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CHROMIUM RESISTANT BACTERIA: IMPACT ON PLANT GROWTH IN SOIL MICROCOSM

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Abstract: Three chromium resistant bacterial strains, *Pseudomonas fluorescens* PF28, Enterobacter amnigenus EA31 and *Enterococcus gallinarum* S34 isolated from tannery waste contaminated soil were used in this study. All strains could resist a high concentration of $K_2Cr_2O_7$ that is up to 300 mg/L. The effect of these strains on clover plants (*Trifolium campestre*) in the presence of two chromium salts $CrCl_3$ and $K_2Cr_2O_7$ was studied in soil microcosm. Application of chromium salts adversely affected seed germination, root and shoot length. Bacterial inoculation improved the growth parameters under chromate stress when compared with non inoculated respective controls. There was observed more than 50% reduction of Cr(VI) in inoculated soil microcosms, as compared to the uninoculated soil under the same conditions. The results obtained in this study are significant for the bioremediation of chromate pollution.

INTRODUCTION

Soil has been contaminated with several heavy metals mostly from mining wastes and industrial discharges. Heavy metals are often toxic in both the chemically combined and the elemental form. Because of its widespread use and adverse impact on the environment, chromium is currently receiving increased interest from various national and international organizations [1]. Even though chromium is an essential component for normal glucose utilization, chromium at high concentration becomes problematic for both fauna and flora [2]. Chromium commonly exists as Cr(III) and Cr(VI), and Cr(VI) is more toxic, mobile and permeable. It can be easily taken up by plants and subsequently can enter into the food chain [3].

Chromium compounds are highly toxic to plants and are detrimental to their growth and development. Cr(VI) is toxic to most higher plants at 100 μM/Kg dry weight [4] and leads to changes in the growth and development pattern of the plant. Among these effects there are reduced germination percentage, decrease in root length and dry weight, increase in root diameter and root hairs, reduction in plant height [5, 6].

In this context, microbial reclamation has been a promising aspect using chromium resistant bacteria to detoxify Cr(VI) in the rhizosphere environment. Detoxification of Cr(VI) can occur directly by enzymatic reduction to less mobile form Cr(III) by Cr(VI) reductase or indirectly through making complexes with metabolites [7–9]. Cr(VI) resistant strain having such detoxifying ability with plant growth promoting features has raised high hope for cost effective and eco-friendly measures for sustainable agriculture in soil tract contaminated with Cr(VI) [10–12]. Therefore, improvement of the interaction between plants and beneficial rhizosphere microbes would be an important component of bioremediation technology in agriculture [13, 14].

The aim of the present study is to check the role of chromium resistant bacterial strains isolated from tannery waste contaminated soil in Fez, Morocco, on germination and growth of clover under Cr(III) and Cr(VI) stress. Thus, soil microcosms experimentally polluted with chromium and bioaugmented with these strains were used to evaluate the ability of these strains to effectively diminish bioavailable chromium from soils. Clover plants were used as bioindicators, to address the success of the bioremediation process.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Three chromium resistant bacterial strains isolated from tannery waste contaminated soil located in Fez (Morocco), PF28, EA31 and S34 were used in the present study. Fez is a striking example of the contamination pressure. Over the last few years, the region of Fez experienced extensive industrial development. Tanneries are among the most polluting industries in the region. Effluents produced daily in tanneries are simply discharged into nearby watercourses without any treatment. Consequently, considerable chromium used in the tanning process, find their way into the Sebou River; the primary source of water for a variety of purposes (drinking, agriculture, industry, recreation).

The strains were isolated as described by Sayel et al. [15] on Luria Broth (LB) agar plates supplemented with 100 mg/L of Cr(VI) as $K_2Cr_2O_7$. Chromium tolerance was checked by transferring morphologically different colonies on LB agar plates amended with 200 and 300 mg/L of Cr(VI). Strains PF28, EA31 and S34 which could grow on plates containing 300 mg/L chromium were used for this study. The LB agar medium consisted of peptone (10 g), sodium chloride (10 g), yeast extract (5 g), agar (15 g) in 1 L distilled water. Strains were normally stored at 4°C.

16S rRNA gene identification, DNA sequencing and phylogenetic analysis of the bacterial strains

DNA of the bacterial strains was extracted by heat shock. The nearly full--length 16S rRNA gene was amplified by PCR using universal primers Fd1 (5'AGAGTTTGATCCTGGCTCAG3') and Rs16 (5'TACGGCTACCTTGTTACGACTT3'). PCR amplification was performed in 20 µL volume containing 10 ng DNA, 10 µM of each primer, 1 mM dNTP, 25 mM $MgCl_2$ and 2 µL of 10x PCR buffer. Amplification cycles (35 cycles in total) consisted of initial denaturation step at 94°C for 5 min, followed by 1 min denaturation step at 94°C, 1 min annealing step at 55°C, 1.5 min elongation step at 72°C and a final extension step at 72°C for 5 min. The PCR products were separated on a 1% agarose gel and purified using Kit MagneSil. DNA sequencing was performed using

ABI 3130; Applied Biosystems according to the manufacture instructions. Sequences were initially analyzed at NCBI server (http://www.ncbi.nlm.nih.gov) using BLAST (blastn) tool and corresponding sequences were downloaded. Parts of the 16S rRNA (920, 464 and 521 bp) corresponding to PF28, EA31 and S34 were submitted to GenBank with accession nos HE590765, FR717599 and FR715561, respectively. A phylogenetic tree was constructed using the maximum likelihood method implemented in the PhyML program at www.phylogeny.fr [16]. Some reference sequences from the GenBank were used in generating the phylogenetic tree for clarification.

Soil microcosms assay

A field soil sample was taken from an uncontaminated site far from any industrial activity. The soil was removed to a depth of 15 cm and collected in bulk quantity. The soil was kept 48 h at room temperature to allow water to equilibrate in the soil. After drying, the soil was sieved (2 mm mesh) and stored in plastic bags at 4°C. For the soil microcosm assay, Petri dishes were filled with 35 g of soil and sterilized by autoclaving (three successive sterilizations 24 h apart, at 100°C for 60 min each).

Chromate reduction in soil microcosms

To observe the effect of strains PF28, EA31 and S34 on chromate reduction, solution of Cr(VI) was added to the soil microcosm up to a final concentration of 22.8 mg/Kg and inoculated with 8 mL of PF28, EA31 and S34 pre-grown in LB to a final concentration of approximately 15x 10^7 CFU/mL. Soil moisture was adjusted to 100% to ensure optimal reduction by bacterial strains and avoid sol driers. Soil microcosms not inoculated were used as controls. All assays were performed in triplicate.

The Petri dishes were incubated at 30°C, and soil samples were taken after 1, 7 and 15 days. Cr(VI) in the soil was extracted by the alkaline digestion method [17]: 2 g of soil sample was digested with 40 mL of solution (NaOH 0.5 M and Na_2CO_3 0.28 M) for 60 min at 90°C with intermittent agitation and the filtrate was titrated with nitric acid for pH between 7 and 8. Chromate reduction efficiency was calculated on the basis of residual Cr(VI) content in the soil as estimated by diphenylcarbazide method (DPC) 0.5% (w/v): 5 mL of filtrate followed by addition of 4 mL of H_2SO_4 (2N) and 0.2 mL of DPC (0.5%) and final volume was made to 10 mL using glass distilled water [18]. Spectrophotometric measurements were made immediately at 540 nm.

Germination and inoculation of clover seeds in soil microcosms

To assess the effect of chromate bioremediation on germination and growth of clover plants (*T. campestre*), seeds were surface sterilized in alcohol for 2 min and then thoroughly washed with sterilized glass distilled water thrice. Two different chromate salts were used (trivalent chromium $CrCl_3$ and hexavalent $K_2Cr_2O_7$). The soil microcosms so prepared were contaminated artificially with Cr(VI) and Cr(III) solutions at different concentrations of 100, 200 and 300 mg/L corresponding to 22.8, 45.7 and 68.5 mg/Kg of soil, respectively, and inoculated with 8 mL of PF28, EA31 and S34 pre-grown in LB to a final concentration of approximately 15×10^7 CFU/mL. The addition of chromate stock solution and inoculum was made in such quantities that the final values of moisture content (w/w, on dry weight basis) was 100%. Soil microcosms not inoculated were used as controls.

Fifty seeds of clover were spread uniformly in Petri dishes containing 35 g of soil. Seeds were germinated in the dark at 30°C. After 7 days, the number of germinated seeds was counted and the seedlings were harvested and shoot and root were measured.

Statistical analysis

All the experiments were carried out in triplicate. Standard errors of the means and LSD were calculated following Steel and Torrie [19].

RESULTS

Bacterial strains

Three chromium resistant bacterial strains (PF28, EA31, and S34) were isolated from the soil contaminated with chromium. All bacterial strains could resist up to 300 mg/L of Cr(VI) on LB agar medium. Based on the biochemical tests and analysis of the 16S rDNA sequence (920bp, 464bp, 521bp) using BLAST function at NCBI database and Ribosomal Database Project, the isolates PF28, EA31 and S34 (NCBI GenBank Accession No. HE590765, FR717599 and FR715561) were identified as *Pseudomonas fluorescens, Enterobacter amnigenus* and *Enterococcus gallinarum*, respectively. The phylogenetic lineage of PF28, EA31 and S34 drawn from 16S rDNA sequence databases of some closely related members is presented in Fig. 1.

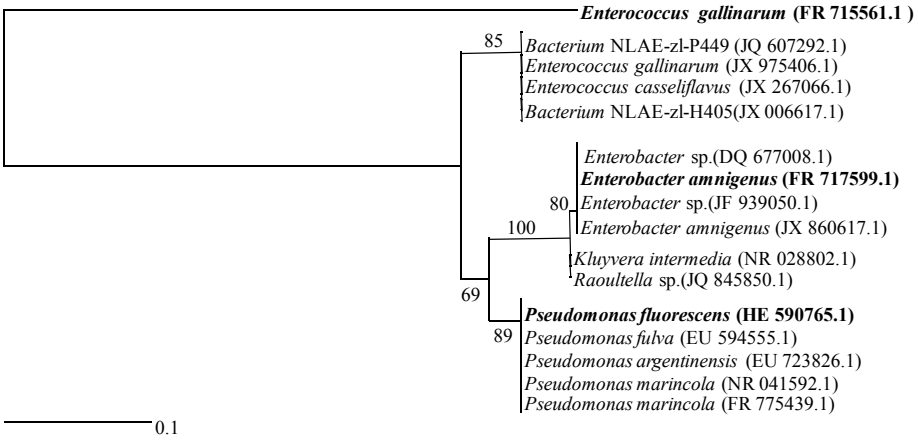

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequence of *Pseudomonas fluorescens* PF28 (HE590765), *Enterobacter amnigenus* EA31 (FR717599) and *Enterococcus gallinarum* S34 (FR715561) using the maximum likelihood method implemented in the PhyML program [16]. Identified bacteria are in bold

Chromate reduction in soil microcosms

To assess the effect of strains on chromate reduction, soil microcosms were supplemented with 100 mg/L (22.8 µg/g soil) of $K_2Cr_2O_7$ and incubated for 15 days at 30°C. Experiments were performed using the sterile soil to eliminate the effect of indigenous microflora on chromate reduction.

Reduction of Cr(VI) in soil microcosm was observed as compared to control, almost 73.4%, 50.7% and 69.6% of Cr(VI) reduced by PF28, EA31 and S34, respectively, within a period of 15 days (Fig. 2). Uninoculated soil microcosms failed to show any significant reduction of chromate.

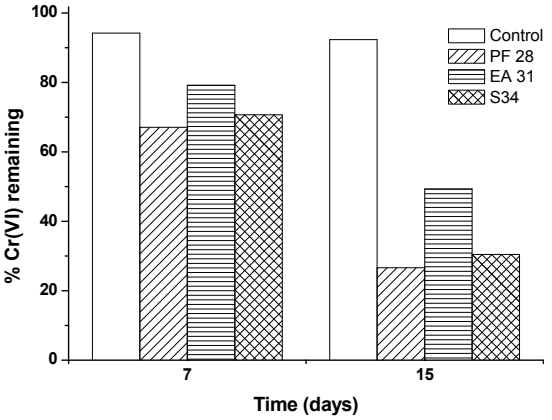

Fig. 2. Reduction of Cr(VI) by chromium resistant bacteria in soil microcosm. Initial Cr(VI) used was 100 mg/L

Plant growth experiments

Application of both chromate salts at different concentrations caused a significant decrease of seed germination especially in the case of Cr(VI) when compared with the control (Table 1). Bacterial inoculation resulted in an enhancement in seed germination both under $CrCl_3$ and $K_2Cr_2O_7$ treatments as compared to control. Both chromate salts adversely affected root and shoot lengths (Tables 2 and 3). A significant decrease in the root length was observed at 100, 200 and 300 mg/L of Cr(VI) (30%, 46% and 66%, respectively). Cr(III) was less toxic and only 20% decrease in root length was observed at 300 mg/L (Table 2). About 17% and 55% decrease in shoot length was observed at 300 mg/L of Cr(III) and Cr(VI), respectively (Table 3).

Interestingly, all the bacterial strains promoted root and shoot length even in the presence of toxic Cr(VI) salt. In the case of the soil microcosm without chromium, a significant enhancement was observed with *P. fluorescens* that improved the root length by 20% and shoot length by 15%, while *E. amnigenus* and *E. gallinarum* had no significant effect. Under 300 mg/L of $K_2Cr_2O_7$, 65%, 35% and 39% enhancement in root length was observed in PF28, EA31 and S34 inoculation, respectively, and 63%, 35% and 18% in shoot length, compared with the respective non-inoculated controls.

DISCUSSION

In the present study, three chromium bacterial strains (*P. fluorescens* PF28, *E. amnigenus* EA31 and *E. gallinarum* S34) were isolated from tannery waste contaminated soil. These strains could grow at very high concentration of $K_2Cr_2O_7$. All bacterial strains

could resist up to 300 mg/L hexavalent chromium in the medium [15]. Megharaj et al. [20] showed that *Arthrobacter* sp. and a *Bacillus* sp., isolated from a long-term tannery waste contaminated soil could grow at concentrations of Cr(VI) up to 100 mg/L in minimal. Micheal et al. [21] also demonstrated that *Desulfomicrobium* sp. was able to endure chromate up to 97 mg/L. While *Pseudomonas aeruginosa* tolerated 40 mg/L Cr(VI) [22].

The strains used in this study not only resisted but also reduced Cr(VI) efficiently in the soil; more than 50% reduction of Cr(VI) was observed in inoculated soil

Table 1. Effect of chromium resistant bacteria on germination of *T. campestre* at 100–300 mg/L of Cr(III) ($CrCl_3$) and Cr(VI) ($K_2Cr_2O_7$). (Means of three replicates)

% Germination							
mg/L	**0**	**100 (CrIII)**	**200 (CrIII)**	**300 (CrIII)**	**100 (CrVI)**	**200(CrVI)**	**300(CrVI)**
Control	100±0.00	100±0.00	98±0.00	91±1.41	85±1.41	80±0.00	76±2.83
PF28	100±0.00	100±0.00	100±0.00	96±2.82	93±1.41	90±2.82	89±1.41
EA31	100±0.00	100±0.00	100±0.00	94±0.00	90±2.65	87±1.41	85±2.94
S34	100±0.00	100±0.00	100±0.00	92±0.00	89±1.41	83±1.41	81±4.24

Table 2. Effect of chromium resistant bacteria on root length of *T. campestre* at 100–300 mg/L of Cr(III) ($CrCl_3$) and Cr(VI) ($K_2Cr_2O_7$). (Means of three replicates)

Root length (cm)							
mg/L	**0**	**100 (CrIII)**	**200 (CrIII)**	**300 (CrIII)**	**100 (CrVI)**	**200 (CrVI)**	**300 (CrVI)**
Control	1.46±0.08	1.40±0.20	1.34±0.06	1.20±0.02	1.02±0.18	0.79±0.02	0.49±0.04
PF28	1.75±0.05	1.56±0.05	1.50±0.00	1.44±0.05	1.19±0.04	1.08±0.07	0.81±0.02
EA31	1.55±0.13	1.53±0.24	1.47±0.05	1.37±0.04	1.15±0.10	0.88±0.06	0.66±0.08
S34	1.56±0.06	1.44±0.05	1.34±0.05	1.20±0.00	1.11±0.13	0.84±0.06	0.68±0.03
LSD at 0.05							
For strains	0.013148						
For treatment	0.006574						

Table 3. Effect of chromium resistant bacteria on shoot length of *T. campestre* at 100–300 mg/L of Cr(III) ($CrCl_3$) and Cr(VI) ($K_2Cr_2O_7$). (Means of three replicates)

Shoot length (cm)							
mg/L	**0**	**100 (CrIII)**	**200 (CrIII)**	**300 (CrIII)**	**100 (CrVI)**	**200 (CrVI)**	**300 (CrVI)**
Control	4.36±0.33	4.00±0.18	3.73±0.08	3.50±0.12	2.98±0.30	2.49±0.27	1.98±0.15
PF28	5.02±0.34	4.44±0.10	4.28±0.20	3.90±0.00	3.6±0.24	3.47±0.22	3.23±0.18
EA31	4.55±0.10	4.34±0.10	3.76±0.06	3.56±0.17	3.7±0.10	3.25±0.27	2.69±0.13
S34	4.40±0.16	3.57±0.50	3.25±0.40	2.76±0.28	3.22±0.34	2.48±0.15	2.34±0.27
LSD at 0.05							
For strains	0.09116						
For treatment	0.06558						

microcosms by the three strains, as compared to the uninoculated soil under the same conditions.

Seed cultures were used as bioindicators to confirm the effective decrease of bioavailable Cr(VI) in the soil microcosms bioaugmented with culture of *P. fluorescens, E. amnigenus* and *E. gallinarum*. Results showed that different growth parameters (% germination, root length, shoot length) of clover were affected drastically under Cr(VI) stress. Similar findings were also observed by Faisal [23] and Riaz [24] where seed germination and growth of *Helianthus annuus* and *Cicer arietinum*, respectively were markedly affected by the presence of chromate salts. Cr(VI) caused decrease in root and shoot length, but inoculation of soil with bacterial strains significantly enhanced growth of seed when compared with non inoculated controls. Our results are in agreement with the findings of several authors. Srivastava et al. [25] demonstrated that the soil microcosm inoculated by *Aspergillus niger*, increased the seed germination and seedling length under chromate stress. Faisal and Hasnain [26] also reported that the *Ochrobactrum intermedium* inoculation caused a decrease in chromate uptake into seedlings as compared to their respective non-inoculated control.

The application of Cr(III) did not result in significant apparent toxic symptoms both in the inoculated and the control soil.

Based on these results, we can suggest that *P. fluorescens, E. amnigenus* and *E. gallinarum* promoted plant growth by reducing toxic Cr(VI) into the less Cr(III) in the soil rhizosphere.

Trifolium campestre showed better visible growth when inoculated with *P. fluorescens* under control conditions, additional Cr supplementation reduced the growth, but the growth retardation was relieved by *P. fluorescens* inoculation, suggesting the potentiality of this strain as plant growth promoter and as an effective bioremediator. Plant growth promoting rhizobacteria can affect plant growth and development either indirectly by preventing some of the deleterious effects posed by some toxic substances, or directly by producing some substances which are useful for the promotion of plant growth. The strain *P. fluorescens* PF28 could, therefore, be utilized for the growth improvements of various economically important cash crops as well as for the bioremediation of chromium polluted soils.

CONCLUSION

In conclusion, the results of this study show that *P. fluorescens, E. amnigenus* and *E. gallinarum* promoted plant growth by reducing toxic Cr(VI) into the less toxic and less bioavailable Cr(III) in the rhizosphe re. Inoculation with *P. fluorescens* resulted in greater clover plants growth as compared to non inoculated control both in chromium stress and unstressed conditions showing the potential of utilization of *P. fluorescens* PF28 for plant growth improvements as well as for Cr(VI) bioremediation.

Results have significance in plant-bacteria interactions in bioremediation technology of chromium polluted soils.

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