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Protective effects of farnesol on a *Rhizobium* strain exposed to cadmium

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ABSTRACT

Soil acts as a repository for many metals that human activity releases into the environment. Cadmium enters agricultural soils primarily from application of phosphate fertilizers and sewage sludge. Among soil bacteria, rhizobia have a great agronomic and environmental significance and are major contributors to a sustainable maintenance of soil fertility. However, the services that this group of microorganisms provides are affected by environmental constraints, such as Cd contamination. Bioactive compounds also influence soil microorganisms. Farnesol is a phytocompound with recognized bioactivity, inducing both beneficial and harmful effects. In this study, *Rhizobium* sp. strain E20-8 was exposed to sole or combined exposure to Cd and farnesol. Results showed that farnesol (25 and 200 μM) did not affect rhizobia; exposure to Cd (μM) inhibited rhizobia growth and induced several biomarkers of oxidative stress; exposure to the combination of farnesol and Cd reduced oxidative damage, and the highest concentration of farnesol tested reduced Cd accumulation and allowed a significant growth recovery. Farnesol protective effects on rhizobia exposed to Cd is novel information which can be used in the development of microbe-based environmental engineering strategies for restoration of metal contaminated areas.

1. Introduction

Soil can be defined as a dynamic natural body having properties derived from the combined effects of climate and biotic activities, acting on parent materials over time, and sustaining a wide variety of organisms adapted to the prevailing conditions. Any disturbance that changes this equilibrium can have serious consequences for the life that soil sustains and the services that soil provides. In the last 200 years, after the onset of industrial era, soil contamination increased continuously, and presently is a widespread problem in the world. According to estimates by the European Environment Agency 250,000 sites may need urgent remediation (European Environment Agency, 2007). Among the most frequent contaminants are metals (Huber and Prokop, 2012). Application of inorganic fertilizers is one of the main anthropogenic activities disrupting soil equilibrium (Solgi and Khodabandelo, 2016). Phosphate fertilizers are a major source of cadmium (Cd) in soils and contribute to soil contamination in addition to industry, sludge amendment, and mining activities (López-Climent et al., 2014; Roberts, 2014). In soil solution, Cd concentrations are usually found between 0.3 μg to 6 mg L^{-1} (1.8–53 μM) (Helmke, 1999; Kabata-Pendias, 2011) but this element can reach concentrations higher than 300 mg L^{-1} (2669 μM) at highly contaminated sites (Itoh and Yumura, 1979). Cd is classified as the seventh more toxic substance on the 2015 Priority List of Hazardous Substances by the Agency for Toxic Substances and Disease Registry (ATSDR, U.S. Department of Health and Human Services). Therefore, even at low concentrations the potential to affect soil communities is high.

Rhizobia are ubiquitous gram-negative soil bacteria that fix N_2 when in endosymbiotic association with legumes (Family Fabaceae), supplying plants with nitrogen, an essential and frequently limiting element for plant growth and metabolism (Denison and Kiers, 2004; Hao et al., 2015). When in free-living form, among the soil microbiome, rhizobia can also play an important role in plant growth promotion, benefiting both legumes and non-legume plants. This positive effect is achieved by solubilizing phosphates, producing phytohormones (auxins, cytokinins and gibberellins) and siderophores (that form complexes with metals such as Fe, Cd, Zn, Cu, Pb and Al), synthesizing compounds with antimicrobial activity against pathogens and enhancing the activity of plant enzymes, namely proteases and lipases (Gopalakrishnan et al., 2014). Other compounds, such as volatile organic compounds (VOCs) synthesized by plants or microorganisms are emerging as growth promoters (e.g. Blom et al., 2011; Kai et al., 2009; Ryu et al., 2003) or stress relievers (Cho et al., 2013; Liu and Zhang, 2015). However the functions this group of microorganisms provides are seriously affected by environmental constraints, such as Cd contamination (Vig et al., 2003).

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Cadmium has high affinity for sulfhydryl groups, and replaces metal cofactors in metalloenzymes, leading to the direct inactivation of important proteins of cell metabolism (Figueiredo-Pereira et al., 1998). Moreover, Cd ions can cause displacement of redox active metals such as Fe or Cu (increasing the concentration of free metal ions), and thus inhibiting electron transport chains and leading to reactive oxygen species (ROS) burst (Cuypers et al., 2010; Pacheco et al., 2008). Highly reactive ROS interact with lipids, proteins and DNA, causing lipid peroxidation (LPO), protein carbonylation (PC) and DNA methylation (Wu and Ni, 2015) and fragmentation (Monteiro et al., 2012), affecting gene and protein expression, membrane fluidity and permeability and enzymes activity, thus compromising cell homeostasis (Cuypers et al., 2010; Monteiro et al., 2012).

In order to survive cells must trigger mechanisms to alleviate Cd toxicity. Cellular mechanisms counteracting this toxicity include intracellular chelation of free Cd ions (Corticeiro et al., 2006). However, the Cd ions remaining free in the cytoplasm can still disturb cell functions (Vestena et al., 2011; Wyszkowska et al., 2012). To reduce Cd effects, cells possess ROS scavenging mechanisms that include low molecular weight antioxidant compounds such as glutathione (GSH). When these compounds are not able to control ROS levels, and although other mechanisms such as volatile organic compounds might be involved (Cardoso et al., 2017), enzymatic mechanisms come into play, such as superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferases (GSTs) (Corticeiro et al., 2013, 2006). SOD catalyzes superoxide radicals dismutation into hydrogen peroxide and is considered the first line of defense against ROS (Cabiscol et al., 2000). Despite being an antioxidant, SOD represents a source of hydrogen peroxide, thus being necessary the coordination of its activity with H_2O_2 reducing enzymes, like CAT, that subsequently detoxifies H_2O_2 to H_2O and O_2 (Cabiscol et al., 2000). GSTs belong to a family of enzymes that catalyze the conjugation of GSH with xenobiotics with exogenous or endogenous origin (Sharma et al., 2004). This enzyme directly neutralizes Cd toxicity by catalyzing the conjugation of GSH to Cd ions, increasing the formation of Cd-GSH conjugates (Cardoso et al., 2018; Corticeiro et al., 2013). Moreover, GSTs use GSH to convert toxic aldehydes, resulting from peroxydized polyunsaturated fatty acids (El-Aal, 2012; Schmidt et al., 2015), to alcohols (Korpi et al., 2009; Schmidt et al., 2015), which are excreted (Sharma et al., 2004). Aldehydes are highly toxic to cells (Ayala et al., 2014; LoPachin and Gavin, 2014), through carbonylation of proteins (Grimsrud et al., 2008; Suzuki et al., 2010) and formation of DNA adducts (Voulgaridou et al., 2011).

Ecosystems are populated by organisms interacting with each other and these relations modulate biogeochemical cycles, regulate ecosystems functioning and productivity and influence the ecology and heath of partners. Bioactive compounds, as those released by plants may affect soil microorganisms. Farnesol, an acyclic sesquiterpene alcohol, is produced by many plants, both in under (roots, rhizomes and bolbs) and aboveground (leaves, flowers) organs in concentrations ranging from a few (4.5 μM in *Aloysia citrodora*) to thousands (5626 μM in *Cymbopogon commutatus*) micromolar (Duke, 2000). Among plants producing farnesol are *Vitis vinifera* (Salomon et al., 2014), *Gingko biloba* (Parveen et al., 2015), *Zea mays* (Schnee et al., 2002), *Oryza sativa* (Cheng et al., 2007), *Syringa vulgaris* (Duke, 2000) and legumes, such as *Acacia farnesiana* (Talapatra et al., 2015) and *Pisum sativum* (Yoshioka et al., 1990). In particular, *Acacia farnesiana* can accumulate high concentrations of farnesol (25% of the essential oil is composed by volatiles, mostly farnesol, geraniol, benzyl alcohol and methyl salicylate) (Khan and Abourashed, 2000). Farnesol has high adsorption and persistence in soil due to its stability. It is not susceptible to photolysis and has low volatility in dry soils (National Center for Biotechnology Information. PubChem Compound Database; CID = 445070). Thus, this compound can easily be found in soils (Duke, 2000; Hawker et al., 2008). Farnesol presents bioactivity in different organisms. Most studies on farnesol bioactivity are as a biocontrol agent against phytopathogenic nematodes (Rowat et al., 2005), as an antimicrobial against bacteria such as *Staphylococcus epidermidis* (Gomes et al., 2009) or yeasts such as *Saccharomyces cerevisiae* (acting as a pro-oxidant compound) (Fairn et al., 2007) or *Candida albicans* (inhibiting filamentation and thus pathogenicity) (Ramage et al., 2002). In *C. albicans* increased hydrogen peroxide resistance (Deveau et al., 2010) and quorum-sensing (Nickerson et al., 2006) were also attributed to farnesol. Farnesol decreased oxidative stress in mice, by decreasing LPO and increasing antioxidant enzymes activity (Jahangir et al., 2005; Khan and Sultana, 2011). Thus, farnesol induces a multiplicity of effects, some of them contradictory. Moreover, tolerance to farnesol varies widely among different species (Colabardini et al., 2010; Gomes et al., 2009; Machida et al., 1998; Nickerson et al., 2006; Savoldi et al., 2008).

Given the high number of plants able to synthesize farnesol, the potential to persist in soils, the contradictory bioactivity, the differences in tolerance between different organisms and the lack of information regarding effects and tolerance of farnesol in agronomic and ecological relevant soil microorganisms, such as rhizobia, the evaluation of farnesol impact in soil microorganisms is of major relevance. Additionally, since soils, especially agricultural ones are subjected to anthropogenic influence, frequently evidencing accumulation of persistent elements, such as metals (European Environment Agency, 2007), investigation of farnesol effects in microorganisms inhabiting Cd contaminated soils is also a relevant subject. In order to respond to these issues a *Rhizobium* strain was exposed to Cd and farnesol alone and in combination, and alterations in growth, metal accumulation, antioxidant responses and cellular damage were evaluated.

2. Materials and methods

2.1. Experimental conditions

Rhizobium sp. strain E20-8 (partial 16S rRNA sequence Genbank accession number KY491644), isolated from *Pisum sativum* L. root nodules grown in a non-contaminated field in Southern Portugal, and previously described as tolerant to Cd (Corticeiro et al., 2013), was grown in tubes containing 5 mL of yeast broth mannitol (YMB) medium (Somasegaran and Hoben, 1994) supplemented with cadmium chloride ($CdCl_2$, Sigma) (0, 7.5, 10 and 20 μM) and farnesol (0, 25, 50, 75, 100, 150 and 200 μM), in order to determine the tolerance to Cd and farnesol and to choose the conditions for further work. Farnesol stock solution was prepared with ethanol and sterilized deionized water (1:1). Ethanol concentration used was confirmed to have no effect on rhizobia growth. Inoculated tubes were incubated at 26 °C in an orbital shaker (150 rpm) until late exponential phase (14 h). Growth was determined by measuring optical density at 620 nm. The relationship between optical density and cell concentration was obtained by direct cell counting in a Neubauer chamber. Cell concentration was expressed in million cells per milliliter (M cells mL^{-1}).

Considering *Rhizobium* tolerance to Cd and farnesol (none of the farnesol concentrations inhibited growth, and only 20 μM Cd inhibited growth significantly compared to control), two Cd (0 and 20 μM) and three farnesol (0, 25 and 200 μM) conditions were chosen for the toxicity tests. Cells were grown in triplicate in YMB medium in a total of six different conditions: 0 μM Cd + 0 μM farnesol (control); 20 μM Cd + 0 μM farnesol; 0 μM Cd + 25 μM farnesol; 0 μM Cd + 200 μM farnesol; 20 μM Cd + 25 μM farnesol; and 20 μM Cd + 200 μM farnesol. Cadmium concentration used (20 μM) is environmental relevant (Kabata-Pendias, 2011). No information regarding farnesol concentration in soil solution was found in the literature, but the concentrations used (25 and 200 μM) did not affect *Rhizobium* growth. Cells were collected after centrifugation at 10,000 × *g* for 10 min at 4 °C, washed twice with deionized water, and frozen at −80 °C for further use.

2.2. Biochemical parameters

Frozen cells were suspended in specific extraction buffers and lysed