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Impact of Long-Term Application of Treated Tannery Effluents on the Emergence of Resistance Traits in *Rhizobium* sp. Isolated from *Trifolium alexandrinum*

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Abstract: A total of 35 *Rhizobium* sp. were isolated from the root nodules of *Trifolium alexandrinum* (Egyptian clover) irrigated with treated tannery effluents and characterised on the basis of morphological, cultural and biochemical characteristics. Rhizospheric soils and plant parts were also analysed for metal concentrations by atomic absorption spectrophotometry. The test soil samples were contaminated with a high level of chromium and also with other heavy metals, i.e. Ni, Zn, Cu, and Cd. The heavy metal analysis of *Trifolium alexandrinum* plant parts revealed different accumulation of these metals in different plant parts, such as root, stem, and leaf. *Trifolium alexandrinum* roots accumulated the highest amount of these metals and this was followed by leaves. All the isolates of *Rhizobium* sp. were tested for their resistance against Cr^{3+}, Cr^{6+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, and Ni^{2+}. The highest minimum inhibitory concentration (MIC) of 1600 µg/ml was observed against Cr^{3+} in 37.1% of the isolates. Some of the metal resistant isolates that showed maximum resistance were also tested for their resistance against 6 commonly used antibiotics, namely tetracycline, ampicillin, gentamycin, kanamycin, chloramphenicol, and nalidixic acid. Forty percent of *Rhizobium* sp. isolates were resistant against nalidixic acid and 33.3% were resistant to chloramphenicol and tetracycline.

Key Words: Antibiotic resistance, metal resistance, soil, tannery effluents, *Trifolium alexandrinum*

Trifolium alexandrinum'dan İzole Edilen *Rhizobium* sp'nin Dayanıklılık Özelliklerinin Ortaya Çıkmasında Dericilik Atıklarının Uzun Süreli Uygulamasının Etkisi

Özet: Dericilik atıklarıyla muamele edilmiş *Trifolium alexandrinum* (Mısır yoncası)'nın kök nodüllerinden toplam 35 *Rhizobium* sp. izole edildi ve morfolojik, kültürel biyokimyasal karakteristiklerine göre karakterize edildi. Rizosferik topraklar ve bitki kısımları aynı zamanda metal derişimleri bakımından atomik absorbsiyon spektrofotometresinde analiz edildi. Test toprak örnekleri yüksek seviyede krom ve aynı zamanda Ni, Zn, Cu ve Cd gibi diğer ağır metallerle kirletilmiştir. *Trifolium alexandrinum* bitki kısımlarının ağır metal analizleri bu metallerin kök, gövde ve yaprak gibi farklı bitki kısımlarında farklı şekillerde biriktirdiğini göstermiştir. *Trifolium alexandrinum* kökleri bu metallerin en yüksek oranlarını biriktirmiştir bunu sırasıyla yapraklar izlemiştir. Tüm *Rhizobium* sp. izolatları Cr^{+3}, Cr^{+6}, Cd^{+2}, Cu^{+2}, Zn^{+2} ve Ni^{+2} metallerine dayanıklılıkları bakımından denenmiştir. En yüksek MIC (Minimal inhibitör derişim) olan 1600 µg/ml, Cr^{+3}'e karşı izolatların % 37,1'inde gözlenmiştir. Maksimum dayanıklılık gösteren metale dayanıklı izolatların bazılarının yaygın olarak kullanılan Tetrasiklin (T), Ampisilin (Am), Gentamisin (G), Kanamisin (K), Kloramfenikol (C) ve Nalidiksik asit (Na) gibi 6 antibiyotiğe karşı dayanıklılıkları da test edilmiştir. *Rhizobium* sp. izolatlarının % 40'ının Nalidiksik asite dayanıklı olduğu % 33' ünün ise Kloramfenikol ve Tetrasikline dayanıklı olduğu gözlenmiştir.

Anahtar Sözcükler: Antibiyotik dayanıklılığı, metal dayanıklılığı, toprak, dericilik atıkları, *Trifolium alexandrinum*

Introduction

Tannery effluents are ranked as the highest pollutants among all the industrial wastes. It is estimated that in India alone about 2000-3000 t of chromium escapes into the environment annually from tannery industries, with chromium concentrations ranging between 2000 and 5000 mg/l in the aqueous effluent compared to the recommended permissible limits of 2 mg/l. The tanning industries are especially large contributors of chromium pollution in India. Two types of effluents are discharged during the tanning process: vegetable tanning, which does not contain chromium, and chrome tanning, which contains chromium (1). Leather processing requires large amount of chemicals like sodium chloride, chromium

sulphate, calcium salts, ammonium salts, sodium sulphide, acids, alkalis, fat, liquor, and organic dyes. However, one of the major emerging environmental problems in the tanning industry is the disposal of chromium contaminated sludge produced as a by-product of wastewater treatment (2). Tannery effluents severely affect the mitotic process and reduce seed germination in extensively cultivated pulse crops (3).

At high concentrations chromium is toxic, mutagenic, carcinogenic, and teratogenic (4). Chromium exists in oxidation states of +2, +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 due to its low water solubility. The hexavalent chromium is much more toxic to many plants, animals, and bacteria inhabiting aquatic environments (5). Chromium resistant micro-organisms from chromium contaminated soil and sediments have been isolated by several investigators (4,6,7). The presence of Cr(VI) in the environment exerts selection pressure on microflora. Most micro-organisms are sensitive to Cr(VI) toxicity but some groups possess resistance mechanisms to Cr(VI) and can tolerate high levels. A relationship was found between the total chromium content of soil and the presence of metal tolerant/resistant bacteria (8).

The availability of selected strains able to resist and reduce chromate elevates the possibility of employing micro-organisms for bioremediation of Cr(VI) contaminated sites in a more economical way with respect to current chemical remediation systems. Our intention was to initiate preliminary work on heavy metal pollution in agricultural soil irrigated with treated tannery effluents and its possible impact on the emergence of metal resistance on N_2-fixing bacteria especially *Rhizobium* sp. isolated from the nodules of *Trifolium alexandrinum*.

Materials and Methods

Sample Collection

Plant and soil samples were collected from agricultural fields irrigated with treated tannery effluents from the Jajmau area of Kanpur. Soil samples were collected in sterilised polyethylene bags with the help of a sterilised spatula as described by Reddy et al. (9). The plants of *Trifolium alexandrinum* (Egyptian clover) were uprooted along with the rhizospheric soil. The plant root nodules of *Trifolium* were obtained along with the rhizospheric soil for isolation of *Rhizobium* sp.

Determination of Soil pH

A 10 g air-dried and sieved soil (<2 mm) sample was taken in a plastic beaker, 25 ml of distilled water was added, and the mixture was stirred for 1 min. The pH of the supernatant was measured as described by Alef and Nannipieri (10).

Isolation and Identification of *Rhizobium* Strains

Rhizobium sp. was isolated from the root nodules of *Trifolium alexandrinum* as described by Vincent (11). The root system of the test legumes was washed under tap water, and well formed, healthy, pink nodules on the tap root were selected. The nodules were surface sterilised with 0.1% mercuric chloride, and then washed with sterilised distilled water and 70% ethyl alcohol for 1 min each respectively. Nodules were finely crushed with a sterile glass rod in a small aliquot of sterilised water. Serial dilutions of the suspensions were prepared and aliquots of the appropriate dilutions were plated on yeast extract mannitol agar supplemented with congo red. Plates were incubated at 28-30 °C for 72-96 h.

Heavy Metal Analysis of Soil and Plant

Heavy metal analysis of soil samples was done as described by Alef and Nannipieri (10). The soil was oven dried (40 °C) and finely ground (<0.1 mm). One gram of soil was then burnt into ashes in a crucible. These ashes were taken and moistened with a little double distilled water. Concentrated HNO_3 and HCl were added successively in a ratio of 3:1. Soil samples in the beaker were then heated gently on a heating plate until the samples were digested, which was indicated by the formation of a clear solution above the soil residue. The mixture was reduced to a volume of 1 ml and diluted with double distilled water and then filtered through Whatman filter no. 42. Double distilled water was added to make the volume up to 100 ml. Digested soil samples were analysed for metal concentration by atomic absorption spectrophotometer (GBC 932 Plus, Australia).

A plant sample was oven dried (40 °C) and finely ground to make fine powder and filtered through muslin cloth. One gram of sample (plant) was taken in a 100 ml beaker. The sample was then digested with perchloric acid on a heating plate until the test solution becomes colourless. The mixture was reduced to a volume of 1 ml

and diluted with double distilled water and filtered through Whatman filter no. 42. Double distilled water was added to make the volume of the filtrate up to 100 ml. Digested plant samples were analyzed for metal concentrations by atomic absorption spectrophotometer (GBC 932 plus, Australia).

Quality Control and Quality Assurance

The standard reference material for metals (J.T. Baker, Phillipsburg, NJ, USA) was used for calibration and quality assurance for each analytical batch. Analytical data quality of metals was ensured through repeated analysis (n = 6) of EPA quality control samples for metals Cd (Lot B40656), Cr (Lot B40691), Cu (Lot B24592), Ni (Lot B06599), and Zn (Lot B06597) in water and the results were found to be within ±1.92% of certified values. The blanks were run in triplicate to check the precision of the method with each set of samples. All the values are means of 6 replicates ± standard deviation.

Determinations of Minimum Inhibitory Concentration (MIC) of Heavy Metals

The MIC of the metal for each bacterial isolate was determined by plate dilution as adopted by Malik and Jaiswal (12) and Aleem et al. (13). The metals Cr^{6+}, Cr^{3+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, and Ni^{2+} were used as $K_2Cr_2O_7$, $CrCl_3.H_2O$, $ZnCl_2$, $CuSO_4.H_2O$, $CdCl_2$, and $NiCl_2$ in varying concentrations from 3.12 to 3200 µg/ml. Stock solutions of the metal salts were prepared in double distilled water and were added to the respective media in varying concentrations. *Rhizobium* cultures grown in yeast extract mannitol broth were then spot inoculated with approximately 3×10^6 organisms on metal supplemented plates with the help of a micropipette. The plates were incubated at 28 °C for 24-72 h. The lowest concentration of the metal that inhibits the growth of microorganisms was considered the MIC of the metal against the strain tested. AB1157 and C600 strains of *E. coli* K-12 were used as control. The characteristics of AB1157 were *thi-1*, *argE3*, *thr-1*, *leuB6*, *proA2*, *hisG4, lacY1*, F^-, Str^r, and I^s (Source: Barbara Bachman, *E. coli* Genetic Stock Center, Dept. of Biology, Yale University, New Haven, USA) and of C600 were *thr-1, leuB6*, *fhuA21*, *lacY1*, *glnV44*, *rfbD1*, *glpR200*, and *thi-1* (Source: Barbara Bachman).

It is well known that there are no currently acceptable concentrations of metal ions that can be used to distinguish metal resistant and metal sensitive bacteria. However, concentrations used in this study have been employed in similar studies reported on eubacteria (12,14,15).

Determination of Antibiotic Resistance

The resistance pattern of different isolates of *Rhizobium* against different antibiotics was determined by disc diffusion method as described by Bauer et al. (16). Concentration of the antibiotics used was in µg/disc. The symbols and concentrations of the respective antibiotics (Source: Hi-media, Bombay, India) are given in parentheses: 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.

Determination of pH Tolerance

pH tolerance was determined by the method described by Tippannavar et al. (17). Media plates of yeast extract mannitol agar with pH ranging from 4 to 9 were prepared. The cultures of *Rhizobium* sp. already grown in their respective broths were then spot inoculated on the plates with approximately 3×10^6 organisms. 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 the isolated strains was determined by the method described by Babak (18). Media plates of YEMA containing various concentrations (1.0%-5.0%, 7.5%, 10%, 12.5%, and 15%) of sodium chloride were prepared. Exponentially growing cultures of *Rhizobium* sp. (3×10^6) were spot inoculated on the NaCl supplemented plates. The plates were incubated at 28 °C for 24-72 h. *E. coli* B was used as the control.

Results and Discussion

The atomic absorption spectrophotometric analysis of heavy metals in soil is summarised in Table 1. Agricultural fields in the Jajmau area (Kanpur) have been receiving industrial wastewater rich in tannery effluent for a long time. For this reason, they are contaminated with different types of heavy metals. The mean concentrations found in the soil were chromium 1178 µg/g, nickel 51.5 µg/g, zinc 39.86 µg/g, copper 21.3 µg/g, and cadmium 1.09 µg/g. The concentration of chromium was very high in the soil as these agricultural lands are irrigated with wastewater rich in tannery effluents, which contains high concentration of chromium sulphate. Other researchers

Table 1. Concentrations (mg/kg) of heavy metals in agricultural soils receiving treated tannery effluents as quantitated by atomic absorption spectrophotometer.

Heavy metals	Agricultural soil irrigated with wastewater rich in tannery effluents (mg/kg)
Cr	1178 ± 132.9
Ni	51.5 ± 10.8
Zn	39.8 ± 9.2
Cu	21.3 ± 3.6
Cd	1.09 ± 0.24

All the values are means of 6 replicates ± SD.

have also reported high concentrations of chromium in the same area (19,20). The concentrations of zinc, copper, and cadmium were comparable to those reported previously in different regions of the country (12,14,21-23).

The heavy metal analysis of different plant parts of *Trifolium alexandrinum* revealed accumulation of such metals in different plant parts such as root, stem, and leaf. The root of *Trifolium alexandrinum* accumulated the highest amounts of these metals (data not shown). Chromium contents in root, leaf, and stem were lower than the content of it in soil. This may be due to the highly immobile nature of Cr^{3+} compounds. Chromium(III) is highly impermeable to the cell membrane as compared to chromium(VI). However, in the soil system the chromium(VI) is reduced to chromium(III) by the organic matter present in the soil as described previously (24,25).

All the bacterial isolates were tested for their resistance against 6 heavy metals, namely Cr^{3+}, Cr^{6+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, and Ni^{2+}. The MIC of 1600 µg/ml was observed against Cr^{3+} in 37.1% of isolates. On the other hand, the isolates were sensitive to nickel and cadmium (Tables 2 and 3). Our findings on Cr^{3+} resistance are comparable to those reported by Pacheco et al. (26), who reported that 57.6% bacteria were resistant to chromium(III) isolated from Mexico City freeway. Malik and Jaiswal (12) reported that *Pseudomonas* strains isolated from metal contaminated soil harbouring resistance to chromium(III) were 71.1%. Our findings showed low levels of resistance against other heavy metals. The high level of resistance against chromium(III) was probably due to the high level of this metallic ion at the sampling site, which might exert selection pressure on the microbial population as reported previously (24,27). Metals and various components of media could interact, thus complicating interpretation of the data. The test of toxicity in solid media could be useful in the evaluation of metal toxicity in sewage sludges and contaminated soil, where conditions of diffusion, complexation, and availability of metals are different from those observed in liquid media (28,29).

Out of 35 isolates, 15 (maximum resistant to metals) of *Rhizobium* sp. were tested for their susceptibility against 6 commonly used antibiotics, namely tetracycline, ampicillin, gentamycin, kanamycin, chloramphenicol, and nalidixic acid. Among the 15 isolates of *Rhizobium* sp., maximum resistance was observed against nalidixic acid (40.0%). Of the isolates, 33.3% were resistant to chloramphenicol and tetracycline. All the isolates were sensitive to gentamycin (Figure). In our study the test isolates were resistant to one or more antibiotics. Among the 6 different tested antibiotics, 5 different antibiotic resistant patterns were observed in the *Rhizobium* isolates (Table 4).

Aleem et al. (13) isolated 57 *Azotobacter chroococcum* from wheat (*Triticum aestivum*) rhizospheric soil irrigated with industrial wastewater and tested for their resistance against 11 commonly used antibiotics/drugs. They found that 91.6% were resistant to nitrofurantoin while 86.4% and 80.5% were resistant to polymyxin-B and co-trimoxazole, respectively. Khan and Malik (30) also reported a high incidence of antibiotic resistance in *E. coli* and *Staphylococci* strains from foodstuffs. Skryabin et al. (31) also found that all the tested strains of *A. chroococcum* were resistant to chloramphenicol and sensitive to tetracycline, ampicillin, streptomycin, and rifampicin. Seven different types of resistance pattern were observed. Of the isolates, 41.3% were resistant to 6 different antibiotics/drugs at a time.

As pointed out by Hsu et al. (32), differences in percentages of bacterial resistance to various antibiotics may reflect the history of antibiotic application and hence drug resistance may be used as an indicator of antibiotic application.

All the isolates showed confluent growth up to a salt concentration of 2%. In our study one of the isolates tolerated a 10% NaCl concentration (Table 5). Our isolates showed high salt tolerance as compared to the isolates reported by Tippannavar et al. (17). One possible

Table 2. MIC of *Rhizobium* sp. against chromium and other heavy metals.

	Metals (µg/ml)					
Isolates	Cr^{6+}	Cr^{3+}	Cd^{2+}	Cu^{2+}	Ni^{2+}	Zn^{2+}
1.	100	1600	12.5	100	25	200
2.	3.12	800	12.5	25	25	100
3.	100	1600	25	50	25	25
4.	3.12	1600	12.5	25	25	100
5.	12.5	1600	25	50	25	200
6.	6.15	1600	25	50	25	200
7.	12.5	1600	12.5	25	25	25
8.	6.15	200	12.5	100	25	25
9.	6.15	100	12.5	100	25	200
10.	12.5	1600	25	50	25	100
11.	12.5	1600	12.5	50	25	100
12.	100	1600	12.5	50	25	100
13.	6.15	200	12.5	50	25	100
14.	6.15	800	12.5	100	25	25
15.	12.5	1600	25	50	25	25
16.	25	1600	12.5	50	25	25
17.	25	400	12.5	100	25	25
18.	25	800	12.5	100	25	25
19.	50	400	12.5	50	25	25
20.	100	400	12.5	50	25	25
21.	50	200	12.5	50	25	25
22.	12.5	800	12.5	50	25	25
23.	50	100	25	100	25	25
24.	3.12	200	12.5	50	25	25
25.	100	1600	25	50	25	25
26.	3.12	100	12.5	25	25	25
27.	25	400	12.5	25	25	25
28.	25	400	12.5	50	25	25
29.	50	400	12.5	25	25	25
30.	50	800	12.5	25	25	25
31.	3.12	1600	12.5	25	25	25
32.	3.12	50	12.5	25	25	25
33.	50	1600	12.5	50	25	25
34.	25	800	12.5	50	25	25
35.	3.12	400	12.5	100	25	25

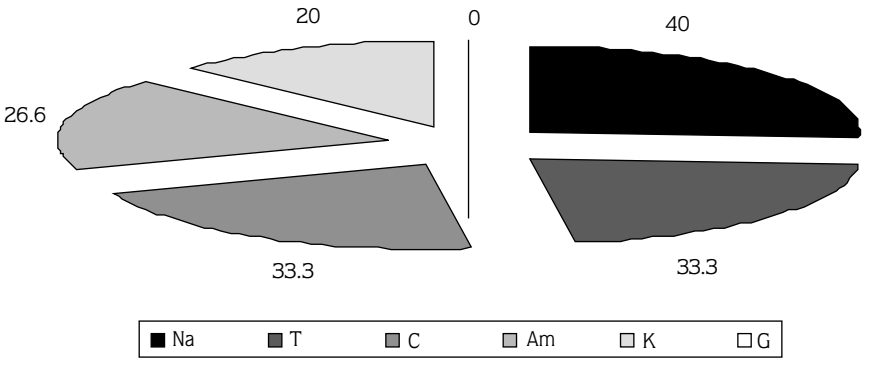

Figure. Percent resistance to antibiotics among 15 *Rhizobium* isolates.

Table 3. Minimum inhibitory concentration of certain heavy metals against *Rhizobium* sp.

Metal Conc. (µg/ml)	No. of Isolates	Isolates (%)
Cr^{3+}		
25	2	5.7
50	3	8.5
100	3	8.5
200	3	8.5
400	6	17.1
800	5	14.2
1600	13	37.1
Cr^{6+}		
3.12	18	51.4
6.25	6	17.1
12.5	5	14.2
25	3	8.5
50	0	0
100	3	8.5
Cu^{2+}		
25	11	31.4
50	17	48.5
100	7	20
Cd_{2+}		
12.5	28	80
25	7	20
Zn^{2+}		
25	26	74.2
50	0	0
100	5	14.2
200	4	11.4
Ni^{2+}		
25	35	100

Total number of isolates = 35

Table 4. Antibiotic resistance pattern in *Rhizobium* sp.

No. of Antibiotics	Resistance Pattern
1	Na (1), K (1), T, (1)
2	Na, C (3), T (1), Am (1)
3	T, Am, K (1)
4	Na, T, C, Am (1)
5	Na, T, C, Am, K (1)

reason for the high salt tolerance is the use of large amounts of salts in the tanning industry.

Neutral pH had no inhibitory effect on the growth of any of the isolates tested, while most of the isolates were tolerant to pH 9 (Table 6). Similar observations were made by Mahmood et al. (33). It is probable that an organism challenged at extreme pH will be less tolerant to toxic substances than under a pH regime that is close to the optimum conditions. Most of the desirable soil microbiological activities of *Rhizobium* are adversely affected as the pH decreases. Therefore, the alkaline pH of the test soil sample made it easier to monitor the effects due to the apparent toxicity of heavy metals (34).

The present finding indicates that the soil irrigated with tannery effluent and industrial wastewater contained high levels of metallic pollutants. At present, many risk assessments and regulations for the application of sewage

Table 5. NaCl tolerance in selected isolates of *Rhizobium* sp.

Salt Conc. (%)	Isolates	Growth Behavior	Total (%)	Result
1	RT3, RT4, RT5, RT6, RT10, RT11, RT13, RT15, RT17, RT25	+	100	Tolerant
2	RT3, RT4, RT5, RT6, RT10, RT11, RT13, RT15, RT17, RT25	+	100	,,
3	RT3, RT4, ,RT6, RT7	+	40	,,
4	RT3, RT4	+	20	,,
5	RT3	+	10	,,
7.5	RT3	+	10	,,
10	RT3	+	10	,,

Table 6. pH tolerance of selected isolates of *Rhizobium* sp.

Tolerant isolates	pH of the medium											
	4.0		5.0		6.0		7.0		8.0		9.0	
	Isolates	Total (%)	Isolates	Total (%)	Isolates	Total (%)	Isolates	Total (%)	Isolates	Total (%)	Isolates	Total (%)
Rhizobium sp.	0	0	RT3, RT4, RT5, RT10, RT13	55.5	RT3 RT4, RT5, RT10, RT13	55.5	RT3, RT4, RT5, RT10, RT13 RT11, RT15,	77.77	RT3 RT4, RT5, RT10, RT13 RT11, RT15, RT17, RT25	100	RT3, RT4, RT5, RT10, RT13 RT11, RT15, RT17, RT25	100

sludge in many countries are based on the total trace metal concentrations in the soils (22,35). However, the present study indicated that, despite these toxic stresses, the *Rhizobium* isolates may have evolved resistance mechanisms to deal with metal toxicity that include volatilisation, extracellular precipitation and exclusion, binding to cell surface, and intracellular sequestration. It is evident from these studies that the test population in the test system responded to the long-term application of industrial wastewater by an increase in resistance to several undesirable agents and maintained physiological traits that could benefit microbial maintenance and survival in contaminated environments.

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