MATERIALS AND METHODS

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The study was conducted to evaluate the effects of Arsenic on morphogenetic diversity of the flora and the ability of selected species to phytoremediate the arsenic from soil. Three arsenic affected villages viz. Jajjal, Giana and Malkana of the Talwandi Sabo block of Bathinda district (Punjab) were chosen for the preliminary study (Fig. I).

Talwandi Sabo block (nearly 77,000 ha) is a part of Indo-gangetic alluvial plain and lies between 29º 46’30” and 30º 10’5” N latitude and 75º 17’08” and 75º 48’34” E longitude. Gradient is generally from NE to SW direction. The major canals flowing through the area are Bhakra Canal (main branch) and Kotla branch. There is improper drainage network in the area and has only one main drain i.e., Lissara Nallah. The climate of the area is of arid type with 300 mm of average rainfall. The morphogenetic observations and phytoremediation potential of plants collected from above arsenic contaminated areas were conducted at Department of Botany, Punjabi University, Patiala during years 2007-2010.

The broad plan of study was as follows:

1. The soils were analysed chemically as well as for their physical characteristics. Further in situ observations and in vivo experiments were carried out selecting the weeds from one site.

2. Soils of three villages of Talwandi Sabo block were tested for genotoxicity.

3. Weed species (with high metal uptake) from study were selected for in situ morphogenetic studies and further in vivo experimentation.

4. In situ observations regarding the morphology and genetic diversity of the selected weeds was done by selecting one site.
5. Phytoremediation potentials of the selected weeds was studied *in vivo*.

Fig. I. Maps Showing Location of Study Area
3.1. ANALYSES OF SOIL

The soils of the study area were analysed for genotoxicity as well as for physiochemical characteristics.

**Collection of samples:**

The soil samples were collected from three villages of the Talwandi Sabo namely, Jajjal, Giana and Malkana. From each village three types of fields i.e., tubewell irrigated, canal irrigated and barren land (non-irrigated) were marked. Five soil samples were taken from each plot at random digging soil upto 20 cm depth. Each sample was about one kg in weight. Five samples of a plot were mixed over a plastic sheet and a final sample of about one kg was taken for tests in lab. The soil samples were sieved for removing pebbles and other inert material.

3.1.1. GENOTOXICITY AND MUTAGENECITY OF SOIL

**Preparation of soil extracts**

Soil samples taken from the three villages of the study area for soil analysis, were used for genotoxicity assays. The soil samples were homogenized with a pestle and mortar. Water extracts of the samples were prepared by adding the sample (Weight/Volume) in distilled water to obtain 1, 5, 10, 20, 50 and 100 % extracts. The mixture thus obtained were thoroughly stirred and kept for 24 h at room temperature. The undissolved solids were later removed by centrifugation and the supernatant was used as soil extracts for experimental purposes.

Genotoxicity of the soil from study areas was tested by two methods: *Allium* Anaphase-telophase chromosome aberration Assay (Grant, 1982; Rank and Nielsen, 1998) and Yeast Assay (Zimmermann *et al*., 1984).

3.1.1.1. *Allium Assay*

The protocol given by Grant (1982) and modified by Rank and Nielsen (1998) was followed for testing the mutagenic potential of sodium arsenate and the soils of study areas.

**Rooting of *Allium sativum* bulbs:**
Medium sized healthy bulblets of common garlic (*Allium sativum*), procured from local market, were used as the test organism. Outer scales were removed carefully to expose root primordia. Test tubes filled with distilled water were taken and bulblets were placed over the test tubes (50 ml capacity) in such a way that the lower portion of the bulblets were dipping in the water (Fig. II). The whole setup was placed at 28±2°C till the roots reach the size of nearly 2 cm in length. Water in the test tubes was changed every 24 hours.

**Treatment of roots**

The germinating bulblets, having 2-3 cm long roots, were placed over the test tubes containing different concentrations of treatment solutions, i.e. water extracts of soil. For treatment bulbs were placed in such a way that only roots were immersed in the test solutions. The bulbs placed over maleic hydrazide solution (0.1mg/ml) and over tap water alone served as positive and negative controls, respectively. After treatments the bulbs were removed from the treatment setups and rinsed with water. The root tips were excised and fixed in Carnoy’s fixative (6 parts ethanol, 3 parts chloroform and one part glacial acetic acid). After 24 hours of fixation the roots were transferred to rectified spirit and stored there till use.

**Staining and scoring of slides**

Mitotic preparations were made by hydrolyzing the root tips in a mixture of 1N HCl and 2% acetocarmine (1: 9), at 60±2°C for 2 h. After maceration the root tip squashes were prepared in 2% acetocarmine and observed under microscope. Each root tip preparation was scanned taking 8-10 observations of cells at random under the microscope. The observations included total number of dividing cells at various stages, analysis of the mitotic index and scoring of cytological abnormalities like fragments, bridges, micronuclei, multipolarity and vagrants indicating various clastogenic and physiological disturbances.
Fig. II. *In vivo* Allium assay
Calculations:

Mitotic Index = \frac{\text{Total number of dividing cells}}{\text{Total number of cells observed}} \times 100

Percent aberration = \frac{\text{Number of Abnormal cells}}{\text{Total number of cells observed}} \times 100

3.1.1.2. Yeast Assay:

Mutagenicity of the soils was tested using Yeast assay as given by Zimmermann et al., (1984).

Tester Strain:

The slant of yeast strain *Saccharomyces cerevisiae* D7 was obtained from Bhabha Atomic Research Centre, Bombay, India. The yeast strain *Saccharomyces cerevisiae* D7 was characterized as heteroallelic at *trp* 5 locus and requires tryptophan in the growth medium. Induction of gene conversion in the *trp* locus results in conversion of auxotrophic cells to tryptophan prototrophy and is detected by the medium lacking tryptophan.

*S. cerevisiae* is a heteroallelic diploid yeast strains carrying two different inactive alleles of the same gene locus. The presence of these alleles causes a nutritional requirement e.g. these heteroallelic diploids grow only in medium supplemented with a specific nutrient such as tryptophan for their survival. Treatment of such strains with mutagenic agents can cause conversion of these alleles back to the wild type condition that allows growth on a medium lacking the required nutrient. When gene conversions occur, a fully active wild type phenotype is produced from these inactive alleles through intragenic recombination. These wild type colonies grow on a medium lacking the specific nutritional requirement. *S. cerevisiae* D7 strain requires a specific nutrient medium.

Yeast cultures were grown in yeast extract peptone glucose medium (YEPG): 1% Difco yeast extract, 2% Difco peptone and 2% glucose (Zimmermann, 1975).
Cell densities were measured using minimal medium containing 2% glucose and 0.67% Difco yeast nitrogen base without amino acids supplemented with adenine sulphate (10mg/l) Isoleucine (60mg/l) and tryptophan (10mg/l). For detecting tryptophan convertants (Trp\(^+\)) Tryptophan was omitted from the minimal medium (Zimmermann, 1975). All media contain 2 x Difco bacto agar.

**Growth of cultures:**

The strain was routinely subcultured in Yeast extract peptone glucose medium (YEPD) broth with a starting cell concentration of 100 cells/ml of broth and were grown at 28±2°C in an incubator for 48 hours. The culture with low background of tryptophan convertants were selected for the experiments. YEPD slant was stored for three weeks in refrigerator and subcultured again repeatedly.

The culture was inoculated from master plates in nutrient broth (1.3%) and incubated at 28°C for 2-3 days, so as to allow the test strain to reach to the required density of 1X10\(^6\) cells per ml. The specific density was determined by turbidity measurements at 660 nm. While performing the experiment on a sterilized laminar flow, the cultures were maintained at a room temperature of 28±2°C in tissue culture laboratory.

**Mutagenicity testing:**

Fresh characterized culture was centrifuged for 15 min at 2000 rpm. The pellet thus obtained was resuspended in fresh YEPG and incubated for 1 h in a shaker at 28°C and 110 rpm. It was then centrifuged and again resuspended 50 mM phosphate buffer (pH 7).

To test the mutagenic potential of soil, different concentrations of the soil extracts were used. A positive control set of maleic hydrazide (0.1mg/ml) and distilled water served as the negative control.

The yeast cells were then kept for 2 h at 28°C in buffer containing soil solutions prepared from soils samples. The treated yeasts were then centrifuged at 2000 rpm and the pellet formed was quenched in 0.9% NaCl. The centrifuging and quenching was repeated for 3-4 times and the culture was finally resuspended in fresh saline.
Plate incorporation assay was performed to detect Trp\textsuperscript{+} convertants. All plating media contained 1.5 % Difco Bacto-agar. The treated yeasts were diluted in saline and surface plated on minimal media to quantify convertants. Plating cell densities were varied to obtain practical countable number of colonies per plate (Fig. III). Plates were incubated at 28°C for 3 days before counting convertants. Frequencies of convertants and were calculated from three replicate plates per treatment.

3.2. PHYSIO-CHEMICAL ANALYSIS OF SOIL

The physical properties of soil samples from the study area were studied in laboratory at Punjab University Patiala.

3.2.1. pH of soil

The pH of the soil samples was determined following Jackson (1967). The oven dried soil sample weighing 12.5 g was suspended in 25 ml of distilled water and stirred continuously. The pH was measured using calibrated pH meter.

3.2.2. Organic content of soil

The total organic content of the soil samples was estimated by using the Walkley-Black (1934) Rapid Titration method i.e. wet combustion method. The soil sample weighing 2 g was taken in a conical flask and to it 10 ml of 1N K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} solution was added and mixed thoroughly. Then 20 ml of concentrated H\textsubscript{2}SO\textsubscript{4} was added by continuously swirling the flask. The contents were allowed to cool. Then 10 ml of orthophosphoric acid and 100 ml of distilled water was added. Ten drops of diphenylamine indicator was added which gives violet colour to the suspension. Titration was done with N/2 ferrous ammonium sulphate (FAS) solution. The end point in this titration changes the colour from violet to bright green. The volume of ferrous ammonium sulphate solution used was noted and thus calculated.
Fig. III. *In vitro* Yeast assay
Calculations:

\[ \text{% organic carbon in the soil (A)} = \frac{x-y}{2} \times 0.003 \times \frac{100}{S} = A \]

\[ \text{% organic carbon in soil (B)} = A \times \frac{100}{76} = A \times 1.31 \]

\[ \text{% organic matter} = B \times 1.724 \]

Where, 
\[ x = \text{Vol. of N/2 FAS solution used for the blank titration} \]
\[ y = \text{Vol. of N/2 FAS solution used for titration the excess } K_2Cr_2O_7 \]
\[ \frac{x-y}{2} \text{ ml} = \text{Vol. of 1N } K_2Cr_2O_7 \text{ used for the oxidation of carbon.} \]
\[ S = \text{Weight of soil taken} \]

3.2.3. Estimation of Cu, Fe, Zn and Mn

For analysis of Cu, Fe, Zn and Mn processing were carried out according to the procedure given by Lindsay and Norvell (1978). The soil samples were air dried. The extractant consists of DTPA solution i.e. 0.005M DTPA (Diethylene triamine penta acetic acid), 0.1M triethyleneamine and 0.01M CaCl\(_2\) with pH 7.3. The soil test consists of shaking 10g of air-dried soil with 20 ml of extractant for 2 hours. The leachate of all the four metals was filtered and thus was measured in the filtrate by atomic absorption spectroscopy.

The standards of the heavy metals were prepared by dissolving respective salts to get 1000 mg metal/L of the solution. For Zn, 4.398g of ZnSO\(_4\).7H\(_2\)O was added to one litre double distilled water. Similarly, for Cu, 3.929g of CuSO\(_4\).5H\(_2\)O, for Fe, 4.977g of FeSO\(_4\).7H\(_2\)O and for Mn, 3.598g of MnSO\(_4\).H\(_2\)O were used.

The absorption of the various heavy metals was done using Atomic Absorption Spectrophotometer (AAS) at 213.9 nm for Zn, 324.8 nm for Cu, 248.3 nm for Fe and 279.5 nm for Mn.

3.2.4. Estimation of Arsenic

For As analysis, the digestion of samples was done by using triacid mixture. To 15 ml of triacid mixture (HNO\(_3\): H\(_2\)SO\(_4\): HCLO\(_4\)::10:1:4) 500mg of dried soil sample
was added and placed overnight. The flasks were then kept over a hot plate with occasional shaking. When dense fumes started appearing, the flasks was removed from the hot plate and allowed to cool and then heated again. During heating the flasks were covered with specially designed coolers containing water. After an hour, the flasks were allowed to cool. The flasks were again heated at 200°C -250°C for about an hour and then allowed to cool again. The coolers were removed to expel the acid fumes during heating. This process was repeated till the solution became nearly colourless and the final volume of the solution left in the flask was about 2-3 ml. Then the residue was made to volume of 50ml with addition of distilled water.

The filtrate left after the digestion was tested for As estimations using GBC Avanta hydride generation system at analysis laboratory at Punjab Pollution Control Board (PPCB), Patiala.

Estimation of arsenic was done at 193.7nm using GBC Avanta hydride generation system with lamp current of 8.0 mA. Arsenic standard was prepared by dissolving 1.3203g of As₂O₃ in the minimum volume of 20% NaOH solution and neutralized with nitric acid. The solution was diluted to 1 litre with distilled water to give 1000µg/ml As. Arsenic estimations were made at analytical lab, PPCB, Patiala.

3.3. MORPHOGENETIC STUDIES

Weed species growing in the fields (Tubewell irrigated, canal irrigated and non-irrigated) were screened for high arsenic uptake. Seven species of weeds, selected on the basis of in situ monitoring of As uptake, that were subjected to further study were:

1. *Achyranthes aspera* vern. Puthkanda
2. *Ageratum conyzoides* vern. Bhakoo
3. *Amaranthus viridis* vern. Chulai
4. *Digera muricata* vern. Tandla
5. *Parthenium hysterophorus* vern. Congress grass
6. *Trianthema portulacastrum* vern. Itsit
7. *Tribulus terrestris* vern. Bhakra
These plants were subjected to morphogenetic analysis covering parameters like plant height, leaf size, chlorophyll content, chromosome number, RAPD analysis.

3.3.1. **Plant Height**

Plant height was measured as length from the surface of soil to the tip of the highest leaf or flowering axis in centimetres. Minimum of ten apparently healthy plants were sampled for each species at random and mean value was calculated.

3.3.2. **Leaf Size**

Observations were made on the length and breadth of the leaf. The length was measured as the distance from base of petiole to the tip of the leaf and the breadth was taken as a measurement of the broadest part of the lamina. For this purpose 10-12 mature healthy leaves per plant from five plants selected at random were measured and mean values were taken.

Cytological studies on the wild plants growing in Jajjal village and Patiala were conducted to determine their chromosome number and pollen fertility.

3.3.3. **Chromosome number**

To determine the chromosome number appropriate sized floral buds were fixed in Carnoy’s fixative (1:3:6::glacial acetic acid: chloroform: absolute alcohol). After 24 hours the materials were transferred to rectified spirit and stored in refrigerator until use.

Meiotic studies were carried out using standard acetocarmine squash technique for which anthers were squashed in 1% acetocarmine. The chromosome count was confirmed from large number of PMCs at different stages of meiosis from temporary preparations.

3.3.4. **Pollen Fertility**

Pollen fertility was studied to estimate the effect of Arsenic in soil on reproductive potential of the chosen species, if any. Pollen fertility was studied by crushing mature anthers in 1:1 glyceroacetocarmine on slide and gently heating for 5
minutes (Marks, 1954). The preparations were examined after 1 hour under the microscope. Well inflated and uniformly stained grains were scored as apparently fertile. Pollen fertility was calculated as follows:

\[
\text{Pollen Fertility} = \frac{\text{Number of fertile pollen grains}}{\text{Total number of pollen grains observed}} \times 100
\]

3.3.5. Estimation of Arsenic accumulation in Plants:

The weeds growing at Jajjal fields were handpicked randomly, packed into plastic bags and brought to the laboratory at Punjabi University, Patiala for analysis. The plants were washed with water to remove debris and then with distilled water. Plant samples were then separated into roots, shoots and leaves. The samples were air dried for 24 hours and then placed in an oven for drying at 60°C±2°C. The dried samples were crushed, packed and labelled separately till further need.

The samples were processed and estimated for total Arsenic content (Ref. 3.2.4).

3.3.6. Molecular Studies
3.3.6.1. Isolation and Purification of Genomic DNA

Genomic DNA of the selected plants was isolated using the CTAB (Cetyl trimethyl ammonium bromide) method as described by Saghai-Maroof et al. (1984). Young leaves from each accession were harvested. Surface of the leaves was cleaned with distilled water and then with absolute alcohol and placed in the polythene bags and stored in freezer. The leaves were ground into fine powder in liquid nitrogen by constant crushing using sterilized pestle and mortar. Five grams of fine tissue powder was transferred into 50 ml oakridge tube containing about 15 ml of pre-heated extraction buffer at 65°C. The homogenate in the oakridge tubes were then incubated in water bath for 30-40 minutes at 65°C.

After incubation 15ml of choloroform: isoamylalcohol (24:1) mixture was added and mixed well for 15-20 minutes by placing the oakridge tubes on a shaker. The tubes were then centrifuged for 20 minutes at 10,000 rpm. The supernatant was transferred to 50ml falcon tube. Ice-cold isopropanol was added to these tubes (a two third volume) and were gently inverted. The precipitated DNA was hooked out by using a sterile bent
Pasteur pipette and transferred into 1.5ml micro-centrifuge tube. DNA was then washed twice with 70% ethanol and air-dried by simply tilting the tubes for 1-1.5 hours. DNA was then dissolved in appropriate quantities of 1X TE buffer. Five µl RNase (10mg/ml) was added to each tube and incubated at 37°C in water bath for 1 hour.

3.3.6.2. Estimation of Quantity and Quality of DNA

Quantity and Quality of DNA samples were determined by gel electrophoresis. The extracted DNA was loaded onto the gel and was run for about 2-3 hours at a constant voltage of 5V/cm in an electrophoresis tank. The gel was then visualized under gel-documentation system (BIO-RAD). The DNA samples were photographed under the UV light. The intensity of fluorescence of each sample was compared with that of the standard marker and then the DNA concentration of each sample was ascertained. The quality of the DNA samples was judged based on whether DNA formed a single high molecular weight band (good quality) or a smear (poor quality).

3.3.6.3. RAPD analysis

Genomic DNA was amplified through Polymerase Chain Reaction (PCR) using decamer oligonucleotide RAPD primers (Saiki et al., 1988). Twenty RAPD primers (OPS-01 to OPS-20) obtained from integrated DNA Technologies, USA. These were first tested on five random samples and only the amplifying primers were used further for RAPD analysis on all genotypes. The primers used for the RAPD analysis study are listed below:
Primers used for RAPD analysis procured from integrated DNA Technologies, USA:

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPS-01</td>
<td>5’-CTACTGCGCT-3’</td>
</tr>
<tr>
<td>OPS-02</td>
<td>5’-CCTCTGACTG-3’</td>
</tr>
<tr>
<td>OPS-03</td>
<td>5’-CAGAGGTCCC-3’</td>
</tr>
<tr>
<td>OPS-04</td>
<td>5’-CACCCCCCTTG-3’</td>
</tr>
<tr>
<td>OPS-05</td>
<td>5’-TTTGGGGGCCT-3’</td>
</tr>
<tr>
<td>OPS-06</td>
<td>5’-GATACCTCGG-3’</td>
</tr>
<tr>
<td>OPS-07</td>
<td>5’-TCCGATGCTG-3’</td>
</tr>
<tr>
<td>OPS-08</td>
<td>5’-TTACGGGTGG-3’</td>
</tr>
<tr>
<td>OPS-08</td>
<td>5’-TCCTGGTCCC-3’</td>
</tr>
<tr>
<td>OPS-09</td>
<td>5’-ACGTTCCAG-3’</td>
</tr>
<tr>
<td>OPS-10</td>
<td>5’-AGTCGGGTCG-3’</td>
</tr>
<tr>
<td>OPS-11</td>
<td>5’-CTGGGTGAGT-3’</td>
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<tr>
<td>OPS-12</td>
<td>5’-GTCGGTCTCTG-3’</td>
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<td>OPS-13</td>
<td>5’-AAAGGGGTCC-3’</td>
</tr>
<tr>
<td>OPS-14</td>
<td>5’-CAGTTTACGG-3’</td>
</tr>
<tr>
<td>OPS-15</td>
<td>5’-AGGGGGTTGC-3’</td>
</tr>
<tr>
<td>OPS-16</td>
<td>5’-TGGGGACCAC-3’</td>
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<td>OPS-17</td>
<td>5’-CTGGCGA ACT-3’</td>
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<td>OPS-18</td>
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<tr>
<td>OPS-19</td>
<td>5’-CTCTGGACCGA-3’</td>
</tr>
<tr>
<td>OPS-20</td>
<td>5’-CTACTGCGCT-3’</td>
</tr>
</tbody>
</table>
PCR amplification and fractionation of RAPD amplification products

*In vitro* amplification using PCR (Saiki *et al.*, 1988) was performed in 96 well microtiter plate in an thermal cycler. The amplification was performed using 20µl volume of the PCR reaction mixture.

**PCR reaction mixture (20µl):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock concentration</th>
<th>Volume (µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>7.8</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>PCR buffer</td>
<td>10X</td>
<td>2.0</td>
<td>1X</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>50mM</td>
<td>1.0</td>
<td>2.5mM</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1mM</td>
<td>3.0</td>
<td>0.15mM</td>
</tr>
<tr>
<td>Primer</td>
<td>10µM</td>
<td>3.0</td>
<td>1.5µM</td>
</tr>
<tr>
<td>Taq Polymersase</td>
<td>5 units/µl</td>
<td>0.2</td>
<td>1 units/µl</td>
</tr>
<tr>
<td>DNA template</td>
<td>5ng/µl</td>
<td>3.0</td>
<td>15ng/µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>20</strong></td>
<td></td>
</tr>
</tbody>
</table>

The reaction mix was overlaid with a drop of low molecular weight mineral oil and placed in 96 well thermocycler. Amplification was performed using temperature profile as follows:

**20 µl PCR Reaction**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation at 94°C</td>
<td>5 minute</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation at 94°C</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>Annealing at 37°C</td>
<td>1 minute</td>
<td>45</td>
</tr>
<tr>
<td>Elongation at 72°C</td>
<td>2 minute</td>
<td></td>
</tr>
<tr>
<td>Extension at 72°C</td>
<td>10 minute</td>
<td>1</td>
</tr>
</tbody>
</table>
After amplification 3µl of 6X loading buffer was added to each of the amplified product and mixed thoroughly. From the mixture 10µl of each sample was loaded in 1.5 agarose gel prepared in 0.5 X TBE buffer. The PCR products were resolved by running gel at 5 V/cm for 3 hours. The gels were visualized under UV light using Gel documentation system and photographed.

3.3.6.4. Scoring of RAPD Profile

The RAPD allele size was determined depending on the position of bands relative to the ladder. Total numbers of alleles were recorded for each RAPD marker in all the four accessions of each species by giving allele number as 1, 2, 3, 4 and so on. The amplified bands were recorded as 1 (band present) or 0 (band absent) in a binary matrix.

3.3.6.5. Estimation of genetic similarity and cluster analysis

The RAPD marker amplification profiles of seven wild species were used to estimate the similarity based on number of shared amplified bands. Software package NTSYS PC 2.02e (Rohlf, 1998) was used for estimation of genetic similarities among the lines using SIMQUAL mode of NTSYS. The similarity matrix value based on Jaccard (1990) coefficient of similarity was used to generate dendrogram. Clustering was done by UPGMA using SHAN module of NTSYS PC version 2.02e.

3.4. PHYTOREMEDIATION POTENTIALS

Phytoremediation potentials of selected denizens were evaluated in vivo by conducting pot and hydroponic experiments. The seeds of all the seven species were collected from inhabitants growing at Jajjal and used for further experimentation. For comparison sake the seeds of these species from local population (Patiala) were used.

3.4.1. Pot Experiments:
The seeds of the selected weed species were collected from the study area (Jajjal) as well as the control area (Patiala) and were grown in separate nursery beds at the Plant Conservatory, Punjabi University, Patiala for 15 days.

Earthen pots of same quality and size (9" diameter) with sealed drainage hole were used for various pot experiments conducted at Plant conservatory section of Botanic gardens, Punjabi University, Patiala. Soil from the conservatory section, which has no record of irrigation and use of agrochemicals, was used for filling the pots. Thus, the potential contamination of soil by metal compounds was anticipated to be low.

Soil used for filling the pots was sun dried and sieved for removing pebbles and other inert material. Each pot was filled with 3 kg of this soil. Arsenic was added in the form of solution of sodium arsenate (Na2HAsO4.7H2O) prepared in 500 ml of water. For preparing 25ppm of arsenic in soil, 18mg of sodium arsenate was added as solution form in 3 kg of soil per pot. Similarly, 36 mg and 72 mg of sodium arsenate were mixed in 3 kg of soil for making 50 ppm and 100ppm of arsenic in soil. Next day each pot was emptied over a polythene sheet. Soil was thoroughly mixed manually and refilled into the same pots. Pots filled with unamended soil served as the control.

The effect of soil borne Arsenic was evaluated on the various morphological and cytological parameters of the selected plant species along with their metal accumulation potential. The observations were made on the morphology of the plant, growth rate, pollen fertility, cytotoxicity (Micronucleus assay, Tetrad analysis) and arsenic accumulation (described earlier).

3.4.1.1. Growth Rate

Effect of As treatment on growth rate of the selected plants was studied from the pot experiments. Five plants grown over each arsenic treatment were carefully uprooted after every 15 days of growth from the time of transplantation. The roots and shoots of plants from each treatment were thoroughly cleaned, excised, measured in length and weighed separately. The plant parts were then dried in hot air oven at 60±2°C for 72 hours and weighed again. Growth rate was calculated as the percent increase in the length and dry matter during this period. The calculations were as follows:


\[ \text{Increase in length (\%) } = \frac{\text{Plant height on (n+15)th day} - \text{Plant height on n\textsuperscript{th} day}}{\text{Plant height on n\textsuperscript{th} day}} \times 100 \]

\[ \text{Increase in dry weight (\%) } = \frac{\text{Dry weight on (n+15)th day} - \text{Dry weight on n\textsuperscript{th} day}}{\text{Dry weight on n\textsuperscript{th} day}} \times 100 \]

3.4.1.2. Micronucleus assay

To study the cytotoxic effects of As uptake in the species *in situ* micronucleus assay was conducted on root tip cells and pollen mother cells following Dash *et al.* (1988).

i) Fixation of Roots:

After Fifteen days of treatment (Arsenic as well as control) seedlings of each species were uprooted from the experimental pots as well as hydroponic treatments taking care so as to avoid any damage to the roots of the seedlings. Roots of seedlings were washed thoroughly in running water. The roots were then surface dried by dabbing with blotting papers, excised and fixed in Carnoy’s fixative (6 Absolute alcohol :3 Choloroform :1 Acetic acid). After 24 h the roots were transferred into rectified spirit till use.

ii) Staining and scoring of slides:

Mitotic preparations from root tips were made following Sharma and Sharma (1972). The fixed root tips were hydrolysed in 1N HCl for 5-7 minutes at 60±2°C. The hydrolysed roots were rinsed in water and then placed in 2% acetocarmine for 1 h at 60±2°C in an oven. Finally the stained root tips of 1-2 mm in size were squashed in 2% acetocarmine.

Evaluation of the Root tip cells (RTCs) for the incidence of MNCs was done by taking 10-12 observations on cells of each meristem at random, under microscope. Observations were made by counting total number of cells and cells with micronuclei in each case. Observations were recorded as MNCs per thousand cells and calculated as:

\[ \text{Increase in dry weight (\%) } = \frac{\text{Dry weight on (n+15)th day} - \text{Dry weight on n\textsuperscript{th} day}}{\text{Dry weight on n\textsuperscript{th} day}} \times 100 \]

3.4.1.3. Tetrad Analysis
Young flower buds of different plants raised on metal amended soils were fixed in Carnoy’s fixative for 24 h and then stored in rectified spirit till use.

Young anthers were crushed in a drop of 2% acetocarmine. Observations were made from temporary mounts. PMCs at tetrad stage were observed. From each slide PMCs from 10-12 focuses were counted. PMCs with various abnormalities like monads, diads, triads, polyads and micronuclei were scored separately. PMCs from five plants per treatment were scored and mean values were taken for further discussion. The scoring was done as follows:

\[
MNCs/1000 \text{ PMCs} = \frac{\text{Total number of micronuclei observed}}{\text{Total PMC observed}} \times 100
\]

\[
\text{Abnormal PMCs} = \frac{\text{Total number of PMCs with abnormalities}}{\text{Total number of PMCs observed}} \times 100
\]

3.4.2. **Hydroponic Experiments**

The Hydroponics experiments were set up to access the arsenic tolerance of plants. Forty day old plants of various species raised in beds from seeds collected from Jajjal and Patiala were transferred to Hydroponic bottles containing treatment solutions. For preparing 25 ppm concentration of As 18 mg of sodium arsenate was dissolved in 500 ml, 50 ppm concentration was prepared by dissolving 32 mg and 100 ppm concentration was prepared by dissolving 72 mg of the salt in 500 ml of distilled water. The treatment solution in each bottle was replaced every 48h and aerated periodically. Tap water was used as control. Each treatment was triplicated and the plants were harvested after four weeks and processed further.

Observations were recorded on parameters like micronuclei in the root tip cells (Ref. 3.4.1.2) and the metal accumulated in plant parts (Ref. 3.3.5).

3.4.3. **Estimations for Phytoremediation Potentials parameters**

Phytoremediation potentials of a species were calculated in terms of its Transfer Factor (TF), Tolerance Index (TI), Bioconcentration Factor (BF) and Phytoextraction Capacity (PC) which were calculated as follows:
a) Transfer Factor (TF) = \frac{\text{Concentrations of Arsenic in shoot}}{\text{Concentrations of Arsenic in root}}

b) Tolerance Index (TI) = \frac{\text{Mean root or shoot size in medium with metal}}{\text{Mean root or shoot size in the control}} \times 100

c) Bioconcentration Factor (BF) = \frac{\text{Concentrations of metal in plant}}{\text{Concentrations of metal in soil}}

d) Phytoextraction capacity (PC) = \text{Plant biomass (DW)} \times \text{Concentration of metal}

3.5. STATISTICAL ANALYSIS

3.5.1. Standard Deviation

Standard Deviation (SD) is a measure of how widely values are dispersed from mean. This was calculated using the formula:

$$SD = \sqrt{\frac{\sum (x - \bar{x})^2}{n}}$$

Where

- $x$ is the value of whole variable or observations
- $\bar{x}$ is the arithematic mean (av)
- $n$ is the total no. of observations
- SD is standard deviation
- $\sum$ is summation

3.5.2. Correlation Coefficient

Correlation coefficient provides the relationship between two or more set of quantitative variables. It was calculated using the following formula:
\[ r = \frac{n \sum xy - (\sum x)(\sum y)}{\sqrt{n \sum x^2 - (\sum x)^2} \sqrt{n \sum y^2 - (\sum y)^2}} \]

Where
- \( r \) is correlation coefficient
- \( n \) is degree of freedom
- \( x \) is one variable
- \( y \) is a second variable

### 3.5.3. Regression Analysis

This is used to analyze how a single dependent variable is affected by the values of one or more independent variables. The formula used was as under:

\[ b = \frac{n \sum xy - \sum x \sum y}{n \sum x^2 - (\sum x)^2} \]

\[ a = \frac{\sum y \sum x^2 - \sum x \sum xy}{n \sum x^2 - (\sum x)^2} \]

Equation for line of linear regression is

\[ y = a + bx \]

Where
- \( x \) is independent variable
- \( y \) is dependent on \( x \)
- \( b \) is slope of linear line
- \( a \) is intercept
- \( n \) is number of observations

### 3.5.4. Student’s t - Test

It was applied to test the mean difference of related samples.

\[ \text{Student’s t} = \frac{\overline{X_d}}{\text{SE}(\overline{X_d})} \]  
(at n-1 df)
where as  $\bar{X} = \text{mean difference between two sets of related samples.}$

$$SE = \frac{SD}{\sqrt{n}}$$

The significance of the t values was then observed from the data table. If $t_{\text{cal}} < t_{0.05}$, the data is considered to be consistent with the hypothesis of an uncorrected population.

### 3.6. STOCK SOLUTIONS AND BUFFERS

#### 3.6.1. STOCK SOLUTIONS

**Stock solution A:**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g / 500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris</td>
<td>60.55 g of Tris base</td>
</tr>
</tbody>
</table>

Dissolve 60.55 g of Tris base in water and make the final volume to 500 ml.

**Stock solution B:**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g / 500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5M EDTA</td>
<td>93.05 g EDTA + 10 g NaOH pellet</td>
</tr>
</tbody>
</table>

Dissolve 93.05 g of EDTA in 400 ml of water. Add 10 g NaOH pellet. Adjust pH to 8.0. Make the final volume to 500 ml.

**Stock solution C:**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g / 500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 M NaCl</td>
<td>146.1 g of NaCl</td>
</tr>
</tbody>
</table>

Dissolve 146.1 g NaCl in 400 ml of distilled water and volume adjusted to 500 ml.

**Stock solution D:**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g / 500ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% Sodium dodecyl sulphate</td>
<td>50.0 g</td>
</tr>
</tbody>
</table>

Dissolve 50.0 g SDS in 150 ml water and heat to 68°C. Adjust pH to 7.2 with conc. HCl and volume was made to 500 ml.
Stock solution E:
Ingredients g/100ml
Ethidium bromide 1g
Add one gram of ethidium bromide to 100 ml of water. Stir on magnetic stirrer for several hours to ensure that dye has dissolved. Wrap container in aluminium foil or transfer solution to dark bottle. Store at room temperature.

Stock solution F:
Ingredients g/100ml
Bromophenol blue 0.25g
Glycerol 40g
Add 0.25g of bromophenol blue and 40g of glycerol in 100 ml of distilled water.

Stock solution G:
Ingredients g/100 ml
Agarose powder 1.5g
10 X TBE 5 ml
Dissolve 1.5g of agarose powder in diluted TBE to make the final volume to 100 ml.

3.6.2. BUFFERS
Preparation of 1X Tris EDTA buffer
Ingredients Per 1000 ml
Stock solution A 10 ml
Stock solution B 2 ml
Dissolved in double distilled water and made the volume to 1000 ml. pH was adjusted to 8.0.

Preparation of 1X Tris Acetate EDTA (TAE) buffer
Ingredients Per 1000 ml
Stock solution A 200 ml
Stock solution B 100 ml
Glacial acetic acid 57.1 ml
Dissolved the above in distilled water and made the volume to 1000 ml. pH was adjusted to 8.0.

**Preparation of 1X Tris Base Acetate EDTA (TBE) buffer**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Per 1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solution A</td>
<td>892 ml</td>
</tr>
<tr>
<td>Stock solution B</td>
<td>40 ml</td>
</tr>
<tr>
<td>Boric acid</td>
<td>55 g</td>
</tr>
</tbody>
</table>

Dissolve and make the final volume to 1000 ml.

**Preparation DNA Extraction buffer**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Per 10 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solution A</td>
<td>500μl</td>
</tr>
<tr>
<td>Stock solution B</td>
<td>500μl</td>
</tr>
<tr>
<td>Stock solution C</td>
<td>600μl</td>
</tr>
<tr>
<td>Stock solution D</td>
<td>1000μl</td>
</tr>
</tbody>
</table>

Make volume to 10 ml by adding autoclaved distilled water.

**Preparation of Cetyl Trimethyl Ammonium Bromide (CTAB) buffer**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solution A</td>
<td>20.0 ml</td>
</tr>
<tr>
<td>Stock solution B</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>Stock solution C</td>
<td>28.0 ml</td>
</tr>
<tr>
<td>10% CTAB</td>
<td>20.0 ml</td>
</tr>
<tr>
<td>Sod.bisulphate</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>1.0 g</td>
</tr>
</tbody>
</table>

Dissolve and make the volume to 100 ml.

**Preparation of Agarose gel**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solution E</td>
<td>5μl</td>
</tr>
<tr>
<td>Stock solution G</td>
<td>100ml</td>
</tr>
</tbody>
</table>
Heat 100 ml of solution G and add 5 µl of ethidium bromide on cooling of the solution. Pour the prepared gel in gel mould.

**Preparation of solution for loading of tank**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solution A</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

Dissolve in distilled water and make the final volume to 100 ml.

**Preparation of 1X double dye for 20 µl PCR Reaction**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solution F</td>
<td>3.5µl</td>
</tr>
</tbody>
</table>

Make the final volume to 100 ml.