to a microorganism's overall tolerance to stress.

Given the different influences that MVOCs have on bacterial cells, the large number of these metabolites and the limited knowledge of their influence on bacterial cells (both in those synthesizing or receiving the compounds), there is still much to unravel on the role that MVOCs may play in the communication and relations among individuals of the same microbial community, and how this dynamic changes when conditions shift, whether being nutrient deficiency and starvation, predation, interaction with plants, drought or contaminants. The importance of MVOCs in the functioning and stabilization of soil

microbial communities as well as their interaction with the surrounding environment is in its infancy, but results reported so far point that VOCs may play an important role and future studies should include new MVOCs, other microbial species and other constraints. This information can allow interventions in different areas such as the restoration of impacted areas, the increase of food security (creation of new agricultural practices for pest control, improved nutrient absorption or plant growth promotion), or the control of bacterial multidrug resistance.

Chapter 5 – Experimental procedures

Rhizobium sp. strain E20-8 (partial 16S rRNA sequence Genbank accession number KY491644), isolated from the nodules of *Pisum sativum* from Elvas, Portugal, was used in the present study. Previous studies described E20-8 strain as tolerant to Cd (Cardoso et al., 2017; Corticeiro et al., 2013; Nunes et al., 2018).

5.1. Growth of E20-8

The isolate was cultured in Petri dishes with 90 mm diameter, 16.2 mm height containing yeast mannitol agar (YMA) medium (Somasegaran and Hoben, 1994) and incubated from 48 h (colonies were visible) until 96 h (stationary phase was reached) at 26 ºC. After checking the behaviour of the cultures, plates were placed at 4 ºC, inhibiting their proliferation.

5.2. Tolerance to cadmium

To determine the IC_{50} of E20-8 strain to Cd a screening was performed by growing in the YMA medium (Somasegaran and Hoben, 1994) with the following concentrations of Cd 0 µM, 50 µM, 100 µM, 150 µM, 200 µM, 300 µM, 400 µM and 600 µM.

5.2.1. Growth medium preparation

YMA medium was prepared according to Somasegaran and Hoben (1994), autoclaved 120 ºC for 20 min. Cd stock solution (0.1 M in 0.05% HNO_3 to prevent Cd precipitation) was sterilized by filtration (0.2 µM pore size) and was added to warm medium in order to obtain the concentrations tested: in the control acid nitric at the same concentration (0.05%) was added. Approximately 20 mL of medium was distributed per Petri dish.

5.2.2. Inoculation

Colonies of E20-8 in log growth (48 h growth) were used to inoculate control (no Cd) and Cd-containing Petri dishes. Inoculation was performed under aseptic conditions (laminar

flow chamber). Twelve colonies were streaked onto each dish and incubated for 96 h at 26 °C. Three independent experiments with 3 dishes each were performed in a total of 108 (12x3x3) colonies per condition.

5.2.3. Growth determination

Plates were photographed at 36, 60, 84 and 96 h of growth, and the colonies diameter measured. At least three independent replicates were performed. Inhibition of the growth was reported as % of inhibition and calculated for each concentration by subtracting the colony diameter at control by the diameter at each concentration and dividing the obtained difference by the colony diameter at control and multiplying the result by 100.

5.2.4. Calculation of IC_{50}

In order to determine the Cd concentration causing 50% inhibition of E20-8 growth values were graphed, and the equation relating Cd concentration and growth determined. The concentration of Cd inhibiting growth by 50% (IC_{50}) was calculated to be 107.54 µM. Consequently, in further work 100 µM Cd was used.

5.3. Influence of the VOCs in the growth of E20-8 in the presence of Cd

5.3.1. Experimental conditions

To evaluate the influence of VOCs in the growth of E20-8 at control (no Cd) and exposed to Cd (IC_{50}=100 µM) the bacterium was grown in YMA supplemented or not with Cd, and exposed to different concentrations of VOCs (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM). The VOCs tested, α-pinene (CAS: 7785-26-4), limonene (CAS: 138-86-3), linalool (CAS: 78-70-6), eucalyptol (CAS: 470-82-6), menthol (CAS: 2216-51-5), methyl thioacetate (CAS: 1534-08-3), dimethyl sulfide (CAS: 75-18-3), dimethyl disulfide (CAS: 624-92-0) and dimethyl trisulfide (CAS: 3658-80-8) were purchased from Sigma.

In order to ensure that the influence on bacterial growth was of a volatile nature, center-divided Petri dishes were used (90 mm diameter, 16.2 mm height, with a septum of

10.0 mm height creating two 2 divisions that share the dish atmosphere). In one side of the plate the YMA medium (supplemented or not with Cd) was inoculated with 18 colonies, while the other side contained a thin layer of the same medium and a sterile paper disc receiving 10 µL of VOC solution (Fig. 14). VOCs used in this study were dissolved in 70% ethanol. Solvent (70% ethanol) was applied in a volume equal to that applied to the disks (10 µL) as control, since it did not influence bacterial growth compared to dishes where no solvent was applied. After inoculation, dishes were incubated 60 h at 26 °C.

Three replicates were performed for each condition. At the end of the incubation period, colonies were collected. All colonies of a plate were considered as a sample. After determining the weight of the pooled colonies, they were stored at -80 °C for further analyses. Growth change compared to control was calculated for each VOC concentration.

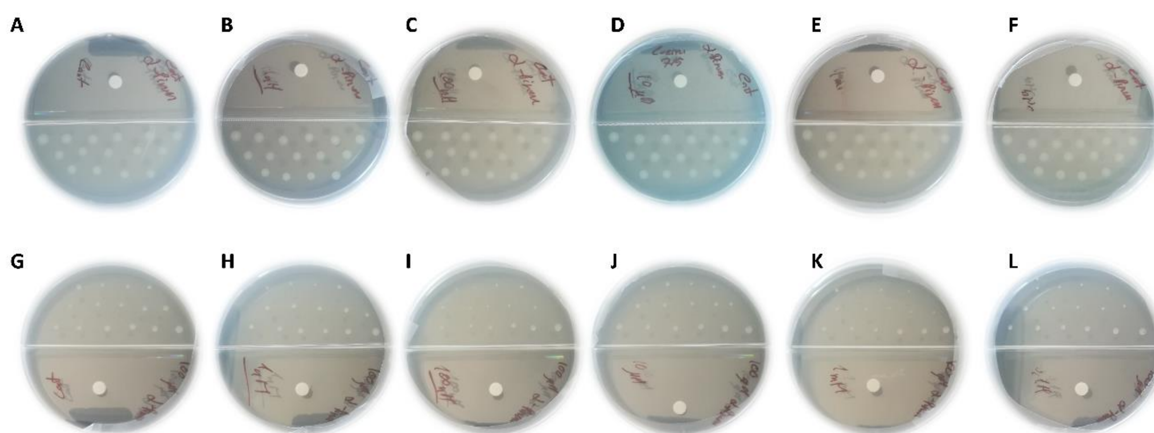


Figure 14 – Growth of *Rhizobium* strain E20-8 in the absence (A to F) and presence (G to L) of Cd when exposed to α-pinene concentration 0 nM (A, G), 1 nM (B, H), 100 nM (C, I), 10 µM (D, J), 1 mM (E, K), 100 mM (F, L), during 96 hours.

5.4. Influence of VOCs in E20-8 biochemistry at 0 and 100 µM Cd

5.4.1 Extraction

To each sample, extraction buffer (potassium phosphate 50 mM, pH 7.0) was added (300 µL to samples <0.02 g, 600 µL to samples ≥0.02 g). Samples were sonicated during 60 s, and centrifuged at 10,000 *g* for 10 minutes at 4 °C. The supernatant was collected to a new microtube and stored at -30 ºC or used immediately. Results were expressed per g of colony

(supplementary tables).

5.4.2. Protein content

The amount of protein was determined using the Biuret method (Robinson and Hogden, 1940). In a microplate, 275 µL of Biuret reagent was added to 25 µL of sample. Absorbance was read at 540 nm. Bovine serum albumin (BSA) was used as standard. Results were expressed in mg protein per g of colony.

5.4.3. Lipid peroxidation

Lipid peroxidation (LPO) was determined according to the protocol described by Buege and Aust (Buege and Aust, 1978), and based on the quantification of thiobarbituric acid reactive substances (TBARS), originated through the reaction of 2-thiobarburoric acid (TBA) with lipid peroxidation products such as malondialdehyde (MDA). In a microtube 112 µL of 20% trichloroacetic acid and 150 µL of 0.5% thiobarbituric acid (in 20% trichloroacetic acid) were added to 38 µL of sample. A blank containing 150 µL of 20% trichloroacetic acid and 150 µL of 0.5% thiobarbituric acid was also prepared. Samples and blank were incubated at 96 °C for 25 minutes. Tubes were cooled in ice. The amount of TBARS on the samples was measured spectrophotometrically at 532 nm and nmol of MDA equivalents per g of colony were calculated using MDA molar extinction coefficient 1.56×10^5 $M^{-1}cm^{-1}$.

5.4.4. Protein carbonylation

Protein carbonylation (PC) was measured by quantification of carbonyl groups (CG), according to the 2,4-dinitrophenylhydrazine (DNPH) alkaline method described by Mesquita et al. (2014), with modifications (Udenigwe et al., 2016). The amount of CG was determinate spectrophotometrically at 450 nm (22,308 mM^{-1} cm^{-1} extinction coefficient). Results were expressed in mU of CG per gram of colony (µmol g^{-1}).

5.4.5. Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined by quantification of nitroblue

tetrazolium (NBT) diformazan formed by the reaction of NBT with superoxide radicals as described by Beauchamp and Fridovich (1971). In microplates, 25 µL of supernatant was mixed with 250 µL reaction buffer (50 mM Tris-HCl (pH 8), 0.1 mM diethylenetriaminepentaacetic acid (DTPA), 0.1 mM hypoxanthine), 68.4 µM (NBT) and 25 µL xanthine oxidase (56.4 mU/mL). The samples absorbance was read at 560 nm after 20 minutes. One unit was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical (Sun et al., 1988). Results were expressed in U per mg of colony.

5.4.6. Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was determined according to the method described by Paglia and Valentine's (1967). In a microplate, for a final volume of 300 µL, 30 µL of supernatant, 112.5 µL dilution buffer, 60 µL GSH (5 mM), 45 µL cumene hydroperoxide (2 mM), 30 µL glutathione reductase (25 U/ml) and 22.5 µl NADPH (2 mM) were mixed. The absorbance was immediately read at 340 nm, with continuous reading at 15 s intervals over 20 minutes. To determine the activity of GPx the molar extinction coefficient 0.00622 μM^{-1} cm^{-1} was used. Results were expressed in U per g of colony.

5.4.7. Glutathione-S-transferases activity

Glutathione-S-transferases (GSTs) activity was determined according to the method described by Habig et al. (1974), by using 1-Chloro 2,4 dinitrobenzene (CDNB) and reduced glutathione (GSH) as co-substrates. In a microplate, 100 µL of sample supernatant was mixed with 200 µL reaction buffer (0.1 M potassium phosphate (pH 6.5), 10 mM GSH, 60 mM CDNB). The absorbance was immediately read at 340 nm with continuous reading at 15 s intervals during 20 minutes. To determine the activity of GSTs the molar extinction coefficient 9.6 mM^{-1} cm^{-1} was used. Results were expressed in mU per g of colony.

5.3.4. Statistical analyses

The data obtained from the growth and the biochemical analysis were subjected to Monte Carlo tests with 9999 number of permutations using the PRIMER 6 & PERMANOVA+

(Anderson, 2017; Anderson and Walsh, 2013). Significant differences were considered for $p \leq 0.05$, being identified in figures and/or supplementary tables with different lowercase (control), uppercase (Cd) letters and asterisk (between conditions for the same concentration of compounds). In order to analyze if the global biochemical response of *Rhizobium* was influenced by the compounds in the presence and absence of Cd, the data (fourth root transformed, normalized and after resemblance matrix calculation (Euclidean distance)) were submitted to an ordering analysis performed by Principal Coordinates (PCO), using the PRIMER 6 & PERMANOVA+. Biochemical data were also analyzed with MetaboAnalyst 4.0 (data was autoscaled) (Chong et al., 2019, 2018) and heatmaps built for the monoterpene compounds.

Chapter 6 – Bibliography

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Annex

Supplemental files

Chapter 2 - Supplemental files

Supplementary Table S1 – Growth, protein content, antioxidant and biotransformation (SOD, GPX, GSTs) activity and damage (LPO) in *Rhizobium* cells exposed to Cd and α-pinene. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) of α-pinene. Values are means of at least 3 replicates ± standard error; different lowercase letters indicate significant differences among α-pinene concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among α-pinene concentrations in Cd condition, bold values indicate significant differences from their control (no Cd or Cd) and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. Considered significantly different values of $p < 0.05$.

α-pinene	Growth (mg/colony)	Protein (mg/g)	LPO (nmol/g)	SOD (U/mg)	GPx (U/g)	GSTs mU/g)
Control	1.93 ±0.11^{a*}	211.34 ±21.00^{a*}	2.04 ±0.56^{a}	0.29 ±0.00^{a*}	9.04 ±2.59^{a}	5.20 ±1.06^{a}
Control 1nM	2.02 ±0.44^{ab*}	241.54 ±67.75^{a}	2.16 ±0.65^{a}	**0.22 ±0.02**^{b*}	10.02 ±2.02^{a}	3.63 ±0.41^{ab}
Control 100nM	2.35 ±0.30^{ab*}	178.77 ±27.12^{ab}	1.84 ±0.81^{a}	0.27 ±0.03^{ab*}	14.32 ±4.16^{ab}	**3.15 ±0.18**^{b}
Control 10µM	2.64 ±0.28^{ab*}	**154.78 ±19.65**^{b*}	1.88 ±0.39^{a*}	0.23 ±0.03^{ab*}	13.91 ±2.16^{ab}	3.90 ±1.38^{ab}
Control 1mM	**2.73 ±0.20**^{b*}	167.66 ±20.07^{ab*}	2.22 ±0.38^{a}	0.23 ±0.02^{ab*}	10.09 ±2.48^{a}	**3.29 ±0.52**^{b}
Control 100mM	2.39 ±0.17^{ab*}	**159.95 ±26.90**^{b*}	1.92 ±0.27^{a}	**0.23 ±0.01**^{b*}	**40.66 ±10.30**^{b}	**2.96 ±0.54**^{b}
Cd	0.87 ±0.07^{A*}	359.86 ±30.24^{A*}	2.77 ±0.49^{A}	0.69 ±0.05^{A*}	13.29 ±3.15^{A}	5.80 ±1.03^{A}
Cd 1nM	0.64 ±0.23^{A*}	365.11 ±9.83^{A}	6.90 ±3.81^{A}	0.59 ±0.07$^{A.B*}$	12.08 ±2.66^{A}	5.41 ±0.54^{A}
Cd 100nM	0.83 ±0.33^{A*}	409.50 ±104.12$^{A.B}$	6.48 ±5.35^{A}	**0.39 ±0.04**$^{B.C*}$	10.21 ±2.76^{A}	5.29 ±1.58^{A}
Cd 10µM	0.94 ±0.16^{A*}	431.34 ±76.17$^{A.B*}$	**11.63 ±1.94**^{B*}	**0.40 ±0.06**^{B*}	8.50 ±1.89^{A}	4.25 ±0.47^{A}
Cd 1mM	0.81 ±0.18^{A*}	427.17 ±93.86$^{A.B*}$	**26.53 ±10.28**^{B}	**0.44 ±0.01**^{B*}	12.89 ±4.80$^{A.B}$	4.83 ±1.06^{A}
Cd 100mM	0.62 ±0.03^{A*}	**536.43 ±14.66**^{B*}	**28.03 ±10.45**^{B}	**0.37 ±0.01**^{C*}	**28.43 ±3.43**^{B}	6.37 ±1.23^{A}

Supplementary Table S2 – Growth, protein content, antioxidant and biotransformation (SOD, GPX, GSTs) activity and damage (LPO) in *Rhizobium* cells exposed to Cd and limonene. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) of limonene. Values are means of at least 3 replicates ± standard error; different lowercase letters indicate significant differences among limonene concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among limonene concentrations in Cd condition, bold values indicate significant differences from their control (no Cd or Cd) and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. Considered significantly different values of $p < 0.05$.

Limonene	Growth (mg/colony)	Protein (mg/g)	LPO (nmol/g)	SOD (U/mg)	GPx (U/g)	GSTs (mU/g)
Control	4.45 ±0.15^{a*}	211.34 ±9.24^{a*}	2.04 ±0.08^{a}	0.29 ±0.05^{a*}	9.04 ±0.78^{a}	5.20 ±1.31^{a}
Control 1nM	4.23 ±0.57^{a*}	240.88 ±30.38^{a}	**1.70 ±0.05^{b}**	**0.35 ±0.11^{b*}**	7.43 ±0.49^{a*}	6.84 ±1.27^{ab}
Control 100nM	4.02 ±0.47^{a*}	244.50 ±25.92^{a*}	1.81 ±0.27^{a}	**0.43 ±0.09^{b*}**	**14.76 ±2.46^{b*}**	7.05 ±1.07^{ab}
Control 10µM	4.42 ±0.45^{a*}	220.43 ±18.88^{a*}	2.59 ±0.51^{ab}	**0.39 ±0.03^{b}**	**14.50 ±0.93^{b}**	**7.61 ±0.27^{b*}**
Control 1mM	4.31 ±0.65^{a*}	234.22 ±28.82^{a}	**3.49 ±0.89^{b}**	**0.38 ±0.05^{b}**	5.82 ±2.32^{a}	**7.20 ±0.00^{b*}**
Control 100mM	3.78 ±0.43^{a*}	258.86 ±29.26^{a*}	**3.35 ±1.66^{b}**	**0.49 ±0.06^{b}**	8.92 ±2.41^{ab}	5.93 ±0.13^{a}
Cd	1.05 ±0.11^{A*}	359.86 ±38.36^{A*}	2.77 ±0.66^{A}	0.69 ±0.03^{A*}	13.29 ±7.96^{A}	5.80 ±0.52^{A}
Cd 1nM	1.00 ±0.17^{A*}	396.90 ±71.61^{AB}	1.91 ±0.19^{A}	**0.93 ±0.03^{B*}**	**31.32 ±5.19^{B*}**	6.26 ±0.65^{A}
Cd 100nM	0.89 ±0.08^{A*}	**428.43 ±44.39^{B*}**	3.04 ±1.01^{A}	0.74 ±0.03^{A*}	**32.61 ±1.44^{B*}**	**7.76 ±1.57^{B}**
Cd 10µM	1.55 ±0.43^{AB*}	**427.17 ±49.66^{B*}**	2.33 ±0.93^{A}	**0.37 ±0.04^{C}**	16.14 ±0.88^{A}	5.53 ±0.18^{A*}
Cd 1mM	**2.19 ±0.40^{B*}**	362.27 ±54.85^{A}	2.70 ±1.81^{A}	**0.40 ±0.09^{C}**	13.62 ±8.07^{A}	3.66 ±0.52^{A*}
Cd 100mM	**1.98 ±0.15^{B*}**	**470.46 ±17.55^{B*}**	3.07 ±1.98^{A}	**0.44 ±0.05^{C}**	18.34 ±5.36^{A}	**7.03 ±0.57^{B}**

Supplementary Table S3 – Growth, protein content, antioxidant and biotransformation (SOD, GPX, GSTs) activity and damage (LPO) in *Rhizobium* cells exposed to Cd and linalool. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) of linalool. Values are means of at least 3 replicates ± standard error; different lowercase letters indicate significant differences among linalool concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among linalool concentrations in Cd condition, bold values indicate significant differences from their control (no Cd or Cd) and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. Considered significantly different values of $p < 0.05$.

Linalool	Growth (mg/colony)	Protein (mg/g)	LPO (nmol/g)	SOD (U/mg)	GPx (U/g)	GSTs (mU/g)
Control	3.73 ±0.43^{a*}	211.34 ±25.80^{a*}	2.04 ±0.49^{a}	0.29 ±0.03^{a*}	9.04 ±1.60^{ac}	5.20 ±0.31^{a}
Control 1nM	2.54 ±0.26^{a*}	186.41 ±27.76^{a*}	**4.19 ±0.73^{b}**	0.31 ±0.08^{a*}	**3.82 ±0.92^{b*}**	**6.93 ±0.65^{b}**
Control 100nM	3.40 ±0.34^{a}	216.85 ±22.23^{a*}	**3.78 ±0.49^{b*}**	0.34 ±0.04^{a*}	5.15 ±0.49$^{a.b}$	**7.32 ±0.35^{b}**
Control 10µM	3.41 ±0.45^{a*}	230.93 ±38.02^{a}	**3.74 ±0.71^{b}**	0.41 ±0.08^{a}	**3.14 ±0.62^{b}**	**7.38 ±0.26^{b}**
Control 1mM	3.34 ±0.11^{a*}	217.60 ±7.60^{a*}	3.30 ±0.70^{ab}	0.32 ±0.00^{a}	7.69 ±2.01^{ab*}	**8.07 ±0.41^{b}**
Control 100mM	2.95 ±0.21^{a}	244.38 ±14.76^{a*}	**4.18 ±0.14^{b}**	0.41 ±0.04^{a*}	9.76 ±0.89^{a*}	**7.34 ±0.21^{b}**
Cd	2.36 ±0.25^{AB*}	359.86 ±38.64^{A*}	2.77 ±0.61^{A}	0.69 ±0.03^{A*}	13.29 ±0.16^{A}	5.80 ±0.39^{A}
Cd 1nM	2.28 ±0.11^{AB*}	405.56 ±20.9^{AB*}	2.02 ±0.33^{AB}	0.71 ±0.08^{A*}	17.73 ±4.53^{A*}	6.68 ±0.25^{AB}
Cd 100nM	2.58 ±0.08^{A}	330.95 ±8.06^{A*}	**1.70 ±0.26^{B*}**	0.56 ±0.03^{A*}	17.92 ±4.68^{A}	6.66 ±0.28^{AB}
Cd 10µM	2.53 ±0.50^{AB*}	362.56 ±77.88^{A}	**4.97 ±0.40^{C}**	0.71 ±0.14^{A}	12.84 ±5.01^{A}	6.68 ±0.76^{AB}
Cd 1mM	2.25 ±0.10^{AB*}	372.80 ±17.53^{A*}	**4.13 ±0.64^{C}**	1.06 ±0.34^{A}	**43.31 ±5.39^{B*}**	**8.04 ±0.60^{B}**
Cd 100mM	**1.46 ±0.20^{B}**	**458.42 ±50.70^{B*}**	**13.31 ±10.70^{C}**	1.17 ±0.26^{A*}	**36.50 ±4.43^{B*}**	**8.82 ±0.83^{B}**

Supplementary Table S4 – **Growth, protein content, antioxidant and biotransformation (SOD, GPX, GSTs) activity and damage (LPO) in *Rhizobium* cells exposed to Cd and eucalyptol.** Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) of eucalyptol. Values are means of at least 3 replicates ± standard error; different lowercase letters indicate significant differences among eucalyptol concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among eucalyptol concentrations in Cd condition, bold values indicate significant differences from their control (no Cd or Cd) and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. Considered significantly different values of $p < 0.05$.

Eucalyptol	Growth (mg/colony)	Protein (mg/g)	LPO (nmol/g)	SOD (U/mg)	GPx (U/g)	GSTs (mU/g)
Control	4.25 ±0.49^{a*}	211.34 ±24.39^{a}	2.04 ±0.44^{a}	0.29 ±0.01^{a*}	9.04 ±0.00^{a*}	5.20 ±1.12^{ab}
Control 1nM	4.59 ±0.13^{a}	187.42 ±8.11^{a}	2.64 ±0.81^{a}	**0.26 ±0.01^{b}**	7.61 ±2.34^{ab}	4.22 ±1.12^{ab}
Control 100nM	4.51 ±0.74^{a}	198.82 ±23.58^{ab}	1.81 ±0.68^{a}	0.27 ±0.06^{ab*}	9.51 ±0.83^{a}	2.66 ±0.40^{a*}
Control 10µM	4.64 ±0.50^{a}	184.86 ±14.72^{ab}	2.19 ±0.50^{a}	0.24 ±0.03^{ab*}	8.85 ±2.38^{ab}	2.20 ±0.39^{a*}
Control 1mM	5.16 ±0.31^{a*}	**166.51 ±2.96^{b*}**	1.70 ±0.36^{a}	**0.23 ±0.02^{b*}**	**6.35 ±1.54^{b}**	3.86 ±0.82^{ab*}
Control 100mM	5.28 ±0.86^{a*}	177.75 ±27.45^{ab}	1.44 ±0.37^{a}	0.30 ±0.05^{ab}	8.19 ±1.36^{a}	**3.86 ±0.07^{b*}**
Cd	2.20 ±0.42^{A*}	359.86 ±80.90^{A}	2.77 ±1.33^{A}	0.69 ±0.10^{A*}	13.29 ±0.79^{A*}	5.80 ±0.94^{A}
Cd 1nM	2.68 ±0.81^{AB}	**202.54 ±42.76^{B}**	3.56 ±2.31^{AB}	**0.36 ±0.04^{B}**	**3.63 ±0.13^{B}**	5.37 ±1.02^{A}
Cd 100nM	**2.89 ±0.07^{B}**	**253.83 ±6.32^{B}**	3.68 ±2.31^{AB}	**0.50 ±0.04^{C*}**	**5.64 ±1.31^{B}**	5.25 ±0.23^{A*}
Cd 10µM	2.72 ±0.80^{AB}	242.23 ±32.10^{AB}	3.42 ±1.17^{AB}	**0.52 ±0.05^{C*}**	**5.10 ±1.38^{B}**	5.68 ±0.67^{A*}
Cd 1mM	3.00 ±0.43^{AB*}	254.00 ±31.48^{AB*}	**5.65 ±2.03^{B}**	0.58 ±0.05^{AC*}	**2.35 ±0.23^{C}**	**10.87 ±0.08^{B*}**
Cd 100mM	2.95 ±0.99^{AB*}	249.01 ±44.77^{AB}	**5.97 ±1.89^{B}**	0.79 ±0.31^{A}	11.72 ±1.33^{A}	**12.59 ±0.58^{C*}**

Supplementary Table S5 – Growth, protein content, antioxidant and biotransformation (SOD, GPX, GSTs) activity and damage (LPO) in *Rhizobium* cells exposed to Cd and menthol. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) of menthol. Values are means of at least 3 replicates ± standard error; different lowercase letters indicate significant differences among menthol concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among menthol concentrations in Cd condition, bold values indicate significant differences from their control (no Cd or Cd) and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. Considered significantly different values of $p < 0.05$.

Menthol	Growth (mg/colony)	Protein (mg/g)	LPO (nmol/g)	SOD (U/mg)	GPx (U/g)	GSTs (mU/g)
Control	4.31 ±0.07^{a*}	211.34 ±5.17^{a*}	2.04 ±0.20^{a}	0.29 ±0.01^{a*}	9.04 ±1.82^{a}	5.20 ±0.39^{a}
Control 1nM	4.24 ±0.49^{a*}	219.81 ±30.74^{a*}	1.79 ±0.38^{a*}	0.25 ±0.08^{a*}	7.60 ±0.75^{a}	**3.59 ±0.38^{b*}**
Control 100nM	3.99 ±0.08^{a*}	**280.59 ±27.48^{c}**	**0.98 ±0.13^{b*}**	**0.32 ±0.03^{ab*}**	8.14 ±0.09^{a*}	3.67 ±0.93^{ab*}
Control 10µM	3.94 ±0.18^{a*}	**332.42 ±5.85^{b}**	**1.41 ±0.19^{b}**	0.28 ±0.02^{a}	9.10 ±1.05^{a}	**2.88 ±0.54^{b*}**
Control 1mM	3.55 ±0.36^{ab*}	**333.37 ±62.07^{bc}**	2.68 ±1.25^{a}	**0.38 ±0.03^{b*}**	**4.48 ±0.34^{b}**	**6.73 ±0.31^{c*}**
Control 100mM	**3.13 ±0.12^{b*}**	**302.14 ±0.26^{c}**	2.47 ±0.73^{a*}	**0.38 ±0.03^{b*}**	7.43 ±1.02^{ab*}	6.20 ±0.95^{ac}
Cd	1.9 ±0.14^{A*}	359.86 ±27.10^{AB*}	2.77 ±0.47^{A}	0.69 ±0.06^{A*}	13.29 ±0.67^{A}	5.80 ±1.21^{A}
Cd 1nM	1.79 ±0.12^{A*}	**383.49 ±8.24^{A*}**	**6.07 ±0.50^{B*}**	0.66 ±0.02^{A*}	11.48 ±2.85^{A}	6.03 ±0.64^{AB*}
Cd 100nM	2.11 ±0.32^{A*}	350.28 ±48.53^{AB}	**10.84 ±0.75^{C*}**	0.68 ±0.09^{A*}	13.67 ±1.44^{A*}	**7.00 ±0.56^{B*}**
Cd 10µM	2.34 ±0.68^{A*}	352.95 ±83.40^{AB}	**6.81 ±2.70^{BC}**	0.65 ±0.14^{A}	10.59 ±1.77^{A}	**6.67 ±0.42^{B*}**
Cd 1mM	2.06 ±0.19^{A*}	**339.21 ±10.72^{B}**	3.66 ±0.66^{A}	0.68 ±0.08^{A*}	13.62 ±2.19^{A}	5.26 ±0.08^{A*}
Cd 100mM	1.60 ±0.23^{A*}	355.05 ±188.30^{AB}	**24.96 ±2.92^{D*}**	0.86 ±0.12^{A*}	16.64 ±2.44^{A*}	**8.14 ±0.93^{B}**

Chapter 3 - Supplemental files

Supplementary Table S6 – Growth, in *Rhizobium* cells exposed to Cd and MTA; DMS; DMDS; DMTS. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) of sulfur compounds. Values are means of at least 3 replicates ± standard error; different lowercase letters indicate significant differences among sulfur compound concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among sulfur compound concentrations in Cd condition, bold values indicate significant differences from their control (no Cd or Cd) and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. Considered significantly different values of $p < 0.05$.

Growth (mg/colony)	MTA	DMS	DMDS	DMTS
Control	3.12 ±0.21^{a*}	3.12 ±0.21^{ab*}	3.12 ±0.21^{ab*}	3.12 ±0.21^{a*}
Control 1nM	3.68 ±0.44^{abc*}	3.11 ±0.45^{ab*}	3.27 ±0.15^{abc*}	3.08 ±0.09^{a*}
Control 100nM	**4.07 ±0.28^{b*}**	3.55 ±0.10^{b*}	3.14 ±0.11^{ab*}	2.91 ±0.14^{a*}
Control 10µM	3.56 ±0.12^{ab*}	3.27 ±0.02^{b*}	3.02 ±0.03^{b*}	2.93 ±0.23^{a*}
Control 1mM	**2.59 ±0.07^{c*}**	2.73 ±0.10^{a*}	**3.60 ±0.02^{c*}**	3.20 ±0.15^{a*}
Control 100mM	**2.40 ±0.22^{c*}**	3.11 ±0.08^{ab*}	3.38 ±0.09^{abc*}	3.01 ±0.18^{a*}
Cd	0.37 ±0.09^{A*}	0.37 ±0.09^{A*}	0.37 ±0.09^{A*}	0.37 ±0.09^{A*}
Cd 1nM	0.20 ±0.06^{A*}	0.47 ±0.05^{A*}	0.44 ±0.12^{A*}	0.39 ±0.05^{AB*}
Cd 100nM	0.40 ±0.06^{AB*}	0.30 ±0.07^{A*}	0.42 ±0.04^{A*}	0.32 ±0.06^{AB*}
Cd 10µM	0.32 ±0.05^{A*}	0.38 ±0.07^{A*}	0.36 ±0.05^{A*}	0.47 ±0.05^{AB*}
Cd 1mM	**0.78 ±0.08^{B*}**	0.37 ±0.05^{A*}	0.29 ±0.00^{A*}	**0.66 ±0.08^{B*}**
Cd 100mM	**0.73 ±0.07^{B*}**	0.43 ±0.05^{A*}	0.28 ±0.02^{A*}	0.40 ±0.03^{AB*}

Supplementary Table S7 – Lipid peroxidation (LPO), in *Rhizobium* cells exposed to Cd and MTA; DMS; DMDS; DMTS. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) of sulfur compounds. Values are means of at least 3 replicates ± standard error; different lowercase letters indicate significant differences among sulfur compound concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among sulfur compound concentrations in Cd condition, bold values indicate significant differences from their control (no Cd or Cd) and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. Considered significantly different values of $p < 0.05$.

LPO (nmol/g)	MTA	DMS	DMDS	DMTS
Control	2.04 ±0.81^{a}	2.04 ±0.21^{a}	2.04 ±0.20^{a}	2.04 ±1.35^{a}
Control 1nM	1.88 ±0.94^{a}	2.33 ±0.33^{ab}	1.74 ±0.19^{a}	2.00 ±0.56^{a}
Control 100nM	0.94 ±0.02^{a}	2.45 ±0.48^{ab}	2.35 ±0.25^{a*}	1.60 ±0.26^{a}
Control 10µM	0.86 ±0.16^{a}	**2.82 ±0.10^{b*}**	2.43 ±0.64^{a}	1.84 ±0.48^{a*}
Control 1mM	0.85 ±0.06^{a}	2.96 ±0.77^{ab*}	1.79 ±0.05^{a}	1.76 ±0.24^{a*}
Control 100mM	0.92 ±0.02^{a}	2.90 ±0.41^{ab*}	2.33 ±0.51^{a}	1.08 ±0.06^{a*}
Cd	2.77 ±1.54^{A}	2.77 ±0.16^{A}	2.77 ±1.50^{A}	2.77 ±0.12^{A}
Cd 1nM	2.93 ±1.08^{A}	2.88 ±0.15^{A}	2.49 ±1.27^{A}	1.90 ±0.89^{AB}
Cd 100nM	1.90 ±0.45^{A}	1.65 ±0.46^{ABC}	0.99 ±0.16^{A*}	**0.46 ±0.19^{B}**
Cd 10µM	1.98 ±0.78^{A}	**0.84 ±0.03^{B*}**	1.25 ±0.24^{A}	**0.33 ±0.16^{B*}**
Cd 1mM	1.60 ±0.54^{A}	**0.51 ±0.11^{C*}**	1.68 ±0.24^{A}	**0.38 ±0.16^{B*}**
Cd 100mM	1.83 ±0.43^{A}	**0.49 ±0.04^{C*}**	1.49 ±0.49^{A}	**0.32 ±0.08^{B*}**

Supplementary Table S8 – Protein carboxylation (PC), in *Rhizobium* cells exposed to Cd and MTA; DMS; DMDS; DMTS. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) of sulfur compounds. Values are means of at least 3 replicates ± standard error; different lowercase letters indicate significant differences among sulfur compound concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among sulfur compound concentrations in Cd condition, bold values indicate significant differences from their control (no Cd or Cd) and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. Considered significantly different values of $p < 0.05$.

PC (mU/g)	MTA	DMS	DMDS	DMTS
Control	12.39 ±1.21^{a}	12.39 ±2.33^{a}	12.39 ±4.10^{a}	12.39 ±3.92^{a}
Control 1nM	11.54 ±1.05^{a*}	11.64 ±2.26^{a}	11.37 ±2.16^{a}	9.32 ±0.14^{a}
Control 100nM	15.36 ±3.09^{a*}	**3.86 ±0.17^{b*}**	7.85 ±0.43^{a}	10.07 ±1.17^{a}
Control 10µM	14.58 ±2.91^{a}	**4.41 ±0.33^{b*}**	5.51 ±1.11^{a*}	9.90 ±2.92^{a}
Control 1mM	22.97 ±4.78^{a*}	3.38 ±1.78^{ab*}	8.40 ±1.96^{a}	10.18 ±3.21^{a}
Control 100mM	19.89 ±5.18^{a*}	6.70 ±1.08^{ab*}	8.41 ±1.31^{a}	7.01 ±0.43^{a}
Cd	27.21 ±2.17^{A}	27.21 ±6.47^{ABC}	27.21 ±7.45^{AB}	27.21 ±5.14^{A}
Cd 1nM	**14.67 ±1.82^{B*}**	15.45 ±2.60^{B}	21.73 ±6.36^{AB}	25.03 ±7.06^{A}
Cd 100nM	**8.42 ±1.13^{C*}**	45.10 ±4.34^{A*}	34.53 ±5.91^{AB}	33.27 ±3.61^{A}
Cd 10µM	**9.82 ±1.27^{BC}**	44.26 ±9.73^{ABC*}	31.92 ±4.31^{A*}	27.05 ±4.43^{A}
Cd 1mM	**13.94 ±0.18^{BC*}**	32.94 ±4.24^{AC*}	21.73 ±4.06^{AB}	20.60 ±3.84^{A}
Cd 100mM	**9.69 ±1.43^{BC*}**	27.80 ±2.23^{C*}	15.22 ±1.80^{B}	18.73 ±2.06^{A}

Supplementary Table S9 – Superoxide dismutase (SOD) activity, in *Rhizobium* cells exposed to Cd and MTA; DMS; DMDS; DMTS. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) of sulfur compounds. Values are means of at least 3 replicates ± standard error; different lowercase letters indicate significant differences among sulfur compound concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among sulfur compound concentrations in Cd condition, bold values indicate significant differences from their control (no Cd or Cd) and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. Considered significantly different values of $p < 0.05$.

SOD (U/mg)	MTA	DMS	DMDS	DMTS
Control	0.29 ±0.03^{a*}	0.29 ±0.02^{a*}	0.29 ±0.02^{ab*}	0.29 ±0.03^{a*}
Control 1nM	0.26 ±0.08^{a*}	0.35 ±0.05^{ab*}	0.28 ±0.03^{ab*}	0.29 ±0.01^{a*}
Control 100nM	0.29 ±0.09^{a*}	**0.37 ±0.02^{c*}**	0.36 ±0.02^{a*}	0.37 ±0.14^{a}
Control 10µM	0.28 ±0.02^{a*}	**0.36 ±0.02^{bc}**	0.36 ±0.08^{ab*}	0.36 ±0.07^{a}
Control 1mM	0.28 ±0.05^{a}	0.27 ±0.04^{ab*}	0.27 ±0.01^{b*}	0.30 ±0.04^{a}
Control 100mM	0.28 ±0.05^{a*}	0.28 ±0.03^{ac*}	0.28 ±0.01^{ab*}	0.30 ±0.03^{a*}
Cd	0.69 ±0.03^{A*}	0.69 ±0.04^{A*}	0.69 ±0.04^{A*}	0.69 ±0.09^{A*}
Cd 1nM	0.74 ±0.13^{AB*}	0.76 ±0.06^{A*}	0.65 ±0.13^{AB*}	0.71 ±0.05^{A*}
Cd 100nM	**1.03 ±0.10^{B*}**	0.76 ±0.14^{A*}	0.73 ±0.07^{A*}	0.82 ±0.12^{A}
Cd 10µM	**0.44 ±0.03^{C*}**	0.65 ±0.10^{A}	0.69 ±0.05^{A*}	0.66 ±0.10^{A}
Cd 1mM	**0.51 ±0.07^{AC}**	0.72 ±0.04^{A*}	**0.94 ±0.00^{B*}**	0.53 ±0.20^{A}
Cd 100mM	**0.47 ±0.06^{C*}**	0.79 ±0.05^{A*}	**1.09 ±0.04^{C*}**	0.58 ±0.00^{A*}

Supplementary Table S10 – Glutathione s-transferases (GSTs) activity, in *Rhizobium* cells exposed to Cd and MTA; DMS; DMDS; DMTS. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) of sulfur compounds. Values are means of at least 3 replicates ± standard error; different lowercase letters indicate significant differences among sulfur compound concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among sulfur compound concentrations in Cd condition, bold values indicate significant differences from their control (no Cd or Cd) and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. Considered significantly different values of $p < 0.05$.

GSTs (mU/g)	MTA	DMS	DMDS	DMTS
Control	5.20 ±0.59^{abc}	5.20 ±0.99^{ab}	5.20 ±0.49^{a}	5.20 ±0.78^{ab}
Control 1nM	5.44 ±0.19^{a}	4.43 ±0.25^{a}	3.69 ±0.52^{ab}	4.72 ±0.01^{a*}
Control 100nM	5.71 ±0.50^{ab}	4.45 ±0.28^{a}	3.93 ±0.06^{a*}	4.58 ±0.54^{ab}
Control 10µM	5.28 ±0.43^{abc}	2.44 ±0.28^{b*}	4.19 ±0.27^{ab*}	4.28 ±0.11^{b*}
Control 1mM	4.66 ±0.18^{bc*}	3.11 ±0.37^{b*}	**3.34 ±0.18^{b*}**	4.44 ±0.50^{ab*}
Control 100mM	4.09 ±0.16^{c}	4.58 ±0.20^{a*}	3.89 ±0.54^{ab*}	4.87 ±0.24^{ab*}
Cd	5.80 ±0.34^{A}	5.80 ±0.91^{A}	5.80 ±0.83^{AB}	5.80 ±0.78^{A}
Cd 1nM	6.10 ±0.65^{A}	6.54 ±0.89^{A}	6.07 ±1.81^{AB}	6.70 ±0.64^{A*}
Cd 100nM	6.24 ±0.87^{AB}	7.27 ±1.36^{A}	6.42 ±0.23^{A*}	7.44 ±2.16^{AB}
Cd 10µM	5.81 ±1.06^{AB}	7.79 ±0.53^{A*}	6.74 ±0.48^{AB*}	**9.69 ±0.84^{B*}**
Cd 1mM	**4.02 ±0.09^{B*}**	6.21 ±0.76^{A*}	8.17 ±0.40^{B*}	9.46 ±1.04^{AB*}
Cd 100mM	4.71 ±0.51^{AB}	6.91 ±0.58^{A*}	7.09 ±0.11^{AB*}	**9.29 ±0.58^{B*}**

Supplementary Table S11 – Glutathione peroxidase (GPx) activity, in *Rhizobium* cells exposed to Cd and MTA; DMS; DMDS; DMTS. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) of sulfur compounds. Values are means of at least 3 replicates ± standard error; different lowercase letters indicate significant differences among sulfur compound concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among sulfur compound concentrations in Cd condition, bold values indicate significant differences from their control (no Cd or Cd) and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. Considered significantly different values of $p < 0.05$.

GPx (U/g)	MTA	DMS	DMDS	DMTS
Control	9.04 ±1.94^{a}	9.04 ±2.53^{a}	9.04 ±0.07^{a*}	9.04 ±2.14^{ab}
Control 1nM	8.64 ±0.84^{a}	11.67 ±3.55^{a}	10.82 ±3.01^{ab}	6.89 ±1.02^{a*}
Control 100nM	10.90 ±0.08^{a*}	18.51 ±5.47^{ab*}	16.82 ±3.46^{a}	9.99 ±0.27^{b*}
Control 10µM	10.01 ±5.33^{ab}	**34.37 ±2.30^{b*}**	16.77 ±2.89^{a}	**15.64 ±0.78^{c}**
Control 1mM	15.76 ±2.87^{ab}	**26.82 ±4.00^{b}**	9.96 ±1.13^{a*}	6.90 ±0.67^{a*}
Control 100mM	**21.36 ±0.88^{b*}**	26.03 ±6.57^{ab}	**5.60 ±0.47^{b*}**	6.31 ±0.45^{a*}
Cd	13.29 ±3.07^{AB}	13.29 ±4.14^{A}	13.29 ±1.51^{AB*}	13.29 ±1.87^{A}
Cd 1nM	14.48 ±3.87^{AB}	23.08 ±1.57^{A}	13.90 ±0.46^{A}	**128.63 ±39.60^{B*}**
Cd 100nM	6.58 ±1.33^{A*}	**36.52 ±1.97^{B*}**	9.96 ±0.10^{B}	**133.36 ±5.44^{B*}**
Cd 10µM	7.93 ±0.38^{A}	**43.77 ±0.95^{C*}**	18.80 ±2.11^{AC}	151.96 ±73.13^{AB}
Cd 1mM	9.17 ±0.82^{AB}	22.52 ±2.43^{A}	**22.48 ±2.52^{C*}**	11.08 ±1.03^{A*}
Cd 100mM	**14.44 ±2.31^{B*}**	18.89 ±1.60^{A}	18.45 ±2.11^{AC*}	17.39 ±2.44^{A*}

Supplementary Table S12 – Protein content, in *Rhizobium* cells exposed to Cd and MTA; DMS; DMDS; DMTS. Cells were simultaneously exposed to 2 Cd conditions (0 and 100 µM) and 6 concentrations (0 nM, 1 nM, 100 nM, 10 µM, 1 mM and 100 mM) of sulfur compounds. Values are means of at least 3 replicates ± standard error; different lowercase letters indicate significant differences among sulfur compound concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among sulfur compound concentrations in Cd condition, bold values indicate significant differences from their control (no Cd or Cd) and asterisks indicate significant differences between conditions (0 and 100 µM Cd) for the same concentration of the same compound. Considered significantly different values of $p < 0.05$.

Protein (mg/g)	MTA	DMS	DMDS	DMTS
Control	211.34 ±26.93^{ab*}	211.34 ±12.04^{a*}	211.34 ±18.89^{a*}	211.34 ±52.22^{a}
Control 1nM	142.52 ±16.73^{ab*}	208.16 ±27.38^{a*}	177.78 ±16.91^{a}	170.78 ±2.30^{a*}
Control 100nM	144.00 ±17.76^{ab}	185.10 ±7.20^{a}	193.93 ±9.90^{a*}	189.21 ±30.31^{a*}
Control 10µM	144.51 ±5.32^{b}	215.97 ±16.15^{a}	204.15 ±29.85^{a*}	207.72 ±32.20^{a*}
Control 1mM	191.46 ±6.07^{a*}	206.44 ±30.54^{a}	196.94 ±28.12^{a*}	166.06 ±0.90^{a*}
Control 100mM	183.52 ±1.20^{a}	199.81 ±17.97^{a}	196.52 ±19.88^{a}	189.96 ±22.34^{a}
Cd	359.86 ±32.11^{AB*}	359.86 ±36.47^{A*}	359.86 ±19.03^{A*}	359.86 ±49.32^{A}
Cd 1nM	288.66 ±49.32^{BC*}	304.07 ±14.20^{A*}	313.61 ±22.34^{A}	**106.18 ±17.56^{BD*}**
Cd 100nM	**164.12 ±46.44^{CD}**	354.27 ±116.65^{A}	396.60 ±37.08^{A*}	**80.09 ±18.50^{BCD*}**
Cd 10µM	**147.28 ±41.02^{CD}**	383.09 ±70.96^{A}	362.00 ±38.19^{A*}	**54.00 ±7.74^{BC*}**
Cd 1mM	**108.48 ±20.27^{D*}**	394.27 ±83.65^{A}	359.25 ±4.64^{A*}	**39.96 ±0.32^{C*}**
Cd 100mM	**195.21 ±57.33^{CD}**	357.94 ±82.92^{A}	341.52 ±66.64^{A}	**113.34 ±19.83^{D}**