In vitro growth and indoleacetic acid production by *Mesorhizobium loti* SEMIA806 and SEMIA816 under the influence of copper ions

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Abstract

The indoleacetic acid produced by symbiotic bacteria is an important phytohormone signaling microbe-plant interaction, being therefore essential for rhizoremediation. In this study, the effect of different concentrations of copper ions on the bacterial growth and indoleacetic acid production was investigated in two strains of *Mesorhizobium loti* in *in vitro* conditions, aiming to determine critical concentrations of this heavy metal for rhizoremediation of contaminated soils using this bacterium. The experiment consisted on a control culture without copper and three treatments supplemented with 10 mg.L^{-1}, 20 mg.L^{-1} or 50 mg.L^{-1} of CuSO$_4$. For both strains, the growth stopped after 48h and no significant difference was observed across treatments. The production of indoleacetic acid by the control treatment without copper was significantly higher in comparison to the copper-containing treatments. *Mesorhizobium loti* SEMIA806 and SEMIA816 are resistant to up to 50 mg.L^{-1} of CuSO$_4$ in the culture medium, presenting effective growth. The synthesis of indoleacetic acid was strongly reduced but not excluded by ions copper in the medium. So, it is expected that environmental copper found in the soil up to the concentration of 50 mg.L^{-1} will not preclude the symbiotic interaction between *M. loti* and leguminous host plant in rhizoremediation enterprises.

Introduction

In recent years, several approaches for bioremediation have been proposed as alternatives for diminishing environmental heavy metal contamination.[1] Bioremediation consists on a group of applications, which involves the detoxification of hazardous substances by means of microbes and plants, instead of transferring the pollutants from one substrate to another. Rhizoremediation is a bioremediation method that consists in the enhancement of the metal extraction process through the root system of plants by its association with microbes.[1,2]

For the effectiveness of rhizoremediation, an efficient microbe-plant interaction is needed. The indole-3-acetic acid (IAA) produced by plant-associated microbes is an important phytohormone signaling this interaction. This phytohormone is involved in many processes of nodule formation, such as founder cell specification, nodule initiation and differentiation, vascular bundle formation, and nodule numbers. Therefore, IAA is essential for the formation of the nodules in the roots.[3] However, sub-lethal levels of copper nanoparticles in the growth medium have been shown to reduce the secretion of secondary compounds by bacteria, including IAA.[4]

Copper is an essential element for all living beings, since it acts as co-factor in several enzymatic reactions. However, exposition to excessive levels of this metal may cause different metabolic problems. In humans, short-term exposure to drinking water contaminated with copper ions may cause gastrointestinal distress, while long-term consumption may cause liver or kidney damage.[5] Therefore, the bioaccumulation of copper causing toxicity in human, animals and plants is an important issue for environmental health and safety, and rhizoremediation is an important alternative to diminish this problem.[1]

Mesorhizobium loti is a soil and rhizosphere bacteria of agronomic importance due to the nitrogen-fixing symbioses formed with leguminous plants. This species is able to form determinant-type globular nodules and to perform nitrogen fixation on several *Lotus* species.[6] *In vitro* investigations have shown the capacity of this bacterial species for growing in culture conditions with high concentrations of copper (Roll *et al.*, unpublished data), suggesting potential for bioremediation of soils contaminated with this metal. In association with fast-growing legume tree species as *Mimosa scabrella* (Leguminosae), *M. loti* may be a quite promissory option for environmental and landscape recuperation in areas polluted with copper, using rhizoremediation. In this study, the interference of different concentrations of copper ions on the bacterial growth and IAA production was investigated in two strains of *Mesorhizobium loti* in *in vitro* conditions, aiming to determine critical concentrations of this heavy metal for rhizoremediation of contaminated soils using this bacterium.

Materials and Methods

Turfs of *Mesorhizobium loti* strains SEMIA806 and SEMIA816 were kindly provided by the State Foundation of Agricultural Research of the Rio Grande do Sul (FEPAGRO), São Gabriel, Brazil. The *M. loti* strains were firstly cultured in Petri dishes with solid YM broth (1.0 g.L^{-1} yeast extract, 0.5 g.L^{-1} K$_2$HPO$_4$, 0.2 g.L^{-1} MgSO$_4$, 0.1 g.L^{-1} NaCl, 1.0 g.L^{-1} CaCO$_3$ and 10 g.L^{-1} of D-mannitol). Before bacterial inoculation, the YM broth was solidified with bacteriological agar (15 g.L^{-1}) and sterilized at 1.2 kg.cm^{-1} at 121°C for 15 min.

For each strain independently, 1.0 g of turf was diluted in 100 mL of saline solution

(0.8% NaCl dissolved in ultrapure water). Subsequently, 100 μL of the dilution from each strain was inoculated in independent Petri dishes with solidified YM broth and cultivated at 28°C during 48 h. Actively growing *M. loti* colonies were isolated from this cultures and employed in the analysis of bacterial growth and IAA synthesis in liquid YM broth with copper.

The experiment consisted on a control culture without copper and three treatments: i) YM broth supplemented with 10 mg.L^{-1} of CuSO$_4$; ii) YM broth supplemented with 20 mg.L^{-1} of CuSO$_4$; and iii) YM broth supplemented with 50 mg.L^{-1} of CuSO$_4$. Before bacteria inoculation, 20 mL of the YM broth was dispensed in 300 mL glass flasks and sterilized at 1.2 kg.cm^{-1} and 121°C for 15 min. Single colonies were added to the YM broth and incubated for five days at room temperature under 100 RPM shaking.

Bacterial growth was evaluated in each treatment by reading the absorbance of the broth at 560 nm (OD$_{560}$ nm) after 0, 24, 48 and 120 h of culture, using a plate reader EnSpire™ 2300 Multilabel Reader (Perkin-Elmer Inc., USA). The IAA production was estimated after five days of culture. For the estimation of IAA production, the broth was centrifuged at 12,000 RPM for 15 min, the cell-free supernatant reserved and the IAA was estimated spectrophotometrically by mixing 1.0 mL of the supernatant with 1.5 mL of the modified ferric chloride-sulphuric acid reagent (1.0 mL of 0.5M FeCl$_3$, 30 mL of H$_2$SO$_4$ and 50 mL H$_2$O) as described by Gordon and Weber (1951)[7]. The optical density was recorded at 530 nm (OD$_{530}$ nm) after 75 min of incubation at room temperature in a dark environment.[7]

Reagents were purchased by Sigma-Aldrich (St. Louis, USA). All experiments were performed in quadruplicate and the inoculation and sample transfer steps were performed within a sterilized airflow chamber Trox model TLF (Trox Technik, Germany). The statistical significance of the difference among the estimated means for bacterial growth and IAA synthesis was determined using the Mann-Whitney test in the software PAST 3.04.[8]

Results and Discussion

Bacterial growth

For both bacterial strains, the growth stopped after 48h (Figure 1) and no statistically significant difference (P>0.27) was observed across treatments in the evaluated periods (0, 24, 48 or 120 h of culture). However, the bacterial growth revealed different patterns for strains SEMIA806 and SEMIA816 (Table 1 and Figure 1). After
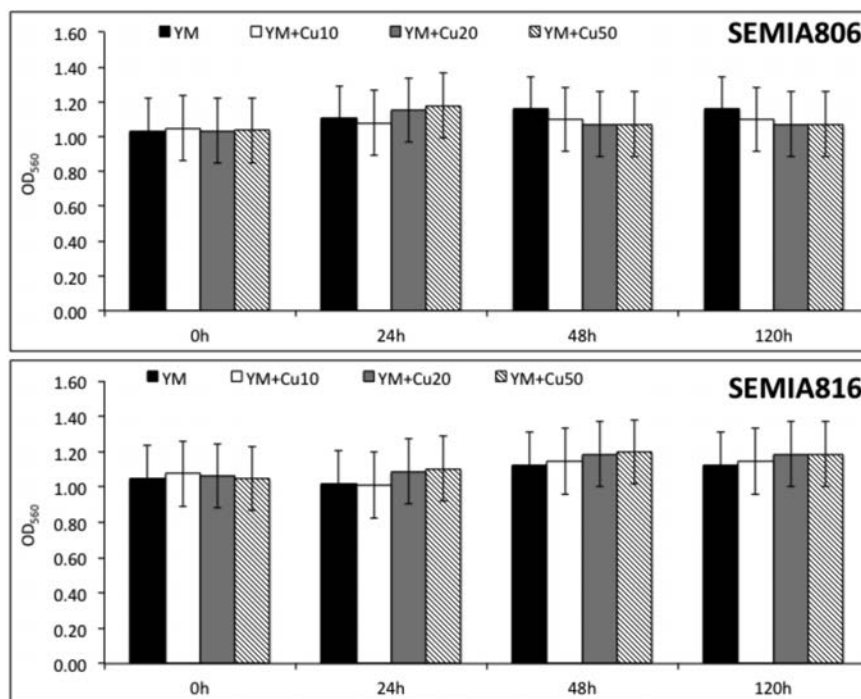
Figure 1. Bacterial growth of *M. loti* strains SEMIA806 and SEMIA816 cultivated in YM broth without and with addition of copper (YM = YM broth without copper addition; YM+Cu10 = YM broth with addition of 10 mg.L^{-1} of CuSO$_4$; YM+Cu20 = YM broth with addition of 20 mg.L^{-1} of CuSO$_4$; YM+Cu50 = YM broth with addition of 50 mg.L^{-1} of CuSO$_4$). Values are means over four replicates.

Table 1. Spectrophotometric lectures of bacterial growth at 560 nm and amount of IAA produced by *M. loti* strains SEMIA806 and SEMIA816. Values are means over four replicates. The polynomial regression and the corresponding R^2 values for the growth curve are given for each treatment.

	Bacterial growth						Amount of IAA produced after 120h of culture (g.mL^{-1})
	O$_{D560}$				Regression	R^2	
	0h	24h	48h	120h			
SEMIA806							
YM*	1.034	1.107	1.158	1.158	y = -0.018x^2 + 0.134x + 0.917	0.99	2.42
YM+10Cu	1.048	1.078	1.099	1.099	y = -0.008x^2 + 0.056x + 0.999	0.99	0.11
YM+20Cu	1.034	1.153	1.072	1.072	y = -0.029x^2 + 0.152x + 0.926	0.47	0.13
YM+50Cu	1.036	1.177	1.072	1.072	y = -0.035x^2 + 0.176x + 0.913	0.45	0.28
SEMIA816							
YM	1.048	1.020	1.124	1.124	y = 0.007x^2 − 0.003x + 1.032	0.67	2.48
YM+10Cu	1.075	1.011	1.146	1.146	y = 0.016x^2 − 0.045x + 1.087	0.56	0.12
YM+20Cu	1.062	1.087	1.186	1.186	y = -0.006x^2 + 0.078x +0.981	0.88	0.14
YM+50Cu	1.048	1.103	1.199	1.186	y = -0.017x^2 + 0.135x +0.922	0.93	0.28

*YM = YM broth without copper addition; YM+Cu10 = YM broth with addition of 10 mg.L^{-1} of CuSO$_4$; YM+Cu20 = YM broth with addition of 20 mg.L^{-1} of CuSO$_4$; YM+Cu50 = YM broth with addition of 50 mg.L^{-1} of CuSO$_4$.

24h of culture, strain SEMIA806 revealed higher growth for treatments with 20 mg.L^{-1} and 50 mg.L^{-1} of CuSO$_4$, in comparison to the control without copper and to the treatment with 10 mg.L^{-1} of CuSO$_4$. At 48h, treatments with 20 mg.L^{-1} and 50 mg.L^{-1} of CuSO$_4$ revealed higher decline in growth. Differently, strain SEMIA816 shown bacterial growth after 24h and 48h of culture in the treatments with 20 mg.L^{-1} and 50 mg.L^{-1} of CuSO$_4$, while the treatment with 10 mg.L^{-1} of CuSO$_4$ revealed decrease in growth after 24h and increased growth after 48h of culture (Table 1 and Figure 1).

Former *in vitro* studies[9-12] have demonstrated that copper is more toxic than zinc and cadmium, inhibiting the growth of *Mesorhizobium* at concentrations lower than 20 mg.L^{-1}. The resistance of *Rhizobium, Bradyrhizobium, Sinorhizobium* and *Azorhizobium* to copper reached concentrations ranging mostly from 20 to 40 mg.L^{-1}.[12] Many microorganisms demonstrate resistance to metals in water, soil and industrial waste. Genes located on chromosomes, plasmids, or transposons encode specific resistance to a variety of metal ions.[13] The *cop* operon responsible by copper resistance has been recorded in *Enterococcus hirae*, while an operon responsible by copper sequestration with similar nomenclature was described in *Pseudomonas*.[13] Such genes can be naturally transferred among bacteria through conjugation and transduction, disseminating this characteristic, which can be quite important for biotechnological purposes. In addition, genetic manipulation can be used to take advantage of metal resistance mechanisms.[1]

The use of microorganisms for the recovery of contaminated areas is an extremely important strategy and has been widely recognized as a very promissory bioremediation method[1]. In that sense, the capacity of growing in a copper-rich environment demonstrated from *M. loti* strains SEMIA806 and SEMIA816 has a significant meaning for the bioremediation of copper-contaminated areas using the rhizoremediation approach.

IAA synthesis

Different from the bacterial growth, the production of IAA was strongly affected by the copper added to the YM broth. The production of IAA by the control treatment without copper was significantly higher (P<0.005) in comparison to the copper-containing treatments. The amount of IAA synthesized by the control treatment was from eight to 20-fold higher than the copper-containing treatments, ranging from 0.11 μg.mL^{-1} (treatment with 10 mg.L^{-1} of

CuSO$_4$) to 2.42 μg.mL^{-1} (control treatment without copper) for SEMIA806, and from 0.12 μg.mL^{-1} (treatment with 10 mg.L^{-1} of CuSO$_4$) to 2.48 μg.mL^{-1} (control treatment without copper) for SEMIA816 (Table 1). Concerning the copper-containing treatments, the highest production of IAA was observed for the 50 mg.L^{-1} of CuSO$_4$, followed by 20 mg.L^{-1} of CuSO$_4$ and 10 mg.L^{-1} of CuSO$_4$ for both bacterial strains (Table 1). In plants, IAA is involved in multiple growth processes and stress responses.[14,15] The higher amount of IAA produced by bacteria cultivated under the higher concentration of copper in this study may be response of the microorganisms to stress. Higher amount of exopolysaccharides production by *M. loti* strains SEMIA806 and SEMIA816 under a more stressful environment concerning pH of the culture media was previously reported.[16] Similar result concerning exopolysaccharides production under stressful conditions was reported for *Bradyrhizobium, Rhizobium, Mesorhizobium, Sinorhizobium* and *Azorhizobium*.[12] Like the IAA, exopolysaccharides are symbiont derived signals essentials for nodulation in legume-rhizobia symbiosis.[17] A positive effect of ion copper in the production of IAA by *Pseudomonas chlororaphis* O6 was also demonstrated.[4] With the addition of thryptophan to the culture medium, the levels of accumulated IAA after 48h was higher than 30 μg.mL^{-1} in cultures with a copper concentration of 43 mg.L^{-1}.

The amount of IAA synthesized by *M. loti* SEMIA806 and SEMIA816 in this study is similar to the extent of IAA produced by different rhizosphere bacteria as *Burkholderia phytofirmans*[18] (0.84±0.33 μg.mL^{-1}), *Rahnella* sp, *Burkholderia* sp, *Pseudomonas* sp.[19] (0.57 to 2.43 μg.mL^{-1}; Khan et al. 2016) and 35 unidentified bacteria isolated from soybean in Indonesia[20] (0.051 to 3.208 μg.mL^{-1}). Considering that all these bacteria were able to interact with the host-plants, the low amount of IAA synthesized by *M. loti* SEMIA806 and SEMIA816 may be sufficient for the establishment of nodules in roots of selected plant species in soils contaminated with copper within the range tested in this study.

Conclusions and perspectives

Factors such as the culture media employed, growth conditions, and incubation period, besides the various possible forms and concentrations of metals used in the tests of tolerance may difficult their standardization and influence the *in vitro* toxicity of the metals. Due to these facts

there are no universally accepted metal concentrations to define bacterial tolerance or resistance.[21] However, it is of extreme importance to determine whether the bacterial isolates intended to be used for bioremediation are able to grow under environmental contamination.

The present study demonstrated that *M. loti* SEMIA806 and SEMIA816 are resistant to up to 50 mg.L^{-1} of CuSO$_4$ in the culture medium, presenting effective growth not significantly different from the control experiment without copper. In addition, it was shown that the synthesis of IAA was strongly reduced but not eradicated by ions copper in the medium. Even though this heavy metal strongly reduced the IAA production for both studied strains of *M. loti*, the amount of hormone synthesis was similar to the quantity produced by different bacterial species able to form nodules in host plants. Therefore, it is expected that environmental copper found in the soil up to the concentration of 50 mg.L^{-1} will not preclude the symbiotic interaction between *M. loti* and leguminous host plant species.

As thryptophan is an efficient physiological precursor of auxins,[22] the presence of this compound in the culture medium improves the bacterial IAA synthesis *in vitro*.[3] Therefore, experiments including the amendment of thryptophan to the culture medium should be performed to evaluate the improvement of IAA synthesis by *M. loti* SEMIA806 and SEMIA816 cultivated with copper. In addition, controlled experiments of the interaction plant-bacteria in contaminated soils are needed in order to evaluate the roots' nodulation in such environments, considering also the interaction copper-soil particles as well as the plant synthesis of IAA.

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