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Original Research

Assessing the Phytotoxicity of Tannery Waste-Contaminated Soil on *Zea mays* (Lin) Growth

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Abstract

Four chromium-resistant bacterial strains (*Bacillus pumilus*-CrK08, *Cellulosimicrobium cellulans*-CrK16, *Exiguobacterium*-CrK19, and *Bacillus cereus*-CrK20) that could resist up to 25 mg·ml^{-1} of Cr (VI) were used to inoculate *Zea mays* (Lin) seeds grown in tannery effluent-contaminated and normal garden soils. Overall, plants growing in tannery-contaminated soil showed slow leaf growth, 40% reduction in shoot length, 73% reduction in dry biomass, burning of leaf margins, delayed flower bud initiation, and feminization of male flowers compared to control. *Zea mays* (Lin) plants growing in tannery-contaminated soil showed an increase in acid phosphatase activity (14-26%), soluble proteins content (17-38%), chlorophyll *a* (34%) and *b* contents (70%), and a decrease in peroxidase (19%) and carotenoid contents (50%) compared to control. Non-inoculated plants have higher chromium uptake (114 mg/kg) as compared to inoculated (49.4 mg/kg).

Keywords: chromium, tannery effluent, *Zea mays* (Lin), *Bacillus*, biofertilizers

Introduction

Soil pollution by heavy metals is an important environmental problem. It has many harmful impacts on agriculture and human health [1]. Heavy metal-containing food-products and crop plants pose the risk of heavy metal accumulation in the food chain even at low contamination levels [2]. High concentrations of heavy metals are present in tannery waste and if this is used for irrigation purposes, then the levels of these toxic heavy metals in soil increases. Long-term exposure to heavy metals results in plant growth inhibition, chlorosis, appearance of apoptotic bodies, induction of oxidative stress, damage to chloroplast, and finally cell death [3]. One of the most common heavy metals present in tannery wastewater affecting soil quality is chromium. It can exist in oxidation states ranging from +2 to +6, but the most frequent forms found in the environment are the trivalent (+3) and hexavalent (+6) states [4]. Hexavalent (VI) is about 100-fold more toxic, mutagenic, and a better known carcinogen than the trivalent (III) form [5]. Adhikari and Singh [6] observed severe interveinal chlorosis, which turns into necrosis at later stages of plants grown under chromium stress. Pandey et al. [7] observed significant increases in the herb yield and bacoside content in brahmi (*Bacopa monnieri*) when grown under Cr stress (10 to 40 mg·kg^{-1} soil).

The traditional physicochemical methods can be used for clean up of metal-contaminated soils, but they are very costly and destructive to the normal properties of the soil [8]. Phytoremediation, the use of plants to extract, sequester, and detoxify pollutants, is an emerging technology and because of its cost-effectiveness and long-term applicability [9]. Many hyperaccumulators, such as *Thlaspi* and *Alyssum* have received much attention, but they usually

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have low growth rates. Therefore, alternative crops such as sunflower, raddish, and the most common maize can be exploited for this purpose because of their high biomass production and adaptive capacity to variable environments [10]. For clean up of heavy metal-contaminated sites, microbes (bacteria) can also be used and these biological methods are not only cheap but also environmentally friendly [11]. Apart from supporting plant growth of the accumulating biomass, rhizosphere bacteria may mobilize heavy metals for enhanced uptake by plant roots [12]. The present study was therefore undertaken to check the metal uptake ability of maize plant and to assess plant growth, promoting the ability of chromium resistant bacterial strains on maize plants.

Materials and Methods

Bacterial Strains Used

Bacterial strains used for this study were isolated from tannery effluent (*Bacillus cereus*-CrK20) and metals-contaminated soil (*Bacillus pumilus*-CrK08, *Cellulosimicrobium cellulans*-CrK16, and *Exiguobacterium*-CrK19) from Kasur, Pakistan. These bacteria were identified by 16S rRNA gene sequence analysis. The gene bank accession numbers of these strains *Bacillus pumilus*-CrK08, *Cellulosimicrobium cellulans*-CrK16, *Exiguobacterium*-CrK19, and *Bacillus cereus*-CrK20 are GQ503326, GQ503328, GQ503330, and GQ503329, respectively. These strains have been characterized and their interaction with plants was also studied in order to determine their potential role in bioremediation and plant growth promotion strategies.

Soil Sampling

For Plant microbe interaction study, soil contaminated by tanneries effluent was collected from Din Gharr, Kasur, Pakistan. The location of the soil sampling site was latitude (N) 3106'18.53" and longitude (E) 7427'34.17". Four soil samples were taken from four different areas from the sampling site (almost up to 9 inches deep), and mixed thoroughly before filling the pots.

Growth Experiments with *Zea mays* (Lin)

Zea mays (Lin) is a popular cereal grain and several investigations have been carried out to find its ability to accumulate metals from polluted sites. Certified seeds of *Zea mays* (lin) var hybrid NK-6326, Syngenta, were obtained from the National Agriculture Research Center (NARC) in Islamabad, Pakistan. The experiment was designed to study the effects of metal-contaminated soil and bacteria inoculation on *Zea mays* (Lin) plants grown under natural daylight and temperature. To check whether any microbial flora were present in contaminated soil, half the amount of soil was sterilized. In this way three treatments of soil were used, which are:

(i) control garden soil (NCS)
(ii) sterilized tannery-contaminated soil (AS)
(iii) unsterilized tannery-contaminated soil (NAS).

Pots were filled with 3 kg of soil and the experiment was done in triplicate. Nine seeds of *Zea mays* (Lin) were sown in their respective pots at equal distances and the pots were kept almost 1 square feet apart. For preparation of bacterial cultures, strains were grown in L-Broth overnight (150 rpm) at 37ºC. Optical density of each culture was made equal by adjusting with sterilizers glass-distilled water. For the preparation of mixed cultures of bacteria equal quantities of bacterial cultures (after adjusting optical density) were taken and mixed to get a uniform number of cells per ml. The inoculum was given to their respective pots prior to sowing of seeds. Un-inoculated seeds were sown as control in natural control soil. Seed germination and emergence was recorded daily. Pots were watered regularly (to field capacity by weighing) and maize plants were allowed to grow till maturity. At maturity plants were harvested and various growth and yield parameters were investigated.

Biochemical Parameters

Soluble protein contents, peroxidase and acid phosphatase activity, pigment analysis (chlorophyll *a*, *b* and xanthophylls) were estimated following the methods of Lowry et al. [13], David and Murray [14], Iqbal and Rafique [15], and Lichtenthaler and Wellburn [16], respectively.

Plant and Soil Analysis for Heavy Metals

Plants were harvested after five and a half months of sowing. Shoots and roots were separated and washed thrice with distil water to remove any soil particles and then were oven-dried at 80ºC. The plant and soil samples were analyzed for heavy metals using inductively coupled plasma spectrometry (ICP-OES) at Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex, Lahore. Methods used and other specifications were consulted from "official method of analysis of AOAC international" (2005) 18th Ed., AOAC international Gaithersburg MD, USA, official method 985.01.

Statistical Analysis

Standard errors of the means were calculated following Steel and Torrie [17].

Results

Bacterial Strains Used

Four chromium-resistant bacterial strains (*Bacillus pumilus*-CrK08, *Cellulosimicrobium cellulans*-CrK16, *Exiguobacterium*-CrK19, and *Bacillus cereus*-CrK20) used in the present study were isolated from tannery effluent of Kasur, Pakistan (Table 1).

Table 1. Description of sites sampled for isolation of chromium-resistant strains.

Strains	Closest matched spp	Cr-resistance mg·ml^{-1}	Accession Number	Locality	Source
CrK08	*Bacillus pumilus*	25	GQ503326	Kasur	soil
CrK16	*Cellulosimicrobium cellulans*	25	GQ503328	Kasur	soil
CrK19	*Exiguobacterium* sp	25	GQ503330	Kasur	soil
CrK20	*Bacillus cereus*	25	GQ503329	Kasur	water

Table 2. Effect of mix cultures bacterial inoculums on germination, root length, shoot length, fresh and dry biomass of *Zea mays* (lin) var NK-6326 plants growing in tannery-contaminated (autoclaved and non-autoclaved) and natural garden soils. (Means of three replicates).

Soil type	Germination (%)	Shoot length cm	Root length cm	Fresh wt. of shoot g/plant	Dry wt. of shoot g/plant	Fresh wt. of root g/plant	Dry wt. of root g/plant
Control	89±0	56.3±3.2	23.8±1.4	17.0±0.8	4.2±0.3	7.4±0.1	1.8±0.1
Auto.P_1	100±00	34±1.7	29±2.0	7.1±0.3	1.1±0.1	7.6±0.1	0.7±0.05
Auto.P_1B	96±0.33	44.5±2	38±3.5	8.5±0.4	1.6±0.07	10.7±0.4	1.2±0.04
P_1	100±00	35±2.6	26±1.3	4.4±0.2	0.9±0.03	6.0±0.2	0.8±0.06
P_1B	96±0.33	45±3.6	23±1.7	10.5±0.6	2.3±0.2	13.7±0.2	2.5±0.13

Control – control garden soil
Auto P_1 – autoclaved tannery-contaminated soil
Auto.P_1B_1 – autoclaved tannery-contaminated soil + bacterial inoculation
P_1 – un-autoclaved tannery-contaminated soil
P_1B_1 – un-autoclaved tannery-contaminated soil + bacterial inoculation

Growth Parameters of *Zea mays* (Lin) Plants

Generally, the effect of tannery-contaminated soil was not very toxic on the seed germination of *Zea mays* (Lin) plants (Table 2). Plants growing in tannery-contaminated soil have shown an 8 to 21% increase in seed germination compared to natural control soil. Compared to natural control soil, plants grown in tannery-contaminated soil have shown a reduction in shoot length. Inoculated plants have shown almost 20% and uninoculated have shown 37 to 39% reduction in shoot length compared to natural control soil (Table 2). An increase in root length was recorded in plants that were sown in contaminated soil while inoculated plants have shown 59% and uninoculated plants had shown 9 to 21% increment in this parameter as compared to natural control soil (Table 2). *Zea mays* plants growing in contaminated soil showed reduction in fresh and dry biomass of shoot in comparison to natural control soil. Overall, bacterial inoculations have a good impact on the growth of *Zea mays* plants in tannery-contaminated soil in comparison to their respective uninoculated plants (Table 2, Fig. 1).

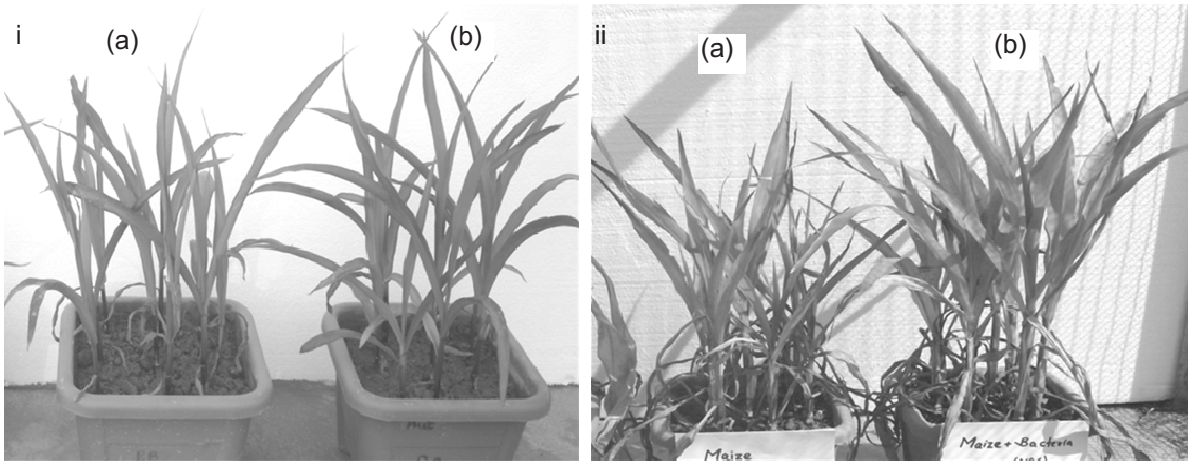

Fig. 1. Growth comparison of uninoculated (a) and inoculated (b) *Zea mays* (lin) plants, growing in tannery-contaminated soil after (i) one month of germination (ii) and at maturity.

Table 3. Estimation of soluble proteins, peroxidase, acid phosphatase, and pigment analysis of *Zea mays* (lin) var NK-6326 plants growing in tannery-contaminated (autoclaved and non-autoclaved) and natural garden soils. (Means of three replicates).

Soil type	Protein content g^{1} fresh wt. of leaf	Peroxidase content Unit g^{-1} of fresh wt. of leaf	Acid phosphatase µg/gm of fresh wt. of leaf	Chlorophyll *a* µg/gm fresh wt. of leaf	Chlorophyll *b* µg/gm fresh wt. of leaf	Total Carotenoids µg/gm fresh wt. of leaf	Amount of total Cr mg/kg plant stem dry wt.
Control	1.65±0.09	2.02±0.14	13.36±0.93	19.46±1.17	9.93±0.29	16.47±1.28	0.0
Auto.P_1	2.06±0.08	0.95±0.06	15.23±0.45	26.06±1.82	16.89±0.68	11.87±0.66	–
Auto.P_1B	1.69±0.14	1.65±0.16	16.81±0.84	20.87±0.63	11.97±0.84	8.11±0.48	–
P_1	1.93±0.09	1.25±0.05	15.50±0.62	24.37±1.38	13.71±0.70	9.36±0.73	114
P_1B	2.28±0.06	1.03±0.08	11.27±1.01	17.87±1.44	9.51±0.71	7.71±0.32	49.35

Control – control garden soil
Auto P_1 – autoclaved tannery-contaminated soil;
Auto.P_1B_1 – autoclaved tannery-contaminated soil + bacterial inoculation
P_1 – un-autoclaved tannery-contaminated soil
$P1B_1$ – un-autoclaved tannery-contaminated soil + bacterial inoculation

Biochemical Parameters

Plants growing in tannery-contaminated soil have shown 17 to 38% and 14 to 26% increases in soluble proteins and acid phosphatase contents, respectively, when compared with plants growing in garden soil. In the same way, 19-53% decreases also were observed in in peroxidase contents of plants growing under contaminated soil compared to control soil. Inoculated plants have shown increased acid phosphatase activity, soluble proteins, and peroxidase contents compared to uninoculated plants growing under contaminated soil (Table 3). Maize plants growing in tannery-contaminated soil have shown 34% and 70% increments in chlorophyll *a* and chlorophyll *b* contents, respectively, and 51% decreases in carotenoid contents compared to control. Inoculated plants have produced less chlorophyll *a*, chlorophyll *b,* and carotenoid contents comared to uninoculated plants (Table 3). For the estimation of total chromium content in maize plants, inductively coupled plasma-spectrometry (ICP-OES) was used. In plants P_1 and P_1B, a decrease in chromium uptake (114 mg/kg to 49.35 mg/kg) was observed (Table 3) in inoculated unsterilized soil (NAS).

Feminization of Male Flower

After 165 days of germination, seed formation started at the basal portion of the tassel, an inflorescence of male flowers and feminization of male flower occurred only in *Zea mays* (lin) plants growing in tannery-contaminated soil (Auto.P_1B, P_1, and P_1B) (Fig. 2).

Soil Analysis

The physico-chemical characteristics of tannery-contaminated soil samples are presented in Table 4. The soil sample has electric conductivity 15.14 ms/cm and organic matter content 7.92%. Soil pH was 7.6, which is one of the most influential parameters controlling the mobility and availability of metals in soils. The total metal content is important because it determines the size of the metal pool in the soil and thus the potential for metal uptake. The concentrations of hexavalent chromium (VI) and total chromium were 4.21 and 24.78 $mg \cdot kg^{-1}$, respectively (Table 4).

Discussion

In the present study three chromium-resistant bacterial strains (*Bacillus pumilus*-CrK08, *Cellulosimicrobium cellulans*-CrK16, and *Exiguobacterium*-CrK19) were isolated

Fig. 2. Feminization of male flower (tassel) in *Zea mays* (lin) plants growing in soil contaminated by tannery effluents.

Table 4. Physico-chemical properties of tannery-contaminated soil and natural garden soil samples.

Soil Type	Electric conductivity	Na	K	Ca	Mg	SO_4	CO_3	HCO_3	Organic matter	PO_4	As	Cr (VI)	Total Cr	Grain Size			
														> 2 mm	500-2mm	63-500 mm	0-63 mm
Contaminated soil	15.1±1.05 ms/cm	1.43±0.128 gm/kg	0.09 gm/kg	1.0±0.05 gm/kg	0.36±0.01gm /kg	3.18±0.28 gm/kg	Nil	0.61±0.01 gm/kg	7.92±0.39%	20.3±0.025 mg/kg	Nil	4.21±0.37 mg/kg	24.8±0.99 gm/kg	23.1±2.07%	24.8±1.24%	26.3±2.89%	25.69±1.79%
Natural Garden Soil	28.7±0.028 ms/cm	5.15±0.36 gm/kg	0.13±0.01 gm/kg	2.32±0.11 gm/kg	0.61±0.06 gm/kg	1.88±0.15g m/kg	Nil	1.76±0.07 gm/kg	5.35±0.32%	35.5±0.71m g/kg	Nil	Nil	Nil	41.3±1.65 %	33.6±3.36 %	23.2±1.16%	1.23±0.11%

from soil contaminated with tannery effluents and one (*Bacillus cereus*-CrK20) from tannery waste water. They showed high-level resistance to potassium chromate K_2CrO_4 (up to 25 mg·ml^{-1}) in nutrient agar medium. Camargo et al. [18] also observed that most of the chromium-resistant bacteria can tolerate up to 2,500 mg·L^{-1} of Cr (VI). Microbial populations in metal-contaminated environments adapt to that situation and become metal resistant [19].

Generally, it was observed that the plants growing in tannery-contaminated soil showed better germination index as compared to the plants growing in ordinary garden soil. It might be due to the better organic matter present in metal-contaminated soil in comparison to garden soil (Table 4). Some other investigators also observed healthy plant growths due to the irrigation of nutrient-rich tannery wastewater having different organic and inorganic pollutants [20]. When comparing the effects of bacterial inoculation, it was observed that bacterial inoculation had no positive effect on germination. Instead they suppressed it to some extent. After 12 days of germination, the color of leaf tips in plants growing in contaminated soil turned yellow and finally whole leaves dried. Because of the toxicity of heavy metals, apoptosis get started in plant tissue and damage to chloroplast occurs, which finally results in cell death. Singh [21] have reported that Cr applied at 60 mg·kg^{-1} or higher will slow down leaf growth, reduce size, and cause burning of leaf margins and tips. Jain et al. [22] reported leaf chlorosis at 40 ppm chromium that turned to necrosis at 80 ppm chromium.

One of the most interesting things observed in this experiment was the feminization of male flowers, which occurred only in those *Zea mays* plants that were growing in soil contaminated by tannery effluents. Plants grown under stress conditions for some time show such irregular behaviors in their growth pattern. A massive decrease in fresh and dry weight of shoots was observed in plants growing in tannery-contaminated soil, as compared to natural garden soil, while bacterial inoculation lessened the intensity of toxicity posed by contaminated soil by improving these parameters to some extent. Generally, plant growth promoting bacteria are known to promote the growth and yields of plants through nitrogen fixation, phosphate solublization, siderophores production, phytohormones production, and synthesis of antimicrobial compounds when ever present in plant rhizosphere [23].

Compared to natural control soil, *Zea mays* (Lin) plants grown under contaminated soil showed some increment (Auto.P_1 26%, Auto.P_1B 3%, P_1 17%, and P_1B 38.4% over control) in soluble proteins content. Inoculated plants produced more protein contents compared to control, even in effluent-contaminated soil. In the case of enzyme peroxidase, some decreases (Auto.P_1 52.8%, Auto.P_1B 18.4%, P_1 38%, and P_1B 49%) were recorded in plants growing in tannery-contaminated soil as compared to garden soil. In another study conducted on willow plants, some reduction in activity of catalase and peroxidase was observed under chromium stress compared with plants growing in control soil [24]. Under stress conditions plants produce more acid phosphatase contents and the same was also observed in the present study, where Auto.P_1 have shown 14.03%, Auto.P_1B 25.84%, and P_1 16.07% increment in acid phosphatase activity compared to normal control garden soil. Plants growing in tannery-contaminated soil have produced more chlorophyll *a* and *b* contents and less carotenoid content compare to *Zea mays* plants growing in natural control.

Conclusions

The results of this investigation showed that metal hyperaccumulator *Zea mays* plants were able to grow in tannery effluent-contaminated soil heavily polluted by heavy metals, especially chromium. Furthermore, the indigenous bacterial strains that have already adapted to this environment can be used as biofertilizers for *Zea mays* crops. Both these candidates (bacteria and maize) can be exploited for the reclamation of tannery effluent contaminated areas.

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