4. Materials and Methods

4.1 Collection of leguminous samples

Different places from three agro-climatic zones (Fig.1) where the flora is representative and naturally luxuriant, were visited in 26 field tours covering all the three seasons of the year and wild leguminous species, identified on the basis of their floral, pod and leaf characteristics, were collected. While digging during the collection of samples, care was taken to retain all the nodules on the root system. Samples were brought to the laboratory and examined carefully for presence of nodules on the roots. The samples showing nodules were identified to species level on the basis of floral characteristics. This list of nodulated leguminous species was checked against the global listing of nodulated and non nodulated legumes (Allen and Allen 1981 and personal communication from E.K.Allen) and those species which were new to the global list were selected for further detailed studies.

4.1.1. Studies on the nodule morphology -

Roots of wild leguminous plants were washed thoroughly with tap water and following observations were made :

1) Relative abundance of the nodules.
2) Location of nodules on the root system - whether on tap root or lateral roots.

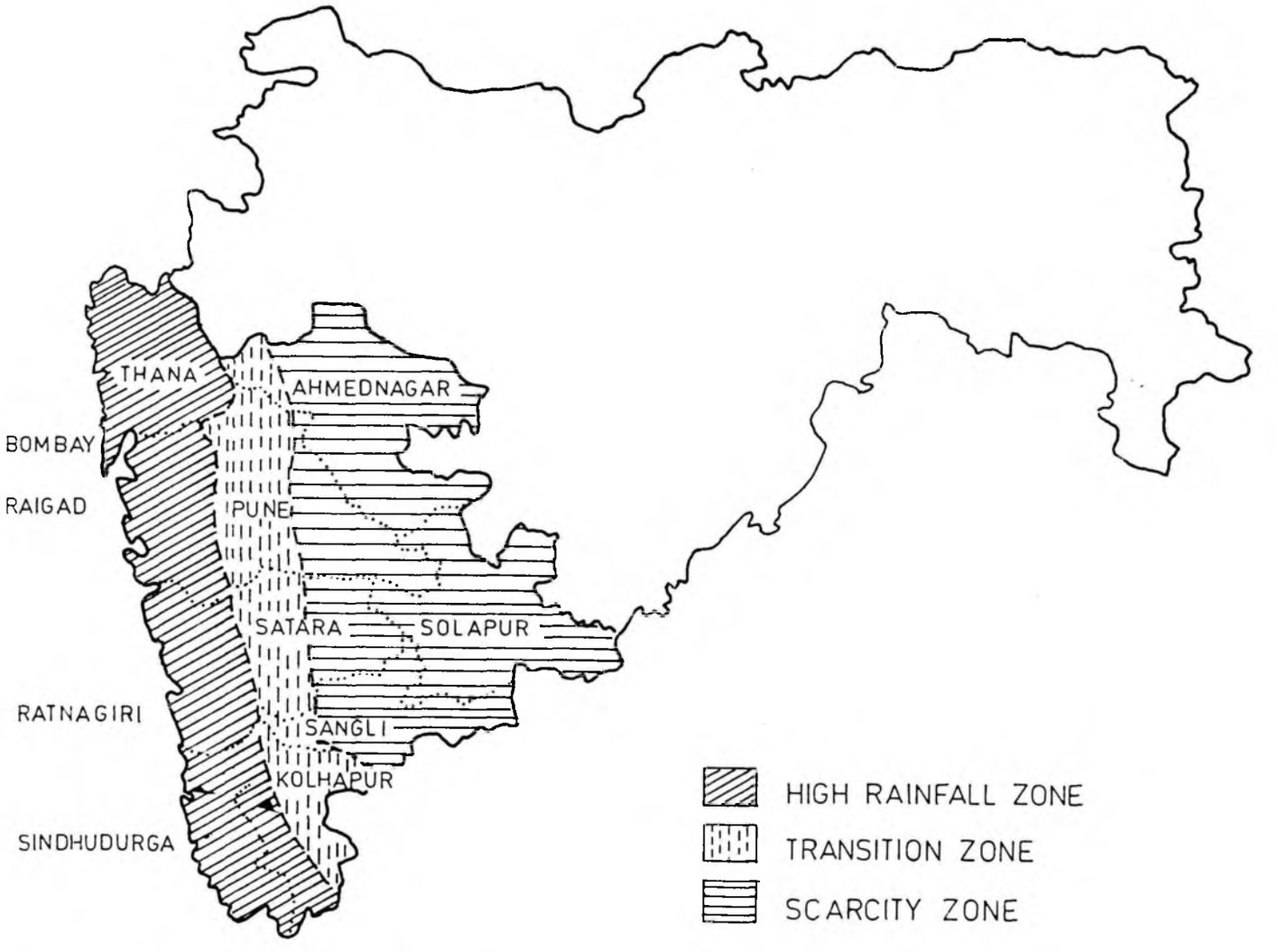

FIG: 1 THREE AGRO - CLIMATIC ZONES OF WESTERN MAHARASHTRA.

3) The shapes of the nodules were described as globose, elongate, bifurcate, fan shaped and palmate, coralloid and siamese by comparing them with the nodule morphology illustrated by Corby (1971) and Allen and Allen (1981).
4) Size of the nodule (Diameter/Length/Width) was measured.
5) External and internal colour of the nodule.
6) Nature of exterior surface of the nodule.

4.2 Isolation and Identification of root nodule bacteria -

4.2.1 Isolation of root nodule bacteria :

A healthy, unbroken and firm nodule of each plant was selected for isolation of root nodule bacteria. It was cut from the root, leaving a small amount of root attached for ease of handling. It was then washed thoroughly in tap water to remove gross surface contamination and then immersed in 0.1% (w/v) acidified $HgCl_2$ for 4 to 5 minutes for surface sterilisation. Finally it was washed thoroughly in six changes of sterile water, to remove the traces of the disinfectant.

Each of the nodule was then crushed in a small aliquot of sterile distilled water in a sterile test tube with the help of a sterile glass rod and the milky fluid was inoculated on three plates of Yeast extract Mannitol Agar, (YEMA). The plates were incubated at 28^{o} C and a typical isolated colony

was picked and agitated in a tube containing little sterile distilled water and again streaked on YEMA plates for purification. The diameter of the well isolated colony was measured.

A well isolated, typical colony from second streaking was picked and transferred to YEMA slants for maintenance and further studies. The slants were preserved at $4^{o}C$ with a regular transfer after 3 months. Each culture was coded according to the host plant from which it was isolated.

4.2.2 Identification of root nodule bacteria :

I) Distinction from Agrobacterium -

In order to distinguish the isolate from Agrobacterium following tests were carried out -

a) Ketolactose test : The production of Ketolactose by the isolate was tested on a modified medium of Bernaerts and Delay (1963).

b) Growth in Hofer's alkaline medium : The ability of the isolates to grow in Hofer's medium (Hofer 1935) with pH 11.0 was tested.

c) Nile blue reduction test : The ability of the isolates to reduce Nile blue was tested by the method of Skinner (1977).

d) Growth on Congo Red medium : The colony morphology of the isolates on the YEMA containing Congo Red (Vincent 1970) was noted.

II) Distinction from nonrhizobial groups -

The isolates were inoculated on Glucose peptone agar slopes (Vincent 1970) and growth observed after 7 days together with the changes in pH brought about by them.

III) Koch's postulates tests -

In order to study the ability of the isolates to nodulate their homologous host plants, the respective leguminous plant was grown in the sterilised soil. Hard coated seeds were treated with concentrated sulfuric acid, if necessary. Before sowing seeds were surface sterilised by treatment with 1:1000 $HgCl_2$. After emergence of the seedling, it was inoculated with 1 ml of culture suspension of the homologous rhizobial strain. Seeds sown without any culture treatment served as a control. After 45 days of growth, the plants were carefully removed and observed for presence of nodules on their root system. Those rhizobial strains showing the ability to nodulate their homologous hosts were considered as Koch's postulates proven strains.

4.3 Morphological and cultural characteristics of root nodule bacteria -

4.3.1 Gram staining : Gram characteristics of the rhizobial strains were determined by the staining method of Tchan (Vincent 1970).

4.3.2 Cultural characteristics : Colony characteristics of the rhizobial strains on Yeast extract Mannitol agar were noted after 8 days of incubation at 28°C.

4.3.3 Rate of Growth in the synthetic medium : 300 ml of synthetic medium of Tan and Broughton (1981) was inoculated with 1 ml of growth of the rhizobial strain in Yeast extract Mannitol broth and the flasks were incubated on a rotary shaker at Room Temperature. Five ml. aliquot of the medium was examined at 12 hours interval for measurement of turbidity on Spectronic 20 at 550 nm. The pH of the medium was measured at 24 hours interval. The generation time was then calculated by plotting a graph of log of optical density against time to determine the fast or slow growing character of the rhizobial strain.

4.4 Biochemical and Physiological characteristics : The rhizobial strains were studied for their biochemical and physiological characteristics as detailed below :

4.4.1 Production of acid or alkali in YEMA :
The ability of the rhizobial strains to change the pH of YEMA was tested by inoculating plates of YEMA containing bromothymol blue as a pH indicator. (Vincent 1970).

4.4.2 Growth and reaction in litmus milk : The tubes of sterile litmus milk, inoculated with the rhizobial strains were incubated at 28°C for 4 weeks and examined for acid or alkali production and formation of a serum zone.

4.4.3 Carbohydrate Utilization : The growth of the rhizobial strains was tested on 19 different sources of carbon, using basal medium of Stowers and Eaglesham (1983).

4.4.4 Utilization of organic acids : Seven organic acids viz. citrate, fumarate, malate, oxalate, pyruvate, succinate and tartarate were tested as a carbon source for the rhizobial strains using basal medium of Elkan and Kwik (1968).

4.4.5 Vitamin requirement : The requirement for the vitamins viz. biotin, calcium pantothenate and thiamine by the rhizobial strains was determined in a synthetic medium of Ahmad et al. (1981).

4.4.6 Antibiotic sensitivity : The sensitivity of the rhizobial strains to 21 different antibiotics was tested by using antibiotic discs of SPAN Diagnostic India (Gupta et al. 1983) on Bergersen's synthetic medium (Bergersen 1961).

4.4.7 Effect of pH of the medium on the growth of root nodule bacteria : Yeast extract mannitol broth, adjusted to different pH values (from 3.5 to 10.0), was inoculated with the rhizobial strains and growth was measured as Optical Density after 7 days of incubation at Room temperature on rotary shaker.

4.4.8 Effect of salt concentration : Yeast extract mannitol broth with different concentrations of Sodium chloride (0.0 - 2.5%) was inoculated with the rhizobial strains and observed for growth after 7 days of incubation at Room temperature on rotary shaker.

4.4.9 Sensitivity to crystal violet : Plates of YEMA containing different concentrations of crystal violet viz. 1:5000, 1:10,000, 1:25,000, 1:50,000 and 1:75,000 were inoculated with the rhizobial strains (Johnson and Allen 1952, Konde 1975) and observed for growth after 7 days of incubation at $28^{o}C$.

4.4.10 Penicillinase activity : The iodometric method of Bhatia et al (1981) was used for detection of penicillinase activity.

4.4.11 Oxidase activity : Oxidase activity of the rhizobial strains was tested by the method of Kovaks (1956).

4.4.12 Urease activity : The slants of Urea agar (Graham and Parker 1964) were inoculated with the isolates and observed for growth after 7 days of incubation at 28°C.

4.4.13 Catalase activity : A loopful of the growth of the rhizobial strain from YEMA slope was stirred in 10% w/v hydrogen peroxide and observed for evolution of gas.

4.4.14 Gelatinase activity : Plates of YEMA containing 0.4% of gelatin were spot inoculated with the rhizobial strains and after 7 days of incubation at 28°C tested for gelatinase activity by flooding with gelatin precipitating reagent (Sadowsky 1983).

4.4.15 Nitrate reductase activity : Nitrate reductase activity of the rhizobial strains was determined by growing them in Yeast extract mannitol broth and in defined medium and by testing the activity under anaerobic and aerobic conditions by the method of Manhart (1979).

4.4.16 Growth on combination of sugars and amino acids : The ability of the rhizobial strains to grow and change the pH of the medium containing different combinations of amino acids viz. aspargine and glutamine and sugars viz. fructose and galactose as nutrients was tested by the method of Tan and Broughton (1981).

4.4.17 Effect of Carbon source on the change of the pH of the medium : Mannitol from Yeast extract mannitol broth was replaced with five different Carbon sources viz., Xylose, Arabinose, Glucose, Rhamnose and Sodium gluconate. The broth tubes were inoculated with the rhizobial strains and after 7 days of incubation, at room temperature on rotary shaker, observed for growth and final pH of the medium.

4.5 Serological characteristics of root nodule bacteria -

4.5.1 Preparation of antisera : Antisera against 19 rhizobial strains were prepared by immunizing inbred albino rabbits weighing about 2.5 - 3.0 kg. The rabbits were bled before immunization to ensure absence of natural antibodies against the antigens under use.

Preparation of antigens : The growth of the organisms in Bergersen's synthetic medium (Bergersen 1961) was washed repeatedly in physiological saline under aseptic conditions to remove extracellular polysacchrides and the suspension having a cell density of 1×10^9 cells/ml was used for immunizing the rabbits.

Inoculation schedule : The inoculation schedule proposed by Schwinghamer and Dudman (1980) was

Agglutination test : Tube agglutination procedure was followed as proposed by Vincent (1970) using two fold dilutions up to 1:6400. The antigen - antiserum suspensions in Dreyer's tubes, together with positive and negative controls were incubated in a water bath at 52°C for 4 hours and examined for agglutination reaction. The serum titer was determined as the reciprocal of the highest dilution at which positive agglutination was evident.

4.5.3 Immunodiffusion -

Preparation of antigens : The cell suspension of organisms under study was prepared, as detailed earlier except for the fact that the final cell density was adjusted to 1 x 10^{10} cells/ml and 3 types of antigens were prepared as detailed below :

i) Unheated antigen - Cell suspension was used as it is as an antigen.

ii) Heated antigens - Cell suspension was heated at 100°C in a waterbath for 30 minutes to dissolve the antigens insoluble at lower temperatures.

iii) Sonicated antigen - The cell suspension was sonicated on ice for 10 minutes at 20 kc/s in order to release the internal antigens.

Preparation of gel for diffusion : To 100 ml of physiological saline, 0.75 g of Difco Noble agar was added and melted by autoclaving. To the melted agar was added 1 ml of a 2.5% (w/v) solution of sodium azide as a preservative. 25 ml of the hot gel was poured in a flat bottom petridish to form a 4 mm thick gel layer and the agar was allowed to solidify.

4 mm diameter wells were cut in the agar gel with the help of a cork borer in a hexagonal pattern, keeping a distance of 5 mm in between two wells. Seventh well was cut in the centre of the hexagonal pattern. The free liquid from the bottom of each well was sucked out with a Pasteur pipette and the bottom sealed with a drop of molten agar.

Immunodiffusion test : The peripheral wells were filled with the antigens and central well with undiluted antiserum, to the top, using Pasteur pipette. During filling the peripheral wells homologous antigens were placed in top and bottom position while heterologous antigens were placed in lateral wells. A negative control using saline and preimmune serum was run along with the experiment.

The petri dishes were incubated at room temperature for seven days in tight lid containers with wet filter paper to keep the atmosphere moist. The plates were observed every day for the formation of precipitin

bands. The homologous titer of the antiserum was also found out using two fold dilutions.

4.6 Symbiotic characteristics of root nodule bacteria -

4.6.1 Nodulation tests on Siratro : To test the ability of the rhizobial strains to nodulate the Siratro (Macroptilium atropurpureum Urb.) plants, Gibson's semienclosed tube system (Gibson 1965) was employed. Jensen's nitrogen free agar medium (Jensen 1965) was used for the growth of the plants and Yeast extract mannitol broth for the growth of inoculum. One ml suspension containing 10^8 cells/ml of the rhizobia was used to inoculate the roots of the siratro plants. Uninoculated plants were run as a control. Quarter strength sterile Jensen's medium was used as a nutrient solution. Tubes were kept in the glass house and observed for initiation and development of nodules during the course of growth. The nodules appeared on the roots were observed and counted after 30 days.

4.6.2 Nodulation tests on cowpea : To study nodulating and nitrogen fixing ability of the rhizobial strains method described by Leonard (1944) was employed using two brown beer bottles for the preparation of jar

assembly, acid washed coarse river sand for the growth of the plant, and Bergersen's nitrogen free mineral solution (Gibson 1980) as a nutrient solution. Cowpea (Vigna unguiculata L.walp) seeds were surface sterilised with 1:1000 $HgCl_2$, inoculated with one ml suspension of the isolate containing 10^8 cells/ml and sown in sterile sand in the Leonard jar. A homologous strain of cowpea viz. VK10 was used for comparison. Seeds sown without any inoculum treatment served as a control. Assemblies were kept in the glass house and volume of the nutrient solution in the reservoir made good during the course of growth.

Plants were removed after 45 days of growth, their roots washed and observations regarding height of the plant, number of nodules/plant, and size of nodules were taken. Nodules were detached from the roots, plant tops were cut off and were dried in the oven at $80^{o}C$ until the material attained a constant weight.

$$\text{Symbiotic effectiveness (S.E.) \%} = \frac{\text{Dry wt. of plant top inoculated with test strain}}{\text{Dry wt. of plant top inoculated with standard strain (VK10)}} \times 100$$

Plant dry weight ratio was determined as follows (Dadarwal et al. 1982):

$$\text{Plant dry weight ratio} = \frac{\text{Dry weight of inoculated plant}}{\text{Dry weight of control plant.}}$$

Symbiotic efficiency (Herridge and Roughley 1975) was expressed as:

$$\frac{\text{treatment plant dry weight - uninoculated control weight}}{\text{nodule dry weight}}$$

4.6.3 Acetylene reduction assay : To measure the acetylene reduction activity of the nodules formed by rhizobial strains under study, procedure described Hardy *et al.* (1968) was followed. The cowpea (*Vigna unguiculata* L.Walp) plants, inoculated with the rhizobia and grown in the Leonard jars as described earlier, were removed after 30 days of plant growth. The nodulated root system was cut and transferred to 65 ml vials and 10% of air from the bottle was replaced with commercial acetylene gas. The nodules were exposed to acetylene for one hour at room temperature and ethylene formed was detected with the help of Gas chromatograph using Porapak column T.

Assay conditions : The following system was used during the gas chromatographic analysis

Gas chromatograph : Chemito 3800

Column : Porapak T, 3 M x 3.1 mm, stainless steel.

Detector : Flame Ionization Detector.

Temperature : Injection Port : 110°C

Oven : 100°C isothermal

Detector : 110°C

Carrier : Nitrogen IOLAR 2 40 ml/min.

Flame : Hydrogen - 30 ml/min

Air - 300 ml/min

Syringe : S.G.E. microlitre 500 ul

Sample value : 500 µl

Retention times : Ethylene - 52 Sec.

Acetylene - 83 Sec.

After the analysis roots were removed from the assay bottles and nodules were detached and weighed. Total acetylene reducing nitrogenase activity was expressed as n moles of ethylene produced/plant/hour while specific acetylene reducing nitrogenase activity was expressed as n moles of ethylene produced/g dry weight of nodules/hour.

4.6.4 Effects of inoculation with the rhizobial strains on the growth of cowpea plants in unsterilized soil : The rhizobial strains showing pronounced nitrogen fixation ability in the Leonard jar experiments were further studied for effect of their inoculation of growth of cowpea (Vigna unguiculata L.walp) plants and for their ability to compete with other rhizobia for nodulation. Experiments were conducted in earthen pots filled with unsterilized soil. Cowpea seeds, surface sterilized with 1:1000 $HgCl_2$, were

inoculated with one ml of rhizobial suspension containing 10^8 cells and sown in the soil. Seeds sown without application of inoculum served as control. The pots were kept in the glass house and watered regularly.

Plants were harvested after 50 days of growth and observations regarding number of nodules, fresh weight of plant top were taken. To determine the nodule occupancy, 20 nodules from each plant were randomly selected. Each nodule was crushed in a little sterile saline, the suspension boiled at $100^{o}C$ in a water bath for 30 min. and tested against the antiserum of the respective strain by immunodiffusion.