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# OCCURRENCE OF HEAVY METAL RESISTANCE IN *Sinorhizobium* sp. ISOLATED FROM ROOT NODULES OF FENUGREEK, TREATED WITH TANNERY EFFLUENT

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## ABSTRACT

**The aim of present study was to initiate preliminary work on heavy metal contamination in farm soil (contaminated with processed tannery effluent) and its potential influence on the development of metal resistance among $N_2$-fixing bacterium, *Sinorhizobium* sp. Contaminated plant samples were analyzed for various metals and minimum inhibitory concentration (MIC) of metals was determined. Fenugreek (*Trigonella* sp.) plants revealed accumulation of these metals in root and leaves. *Sinorhizobium* sp. were isolated (25) from the root nodules of fenugreek-treated with processed tannery wastewater and characterized morpho-biochemically. All isolates were evaluated for their resistance against $Cr^{3+}$, $Cr^{6+}$, $Cd^{2+}$, $Cu^{2+}$, $Zn^{2+}$ and $Ni^{2+}$. The maximum MIC of 1600 µg $mL^{-1}$ was noticed against $Cr^{3+}$ in 64% isolates. Among all the isolates, the lowest MIC of 25 µg $mL^{-1}$ was detected against $Ni^{2+}$. Some metal resistant isolates were evaluated for their resistance against frequently used antibiotics *viz*., tetracycline, ampicillin, gentamycin, kanamycin, chloramphenicol and nalidixic acid. About 46% *Sinorhizobium* isolates were resistant to nalidixic acid whereas 26.6% showed resistance to ampicillin and kanamycin. The isolates demonstrated confluent growth upto a salt concentration of 3%; whereas isolates (SM17, SM24) tolerated 10% NaCl. Acidic pH eliminated almost all the test population and neutral pH had no suppressive effect on growth while majority were tolerant to pH 9.**

**Keywords:** Fenugreek, **m**etal resistance, *Sinorhizobium*, tannery effluent

## INTRODUCTION

Tannery industry is one of the highest polluting industries in India, causing environmental problems that directly affect plant, animal and microbes. In most towns of India, the treated industrial and domestic wastewater is used for irrigating the agricultural fields. The agronomic and economic benefits related to the application of treated tannery effluent are clear; on the other hand, its long-term use could lead to the build-up of contaminants in soil which significantly may contribute to the soil pollution (Cooman *et al.*, 2003). India is one of the main manufacturers of leather in the world and approximately 2787 billion $cm^2$ raw leather $year^{-1}$ are produced (https://leatherindia.org/indian-leather-industry/). The processing of one metric ton rawhide generates 200 kg tanned leather, 190–350 kg non-tanned waste, 200–250 kg tanned leather waste, and 50,000 kg wastewater (Sundar *et al.*, 2011). These tannery industries discharge chromium ranging from 2000 and 5000 mg $L^{-1}$ in the aqueous effluent in contrast to the recommended permissible limits of 2 mg $L^{-1}$ (Baldiris *et al.*, 2018).

There are about 400 tanneries in the Jajmau region of Kanpur, India (Gupta *et al.,* 2018). At high concentrations, chromium is toxic, mutagenic, carcinogenic, and teratogenic (Lee *et al*., 2008). Chromium exists in oxidation states of +3, and +6. The trivalent oxidation state is the most stable form of chromium and is essential to mammals in trace concentration and relatively immobile in the aquatic system as Cr (III) precipitates easily at the average pH of natural waters. The hexavalent chromium is highly soluble and about 300 times much more toxic than Cr (III). Tannery wastewater contains mainly Cr (III) (Cervantes *et al*., 2001).

Chromium tolerant microbes from chromium polluted soil have been reported by several worker (Viti *et al*., 2003; Gupta *et al*., 2018). The occurrences of Cr (VI) in the atmosphere apply selection force on microorganisms. A good number of microbes are susceptible to Cr (VI) toxicity. However, several groups have resistance systems to Cr (VI) and capable of tolerating high concentration. The accessibility of special strains capable to resist and diminish chromate increases the likelihood of utilizing microorganisms for bioremediation of Cr (VI) polluted sites in a more efficient method with respect to present chemical remediation systems. The aim of this study was to have preliminary information on heavy metal contamination in farming soil contaminated with tannery effluents, isolate potential nitrogen fixing *Sinorhizobium* sp. and assess metal resistance in them.

## MATERIALS AND METHODS

### *Collection of samples*

Plant samples were collected from different farming land of Jajmau area of Kanpur (India) receiving treated tannery effluent. The plants of fenugreek (*Trigonella foenum-graecium*) were collected from filed along with their rhizospheric soil. The root nodules of fenugreek were separated for the isolation of *Sinorhizobium* sp.

### *Isolation and identification of Sinorhizobium isolates*

*Sinorhizobium* sp. was isolated from the root nodules of fenugreek as per Vincent (1970). Mature, healthy and pink nodules were surface sterilized with 0.1% mercuric chloride and 70% ethyl alcohol for 1 min each and crushed with a sterile glass rod in a small aliquot of sterilized water. Appropriate dilutions were prepared and spread plated on yeast extract mannitol agar supplemented with Congo red (CRYEMA) followed by incubation at 28-30°C for 3-5 days. The *Sinorhizobium* isolates were characterized morphologically and biochemically as per Somasegaran and Hoben (1994).

### *Heavy metal analysis of different plant parts and tannery effluent*

Plant samples were dried at 40°C, converted to fine powder and sieved through a muslin cloth. Plant sample (1 g) was taken in a 100 mL beaker and digested using perchloric acid on a heating plate till the solution became colourless. The resulting concoction was concentrated to 1 mL and diluted with double distilled water before filtering with Whatman filter No.42. The volume of the filtrate was made upto 100 mL by double distilled water. Digested plant samples were examined for metal concentration through atomic absorption spectrophotometer (GBC 932, Avanta Sigma Plus, Australia). For determination of heavy metals in tannery wastewater, the collected samples were digested in perchloric acid and analyzed by spectrophotometer as described above.

### *Evaluation of minimum inhibitory concentration (MIC) of heavy metals*

The MIC of heavy metal for each bacterial isolate was evaluated through plate dilution method (Mondaca *et al.,* 1998). Heavy metals $Cr^{6+}$, $Cr^{3+}$, $Cu^{2+}$, $Cd^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ were used as $K_2Cr_2O_7$, $CrCl_3.H_2O$, $CuSO_4.H_2O$, $CdCl_2$, $NiCl_2$ and $ZnCl_2$ in different concentrations ranging from 3.12 to 3200 µg $mL^{-1}$. Stock solutions of these metal salts of different concentration were mixed to the respective media. *Sinorhizobium* sp. cultures were spot inoculated on metal amended plates and

incubated at 28°C for 24-72 h. The lowest concentration of metal that inhibited the growth of bacteria was considered the MIC of that metal against the strain tested. AB1157 and C600 strains of *Escherichia coli* K-12, procured from Genetic Stock Centre, Department of Botany, Yale University, USA), were used as control. Presently there are no standard concentrations of metal ions that can be used to differentiate among metal resistant and metal sensitive bacteria. All the experiments were conducted in a completely randomized design and replicated four times.

***Determination of antibiotic resistance***

The isolates of *Sinorhizobium* sp. were examined for different resistance pattern against several antibiotics through disc diffusion technique (Bauer *et al*., 1966). The symbols and concentrations (µg $disc^{-1}$) of relevant antibiotics (Hi-media, India) are given in parenthesis: ampicillin (Am, 10), chloramphenicol (C, 30), gentamycin (G, 10), kanamycin (K, 30), tetracycline (T, 30) and nalidixic acid (Na, 30). *E. coli* B was used as a sensitive strain. The plates were counted for resistance or sensitivity after 24-72 h of growth by comparing the chart depending on the zone of inhibition diameter as provided by the company.

***Determination of pH tolerance***

Tolerance to pH was evaluated as per Nag *et al.* (2018). *Sinorhizobium* sp. were spot inoculated on yeast extract mannitol agar (having different pH: 4, 5, 6, 7, 8 and 9) plates with $3 \times 10^6$ bacteria. The plates were incubated at 28°C for 24-72 h. *E. coli* B was used as the control.

***Determination of salt tolerance***

Salt tolerance among selected isolates was evaluated through the process explained by Babak (1966). YEMA medium having varying concentrations of sodium chloride (1.0, 5.0, 7.5, 10.0, 12.5 and 15.0%) were prepared. Exponentially growing cultures of *Sinorhizobium* sp. ($3 \times 10^6$) were spot-inoculated on NaCl-amended YEMA plates and incubated at 28°C for 24-72 h. *E. coli* B was used as the control.

## RESULTS AND DISCUSSION

The pure cultures of *Sinorhizobium* sp. grown on YEMA medium formed white, raised and opaque colonies with smooth margin. Twenty-five isolates were selected for further study. The farming land in Jajmau region of Kanpur (India) receives tannery effluent since long which contributes to the heavy metal pollution of soil. The mean heavy metal levels detected in soil was high, especially with respect to chromium (Altaf *et al*., 2008). These lands routinely receive tannery effluent through irrigation water which have high concentration of chromium sulphate (Table 1). This high heavy metal content in effluents contribute to chromium accumulation in agricultural soil. As per Central Pollution Control Board of India (2001), the permissible limit of total chromium in treated tannery effluent discharge to surface water is < 2 mg $L^{-1}$, while the traditional chrome tanning contributes effluents having as high as 1500-3000 ppm of chromium (Suresh *et al*., 2001; Paul *et al*., 2015; Gupta *et al.*, 2018). The heavy metal evaluation of fenugreek depicted the accumulation of metals in various plant parts like root, stem and leaf. The metal absorption by plants revealed the highest accumulation of Zn (4.2 mg $kg^{-1}$), Ni (2.0 mg $kg^{-1}$) and Cr (0.9 mg $kg^{-1}$) within roots and Cu (5.5 mg $kg^{-1}$), Zn (4.1 mg $kg^{-1}$), and Ni (2.1

**Table 1: Concentrations of heavy metals (mg $L^{-1}$) in tannery effluent as quantitated by atomic absorption spectrophotometer**

| Heavy metals | Tannery effluent (mg $L^{-1}$) |
|---|---|
| Cr | 90.2 ±13.50 |
| Ni | 15.6 ± 2.32 |
| Cu | 5.33 ± 0.92 |
| Cd | 0.23 ± 0.01 |
| Zn | 1.43 ± 0.25 |

All the values are means of 6 replicates ± SD

mg $kg^{-1}$) inside the leaves. The concentration of Cr observed in plant samples were above the permissible limit of WHO (1988) i.e. 0.19 mg $kg^{-1}$. This could be attributed to the extremely immobile nature of trivalent ($Cr^{3+}$) compounds. Trivalent chromium ($Cr^{3+}$) is extremely impervious to the cell membrane in contrast to hexavalent chromium ($Cr^{6+}$). Cr is typically found as $Cr^{3+}$ in soils. However, oxidation-reduction reactions reciprocally transform $Cr^{6+}$ to $Cr^{3+}$ (Jobby *et al.*, 2019). The entire bacterial isolates were evaluated for their resistance against six heavy metals, specifically $Cr^{3+}$, $Cr^{6+}$, $Cd^{2+}$, $Cu^{2+}$, $Zn^{2+}$, and $Ni^{2+}$. The MIC of 1600 µg $mL^{-1}$ was detected against $Cr^{3+}$ in 64% isolates. Conversely, the isolates were found sensitive to Ni and Cd (Tables 2). Similar results related to chromium (III) have been reported by Khan *et al* (2015). Further, low levels of resistance were also observed against other heavy metals. The high intensity of resistance against chromium (III) was possibly attributed to the high level of this metallic salt at the sampling place, which apply deciding force on the native microbial inhabitants of that soil (Cervantes *et al*., 2001; Viti *et al*., 2003). Metals and different constituents of media might intermingle which complicates the elucidation of data. The toxicity test in solid media might be useful in assessing the metal toxicity within sewage sludge and contaminated soil, where the environment of metal diffusion, complexation and availability are dissimilar from those noticed in liquid media (Nahurira *et al.*, 2019).

**Table 2: Minimum inhibitory concentration of certain heavy metals against 25 isolates of *Sinorhizobium* sp.**

| Metal | Concentration (µg $mL^{-1}$) | No. of Isolates | Isolates (%) |
|---|---|---|---|
| $Cr^{3+}$ | 25 | 3 | 12 |
| | 50 | 2 | 8 |
| | 100 | 1 | 4 |
| | 400 | 3 | 12 |
| | 1600 | 16 | 64 |
| $Cr^{6+}$ | 100 | 7 | 28 |
| | 200 | 18 | 72 |
| $Cu^{2+}$ | 25 | 19 | 76 |
| | 50 | 3 | 12 |
| | 100 | 3 | 12 |
| $Cd^{2+}$ | 12.5 | 10 | 40 |
| | 25 | 4 | 16 |
| | 50 | 11 | 44 |
| $Zn^{2+}$ | 50 | 12 | 48 |
| | 100 | 13 | 52 |
| $Ni^{2+}$ | 25 | 25 | 100 |

**Table 3: Antibiotic resistance pattern in *Sinorhizobium* sp.**

| No. of antibiotics | Resistance pattern |
|---|---|
| 1. | Na (2), Am, K (1) |
| 3. | Na, K (2), C (1), Am (1) |
| 4. | Na, T, C (1) |
| 6. | Na, T, C, Am, K, G (1) |

Am: Ampicillin, C: Chloroamphenicol, G: Gentamycin, K: Kanamycin, Na: Nalidixic acid, and T: Tetracycline

Some selected isolates of *Sinorhizobium* sp. were examined for their sensitivity against six regularly used antibiotics *viz*., chloramphenicol, ampicillin, gentamycin, kanamycin, nalidixic acidand tetracycline. Within the 15 isolates of *Sinorhizobium* sp., highest resistance was observed against nalidixic acid (46.6%). Of the isolates, 26.6% were resistant to ampicillin and kanamycin. The entire isolates were susceptible to gentamycin. Our study revealed that the test isolates were resistant to one or more antibiotics. Within the six different tested antibiotics, four different antibiotic resistant patterns were found in *Sinorhizobium* isolates (Table 3). Seven different kinds of antibiotic resistance pattern were detected. Among the test isolates 41.3% were resistant to 6 different antibiotics at one time. The concurrence of metal and antibiotic resistance has been reported by Viti *et al*. (2003) and Satarupa and Paul (2013). It might be assumed that antibiotic resistance in these bacteria may have been induced by the stress due to metal contamination and horizontal movement of related antibiotic resistance genes. All the selected isolates demonstrate good growth up to salt concentration of 3%. These finding revealed that two isolates tolerated a 10% NaCl concentration (Table 4). Our isolates demonstrated improved salt tolerance in contrast to the isolates stated by Chaudhary *et al*. (2013). One likely reason for high salt tolerance appears the high saline nature of tannery wastewaters. Neutral pH had no suppressing impact on the growth of any tested isolates, whereas the majority of isolates were tolerant to pH 9 (Table 5). Possibly a bacterium exposed to extreme pH may be

**Table 4: NaCl tolerance among some isolates of *Sinorhizobium* sp.**

| Salt conc. (%) | Isolates | Growth behavior | Total (%) | Result |
|---|---|---|---|---|
| 1 | SM1, SM3, SM4, SM5, SM6, SM7, SM10, SM13, SM17, SM24 | + | 100 | Tolerant |
| 2 | SM1, SM3, SM4, SM5, SM6, SM7, SM10, SM13, SM17, SM24 | + | 100 | ,, |
| 3 | SM1, SM3, SM4, SM5, SM6, SM7, SM10, SM13, SM17, SM24 | + | 100 | ,, |
| 4 | SM10, SM17, SM24 | + | 30 | ,, |
| 5 | SM17, SM24 | + | 20 | ,, |
| 7.5 | SM17, SM24 | + | 20 | ,, |
| 10 | SM17, SM24 | + | 20 | ,, |

less tolerant to toxic substances than under a pH regime that is close to the optimum environment. The reduction in pH severely affected the activities of *Sinorhizobium* sp. As a result, the alkaline pH of test soil made it easier to assess the impacts owing to the clear toxicity of heavy metals (Baath, 1989; Bahig *et al.,* 2008).

**Table 5: pH tolerance among some isolates of *Sinorhizobium* sp.**

| pH | Isolates | Total (%) |
|---|---|---|
| 4.0 | 0 | 0 |
| 5.0 | SM2, SM3 and SM5 | 30.3 |
| 6.0 | SM2, SM3, SM5, SM7 and SM10 | 50.5 |
| 7.0 | SM2, SM3, SM5, SM7, SM10, SM12, SM13, SM17 and SM24 | 100 |
| 8.0 | SM2, SM3, SM5, SM7, SM10, SM12, SM13, SM17 and SM24 | 100 |
| 9.0 | SM2, SM3, SM5, SM7, SM10, SM12, SM13, SM17 and SM24 | 100 |

The present study revealed that the soil irrigated with tannery effluents accumulate high contents of heavy metals. Further, the resident bacterial isolates react to the long-term use of tannery effluent through enhanced resistance to several heavy metals and sustain physiological characters that possibly promote their survival in polluted atmosphere. Therefore, the root nodulating bacteria can be used for the designing of system for removal of heavy metals.

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