Universidade de Aveiro Departamento de Biologia
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Etelvina Maria de Almeida Paula Figueira, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro, e co-orientação da Doutora Sílvia Maria da Rocha Simões Carriço, Professora Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Rosa de Fátima Lopes de Freitas, Equiparada a Investigadora Auxiliar do Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro

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agradecimentos

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palavras-chave *Rhizobium*, cádmio, metabolómica, metabolitos voláteis

resumo

Nos sistemas agrícolas, o uso de fertilizantes que contêm fosfatos é uma importante via de entrada de Cd nos solos, podendo ser atingidas concentrações que interferem com os microrganismos do solo. O metabolismo secundário pode fornecer pistas e/ou biomarcadores que poderão ser usados para detectar, com elevada sensibilidade, modificações nos organismos induzidas pelo ambiente, tais como efeitos resultantes da exposição a metais. Neste trabalho, a técnica de cromatografia bidimensional acoplada a espectrómetro de massa “time-of-flight” em combinação com a microextração em fase sólida de “headspace” foi usada para estudar as alterações induzidas no metaboloma volátil de rizóbio exposto a cádmio. Os resultados mostram que a exposição ao metal alterou o número e quantidade dos metabolitos, quer intracelular, quer extracelularmente. Em particular, as áreas dos picos correspondentes a álcoois alifáticos de cadeia curta aumentaram linearmente com a concentração de metal, reflectindo o ambiente oxidante induzido pela exposição ao metal. Os resultados sugerem ainda que o metabolito volátil acetato de etilo poderá ser um óptimo marcador de stress induzido por exposição a metais em bactérias. Além dos dados acerca dos efeitos da exposição a metais no metaboloma volátil em bactérias, este trabalho permitiu também detectar compostos com bioactividade descrita na literatura, e que podem contribuir para alargar o papel ecológico desta bactéria do solo para além da fixação biológica de azoto.

keywords *Rhizobium*, cadmium, metabolomics, volatile metabolites

abstract In agricultural systems the use of phosphate fertilizers is a major input of Cd into the soil, and Cd may reach concentrations interfering with soil microorganisms. The secondary metabolism might provide clues or even deliver sensitive biomarkers that could be used to detect small modifications induced by the environment, such as metal exposure. In this work, comprehensive two-dimensional gas chromatography coupled to mass spectrometry with a high resolution time of flight analyzer (GC×GC–ToFMS) combined with headspace solid phase microextraction (HS-SPME) was used to screen for alterations in the volatile metabolome of rhizobia exposed to cadmium. The results showed that exposure to cadmium altered the number and amount of metabolites, both intra and extracelullarly. Specifically, peak areas of low chain aliphatic alcohols increased linearly with the increment of cadmium concentration, reflecting the intracellular redox environment of the bacteria. Ethyl acetate emerged as an excellent biomarker for metal stress in bacteria. Apart from providing data on the effects of metal exposure in the volatile metabolome of bacteria, data about bioactive compounds synthesized by this group of soil bacteria that interact with other organisms was also collected, thus widening the role of this group of bacteria beyond nitrogen fixation and legumes.

Index

Abbreviations

^{1}D	First dimension
$^{1}t_R$	Retention time of the first dimension
^{2}D	Second dimension
$^{2}t_R$	Retention time of the second dimension
BNF	Biological nitrogen fixation
DTIC	Deconvoluted total ion current
GCxGC	Two-dimensional gas chromatography
GCxGC-ToFMS	Two-dimensional gas chromatography coupled to time of flight mass spectrometry
HSPME	Headspace solid-phase microextraction
MS	Mass spectrometry
mVOC	Microbial volatile organic compound
NMR	Nuclear magnetic resonance
PCO	Principal coordinates method
PGPR	Plant growth promoting rhizobacteria
RI	Retention index
ROS	Reactive oxygen species
rpm	Revolutions per minute
TIC	Total ion chromatograms
ToFMS	Time-of-flight mass spectrometry
VOC	Volatile organic compound
VSC	Volatile sulfur compound
YMB	Yeast mannitol broth

Chapter 1
Background

1.1. Metabolomics

Metabolomics has been a focus of much attention in recent years as it is, along with proteomics and transcriptomics, an useful tool to complement the data gathered from genomics, providing insights on the genotype-phenotype relationship (Fiehn, 2002; Goodacre et al., 2004). It is known that from the same genetic information different proteins can be made (e.g. due to splicing processes) implying different metabolic activities. The advantage of studying metabolites is that, instead of studying the nucleotide sequence that is the source for multiple different products, the metabolite can be considered as a final state of expression of a gene or set of genes, and therefore, any alterations detected in the metabolome might be the reflection of genetic or environmental changes (Fiehn, 2002). This makes the pool of metabolites produced by organisms an excellent source of robust biomarkers of disease, stress or virtually any response to environmental variables of interest. It is important, however, to keep in mind that, even if the concentration of certain metabolites is altered as a response to a variable, further validation is necessary for a metabolite to be considered a biomarker (Koulman et al., 2009).

The "omics" technologies generate big amounts of data, a fact that is shifting the biological sciences from a qualitative to a quantitative paradigm, and one that is responsible for the development of databases, necessary to analyze the data through bioinformatics platforms (Hummel et al., 2007). Several databases containing metabolomics data are available. Some databases were developed thinking specifically on this type of data, such as the Golm database (Kopka et al., 2005), the Human Metabolome Project (Wishart et al., 2007), and more recently, mVOC (Lemfack et al., 2014). While the first two databases are focused on plant and human metabolites, respectively, the latter receives data concerning microbial metabolites. Other databases, not specific for metabolomics, also include data concerning metabolites and metabolic pathways. Such is the case of the KEGG database (Kanehisa and Goto, 2000).

The advancements achieved so far have only been possible thanks to the advancements in the instrumentation, with the advent of high-throughput nuclear magnetic resonance (NMR) spectroscopy or liquid/gas-chromatography coupled to mass-spectrometry (Pan and Raftery, 2007). The large majority of published studies in the field of metabolomics are based either in NMR spectroscopy or mass spectrometry (MS) platforms. As with all techniques, each displays strengths and limitations. Moreover, a technique or framework that covers all metabolites of an organism does not exist (Dunn et al., 2005). Two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GCxGC-ToFMS) is a state of the art technique that benefits both from the high resolution of GCxGC and from the high sensitivity of the time-of-flight mass spectrometer (ToFMS) to yield a broad analysis of the volatile metabolites that can be comprehensively extracted from biological matrices through headspace solid-phase microextraction (HSPME).

1.2. Metabolite volatile organic compounds produced by rhizobacteria

Many organisms are able to produce biogenic volatile organic compounds that work as metabolites, known as microbial VOCs (mVOCs). Bacteria are able to produce and release mVOCs from many different chemical classes, including fatty acid derivatives (hydrocarbons, alcohols, ketones, aldehydes), nitrogen containing compounds and volatile sulfur compounds (VSC) (Schulz and Dickschat, 2007).

Strategies to maximize production in agriculture are on demand due to several reasons, such as the high prices of food, the expected population growth and the degradation of soils due to intensive farming and climatic changes. There has been extensive research regarding the potential use of soil bacteria to improve the productivity of many plants with agricultural interest. These studies allowed several species of bacteria in the rhizosphere to be classified as plant-growth-promoting-rhizobacteria (PGPR). Rhizobia are one of the recognized groups of species of bacteria that are classified as PGPR (Ahemad and Kibret, 2014; Antoun et al., 1998; Lugtenberg and Kamilova, 2009).

Technological advancements allowed the screening of mVOCs of bacteria in the soil and revealed their potential to improve agricultural productivity. Mechanisms for the improvement of plant productivity are widely studied and include the improvement of the

availability of nutrients, the production of phytohormones or the prevention of the action of deleterious microorganisms (Lugtenberg and Kamilova, 2009). However, the potential role of bacterial volatiles to induce change in the primary and secondary metabolism of plants has not received the same amount of attention (Ryu et al., 2004). The results from the report (Ryu et al., 2003) showed that certain *Bacillus* strains were able to release a blend of mVOCs that promoted growth of *Arabidopsis thaliana* seedlings, with 2,3-butanediol and acetoin being released exclusively from the strains with the greatest growth inducing effect. Since that study was published, more work has been carried on this topic. The same team reported in 2004 the ability of mVOCs produced by *Bacillus* strains to induce systemic resistance in *Arabidopsis* (Ryu et al., 2004), and many papers have been published concerning the potential plant growth effects of volatiles released by rhizobacteria, and allowed, not only to establish volatiles as features of PGPR, but also to discover new functions of rhizobacterial volatiles, such as the inhibition of growth of phytopathogenic fungi (e.g. (Kai et al., 2007)). Interestingly, there are also cases of deleterious volatile metabolites that can negatively affect plants or other organisms (e.g. Vespermann et al., 2007). In fact, many studies have shown that rhizobacterial volatiles can have beneficial or deleterious effects in plants and fungi (Kai et al., 2009). In what concerns to rhizobia, it has been shown that rhizobia colonization affects the emission of VOCs by the host plant, which in turn affects the plant selection by an insect herbivore (Ballhorn et al., 2013). Nonetheless, until the moment, no studies have focused on the production of mVOCS by rhizobia.

1.3. The metabolome of bacteria under metal-induced stress

In the environment, bacteria might be subjected to many different pressures. Pollution and intensive farming practices, climatic events, salt, drought and metal induced stress are some of the most common challenges that bacterial populations must face in agricultural systems. It is known that several metabolites produced by rhizobacteria play roles in helping plants to cope with abiotic stresses (Yang et al., 2009). Yet, since research is devoted for the abilities to induce plant growth, works trying to disclose potential effects that these stresses might carry to the metabolism of the bacterial side are scarce.

Exposure to cadmium (Cd) and other non-essential metals often leads to the generation of reactive oxygen species (ROS) (Stohs and Bagchi, 1995) that can damage lipids (thus affecting membranes), nucleic acids, and also oxidize proteins. Therefore, the effects on the cell may include lipid peroxidation, damages in the DNA molecule, oxidation of sulfhydryl groups, reduction of difulfides, oxidative adduction of amino acid residues close to metal-binding sites, among others (Cabiscol et al., 2000). This raises the possibility of disruption of key enzymes or other components of the cellular metabolism. The bacterial metabolism under stress was studied for a strain of *Pseudomonas* exposed to the metalloid tellurite, with the aim of discovering the mechanism of tolerance, which was unknown (Tremaroli et al., 2009). The authors were able to correlate the resistance with the induction of oxidative stress, resistance to membrane perturbation and, most interestingly, reconfiguration of the cellular metabolism (Tremaroli et al., 2009). These exciting results suggest an undisclosed role of the metabolism in the resistance to non-essential metals and metalloids and indicate that metabolomics could be a powerful tool in this topic of research. However, so far this is the only metabolomics study of bacteria under metal stress, and did not include mVOCs.

1.4. The importance of rhizobia

Rhizobia are a group of rhizobacteria capable of nodulating legumes and in the specific conditions inside the nodule, fix atmospheric nitrogen and convert it to forms that the plant can use, in a process known as biological nitrogen fixation (BNF). This ecological and agricultural service, although not performed exclusively by this group of microorganisms, places this symbiosis as one of the most studied processes in Biology. The genome sequence of *Rhizobium leguminosarum*, a species that nodulates *Pisum sativum*, was published almost ten years ago (Young et al., 2006). The genome is surprisingly big for a bacteria, an observation that can possibly be explained due to the accumulation of genetic material that can increase the organism's plasticity and be useful to face the dynamic nature of soil and its dynamic substrates (Young et al., 2006).

Persistence of *Rhizobium* in the soil and effectiveness of nodulation may be affected by a number of environmental factors, such as drought (Athar, 1998), salinization (Figueira, 2000) or contamination by non-essential metallic elements ((Broos et al., 2005; Wani et al., 2008). Anthropogenic activities such as intensive farming account for a part of

the contamination by non-essential metals in agricultural fields. In the particular case of contamination by Cd, the use of phosphate based fertilizers is one of the major inputs of the metal to the soil (Smolders and Mertens, 2013). This is due to the fact that manufacture of these fertilizers is based on the use of phosphate rocks, which, depending on their origin, might be rich in Cd.

1.5. Aims of the dissertation

This dissertation work aims to test the hypothesis that exposure to Cd induces alterations in the volatile metabolome of *Rhizobium.* Using an assay to test the tolerance of an isolate of *Rhizobium* to Cd as premise, this work might provide the first insights into the intracellular and excreted volatile metabolomes of bacterial cells under the presence of a metal stressor. The potential results from this approach will constitute clues about the importance of mVOCs in coping with a metal stress or as biomarkers of stress. Hopefully, this dissertation thesis will also constitute a pioneer foundation stimulating other research teams to investigate the intracellular volatile domain of bacteria, since research is currently mainly devoted to the released mVOCs.

Chapter 2

Analysis of the volatile metabolome of the *Rhizobium leguminosarum* strain E20-8 exposed to cadmium

2.1. Introduction

Metabolism has been proposed as a way to study the mechanisms behind the toxicity of metals towards bacteria, based on the conservation of the primary metabolites and metabolic pathways found across the different groups of organisms (Tremaroli et al., 2009). Likewise, the secondary metabolism might provide clues or even deliver sensitive biomarkers that could be used to detect small modifications induced by metal exposure. These alterations are hard to detect using conventional methodologies due to the small molecular weight and low concentration of many of the secondary metabolites, but might have strong impacts on the cell, organism or even higher levels of biological organization (Peñuelas and Sardans, 2009).

A wide range of mVOCs are products of the secondary metabolism of organisms including bacteria (Schulz and Dickschat, 2007). Bacteria are capable of producing mVOCs which are important in economic activities such as the production of food and beverages. Therefore, literature regarding bacterial mVOCs is mostly concerned with the improvement of the sensorial characteristics induced by them. mVOCs display a high variety of structures and compositions, and are therefore organized into several chemical families, such as terpenes, alkanes, alcohols, esters and acids (Kesselmeier and Staudt, 1999). This diversity of structures might be a reflection of the different functions they assume, since VOCs are involved in key ecological functions among different organisms, which include biochemical and physiological but also ecological functions (Peñuelas and Sardans, 2009).

The biochemical mechanisms behind tolerance to non-essential metallic elements in rhizobia and other soil bacteria have been widely studied (Bruins et al., 2000; Silver, 1996), but so far no studies have focused on the effects of metal exposition in the volatile metabolome of bacteria. There are also no published studies aiming for the simultaneous characterization of both the intra and extracellular mVOCs of any species of rhizobia. In

fact, only one report aiming for the profiling of rhizobial metabolites was published in 2004 and was not focused in the detection of VOCs (Barsch et al., 2004). This is a considerable gap in the knowledge of such an important group of soil bacteria, which in addition to their ability to fix N_2 in symbiosis with legumes can also promote plant growth beyond the Fabaceae family, being able to help plants to tolerate abiotic stresses (Yang et al., 2009) and to prevent plant diseases through the production of biocidal volatiles (Compant et al., 2005).

The present chapter aims characterizing the mVOCs fraction of the metabolome of *Rhizobium* grown in culture medium (control) or in culture medium supplemented with a range of Cd concentrations (2.5, 5.0, 7.5 µM), inducing different levels of growth inhibition. Intra and extracellular (compounds released to the growth medium) mVOCs were extracted using headspace solid phase micro extraction (HS-SPME) and analyzed using comprehensive two-dimensional gas chromatography coupled to mass spectrometry with a high resolution time-of-flight analyzer (GC×GC–ToFMS). Data analysis using bioinformatics tools allowed to compare the volatile metabolome of *Rhizobium* exposed to the four conditions tested and to infer the alterations induced by the different levels of stress imposed.

2.2. Material and methods

2.2.1. Preparation of the material

Before each trial, flasks were immersed overnight in an acid bath (10% of nitric acid). Then, flasks were washed by immersion overnight in 1:1 ethanol/deionized water bath, followed by two rinses of deionized water, and left immersed overnight in a deionized water bath.

2.2.2. Exposure of E20-8 to cadmium

The strain used in this work (E20-8) was previously described as tolerant to Cd (Corticeiro et al., 2006; Figueira et al., 2005; Lima et al., 2006).

In order to determine a time period of growth that would both provide sufficient amount of cells for mVOCs detection and also ensure that cells were physiologically robust (exponential phase), a growth curve until the end of the exponential phase was constructed. For that, cells were grown in YMB medium for 24 h, and absorbance at 600 nm was recorded in time intervals of 2 h as an estimation of growth. The growth medium used was YMB (Somasegaran and Hoben, 1994) with the following composition: 1 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl and 0.5 g yeast extract, per liter of distilled water.

For the exposure assays, three independent trials were performed. In each trial, cells were grown during 14 h (overnight) in freshly prepared 400 mL YMB (with 1 g of mannitol) supplemented with cadmium chloride (purchased from Sigma) in the concentrations 2.5, 5.0 and 7.5 µM, and in a control condition (no cadmium chloride). These concentrations were chosen based in previous studies regarding tolerance of E20-8 to Cd (Corticeiro, 2012). At the end of the growth period, optical density at 600 nm was recorded to be used as a measure of growth. Since *Rhizobium* cells release exopolissacharids to the growth medium, and this could affect the reliability of the measurement of the optical density, an aliquot of each sample was also plated to determine the most probable number of cells.

2.2.3. Extracellular fraction

Cells were harvested by centrifugation at 10 000 g for 10 min at 4ºC. During the handling, flasks were kept in ice and were always capped, to prevent loss of volatiles. The supernatant was considered the extracellular fraction and 2 mL were collected and frozen at -80 ˚C until further analysis. The pellet resulting from the centrifugation and containing the cells was washed by the addition of 2 mL of deionized water followed by centrifugation at 10 000 g for 10 min at 4 ºC and the supernatant was discarded. This

washing procedure was repeated three times, to ensure that the intracellular fraction was not contaminated with extracellular compounds. The resulting pellet was then suspended in 2 mL of deionized water before cells were disrupted.

2.2.4. Sonication

To allow analysis of intracellular volatiles, cells were disrupted by sonication. Different sonication approaches were tested to ensure an effective disruption of cells and to prevent the loss of volatiles. Cells were disrupted by using an ultrasonic homogenizer U 200 S Control (IKA-WERKE). To achieve a rapid disruption, and therefore minimize the loss of volatiles, cycle and amplitude parameters were set at the highest values, 1 and 100%, respectively, and tubes were always kept in ice to prevent loss of volatiles due to heating. The duration of sonication was optimized in order to allow lysis of most of the cells but at the same time minimizing the loss of mVOCs. To determine the optimal conditions, a test suspension of cells in 2 mL of deionized water was disrupted for 8 min. An aliquot of 100 µL was collected at 1 min intervals to monitor protein content using the Pierce™ BCA Protein Assay Kit. Estimation of protein content served as an indicator of cell disruption. Based on the results of this approach, sonicating for 1 min, followed by a rest in ice during 1 min before a second sonication period of 1 min, was the procedure applied to cells from the exposure assays. After sonication, samples were frozen at -80 ºC for further analysis by HSPME and GCxGC-ToFMS.

2.2.5. HSPME and GCxGC-ToFMS analysis

The SPME holder for manual sampling and fiber coating was purchased from Supelco (Aldrich, Bellefonte, PA, USA). For each HS-SPME assay, an aliquot of 2 mL of each *Rhizobium* fraction (extracellular or intracellular) was placed into a 8 mL glass vial. After the addition of NaCl (0.4 g) and stirring (0.8 × 0.3 cm bar) at 500 RPM, the vial was capped with a PTFE septum and a screw cap (Chromacol, Hertfordshire, UK). The vial was placed in a bath adjusted to 50.0±0.1 ºC, and the SPME fiber was inserted in the headspace for 60 min. In order to avoid any cross-over contamination due to the fiber coating, blanks were prepared, and an analysis of the fiber coating not submitted to any extraction procedure, were run between every set of three analyses. Three independent

aliquots of each sample, plus non-inoculated medium, were analyzed for each condition of both extracellular and intracellular fractions.

After the extraction/concentration step, the SPME fiber was manually introduced into the GC×GC–ToFMS injection port at 250°C and kept for 30 s for desorption. The injection port was lined with a 0.75 mm I.D. splitless glass liner. Splitless injections were used (30 s). The LECO Pegasus 4D (LECO, St. Joseph, MI, USA) GC×GC–ToFMS system consisted of an Agilent GC 7890A gas chromatograph, with a dual stage jet cryogenic modulator (licensed from Zoex) and a secondary oven. The detector was a high-speed ToF mass spectrometer. AnEquity-5 column (30 m × 0.32 mm I.D., 0.25 µm film thickness, Supelco, Inc., Bellefonte, PA, USA) was used as ^{1}D column and a DB-FFAP (0.79 m × 0.25 mm I.D., 0.25 m film thickness, J&W Scientific Inc., Folsom, CA, USA) was used as a ^{2}D column. The carrier gas was helium at a constant flow rate of 2.50 ml/min. The GC×GC–ToFMS injection port was at 250°C. The primary oven temperature program was: initial temperature 35°C (hold 1 min), raised to 40°C (1°C min^{-1}), and then to 210°C (7°C min^{-1}) (hold 1 min). The secondary oven temperature program was 15°C offset above the primary oven. The MS transfer line and MS source temperatures were 250°C. The modulation time was 6 s; the modulator temperature was kept at 20°C offset (above primary oven). A 6 s modulation time with a 15°C secondary oven temperature offset was chosen to be a suitable compromise as it maintained the ^{1}D separation, maximized the ^{2}D resolution, and avoided the wrap-around effect (i.e. when the elution time of a pulsed solute exceeds the modulation period) for compounds that were late to elute from the ^{2}D. Ideally, all peaks must be detected before the subsequent re-injection and, hence, $^{2}t_{R}$ must be equal or less than the modulation period (Dallüge et al., 2003; Mondello et al., 2008). The ToFMS was operated at a spectrum storage rate of 125 spectra/s. The mass spectrometer was operated in the EI mode at 70 eV using a range of m/z 35–350 and the detector voltage was −1522 V. Total ion chromatograms (TIC) were processed using the automated data processing software ChromaTOF® (LECO) at signal-to-noise threshold of 100. Contour plots were used to evaluate the separation general quality and for manual peak identification. For identification purposes, the mass spectrum and retention times (^{1}D and ^{2}D) of the analytes were compared with standards, when available. Also, the mass spectrum of each compound detected was compared to those in mass spectral libraries, which included an in-house library of standards and two commercial databases (Wiley275

and US National Institute of Science and Technology (NIST) V.2.0 – Mainlib and Replib). The identification was also supported by experimentally determined retention index (RI) values that were compared, when available, with values reported in the bibliography for chromatographic columns similar to the one used in the present work as the ^{1}D column. RI values were determined using a C_8-C_{20} n-alkanes series (the solvent n-hexane as used as C_6 standard) and calculated according to the van den Dool and Kratz equation (van Den Dool and Dec. Kratz, 1963). The majority (>90%) of the identified compounds presented similarity matches >900. The DTIC (Deconvoluted Total Ion Current) GC×GC area data were used as an approach to estimate the relative content of each volatile component of *Rhizobium*.

2.2.7. Data analysis

Data from the growth inhibition of cells exposed to Cd was submitted to a One-Way ANOVA analysis in R (R Core Team, 2014) using the Rcmdr package (Fox, 2005). This allowed rejecting the null hypothesis of: no significant differences exist on the growth inhibition between conditions. To check for pair-wise significant differences, a post-hoc analysis using the Tukey's HSD (honest significant difference) test was performed.

Data from the chromatograms were exported from ChromaTOF (LECO) to the software Guineu (Castillo et al., 2011) for peak table data alignment. At this point, compounds detected in the non-inoculated growth medium were excluded from the matrices before further analysis, because since they already exist in the non-inoculated growth medium, it is unclear whether in the tested conditions they are also produced by the bacteria or not (Schulz and Dickschat, 2007).

The matrices resulting from the previous step were submitted to principal ordination analysis using the Principal coordinates analysis (PCO) from the software Primer v6. Data (mVOCs peak areas) were fourth root transformed and represented through Euclidean distance matrices among samples.

Venn diagrams were built using the Venn Tool from the Bioinformatics Evolutionary Genomics of the University of Gent (http://bioinformatics.psb.ugent.be/webtools/Venn/).

The heatmaps were built using R (R Core Team, 2014) and the R package gplots (Warnes et al., 2014). To diminish differences on compound peak areas, a fourth root transformation was applied to the compound peak areas, since they are present in different values of magnitude. For both conditions and mVOCs, the dendrograms were built based on the hierarchical clustering (complete linkage clustering) of the dissimilarity (Euclidean distance) between conditions or mVOCs.

2.3. Results

2.3.1. Exposure to cadmium inhibits E20-8 growth

The partial growth curve of E20-8, under control conditions, is represented in **Figure 1**. A growth period of 14 h was defined as suitable for the exposure assays, since at this stage cell growth is already considerable and the cells are still in the exponential growth phase.

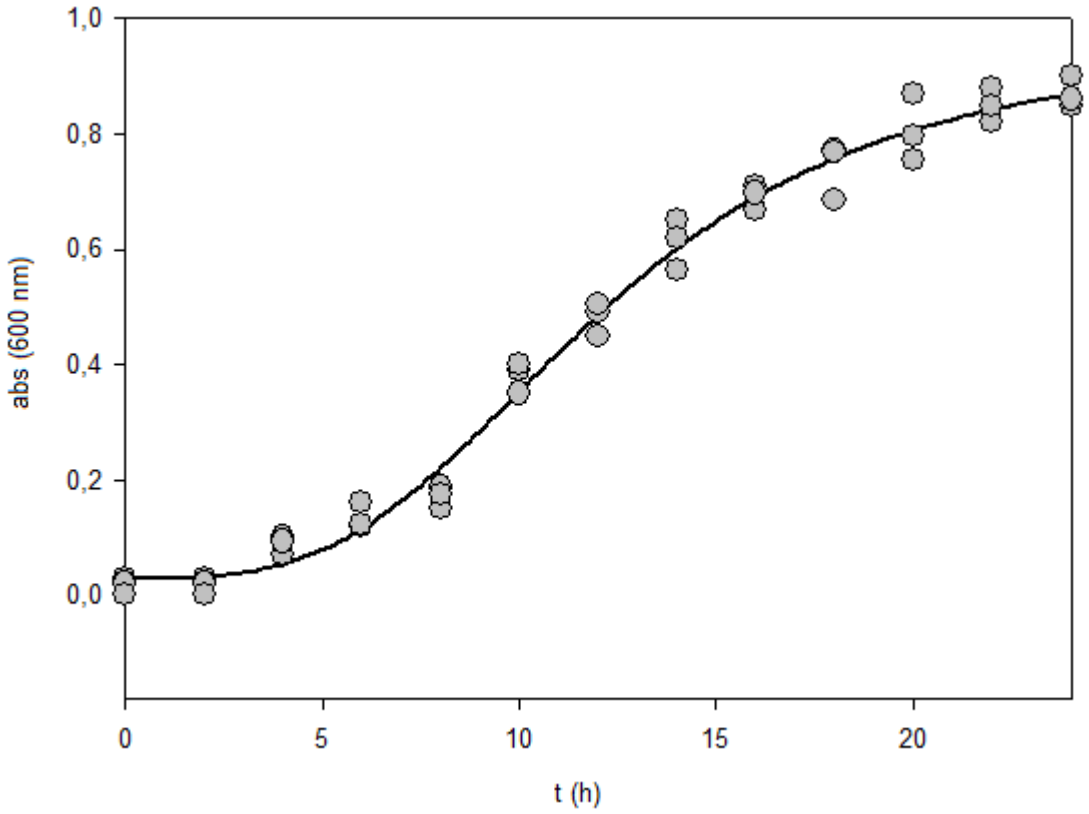

Figure 1 – Growth curve of E20-8. Absorbance at 600 nm at 2 h intervals was plotted for 3 replicates. Nonlinear regression model (four parameter logistic curve). Standard error of estimate: 0.03; R^2=0.99.

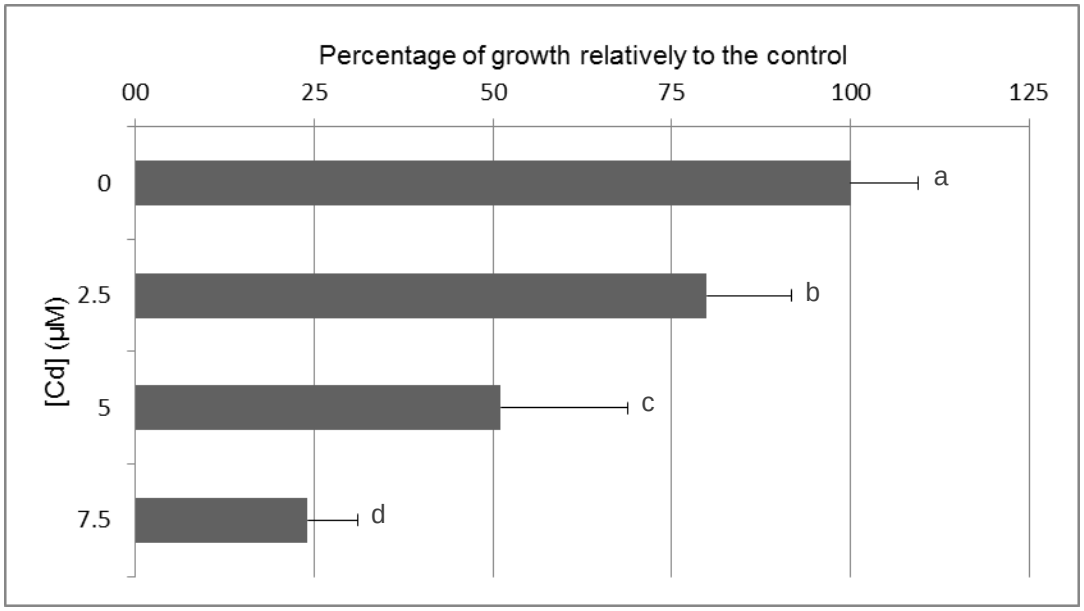

Figure 2 – Percentage of growth relatively to the control of E20-8 exposed to a range of Cd concentrations. The results are the mean values of three replicate experiments. Different letters represent statistically significant differences ($p \leq 0.05$).

Inhibition of E20-8 growth induced by Cd at 14 h was found to be statistically different ($p<0.005$) between the control and the three Cd concentrations tested (2.5, 5 and 7.5 µM). The results from this exposure assay are represented in **Figure 2**.

2.3.2. Sonication procedure

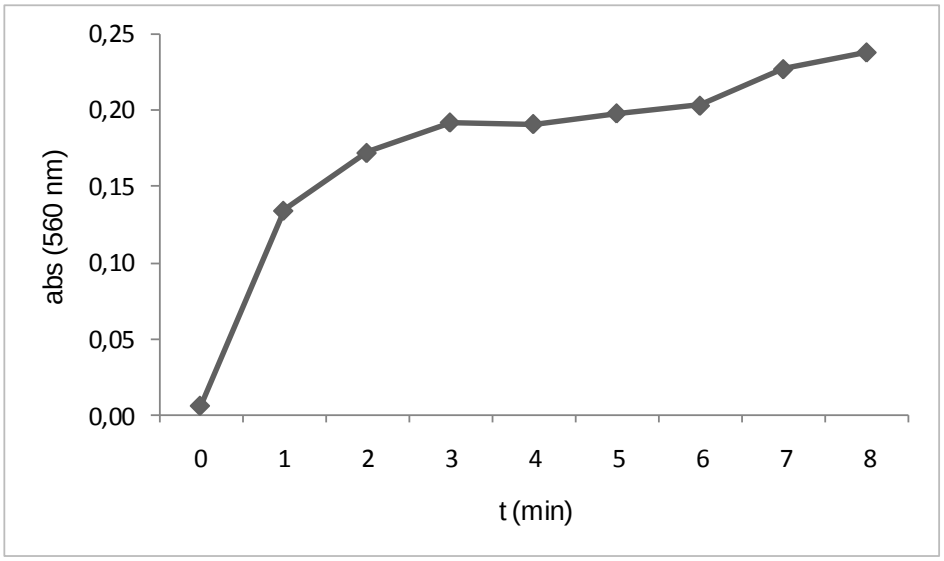

Figure 3 – Assesment of the success of the disruption of cells through estimation of protein content using a Pierce™ BCA Protein Assay Kit.

In order to optimize the sonication procedure, necessary to disrupt cells for the analysis of the intracellular mVOCS, a test was performed prior to the exposure assays. For this, protein content was estimated in intervals of sonication periods of 1 min. The results of this test (**Figure 3**) suggest that 2 min of sonication provide a good compromise between time of sonication and disruption yield.

2.3.3. Intracellular metabolites

The principal components ordination analysis generated from the intracellular mVOCs data shows a distinction between all conditions tested (**Figure 4**). The PCO axis 1 explained 59.7% of the total variance among conditions, revealing a clear distinction between the highest concentration of Cd tested (7.5 µM) and the remaining concentrations (0, 2.5 and 5 µM). While this condition is placed in the negative side of the PCO axis 1, the control condition (0 µM) and the lowest concentration tested (2.5 µM) are both placed in the positive side of the PCO1 axis. The intermediate concentration tested (5 µM) is placed at an intermediate distance between the highest and the lowest concentrations.

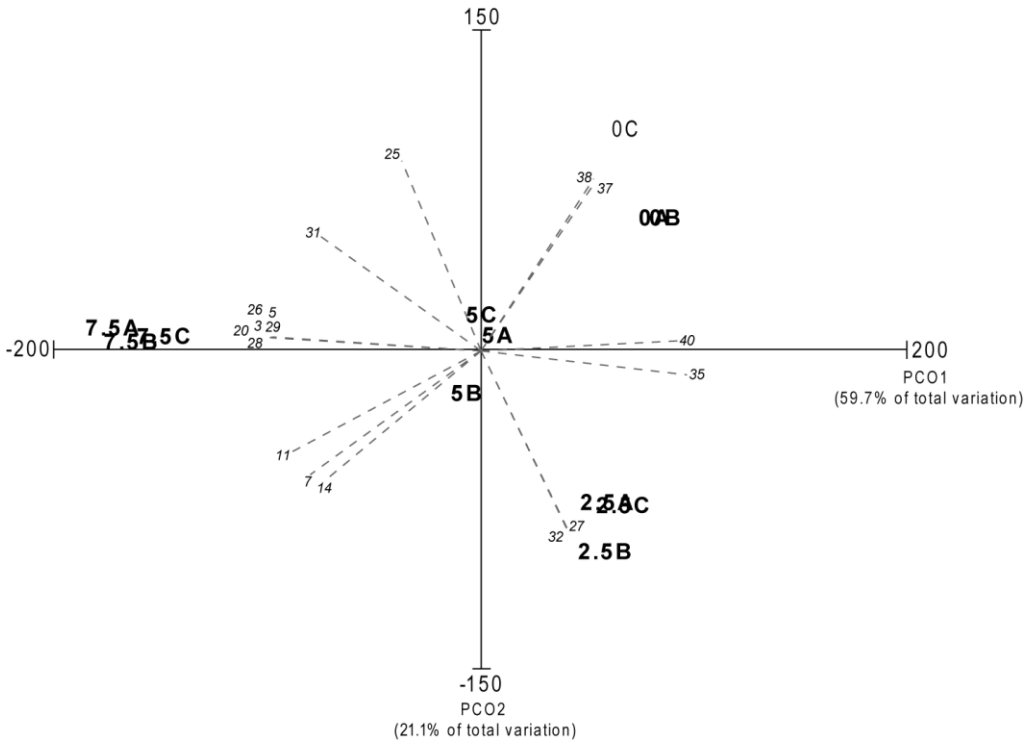

Figure 4 – Principal ordination analysis of the fourth root transformed peak areas of the intracellular mVOCs. Compounds represented as gray numbers are the most correlated (R >0.90). See Table I (Annex 1) for assignment.

The mVOCs methylcyclohexane (3), 1-Propanol (5), ethyl propionate (20), tetrahydrofuran (26), limonene (28), dihydrolinalool (29), towards the negative PCO1, are exclusive to the 7.5 µM Cd condition. PCO axis 2 separates the conditions with Cd from the control condition, explaining approximately 21% of the total variation among conditions. This axis clearly separates the control condition and the lowest Cd concentration tested (2.5 µM Cd), with mVOCs 37 and 38 being exclusive of the control condition, and *p*-Cimene (27) and 32 exclusive of the 2.5 µM Cd. It is also worth to note mVOCs 1-Pentanol (7), 1-Heptanol (11) and 2-Octenal (14), that exist in the presence of Cd but that are not detected in the control.

The methodology employed allowed the detection of 15 intracellular mVOCs in the control condition (**Table I – Annex 1**). The number of metabolites increased when cells were grown in the presence of Cd and the highest number (24 mVOCs) was found at the highest concentration tested (7.5 µM). More detailed information on the number of mVOCs is represented in the Venn diagram (**Figure 5**). Eight mVOCs appeared in all conditions, corresponding to more than 50% of the metabolites in control, about 40% at 2.5 and 5.0 µM and 33% in 7.5 µM Cd. The number of exclusive metabolites of a condition is higher (8) at 7.5 µM, representing 25% of the mVOCs of this condition, while in the remaining conditions the percentage varies between 5 and 15%. Regarding the number of metabolites that appear simultaneously in two or three conditions the number is low (≤2), except for mVOCs common to all three conditions with Cd (4).

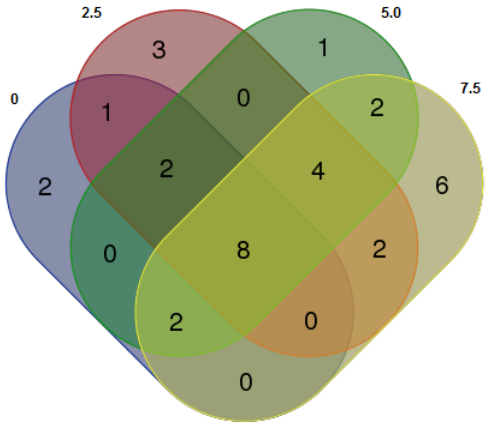

Figure 5 – Venn diagram of rhizobial intracellular metabolite volatile organic compounds (mVOCs), representing the number of exclusive and shared mVOCs between the control (0) and three Cd exposure conditions (2.5, 5.0 and 7.5 µM).

The total area of the metabolites (sum of peak areas of all the metabolites) also varied, increasing along with Cd concentration, being highest at 7.5 µM. The sum of the peak areas of all mVOCs are 1.18E+08 (control), 1.00E+08 (2.5 µM Cd), 1.88E+08 (5.0 µM Cd) and 7.31E+08 (7.5 µM Cd). Such a high value is explained not only by higher peak areas for many mVOCs, but also by the higher number of mVOCs.

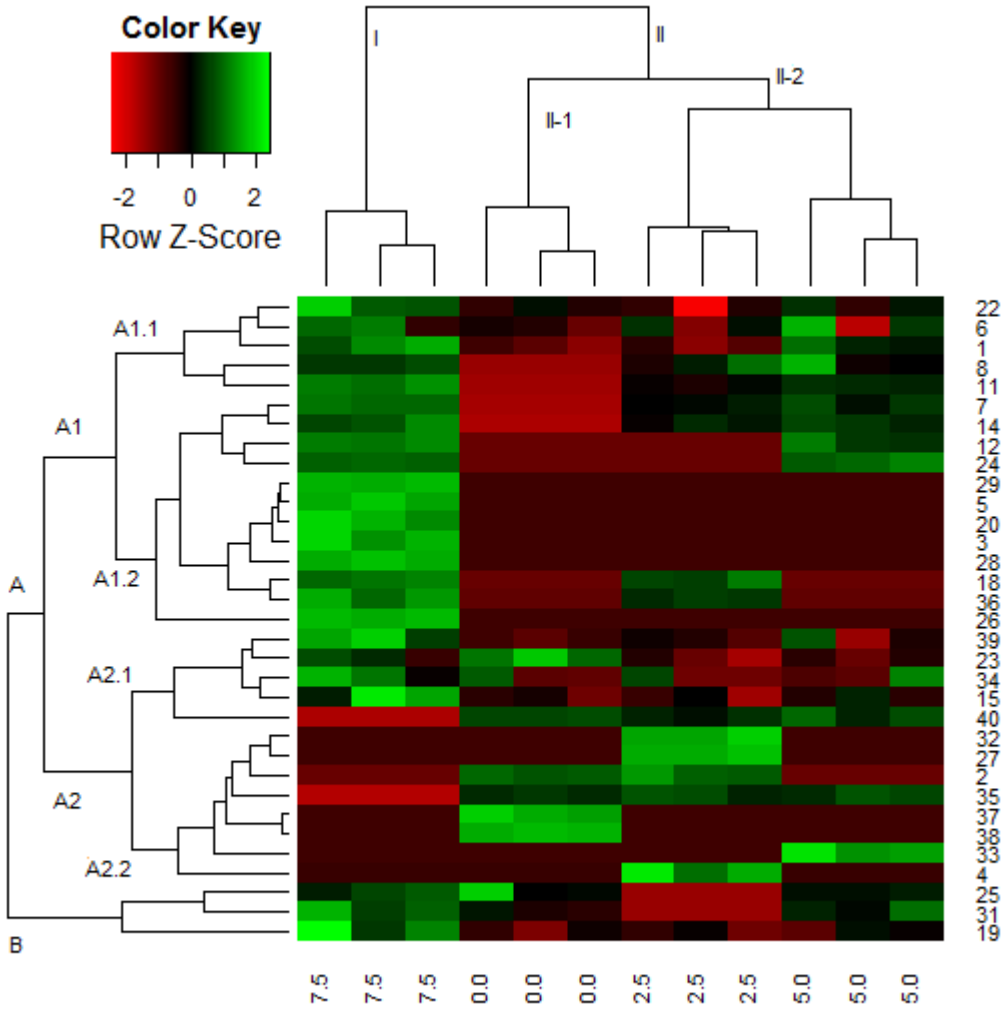

Figure 6 – Heatmap built using the fourth root transformation of the peak areas of intracellular metabolites in the three replicate experiments. Dendrograms were calculated using the Euclidean distance to the mean of metabolite peak areas of each row (mVOCs) and column (samples). Peak number assignment in Table I (Annex 1).

After testing several transformations to the data, a heatmap using the fourth root transformation of the intracellular metabolites peak areas was built (**Figure 6**). The hierarchical clustering dendrogram of the conditions grouped together all replicates of each condition, an indication that the variations detected in peak areas are due to the imposed condition and not to variations between the independent trials. A clear distinction between the volatile metabolome of the highest concentration (7.5 µM) (cluster I) and the other three conditions (cluster II) is noticed, evidencing the higher similarity between the mVOCs profiles of 0, 2.5, 5.0 than between these conditions and 7.5 µM Cd. Nevertheless, mVOCs of cells under low (2.5 µM) and moderate Cd stress (5.0 µM) are more similar to each other than any of them to the control condition. The dendrogram built with the variations among the metabolites clearly shows the division into different groups, with several clusters being formed. Two main clusters (A and B) appeared. Cluster A is divided in two sub-clusters, A1 and A2: A1 contains mVOCs which are exclusive (A1.2) or have higher peak areas (A1.1) for 7.5 µM than for the other conditions; A2.1 includes 4 mVOCs with generally higher peak area for 7.5 µM, although the mVOCs are also present in the other conditions, while A2.2 presents several mVOCs which are characteristic of each condition, namely hexadecanoic acid (4), p-Cimene (27) and 32 for 2.5 µM, 33 for 5.0 µM, and 37 and 38 for control. Cluster B contains mVOCs which generally are detected in most conditions, but which have higher areas for 7.5 µM, particularly, ethyl acetate (19).

2.3.4. Extracelullar metabolites

The PCO generated from the released mVOCs matrix (**Figure 7**) showed that 57.7% of the variation between conditions is explained by the PCO1, with the highest concentration tested being represented in the negative side of PCO axis 1, and the remaining conditions in the positive side. Similarly to the PCO obtained with intracellular samples, the 5 µM Cd concentrations also occupy an intermediate position on this axis. However, while for the intracellular samples there is a clear separation between the control and 2.5 µM Cd conditions, the extracellular metabolome of these two conditions are grouped together. The mVOCs 5-Methyl-1-hexene (2), 3,4-Dimethyl-3-hexanol (9), 3,4-Dimethyl-2-hexanone (17), ethyl propionate (20), dimethyl trisulfide (24) and dihydrolinalool (29) characterized the 7.5 µM Cd condition. mVOC 3-Methyl-1-butanol (6) increases as the Cd concentration increases, while methanethiol (22) is only detected in

the two highest concentrations tested. mVOCs 3-Buten-2-one (15), 4-Heptanone (16), ethyl propionate (20) and 40 are absent from 7.5 µM Cd, and therefore are also responsible for the distance observed between this condition and the other conditions tested. The 5 µM condition is placed towards the positive side of PCO2, which explains approximately 24 % of the variance, displaying mVOCs pentane (1), 1-Octanol (12), isopropyl laurate (21), bornyl acetate (30) and 37 as characteristic of this condition.

In the control condition, 8 mVOCs were detected extracelullarly, the same number found at 2.5 µM Cd (**Table I – Annex 1**). The number of mVOCs increased in the moderate (5.0 µM) and high (7.5 µM) Cd concentrations, to 12 and 11 mVOCs, respectively.

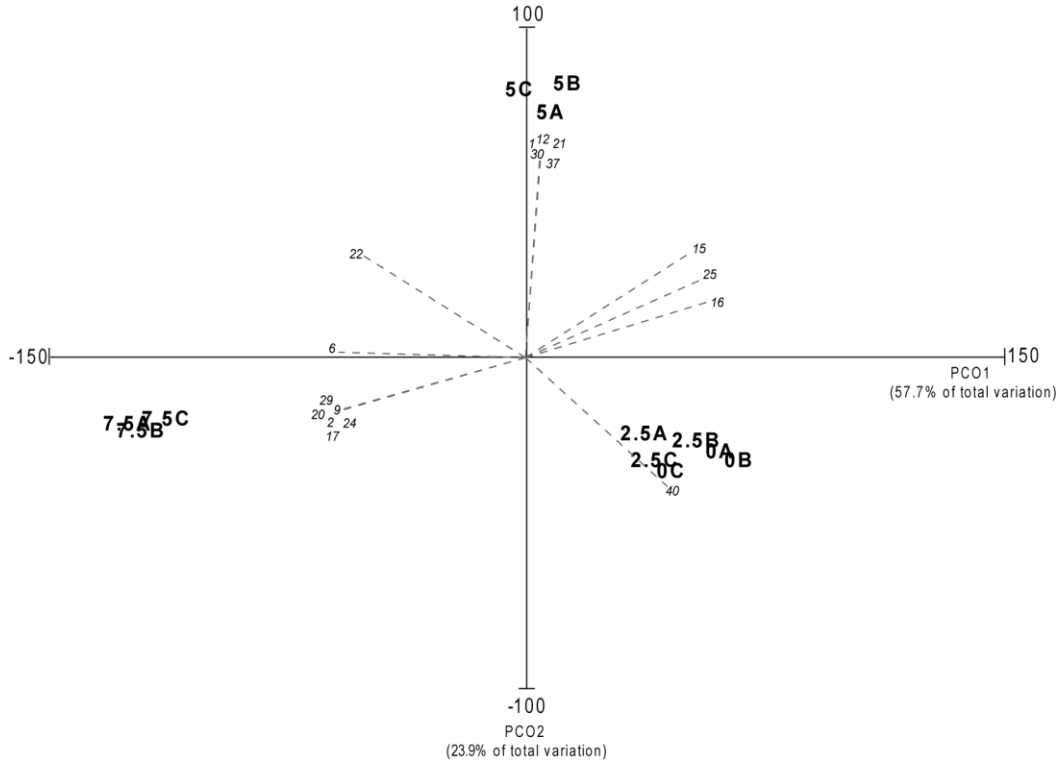

Figure 7 - Principal ordination analysis build using the fourth root transformed peak areas of the released mVOCs. Compounds represented as gray numbers are the most correlated (R >0.90). See Table I (Annex 1) for compound name assignment.

Extracellular mVOCs unique to one or common to more than one condition are shown in **Figure 8**. The number of unique metabolites is clearly lower (1) in the control and 2.5 µM Cd compared to the highest concentrations (5.0 and 7.5 µM), with six mVOCs

each, corresponding to 50% and 54% of the total number, respectively, and showing clearly different mVOCs profiles.

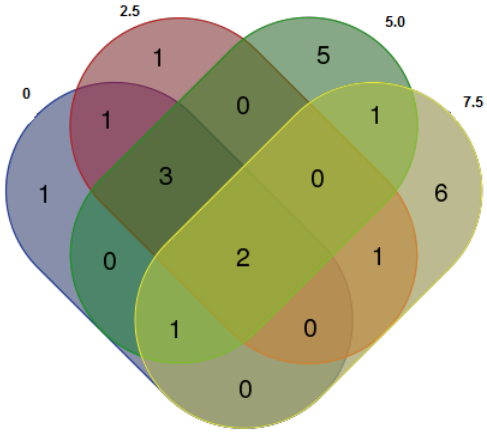

Figure 8 – Venn diagram of rhizobial released metabolite volatile organic compounds (mVOCs), representing the number of exclusive and shared mVOCs between the control (0) and three Cd exposure conditions (2.5, 5.0 and 7.5 µM).

The hierarchical clustering of the conditions generated from the data of the released mVOCs (**Figure 9**) reveals that, as with the intracellular samples, replicates of the same condition are grouped together, supporting once again that differences detected are originated on metabolic differences induced by each condition and not on variations between independent trials. A clear distinction between the volatile metabolome of the 7.5 µM Cd (cluster I) and the volatile metabolomes of the remaining three conditions (cluster II) is noticed. However, contrary to the intracellular clustering, the volatile metabolome of the control and 2.5 µM are more similar to one another than to 5.0 µM Cd. The dendrogram built with the variations among mVOCs clearly shows the highest Cd concentration as the condition in which cells displayed the most distinct metabolome, followed by the concentration 5 µM. Clustering of the mVOCs shows the formation of two distinctive signature clusters, A and B, for concentrations 7.5 and 5 µM, respectively. Cluster A1.3 represents two mCOVCs, 3-Buten-2-one (15) and 4-Heptanone (16), that exist either in the control, 2.5 µM, and 5 µM Cd, but not at 7.5 µM Cd. Cluster A2 includes mVOCs which are characteristic of control (tetrahydrofuran (26)) and 2.5 µM (limonene(28)). Cluster B is composed of two mVOCs which exist in all conditions but in much higher quantity for 7.5 µM Cd, 3-Methyl-1-butanol (6) and ethyl acetate (19). The latter also displays the same pattern of response intracellularly.

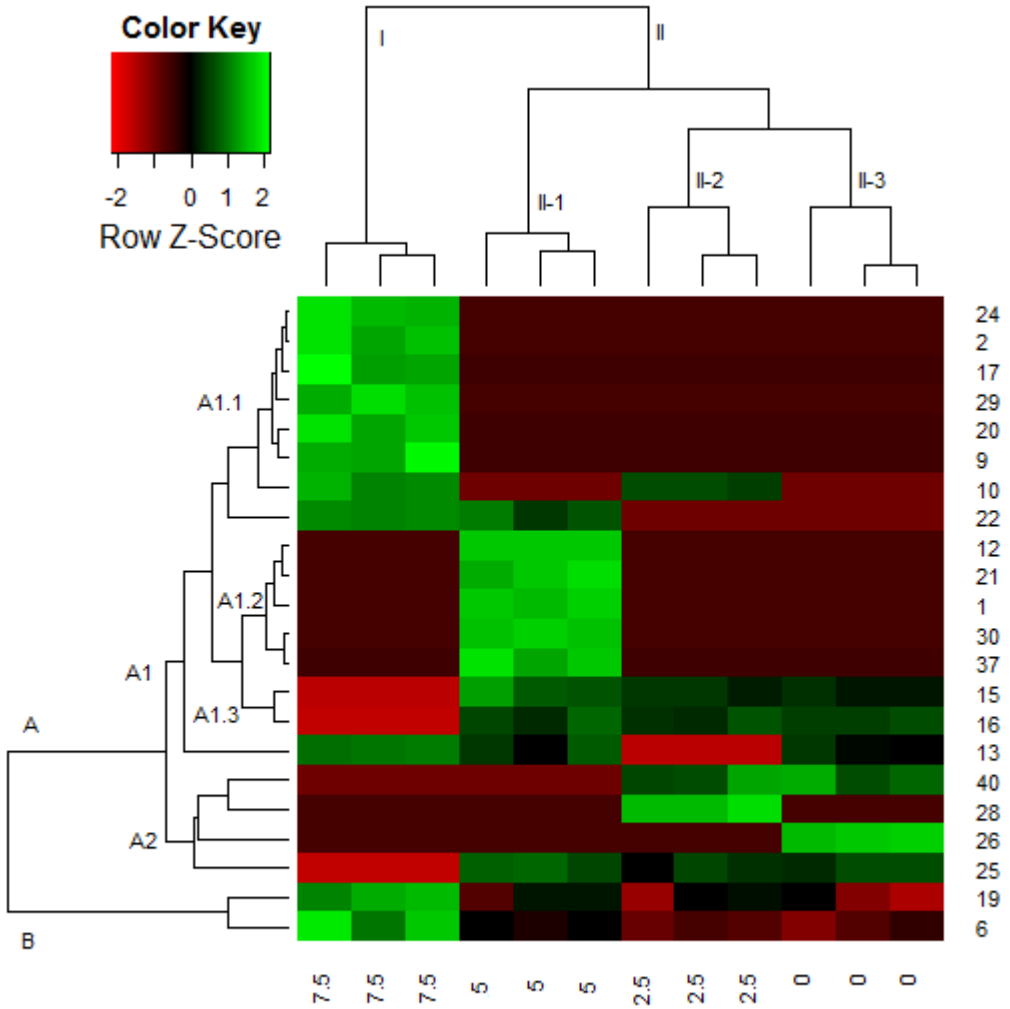

Figure 9 - Heatmap built using the fourth root transformation of the peak areas of released compounds present in the three replicate experiments. Dendrograms were calculated using the Euclidean distance to the mean of compound peak areas of each row (mVOCs) and column (samples). Peak number assignment in Table I (Annex 1).

The sum of the peak areas of the extracellular mVOCs increased along the increment of Cd concentration: 2.29E+08 (control), 2.67E+08 (2.5 µM Cd), 4.11E+08 (5.0 µM Cd) and 1.43E+09 (7.5 µM Cd).

2.4. Discussion

The PCO results confirmed the validity of the conditions used for the exposure assays and of the methodology used for HSPME and GCxGC-ToFMS analysis, since the

replicates were grouped together and there was a clear distinction between the concentrations tested. It is worth to note that the wash routine and the sonication procedure were efficient, allowing for a clear distinction between the intracellular samples and extracellular samples.

Taken together, the results show that in the presence of Cd, especially at the highest concentration tested (7.5 µM), cells produce higher number and especially higher amounts of mVOCs, which are accumulated intracellularly or excreted to the culture medium, evidencing the changes in the secondary metabolism induced by Cd.

The metabolites that are exclusive of the control condition certainly are negatively affected by the stress caused by Cd toxicity. Some enzymes are particularly vulnerable to Cd, since it shares some chemical and physical properties with neighboring elements in the periodic table, such as Zn, Cu, Fe, or Mn, it can displace Zn and Cu from metalloenzymes (Price and Morel, 1990), leading to inhibition of their activity and interfering with normal cell metabolism (Branco et al., 2010; Santos et al., 2013; Torres et al., 2000). Cd strongly inhibits the activity of enzymes containing sulfhydryl groups in their active sites due to the high affinity to these chemical groups (Lehninger et al., 2008).

On the other hand, the exclusive metabolites of metal-exposed cells might originate from new pathways, as a response to the new conditions imposed by an increase of Cd ions. The new mVOCs appear at 5.0 and 7.5 µM Cd, and might be related to the induction of repair mechanisms or to the failure of cell processes, since at these concentrations the interference with cellular metabolism should be high, as evidenced by the high inhibition of growth of the cells exposed to these concentrations of Cd (Figure 1). Two VSC were detected, methanethiol (22) and dimethyl trisulfide (24), both found intra and extracelullarly especially at the highest Cd concentrations. These metabolites are originated from the metabolism of the amino acid methionine and have been reported in bacteria (Schulz and Dickschat, 2007). Methanethiol is highly toxic and hard to detect due to its high volatility (Levitt et al., 1999; Schulz and Dickschat, 2007). Methanethiol is present in all conditions intracellularly, but higher peak areas are observed at 5.0 and 7.5 µM Cd, and is only detected in the culture medium at these concentrations, with higher peak area at 7.5 µM when compared to 5.0 µM Cd. Dimethyl trisulfide is not present intracellularly in the control and in 2.5 µM of $CdCl_2$ but at 5.0 and 7.5 µM of Cd is.

Extracelullarly, it is only present at 7.5 µM of Cd. Dimethyl trisulfide is derived from methanethiol (Schulz and Dickschat, 2007), and is also toxic (Beattie and Torrey, 1986). Taking into consideration that the two mVOCs are originated from methionine, the results suggest that in the conditions of highest exposures to Cd there is an increment in the degradation of the amino acid methionine. In fact, the accumulation of methanethiol, a product of methionine metabolism (Finkelstein and Benevenga, 1986), may occur due to hydrolysis of proteins, which owing to the prevailing oxidative conditions at 5.0 and 7.5 µM Cd concentrations, are oxidized in an extent that the mediated repair and refolding of proteins are no longer possible and degradation is the plausible end (Ezraty et al., 2005). The degradation of the proteins frees methionine residues, whose metabolization originates methanethiol and dimethyl trisulfide.

Ethyl acetate (19) is hard to detect due to its high volatility (Schulz and Dickschat, 2007) but was detected in our study. Its detection may be explained due to the use of a high efficient microextraction technique (SPME) combined with an advanced and high sensitive gas chromatographic methodology, such as (GC×GCToFMS). This mVOC exists both intra and extracelullarly, in all conditions, but peak areas increase to about 10 times (intracellularly) and 12 times (extracelullarly) the peak area of the control at the highest Cd concentration (7.5 µM). Apart from fatty acids metabolism, ethyl acetate might also be originated from sugar and amino acids catabolism (Nardi et al., 2002) Thus, in our experiment ethyl acetate might be produced from several pathways which may all be potentially induced by oxidative stress. One of the possible pathways could involve the catabolism of peroxidized lipids, since the synthesis of esters use alcohols and thiols as substrates (Nardi et al., 2002) and an increment of alcohols should increase ethyl acetate synthesis. In fact, the sum of the peak areas of the low chain aliphatic alcohols (C_5-C_{10}) detected in the intracellular fraction reveals that there is an increment in the peak areas with exposition to increasing Cd concentrations. The sum of the peak areas for 2.5, 5.0 and 7.5 µM Cd increased 5, 10 and 23 times compared to the control, respectively (Table I – Annex 1). Low chain aliphatic alcohols (C_5-C_{12}), can arise from the degradation of peroxidized polyunsaturated fatty acids by the activity of hydroperoxide lyases, enzymes belonging to the cytochrome P450 family. The high increase of these alcohols peak areas may reflect the high peroxidation of unsaturated fatty acids do to an intracellular environment increasingly oxidated. Another possible pathway of ethyl acetate and other

esters generation could be methanethiol (Nardi et al., 2002), which was already described as increasing at high Cd concentrations. Aliphatic alcohols such as 1-Pentanol (7) are volatile products of lipid peroxidation. Aliphatic alkanes, namely pentane (1) were detected in intra and extracellular mVOCs, which further contribute to support the hypothesis that most of the alterations of the metabolome detected in this work are due to oxidative stress. The alkane pentane can be originated from lipid peroxidation (Pyo et al., 2008). Methylciclohexane (3), a cyclic alkane, is only present intracellularly at the higher degree of stress (7.5 µM Cd), while pentane is present in all conditions but with peak areas 3 and 5 times higher at 5 and 7.5 µM Cd, respectively, when compared to the control.

Several metabolites were detected in this work which that bioactivity reported in the literature. 1-Hexanol (8) has been reported as a compound that can be used to control *Botrytis* in strawberry (Archbold et al., 1997). This mVOC was also reported as a potent inhibitor of *Escherichia coli* growth (Ingram and Vreeland, 1980). Therefore, it may be inferred that in the present study, 1-Hexanol might be a key inhibitor of cell growth in the presence of Cd. 1-Hexanol was described to increase membrane fluidity but not to induce lysis (Ingram and Vreeland, 1980). Interestingly, lipid peroxidation was reported to decrease membrane fluidity (Cabiscol et al., 2000), so hypothetically 1-Hexanol could be counteracting the decrease in membrane fluidity. Dimethyl trisulfide can be produced by *Pseudomonas* and is able to work as antifungal against *Sclerotinia sclerotiorum*, with a complete inhibition of mycelia and sclerotia formation (Fernando et al., 2005). The mVOC 3-methyl-1-butanol- (6) was detected in fungal endophytes which were able to inhibit *Fusarium oxysporum* (Ting, 2010), and 3-methyl-1-butanol and methanethiol were produced by *Brevibacterium linens* in concentrations that inhibited the germination of spores of *Penicillium expansum* (Beattie and Torrey, 1986). Although this is not the focus of this work, the detection of volatiles with bioactivity in the *Rhizobium* strain E20-8 adds new information about its ability to produce compounds capable of controlling microorganisms pathogenic to plants, contributing with new scientifically relevant information, strengthening the role of this group of bacteria as PGPRs, and thus increasing its economic and ecological relevance.

Chapter 3
Conclusion and future prospects

The impact of free Cd ions in the cytosol is so great that the volatile metabolome suffered profound changes compared to the control. The presence of Cd induced not only qualitative (number of metabolites) but also quantitative (expressed as peak area) changes, leading to the synthesis of 27% to 60% new metabolites and increasing the total peak areas when comparing to the control. Some families of metabolites, the low chain aliphatic alcohols in particular, underwent major changes. The total area of low chain aliphatic alcohols increased nearly exponentially with the increase of Cd concentration ($R^2 = 0.96$) along the Cd concentration. This slope certainly reflects the intracellular redox state, since the increased stress oxidation induces the increase of lipid peroxidation, whose degradation gives, among other compounds, low chain aliphatic alcohols. This family of compounds thus shows potential to be an excellent redox state biomarker of the bacteria cytoplasm.

On the other hand, ethyl acetate may reflect the oxidative effects of Cd ions in a more comprehensive mode, since its synthesis results not only from the catabolism of fatty acids but also from amino acids and sugars. The synthesis of ethyl acetate mainly increases at higher Cd concentrations (5 and 7.5 uM), indicating that this mVOC is less sensitive than aliphatic alcohols to the effects of Cd ions. The increase of ethyl acetate occurs both intra and extracelullarly, so it may be a good biomarker of moderate and high stress in bacteria. Since this metabolite can be detected extracelullarly, it could be used as a non-destructive method to detect stress, and may be a good option for overtime studies or when biomass use should be avoided.

In this study, it was not tested if the cells exposed to Cd would express the same metabolic pattern observed once metal stress was relieved. It could be interesting to investigate the possibility of *Rhizobium* to preserve a "memory" and be able to maintain the metabolic alterations, possibly implicating changes in the genomic and transcriptomic expressions. A study with a strain of Pseudomonas alcaligenes resistant to tellurite showed that the metabolic changes induced by exposure to the metalloid turned the cells into a state in which resistance to other metals was also kept activated, along with a series of

physiological adaptations, such as alterations in the glutathione levels (Tremaroli et al., 2009).

It could also be interesting to test different media formulations and different types of stress. The genome of *R. leguminosarum* biovar *viciae* strain 3841 is large, a fact that is thought to be related with the potential advantage of having extra genetic material to face the challenges of the heterogeneous soil environment (Young et al., 2006). Many research questions could be addressed on this matter. For instance, if faced with the same stress induced by Cd but with a different substrate, would the most affected metabolites be the same as in this work? And what would happen if along with the metal induced stress cells were also deprived for key nutrients or had to cope with another stress variable, such as a non-optimum growth temperature? The potential results could help understanding if the plasticity found in the genome is reflected on the volatile metabolome.

The study of the interactions between bacteria and other organisms has become an interesting topic of research, particularly if variables such as environmental changes and pollution are considered. It is consensual that many agricultural systems are contaminated with xenobiotic substances such as non-essential metals. Therefore, it is important to try to understand the effects that these contaminants may carry to the key players in these biological and economically important interactions. Although extensive research has been published concerning the effects at physiological or higher levels of biological organization, our knowledge regarding molecular pathways is still limited. Information at the molecular level is important since alteration might have reflections at superior levels, potentially being able to affect communities and ecosystems, and this information is necessary to understand the processes under an integrative Systems Biology perspective.

Chapter 4
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Annex 1

Table 1 – Means of fourth root transformed peak areas of mVOCs of 0 (control) 2.5, 5 and 7.5 µM Cd conditions.

Compound no.	^1t_R [a]	^2t_R [b]	Compound name	CAS	Formula	RI_{calc} [c]	RI_{lit} [d]	Intracellular				Extracellular			
								0	2.5	5.0	7.5	0	2.5	5	7.5
Hydrocarbons															
1	78	0.310	Pentane	109-66-0	C5H12	573	500	51.5	52.8	69.8	78.4	-	-	46	-
2	144	0.360	5-Methyl-1-hexene	3524-73-0	C7H14	649	639	22.4	24.5	-	-	-	-	-	36.9
3	168	0.370	Methylcyclohexane	108-87-2	C7H14	676	718	-	-	-	35.8	-	-	-	-
Fatty acid															
4	1734	2730	Hexadecanoic acid	57-10-3	C16H32O2	1960	1960	-	60.4	-	-	-	-	-	-
Alcohols															
5	90	0.770	1-Propanol	71-23-8	C3H8O	587	595	-	-	-	41.1	-	-	-	-
6	186	1630	3-Methyl-1-butanol	123-51-3	C5H12O	698	727	48.6	50.6	53.2	56.4	98.7	99.1	111	143
7	234	2030	1-Pentanol	71-41-0	C5H12O	754	756	-	37.9	45.6	58.7	-	-	-	-
8	444	2360	1-Hexanol	111-27-3	C6H14O	877	878	-	62.1	68.5	70.6	-	-	-	-
9	582	0.990	3,4-Dimethyl-3-hexanol	19550-08-4	C8H18O	946	-	-	-	-	-	-	-	-	43.2
10	612	1200	4-Methyl-2-heptanol	56298-90-9	C8H18O	962	-	-	-	-	-	-	24.8	-	35.4
11	642	1620	1-Heptanol	111-70-6	C7H16O	978	972	-	46.2	61	87.8	-	-	-	-
12	804	1330	1-Octanol	111-87-5	C8H18O	1078	1078	-	-	51.8	66.2	-	-	38.4	-
Aromatic alcohols															
13	882	3920	Phenylethyl Alcohol	60-12-8	C8H10O	1135	1141	-	-	-	-	49.6	-	55.8	73.4
Aldehyde															
14	780	0.950	2-Octenal	2363-89-5	C8H14O	1062	1061	-	36.3	43.3	51.6	-	-	-	-
Ketones															
15	96	0.530	3-Buten-2-one	78-94-4	C4H6O	594	-	28.4	27.5	30.9	39.6	24.4	26.3	33.8	-
16	444	0.800	4-Heptanone	123-19-3	C7H14O	876	869	-	-	-	-	24.4	23.4	24.4	-
17	498	0.780	3,4-Dimethyl-2-hexanone	19550-10-8	C8H16O	900	-	-	-	-	-	-	-	-	33.3
18	666	0.730	3-Octanone	106-68-3	C8H16O	991	989	-	28.9	-	33.7	-	-	-	-

Table I (continued) - Means of fourth root transformed peak areas of mVOCs of 0 (control) 2.5, 5 and 7.5 µM Cd conditions.

Compound no.	$^{1}t_R$ [a]	$^{2}t_R$ [b]	Compound name	CAS	Formula	RI_{calc} [c]	RI_{lit} [d]	Intracelullar				Extracelullar			
								0	2.5	5.0	7.5	0	2.5	5.0	7.5
Esters															
19	108	0.480	Ethyl acetate	141-78-6	C4H8O2	607	608	72.2	73.7	81.6	129	95.5	112	122	176
20	162	0.540	Ethyl propionate	105-37-3	C5H10O2	670	691	-	-	-	43.3	-	-	-	45.8
21	1440	0.590	Isopropyl Laurate	10233-13-3	C15H30O2	1626	1618	-	-	-	-	-	-	34.7	-
Sulfur containing compounds															
22	72	0.400	Methanethiol	74-93-1	CH4S	566	500	51.5	41.4	54.7	68.5	-	-	43.5	54.8
23	150	0.780	Methyl thioacetate	1534-08-3	C3H6OS	656	699	55	31.2	35.2	43.3	-	-	-	-
24	618	1300	Dimethyl trisulfide	3658-80-8	C2H6S3	965	964	-	-	44	42.6	-	-	-	37.2
Furans															
25	108	0.410	2,3-Dihydrofuran	1191-99-7	C4H6O	607	-	77.7	-	58.4	73.8	54.8	49.8	60.5	-
26	114	0.420	Tetrahydrofuran	109-99-9	C4H8O	614	621	-	-	-	112	73.5	-	-	-
Terpenes															
27	720	0.670	*p*-Cimene	99-87-6	C10H14	1024	1024	-	20.2	-	-	-	-	-	-
28	726	0.560	Limonene	5989-54-8	C10H16	1027	1027	-	-	-	59.1	-	32.2	-	-
29	840	0.770	Dihydrolinalool	78-69-3	C10H22O	1101	1097	-	-	-	43.8	-	-	-	38
30	1080	0.710	Bornyl acetate	76-49-3	C12H20O2	1286	1286	-	-	-	-	-	-	27.4	-
Others															
31	78	0.330	*m/z* (43, 59, 74, 45)	-	C6H14O2	573	-	37.7	-	56.5	74.1	-	-	-	-
32	102	0.720	*m/z* (45, 75, 59, 43)	-	C4H10O3	601	-	-	30.6	-	-	-	-	-	-
33	120	0.330	*m/z* (57, 41, 42, 43)	-	C8H12O3	621	-	-	-	33.7	-	-	-	-	-
34	180	0,.450	*m/z* (45, 73, 43, 47)	-	C6H14O2	690	-	29.1	27.4	30.7	40	-	-	-	-
35	468	0.750	*m/z* (43, 81, 79, 39)	-	C8H12O	887	-	23.4	25.4	25.5	-	-	-	-	-
36	534	0.760	*m/z* (43, 70, 61, 42)	-	C7H14O2	920	-	-	20.5	-	32.6	-	-	-	-
37	798	0.670	*m/z*(58, 57, 85, 41)	-	C10H20O	1074	-	25.7	-	-	-	-	-	26.2	-

Table I (continued) - Means of fourth root transformed peak areas of mVOCs of 0 (control) 2.5, 5 and 7.5 µM Cd conditions.

Compound no.	^1t_R [a]	^2t_R [b]	Compound name	CAS	Formula	RI_{calc} [c]	RI_{lit} [d]	Intracelullar				Extracelullar			
								0	2.5	5.0	7.5	0	2.5	5.0	7.5
Others															
38	810	0.460	*m/z (69, 41, 55, 39)*	-	C10H16O	1081	-	23.6	-	-	-	-	-	-	-
39	1284	0.860	*m/z* (43, 54, 67, 41)	-	C16H30O2	1471	-	38.4	39.8	40.7	51.2	-	-	-	-
40	1512	0.760	*m/z* (55, 74, 41, 67)	-	C19H36O2	1701	-	65.5	55.8	66	-	49.5	46.8	-	-

[a] Retention times of first dimension

[b] Retention times of second dimension

[c] RI: retention index obtained through the modulated chromatogram

[d] RI: retention index reported in the literature for one-dimensional GC with 5%-phenyl-methylpolysiloxane GC column or equivalent (Adams, 2007; Bylaite and Meyer, 2006; Carrapiso et al., 2002; Flamini et al., 2005; Guichard and Souty, 1988; Lucero et al., 2006; Pino et al., 2005; Ramarathnam et al., 1993; Rembold et al., 1989; Rodríguez-Burruezo et al., 2004; Rout et al., 2007; Saroglou et al., 2007; Song et al., 2003; Verdier-Metz et al., 1998; Weissbecker et al., 2004; Wu et al., 2005; Wu and Cadwallader, 2002; Xu et al., 2003)