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Isolating, screening and applying chromium reducing bacteria to promote growth and yield of okra (*Hibiscus esculentus* L.) in chromium contaminated soils

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ABSTRACT

Hexavalent chromium [Cr (VI)], extensively used in different industries, is one of the most toxic heavy metals. The Cr (VI) reducing bacteria could be helpful in decreasing its toxic effects. The present study was conducted to evaluate the potential of Cr (VI) reducing bacteria to improve growth and yield of okra (*Hibiscus esculentus* L.) in Cr-contaminated soils. Most of the selected bacterial isolates significantly increased the growth and yield of okra. Maximum response was observed in the plants inoculated with the isolate K12 where plant height, root length, fruit weight and number of fruits per plant increased up to 77.5 percent, 72.6 percent, 1.4 fold and 2.9 fold, respectively. Moreover, inoculation with bacteria caused significant decrease in Cr (VI) concentration in soil and plant parts across all treatments. The maximum decrease of 69.6, 56.1 and 40.0 percent in Cr (VI) concentrations in soil, plant vegetative parts and plant reproductive parts, respectively, was observed in the treatment inoculated with the strain K12. Based on amplification, sequencing and analysis of 16S rDNA sequence, the strain K12 was found belonging to genus *Brucella* and was designated as *Brucella* sp. K12. These findings suggest that the strain K12 may serve as a potential bioresource to improve crop production in Cr-contaminated soils.

1. Introduction

Chromium (Cr) is a heavy metal that is released into the environment from a number of industries including leather tanning, metal cleaning, alloy formation, textile dyeing and chromate plating (Mishara and Doble, 2008; Ilias et al., 2011; Pal and Vimala, 2011; Tripathi et al., 2011). Cr exists in several oxidation states; however, trivalent [Cr (III)] and hexavalent chromium [Cr (VI)] are relatively more stable and dominant forms existing in the environment (Pellerin and Booker, 2000; Ackerley et al., 2004). Cr (VI) is considered relatively more toxic given its higher solubility and mobility in nature than Cr (III) (Zahoor and Rehman, 2009; Datta et al., 2011). It is a strong oxidizing agent, mutagenic as well as teratogenic in nature and has been listed as class-A human carcinogen by the US-EPA (Costa and Klein, 2006; Desai et al., 2008). Several human diseases and disorders including vomiting, skin allergy, diarrhea, dermatitis, gastrointestinal bleeding, birth defects, lung cancer, brain damage and premature death are associated with exposure to Cr (VI) (Dakiky et al., 2002; Iyer and Mastorakis, 2010). Cr (VI) has also been reported to affect a number of metabolic processes in plants leading to poor seed germination, stunted root growth, chlorosis, photosynthetic impairing and the death of plant (Kocik and Havsky, 1994; Gardea-Torresday et al., 2004; Datta et al., 2011). Keeping the toxic and detrimental effects of Cr (VI) in view, there is a need to remediate this contaminant from the environment.

Biological reduction of Cr (VI) to relatively less toxic Cr (III) by living and inactivated microbial biomass is considered one of the most practical and useful methods for reducing Cr (VI) toxicity (Ganguli and Tripathi, 2002; Humphries and Macaskie, 2002; Amoozegar et al., 2007). A number of bacterial strains have been reported to possess Cr (VI) reduction potential e.g. *Intrasporangium* sp. (Yang et al., 2009), *Bacillus* sp. (Camargo et al., 2003; Elangovan et al., 2006; Rehman et al., 2008), *Arthrobacter* sp. (Megharaj et al., 2003), *Escherichia coli* (Bae et al., 2004), *Shewanella* sp. (Desai et al., 2008), *Serratia marcescens* (Mondaca et al., 2002), *Vogococcus fluvialis* (Mistry et al., 2010), *Arthrobacter aurescens* (Horton et al., 2006), *Burkholderia cepacia* (Wani et al., 2007), *Microbacterium* sp. (Pattanapipitpaisal et al., 2001), *Pseudomonas* sp. (McLean and

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Beveridge, 2001; Jimenez-Mejia et al., 2006; Rahman et al., 2007) and *Pseudomonas ambigua* (McLean and Beveridge, 2001). Recently, Ilias et al. (2011) reported the reduction of Cr (VI) to Cr (III) in the presence of *Staphylococcus aureus* and *Pediococcus pentosaceus* isolated from tannery effluents. These strains showed considerable growth even in the solutions containing 2000 mg L^{-1} Cr (VI). Similarly, Essahale et al. (2012) examined the potential of *Acinetobacter* sp. to reduce Cr (VI) under varying environmental conditions including pH, temperature, exposure time and resistance to Cr (VI). Results revealed that *Acinetobacter* sp. had substantial ability to tolerate 400 mg L^{-1} Cr (VI).

A number of researchers have tried to exploit the potential of bacteria to reduce Cr (VI) and improve growth and yield of crops in Cr contaminated soils. For example, Wani and Khan (2010) reported that plant growth, nodulation and yield were significantly increased in bacterial inoculated treatments compared to un-inoculated controls of Cr (VI) contaminated soils. Inoculation with bacteria also caused significant decrease in Cr (VI) concentrations in roots, shoots and grains of chickpea. *Mesorhizobium* strain, isolated from nodules of chickpea plants, was found to tolerate Cr up to 500 mg L^{-1}. Although Cr was toxic to chickpea at 136 mg kg^{-1} but inoculation with *Mesorhizobium* strains significantly increased number of nodules, dry matter production, seed yield and protein contents with reduced Cr contents in roots, shoots and grains. Despite that a number of bacterial strains have been reported for reduction of Cr (VI), there is a growing need to find novel microbial resources that can help improve growth and yield of crops in Cr-contaminated soils and reduce Cr (VI) to Cr (III). Therefore, the present study was designed to isolate and screen Cr (VI) reducing bacteria from Cr-contaminated soils, and to assess the potential of Cr (VI) reducing bacteria to promote plant growth and yield in contaminated soil using okra as a model crop.

2. Materials and methods

2.1. Soil sampling and preparation

The surface soil samples (0–15 cm) were collected from ten sampling points from an agricultural field in Kasur, Pakistan (31.1170°N, 74.4502°E). Soils have frequently been irrigated with Cr loaded effluents of leather industry for many years. Individual samples were hand-sorted in the field to remove stones and residual intact live and dead vegetation before they were transferred into clean plastic bags and transported to the laboratory. In the laboratory, soil samples were passed through a 2 mm sieve and ten soil samples were mixed thoroughly to form a homogenized composite sample. Three analytical sub-samples were taken from the composite sample for physicochemical analyses including pH, EC, texture, organic matter content, total nitrogen and Cr (VI) at the start of the experiment (Table 1).

2.2. Soil physical and chemical analysis

Soil pH and EC were determined at 1:5 soil–water ratio (w/v) on field moist samples. The soil textural analyses were performed on the soil samples at the start of the experiment using the method of Gee and Bauder (1986). Soil organic matter was measured by the method of Walkley and Black (1934). Total soil N was estimated by Kjeldahl method (Bremmer, 1965).

Table 1
Physico-chemical properties of soil collected from Kasur.

Soil property	Value
pH	7.81
EC (dS m^{-1})	2.01
Sand (g kg^{-1})	540
Clay (g kg^{-1})	280
Texture class	Sandy clay loam
Organic matter (g kg^{-1})	5.40
Total nitrogen (g kg^{-1})	1.20
Cr (VI) (mg kg^{-1})	30.46

2.3. Isolation and screening of Cr (VI) reducing bacterial isolates

Cr (VI) reducing bacteria were isolated from the collected soil samples using the dilution plate technique on glucose agar medium [Glucose, 1.5 g L^{-1}; Peptone, 0.5 g L^{-1}; K_2HPO_4, 0.5 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.1 g L^{-1}; $FeSO_4 \cdot 7H_2O$, traces; $(NH_4)_2SO_4$, 0.5 g L^{-1}; agar (for solid medium), 20 g L^{-1}] supplemented with $K_2Cr_2O_7$ (15 mg L^{-1}). From 57 initially isolated bacteria, twenty strains were selected on the basis of their morphology and relatively proliferated growth rates.

2.4. Determination of minimum inhibitory concentration (MIC) and Cr (VI) reduction potential

The selected bacterial isolates were tested for their resistance against Cr (VI) by estimating the minimum inhibitory concentrations (MIC) of the Cr (VI) for each bacterial isolate using dilution plate method. For this purpose, the bacterial isolates were subjected to different Cr (VI) concentrations ranging from 0 to 100 mg L^{-1} in Nutrient Agar (NA) medium [Beef extract, 3 g L^{-1}; Peptone, 5 g L^{-1}; Agar, 15 g L^{-1}]. MIC for Cr (VI) was evaluated by observing growth of each bacterial isolate after five days of incubation at 30 °C. Digital colony counter (Humanlab, Korea) was used to monitor the growth of the bacterial isolates. The minimum concentration which inhibited the growth of bacteria was considered as MIC.

Cr (VI) reduction potential of the selected bacterial isolates was estimated by diphenylcarbazide method (Zahoor and Rehman, 2009). Bacterial isolates were grown in liquid glucose media containing 15 mg L^{-1} Cr (VI). The bacterial cultures and un-inoculated control were incubated in triplicate at 150 rpm and 30 °C in an orbital shaking incubator. After 72 h, 1 ml aliquot from each culture and control was taken and centrifuged at 7000 rpm. The supernatants were collected in test tubes to which 1 ml of diphenylcarbazide solution (0.25 g diphenylcarbazide dissolved in 100 mL acetone) was added followed by the addition of one drop of concentrated H_3PO_4 to lower the pH up to 2 ± 0.5. The solutions were kept at room temperature for 5 min to allow color development and the concentration of the remaining Cr (VI) was estimated by spectrophotometer (Shimadzu UV/VIS) at 540 nm (Zahoor and Rehman, 2009). Spectrophotometer was standardized using analytical grade $K_2Cr_2O_7$ solutions and development of standard curve. The Cr (VI) reduction was estimated using the following equation:

$$\text{Cr(VI) reduction } (\%) = \frac{A-B}{A} \times 100$$

where A and B represent absorbance for the un-inoculated control and samples inoculated with bacterial isolate respectively. For further use in the pot trial, ten bacterial isolates were screened based on their MIC (> 25 ppm) and reduction (> 50 percent) potential for Cr (VI).

2.5. Pot experiment

Impact of Cr-reducing bacteria on growth and yield of okra (*Hibiscus esculentus* L.) was assessed by inoculating and sowing okra seeds with ten selected bacterial isolates in Cr-contaminated soils [30.46 mg kg^{-1} Cr (VI)]. In order to inoculate seeds of okra with selected bacterial isolates, glucose broth medium was prepared. After autoclaving, flasks containing glucose broth medium were inoculated with the selected bacterial isolates separately and incubated at 30 °C. Uniform cell density (10^7–10^8 CFU mL^{-1}) was maintained by maintaining optical density (OD_{600} = 0.5) in each inoculated flask. Then the inoculum of each bacterial isolate was injected into sterile peat (100 ml kg^{-1}) and was incubated at 30 °C for 24 h before using it for seed coating. For seed inoculation, seed dressing was carried out with inoculated peat mixed with clay and ten percent sugar solution. In case of the un-inoculated control, the seeds were coated with the same type of material but containing autoclaved distilled water instead of bacterial culture. The okra seeds inoculated with ten different bacterial isolates were sown in chromium contaminated soil. Un-inoculated Okra containing pots were used as controls. The treatment plan for of this study was: T0 = Control (No inoculation), whereas, the treatments T1, T2, T3, T4, T5, T6, T7, T8, T9 and T10 represented the inoculation with the isolates K4, K5, K6, K7, K8, K11, K12, K16, K18 and K20, respectively. The treatments were replicated three times and experiment was laid out in completely randomized design (CRD). Each pot having 10 kg Cr-contaminated soil was fertilized with N (urea), P (di-ammonium phosphate) and K (sulfate of potash) @ 120, 60 and 60 kg ha^{-1} respectively. After germination, thinning was done to maintain one okra plants per pot for each replicate of all the treatments. The pots were irrigated with water having electrical conductivity (EC) 0.07 dS m^{-1}; sodium absorption ratio (SAR) 0.36 (mmol $l^{-1})^{1/2}$ and residual sodium carbonate (RSC) zero. Data regarding plant height (cm), root length (cm), number of fruits and fruit weight (g) were recorded after harvesting. Soil and plant samples were also analyzed for Cr (VI) contents at the end of the experiment.