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Rhizoremediation of phenol and chromium by the synergistic combination of a native bacterial strain and *Brassica napus* hairy roots

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ABSTRACT

A bacterial strain resistant to phenol and Cr (VI) was isolated from an industrial polluted soil of Córdoba province (Argentina), which was identified as *Pantoea* sp. FC 1. This microorganism was able to use phenol as sole carbon source. In addition it was capable of reducing Cr (VI) to Cr (III) in mineral and nutrient media. The isolated strain exhibited some properties as plant-growth promoting bacterium (PGPB), such as production of Indole Acetic Acid (IAA) and synthesis of siderophores, as well as being capable of solubilizing inorganic phosphates. A rhizoremediation system using the association *Pantoea* sp. FC 1-*Brassica napus* hairy roots (HRs) was tested for phenol and Cr (VI) removal in a hydroponic system. Microbial inoculation improved both phenol removal and chromium accumulation efficiency by HRs, showing a significant increase in Cr (III) accumulation compared to non-inoculated HRs, exceeding 1000 mg kg^{-1}. Cr (III) was detected in HR biomass and supernatants, suggesting a possible Cr (VI) reducing activity of *B. napus* HRs. Basic studies in plant model systems, such as HRs, provide additional useful information that could facilitate the transition of this technology into plants suitable for practical rhizoremediation applications.

1. Introduction

Several industrial operations produce highly dangerous organic and inorganic compounds which are released into the environment. Among them, phenol and chromium (Cr) are listed as priority pollutants by the US Environmental Protection Agency (EPA) due to their toxicity and persistence. Although conventional physicochemical treatments have been developed for environmental restoration of polluted areas, they are usually quite expensive and cumbersome (Malik, 2004). Therefore, alternative biological methods such as bacterial bioremediation have received considerable interest in recent years (Guo et al., 2010; Megharaj et al., 2011). One of the main advantages of xenobiotic bioremediation is the fact that it frequently results in complete mineralization of toxic substances. In contrast, heavy metals cannot be degraded and, therefore, bioremediation mechanisms involve sequestration, biosorption or changes in their oxidation states, such as Cr (VI) to Cr (III) reduction (Patra et al., 2010; Singh et al., 2011). Biological reduction of Cr (VI) to the less toxic insoluble Cr (III) is probably the most studied microbial mechanism for chromium bioremediation (Ramírez-Díaz et al., 2008). However, the microbial remediation of metal species present in the environment is limited, especially when dealing with sediment contamination (Yu et al., 2008). In contrast to microorganisms, plants are capable of removing and store high concentrations of heavy metals that may undergo speciation into their biomass, a kind of phytoremediation called phytoextraction (Abhilash et al., 2009).

A convergence of phytoremediation and microbial bioremediation strategies led to a new and more successful approach to remediate contaminants called rhizoremediation or bacterially assisted phytoremediation (Glick, 2010). The use of this technology for rehabilitation of contaminated environments is an emerging area of interest. This strategy employs rhizospheric microorganisms, like plant growth promoting bacteria (PGPB) capable of stimulating growth of host plants through different mechanisms such as siderophore or phytohormone production and mineral solubilization. Furthermore, there are some plant-associated bacteria able to degrade organic contaminants and to tolerate/detoxify heavy metals that can assist plants in pollutant remediation, as well as to improve plant biomass production (Weyens et al., 2009; Rajkumar et al., 2010). Although rhizoremediation occurs naturally,

Abbreviations: Cr (III), Chromium III; Cr (VI), Chromium VI; HRs, Hairy roots; PGP, Plant growth promoting; PGPB, Plant growth promoting bacteria; NM, Nutrient Medium.

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it can also be optimized by using suitable plant-microbe partnerships, providing an ecologically safe method for restoration and remediation (Gerhardt et al., 2009).

Different plant model systems have been employed in the field of phytoremediation research. However, the use of hairy roots (HRs) has gained relevance in recent years because they have contributed to our knowledge of the complex biochemical and molecular mechanisms involved in phytoremediation of organic and inorganic pollutants (Agostini et al., 2013). In this sense, HRs obtained from species of the hyperaccumulator genus *Brassica*, such as *Brassica napus*, are among the most efficient HRs involved in phytoremediation (Agostini et al., 2003; Guillon et al., 2006). However, to our knowledge no reports are available on chromium removal by HRs, and only few reports describe the effect of bacteria-HRs association on pollutants removal (González et al., 2013).

Consequently, the aims of the present work were a) to evaluate the capability of a bacterial strain isolated from a contaminated area to tolerate and remove phenol and Cr (VI); b) to study the bacterium PGPB properties; and c) to analyze the removal efficiency of this microorganism and the partnerships HRs-bacterium interaction, as model system for future rhizoremediation studies.

2. Materials and methods

2.1. Sample collection

Sediment samples from a discharge channel of effluents belonging to chemical and petrochemical industries from Río Tercero, Córdoba, Argentina (32° 9′7.39″S 64° 6′2.23″W) were collected in plastic bags. They were kept at 4 °C and employed for the isolation of phenol and Cr (VI) resistant bacteria as is described in next section.

2.2. Bacterial isolation, characterization and identification

For selection of phenol or Cr (VI) resistant strains, appropriately diluted soil samples (10^{-1} to 10^{-3}) were spread on agar plates with a nutrient medium TY (NM) (Beringer, 1974) (Tryptone 5.0 g L^{-1}; yeast extract 3.0 g L^{-1}; $CaCl_2$ 0.65 g L^{-1}, Agar 13.0 g L^{-1}) supplemented with 100 mg L^{-1} phenol or 25 mg L^{-1} Cr(VI) as $Cr_2O_7K_2$. Incubation was carried out for 48 h at 28 ± 2 °C. Morphologically different colonies obtained from the plates were transferred to NM containing higher concentrations of the contaminants. On the basis of their tolerance (Section 2.3) a Gram negative bacterial strain denominated FC 1 was selected. FC 1 was characterized based on its morphological, biochemical and genetic features. Oxidase and catalase test were performed using standardized methods. In addition, biochemical analysis was performed using API 20 NE and API 20 E kit systems (BioMerieux® SA).

The identification through amplification of 16S rRNA gene was done by MACROGEN Company (Seoul, Korea). The sequence was compared using BLAST program (http://www.ncbi.nlm.lih.gov) and the results were deposited in GenBank, under accession number KC478287.

The bacterium was maintained on solid NM at 28 °C and subcultured weekly.

2.3. Evaluation of phenol and chromium bacterial tolerance

For tolerance test, FC 1 strain was spread on agar plates containing NM and MM9 mineral medium with different phenol (100–1000 mg L^{-1}) or Cr (VI) (50–500 mg L^{-1}) concentrations. MM9 contained Na_2 H PO_4 2.0 g L^{-1}; K H_2PO_4 9.0 g L^{-1}; NaCl 2.5 g L^{-1}; NH_4Cl 1.0 g L^{-1}; with 13.0 g L^{-1} Agar added. Only for Cr (VI) tolerance assays, glucose (10 g L^{-1}) was used as carbon source.

Plates were incubated for a week at 28 °C, and maximum tolerated concentration (MTC) for each compound was determined. MTC was defined as the highest phenol or Cr (VI) concentration that allowed bacterial growth.

2.4. Phenol degradation and Cr (VI) removal assays

Phenol degradation and Cr (VI) reduction assays were performed in Erlenmeyer flasks containing 20 mL of liquid medium, with different phenol (100, 200 and 500 mg L^{-1}) or Cr (VI) (10, 20, 50 and 100 mg L^{-1}) concentrations.

For phenol removal assays, MM9 medium with phenol as only carbon source was used. Cr (VI) removal assays were carried out using NM and MM9 medium which was supplemented with glucose (3 g L^{-1}) as carbon source.

All flasks were inoculated with a bacterial culture grown in NM, to achieve an initial absorbance of 0.05 at 600 nm and then, they were incubated in 150 rpm and 28 ± 2 °C.

Abiotic controls were performed using non-inoculated media supplemented with phenol or Cr (VI). Inoculated MM9 media containing glucose 3 g L^{-1} and NM without pollutants were employed as growth controls.

Daily, aliquots of 1 mL were withdrawn for bacterial growth evaluation and residual phenol or Cr (VI) determination (Section 2.5). Growth was monitored by measuring absorbance at 600 nm. Then, these samples were centrifuged at 10,000 rpm for 5 min and residual phenol or Cr (VI) was determined in supernatants. The experiments were conducted in triplicate.

2.5. Analytical methods

2.5.1. Phenol determination

Residual phenol concentration was spectrophotometrically evaluated in supernatants according to Wagner and Nicell (2002) using Beckman DU640 spectrophotometer. Briefly, samples of 100 μl were mixed with 700 μl of sodium bicarbonate (pH 8), 100 μl of 4-aminoantipyrine (20.8 mM) and 100 μl of potassium ferricyanide (83.4 mM). After 5 min, absorbance at 510 nm was measured. The absorbance data were converted to phenol concentrations using a calibration curve from 0 to 100 mg L^{-1} with a r^2 of 0.995. The results were expressed as percentage of removed phenol.

2.5.2. Chromium determination

Cr (VI) was determined at 540 nm after reaction with diphenylcarbazide in acid solution. The reaction mixture contained 500 μl of H_2SO_4 0.2 N, 200 μl of diphenylcarbazide (5 mg mL^{-1}); 500 μl of each sample were added at a final volume of 5 mL, which was obtained by adding distilled water, according to APHA (1995) modified method. The absorbance data were converted to Cr (VI) concentrations using a calibration curve from 0 to 10 mg L^{-1}, with a r^2 of 0.988.

For total Cr [Cr (VI) plus Cr (III)] determination, samples were first digested in HNO_3 and chromium accumulated in biomass was analyzed by Atomic Absorption Spectrometry using a Perkin Elmer Analyst 400 (AAS).

In supernatants, total chromium concentration was determined by AAS at the end of the assay (APHA, 1989). Cr (III) concentration was calculated by difference between total chromium and Cr (VI).

2.6. Determination of plant growth promoting (PGP) properties

2.6.1. Indol acetic acid (IAA) production

IAA was analyzed following Glickmann and Dessaux's (1995) method. The bacterial strain was cultured in the dark during 72 h in Luria Bertani medium, with and without tryptophan

(0.5 mg mL^{-1}). After centrifugation (10,000 rpm, 10 min), the supernatant was mixed with Salkowski's reagent and the absorbance was measured at 530 nm. Final IAA concentrations were calculated using a calibration curve and were expressed as μg mL^{-1}.

2.6.2. Siderophore production

Siderophore production was tested using Schwyn and Neilands's (1987) method. Petri dishes with Chrome-azurol S (CAS) medium were inoculated with FC 1 strain and they were incubated for 7 d in the dark at 28 °C. CAS medium contains the ternary complex azurol S/ferrum III/trimethylammonium bromide (HTDMA) that changes from blue to orange when siderophores remove iron from the medium. An orange halo around the colony indicates a positive result for siderophores production.

2.6.3. Phosphate solubilization

For this determination, the strain was spread in NBRIP-BPB medium and incubated for a week at 28 °C (Mehta and Nautiyal, 2001). This medium contained bromothymol blue as redox indicator and Ca_3 $(PO_4)_2$. If bacteria are capable of solubilizing $Ca_3(PO_4)_2$, the medium is acidified and a color change from blue to yellow is observed.

2.6.4. ACC (1-aminocyclopropane-1-carboxylate) deaminase activity

The capability of the bacterial strain to grow with ACC as the only source of nitrogen was evaluated (Glick et al., 1995). The microorganism was cultured during 24 h in NM and then, it was inoculated in liquid medium with and without ACC (0.3 g L^{-1}). Media were incubated during 72 h at 28 °C and 150 rpm. After the incubation period, bacterial growth was determined measuring the absorbance at 600 nm.

2.7. Association B. napus HRs and FC 1 strain for removal studies

2.7.1. Hairy roots cultures

HRs cultures of *B. napus* were obtained inoculating sterile leaf explants with *Agrobacterium rhizogenes* strain LBA 9402 as previously described (Agostini et al., 1997). They were subcultured every 30–35 d in Murashige-Skoog (MS) liquid medium (Murashige and Skoog, 1962) enriched with vitamins and kept in an orbital shaker at 100 rpm at 25 ± 2 °C in the darkness.

2.7.2. Phenol and Cr (VI) removal assays

Root samples (100 mg) of *B. napus* HR were grown in MS medium during 14 d. At this time, HR cultures were inoculated with the FC 1 strain to obtain an initial A_{600nm} of 0.05. Filter-sterilized phenol or Cr (VI) solutions were added to the cultures to reach a final concentration of 100 mg L^{-1} or 10 mg L^{-1}, respectively.

HRs growing in MS medium plus phenol or Cr (VI) were employed to analyze contaminant removal by HRs without microorganisms. In addition, phenol and Cr (VI) removal by the strain FC 1 in medium MS was evaluated. Abiotic controls (MS medium with each pollutant) were used to discard possible losses by physical mechanisms.

Samples of 1 mL were taken every day during 15 d. After centrifugation at 10,000 rpm for 5 min, supernatants were used to determine residual phenol or Cr (VI) as was described in Section 2.4. After 15 d, HRs were harvested and dry weight was measured. Cr (III) and Cr (VI) accumulation by HRs was analyzed by AAS in a specialized laboratory. In supernatants, total chromium concentration was determined by AAS at the end of the assay. Cr (III) concentration was calculated by difference between total chromium and Cr (VI).

The results were expressed as removal efficiency (%), removal rate for each pollutant (mg L^{-1} h^{-1}) and accumulated chromium in HRs (mg kg^{-1}). It was also calculated as percentage (mg accumulated chromium/mg added Cr × 100).

2.8. Reagents

All reagents used in the present experiments were of analytical grade and were purchased from Merck and Sigma–Aldrich. Phenol and Cr (VI) have purity in excess of 99.5%. All solutions and culture media were prepared using deionized water.

2.9. Statistical analysis

All the experiments were done at least three times in triplicate. Data were analyzed using ANOVA, followed by the posteriori Multiple Range test ($p < 0.05$), through Stargraphic Plus (7.1).

3. Results and discussion

3.1. Characterization and identification of FC 1 strain

The isolated strain FC 1 was characterized as a Gram negative bacillus, catalase-positive and oxidase-negative. According to biochemical analysis, the bacillus was capable of employing various carbon sources such as glucose, mannitol, citrate, arabinose and sucrose. Results of 16S rRNA gene sequence amplification allowed us to identify this strain as *Pantoea* sp. The isolate FC1 presented high sequence similarity with *Pantoea agglomerans* strains and grouped in a cluster with *P. agglomerans* 1BJN10. In this sense, some *P. agglomerans* have been described according to their capacity to tolerate and remove different toxic compounds. This species also showed properties as PGPB (Francis et al., 2000; Asis and Adachi, 2004; Escalante et al., 2009).

3.2. Phenol and chromium (VI) bacterial tolerance

MTC for each pollutant was evaluated in NM and MM9 medium. The results showed that *Pantoea* sp. FC 1 strain was capable of tolerating high phenol and Cr (VI) concentrations; however, for both contaminants the tolerance was higher in NM than in MM9 medium. For instance, MTC for phenol was 1000 mg L^{-1} in NM, whereas it was 500 mg L^{-1} in MM9. Regarding to tolerance assays using Cr (VI), MTC values obtained in NM and MM9 medium were 200 and 100 mg L^{-1} respectively. Maybe the high content of nutrients in NM could promote growth more than MM9 medium. According to Polti et al. (2007), organic constituents in the medium could either adsorb or chelate the pollutants, thereby decreasing its bioavailability and toxicity.

Many microorganisms are capable of growing in the presence of high concentrations of different organic or inorganic compounds, however this fact does not mean that the bacterial strain have ability to detoxify these contaminants. In this regard, several mechanisms of tolerance for organic and metal compounds, such as energy-dependent efflux system and low membrane permeability were described (Ramírez-Díaz et al., 2008; Fillet et al., 2012). Based on these backgrounds, we proceed to analyze the capability of *Pantoea* sp. FC 1 to remove different phenol and Cr (VI) concentrations in liquid medium.

3.3. Growth and phenol degradation

Growth and phenol degradation were checked by adding different concentrations of this pollutant (100, 200 and 500 mg L^{-1}) in MM9 medium as sole carbon source. Media with glucose and

without phenol were employed as growth control. Other growth control was performed using MM9 medium without carbon source. The results demonstrated that this strain was capable to grow when glucose was added in the medium (Absorbance$_{600nm}$ of 0.65, Fig. 1) whereas a slight growth (Absorbance$_{600nm}$ 0.1) was observed without carbon source addition, which could be due to the presence of trace amount of carbon source of TY medium, present in the initial inoculum.

As shown in Fig. 1, *Pantoea* sp. FC 1 was able to grow in the presence of all tested phenol concentrations as sole carbon source in a mineral medium. Although phenol notably affected bacterial growth with respect to control conditions ($p < 0.05$), this growth decrease was dose-independent for concentrations between 100 and 500 mg L^{-1}. These negative effects of phenol in bacterial growth must be related with the effect of inhibition by substrate, previously described for another genus (El-Sayed et al., 2003; Afzal et al., 2007). Phenol toxicity has been associated with the binding of active phenol species to the bacterial cell surface, which causes disturbance in lipids and proteins and increasing of cellular membrane permeability, resulting in growth reduction and, sometimes, microbial death (Keweloh et al., 1999).

However, *Pantoea* sp. FC 1 was not only able to tolerate high phenol concentrations but also to completely degrade 100 mg L^{-1}. When higher phenol concentrations were used, a partial removal was observed after 12 d. The ability of *Pantoea* sp. FC 1 to grow in the presence of phenol as sole carbon source and remove it, suggests a possible metabolic pathway involved in phenol detoxification. Although in a recent study Dastager et al. (2009) described for the first time a *Pantoea* strain ability to remove high phenol concentrations, it is not yet known the mechanism by which bacteria of this genus could degrade this compound.

As it could be seen, the isolated strain was able to tolerate and remove phenol concentrations commonly found in the polluted areas to be treated. For example, it has been reported that in wastewaters from resin plants phenol concentrations are frequently in the range of 12–300 mg L^{-1} (Mukherjee et al., 2008).

3.4. Growth and Cr (VI) removal

Growth and Cr (VI) removal were evaluated in both NM (Fig. 2A) and MM9 medium amended with glucose (Fig. 2B) at different initial Cr (VI) concentrations (10 to 100 mg L^{-1}).

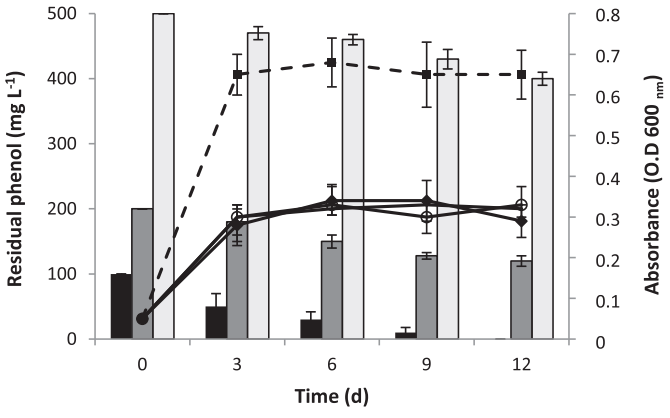

Fig. 1. Time-course of phenol degradation and growth by *Pantoea* sp. FC 1 in liquid MM9 medium containing different phenol concentrations. Bars represent the removal of different phenol concentrations (mg L^{-1}): 100 (■), 200 (■), 500 (□). Phenol concentrations in uninoculated controls remained constant along the time. Curves indicate bacterial growth at different initial phenol concentrations (mg L^{-1}): 500 (-○-), 200 (-◆-), 100 (—), 0 (---). The error bars represent standard errors.

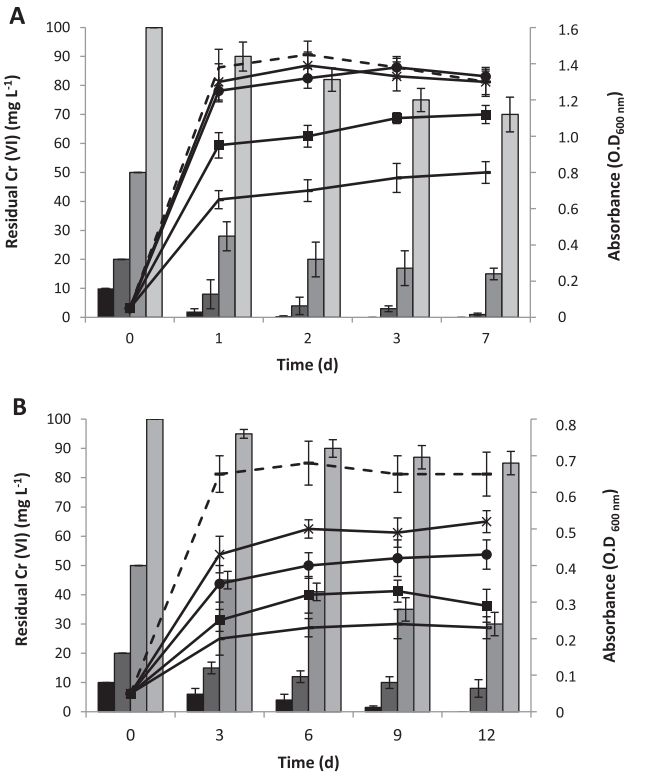

Fig. 2. Time-course of Cr (VI) removal and growth by *Pantoea* sp. FC 1 at different initial Cr (VI) concentrations. A: NM after 7 d treatment. B: MM9 medium after 12 d treatment. Bars represent removal of different Cr (VI) concentrations (mg L^{1}): 10 (■), 20 (■), 50 (■), 100 (□). Cr (VI) concentrations in uninoculated controls remained constant along the time. Curves indicate bacterial growth at different initial Cr (VI) concentrations (mg L^{-1}): 10 (-X-), 20 (-●-), 50 (-■-), 100 (—), 0 (---). The error bars represent standard errors.

The strain FC 1 was able to tolerate high Cr (VI) concentrations and grew in both NM and MM9 medium. Growth in the NM with 10 and 20 mg L^{-1} of Cr (VI) was slightly lower than that achieved in the control without Cr (VI). However, growth significantly decreased at higher metal concentrations (50 and 100 mg L^{-1}) (Fig. 2A).

Fig. 2B shows that the microbial development achieved in MM9 was lower than that in the NM. In MM9 all Cr (VI) concentrations significantly affected the bacterial growth ($p < 0.05$) compared to the control without Cr (VI). In both culture media, growth was concentration-dependent.

This negative effect of high Cr (VI) concentrations over microbial growth has been mostly associated with oxidative stress as well as with protein and DNA damage (Liu et al., 2006; Ramírez-Díaz et al., 2008).

Regarding to Cr (VI) removal, a complete reduction of 10 and 20 mg L^{-1} Cr (VI) was observed approximately at 48 h in NM (Fig. 2A). Removal percentages for higher Cr (VI) concentrations were 70% and 25% for 50 and 100 mg L^{-1} after 3 d of incubation, respectively. After 7 d, the removal levels of Cr (VI) were not altered significantly. The rate of Cr (VI) reduction increased with Cr (VI) concentration of 50 mg L^{-1}, and the highest removal rate (0.7 mg L^{-1} h^{-1}) was obtained over the initial 48 h of incubation. However, the reduction rate decreased with time regardless of the initial Cr (VI) concentrations used. This effect could be associated to consumption of electron sources in the medium and, also, to the high toxicity of the metal to bacterial cells, which probably produced oxidative stress, death and loss of active cells (Ramírez-Díaz et al., 2008).

Cr (VI) reduction in MM9 medium was lower than in NM. For instance, in MM9 medium, 10 mg L^{-1} were completely removed, whereas 20, 50 and 100 mg L^{-1} were partially removed after 12 d. This lower removal capability and the longer time required for Cr (VI) removal in MM9, suggest that *Pantoea* sp. FC 1 needs both a rich source of nutrients and electron donors to achieve high removal efficiency, since MM9 only contains glucose as carbon source, while NM is a complex nutrient broth. Additionally, total chromium concentration was evaluated in supernatants, after removal. Both stable chromium species (VI and III) were identified in the analyzed samples. However when total removal was achieved, only Cr (III) was detected. This fact would be interesting for bioremediation processes, since Cr (III) is less toxic than Cr (VI) (Polti et al., 2010). These results could indicate the presence of mechanisms involved in Cr (VI) reduction in *Pantoea* sp. FC 1, such as those catalyzed by reductases, which were described in various bacterial species (Ramírez-Díaz et al., 2008).

Although many microorganisms are capable of tolerating organic or inorganic pollutants, few bacterial strains are known to be suitable for the removal of both kinds of compounds (Song et al., 2009). According to our results, the capability of the studied strain to tolerate and remove high phenol and Cr (VI) concentrations would be an important advantage for bioremediation purposes, taking into account that many polluted areas are co-contaminated with both heavy metals and organic compounds.

3.5. Determination of PGP properties

The PGPB may benefit plant growth either by improving plant nutrition and/or by producing plant growth regulators. These microorganisms are very important in agriculture and in recent years they became suitable for bioremediation purposes, because in polluted environments bacteria with PGP properties could play important roles in plant growth. Therefore, the isolate FC 1 was screened for siderophore production, phosphate solubilization, IAA synthesis and ACC deaminase activity.

The strain showed positive results for siderophore production, which was evidenced by a characteristic orange halo around the colony in CAS medium. Glick (2010) reported that siderophores help the plants to acquire sufficient iron in the presence of elevated amounts of some potentially competing metals. Furthermore, bacterial siderophores are also able to bind metals other than iron and thus enhancing their bioavailability in plant rhizosphere (Rajkumar et al., 2010).

Similarly, inorganic phosphate solubilization was detected. The color change around the colony due to pH decrease clearly indicates the acid production, which is considered to be responsible for phosphate solubilization (Bano and Mussarat, 2003). It is well established that, as a result of phosphate solubilization, the increased availability of phosphorous improves plant nutrition and, hence, its growth and root development. Also, plants inoculated with phosphate solubilizing bacteria can increase productivity and reduce additional phosphorus incorporation in soil (Hameeda et al., 2008).

Furthermore, the strain FC 1 showed ability to produce IAA both in the absence and in the presence of tryptophan (8 and 24 μg mL^{-1} in tryptophan absence and presence, respectively). This result could indicate that the strain utilized L-tryptophan as precursor for IAA production, since the IAA production was higher when tryptophan was added to the culture medium. This was an interesting finding considering that some IAA producer bacteria can be able to improve plant growth, indirectly favoring the pollutant accumulation by increasing the plant biomass (Jiang et al., 2008).

In contrast, *Pantoea* sp. FC 1 was not able to produce ACC deaminase. Furthermore, as the bacteria did not grow in the medium without nitrogen source, it probably did not present free living capability to fix nitrogen.

Bacterial plant growth promotion is a complex process which is often achieved by the activities of more than one trait. In this study, we demonstrated that the isolate FC 1 possessed various properties as PGPB, which are advantageous for practical applications. Huang et al. (2004) demonstrated that the combination of PGPB and pollutant detoxifying microorganisms not only enables plant growth in contaminated environments but also enhance their removal capability. However, if a microorganism simultaneously presents both properties, it would have an interesting potential for rhizoremediation. In this sense, Muratova et al. (2005) reported that *Azospirillum brasiliense* SR 80 (an IAA-producing strain) led to a significant increase in oil phytoremediation efficiency when it was inoculated in a hydroponic system with wheat plants. In addition, *Staphylococcus arlettae* NBRIEAG-6, improved arsenic (As) accumulation in *Brassica juncea*, possibly due to its PGP properties and As removal capability (Srivastava et al., 2012).

Considering that *Pantoea* sp. FC 1 showed high phenol and Cr (VI) tolerance and removal capability and was also able to synthesize some PGP compounds, we suppose that a combination of this strain and plants would be a promising strategy for phytoremediation purposes. In this context, a first approximation would be provided by the use of HRs as model system.

3.6. Association B. napus HRs/Pantoea sp. FC 1 in assisted phytoremediation

HRs cultures have demonstrated a great potential as model plant system for phytoremediation studies, because they present most advantages related to their aseptic growth and stability, regarding to whole plant (Agostini et al., 2013).

Several authors have reported that HRs from different plant species are able to remove phenolic compounds (Sosa Alderete et al., 2009). However, few studies related to the use of HRs for metal detoxification have been published, and even less, related to the implication of HRs-bacterial association in remediation (González et al., 2013; Nedelkoska and Doran, 2000). Thus, in the present work the association of *Pantoea* sp. FC 1 with *B. napus* HRs for bioremediation studies of both contaminants was investigated.

For removal experiments, 14 d old HRs were treated with phenol (100 mg L^{-1}) or Cr (VI) (10 mg L^{-1}), and then, they were inoculated with the strain FC 1. The capability of HRs alone and co-inoculated HRs-*Pantoea* sp. FC 1 to remediate phenol and Cr (VI) was analyzed. Table 1 summarizes phenol removal efficiency (%) and maximum removal rate achieved by HRs, *Pantoea* sp. FC 1 and the association of both partnerships.

B. napus HRs *per se* achieved a removal efficiency of 60%, after 7 d. However, a significant enhancement of phenol degradation efficiency ($p < 0.05$) was reached by HRs inoculated with *Pantoea* sp. FC 1 in the same period of time. In addition, removal rate was higher in the presence than in the absence of this microorganism, indicating the positive effect of co-inoculation in the phenol bioremediation process. This effect could be probably due to the synergistic effect of PGP properties and the ability of this bacterium to tolerate and remove phenol in MS growth medium. These results are in agreement with those presented by González et al. (2013), which showed that the interaction between *B. napus* HRs and phenol degrading rhizospheric microorganisms improves HRs removal process. Similarly, other plant-microorganism systems have shown to be more efficient than plants alone for organic pollutant bioremediation, such as the association between *Arabidopsis* sp. and *Pseudomonas putida* PML2 in PCB degradation (Narasimhan et al., 2003).

Table 1
Phenol removed and maximum removal rate by *Pantoea* sp. FC 1, *B. napus* HRs and its association.

System	Phenol removed (mg L^{-1})	Maximum removal rate (mg L^{-1} d^{-1})
Pantoea sp. FC 1	30 (±6.8)[a]	4.7 (±1.7)[a]
HRs	60 (±5.3)[b]	6.5 (±2.4)[b]
HRs + *Pantoea* sp. FC 1	85 (±7.8)[c]	7.3 (±1.8)[b]

Different letters denote significantly differences between treatments ($p < 0.05$).

For chromium removal, both HRs as well as the association of HRs-microorganisms were capable of completely removing 10 mg L^{-1} of Cr (VI), being these systems more efficient than *Pantoea* sp. FC 1 alone However, HRs themselves were significantly slower to remove Cr (VI) than those inoculated with the microorganism, reaching 100% removal after 6 and 3 d, respectively (Table 2).

Furthermore, chromium species [Cr (VI) and Cr (III)] were determined in the supernatant and in the HRs biomass, before and after removal experiments. In this sense *B. napus* HRs accumulated 570 mg kg^{-1} Cr (III), corresponding to 12.5% of total chromium initially present in the medium as Cr (VI). In addition, the remaining chromium detected in supernatants was found as Cr (III). Hence, we may suggest the ability of *B. napus* HRs to reduce and accumulate high chromium concentrations, in concordance with previous reports, which described *Brassica* as a hyperaccumulator genus (Vara Prasad and Oliveira Freitas, 2009).

Some plants growing hydroponically have been described as efficient systems involved in chromium phytoextraction, however the accumulated metal is usually stored within the roots, with little translocation to leaves and stems (Yu et al., 2007), suggesting that the chromate reductase enzymes would be largely located at the root biomembrane rather than leaf biomembrane. In this sense, *B. napus* HRs would be an interesting system to study the mechanisms involved in Cr (VI) reduction by plant roots, which still remain unclear.

As can be seen in Table 2, inoculated HRs achieved significantly higher accumulation capability (21%) than non-inoculated HRs, indicating that *Pantoea* sp. FC 1 not only facilitated the removal process, but also increased chromium incorporation by root biomass. The benefit of this microorganism could be related to its PGP properties, as well as to its capability to reduce Cr (VI) to Cr (III), a less toxic and more easily bioaccumulable metal form for many plant species (Yu et al., 2008). In a similar way, several research works demonstrated metal phytoextraction improvement through the incorporation of PGPB in soils (Wani et al., 2007; Jiang et al., 2008). In addition, in hydroponic cultures containing Cd, Se and Hg, the positive effect of plant–rhizobacteria interaction in heavy metal phytoaccumulation, has also been described (Souza et al., 1999; Wu et al., 2006). However, according to our knowledge, this is the first study which evaluates Cr (VI) phytoremediation in liquid media by a HRs-bacterium system.

Table 2
Chromium removed (%), maximum removal rate and Cr (III) accumulation.

System	Cr (VI) removal		Accumulation in HRs biomass as Cr (III)	
	Cr (VI) removed (mg L^{-1})	Max. removal rate (mg L^{-1} d^{-1})	(%)	(mg kg^{-1})
Pantoea sp. FC 1	5.9 (±1.3)[a]	0.9 (±0.6)[a]	–	–
HRs	10[b]	1.7 (±0.2)[b]	12.5 (±3.1)[a]	570 (±150)[a]
HRs + *Pantoea* sp. FC 1	10[b]	3.3 (±0.5)[c]	21.0 (±5.0)[b]	1013(±250)[b]

Different letters denote significantly differences between treatments ($p < 0.05$).

Thus, the use of native species like *Pantoea* sp. FC 1 with both PGP properties and pollutant degrader ability could enhance plant growth under stress conditions like those found in contaminated areas, particularly when used in association with tolerant plant species, such as those belonging to *Brassica* genus. An advantage of using this plant–bacterium association is that chromium would be removed by root harvesting. This feature is particularly important because unlike organic pollutants which are frequently enzymatically degraded, the sequestered heavy metals must be physiologically removed.

4. Conclusions

Phenol and Cr (VI) tolerance and removal capabilities, combined with some plant growth promoting properties exhibited by *Pantoea* sp. FC 1, might provide certain advantages to this strain, which could be applied for bioremediation of co-polluted environments with such compounds, making it a good candidate for phytoremediation assisted by microorganisms via multiple models of action.

Moreover, greater efficiency in phenol and Cr (VI) phytoremediation by *B. napus* HRs inoculated with *Pantoea* sp. FC 1 than in non-inoculated HRs indicated that *B. napus* HRs – *Pantoea* sp. FC 1 would be an efficient and innovative system for phenol and Cr (VI) biotransformation and chromium immobilization, being both important activities from a biotechnological viewpoint.

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