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Assessing the Toxic Effects of Nickel, Cadmium and EDTA on Growth of the Plant Growth-Promoting Rhizobacterium *Pseudomonas brassicacearum*

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Abstract Plant growth-promoting rhizobacteria (PGPR) play an important role in the biodegradation of natural and xenobiotic organic compounds in soil. They can also alter heavy metal bioavailability and contribute to phytoremediation in the presence or absence of synthetic metal chelating agents. In this study, the inhibitory effect of Cd^{2+} and Ni^{2+} at different concentrations of Ca^{2+} and Mg^{2+}, and the influence of the widely used chelator EDTA on growth of the PGPR *Pseudomonas brassicacearum* in a mineral salt medium with a mixture of four main plant exudates (glucose, fructose, citrate, succinate) was investigated. Therefore, the bacteriostatic effect of Cd^{2+}, Ni^{2+} and EDTA on the maximum specific growth rate and the determination of EC50 values was used to quantify inhibitory impact. At high concentrations of Ca^{2+} (800 μmol L^{-1}) and Mg^{2+} (1,250 μmol L^{-1}), only a small inhibitory effect of Cd^{2+} and Ni^{2+} on growth of *P. brassicacearum* was observed (EC50 Cd^{2+}, 18,849±80 μmol L^{-1}; EC50 Ni^{2+}, 3,578± 1,002 μmol L^{-1}). The inhibition was much greater at low concentrations of Ca^{2+} (25 μmol L^{-1}) and Mg^{2+} (100 μmol L^{-1}) (EC50 Cd^{2+}, 85±0.5 μmol L^{-1} and EC Ni^{2+}, 62±1.8 μmol L^{-1}). For the chosen model system, a competitive effect of the ions Cd^{2+} and Ca^{2+} on the one hand and Ni^{2+} and Mg^{2+} on the other hand can be deduced. However, the toxicity of both, Cd^{2+} and Ni^{2+}, could be significantly reduced by addition of EDTA, but if this chelating agent was added in stoichiometric excess to the cations, it also exhibited an inhibitory effect on growth of *P. brassicacearum*.

Keywords Heavy metal ions · EDTA · Growth inhibition · *Pseudomonas brassicacearum*

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1 Introduction

The rhizosphere is the root–soil contact zone which is most important for the regulation of soil organic matter decomposition and nutrient cycling, particularly due to the biodegradation of natural and xenobiotic organic compounds. It is a highly dynamic area for interactions between plant roots and, among others, beneficial soil microbes such as plant growth-promoting rhizobacteria (PGPR). One of the most important natural influences on microbial communities in the rhizosphere is the

release of a wide range of various compounds by plant roots, referred to as exudation (Lugtenberg et al. 1999) or rhizodeposition (Costa 2006). Growing plants release up to 40% of assimilated carbon as exudates into the rhizosphere (Liljeroth et al. 1994; Neumann and Römheld 2000; Kumar et al. 2006). This results in highly active and diverse heterotrophic microbial populations with bacterial densities 10 to 200 times higher than in surrounding bulk soil. In turn, PGPR essentially contribute to plant growth and health. Direct beneficial effects are, for example, biofertilisation (e.g., solubilising mineral nutrients), production of phytohormones, stimulation of root growth, rhizoremediation and enhancing plant stress resistance (Lynch 1990; Bowen et al. 1999; Cook 2000; Jing et al. 2007; Lugtenberg and Kamilova 2009).

The contamination of ecosystems by heavy metals, in particular, soils used for agriculture, represents one of the long-standing major environmental problems due to their prevalent existence and potential toxicity to plants, microorgansims, animals and humans. In this study, the term "heavy metal" refers to the definition of Passow et al. (1961). They classify elements according to their density, in which heavy metals show a density greater than 5 $g cm^{-3}$.

One of the main roles of rhizobacteria in the phytoremediation of heavy metal-contaminated soil using hyperaccumulating plants is the increased heavy metal mobility and availability to the plant caused by the release of naturally occurring chelating agents, acidification, phosphate solubilization and redox changes (Lynch 1990; Bowen et al. 1999; Whiting et al. 2001; Abou-Shanab et al. 2003; Kamnev 2003; Jing et al. 2007; Zhuang et al. 2007; Khan et al. 2009). An additional increase of heavy metal availability to the plant can be achieved by the addition of a synthetic chelating agent such as EDTA to the contaminated soil. Consequently, chelator-enhanced phytoremediation of heavy metal contaminated soil has gained considerable attention within the last two decades (Alkorta et al. 2004; Lestan and Finzgard 2007). For example, Chen and Cutright (2002) showed that inoculation of sunflower seeds and surrounding soil with heavy metal-resistant rhizosphere bacteria caused a significant increase of heavy metal phytoremediation in the presence of EDTA. The exposure to EDTA without inoculation with rhizobacteria caused an increased specific bioaccumulation, but also a reduced formation of biomass. In the presence of the rhizobacteria, however, a high biomass yield together with an increased accumulation of cadmium, chrome or nickel was observed.

Nevertheless, the multiple and complex interactions between rhizobacteria, heavy metal ions and synthetic chelating agents are still not fully investigated and understood to date. In addition, many rhizobacteria, including xenobiotics-degrading bacteria, are very sensitive to heavy metals (Duxbury 1985; Baath 1989; Jansen et al. 1994; McGrath et al. 1995; Giller et al. 1998; Oliveira and Pampulha 2006; Giller et al. 2009). Amongst other reasons, this toxicity is due to the displacement of essential macroelements such as Ca^{2+} and Mg^{2+} from biomolecules, and result in a substantial reduction of viability, activity, diversity and density of microbial rhizosphere populations (Pennanen et al. 1996; Giller et al. 1998; Sandaa et al. 1999; Liao et al. 2005). As the contamination of soils with heavy metals represents one of the long-standing major environmental problems, a better understanding of the manifold interactions between plants, rhizobacteria, heavy metals, essential macroelements and additional synthetic chelating agents is required.

Strains of *Pseudomonas brassicacearum* represent an interesting group of PGPR which belong to the major root zone colonisers of *Arabidopsis thaliana* and *Brassica napus* (Achouak et al. 2000; Achouak et al. 2004). In this study, *P. brassicacearum* (type strain CFBP 11706) was chosen as a representative and attractive model rhizobacterium to obtain a better understanding of the interactions between rhizobacteria, metal ions and synthetic chelating agents by investigating the inhibitory effect of Cd^{2+} and Ni^{2+}, two of the environmentally most important and hazardous heavy metals, and in return, a possible protective influence of Ca^{2+}, Mg^{2+} and EDTA.

2 Material and Methods

2.1 Strain and Culture Conditions

P. brassicacearum (CFBP 11706) was obtained as a lyophilisate from the "German Collection of Microorganisms and Cell Cultures" (DSMZ) with DSM number 13227.

It was grown on a mineral medium based on the four characteristic plant exudates glucose, fructose (Sigma), citrate (Merck) and succinate (Fluka Biochemika), each

at 2 mmol L^{-1} (so-called 4C medium). Additional components were 6 mmol L^{-1} $(NH_4)_2SO_4$, 0.8 mmol L^{-1} $CaCl_2$, 1.25 mmol L^{-1} $MgSO_4$, 60 μmol L $FeCl_3$, 1 ml L^{-1} trace element solution SL6 (according to Pfennig (1974)), and 25 ml L^{-1} of 1 mol L^{-1} phosphate buffer (as reported by Pack (1996)). The pH value was adjusted to 7.5, which was identified to be optimal for bacterial growth in preliminary experiments.

Precultivations of *P. brassicacearum* were conducted in a tenfold diluted tryptic soy broth (TSB) medium (as described by Achouak et al. 2000). Typically, these cultivations were performed in 1,000-mL shaking flasks containing 200 mL of TSB/10 medium. The medium was inoculated with 2 mL of *P. brassicacearum* cryo-culture and incubated overnight (12 h) at 30°C and 120 min^{-1}. After that, 180 mL of sterile 4C medium (1,000-mL shaking flasks) were inoculated with 20 mL of the preculture. Ni^{2+}, Cd^{2+} and/or EDTA were added to culture medium directly after inoculation. Cultivations continued at 30°C with a shaking rate of 120 min^{-1}. Bacterial growth was monitored by measuring absorbance at 546 nm, which was adopted from experiments described in literature (Belimov et al. 2007)

Screening experiments were carried out in 96-well microtiter plates containing 200-μL 4C medium per well. For this purpose, 180 mL sterile 4C medium was prepared in 1,000-mL shaking flasks. Subsequently, shaking flasks (TSB/10) were inoculated with 20 ml of the preculture, and 200 μL of this culture were transferred to microtiter wells. Cultivations in 96-well microtiter plates were performed using the microplate reader Sunrise (Tecan). Contaminations could be avoided by sterilisation of the microtiter plates with UV light for 20 min before adding culture medium. Cycles of the microplate reader were adjusted to 15-min measurement intervals and medium agitation intensity in the meantime. Absorbance measurements were conducted at 620 nm. This wavelength differs from the one used in the shaking-flask experiments due to the fact that the microtiter plate reader used in this study only supports certain wavelengths. However, *P. brassicacearum* measurements showed almost identical values at both wavelengths. All cultivations were performed as triplicates of at least two independent experiments. The respective maximum specific growth rate was evaluated by plotting logarithmical optical density values versus time, followed by linear regression of the data from the exponential growth phase. The slope of this regression line is referred to as maximum specific growth rate μ_{max} [per hour]. It was used as characteristic parameter giving hints on bacteriostatic effects of Cd^{2+} and Ni^{2+} on bacterial growth.

2.2 Analytics

After filtration of the samples using 0.2 μm sterile filters, sugars and organic acids in the medium were analysed by High-Performance Liquid Chromatography (HPLC) using a VWR-Hitachi LaChrom Elite system. The HPLC system was equipped with an UV- (detection wavelength 210 nm) and an RI-detector. A Varian Metacarb 87C column was utilised for determination of glucose and fructose concentrations, a Varian Metacarb 67H column served for analysis of citrate and succinate. Sulphuric acid (15 mmol L^{-1}) was used as mobile phase in an isocratic HPLC method. Additionally, glucose was quantified with an YSI 2700 Select Biochemistry Analyzer, allowing quick analysis of cultivation samples with identical concentrations in comparison to the HPLC analyses (data not shown).

2.3 Determination of EC50 Values

In toxicology, the half-maximal effective concentration (EC50) is defined as the concentration of a toxicant that leads to a response halfway between the baseline and the maximum effect. To determine the EC50 value, the logarithmical value of the toxicant concentrations added to the cultivations was plotted against the respective maximum specific growth rate of *P. brassicacearum* (determined as described in Section "Strain and Culture Conditions"). Afterwards, a linear regression of the data points was performed. From the regression line's equation, the logarithmical concentration of toxicants could be calculated which lead to a half-maximal specific growth rate. From these data, the EC50 values can be derived.

3 Results

3.1 Medium Development and Determination of Kinetic Parameters K_S- [mmol L^{-1}] and μ_{max} [per hour].

Sugar and organic acid exudates provide the main carbon and energy sources for rhizosphere colonising

microorganisms (Lugtenberg et al. 1999). Prior to the experiments with the 4C medium containing the four characteristic plant exudates glucose, fructose, citrate and succinate, the kinetic parameter K_S [millimoles per litre] and μ_{max} [per hour] for growth of *P. brassicacearum* with each of these compounds (0–2.5 mmol L^{-1}) as a sole source of carbon and energy were determined. The results clearly displayed a Monod-like growth kinetic. Significant differences in the kinetic data for the single components were observed. The maximum growth rates were, in descending order: succinate (0.4±0.04 h^{-1})>glucose (0.36±0.03 h^{-1})>fructose (0.18±0.01 h^{-1})>citrate (0.1±0.01 h^{-1}). Substrate affinity (given by the K_S value) increased in reverse order: succinate (0.64±0.13 mmol L^{-1})<glucose (0.24±0.06 mmol L^{-1})<fructose (0.1±0.03 mmol L^{-1})<citrate (0.09±0.03 mmol L^{-1}). At an initial substrate concentration of 2 mmol L^{-1}, growth with each of the single-carbon sources occurred approximately with the respective maximum rate, and so a model 4C medium with 2 mmol L^{-1} of each of the plant exudates was chosen for the further investigations.

3.2 Influence of Cd^{2+} and Ni^{2+} on Growth of *P. brassicacearum* in a Standard Mineral Medium

In a previous study with cells of *P. brassicacearum* strain NFM421, Pagès et al. reported a maximum tolerable Cd^{2+} concentration of 25 µmol L^{-1} when using a tenfold diluted TSB/10 medium for the cultivations (Pages et al. 2007). In the present study, however, the synthetic mineral 4C medium was used to specifically investigate the influence of Cd^{2+} and Ni^{2+} on growth of *P. brassicacearum* (type strain CFBP 11706) under various defined conditions. In the first experiments, the mineral salt composition corresponded to that of typical mineral media used for growth experiments; it contained, amongst others, 1.25 mmol L^{-1} of $MgSO_4$ and 0.8 mmol L^{-1} of $CaCl_2$ (see Material and Methods), and the concentrations of Cd^{2+} or Ni^{2+} were 0 µmol L^{-1} or adjusted to 100 µmol L^{-1} and 500 µmol L^{-1}, respectively. Under these conditions, only a slight growth inhibition even at relatively high concentrations of 500 µmol L^{-1} of Cd^{2+} (EC50 15,849±80 µmol L^{-1}) or Ni^{2+} (EC50 3,578±1002 µmol L^{-1}) was observed (Fig. 1a and b). Figure 1 also exhibits a lower maximum optical density in presence of heavy metal ions, but however, in comparison to the high amount of metal ions, this effect is relatively small. Since the inhibitory or toxic effect of heavy metal ions might be caused by their competition with essential macroelements, it was assumed, that the high concentrations of Mg^{2+} and Ca^{2+} in the 4C medium could alleviate the toxicity of Cd^{2+} and Ni^{2+} based on similar ion radii of Mg^{2+} (72 pm) and Ni^{2+} (69 pm), and of Ca^{2+} (100 pm) and Cd^{2+} (95 pm) (Lide 1994).

3.3 Influence of Cd^{2+} and Ni^{2+} on Growth of *P. brassicacearum* at Low Mg^{2+} and Ca^{2+} Concentrations

To examine, if the given concentrations of Mg^{2+} and Ca^{2+} might have significantly influenced the inhibitory effect of Cd^{2+} or Ni^{2+} on the growth of *P. brassicacearum*, the Mg^{2+} and Ca^{2+} concentrations in 4C medium were lowered to a minimum level, which still allowed growth of the microorganisms at the maximum rate. This required a screening experiment which is performed in microtiter cultivations using the standard 4C medium with different concentrations of $MgSO_4$ (0–1 mmol L^{-1}) and $CaCl_2$ (0–0.8 mmol L^{-1}). The results of this experiment are depicted in Fig. 2. As expected, no bacterial growth is observed in the absence of the essential elements Mg^{2+} and Ca^{2+}. Additionally, the respective maximum growth rates indicate a higher dependency from Mg^{2+} concentration rather than from Ca^{2+} concentrations while remaining almost constant even at low concentrations of $CaCl_2$, it declines rapidly at decreased $MgSO_4$ concentrations. As a consequence, the initial concentrations of $MgSO_4$ and $CaCl_2$ in the 4C medium were reduced from 1.25 mmol L^{-1} to 100 µmol L^{-1} ($MgSO_4$) and from 0.8 mmol L^{-1} to 25 µmol L^{-1} ($CaCl_2$) ensuring an only slightly reduced maximum specific growth rate of approximately 0.4 h^{-1} (Fig. 2).

At these lower concentration of Mg^{2+} (100 µmol L^{-1}) and Ca^{2+} (25 µmol L^{-1}) in the 4C medium, a strong inhibitory effect of Cd^{2+} (EC50 85±0.5 µmol L^{-1}) and Ni^{2+} (EC50 62±1.8 µmol L^{-1}) on the maximum growth rate of *P. brassicacearum* was observed (Fig. 3a and b). While in the previous experiment at high Mg^{2+} and Ca^{2+} concentrations in the medium, inhibition was almost negligible at heavy metal concentrations of 100 µmol L^{-1} (see Fig. 1), a similar concentration at low Mg^{2+} and Ca^{2+} contents led to a dramatic decrease of bacterial growth in both experimental setups. In addition, Fig. 3 and the EC50 values

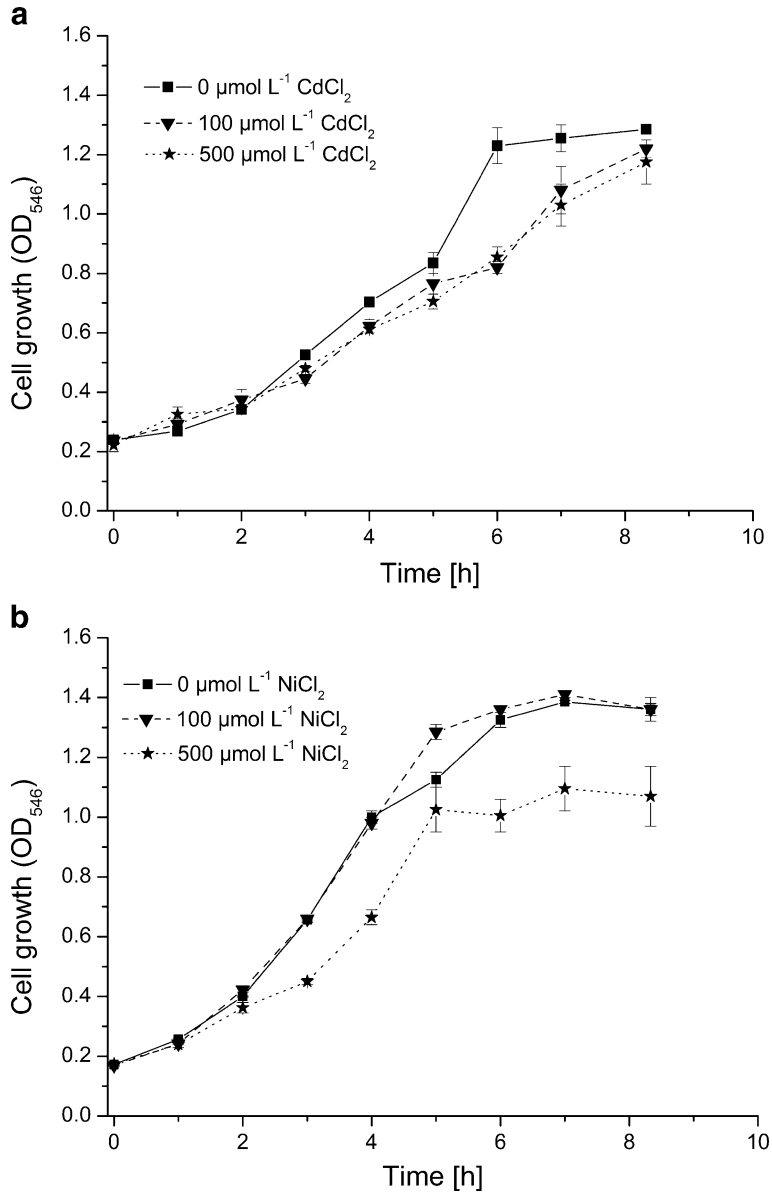

Fig. 1 Growth curves of *P. brassicacearum* based on synthetic mineral salt medium 4C in the presence of: **a** 0, 100 or 500 µmol L^{-1} $CdCl_2$; 1,250 µmol L^{-1} $MgSO_4$ and 800 µmol L^{-1} $CaCl_2$. **b** 0, 100 or 500 µmol L^{-1} $NiCl_2$; 1,250 µmol L^{-1} $MgSO_4$ and 800 µmol L^{-1} $CaCl_2$

derived from this experiment (given in Table 1) indicate a higher toxicity of Ni^{2+} compared to Cd^{2+}.

3.4 Combined Effect of Cd^{2+} and Ni^2 on Growth of *P. brassicacearum* at Low Mg^{2+} and Ca^{2+} Concentrations

Plants and soil microorganisms growing in heavy metal contaminated soils are usually exposed to mixtures of different pollutants. After studying the growth inhibiting impact of Cd^{2+} or Ni^{2+} separately, the combined effect of these metals at different particular concentrations is investigated. The curve progression of Fig. 4 shows a sharp decline of μ_{max} [per hour] with increasing concentrations of Cd^{2+} and Ni^{2+} present in 4C medium. Bacterial growth of *P. brassicacearum* was strongly inhibited by equimolar mixtures of Ni^{2+} and Cd^{2+} (EC50 Cd^{2+}/Ni^{2+} 40± 0.4 µmol L^{-1}). The combined toxicity effect of Cd^{2+} and Ni^{2+} on μ_{max} values [per hour] of *P. brassicacearum* can therefore be described as additive. The course of isolines illustrates again the higher toxicity of Ni^{2+} towards the bacteria than the same concentration of Cd^{2+}.

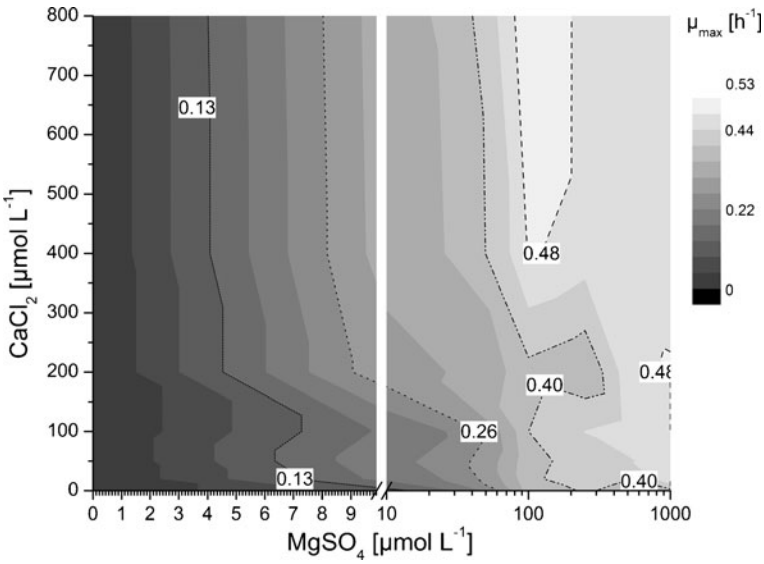

Fig. 2 Dependency of μ_{max} [per hour] on Mg^{2+} and Ca^{2+} concentration, 100 μmol L^{-1} $MgSO_4$ and 25 μmol L^{-1} $CaCl_2$ present in 4C medium ensure a maximum specific growth rate of 0.4 h^{-1}

3.5 Effect of EDTA in the Absence or Presence of Cd^{2+} or Ni^{2+}, Respectively

As EDTA is widely used in remediation processes for heavy metal contaminated soils (Maki et al. 2009), the effect of EDTA on Cd^{2+} and Ni^{2+} toxicity for growth of *P. brassicacearum* was examined. For this purpose, screening experiments in microtiter scale were carried out using the developed 4C medium with low concentrations of Ca^{2+} and Mg^{2+}, varying concentrations of Cd^{2+} or Ni^{2+} (0–200 μmol L^{-1}), respectively, and of non-complexed EDTA (0–1,000 μmol L^{-1}). As indicated by Fig. 5a and b, no significant inhibitory of Cd^{2+} or Ni^{2+} was observed when EDTA was present at respective stoichiometric concentrations (EC50 Cd^{2+}/EDTA 19,953±1,852 μmol L^{-1}; EC50 Ni^{2+}/EDTA 4,148±616 μmol L^{-1}). At equimolar concentrations of EDTA and Cd^{2+} (Fig. 5a) or Ni^{2+} (Fig. 5b), the maximum growth rates were in the range between 0.4 and 0.53 h^{-1}, independent of the respective heavy metal concentration. This indicates that the heavy metals are completely masked by the formation of strong and non-inhibitory EDTA chelates. However, not only an excess concentration of either heavy metal ions (EC50 Cd^{2+} 85±0.5 μmol L^{-1}; EC50 Ni^{2+} 62± 1.8 μmol L^{-1}, see Fig. 5 along *x*-coordinate) but also of non-complexed EDTA (EC50 EDTA 116± 4.6 μmol L^{-1}, see Fig. 5 along *y*-coordinate) resulted in decreasing maximum specific growth rates of *P. brassicacearum*.

Table 1 summarises the EC50 values which could be obtained by graphical determination. As discussed before it becomes obvious, that high concentrations of either Mg^{2+} or Ca^{2+} significantly reduce toxicity of the heavy metal ions Cd^{2+} and Ni^{2+} towards *P. brassicacearum*. The aforementioned higher impact of nickel in comparison to cadmium also is described in this table, while the addition of both heavy metal ions results in an increased effect in comparison to addition of only one heavy metal species. Additionally, the EC50 values for the experiments containing EDTA are depicted in Table 1.

In Table 2 some physicochemical properties of the considered metal ions and observations concerning toxicity are listed. The toxic effects are subdivided into three classes: absence of EDTA, equimolar concentrations and excess of EDTA. Each class showed different impact on bacterial growth.

The similar ion radii of Ni^{2+} and Mg^{2+} on the one hand and Cd^{2+} and Ca^{2+} on the other hand (see Table 2) as well as similar ionic indices lead to the conclusion that toxic effects are based on competitive interactions. The comparably high complexation constants (log KB) of heavy metal ions with EDTA explain the fact that equimolar concentrations of heavy metal ions and EDTA result in almost no inhibition effect on *P. brassicacearum*: heavy metal complexation is preferred compared to complexation of Ca^{2+} and Mg^{2+}. The toxicity of both compounds is masked due to the complexation.

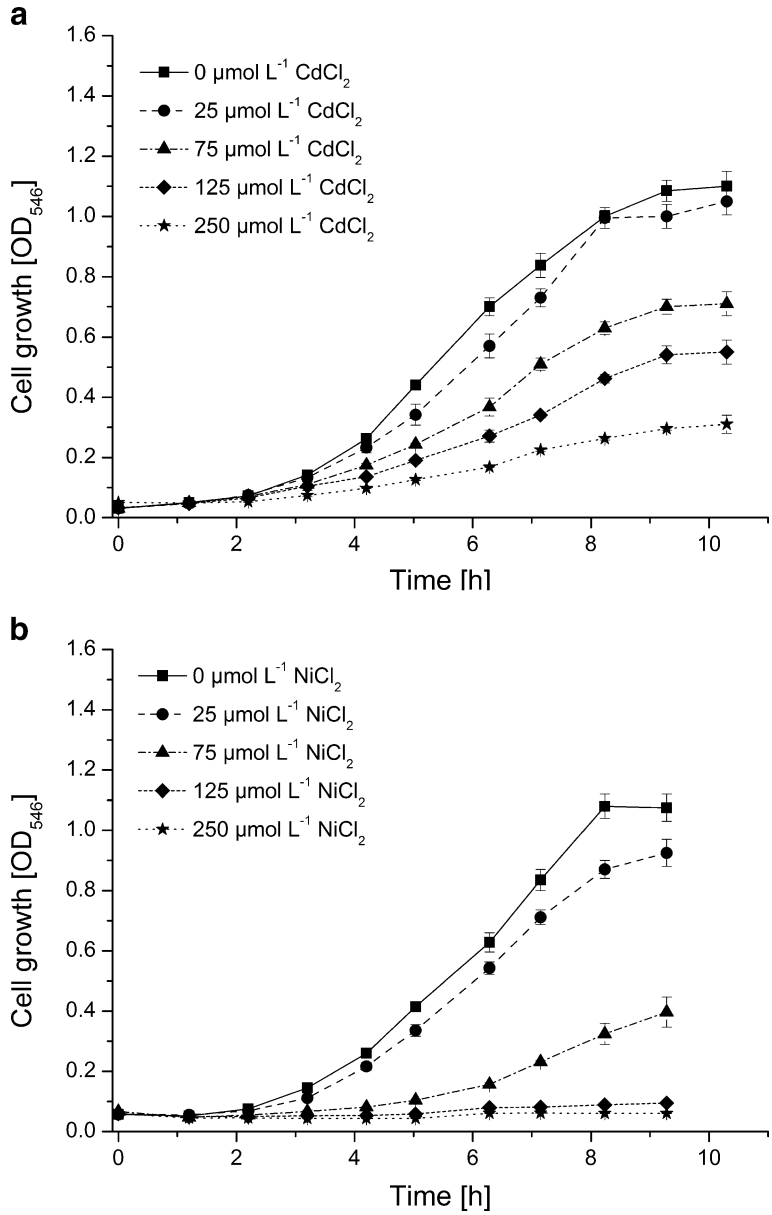

Fig. 3 Growth of *P. brassicacearum* based on mineral salt medium 4C in the presence of: **a** 0, 25, 75, 125 or 250 µmol L^{-1} CdCl2; 100 µmol L^{-1} MgSO4 and 25 µmol L^{-1} CaCl2. **b** 0, 25, 75, 125 or 250 µmol L^{-1} NiCl2; 100 µmol L^{-1} MgSO4 and 25 µmol L^{-1} CaCl2

4 Discussion and Conclusions

The present study focuses on the interactive effects of environmentally important heavy metals (Cd^{2+} and Ni^{2+}), essential macroelements (Ca^{2+} and Mg^{2+}) and a synthetic metal chelating agent (EDTA) on growth of a representative PGPR (*P. brassicacearum*) in mineral salts media. Complex growth media, soil waters and

Table 1 Comparison of EC50 values of Cd^{2+}, Ni^{2+} and EDTA

Mg^{2+}/Ca^{2+}	High	High	Low	Low	Low	Low	Low	Low
Heavy metal/chelator	Cd^{2+}/–	Ni^{2+}/–	Cd^{2+}/–	Ni^{2+}/–	Cd^{2+}/Ni^{2+}/–	Cd^{2+}/EDTA	Ni^{2+}/EDTA	–/EDTA
EC50 [µmol L^{-1}]	15,849±80	3,578±1,002	85±0.5	62±1.8	40±0.4	19,953±1,852	4.148±616	116±4.6

High 800 µmol L^{-1} (Ca^{2+})/1,250 µmol L^{-1} (Mg^{2+}), *Low* 25 µmol L^{-1} (Ca^{2+})/100 µmol L^{-1} (Mg^{2+})

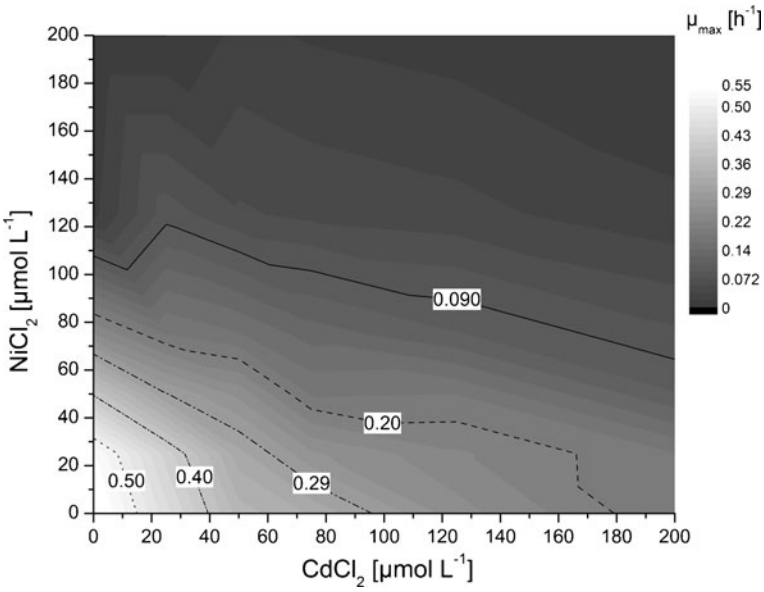

Fig. 4 Combined effect of heavy metal toxicity on μ_{max} [per hour] of *P. brassicacearum* 4C medium in presence of $CdCl_2$ and $NiCl_2$; Ni^{2+} (75 μmol L^{-1}): μ_{max}=0.24 h^{-1}; Cd^{2+} (75 μmol L^{-1}): μ_{max}= 0.32 h^{-1}; Ni^{2+} and Cd^{2+} (each 75 μmol L^{-1}): $\mu_{max,\ combined}$=0.14 h^{-1}; reference (no heavy metal) μ_{max}=0.53 h^{-1}

sediment pore waters can contain a wide variety of dissolved inorganic and organic constituents which may drastically alter the speciation of metals in solutions and thus mask the effective interactions between heavy metals, essential cations and additional chelators. In order to avoid or minimise such undesired interactions, the investigations are carried out under defined conditions in liquid cultures with mineral salts media containing either one or all of four plant exudate compounds (glucose, fructose, succinate and citrate, each 2 mmol L^{-1}).

Although laboratory conditions do not adequately represent field settings, they are necessary to gain a better understanding of the influence of major essential cations and chelating agents on the toxicity of heavy metals towards rhizobacteria. Independent of the actual chemical speciation of Cd^{2+} and Ni^{2+} in the mineral media used for this study, the defined and comparable conditions should thus supply significant information about the interactive influence of Ca^{2+} and Mg^{2+} at different concentrations as well as of EDTA on growth of *P. brassicacearum* and on the inhibitory effect caused by Cd^{2+} and Ni^{2+}. Since the toxic effect of Cd^{2+} on *P. brassicacearum* has been characterised as a bacteriostatic inhibition of growth, but not as a bacteriocidal inactivation (Pages et al. 2007), the respective maximum specific growth rate μ_{max} [per hour] of this organism is used as an indicator for the magnitude of toxicity caused by Cd^{2+}, Ni^{2+} and EDTA.

Assuming that the displacement of essential metal ions from biomolecules by heavy metal ions does not necessarily result in the inactivation of the biomolecule, the inhibitory effect of Cd^{2+} and Ni^{2+} on growth of *P. brassicacearum* should thus, at least in part, be based on their competition with Ca^{2+} and Mg^{2+}. According to this, the respective concentrations of Ca^{2+} and Mg^{2+} are supposed to have an important influence on the heavy metal toxicity. This assumption is confirmed by comparative investigations which were carried out using two different growth media which contained the same components but a different concentration of Ca^{2+} and Mg^{2+}, respectively. When a mineral salt medium containing 1.25 mmol L^{-1} of Mg^{2+} and 0.8 mmol L^{-1} of Ca^{2+} is used, only a minor inhibitory effect of Cd^{2+} and Ni^{2+} on growth of *P. brassicacearum* is observed even at a high concentration of 500 μmol L^{-1} of Cd^{2+} (EC50 15,849± 80 μmol L^{-1}) or Ni^{2+} (EC50 3.578±1,002 μmol L^{-1}). In contrast, at minimum concentrations of Mg^{2+} (100 μmol L^{-1}) and Ca^{2+} (25 μmol L^{-1}) which still enable growth at a maximum rate in the absence of heavy metals, an expected strong toxic effect is detected (EC50 Cd^{2+} 85±0.5 μmol L^{-1}; EC50 Ni^{2+} 62±1.8 μmol L^{-1}). This is in accordance with other studies describing a protective effect of Ca^{2+} or Mg^{2+} (respectively, the water hardness) against heavy metal toxicity on pro- and eukaryotic organisms (Aimal and Khan 1984; Hong et al. 1995; Heijerick et al. 2003; Slaveykova et al. 2009). Although it is known that the

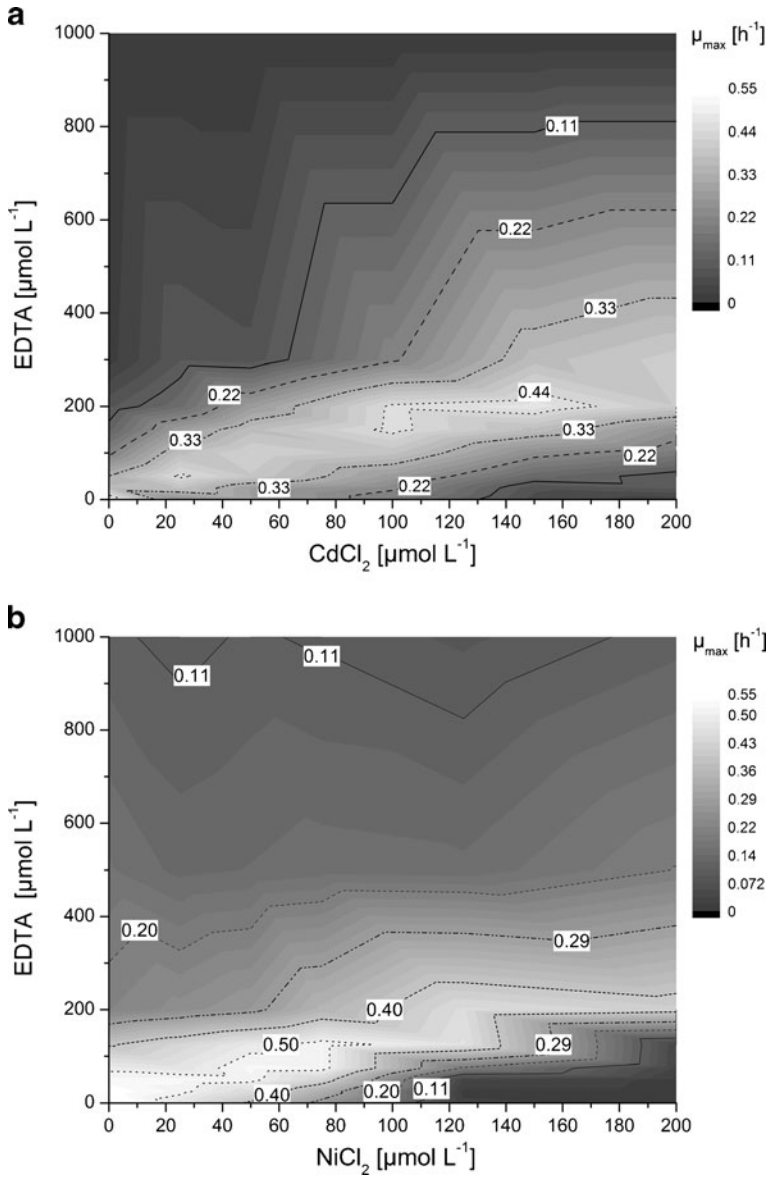

Fig. 5 Influence of chelating agent EDTA on heavy metal toxicity: **a** Variation of $CdCl_2$ (0–200 µmol L^{-1}) and EDTA (0–1,000 µmol L^{-1}). **b** Variation of $NiCl_2$ (0–200 µmol L^{-1}) and EDTA (0–1,000 µmol L^{-1})

Table 2 Comparison of ion radii and complexation constants of different cations (Ringbom 1963; Lide 1994)

Element	Electro-negativity	Ionic index	Ion radius [pm]	log K_B[a] EDTA	Toxic effect		
					Without EDTA	Equimolar EDTA	Excess EDTA
Ni^{2+}	1.9	5.8	69	18.62	++	−	++
Mg^{2+}	1.31	5.6	72	8.79	−	+	++
Cd^{2+}	1.69	4.2	95	16.46	+	−	++
Ca^{2+}	1.00	4.0	100	10.69	−	+	++

++ highest toxicity, + high toxicity, − no toxicity

[a] Stability constant with EDTA

toxicity may be reduced if dissolved cations such as Ca^{2+} or Mg^{2+} compete with metal cations for uptake into the organism (Campbell 1995; Santore et al. 2002), the observed differences in the EC50 values for Cd^{2+} and Ni^{2+} at different Ca^{2+} or Mg^{2+} concentrations (Table 1) are surprisingly high and indicate that the influence of essential macroelements on the heavy metal toxicity is even stronger than expected.

Although phosphate, which was used as pH buffer in this medium, tends to form insoluble salts with certain metal species and hence, reduces the effective concentration of bioavailable heavy metal ions, control experiments with different concentrations of phosphate or HEPES buffer instead of phosphate gave no hint of a strong chelation or potential precipitation of insoluble Cd^{2+} or Ni^{2+} salts (data not shown).

In contrast to Cd^{2+}, the transition metal nickel is an essential cofactor for a number of enzymatic reactions. At elevated concentrations, however, Ni^{2+} is also highly toxic (Babich and Stotzky 1983). Under artificial conditions, i.e. at concentrations that are much higher than in the natural environment, Ni^{2+} (ion radius, 95 pm) may be taken up by microbial Mg^{2+} (ion radius, 72 pm) transportation systems such as the fast and unspecific CorA magnesium transporter described for Gram-negative bacteria like *Salmonella typhimurium* (Tao et al. 1995; Eitinger and Friedrich 1997; Kehres et al. 1998; Smith and Maguire 1998). In these systems, Mg^{2+} seems to be a competitive inhibitor of Ni^{2+} transport so that the concentration of Mg^{2+} may have a significant impact on Ni^{2+} toxicity.

At comparable concentrations, Ni^{2+} exhibits a stronger inhibitory effect (EC50 62±1.8 μmol L^{-1}) on growth of *P. brassicacearum* than Cd^{2+} (EC50 85± 0.5 μmol L^{-1}). This higher toxicity of Ni^{2+} could possibly be explained by a missing or weak detoxification mechanism, or by its competition with the metabolically higher importance of Mg^{2+} compared to that of Ca^{2+}. Magnesium acts as cofactor in more than 300 enzymatically catalysed reactions, affecting energetic metabolism, transmembrane ion movements, e.g. Na,K-ATPase, and synthesis of DNA and RNA (Jorgensen et al. 2003; Haber 2004). This would also explain why, in the absence of heavy metal ions, the dependency of the maximum growth rate on the Mg^{2+} concentration is significantly higher than on the calcium concentration (Fig. 2). Hence, inhibition of Mg^{2+}-based reactions by structural related Ni^{2+} seems to have a greater influence on bacterial metabolism than inhibition of Ca^{2+}-based reactions by Cd^{2+}. The additive toxic effect (EC50 Cd^{2+}/Ni^{2+} 40±0.4 μmol L^{-1}) on μ_{max} [per hour] of *P. brassicacearum* by facing Ni^{2+} and Cd^{2+} corroborate the hypothesis of different target sites within bacterial cells.

However, the poor biodegradability of EDTA becomes advantageous for investigations of metal-

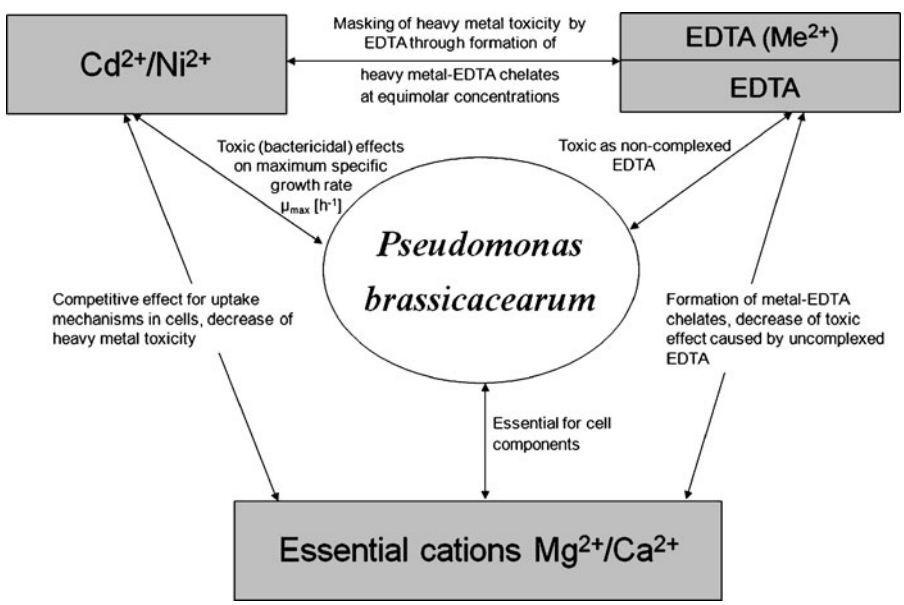

Fig. 6 Summary of mutual interactions between heavy metals Cd^{2+} and Ni^{2+}, essential divalent cations Ca^{2+} and Mg^{2+}, chelator EDTA and plant-growth promoting rhizobacteria *P. brassicacearum* which could be observed in this study

chelating effects on the inhibition of growth of rhizobacteria, as its total concentration remains constant during the experiments. When EDTA is added to *P. brassicacearum* cultivations in 4C medium containing toxic nickel or cadmium concentrations, a noticeable increase in the EC50 values is observed (Fig. 5a and b). At equal concentration of heavy metal ions and EDTA, growth of *P. brassicacearum* is not inhibited which can be explained by the formation of the corresponding Cd- or Ni-EDTA chelates. Compared to Ca- or Mg-EDTA, these are thermodynamically highly stable (compare complexation constants K_B in Table 2), leading to very low concentrations of free heavy metal ions in the solution. In summary, this results in a negligible or highly reduced impact of Cd^{2+} and Ni^{2+}. However, if concentrations of EDTA exceed that of Cd^{2+} or Ni^{2+}, the chelator also exhibits an inhibitory effect, most probably due to removal of Ca^{2+} and Mg^{2+} from the outer cell membrane which affects membrane integrity (Chavez de Paz et al. 2010).

It was shown for a representative PGPR that the heavy metal toxicity depends essentially—even stronger than expected—on environmental conditions like concentrations of Mg^{2+} and Ca^{2+}, as well as on the presence of chelating agents such as EDTA. Similar ion radii of Mg^{2+} and Ca^{2+} and their toxic antagonists Ni^{2+} and Cd^{2+} can be assumed responsible for the observed competition, either in uptake mechanism into cells, in toxic effects at cellular targets or for detoxification mechanisms.

Figure 6 summarises the results of this study and illustrates the interactions between the essential divalent cations Ca and Mg, the heavy metal ions Cd and Ni, the chelator EDTA and the observed plant-growth promoting bacteria *P. brassicacearum*, which could be observed in this study .

Additionally and as a further conclusion, the application of EDTA for remediation of heavy metal contaminated soil can be seen from two sides: on the one hand, EDTA has proven to build highly stable complexes with both heavy metal ions tested in this study, leading to a reduced toxicity onto soil bacteria such as *P. brassicacearum*. On the other hand, EDTA itself poses toxic properties towards bacteria in the uncomplexed form. Besides its poor biodegradability, this observation should lead to careful evaluation of synthetic chelating agents for soil remediation purposes.

For future approaches regarding interactions between soil bacteria and contaminants such as complexing agents and heavy metal ions, a transfer of the experiments discussed in this work into a soil system, e.g. soil column or lysimeter, might be a feasible advance.

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