PHYSIOLOGICAL AND GENETIC STUDIES ON GUAR *(CYAMOPSIS TETRAGONOLOBA)* RHIZOBIA WITH PARTICULAR REFERENCE TO PLASMIDS

BY

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1987

to

my beloved

dahiya family

CERTIFICATE-I

This is to certify that this dissertation entitled "physiological and genetic studies on Guar (*Cyamopsis tetragonoloba*) rhizobia with particular reference to plasmids", submitted for the degree of Doctor of Philosophy in Microbiology of the Haryana Agricultural University, is a bonafide research work carried out by Miss Sangeeta Mand under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE-II

This is to certify that the dissertation entitled "Physiological and genetic studies on Guar (*Cyamopsis tetragonoloba*) rhizobia with particular reference to plasmids" submitted by Miss Sangeeta Mand to the Haryana Agricultural University in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Microbiology has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.

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CONTENTS

INTRODUCTION

All plants require nitrogen for growth and in most environments the amount of available fixed nitrogen is low as to cause limitations in yield. Biological nitrogen fixation is the enzymatic reduction of atmospheric dinitrogen (N_2) to ammonia and its importance to plants is now widely realized. This awareness is largely due to the world wide energy crisis. Ammonium fertilizers are produced by high energy requiring chemical means. Each day more than 2×10^6 barrels of oil and huge amounts of electricity are consumed for this purpose. The increasing scarcity in the availability of oil and natural gas and alternate uses of electricity, necessitate increased utilization of biological nitrogen fixation.

The ability to fix dinitrogen is distributed among several prokaryotic taxonomic groups, including Azotobacteriaceae, Enterobacteriaceae, Rhodosporillaceae, Bacillaceae, Rhizobiaceae, Actinomycetaceae and Cyanobacteria (Burns and Hardy, 1975). The physiological conditions under which fixation of nitrogen occurs among these organisms vary but the enzymatic apparatus involved in nitrogen fixation appears to be largely similar.

According to quantitative analyses, leguminous plants in symbiosis with rhizobia, can fix nitrogen in a range of 50-300 kg per hectare per year (Phillips 1980). These amounts of nitrogen are sufficient to support good plant growth and yield. If greater reliance on biological nitrogen fixation is made, it may become unnecessary to apply expensive nitrogen fertilizers which imbalance ecosystem and create health hazards. These facts have stimulated scientists to exploit the biological process of nitrogen fixation.

Different species of rhizobia were defined earlier on the basis of the legumes they nodulated and were included under the same genus *Rhizobium* irrespective of their growth rate. Rhizobia are of two types, slow growing and fast growing, having generation times 6-13 h and 2-4 h respectively. According to the recent classification, root nodule bacteria are split into two genera (Jordon, 1982). The genus *Rhizobium* includes all the fast growers and the genus *Bradyrhizobium* includes slow growers. The genus *Rhizobium* has three species, *R. leguminosorum* (biovars *trifolii*, *phaseoli*, *viceae*), *R. meliloti* and *R. loti*. Many bradyrhizobia occur and except one recognized species, *B. japonicum*, all others are designated by the host plant eg., *Bradyrhizobium* sp. (*Vigna*), *Bradyrhizobium* sp. (*Lupinus*) etc.

Normal hosts of *Bradyrhizobium* species include many of the important grain legumes eg. *Arachis hypogea*, *Cicer arietinum*, *Glycine max*, *Psophocarpus tetragonolobus*, *Vigna unguiculata* as well as most other tropical clover and pasture plants. Fast growing rhizobia, however, exist that are capable of forming effective nodules with many of the hosts normally associated with *Bradyrhizobium* species (Trinick, 1982; Broughton *et al*., 1984).

Performance of a *Rhizobium* strain as a biofertilizer for a legume plant depends on genetic constitution of both the partners. To be used as a biofertilizer, *Rhizobium* should have, such qualities as specificity to a crop, effectivity in nitrogen fixation, competitive ability against other rhizosphere micro-organisms for better survival and growth under stressful conditions. To increase the efficiency of *Rhizobium*-legume symbiosis, or to extend the symbiotic relation to new crops, or even to integrate the functional nitrogen fixation genes within the plant genome, it is important to understand clearly the genetics of symbiotic and adaptive functions.

The molecular basis of nodulation and nitrogen fixation has been under investigation for a long time. During the last 10-15 years, great progress has been made in *Rhizobium* genetics. It has been shown that rhizobia carry large and small plasmids and the genetic determinants for symbiotic function are largely plasmid borne (Dunican and Cannon, 1971). Several *nod* and *nif* genes of the symbiotic apparatus have been defined in rhizobia (Banfalvi *et al*., 1983). Some of these plasmids have been shown to be self transferable (Johnston *et al*., 1978). Circular maps of the chromosomes of *R. meliloti*, *R. leguminosarum*, *R. phaseoli* and *R. trifolii* have been given in some detail (Beringer *et al*., 1978b,1980). Gene Banks have been constructed for several strains of *Rhizobium* (Friedman *et al*., 1982).

Although much is known about the regulation of nitrogenase in *Azotobacter* and *Klebsiella pneumoniae*, very little is known in case of rhizobia. This is due to the fact that this phenotype is restricted to symbiotic bacteroid form for most strains and thus involves a complex experimental system. Although symbiotic nitrogen fixation has been shown in *Bradyrhizobium* by a number of workers, progress in understanding the molecular biology of nitrogen fixation and nodulation is slow because of their long generation time and less amenability to genetic manipulations (Kuykendall, 1979). Also, the procedures established for preparation of plasmids for fast growers are not useful for slow growers (Cantrell *et al*., 1982).

The reports on the presence of *ex planta* nitrogenase activity in fast growing rhizobia are very rare (Dreyfus *et al*., 1983; Bender *et al*., 1986; Urban *et al*., 1986). Highly specific and regulated conditions are required for its expression.

Clusterbean, (*Cyamopsis tetragonoloba*) commonly known as guar, is a drought tolerant crop of Indian arid zone. It is widely cultivated in North West India under rainfed conditions and covers 2.81 million hectares of land with an annual production of 1.11 million tonnes of guar seed. Guar is grown not only for grain, vegetable and fodder purposes but also for its soil building properties. In recent years, it has gained great industrial importance because of the presence of gum in its endosperm which constitutes 35-40 % of the seed weight. Guar gum is in great demand in the world market because of its multipurpose use in textiles, foods, cosmetics, mining, explosives and oil industries.

Guar is nodulated by Rhizobium strains belonging to cowpea miscellany group. Nodulation status of guar rhizobia is generally poor due to many factors which include, plant genotype, moisture, pH, salinity, temperature and survival of rhizobia in soil. The amount of nitrogen fixed under field conditions by guar rhizobia may vary from 50-150 kg N/ha (Mal, 1969).

Broad host range, fast growth, presence of ex planta nitrogenase activity and presence of sym plasmid are important characteristics for using rhizobia to understand the genetic basis of nodulation, nitrogen fixation and cross-infectivity in cowpea rhizobia. Keeping these points in view, the present investigations were undertaken with the following objectives:

(1) Isolation of a large number of rhizobia from nodules of clusterbean (Cyamopsis tetragonoloba) collected from different regions of Haryana and establishment of purity of isolates by plant infection.

(2) Grouping of the isolated rhizobia on the basis of their growth rate and study of their antibiotic resistance pattern.

(3) Screening of isolates for the expression of ex planta nitrogenase activity.

(4) Testing of representative strains for their host range.

(5) Prevalence and nature of plasmids by agarose gel electrophoresis.

(6) Attempts to study the role of plasmids in nodulation, nitrogen fixation by using methods such as curing, mutagenesis etc.

REVIEW OF LITERATURE

The symbiotic relationship between legume and bacteria was discovered by Hellriegal and Wilfarth in 1888. In 1888 Beijerinck isolated the nodule bacterium and named it *Bacillus radicicola*. Later this name was changed into *Rhizobium* (Frank, 1889). In the following 50 years all the fundamental aspects of *Rhizobium*-legume symbiosis were understood. These were published by Fred *et al*. (1932) and Wilson (1940). The next two decades of research were devoted to understand the biochemical aspects of nitrogen fixation (Dixon, 1969), but much was not studied about its genetics.

Although the history of *Rhizobium* genetics may be traced back to 1941 (Krasilnikov, 1941a) it is only in the last few years that such studies have developed sufficiently to be able to form a framework for the analysis of the *Rhizobium* genes that determine symbiotic functions. Three findings have greatly influenced the direction of genetic research. They are:-

(i) Conjugation mediated by R plasmids with wide host range.

(ii) Large *Rhizobium* plasmids present in fast growers carry genes for nodulation and nitrogen fixation.

(iii) Hybridization of *Rhizobium* DNA sequences to cloned *K. pneumoniae nif* genes.

The present review will cover literature concerning presence and role of plasmids in rhizobia, physiology of *ex planta* nitrogenase, and Tn5 mutagenesis.

2.1. Plasmids:

Following the discovery of the tumour inducing plasmid of *Agrobacterium tumefaciens* (Van Larebeke *et al.*, 1974), attempts were made to examine for the presence of extrachromosomal DNA in *Rhizobium* strains and with the progressive refinement of techniques plasmids were eventually found (Nuti *et al.*, 1977; Uozumi *et al.*, 1980). Subsequently they were encountered in almost all *Rhizobium* strains, including slow growers (Rosenberg *et al.*, 1982; Masterson *et al.*, 1982).

2.1.1 Plasmids in fast growing rhizobia:

Most strains contain more than one plasmid species, ranging in size upto 300 megadaltons or more. *R. meliloti* strains, in particular, seem to have a very large plasmid with symbiotic determinants (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1982).

One of the functions first assigned to a plasmid was the host range of a *Rhizobium* strain. Higashi (1967) demonstrated transfer of clover infectivity from *R. trifolii* to *R. phaseoli*. Johnston *et al.* (1978) showed that transfer of derivatives of the plasmid pRLIJI from *R. leguminosarum* to strains of *R. trifolii* and *R. phaseoli* conferred to the transconjugants the ability to nodulate peas besides *Trifolium* and *Phaseolus*, the original hosts. Genes required for bacterial-plant recognition (*nod* genes) are shown to be located on plasmids of *Rhizobium*. Heat treated *R. trifolii* strains were enriched for non-nodulating strains (Zurkowski and Lorkiewicz, 1978). The derivative of *R. leguminosarum* strain A171 that has been cured of its smallest plasmid fails to nodulate peas (Casse *et al*., 1979). In *R. leguminosarum* strain 300, the mutant strain 6007, which carries a deletion in the 205 Md plasmid, is *nod*$^-$ and is restored to *nod*$^+$*fix*$^+$ by the introduction of the plasmid pRLIJI (Johnston *et al*., 1978). *R. leguminosarum* strain TOM was found to carry three plasmids (Brewin *et al*., 1980). The ability to nodulate was transferred from this strain to a non-nodulating strain 16015 by conjugation. The transfer of *nod*$^+$ character was associated with the transfer of a plasmid pRL5JI of MW 160 Md. In addition to genes for nodulation and host-range, the plasmid pRL5JI carried genes for nodule function (*fix*$^+$) that were absent from strain 16015.

In several *Rhizobium* species, genes that code for nitrogenase are located on large indigenous plasmids, (Nuti *et al*., 1979; Ruvkun and Ausubel, 1980 and Prakash *et al*., 1981). By DNA-DNA hybridization certain *Rhizobium* plasmids were shown to contain sequences homologous to *nif H* and *nif D* of *Klebsiella pneumoniae* (Rosenberg *et al*., 1982; Broughton *et al*. 1984 and Bender *et al*., 1986). In some *Rhizobium* species, *nif* and *nod* genes are also linked on the same large plasmid (Rosenberg *et al*., 1981, Long *et al*., 1982).

A plasmid which carries such *nif* homology on nodulation determinants is often called the *sym* plasmid of the strain. So far, plasmids with these characters have been found only in fast growers. Although symbiotic characters may be confined to only one of several plasmids in a given strain, this plasmid is not the same in all strains. Krol *et al.* (1982) isolated DNA from six strains of *R. leguminosarum* and found a large variation in the number and size of plasmids. The molecular weights of the *nif* plasmids ranged between approximately 130×10^6 to 550×10^6. Moreover, *sym* plasmids of the closely related *R. leguminosarum*, *R. trifolii* and *R. phaseoli* may share two-thirds or less of the same sequences (Prakash *et al.*, 1981). This indicates that specific DNA sequences which carry the structural genes for nitrogenase, are highly conserved on the *sym* plasmid. Considerable amount of work has been done on *sym* plasmids particularly in *R. meliloti*. In *R. meliloti* 41, some *nod* and *fix* genes have been located on PRme41b using heat curing of plasmid (Banfalvi, *et al.*, 1981). Genes controlling early and late functions in symbiosis have also been located on megaplasmid in *R. meliloti* (Rosenberg *et al.*, 1981). Pankhurst *et al.* (1983) carried out studies on megaplasmids of *R. meliloti* to confirm their role in nodule formation and nitrogen fixation. Transfer of *R. meliloti* *sym* genes to other rhizobia and *Agrobacterium* has also been carried out (Hirsch *et al.*, 1984; Fisher *et al.*, 1985; Long *et al.*, 1985). *Sym* functions in relation to *sym* plasmids have been studied also in *R. trifolii* (Hooykaas *et al.*, 1981; Zurkowski, 1982), *R. leguminosarum* (Hombrecher *et al.*, 1981; Johnston *et al.*, 1982; Brewin *et al.*, 1983), *R. phaseoli* (Lamb *et al.*, 1982; Martinez *et al.*, 1985) and other rhizobia (Morrison *et al.*, 1983, 1984; Pankhurst *et al.*, 1986).

Prakash et al. (1982) have shown that only relatively small portions of a sym plasmid are expressed in the nodule. Therefore, inspite of the excitement over its role in symbiosis, it remains possible that most of the plasmid DNA is for other purposes, especially the DNA of plasmids other than the sym plasmid (Krol et al., 1982).

Many plasmids are not necessary for growth in laboratory media either (Zúrkowski and Lorkiewicz, 1978). Growth and survival in soil poses nutrient, temperature and pathogenic challenges besides competition for nodulation among strains of the same species. Plasmids might carry functions to meet these stresses. Much more work is required to clarify the role of each plasmid. Variants which have been cured of a plasmid altogether are important in elucidating the role of that plasmid. Zurkowski and Lorkiewicz (1978) obtained non-nodulating mutants of R. trifolii after incubation for seven days at 35°C. Similarly Casse et al. (1979) after heat treatment of a strain of R. leguminosarum, obtained a non-nodulating mutant that lacked one of the three large plasmids of the parent strain. Higashi (1967) found that the ability of R. trifolii to nodulate clover was lost at high frequency following treatment with acridine orange. However, neither heat treatment nor acridine orange is universally successful, and no strain of Rhizobium exists that has been cured of all its plasmids (Beringer et al., 1980).

2.1.2 Plasmids in *R. japonicum* :

Parallel to the detection of plasmid DNA in many species of fast-growing rhizobia, studies on slow growing *R. japonicum* strains for plasmids were also carried out. Some strains of *R. japonicum* were found to contain plasmid DNA (Klein *et al.*, 1975; Luyindula *et al.*, 1975; Nuti *et al.*, 1977) but the number and molecular weight was not determined and no phenotypic character was correlated with the presence of these plasmids. Gross *et al.* (1979), later on reported the presence of plasmids of MW 49 to 118 Md in many *R. japonicum* strains. Further progress in the genetic studies of *R. japonicum* has been extremely slow. Obviously, their slow growth rate makes them less attractive for routine genetic manipulation. They have proved refractive to molecular biological studies due to the difficulty in detection of *sym* plasmid.

Keyser *et al.* (1982) reported the presence of fast growing *R. japonicum* strains. In contrast to a generation time of 6–13 h of the slow growing *R. japonicum* strains the fast growing strains have a generation time of 2–4 h.

The discovery of the fast growing *R. japonicum* strains allowed a rapid analysis of organisation of symbiotic genes in strains infecting soybean. Plasmids were detected in both the slow and fast growing *R. japonicum* strains. The slow growing strains usually contained one large plasmid, whereas the fast growing strains had one to three plasmids. Hybridization studies with *K. pneumoniae nif* genes showed that all fast growing strains, except PRC194 contained *nif* genes on large plasmids while slow growing *R. japonicum* strains carried them on chromosome (Masterson *et al.*, 1982).

Several fast growing strains were also found to contain one to four plasmids with MW 100 Md or more (Sadowsky and Bohlool, 1983). In one of the strains acridine orange produced mutants cured of largest plasmid and the mutants also lost the ability to nodulate soybean showing that the genes for nodulation were plasmid borne; high temperature curing of smaller plasmids did not affect nitrogen fixation. Applebaum *et al.* (1985b) showed that in soybean specificity for nodulation and cultivar specificity for nitrogen fixation were plasmid encoded.

Recently Barbour *et al.* (1985) isolated a fast growing rhizobial strain *R. fredii*. This strain carried both *nif* and *nod* genes on a 197 Md plasmid. Hybridization studies revealed that it carried multiple copies of *nif* genes located on chromosomal DNA, on *sym* plasmid and also on at least one of the smaller plasmids. Mathis (1985) obtained a mutant USDA206c by passage on acridine plates and observed that there was a correlation between loss of *sym* plasmid borne *nif* gene copies and reduction of overall symbiotic effectiveness.

Although some fast growing strains of *R. japonicum* have been studied in detail, with respect to *nod* and *nif* gene organisation, detailed studies of *nif* genes have been carried out only in one slow growing strain USDA 110 (Hennecke, 1981; Kaluza *et al.*, 1983; Hahn and Hennecke, 1984; Adams *et al.*, 1984; Fuhrmann and Hennecke, 1984; Hennecke *et al.*, 1985).

The slow growing strains are given a new generic name *Bradyrhizobium japonicum*,to account for the many differences by which these can be distinguished from the fast growing rhizobia (Jordon, 1982).

On the basis of hybridization experiments, it was reported recently (Masterson et al., 1985) that all R. japonicum strains examined carried nif and nod sequences on plasmid DNA with the exception that the strain USDA 194 contained these sequences on the chromosome. All the Bradyrhizobium japonicum strains showed the location of nif and nod genes on the chromosomal DNA and the genes were homologous to those of R. meliloti.

2.1.3 Plasmids in Rhizobium spp. cowpea:

Rhizobia belonging to the cowpea miscellany group include those strains that form symbiotic relationship with the roots of a large number of tropical legumes. It also includes strains of rhizobia that can fix nitrogen in symbiosis with the members of the non-legume Parasponia. Cowpea rhizobia have slow growth rate like R. japonicum rhizobia. Trinick (1982) however, reported fast growing rhizobia exist that are capable of forming effective nodules with many of the hosts normally associated with slow-growing strains.

Rhizobium ANU289 is a slow growing cowpea Rhizobium strain capable of effective nodulation with Parasponia. Scott et al. (1983) isolated the structural genes for nitrogenase from a Lambda genomic library of the DNA of this strain by hybridization with the cloned nitrogenase structural genes of Klebsiella. Weinman et al. (1984) studied the organisation and primary structure of *Rhizobium* strain ANU289 by hybridization analysis and revealed that there was only one copy of *nif* structural genes. *nif* H was seperated from *nif* D and *nif* K by at least 10 kb of DNA.

Hadley *et al*. (1983) identified DNA regions that were homologous to structural genes of nitrogenase by hybridization in several slow growing cowpea rhizobia isolated from various geographical locations. One such strain IRC78 was analysed by Jagadish and Szalay (1984). Analysis of different symbiotic mutants showed that a 12 Kb fragment consisted of 2kb *nif* K DNA adjacent to *nif* D. Noti *et al*. (1985) isolated a 11.76 Kb segment of DNA from this strain IRC78 (now named as *Bradyrhizobium* sp. (*Vigna*) strain IRC78) that hybridized to nodulation genes of *R. meliloti* 41. When this segment was transferred to *nod*$^-$ mutants of *R. meliloti*, it restored their ability to nodulate. Thus this segment carried common nodulation genes. Since no plasmids were found in this strain by Jagadish and Szalay (1984) *nif* and *nod* genes can be presumed to be located on the chromosome.

Bradyrhizobium strain Rp501 infecting *Parasponia* was found to have *nif* H at 25-30 Kb from *nif* DK. DNA sequences homologous to *R. meliloti fix* ABC genes were found to be located near *nif* H. Preliminary experiments with translation fusion of *nif* H with *lac z* suggested that *nif* H derepression in free living cells of this strain occured by a different regulatory circuit than derepression in nodules.(Ausubel *et al*., 1985).

ORS571 is a fast growing strain isolated from stem nodules of tropical legume Sesbania rostrata (Dreyfus and Dommergues, 1981). It can fix atmospheric nitrogen and grow in free living state. Elmerich et al. (1982) characterized some nif mutants which were unable to form colonies on plates under conditions of nitrogen fixation. All of them which appeared as fix$^-$ in planta were found to be nif$^-$ mutants. A 13 Kb Bam H1 fragment from the wild type strain complemented the nif mutant 5740 indicating that this was the DNA region carrying structural genes for mitrogenase. It is not known if the nif genes are plasmid borne or are located on the chromosome.

Bender et al. (1984) studied the plasmid profile of a fast growing Rhizobium strain IHP100 isolated from pigeonpea nodules collected in India by Kumar Rao of ICRISAT. The strain is capable of in vitro acetylene reduction. This strain harboured four plasmids with approximate Mwts of 500, 450, 300 and 115 Md. Probes containing nod genes from R. trifolii strain 843 hybridized to the 300 Md plasmid. The same plasmid showed hybridization with the nif probes from Klebsiella and R. trifolii 843, thus indicating that it was sym plasmid.

Broughton et al. (1984) analysed 49 fast growing rhizobial strains from the nodules of 26 different tropical legumes. All these strains were found to contain plasmids varying from one to five. Hybridization studies revealed that nif-nod genes were linked on a single sym plasmid in these strains. One of the strains NGR234 containing a single plasmid showed that an 80 Kb EcoRI fragment carried both *nif* and *nod* genes. When this plasmid was mobilized to *nod*$^{-}$ *R. meliloti* and *R. leguminosarum*, the transconjugants were *nod*$^{+}$.

Morrison *et al.* (1984) obtained nod^{-} mutants of the strain ANU240 (a streptomycin resistant spontaneous mutant of NGR 234). *Nod*$^{+}$ strains were found to contain three plasmids of M.W. 200, 20 and 25 Md. The *nod*$^{-}$ strains lacked 200 Md plasmid. This 200 Md plasmid was required for the formation of infection threads and root hair curling on siratro whereas it caused nodulation of a broad range of different legumes and the non-legume *Parasponia*. Hybridization ~~Hybridization~~ experiments showed that nitrogenase enzyme complex genes were also on this plasmid.

2.2. Ex planta Nitrogenase activity:

Biological nitrogen fixation (BNF) depends on the functioning of an enzyme complex called nitrogenase which is present only in prokaryotic microorganisms. The legume *Rhizobium* symbiosis is the major single supplier of biologically fixed nitrogen. Nitrogenase function represents the final operative phenotype of the legume root-nodule symbiosis.

Elucidation of the various physiological processes involved, and understanding their genetic regulation, will help in seeking ways to increase N_2 fixation by legumes. For improving nitrogen input into agricultural soils through exploitation of biological nitrogen fixation, two approaches are under investigation.

(i) Improvements in tho available nitrogen fixing associations, and

(ii) transfer of important characteristics of nodulating symbiosis to non-nodulating plants through genetic manipulations which is a long term research project. But many experimental approaches in understanding plant-microbe interactions have been hampered by the complexity of whole plant system. Development of *in vitro* systems have an important role in both these strategies.

2.2.1 Legume-Tissue culture Associations:

As a means of attempting to elucidate the factors controlling infection, nodule formation and induction of nitrogenase activity, Holsten *et al*. (1971) developed a soybean cell tissue culture-*Rhizobium japonicum* system. Structures like infection threads were found between cells. Upto 1-10 % of the cells were filled with bacteria enclosed in vesicles and at times nitrogen fixation was also detected. Subsequently, a number of reports appeared showing C_2H_2 dependent C_2H_4 production in tissue culture associations. Child and LaRue (1974) and Phillips (1974a) found that soybean cells induced nitrogenase activity in some strains of *Rhizobium japonicum*. A broad correlation between the activity in nodules and in tissue culture associations was perceived but it was not investigated further. Enhanced nitrogenase activity was observed when various nitrogen sources, especially glutamine and citric acid cycle intermediates were included in agar medium (Phillips, 1974b, 1976; Child and LaRue, 1976). In all cases the cultures were grown under air, and 0.2 atm oxygen of air in the assay gas mixture promoted highest activity.

Associations were shown for a range of legumes and rhizobia normally capable of nodulating these legumes *viz.*, cowpea (Scowcroft and Gibson, 1975), Lupins (Werner and Oberlies, 1975), *Stylosanthes gracilis* and red clover (Ranga Rao, 1976). Using strains of cowpea rhizobia and different sources of host cells (peas, sweet clover, *Vicia hajastane*). Child (1975) confirmed that the ability of a strain to nodulate a host was not essential for the induction of nitrogenase in the bacteria.

2.2.2 Non legume - Tissue culture Associations:

Child (1975) and Scowcroft and Gibson (1975) simultaneously demostrated the successful induction of nitrogenase activity in strain 32H1, in association with cultured cells of non-legumes, Child worked on callus cultures of wheat, brome grass and rapeseed. Scowcroft and Gibson (1975) worked with tobacco and showed 15N_2 incorporation into the callus, particularly in bacteria. Cultures of 32H1 grown alone on the agar medium did not develop the nitrogenase activity. The results indicated that the above non-legume possessed the factor(s) necessary for induction of nitrogenase and the factor(s) were not an unique property of legumes. Furthermore, it was shown that nitrogenase activity could be induced in strain 32H1 by culturing bacteria adjacent to, but not in contact with tobacco (Scowcroft and Gibson, 1975) and rice (LaRue *et al.*, 1975) cells.

2.2.3 N_2 fixation in cultured rhizobia:

The quest for nitrogen fixation by cultured rhizobia has been long and frustrating. In parallel with the tissue culture studies, circumstantial evidence accumulated which indicated that rhizobia possessed all the genes necessary for nitrogenase synthesis and function. Close similarity between Mo-Fe protein fractions of nitrogenase isolated from bacteroids of the same strain but taken from nodules on genetically different legumes was shown by Phillips *et al.* (1973) and Whiting and Dilworth (1974). Phillips *et al.* (1973) concluded that the genetic information for the synthesis of Mo-Fe protein was probably provided by the *Rhizobium*. Dunican and Tierney (1974) reported the transfer of *nif* genes from *Rhizobium trifolii* to a non-nitrogen fixing strain of *K. aerogenes*. Bishop *et al.* (1975) detected cross-reactivity between antisera prepared against Mo-Fe protein and extracts of *R. japonicum* cells grown and assayed under various conditions. Against this background of genetical, biochemical and tissue culture evidence, five simultaneous reports provided direct evidence that some strains of cultured rhizobia can reduce atmospheric nitrogen (Pagan *et al.*, 1975) or reduce acetylene on defined agar medium under air (Kurz and LaRue, 1975; McComb *et al.*, 1975; Pagan *et al.*, 1975) or in liquid medium under a low oxygen tension (Keister, 1975; Tjepkema and Evans, 1975). The finding that strain 32H1 reduces acetylene on laboratory media directly establishes that genes for nitrogenase are present in Rhizobium and renders invalid the theory proposed by Dilworth and Parker (1969), that part of the nitrogenase in the legume nodules might be specified by plant DNA (McComb et al., 1975).

All test systems used by above workers contained the inorganic salts, inositol and vitamins. Also, all the workers used Rhizobium sp. strain 32H1. An extensive study on nutritional and physical conditions affecting nitrogenase activity in the strain 32H1 was done by Gibson et al. (1976). Neither inositol nor vitamins were essential for the activity. Most of the systems contained two carbon sources along with pentoses and citric acid cycle intermediates, but one carbon source was also adequate (Tjepkema and Evans, 1975; Gibson et al., 1976). A range of combined nitrogen sources from inorganic NO_3^- and NH_4^+ salts to casamino acids could be used. Glutamine, at 1-2mM, appears to promote the highest activity (Gibson et al., 1976). High phosphate enhanced activity in 32H1 (Keister, 1975; Gibson et al., 1976b). Nitrogenase activity could be substantially increased and also sustained for longer periods by culturing bacteria at 25°C. All agar cultures were grown under air and maximum nitrogenase activity was at $pO_2 = 0.2$ atm. In liquid cultures, microaerobic conditions were required. The oxygen requirements for acetylene reduction were lower than any other known aerobic nitrogen fixing bacteria. Only by carefully balancing the oxygen (Berndt *et al.* 1976) concentration and the cell density, high rates of acetylene reduction be attained (Keister and Evans, 1976). In chemostat cultures of strain 32H1, activity was not observed until the oxygen concentration in solution fell to 1 µm (Bergersen *et al.*, 1976).

The induction of nitrogenase activity in free living cells of *Rhizobium* has mainly been demostrated in certain strains of slow growing group *R. japonicum* and the *cowpea* rhizobia .

Keister and Ranga Rao (1977) observed that the development of *ex planta* nitrogenase activity by *Rhizobium japonicum* was stimulated by carbondioxide and this was related to pH buffering effect of carbon-dioxide. However, Anguilar and Favelukes (1982) showed the expression and maintainence of nitrogenase activity in growing microerobic liquid cultures of *R. japonicum* 311b110 was stringently dependent on the sustained supply of carbon-dioxide but was not related to effects on pH.

Gibson *et al.* (1976) proposed that the mucoid material produced by rhizobia might aid nitrogenase activity by lowering the speed of transfer of O_2 as suggested by Postgate (1971) for other bacteria. Nitrogenase activity and slime production by *R. japonicum* 61-A-101 growing on solid medium were investigated by Wilcockson and Werner (1978). They found that slime production varied in different media but there was no simple correlation between slime production and high nitrogenase activity.

Agarwal and Keister (1983) studied thirty nine wild type strains of R. japonicum for their ability to synthesize nitrogenase ex planta in defined liquid media. The strains derepressable for activity had slower growth, lower oxygen consumption and lower extracellular polysaccharide production than non-derepressible strains. This reciprocal correlation between EPS production and nitrogeenase activity is just the opposite of the suggestions of Kurz and LaRue (1975) and Pagan et al. (1975),which were based on results with semi-solid agar medium. Agarwal and Keister (1983) proposed that in liquid cultures under very low oxygen concentrations, slime, instead of being beneficial, may limit availability of oxygen to the growing cells. Upchurch and Elkan (1977) also showed that non-slimy strain of Rhizobium japonicum developed more ex planta acetylene reduction activity than did the slimy derivative strain.

Kundu et al. (1981) showed ex planta nitrogenase induction on slopes of yeast extract mannitol glutamate-malate agar(YEMGMA) medium in slow growing Rhizobium strains of greengram.

Pankhurst and Craig (1978) observed that free living rhizobial cultures of 32H1 grown under nitrogen fixing conditions display pleomorphism similar to that of bacteroids; but not all pleomorphic cultures yielded acetylene reducing (AR) activity (Pankhurst and Craig, 1978 and Van Brussel et al., 1979). Kaneshiro et al. (1983) confirmed that Rhizobium sp. strain 32H1 and R. japonicum USDA26 and USDA110 grown on a glutamate-mannitol-gluconate agar medium showed increase in the number of pleomorphic cells coincident with their AR activity but strain USDA10 consistently failed to reduce acetylene, even though it could grow and yield pleomorphic cells under various conditions.

Ludwig (1984) proposed a model for free living nitrogen fixation in *Rhizobium* Sp. 32H1 (RC 3200) which asserts that this process occurs in non-growing cells. In nitrogen-limited liquid suspension cultures, cooperative growth of N_2 fixing and vegetative cells occur. The ammonium that is produced and exported by non-growing, N_2 fixing cells is transported to vegetative cells. This model implies the creation of metabolically specialized cells like cyanobacteria.

2.2.4 Ex planta N_2ase activity in fast growing rhizobia:

Reports on the presence of *ex planta* nitrogenase activity in fast growing rhizobia are few. Kurz and LaRue (1975) were the first to induce free living nitrogen fixation in fast growing *R. leguminosarum* TA101 but the activity was low as compared to 32H1. Bednarski and Reporter (1978) showed for the first time the expression of nitrogenase activity in free living cultures of various species of rhizobia *R. trifolii*, *R. meliloti*, *R. lupini* and *R. leguminosarum* but the fast growing rhizobia could be derepressed by the use of plant cell conditioned medium (PCM) or appropriate substances isolated from plant cell culture media with the aid of dialysis cultures. Hollander (1981) and Stam *et al.* (1983) were able to induce nitrogenase activity *ex planta* in *Rhizobium trifolii* and *R. leguminosarum* respectively in nitrogen-limited chemostat cultures at very low oxygen concentrations. These strains were less suitable for genetic studies requiring a simple rapid assay; such that used for the expression of *ex planta* nitrogenase activity by *Bradyrhizobium*.

A notable exception is the *Sesbania rostrata* stem nodule isolate ORS571. (Dreyfus and Dommergues, 1981). It resembles *Klebsiella* spp. with regard to its high rate of free living nitrogen fixation and also in its ability to use N_2 as the sole nitrogen source for growth in liquid medium (Dreyfus *et al*., 1983).

Recently, Urban *et al*. (1986) showed that *Rhizobium trifolii* 0403 when treated with 16.6mM succinate and other nutrients can be induced to grow in nitrogen free medium. The effects of oxygen tension and energy availability on fixation rates suggest that it is typical of other nitrogen-fixing bacteria, including *Azotobacter* spp. The rate of fixation is intermediate between that of *Azotobacter* spp. and *Bradyrhizobium*.

Bender *et al*. (1986) showed nitrogenase activity in *Rhizobium* strain IHP100 isolated from root nodules of *Cajanus cajan*. Nitrogenase activity in agar culture under air occured at a rate similar to that found for *Bradyrhizobium* strain CB756 but lower than that for *Rhizobium* strain ORS571.

2.3. Transposon mutagenesis:

Transposons are discrete segments of DNA which are capable of movement, or transposition from site to site. The movable elements were discovered in maize by Barbara McClintock in the 1940's who then proceeded to demonstrate a remarkable array of properties associated with them, such as controlled chromosomal breakage, effect on gene expression, localized chromosomal breakage, effect on gene expression, localized mutagenicity etc. (Mc Clintock, 1951). Elements with similar properties were discovered in bacteria around 1968. Most of them carry a drug resistance gene which can be used to mark their location in the bacterial DNA. Transposons cause mutations in genes into which they insert themselves. As a result, transcription is interrupted, and downstream genes are not expressed in the operon. The use of transposons as mutagens has helped to largely overcome the problems associated with genetic analysis of symbiotic determinants, (Beringer et al., 1980; Meade et al., 1982; Forrai et al., 1983).

2.3.1 Transposon Tn5:

Resistence to various antibiotics has been found to be encoded by a number of bacterial transposons. Transposon Tn5 carries the marker for Kanamycin and Neomycin resistance (Kleckner, 1977). Transposon Tn5 is particularly useful because of its ability to insert 'randomly' into a genome.

Tn5 DNA is known to encode the following functions. (i) The enzyme neomycin phosphotransferase II, which results in resistance to several aminoglycoside antibiotics such as Kanamycin (Km) and Neomycin (Nm) (ii) Transposition functions.

Tn5 has been extensively used as a mutagenic tool in the genetic analysis of Rhizobium legume symbiosis and nitrogen fixation (Meade et al., 1982; Ausubel, 1982; Forrai et al., 1983; Noel et al., 1984). Devos et al. (1984) reported another advantage in the use of Tn5. They have found Tn5 specific streptomycin resistance in Rhizobium. This host mediated differential expression of Str^r of Tn5 provides an additional marker for genetic manipulations in Rhizobium.

2.3.2 Suicide plasmids in transposon mutagenesis:

The use of transposons was pioneered in *E. coli* and the techniques are well established for enteric bacteria (Kleckner, 1977). In transposon mutagenesis once a transposon has been introduced into a particular host using a suitable vector molecule the problem is the elimination of the vector.

It has been noticed that P-type plasmids that carry an inserted mutagen have a reduced ability to become stably inherited in Rhizobium or Agrobacterium strains (Boucher *et al.*, 1977). Taking advantage of the unstable "plasmid suicide effects", the transposon Tn5 has been inserted into the genome of different rhizobia (Beringer *et al.*, 1978a). However, the elimination of RP4 Mu vectors is not always equally efficient. In *R. meliloti* the elimination is very efficient (Simon, 1984). Till 1983 the suicide vehicles used for the members of Rhizobiaceae family were the cointegrates of a conjugative, Inc-P group plasmid and bacteriophage Mu, with the Mu genome conferring the suicidal property. The suicide plasmid pJB4JI (Beringer *et al.*, 1978a) is such a plasmid which has been extensively used in mutagenesis experiments in Rhizobium (Ruvkun *et al.*, 1980; Meade *et al.*, 1982; Casey *et al.*, 1983).

Selvaraj and Iyer (1983) found that in addition to inefficient elimination of Mu containing suicide plasmids, these vehicles also induce secondary genetic changes incited by Mu itself. These observations suggested the need of developing a suicide vehicle that did not contain Mu DNA. Selvaraj and Iyer (1983) constructed such a set of plasmid vectors Tn1, Tn5 and Tn9 that are suicidal in Rhizobium. These constructed vectors have a þ 15A-type of replicon and an N type of bacterial conjugation system which is very efficient in matings between E. coli and Rhizobium species. In this way a suicidal plasmid vector for transposon Tn5, þGS9 was constructed. By cloning a region of N group plasmid þCU1 specifying tra genes into multicopy vector þACYC 184, resultant þCU101 was obtained. HB101 rec A (þCU101) was infected with λ :: Tn5 to obtain þGS9 having "tra" region from þCU1 (N type) in þ15A group plasmid having Tn5. Because of þ15A replicon, þGS9 cannot replicate in Rhizobium and have function like suicide plasmid.

2.3.3 Transposon induced mutants : nature and use in genetic studies:

The advent of bacterial transposable elements coding for antibiotic resistance and causing mutagenesis in Rhizobium made possible the genetic analysis of symbiotic determinants (Beringer et al., 1980). Most mutants made by insertion of a transposon are non-leaky. Every insertion had a strong polar effect on the expression of the distal genes in the operon and were stable. However, reversion by excision occured but at a low frequency (Meade et al., 1982).

There are two approaches for finding genetic variants which affect the symbiosis by the use of transposons.

(i) Mutants having a variety of phenotypes can be induced, in free living <u>Rhizobium</u> cells, such as those harbouring alterations in related processes like exoploysaccharide production, phage resistance or synthesis of metabolites *and these can then be tested for changes in the symbiotic* properties. (Denarie <u>et</u> <u>al</u>., 1976 and Sanders <u>et</u> <u>al</u>., 1978).

(ii) A large number of random mutants can be raised on the basis of requirement of selectable marker of transposon which can then be screened to isolate those altered for symbiotic properties.

Both these approaches have been widely used and a variety of phenotypic mutants and symbiotic mutants have been obtained by Tn5 mutagenesis by various workers.

In <u>Rhizobium</u>, various Tn5 mutants defective in symbiotic properties have been isolated in various <u>Rhizobium</u> species such as <u>R</u>. <u>leguminosarum</u> (Downie <u>et</u> <u>al</u>., 1983), <u>R</u>. <u>trifolii</u> (Rolfe <u>et</u> <u>al</u>., 1983), <u>R</u>. <u>phaseoli</u> (Noel <u>et</u> <u>al</u>., 1984), <u>R</u>. <u>loti</u> (Chua <u>et</u> <u>al</u>., 1985) and a large number in <u>R</u>. <u>meliloti</u> (Meade <u>et</u> <u>al</u>., 1982; Forrai <u>et</u> <u>al</u>., 1983).

Transposon Tn5 has been used to mutagenize the entire genomes (Meade <u>et</u> <u>al</u>., 1982; Scott <u>et</u> <u>al</u>., 1982; Forrai <u>et</u> <u>al</u>., 1983 and Noel <u>et</u> <u>al</u>., 1984), or transmissible indigenous plasmids (Buchanan-Wollaston <u>et</u> <u>al</u>., 1980). Another additional advantage of Tn5 mutagenesis has been that it can be used for site-specific mutagenesis of <u>Rhizobium</u> DNA regions that have been cloned in *E. coli*. This can help in the isolation of mutants in the pathways involved in establishing a complex process like nodule formation or nitrogen fixation. The technique has been applied in *R. meliloti* (Ditta *et al.*, 1983 and Anguilar *et al.*, 1985), *R. trifolii* (Djordjevic *et al.*, 1985), *R. leguminosarum* (Hombrecher *et al.*, 1984), *R. japonicum* (Hahn and Hennecke, 1984) and cowpea *Rhizobium*(Jagadish and Szalay, 1984; Noti *et al.*, 1985).

MATERIAL AND METHODS

Materials

3.1 Bacterial strains

Escherichia coli WA 803 (pGS 9::Tn5) strain of Selvaraj and Iyer (1983) was procured from Dr. Susheel Kumar, IARI, New Delhi. The rhizobial strains used were isolated during the course of this study.

3.2 Host cultivars

Different legume cultivars used in the present investigation were :

(1) Guar (*Cyamopsis tetragonoloba* (L.) Taub) CV. HG-75

(2) Pigeon pea (*Cajanus cajan* (L.) Millsp.) CV. UPAS-120

(3) Mung bean (*Vigna radiata* (L.) Wilczok) CV. K-851

(4) Urd bean (*Vigna mungo* (L.) Hepper) CV. T-9

(5) Rice bean (*Vigna umbellata* (Thunb) Ohwi & Ohashi) CV. RB53

(6) Soybean (*Glycine max* (L.) Merrill.) CV. Bragg

(7) Chickpea (*Cicer arietinum* (L.) CV. H75-35

The seeds of different cultivars were obtained from Department of Plant Breeding, Haryana Agricultural University, Hisar.

3.3 Chemicals

Agarose, sodium dodecyl sulphate, ficoll, ethidium bromide, ethylene diamine tetra acetate, lauryl sarcosinate, trizma base, boric acid, bovine serum albumin, naladixic acid, erythromycin, chloramphenicol, streptomycin, neomycin, kanamycin, ampicillin, tetracycline, lysozyme, pronase, RNase I were from

Sigma Chemical Co., U.S.A. Most of the media components such as yeast extract, mannitol, bactotryptone, bactoagar and other chemicals were purchased from manufacturers in India. The reagents used were of Analar grade from Glaxo labs, E. Merck, Himedia, Ranbaxy, Sarabhai, SD's and Bengal Chemicals and Pharmaceuticals Ltd.,India.

3.4.Preparation of media

All the media were prepared in distilled water. Unless otherwise stated, the sterilization was done at 15 lbs/sq inch pressure for 20 min. Heat labile components were filter sterilized through 0.45 µm nitrocellulose membranes and later added to cooled media. Composition of different media used during the course of this work is given below:

(i) Yeast extract mannitol agar medium (YEMA) (Fred *et al*.,1932):

	g/l
Mannitol	10.0
$K_2HPO_4.3H_2O$	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
$CaCO_3$	1.0
Yeast extract	0.5
Congo red (1:400)	5 ml
Agar-Agar	15-20
pH	6.8-7.0

(ii) Sloger's nitrogen free nutrient solution (Sloger, 1969):

	g/l
$K_2HPO_4.3H_2O$	0.2
$MgSO_4.7H_2O$	0.2
NaCl	0.2

$MnCl_2.H_2O$	0.004
$CuSO_4.5H_2O$	0.0002
Na_2EDTA	0.03
H_3BO_3	0.00025
$CaHPO_4$	1.0

(iii) Minimal medium (Van Egeraat, 1972):

	g/1
$K_2HPO_4.3H_2O$	0.5
$MgSO_4.7H_2O$	0.2
$Ca\ Cl_2.2H_2O$	0.04
$FeCl_3.6H_2O$	0.005
Agar	10.0
Micronutrient solution	5.0 ml
pH	6.8

Composition of micronutrient solution

	g/1
$MnSO_4.7H_2O$	0.2
$ZnSO_4.7H_2O$	0.2
$CuSO_4.5H_2O$	0.02
$CaCl_2.6H_2O$	0.002
H_3BO_3	0.2
$Na_2MoO_4.2H_2O$	0.2

(iv) Rhizobium minimal medium (RMM) :

First stock solution 'A' and 'B' were prepared. The solution 'A' was 20% glucose or any other sugar at 20%. The solution 'B' called 2xR salt solution (2xR S S) had the following composition.

Salt	Stock solution	Amount of stock solution added/l	Final concentration obtained per litre
$Na_2HPO_4 \cdot 12H_2O$	4.5%	20 ml	0.90g
$(NH_4)_2SO_4$	20%	20 ml	4.0g
$FeCl_3.6H_2O$	1%	0.4 ml	4.0mg
$MgSO_4 \cdot 7H_2O$	1 M	0.4 ml	0.2g
$CaCl_2 \cdot 2H_2O$	1 M	0.2 ml	0.08g
H_2O to make 1 litre volume			

One litre of RMM, was prepared as follows : To 500 ml of H_2O, 16g of agar and 10 ml of solution 'A' were added and autoclaved. Separately, 500 ml of 2xRSS was also autoclaved, both, cooled to 50°C and mixed. At this stage, supplements were added, if required and the medium poured in sterile plates. To make liquid RMM medium, the procedure described above was used except that agar was omitted.

(v) Tryptone Yeast extract medium (TY) (Beringer, 1974):

	g/1
Tryptone	5.0
Yeast extract	3.0
$CaCl_2.6H_2O$	1.3
Agar-agar	15-20

$CaCl_2$ was sterilized seperately and mixed later.

(vi) Nitrogenase Induction medium (NIM) (Kundu *et al.*, 1981):

	g/1
Mannitol	10
$K_2HPO_4.3H_2O$	0.25

$MgSO_4.7H_2O$	0.2
NaCl	0.1
$CaCO_3$	0.5
Yeast extract	0.2
Sodium glutamate	0.32
Sodium malate	1.6
Inositol	0.1
Sodium molybdate	0.00025
Thiamine HCl	0.0001
Nicotinic acid	0.0001
Pyridoxal HCl	0.0001
$FeSO_4.7H_2O$	0.00028
Na_2EDTA	0.00032
$MnSO_4$	0.0001
$ZnSO_4.7H_2O$	0.0002
$CuSO_4.5H_2O$	0.00004
H_3BO_3	0.0003
$CoCl_2.6H_2O$	0.000025
KI	0.00078
Agar-agar	15
pH	7.5

(vii) <u>Modified Burk's medium</u>:

	g/l
Mannitol	20.0
$KH_2PO_4.3H_2O$	0.2
K_2HPO_4	0.8
$MgSO_4.7H_2O$	0.2
$FeSO_4.7H_2O$	0.1

NaCl	0.2
$CaCl_2.2H_2O$	0.09
Na_2MoO_4	0.05
Inositol	0.1
Thiamine HCl	0.0001
Nicotinic acid	0.0001
Pyridoxal HCl	0.0001
Agar-agar	15
pH	7.0

Methods

3.5. Isolation of rhizobia from nodules: (Vincent, 1970)

Nodules were obtained from roots of guar plants collected from Tosham, Bhiwani, Dadri, Rewari, Bawal, Gurgaon, Samaspur, Tikri, Sohna, Palwal, Ballabhgarh, Rohtak, Hansi and different locations around Hisar. They were stored until use in sterile tubes containing anhydrous calcium chloride. The nodules were activated in the laboratory by putting them in sterile distilled water for two h and washed thoroughly. The nodules were surface sterilized by dipping in 0.2% mercuric chloride for 3 min followed by keeping in alcohol for 3 min. The surface sterilized nodules were washed with sterile distilled water five to six times so as to remove the traces of mercuric chloride and alcohol. Each nodule was cut aseptically and a loopful of its inside sap streaked on YEMA medium plates. The plates were incubated at 28±2°C for 6-15 days. Single colonies appearing white and mucoid were picked and restreaked. Each isolate was purified by repeated streaking.

3.6. Maintenance of rhizobial isolates:

All the isolates were transferred to slants of YEMA medium, grown for 6-8 days at 28±2°C and finally stored at 4°C in a refrigerator. Each isolate was subcultured periodically.

3.7. Plant infection test in chillum jars:

Chillum jar assemblies containing washed sand were autoclaved twice on successive days for 2 h at 15 lbs/sq inch pressure. Surface sterilized seeds of guar were germinated on soft agar plates, treated with *Rhizobium* isolates (10^6-10^7cells ml^{-1}), and transferred to *chillum* jars. Each *chillum* jar containing four seeds was later kept in the pot house. The plants were supplied with sterilized water daily and Sloger's nitrogen free nutrient solution once a week. Three replicates were kept for each isolate. For testing the performance of these isolates on other crops the seeds of urdbean, mungbean, pigeonpea, ricebean, soybean and chickpea were also used.

3.8. Plant infection test under test tube conditions:

Twenty five ml of Van-Egeraat medium was taken in 150x25 mm tubes and autoclaved. Seeds were surface sterilized and germinated on soft agar. The germinated seeds were then transferred to test tubes and their redicle were inserted in the medium of the tube. One ml of log phase culture (10^6-10^7 cells ml^{-1}) was added over the germinated seeds. The tubes were wrapped in black carbon paper, placed in test tube stand and then transferred to growth chamber fitted with flourescent tube and bulbs of incandescent light. Temperature of the growth room was maintained at 28±2°C with 14h light and 10h dark periods.

The plants were removed for observation after 30-35 days of growth for nodulation.

3.9. Assay for nitrogenase:

(i) Ex planta nitrogenase: (Kundu *et al*., 1981)

Test tubes (15x125 mm) containing 5 ml of nitrogenase induction medium or YEMA or Burk's medium were sterilized and slants prepared. The cultures to be tested were inoculated on the slants and incubated for 4 to 6 days at 28±2°C. Plugs were then replaced by serum stoppers. One ml of air from each tube was replaced with one ml of acetylene. The tubes were again incubated at 28±2°C for 48 h. Gas sample (0.5 ml) from each tube was analysed by Nucon Gas liquid chromatograph fitted with flame ionization detector and Porapak N column. N_2 at a flow rate of 45 ml/min and H_2 at a flow rate of 25 ml/min were used as carrier and fuel gases, respectively. The oven temperature was maintained at 100°C. Standard ethylene (110 vpm) was used for calibration purpose. Nitrogenase activity was expressed as ARA (n moles h^{-1}mg protein^{-1}). In case of liquid medium, the composition was same excepting that agar-agar was omitted. The medium was inoculated and kept on shakar at 28±2°C for 4 to 6 days, and subjected to same procedure as described for solid slants.

(ii) In planta nitrogenase: (Hardy *et al*., 1968)

Plants were harvested gently from the chillum jars after 40 days of growth. The nodulated roots were transferred to B-24 neck conical flasks fitted with serum stoppers. A 10 % atmosphere of acetylene was created in the flasks thereafter they were incubated at 30°C for 1 to 2 h. Ethylene in 0.5 ml of gas samples was determined by gas liquid chromatograph as described earlier. The nodules were later detached from the roots, washed with water, dried in the folds of filter paper and weighed. Dry matter accumulation was determined by weighing the shoot portions after drying at 70°C for 5 days. The nitrogenase activity was expressed as ARA (μ moles h^{-1} $plant^{-1}$).

3.10. Estimation of protein in whole cells:

The method described by Herbert et al. (1971) was followed

(i) Na_2CO_3 (5% W/v)

(ii) $CuSO_4.5H_2O$ (0.5 % W/v) in 1% (W/v) sodium potassium tartarate.

(iii) Alkaline copper reagent prepared freshly by mixing 50 ml of reagent (i) and 2 ml of reagent (ii)

(iv) Dilute Folin Ciocalteu reagent (1N):

(v) Protein standard solution (Bovine serum albumin) 250 μg ml^{-1}.

Procedure

After the nitrogenase assay, the bacterial growth from each slant was collected in a total of 5 ml of sterile distilled water. To a suitable aliquot (0.5 ml) in triplicate, 0.5 ml of 1N NaOH was added and placed in a boiling water bath for 5 min. After cooling the tube, 2.5 ml of alkaline copper reagent was added, mixed thoroughly and allowed to stand for 10 min. This was followed by a rapid addition of 0.5 ml of dilute Folin ciocalteu reagent with immediate mixing. After 30 min the absorbance was read at 750 nm against reagent blank using a spectrophotometer.

3.11. Intrinsic resistance to antibiotics:

Antibiotic resistance was determined by spot tests. Logarithmically growing cultures were used for spotting. A loopful of the culture was spotted on the plates of YEMA medium containing varying concentrations of antibiotics. The plates were incubated at 28±2°C for 6 to 8 days and observed for growth.

3.12. Growth on various sugars and organic acids:

Sugar and organic acid utilization was determined by spot tests. The sugars were added in RMM at a concentration of 10 g per litre and organic acids were added at the rate of 2 g l^{-1}. Growth was seen after 6 days of incubation at 28±2°C.

3.13. Plasmid detection by gentle lysis:

Horizontal slab gel electrophoresis:

The method originally described by Eckhardt (1978) and as modified by Rosenberg *et al.* (1982) was used. Cells were grown in 20 ml of TY broth on shaker till absorbance reached between 0.4 to 0.5. The cells were harvested, washed with 0.1 % sarkosyl solution in TE 8 buffer (50mM Tris 20mM EDTA, pH8), rinsed with TE8 and suspended in 2 ml of TE8 buffer.

Cell suspension (100 µl) was taken in an Eppendorf tube and centrifuged at 15,000 g for 2 min. The cell pellet obtained was suspended in 40 µl of lysing mixture (Lysozyme, 7500 units/ml; RNase I, 0.3 K units/ml; Bromophenol blue 0.05 % and Ficoll 400, 20 %) in TBE buffer (89 mM Tris base - 2.5 mM disodium EDTA-89 mM boric acid, pH 8.2). The cells were mixed with the lysing mixture with a sterile tooth pick and the suspension was kept at room temperature for 5 min to allow lysozyme action.

Lysis was completed by adding 40 µl of SDS mixture (SDS, 0.2 % and Ficoll 400, 10 % in TBE). The two layers of lysing mixture and SDS were mixed gently by tipping and kept for 5 min at room temperature. Now 5 µl of pronase solution (5 ug ml^{-1} of TBB buffer) was added, mixed gently by tipping and kept for 5 min at room temperature.

The lysate (85 µl) was then loaded into the slot of a horizontal agarose gel (0.6 % in TBB). The slots were then sealed with 0.6 % agarose in TBB and allowed to solidify. Both the chambers of horizontal gel electrophoresis apparatus were filled with electrophoresis buffer (TBB) and connected to power supply. The gel was run at 15mA for 2 h and 40 mA for 20 to 25 hours.

After the run was over, the gel was taken out and stained for 15-20 min in aqueous solution of ethidium bromide (0.4 µg ml^{-1}). The gel was visualized under short wave UV light (300nm) transilluminator. Photographs were taken with an Olympus, OM-2 camera using 400 ASA film (ORWO,FDR Germany).

3.14. A rapid alkaline extraction procedure for screening plasmid DNA: Birnboim and Doly (1979)

Cells were grown overnight in 5 ml of TY broth, the culture was transferred to 1.5 ml Eppendorf tube and centrifuged to get sufficient cell pellet. The cell pellet was suspended in 100 µl of solution I (2 mg/ml lysozyme, 50mM glucose, 10mM EDTA, 25mM Tris Hcl pH 8.0). After a 30 min period of incubation at 0°C, 200 µl of solution II was added (0.2 N NaOH, 1% SDS) and the tube was gently vortexed. The suspension became almost clear and slightly viscous. The tube was kept for 5 min at $0^{o}C$ and then 150 µl of solution III (3M sodium acetate, pH 4.8) was added. The contents of the tube were gently mixed by inversion for a few seconds. The tube was maintained at $0^{o}C$ for 60 min to allow most of the protein, high mol. wt. RNA and chromosomal DNA to precipitate. The mixture was centrifuged for 5 min. The clear supernatant was transferred to a second centrifuge tube, one ml of cold ethanol ($-20^{o}C$) was added and finally kept overnight in deep freeze ($-70^{o}C$). The precipitate was collected by centrifugation for 2 min and the supernatant removed by aspiration. The pellet was dissolved in 100 µl of 0.1 M sodium acetate/0.05 M Tris Hcl (pH 8.0) and reprecipitated with 2 volumes of cold ethanol. After 10 min at $-20^{o}C$, the precipitate was again collected by centrifugation as before. It was dissolved in 20 ul of solution I and loading buffer was charged into agarose wells and run at 10mA for 1 h and 40mA for 24-25 h as before.

3.15. Effect of temperature on ex planta nitrogenase activity:

Two approaches were followed to see the effect of temperature on the synthesis and activity of nitrogenase.

(i) To see the effect on synthesis of nitrogenase enzyme YEMA slants were streaked with the culture and the slants were kept at different temperatures i.e. $25^{o}C$, $30^{o}C$, $35^{o}C$, $40^{o}C$ and $45^{o}C$. After 96 h, 10 % C_2H_2 atmosphere was created. Slants were again incubated at the respective temperatures for an additional 48 h and *ex planta* nitrogenase activity determined.

(ii) To see the effect on activity of nitrogenase enzyme YEMA slants were streaked with the culture and incubated at 30^oC for 96 h. A 10 % acetylene atmosphere was created and the slants incubated at different temperatures for 48 h. Five slants each were kept at 25^oC, 30^oC, 35^oC, 40^oC and 45^oC and acetylene reducing activity was measured after incubation. Nitrogenase activity was expressed as ARA (n moles h^{-1} mg $protein^{-1}$).

3.16. Effect of combined nitrogen sources on nitrogenase activity:

Two nitrogen sources, potassium nitrate and ammonium chloride, were used for these studies. Yeast extract mannitol agar medium (5 ml) slants as well as YEM broth (5 ml) were prepared with 1, 2, 5, 10, 20, 50 and 100mM concentrations of KNO_3 per ml. The slants and broth were inoculated with log phase culture and incubated for 96 h. A 10 % acetylene level was created and then incubated for 48 h. Nitrogenase activity was measured and expressed as ARA(n moles h^{-1} mg $protein^{-1}$). Similar procedure was adopted for using ammonium chloride as nitrogen source.

3.17. Growth and expression of nitrogenase activity on nitrogen free medium:

Burk's medium was modified by substituting sucrose with mannitol and further adding inositol and other vitamins. As described above, slants (5 ml agar medium) as well as broth (5 ml) were used. The culture was scrapped from YEMA plates, washed twice with nitrogen free Burk's medium, suspended in

Burk's medium and used for inoculation. A loopful of culture was spread on slants while 0.1 ml of cell suspension was used for inoculation of broth.

The slants were incubated at 28±2°C for 2, 4 and 6 days and ARA was measured as described earlier. Tubes containing nitrogen free broth were inoculated and kept on shaker at 28±2°C. Samples were removed on 2, 4 and 6 days and nitrogenase activity was determined.

3.18. Plasmid curing:

High temperature and certain chemicals eliminate the plasmids from the cells by preferentially inhibiting the replication of plasmid DNA. Since the concentration of the curing agent should not affect the replication of chromosomal DNA, the non-growth inhibitory concentration was determined.

(i) Temperature :

The cultures were inoculated (1 % inoculum) in 100 ml of YEM broth in 250 ml conical flasks. One set of flasks were incubated at normal temperature for growth(28±2°C) and other sets at different higher temperatures. The change in optical density was followed after 48 h of incubation. The maximum temperature at which there was no inhibition of growth was taken as non-growth inhibitory high temperature.

The culture was grown at selected high temperature for 6-8 days, a small amount of the culture appropriately diluted and plated on YEMA plates to obtain single colonies. Each clone was transferred in duplicate on nitrogenase induction medium and *ex planta* nitrogenase activity was determined.

(ii) Chemical agents:

Two chemicals, Ethidium bromide (EtBr) and Sodium dodecyl sulphate (SDS) were used for curing of plasmid DNA. The non-growth inhibitory concentration of each chemical was determined as follows:

Flasks containing different concentrations of curing agents in YEM broth were inoculated at 28±2°C. The maximum concentration of the agent at which there was no inhibition of growth compared to the growth in control flask was taken as non-growth inhibitory concentration.

The culture was inoculated and incubated in the medium containing the curing agents at their respective non-growth inhibitory concentrations. The cultures were plated to get single clones, all of which were then individually tested for the expression of <u>ex planta</u> nitrogenase in the same manner as in case of clones obtained after incubation at high temperature.

(iii) Serial transfer in SDS and high temperature:

The culture was grown in YEM broth containing 100 μg/ml of SDS at 39°C. An aliquot from this was inoculated to fresh medium flasks containing SDS, allowed to grow for 3-4 generations and again transferred. The serial transfer was continued for a large number of generations and at the time to 5th, 10th and 15th transfer, an aliquot of culture was appropriately diluted and plated to get single clones. Each clone was then tested for the expression of <u>ex planta</u> nitrogenase activity.

3.19. Transposon mutagenesis:

Modified procedure of Selvaraj and Iyer (1983) was used to obtain Tn5 mutants, WA803 (pGS9::Tn5) (Km^r Nm^r) were mixed in 1:1 ratio and seven patches were made on TY plates using 20 µl of bacterial suspension. After the patches got dried, the plates were incubated at 30°C for 24 h. Later the growth of each patch was suspended in one ml of TY broth in a tube. Each of the suspension so obtained was plated directly and also after tenfold dilution on TY agar medium containing 100 µg ml^{-1} kanamycin and 25 µg ml^{-1} naladixic acid. The plates were incubated at 30°C for 5-6 h to obtain antibiotic ($Km100^r$ $Nx25^r$) resistant Tn_5 mutants. The Tn_5 mutants obtained were tested for the expression of *ex planta* nitrogenase activity on solid nitrogenase induction medium and also for nodulation in plastic pouches and test tubes as described earlier.

EXPERIMENTAL RESULTS

During the course of present investigations, one hundred rhizobia were isolated from root nodules of guar Cyamopsis tetragonoloba collected from different agroclimatic regions of Haryana. Sixty four of them were slow growers while thirty six were fast growing Rhizobium isolates.

The nodulation status of guar in Haryana is generally poor. Keeping this in view, the overall objective of the present study has been kept as to carry out investigations which will help in the understanding of physiology and genetics of guar rhizobia.

The proposed physiological studies included determination of growth rate, efficiency, host range, carbohydrate utilization, antibiotic resistance pattern and ex planta nitrogenase activity of selected strains. Genetic studies included curing treatments and Tn5 mutagenesis.

4.1 Screening of guar rhizobia isolates in chillum jars:

All the isolates were screened in chillum jar assemblies under pot house conditions to select efficient strains for further physiological and genetic studies. The plants were uprooted after forty days and observations on per plant basis for nodule number, nodule fresh weight, shoot dry weight and nitrogenase activity as acetylene reducing activity (ARA) were recorded. Frequency of variation with respect to each of these characters is given in Table 1. The range of nodule

Table 1: Frequency of variation in various characters of guar rhizobia isolates

Character	Class Interval	Frequency
Nodules plant^{-1}	1-10	30
	11-20	39
	21-30	18
	31-40	11
	41-50	2
Nodule fresh wt. plant^{-1} (mg)	1-100	51
	101-200	16
	201-300	11
	301-400	15
	401-500	5
	501-600	2
Shoot dry wt. plant^{-1} (mg)	301-400	19
	401-500	37
	501-600	12
	601-700	22
	701-800	10
ARA (μ moles h^{-1} plant^{-1})	0.1-0.500	36
	0.501-1.000	42
	1.001-1.500	8
	1.501-2.000	6
	2.001-2.500	5
	2.501-3.000	2
	3.001-3.500	1

number plant^{-1}, nodule fresh weight plant^{-1}, shoot dry weight plant^{-1} and nitrogenase activity plant^{-1} were found to be 1-49, 1-585 mg, 310-789 mg and 0.1-3.4 ARA (μ moles h^{-1} plant^{-1}) respectively. Only 2% of the isolates showed high nodule number plant^{-1} (41-50) whereas most of them (69 %) had lesser number of nodules ($\leq$ 20 plant^{-1}). Similarly higher nodule fresh weight (501-600 mg plant^{-1}) was observed in 2% plants while 67 % had lesser nodule fresh weight ($\leq$ 200 mg plant^{-1}). Highest shoot dry weight was seen in 10 % of plants (>700 mg) while the maximum shoot dry weight of the isolates was observed within the range 401-500 mg. Maximum number of isolates had nitrogenase activity between 0.501-1.000 ARA (μ moles h^{-1} plant^{-1}) while 3 % of the isolates expressed maximal nitrogenase activity $\geq$2.5 ARA (μ moles h^{-1} plant^{-1}).

On the basis of these results ten cultures each of slow and fast growers were selected. The detailed results of these isolates with respect to each of the above mentioned characters are represented in tables 2 and 3. The values seen in all the fast growers (Table 2) were generally lower as compared to those seen in case of their slow growing counter parts (Table 3). Fast growers showed more variability in all characters, as evident from their high CV% with the exception in case of the nodule fresh weight where the CV% was almost identical. Maximum variability was noted for nitrogenase activity in fast growers (CV% = 31.32) and minimum variability was observed in number of nodules plant^{-1} for slow growers (CV% = 13.58). Maximum number of nodules plant^{-1} (36) and

Table 2: Evaluation of fast growing *Rhizobium* isolates in *chillum* jars

Isolate	Shoot dry wt. plant^{-1} (mg)	Number of nodules plant^{-1}	Nodule fresh wt. plant^{-1} (mg)	ARA (μ moles h^{-1} plant^{-1})
G-9	645	28	325	1.895
G-14	540	21	313	1.972
G-20	625	22	368	2.104
G-28	580	23	350	1.210
G-34	406	13	307	1.021
G-54	452	16	208	1.009
G-73	506	19	213	1.265
G-74	455	18	256	1.106
G-76	322	21	209	0.983
G-78	461	24	342	1.792
Mean	499	21	289	1.436
CV %	20.15	20.73	21.51	31.32
Control	285	5	109	0.531

(Average of six plants)

Table 3: Evaluation of slow growing *Rhizobium* isolates in *chillum* jars

Isolate	Shoot dry wt. plant^{-1} (mg)	Number of nodules plant^{-1}	Nodule fresh wt. plant^{-1} (mg)	ARA (μ moles h^{-1} plant^{-1})
G-2	402	27	321	1.795
G-3	651	27	351	2.460
G-4	798	28	520	3.019
G-5	752	27	296	2.781
G-6	686	32	360	1.982
G-11	725	36	553	2.098
G-12	701	24	308	2.103
G-13	650	34	351	2.182
G-17	620	35	359	1.904
G-18	709	32	342	2.592
Mean	669	30	376	2.292
CV %	16	13.58	23.29	17.61

Control as in table 2

(Average of six plants)

and nodule fresh wt. plant^{-1} (553 mg) was seen in G-11 among slow growers. For dry wt. plant^{-1} and nitrogenase activity, the maximum values (798 (mg) and 3.019) ARA (μ moles h^{-1} plant^{-1}) were found in G-4.

G-2 (402 mg), G-12 (24), G-5 (296 mg) and G-17 (1.904 μ moles of C_2H_2 red. h^{-1} plant^{-1}) were found to have minimum values for plant dry weight, nodule number, nodule fresh weight and nitrogenase activity respectively.

4.2 Carbohydrate utilization by fast and slow growing Rhizobium isolates:

A broad distinction has been made between fast and slow growing rhizobia in their ability to use different carbon sources.

All the ten fast and slow growing Rhizobium strains were tested for growth by spot tests on different carbon sources. These carbon sources were supplemented individually in Rhizobium minimal agar medium. The carbon sources tested were pentoses, hexoses, disaccharides, trisaccharides and organic acids.

Fast growing rhizobia were able to use a broad range of carbon substrates, whereas slow growing rhizobia showed restricted ability to use diverse carbon sources (Table 4).

All the ten fast growing isolates tested were found to utilize ribose, xylose, glucose, fructose, mannose, sucrose, lactose, maltose, mannitol, pyruvate, malate and succinate. Arabinose, raffinose and fumarate were utilized by eight isolates only, while galactose and rhamnose were utilized by nine isolates. All the slow growers could grow only on

Table 4: Carbohydrates utilization by fast-and slow growing <u>Rhizobium</u> isolates

Rhizobium isolates	CARBOHYDRATES								
	Ribose	Arabinose	Xylose	Glucose	Fructose	Galactose	Mannose	Rhamnose	Raffinose
1	2	3	4	5	6	7	8	9	10
Slow growers									
G-2	−	−	−	+	−	−	−	−	−
G-3	−	−	−	+	+	+	−	−	−
G-4	−	−	−	+	−	−	−	−	−
G-5	+	+	+	+	+	+	+	−	−
G-6	−	−	−	+	+	+	−	−	−
G-11	−	−	−	−	+	−	−	−	−
G-12	+	+	+	+	−	+	−	−	−
G-13	+	+	+	+	+	−	+	−	−
G-17	+	+	+	+	+	+	−	+	−
G-18	+	−	+	+	−	+	−	−	−
Fast growers									
G-9	+	+	+	+	+	+	+	+	+
G-14	+	+	+	+	+	+	+	+	+
G-20	+	−	−	+	+	+	+	+	+
G-28	+	+	+	+	+	+	+	+	+
G-34	+	+	+	+	+	+	+	−	+
G-54	+	+	+	+	+	+	+	+	+
G-73	+	−	−	+	+	−	+	+	+
G-74	+	+	+	+	+	+	+	+	−
G-76	+	+	+	+	+	+	+	+	−
G-78	+	+	+	+	+	+	+	+	+

Contd...

Contd... table 4.

Rhizobium isolates	CARBOHYDRATES							
	Sucrose	Lactose	Maltose	Mannitol	Pyruvate	Malate	Succinate	Fumarate
11	12	13	14	15	16	17	18	19
Slow growers								
G-2	-	-	-	+	-	-	-	-
G-3	-	-	-	+	-	-	-	-
G-4	-	-	-	+	-	-	-	-
G-5	-	+	+	+	-	-	-	-
G-6	-	-	-	+	-	-	-	-
G-11	-	-	-	+	-	-	-	-
G-12	-	+	-	+	-	+	+	-
G-13	-	-	-	+	-	+	+	-
G-17	-	-	-	+	-	-	-	-
G-18	-	-	-	+	-	-	-	-
Fast growers								
G-9	+	+	+	+	+	+	+	+
G-14	+	+	+	+	+	+	+	+
G-20	+	+	+	+	+	+	+	-
G-28	+	+	+	+	+	+	+	+
G-34	+	+	+	+	+	+	+	+
G-54	+	+	+	+	+	+	+	+
G-73	+	+	+	+	+	+	+	-
G-74	+	+	+	+	+	+	+	+
G-76	+	+	+	+	+	+	+	+
G-78	+	+	+	+	+	+	+	+

glucose and mannitol, while raffinose, sucrose, pyruvate and fumarate were not utilized by any of the slow growers (Table 5).

4.3. Intrinsic antibiotic resistance pattern of guar Rhizobium isolates:

Antibiotic resistance profile is a strain specific character, which helps in the identification of the derivatives of wild type. With a view to eventually select the right strain for Tn_5 mutagenesis, ten isolates each of fast and slow growing rhizobia were subjected to test for resistance to different antibiotics. Yeast extract mannitol agar plates containing different concentrations (10, 25, 50, 100 µg/ml) of Kanamycin (Km), Neomycin (Nm), Ampicillin (Ap), Erythromycin (Er), Naladixic acid (Nx), Tetracycline (Tc), Chloramphenicol (Cm) and Streptomycin (Sm) were used for testing the resistance pattern. The antibiotic resistance pattern of fast and slow growing isolates has been shown in tables 6 and 7, respectively.

Fast and slow growing isolates exhibited different antibiotic resistance patterns. G-34, G-54 and G-28 among fast growers and G-4 and G-5 among slow growers were found to be resistant to all the antibiotics tested.

4.4. Performance of fast and slow growing isolates on different hosts:

Host range of the selected slow and fast growing (10 from each group) isolates of Rhizobium was studied by inoculating various legumes like guar (HG-75), Urdbean (T-9), mungbean (K-851), pigeonpea (UPAS-120) and ricebean (RB53).

Table 5: Carbohydrate utilization by selected fast and slow growing *Rhizobium* isolates

Carbohydrates	Number of isolates utilizing a particular carbon source	
	Fast growers	Slow growers
Ribose	10	5
Arabinose	8	4
Xylose	10	5
Glucose	10	10
Fructose	10	6
Galactose	9	6
Mannose	10	2
Rhamnose	9	1
Raffinose	8	-
Sucrose	10	-
Lactose	10	2
Maltose	10	1
Mannitol	10	10
Pyruvate	10	-
Malate	10	2
Succinate	10	2
Fumarate	8	-

Table 6: Intrinsic antibiotic resistance pattern in fast growing *Rhizobium* isolates

Isolate	Km	Nm	Ap	Er	Nx	Tc	Cm	Sm
G-73	50	S	100	100	100	S	100	50
G-20	50	S	S	100	100	S	50	S
G-14	S	S	100	100	100	10	100	S
G-7	25	25	S	100	50	S	100	100
G-76	50	25	S	100	50	S	50	25
G-54	100	100	100	100	100	25	100	100
G-34	25	25	100	25	25	25	100	25
G-74	S	25	50	100	100	S	100	S
G-28	25	25	100	50	50	10	100	25
G-9	S	S	100	100	50	25	100	S

S Susceptible

Table 7: Intrinsic antibiotic resistance pattern in slow growing Rhizobium isolates

Isolate	Km	Nm	Ap	Er	Nx	Tc	Cm	Sm
G-2	100	100	S	50	100	25	100	S
G-3	100	100	S	S	100	10	50	S
G-4	100	100	100	100	100	25	100	50
G-5	100	100	100	100	100	25	100	100
G-6	S	S	S	S	50	S	100	S
G-11	100	100	100	S	100	10	100	S
G-12	100	100	S	S	100	10	50	S
G-13	100	100	100	S	100	10	50	S
G-17	100	50	100	S	100	10	100	S
G-18	100	50	100	S	50	10	50	S

S Susceptible

Plants were raised in chillum jars separately after inoculation with all the isolates individually. Plants were uprooted after 40 days to record observations for number of nodules, nodules dry wt, plant dry weight and nitrogenase activity per plant. The performance of slow and fast growing isolates with each host is presented in Table 8 and 9 respectively.

The results showed that slow growing rhizobial isolates viz., G-4, G-5, G-11 and G-18 are good for all the five host crops (Table 8). Highest values of each character were obtained in all the host crops. However, the performance of G-2, G-13 and G-17 was found to be poor as compared to above mentioned isolates. Performance of G-6 and G-12 isolates was intermediate. Isolate G-4 was exceptionally better on guar, urdbean, mungbean and ricebean. Similarly isolate G-18 performed better on pigeonpea and ricebean while G-5 performed better on guar, urdbean and mungbean.

Among fast grower isolates G-9, G-20 and G-28 showed maximum values of each character in all the crops tested(Table 9). Isolates G-73, G-74 and G-76 proved to be intermediate while G-34 was found to be the poorest one. Among all the isolates tested G-14 was better while G-7 and G-54 were poor for guar.

4.5. Ex planta nitrogenase expression by slow and fast growing Rhizobium isolates:

The normal practice to screen for nif/nod mutations in Rhizobium is by plant infection test. This test is tedious and time consuming and may become impracticable when a large number of clones are to be screened. A strain showing ex planta nitrogenase activity will be highly advantageous.

When tested for the expression of ex planta nitrogenase

Table 8: Performance of slow growing *Rhizobium* isolates on different hosts

	Guar		Urdbean		Mungbean		Pigeonpea		Ricebean	
1	High 2	Low 3	High 4	Low 5	High 6	Low 7	High 8	Low 9	High 10	Low 11
Number of nodules plant^{-1}	85.0 (G-4)	6.7 (G-17)	76.0 (G-4)	8.5 (G-2)	101 (G-4)	9.3 (G-2)	88.5 (G-11)	6.0 (G-12)	12.2 (G-11)	4.2 (G-2)
	42.0 (G-5)	12.7 (G-3)	66.6 (G-5)	12.1 (G-13)	72 (G-11)	13.3 (G-13)	55.5 (G-4)	13.2 (G-13)	96.0 (G-4)	19.0 (G-13)
	41.3 (G-11)	15.3 (G-13)	42.0 (G-11)	15.0 (G-12)	61 (G-5)	13.6 (G-17)	40.1 (G-18)	13.3 (G-17)	82.0 (G-18)	27.0 (G-12)
Nodule fresh wt plant^{-1} (mg)	616 (G-4)	85 (G-17)	614 (G-4)	41 (G-13)	568 (G-5)	38 (G-13)	680 (G-18)	41 (G-13)	523 (G-18)	104 (G-13)
	460 (G-5)	139 (G-13)	593 (G-5)	86 (G-2)	351 (G-4)	50 (G-2)	482 (G-6)	86 (G-12)	498 (G-4)	116 (G-2)
	417 (G-11)	140 (G-3)	416 (G-18)	103 (G-12)	322 (G-18)	104 (G-17)	352 (G-11)	97 (G-2)	490 (G-11)	143 (G-12)
Shoot dry wt plant^{-1} (mg)	890 (G-4)	158 (G-17)	902 (G-5)	100 (G-2)	938 (G-4)	166 (G-2)	867 (G-11)	114 (G-2)	798 (G-11)	250 (G-2)
	671 (G-5)	226 (G-6)	882 (G-4)	162 (G-12)	933 (G-5)	200 (G-13)	860 (G-5)	183 (G-13)	626 (G-4)	287 (G-13)
	567 (G-11)	258 (G-3)	808 (G-11)	200 (G-13)	831 (G-11)	298 (G-18)	820 (G-18)	208 (G-12)	600 (G-18)	337 (G-12)

Contd...

Contd.... table 8.

1	2	3	4	5	6	7	8	9	10	11
ARA (μ moles h^{-1} plant^{-1})	5.046 (G-4)	0.693 (G-17)	5.502 (G-4)	0.326 (G-13)	7.279 (G-5)	0.104 (G-13)	7.774 (G-18)	0.169 (G-13)	8.912 (G-18)	0.170 (G-13)
	4.111 (G-5)	1.431 (G-3)	5.372 (G-5)	1.042 (G-2)	5.210 (G-4)	0.318 (G-2)	5.567 (G-6)	0.344 (G-2)	8.840 (G-4)	0.338 (G-2)
	3.381 (G-11)	1.914 (G-12)	3.815 (G-11)	1.579 (G-12)	4.538 (G-6)	0.538 (G-17)	3.488 (G-11)	1.130 (G-12)	1.894 (G-11)	1.105 (G-17)

Table 9: Performance of fast growing <u>Rhizobium</u> isolates on different hosts

	Guar		Urdbean		Mungbean		Pigeonpea		Ricebean	
	High 2	Low 3	High 4	Low 5	High 6	Low 7	High 8	Low 9	High 10	Low 11
1										
Number of nodules plant^{-1}	57.5 (G-9)	6.0 (G-34)	60.5 (G-20)	6.5 (G-34)	49.3 (G-28)	3.7 (G-34)	82.0 (G-20)	7.6 (G-34)	85.0 (G-20)	8.3 (G-34)
	28.5 (G-73)	8.5 (G-7)	41.6 (G-76)	8.2 (G-73)	35.5 (G-9)	5.7 (G-54)	52.0 (G-28)	10.4 (G-76)	81.5 (G-28)	12.1 (G-73)
	25.5 (G-14)	13.5 (G-74)	30.2 (G-9)	13.0 (G-74)	28.0 (G-20)	12.0 (G-7)	35.2 (G-9)	11.9 (G-73)	71.0 (G-9)	23.2 (G-76)
Nodule fresh wt plant^{-1} (mg)	475 (G-9)	69 (G-7)	446 (G-20)	43 (G-34)	486 (G-28)	32 (G-34)	517 (G-20)	28 (G-34)	463 (G-14)	25 (G-34)
	441 (G-14)	120 (G-34)	425 (G-9)	63 (G-74)	340 (G-9)	53 (G-76)	359 (G-28)	36 (G-76)	356 (G-20)	80 (G-7)
	350 (G-20)	137 (G-54)	399 (G-76)	87 (G-54)	285 (G-73)	119 (G-7)	312 (G-9)	59 (G-7)	262 (G-28)	89 (G-74)
Shoot dry wt plant^{-1} (mg)	680 (G-9)	117 (G-7)	610 (G-20)	90 (G-34)	860 (G-28)	133 (G-74)	770 (G-28)	103 (G-34)	742 (G-28)	232 (G-34)
	560 (G-14)	221 (G-74)	598 (G-14)	97 (G-74)	850 (G-14)	135 (G-76)	760 (G-9)	152 (G-7)	597 (G-20)	269 (G-7)
	492 (G-28)	242 (G-54)	576 (G-9)	117 (G-7)	840 (G-9)	267 (G-7)	746 (G-20)	200 (G-76)	589 (G-14)	275 (G-54)

Contd ..

Contd... table 9.

1	2	3	4	5	6	7	8	9	10	11
ARA (μ moles h^{-1} $plant^{-1}$)	4.336 (G-9)	0.701 (G-7)	4.583 (G-76)	0.168 (G-34)	4.642 (G-28)	0.169 (G-34)	7.594 (G-28)	0.143 (G-34)	4.196 (G-14)	0.162 (G-34)
	3.758 (G-14)	1.412 (G-34)	2.764 (G-20)	0.184 (G-74)	2.715 (G-14)	0.174 (G-76)	3.558 (G-20)	0.182 (G-76)	3.105 (G-28)	0.579 (G-74)
	3.012 (G-20)	1.520 (G-54)	2.755 (G-9)	0.904 (G-73)	2.137 (G-9)	0.705 (G-73)	3.392 (G-9)	0.288 (G-73)	3.092 (G-20)	1.094 (G-54)

activity on nitrogenase induction medium, all the slow growing isolates reduced acetylene under free-living conditions (Table 10). Only two fast growing isolates expressed <u>ex planta</u> ARA. Significantly the level was far higher in fast growing isolates as compared to the slow growers.

4.6 <u>Plasmid profile of guar rhizobia</u> :

The genes responsible for symbiosis may be present on chromosome or on plasmids. In order to carry out molecular biological studies, it is essential to know the plasmid profile of an isolate.

Two different techniques were used for visualizing the plasmid DNA. One of the methods involved the isolation of plasmid DNA by alkaline extraction and then determining the number of plasmids by agarose gel electrophoresis. The other technique involved gentle lysis of cells directly in the slots of an agarose gel.

Fast and slow growing isolates showed different plasmid profiles. Among slow growers, maximum number of plasmid bands were observed in G-3, G-4 and G-17 each of which showed two plasmids. G-6, G-13 did not show any plasmid band, rest of the slow growers showed one plasmid each (Table 11; Plate 1).

G-54 among fast growers showed the maximum number of plasmids i.e. 3 plasmid bands. G-76 and G-9 had one plasmid each. The remaining fast growers harboured two plasmids each. Unlike the slow growers, all the fast growing isolates showed plasmids (Plate 1).

Table 10: *Ex-planta* nitrogenase activity of slow-and fast growing *Rhizobium* isolates

Isolate	ARA (n moles h^{-1} mg protein^{-1})
Slow growers	
G-2	2.72
G-3	4.76
G-4	3.74
G-5	4.12
G-6	4.25
G-11	4.50
G-12	1.24
G-13	3.83
G-17	3.91
G-18	4.08
Fast-growers	
G-9	0.00
G-14	0.00
G-20	46.83
G-28	0.00
G-34	0.00
G-54	0.00
G-73	45.12
G-74	0.00
G-76	0.00
G-78	0.00

Table 11: Plasmid profile of slow-and fast growing *Rhizobium* isolates

Rhizobium isolates	Number of plasmids observed
Slow growers	
G-2	1
G-3	2
G-4	2
G-5	1
G-6	-
G-11	1
G-12	1
G-13	-
G-17	2
G-18	1
Fast growers	
G-73	2
G-20	2
G-14	2
G-7	2
G-76	1
G-54	3
G-34	2
G-74	2
G-28	2
G-9	1

A) Plasmid pattern of slow growing *Rhizobium* isolates

1	G-12	7	G-2
2	G-17	8	G-3
3	G-11	9	G-4
4	G-13	10	G-5
5	G-6	11	G-18
6	*E.Coli*	12	*E.Coli*

B) Plasmid pattern of fast growing *Rhizobium* isolates

1	G-9	6	G-76
2	G-28	7	G-7
3	G-74	8	G-14
4	G-34	9	G-20
5	G-54	10	G-73

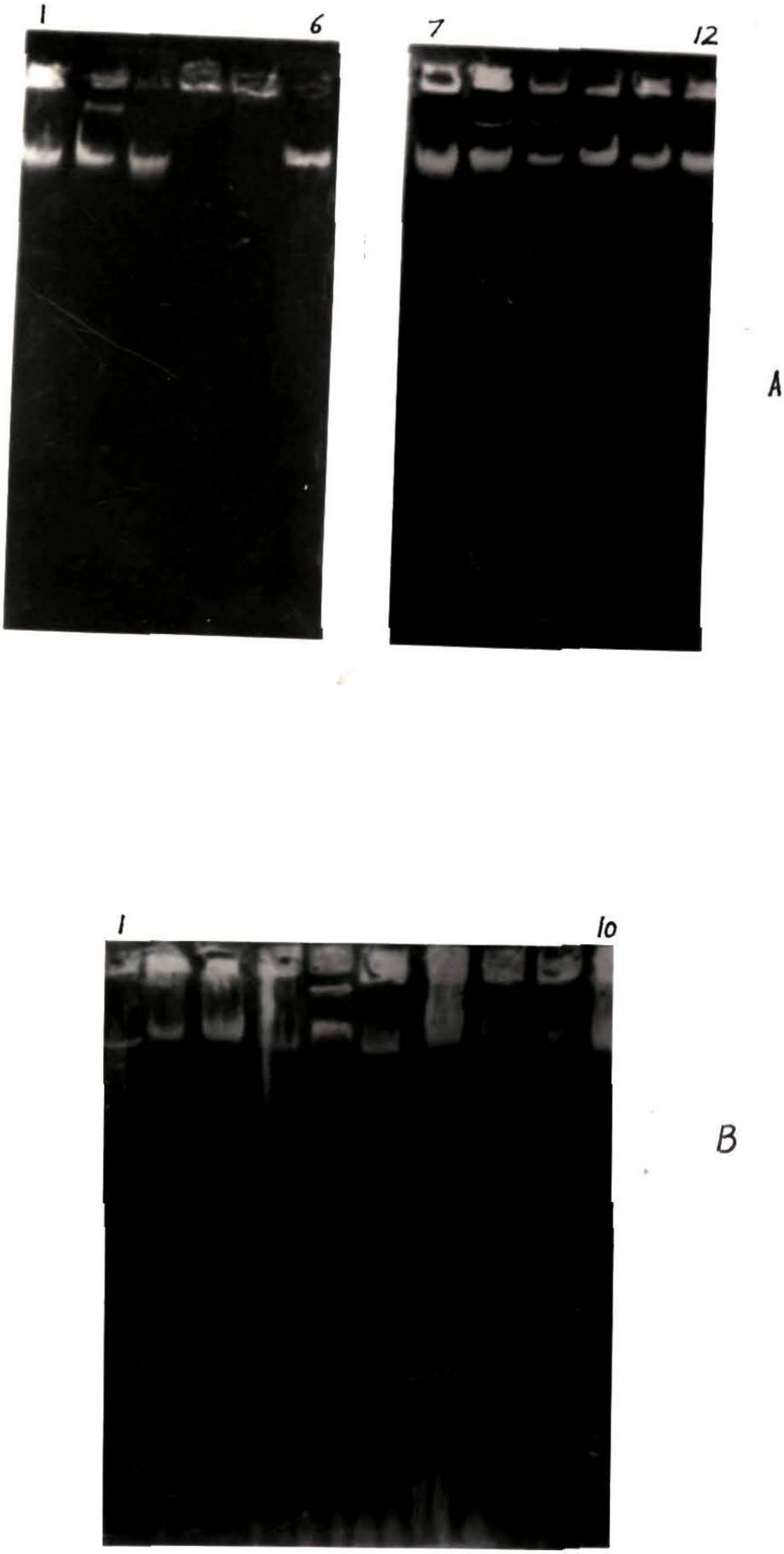


PLATE 1

4.7. Physiological and genetic studies:

For further physiological and genetic studies on guar rhizobia, isolate G-20 was chosen. It is an efficient strain, showing a broad host range, it expresses high level of *ex planta* nitrogenase activity, it harbours two plasmids and is also suitable for Tn5 mutagenesis by virtue of the antibiotic resistance marker.

Colony types of Rhizobium isolate G-20:

Colonial variation is generally observed in slow as well as fast growing isolates. When spread on media plates, they may give rise to big and small colonies or slimy or non-slimy colonies.

Two types of colonies were observed in the original culture of G-20, when spread on YEMA plates. One colony type was slimy which was designated as G-20 (S) and another was mucoid, designated as G-20 (M) based on their external morphology. The characteristics of these colony types are represented in Table 12;

4.8. Kinetics of acetylene reduction by G-20 (S) and G-20 (M) cultured on solid nitrogenase induction medium and yeast extract mannitol agar medium:

Expression of *ex planta* nitrogenase activity depends on the genotype of the strain, composition and state of the media for the induction of nitrogenase and age of the culture.

Nitrogenase activity was determined for two colony types of strain G-20 i.e. G-20(S) and G-20 (M) at different intervals of time, on nitrogenase induction medium (NIM) and

Table 12: Characteristics of G-20(S) and G-20(M) colony types of *Rhizobium* isolate G-20

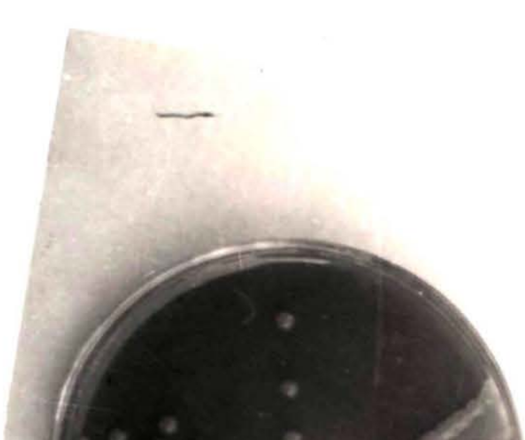
Character	G-20(S)	G-20(M)
Colonial type	Slimy	Mucoid
Shape	Flat colonies	Raised colonies
Appearance	Dull	Glistening
Periphery	Irregular	Circular
Colour on ageing	Turns pinkish	No change
EPS* content	1.189	6.519

* Exo poly saccharide in terms of mg glucose mg protein^{-1}

A) Colonial morphology of slimy colony type G-20(S)

B) Colonial morphology of mucoid colony type G-20(M)

A



B

PLATE 2

yeast extract mannitol agar (YEMA). Activity was seen till 26 days in fourteen batches (Table 13).

NIM was found better than YEMA for ARA expression by both the colony types (Fig.1). The ARA increased from 3rd day onwards and attained the maximum value on 4th day in all the cases, whereafter it declined slowly and reduced to zero on 24th day. In both the media tested G-20 (S) showed higher *ex planta* nitrogenase activity than mucoid colony type G-20 (M).

4.9. Kinetics of acetylene reduction by G-20 (S) and G-20 (M) in liquid nitrogenase induction medium and yeast extract mannitol medium:

The trend of ARA in liquid medium was similar to that of solid medium. The activity reached maximum on fourth day in all the cases (Table 14) and subsequently decreased to almost zero on 16th day. No activity was observed beyond the sixteenth day. ARA in liquid medium, in general, was lesser than in the solid medium. The slimy colony type G-20 (S) showed higher activity than the mucoid colony type (Fig. 2). As observed earlier in solid medium, in liquid medium also, the activity was higher in nitrogenase induction medium than the yeast extract mannitol agar medium.

4.10. Effect of temperature on nitrogenase activity in G-20 (S) and G-20(M) in solid nitrogenase induction medium and yeast extract mannitol medium:

The temperature may affect the synthesis of nitrogenase enzyme or it may modulate the enzyme activity. The expression of nitrogenase is maximum at a particular temperature.

To see the effect of temperature on the synthesis of

Table 13: Kinetics of acetylene reduction by G-20(S) and G-20(M) cultured on nitrogenase induction medium (NIM) and yeast extract mannitol medium (YEM) agar slopes

Days	ARA (n moles h^{-1} mg protein^{-1})			
	NIM		YEMA	
	G-20(S)	G-20(M)	G-20(S)	G-20(M)
2	2.96	0.29	1.52	0.28
3	42.56	12.83	12.88	11.76
4	61.94	25.99	39.83	20.04
6	44.78	19.24	31.54	12.36
8	34.69	17.88	21.11	11.06
10	32.30	15.23	18.23	8.00
12	21.88	14.45	11.61	4.06
14	16.87	10.93	7.24	3.06
16	15.06	8.89	4.43	2.86
18	13.70	8.16	1.44	2.73
20	13.12	6.11	0.70	1.63
22	0.23	0.96	0.08	1.48
24	-	-	-	-
26	-	-	-	-

Fig.1 Kinetics of acetylene reduction by G-20(S) and G-20(M) cultured on solid nitrogenase induction medium and yeast extract mannitol medium

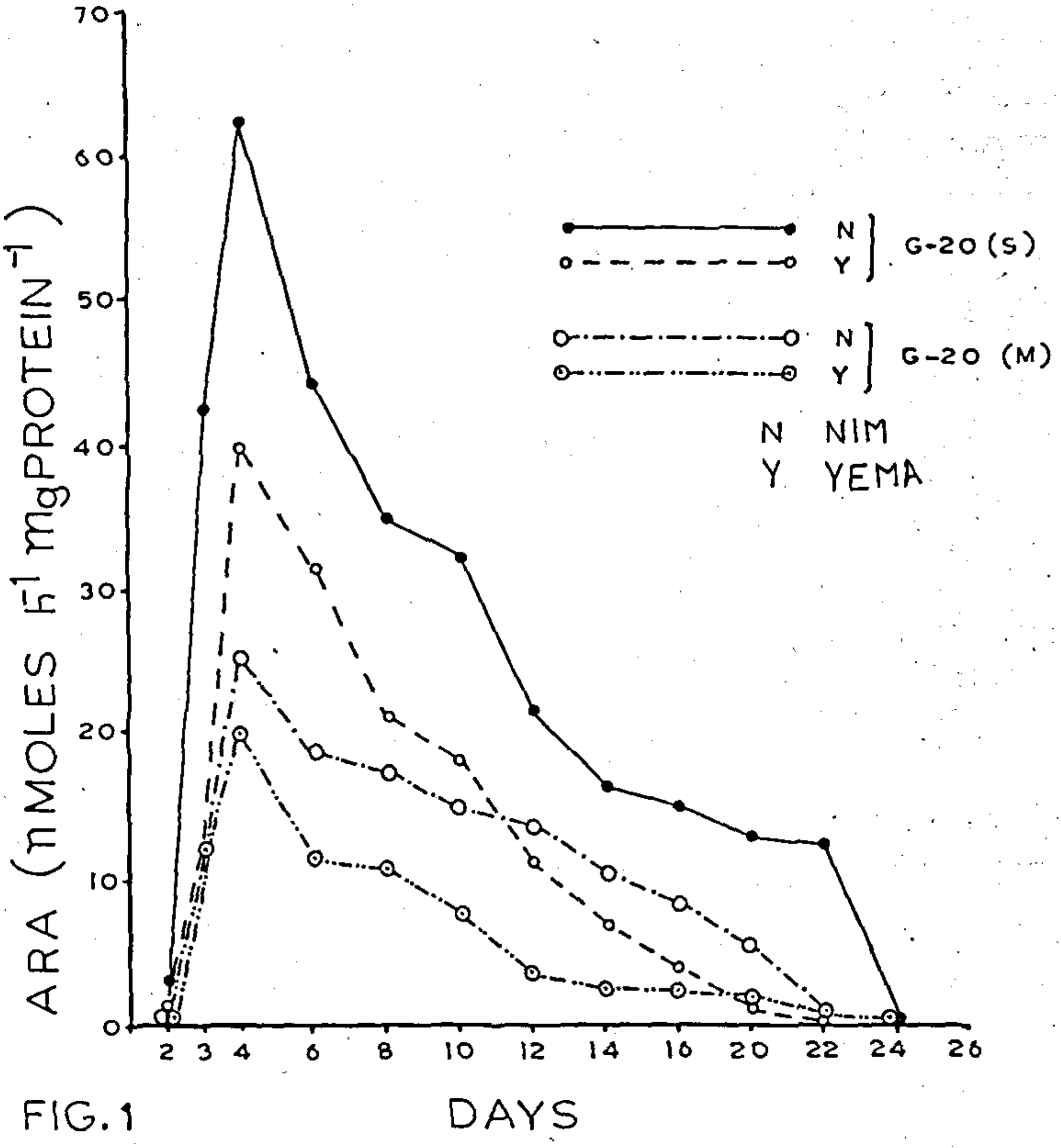

ARA (nMOLES h⁻¹ mgPROTEIN⁻¹)
0
10
20
30
40
50
60
70
2
3
4
6
8
10
12
14
16
20
22
24
26
DAYS
N
Y
G-20 (S)
N
Y
G-20 (M)
N NIM
Y YEMA

FIG. 1

Table 14: Kinetics of acetylene reduction by G-20(S) and G-20(M) cultured on liquid nitrogenase induction medium (NIM) and yeast extract mannitol (YEM) medium

Days	ARA (n moles h^{-1} mg $protein^{-1}$)			
	NIM		YEM	
	G-20(S)	G-20(M)	G-20(S)	G-20(M)
2	2.93	1.82	1.64	0.95
3	8.33	5.36	4.66	3.53
4	12.63	6.19	5.81	4.30
6	11.41	5.18	5.44	2.65
8	10.52	2.65	4.90	1.26
10	6.46	2.12	2.66	1.25
12	2.11	1.05	1.45	0.99
14	1.22	0.92	1.21	0.07
16	0.09	0.10	0.01	0.04
18	-	-	-	-

Fig.2 Kinetics of acetylene reduction by G-20(S) and G-20(M) cultured on liquid nitrogenase induction medium and yeast extract mannitol medium

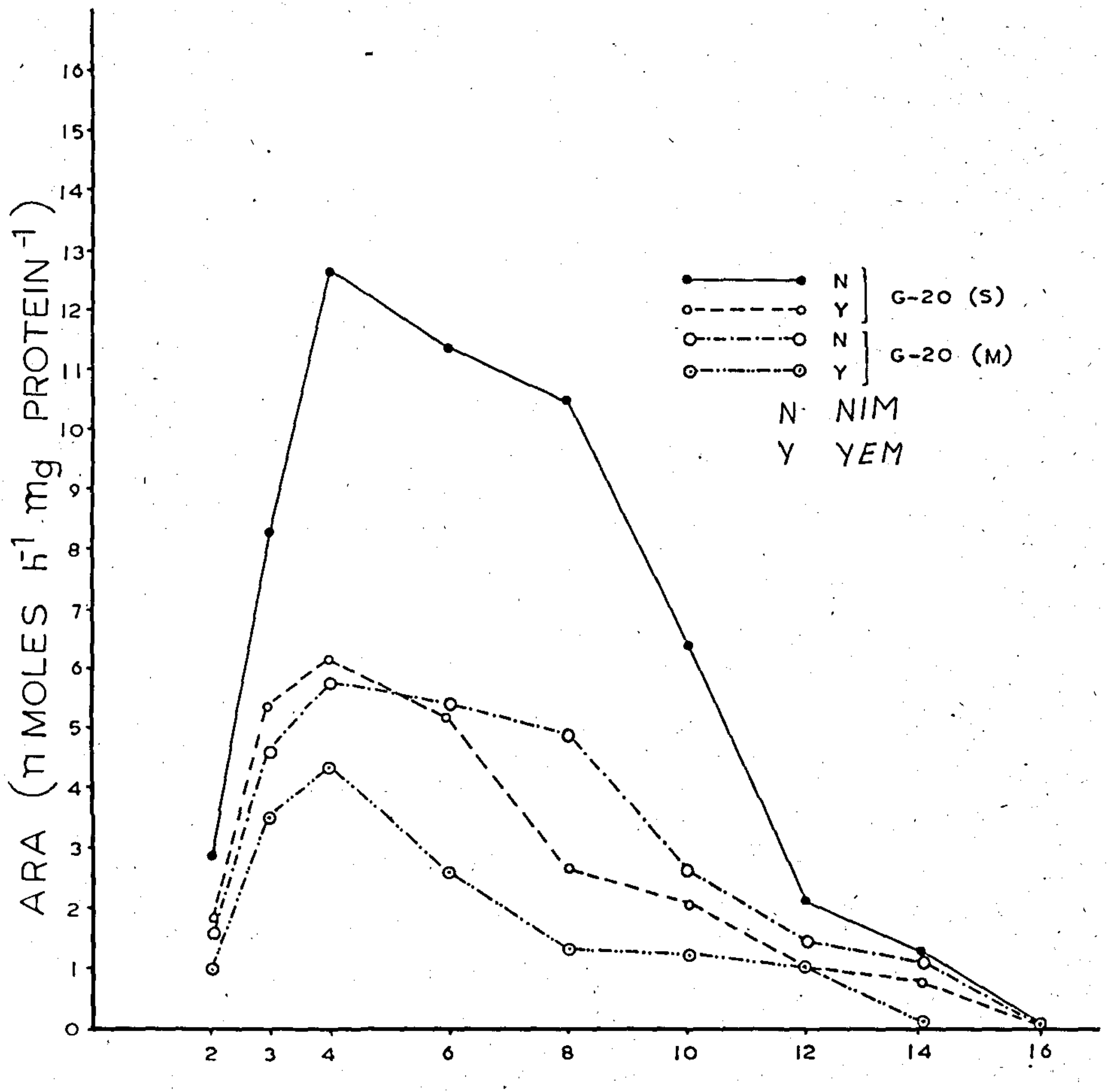

ARA (n MOLES h^{-1} mg PROTEIN^{-1})
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
2
3
4
6
8
10
12
14
16
DAYS
N
Y
G-20 (S)
N
Y
G-20 (M)
N NIM
Y YEM

FIG. 2

nitrogenase enzyme, the culture tubes were incubated for four days at 25, 30, 35, 40 and 45°C and then the activity was also recorded on the respective temperatures. The results are shown in table 15.

A gradual increase in ARA was seen in slimy as well as mucoid colony types. Maximum value was observed at 35°C. The values for G-20 (S) were 33.96 and 25.35 n moles of acetylene reduced h^{-1} mg $protein^{-1}$ in NIM and YEMA medium respectively. G-20 (M) on the other hand showed similar activities of ARA, 11.76 and 11.73 n moles of ARA h^{-1} mg $protein^{-1}$ in both the media tested. Further, for G-20 (M) the difference in activity in nitrogenase induction medium was not much at 30 and 35°C. Beyond 35°C the ARA decreased abruptly in all the cases. Activity seen at 45°C (Fig. 3) was very low.

To see the effect of temperature on the activity of nitrogenase enzyme the culture tubes were initially incubated at 30°C for four days and then after acetylene injection the tubes were further incubated at 25, 30, 35, 40 and 45°C for recording the ARA.

The results represented in Table 16 indicated higher activity on YEMA than on NIM at all the temperatures, except for G-20 (S) at 45°C. Activity was higher in slimy colony type G-20 (S) as compared to mucoid colony type G-20 (M) barring the higher values of G-20(M) at 40°C in YEMA.

In case of G-20 (S) <u>ex planta</u> nitrogenase activity was maximum at 35°C. On NIM and YEMA media, 13.83 and 16.38 ARA

Table 15: Effect of temperature on synthesis of nitrogenase in G-20(S) and G-20(M) on NIM and YEM agar slopes

Temperature (^{o}C)	ARA (n moles h^{-1} mg protein^{-1})			
	NIM		YEMA	
	G-20(S)	G-20(M)	G-20(S)	G-20(M)
25	4.71	0.15	5.82	0.18
30	22.65	9.06	15.84	4.41
35	33.96	11.76	25.35	11.73
40	2.79	1.95	2.58	0.39
45	1.35	0.12	0.12	0.24

Fig.3 Effect of temperature on _ex planta_ nitrogenase synthesis in G-20(S) and G-20(M) in solid NIM and YEMA

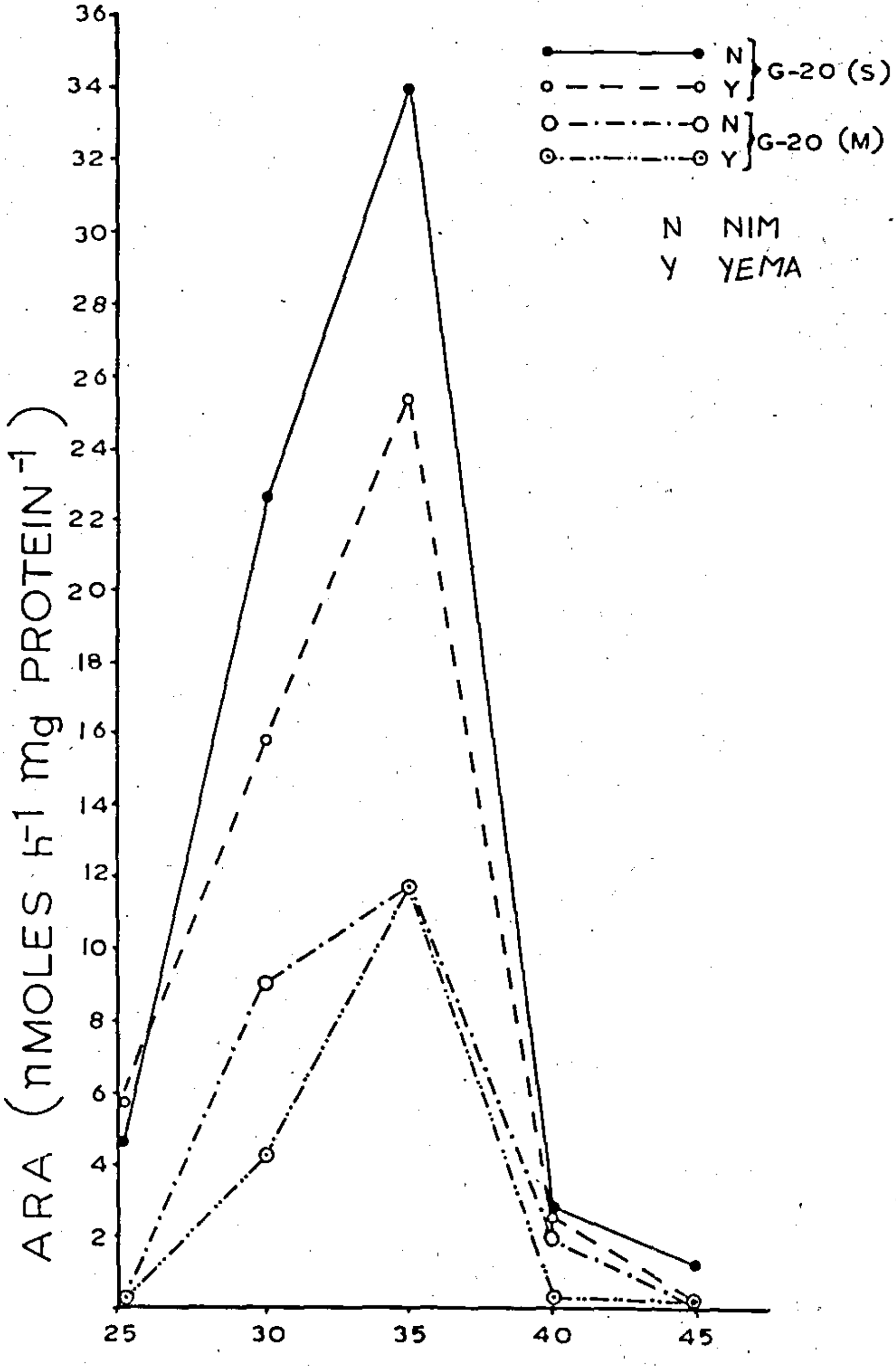
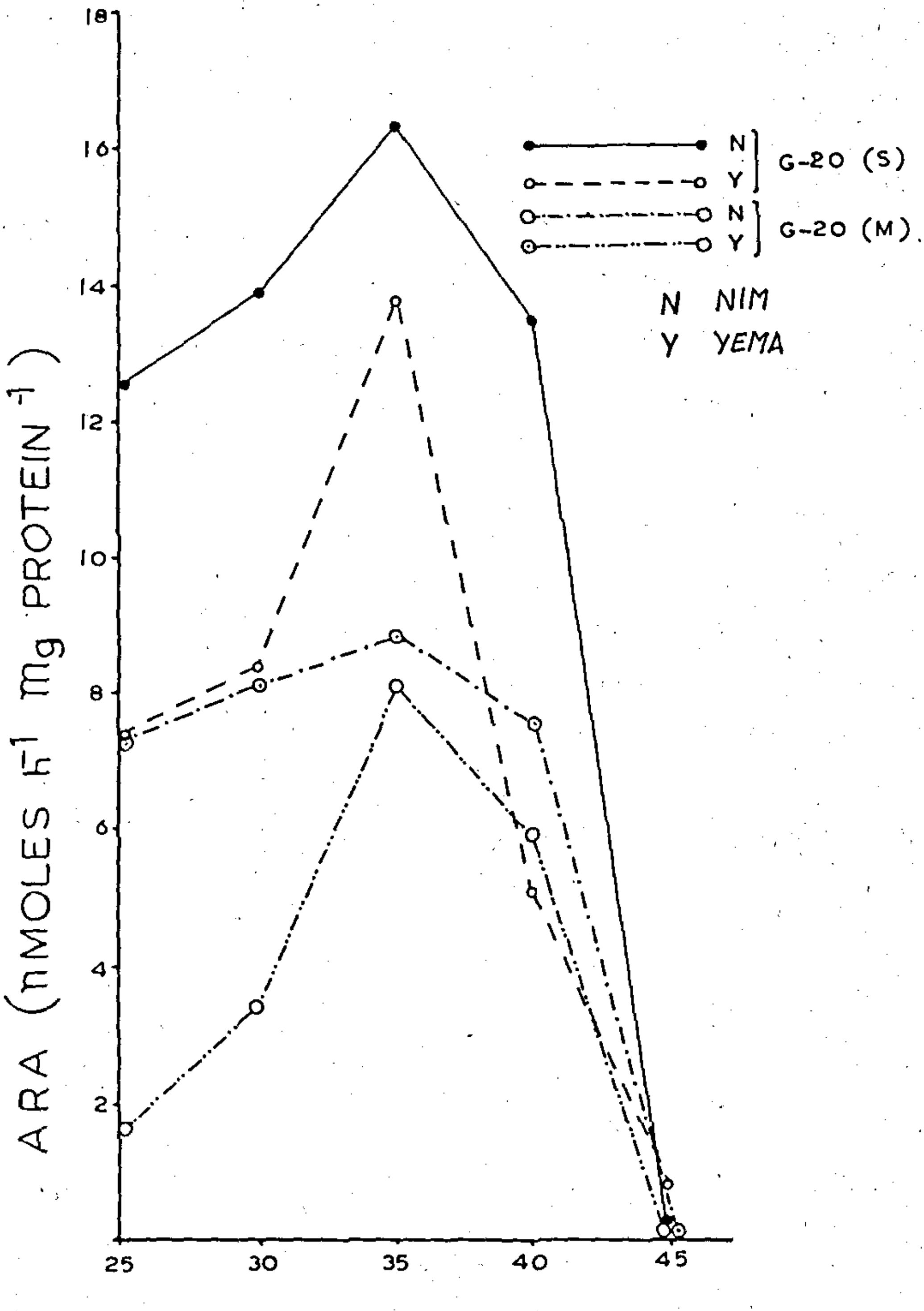


FIG. 3 TEMPERATURE °C

Table 16: Effect of temperature on ex planta nitrogenase activity G-20(S) and G-20(M) on NIM and YEM agar slopes

Temperature (°C)	ARA (n moles h^{-1} mg protein^{-1})			
	NIM		YEMA	
	G-20(S)	G-20(M)	G-20(S)	G-20(M)
25	7.38	1.62	12.60	7.41
30	8.40	3.48	13.98	8.25
35	13.83	8.10	16.38	8.91
40	5.07	5.91	13.53	7.62
45	0.81	0.21	0.33	0.36

Fig.4 Effect of temperature on *ex planta* nitrogenase activity in G-20(S) and G-20(M) in solid NIM and YEMA



18
16
14
12
10
8
6
4
2
ARA (nMOLES h⁻¹ mg PROTEIN⁻¹)
25
30
35
40
45
N
Y
G-20 (S)
N
Y
G-20 (M)
N NIM
Y YEMA

FIG.4 TEMP. °C

(n moles h^{-1} mg protein^{-1}) were observed respectively. In case of G-20 (M) the values were 8.10 and 8.91 ARA (n moles h^{-1} mg protein^{-1}). An abrupt reduction in the activity was seen at 40°C which was further lowered at 45°C (Fig. 4). On comparing the results of both the approaches it is evident that -

(1) At 35°C the corresponding activities were higher in the first method where the tubes were incubated at 35°C throughout the experiment, than the second method of approach where there was temperature shift from 30°C to 35°C.

(2) The reduction of ARA was higher in the first method of approach at 40°C.

(3) At 25°C the ARA was higher in the second method of approach of temperature shift, than the first method.

4.11 Effect of NH_4Cl on ex planta nitrogenase activity in solid and liquid cultures:

Combined nitrogen sources have been reported to have an inhibitory effect on nitrogenase activity. To see the effect on the expression of ex planta nitrogenase activity in slimy G-20 (S) and mucoid G-20 (M) colony types, NH_4Cl was substituted at different concentrations (1, 2, 5, 10, 20, 50 and 100mM) in solid as well as liquid yeast extract mannitol medium.

The results presented in Table 17 show a negative correlation between NH_4Cl concentration and ARA, in solid as well as liquid medium for both the colony types. Inhibition in ARA shown by G-20 (M) and G-20 (S) at 1mM concentration of NH_4Cl was higher in liquid medium (98.5 % and 72.1 % respectively) as compared to solid medium, (76.94 % and

Table 17: Effect of NH_4Cl on ex *planta* nitrogenase activity of G-20(S) and G-20(M) in solid and liquid cultures

NH_4Cl concentration (mM)	ARA (n moles h^{-1} mg protein^{-1})			
	Solid		Liquid	
	G-20(S)	G-20(M)	G-20(S)	G-20(M)
0	27.30 (0.00)	23.85 (0.00)	5.13 (0.00)	4.01 (0.00)
1	16.97 (37.84)	5.50 (76.94)	1.40 (72.71)	0.06 (98.50)
2	15.96 (41.54)	5.15 (78.41)	0.00 (100.00)	0.00 (100.00)
5	0.33 (98.79)	0.00 (100.00)	0.00 (100.00)	0.00 (100.00)
10	0.00 (100.00)	0.00 (100.00)	0.00 (100.00)	0.00 (100.00)
20	0.00 (100.00)	0.00 (100.00)	0.00 (100.00)	0.00 (100.00)
50	0.00 (100.00)	0.00 (100.00)	0.00 (100.00)	0.00 (100.00)
100	0.00 (100.00)	0.00 (100.00)	0.00 (100.00)	0.00 (100.00)

Figures in parentheses indicate per cent inhibition of nitrogenase activity

Fig.5 Effect of NH_4Cl on *ex planta* nitrogenase activity of G-20(S) and G-20(M) in solid and liquid cultures

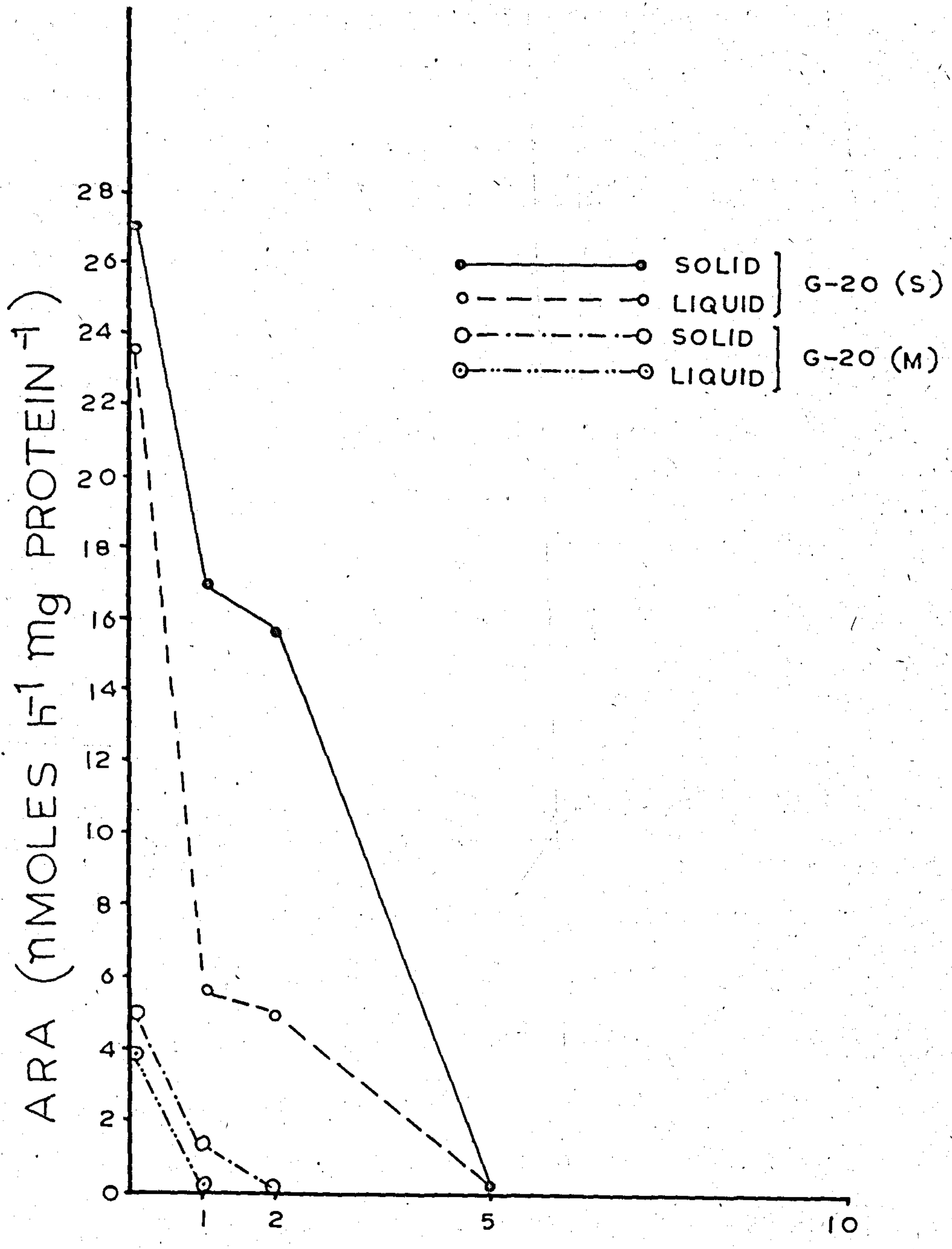


FIG.5 NH$_4$ CL (mM)

37.84 % respectively). There was complete repression of (Fig.5) activity from 10mM concentration onwards, though the growth was observed upto 100mM concentration of NH_4cl. At higher concentration the colour of the culture also changed to pale yellow.

4.12 Effect of K NO_3 on ex planta nitrogenase activity of G-20 (S) and G-20 (M) in solid and liquid cultures :

Both colony types showed higher *ex planta* nitrogenase activity in solid medium than in liquid medium. Among the two types, it was higher in G-20 (S) than in G-20 (M) (Table 18). In liquid medium ARA was negatively associated with KNO_3 concentration (Fig. 6). The activity was nil at 20mM concentration in G-20 (M) colony type in liquid medium, while it was retained till 50mM concentration by G-20 (S) in solid medium. In solid medium the ARA was relatively higher at lower concentrations of KNO_3 (Fig. 7).

4.13 Effect of yeast extract on ex planta nitrogenase activity of G-20(S) and G-20(M) in solid and liquid culture:

Yeast extract (Y.E.) is a source of combined organic nitrogen and vitamins. To study its effect on *ex planta* nitrogenase activity, different concentrations of yeast extract (0.05, 0.20, 0.50, 1.0, 2.0, 3.0 and 4.0 g/l) were taken in yeast extract mannitol medium. The results are shown in Table 19. The ARA was higher in solid medium as compared to the liquid medium. In both solid and liquid media, G-20(S) colony type showed higher ARA as compared to G-20 (M) except at 0.05 g/l concentration, where G-20 (M) showed higher values.

Table 18: Effect of potassium nitrate on *ex planta* nitrogenase activity of G-20(S) and G-20(M) in liquid and solid cultures.

KNO_3 (mM)	ARA (n moles h^{-1} mg $protein^{-1}$)			
	G20(S)		G-20(M)	
	Solid	Liquid	Solid	Liquid
0	37.75	5.90	21.01	4.30
1	69.03	4.92	23.00	3.47
2	45.73	4.65	23.92	2.71
5	42.57	2.18	16.87	1.73
10	30.85	0.37	7.67	0.21
20	11.43	0.28	0.61	0.00
50	5.61	0.00	0.15	0.00

Fig.6 Effect of KNO_3 on *ex planta* nitrogenase activity of G-20(S) and G-20(M) in liquid culture

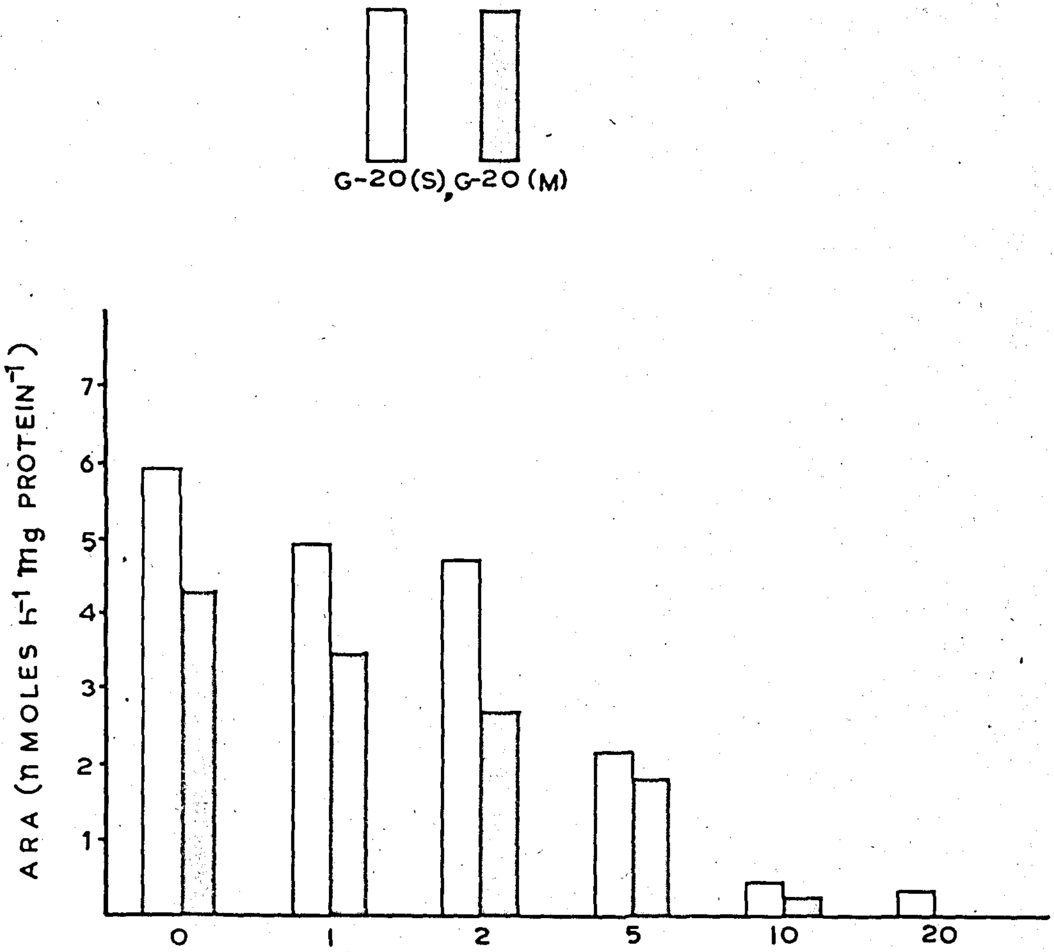


FIG. 6 KNO$_3$ (mM)

Fig.7 Effect of KNO_3 on ex planta nitrogenase activity of G-20(S) and G-20(M) in solid medium

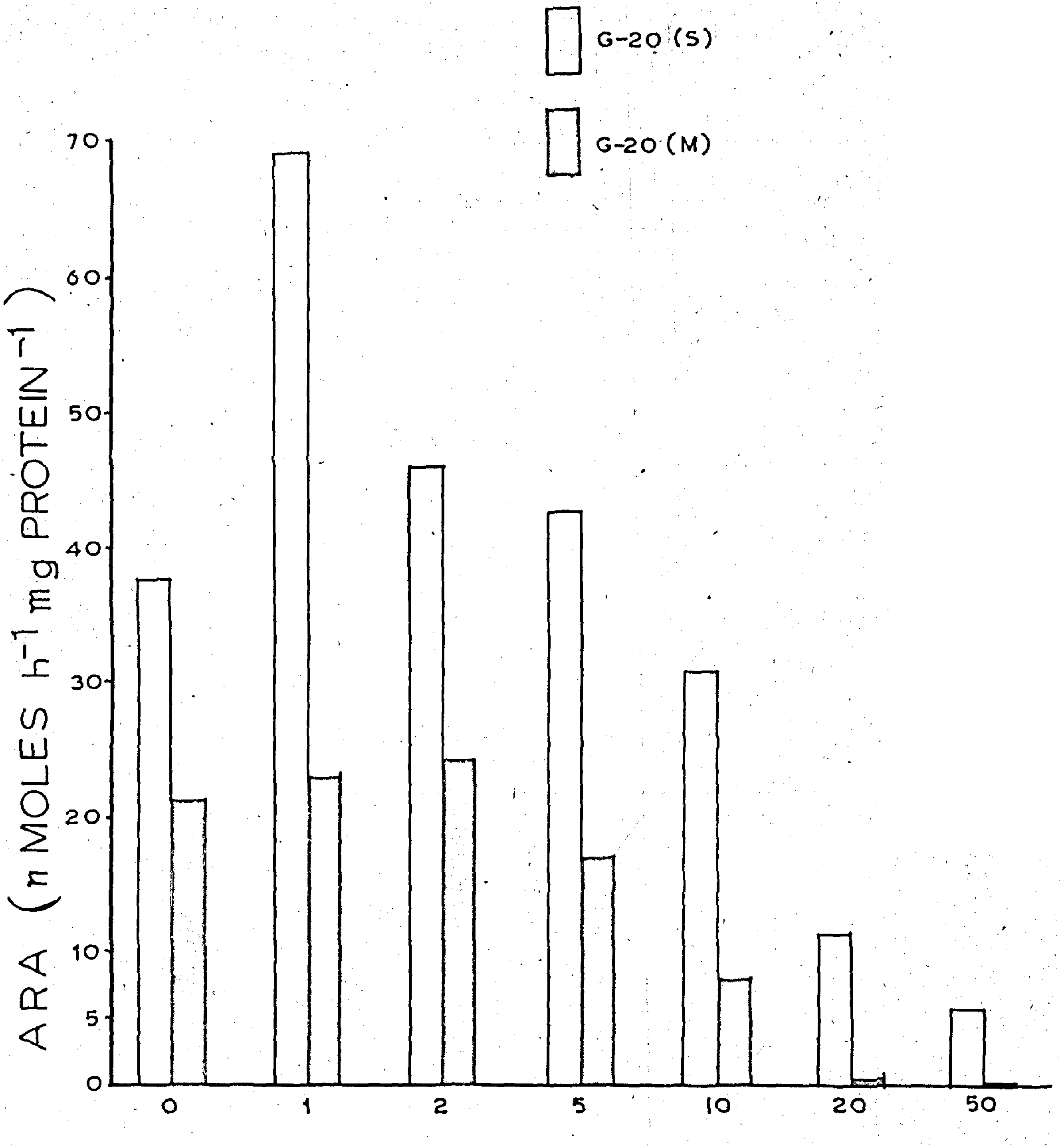

G-20 (S)
G-20 (M)
ARA (n MOLES h^{-1} mg PROTEIN^{-1})
70
60
50
40
30
20
10
5
0
0
1
2
5
10
20
50
KNO$_3$ (mM)

FIG. 7

Table 19: Effect of yeast extract on ex *planta* nitrogenase activity of G-20(S) and G-20(M) in solid and liquid cultures

Yeast extract g/l	ARA (n moles h^{-1} mg protein^{-1})			
	Solid		Liquid	
	G-20(S)	G-20(M)	G-20(S)	G-20(M)
0.05	20.96	14.69	2.14	5.14
0.20	32.59	18.37	7.58	6.62
0.50	38.60	22.03	8.08	7.04
1.00	23.56	16.49	4.59	2.04
1.50	11.06	9.80	0.22	0.21
2.00	10.04	1.28	0.00	0.00
3.00	0.56	0.00	0.00	0.00
4.00	0.41	0.00	0.00	0.00

Maximum values were observed at 0.5 g/l of Y.E. in all the cases, whereafter the activity decreased progressively.

While in liquid medium there was a total cessation of activity at 2 g/l concentration of yeast extract for both the colony types, in solid medium complete inhibition occured at 3 g/l concentration in case of G-20(M).

4.14. Expression of ex planta nitrogenase activity on liquid and solid nitrogen free medium by G-20:

Generally, the amount of nitrogen fixed under free living conditions is not enough to sustain growth. The combined nitrogen source is therefore necessary for the growth of *Rhizobium* culture. For the induction of *ex planta* nitrogenase activity a source of combined nitrogen is always included in the medium used. To see if there is any expression of *ex planta* nitrogenase activity under nitrogen free conditions, modified Burk's medium was used. Sucrose was substituted in Burk's medium with mannitol in one experiment and with malate in another. The activity was seen both in liquid as well as in solid media after 2, 4, 6 and 9 days. The activity in general was more in solid medium than in liquid medium (Table 20). The activity increased from second day onwards to reach a maximum on the sixth day in all the cases (Fig. 8&9).

The activity was higher in the malate substituted medium than in mannitol substituted medium upto fourth day.

4.15. Non-growth inhibitory concentrations of different plasmid curing agents:

Most of the curing agents are known to eliminate plasmids from cells by preferentially inhibiting the replication of plasmid DNA. Thus the concentration of the agent

Table 20: *Ex planta* nitrogenase activity by G-20 in solid and liquid mannitol and malate substituted Burk's medium

Incubation (Days)	ARA (n moles h^{-1} mg protein^{-1})			
	Mannitol substitution		Malate substitution	
	Liquid	Solid	Liquid	Solid
2	2.103	8.063	11.782	10.867
4	5.405	10.687	15.960	24.391
6	29.148	48.238	27.338	40.582
9	3.193	39.713	7.162	24.147

Fig.8 Expression of *ex planta* nitrogenase activity by G-20 in solid and liquid nitrogen free mannitol substituted Burk's medium

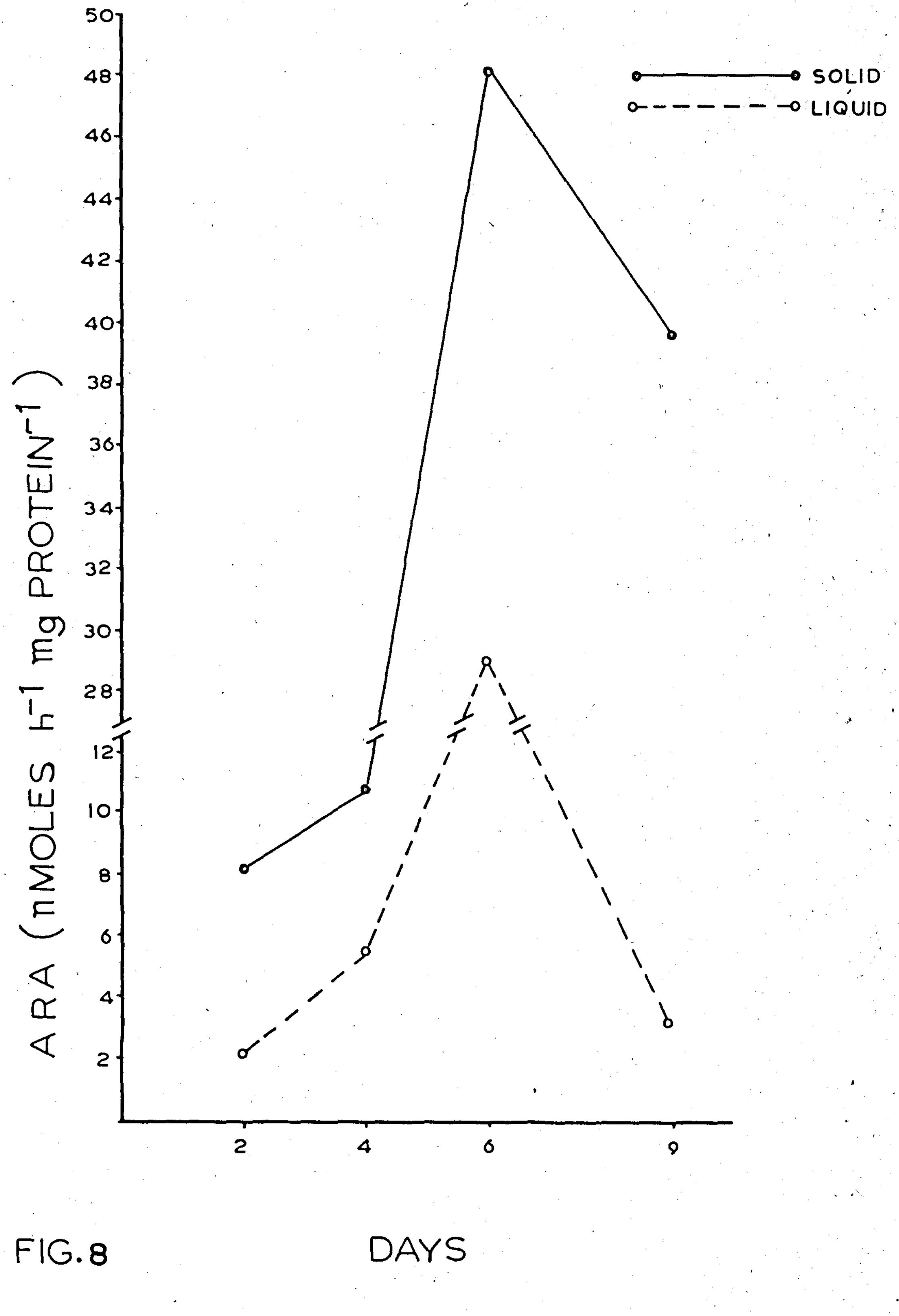

SOLID
LIQUID
ARA (nMOLES h⁻¹ mg PROTEIN⁻¹)
50
48
46
44
42
40
38
36
34
32
30
28
12
10
8
6
4
2
2
4
6
9
DAYS

FIG. 8

Fig.9 Expression of *ex planta* nitrogenase activity by G-20 on in solid and liquid nitrogen free malate substituted Burk's medium

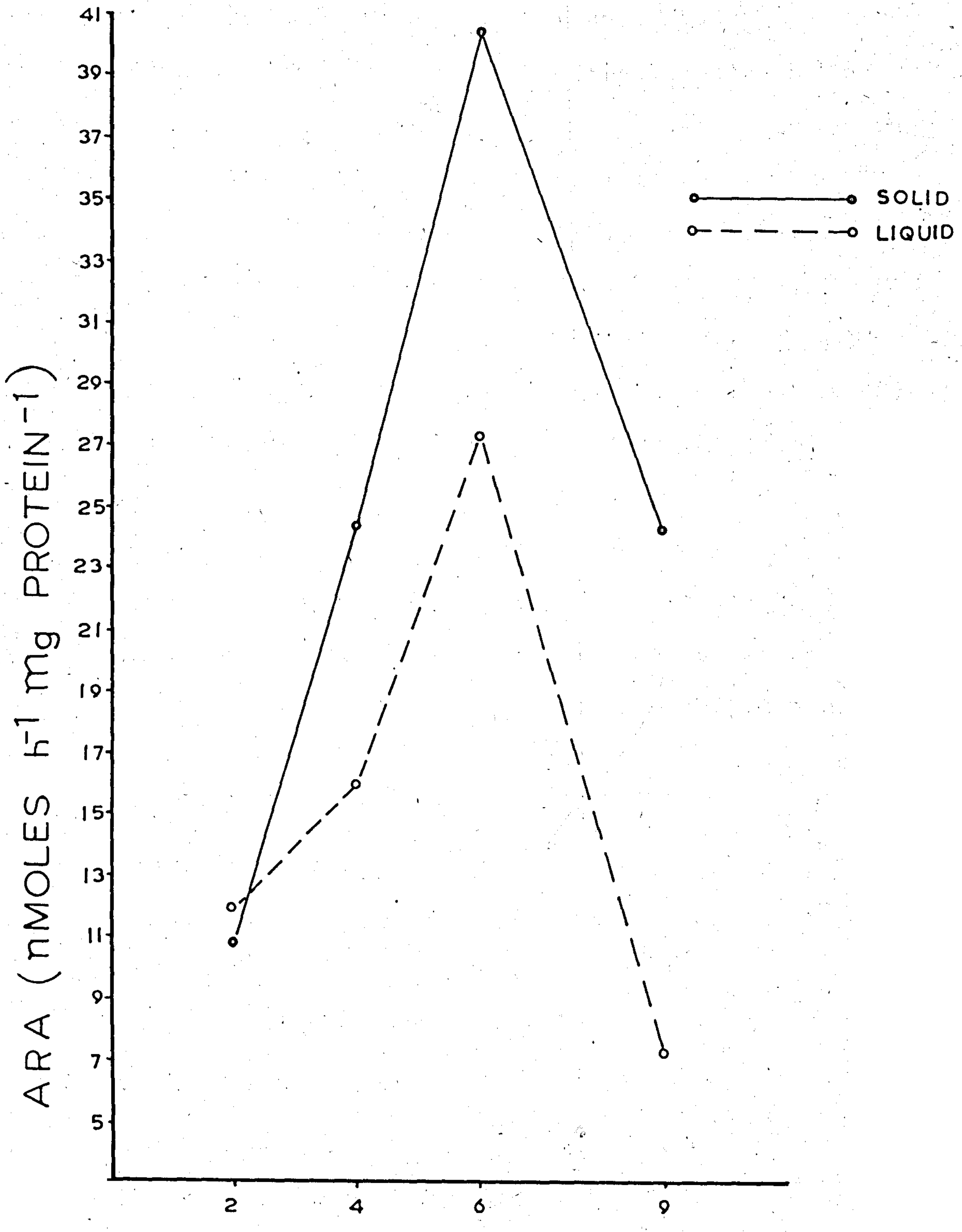

ARA (nMOLES h^{-1} mg PROTEIN^{-1})
41
39
37
35
33
31
29
27
25
23
21
19
17
15
13
11
9
7
5
2
4
6
9
SOLID
LIQUID

FIG.9 DAYS

to be used for curing plasmid DNA should be the one that does not interfere with the replication of chromosomal DNA and hence has no lethal effect on the organism under study.

During the present study, Ethidium bromide and sodium dodecyl sulphate were used as chemical agents for curing plasmids in G-20. In addition, high temperature, a physical agent that has been widely reported to be very effective in curing plasmid DNA in many bacterial systems, was also used. Different combinations of chemical and physical agents were also tried.

(i) Temperature:

G-20 was incubated in liquid culture at 30, 35, 39 and 42°C. It was observed that upto 39°C there was no inhibition of growth, while at 42°C the growth was inhibited after 48 hours (Table 21). For further studies 39°C was selected as the non-growth inhibitory high temperature.

(ii) Sodium dodecyl sulphate:

G-20 was incubated in media containing SDS at 25, 50, 100, 150 and 200 µg/ml concentrations. Inhibition of growth was seen at 150 µg/ml concentration. Hence 100 µg/ml concentration of SDS was chosen as the non-growth inhibitory concentration for G-20 (Table 22).

(iii) Ethidium bromide (EtBr):

G-20 was incubated in liquid culture in presence of EtBr at concentrations of 0.5, 1.0 and 1.5 µg/ml. Relatively less growth was seen at 1.0 µg/ml, and hence 1.0 µg/ml concentration of EtBr was chosen as non-growth inhibitory concentration (Table 22).

Table 21 : Growth of G-20 at different temperatures after 48 hours (Optical density)

Time (h)	Temperature (°C)			
	30	37	39	40
0	0.042	0.041	0.040	0.041
48	0.612	0.618	0.615	0.501

Table 22 : Growth of G-20 in the presence of different curing agents

Curing agent	Concentration µg/ml	Optical density	
		0 h	48 h
SDS	25	0.034	0.688
	50	0.035	0.694
	100	0.032	0.685
	150	0.032	0.482
	200	0.034	0.270
Et Br	0.5	0.084	0.753
	1.0	0.085	0.748
	1.5	0.084	0.489
	2.0	0.086	0.239
	2.5	0.083	-

4.16. Elimination of nif character:

The parent strain G-20 was subjected to curing treatments, at non-growth inhibitiory temperature and non-growth inhibitory levels of SDS and EtBr. The parent was grown in liquid culture in presence of curing agents for 6-7 days under stationary conditions. The flasks were manually shaken 4-5 times a day for about 30 seconds each time. An aliquot of the culture was plated on YEMA plates and single clones were obtained. Table 23 lists different curing treatments given and the number of clones screened for the expression of nitrogenase activity under free living conditions against each treatment for Rhizobium sp. G-20. The data presented in Table 23 show that no clone was found to be lacking nitrogenase expression under free living conditions.

In case of bacterial systems where the frequency of plasmid elimination is very low, serial transfer in the presence of SDS is recommended as it results in the enrichment of plasmid-cured cells. The wild type strain was grown in liquid medium containing 100 µg/ml SDS at 39°C. An aliquot from this was later inoculated into fresh medium and allowed to grow for 3-4 generations. The serial transfer was repeated.

The serial transfer was continued for a large number of generations. At the time of 5th, 10th and 15th transfer, an aliquot of culture was appropriately diluted and plated to get single clones, each of which was then tested for the expression of *ex planta* nitrogenase activity.

Table 23: Elimination of *ex planta* nitrogenase activity in G-20 by different curing agents

Treatment	No. of clones tested	*Ex planta* nitrogenase negative clones
High temperature ($39^{\circ}C$)	147	Nil
SDS	160	Nil
EtBr	180	Nil
High temperature ($39^{\circ}C$) + SDS + EtBr	208	Nil

No *ex planta* nitrogenase negative clone was detected among a total of 307 clones screened. The data are presented in table 24.

4.17. Isolation and characterization of Tn5 mutants of *Rhizobium sp.* G-20:

Transposon Tn5 has the property of integrating randomly in the bacterial genome, which can result in a series of symbiotic mutants. Great advantage of transposon mutagenesis is that it marks the gene in which it inserts. Various *nif*$^{-}$ and *nod*$^{-}$ mutants thus obtained may help in understanding of number, structure and location of the genes involved in nodulation and nitrogen fixation.

Tn5 mutagenesis was carried out according to a modified method described by Selvaraj and Iyer (1983). Patch mating of WA 803 (PGS9) and strain G-20, was carried out and 1,860 Tn5 mutants showing (Nal^{R}25 Nm^{R}50 Km^{R}100), were isolated. But of the above, 1200 mutants were tested for the expression of *ex planta* nitrogenase activity and also for nodulation and nitrogenase activity *in planta*. Four categories of mutants were isolated (Table 25).

Group 1 : Consisted of 0.58% of *ex planta* nitrogenase activity negative (*ex nif*$^{-}$) nodulation negative (*nod*$^{-}$) mutants.

Group II: Included 0.75% of (*ex nif*$^{+}$ *nod*$^{-}$) mutants.

Group III: Included 0.5% of (*ex nif*$^{+}$ *nod*$^{+}$ *fix*$^{-}$) mutants.

Group IV : Consisted of 1.5% of (*ex nif*$^{-}$ *nod*$^{+}$ and *fix*$^{+}$)

Table 24: Elimination of *ex planta* nitrogenase activity of G-20 by serial transfer in SDS medium at high temperature*

Serial transfer	Total No. of clones tested	*Ex planta* nitrogenase negative clones
5th	86	Nil
10th	102	Nil
15th	119	Nil

* SDS 100 mg/ml at 39°C

Table 25: Nature of Tn5 mutants

Types of mutants	% of mutants
ex *nif*$^-$ *nod*$^-$	0.58
ex *nif*$^+$ *nod*$^-$	0.75
ex *nif*$^+$ *nod*$^+$ *fix*$^-$	0.50
ex *nif*$^-$ *nod*$^+$ *fix*$^+$	1.50

4.18. In planta and ex planta nitrogenase activity of Tn5 derivatives:

The *in planta* and *ex planta* nitrogenase activity of eight Tn5 mutants is represented in Table 26.

In all the eight Tn5 mutants the *ex planta* nitrogenase activity was found lower than the parent strain. It was maximum in t-233 (9.5) and minimum in t-165 (5.6). *In planta* nitrogenase activity also was low in t-163 and t-251 than the parent strain, which showed 4.98 ARA (u moles h^{-1} $plant^{-1}$).

4.19. Plasmid pattern of Tn5 mutants:

To know if the transposon mutagenesis has caused some change in the plasmid pattern of the parent strain. The plasmid profile of the eight Tn5 mutants was checked.

The plasmid pattern of Tn5 mutants has been shown in Table 27. All the Tn5 mutants showed two plasmid bands each, like the parent strain, except the mutant t-49 (*ex nif*$^-$ *nod*$^-$) in which only one plasmid band was detected (Plate 3).

Table 26: *In planta* and *ex planta* nitrogenase activity of parents and Tn5 derivatives

Strain	Genotype	ARA	
		ex planta^a	*in planta*^{b}
t-49	*ex nif*$^-$ *nod*$^-$	-	-
t-237	*ex nif*$^-$ *nod*$^-$	-	-
t-233	*ex nif*$^+$ *nod*$^-$	9.5	-
t-90	*ex nif*$^+$ *nod*$^-$	6.4	-
t-165	*ex nif*$^+$ *nod*$^+$ *fix*$^-$	5.6	-
t-264	*ex nif*$^+$ *nod*$^+$ *fix*$^-$	8.3	-
t-163	*ex nif*$^-$ *nod*$^+$ *fix*$^+$	-	2.18
t-251	*ex nif*$^-$ *nod*$^+$ *fix*$^+$	-	3.02
Parent G-20	ex nif$^+$ nod$^+$ fix$^+$	38.61	4.98

nod = nodulation

ex nif = *ex planta* nitrogenase

fix = *In planta* nitrogenase

a = ARA (n moles h^{-1} mg $protein^{-1}$)

b = ARA (µ moles h^{-1} $plant^{-1}$)

Table 27: Plasmid pattern of Tn5 mutants

Mutant	Plasmid bands
t-49	1
t-237	2
t-233	2
t-90	2
t-165	2
t-264	2
t-163	2
t-251	2
Parent	2

Plasmid pattern of Tn5 mutants

1	t-251
2	t-163
3	t-264
4	t-165
5	t-90
6	t-233
7	t-237
8	t-49
9	*E. Coli*

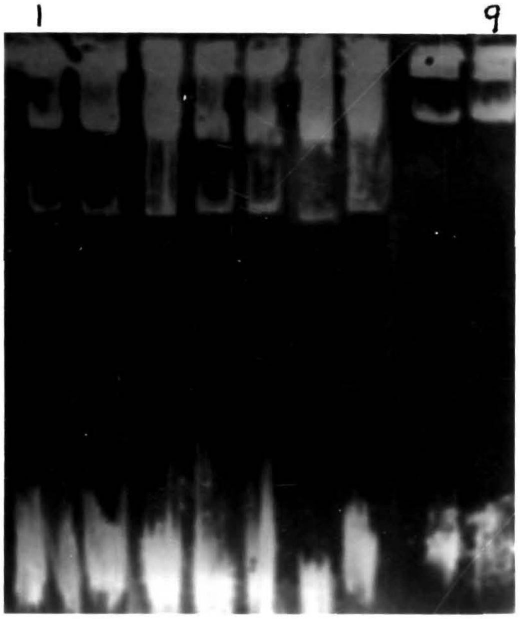


PLATE 3

DISCUSSION

Guar, an important *Kharif* legume of Indian arid zone is nodulated by rhizobia that are taxonomically grouped with cowpea miscellany group (Richmond, 1926 and Wilson, 1939). The survey of guar growing area has revealed that the nodulation status with regard to native rhizobia under field conditions, is generally poor. To improve the nodulation status, it is therefore important that efficient *Rhizobium* cultures are introduced as biofertilizers. Not much work has been reported on the physiological and genetic aspects of guar rhizobia.

In the present studies, guar nodules were collected from different agroclimatic regions of Haryana and one hundred rhizobial isolates were obtained. These isolates were identified as slow growers (64 isolates) and fast growers (36 isolates). Fast growers appeared on YEMA plates between 2-4 days, while slow growers took 5-10 days. This indicates both slow and fast growing rhizobia can nodulate guar. Numerous reports have so far appeared showing that an individual host legume can be nodulated by both types of rhizobia (*Lotus penduncolatus* : Pankhurst, 1977; *Vigna sinensis* : Broughton and Dilworth, 1971; *Glycine max* : Keyser *et al*., 1982). These rhizobia must contain, atleast, a similar complement of *sym* genes plus other ancillary genes required for nodulation of the particular host. Cross hybridization studies between such strains may assist in identifying and characterizing such genes. In addition, analysis of components present on the surface of both types of rhizobia may help in detecting a common factor required for nodulation.

In order to carry out detailed studies, ten fast and ten slow growing representative isolates were chosen. The choice was based on the data obtained in pot house studies on guar. Isolates showing high plant dry weight and high nitrogenase activity were selected.

When the fast and the slow growing isolates were compared for the metabolic differences a clear distinction was observed in carbohydrate utilization (Table 4 and 5). The fast growers resembled the classical fast growers like R. meliloti, R. trifolii and R. leguminosarum in carbon utilization, while the slow growers resembled the Bradyrhizobium. The fast growers were able to use a broad range of hexoses, pentoses, disaccharides, trisaccharides and organic acids while slow growers had limited ability to use diverse carbon sources. Differences in carbon utilization between fast and slow growing rhizobia were reported by Graham (1964), Chakrabarti et al. (1981) and Stowers [illegible] (1985). The observations made in the present studies are similar to those recorded by Broughton et al. (1984) in fast and slow growing rhizobia belonging to cowpea miscellany group.

Intrinsic drug resistance is widely seen in rhizobia. It probably imparts survival value to the rhizobia in natural environment. Drug resistance markers help in strain identification in ecological and in genetic experiments. In the present studies also multiple drug resistance was observed in all fast and slow growing *Rhizobium* isolates (Table 6 and 7).

Trinick (1980, 1982) observed that many fast and slow growing *Rhizobium* sp., display a broad host range, and that some strains have the capacity even to nodulate the non-legume *Parasponia andersonii* (Trinick and Galbraith, 1980).

During the present investigations, it was observed that when tested for nodulation on different crops, both slow and fast growing rhizobia exhibited a broad host range and nodulated guar, pigeonpea, mungbean, urdbean and ricebean but not soybean and chickpea. Among slow growing rhizobial strains, (Table 8) G-4, G-5, G-11 and G-18 showed uniformly good performance on all the five host crops, and among fast growers (Table 9) G-9, G-20 and G-28 were found better for all the crops tested. Strains showing wide host range can prove useful in studying various aspects of nodule development such as identifying different steps incurred and number of plant genes involved in the development. The nodules formed on ricebean were very small while those formed on guar and pigeonpea were large. Detailed comparisons of the nodulins (Verma *et al.*, 1981) they contain must prove useful.

Since the discovery by five independent groups of workers that some rhizobia express nitrogenase activity on defined laboratory media in the absence of plant host (Keister, 1975; Kurz and LaRue, 1975; Mc Comb *et al.*, 1975; Pagan *et al.*, 1975; Tjepkema and Evans, 1975), many reports have appeared on the expression of *ex planta* nitrogenase activity. These studies are mainly on slow growing strains which show free living nitrogen fixation under defined cultural conditions. Attempts to extend derepression of nitrogenase to other rhizobia have largely proved refractory (Mohapatra and Gresshoff, 1983). Most of the strains showing *ex planta* nitrogenase belong to *R. japonicum* and slow growing cowpea strains. Also, highly specific and regulated conditions are reported to be required for its expression. Although the conditions were standardized for cowpea strain 32H1 and some *Rhizobium japonicum* strains for the *ex planta* nitrogenase activity, many other strains could not be derepressed. Reports on the presence of *ex planta* nitrogenase activity in fast growing rhizobia are very limited and only recent (Dreyfus *et al.*, 1983; Bender *et al.*, 1986; Urban *et al.*, 1986).

During the course of present studies all the ten slow growing isolates tested showed *ex planta* nitrogenase activity on nitrogenase induction medium (NIM). All the available fast growers were tested for the activity, but only two isolates G-73 and G-20 expressed free living acetylene reduction activity. The activity was also very high as compared to that seen in slow growing isolates (Table 10).

The reason for not observing the *ex planta* nitrogenase activity in other fast growing isolates may be that the conditions were not optimal for the derepression of nitrogenase in these isolates. Gibson *et al.* (1976), Pankhurst (1981) and Kaneshiro and Kurtzman (1982) have reported that the requirements for the expression of *ex planta* nitrogenase in *Rhizobium* strains are different. Alternatively, the derepression of nitrogenase in the laboratory cultures may be under genetic control and it may be strain specific as proposed by Mohapatra and Gresshoff (1983).

All the classical fast growing rhizobia (*R. meliloti*, *R. leguminosarum*, *R. trifolii*, *R. phaseoli*) have been shown to contain plasmids (Prakash *et al.*, 1981; Banfalvi *et al.*, 1981). The fast growing rhizobia infecting soybeans and cowpea miscellany plants resemble the classical fast growing rhizobial species in regard to presence of plasmids as well as location of *nod* and *nif* genes (Masterson *et al.*, 1982; Sadowsky *et al.*, 1983; Broughton *et al.*, 1984; Prakash and Atherly, 1984; Applebaum *et al.*, 1985b). During the present studies, 1-3 plasmids were observed in fast growing isolates of rhizobia. Eight slow growing isolates showed 1-2 plasmids but two others did not harbour any (Table 11). *Bradyrhizobium* have not always been found to harbour plasmids suggesting that the genes controlling nodulation and nitrogen fixation might be located on the chromosomal DNA (Cantrell *et al.*, 1982; Jagadish and Szalay, 1984; Hennecke *et al.*, 1985; Noti *et al.*, 1985).

All detailed physiological and genetic studies were carried out on a fast growing *Rhizobium* isolate G-20 which in addition to expressing ex planta nitrogenase activity showed kanamycin and neomycin sensitivity thus making it suitable for Tn5 mutagenesis as well.

When G-20 was spread on yeast extract mannitol agar plates it gave rise to large and small colonies and also slimy and mucoid colonies. The slimy and mucoid colonies differed in certain characteristics (Table 12). Such colonial variation is reported to occur in almost all cultures of slow growing rhizobia and was seen when the cultures were spread on media plates containing sugar or sugar derivatives (Agarwal and Keister, 1983). On repeated isolation and replating, most of R. japonicum colony types were found to be unstable and gave rise to another population. The derivatives isolated by Kuykendall and Elkan (1976) and Upchurch and Elkan (1977) were, however, stable in R. japonicum. In fast growing species also the colonial variation was reported by Bender et al. (1986) who observed two colony types in pigeonpea isolate IHP100.

Both slimy G-20(S) and mucoid G-20(M) colony types were selected to see the effect of different factors on ex planta nitrogenase activity. The expression of ex planta nitrogenase activity was observed on different media agar slopes or liquid cultures incubated on shaker. Interestingly, G-20 showed ex planta nitrogenase activity on simple YEMA slants and YEM liquid culture. No specific regulated conditions of cell density (Keister and Evans, 1976) or control of gaseous atmosphere was required (Agarwal and Keister, 1983; Dreyfus et al., 1983). No such results have so far been reported.

Slimy colony type expressed high level of *ex planta* nitrogenase activity as compared to its mucoid counterpart. The activity was more on nitrogenase induction medium than yeast extract mannitol agar medium. Also, in all experiments the activity was low in liquid cultures as compared to agar slopes (Tables 13 and 14).

Production of EPS is a characteristic of many nitrogen fixing bacteria. It is possible that EPS production is related to derepression of nitrogenase. Kurz and LaRue (1975), Pagan *et al*. (1975) and Pankhurst and Graig (1978) proposed that the mucoid material produced by rhizobia might aid nitrogenase activity by lowering the speed of oxygen transfer. Wilcockson and Werner (1978) could establish no clear correlation between EPS and acetylene reduction. In isolate G-20, a negative correlation was observed between EPS and nitrogenase activity. Upchurch and Elkan (1977) also found a reciprocal correlation between the two. In every case they studied, the strain with less EPS developed more *ex planta* acetylene reduction than did the strain with high content of EPS. Similar results were obtained by Agarwal and Keister (1983) in 39 original isolates of *R. japonicum*. The reciprocal relationship observed during the present studies can be explained by the fact that both nitrogen fixation and EPS synthesis demand cellular energy and under microaerobic conditions necessary for the synthesis and expression of nitrogenase, bacteria are energy limited. The available ATP may be directed towards the synthesis of EPS instead of nitrogenase.

Higher activity observed on nitrogenase induction medium could be attributed to the presence of malate, inositol, micronutrients and vitamins which are not present in the yeast extract mannitol medium. Nitrogenase induction medium being a rich medium compared to YEMA, ex planta nitrogenase activity is higher in the former medium.

Ex planta nitrogenase activity is found to be more on agar slopes than in liquid culture. Pankhurst and Craig (1978) reported that in the solid medium there is protection from oxygen. The upper layer of cells do not synthesize nitrogenase. Cells present in lower strata are protected from oxygen effect and hence fix nitrogen. Although in liquid media, nitrogenase activity is subject to oxygen effect due to continous shaking, respiratory protective mechanism can operate due to high cell respiration thereby allowing some nitrogenase activity. Bergersen and Tuner (1976) observed such 'O_2 shock' effect in chemostat cultures of Rhizobium sp. strain 32H1. The maximum activity was observed at 35°C. Below and above this temperature the activity was low. Although, growth was seen at 45°C, very little activity was observed. (Table 15 and 16). Capacity of G-20 to express ex planta nitrogenase activity at high temperature is probably because of the fact that guar rhizobia are adapted to conditions of high temperature. High temperature is prevalent during summer season when sowing of guar is generally done. Nitrogenase of Azotobacter ,Clostridium and Bacillus are also stable at 50-60°C (Burns and Hardy, 1975), for 10-15 min. Low activity above 35^{o}C in G-20 indicates that some of the nif genes are probably not expressed as reported by Hennecke and Shanmugam (1979) in Klebsiella pneumoniae.

Pankhurst and Craig (1978) observed that increasing the incubation temperature of Rhizobium strain 32H1 decreased or inhibited the nitrogenase activity and that this was due to the disruption of specialized cells expressing nitrogenase activity. This was analogous to the disruption of bacteroid development in clover nodules at high temperature (Pankhurst and Gibson, 1973). Detailed studies on proteins and ultrastructure of cells are needed, under conditions of nitrogen fixation, to come to definite conclusion on the effect of temperature on ex planta nitrogenase activity.

Contradictory reports are present in the literature on the effect of ammonium on ex planta nitrogenase activity. Effect of ammonium on nitrogenase activity is strain specific. It is further affected by type of carbon sources, oxygen levels, culture regime and presence of other nitrogenous compounds. (Mohapatra and Gresshoff, 1983). Ammonium is found to inhibit ex planta nitrogenase activity in G-20 but favoured growth (Table 17). These results are similar to those observed by Pankhurst and Craig (1978) who found that increase in nitrogen content of media repressed nitrogenase activity and stimulated growth. Keister and Ranga Rao (1977) observed that nitrogenase activity was slightly repressed by NH_4^+ but they attributed it to metabolic effect rather than repression.

Experimental evidence has been presented by some workers to implicate the role of glutamine synthetase (GS) in ammonia assimilation in *Rhizobium*. Ludwig and Signer (1977) found glutamine auxotrophs of *Rhizobium* strain 32H1 failed to derepress synthesis of nitrogenase both in culture and *in planta*. Reversion to prototrophy simultaneously recovered nitrogenase derepression ability. Whether inhibition of *ex planta* nitrogenase activity is due to repression or simply modulation of the activity, can be revealed only by further studies on polypeptide synthesis in the presence of ammonia.

Nitrate was stimulatory at lower concentrations but higher concentrations inhibited the *ex planta* nitrogenase activity (Table 18). It is probable that nitrate is used as terminal electron acceptor in place of oxygen, leading to favourable growth (Pankhurst, 1981) but at higher concentrations it inhibits nitrogenase activity by probably switching off *nif* operon, as nitrogen fixation in the presence of a nitrogen source is energy expensive. Low levels of KNO_3 were found beneficial for *in planta* nitrogenase activity also(Mand and Chahal, 1987).

The effect of yeast extract was also analogous to the effect of nitrate, low levels being stimulatory to the nitrogenase activity. Highest nitrogen fixation was seen at 0.5 g/l yeast extract concentration (Table 19). The stimulation of nitrogenase activity could be due to the availability of growth factors, rare carbon sources and nitrogen sources that promote growth without inhibiting acetylene reduction. Inhibition of nitrogenase activity at higher concentrations could be due to amino acids. The intracellular pool of amino acids cause repression of nitrogenase activity due to release of ammonia from them (Rennie, 1981; Das and Mishra, 1982).

For the expression of *ex planta* nitrogenase activity a source of combined nitrogen is required in the medium which supports the growth of *Rhizobium*. To date, there are only two reports of free-living nitrogen fixation without combined nitrogen source. Dreyfus *et al*. (1983) isolated a *Rhizobium* strain from stem nodules of the legume (*Sesbania rostrata*)which is able to grow on atmospheric nitrogen (N_2) as the sole nitrogen source. Urban *et al*. (1986) showed that strain 0403 from *Rhizobium trifolii* is able to grow without combined nitrogen.

Interestingly, the *Rhizobium* isolate G-20 is able to grow and fix nitrogen in modified Burk's nitrogen free medium (Table 20). When Burk's medium containing sucrose as carbon source was used G-20 showed a slight growth on it, but on modified Burk's medium in which sucrose is substituted with either mannitol or malate, G-20 grew better and expressed better *ex planta* nitrogenase activity. High activity was observed on agar slopes and liquid cultures. Moreover, no control of gaseous atmosphere as reported by Dreyfus *et al*. (1983) or treatment of cells with succinate or other nutrients for the induction of nitrogenase in nitrogen free medium (Urban *et al*., 1986) was necessary.

Plasmid cured derivatives have been obtained by growth at elevated temperatures (Zurkowski and Lorkiewicz, 1979) or in ethidium bromide or SDS (Dunican and Cannon 1971; Zurkowski *et al*., 1973). The plasmid curing agents when employed singly or in combination did not yield any *nif* derivatives (Table 21). Serial transfer in SDS at high temperature spanning many generations was done but no *nif*$^-$ clone could be obtained (Table 22).

The results of the present study indicate that either the genes for nitrogen fixation are borne on the chromosome in *Rhizobium* isolate G-20, like many classical slow growers (Cantrell *et al*., 1982 and Noti *et al*., 1985) or the plasmids do not carry *nif* and *nod* determinants (Masterson *et al*., 1982 and Masterson *et al*., 1985). Alternatively, *nif* genes may be located on a megaplasmid which could not be cured by the above methods. For definite conclusions detailed studies such as involving DNA hybridization are necessary.

During the present studies, transposon mutagenesis of *Rhizobium* isolate G-20 led to the isolation of symbiotic mutants. After screening twelve hundred putative Tn5 mutants, for *ex planta* nitrogenase activity, for nodulation and *in planta* nitrogenase activity (Table 23) four categories of mutants have been obtained *viz*., *ex* *nif*$^-$ *nod*$^-$, *ex* *nif*$^+$ *nod*$^-$, *ex* *nif*$^+$ *nod*$^+$ *fix*$^-$, *ex* *nif*$^-$ *nod*$^+$ *fix*$^+$.

The *ex nif*$^-$ *nod*$^+$ *fix*$^+$ derivatives which were *ex planta nif*$^-$ but could form nodules and fix nitrogen *in planta* do not seem to be defective in the structural genes for nitrogenase. It is likely that these were regulatory mutants defective in a gene whose product is required for the *ex planta* expression of nitrogenase. One such gene product (a 15 Kd respiratory regulatory protein) has been identified recently in case of *R. japonicum* mutants which are incapable of fixing nitrogen under *ex planta* conditions (Hennecke *et al.*, 1985).

The derivatives *ex nif*$^+$ *nod*$^+$ *fix*$^-$ again may be mutants defective in regulatory genes of nitrogen fixation but not the structural genes, as these could fix nitrogen *ex planta*, but not *in planta*. Alternatively, the control of nitrogenase activity *in vitro* and *in planta* could be different. (Cen *et al.*, 1982). Unlike the nodules from plants inoculated with parent strain, the nodules from the plants inoculated with these Tn5 mutants were small and pale. This indicates that the inserted Tn5 has caused defect in the genes associated with heme synthesis. Maier and Brill (1976) obtained a mutant strain of *R. japonicum* which produces small ineffective nodules lacking leghaemoglobin and incapable of fixing nitrogen. However, the same strain could be induced to fix nitrogen in culture. Isolation of Tn5 mutants which are *ex nif*$^+$ *nod*$^-$ indicates that the transposon is inserted in the nodulation genes, giving rise to *nod*$^-$ phenotype.

The parent G-20 showed two plasmids while an *ex nif*$^-$ *nod*$^-$ mutant t-49 showed only one plasmid (Table 27). The *ex nif*$^-$ *nod*$^-$

mutant t-49 may represent a cured derivative due to the insertion of Tn5 in the replicon of the sym plasmid carrying nif and nod genes. Alternatively, the transposon got inserted in the region of nif and nod genes, which indicates, they are closely linked (Long et al., 1982; Banfalvi et al., 1983; Broughton et al., 1984). Examination of the mutants with current molecular biology technique should enable an analysis of the bacterial genes involved in nodulation and nitrogen fixation.

SUMMARY

Guar is a drought tolerant crop widely cultivated in north-west India. It is an economically important crop and is grown for gum, grain, vegetable and fodder purposes. Guar is nodulated by Rhizobium strains belonging to coupea miscellany group. The nodulation status of this crop is poor. Also, genetic, physiological and biochemical basis pertaining to nitrogen fixation, nodulation and cross infectivity in guar rhizobia is not known. The present studies were aimed at identifying best nodulating and efficient strains and carrying out studies on the physiology and genetic make up of the important isolates.

During the course of present study, 64 slow growing and 36 fast growing rhizobia were isolated from nodules collected from guar growing areas in Haryana state. For further studies, ten of each slow and fast growing isolates were selected on the basis of efficiency and best nodulation on guar plants. All exhibited a multiple antibiotic resistance pattern. A broad distinction was observed between fast and slow growing isolates in the ability to use different carbon sources. All the isolates had a wide host range and nodulated guar, pigeonpea, mungbean, urdbean and ricebean, but not soybean and chickpea.

The isolates were tested for *ex planta* nitrogenase activity. All slow growing isolates expressed a low level of *ex planta* nitrogenase activity. Among fast growers two (G-20, G-73) isolates expressed a high level of *ex planta*

nitrogenase activity. Slow growing isolates showed 0-2 plasmids while fast growing isolates showed 1-3 plasmids.

One of the fast growing *Rhizobium* isolates, G-20, expressed high levels of *ex planta* nitrogenase activity in liquid culture and agar slopes of both nitrogenase induction medium and yeast extract mannitol medium. No specific regulated conditions were required. No such report has appeared in *Rhizobium* so far. The maximum *ex planta* nitrogenase activity was observed at 35°C. Ammonium chloride depressed the nitrogenase activity even at low concentrations (1 mM). Lower doses of potassium nitrate (1-2 mM) accelerated the *ex planta* nitrogenase activity, nitrate probably acting as terminal electron acceptor under the microaerobic conditions. In case of yeast extract mannitol medium, yeast extract at the concentration of 0.5 g/l was found optimum. G-20 also expressed *ex planta* nitrogenase activity on modified Burk's nitrogen free medium requiring no specific regulated conditions in both liquid culture and on agar slopes.

Tn5 mutagenesis of G-20 led to the isolation of four types of mutants namely, (*ex* *nif*$^{-}$*nod*$^{+}$*fix*$^{+}$), (*ex* *nif*$^{+}$*nod*$^{+}$*fix*$^{-}$), (*ex* *nif*$^{+}$ *nod*$^{-}$) and (*ex* *nif*$^{-}$ *nod*$^{-}$). They are either structural or regulatory mutants and indicate that the control of *ex planta* and *in planta* nitrogenase activities is different. All symbiotic mutants showed two plasmids like the parent strain, except mutant t-49 in which one plasmid was observed.

Curing was attempted by using SDS, Ethidium bromide and high temperature but no *ex planta nif* negative derivative was obtained. The results indicate that nif and nod genes are probably borne on a megaplasmid which is not curable. Alternatively, they may be located on the chromosome.

The Rhizobium isolate G-20 occupies a unique position among rhizobia. It has the ability to reduce acetylene in liquid as well as in agar medium in a manner similar to that of Bradyrhizobium. Further, as reported in strain ORS 571 from stem nodules of Sesbania rostrata and Rhizobium trifolii 0403, it can grow without combined nitrogen. Further studies on it can help in understanding nitrogen fixation under free living and symbiotic conditions.

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PHYSIOLOGICAL AND GENETIC STUDIES ON GUAR (*CYAMOPSIS TETRAGONOLOBA*) RHIZOBIA WITH PARTICULAR REFERENCE TO PLASMIDS

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Sixty-four slow growing and thirty-six fast growing rhizobia were isolated from nodules collected from field grown guar plants in Haryana State. For further studies, ten each of slow and fast growing isolates were selected on the basis of efficiency and best nodulation on guar plants. All isolates exhibited multiple antibiotic resistance pattern. Compared to slow growers, fast growers used a broad range of carbohydrates. In addition to guar, all isolates nodulated pigeonpea, mungbean, urdbean and ricebean but not soybean and chickpea. All slow growers expressed a low level of *ex planta* nitrogenase activity. Only two of the fast growers (G-20, G-73) expressed a high level of *ex planta* nitrogenase activity. Slow growing isolates showed 0 to 2 plasmids as compared to 1 to 3 of the fast growers.

G-20 expressed high *ex planta* nitrogenase activity on nitrogenase induction medium, yeast extract mannitol medium and modified Burk's nitrogen free medium. The activity was seen on solid as well as liquid medium. In case of yeast extract mannitol medium, the optimal concentration of yeast extract was 0.5 g/l. The activity was maximum at 35°C. Ammonium chloride depressed the activity even at low levels, but KNO_3 stimulated the activity at low levels.

No *ex planta nif*$^-$ derivatives were obtained after curing with SDS, ethicium bromide and high temperature.

Tn5 mutagenesis of G-20 led to the isolation of *ex nif*$^-$*nod*$^+$*fix*$^+$, *ex nif*$^+$ *nod*$^+$*fix*$^+$, *ex nif*$^+$ *nod*$^-$ and *ex nif*$^-$*nod*$^-$ symbiotic mutants. All mutants showed two plasmids like the parent strain except t_{49} which showed only one plasmid.