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

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

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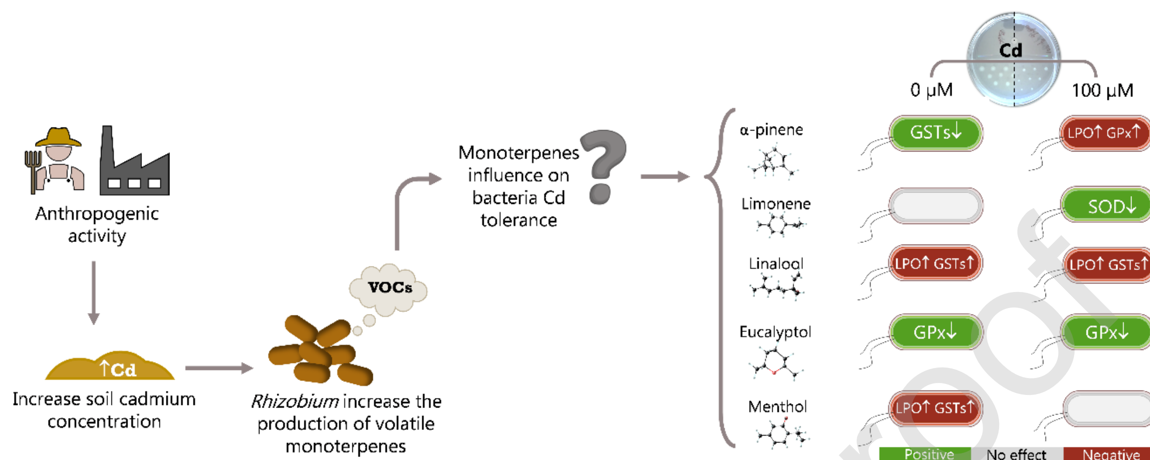
Graphical abstract



Highlights

- Different monoterpenes have distinct influences on *Rhizobium* E20-8
- Eucalyptol and α-pinene evidenced antioxidant properties in the absence of stress
- Majority of the monoterpenes did not minimize the effects of Cd
- Most monoterpenes did not alleviate oxidative stress
- Limonene and eucalyptol induce *Rhizobium* growth in the presence of Cd

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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

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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.

Keywords

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

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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 [1]. For this reason, *Rhizobium* strains are focus of great agricultural and economic interest for promoting plant growth, increasing productivity [2] and reducing inorganic nitrogen fertilization [3]. *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 [4].

Microorganisms can communicate with each other by quorum sensing (QS) [5] or quorum quenching [6] allowing a collective response to environmental changes [7]. Microorganisms are also capable of producing and releasing a wide range of volatile organic compounds [8,9], which can diffuse through the complex soil matrix [8]. Microbial volatile organic compounds (MVOCs) are important in the interactions of microbial communities at-a-distance [8,10]. 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 [11]. To date, approximately 2000 microbial volatile compounds were found in almost 1000 species studied [12]. Most MVOCs are alkanes, alcohols, ketones, aldehydes, volatile sulfur compounds, aromatic compounds and terpenoids [9,10]. However, due to the small number of species studied, compared to the 1 trillion (10^{12}) microbial species estimated to exist [13], 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) [14]. For example, plant volatiles can influence the microorganism's communication (QS) [15], demonstrating a volatile interaction between these different two life kingdoms. VOCs

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can also play a role in the communication between bacteria, influencing their motility and drug resistance [16]. Previous studies [17,18] have reported antioxidant properties of VOCs in animal and plant cells, however knowledge on the influence of these compounds in soil bacteria is scarce [19].

Monoterpenes may play a role in nutrient cycling and in the rhizosphere community, some can be used as substrates for bacterial growth [14], and they might be also involved in microbial interactions [10]. Plant monoterpenes can be synthetized and accumulated in roots and rhizomes of numerous plant species [20,21] and were reported to induce the production of violacein (which has antibiotic activity) and pyocyanin (a toxin) [15]. Bacteria are also capable of producing terpenoid compounds and are considered an unexplored source of new natural products [22]. The concentrations of monoterpenes in the environment depend on the number and proximity of monoterpene 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 [14,23,24]. Monoterpenes were reported to have antimicrobial and antiherbivore activities, providing a belowground protection to the plants capable of accumulating them [25–27]. Monoterpenes released by microorganisms might also have identical properties [25].

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 [28,29]. Among the many factors that influence soil microbial communities toxic elements are one of the most detrimental, due to their high toxicity and persistence [30]. Cadmium (Cd) is a toxic metal that occurs in nature at low concentrations as a component of rocks, sediments, soils and dusts [31]. However, anthropogenic activities

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such as industrial production of batteries, plastics, alloys and synthetic materials lead to an increase of natural levels of Cd in the soil [32] by releasing Cd into the environment through atmospheric emissions, effluents, sludges, or solid waste [31]. In agricultural soils the main route of Cd contamination is the application of phosphate fertilizers [33]. Usually, in soil, 1.8–53 μM of Cd can be found [34]. However, the concentration can surpass 2669 μM in extremely contaminated sites [35], with impacts to microbial communities [36].

A study performed by Cardoso et al. [37] 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 of different concentrations of α-pinene, limonene, eucalyptol, linalool and menthol. Growth and biochemical status were assessed in the presence and absence of Cd.

2. Experimental Procedures

2.1. Experimental conditions

Rhizobium leguminosarum strain E20-8 (partial 16S rRNA sequence Genbank accession number KY491644), isolated from the nodules of *Pisum sativum* from Elvas, Portugal, and previously described as tolerant to Cd [37–39], was used in the present study. To evaluate the influence of VOCs in the growth of E20-8 when exposed to Cd (EC_{50}=100 μM) a screening was made by growing the bacteria in yeast extract mannitol (YMA) medium [40] and YMA supplemented with Cd, exposed to different concentrations of VOCs (0 nM,

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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) and menthol (CAS: 2216-51-5) 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. In one side of the plate the medium was inoculated with 18 colonies, while the other side contained a thin layer of medium and a disc which received 10 µL of a VOC solution. VOCs used in this study were dissolved in 70% ethanol. Solvent exposure was also tested in a volume equal to that applied to the disks (10 µL), being used as control since it did not influence bacterial growth. After inoculation, the plates were placed in an incubator at 26 °C, for 60 hours. Three divided Petri dishes were performed for each condition. At the end of the incubation period, the plates were photographed, and colonies collected. All colonies of a plate were pooled and 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 concentration of VOC.

2.2. Extraction

Extraction buffer (potassium phosphate 50 mM, pH 7.0) was added to each sample (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).

2.3. Protein content

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The Biuret method was used to determine the protein content [41]. In a microplate, 275 μL of Biuret reagent was added to 25 μL of supernatant. The microplates were incubated for 10 minutes at room temperature and absorbance was read at 540 nm. Bovine serum albumin (BSA) was used as standard. Results were expressed in mg protein per g of colony.

2.4. Oxidative damage (lipid peroxidation)

Lipid peroxidation (LPO) was measured by quantification of thiobarbituric acid reactive substances (TBARS), according to the protocol described by Buege and Aust [42]. To 37.5 μL of sample, 112.5 μL of 20% trichloroacetic acid and 150 μL of 0.5% thiobarbituric acid (in 20% trichloroacetic acid) were added. A blank containing 115 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 absorbance was read at 532 nm and nmol of MDA equivalents per g of colony were calculated using the molar extinction coefficient 1.56×10^5 M^{-1} cm^{-1}.

2.5. Superoxide dismutase

Superoxide dismutase (SOD) activity was determined by the quantification of nitroblue tetrazolium (NBT) diformazan formed by the reaction of NBT with superoxide radicals as described by Beauchamp and Fridovich [43]. In microplates, 25 μL of supernatant and 250 μL reaction buffer (50mM Tris-HCl (pH 8), 0.1mM diethylenetriaminepentaacetic acid (DTPA), 0.1mM hypoxanthine), 68.4 μM (NBT) and 25 μL xanthine oxidase (56.4 mU/mL). The absorbance was read at 560 nm. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Results were expressed in U per mg of colony.

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2.6. Glutathione peroxidase

The activity of glutathione peroxidase (GPx) was determined according to the method described by Paglia and Valentine's [44]. In microplates 30 μL of supernatant, 112.5 μL dilution buffer [44], 60 μL GSH (5 mM), 45 μL cumene hydroperoxide (2 mM), 30 μL glutathione reductase (25U/mL) and 22.5 μL NADPH (2 mM) were added. 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.

2.7. Glutathione-S-transferases

Glutathione-S-transferases (GSTs) activity was determined using 1-chloro 2,4 dinitrobenzene (CDNB) and reduced glutathione (GSH) as co-substrate, based on the method described by Habig et al. [45]. In microplates 100 μL of sample supernatant was mixed with 200 μL reaction buffer. 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.

2.8. 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+ [46,47]. Significant differences were considered for $p \leq 0.05$ and identified in figures and supplementary tables with different lowercase (control), uppercase (Cd) letters and asterisk (between conditions for the same concentration of compounds). In order to

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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 and normalized) was used to calculate Euclidean distance data matrices which were submitted to an ordering analysis performed by Principal Coordinates (PCO), using the PRIMER 6 & PERMANOVA+ software. Biochemical data were also analyzed with MetaboAnalyst 4.0 to build the heatmaps (data was autoscaled) [48,49].

3. Results

No differences on colony macroscopic characteristics (shape, color) besides colony size were noticed when comparing colonies exposed and not exposed to the monoterpenes. 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 (Figure 1 - α-pinene, Figure 2 - limonene, Figure 3 - linalool, Figure 4 – eucalyptol and Figure 5 - menthol) for two Cd conditions (0 and 100 µM).

3.1. Sole exposure to Cd

When exposed to Cd a 55% reduction in growth of E20-8 compared to control condition is observed (Fig. 1A, Fig. 2A., Fig. 3A, Fig. 4A and Fig. 5A). Cd also induced alterations in all of the biochemical parameters determined (Fig. 1B, Fig. 2B, Fig. 3B, Fig. 4B and Fig. 5B). Results showed a considerable increase of the protein content and antioxidant activity (SOD and GPx). Regarding LPO and GSTs a small increase was observed.

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3.2. 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. 1A). Maximum growth is 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 high concentrations (less 25%) (Fig.1B; 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. 1B; Supplementary Table S1).

In the presence of Cd, α-pinene did not alleviate the growth decrease imposed by Cd (Fig. 1A). In the combined exposure to Cd and α-pinene protein and LPO were increased by α-pinene especially at high concentrations (Fig. 1B 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. 1B and Supplementary Table S1).

The Principal Components Ordination (PCO) of α-pinene data (Figure 1C) shows that PCO1 is responsible for 71.1% of the differences separating bacteria exposed and not exposed to Cd based on their different biochemical behavior. PCO2 is responsible for 14.8% of the variation showing that GPx activity is the main mechanism of distinction between α-pinene concentrations (Fig. 1C).

3.3. 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. 2A). Limonene did not change protein

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content but increased LPO levels and enzymes activity (SOD, GSTs and GPx) (Fig. 2B and Supplementary Table S2).

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. 2A). LPO was not changed by limonene compared to sole exposure to Cd (Fig. 2B; 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. 2B; Supplementary Table S2). GPx activity was increased by the lower limonene concentrations (1 nM, 100 nM), but remained similar at the higher concentrations (10 µM, 1 mM and 100 mM).

PCO analysis demonstrates that the abscissa axis is 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 is responsible for 17.6% of the differences, pointing GSTs activity as the main mechanism of distinction between the concentrations of limonene in the presence of Cd (Fig. 2C).

3.4. 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 higher concentrations, where a significant decrease was observed (Fig. 3A). Sole exposure 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. 3B 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. 3B

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and Supplementary Table S3). Furthermore, linalool decreased LPO at low concentrations (1 nM, 100 nM) and increased at high concentrations (10 μM, 1 mM and 100 mM) compared to sole exposure to Cd (Fig. 3B).

PCO analysis shows that the abscissa axis is responsible for 78% of the differences separating bacteria exposed and not exposed to Cd based on their different biochemical traits (Fig. 3C). The ordinate axis is 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.

3.5. 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. 4A).

Eucalyptol decreased protein (only significantly at 1mM), LPO (100 mM) and enzymes activity for one (GPx) or more concentrations (SOD, GSTs) of the compound (Fig. 4B 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. 4C) demonstrates that the abscissa axis is 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 is responsible for 12.6% of the differences, showing that GPx activity is negatively influenced by eucalyptol both in the presence and absence of Cd.

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3.6. 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. 5A).

The increase in menthol concentrations led to protein increase (Fig. 5B and Supplementary Table S5). LPO decreased significantly at 100 nM and 10 μM. SOD activity increased at higher concentrations. GPx activity decreased at 1mM and GSTs decreased at low concentrations (1 nM to 10 μM) and increased at high concentrations (1 mM and 100 mM). The presence of menthol in Cd stressed cells lead 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. 5B and Supplementary Table S5).

PCO analysis (Figure 5C) shows that the abscissa axis is responsible for 78.5% of the differences separating the bacteria exposed and not exposed to Cd based on their different biochemical features. It is also possible to see that SOD and GSTs activity and protein content are the main mechanisms that discriminate the influence of menthol (ordinate axis).

4. Discussion

Previous studies demonstrated that Cd decreases the diversity and number of microorganisms found in contaminated soils [50,51], 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* [52,53]. 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. Although alpha-pinene and limonene do not have any of these groups, they are aromatic compounds. Menthol and linalool both contain an alcohol functional group. However, menthol has a cyclic structure while linalool is linear. Eucalyptol is a cyclic ether. Bioactivity of monoterpenoids (monoterpenes with functional groups) depends on the nature and position of functional groups and molecular configuration [54]. Monoterpene hydrocarbons are antioxidants, however oxygenated monoterpenes are more powerful antioxidants [55]. Regarding antibacterial activity, terpenoids that contain alcohols possess higher activity than the corresponding carbonyl compounds [55]. 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 [55]. Eucalyptol and linalool have been reported as inhibitory of bacterial growth [55]. 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 [56]. It is also important to note that, as opposed to inhibition studies which used relatively high concentrations and tested direct contact [55], 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

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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 [9,22,37].

In our study, α-pinene increased colony growth. Previous studies have found that α-pinene can be used as a carbon source by microorganisms [57]. It is known that LPO is one of the main effects on cells under oxidative damage [58]. 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 [59] 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 [52,53]. Glutathione peroxidase (GPx) plays a key role in cellular antioxidant activity by catalyzing the reduction of hydroperoxides using GSH as a reducing agent [60]. 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 [61] and has also been detected in the headspace of microorganisms [37,61]. 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 was observed at the higher concentrations. Although limonene may be used by some bacteria as a carbon source [57], it is mainly known for its antimicrobial activity [15,62]. 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 [63], 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 [64]. 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 [65,66] that decreases free Cd ions concentration in cells thus reducing their toxicity [66]. PCO brings out 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*) [67] and its main constituent is linalool [68]. 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 [68,69] is also observed and may might be due to the

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biochemical response of the cell to linalool, evidencing an increase in oxidative stress levels. Van Bogelen et al. [70] 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 [71], evidencing that the most efficient mechanisms are those avoiding the buildup of oxidative stress [52]. 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. [72]. 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. [67], 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

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formation of Cd-GS complexes, turning cells less vulnerable to Cd, and supporting higher growth than when cells were only exposed to Cd.

Menthol is widely used in food, cosmetic and pharmaceutical industries [73,74]. This compound is not common in the VOCs matrix released by bacteria [75], and little is known about its individual effect 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 [76,77]. 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 [78], 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.

5. Concluding Remarks

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Airborne exposure of *Rhizobium* colonies to monoterpenes evidenced differences among compounds, both in cells exposed to Cd and in cells not exposed to Cd. 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, leaving 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 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 when applied at 1 and 100 mM.

Our study 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.

CRediT statements

Carina Sá: Formal analysis, Investigation, Writing-Original draft, Writing-Review and Editing, Visualization **Diana Matos:** Investigation **Adília Pires:** Formal analysis, Writing - Review & Editing, Visualization, Supervision **Paulo Cardoso:** Writing-Original draft, Writing-Review and Editing **Etelvina Figueira:** Conceptualization, Methodology, Resources, Writing-Original draft, Writing-Review and Editing, Visualization, Supervision.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Figure legends

Figure 1 - 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 terpene 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 terpene and not to Cd (dashed line); to the terpene and to Cd (full line). Values are means of 3-6 replicates ± standard errors. Different lowercase letters indicate significant differences among terpene concentrations in no Cd (0 µM) condition; uppercase letters indicate significant differences among terpene 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 (prot); glutathione peroxidase (GPx); glutathione S-transferases (GSTs); and superoxide dismutase (SOD). 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 the terpene and not to Cd (open circles); terpene and Cd (closed circles); detailed color scheme in the figure. Pearson correlation vectors were imposed: LPO, prot, SOD, GSTs and GPx ($r \geq 0.70$).

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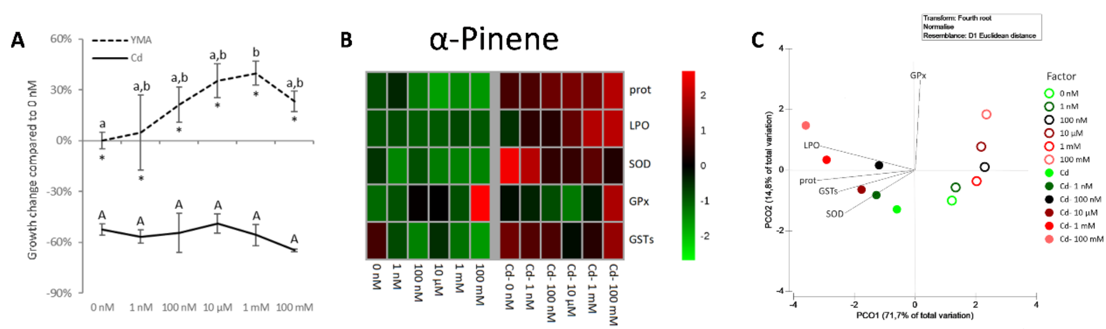

Figure 2 - Growth, antioxidant and biotransformation activity and damage in *Rhizobium* cells exposed to Cd and limonene. (A) growth variation relatively to control (no Cd, no compounds). (B) Heatmap of the biochemical determinants for each condition. For mean values, standard errors and statistical significance see Supplementary Table S2. (C) Principal Coordinates with centroids ordination (PCO) of the biochemical determinants for each condition. For detailed information see Figure 1 legend.

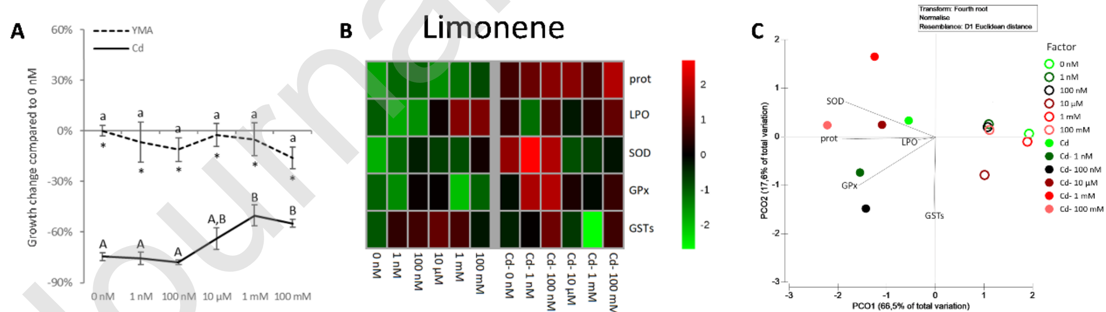

Figure 3 - Growth, antioxidant and biotransformation activity and damage in *Rhizobium* cells exposed to Cd and linalool. (A) growth variation relatively to control (no Cd, no compounds). (B) Heatmap of the biochemical determinants for each condition. For

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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. For detailed information see Figure 1 legend.

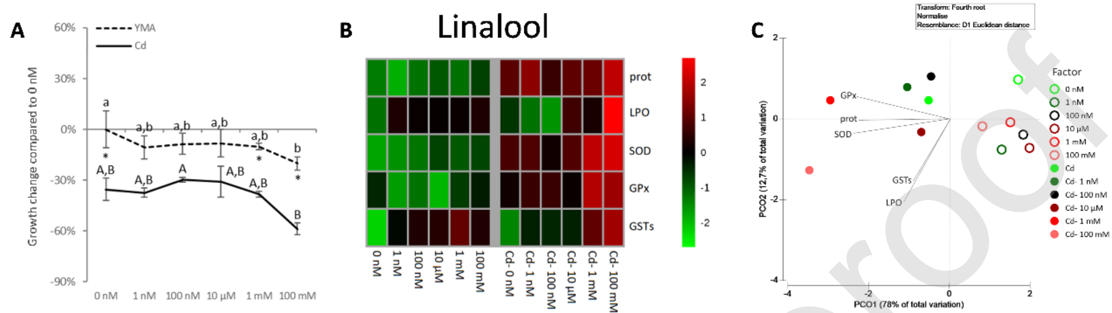


Figure 4 - Growth, antioxidant and biotransformation activity and damage in *Rhizobium* cells exposed to Cd and eucalyptol. (A) growth variation relatively to control (no Cd, no compounds). (B) Heatmap of the biochemical determinants for each condition. For mean values, standard errors and statistical significance see Supplementary Table S4. (C) Principal Coordinates with centroids ordination (PCO) of the biochemical determinants for each condition. For detailed information see Figure 1 legend.

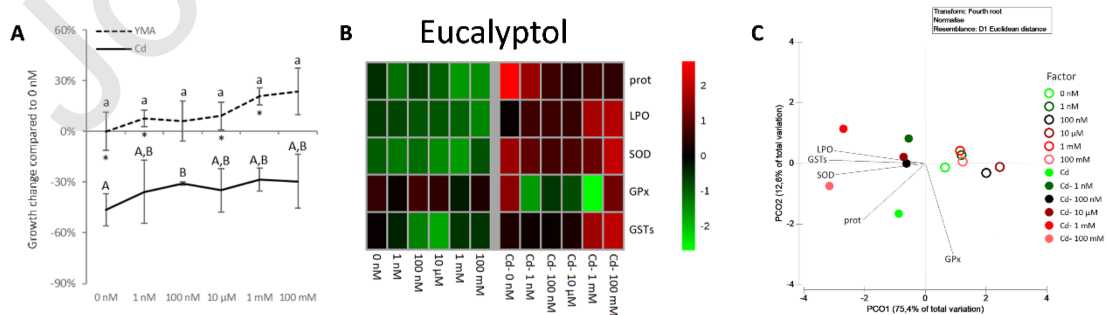
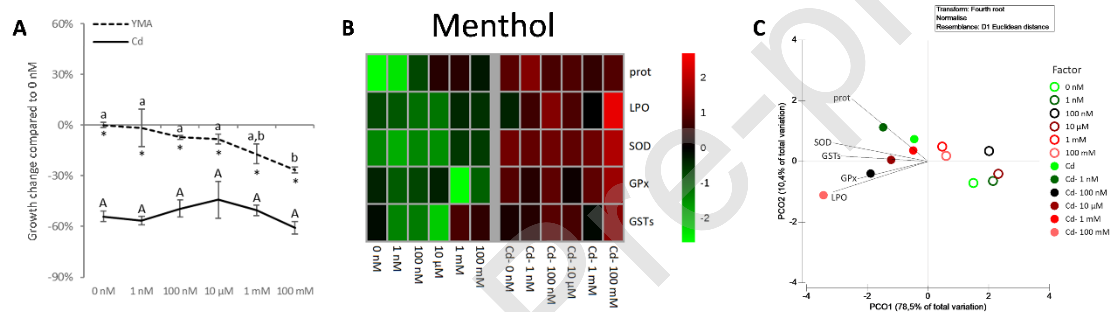


Figure 5 - Growth, antioxidant and biotransformation activity and damage in *Rhizobium* cells exposed to Cd and menthol. (A) growth variation relatively to control (no Cd, no compounds). (B) Heatmap of the biochemical determinants for each condition. For mean values, standard errors and statistical significance see Supplementary Table S5. (C) Principal Coordinates with centroids ordination (PCO) of the biochemical determinants for each condition. For detailed information see Figure 1 legend.



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Supplemental files legends

Supplementary Table S1 – Growth, protein, 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, and asterisks indicate significant differences between conditions (0 and 100 μM Cd) for the same concentration of the same compound ($p < 0.05$).

Supplementary Table S2 – Growth, protein, 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, and asterisks indicate significant differences between conditions (0 and 100 μM Cd) for the same concentration of the same compound ($p < 0.05$).

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

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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, and asterisks indicate significant differences between conditions (0 and 100 μM Cd) for the same concentration of the same compound ($p < 0.05$).

Supplementary Table S4 – Growth, protein, 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, and asterisks indicate significant differences between conditions (0 and 100 μM Cd) for the same concentration of the same compound ($p < 0.05$).

Supplementary Table S5 – Growth, protein, 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

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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 ($p < 0.05$).

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