Investigations on the Effect of Tungsten Resistant Soil Bacteria against the Toxicity of Tungsten on *Spinacea oleracea*: a Case Study

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Citation

ABSTRACT

A vegetation experiment was performed to test the bioaccumulation capabilities of two Tungsten Resistant Soil Bacteria (TRSBs) as a consortium - *Proteus mirabilis* (2K) and *Bordetella* sp. (3K) – based on their tungsten tolerating and accumulating capacities measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) in a previous study. The bioremediation effect was observed against toxicity of tungsten on the spinach plants (*Spinacia oleracea*), as tungsten is believed to enter the food chain through vegetation. Sodium tungstate (Na₂WO₄·2H₂O) in two concentrations (10 g/kg and 15 g/kg) was used as a source of tungsten in the soil of the potted plants. Isolated bacterial broth cultures of the TRSBs were used to populate the soil in the experimental pots to observe their prospective tungsten accumulation capacities. Tungsten was observed to cause a progressive decrease in the growth, fresh weight and chlorophyll content of the plants when grown at increasing tungsten concentrations of 10 g/kg and 15 g/kg. Whereas, the experimental plants grown under similar conditions, but populated with TRSBs showed satisfactory growth.

Keywords: Tungsten, *Proteus mirabilis*, *Bordetella* sp., Bacterial Accumulation, Spinach Plants, Bioremediation
1. INTRODUCTION

Anthropogenic activities are a cause of major heavy metal pollution in the environment. Industrial effluents, sewage waste, agriculture waste, chemical fertilizers, abandoned metal mining (Backstrom, 2003), surface run-offs into nearby farm areas, smelting, landfills with discarded batteries and paints among others are some of the leading causes of tungsten (W) pollution in the environment. Military activities like heavy shelling has also resulted in large amounts of W being deposited in the soil (Koutsospyros, 2006). This has lead to widespread contamination of soil and groundwater, eventually making these sources unfit for the healthy existence of microbes and plants, and to a large extent for the survival of animals and humans as well. It has invariably put a strong survival pressure on microbes and plants and has lead to their constant adaptations in the form of tolerance or resistance. Presence of high concentration of the metals due to anthropogenic activities has also lead to a disruption in the metal’s geochemical cycle in the nature (ASTDR, 2003).

Tungstates polymerize in solutions having a pH lower than 6.2 that results in the formation of polytungstates (Dermatas, 2004). Polytungstates were discovered in aged soils that previously contained only tungsten particles, and were established as the key bioavailable species of W. Size exclusion chromatography (SEC) interfaced to ICP-MS (Bednar, 2009a) helped in the confirmed demonstration of the bioavailability and abundance of polytungstates in the environment (Johnson, 2006). Tungsten toxicity is known to be variable for each soil and depends on soil properties, such as its clay content and buffering capacity (Strigul, 2005). It was also widely predicted by geochemists that polytungstates are common in environmental systems as decomposition of W minerals is usually accompanied by acidification.

These were also shown to accumulate in the plant tissues (Bednar, 2009b). W has been known to accumulate, but inhibit the nitrate reductase activity in the leaves of Spinacea oleracea L. The reduction in activity results from the formation of a tungstoprotein analogue, lacking nitrate reductase activity, but active as a diaphorase. Super-induction of NADH-diaphorase by W leads to an increased nitrate accumulation in the leaves as well (Barber et al., 1990).

In this experiment, the bioremedial effects of the prospective TRSBs previously isolated were checked in terms of the growth performance, W accumulation and chlorophyll content in the spinach plants. Besides, phytochemical effects of spinach, if any, were also assessed as a ‘polishing technique’ that could be used in tandem with other techniques of bioremediation in the future.

2. MATERIALS AND METHODS

The experiments were set up during the month of March. The plants were grown for a period of three weeks. The experiments presented in this work were established in a greenhouse at Rajiv Gandhi Biotechnology Centre, RTM Nagpur University. They were arranged in a complete randomized design in duplicates. At the end of the experiments plant tissue samples were harvested and analyzed further. The soil used in this experiment was clay-loam in nature, the physico-chemical characteristics of which are mentioned in Table 1.

2.1. Chemicals and Media

Luria Bertani (LB) broth [Hi-Media] was used as the preferred medium of growth for the TRSBs. Analytical grade Sodium Tungstate (Na2WO4·2H2O) [Hi-Media] was used as a source of W.

2.2. Bacterial Culture Used

A consortium of Proteus mirabilis (2K) and Bordetella sp. (3K) was used in this experiment. These strains were established as potential W tolerating bacterial species in previous experiments performed for their molecular isolation, characterization and identification.

2.3. Preparation of soil for Vegetation Experiment

The soil sample was collected from the premises of Rajiv Gandhi Biotechnology Centre, RTM Nagpur University, which was homogenized, dried, disaggregated with a porcelain pestle and mortar, and sieved through a 5 mm sieve. It was then filled into autoclavable plastic bags, sealed and autoclaved at 121°C and 15 psi pressure for 20 minutes. Sterile soil sample was used in the vegetation experiments in order to observe the bioremediation effect, if any, of the TRSB consortium used. The soil was stored at 4°C until further use in experimentation. Two concentrations of W were applied as sodium tungstate (Na2WO4·2H2O). The concentration was prepared separately by taking corresponding amount (calculated on the basis of molecular weight) of chemical/ kg of air dried soil. 10 g sodium tungstate/ kg of dried soil and 15 g sodium tungstate/ kg of dried soil were the concentrations used. This W range was chosen to be representative of the values at which the TRSBs exhibited tolerance to W individually in experiments performed previously.
2.4. pH Measurement of the Experimental Soil
2g of W contaminated and uncontaminated soil samples were separately placed into plastic, chemically inert, 15 ml centrifuge tubes and were mixed with distilled water in a 1:10 ratio. After shaking for 30 min, the tubes were centrifuged at 6000 rpm. The clear supernatant was used for pH measurement using a pH meter.

2.5. Experimental Design for Pot Experiment
Spinach seeds were sown into 5 plastic containers (7 cm height and 13.5 cm diameter) with approximately 450 g of the autoclaved soil in each. A hole was drilled into the bottom of each of the containers to drain off excess water. This experiment was conducted in duplicates. The details are given in Table 2.

- Control Container: Uncontaminated Soil [no sodium tungstate or bacterial broth]
- Container 1A (C1A): Sodium Tungstate (10 g/kg of soil) + Bacterial Broth (20 ml)
- Container 2A: (C2A) Sodium Tungstate (15 g/kg of soil) + Bacterial Broth (20 ml)
- Container 1B (C1B): Sodium Tungstate (10 g/kg of soil)
- Container 2B (C2B): Sodium Tungstate (15 g/kg of soil)

30 seeds of the test plant were sown at 2-3 cm depth in each plastic container. 30 ml water was provided daily in the morning to compensate for the transpirational losses. The plants were maintained outdoors for a period of 21 days (3 weeks).

2.6. Detection of Growth Parameters and Fresh Weight
The root and shoot lengths were measured weekly. Fresh weight of the harvested leaves, root and shoot parts of the plant was measured. The morphological characters of the plants were also noted.

2.7. Determination of Chlorophyll Content
Chlorophyll a (Chl a) and Chlorophyll b (Chl b) concentration is an important parameter for indicating the effect of specific heavy metal interventions (Manios, 2003). The method of Arnon (Arnon, 1949) was used to determine the chlorophyll contents in the leaves of test plant samples of spinach that were harvested after 3 weeks. Spinach leaves were cut into small pieces. Major veins and any tough, fibrous tissues were discarded. 50 g of leaf material was used for grinding. The tissue was homogenized in a porcelain mortar and pestle with 10 ml of 80% acetone (acetone: water 80:20 v/v). The extracts were centrifuged at 2000g for 10 min and the supernatant was collected in test tubes. 80% acetone was used as blank for spectrophotometric determination. Measurements were carried out using spectrophotometer UV/VIS. The absorbance at 663 nm and 645 nm for Chl a and Chl b, respectively, was determined.

Calculations:

\[
\text{Total Chlorophyll Content (µg/mL)} = 17.3 \, A_{663} + 7.18 \, A_{645} \\
\text{Chlorophyll a (µg/g)} = 12.21 \, A_{663} - 2.81 \, A_{646} \\
\text{Chlorophyll b (µg/g)} = 20.13 \, A_{646} - 5.03 \, A_{663}
\]

2.8. Quantification of tungsten using inductively Coupled Plasma-Mass Spectrometry (ICP-MS)
2.8.1. Detection of Residual Tungsten in Soil
After the 3 week incubation period, experimental soil from the containers (C1A, C1B, C2A, C2B) were analyzed separately for residual W by ICP-MS. The soil samples were sent to Anacon labs Pvt Ltd, Nagpur, for detection of W content by ICP-MS.

2.8.2. Detection of Tungsten in Spinach Leaves
Dried plant samples (80°C in the convection oven) were sent to Anacon Labs Pvt Ltd, Nagpur, for the detection of tungsten content by ICP-MS.

2.9. Statistical Analysis
The statistical analysis was done in MS Excel.
3. RESULTS

3.1. Measurement of pH

pH of the potted soils was measured before sowing the seeds and after harvesting the spinach plants. The results of which are given in Table 3.

3.2. Pot experiment

Spinach plants were successfully grown for 3 weeks (Figure 1) and their growth parameters were monitored weekly. At the end of the growth period, samples of leaves, shoots and roots were harvested and stored at -20 °C in ultra-refrigerator in order to be used for further plant physiological and biochemical analyses.

3.3. Effect of Tungsten on Morphological, Physiological and Biochemical Parameters of Spinacia oleracea

3.3.1. Fresh Weight of the Experimental Leaves of Spinacia oleracea

Leaves of the plants in C1A and C2A pots showed signs of necrosis on their edges. The plants showed stunted growth and wilting. Delayed seed germination was observed in C2B with 15 g/kg of W (after 2-3 days) and in C1B with 10 g/kg of W (after 1 day) as compared to the plants in C2A and C1A, respectively. Till week one, the leaf sizes in C1B and C2B were seen to be relatively smaller than those in C1A and C2A. These were the visible symptoms of W stress observed in the potted plants. Fresh weight of the harvested leaves, roots and shoots was calculated, the results of which are displayed in Figure 2. It was observed that the fresh weights of the leaves in C2B and C1B were 14.28 % and 11.76 % lesser than the fresh weight of leaves in C2A and C1A. Fresh weights of shoots in C2B and C1B were 21.05 % and 17.94 % lesser than those in C2A and C1A. And the fresh weights of roots in C2B and C1B were 11.64 % and 11.76 % lesser than those in C2A and C1A. The above observations suggest that morphology observed for the plants in C1B and C2B (no bacterial broth) was poor in quality with respect to that of the leaves in C1A and C2A.

3.3.2. Growth Parameters of the Experimental Potted Plants

The growth parameters in C1A were almost identical to that of the control container. The shoot and root lengths were comparable to that of the control plants. Whereas, in case of C1B, the shoot and root lengths were observed to be slightly inferior to those in C1A and in the control. The shoot and root lengths of the plants growing in C2A were measured and found to be slightly inferior to that of plants in the control container. Whereas, in the case of C2B (15 g/kg W), the shoot and root lengths were found to be considerably inferior to those of C2A and Control. The density of plants growing in the pots was also found to be very less. This shows that a W concentration of 15 g/kg of soil is detrimental to the growth of spinach plants. The result is given in Figure 3.

3.3.3. Chlorophyll a and b Content in leaves

The chlorophyll content of the experimental spinach plants was investigated spectrophotometrically. The Chl a and b content in the leaves of C1A, C2A, C1B and C2B was found to be lower than in the leaves of the control plants. The Chl a content in C1B was calculated to be 13.33 % lower than in C1A. Whereas, the Chl a content in C2A and C2B was found out to be similar. The Chl b content in C1B was 3.125 % lower than in C1A, whereas the Chl b conc in C2A was 19.04 % lower than in C2B. The results have been illustrated in Figure 4.

3.4. Elemental Analysis of Tungsten by ICP-MS

In this study, W accumulated in spinach plants and residual W in the potted soil was investigated. It was found that the potted soil in C1A and C2A (with TRSB consortia) contained 15.73 % and 13.76 % lower residual W concentration than the amount present in the soil of C1B and C2B (without TRSB consortia), respectively. Accumulation of W in plant tissue was also investigated in the spinach plants at two different W concentrations. The level of W accumulation in the plant tissues of C1A and C2A (with TRSB consortia) was 22.23 % and 25 % lower than the amount accumulated in C2A and C2B (without TRSB bacterial broth), respectively. The results are shown in Table 4.

4. CONCLUSION

In this study, the effect of the consortium of Proteus mirabilis (2K) and Bordetella sp. (3K) was observed against two concentrations of W in Spinacia oleracea plants. The response of the plant species to W toxicity was evaluated in terms of growth and morphological characteristics and chlorophyll content. It was observed that W significantly affected all the parameters tested. The following conclusions were drawn.

The residual W concentration calculated for the soil of the potted plants of C1A and C2A (10 g/kg and 15 g/kg W + 20 ml TRSB bacterial consortia) was greater than the W residual concentration in C1B and C2B soil (10 g/kg and 15
and the spinich plants combined - accumulated greater amount of tungsten. Thus, for plants of C1A and C2A, we can conclude that the bacterial species contributed to W bioremediation in soil and reduced the unnecessary accumulation of W in the plants.

The concentration of W accumulated by C1A and C2A plants was greater as compared to that in plants of C1B and C2B, respectively, which indicates that plants experienced greater W toxicity as there was no bacterial consortium to reduce the load of W toxicity. This resulted in impeded growth of the plants.

This claim is further strengthened by the fact that, the growth, morphological and photosynthetic efficiency (chlorophyll content) characteristics observed for the experimental plants suggested that plants grown in tungsten contaminated soil populated with the TRSB consortium (C1A and C2A), showed improved growth characteristics as compared to those (C1B and C2B) without any bacterial supplement. Furthermore, considerable growth impairment was observed in C1B and C2B. 15 g/kg of W concentration proved to be highly toxic to the plants of C2B. Thus the combined system of the TRSB consortium and spinach plants (phytoremediation to some extent) has proved to be an effective system for remediation of W. This study is a preliminary analysis that proves that Proteus mirabilis and Bordetella sp. may be used as a potential agent of bioremediation of W contaminated fields growing spinach plants in tandem with the other soil remediation techniques already in use. Besides, it is an eco-friendly and an economical in-situ alternative to expensive ex-situ W remediation techniques. Further, studies need to be carried out to fully realize the potential of the consortium’s W accumulation capacities and must be tested in extensive field-like conditions, while taking into account other concentrations of W.

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REFERENCE

Table 1
Physico-chemical properties of the soil used

<table>
<thead>
<tr>
<th>Element</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Nitrogen (%)</td>
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<tr>
<td>Carbon (%)</td>
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<tr>
<td>Phosphorous (mg/kg)</td>
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<tr>
<td>Potassium (mg/kg)</td>
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<tr>
<td>Tungsten (mg/kg)</td>
<td>Undetected</td>
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<td>Iron (mg/kg)</td>
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</table>

Table 2
Specifications of Experimental Pots

<table>
<thead>
<tr>
<th></th>
<th>Container 1A (C1A)</th>
<th>Container 1B (C1B)</th>
<th>Container 2A (C2A)</th>
<th>Container 2B (C2B)</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>Sodium Tungstate (g/kg of Soil)</td>
<td>10</td>
<td>10</td>
<td>15</td>
<td>15</td>
<td>-Nil-</td>
</tr>
<tr>
<td>Bacterial LB Broth (ml)</td>
<td>20</td>
<td>-Nil-</td>
<td>20</td>
<td>-Nil-</td>
<td>-Nil-</td>
</tr>
</tbody>
</table>

Table 3
pH of Potted Soil

<table>
<thead>
<tr>
<th>Potted Soil</th>
<th>pH (Day 0)</th>
<th>pH (Day 21)</th>
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<tbody>
<tr>
<td>Control</td>
<td>7.1</td>
<td>7.0</td>
</tr>
<tr>
<td>C1A</td>
<td>6.6</td>
<td>6.4</td>
</tr>
<tr>
<td>C1B</td>
<td>6.4</td>
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</tr>
<tr>
<td>C2A</td>
<td>6.7</td>
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<tr>
<td>C2B</td>
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Table 4
Results of detection of tungsten concentration in Spinacia oleracea plants and potted soil by ICP-MS

<table>
<thead>
<tr>
<th>Plant Sample</th>
<th>W Conc in Potted Soil (g/L)</th>
<th>W accumulation in Plant Tissue (g/L)</th>
</tr>
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<tbody>
<tr>
<td>C1A</td>
<td>8.5</td>
<td>0.3</td>
</tr>
<tr>
<td>C1B</td>
<td>7.9</td>
<td>0.8</td>
</tr>
<tr>
<td>C2A</td>
<td>13.8</td>
<td>0.6</td>
</tr>
<tr>
<td>C2B</td>
<td>11.9</td>
<td>1.4</td>
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</table>
Figure 1
(a) Spinach plants growing in soil contaminated with 15 g/kg Sodium Tungstate. (b) Spinach plants growing in soil contaminated with 30 g/kg Sodium Tungstate.
Figure 2
Fresh weigh of *Spinacea oleracea* was measured in grams.

Figure 3
(a-b) Growth Characteristics (a) Growth of shoots (b) growth of roots.
Chlorophyll a and b content in the leaves of the experimental pots harvested after 3 weeks.