CHAPTER-II
MATERIALS AND METHODS
Twenty strains of *Rhizobium meliloti* (RM) were taken for the present study. Among the twenty strains used, thirteen strains (RM-1 to RM-17) were isolated and identified as described by Vincent (1). The remaining three strains namely., RM-4014, RM-4015 and RM-4020, were kindly supplied by Dr. N.S. Subba Rao, Indian Agricultural Research Institute, New Delhi, India.

The stock cultures of *R. meliloti* were maintained by weekly subculturing on mannitol yeast extract agar (YMA) slants, having the following composition (in gm %): K$_2$HPO$_4$, 0.5; MgSO$_4$. 7H$_2$O, 0.2; NaCl, 0.1; Mannitol, 10.0; yeast extract (Difco), 1.0; Davis agar, 1.5. The pH of the medium was adjusted to 7.2. The culture slants were stored after sealing with wax, for 3 - 6 months at 0-4°C.

For the work on ammonia assimilatory enzymes, the medium described by Kurz and LaRue (2) was used having the following ingredients in mgs/1000 ml medium; KNO$_3$, 1000; MgSO$_4$. 7H$_2$O, 250; Na$_2$HPO$_4$. H$_2$O, 150; CaCl$_2$. 2H$_2$O, 150; Fe (330), 28; MnSO$_4$, 10; H$_3$BO$_3$, 3; ZnSO$_4$. 7H$_2$O, 2; Na$_2$ MoO$_4$. 2H$_2$O, 0.25; CuSO$_4$, 0.025; CoCl$_2$. 6H$_2$O, 0.025; KI, 0.78; meso-inositol, 100; thiamine - HCl, 10; nicotinic acid, 1; pyridoxine, 1 and sucrose, 1000. The pH of the medium was adjusted to 7.2. Unless or, otherwise mentioned, potassium nitrate was replaced by ammonium sulphate, and sucrose by glucose and sodium.
succinate. Potassium nitrate, ammonium sulphate, sucrose, glucose and sodium succinate were separately autoclaved and then added in the medium.

**Testing the efficiency of cultures:**

Healthy seeds of *Trigonella foenum-graecum* (obtained from National Seed Corporation, New Delhi, India; Var. Early bunchy) were surface sterilized by immersing them in concentrated H\textsubscript{2}SO\textsubscript{4} for five minutes and washing successively with sterilized water under aseptic conditions, till the pH of decanted water reached neutral. The seeds were inoculated with individual culture as described by Burton (3). The plants were grown in Leonard jars. Uninoculated seeds were kept as controls. The Leonard jars containing quartz sand, were sterilized in an autoclave for 4 hrs. at 15 lb. pressure. Equal quantity of cooled water after boiling, was provided throughout the experiment to each pot. Fifteen healthy and uniform seeds were selected and inoculated with individual *R. meliloti* strain, in replicates of four. The uninoculated seeds were kept as controls. After four weeks, the plants were carefully taken out and the adhering sand washed off. Table 1, summarises data of various characteristics examined.

The data was subjected to statistical analysis with the help of Burrough - 4700 (U.S.S.R.) computer at
TABLE 1: Mean response of different characters under study by *Rhizobium meliloti* (RM)

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Number of nodules</th>
<th>Number of leaves</th>
<th>Length of shoot (cm)</th>
<th>Length of root (cm)</th>
<th>Number of lateral roots</th>
<th>Ethylene produced per plant (µg)</th>
<th>Dry weight per plant (mg)</th>
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<tr>
<td>Control</td>
<td>-</td>
<td>4.7</td>
<td>1.5</td>
<td>3.7</td>
<td>12.3</td>
<td>-</td>
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<td>RM - 1</td>
<td>16.7</td>
<td>6.0</td>
<td>1.7</td>
<td>6.7</td>
<td>16.7</td>
<td>59.2</td>
<td>12.9</td>
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<td>18.6</td>
<td>6.6</td>
<td>1.6</td>
<td>7.0</td>
<td>12.4</td>
<td>73.2</td>
<td>15.0</td>
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<td>11.2</td>
<td>6.4</td>
<td>1.9</td>
<td>5.7</td>
<td>12.1</td>
<td>74.1</td>
<td>12.3</td>
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<td>9.5</td>
<td>6.1</td>
<td>2.1</td>
<td>6.0</td>
<td>11.2</td>
<td>38.6</td>
<td>13.5</td>
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<td>17.7</td>
<td>5.2</td>
<td>2.1</td>
<td>5.2</td>
<td>16.7</td>
<td>98.7</td>
<td>12.6</td>
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<td>10.7</td>
<td>5.3</td>
<td>2.4</td>
<td>5.0</td>
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<td>7.7</td>
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<td>85.3</td>
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<td>4.6</td>
<td>16.8</td>
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<td>1.5</td>
<td>7.6</td>
<td>17.3</td>
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<td>6.2</td>
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<td>13.3</td>
<td>80.7</td>
<td>16.0</td>
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<td>12.7</td>
<td>72.3</td>
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<td>7.3</td>
<td>15.6</td>
<td>63.9</td>
<td>14.2</td>
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</tbody>
</table>

G.M.  15.9  6.0  1.9  6.1  14.6  66.0  13.2

L.S.D.  7.6  L.S.D.  1.0  S.E. for 10.2  L.S.D.  1.1  S.E. for 2.0  L.S.D.  14.6  L.S.D.  4.0

at 5%  at 5%  at 5%  at 5%  at 5%  at 5%
Indian Agricultural Statistical Research Institute, New Delhi, India. The RM-13 was selected for further studies. Plate 1(A) shows the growth of *Trigonella foenum-graecum* plants, in the presence and absence of seeds inoculated with RM-13. Plate 1(B) shows the nodules formed on *Trigonella foenum-graecum* roots, after inoculation with RM-13 and uninoculated control plant.

Preparation of cell free extracts of *R. meliloti* and *Trigonella foenum-graecum*, for enzyme studies:

(A) Cell free extracts of *R. meliloti* - The cells were harvested, after growing for 24 h at 30°C on a rotary shaker (180 rpm), by centrifugation for 15 min at 10,000 x g at 0 - 4°C. They were washed twice with 0.05M Tris - HCl buffer (pH 7.2), resuspended in a small volume of the same buffer, and were disrupted at 0 - 4°C by twelve 30 - s bursts (separated by 30 - s intervals to prevent overheating) in an ultrasonicator with an operating frequency of 25 Kc/second of 120 - w power output. The buffer was added to the sonicated preparation to obtain 10% (W/V) cell homogenate. The cell debris was removed by centrifugation at 15,000 x g for 20 min. at 0 - 4°C. The resultant supernatant unless or otherwise mentioned, was applied to a precalibrated column of sephadex G-15 (1.5 x 15 cm) and eluted with buffer (Tris - HCl, 0.05M, pH 7.5), before assaying for enzyme activities.

(B): Nodules formed on *Trigonella foenum-graecum* roots, after inoculation with *Rhizobium meliloti* - 13 (RM - 13) and uninoculated control plant.
(B) Cell free extracts of *Trigonella foenum-graecum* - All the isolation steps were performed at 0-4°C. Nodules were ground in a precooled mortar and pestle to 30% (w/v) tissue concentrations. Grinding medium consisted of polyvinyl-polypyrrolidone (1/3 amount of nodules; w/w), 5 µM dithiothreitol and glycerol (25%, V/V) in the extraction buffer. The extraction buffer used throughout the experiment was Tris-HCl (pH 7.5; 0.05M). Extracts were filtered through four layers of cheese cloth and centrifuged at 600 x g for 10 min. to remove the cell debris the supernatant was recentrifuged at 6,000 x g for 10 min. to yield the crude bacteroidal pellet and a supernatant, which was recentrifuged at 32,000 x g for 20 min; the supernatant thus obtained, was used for assaying nodule cytosol enzymes. The bacteroidal pellet was resuspended in extraction buffer and after subjecting to ultrasonic treatment was centrifuged at 32,000 x g for 20 min; the supernatant thus obtained, was used for assaying bacteroidal enzymes. Extraction procedure for the root cytosolic enzymes was essentially the same as described for the nodule cytosol. The crude enzyme extracts thus obtained were applied on a precalibrated column of sephadex G-15 (1.5 x 15 cm) and eluted with extraction buffer, before assaying for enzyme activities. Unless or otherwise mentioned the activities of all the enzymes assayed are expressed in terms of specific activities.
(Units/mg protein) and are the average values of five determinations.

**Isolation of chloroplasts:**

Chloroplasts from the leaves of *Trigonella foenum-graecum* were isolated by following the method of Cockburn et al (4).

**Protein estimation:**

Protein was estimated by the method of Lowry et al (5) using bovine serum albumin as the standard. It was estimated in the purified fractions by following the spectrophotometric method (6).

**Enzyme assays:**

Glutamine synthetase (E.C. 6.3.1.2):

The enzyme was assayed by the method of Elliot (7). The standard assay system contained in a total volume of 3.0 ml, Tris - HCl buffer (pH 7.2), 200 µmoles; sodium glutamate, 50 µmoles; hydroxylamine (used as ammonia donor), 20 µmoles; ATP, 10 µmoles; unless or otherwise mentioned manganese sulphate was used as a metal ion, 10 µmoles and an appropriate concentration of enzyme. The system was incubated at 30°C for 30 min. The reaction was stopped by adding 0.5 ml of FeCl₃ reagent (equal volumes of 10% FeCl₃, 6H₂O in 0.2 N HCl, 24% TCA, and 50% V/V HCl were mixed together) to each tube, and the precipitates were removed by centrifuging at 10,000 x g for 10 min. The γ-glutamyl hydroxamate in the supernatant was colorimetrically estimated at 540 nm.
The unit of enzyme was defined as the amount of enzyme that brings about the formation of 1.0 μmole of the product (γ-glutamyl hydroxamate) per 30 min at 30°C.

**Glutamate synthase (E.C. 2.6.1.53):**

The method described by Roon et al. (8) was used to assay glutamate synthase activity. Assay system contained, 200 μmoles of potassium phosphate buffer, (pH 7.0); 15 μmoles of α-ketoglutarate; 10 μmoles of glutamine; 0.5 μmole of NADH and a suitable concentration of enzyme in a total volume of 2 ml.

The unit for the enzyme activity was defined as the amount of enzyme which brings about a change of 0.01 O.D. at 340 nm per min at 30°C.

**Glutamate dehydrogenase (E.C. 1.4.1.2):**

The enzyme was assayed by following the method of Thomulka and Moat (9). The reaction mixture consisted of 200 μmoles potassium phosphate buffer (pH 7.5); 15 μmoles of α-ketoglutarate; 15 μmoles of ammonium sulphate and 0.5 μmole of NADH and an appropriate concentration of enzyme in a total volume of 2.0 ml.

The unit of enzyme activity was defined as the amount of enzyme which brings about a change of 0.01 O.D. at 340 nm per min at 30°C.
Purification of glutamate synthase and glutamate dehydrogenase:

All operations of purification were carried out at 0 - 4°C. Five week-old *Trigonella foenum-graecum* root nodules were used in the isolation of the enzymes from the nodule cytosol. To the cell free extract prepared, as described earlier, crystalline ammonium sulphate was gradually added to give a final concentration of 40%. The solution was left for 6 hrs. and then centrifuged at 15,000 x g for 10 min. The precipitates were discarded after testing for activities and to the resultant supernatant, more ammonium sulphate was added to give a final concentration of 70%. The mixture was left overnight after dissolving the ammonium sulphate. The mixture was centrifuged at 15,000 x g for 15 min. The supernatant was discarded after checking the activities. The precipitates obtained were dissolved in 0.05M Tris-HCl buffer (pH 7.5) and dialysed against the same buffer for 4 hrs. The enzyme preparation was concentrated by dialysis against solid sucrose. The concentrated enzyme preparation, mentioned above was applied to a sephadex G-100 column (2.5 x 30 cm) previously equilibrated with 0.05M Tris-HCl buffer, pH 7.5 and 5 ml fractions were collected. Both, enzyme activity and protein content were monitored in the fractions.

Nitrogenase:

The nitrogenase was assayed according to the method described by Kurz et al (10). Roots, separated from the upper plant were freed of debris and sealed in 15 ml test tubes.
Acetylene (2%) was added and ethylene production was measured by gas chromatograph and expressed as μLt/hr/plant.

Superoxide dismutase (E.C. 1.15.1.1):

The method described by Beanchamp and Fridovich (11) was used to assay superoxide dismutase activity. The standard assay system contained in a total volume of 3.0 ml, potassium phosphate, 0.05 M; riboflavin, \(1.17 \times 10^{-6}\) M; potassium cyanide, \(2 \times 10^{-5}\) M; nitro blue tetrazolium (NBT), \(5.6 \times 10^{-5}\) M; methionine, 0.01 M and an appropriate concentration of enzyme.

The unit of enzyme was defined as the amount of enzyme, which brings about a reduction of 0.1 O.D. at 560 nm per min at 30°C of NBT.

Catalase (E.C. 1.11.1.6 Hydrogen peroxide: hydrogen peroxide oxidoreductase):

Catalase activity was determined by the method of Euler and Josephson (12). The system contained 50 ml of 0.01M \(H_2O_2\) in M/150 phosphate buffer (pH 6.8) and 1.0 ml of the enzyme extract. The incubation was carried out at 30°C and 5.0 ml samples were withdrawn at 0, 5, 10, 15 and 20 min. intervals and pipetted into 5.0 ml of 2N \(H_2SO_4\) to stop the reaction. The residual \(H_2O_2\) was titrated against 0.01 N \(KMnO_4\).

A unit of catalase was defined as the amount of enzyme which oxidizes one μ mole of \(H_2O_2\) per min at 30°C.
Peroxidase (E.C. 1.11.1.7 Donor: H₂O₂ oxidoreductase):

Peroxidase activity was determined by the method described in Worthington and Teller's manual (13). The rate of decomposition of H₂O₂ (in 0.1M acetate buffer, pH 5.0) by peroxidase with D-dianisidine as the hydrogen donor is determined by measuring the rate of colour developed at 460 nm.

One unit of peroxidase activity is that amount of enzyme which decomposed one μ mole of peroxide per min. at 30°C.

Purification of peroxidase inhibitor(s):

For inhibitor - purification the following procedure was used. All the operations were carried out at 0-4°C. Five week-old Trigonella foenum-graecum root nodules were used to isolate the peroxidase inhibitor from the nodule cytosol. Cell free extract was prepared as described earlier. Five ml nodule cytosol cell-free extract was applied to a sephadex G-100 column (2.5 x 30 cm) and was eluted with 0.05 M tris HCl - buffer (pH 7.2) and 5 ml fractions were collected.

The inhibitory activity of the fractions was measured by adding graded amounts to the root cytosol peroxidase, and after preincubation for 30 min at 0 - 4°C, the system was analysed for the residual peroxidase activity. Controls contained buffer instead of the inhibitor preparation which was incubated with the root cytosolic peroxidase.
One unit of the inhibitor is defined as the amount of inhibitor necessary to inhibit one unit of enzyme activity under the experimental conditions.

Pooled fractions showing maximum inhibitor activity, obtained from sephadex G-100 column as described above (fractions 15-30), were concentrated by dialysis against solid sucrose, to a volume of about 5.0 ml. This concentrated inhibitor preparation was applied to a DEAE-cellulose column (2.5 x 25 cm) and was eluted with an increasing gradient of sodium chloride (0.05 - 0.6M), after washing with 0.05 M Tris-HCl buffer (pH 7.2) used for equilibration. The effluent was collected in 10 ml fractions and was dialysed against distilled water for four hrs. before using for further experimentation.

Polyacrylamide gel electrophoresis:

Polyacrylamide gel electrophoresis of partially purified peroxidase inhibitor was carried out by the method of Davis (14). Proteins on the gel were stained with 0.25% (W/V) coomassie blue in methanol-acetic acid - water (5:7:88 by volume) and destained in methanol - acetic acid - water (5:7:88 by volume), by soaking the gels at 30°C (15).

Glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.13):

The enzyme was assayed by the method of Senior and Dawes (16). The assay system contained, 100 μ moles of sodium phosphate buffer, pH 7.8; cysteine, 4.0 mM;
As Na₂H₂AsO₄, 20 μ moles; NADP, 0.25 μ moles; fructose-1,6-diphosphate, 5.0 μ moles and an appropriate amount of enzyme, in a total volume of 2.0 ml.

One unit of the enzyme was defined as the amount of enzyme which brings about a change of 0.01 O.D. at 340 nm per min. at 30°C.

Invertase (E.C. 3.2.1.26 - D-Fructofuranoside fructo-hydrolase):

Invertase activity was assayed by measuring quantitatively, the hexose formed by the method of Nelson (17). The reaction mixture contained in μ moles, potassium phosphate buffer (pH 8.0), 100; sucrose, 25 and an appropriate amount of enzyme extract. Blanks were run without the substrate which was added after the reaction was stopped. Incubation was carried out at 37°C for one hour and the reaction was stopped by adding 3 ml of 0.5 M dibasic sodium phosphate and heating the tubes in a waterbath for two minutes. The precipitated proteins were filtered off and the filtrates were analysed for reducing sugars by the method of Pressey (18).

A unit of invertase activity was that amount of enzyme which liberated one milligram of reducing hexoses per hour under the assay conditions.
Malate dehydrogenase (E.C. 1.1.1.41):

Malate dehydrogenase was assayed as described by Ochoa (19). The test system contained in a total volume of 2.0 ml as follows: potassium phosphate buffer (pH 7.2), 100 μ moles; NADH, 0.2 μ moles; oxaloacetate, 0.4 μ moles and an appropriate amount of enzyme.

One unit of malate dehydrogenase was defined as the amount of enzyme which brings about a change of 0.01 O.D. at 340 nm per min at 30°C.

Isocitrate dehydrogenase (E.C. 1.1.1.42):

The enzyme was assayed according to the method described by Ochoa (20). The assay system contained in a total volume of 2.0 ml, Tris-HCl buffer (pH 8.5) 200 μ moles; MnCl₂; 10 μ moles; NADP, 0.3 μ mole; DL-isocitrate, 0.1 μ mole and an appropriate concentration of enzyme.

The unit of the enzyme was defined as the amount of enzyme which brings about a change of 0.01 O.D. at 340 nm per min at 30°C.

Purification of isocitrate dehydrogenase:

All operations of purification were carried out at 0 - 4°C. Five week-old Trigonella foenum-graecum root nodules were used in the isolation of the enzyme from the nodule cytosol. To the cell free extract prepared as
described earlier, crystalline ammonium sulphate was gradually added with constant slow stirring until 50% saturation was attained. The mixture was kept for an additional 6 hrs. and centrifuged at 15,000 x g for 10 minutes. The precipitates were discarded after testing for activity and, to the resultant supernatant, more ammonium sulphate was added, to obtain 75% saturation. The mixture was left overnight at 0 - 4°C and was centrifuged at 15,000 x g for 15 min. The supernatant was discarded after checking the activity. The precipitates obtained were dissolved in 0.05 M Tris-HCl buffer (pH 7.5) and dialysed against the same buffer for 4 hrs. This enzyme preparation was concentrated by dialysis against solid sucrose. The concentrated enzyme preparation was passed through a sephadex G-100 column (2.5 x 30 cm) previously equilibrated with 0.05 M Tris-HCl buffer, pH 7.5 and 2 ml fractions were collected. Both, the enzyme activity and protein content were monitored in the fractions.

Malic enzyme (E.C. 1.1.1.40):

The method of Ochoa (21) was used to determine malic enzyme activity. The assay system in a total volume of 2.0 ml contained Tris-HCl buffer (pH 7.2), 100 μ moles; malate, 5 μ moles; MnCl₂, 5H₂O, 10 μ moles; NADP, 0.5 μ mole, and an appropriate amount of enzyme.
One unit of malic enzyme was defined as the amount of enzyme which brings about a change of 0.01 O.D. at 340 nm per min. at 30°C.

Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.41):

Glucose-6-phosphate dehydrogenase was assayed according to the method of Kornberg and Horecker (2.2). The reaction mixture contained Tris-HCl buffer (pH 7.2), 200 μ moles; MgCl₂, 5 μ moles; Glucose-6-phosphate, 6 μ moles; NADP 1.5 μ moles and an appropriate concentration of enzyme in a final volume of 2.0 ml.

One unit of glucose-6-phosphate dehydrogenase activity was defined as the amount of enzyme which brings about a change of 0.01 O.D. at 340 nm per min at 30°C.

6-phosphogluconate dehydrogenase (E.C.1.1.1.43):

6-phosphogluconate dehydrogenase was assayed according to the method of Kornberg and Horecker (22). The reaction mixture contained Tris-HCl buffer (pH 7.5) 200 μ moles; 6-phosphogluconate, 6 μ moles; NADP, 1.5 μ moles and an appropriate amount of enzyme in a final volume of 2.0 ml.

One unit of 6-phosphogluconate dehydrogenase activity was defined as the amount of enzyme which brings about a change of 0.01 O.D. at 340 nm per min. at 30°C.
α-ketoglutarate estimation:

The concentration of α-ketoglutarate was measured according to the method of Theodore and Friedman (23).

Ammonia estimation:

Ammonia was estimated as described by Fawcett and Scott (24).

Sucrose estimation:

The amount of sucrose was determined by the method of Emil-Van-Mandel (25).

Glucose estimation:

The concentration of glucose was determined by the glucose oxidase method of Dahlquist (26).

Fructose estimation:

Fructose was estimated following the method of Roe et al (27).

NAD(P)H Oxidation:

The amounts of NADPH and NADH oxidized were determined as described by Horecker and Kornberg (28), and Kornberg (29) respectively.
REFERENCES