3. MATERIALS AND METHODS

3.1 Soil collection

Black soil from agricultural fields of Jangalapalle village of Anantapur district, Andhra Pradesh, was collected. The soil was air-dried and after breaking the clods sieved through a 2-mm mesh and stored in polyethylene bags.

The physico-chemical properties of the soil presented in Table 1 were determined by the following methods:

Soil pH was measured by 1:1.25 soil to water ratio using Elico digital pH meter with calomel-glass electrode assembly.

Organic carbon in the soil was determined by Walkley-Black method and organic matter was calculated by multiplying the organic carbon values with 1.72 (Jackson, 1971).

The soil was analysed for sand, silt and clay employing Bouyoucos hydrometer method (Black, 1971).

The cation exchange capacity (CEC) of soil was
Table 1. Characteristics of the soil used in the experiments.

<table>
<thead>
<tr>
<th>Location</th>
<th>Texture</th>
<th>pH (%)</th>
<th>Sand (%)</th>
<th>Silt (%)</th>
<th>Clay (%)</th>
<th>Organic matter (%)</th>
<th>Total Nitrogen (%)</th>
<th>CEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jangalapalle</td>
<td>Black</td>
<td>7.62</td>
<td>67.4</td>
<td>20.1</td>
<td>12.5</td>
<td>1.12</td>
<td>0.17</td>
<td>14.2</td>
</tr>
</tbody>
</table>

1. Measured by taking 1:1.25 soil water slurry
2. Bouyoucos hydrometer method
3. Estimated by Walkley-Black method (Jackson, 1971)
4. Estimated by microkjeldahl method (Jackson, 1971)
5. Estimated by normal ammonium acetate pH 7.0
determined by ammonium acetate pH 7.0 method (Jackson, 1971).

The total nitrogen content of the soil was estimated by microkjeldahl method (Jackson, 1971).

3.2 Pot experiments

Soil samples (5 kg) were placed in earthen pots and foxtail millet plants were grown in the pots. A variety of foxtail millet viz., Lepakshi-AK - 132-1 collected from the Regional Agricultural Research Station at Rekulakunta (Andhra Pradesh Agricultural University) near Anantapur, was sown in January, 1991.

Plants were grown under glass house conditions (31 °C ± 9 °C day / 26 °C ± 2 °C night) up to 13 weeks with different pots (5 replicates for each sampling) sampled at 15-day intervals up to 90-days. Plant roots were dugout of each pot and shaken gently to remove most of the adhering soil and washed gently in tap water. Roots were then cut at the soil line and weighed.

3.3 Soil sampling and dilution

3.3.1 Rhizosphere soil

Roots plus soil still adhering after gentle shaking
and washing is considered rhizosphere sample and the same was used as the starting material for isolation of bacteria (Subba Rao, 1986). One gram portions of the rhizosphere soil (roots plus adhering soil) ground in a mortar with 10 ml of sterilized distilled water was -1 considered to be the 10 dilution. Further 10-fold serial dilutions (up to 10 ) of the rhizosphere sample were prepared aseptically.

3.3.2 Non-rhizosphere soil

Unplanted pots containing the same black soil (5 kg) and treated in the same way as the planted pots served as the non-rhizosphere soil (control).

3.4 Enumeration

-3 -6 Dilutions (10 to 10 ) of the rhizosphere and non-rhizosphere soil samples of foxtail millet, were macerated and plated on UAT 4C medium (Omar et al., 1989) having the following composition (g/l):

I SOLUTION A

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boric acid</td>
<td>0.750</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>0.550</td>
</tr>
<tr>
<td>Cobaltous sulphate</td>
<td>0.350</td>
</tr>
<tr>
<td>Cupric sulphate</td>
<td>0.025</td>
</tr>
</tbody>
</table>
**Manganous chloride**  - 0.020

**Distilled water**  - 1000 ml

---

### II  SOLUTION B

- **Ferrous sulphate**  - 0.800
- **Magnesium sulphate**  - 4.000
- **Sodium molybdate**  - 0.118
- **Calcium chloride**  - 4.000
- **Ethylene Diamine Tetra Acetic acid**  - 0.800

**Solution A**  - 4 ml

**Distilled water**  - 1000 ml

---

### III

- **Potassium dihydrogen orthophosphate**  - 1.800
- **Potassium phosphate Dibasic**  - 2.700
- **Solution B**  - 50 ml
- **Starch**  - 5.000
- **Glucose**  - 5.000
- **Mannitol**  - 5.000
- **Malic acid**  - 3.500
- **Yeast extract**  - 0.100
- **Agar agar**  - 18.000
- **Distilled water**  - 1000 ml
Final pH of the medium was adjusted to 6.8 using potassium hydroxide solution. Then the medium was sterilized at 110°C for 20 min. The sterilized medium was distributed into previously sterilized petri dishes which were inoculated with 0.1 ml of serial dilutions (10⁻³ to 10⁻⁶). The inoculum was spread uniformly and the plates were incubated at 37°C for 3 days. Five replicates were maintained for each dilution. In the plates containing growth, bacterial colonies alone were recorded and their population was calculated using the Most Probable Number (MPN) method (Heulin et al., 1980, 1982; Thomas-Bauzon et al., 1982; Bally et al., 1983; Heulin et al., 1983; Charyulu et al., 1985; Omar et al., 1989; Berge et al., 1991).

3.5 Isolation and purification of bacterial isolates

Some of the individual colonies (morphologically different and pigmented) from plates of different dilutions of rhizosphere and non-rhizosphere samples were picked up. These colonies were purified following standard procedures (Krieg, 1981). The selected single colonies were streaked repeatedly on the nutrient agar plates until isolated single colonies could be obtained. Further purification of the single colonies was carried out on nutrient agar slants. The single
colonies thus purified were preserved for further studies. All the bacterial isolates were maintained on nutrient agar slants. Before use, they were checked for purity by streaking on nutrient agar plates. After confirmation of their purity, the isolates were used in the subsequent experiments.

3.6 Estimation of nitrogen fixation by pure cultures (isolates) of bacteria

The ability of isolated bacteria (67 isolates) to fix nitrogen was tested by the microkjeldahl method (Jackson, 1971) in mineral salts medium which is free from nitrogen. A loopful of bacterial inoculum (of each isolate separately) was inoculated into an Erlenmeyer flask (100 ml) containing 25 ml of sterilized mineral salts medium which had the following composition (g/l):

- Potassium phosphate Dibasic - 0.100
- Magnesium sulphate - 0.200
- Ferrous sulphate - 0.001
- Glucose - 5.000
- Distilled water - 1000 ml

Uninoculated medium served as control. All the culture flasks (3 replicates for each isolate)
including controls were incubated at 37°C. Triplicate samples were analysed for total nitrogen by microkjeldahl method after 10 days of incubation. The amount of nitrogen fixed by each isolate was expressed as mg N fixed / g of glucose after deducting the amount of nitrogen in control samples.

3.6.1 Estimation of nitrogen fixation by Kjeldahl method

The sample (25 ml of culture) was taken in a 100ml Kjeldahl digestion flask to which a pinch of digestion mixture (containing potassium sulphate, copper sulphate and selenium powder in the ratio of 10 : 1 : 0.1) and 10 ml of concentrated sulphuric acid were added. The contents were mixed thoroughly by swirling the flask carefully and digested on an electric digestion rack slowly at first to avoid frothing. The digestion was continued for at least 3h and until the solution has cleared. The entire sample was transferred to a distillation flask after washing 3-4 times with distilled water. The contents of the distillation flask were made alkaline with adequate amount of 40% sodium hydroxide solution. The ammonia in the sample was completely distilled into 10 ml of 3% boric acid and titrated with 0.01 N hydrochloric acid using bromocresol green and methyl red mixed indicator.
Complete distillation of ammonia from the sample was ensured by frequent checking with red litmus paper. The amount of nitrogen fixed was calculated by using the following formula:

Total Nitrogen = \((T - B) \times N \times 0.14\)

\[ \frac{S}{\text{ }} \]

where

- \(T\) = Sample titration, ml standard acid
- \(B\) = Blank titration, ml standard acid
- \(S\) = Sample weight, g
- \(N\) = Normality of standard acid

3.7 Morphological and biochemical tests of bacterial isolates

Morphological and biochemical tests of the diazotrophic bacterial isolates (22 in number) were carried out as per the published procedures (Collins and Lyne, 1984) and the results were tabulated.

3.8 Selection of seeds of foxtail millet

Seeds of the recommended variety of foxtail millet Lepakshi - AK - 132 -1 were grouped on the basis of weight into first group (upto 1 mg), second group (1 to 2 mg), third group (2 to 3 mg) and fourth
group (3 to 4 mg) per 1000 seeds. The most abundant group (2 to 3 mg) among them was selected to conduct experiments using spermosphere model.

3.9 Spermosphere model

Seeds of foxtail millet in the range of 2-3 mg were surface sterilized by soaking successively in saturated calcium hypochlorite, (2h with constant shaking followed by rinsing 3 to 4 times in sterilized distilled water) and in hydrogen peroxide (9 volumes for 20 min). The seeds were once again rinsed 5 to 6 times with sterilized distilled water. They were introduced aseptically on to the surface of 7 ml of semi-solid (0.3% agar) nitrogen free WAT medium (Omar et al., 1989), in a Pankhurst tube (Fig. 1). The tubes were inoculated and incubated at 37°C for one week in dark. Triplicate samples were maintained for each sampling. Uninoculated tubes served as control.

After one week, when the coleoptiles were 1 cm high, the Pankhurst tube was inoculated with a known quantity of bacterial suspension of isolate (0.5 ml of bacterial isolate containing 10^8 cells/ml). Three selected isolates (75 R5, 15 R4, 30 R5) of diazotrophic bacteria were used. All the Pankhurst tubes were
Figure 1. The spermsphere model. It consists of a Pankhurst tube containing 7 ml of WAT medium (without carbon and nitrogen). The side tube contains sodium hydroxide (1N) to trap CO₂.
incubated in dark throughout the experiment. The delay in inoculation allowed for the identification of contamination by insufficiently sterilized seeds. The amount of nitrogen fixed by three isolates was estimated as described earlier (Section 3.6) and was expressed as mg N/7ml of WAT medium after deducting the amount of nitrogen in control samples.

3.10 Statistical analysis

In all the cases, analyses of significant differences of \( P \leq 0.05 \) between values of each sampling and treatment were performed using Duncan's New Multiple Range (DMR) Test (Duncan, 1955).