MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Study Material

Pithecellobium dulce Benth. belongs to the sub-family Mimosoideae of Leguminosae. It is a large tree with spines and widely distributed in the tropical and subtropical regions. It thrives well in any fair garden soil in the dry districts and can be propagated through seeds sown during rains. It produces club shaped, elongated nodules on the roots of the seedlings and trees.

Erythrina indica Lam. belongs to the sub-family Papilionoideae of Leguminosae. It is a medium sized large tree with prickle covered shoots. They are widely distributed in the tropical and subtropical regions, and can be propagated through cuttings or seeds. Nodules are formed on the roots of seedlings and trees.

Sesbania grandiflora Pers. belongs to the sub-family Papilionoideae of Leguminosae. It is a short lived, fast growing, drought resistant soft wood tree grown in many parts of India and also distributed in tropical regions of the world. It can be propagated through seeds. Nodules are found on the roots of seedlings and trees.

3.2. Morphology of Nodules

Nodules were collected from three different leguminous trees at three different localities, by using standard techniques (Vincent, 1970). Their morphometric characteristics such as size, shape, colour, nodule number per plant and nodule weight were studied. In addition nitrogenase activity and leghaemoglobin content of the nodules were also estimated.

3.3. Estimation of Leghaemoglobin (Wilson and Relsennayer, 1963)

Drabkin's solution was prepared by mixing potassium cyanide, 58 mg; potassium ferricyanide, 198 mg and sodium bicarbonate, 1 g in 1000 ml of distilled water. The root nodules were washed thoroughly in tap water and blotted dry using whatman filter paper. 0.5 g of root nodules were taken and ground in a mortar with pestle adding 3 ml of Drabkin's solution. The extract was centrifuged at 5000x g for 5 minutes, and the supernatant was decanted and stored. The residue was again extracted with Drabkin's solution and centrifuged. The supernatant were pooled and the volume was made upto 10 ml. The absorbance was measured in a spectrophotometer at 540 nm.

3.4. Nitrogenase activity (Stewart *et al.*, 1967)

Nitrogenase activity was measured by the acetylene reduction assay method. The collected nodules were aseptically transferred into a 13 ml sterilized vial. These vials were sealed with alcohol sterilized suba seals. A volume of air, equal to the volume of acetylene to be injected, was removed from the vials with a syringe. The reaction was initiated by the injection of acetylene. The vials were incubated in a shaker at 100 rpm for three hours. After the incubation period, 0.1 ml of gas from the vial was analysed with Hewlett Packard 5890 Gas Chromatograph, fitted with Poropak T column and a flame ionization detector. The carrier gas was N_2 and supplied at the rate of 30 ml min^{-1}. The detector gas flow rates were 30 ml of H_2 min^{-1} and 300 ml of air min^{-1}. The chromatograph oven was set at 75°C, injection port at 110°C and the detector at 120°C. From the peak area of ethylene formed, the amount of ethylene (n moles) was calculated using calibrated ethylene standard. The results were expressed as n moles of ethylene formed per gram nodule per hr at 28°C.

3.5. Isolation of Rhizobium (Vincent, 1970)

The selected nodules were properly washed with running water to remove the soil particles. Then the undamaged root nodules were selected and again washed with distilled water. These selected nodules were kept immersed in 0.1% acidified potassium chloride solution for 5 minutes and washed repeatedly with sterile water. Then, they were immersed in ethyl alcohol solution. This treatment was followed by repeated washing with sterile distilled water. These sterilized root nodules were crushed simply with pestle and mortar. The homogenate was sieved through a fine sieve. The extract was serially diluted with distilled water and inoculated into a Yeast Extract Mannitol Agar (YEMA) medium (Vincent, 1970).

Composition of YEMA medium

Agar	-	20 g
Mannitol	-	10 g
Yeast Extract	-	1.0 g
K_2HPO_4	-	0.5 g
$MgSO_4.7H_2O$	-	0.2 g
NaCl	-	0.1 g
Distilled water	-	1000 ml
Congo red (1%)	-	2.5 ml (only for solid medium during isolation)

The pH of the medium was adjusted to 6.8 before adding agar.

The plates were incubated at 28 $\pm$ 2°C for 2 to 3 days. This medium allowed ***Agrobacterium*** and ***Rhizobium*** to grow and develop into colonies. The rhizobial colonies appeared as white translucent, elevated colonies on YEMA medium. They were removed and purified by repeated streaking. Pure rhizobial cultures were maintained on YEMA slants.

3.6. Identification of *Rhizobium*

The bacterium was identified by the following tests.

Growth on media: The pure culture from the slants was spread on peptone glucose agar and on YEMA media and the growth was observed.

Gram staining: The bacterial cultures were subjected to gram staining procedures and observed.

Congo red test (Hahn, 1966): An aliquot of 2.5 ml of 1 per cent solution of the dye in water was added to a litre of YEMA. The isolated bacterial culture was inoculated on to the plated YEMA and observed.

Hofer's alkaline broth test (Hofer, 1941): The isolated bacterial cultures were spread on YEMA with high pH 11 and observed for growth.

Lactose Agar Test (Bernaerts and Delay, 1963): Isolated nodule bacteria were grown on lactose plates for 4-10 days and the plates were flooded with Benedict's reagent. Finally the plates were observed for colour development.

Staining of poly β-hydroxy butyrate (PHB) (Mc Kinney, 1953): A loopful of selected rhizobia were spread out on a microscope slide in a drop of water and allowed to dry in air. The cooled smear was flooded with 5 times diluted carbol fuchsin for 30 seconds. The slide was washed in running water and allowed to air dry. The pinkish PHB granules were observed under the oil immersion microscope.

3.7. Cross inoculation studies

The economically important leguminous seeds of ***Pisum sativum*** belongs to the cross inoculation pea group, ***Phaseolus vulgaris*** bean group, ***Trigonella foenum-graecum*** alfalfa group, ***Glycine max*** soy bean group, ***Trifolium repens*** clover group and ***Vigna mungo, Vigna radiata, Vigna unguiculata, Arachis hypogaea*** and ***Cicer arietinum*** of cowpea

group were selected for the cross inoculation studies. The nodule isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** were tested with various legumes belonging to different cross inoculation groups (Table 1).

Cross inoculation test

The seeds of the above cross inoculation groups were scarified with concentrated sulphuric acid, treated with 90 per cent alcohol for 3 minutes and surface sterilized with 1 per cent $HgCl_2$ for 5 min. After acid and $HgCl_2$ treatments, the seeds were washed with sterile water until all traces of acid and $HgCl_2$ were removed. The surface sterilized seeds were transferred to the sterile petridishes containing one per cent aqueous agar and incubated for 24-48 hrs so as to allow the germination of seeds. Atleast three replicates were maintained in each case. On germination, the seedlings were removed and treated separately with isolates of ***P. dulce, E. indica*** and ***S. grandiflora***. Then they were transferred to culture tubes (25 x 200 mm) containing sterile N_2-free Jensen's Seedling Agar medium (JSAM) and kept for observation. Control seedlings were also maintained. Jensen's N_2 free nutrient solution and distilled water were occasionally added to the respective tubes.

Composition of Jensen's N_2 free nutrient solution

	Composition	**g l^{-1}**
a)	Potassium chloride	0.0745
b)	Potassium hydrogen phosphate	0.175
c)	Calcium sulphate	0.344
d)	Magnesium sulphate	0.246
e)	Trace elements solution	
	1. Copper sulphate	0.78
	2. Zinc sulphate	2.22
	3. Manganese sulphate	2.03

	4. Ammonium molybdate	0.01
	5. Boric acid	1.43

Solution (e) was prepared as stock consisting of trace elements per litre. From the stock 0.5 ml was added per litre.

f)	Iron solution	g/100 ml
	1. Ferrous sulphate	5.0
	2. Citric acid	5.0

Solution (f) was prepared as 100 ml of stock. From this 0.5 ml was added per litre.

From the cross inoculation studies, nodulation ability and morphometric studies such as height of the plant, number and weight of the nodules and fresh weight of whole plant were carried out in both inoculated and uninoculated plants.

3.8. Characterization studies on *Rhizobium*

3.8.1. Morphological characteristics

Size and shape

The size and shape of the cells of rhizobia were studied by using micrometric and gram's staining techniques.

Capsular staining (Mc Kinney, 1953)

The bacterial smear was prepared, heat fixed and stained with 1 per cent alcian blue in 95 per cent ethanol for 1 minute. The excess stain was washed with distilled water and allowed to air dry. Zihel-Neelsen carbol fuchsin was used as counter stain and washed with tap water and examined under oil immersion.

Motility test

A ring of petroleum jelly was applied around the concavity of the depression slide. A loopful of rhizobial isolates of ***P. dulce, E. indica*** and

S. grandiflora were placed separately in the centre of a clean coverslip by using sterile technique. The depression slide was placed with the concave surface facing down over the coverslip and pressed gently to form a seal between the slide and coverslip. Then the slide was quickly turned the right side up. So that the drop continues to adhere to the inner surface of the coverslip. Then the slide was observed through the oil immersion microscope.

3.8.2. Ecological characteristics

Effect of temperature on growth

Rhizobial isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** were inoculated into YEM broth tubes separately. Initial population was found out by taking initial optical density (OD) using a spectrometer at 620 nm. Then these test tubes containing rhizobial isolates were incubated at different temperatures such as 5°, 15°, 20°, 25°, 30°, 40° and 45°C for 24 hours. Final reading was taken after 24 hours. Growth was expressed interms of optical density.

Effect of pH on growth

The pH of the YEM broth was adjusted to pH 3, 4, 5, 6, 7, 8 and 9 by using 0.1 N NaOH and 0.1 N HCl. Then the pH adjusted broth was distributed into test tubes of 10 ml each and sterilized. Then 0.1 ml of 24 hours old rhizobial culture of ***P. dulce, E. indica*** and ***S. grandiflora*** were inoculated separately into each test tube of YEM broth, having different pH values. The initial population was found out by taking initial O.D. at 620 nm. Then the tubes were incubated at 28 $\pm$ 2°C for 24 hours, and the growth was measured by taking O.D. at 620 nm.

Effect of salinity tolerance on growth

The rhizobial isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** were inoculated separately into YEM broth tubes containing various concentrations of NaCl such as 0.1, 0.2, 0.3, 0.5, 0.8, 1, 2, 4, 10 and 20% and incubated at 28 $\pm$ 2°C for 24 hours and the growth was measured by taking O.D. at 620 nm.

3.8.3. Biochemical characteristics

The biochemical tests were conducted by the following methods of Josey *et al.* (1979), as described by Cappuccino and Sherman (1999) to identify the bacteria.

Mac Conkey agar test

Mac Conkey agar medium was prepared by mixing bacto peptone, 17.0 g; protease peptone, 3.0 g; lactose, 10.0 g; bile salts mixture, 1.5 g; sodium chloride, 5.0 g; agar, 13.5 g; neutral red, 0.03 g and crystal violet, 0.001 g in 1000 ml of distilled water. The pH of the medium was adjusted to 6.5 before adding agar and sterilized. The sterile medium was poured into the sterile petri plates and allowed to solidify. The plates were inoculated with the isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** separately. The inoculated plates were incubated at 28 $\pm$ 2°C for 3 to 5 days and observed.

Indole test

Sim's agar broth was prepared by mixing peptone, 30.0 g; beef extract, 30.0 g; ferrous ammonium sulphate, 0.2 g; sodium thiosulfate, 0.025 g and agar, 3.0 g in 1000 ml of distilled water. The pH of the medium was adjusted to 7.3 before adding agar and sterilized. The test tubes containing sterile Sim's agar broth were inoculated with isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** separately and the uninoculated

broth was maintained as control. Inoculated and uninoculated tubes were maintained at 28 ± 2°C for 48 hrs. One ml of Kovac's reagent was added to each tube, including control, after 48 hrs. The tubes were gently shaken at an interval of 10 to 15 minutes and allowed to stand until the reagent reaches the top. Production of a red reagent layer is the indicative of indole positive.

Methyl red test

MR-VP broth was prepared by mixing peptone, 7.0 g; dextrose, 5.0 g and potassium phosphate, 5.0 g in 1000 ml of distilled water. The pH of the medium was adjusted to 6.9. Five ml of the broth was poured into each tube and sterilized. MR-VP tubes were inoculated with the isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** separately and the controls were maintained. All the tubes were incubated at 28 ± 2°C for 48 hours. After 48 hrs, five drops of methyl red indicator was added to the tube of each set. The methyl red indicator at pH 4 will remain red (throughout the tube), which indicates the positive test, while turning of methyl red to yellow is a negative test.

Voges Proskauer test

Five ml of MR-VP broth was poured into different tubes and sterilized. These tubes were inoculated separately with isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** the uninoculated tubes were maintained as control. All the tubes were incubated at 28 ± 2°C for 48 hrs. A few drops of Barritt's reagent was added to each tube and observed for the appearance of deep rose colour.

Citrate utilization test

Simmon's citrate agar slants were prepared by mixing ammonium dihydrogen phosphate, 1.0 g; dipotassium phosphate, 1.0 g; sodium

chloride, 5.0 g; sodium citrate, 2.0 g; magnesium sulfate, 0.2 g; bromothymol blue, 0.08 g and agar, 15.0 g in 1000 ml of distilled water. The pH of the medium was adjusted to 6.9 before adding agar and sterilized. These slants were inoculated with the isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** and control was maintained without inoculation. They were then incubated at 28 $\pm$ 2°C for 48 hours and observed for colour change from green to blue.

l

Triple Sugar-Iron Agar test

Triple sugar-iron agar slants were prepared, and the rhizobial isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** were inoculated into its appropriately labelled tube by means of a stab and streak inoculation by using sterile technique and incubated for 24 hours at 28 $\pm$ 2°C and examined.

Starch hydrolysis test

Starch agar medium was prepared by mixing soluble starch, 10.0 g; beef extract, 3.0 g and agar, 12.0 g in 1000 ml of distilled water. The pH of the medium was adjusted to 7.5 before adding agar at 28 $\pm$ 2°C. The sterilized medium was poured into the sterile petri plates, inoculated with the isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** and observed for colour change. A clear zone of hydrolysis surrounding the growth of the organism is a positive result.

Urea hydrolysis test

Urea agar medium was prepared by mixing peptone, 1.0 g; sodium chloride, 5.0 g; potassium monohydrogen phosphate, 2.0 g and agar 20.0 g in 1000 ml of distilled water. The pH of the medium was adjusted to 6.8 before adding agar and sterilized. The medium was poured into the sterile petri plates and allowed to solidify. The petri plates were then inoculated

with the isolate of ***P. dulce, E. indica*** and ***S. grandiflora*** and incubated at 28 ± 2°C for 48 hrs. Appearance of deep pink colour is the positive result.

Gelatin hydrolysis test

Gelatin agar medium was prepared by mixing peptone 0.5 g, beef extract 0.3 g, gelatin 12 g in 100 ml of distilled water in a 250 ml conical flask thoroughly and was distributed in all test tubes about 5 ml each and plugged with cotton. Then the tubes were sterilized and allowed to cool. After cooling the tubes were inoculated with the isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** separately, incubated at 28 ± 2°C for 2 to 3 days and examined.

Nitrate reduction test

Nitrate broth was prepared by mixing peptone, 5.0 g; beef extract, 3.0 g and potassium nitrate, 5.0 g in 1000 ml of distilled water. After adjusting the pH of the medium to 7.2 was poured into test tubes and sterilized. The tubes were inoculated separately with three different bacterial cultures after cooling. The uninoculated tubes were maintained as control. They were then incubated at 28 ± 2°C for 48 hrs. After incubation period, 3 drops of sulphanilic acid reagent and 3 drops of alphanaphthylamine reagent were added and observed for cherry red colour.

Hydrogen sulfide production test

Sim's agar medium was prepared by mixing peptone, 30.0 g; beef extract, 3.0 g; ferrous ammonium sulfate, 0.2 g; sodium thiosulfate, 0.025 g and agar 3.0 g in 1000 ml of distilled water. The pH of the medium was adjusted to 7.3 before adding agar. The medium was poured into test tubes and sterilized. The sterilized tubes were inoculated with the isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** separately. They were then

incubated at 28 ± 2°C for 48 hours and observed for hydrogen sulfide production.

Arginine hydrolase test

Rhizobial isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** were inoculated separately into the plates containing arginine agar, incubated at 28 ± 2°C for 24 hours and examined. A bright magenta colour formation indicates the positive reaction.

Cytochrome oxidase test

A piece of filter paper was moistened with a few drops of freshly prepared 1 per cent solution of tetramethyl-p-phenylene diamine dihydrochloride. The isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** were picked up aseptically from the slant with a sterile wooden applicator stick for smear. Then it was smeared on the moistened filter paper and observed immediately for the development of a violet colour as positive result.

Oxidase test

Tripticase soy agar plates were prepared, and single line streak inoculation of rhizobial isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** were made separately on the agar surface, and incubated at 28 ± 2°C for 24 hours. Then 2 or 3 drops of P-aminodimethylaniline oxalate were added to the surface of the inoculated plates and observed for the colour change.

Catalase test

Cultures of ***P. dulce, E. indica*** and ***S. grandiflora*** isolates were picked up aseptically from the slant and made smear on a clean glass

slide. Then a drop of hydrogen peroxide was pipetted out into the slide with bacterial culture and observed for the production of gaseous bubble.

Carbohydrate fermentation test

Tryptone 0.5 g, yeast extract 0.25 g, K_2HPO_4 0.1 g, carbohydrate 0.25 g (glucose or lactose or sucrose) were mixed with 50 ml distilled water in a 250 ml conical flask was prepared. Then 0.1 ml of Bromocresol purple reagent was added to the prepared medium (dissolved 160 mg BCP in 5 ml of 95% ethanol and make upto 10 ml with distilled water). The indicator containing medium was distributed to test tubes of 5 ml each. Similarly, other sugar containing medium was also distributed to each test tubes. Small tubes were inserted in the inverted position, so that the media levels were well above the small tubes. Then the tubes were plugged with cotton and sterilized. Rhizobial isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** were inoculated separately into the test tubes and an uninoculated tube was kept as control. All the tubes were incubated at 28 $\pm$ 2°C for 48 hours and observed for colour change.

Carbon utilization test

Solutions of carbohydrate (0.1%) and its derivatives namely adonitol, arabinose, acetate, arabitol, aconitate, arbutin, amygdalin, ammonium tartarate, N-acetyl glucosamine, benzoate, caprate, cellobiose, citrate, casein hydrolysate, dulcitol, erythrose, fructose, galactose, glucose, glutarate, gluconate, glycerate, glycerol, inositol, inulin, lactose, lactate, D-lyxose, maltose, maleate, malate, mannitol, mannose, melibiose, mesotartarate, oxalate, propionate, pyruvate, pimelate, raffinose, ribose, rhamnose, salicin, sorbitol, sodium acetate, suberate, succinate, sucrose, L-tartrate and xylose were prepared separately and filtered. One ml aliquots of the specific carbon source was added to each of the phenol red broth tubes. These media were added with tripticase as a protein source to

support the bacterial growth. The pH indicator, phenol red, turns from red to yellow at acid pH. The test bacterium was inoculated to each set of carbon source supplemented tubes and incubated at 28 $\pm$ 2°C for 48 hrs. After incubation the colour of the tubes was observed. Yellow colour indicates the acid production. Partial filling of the Durham tube indicates positive gas production.

3.8.4. Estimation of biochemical components

Estimation of total polysaccharide

Reagent

Ethanol (50%)

Procedure

500 mg of the bacterial cells were weighed and ground with the pestle and mortar. This was continued for 15 minutes. The mixture was centrifuged for 15 min at 7,000x g. To the supernatant 15 ml of ethanol was added and again centrifuged for 30 min. The pellet was taken in the pre-weighed bottle and total amount of polysaccharide formed was found out.

Estimation of total protein (Lowry *et al.*, 1951, modified by Tandeau de Marsac and Houmard, 1988)

Reagents

A. 10 per cent TCA

B. 1 N NaOH

C. Alkaline sodium carbonate solution (2 per cent $NaCO_3$ in 0.1 N NaOH).

D. Copper sulphate – sodium potassium tartrate solution.

0.5 per cent $CuSO_4.5H_2O$ in 1 per cent sodium potassium tartrate – prepared freshly every time.

E. Alkaline copper reagent

Prepared freshly by mixing 50 ml of reagent C and 1 ml of reagent D.

F. Folin-Ciocalteu reagent

Commercially available reagent was diluted with equal volume of water just prior to use.

Procedure

The cultures were centrifuged at 10,000x g for 10 min. From the pellet 500 mg was treated with reagent A and centrifuged at 10,000x g for 10 min. The resulting pellet was resuspended in reagent B and boiled for 30 min, cooled and then recentrifuged to eliminate light scattering materials. The supernatant was made upto a known volume. To 0.1 ml of the supernatant, 5 ml of reagent E was added and allowed to stand for 10 min. Finally 0.5 ml of reagent F was added. The absorbency was measured after 30 min at 750 nm in spectronic 20 D. The amount of protein was calculated with a standard curve prepared using Bovine Serum Albumin (BSA).

Estimation of Free Amino acid (Jayaraman, 1981)

Reagents

Ethanol (80%)

Ethanol (50%)

Ninhydrin reagent

Prepared by dissolving 2 g of ninhydrin in 25 ml of acetone. To this 25 ml of 0.2 M acetate buffer, pH 5.5 was added and stored in brown bottle.

Procedure

The rhizobial cultures were centrifuged at 10,000x g for 10 min. From the pellet, 500 mg was homogenized with 80% ethanol in pestle and mortar. The homogenate was centrifuged at 10,000x g. The clear supernatant was pipetted out into a test tube and diluted to 4 ml with distilled water. To this 1 ml of ninhydrin reagent was added and kept in boiling water bath for 15 min. The tubes were then cooled and 1 ml of 50% ethanol was added. The purple colour developed was measured in spectronic 20D at 540 nm. Standard graph was made using a mixture of alanine, aspartic acid, tryptophan, proline and lysine.

Estimation of total lipids (Sato, 1988)

Reagents

Chloroform : Methanol 2:1 (v/v).

Procedure

Rhizobial cultures were centrifuged at 10,000x g for 10 min. Equal amount of samples from each of the culture isolate was homogenized in a mortar and pestle with extraction solvent (Chloforom : Methanol) and filtered through filter paper. The filtrate was vortexed with sodium sulphate to remove moisture. Then it was taken in a pre-weighed bottle and dried by a stream of nitrogen. The dried extracts were weighed and the total lipids were estimated by subtracting the initial from the final weight. The amount of total lipid was expressed as mg g^{-1} dry weight.

3.8.5. Molecular characteristics

Gel Electrophoresis

Extraction of Protein (Scanlan and Carr, 1988)

Reagents

A. Sodium phosphate buffer	-	0.1 M, pH 7.4
B. Tris-HCl	-	0.5 M, pH 6.8

The rhizobial isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** were centrifuged at 10,000x g for 10 min in refrigerated centrifuge. Equal amount of samples on dry weight basis was homogenized well with reagent A. The homogenate was centrifuged at 15,000x g for 10 min at 4°C. From the supernatant total protein was precipitated by the addition of finely powdered ammonium sulphate (47.2 g/100 ml, yielding a 70 per cent saturated solution) at 4°C over 10-15 min with stirring, and was allowed to continue for a further 30 min before spinning in transparent tubes at 8,500x g for 40 min at 4°C. The supernatant was poured off, and the pellet was resuspended in reagent B. The sample was then subjected to SDS-PAGE.

SDS-PAGE

Analysis of proteins was carried out by SDS-PAGE according to the method of Laemmli (1970).

(A) Sample buffer

1 M Tris-HCl, pH 6.8	-	60 µl
β-mercaptoethanol	-	50 µl
10 per cent SDS	-	200 µl
100 per cent glycerol	-	100 µl
1 mg ml^{-1} bromophenol blue	-	2 µl

(B) Separation gel buffer – 4x Tris-HCl, pH 8.8

Tris	-	18.2 g
SDS	-	0.4 g
Distilled water	-	60 ml

pH was adjusted to 8.8 with 1 N HCl. Final volume was made up to 100 ml with distilled water.

(C) Stacking gel buffer – 4x Tris-HCl, pH 6.8

Tris	-	6.0 g
SDS	-	0.4 g
Distilled water	-	80 ml

pH was adjusted to 6.8 with 1 N HCl. Final volume was made to 100 ml with distilled water.

(D) Acrylamide – 30 g

N, N'-methylene bis acrylamide	-	0.8 g

made upto 100 ml with distilled water.

(E) Electrode buffer – Stock

5 x Tris-HCl	-	15.1 g
Glycine	-	72.0 g
SDS	-	5.0 g
Distilled water	-	1,000 ml

The stock was diluted to 1x before use.

Preparation of separating gel

Separating gel was prepared in the following manner.

Reagent B	-	11.25 ml
Reagent D	-	18.00 ml
Distilled water	-	15.75 ml

After degassing the acrylamide mixture, 150 µl of 10 per cent ammonium persulphate (APS) and 30 µl of N,N,N,N-tetramethyl ethylene-diamine (TEMED) were added. Using a gel maker, a linear slab gel was prepared.

Stacking gel was prepared in the following manner.

Reagent B	-	3 ml
Reagent C	-	5.0 ml
Distilled water	-	12.0 ml
APS	-	100 ml
TEMED	-	20 µl

The solution was layered on the top of the separating gel after introducing a comb and allowed the gel to set. The comb was then removed without distorting the shapes of the wells. The gel was installed in the electrophoretic apparatus carefully. The sample extract (25 – 50 µl) was injected into the wells. The electrode buffer was filled in tank and trapped air bubbles at the bottom of the gel were removed. The gel was run initially at 15 mA until the tracking dye travelled through the stacking gel. Then the current was increased to 30 mA until the tracking dye (bromophenol blue) reached the bottom of the gel. The gel was carefully removed and stained.

Isolation of plasmid DNA (Eckhardt, 1978)

Reagents

Solution – I

15% glucose

25 mM Tris (pH 8.0)

10 mM EDTA

Solution – II

0.2 N NaOH

1% SDS

Solution – III

3 M sodium acetate

Procedure

A known volume (1.5 ml) of rhizobial culture of ***P. dulce, E. indica*** and ***S. grandiflora*** were taken separately in eppendorf tubes and spin down for 5 minutes. Then the pellets were resuspend, in 100 µl of solution I by vigorous vortexing. To this 200 µl of freshly prepared solution II was added and the tubes were closed tightly. Then the contents were mixed rapidly by inverting the tubes five times. To the tubes 150 µl each of solution III was added; mixed the contents by inverting the tubes and kept for 5 minutes. Then the contents were centrifuged in a microfuge for 10 minutes and the supernatant was transferred to a fresh eppendorf. To the supernatant, 450 µl of isopropanol was added to precipitate the DNA and kept at room temperature for 2 to 5 minutes. The pellets were separated by centrifugation for another 10 minutes, and rinsed with 70% ethanol. Finally the pellets were dried for 10 minutes and then dissolved in 20 µl of 1 x TE buffer. To remove the RNA, 1 µl of RNase was added at 42°C for 5 minutes. Then the plasmids were separated by agarose gel electrophoresis and visualized with ethidium bromide.

Analysis of fatty acids (Miller and Berger, 1985)

Reagents

(a) Saponification reagent

Prepared by dissolving 45 g of NaOH in 300 ml of methanol, water moisture (1:1 v/v).

(b) Methylation reagent

Prepared by mixing 325 ml of 6 N HCl with 275 ml methanol.

(c) Extraction solvent

Prepared by mixing 200 ml of hexane with 200 ml of anhydrous diethyl ether.

(d) Base wash

Prepared by mixing 10.8 g of NaOH in 900 ml.

Procedure

100 mg sample from each flask after centrifugation was taken in separate screw cap test tubes. To them 1 ml of reagent A was added and tightly sealed with Teflon-lined screw cap. Then the tubes were vortexed for 10 second and kept in a boiling water bath for 5 minutes. Again the tubes were vortexed 10 seconds. Then the tubes were kept in the water bath for the additional 20 minutes. After a total of 30 min of saponification, the tubes were removed from the water bath and cooled to room temperature. To each tube, 2 ml of reagent B was added by uncapping the tubes. After vortexing for 10 sec, the tubes were placed in a water bath set at 80°C for 10 min. Finally 1.25 ml of reagent C was added to each cooled tube. Then the tubes were tightly closed and rotated end-over-end for 10 min. From the tubes, the lower aqueous phase was removed and discarded. To the upper phase, 3 ml of reagent D was added and rotated end-over-end for 5 minutes. With the help of clean Pasteur pipette 2/3 of the organic extract from each tube was transferred to GC vials and kept in deep freezer by capping with Teflon-lined septum. From each vial 2 µl of sample was analysed with the following settings.

Column	-	DEGS
Column temperature	-	180°C
Injection Port temp	-	200°C
Detector temp	-	230°C
Carrier gas	-	Nitrogen

Sample volume	-	½ µl
Detector	-	FID
Integrator settings		
Zero	-	10
Attenuation	-	6
Chart speed	-	1
Peak width	-	0.04
Thrush	-	4
Chart speed	-	0.5, time 6
Peak width	-	0.64, time 5

From the peak area of fatty acid, the amount of fatty acid was calculated using respective standards.

3.9. Numerical analysis

To determine the exact taxonomic position of N_2 fixing, root nodulating rhizobial strains of leguminous trees such as ***P. dulce,*** ***E. indica*** and ***S. grandiflora***, the nodules were collected from these plants belonging to three different geographical areas and the strains were compared with the informations so obtained from the studies such as proteins, plasmids and fatty acid profiles. The results were then disposed in a numerical classification, to ascertain whether any correlation obtained between such a classification and the species of host.

Characters were coded 1 for positive and 0 for negative. The final n x t matrix contained nine strains and thirty four characters. The similarity between all possible pairs of traces was expressed by the Pearson product moment correlation co-efficient (r). After calculation of similarities among these strains, clusters were formed by using the complete-linkage clustering method between strains with the help of SPSS

(Statistical Package for Social Science). These clusters were represented by means of a dendrogram.

3.10. Pot culture experiments (Dahiya and Khurana, 1981)

Mass culture and inoculation

One isolate from each legume was selected to study the biofertilizer potential of ***Rhizobium*** on some pulses. The selected rhizobial isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** were cultured separately in YEMA medium for about 3 days in order to establish better growth of the bacteria and subsequently, subcultured in test tubes containing YEM broth. Finally the cultures were transferred aseptically to 250 ml conical flask and incubated at 28 $\pm$ 2°C on a rotary shaker for 2-3 days. From the conical flask, the well grown rhizobial culture was transferred to a fermenter vessel containing the sterile medium. The cultures were maintained at constant temperature and pressure for about 3-6 days. The cultured rhizobial isolates were mixed with finely powdered sterilized lignite as carrier material (Kandasamy and Prasad, 1991) and kept for curing.

The seeds of economically important legumes such as ***Vigna mungo, Vigna radiata*** and ***Arachis hypogaea*** obtained from the depot of the Agricultural department, Thanjavur, Tamil Nadu, were used throughout the study. The seeds of the above species were scarified and surface sterilized. The sterilized seeds were germinated in pots containing sterilized garden soil. The experiment was performed in replicates under natural conditions. After 15 days, from the date of sowing the seeds, the selected rhizobial isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** were inoculated separately into the pots containing the seedlings of ***Vigna mungo, V. radiata*** and ***A. hypogaea***. Controls were also maintained with out rhizobial inoculation.

Morphometric studies of the inoculated plants

The following morphometric analysis were done from matured plants of each species tested. They were,

1. Height of the plant (cm)
2. Number of nodules / plant
3. Root biomass (g / plant)
4. Shoot biomass (g / plant)
5. Total number of seeds / plant
6. Seed biomass (g / plant)

Statistical analysis

Analysis of independent test was also carried out by using the SPSS package.