EFFECT OF ACRIDINE ORANGE AND ULTRAVIOLET RADIATION ON MUTAGENESIS, CULTURAL, PHYSIOLOGICAL AND SYMBIOTIC CHARACTERS OF *Bradyrhizobium* spp.

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CERTIFICATE

This is to certify that the thesis entitled "**EFFECT OF ACRIDINE ORANGE AND ULTRAVIOLET RADIATION ON MUTAGENESIS, CULTURAL, PHYSIOLOGICAL AND SYMBIOTIC CHARACTERS OF *Bradyrhizobium* spp.**" submitted by **Miss V. SRIDEVI** in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE IN AGRICULTURAL MICROBIOLOGY to the University of Agricultural Sciences, Bangalore, is a record of research work carried out by her during the period of her study in this university under my guidance and supervision, and that no part of thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or other similar titles.

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V. Sridevi
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CONTENTS

LIST OF TABLES

LIST OF TABLES (contd...)

LIST OF TABLES (contd...)

LIST OF TABLES (contd...)

LIST OF FIGURES

LIST OF PLATES

INTRODUCTION

I. INTRODUCTION

In the process of resource management in agricultural production, biological nitrogen fixation has rightly gained it due attention in recent years mainly because of its economic and ecological significance.

Biological nitrogen fixation (BNF) is a unique character possessed by only a few genera of prokaryotic organisms that contain the genetic information to synthesize the enzyme nitrogenase which catalyses the conversion of atmospheric dinitrogen to ammonia. The most efficient and agriculturally important BNF system is *Rhizobium* - legume symbiosis. Legume-*Rhizobium* symbiosis is known to contribute around 80 million tonnes of nitrogen annually from the vast free supply in the atmosphere.

There has been constant research efforts to improve the efficiency of symbiotic nitrogen fixation through the use of more efficient rhizobial strains. The main criteria for good inoculant strain include (Brockwell et al., 1982):

a) effective nodulation and nitrogen fixation over a range of environmental conditions.

b) competitive ability against other strains.

c) ability to multiply in broth and survive in peat.

d) survival in seed pellets.

e) persistence in soil.

f) ability to migrate in soil and colonize it.

g) ability to survive adverse environmental conditions.

h) strain stability during storage.

i) nodule formation and nitrogen fixation in the presence of soil nitrogen, as intercropping legumes with non-legumes is a common cropping system in the tropics.

Generally promiscuous rhizobial strains have been found to be less effective, while strains which are highly specific in their host requirement are more efficient. However, promiscuity of rhizobia is advantageous when two or more legumes are taken up as intercrop so that inoculation of a promiscuous strain can nodulate all the legume components in an intercrop.

Conventionally an effective rhizobial strain was obtained by making large number of isolates from soil or legume root nodules formed naturally and screening them for symbiotic efficiency which was laborious and time consuming. Presently the emphasis is to use genetic techniques to manipulate the characteristics of *Rhizobium*. Mutations are considered as an alternative to naturally occurring variation and also an alternative to hybridization (Brock, 1971).

Mutagens have proved to be wonderful tools in the hands of scientists to allow the tailoring of rhizobia. Mutants of *Rhizobium* with altered symbiotic interactions have been reported (Denarie et al., 1976 ; Kuykendall, 1981).

Rhizobial genetics has progressed rapidly with *Rhizobium* strains due to the presence of symbiotic genes on plasmids in fast growers. On the other hand slow growth rate of *Bradyrhizobium* has made them less attractive for routine genetic manipulations, however considering the agronomic importance of some of their hosts like soybean, groundnut, pigeonpea and cowpea it would be equally desirable to apply genetic manipulations in *Bradyrhizobium* spp.

Therefore the present study was undertaken to determine the effect of two mutagens, acridine orange and ultraviolet rays on *Bradyrhizobium* spp with the following objectives;

a) To determine the effect of mutagens on morphological, cultural and physiological characters of *Bradyrhizobium* spp.

b) To determine the effect of mutagens on the host specificity and symbiotic characteristics of *Bradyrhizobium* spp.

c) To screen mutants of *Bradyrhizobium* spp for nitrate tolerant symbiosis.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Nitrogen is one of the major nutrient element required for crop production, which is supplied largely through chemical fertilizers. Although nitrogen constitutes 78 per cent of the earth's atmosphere not all life forms can assimilate gaseous nitrogen. The ability to convert atmospheric dinitrogen gas to ammonia is strictly limited to a few prokaryotes. The predominant group is the rhizobia, the bacteria which form symbiotic association with plants belonging to the family Leguminosae (Fabaceae) with the exception of one non-legume *Parasponia* (Family Ulmaceae), resulting in the formation of a novel plant organ called nodules where they fix atmospheric nitrogen. The ecological and economic importance of biological nitrogen fixation has justly earned research attention for legume - rhizobial symbiosis.

RHIZOBIA

Legume - *Rhizobium* symbiosis was discovered more than a hundred years ago when Hellrigeal and Willfarth (1888) showed that legumes obtain nitrogen from atmosphere rather than from soil. However the role of bacteria in nitrogen fixation was firmly established by Beijerinck(1888) who isolated them from root nodules and called them *Bacillus radicicola* and stated their important roles. Since then there has been constant effort to exploit the full potential of symbiotic nitrogen fixation in legumes.

Rhizobia are chemoorganotrophs, gram-negative non-sporing, motile rods (0.5 - 0.9 x 1.2-3.0 µm in size) aerobic, soil bacteria and belong to the family Rhizobiaceae. They characteristically produce colorless, white or cream coloured colonies on yeast extract mannitol agar (YEMA) medium with congored.

The fast growers use many sugars and organic acids whereas slow growers are more specific and commonly prefer pentoses (Fred et al., 1932 ; Ishizawa, 1953). Glucose galactose, fructose, arabinose, xylose and mannitol are reasonably well utilised by fast and most slow growers. Rhamnose, sucrose, lactose, trehalose and raffinose are poorly utilised by slow growers.

Many strains can use nitrate, ammonium or an amino acid as sole source of nitrogen although Vitamin free casein hydrolysate was still better than any tested combination of amino acids for growth of *Rhizobium japonicum*. Only 9 out of 29 other rhizobia comprising of *Rhizobium meliloti*, *R. lupini*, *Bradyrhizobium japonicum* and the cowpea rhizobia require any Vitamin and these were satisfied with biotin alone (Elkan and Kwik, 1968).

The term rhizobia is collectively used for bacteria belonging to the genera *Rhizobium* , *Bradyrhizobium* , and *Azorhizobium* . Few main differences between the three genera are presented in Table 1.

An important classification of rhizobia is based on host range and specificity as the symbiosis is specific within legumes. Host dependent cross-inoculation group system of classification has been subjected to much criticism because it is not a taxonomic one, but it is the best practiced system currently available. Rhizobia have been divided into seven cross inoculation groups as shown in table 2.

The genus *Bradyrhizobium* contains only one named species viz., *B. japonicum*. All other strains belonging to this genus are designated as *Bradyrhizobium* sp. followed by the name of the host plant from which they were isolated.

Degree of specificity varies greatly among rhizobia. Most isolates of *Rhizobium leguminosarum* biovar *viciae* even differentiate between genotypes nodulating European pea varieties but not certain peas from Afghanistan (Denarie et al., 1992). In contrast, some tropical *Bradyrhizobium* strains nodulate legumes in different tribes and sub families (Young and Johnston, 1989) while *Rhizobium* sp. strain NGR 234 nodulates atleast 35 legume genera (Denarie et al., 1992). *Rhizobium* species are defined in terms of the host legume that they can nodulate.

EXTENSION OF HOST RANGE OF RHIZOBIA

Promiscuity of rhizobia would be advantageous when two or more different legumes are intercropped so that addition of just one promiscuous rhizobia could

Table 1: Main differences between the three rhizobial genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*.

Feature/Genera	*Rhizobium*	*Bradyrhizobium*	*Azorhizobium*
Growth rate	Usually fast growth. 1mm colonies in 2 days	usually slow growth. 1mm colonies in 6-10 days	usually fast growth. 1mm colonies in 2 days
Flagella	Peritrichous	one polar or subpolar flagella	peritrichous
% G + C of DNA	59 - 64	62 - 66	66
N_2 fixation explanta	Rare	Some strains	Generic character
Location of nod and nif genes	plasmid	chromosomal	Probably
Host specificity	usually narrow	often broad	Few species

Table 2 : Cross inoculation groups in rhizobia

rhizobia	some of the hosts nodulated
Rhizobium	
R. leguminosarum biovar *viciae*	Pea (*Pisum*), Vetch (*Vicia*)
bv. *phaseoli*	bean (*Phaseolus*)
bv. *trifolii*	clover (*Trifolium*)
R. meliloti	Alfalfa (*Medicago*)
R. loti	Trefoil (*Lotus*)
R. fredii	Soybean (*Glycine*)
Bradyrhizobium	
B. japonicum	Soybean (*Glycine*)
B. spp	Peanut (*Arachis*)
	Cowpea (*Vigna*)
Azorhizobium	
A. caulinodans	*Sesbania*

nodulate different legumes in an intercrop.

Schwinghamer (1962) increased the percentage of pea plants nodulated with *Rhizobium trifolii* from 15 per cent to as high as 96 per cent by various mutagenic treatments of the bacterium, but the pea nodules were ineffective. O'Gara and Shanmugam (1978) isolated mutant strains of *R. trifolii* which unlike the wild type effectively nodulated both soybean and mungbean.

Djordjevic et al., (1985) identified a gene hsnA on a 14 kb fragment of *Rhizobium leguminosarum* bv. *trifolii* Sym plasmid that encode host specific nodulation functions. Tn5 mutations in the hsnA region of *Rhizobium leguminosarum* bv. *trifolii* strain ANU 843 displayed extended host range. Phenotypically, these mutants could not nodulate *Trifolium repens*, but could nodulate *Trifolium subterraneum*. More importantly unlike wild type parent, the hsnA mutants could nodulate *Pisum sativum*.

In another genetic study Bender *et al.*, (1988) transfered a nodD gene from a *Rhizobium* strain with an extended host range, including *Parasponia* to a strain from clover after which this *Rhizobium* too could nodulate *Parasponia*. In this transgenic strain the transcription of nodD was induced by many more flavonoids than the original strain.

Point mutation in the nodD gene of a strain of *R. leguminosarum* bv *trifolii* in which the host range was extended even to the non-legume *Parasponia* (Melver et al., 1989).

RHIZOBIAL SYMBIOSIS

The induction and development of nitrogen fixing root nodules on legumes is a highly complex process which requires a highly regulated expression of genes in an eucaryotic (macrosymbiont) legume plant and its prokaryotic (microsymbiont) rhizobia.

Nodules develop in a complex series of steps (Long, 1989; Sprent, 1989). *Rhizobium* is chemotactic towards plant roots probably, due in part to specific plant attractants especially flavonoids. At the surface of the root, bacteria alter the growth of the epidermal hairs on the root such that they grow deformed, even curled. As this happens, the cells of the root cortex, under the epidermis begin dividing trapped in a curled root hair, or between a hair and another cell, proliferate and begin to infect the outer plant cells, as they do, the invaded plant cell is stimulated to produce a cell wall sheath, "infection thread". A cell division in the plant root establish the body of the nodule, infection threads ramify and penetrate individual target cells within the nodule. Bacteria are released into the plant cytoplasm itself, enveloped in plant plasma membrane called peribacteroid membrane. A central tissue is developed with reduced access to atmospheric oxygen within which the oxygen sensitive nitrogen fixation reaction can take place. The bacteria then differentiate into nitrogen fixing form, the bacteroids and symbiotic nitrogen fixation begins which is catalysed by nitrogenase enzyme to form ammonia and is exported to the plant for assimilation, the plant reduces carbon dioxide into sugar during photosynthesis and translocates these to bacteroids which they use as fuel. (Sutton et al., 1981).

GENETICS OF RHIZOBIA

Rhizobial genetics plays an important role in the understanding and improvement of symbiotic nitrogen fixation. Firstly it can be used as an analytical tool to identify the important biochemical steps involved in development and functioning of the root nodules. Secondly it can be used as a breeding method for the construction of new strains of Rhizobium of potential agricultural value.

Rhizobial genes required for symbiotic nitrogen fixation include those involved in Nod factor synthesis, nodule development, synthesis of the nitrogen-fixing apparatus and bacteroid metabolism. On the other hand, a number of plant genes (nodulin genes) whose expression is specifically induced in root tissue as a consequence of the interaction with rhizobia are also known. Hence the formation of effective (nitrogen fixing) root nodules requires a coordinated temporal and spatial expression of both plant and bacterial genes (Hirsch, 1992).

In *Rhizobium* plasmids are the primary sites of symbiotic genes (Beringer *et al.*, 1987). The arrangement of symbiotic genes differs in various species. *Rhizobium meliloti* carries genes necessary for symbiosis on two extremely large plasmids termed mega plasmid of about 1400 and 1700 kilobase pairs (Burkhardt et al., 1987) designated as pSym-a and pSym-b. while a single *R. leguminosarum* bv *phaseoli* 410 kb plasmid (pCFN 299) bears all the genes needed to confer nodulation and nitrogen fixation on recipient *Agrobacterium tumefaciens* (Martinez et al., 1987). Even tighter clustering is shown by *R. leguminosarum* bv *trifolii*, a 32 kb segment of the symbiotic plasmid has all the genes sufficient for host-specific nodulation and nitrogen fixation, if placed in a plasmid cured *Rhizobium* background (Innes et al., 1988). In the other two rhizobial genera *Bradyrhizobium* and *Azorhizobium* symbiotic genes appear to be chromosomally located (Kondorosi et al., 1984a ; Kondorosi, 1989).

Symbiotic nitrogen fixation genes in the broadest sense can be divided into nod/nol, nif and fix genes.

Over 30 nodulation genes have been identified and sequenced. Certain nod genes were detected in all rhizobial strains. These nod genes were designated as common nod genes, which include the nodABC and nodD genes (Kondorosi *et al.*, 1984b). Mutations in common nodABC genes abolish early interactions, root hair curling and nodule initiation and can be complemented by the corresponding genes from other strains or species and their nucleotide sequence is also highly conserved. The nodD gene is also common in the sense that it is present in all strains but it functions in a host plant-dependent manner (Kondorosi, 1989). Legume species exude a characteristic spectrum of flavonoid compounds which induce the transcription of an important set of nodulation gene in *Rhizobium* (Peters et al., 1986) and *Bradyrhizobium* (Kosslak et al., 1987). The nodD product from different species of rhizobia recognize particular flavonoids preferentially. This molecular recognition is an important determinant of host-*Rhizobium* specificity (Horvath *et al.*, 1987). In the presence of an appropriate flavonoid NodD activates transcription of all the other nod genes by binding to a conserved 60 bp region of DNA sequence in each nod gene promoter known as nod box (Fisher and Long, 1989).

All the other nod genes differ from the common nod genes in that they are not found in all strains and species and they cannot be complemented by nod genes from other species. These genes were designated as host specific nodulation genes (hsn) (Kondorosi *et al.*, 1984b). The hsn genes contribute to nodulation only of restricted plant hosts or in some cases of specific genotypes. Mutation in these genes cause only delay of nodulation and root hair curling.

Rhizobial host range determinants have been defined by two means. First by the examination of nodulation mutants that have altered host range properties. These are mutants that cannot be complemented by DNA regions from other rhizobia. Second hsn loci have been defined as genes when transferred to a suitable strain render the recipient capable of nodulating the donors host plant (Djordjevic et al., 1987a; Djordjevic et al., 1987b).

Genes for nitrogen fixation in Rhizobium are generally divided into two groups. Those with homologs in free living nitrogen fixing systems such as *Klebsiella* are referred to as nif genes and those that are shown to be required for symbiotic nitrogen-fixation, but whose functions are not known to be analogous to a free living system, are referred to as fix genes. Both nif and fix gene mutants are able to cause nodule development, but the nodules do not fix nitrogen (Nod^+ Fix^-).

The nif and fix genes of *Rhizobium meliloti, Bradyrhizobium japonicum* and *Azorhizobium caulinodans* are organised in distinct clusters whose structure and genomic location are species specific (Fig 1). In *R. meliloti* both cluster I (nifHDKE, nifN, fixABCX, nifA, nifB, frdX) and cluster II (fixLJ, fixK, fixNOQP, fixGHIS) (fig 1A) are located on megaplasmid 1 (Batut, et al., 1985 ; David et al., 1987). The cluster II genes map at about 220 kb down stream of the nifHDKE operon and are transcribed in opposite orientation to it. A cluster of nod genes including the common nod genes (nod ABC) is located in the 30 kb region

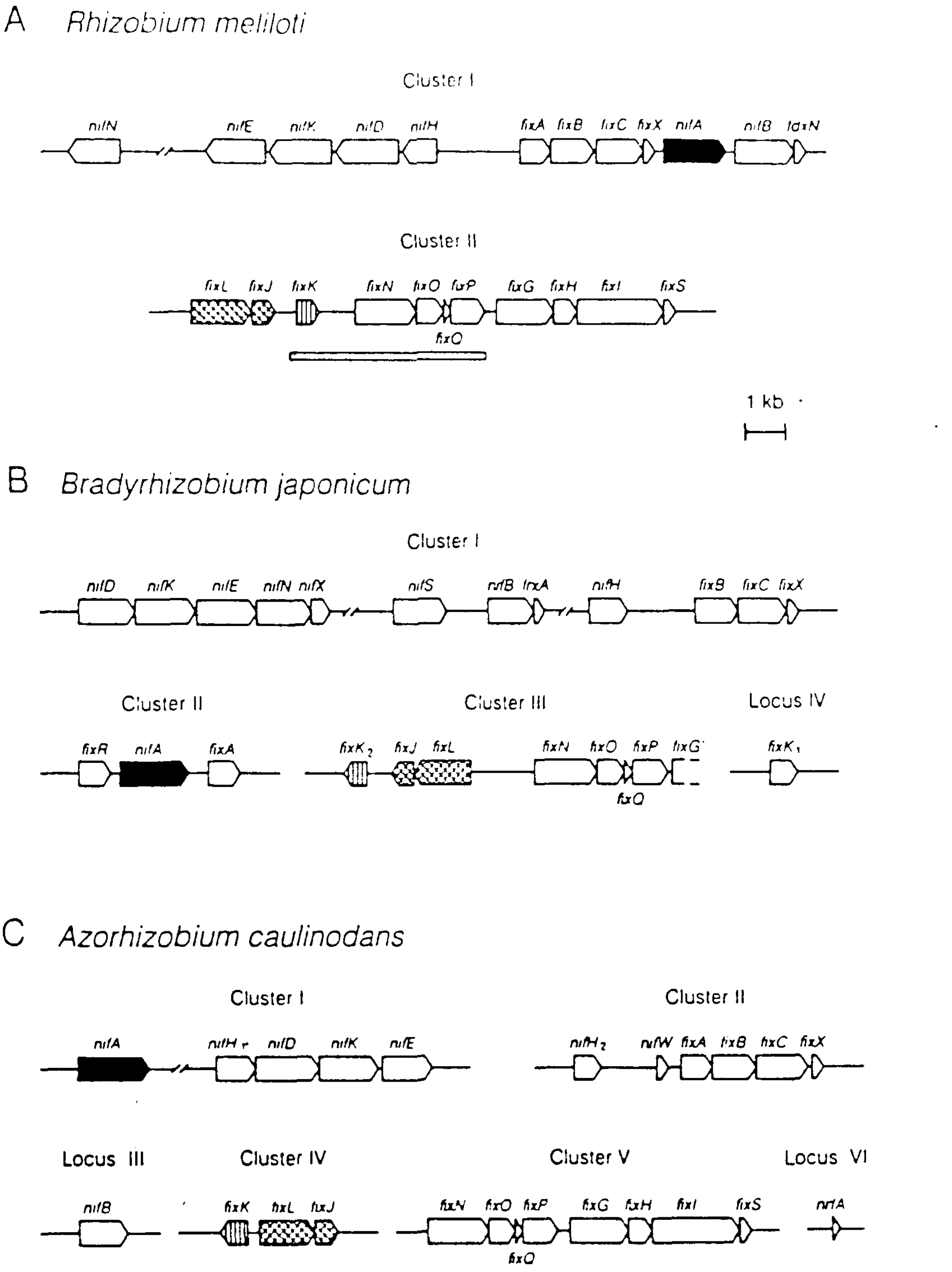

Fig 1: Organization of nif and fix gene clusters in *R. meliloti* (A), *B. japonicum* (B) and *A. caulinodans* (C). Homologous regulatory genes are marked by identical patterns (Fischer, H.M, 1994).

between nifE and nifN (Long, 1989). Additional genes required for an effective symbiosis are located on megaplasmid 2 (Hynes et al., 1986; Honeycutt et al., 1993) and on chromosome (Honeycutt et al., 1993).

In *B. japonicum* and *A. caulinodans* the nif, fix and nod genes are located on the chromosomes and are organised as depicted in Fig 1B and 1C. In *B.japonicum* three clusters of nif and fix genes have been characterised. Cluster II of *B. japonicum* which includes the fixRnifA operon, fixA and atleast 11 nod genes is located at a distance of ca.240 kb away from Cluster I which contains the nitrogenase structural genes. Cluster III, harboring fixLJ, $fixK_2$ and the fixNOQP operon, maps at ca 770 kb away from cluster II. Hence, in *B.japonicum* all known nif and fix genes required for symbiotic nitrogen fixation and the common nod genes are located within about 1,000 kb on the chromosome. In *A. caulinodans* four clusters of nif or fix genes and two additional loci carrying single nitrogen fixation genes (nifB, nfrA) have been characterised (Donald et al., 1986; Denefle et al., 1987 ; Pawlowski et al., 1987).

The nifD and nifK genes specify the α and β sub units, respectively of the $\alpha_2\beta_2$ FeMo protein (component I or dinitrogenase). The homodimeric Fe protein (component II or dinitrogenase reductase) is encoded by nifH.

It has been proposed that the fixABCX gene products might be involved in electron transport to nitrogenase (Gubler and Hennecke, 1986). The fixNOQP genes are organised in an operon and mutant strains deleted for fixNOQP are defective in symbiotic nitrogen fixation (Preisig et al., 1993 ; Mandon et al., 1994).

All four fixGHIS gene products are predicted to be transmembrane proteins and transposon insertions in any one of them are unable to fix nitrogen in *R. meliloti*. It is speculated that fixI is a symbiosis specific cation pump whose function is coupled to a redox reaction catalysed by the fixG sub unit (Kahn, et al., 1989).

STRESS TOLERANCE IN RHIZOBIA

Few agriculturists have the luxury of production under ideal conditions. For a great majority and particularly in the third world stresses of one from or another are an integral part of the crop cycle and many pose a severe yield constraint. Studies of environmental stress and the legume - *Rhizobium* symbiosis were restricted to defining the problem and to using physical or chemical amendments to overcome it. The identification of bacterial strains and in some cases host cultivar that are tolerant to these stresses opens the ways for alternate, lower cost solutions to these problems. Acidity, salinity, high temperature, presence of combined nitrogen are a few stress conditions rhizobia encounter.

SOIL ACIDITY

Soil acidity affects all aspects of nodulation and nitrogen fixation from survival and multiplication of the rhizobia in soil, through infection and nodulation to nitrogen fixation.

The lowest pH at which rhizobia grow varies with cultural conditions, but when pH of the medium was adjusted after autoclaving and the inoculant density was low, a few strains could grow below pH 4.5 (Date and Halliday, 1979; Keyser and Munns 1979; Graham et al., 1982; Ayanaba et al., 1983; Lowendorf and Alexander, 1983; Karanja and Wood, 1988). Fast growing rhizobia have generally been considered less tolerant to acid pH than bradyrhizobia, a few of which can tolerate pH 4.0.

Strains identified as acid tolerant under laboratory conditions have been shown to more readily colonize an acid soil and subsequently improve growth of the host plant (Thornton and Davey, 1983).

Vargas and Graham (1988) identified several acid tolerant bean cultivars and in studies with both acid tolerant and sensitive rhizobia, observed that only one of the symbionts needed to be acid tolerant for good nodulation at pH 4.5.

Agar plate methodologies (Graham et al., 1982 ; Ayanaba et al., 1983) have provided a convenient means for comparison of strain differences in pH tolerance.

Some bradyrhizobia have been found to grow better at acid pH than at pH 7 and on isolation from nodules need to be cultured in medium of pH 4.5 - 5.5 (Date and Halliday 1979 ; Gomez de souza et al., 1984 ; Sylvester-Bradley et al., 1988). The basis for pH tolerance in neutrophilic species is through the regulation of cytoplasmic pH, with cells maintaining an internal pH of 7.5 to 8.0 against a pH differential with the outside environment of as much as 3 pH units (Booth 1985).

For the rhizobia, the basis of pH tolerance remains uncertain. For an external pH of 6.15 - 8.45, Gober and Kashket (1984) found the cytoplasmic pH (pH_i) of *Bradyrhizobium* sp 32 H1 to range from pH 7.60 to 8.90 at 20 per cent oxygen and from 6.15 to 8.90 at 0.2 per cent oxygen, with pHi always more alkaline than external pH. In the acid tolerant *R. meliloti* strain WSM 419 decline in pH as the external pH fell from 7.0 to 5.5 was less than 0.3 pH units but in an acid sensitive Tn5 mutant derived from it cytoplasmic pH fell from 7.3 to 6.1 over the same external pH range (O'Hara et al., 1989).

Ayanaba et al., (1983) noted considerable ammonia production in acid-tolerant cowpea rhizobia cultured in media of pH 4.5, whereas little was produced at pH 7.0 or by an acid-sensitive strain. Cells subjected to acidity maintained higher levels of potassium (Aarons and Graham, 1991).

Studies on the genetic basis of tolerance to low pH suggest a chromosomal rather than a plasmid location for pH genes (Chen et al., 1991). "Acid-shock" proteins have also been shown in both TA1 (Glenn et al., 1986) and UMR 1899 (Aarons and Graham, 1991) strains of *Rhizobium*.

A practical approach to the development of more effective acid tolerance strain is that of chen et al., (1991) who first cured an acid tolerant clover strain of its Sym plasmid, then transferred to it the Sym plasmid of a superior nitrogen fixing strain.

Richardson and Simpson (1988) obtained exudates from cultivars of subterranean clover grown in media of pH 3.0 - 8.0 and showed that ability to induce nod gene activity in clover rhizobia was maximal in samples grown at pH 5.0 - 6.0 and was significantly reduced at pH 4.5. Graham et al., (1982) obtained a strong response to inoculation with an acid tolerant strain under conditions where an equally effective but acid-sensitive strain gave no response.

SALT TOLERANCE

Rhizobium and *Bradyrhizobium* strains show marked variation in salt tolerance. They are inhibited by 100 mM salt (0.6 per cent) (Singleton et al., 1982; Yelton et al., 1983; Zhang et al., 1991). But growth at salt concentrations of more than 300 mM has been reported with strain of *Rhizobium meliloti* (Graham and Parker 1964; Sauvage et al., 1983), *R. fredii* (Yelton et al., 1983) and some alfalfa, *Acacia, Prosopis*, Leucerne, gram, groundnut, and cowpea strains have tolerated 500 mM NaCl (Yadav and Vyas, 1971; Basak and Goyal, 1980; Sauvage et al., 1983; Zhang et al., 1991).

Legume and the process of nodule initiation are both more highly sensitive to salt or osmotic stress than are the rhizobia (Tu, 1981; Zahran and Sprent, 1986). Soybean root hairs showed little curling or deformation when inoculated with *Bradyrhizobium japonicum* in the presence of 170 mM NaCl and nodulation was completely suppressed by 210 mM NaCl (Tu, 1981). Zahran and Sprent (1986) reported that bacterial colonization and root hair curling of *Vicia faba* reduced in the presence of 50 - 100 mM NaCl.

Rai and Prasad (1983) identified two nitrosoguanidine mutants of the salt sensitive strain RL 5 which showed significantly enhanced nodulation and nitrogen fixation under salt stress.

PRESENCE OF NITRATE

Mineral nitrogen is known to suppress both nodule formation and symbiotic nitrogen fixation of several legumes (Harper and Gibson, 1984; Harper 1987; Streeter, 1988). Most of the research in this area has involved nitrate form of nitrogen because various chemical forms of nitrogen when applied to soil are rapidly converted to nitrate.

When plants have a choice between mineral nitrogen and nitrogen fixation, the former is preferred. Thus as the level of combined N is increased the plant substitutes combined nitrogen for fixed nitrogen (Pal and Saxena, 1976). This effect of nitrate on symbiotic efficiency has important agronomic consequence (Herridge, 1985).

Nitrogen application in crop production are generally high enough to result in inhibitory nitrate concentration in the soil solution atleast for a brief period. Circumstances often arise in crop management where it would be advantageous to apply combined nitrogen without any chance of inhibition of nodule production and function. For example when legume, is intercropped with a non legume application of mineral nitrogen to non-legume is required for maximising yield and may be economically justified. For maximum exploitation of symbiotic nitrogen fixation and for maximum flexibility in crop management, overcoming the negative effects of nitrate on nodules is a highly important goal.

Combined nitrogen has multiple and complex effects on both establishment and functioning of nodules. Nitrate inhibits the infection process, development of nodules, and the expression of nitrogenase activity as well as hasten the breakdown of nodule tissue.

a. Inhibition of infection

Thornton (1936) reported that nitrate affects a broad range of infection events, including a decrease in root hair deformation, a decrease in the binding of rhizobia to root hairs (Dazzo and Brill, 1973), a decrease in the number of infection threads (Darbyshire, 1966; Munns, 1968) by inhibiting formation of new root hairs and an increase in the number of aborted infection events and time of appearance of first nodule (Munns, 1968). Small amounts of nitrate and nitrite (10 μg N) prolonged infection of the roots, resulting in a high number of infected root hairs (Darbyshire, 1966). High nitrate (18mM) completely inhibited nodule formation, a delay of only five days in nitrate treatment after inoculation allows some infections to generate functional nodules.

Relatively few attempts to elucidate molecular mechanisms involved were made. Dixon (1969) observed that indole acetic acid (IAA) is required for infection and IAA is destroyed by nitrite formed from supplied nitrate. The key piece of evidence is that IAA added with nitrate, partially offset the negative effect of nitrate on infection (Valera and Alexander, 1965 ; Dixon, 1969). Sherwood et al., (1984) found that 15 mM nitrate does not repress the synthesis of pectin but does reduce the rhizobia binding activity of pectin on the root surface.

b. Inhibition of nodule growth

At nitrate concentrations greater than 2 mM, both nodule growth and nodule activity are depressed (Streeter, 1981 ; Eardly *et al.*, 1984). With long term (several weeks) exposure of plants to 4 to 8 mM NO_3, effects of NO_3 on nodule growth are clearly greater than effects of NO_3 on specific acetylene reduction activity (Streetor, 1981 ; Ursino, et al., 1982 ; Sawhney et al., 1985). Studies with field grown plants also indicate much greater affect on nodule growth than on specific nodule activity.

Several innovative approaches have been made to understand inhibition of nodule growth by nitrate. The root system was divided and only one half of the root system was exposed to NO_3. The nodule growth was inhibited only on the roots directly exposed to nitrate (Van Schrewven, 1958) but at very high (20mM) NO_3 concentration, nodule size on the unexposed half of the root system was depressed (Hinson, 1975). A second approach involved a comparison of four nitrate concentrations and time combinations, all of which resulted in the same nitrate dose (Davidson and Robson, 1986). Plants suppled with continuous low nitrate concentration absorbed more nitrate and had lower nodule mass than plant supplied with relatively high nitrate for short periods.

Thornton and Rudorf (1936) studied the inhibition of nodule growth by NO_3 as accompanied by effects of nitrate on nodule structure and observed that nodules grown in the presence of nitrate showed wall thickening in the endodermis.

c. Inhibition of nitrogenase activity

The inhibitory effects of nitrogenase activity was proportional to the amount of nitrogen supplied, time of application, type of host plant and bacterial strain used and was independent of rooting medium and legume species (Allos and Bartholomew, 1959; Harper and Gibson, 1984; McNeil, 1982).

The effect of nitrate on nitrogenase activity has been shown under a wide range of conditions. Nitrogenase activity in rhizobia induced to fix nitrogen in agar culture was inhibited by nitrate and bacterial morphology associated with active nitrogenase, was absent in the presence of nitrate (Pankhurst and Craig, 1978). Large majority of studies have been done with plants grown in non-soil media in controlled environment. Supply of 10 to 20 mM nitrate is typical and the inhibition of acetylene reduction activity is apparent after one day, whereas the inhibition of nodule growth is not evident until two or three days after the initiation of nitrate treatment (Skrdleta et al., 1980; Steeter, 1985).

Rhizobia in free living form tolerate high soil nitrogen conditions better than their symbiosis with host plants. The reduced nodule mass and acetylene reduction activity due to the nitrate effect suggest that interactive stages between host plant and rhizobia are very sensitive to nitrogen.

Possible mechanisms for inhibitory effects of nitrate

Although the exact mechanism of inhibition by nitrate on nodule development and nitrogenase activity is not clear, it is frequently attributed to two hypothetical mechanisms which have received the most attention. They are carbohydrate deprivation and nitrite toxicity.

Carbohydrate deprivation states that when nitrate was supplied, its reduction and assimilation requires enough reducing power, so that supply of carbohydrate available for distribution to nodules will be diminished. Orcutt and Wilson (1935) indicated that a high nitrate supply lowered the concentration of reducing sugars and sucrose in soybean leaves, stem and roots showing that the presence of combined nitrogen lowers the sugar content which affects nodule development.

Fred and Wilson (1934) showed that CO_2 enrichment could partially alleviate the negative effect in nodule mass per plant. Added sucrose, mannitol and arabinose were reported to stimulate nodule formation (Raggio et al., 1965). Alteration by sucrose of nitrate inhibition of nitrogenase activity has been reported for detached soybean nodules (Stephens and Neyra, 1983) and for intact pea plants (Houwaard, 1980).

Wong (1980) reported that fructose, glucose and sucrose were all active in offsetting the inhibitory effect of nitrate in lentil. The sugar treated plants contained only 10 per cent as much nitrate and 7 per cent as much nitrate reductase as control plants.

The carbohydrate deprivation hypothesis was also supported by the evidence, that transport of ^{14}C-labelled photosynthate to nodules is decreased by nitrate. This has been confirmed for cowpea (Kahn and Kahn, 1981) and numerous times for soybean (Rabie et al., 1980; Kouchi et al., 1986).

For soybean plants, supplied with nitrate concentration high enough to depress nodule weight per plant by 70 per cent, sugar concentration in nodules were depressed only 12 per cent (Streeter, 1981).

Trinchant and Rigaud (1980), suggested that nitrite produced from nitrate via nitrate reductase, present in the legume or the rhizobia, retards nitrogenase activity in bacteroids (Kennedy et al., 1975) and in nitrogen fixing cultures of rhizobia (Pagan et al., 1977).

An important approach in this direction was the use of rhizobium mutants lacking nitrate reductase which can be obtained by chlorate resistance as shown by Pagan et al., (1977). Acetylene reduction activity of isolated *Bradyrhizobium japonicum* bacteroids lacking nitrate reductase was not inhibited by nitrate, whereas wild type bacteroids were inhibited after a lag of 30 minutes (Stephens and Neyra, 1983). In contrary, acetylene reduction activity of nodules formed by nitrate reductase deficient rhizobium mutants was still inhibited by nitrate (Manhart and Wong, 1980). Growth of nodules was also inhibited by nitrate whether or not bacteroids had nitrate reductase (Streeter, 1982).

Nitrite concentration in the nodules are probably higher in cytosol (the source of nitrite) than in bacteroids and bacteroids have an active nitrite reductase. It is clear that nitrate reductase in bacteroids is not required for nodules to be inhibited by nitrate and if nitrite is involved in inhibition, it must be coming from some other source.

Attempts to circumvent the inhibitory effects of nitrate

Three approaches have been followed by different research groups to develop mineral nitrogen tolerant symbiosis in legumes : through identification of rhizobia, host cultivars and mutants of rhizobia and of host cultivars.

Using the isotope - dilution method Rennie and Kemp, (1983) reported that the strains of rhizobia influenced the quantity of soil nitrogen taken up by common bean (*Phaseolus vulgaris* L.). Two strains of rhizobia provided approximately 70 kg Nha^{-1} to the plants but the total N contents of "Aurora" nodulated by strains 255 and 904 were 140 and 117 kg Nha^{-1} respectively. Different rhizobial strains nodulating cowpea "California No.5 Blackeye" provided from 70 to 98 per cent of the plant shoot nitrogen from biological nitrogen fixation although total nitrogen contents of plant shoots were not significantly different (Elhassan and Focht, 1986). This leads to the conclusion that strains of rhizobia influence plant uptake of mineral nitrogen.

Estimates of symbiotic nitrogen fixation capacities of soybean under normal field conditions ranged upward of 160 kg N/ha with average estimate indicating 100 kg N/ha (Vest et al., 1973). These estimates indicate about 25 to 60% of total nitrogen in mature plant is derived from symbiotic nitrogen fixation, the rest is soil derived. Lower estimates of symbiotic nitrogen fixation contribute to total plant nitrogen (range of 1 to 24 per cent) have been reported when soil nitrogen was increased through nitrogen fixation (112 kg N/ha) during a 3 year test using soybean (Weber, 1966).

Harper (1974) reported that seed yield of plants totally dependent on atmospheric nitrogen, was less than one half the yield of plants utilizing both nitrate and atmospheric nitrogen under hydroponic growth conditions. Plants grown on low nitrate (0.75 mM) had higher symbiotic nitrogen fixation rates than those grown in no nitrate. Seed yield of plants grown in hydroponics on high NO_3 (7.5mM) which inhibited symbiotic fixation was less than yield of plants utilizing both nitrate and atmospheric nitrogen, thus indicating that both symbiotic nitrogen fixation and nitrate utilization is essential for maximizing yield.

McNeil (1982) investigated difference in sensitivity to nitrate of 16 different strains of soybean rhizobium, 10mM nitrate present during establishment of nodules equally inhibited all symbioses, whereas specific strain effects appeared at low (0.5 mM) to medium (2.0mM) levels of NO_3. At low NO_3 level symbiosis with CB:1809 increased total nodule mass by 30 per cent relative to nitrate free controls. However at 2 mM NO_3 it maintained total nodule mass. In the presence of 10mM NO_3 CB:1809 occupied 1.75 times as many nodules as USDA 110.

Gibson and Harper (1985) observed minor differences in tolerance of nodulation to nitrate among eight strains of *Bradyrhizobium japonicum*. Nodule development was delayed by all nitrate treatment conditions. Treatment conditions providing similar rates of NO_3 concentration (4.0 mM rundown Vs 0.5mM maintained) resulted in a more marked inhibition of nodule appearance at higher level of solution NO_3 . Maintaining solution concentration of 1 mM after appearance of nodules retarded or prevented nitrogenase activity.

Harper and Gibson (1984) observed differential nodulation tolerance to NO_3 among legume species. Visible nodule appearance of lupin, pea, subterranean clover, chickpea and siratro was less sensitive to NO_3 than soybean, lablab bean or barrel medic. The latter species showed marked inhibition of nitrogenase activity with 1.0 mM NO_3 treatment, other five species showed similar or higher nitrogenase activity at 1.0mM NO_3. Of the species compared nitrogenase activity of sub clover was least affected by the 4.0 mM nitrate treatment. Hardarson et al., (1984) reported a soybean cultivar Dunadja from Romania as having similar level of nitrogen fixation both with application of 20 and 100 kg Nha^{-1} than other seven cultivars.

Super nodulating mutants of soybean cultivar Bragg developed by Carroll et al., (1985) and in *Pisum sativum* by Jacobsen and Feenstra (1984) characterised as nitrate-tolerant symbionts (nts), were found sensitive to nitrate in the studies of Eskew et *al.*, (1989) as were the hyper nodulating mutants from cultivar Williams of soybean (Wu and Harper, 1991). Most supernodulating mutants had symbiosis superior to the parent lines at low N but at high N, it was not convincing, that their symbiosis were better than parent lines. Most supernodulating materials including those of *Phaseolus vulgaris* (L.) produced significantly lower biomass and/or grain yield than their respective parent lines (Buttery et al., 1990; Eskew et al., 1989; Wu and Harper 1991).

Gibson and Pagan (1977) observed that nitrate retarded initial nodulation and nitrogen fixation activities of cowpea nodules induced by nitrate reductase deficient mutants of cowpea rhizobium 32H1 and *Rhizobium trifolii* (TA1) to an extent similar to that found with parent strains in the presence of 0.7, 2.3 and 7.0 mM NO_3. These observations have recently been extended to *Vigna unquiculata*, *Lupinus angustifolius* and soybean nodules (Manhart and Wong, 1980 ; Malik et al., 1987).

Bradyrhizobium japonicum mutants SH31 and SH35 derived after nitrosoguanidine mutagenesis of wild type inoculant strain, formed significantly more nodules which resulted in greater acetylene reduction activity per plant than wild type nodules and this improved nitrogenase activity was apparent even when plants were grown in 10mM nitrate or 1mM ammonia (Maier and Brill, 1978).

MUTATION

Mutations are invaluable to the process of evolution as they provide the raw material required for its occurance. Mutations are considered as an alternative to naturally occuring variation and also alternative to hybridization (Brock, 1971). Without mutations, all genes would exist in only one form. Alleles would not exist and thus genetic analysis would not be possible. Most important, organisms would not be able to evolve and adapt to environmental changes. Some level of mutation is essential to provide new genetic variability to allow organisms to adapt to new environment.

DeVries (1901) for the first time used the term mutation. Mutations are sudden heritable changes in the genetic material while mutagenesis is the process by which a gene undergoes heritable alteration. Mutant is an individual bearing an allele that has undergone mutation. Mutagen is a mutation causing agent.

Even though most mutations make the organism less efficient, the possibility of developing new desirable traits through induced mutations has intrigued many scientists. Mutations have been used extensively to elucidate biochemical pathways. Mutagenesis is also used as a method for obtaining strains of bacteria that synthesize increased levels of microbially produced metabolites. When penecillin was first discovered the yield was low and production was seriously limited in the mold *Penecillium*. Then millions of spores were irradiated and a few of the surviving mutant colonies produced considerably more penecillin than the average. Such mutant overproducers of penecillin are important in commercial production of this antibiotic.

Mutations can be classified as spontaneous and induced. Spontaneous mutations occur in the absence of any known mutagenic agent which may be due to mistakes during DNA replication or may be caused by mutagenic agents present in the environment. The frequency of spontaneous mutations in bacteria is low, about one in million. This rate can be increased by the use of mutagens. Induced mutations are those resulting from exposure of organisms to mutagenic agents, thereby increasing frequency of mutations.

The first evidence, that external agents could increase mutation rate was presented by Muller (1927), who showed that X-rays are mutagenic in *Drosophila*. Since then a large number of physical and chemical mutagens have been shown to increase mutation rate.

Mutagens can be broadly classified as physical and chemical. Physical mutagens include ionising radiations (X-rays, ß-particles and gamma rays) and non-ionising radiations (ultra violet rays).

Ionising radiations such as X-rays (0.1 to 1 nm) are of high energy and can penetrate living tissues. In the process of penetration, these high energy rays collide with atoms and cause the release of electrons leaving positively charged ions, these in turn collide with other molecules, including DNA which results in mutagenic effect. The net result is that a core of ions is formed along the track of each high energy ray as it passes through living tissues. Because these rays cause ionization hence the name ionizing radiation.

Ultraviolet rays (UV) do not possess sufficient energy to induce ionization because of their lower energy, they penetrate tissues only slightly. Thus UV rays proved to be an excellent mutagen for the easily penetrated cells of microorganisms. UV rays are readily absorbed by certain substances such as purines and pyrimidines which then enter a more reactive or excited state. Maximum absorbtion of UV by DNA is at wavelength of 254nm. *In vitro* studies show that the pyrimidines (especially thymine) absorb strongly at 254 nm and becomes very reactive. The major products of UV absorbtion are covalently joined thymine called thymine dimers, from thymine that are adjacent in the same polynucleotide strand. This chemical linkage brings the bases closer together and no longer form hydrogen bonds with corresponding purine of the base pair, causing a distortion of the helix which blocks transcription and transiently blocks DNA replication (Hall and Mount, 1981).

The first chemical mutagen, mustard gas was discovered by Auerbach and her associates during world war II.

Chemical mutagens can be divided into two classes.

(1) Those mutagenic to both replicating and non replicating DNA, such as the alkylating agents and nitrous acid.

(2) Those mutagenic only to replicating DNA, which includes acridines and base analogues.

Alkylating agents such as nitrogen and sulfur mustards, methyl and ethyl methanesulfonate, nitrosoguanidine and many others are mutagenic. One major mechanism of mutagenesis by alkylating agents is the transfer of alkyl groups like methyl or ethyl groups to the DNA bases, so that their base pairing potentials are altered and transition occurs. In case of EMS ethylation at 7th-N position and 6th-O position are believed to be the two effects. (Brookes and Lawley, 1961).

Acridines such as proflavin and acridine orange are very powerful chemical mutagens. Dimensions of acridine molecules are roughly same as those of a purine-pyrimidine pair. These positively charged acridines insert between adjacent base pairs of DNA, a process called intercalation (Lerman, 1961). As a result adjacent base pairs move apart by a distance roughly equal to the thickness of one base pair. In doing so they increase the rigidity and alter the confirmation of the double helix causing slight kinks in molecules. When DNA containing intercalated acridines replicates, the template and daughter DNA strands can misalign, the result is that a nucleotide is either added or deleted in the daughter strand causing frame shift mutation.

Of late transposable element mutagenesis is followed. Transposon is a DNA sequence able to insert itself at new locations in the genome without any sequence relationship with target locus. When a transposon inserts into an essential region of the genome it interrupts the coding region. Even if transcription proceeds through the element the phenotype will be a mutant because coding region will contain incorrect sequences.

In rhizobial mutagenesis transposons like Tn5, Tn7 and Tn9 are being used extensively (Hagedorn, 1988; Maier and Graham, 1990). Three features of transposon make them useful for genetic analysis. First, they insert at a large number of potential target sites that are random in their distribution around the genome. Second many transposons code their own transposase and thirdly some transposons contain one or more genes for antibiotic resistance serving as genetic markers (Kanamycin resistance in Tn5).

MUTATIONS IN RHIZOBIA

Mutations in rhizobia play an important role in improvement of symbiotic nitrogen fixation. Firstly mutation can be used as an analytical tool to identify important biochemical steps involved in developing and functioning of the root nodules. Secondly, it can be used as a breeding method for the construction of new strains of Rhizobium of potential agricultural value. Some of the desirable traits for rhizobia include enhanced nitrogen fixation, increased tolerance to environmental stress such as pH, metal - ion toxicity, nitrate tolerance and promiscuity of rhizobia.

The genetics of rhizobia are based largely on metabolic mutants. Three types of such mutants are

i. Auxotrophs which have a requirement for a metabolite not needed by the prototrophic wild type.
ii. Resistant mutants are resistant to radiation, chemicals, extreme temperature.
iii. Utilization mutants which differ from wild type by being able or unable to use specific compounds as carbon or nitrogen source for growth.

The first report of an artificially induced mutation in rhizobia was that of Jordan (1952) who noted increased effectiveness in three X-ray induced colonial mutant strains of *Rhizobium meliloti* at a dosage of 45,000r for 32 minutes. Dygdala (1962) studied morphological and cytological changes of *R. leguminosarum* influenced by gamma rays and observed that the cell division was considerably delayed or entirely stopped by irradiation. Some of the cells had altered into bacteroidal or teratologic forms. Hamatova (1964) also used gamma rays at dose of 50,000 r to irradiate two strains of *Rhizobium japonicum*. Mutants with increased virulence, nitrogen fixation and dry matter production in host plants was observed.

Imshenetskii et al., (1970) observed that out of the twelve mutants of *Rhizobium meliloti* induced by nitroso methyl urea and resistant to neomycin, two strains NN5 and NN39 possessed increased activity estimated from weight of plants and all twenty spontaneous mutants of *R. meliloti* resistant to streptomycin were active and four mutants possessed higher activity than wild type strain.

Scherrer and Denarie (1971) isolated auxotrophic mutants from two strains of *R. meliloti* after mutagenesis with N-methyl - N′-nitro-N-nitrosoguanidine and penicillin enrichment and studied effectiveness in nitrogen fixation on *Medicago sativa* (Var 'Dupuits'). Glycine dependent mutants were more effective than the wild type strain while purine and pyrimidine dependent mutants were ineffective. However Imshenetskii et al., (1970) and Bose and Venkataraman (1972) couldnot obtain auxotrophic mutant following exposure to UV rays, nitrosomethylurea, ethylenimine and nitrous acid.

Bose and Venkataraman (1972) isolated UV mutants of *R. leguminosarum*. Except for mutants mRh/6 and mRh/7 which had enlarged cells, ellipsoidal shape and were unable to ferment mannitol, galactose and arabinose while the rest shared morphology of the wild strain and could not ferment lactose, sucrose and xylose. Mutant strains mRh/1, mRh/3, mRh/4 and mRh/5 were tolerant to 1.8% NaCl.

Maier and Brill (1976) used 160 µg/ml nitrosoguanidine to mutagenize *Rhizobium japonicum* cells. Mutants SM1 and SM2 did not form nodules on host plant. SM3 and SM4 produced small nodules that lacked pink colored pigment. In another experiment conducted by Maier and Brill (1978) a commercial inoculant of *R. japonicum* was mutagenised with nitrosoguanidine as before and screened for effectiveness on soybean plants. Two mutants SM31 and SM35 nodulated earlier than wild type and also expressed greater symbiotic nitrogen fixing activity than wild type in the absence and presence of 10 mM KNO_3 and 1 mM NH_4^+ acetate. SM35 formed more nodules than wild type strain. Plants inoculated with these two mutants had increased dry weight (60 per cent) and nitrogen content (100 per cent).

EMS, 0.2M was used to isolate auxotrophic mutants of *Rhizobium japonicum* S_{10} and *R. meliloti* L5 - 30 (Kalra et al., 1977; Fedorov and Zaretskaya, 1978). Arginine, tryptophan and Thiamine requiring mutants were effective with respect to nitrogen fixation and yield of alfalfa (Fedorov and Zaretskaya, 1978).

Ronson and Primrose (1979) isolated carbohydrate negative mutants of *Rhizobium trifolii* by treating with 200 µg/ml nitrosoguanidine and subjected for enrichment with ampicillin 25 µg per ml and characterised as deficient in glucokinase, fructose uptake and pyruvate carboxylase. All mutants formed effective symbiosis on red clover suggesting that neither, glucose, fructose nor sucrose were used by bacteroids to provide ATP and reductant for nitrogen fixation.

Tn5 induced carbohydrate mutants of *Rhizobium meliloti.* MD2, MD3, MD5 and MD6 were unable to grow on mannitol, L-arabinose, ribose, and xylose respectively, while MD7 was unable to grow on fructose, mannitol and sorbitol. All mutants were able to nodulate alfalfa plants and L-arabinose mutant showed either late or no nitrogen fixing ability (Duncan, 1981). Mainra et al., (1984) also isolated mutants of *R. meliloti* differing in their carbohydrate utilization pattern by nitrosoguanidine treatment. Lafontaine et al., (1989) reported that arabinose, fructose, glucose and pyruvate utilization mutants of *Rhizobium* formed nodules similar in shape and size to nodules formed by wild type strain. All C_4 dicarboxylic acid utilization mutants formed ineffective nodules.

Pain (1979) used 0.1M nitrous acid for three minutes to isolate auxotrophic mutants of *Rhizobium leguminosarum.* Streptomycin, chloramphenicol, neomycin, cycloserine and Kanamycin resistant mutants formed effective nodulation.

Williams (1981) examined the effect of 150 µg per ml nitrosoguanidine on cowpea Rhizobium strain 101 and isolated two ineffective mutants (M_1 and M_2) and an effective mutant (M_3). Plants inoculated with M_3 nodulated earlier, produced more nodules (58 per cent) had increased dry weights (26 per cent) and roots expressed greater acetylene reduction activity (39 per cent) than plants inoculated with wild type.

Staley et al., (1982) isolated several mutants defective in nodulation from

Rhizobium japanicum strain 311 b 110 and 61A76. Mutants of class I formed nodules on soybean at 28 days after inoculation while class II formed fewer nodules and class III mutants did not nodulate.

Khare et al., 1982 examined the comparative efficiency of chemical and radiation mutagens on morphology, nutritional totipotence and symbiotic characters of two *Cicer arietinum* rhizobium. The mutants produced by radiation (Gamma and UV rays) differed from parents in that the mutant colony size was bigger, gelatinase activity higher, were not deficient nutritionally had wider host range and size and the number of nodules produced were high. Radiation mutagenic effects were more pronounced than chemical mutagens (acridine orange and 5-bromouracil).

Fedorov et al., (1983) and Ghai *et al.*, (1983) used UV radiation to isolate mutants of rhizobium. Fedorov et al., (1983) observed that low doses of UV cause the maximum induction of auxotrophic mutants while higher doses gave more number of morphological mutants. Of the nine UV mutants isolated by Ghai *et al.*, (1983) only one strain (21M11B) produced more water soluble polysaccharide and also induced formation of maximum number of nodules.

Rai and Prasad (1983) used an effective rhizobium strain RL5 which nodulates lentil to mutagenise with nitrosoguanidine 30μg/ml for 20 minutes. Mutants LM4 and LM1 tolerated 200 μg/ml streptomycin and 1.5 per cent NaCl, had significantly increased number and dry weight of nodules per plant,dry matter yield and nitrogen fixation. Similar increases in dry weight and N content of soybean by 40 per cent was also observed by Williams and Phillips (1983) by a mutant strain C33, of *Rhizobium japonicum* strain USDA110, under controlled environment.

Mroz et al., (1984) isolated a mutant of *R. meliloti* by treating with nitrosoguanidine (1500 μg/ml) for 30 minutes, which showed pleiomorphic character. Neither the plasmid content nor symbiotic properties were affected.

Kohli and Vashishat, (1985) used a combination of ampicillin with lysozyme to enrich the auxotrophs out of the three mutagens (UV, EMS and ECH) in slow growing mungbean *Rhizobium*. Only EMS (0.08M) was found to be effective in inducing auxotrophs of which lysine and valine requiring mutants were infective.

Ozawa and Yamaguchi (1986) examined the effect of acridine orange (AO) on *Bradyrhizobium japonicum*. Symbiotic nitrogen fixation activity of strains 311 b - 29, - 311, - 110 and - 138 increased when cultured in medium containing 20, 5, 20 and 2 μg ml^{-1} of AO respectively. Except strain 311b - 29, AO at concentrations below 20 μg ml^{-1} did not affect total weight of nodules per plant. Hagedorn (1988) isolated transposon derived mutants of *B. japonicum* strain I-110 by introduction of Tn5. Five mutants were identified with highest elevation in nodulation (40 - 55 nodules / plant Vs 20 nodules/ plant for I - 110). Three mutants (VT1, VT2 AND VT4) exhibited 50 per cent greater nitrogen fixation activity both *in vitro* and *in plant* compared to parent strain.

Hunter and Kuykendall (1990) reported that plants inoculated with *Bradyrhizobium japonicum* TA11 NOD^+ (prototrophic revertant) contained significantly more nitrogen per plant than did plants inoculated with wild type bacteria (275.9 ± 35 versus 184 ± 18 mg). Plants that received NOD^+ strain had 56 per cent more nodules and 41 per cent more nodule mass than did control plants.

Maier and Graham (1990) isolated mutant of *Bradyrhizobium japonicum* that required higher levels of molybdate than wild type strain for growth on NO_3 containing medium by Tn5 mutagenesis of wild type strain (JH). However, soybeans inoculated with mutant strain JH 359 and grown under Mo deficient condition had 35 per cent greater acetylene reduction rates and 58 per cent greater fresh weights compared with wild type inoculated plants.

Paau (1989) used indigenous *Rhizobium* population to develop *Bradyrhizobium japonicum* inoculants which could out compete strains present in existing commercial inoculants. The mutants were derived from indigenous wild type strains that are capable of high level occupancy of nodules in soybean fields by mutagenizing with nitrosoguanidine. The inoculant contained a mixture of mutants selected for increased nitrogen fixation ability. Field tests in Mississippi delta region averaged a 169 kg per ha increased grain yield.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

The experiments on the effect of chemical and physical mutagens on *Bradyrhizobium* spp were conducted at the Department of Agricultural Microbiology, G.K.V.K. Campus, University of Agricultural Sciences, Bangalore. The materials used and the methods followed are described below.

Bradyrhizobium sp. (*Vigna*) strain P-132 and *Bradyrhizobium* sp (*Arachis*) strain TAL-1000 obtained from the Department of Agricultural Microbiology, G.K.V.K., Bangalore, were the two parent strains used for the present study.

A loopful of the two rhizobial cultures were streaked separately on yeast extract mannitol agar (YEMA) medium with congo red and incubated for 7 days at 28°C. Well isolated white colonies were streaked on YEMA slants labelled and stored at 4°C which were used for further study.

Composition of YEMA is given in Appendix.

3.0 CHARACTERISATION OF PARENT STRAINS

The two *Bradyrhizobium* spp strain P-132 and TAL-1000 were subjected to the following determinative tests to authenticate and characterise as *Rhizobium* species.

3.1 Gram staining

Gram staining of the two parent strains was conducted following the method of Somasegaran and Hoben (1985).

3.2 Growth on YEMA medium

The growth of wild strains on YEMA congored medium was studied as per the procedure given by Somasegaran and Hoben (1985).

The YEMA medium with 10 ml of 0.25 per cent congored per litre was prepared. Sterile agar plates of this medium were prepared by dispensing 15 ml of the sterile YEMA medium into sterile petriplates. Young cultures of the two strains were streaked on the solidified medium and incubated for seven days at 28°C. Little or no absorption of congored by young colonies was confirmatory to *Rhizobium*. The number of days for colonies to appear, the colony diameter and character were also recorded.

3.3 Growth on YEM-BTB broth

About 10ml of freshly prepared YEM broth having pH 7.0 and BTB indicator which gives a green color to medium was poured into test-tubes and sterilized. Seven day old cultures (0.1ml) were used for inoculation and incubated for 7 days. Change in colour of broth to blue indicated alkaline reaction whereas acid reaction was indicated by change in broth colour to yellow (Somasegaran and Hoben, 1985).

3.4 Growth on glucose peptone agar

The method described by Somasegaran and Hoben (1985) was followed. Young cultures were streaked on the freshly prepared glucose peptone agar plates and incubated at 28°C. Little or no growth and no change in pH of medium was conformity to *Rhizobium*.

Composition of glucose peptone agar is given in Appendix.

3.5 Ketolactose test

Test isolates were streaked on the ketolactose medium and these plates were incubated at 28°C for 7 days. After the incubation period the plates were flooded with Benedict's solution in order to test for the production of lactonic acid. The change in colour of Benedict's solution from blue to yellow around the colonies indicated conversion of lactose to lactonic acid. Strains exhibiting negative reaction for the test were considered positive for *Rhizobium* (Bernartz and Daley, 1963).

Composition of ketolactose medium is given in Appendix.

3.6 Hofers alkaline broth test

The pH of YEM broth was raised to 11.0 by adding 0.1N KOH. Ten ml of this broth was dispensed into test tubes and autoclaved at 121°C, 15 lbs for 15 minutes. One tenth of a ml of 7 day old culture in YEM broth was used to inoculate Hofers alkaline broth and incubated at 28°C for 7 days. Inability of these isolates to grow on this medium is confirmatory for *Rhizobium* (Subba Rao, 1982).

3.7 Nodulation Test

Two wild type strains P-132 and TAL-1000 were confirmed as Rhizobia by the earlier described tests were used for this final confirmatory test. The method described by Somasegaran and Hoben (1985) was followed.

3.7.1. Preparation of seedling agar tubes

Corning test-tubes of 200 x 30 mm size containing 40ml of sterile Jensen's seedling agar slopes were used for the enclosed culturing of the seedlings.

Composition of Jensen's seedling agar is given in appendix.

3.7.2 Sowing of seeds

Clean, healthy seeds of Siratro (*Macroptilium atropurpureum*) which is test host for cowpea miscellany group of rhizobia were used. Seeds were acid scarified and sterilised with concentrated sulphuric acid for 10 minutes. Sulphuric acid was decanted and seeds were washed in 6-8 changes of sterile distilled water. These surface sterilised seeds were then aseptically placed on petri plates containing one per cent water agar medium and incubated for two days so that the radical size was 0.5-1 cm long and straight.

The germinated seeds were placed on the edge of seedling agar slope with sterile forceps. The radicle of the seed was made to adhere to the agar slope by making a slight depression over it. The test-tubes were rested on their sides for a day to allow the radicles to anchor into the plant nutrient agar.

3.7.3 Preparation of inoculant

Test-tubes containing 10 ml of YEM broth were sterilised at 121°C, 15 lbs for 15 minutes, then a loopful of pure culture of test isolates were inoculated to the broth and incubated at 28°C for 7 days. Two ml of this broth was used as inoculant for the enclosed culture.

3.7.4 Inoculation

A day after planting the tubes, the seedlings were inoculated with test rhizobium cultures. Two ml culture of the respective test isolates was poured into the seedling tubes. The lower portion of the tubes were covered with black paper to facilitate unimpeded root growth. The tubes were then placed in seedling tube stands and incubated under green house condition with an average daylength of 9-10 hours. Three replicates were maintained for each isolate with a control (no inoculation). Seedlings were observed for nodulation till the end of five weeks.

3.8Intrinsic Streptomycin resistance level of parent strains

Intrinsic streptomycin resistance level (maximum level of natural resistance) of parent strains, P-132 and TAL-1000 was investigated.

Stock solution of the antibiotic streptomycin (Indian drugs and pharmaceuticals Ltd, Rishikesh) was prepared by dissolving 1g of streptomycin in 100ml of distilled water to give a concentration of 10,000 µg/ml. The stock solution was filter sterilized (0.45 µm Millipore filters) and appropriate amounts of stock solution was added to 200ml YEMA flasks which were previously autoclaved and cooled to 40-50°C to give a working concentration of 0, 1, 2, 4, 6, 8, 10, 15, 20, 40, 60, 80 and 100 µg ml^{-1}. Flasks were gently shaken and poured into sterile petriplates. Three replications were maintained for each concentration of antibiotic in YEMA. Seven day old broth cultures of strains P-132 and TAL-1000 were used to spot inoculate on the YEMA plates containing antibiotic in a series of concentration. All the plates were incubated at 28°C. (Schwinghamer, 1967).

3.9 USE OF MUTAGENS FOR STRAIN IMPROVEMENT

Acridine orange (Chemical mutagen) and ultraviolet rays (physical mutagen) were used in the present study to mutagenise P-132 and TAL-1000 strains of

Bradyrhizobium spp.

3.9.1 Acridine orange mutagenesis

Acridine orange (AO) mutagenesis was done to obtain mutants with desirable traits. The method followed is based on the procedure followed by Ozawa and Yamaguchi, (1986).

Stock solution of AO (Hi media Lab. Pvt Ltd., Bombay) was prepared by dissolving 0.01g in 100ml distilled water and filter sterilized (millipore filter 0.45 µm).

Appropriate amount of sterilized AO stock solution was added to sterilized 5ml YEM broth tubes to give AO concentration of 0, 2, 5, 20 and 50 $\mu g ml^{-1}$ of medium. 0.05 ml mid log phase culture (3 day old) of the test strains was added to YEM broth tubes containing five different concentrations of AO and grown for 14 days at 28°C except YEM broth containing 50 µg/ml of AO, which was incubated only for 24 hrs. Mutagenised cells were then washed two to three times with 5 ml, 0.1M sterile potassium phosphate buffer saline (0.85% NaCl; pH 7) by centrifugation at 6000 rpm for 20 minutes to discard supernatant, containing medium and AO. Bacterial pellet was dissolved in 1 ml of 0.1M sterile phosphate buffer saline and transferred to 4 ml sterile YEM broth in test tubes, and incubated for seven days at 28°C for mutants to multiply and express.

3.9.2. Ultra Violet (UV) rays mutagenesis

The two *Bradyrhizobium* strains P-132 and TAL-1000 were mutagenised with UV. The procedure used for UV mutagenesis was similar to the procedure followed by Noel et al., 1982 and Ghai et al., (1983).

Strains P-132 and TAL-1000 were grown in 100 ml YEM broths for 3 days (mid log phase) and pelleted by centrifugation at 6000 rpm for 20 min. Supernatent containing media was discarded. The bacterial pellet was washed with and resuspended in sterile phosphate buffer saline (0.1M PBS) to a final concentration of approximately 10^8 cells per ml. Five ml of this cell suspension was irradiated with UV as a thin layer in 9 cm petriplates in a laminar flow chamber. The UV light source was a 30 W phillips tube placed at a distance of 32.5 cm above the cells. The cells were exposed to different durations (0, 1, 2, 4, 6, 8, 10, 30 and 60 seconds) of UV. One ml of the mutagenised cells were diluted into 9 ml YEM broth in test tubes and incubated in dark to avoid photoreaction of cells for three days at 28°C for the growth of mutants.

3.9.3. Isolation of mutants

Streptomycin was used to isolate mutants after AO and UV mutagenesis.

One tenth of a ml mutagenised cultures were plated on sterile YEMA congo red medium containing 30 µg/ml of streptomycin which is more than three times the concentration of antibiotic able to prevent growth of the parent strains and spread using a sterilized glass spreader. All the plates were incubated at 28°C.

After incubation about two to three streptomycin resistant colonies were randomly picked from each AO or UV treatment, transfered to YEM agar slants and stored at 4°C after carefully labelling. These mutants were used for further study.

3.10 NODULATION TEST

All the AO and UV mutants isolated were first tested for ability to form root nodules on siratro (*Macroptilium atropurpureum*) the indicator host for cowpea miscellenary group of rhizobia.

The method of preparation of seedling agar tubes, sowing of seeds, preparation of mutant inoculant and inoculation was similar to the procedure described earlier in section 3.7.

Only those mutants which formed increased number of medium to big sized pink nodules were used for further study.

3.11 Isolation of *Rhizobium* mutants from root nodules

The isolation of *Rhizobium* from the nodules was carried out as per the procedure described by Somasegaran and Hoben (1985). Only those mutants which formed medium to big sized pink nodules were selected for isolation. The roots of the plants along with nodules were washed under running water to clean them off the agar medium. The nodules were detached with a root bit and subjected to surface sterilization by momentary rinsing in absolute alcohol followed by treatment in 0.1 per cent mercuric chloride for three minutes. The surface sterilized nodules were washed in 6 to 8 changes of sterile water taken in sterile petriplates. The sterilized nodules were transferred aseptically to sterilized petriplates with a drop of water and crushed with sterile blunt end forceps to get a nodule suspension. A loopful of nodule suspension was streaked on yeast extract mannitol agar medium containing 30 μgml^{-1} of streptomycin and incubated at 28°C in an incubator.

Finally well isolated colonies from these plates were transferred to YEMA slants and stored and used for further study.

Mutant strains were subjected to the following determinative tests.

3.12 Morphological Studies

3.12.1 Gram staining

Gram staining of mutants was conducted following the method described by Somasegaran and Hoben (1985) to observe the gram reaction, shape and size.

3.13 Cultural studies

3.13.1 Growth on YEMA congored medium

The growth of mutant strains was compared with wild type strains on YEMA congo red medium containing 30 µg/ml streptomycin (Somasegaran and Hoben 1985).

YEMA medium with 10ml of 0.25 per cent congo red per litre was autoclaved at 121°C, 15 lbs for twenty minutes and 3 ml of 10,000 $\mu g\ ml^{-1}$ stock of streptomycin was added to sterilized and cooled (50°C) one litre YEMA medium, shaken gently to mix the contents and dispensed to sterile petriplates. Young cultures of the parent and mutant strains were streaked on solidified medium and incubated for 7 to 10 days at 28°C. The characters considered were number of days for colonies to appear, colony colour, size, elevation, transparency and pigmentation.

3.13.2 Growth in YEM - BTB broth

The ability of mutants to grown in YEM - BTB broth and change in colour of broth was studied as described earlier in section 3.3. Each mutant culture was inoculated to YEM BTB broth in triplicate.

3.13.3 Growth on glucose peptone agar

The growth of mutants on GPA was compared with the parent strains as described by Somasegaran and Hoben (1985) and explained in section 3.4.

3.13.4 Hofers alkaline broth test

The growth of mutants in Hofers alkaline broth was tested as mentioned earlier in section 3.6. Seven day old mutant culture (0.1 ml) in YEM broth was inoculated in Hofers alkaline broth tubes maintained in triplicate.

3.14 Physiological studies

AO and UV mutants of strain P-132 and TAL-1000 were subjected to various biochemical studies and compared with wild type strain.

3.14.1 Carbohydrate Utilization studies

Fifteen AO mutants and twenty two UV mutants were subjected for this study. Carbon sources used in this study were xylose, arabinose (Pentoses); mannitol, glucose, galactose (Hexoses); sucrose, maltose, lactose (dissacharides); starch (polysaccharide) ; glycerol (C_3 - dicarboxylates).

Except xylose and arabinose, all the other carbon sources were added to get a final concentration of one per cent in the basal medium (YEMA minus mannitol) and autoclaved at 121°C, 15 lbs for 15 minutes. Xylose and arabinose were filter sterilized (0.45µm Millipore filter) and added to sterilized and cooled (50°C) basal medium to get a final concentration of 1 per cent, and poured into sterile petriplates. A loopful of seven day old broth culture of mutants and parent strains were streaked on different carbon source and each treatment was maintained in duplicate. Observations were made after seven days incubation at 28°C.

3.14.2 pH tolerance range

YEM broth with pH ranging from 3 to 10 were prepared by addition of 0.1N HCl or 0.1N KOH and adjusted using digital pH meter. Ten ml of each pH level was dispensed into test-tubes and autoclaved at 121°C, 15 lbs for 15 minutes. Seven day old broth culture (0.1ml) of each test isolate was added to thirteen different levels of pH maintained in duplicate and incubated for seven days. The growth of parent strains and mutants was determined by measuring optical density at 540 nm using spectronic - 20 spectrophotometer (Somasegaran and Hoben, 1985).

3.14.3 Salt tolerance range

YEM broth with different concentrations of NaCl was prepared in separate 10 ml test tubes 0.01, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 per cent NaCl was prepared by adding 0.05, 0.5, 2.5, 5.0, 7.5, 10, 12.5 and 15g of NaCl per 500 ml of basal medium respectively. One tenth ml of mutant and wild strains were inoculated using sterile pipettes and incubated for 7 days at 28°C. The presence or absence of growth was determined by recording turbidity in terms of optical density at 540 nm using Spectronic - 20 spectrophotometer.

3.15 CROSS - INOCULATION STUDIES

Specificity and promiscuity in symbiosis are studied in cross inoculation experiments. Two legumes, soybean (*Glycine max* L.) cv. KHSB-2 and chickpea (*Cicer arietinum* L.) local variety were used in this study.

Eight AO mutants and twelve UV mutants selected after morphological, cultural and physiological studies along with their wild type strains were examined for their ability to nodulate soybean and chickpea.

3.15.1 Preparation of inoculum

Hundred ml conical flasks containing 25 ml YEM broth were prepared. These flasks were sterilized at 121°C, 15 lbs for 15 minutes and a loopful of pure culture of the test isolates was added and incubated at 28°C for 7 days in an incubator with periodic shaking.

3.15.2 Preparation of Growth pouches

The pouches used were made of polypropylene (19 x 15 cm) with germination paper wick liner and a trough was made at the top to place the germinated seedling. Growth pouches served well as inexpensive space saving substitutes for Leonard jars. Growth pouches were sterilized by wrapping in aluminium foil and autoclaved at 121°C, 15 lbs for 20 minutes (Somasegaran and Hoben, 1985).

3.15.3 Surface sterilization of seeds.

About 100 seeds of soybean and chickpea were surface sterilized. Seeds were placed in 250 ml sterile conical flask and rinsed with 95 per cent alcohol for 10 sec to remove waxy material and trapped air. Alcohol was then drained off and sterilant $HgCl_2$ (0.1 per cent) was added for 3 min. The sterilant was drained and seeds were washed with 6 to 8 changes of sterile distilled water. Seeds were then placed in fridge for two hours to imbibe water, after which seeds were again washed and placed on sterile water agar (1 %) plates and incubated for 2-3 days at 25°C.

3.15.4 Planting and inoculation

Thirty ml of sterile N - free nutrient solution (composition in Appendix) was dispensed into each of the sterile growth pouches and arranged in racks. About 69 pouches were used for each test host. Sixty nine well germinated seeds of similar size and radical length (1 - 1.5 cm) were selected and one seedling per pouch was placed aseptically in the trough of the paper wick. To prevent the growing radical from pushing seed out of the pouch a hole was made in the trough of wick with a sterile fine tipped forceps and radical was inserted into the hole.

The seedlings were then inoculated with 2 ml broth culture (approximately 10^8 cells/ml) of the parent and mutant strains of rhizobium by pouring on the root system. Each treatment (host - strain combination including control with no inoculation) was maintained in triplicate. These racks were placed in green house and observed periodically to observe the number of days for nodule initiations. Nutrient solution was replenished when necessary (Somasegaran and Hoben, 1985). Plants were examined over a period of five weeks for nodulation. The nodule size, number and color were recorded after 5 weeks.

3.16 SCREENING MUTANTS FOR NITRATE TOLERANT SYMBIOSIS

Twenty mutants (8 AO mutants and 12 UV mutants) selected after various morphological, cultural, physiological and nodulation studies, were further used to screen mutants along with their parent strains for nitrate tolerant symbiosis with cowpea in growth pouches. Tolerance in the presence of four levels of nitrate (NO_3) viz 0, 0.5, 1.5 and 5mM nitrate, corresponding to 0, 16, 47 and 157 kg N/ha respectively, was examined.

3.16.1 Preparation of inoculum

Eight AO mutants, twelve UV mutants along with their parent strains P-132 and TAL-1000 were used in the present investigation. A loopful of pure culture from each of the test isolates was inoculated to sterile 50ml YEM broth in 100 ml conical flasks and incubated for 7 to 8 days at 28°C with periodic shaking.

3.16.2 Surface sterilization of cowpea seeds

The seeds of cowpea (*Vigna unguiculata* L.) var TVX was used. Healthy, uniform sized seeds were surface sterilized with mercuric chloride (0.1 per cent) and germinated as described earlier in section 3.15.3.

3.16.3 Preparation of nutrient solution

Stock solution of nitrate was prepared by dissolving 5.05g of KNO_3 in 100 ml distilled water to give 500 mM NO_3.

Ten litres of N-free nutrient solution was prepared (composition in Appendix) and divided into four equal parts of 2.5 litres each. 0, 2.5, 7.5 and 25ml of stock solution of NO_3 was added to each 2.5 litre nitrogen free plant nutrient solution to give a final concentration of 0, 0.5, 1.5 and 5.0 mM NO_3 in nutrient solution respectively. The pH was adjusted to 6.8 and autoclaved at 121°C 15 lbs for 30 minutes.

3.16.4 Preparation of growth pouches

Growth pouches of size 18 x 12.5 cm were sterilized as mentioned earlier in section 3.15.2.

3.16.5 Planting and inoculation

Each isolate was tested in the presence of four levels of nitrate viz. 0, 0.5, 1.5 and 5.0 mM NO_3 maintained in triplicate.

About 30ml of nutrient solution containing different concentrations of nitrate was dispensed into sterile growth pouches and labelled. Planting of cowpea seeds in growth pouches and inoculation of parent and mutant *Bradyrhizobium* strains was similar as mentioned earlier in section 3.15.4. Uninoculated plants were used in between the inoculated plants as controls to check for possible contamination. Growth pouch racks were transferred to green house and observed periodically to note the number of days taken for nodule initiation by different mutant inoculated plants. Growth pouches were replenished with plant nutrient solution containing appropriate concentrations of nitrate, once in three to four days.

Treatments

The two parent strains P-132 and TAL-1000 and ten mutants derived from each parent were examined for altered symbiosis in the presence of four levels of NO_3 (0, 0.5, 1.5 and 5 mM NO_3). Treatments were replicated thrice.

Observations recorded:

Number of days for nodule initiation. Shoot colour, plant height, number of trifoliate leaves, leaflet length and width, nodule size and nodule number were recorded at 35 days after planting. Dry weight of nodules per plant (dried at 60°C for 48 hrs) and unit weight of nodule were also recorded.

Unit weight of nodule was computed by the formula

$$\text{Unit wt of nodule} = \frac{\text{dry weight of nodules per plant}}{\text{number of nodules per plant}}$$

Statistical analysis

The data obtained was subjected to analysis of variance by factorial completely randomized design using Microvax system of Digital Equipment Corporation, U.S.A. (VAX/VMS Version 5.4)

Treatment means were separated by Duncans Multiple Range test (DMRT) at 5 per cent level of significance (Little and Hills, 1978).

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

The results of various experiments conducted in order to mutagenise and characterize the mutants deriverd from two *Bradyrhizobium* spp strain P-132 and TAL-1000 (parent strains) and to select effective rhizobial mutants with broader host range and nitrate tolerant symbiosis is presented below.

The two *Bradyrhizobium* spp strain P-132 and TAL-1000 obtained from the Department of Agricultural Microbiology, U.A.S., Bangalore were checked for purity and viablity by streaking on sterile yeast extract mannitol agar congo red medium (YEMA) containing plates. Well isolated colonies from the YEMA plates were streaked on YEMA slants, stored at 4°C and used for further study.

4.1 CHARACTERISTICS OF PARENT STRAINS

The two parent strains, P-132 and TAL-1000 were characterized as follows. The details regarding the characters of rhizobial isolates are given in Table 3.

4.1.1 Morphological characters

Morphologically strain P-132 and TAL-1000 were gram negative and short rods (Table 3).

4.1.2 Cultural characters

Table 3 gives cultural characters of the parent strains used in the present study. The details regarding the growth of the rhizobial isolates in different media are given below.

Growth on YEMA with congo red medium

Strains P-132 and TAL-1000 did not absorb congo red. Well isolated colonies of one mm size were observed only after seven days of incubation which were white, opaque convex shaped, smooth and mucoid.

Production of acid or alkali

The results indicate that strains P-132 and TAL-1000 are alkali producers.

Table 3 :Morphological, cultural and physiological characters of parent rhizobial strains.

Strains (original host)	Gram stain	Growth on YEMA	Colony characters on YEMA congored medium	Growth in YEM-BTB broth	Growth in glucose peptone agar	Production of lactonic acid	Growth in Hofers broth	Nodulation Test*
P-132 (cowpea)	Gram-ve rods	Slow (7 days)	White, opaque	alkali	-	-	-	+
TAL-1000 (peanuts)	Gram-ve rods	Slow (7 days)	White, opaque	alkali	-	-	-	+

- = no growth ; + = positive for nodulation ;* = Siratro used as test host

Table 4 : Intrinsic streptomycin resistance of parent rhizobial strains.

Parent strains	Streptomycin (μg/ml)												
	0	1	2	4	6	8	10	15	20	40	60	80	100
P-132	+	+	+	+	+	+	+	-	-	-	-	-	-
TAL-1000	+	+	+	-	-	-	-	-	-	-	-	-	-

+ = growth ;- = no growth

Growth on glucose peptone agar (GPA)

Strains P-132 and TAL-1000 did not show any growth on GPA medium.

Production of lactonic acid

Neither of the two strains grown on ketolactose medium tested positive for lactonic acid production.

Growth on Hofers alkaline broth

Both the strains did not show any growth in Hofers alkaline medium.

4.1.3 Plant infection studies

Both the strains tested for plant infection gave positive nodulation on siratro (Table 3). The nodules formed were medium sized, globose in shape and pink inside.

4.1.4 Intrinsic streptomycin resistance

The intrinsic streptomycin resistance level (maximum level of natural resistance) was found to be 10 µg/ml for strain P-132 and 2 µg/ml for strain TAL-1000 (Table 4).

4.2 MUTAGENESIS

The two strains, P-132 and TAL-1000 were mutagenized with acridine orange (chemical mutagen) and ultra violet rays (physical mutagen) as described earlier.

4.2.1 Acridine orange (AO) mutagenesis and isolation of mutants

Strains P-132 and TAL-1000 were mutagenized with AO as described earlier in materials and methods and plated on sterile YEMA plates containing 30 µg/ml of streptomycin which is three times above the intrinsic streptomycin resistance level for strain P-132 and 15 times above intrinsic streptomycin resistance level for TAL-1000.

AO at 0 µg/ml (control treatment) did not result in the production of any mutants from both parents when plated on streptomycin containing YEMA plates. None of the other four AO treatments (2, 5, 20 and 50 µg/ml) completely inhibited growth of the two strains. Two to three streptomycin resistant colonies were picked from each concentration treatment of AO (2 to 50 µg/ml), totalling to 27 mutants from the two strains. These mutants were labelled and maintained on YEMA slants.

4.2.2 Ultraviolet (UV) rays mutagenesis and isolation of mutants

Strains P-132 and TAL-1000 were irradiated with UV rays as described earlier in materials and methods and plated on sterile YEMA plates containing 30 µg/ml of streptomycin.

No exposure to UV (0 sec UV or control treatmemt) did not result in production of any mutants from both the strains on stereptomycin containing YEMA plates. Sixty sec UV exposure was lethal to both the strains, while two to three well isolated mutant colonies were isolated from all the other durations of UV exposure (1, 2, 4, 6, 8, 10 and 30 sec). A total of 37 UV mutants were isolated from both the parent strains and maintained on YEMA slants after labelling.

4.2.3 Plant infection studies

All the mutants isolated were subjected to plant infection studies in seedling agar tubes as described earlier and the data is presented in Tables 5a, 5b, 6a and 6b.

Except AP5M$_3$, AP5M$_5$ and AT20M$_1$, all other AO mutants tested formed nodules on *Macroptilium atropurpureum* (siratro). Time taken for nodulation in seedling agar tubes varied from 10 to 20 days. Mutants AP2M$_1$, AP2M$_3$, AP5M$_2$, AP5M$_4$, AP20M$_2$, AP20M$_3$, AP50M$_1$, AP50M$_3$, AT2M$_2$, AT2M$_3$, AT5M$_2$, AT50M$_1$ AND AT50M$_2$ formed medium to big sized nodules and were pink inside (Tables 5a and 5b).

Among the UV mutants, except UP2M$_1$, UP6M$_1$, UP30M$_1$, UP30M$_2$, UP30M$_3$, UT1M$_2$, UT4M$_2$, UT6M$_1$, UT1OM$_1$, UT10M$_2$, and UT30M$_1$, all the other mutants nodulated siratro. Number of days taken for nodulation varied from 13 to 23 days. UV mutants UP1M$_1$, UP1M$_3$, UP2M$_2$, UP2M$_3$, UP4M$_1$, UP6M$_2$, UP6M$_4$, UP8M$_1$, UP10M$_2$, UT1M$_1$, UT1M$_3$, UT2M$_1$, UT4M$_3$, UT6M$_2$, UT8M$_1$

Table 5a : Nodulation test of acridine orange mutants derived from *Bradyrhizobium* sp. strain P-132.

Mutants	Nodulation	Days for nodule initiation	Nodule number per plant	Nodule character
P-132	+	18	6	M, G, P
AP2M$_1$	+	12	8	M, G, P
AP2M$_2$	+	14	5	M, G, W
AP2M$_3$	+	11	9	M, G, P
AP2M$_4$	+	16	5	M, G, P
AP5M$_1$	+	15	3	S, R, W
AP5M$_2$	+	14	6	B, G, P
AP5M$_3$	-			
AP5M$_4$	+	13	10	M, G, P
AP5M$_5$	-			
AP5M$_6$	+	14	10	M, G, P
AP20M$_1$	+	11	6	M, R, P
AP20M$_2$	+	10	8	B, G, P
AP20M$_3$	+	10	8	M, G, P
AP50M$_1$	+	13	10	B, G, P
AP50M$_2$	+	13	9	M, R, P
AP50M$_3$	+	15	8	B, G, P

+ = positive for nodulation ; - = no nodulation

Nodule size	Color inside the nodule	Nodule shape
Big (B)	Pink (P)	Globose (G)
Medium (M)	White (W)	Round (R)
Small (S)		

Table 5b : Nodulation test of acridine orange mutants derived from *Bradyrhizobium* sp strain TAL-1000

Mutants	Nodulation	Days for nodule initiation	Nodule number per plant	Nodule character
TAL-1000	+	18	8	M, G, P
AT2M$_1$	+	16	2	M, G, P
AT2M$_2$	+	16	6	B, G, P
AT2M$_3$	+	14	8	B, G, P
AT2M$_4$	+	16	4	S, R, W
AT2M$_5$	+	18	3	M, G, P
AT5M$_1$	+	20	6	M, G, P
AT5M$_2$	+	14	10	B, G, P
AT5M$_3$	+	16	2	S, R, W
AT20M$_1$	-			
AT50M$_1$	+	12	10	B, G, P
AT50M$_2$	+	19	12	B, G, P

+ = positive for nodulation ; − = no nodulation

Nodule size	Color inside the nodule	Nodule shape
Big (B)	Pink (P)	Globose (G)
Medium (M)	White (W)	Round (R)
Small (S)		

Table 6a: Nodulation test of UV mutants derieved from *Bradyrhizobium* sp strain P-132.

Mutants	Nodulation	Days for nodule initiation	Nodule number per plant	Nodule character
P-132	+	18	5	M, G, P
UP1M$_1$	+	18	6	S, R, P
UP1M$_2$	+	20	3	S, R, W
UP1M$_3$	+	18	5	S, R, P
UP1M$_4$	+	18	8	S, R, W
UP2M$_1$	-			
UP2M$_2$	+	16	5	M, G, P
UP2M$_3$	+	13	2	S, R, P
UP4M$_1$	+	17	14	M, G, P
UP4M$_2$	+	18	3	S, R, W
UP4M$_3$	+	21	3	M, R, P
UP6M$_1$	-			
UP6M$_2$	+	18	4	M, G, P
UP6M$_3$	+	20	8	S, R, W
UP6M$_4$	+	19	4	S, R, P
UP8M$_1$	+	20	4	B, G, P
UP8M$_2$	+	22	3	M, G, P
UP10M$_1$	+	18	6	S, R, W
UP10M$_2$	+	18	8	M, G, P
UP10M$_3$	+	20	6	M, G, P
UP30M$_1$	-			
UP30M$_2$	-			
UP30M$_3$	-			

+ = positive for nodulation ; - = no nodulation

Nodule size	Color inside the nodule	Nodule shape
Big (B)	Pink (P)	Globose (G)
Medium (M)	White (W)	Round (R)
Small (S)		

Table 6b :Nodulation test of UV mutants derived from *Bradyrhizobium* sp strain TAL-1000

Mutants	Nodulation	Days for nodule initiation	Nodule number per plant	Nodule character
TAL-1000	+	18	4	M, G, P
UT1M$_1$	+	23	5	M, G, P
UT1M$_2$	-			
UT1M$_3$	+	20	3	M, G, P
UT2M$_1$	+	16	5	M, G, P
UT4M$_1$	+	22	6	M, G, P
UT4M$_2$	-			
UT4M$_3$	+	15	6	M, G, P
UT6M$_1$	-			
UT6M$_2$	+	18	6	B, G, P
UT8M$_1$	+	15	6	M, G, P
UT10M$_1$	-			
UT10M$_2$	-			
UT10M$_3$	+	18	4	M, G, P
UT10M$_4$	+	20	2	M, G, P
UT30M$_1$	-			

+ = positive for nodulation ; - = no nodulation

Nodule size	Color inside the nodule	Nodule shape
Big (B)	Pink (P)	Globose (G)
Medium (M)	White (W)	Round (R)
Small (S)		

and UT10M$_3$ formed medium to big sized nodules which were pink inside, indicating that they formed effective symbiosis (Tables 6a and 6b).

Only those mutants which formed effective symbiosis with *Macroptilium atropurpureum* were isolated from nodules on YEMA plates containing 30 µg/ml streptomycin and used for further studies.

4.3 CHARACTERISTICS OF RHIZOBIAL MUTANTS

Only those AO and UV mutants which effectively nodulated siratro were further characterized as follows.

4.3.1 Morphological characters

The rod shaped morphology and gram negative property of the two parent strains P-132 and TAL-1000, were shared by all the AO and UV mutants studied (Tables 7a and 7b).

4.3.2 Cultural characters

The details regarding growth of *Bradyrhizobium* mutants in different media are given below

- Growth on YEMA congored medium with streptomycin (30 µgml^{-1})

The details regarding the growth of parent strains and all the mutants on YEMA congored with streptomycin are presented in Tables 7a and 7b.

Parent strains P-132 and TAL-1000 formed 1 mm colonies on YEMA congo red medium after 7 days (Table 3) but were inhibited on YEMA plates containing 30 µg ml^{-1} streptomycin (Tables 7a and 7b and plate 1). However all AO and UV mutants showed growth on YEMA plates containing 30 µg ml^{-1} streptomycin after 7-10 days incubation. None of the young colonies of mutants absorbed congo red and formed white, opaque, convex shaped smooth colonies on YEMA with streptomycin, but the size of colonies varied among mutants between 0.5 to 2.0mm.

AO mutants AP5M$_2$, AP20M$_2$, AP50M$_3$, AT2M$_2$, AT2M$_3$, AT50M$_1$, AT50M$_2$ and UV mutants UP6M$_2$, UP10M$_2$, UT1M$_3$, UT2M$_1$, UT4M$_3$ and UT1OM$_3$

Table 7a : Morphological and cultural characters of acridine orange mutants of two *Bradyrhizobium* spp

Mutants	Gram stain	Cell shape	colony characters on YEMA congored medium		
			with 30 µg/ml streptomycin size (mm)	Colony, colour	Shape, texture
P-132	Gram -ve	rods	-	White, opaque	Convex, smooth
AP2M$_1$	Gram -ve	rods	1.5	White, opaque	Convex, smooth
AP2M$_3$	Gram -ve	rods	1.0	White, opaque	Convex, smooth
AP5M$_2$	Gram -ve	rods	2.0	White, opaque	Convex, smooth
AP5M$_4$	Gram -ve	rods	1.5	White, opaque	Convex, smooth
AP20M$_2$	Gram -ve	rods	2.0	White, opaque	Convex, smooth
AP20M$_3$	Gram -ve	rods	1.5	White, opaque	Convex, smooth
AP50M$_1$	Gram -ve	rods	1.0	White, opaque	Convex, smooth
AP50M$_3$	Gram -ve	rods	2.0	White, opaque	Convex, smooth
TAL-1000	Gram -ve	rods	-	White, opaque	Convex, smooth
AT2M$_2$	Gram -ve	rods	2.0	White, opaque	Convex, smooth
AT2M$_3$	Gram -ve	rods	2.0	White, opaque	Convex, smooth
AT5M$_2$	Gram -ve	rods	1.0	White, opaque	Convex, smooth
AT50M$_1$	Gram -ve	rods	2.0	White, opaque	Convex, smooth
AT50M$_2$	Gram -ve	rods	2.0	White, opaque	Convex, smooth

Table 7b : Morphological and cultural characters of UV mutants of two *Bradyrhizobium* spp

Mutants	Gram stain	Cell shape	colony characters on YEMA congored medium		
			with 30 µg/ml streptomycin size (mm)	Colony, colour	Shape, texture
P-132	Gram -ve	rods	-	White, opaque	Convex, smooth
UP1M$_1$	Gram -ve	rods	1.0	White, opaque	Convex, smooth
UP1M$_3$	Gram -ve	rods	1.5	White, opaque	Convex, smooth
UP2M$_2$	Gram -ve	rods	1.5	White, opaque	Convex, smooth
UP2M$_3$	Gram -ve	rods	0.5	White, opaque	Convex, smooth
UP4M$_1$	Gram -ve	rods	0.5	White, opaque	Convex, smooth
UP6M$_2$	Gram -ve	rods	2.0	White, opaque	Convex, smooth
UP6M$_4$	Gram -ve	rods	1.5	White, opaque	Convex, smooth
UP8M$_1$	Gram -ve	rods	0.5	White, opaque	Convex, smooth
UP10M$_2$	Gram -ve	rods	2.0	White, opaque	Convex, smooth
TAL-1000	Gram -ve	rods	-	White, opaque	Convex, smooth
UT1M$_1$	Gram -ve	rods	0.5	White, opaque	Convex, smooth
UT1M$_3$	Gram -ve	rods	2.0	White, opaque	Convex, smooth
UT2M$_1$	Gram -ve	rods	2.0	White, opaque	Convex, smooth
UT4M$_3$	Gram -ve	rods	2.0	White, opaque	Convex, smooth
UT6M$_1$	Gram -ve	rods	0.5	White, opaque	Convex, smooth
UT6M$_2$	Gram -ve	rods	0.5	White, opaque	Convex, smooth
UT8M$_1$	Gram -ve	rods	1.5	White, opaque	Convex, smooth
UT10M$_3$	Gram -ve	rods	2.0	White, opaque	Convex, smooth
UT10M$_4$	Gram -ve	rods	1.0	White, opaque	Convex, smooth

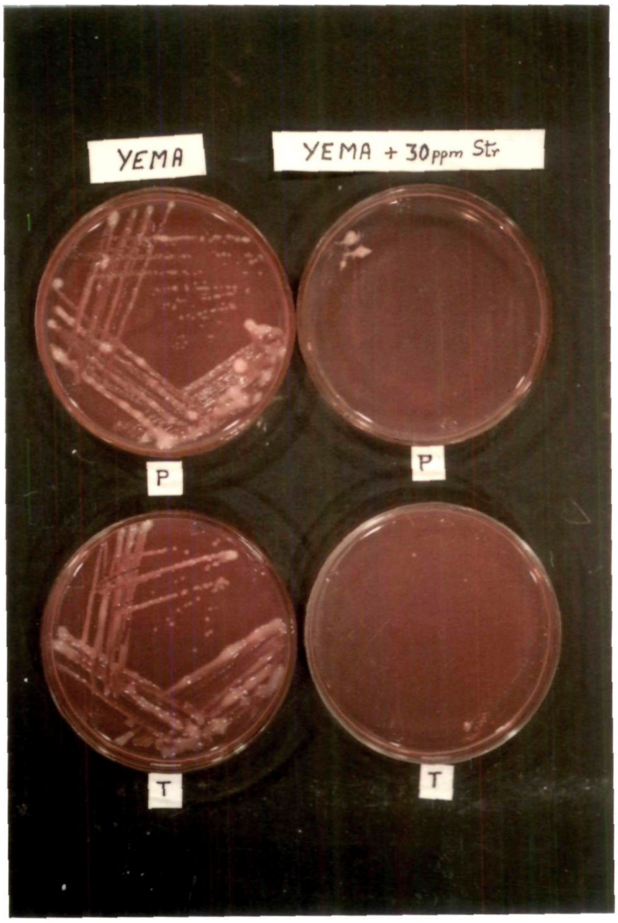

Plate 1: Growth comparison between parent *Bradyrhizobium* spp. strains P-132 (P) and TAL-1000 (T) on YEMA and YEMA + 30 µg/ml streptomycin medium.

formed big colonies (2mm) on YEMA plates containing streptomycin 30 µg ml^{-1}(plates 2, 3, 4 and 5). Mutants UP2M$_3$, UP4M$_1$, UP8M$_1$, UT1M$_1$, and UT6M$_2$ formed small colonies (0.5 mm) on YEMA containing streptomycin (30 µg ml^{-1}).

Growth in YEM - BTB broth

The results indicate that mutants AP2M$_1$, AP5M$_2$, AP20M$_2$ AP20M$_3$, AP50M$_3$, AT2M$_2$, AT5M$_2$, AT5M$_3$ AND AT50M$_2$ are acid producers among the AO mutants (Table 8a). UV mutants UP1M$_1$, UP2M$_3$, UP4M$_1$, UP6M$_2$, UP8M$_1$ and all the UV mutants derived from TAL-1000 were acid producers (Table 8b). All the remaining mutants were alkali producer like the parent strains.

Growth on Glucose peptone agar (GPA)

Neither the two parent strains nor the AO and UV mutants except UP6M$_2$, UT1M$_1$ showed any growth on GPA medium. UP6M$_2$ and UT1M$_1$ formed white colonies on GPA after 6 days changing the colour of GPA medium from purple to violet (Tables 8a and 8b) (Plate 6).

Growth in Hofers alkaline medium

Most of the mutants like the parent strains did not show any growth in Hofers alkaline medium. However mutants AP2M$_1$, AP2M$_3$, AP5M$_2$, AP20M$_2$, AP20M$_3$, AT50M$_1$, UP6M$_2$ and UT10M$_3$ showed faint growth. (Tables 8a and 8b).

4.3.3 Physiological studies

Utilization of different carbohydrates by rhizobial mutants

Ten different carbohydrates inclusive of mono, di and polysaccharides were used for this study. Ability to utilize these carbohydrates and their growth were studied.

Glycerol, arabinose, glucose, mannitol were the most preferred carbohydrates by parent strains and all AO mutants. Though the parent strains showed good growth on xylose and galactose too, most mutants showed only moderate growth. AP5M$_4$, and AP20M$_2$ showed faint growth on xylose while

Table 8a : Cultural characters of parent strains and acridine orange mutants of *Bradyrhizobium* spp.

Mutants	Growth on GPA	Growth in Hofers alkaline medium	Growth in YEM-BTB broth
P-132	-	-	alkali
AP2M$_1$	-	*	acid
AP2M$_3$	-	*	alkali
AP5M$_2$	-	*	acid
AP5M$_4$	-	-	alkali
AP20M$_2$	-	*	acid
AP20M$_3$	-	*	acid
AP50M$_1$	-	-	alkali
AP50M$_3$	-	-	acid
TAL-1000	-	-	alkali
AT2M$_2$	-	-	acid
AT2M$_3$	-	-	alkali
AT5M$_2$	-	-	acid
AT5M$_3$	-	-	acid
AT5OM$_1$	-	*	alkali
AT50M$_2$	-	-	acid

- = no growth

* = faint growth

Table 8b : Cultural characters of Parent strains and UV mutants of *Bradyrhizobium* spp.

Mutants	Growth on GPA	Growth in Hofers alkaline medium	Growth in YEM-BTB broth
P-132	-	-	alkali
UP1M$_1$	-	-	acid
UP1M$_3$	-	-	alkali
UP2M$_2$	-	-	alkali
UP2M$_3$	-	-	acid
UP4M$_1$	-	-	acid
UP4M$_3$	-	-	alkali
UP6M$_2$	+	*	acid
UP6M$_4$	-	-	alkali
UP8M$_1$	-	-	acid
UP10M$_2$	-	-	alkali
TAL-1000	-	-	alkali
UT1M$_1$	+	-	acid
UT1M$_3$	-	-	acid
UT2M$_1$	-	-	acid
UT4M$_3$	-	-	acid
UT6M$_1$	-	-	acid
UT6M$_2$	-	-	acid
UT8M$_1$	-	-	acid
UT10M$_3$	-	*	acid
UT10M$_4$	-	-	acid

- = no growth; + = Growth after 6 days; * = faint growth

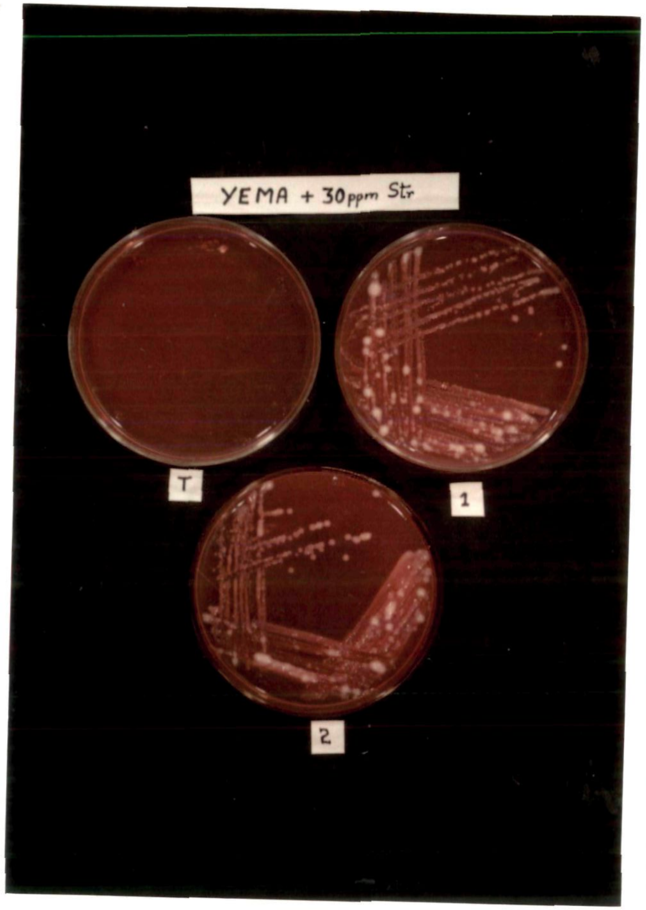

Plate 3: Growth comparison between parent *Bradyrhizobium* sp. strain TAL-1000 and its AO mutants on YEMA+30 µg/ml streptomycin medium. T=TAL-1000; 1=AT2M_3; 2=AT50M_2.

Plate 2: Growth comparison between parent *Bradyrhizobium* sp. strain P-132 and its AO mutants on YEMA + 30 µg/ml streptomycin medium. P= P-132; 1=AP5M_2; 2=AP20M_2

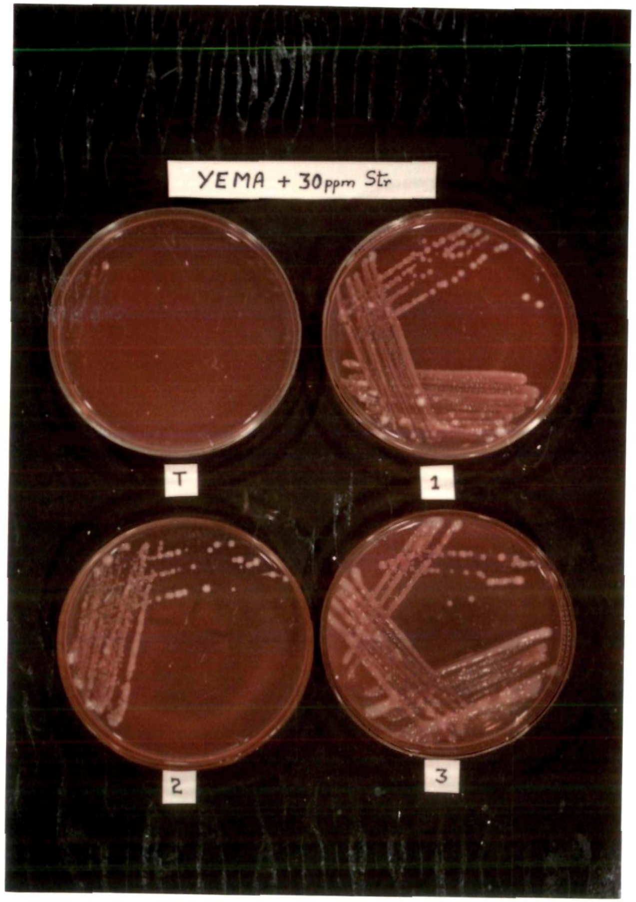

Plate 5: Growth comparison between parent strain of *Bradyrhizobium* sp. strain TAL-1000 and its UV mutants on YEMA + 30 µg/ml streptomycin medium. T=TAL-1000; 1=UT2M_1; 2=UT4M_3; 3=UT10M_3.

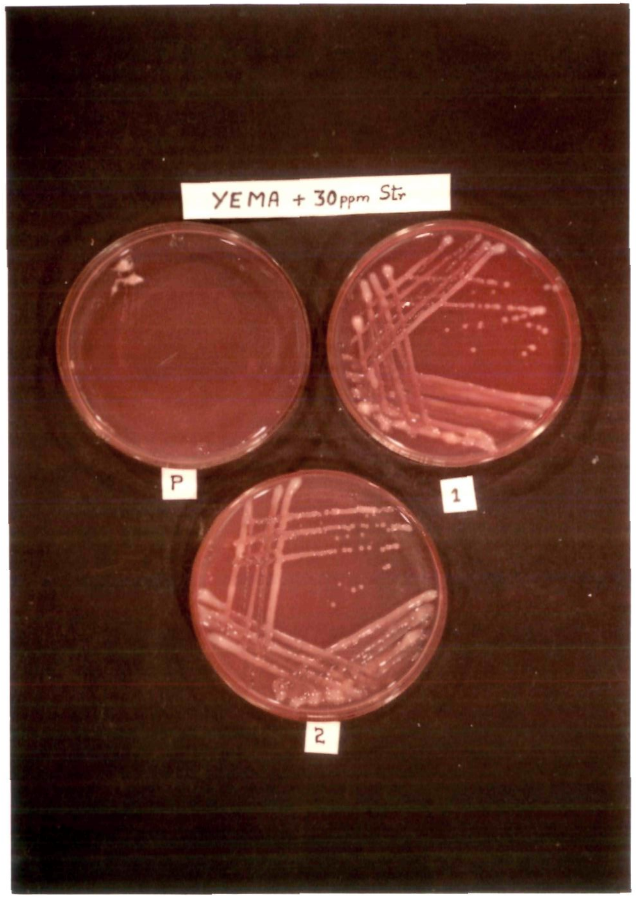

Plate 4: Growth comparison between parent strain of *Bradyrhizobium* sp. strain P-132 and its UV mutants on YEMA + 30 µg/ml streptomycin medium. P=P-132; 1=UP6M_2; 2=UP10M_2

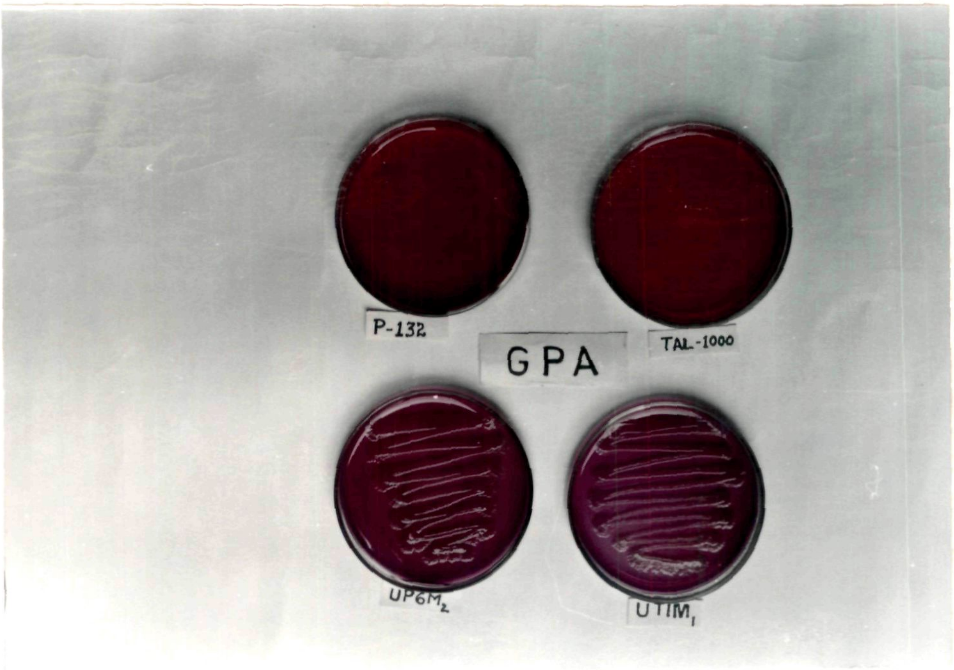

Plate 6: Growth of UV mutants UP6M$_2$ and UT1M$_1$ on glucose peptone agar medium.

AP20M$_3$ showed faint growth on both the pentoses. AT50M$_1$ and AT50M$_2$ showed moderate growth on maltose unlike their parent strains. Both the wild strains and all AO mutants were unable to grow on sucrose, lactose and starch (Table 9a).

Glycerol, xylose, arabinose, mannitol glucose and galactose were the most prefered carbohydrates of the UV mutants and parent strains. Some UV mutants showed faint growth on sucrose and maltose although P-132 was unable to grow on them. Neither parents nor UV mutants were able to utilize lactose and starch (Table 9b).

pH tolerance range of mutants

The optimum pH for growth of AO and UV mutants was 6 to 7 like the parents, except UP6M$_2$ which showed better growth at pH 8. Like the wild strains, all the UV mutants were inhibited at pH4 but all AO mutants were tolerant and AP20M$_2$ showed maximum growth among the mutants. pH 3.5 was inhibitory to all the strains. On the contrary most of the mutants exhibited growth at pH 10 which was inhibitory to P-132, UP8M$_1$ and UP10M$_2$ (Table 10a) and TAL-1000, AT50M$_2$, UT1M$_1$, UT10M$_4$ (Table 10b). Mutant UT1M$_3$ was inhibited at pH 9 itself.

Salt tolerance range of mutants

The data on the salt tolerance range of the mutants are presented in tables 11a and 11b.

Most of the AO and UV mutants studied exhibited maximum growth at 0.01 per cent NaCl. However, mutants AP5M$_2$, UP6M$_4$, AT5M$_2$, UT2M$_1$, and UT6M$_1$ had maximum growth at 0.1 per cent NaCl. Most of the mutants were inhibited at 2 per cent NaCl like the parents, however mutant UT8M$_1$ was inhibited at 1 per cent NaCl.

Mutants AP2M$_1$, AP5M$_4$, UP6M$_4$, AT2M$_2$ AT50M$_1$, AT50M$_2$, and UT10M$_4$ were tolerant to 2 per cent NaCl (Tables 11a and 11b). However mutants AP50M$_1$, UP1M$_1$ and UP6M$_2$ could survive even 2.5 per cent NaCl (Table 11a).

Table 9a :Utilization of different carbohydrates by two parent *Bradyrhizobium* strains and AO mutants derived from them.

Mutants	Carbhohydrates									
	Glycerol	Xylose	Arabinose	Mannitol	Glucose	Galactose	Sucrose	Maltose	Lactose	Starch
P-132	3 +	3 +	3 +	3+	3+	3 +	-	-	-	-
AP2M$_1$	3 +	2 +	3 +	3+	3 +	2 +	-	-	-	-
AP2M$_3$	3 +	2 +	3 +	3 +	3 +	3 +	-	1 +	-	-
AP5M$_2$	3 +	2 +	3 +	3+	3 +	2 +	-	-	-	-
AP5M$_4$	3 +	1 +	3 +	3+	3 +	2 +	-	-	-	-
AP20M$_2$	3 +	1 +	3 +	3+	3 +	2 +	-	-	-	-
AP20M$_3$	3 +	1 +	1 +	3+	2 +	2 +	-	-	-	-
AP50M$_1$	3 +	2 +	3 +	3+	2 +	2 +	-	-	-	-
AP50M$_3$	3 +	2 +	3 +	3+	3 +	3 +	-	-	-	-
TAL-1000	3 +	3 +	3 +	3+	3 +	3 +	-	1 +	-	-
AT2M$_2$	3 +	2 +	3 +	3+	3 +	2 +	-	1 +	-	-
AT2M$_3$	2 +	2 +	3 +	3+	3 +	2 +	-	1 +	-	-
AT5M$_2$	2 +	2 +	3 +	3+	3 +	2 +	-	1 +	-	-
AT50M$_1$	3 +	2 +	3 +	3+	3 +	3 +	-	2 +	-	-
AT50M$_2$	3 +	3 +	3 +	3+	3 +	3 +	-	2 +	-	-

3 + = Good growth; 2 + moderate growth; 1 + = faint growth; - = no growth.

Table 9b : Utilization of different carbohydrates by two parent *Bradyrhizobium* strains and UV mutants derived from them.

Mutants	Carbohydrates									
	Glycerol	Xylose	Arabinose	Mannitol	Glucose	Galactose	Sucrose	Maltose	Lactose	Starch
P-132	3 +	3 +	3 +	3+	3+	3 +	-	-	-	-
UP1M$_1$	3 +	3 +	3 +	3+	3 +	2 +	-	1 +	-	-
UP1M$_3$	3 +	3 +	3 +	3 +	3 +	2 +	-	-	-	-
UP2M$_2$	3 +	3 +	3 +	3+	3 +	2 +	-	1 +	-	-
UP2M$_3$	3 +	3 +	3 +	3+	3 +	2 +	1 +	1 +	-	-
UP4M$_1$	3 +	3 +	3 +	3+	3 +	2 +	-	1 +	-	-
UP6M$_2$	3 +	3 +	3 +	3+	3 +	2 +	1 +	1 +	-	-
UP6M$_4$	3 +	3 +	3 +	3+	3 +	3 +	-	1 +	-	-
UP8M$_1$	3 +	3 +	3 +	3+	3 +	2 +	-	1 +	-	-
UP10M$_2$	3 +	3 +	3 +	3+	3 +	2 +	-	1 +	-	-
TAL-1000	3 +	3 +	3 +	3+	3 +	3 +	-	1 +	-	-
UT1M$_1$	3 +	3 +	3 +	3+	3 +	3 +	1 +	-	-	-
UT1M$_3$	3 +	3 +	3 +	3+	3 +	3 +	2 +	1 +	-	-
UT2M$_1$	3 +	3 +	3 +	3+	3 +	3 +	2 +	1 +	-	-
UT4M$_3$	3 +	3 +	3 +	3+	3 +	3 +	-	1 +	-	-
UT6M$_1$	3 +	3 +	3 +	3+	3 +	3 +	1 +	-	-	-
UT6M$_2$	3 +	3 +	3 +	3+	3 +	3 +	1 +	-	-	-
UT8M$_1$	3 +	3 +	3 +	3 +	3 +	3 +	-	-	-	-
UT10M$_3$	3 +	3 +	3 +	3 +	3 +	3 +	-	1 +	-	-
UT10M$_4$	3 +	3 +	3 +	3 +	3 +	3 +	-	1 +	-	-

3 + = Good growth ; 2 + moderate growth; 1 + = faint growth ; - = no growth.

Table 10a : pH tolerance range of *Bradyrhizobium* sp strain P-132 and its acridine orange and ultraviolet rays mutants.

Mutants	Optical density values (540 nm) at different pH levels												
	3.0	3.5	4.0	4.5	5.0	5.5	6.0	7.0	8.0	8.5	9.0	9.5	10.0
P-132	0	0	0	0.119	0.174	0.187	0.200	0.268	0.180	0.130	0.076	0	0
AP2M$_1$	0	0	0.108	0.114	0.119	0.174	0.187	0.194	0.174	0.174	0.174	0.155	0.066
AP2M$_3$	0	0	0.076	0.108	0.125	0.130	0.200	0.168	0.155	0.130	0.114	0.097	0.080
AP5M$_2$	0	0	0.046	0.155	0.161	0.208	0.230	0.125	0.108	0.097	0.097	0.086	0.070
AP5M$_4$	0	0	0.066	0.070	0.080	0.120	0.168	0.155	0.114	0.076	0.066	0.066	0.046
AP20M$_2$	0	0	0.155	0.161	0.180	0.187	0.292	0.296	0.276	0.260	0.180	0.155	0.125
AP20M$_3$	0	0	0.070	0.114	0.155	0.174	0.194	0.208	0.174	0.143	0.125	0.119	0.119
AP50M$_1$	0	0	0.027	0.097	0.108	0.125	0.125	0.125	0.097	0.097	0.086	0.070	0.055
AP50M$_3$	0	0	0.022	0.050	0.060	0.060	0.102	0.114	0.097	0.097	0.086	0.080	0.055
UP1M$_1$	0	0	0	0.076	0.143	0.180	0.187	0.215	0.208	0.187	0.097	0.070	0.022
UP1M$_3$	0	0	0	0.143	0.143	0.167	0.180	0.230	0.208	0.108	0.086	0.056	0.036
UP2M$_2$	0	0	0	0.155	0.168	0.174	0.187	0.237	0.230	0.137	0.114	0.027	0.018
UP2M$_3$	0	0	0	0.130	0.200	0.222	0.230	0.260	0.194	0.194	0.174	0.097	0.009
UP4M$_1$	0	0	0	0.009	0.149	0.194	0.194	0.268	0.200	0.194	0.097	0.097	0.018
UP6M$_2$	0	0	0	0.187	0.292	0.301	0.319	0.328	0.347	0.187	0.155	0.125	0.086
UP6M$_4$	0	0	0	0.060	0.131	0.155	0.155	0.187	0.168	0.125	0.108	0.061	0.009
UP8M$_1$	0	0	0	0.143	0.149	0.187	0.208	0.161	0.149	0.125	0.108	0.061	0
UP10M$_2$	0	0	0	0.125	0.155	0.168	0.208	0.161	0.155	0.113	0.036	0.027	0

Table 10b : pH tolerance range of *Bradyrhizobium* sp strain TAL-1000 and its acridine orange and ultraviolet rays mutants.

Mutants	Optical density values (540 nm) at different pH levels												
	3.0	3.5	4.0	4.5	5.0	5.5	6.0	7.0	8.0	8.5	9.0	9.5	10.0
TAL-1000	0	0	0	0.097	0.114	0.180	0.198	0.221	0.187	0.137	0.081	0.009	0
AT2M$_2$	0	0	0.046	0.060	0.066	0.080	0.119	0.145	0.108	0.097	0.092	0.056	0.046
AT2M$_3$	0	0	0.046	0.066	0.070	0.086	0.149	0.187	0.108	0.092	0.076	0.056	0.056
AT5M$_2$	0	0	0.056	0.060	0.066	0.114	0.150	0.187	0.119	0.114	0.097	0.076	0.046
AT50M$_1$	0	0	0.114	0.187	0.215	0.215	0.222	0.284	0.276	0.166	0.119	0.086	0.076
AT50M$_2$	0	0	0.096	0.097	0.097	0.108	0.114	0.130	0.125	0.108	0.060	0.018	0
UT1M$_1$	0	0	0	0.215	0.260	0.260	0.284	0.301	0.244	0.120	0.092	0.018	0
UT1M$_3$	0	0	0	0.086	0.097	0.149	0.187	0.222	0.149	0.125	0	0	0
UT2M$_1$	0	0	0	0.097	0.131	0.143	0.174	0.230	0.143	0.137	0.050	0.036	0.013
UT4M$_3$	0	0	0	0.086	0.097	0.137	0.208	0.260	0.137	0.125	0.066	0.032	0.018
UT6M$_1$	0	0	0	0.027	0.060	0.092	0.125	0.131	0.022	0.009	0	0	0
UT6M$_2$	0	0	0	0.041	0.066	0.092	0.155	0.161	0.155	0.102	0.027	0	0
UT8M$_1$	0	0	0	0.071	0.120	0.155	0.180	0.222	0.194	0.180	0.137	0.092	0.013
UT10M$_3$	0	0	0	0.036	0.071	0.092	0.125	0.187	0.180	0.125	0.097	0.092	0.080
UT10M$_4$	0	0	0	0.056	0.060	0.097	0.125	0.161	0.155	0.081	0.056	0.056	0

Table 11a : NaCl tolerance range of *Bradyrhizobium* sp strain P-132 and its acridine orange and ultraviolet rays mutants.

Mutants	Optical density values (540nm) at different conc of NaCl							
	0.01%	0.1%	0.5%	1.0%	1.5%	2.0%	2.5%	3.0%
P-132	0.156	0.086	0.055	0.036	0.009	0	0	0
AP2M$_1$	0.275	0.187	0.070	0.036	0.032	0.022	0	0
AP2M$_3$	0.494	0.222	0.125	0.031	0.031	0	0	0
AP5M$_2$	0.222	0.237	0.075	0.027	0.017	0	0	0
AP5M$_4$	0.194	0.174	0.050	0.027	0.022	0.013	0	0
AP20M$_2$	0.237	0.237	0.055	0.031	0.027	0	0	0
AP20M$_3$	0.260	0.222	0.097	0.046	0.031	0	0	0
AP50M$_1$	0.200	0.137	0.046	0.036	0.036	0.031	0.013	0
AP50M$_3$	0.215	0.194	0.180	0.031	0.031	0	0	0
UP1M$_1$	0.252	0.187	0.180	0.155	0.143	0.119	0.045	0
UP1M$_3$	0.137	0.075	0.070	0.009	0	0	0	0
UP2M$_2$	0.131	0.108	0.046	0.018	0	0	0	0
UP2M$_3$	0.070	0.070	0.022	0.022	0	0	0	0
UP4M$_1$	0.143	0.070	0.056	0.018	0.004	0	0	0
UP6M$_2$	0.194	0.186	0.180	0.167	0.155	0.097	0.017	0
UP6M$_4$	0.097	0.125	0.070	0.046	0.022	0.013	0	0
UP8M$_1$	0.137	0.125	0.080	0.036	0	0	0	0
UP10M$_2$	0.108	0.076	0.036	0.027	0	0	0	0

Table 11b : NaCl tolerance range of ***Bradyrhizobium*** sp strain TAL-1000 and its acridine orange and ultraviolet rays mutants.

Mutants	Optical density values (540nm) at different conc of NaCl							
	0.01%	0.1%	0.5%	1.0%	1.5%	2.0%	2.5%	3.0%
TAL-1000	0.180	0.091	0.080	0.031	0.027	0	0	0
AT2M$_2$	0.263	0.184	0.130	0.027	0.027	0.026	0	0
AT2M$_3$	0.276	0.237	0.143	0.036	0.022	0	0	0
AT5M$_2$	0.187	0.237	0.222	0.070	0.031	0	0	0
AT5OM$_1$	0.268	0.222	0.097	0.031	0.027	0.027	0	0
AT50M$_2$	0.252	0.180	0.055	0.031	0.031	0.022	0	0
UT1M$_1$	0.137	0.130	0.070	0.070	0	0	0	0
UT1M$_3$	0.108	0.108	0.097	0.070	0.009	0	0	0
UT2M$_1$	0.125	0.155	0.036	0.027	0.027	0	0	0
UT4M$_3$	0.222	0.167	0.086	0.036	0.018	0.008	0	0
UT6M$_1$	0.076	0.080	0.055	0.046	0.022	0	0	0
UT6M$_2$	0.200	0.174	0.108	0.046	0.027	0	0	0
UT8M$_1$	0.174	0.130	0.097	0	0	0	0	0
UT10M$_3$	0.155	0.149	0.055	0.027	0.004	0	0	0
UT10M$_4$	0.125	0.076	0.070	0.070	0.036	0.027	0	0

4.4 CROSS INOCULATION STUDIES

Ten mutants each from the two parent strains P-132 and TAL-1000 were selected for cross inoculation studies on two legume hosts soybean and chickpea . None of the twenty mutants studied nor their parent strains were able to nodulate chickpea. Soybean was nodulated by P-132 and all the ten mutants derived from it, but TAL-1000 and UT6M$_2$ did not nodulate soybean. However all the other mutants derived from TAL-1000 used in the present study nodulated soybean (Table 12).

Among the mutants derived from P-132, AP20M$_2$ and UP10M$_2$ initiated nodulation significantly earlier than the wild type strain (14 days) and also formed bigger nodules (2.93mm and 3.0mm respectively). Mutant UP10M$_2$ inoculated soybean plants produced more number of nodules per plant (18.66 nodules per plant) followed by AP20M$_2$ inoculated plants (14.66 nodules per plant) (Table 13a). Mutant UP1M$_1$ inoculated soybean plants initiated nodulation very late (23.66 days after inoculation) and formed small nodules (1.66mm). Mutant UP4M$_1$ inoculated soybean plants had least number of nodules (5.66 nodules/plant).

Among the mutants derived from TAL-1000, AT2M$_3$ followed by UT8M$_1$ initiated nodulation significantly earlier than other mutants (14 days and 15 days respectively). Though UT2M$_1$ inoculated soybean plants formed significantly more number of nodules (27.66 nodules/plant) they were initiated very late (22.33 days) and small in size (2.00 mm). Soybean plants inoculated with mutants AT2M$_3$ and UT8M$_1$ formed significantly more nodules (25.33 and 25.0 nodules/plant) whose size was also bigger (2.83mm and 3.16mm respectively) than all other mutants(Table 13b).

4.5 SCREENING MUTANTS FOR NITRATE TOLERANT SYMBIOSIS

The experiment was conducted in growth pouches with four levels of NO$_3$ 0, 0.5mM (low), 1.5mM (medium) and 5 mM (high) (corresponding to 0, 16, 47 and 157 kg N/ha respectively) on cowpea plants inoculated with ten mutants from

Table 12: Cross- inoculation studies of two *Bradyrhizobium* strains and their mutants on soybean and chickpea

Mutants	Nodulation on soybean	Nodulation on chickpea
P-132	+	-
AP2M$_3$	+	-
AP5M$_2$	+	-
AP20M$_2$	+	-
AP50M$_1$	+	-
UP1M$_1$	+	-
UP2M$_2$	+	-
UP4M$_1$	+	-
UP6M$_2$	+	-
UP8M$_1$	+	-
UP10M$_2$	+	-
TAL-1000	-	-
AT2M$_2$	+	-
AT2M$_3$	+	-
AT5M$_2$	+	-
AT50M$_2$	+	-
UT1M$_1$	+	-
UT2M$_1$	+	-
UT4M$_3$	+	-
UT6M$_2$	-	-
UT8M$_1$	+	-
UT10M$_3$	+	-

+ = Positive for nodulation ; - = no nodulation

Table 13a : Effect of *Bradyrhizobium* strain P-132 and its mutants on nodule characteristics of soybean

Mutants	Days taken for nodule initiation	nodule number per plant	Average nodule size (mm)
P-132	15.00 D	13.66 C	2.5 BC
AP2M$_3$	16.33 C	9.66 E	2.63 ABC
AP5M$_2$	15.00 D	13.66 C	2.33 CD
AP20M$_2$	14.00 E	14.66 B	2.93 A
AP50M$_1$	16.66 C	8.33 F	2.66 ABC
UP1M$_1$	23.66 A	8.00 FG	1.66 E
UP2M$_2$	15.00 D	7.33 G	2.83 AB
UP4M$_1$	15.00 D	5.66 H	2.5 BC
UP6M$_2$	15.00 D	11.66 D	2.56 BC
UP8M$_1$	19.00 B	12.00 D	2.16 D
UP10M$_2$	14.00 E	18.66 A	3.0 A

Values followed by different letters were significantly different at 5% level of significance.

Table 13b : Effect of *Bradyrhizobium* strain TAL-1000 and its mutants on nodule characteristics of soybean

Mutants	Days taken for nodule initiation	nodule number per plant	Average nodule size (mm)
TAL-1000	-	-	-
AT2M$_2$	17.33 C	15.66 D	2.43 B
AT2M$_3$	14.00 F	25.33 B	2.83 B
AT5M$_2$	16.33 D	6.66 F	1.93 C
AT50M$_2$	16.00 D	14.00 E	2.50 B
UT1M$_1$	16.33 D	22.00 C	2.66 B
UT2M$_1$	22.33 A	27.66 A	2.00 C
UT4M$_3$	19.00 B	15.00 DE	2.53 B
UT6M$_2$	-	-	-
UT8M$_1$	15.00 E	25.00 B	3.16 A
UT10M$_3$	18.33 B	15.66 D	2.56 B

Values followed by different letters were significantly different at 5% level of significance.

each of the two parent strains, to screen for mutants which form symbiosis in the presence of high levels of nitrate.

4.5.1 Effect of nitrate on the number of days taken for nodule initiation

The data on the number of days taken for nodule initiation by P-132 and its mutants and TAL-1000 and its mutants, as influenced by different levels of nitrate on cowpea are presented in Table 14a and 14b respectively.

***Bradyrhizobium* strain P-132 and mutants derived from it**

All the isolates initiated nodulation in the presence of four nitrate levels tested. In general, nodule initiation was delayed in the presence of 5 mM NO_3 by all isolates except $AP20M_2$ (22.33 days), $UP1M_1$ (18.33 days) and $UP2M_2$ (20.57 days), which initiated nodulation significantly earlier in the presence of 5mM NO_3 than when grown in nitrate free medium. $UP4M_1$ inoculated plants nodulated significantly earlier than plants inoculated with all other isolates in the presence of 1.5 mM NO_3. In the presence of 5 mM NO_4, $UP4M_1$ and $UP2M_2$ (20.0 and 20.57 days respectively) inoculated plants nodulated significantly earlier than all other isolates including the parent strain P-132. $AP5M_2$ initiated very late nodulation (26.0 days) (Table 14a).

***Bradyrhizobium* strain TAL-1000 and mutants derived from it**

In general all the strains showed a delay in nodule initiation as the concentration of nitrate was increased except strain $AT50M_2$ which initiated nodules earlier in the presence of 5 mM NO_3 than when grwon in nitrate free control.

In the presence of 1.5 mM NO_3, $UT10M_3$ inoculated plants initiated nodulation significantly earlier (15.67 days) than all other strains tested. In the presence of 5 mM NO_3, $AT50M_2$ inoculated plants nodulated significantly earlier (18.67 days) than TAL-1000 and all other mutants inoculated plants. TAL-1000 inoculated plants initiated very late nodulation in the presence of 5 mM NO_3 (27.33 days) (Table 14b).

Table 14a : Effect of nitrate on the number of days taken for nodule initiation on cowpea plants inoculated with *Bradyrhizobium* strain P-132 and its mutants.

Mutants	NO_3 conc.(mM)			
	0	0.5	1.5	5.0
P-132	19.33 Gc	23.00 Bb	24.67 Aa	25.00 Ba
$AP2M_3$	17.00 Id	21.00 Dc	22.00 Cb	24.00 Ca
$AP5M_2$	20.33 Fc	22.00 Cb	22.00 Cb	26.00 Aa
$AP20M_2$	23.00 Cb	25.00 Aa	22.00 Cc	22.33 Ec
$AP50M_1$	16.67 Id	21.00 Dc	22.00 Cb	23.00 Da
$UP1M_1$	22.00 Da	20.00 Eb	20.00 Eb	18.33 Gc
$UP2M_2$	24.00 Ba	23.00 Bb	21.00 Dc	20.57 Fc
$UP4M_1$	18.33 Hb	18.00 Fb	16.00 Fc	20.00 Fa
$UP6M_2$	21.33 Ec	22.00 Cb	22.33 Cb	24.33 Ca
$UP8M_1$	27.67 Aa	21.67 Cd	23.00 Bc	25.00 Bb
$UP10M_2$	21.67 DEb	20.00 Ec	23.00 Ba	23.33 Da

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical column.

Table 14b : Effect of nitrate on the number of days taken for nodule initiation on cowpea plants inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants.

Mutants	NO_3 conc.(mM)			
	0	0.5	1.5	5.0
TAL-1000	18.00 Fd	21.33 Dc	25.00 Ab	27.33 Aa
$AT2M_2$	16.00 Gd	23.33 Cc	25.00 Ab	26.67 Ba
$AT2M_3$	14.00 Hc	23.00 Cb	25.00 Aa	25.00 Ca
$AT5M_2$	19.00 Ec	23.00 Cb	25.33 Aa	22.33 Fb
$AT50M_2$	28.00 Aa	28.00 Aa	25.00 Ab	18.67 Gc
$UT1M_1$	27.00 Ba	23.67 Cb	20.67 Cc	26.67 Ba
$UT2M_1$	17.33 Fc	23.00 Ca	20.00 CDb	23.00 Ea
$UT4M_3$	17.67 Fc	20.00 Eb	19.67 Db	25.00 Ca
$UT6M_2$	24.00 Cb	24.00 Cb	25.00 Aa	23.33 Eb
$UT8M_1$	15.33 Gd	21.00 Dc	23.00 Bb	24.00 Da
$UT10M_3$	21.33 Db	25.00 Ba	15.67 Ec	24.67 Ca

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical column.

4.5.2. Effect of nitrate on nodule size of parent strains and their mutants

The data on nodule size formed by P-132 and its mutants, and TAL-1000 and its mutants in the presence of different levels of nitrate are presented in tables 15a and 15b respectively.

Nodule size of *Bradyrhizobium* strain P-132 and mutants derived from it

In general nodule size was decreased in the presence of 5mM NO_3^- for all the isolates tested except $AP20M_2$ and $AP50M_1$ which formed bigger sized nodules in the presence of 5mM NO_3 (2.43 mm and 2.50 mm) compared to nitrate free controls (1.87 mm and 2.00 mm respectively). Among the plants grown in the presence of 1.5 mM nitrate $AP20M_2$, $UP2M_2$, $UP4M_1$, $UP6M_2$ and $UP10M_2$ (2.6 - 2.43 mm) inoculated plants formed bigger nodules which were also significantly bigger than P-132 inoculated plants (1.33mm). In the presence of 5mM NO_3, $AP20M_2$ and $AP50M_1$ inoculated plants formed big nodules and $UP8M_1$ inoculated plants formed small sized nodules (0.40 mm) (Table 15a).

Nodule size of *Bradyrhizobium* strain TAL-1000 and its mutants

In general nodule size of all the inoculated plants was decreased when plants were grown in the presence of 5 mM nitrate. Under nitrate free condition $AT2M_3$ and inoculated plants formed big nodules (4.0mm) (plate 7). Mutants $AT5M_2$, $UT1M_1$ AND $UT10M_3$ inoculated plants formed bigger nodules in the presence of 1.5 mM nitrate than when grown in N-free condition. In the presence of 1.5mM NO_3 plants inoculatd with mutants $UT2M_1$ and $UT10M_3$ formed big sized nodules (3.93mm and 3.87mm respectively).

In the presence of 5 mM nitrate, $UT4M_3$ inoculated plants formed bigger nodules (2.97mm) which was statistically higher than all other mutants and TAL-1000. $AT2M_3$ inoculated plants formed smaller nodules (0.83 mm) (Table 15b, plate 8).

4.5.3 Effect of NO_3 on nodule number of plants inoculated with parent strains and their mutants

The data on the number of nodules formed on cowpea plants inoculated

Table 15a : Effect of nitrate on the nodule size (mm) of cowpea plants inoculated with *Bradyrhizobium* strain P-132 and its mutants.

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
P-132	1.83 Fa	1.93 Ea	1.33 Cb	1.07 Dc
$AP2M_3$	2.67 Ba	2.33 CDb	2.20 Bb	2.00 Bc
$AP5M_2$	2.07 CDEb	2.90 Aa	2.00 Bb	2.00 Bb
$AP20M_2$	1.87 EFc	2.07 Eb	2.53 Aa	2.43 Aa
$AP50M_1$	2.00 DEFb	2.07 Eb	2.13 Bb	2.50 Aa
$UP1M_1$	1.97 DEFb	2.47 Ca	2.00 Bb	2.00 Bb
$UP2M_2$	2.03 DEFb	2.13 DEb	2.60 Aa	1.53 Cc
$UP4M_1$	3.00 Aa	2.70 Bb	2.57 Ab	2.00Bc
$UP6M_2$	2.27 Cb	2.40 Cab	2.53 Aa	1.50 Cc
$UP8M_1$	1.47 Gc	2.90 Aa	2.00 Bb	0.40 Ed
$UP10M_2$	2.13 CDb	2.37 Ca	2.43 Aa	2.10 Bb

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

Table 15b : Effect of nitrate on the nodule size (mm) of cowpea plants inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants.

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
TAL-1000	2.20 Da	2.07 Ea	2.00 Ea	0.83 Db
$AT2M_2$	3.93 Aa	2.00 Eb	1.10 Fc	1.00 Dc
$AT2M_3$	4.00 Aa	2.70 Db	1.00 Fc	0.83 Dc
$AT5M_2$	2.57 Cb	2.67 Db	3.00 Ca	2.00 Bc
$AT50M_2$	0.60 Ed	2.97 Ca	2.40 Db	2.00 Bc
$UT1M_1$	0.53 Ec	1.93 Ea	1.97 Ea	1.50 Cb
$UT2M_1$	3.93 Ab	4.33 Aa	3.93 Ab	2.00 Bc
$UT4M_3$	3.83 Aa	3.40 Bb	3.07 Cc	2.97 Ac
$UT6M_2$	3.87 Aa	2.07 Ec	3.47 Bb	1.00 Dd
$UT8M_1$	3.00 Ba	2.00 Eb	2.00 Eb	2.00 Bb
$UT10M_3$	2.00 Db	2.00 Eb	3.87 Aa	1.93 Bb

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

Plate 7: Nodulation of cowpea by *Bradyrhizobium* strain TAL-1000 and its AO mutants in nitrate free condition in growth pouches. T=TAL-1000; 1=AT2M_2; 3=AT2M_3.

Plate 8: Growth and nodulation of cowpea by *Bradyrhizobium* strain TAL-1000 (T) and AT2M_3(3) under 0-NO_3 and 5mM NO_3 condition in growth pouches.

with parent strains and their respective mutants in the presence of different nitrate levels are presented in Tables 16a and 16b.

Nodule number of *Bradyrhizobium* strain P-132 and its mutants

Plants inoculated with P-132, AP2M$_3$, AP5M$_2$ and UP2M$_2$ formed significantly more nodules in the presence of 1.5mM nitrate (24.0, 42.67, 31.33 and 39.00 nodules/plant respectively) than when grown in the absence of nitrate (21.00, 30,33, 21.67 and 21.33 nodules/plant respectively) with AP2M$_3$ forming significantly more number of nodules(42.67 nodules/plant) but reduced at higher concentrations of nitrate.

Similarly AP20M$_2$, UP1M$_1$, UP4M$_1$ and UP8M$_1$ formed significantly more number of nodules in the presence of 5 mM NO$_3$ (37.00, 34.67, 35.33 and 34.67 nodules/plant respectively) than when grow in nitrate free medium (29.67, 29.33, 26.67 and 18.33 nodules/plant respectively). However for all other mutant inoculated plants presence of 5 mM NO$_3$ caused a reduction in nodule number.

In the presence of 5 mM nitrate, AP2M$_3$ inoculated plants formed more number of nodules per plant (39.00 nodules/plant) followed by UP10M$_2$ and AP20M$_2$ (37.67 and 37.00 nodules / plant respectively). AP50M$_1$ inoculated plants formed least number of nodules in the presence of 5 mM NO$_3$ (17.67 nodules per plant) (Table 16a).

Nodule number of *Bradyrhizobium* strain TAL-1000 and its mutants

The mutants AT2M$_3$, AT50M$_2$, UT1M$_1$, UT4M$_3$, UT6M$_2$ and UT10M$_3$ showed an increase in nodule number per plant as the concentration of nitrate in rooting medium was increased from 0mM NO$_3$ to 5.0mM NO$_3$. However the mutant AT5M$_2$ and UT2M$_1$ inoculated plants exhibited a decrease in nodule number when the concentration of NO$_3$ was increased in the medium.

In the presence of 5 mM NO$_3$ in rooting medium UTIM$_1$ inoculated plants produced maximum number of nodules per plant (50 nodules/plant) followed by AT2M$_3$ (41.67 nodules/plant) and least number of nodules were produced by UT10M$_3$ inoculated plants (12.33 nodules/plant) (Table 16b).

Table 16a : Effect of nitrate on number of nodules per cowpea plant inoculated with *Bradyrhizobium* strain P-132 and its mutants.

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
P-132	21.00 EFb	22.67 Dab	24.00 Ga	20.33 EFb
$AP2M_3$	30.33 Cc	31.00 Bc	42.67 Aa	39.00 Ab
$AP5M_2$	21.67 Ec	27.33 Cb	31.33 DEa	21.67 Ec
$AP20M_2$	29.67 CDc	31.67 Bbc	34.00 CDb	37.00 ABCa
$AP50M_1$	39.33 Ba	31.00 Bc	35.33 Cb	17.67 Fd
$UP1M_1$	29.33 CDc	31.33 Bdc	34.33 CDa	34.67 CDa
$UP2M_2$	21.33 Ec	37.00 Aa	39.00 Ba	33.00 Db
$UP4M_1$	26.67 Dc	28.67 BCbc	30.33 EFb	35.33 BCDa
$UP6M_2$	43.67 Aa	29.67 BCb	19.33 Hc	32.33 Db
$UP8M_1$	18.33 Fc	19.00 Ec	22.67 Gb	34.67 CDa
$UP10M_2$	44.33 Aa	28.67 BCc	28.00 Fc	37.67 ABb

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

Table 16b : Effect of nitrate on number of nodules per cowpea plant inoculated with *Bradyrhizobium* strain TAL-1OOO and its mutants.

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
TAL-1000	16.33 Eb	28.00 Ca	15.67 Fb	28.67 Ea
$AT2M_2$	23.67 Cc	34.33 Aa	26.67 Db	18.33 Hd
$AT2M_3$	19.33 Dd	22.67 Ec	37.67 Ab	41.67 Ba
$AT5M_2$	31.67 Aa	16.67 Gb	15.00 Fbc	14.00 Ic
$AT50M_2$	11.33 Fc	12.00 Hc	35.00 Bb	37.67 Ca
$UT1M_1$	6.67 Gd	29.33 Cc	36.67 Ab	50.00 Aa
$UT2M_1$	27.33 Bb	31.00 Ba	28.00 Db	22.00 Gc
$UT4M_3$	12.00 Fb	19.67 Fa	18.67 Ea	19.00 Ha
$UT6M_2$	22.33 Cb	21.67 Eb	33.00 Ca	34.00 Da
$UT8M_1$	23.00 Cc	26.33 Dab	28.00 Da	25.00 Fb
$UT10M_3$	4.33 Hc	11.67 Hb	18.33 Ea	12.33 Ib

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

4.5.4 Effect of nitrate on weight of nodules per plant inoculated with parent strain and their mutants

The data on the dry weight of nodules per plant inoculated with the two parent strains and their respective mutants in the presence of four different nitrate levels are presented in tables 17a and 17b and Figs. 2 and 3.

Variation was observed within the mutants when they were grown in the presence of different levels of nitrate. In general all mutants showed a decrease in dry weight of nodules per plant when grown in the presence of 5mM nitrate. However strain variation was observed at lower concentrations.

Weight of nodules per plant inoculated with *Bradyrhizobium* strain P-132 and its mutants

Plants inoculated with mutants $AP2M_3$, $AP5M_2$, $AP20M_2$ $AP50M_1$, $UP1M_1$, $UP2M_2$ and $UP4M_1$ resulted in more nodule weight per plant in the presence of 1.5 mM nitrate compared to when they were grown in nitrate free medium.

In the presence of 1.5 mM nitrate, mutants $AP20M_2$ and $AP50M_1$ produced maximum weight of nodules per plant (20.37 mg/plant and 19.80 mg/plant respectively) which was significantly higher than that produced by P-132 and its other mutants. In the presence of 5mM nitrate $AP20M_2$ and $UP10M_2$ inoculated plants produced significantly higher weight of nodules per plant (15.63 mg/plant and 15.73 mg/plant respectively) and $UP8M_1$, inoculated plants produced the least nodule weight per plant (4.87 mg/plant) (Table 17a).

Weight of nodules per plant inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants

In nitrate free medium cowpea plants inoculated with $AT2M_3$ produced maximum weight of nodules per plant (29.67 mg/plant) which was significantly higher than all isolates tested. Plants inoculated with mutants $AT5M_2$, $AT50M_2$, $UT1M_1$, $UT4M_3$, $UT6M_2$ and $UT10M_3$ produced more nodule dry weight per plant in the presence of 1.5mM NO_3 in rooting medium compared to when grown in nitrate free medium.

Table 17a : Effect of nitrate on the weight of nodules (mg) per cowpea plant inoculated with *Bradyrhizobium* strain P-132 and its mutants

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
P-132	8.07 Ib	11.93 Ea	7.93 Eb	5.97 Ec
AP2M$_3$	14.77 Eb	15.03 Db	16.80 Ba	11.37 Bc
AP5M$_2$	9.97 Gc	12.50 Eb	14.77 Ca	12.10 Bb
AP20M$_2$	17.70 Cc	18.93 Ab	20.37 Aa	15.63 Ad
AP50M$_1$	11.23 Fc	15.60 CDb	19.80 Aa	10.20 Cd
UP1M$_1$	11.83 Fb	11.83 Eb	14.90 Ca	11.23 Bb
UP2M$_2$	11.77 Fb	16.33 BCa	17.07 Ba	11.53 Bb
UP4M$_1$	15.97 Db	17.17 Ba	17.50 Ba	9.70 Cc
UP6M$_2$	23.80 Ba	15.40 CDb	11.37 Dc	6.97 Dd
UP8M$_1$	8.97 Hb	12.67 Ea	7.40 EC	4.87 Fd
UP10M$_2$	26.50 Aa	15.63 CDb	11.50 Dc	15.73 Ab

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

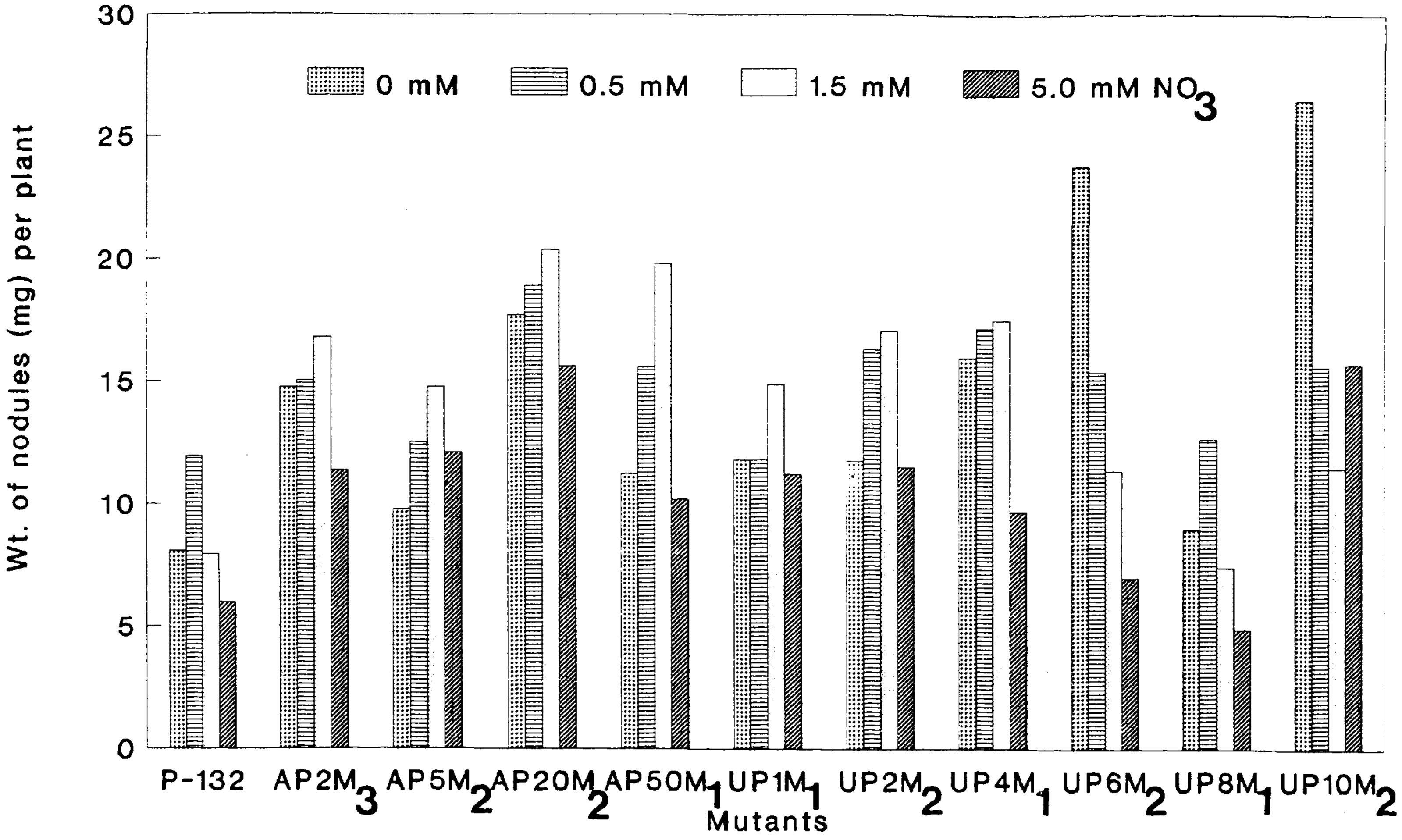

Fig. 2: Effect of nitrate on the weight of nodules (mg) per cowpea plant inoculated with *Bradyrhizobium* strain P-132 and its mutants (CD:0.90).

Table 17b : Effect of nitrate on the weight of nodules (mg) per cowpea plant inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
TAL-1000	17.23 Db	18.53 Ba	9.13 Ec	10.03 Gc
$AT2M_2$	26.67 Ba	20.93 Ab	8.07 Fc	5.63 Hd
$AT2M_3$	29.67 Aa	19.03 Bb	16.50 Dc	10.97 FGd
$AT5M_2$	17.70 Db	20.17 Aa	20.20 Ba	17.83 Ab
$AT50M_2$	5.17 Gd	11.97 Ec	16.70 CDa	14.43 Cb
$UT1M_1$	2.97 Hc	13.33 Db	16.33 Da	13.53 CDb
$UT2M_1$	17.63 Da	18.67 Ba	15.70 Db	11.93 EFc
$UT4M_3$	12.90 Fc	14.47 Cb	16.50 Da	12.13 Ec
$UT6M_2$	14.63 Eb	18.67 Ba	19.60 Ba	12.53 DEc
$UT8M_1$	18.83 Ca	18.87 Ba	17.63 Cb	15.87 Bc
$UT10M_3$	3.03 Hd	7.17 Fc	23.67 Aa	10.77 Gb

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

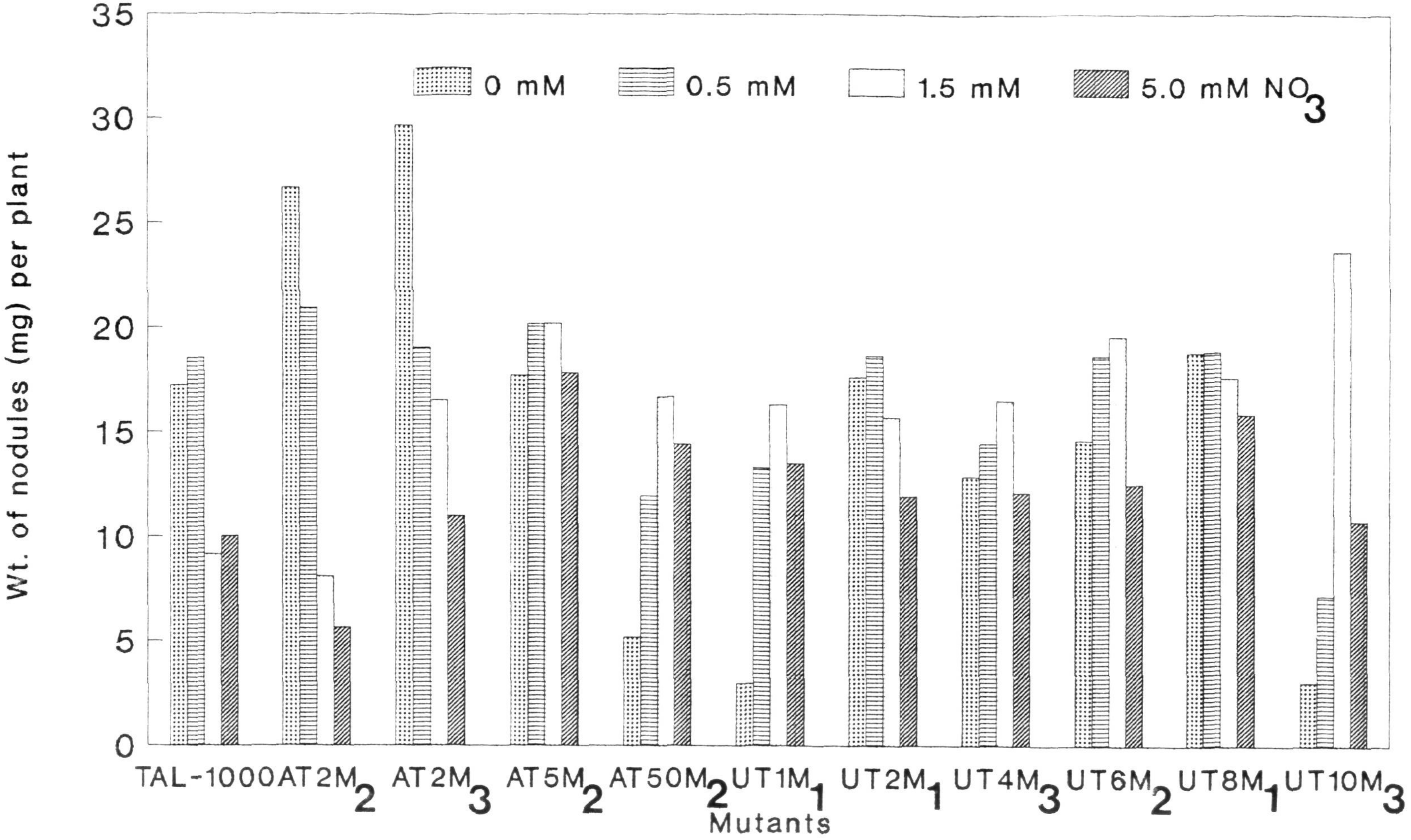

Fig 3: Effect of nitrate on the weight of nodules (mg) per cowpea plant inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants (CD:1.03).

In the presence of 1.5 mM nitrate in the rooting medium mutant UT10M_3 inoculated plants produced significantly more weight of nodules per plant (23.67 mg/plant) followed by AT5M_2 (20.20mg/plant). However in the presence of 5mM nitrate AT5M_2 inoculated plants produced maximum weight of nodules per plant (17.83 mg/plant) and AT2M_2 inoculated plants produced least dry weight of nodules per plant (5.63 mg/plant) (Table 17b).

4.5.5 Effect of nitrate on unit weight of nodule of plants inoculated with parent strains and their mutants

The data on the unit weight of nodule formed on cowpea plants inoculated with parent strains and mutants derived from them in the presence of different levels of nitrate are presented in Tables 18a and 18b.

Unit weight of nodules of plants inoculated with *Bradyrhizobium* strain P-132 and its mutants

Plants inoculated with mutants AP20M_2 and UP4M_1 maintained significantly higher unit weight of nodule in the presence of 0, 0.5 and 1.5mM NO_3 and decreased in the presence of 5mM NO_3. However plants inoculated with mutants AP5M_2 and AP50M_1 formed significantly more unit weight of nodule in the presence of 5mM NO_3 compared to when grown in nitrate free condition.

In the presence of 1.5mM NO_3 in the rooting medium AP20M_2 inoculated plants produced maximum unit weight nodule (0.60 mg/nodule) which was on par with mutants AP50M_1, UP4M_1, and UP6M_2 inoculated plants (0.56, 0.58, 0.59 mg/nodule respectively), but significantly superior to other mutant inoculated plants at 1.5mM NO_3.

In the presence of 5 mM NO_3 plants inoculated with AP5M_2 and AP50M_1, produced maximum unit weight of nodule which were on par with each other (0.56 and 0.58 mg/nodule respectively) followed by plants inoculated with AP20M_2 and UP10M_2 (0.42 and 0.41 mg/nodule respectively). The lowest unit

Table 18a : Effect of nitrate on unit weight of nodules (mg) of cowpea inoculated with *Bradyrhizobium* strain P-132 and its mutants

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
P-132	0.38 Db	0.53 CDa	0.33 Ec	0.29 Dc
$AP2M_3$	0.49 Ca	0.48 EFa	0.39 Db	0.29 Dc
$AP5M_2$	0.47 Cb	0.46 FGb	0.47 Bb	0.56 Aa
$AP20M_2$	0.60 Aa	0.60 Ba	0.60 Aa	0.42 Bb
$AP50M_1$	0.29 Ec	0.50 DEb	0.56 Aa	0.58 Aa
$UP1M_1$	0.40 Dab	0.38 Hb	0.44 BCa	0.32 CDc
$UP2M_2$	0.55 Ba	0.44 Gb	0.44 BCb	0.35 Cc
$UP4M_1$	0.60 Aa	0.59 Ba	0.58 Aa	0.28 Db
$UP6M_2$	0.55 Bab	0.52 CDb	0.59 Aa	0.21 Ec
$UP8M_1$	0.49 Cb	0.67 Aa	0.33 Ec	0.14 Fd
$UP10M_2$	0.60 Aa	0.55 Cb	0.41 CDc	0.41 Bc

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

Table 18b: Effect of nitrate on unit weight of nodules (mg) of cowpea plant inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
TAL-1000	1.00 Ca	0.66 DEb	0.58 Cb	0.35 FGc
$AT2M_2$	1.13 Ba	0.61 Eb	0.30 Ec	0.30 FGc
$AT2M_3$	1.54 Aa	0.84 Cb	0.44 Dc	0.27 Gd
$AT5M_2$	0.56 Fc	1.21 Ab	1.35 Aa	1.27 Aab
$AT50M_2$	0.51 FGb	0.99 Ba	0.48 Db	0.38 Fc
$UT1M_1$	0.44 Ga	0.46 Fa	0.44 Da	0.27 Gb
$UT2M_1$	0.65 Ea	0.60 Eab	0.56 Cb	0.55 DEb
$UT4M_3$	1.08 Ba	0.74 Dc	0.88 Bb	0.64 Cd
$UT6M_2$	0.66 Eb	0.86 Ca	0.59 Cb	0.37 Fc
$UT8M_1$	0.82 Da	0.71 Db	0.63 Cb	0.63 CDb
$UT10M_3$	0.71 Ec	0.61 Ed	1.30 Aa	0.87 Bb

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differnces in horizontal rows and capital letters indicate differences in vertical columns.

Table 19 : Effect of nitrate on shoot colour of cowpea plants inoculated with *Bradyrhizobium* parent strains and their mutants.

	NO_3 conc. (mM)			
Mutants	0	0.5	1.5	5.0
P-132	2+	2+	2+	2+
AP2M$_3$	3+	2+	2+	2+
AP5M$_2$	2+	3+	3+	3+
AP20M$_2$	3+	3+	3+	3+
AP50M$_1$	2+	3+	3+	3+
UP1M$_1$	2+	2+	3+	3+
UP2M$_2$	2+	2+	2+	3+
UP4M$_1$	3+	3+	3+	3+
UP6M$_2$	2+	2+	3+	2+
UP8M$_1$	2+	2+	2+	1+
UP10M$_2$	3+	3+	3+	3+
TAL-1000	2+	2+	2+	2+
AT2M$_2$	3+	3+	2+	2+
AT2M$_3$	3+	3+	2+	1+
AT5M$_2$	3+	3+	3+	3+
AT50M$_2$	2+	3+	3+	3+
UT1M$_1$	2+	2+	2+	1+
UT2M$_1$	2+	2+	3+	3+
UT4M$_3$	3+	2+	3+	3+
UT6M$_2$	2+	3+	2+	2+
UT8M$_1$	3+	3+	3+	3+
UT10M$_3$	2+	2+	3+	3+

Shoot color : 3+ = dark green, 2+ = green, 1+ = greenish yellow weight of nodule was produced by plants inoculated with mutant $UP8M_1$ (0.14 mg/nodule) (Table 18a).

Unit weight of nodules of plants inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants

When plants were grown in nitrogen free medium, mutant $AT2M_3$ inoculated plants produced maximum unit weight of nodule (1.54 mg/nodule) which was significantly superior to all other isolates tested including the parent TAL-1000 (Plate 7). However unit weight of nodules decreased drastically in the presence of NO_3 in the rooting medium (Table 18b) (Plate 8).

In general all the mutant and parent strain inoculated plants showed a decrease in unit weight of nodule in the presence of 1.5 and 5mM NO_3 except mutant $AT5M_2$ and $UT10M_3$ inoculated plants which exhibited an increase in the unit weight of nodule when grown in the presence of 1.5mM NO_3. In the presence of 5mM nitrate in the rooting medium, $AT5M_2$ inoculated plants had maximum unit weight of nodule which was significantly higher than all other isolates tested (1.27mg per nodule) and $AT2M_3$ and $UT1M_1$ inoculated plants produced least dry weight per nodule (0.27 mg/nodule).

4.5.6 Effect of nitrate on height (cm) of cowpea plants inoculated with parent strains and their mutants

The data on the plant height (cm) of cowpea inoculated with parent strains and their respective mutants in the presence of different levels of nitrate are presented in Tables 20a and 20b.

Height of cowpea plants inoculated with *Bradyrhizobium* strain P-132 and its mutants

In general there was an increase in plant height for all the mutant inoculated plants in the presence of 1.5mM and 5.0mM NO_3 except $UP2M_2$ and $UP8M_1$, which showed a decrease in height in the presence of 5mM NO_3 compared to nitrate free control condition.

In the presence of 5.0mM nitrate plants inoculated with $AP20M_2$ were

Table 20a : Effect of nitrate on plant height (cm) of cowpea inoculated with *Bradyrhizobium* strain P-132 and its mutants.

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
P-132	20.47 CDab	20.87 Fa	19.47 Fb	20.77 Ga
$AP2M_3$	21.17 Cc	22.83 CDEb	25.83 BCa	24.97 CDa
$AP5M_2$	19.37 Db	22.60 DEa	22.80 Ea	23.57 Ea
$AP20M_2$	23.10 Bb	23.03 CDb	24.30 Db	29.67 Aa
$AP50M_1$	22.80 Bb	26.30 Aa	26.27 Ba	26.83 Ba
$UP1M_1$	21.13 Cb	24.03 BCa	24.83 CDa	23.73 DEa
$UP2M_2$	24.87 Ab	24.90 Bb	27.73 Aa	20.43 Gc
$UP4M_1$	22.93 Bb	24.93 Ba	25.77 BCa	25.93 BCa
$UP6M_2$	20.07 CDb	21.67 EFa	22.13 Ea	21.80 FGa
$UP8M_1$	22.77 Bb	23.83BCDab	24.47 CDa	20.93 Gc
$UP10M_2$	24.77 Aa	24.93 Ba	22.30 Eb	23.03 EFb

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

Table 20b : Effect of nitrate on plant height (cm) of cowpea inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants.

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
TAL-1000	21.10 Eb	23.20 Ca	19.27 Fc	20.63 Db
$AT2M_2$	24.80 Ba	25.43 Ba	23.03 DEb	19.63 Dc
$AT2M_3$	27.30 Ab	29.53 Aa	21.93 Ec	19.43 Dd
$AT5M_2$	21.60 DEb	22.03CDEFb	27.77 Aa	26.53 Aa
$AT50M_2$	15.47 Gc	20.97 EFa	18.43 Fb	22.17 Ca
$UT1M_1$	18.67 Fb	22.30 CDEa	21.83 Ea	22.43 Ca
$UT2M_1$	22.33 Ca	22.83 CDa	23.53 CDa	23.97 Ba
$UT4M_3$	22.57 CDb	23.10 Cb	24.50 Ca	25.77 Ba
$UT6M_2$	22.93 Cb	22.50 CDEb	26.27 Ba	22.90 BCb
$UT8M_1$	24.63 Bab	21.53 EFc	23.80 CDb	25.67 Aa
$UT10M_3$	16.00 Gd	20.77 Fc	27.73 Aa	24.13 Bb

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

tallest (29.67cm) (Plate9) and was significantly taller than all other mutant inoculated plants. Plants inoculated with UP2M_2 were shortest (20.43 cm) and was on par with plants inoculated with isolates P-132 and UP8M_1 (20.77 and 20.93 cm respectively).

Height of plants inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants

In general there was an increase in plant height in all the mutant inoculated plants in the presence of 1.5mM or 5.0mM NO_3 except strain TAL-1000, AT2M_2 and AT2M_3.

AT2M_3 inoculated plants were tallest (27.30 cm), had dark green shoot and were significantly taller than other mutants studied including parent TAL-1000 inoculated plants under nitrate free condition (Table 19, 20b and Plate 8).

In the presence of 1.5mM and 5.0mM nitrate, plants inoculated with AT5M_2 were tallest (27.77 cm and 26.53 cm respectively, Plate 10) plants inoculated with AT2M_3 were shortest (19.43 cm) and on par with plants inoculated with TAL-1000 and AT2M_2 (20.63cm and 19.63cm respectively) in the presence of 5mM nitrate.

4.5.7 Effect of nitrate on the number of trifoliate leaves of plants inoculated with parent strain and mutants

The data on the number of trifoliate leaves formed on cowpea inoculated with the two parent strains and mutants derived from them in the presence of different levels of nitrate are presented in tables 21a and 21b.

Number of trifoliate leaves of plants inoculated with *Bradyrhizobium* strain P-132 and its mutants

In general most of the strains did not show any significant variation in the number of trifoliate leaves in the presence or absence of nitrate, except AP50M_1, UP4M_1, and UP10M_2 inoculated plants which showed an increase in leaf number in the presence of 5 mM nitrate.

Table 21a : Effect of nitrate on the number of trifoliate leaves per plant of cowpea inoculated with *Bradyrhizobium* strain P-132 and its mutants.

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
P-132	4.0 ABa	4.0 Aa	4.0 Aa	4.33 Aa
$AP2M_3$	4.0 ABa	4.0 Aa	4.0 Aa	4.33 Aa
$AP5M_2$	4.0 ABa	4.0 Aa	4.0 Aa	4.0 Aa
$AP20M_2$	4.33 Aab	4.0 Ab	4.33 Aab	4.67 Aa
$AP50M_1$	3.33 Bb	3.67 Ab	3.67 Ab	4.33 Aa
$UP1M_1$	4.0 ABa	4.0 Aa	4.0 Aa	4.33 Aa
$UP2M_2$	4.33 Aab	4.0 Ab	4.33 Aab	4.67 Aa
$UP4M_1$	4.0 ABb	4.0 Ab	4.0 Ab	4.67 Aa
$UP6M_2$	3.67 Bb	3.67 Ab	4.0 Aab	4.33 Aa
$UP8M_1$	4.0 ABa	4.0 Aa	4.0 Aa	4.0 Aa
$UP10M_2$	4.0 ABb	4.0 Ab	4.0 Ab	4.67 Aa

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

Table 21b : Effect of nitrate on the number of trifoliate leaves per plant of cowpea inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants.

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
TAL-1000	4.0 BCa	4.00 ABa	4.33 Aa	4.33 ABa
AT2M$_2$	4.33 ABa	4.00 ABa	4.00 Aa	3.67 Ba
AT2M$_3$	5.00 Aa	4.33 Aa	4.33 Aa	4.33 ABa
AT5M$_2$	4.00 BCa	4.0 ABa	4.33 Aa	4.67 Aa
AT50M$_2$	3.00 Db	4.0 ABa	4.00 Aa	4.00 ABa
UT1M$_1$	4.00 BCA	4.0 ABa	4.00 Aa	4.33 ABa
UT2M$_1$	4.33 ABa	4.0 ABa	4.67 Aa	4.33 ABa
UT4M$_3$	4.67 ABa	4.0 ABa	4.33 Aa	4.33 ABa
UT6M$_2$	4.00 BCab	3.33 Bb	4.33 Aa	4.0 ABab
UT8M$_1$	4.33 ABa	4.00 ABa	4.00 Aa	4.33 ABa
UT10M$_3$	3.33 CDb	4.00 ABab	4.33 Aa	4.33 ABa

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

Plate 9: Growth and nodulation of cowpea by *Bradyrhizobium* strain P-132(P) and mutant AP20M$_2$ (7) under 0-NO$_3$ and 5mM NO$_3$ condition in growth pouches.

Plate 10: Growth and nodulation of cowpea by *Bradyrhizobium* strain TAL-1000 (T) and mutant AT5M$_2$ (9) under 0-NO$_3$ and 5mM NO$_3$ condition in growth pouches.

In the presence of 0.5 mM, 1.5 mM and 5.0 mM nitrate there was no significant difference in the number of leaves among parent strain P-132 and its mutants although some variation was observed under nitrate free condition. In the presence of 5 mM nitrate, plants inoculated with AP20M$_2$, UP2M$_2$, UP4M$_1$ and UP10M$_2$ formed higher number of trifoliate leaves (4.67 leaves/plant) and plants inoculated with AP5M$_2$ and UP8M$_1$, had least number of leaves (4.0 leaves per plant) though the difference was not significant (Table 21a).

Number of trifoliate leaves of plants inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants

In general most of the strains did not show any significant variation in the number of trifoliate leaves either in the presence or absence of nitrate in rooting medium except strain UT10M$_3$, which formed more leaves in the presence of nitrate than under nitrate free condition.

When plants were grown in nitrogen free medium, AT2M$_3$ inoculated plants produced maximum number of trifoliate leaves (5 leaves/plant) and AT50M$_2$ formed the least number (3 leaves per plant). However in the presence of nitrate not much variation was observed in leaf number. In the presence of 5 mM nitrate, plants inoculated with AT5M$_2$ produced maximum number of trifoliate leaves (4.67 leaves/plant) and AT2M$_2$ inoculated plants produced least number (3.67 leaves/plant) (Table 21b)

4.5.8 Effect of nitrate on length of leaflets (cm) of cowpea inoculated with parent strains and their mutants

The data on length of leaflets of cowpea plants inoculated with parent strains and mutants derived from them in the presence of different levels of nitrate are shown in tables 22a and 22b.

Leaflet length of cowpea plants inoculated with *Bradyrhizobium* strain P-132 and its mutants

In general, plants inoculated with most of the mutants and parent strain formed longer leaflets in the presence of 1.5 mM or 5 mM nitrate except plants

Table 22a : Effect of nitrate on length of leaflet (cm) of cowpea plants inoculated with *Bradyrhizobium* strain P-132 and its mutants.

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
P-132	4.23 Db	4.70 BCa	4.27 Eb	4.87 Ba
$AP2M_3$	4.63 Ca	4.70 BCa	4.73 CDa	4.83 BCa
$AP5M_2$	4.03 DEc	4.50 CDb	5.03 ABa	4.80 BCa
$AP20M_2$	4.13 DEc	4.77 ABb	4.90 BCb	5.63 Aa
$AP50M_1$	3.90 Ed	4.33 Dc	4.60 Db	4.87 Ba
$UP1M_1$	4.13 DEc	4.37 Db	4.70 CDa	4.77 BCa
$UP2M_2$	4.60 Cc	5.0 Aab	5.07 ABa	4.80 BCbc
$UP4M_1$	5.1 Aa	4.57 BCDb	4.77 CDb	4.90 Ba
$UP6M_2$	3.97 Ec	4.80 ABb	5.17 Aa	4.83 BCb
$UP8M_1$	4.83 BCa	4.53 CDb	4.87 BCa	4.63 Cb
$UP10M_2$	4.87 Ba	4.77 ABab	4.67 CDab	4.60 Cb

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

Table 22b: Effect of nitrate on length of leaflets (cm) of cowpea inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants.

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
TAL-1000	4.53 Eb	4.53 Eb	4.37 Fb	4.93 CDa
$AT2M_2$	6.43 Ba	5.63 Bb	4.77 DEc	4.73 DEc
$AT2M_3$	6.67 Aa	6.57 Aa	4.80 DEb	4.67 Eb
$AT5M_2$	4.63 Eb	4.60 DEb	5.53 Aa	5.47 Aa
$AT50M_2$	3.77 Gc	4.43 Eb	4.60 Eb	4.87 CDEa
$UT1M_1$	4.20 Fb	4.80 CDa	4.80 DEa	4.90 CDEa
$UT2M_1$	5.43 Cb	5.77 Ba	4.90 CDc	4.93 CDc
$UT4M_3$	5.13 Da	5.00 Ca	5.00 BCDa	5.20 Ba
$UT6M_2$	5.00 Da	4.43 Ec	5.13 Ba	4.67 Eb
$UT8M_1$	4.93 Dab	4.83 Cb	5.10 BCa	4.97 Cab
$UT10M_3$	2.83 Hd	3.20 Fe	5.50 Aa	4.70 Eb

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

inoculated with mutants UP4M$_1$, and UP10M$_2$ which expressed greater leaflet length in nitrate free medium.

In the presence of 5mM nitrate, plants inoculated with AP20M$_2$ produced longer leaflets (5.63cm) which was significantly greater than all other mutant inoculated plants and parent P-132 inoculated plants, While UP10M$_2$ inoculated plants produced shortest leaflets in the presence of nitrate (4.60 cm) (Table 22a).

Leaflet length of cowpea plants inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants

In general most of the isolates exhibited increased leaflet length in the presence of 1.5 mM or 5 mM nitrate. However mutants AT2M$_2$ andAT2M$_3$ formed longer leaflets when grown in nitrogen free medium and decreased in presence of nitrate. AT2M$_3$ formed longest leaflets when grown in nitrogen free medium (6.67 cm) (plate 8).

In the presence of 1.5 mM and 5.0 mM nitrate AT5M$_2$ formed longest leaflets (5.53cm and 5.47 cm respectively) (Table 22b).

4.5.9 Effect of nitrate on the width of leaflet (cm) of cowpea plants inoculated with parent strains and their mutants

The data on the width of leaflets of cowpea plants inoculated with parent strains P-132 and TAL-1000 and mutants derived from them are illustrated in Tables 23a and 23b.

Leaflet width of plants inoculated with *Bradyrhizobium* strain P-132 and its mutants

In general plants inoculated with most of the P-132 mutants studied formed wider leaflets in the presence of 1.5 or 5 mM nitrate, except UP8M$_1$ and UP10M$_2$ which formed wider leaves in the absence of nitrate in rooting medium.

Mutant AP20M$_2$ inoculated plants produced broader leaflets at 0, 0.5 mM, 1.5 mM and 5 mM nitrate (3.50 cm, 4.77 cm, 4.50 and 4.50 cm respectively) which was significantly broader than all other isolates tested. UP8M$_1$ inoculated plants

Table 23a : Effect of nitrate on width of leaflet (cm) of cowpea plants inoculated with *Bradyrhizobium* strain P-132 and its mutants.

Mutants	NO_3 conc. (mM)			
	0	0.5	1.5	5.0
P-132	2.40 Eb	2.53 Fb	2.40 Eb	2.77 DEa
$AP2M_3$	2.67 CDa	2.77 CDa	2.77 Ca	2.80 DEa
$AP5M_2$	2.43 Eb	2.93 Ca	2.63 CDb	2.90 CDa
$AP20M_2$	3.50 Ac	4.77 Aa	4.50 Ab	4.50 Ab
$AP50M_1$	2.37 Ec	2.57 EFbc	2.67 CDb	2.93 CDa
$UP1M_1$	2.33 Eb	2.73 CDEa	2.70 Ca	2.77 DEa
$UP2M_2$	2.47 DEc	2.73 CDEb	2.47 DEc	3.07 Ca
$UP4M_1$	3.17 Bb	3.40 Ba	3.43 Ba	3.37 Bab
$UP6M_2$	3.43 Aa	3.53 Ba	2.47 DEb	2.67 Eb
$UP8M_1$	2.73 Ca	2.67 DEFa	2.37 Eb	2.57 Eab
$UP10M_2$	3.50 Aa	3.37 Ba	2.37 Ec	2.80 DEb

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

Table 23b : Effect of nitrate on width of leaflet (cm) of cowpea inoculated with *Bradyrhizobium* strain strain TAL-1000 and its mutants.

Mutants	NO$_3$ conc. (mM)			
	0	0.5	1.5	5.0
TAL-1000	2.10 Ed	2.43 EFc	2.63 Fb	2.90 Ca
AT2M$_2$	3.23 Ba	3.33 Ba	2.10 Gc	2.67 DEb
AT2M$_3$	4.17 Aa	3.80 Ab	3.53 Ac	2.93 BCd
AT5M$_2$	2.27 Dc	2.37 EFc	2.80 EFb	3.30 Aa
AT50M$_2$	1.83 Fd	2.33 Fc	3.07 Db	3.30 Aa
UT1M$_1$	2.03 Ed	2.47 EFc	3.47 Ba	2.97 BCb
UT2M$_1$	3.27 Ba	3.03 Cb	2.93 DEb	2.90 Cb
UT4M$_3$	3.27 Bab	2.93 CDc	3.37 BCa	3.10 Bbc
UT6M$_2$	2.30 Dc	2.47 EFbc	3.43 BCa	2.53 Eb
UT8M$_1$	2.57 Cc	2.80 Db	3.27 Ca	2.80 CDb
UT10M$_3$	1.80 Fc	2.57 Eb	3.43 BCa	2.60 DEb

Values followed by different letters were significantly different at 5% level of significance. Small letters indicate differences in horizontal rows and capital letters indicate differences in vertical columns.

formed leaves having least width (2.57cm) in the presence of 5mM nitrate (Table 23a).

Width of leaflets of cowpea plants inoculated with *Bradyrhizobium* strain TAL-1000 and its mutants

Variations were observed within mutants when they were grown in the presence of different concentration of nitrate. In general most of the isolates formed wider leaflets in the presence of 1.5 mM or 5 mM nitrate however plants inoculated with mutants AT2M$_3$ and UT2M$_1$ formed wider leaves under nitrogen free condition. AT2M$_3$ inoculated plants formed widest leaflets (4.17 cm) which was significantly higher than all other isolates tested under zero nitrate condition (plate 8). In the presence of 5mM nitrate in the rooting medium AT5M$_2$ and AT50M$_2$ inoculated plants formed wider leaflets (3.30 cm) and UT6M$_2$ inoculated plants had the least width of leaflets (2.53 cm) (Table 23b).

DISCUSSION

V. DISCUSSION

Mutations are invaluable to the process of evolution since they provide the raw material required for its occurrence. Without mutations, alleles of genes would not exist and thus genetic analysis would not be possible and organisms would not be able to evolve and adapt to environmental changes.

Mutagens have proved to be wonderful tools in the hands of microbiologists to allow the tailoring of micro organisms to produce a whole range of new products at economic levels and synthesize increased levels of microbially produced metabolites.

Mutations have played a crucial role in both research and applied science. Mutations in *Rhizobium* producing specific defects in symbiotic capacity are being used as genetic markers to map and identify genes required for infection, nodule formation and nitrogen fixation. Mutations could also be used to isolate strains with desirable characters like an increase in the range of hosts nodulated effectively, increased tolerance to environmental hazards such as temperature, pH, metal ion toxicity or tolerance to nitrate and with enhanced symbiotic nitrogen fixing ability (Bergersen et al., 1971).

The survey of literature on induced mutagenesis indicates that *Bradyrhizobium* sp has not gained its due attention by mutation scientists. Obviously their slow growth makes them less attractive for routine genetic manipulation and there also appears to be difficulties in the isolation of auxotrophic mutants. Given the agronomic importance of some of their hosts like soybean, groundnut, pigeonpea and cowpea, it would be desirable obviously to be able to handle the slow growing *Bradyrhizobium* sp genetically.

In this context, two *Bradyrhizobium* spp strain P-132 and TAL-1000 were mutagenized with acridine orange (chemical mutagen) and ultraviolet rays (physical mutagen) and morphological, cultural and physiological properties of mutants were examined. A study was also taken up to know the cross inoculation properties and nitrate tolerance of mutants. The results of various experiments are discussed here.

The parent *Bradyrhizobium* strain P-132 exhibited an intrinsic streptomycin resistance level of 10 µg/ml while strain TAL-1000 exhibited much lower level of 2 µg/ml (Table 4). Such low intrinsic streptomycin resistance levels was reported by Schwinghamer (1967) and Brunel et al., (1988) in Rhizobia.

MUTAGENESIS

The two *Bradyrhizobium* strains, P-132 and TAL-1000 were mutagenized with acridine orange and UV rays. 30 µg/ml of streptomycin in YEMA medium was used to isolate mutants which was three times above the intrinsic streptomycin resistance level of strain P-132 and 15 times above the intrinsic streptomycin resistance level of strain TAL-1000.

Streptomycin was used to isolate mutants because firstly it acts as a marker and secondly, strains resistant to streptomycin rarely lose their symbiotic effectiveness (Schwinghamer, 1967).

Acridine orange mutagenesis

The two *Bradyrhizobium* strains P-132 and TAL-1000 were mutagenized with acridine orange as described in materials and methods. Control treatment of 0 µg/ml AO did not produce any mutants on YEMA medium containing 30 µg/ml streptomycin, indicating that parent strains are sensitive to 30 µg/ml of streptomycin. A total of 27 acridine orange mutants were isolated after mutagenesis of strain P-132 and TAL-1000 with four concentrations (2, 5, 20 and 50 µg/ml) of acridine orange.

On subjection of mutants to plant infection test on siratro, three mutants were non-nodulating (AP5M$_3$, AP5M$_5$ and AT20M$_1$) while the remaining 24 mutants formed nodules varying in size from small to big and having white or pink interior on dissection of nodules, which is also one of the criterion to indicate effectiveness of symbiosis (Tables 5a and 5b). Such non-nodulating and ineffective mutants are frequently isolated by acridine orange treatment (Khare et al., 1982)

which could be due to some damage caused by mutations with acridine orange,to nod genes of *Bradyrhizobium* sp which are borne on chromosomes as it is known to cause frame shift mutations. Long (1989) reported that mutants in nodA, nodB and nodC are completely Nod$^-$ (no nodules are formed).

Only 13 among the Nod$^+$ mutants which formed medium to big sized, pink nodules caused early nodulation and produced comparatively more number of nodules than parent strains were selected for further study.

The rod shaped morphology and gram negative property of the two parent strains were shared by all AO mutants. All the AO mutants were slow growers like parent strains. Colony morphology of AO mutants and parent strains were similar except for the colony size. Mutants AP5M$_2$, AP20M$_2$, AP50M$_3$, AT2M$_2$, AT2M$_3$, AT50M$_1$, and AT50M$_2$ produced big colonies (2mm diameter) on YEMA medium containing 30 µg/ml streptomycin which inhibited growth of parent strains (Table 7a and plates 2 and 3). Khare et al.,(1982) also isolated mutants forming big colonies after acridine orange treatment of chickpea rhizobia.

UV mutagenesis

Two *Bradyrhizobium* spp strains P-132 and TAL-1000 were irradiated with ultraviolet rays for different time lengths (0 to 60 seconds) as described earlier in materials and methods.

Control treatment of no exposure to UV rays did not produce mutants on YEMA medium containing 30 µg/ml streptomycin indicating that parent strain are inhibited at 30 µg/ml streptomycin. About two to three mutants were randomly isolated from each duration of UV exposure on YEMA plates containing 30 µg/ml streptomycin, however sixty seconds UV exposure was lethal to both the strains studied and hence did not produce any mutants. Bose and Venkataraman (1972) also observed that rhizobium were killed by increasing the time of UV exposure.

Totally 37 UV mutants were isolated from the two parent strains and subjected to plant infection test in seedling agar tubes as described earlier.

Eleven of the UV mutants lost their nodule forming ability which included all the mutants obtained from 30 seconds UV exposure, probably due to some mutations in nod genes.

Mutants UP1M$_2$, UP1M$_3$, UP1M$_4$, UP4M$_2$, UP6M$_3$ and UP10M$_1$ inoculated siratro plants formed small nodules and were white inside on dissection which were considered to be due to ineffective symbiosis.

Similar observations were reported earlier by Maier and Brill (1976) following nitrosoguanidine mutagenesis of *Bradyrhizobium japonicum*. Mutants SM1 and SM2 were non nodulating, while SM3 and SM4 formed small nodules which lacked characteristic pink pigment inside, thus lacked acetylene reducing activity. Williams (1981) mutagenized a cowpea rhizobium with nitrosoguanidine and observed that mutant M1 produced more but small sized white nodules while mutant M3 nodulated earlier, produced more nodules (58%) than plants inoculated with wild type strain. In another study, Ghai et al., (1983) irradiated *Rhizobium trifolii* strain 11B with UV and reported that five of the UV mutants were Nod$^-$, while the mutants 6M11B, 12M11B, 19M11B and 21M11B resulted in the development of medium sized pink nodules like wild strain on its host.

Mutants UP2M$_2$, UP4M$_1$, UT2M$_1$, UT4M$_3$ and UT8M$_1$, nodulated early and also formed more nodules than wild strain on test host siratro. (Tables 6a and 6b). Mutants which formed medium to big sized nodules, having pink interiors indicating the presence of leghaemoglobin (Appleby, 1974) were selected for further study.

In the present study UV mutants retained rod shaped morphology and gram negative property like the parent strains. Similar observations were made by Bose and Venkataraman (1972) for UV mutants derived from parent strains of *Rhizobium leguminosarum*.

All the UV mutants in this study were slow growers like the parent strains of *Bradyrhizobium*. UV mutagenesis of the two parent strains did not alter the colony morphology of mutants from parent strains except for size of mutants.

UV mutants $UP2M_3$, $UP4M_1$, $UP8M_1$, $UT1M_1$, $UT6M_1$ and $UT6M_2$ formed small colonies (0.5mm diameter) while mutants $UP6M_2$, $UP10M_2$, $UT1M_3$, $UT2M_1$, $UT4M_3$ and $UT10M_3$ formed big colonies (2mm diameter) on YEMA medium containing 30 $\mu g\ ml^{-1}$ streptomycin, unlike the parents which were unable to grow (Table 7b and plates 4 and 5). Khare et al., (1982) also segregated UV mutants into four types based on colony size.

Cultural characters

None of the acridine orange mutants nor UV mutants grew within 48 hours on glucose peptone agar medium (GPA) like the parent strains, however only two UV mutants $UP6M_2$ and $UT1M_1$ showed growth on GPA medium after 6 days of incubation (plate 6) which could be due to their ability to utilize peptone as a result of mutation, which is normally not well utilized by rhizobia except for isolates from *Lotononis* (Krieg and Holt, 1984). Okon et al., (1972) also reported that two *Cicer* rhizobia grew well on GPA.

A few mutants $AP2M_1$, $AP2M_3$, $AP5M_2$, $AP20M_2$, $AP20M_3$, $AT2M_3$, $AT50M_1$, $UP6M_2$ and $UT10M_3$ unlike the parent strains showed faint growth in Hofers alkaline medium. It was also observed that most of these strains were acid producers as indicated by their growth in YEM-BTB broth (Table 8a and 8b). The ability of these mutants to survive in high pH (11 pH) condition of Hofers alkaline medium could be partly due to their neutralizing action of alkalinity in medium by acid production by mutants in the medium.

Mutant strains $AP2M_3$, $AP5M_4$, $AP50M_1$, $AT2M_3$, $AT50M_1$, $UP1M_3$, $UP2M_2$, $UP4M_3$, $UP6M_4$, $UP10M_2$ and $UP10M_3$, were alkali producers like the parent strains, while the rest of the mutants studied were acid producers (Table 8a and 8b). This is contradictory to the hypothesis of Norris (1965) who concluded that slow growing rhizobia produce alkaline reaction.

Physiological studies

Mutants were further subjected to physiological studies.

Carbohydrate utilization has been used to some extent in the subdivision of species of rhizobia (Fred et al., 1932). Free living rhizobia can utilize a wide range of carbohydrates for growth (Graham, 1964; Ronson and Primrose, 1979). Carbon source dependent variation in growth of rhizobium are known (Meyer and Pueppke, 1980) which could be either due to the differential uptake and/or metabolism.

Carbohydrate utilization study with acridine orange mutants indicated that mutants showed maximum growth on glycerol, arabinose, mannitol, glucose and no growth was observed on sucrose, lactose and starch like the parent strains. Some acridine orange mutants showed moderate to faint growth on xylose unlike parent strains, which exhibited good growth. Mutant AP20M$_3$ showed faint growth on both the pentoses tested, which is due to some mutation in genes required for utilization of pentoses. Mainra et al., (1984) also observed that nitrosoguanidine mutants of *R. meliloti* unable to utilize glycerol, arabinose, xylose and gluconate had low acetylene reduction activity. Though TAL-1000 exhibited only faint growth on maltose, mutants AT50M$_1$ and AT50M$_2$ derived from it showed moderate growth on maltose (Table 9a).

Like the parent strains UV mutants too showed good growth on glycerol, xylose, arabinose, mannitol, glucose and galactose and no growth was observed on lactose and starch. Some UV mutants due to mutation showed moderate to faint growth on disaccharides, sucrose and maltose unlike parents. These finding are in accordance with the observations of Graham (1964), Graham and Parker (1964) and Sadowsky et al., (1983) on slow growing root nodule bacteria (Table 9b).

As pointed out by Glenn and Dilworth, (1981) slow growing rhizobia seem to lack both uptake systems and catabolic enzymes for disacharides. Bose and Venkataraman (1972) observed that none of the UV mutants like their wild strain *Rhizobium leguminosarum* fermented lactose, sucrose and xylose, but wild and mutants mRh/1, mRh/6 and mRh/7 could ferment glucose.

Rhizobium meliloti mutants MD2, MD3, MD5 and MD6 were specifically unable to grow on mannitol, L-arabinose, ribose and xylose while MD7 was unable to grow on several sugars. Though all mutants nodulated alfalfa plants, L-arabinose mutant showed either late or no nitrogen fixing ability (Duncan, 1981).

Mutants derived after acridine orange and UV mutagenesis varied considerably in their tolerance to pH. The pH range congenial for the maximum growth of all mutants was between 6 to 7 (tables 10a and 10b) as reported earlier by Yadav and Vyas (1971) Basak and Goyal (1980).

Though pH 4.0 was inhibitory to both the parent strains of *Bradyrhizobium* and all UV mutants, but acridine orange mutants showed growth as a result of mutation which was also observed for a few *Bradyrhizobium* spp by Graham and Parker (1964).

Mutant UT1M$_3$ and UT6M$_1$ were inhibited at pH-9 as observed by Sadowsky et al., (1983) for slow growing *Bradyrhizobium japonicum*. Most of the mutants were tolerant to pH 10.0 which was inhibitory to parent strains. O'Hara et al., (1989) observed that when the external pH is low, the intracellular pH of *Rhizobium* is high and vice versa . The mutagenesis could have induced such balance, thus making the mutants able to grow in adverse pH conditions.

This variation in the ability of different mutants to thrive at varying pH, indicate the importance of strain selection for varying soil conditions.

Salt tolerance studies indicated that mutants AP50M$_1$, UP1M$_1$ and UP6M$_2$ showed growth at NaCl concentration of 2.5 per cent, while AP2M$_1$, AP5M$_4$, UP6M$_4$, AT2M$_2$, AT50M$_1$, AT50M$_2$ and UT10M$_4$ could tolerate 2.0 per cent which was inhibitory to parent strains used in this study. (Tables 11a and 11b). Thus, mutants were induced to tolerate higher salt condition. It is generally observed that *Rhizobium* isolated from saline areas or salt stressed areas are more resistant to salt concentration than other Rhizobia (Douka et al., 1983). There could be possibly a factor which could be similar to that of salt stressed *Rhizobium*, that is induced due to mutagenesis making the mutants to grow in adverse concentrations of NaCl.

Graham and Parker (1964) found 2% NaCl lethal for most *Bradyrhizobium* spp, but Yadav and Vyas (1971) observed that isolates from gram, peanut and cowpea survived 3% NaCl.

UV mutant $UT8M_1$ became sensitive to 1 per cent NaCl as a result of mutation. In general mutants showed better growth at 0.01 per cent NaCl except mutants $AP5M_2$, $UP6M_4$, $AT5M_2$, $UT2M_1$ and $UT6M_1$ which showed better growth in the presence of 0.1 per cent NaCl.

The ability of some of these mutants to survive NaCl concentration of 2 to 2.5 per cent calls for a further investigation of their actual performance in field situation. If field studies confirm lab observations then some of these mutants could become enterprising inoculants for acids soils and salt affected waste lands.

The entire study of morphological, cultural and physiological characters of various mutants indicate that all these isolates are morphologically alike, but their cultural and physiological characters show differences.

Cross-inoculation studies

Speciation of rhizobia is based on the cross-inoculation grouping suggested by the classical studies of Fred et al., (1932) which has been widely adopted as a workable and practical method to differentiate rhizobia. Seven cross inoculation groups have been identified. There are several groups of leguminous plants which are all nodulated by rhizobia common to them and each group is called a cross inoculation group. *Rhizobium* species generally infect only a few closely related legumes whereas *Bradyrhizobium* species of the cowpea cross-inoculation group can infect a broad range of diverse legume hosts (Elkan, 1981). Cross nodulation between bacteria belonging to one group and plants belonging to the other group is extremely rare. Symbiotic promiscuity between cross inoculation groups would be an advantage when two or more legumes are grown as intercrop in field.

In the present study AO and UV mutants derived from a cowpea strain, P-

132 and groundnut strain, TAL-1000 were screened for promiscuity on soybean (*Glycine max*(L.) Merr.) cv KHSB-2 and chickpea (*Cicer arientinum* L.) local variety.

Neither the two wild strains nor their mutants nodulated chickpea. Chickpea is known to be very specific in its rhizobial interaction.

Though Doku (1969) and Eaglesham et al., (1984) observed that soybean plants did not nodulate when inoculated with cowpea and groundnut rhizobia. However in the present investigation, strain P-132 and all mutants derived from it nodulated soybean with mutants UP10M$_2$ and AP20M$_2$ initiating nodulation significantly earlier (14 days). Mutants UP10M$_2$ and AP20M$_2$ also formed significantly bigger nodules (3.0 mm and 2.93 mm respectively). UP10M$_2$ formed maximum number of nodules (18.66 nodules per plant) followed by AP20M$_2$ (14.66 nodules per plant) (Table 13a).

Although parent strain TAL-1000 and one of its UV mutants UT6M$_2$ could not nodulate soybean, however other mutants derived from TAL-1000 nodulated soybean as a result of mutation (Table 12). Among the mutants of strain TAL-1000 that nodulated soybean, AT2M$_3$ followed by UT8M$_1$ nodulated significantly earlier than other mutants (14 days and 15 days respectively) and they formed significantly more nodules (25.33 and 25.00 nodules / plant respectively), whose nodule size was also significantly big (2.83mm and 3.16mm respectively) (Table 13b). Such observations of extended host range of rhizobia by mutations were reported by Schwinghamer (1962) who isolated mutants of *Rhizobium trifolii* capable of ineffectively nodulating pea seedlings. O'Gara and Shanmugam (1978) isolated mutants DT 72 and DT 127 of *R. meliloti* which unlike parent strain, nodulated soybean and mungbean plants producing effective nodules.

A few hsn genes have essential function in extending the host range for a single species or genotype (Horvath et al., 1987). Bacteria carrying mutations in such genes sometimes affect hosts that would normally be unresponsive to them. For example in *R. meliloti* mutation in nodH and nodQ lead to the production of a signal substance that elicits root hair deformation on vetch (*Vicia*) but not an alfalfa (Lerouge et al., 1990).

Screening mutants for nitrate tolerant symbiosis

Nitrate is known to suppress nodulation and biological nitrogen fixation in several legumes (Harper and Gibson, 1984 ; Streeter, 1988) but the exact mechanism of suppression has not been elucidated. It may be agronomically important to find symbiosis that continue to fix nitrogen in the presence of fertilizer nitrogen. One of the criterion for good inoculant strain is its ability to form nodules and fix nitrogen in the presence of soil nitrogen (Brockwell et al., 1982).

One of the approaches to develop nitrate tolerant symbiosis in legumes could be through identification of mutants of rhizobia which produce symbiosis less affected by external nitrate or which recover more rapidly from such effects. The resultant increase in residual soil nitrogen could benefit non-fixing component of the system.

In the present study, ten mutants from each of the two *Bradyrhizobium* parent strains were selected after various cultural, physiological and plant infection studies, were further screened for their tolerance to four concentrations of nitrate 0, 0.5mM, 1.5mM and 5.0mM (corresponding to 0, 16, 47 and 157 kg N/ha respectively) in the nutrient solution of cowpea plants grown in growth pouches.

Cowpea was chosen for this test because the parent strains P-132 and TAL-1000 effectively nodulate cowpea, it is an important grain legume, data are not available for this plant, it is well suited to grow under glass house condition and it grows to maturity in a relatively short time. After 35 days of growth in greenhouse, performance of each mutant was compared with wild type inoculated plants. The parameters used to determine effective symbiosis were number of days for nodule initiation, nodulation characteristics like size of nodules, number of nodules per plant, dry weight of nodules, shoot colour, plant height, number of trifoliate leaves and leaflet length and width. Hardarson and Danso (1993) stated that nodule number and weight have often been found to be positively correlated to the amount of nitrogen fixed.

Screening of these mutants for nodulation and plant growth indicated a marked variation in the symbiotic response of these mutants over the wild type strains at different levels of nitrate in the parameters studied.

Two rhizobial mutants inoculated soybean plants (*Glycine max* (L) merr.) exhibited significantly higher nodulation and symbiotic nitrogen fixation than parent strain inoculated plants, both in the presence and absence of nitrate (Maier and Brill, 1978). Similar results were evident in our present study.

In general, all mutants like the two *Bradyrhizobium* parent strains exhibited a delay in nodule initiation when the concentration of nitrate in the rooting medium was increased to 5mM, except mutants AP20M_2, UP1M_1, UP2M_2 and AT50M_2 which initiated nodulation early on plants when grown in the presence of 5mM NO_3 (22.33 days, 18.33 days, 20.57 days and 18.67 days respectively) than when receiving no nitrogen.

Mutant UP1M_1 nodulated significantly early compared to other mutants of strain P-132 in the presence of 5mM nitrate (18.33 days). Except AP5M_2 and UP8M_1 inoculated plants, all other mutants of P-132 nodulated significantly earlier than parent strain P-132 inoculated plants when grown in the presence of 5mM nitrate (Table 14a).

AT50M_2 followed by AT5M_2 nodulated significantly earlier than other mutants of TAL-1000 in the presence of 5mM NO_3 (18.67 days and 22.33 days respectively) (Table 14b) as a result of mutation.

These results are in accordance with earlier reports by Gibson and Pagan (1977) who observed a delay in appearance of nodules on siratro plants inoculated with cowpea strain 32H1 and its mutant in the presence of 0.7, 2.3 and 7 mM nitrate. But Rawthorne et al., (1985) reported that nitrate at 0.71 mM stimulated early nodulation and nodule growth, but 1.43 and 2.86 mM hastened nodule senescence.

McNeil (1982) observed variation in ability of *Bradyrhizobium japonicum* strains to nodulate soybean in the presence of nitrate. At 1.5mM nitrate concentration, days for nodule initiation varied from less than 10 (CB 1809, Nit 61A118) to more than 16 days (11 of the 16 strains tested). Similar delay in nodule initiation at 1.0mM NO_3 was observed among strains by Gibson and Harper (1985) and reported that strain USDA 110 was least affected.

Proposed mechanisms to explain the inhibition of infection in different legumes include the catalytic destruction of 1AA by nitrite (Tanner and Anderson, 1964), a decrease in the level of lectin production in the presence of nitrate.

Sanjuan and Olivares, (1989) identified a 5 kb region on a cryptic plasmid of *R. meliloti* GR4, pRme GR 4b mutation in this region referred to as nfe, delay nodule formation by one or two days.

Most of the mutants studied and their parent strains formed bigger nodules in the presence of low (0.5mM) or medium (1.5mM) concentrations of nitrate, but decreased at high (5mM) concentrations of nitrate except mutants $AP20M_2$ and $AP50M_1$, which formed bigger nodules in the presence of 5mM nitrate (2.43mm and 2.50mm respectively) compared to when grown under nitrate free condition (1.87mm and 2.00mm respectively). They formed maximum sized nodules at 5mM nitrate among the mutants of P-132 inoculated plants. All mutants of P-132 except $UP8M_1$ formed significantly bigger nodules than the parent strain P-132 inoculated plants in the presence of 5mM nitrate (Table 15a).

Mutant $UT4M_3$ inoculated plants formed maximum sized nodules (2.97mm) which were significantly bigger than its parent TAL-1000 inoculated plants (0.83mm) in the presence of 5mM nitrate. Mutant $UT2M_1$ formed maximum sized nodules in the presence of 0.5 and 1.5mM nitrate (4.33mm and 3.93mm respectively) (Table 15b). Mutants shared the same nodule morphology of parent strains in being globose shaped, streaked on the surface and were pink inside on dissection of nodules except $UP1M_1$ indicating the presence of leghaemoglobin.

Schiffman and Lobel (1973) suggested that quantitative determination of leghaemoglobin during certain stages of plant growth could be used as an indicator of nitrogen fixation.

Mutants AP20M$_2$, UP1M$_1$, UP4M$_1$ and UP8M$_1$ inoculated plants formed more nodules in the presence of 5mM nitrate than when grown under nitrate free condition. The same was seen at 1.5mM nitrate for mutant AP2M$_3$, AP5M$_2$, UP2M$_2$ and parent P-132 inoculated plants, but decreased in the presence of 5mM nitrate. Mutants AP2M$_3$, AP20M$_2$, UP10M$_2$ inoculated plants formed more nodules in the presence of 5mM nitrate and were on par with each other (39.00, 37.00 and 37.67 nodules per plant respectively) (Table 16a).

Plants inoculated with mutants AT2M$_3$, AT50M$_2$, UT1M$_1$, UT4M$_3$ and UT6M$_2$ like TAL-1000 formed significantly more nodules in the presence of 5mM nitrate than when they were inoculated to cowpea plants grown in nitrogen free medium. UT1M$_1$ inoculated plants formed more nodules at 5mM nitrate concentration (50 nodules/plant) whose number was significantly higher than other mutants derived from TAL-1000 but they were small and white coloured. Although the plants inoculated with AT5M$_2$ and UT10M$_3$ had fewer nodules in the presence of 5mM nitrate (Table 16b) they were big sized and were pink inside indicating their effectiveness.

Ralston and Imsande (1983) also reported that nitrate concentration equal to or greater than 2mM nitrate restricted nodule development in hydroponically grown soybean plants.

Gibson and Harper (1985) observed that *Bradyrhizobium japonicum* strain USDA 110 exhibited greater inhibition in nodulation in the presence of 0.5 and 1.0mM nitrate.

Maier and Brill (1978) reported that symbiotically effective mutant strain SM35 formed significantly more number of nodules than wild type inoculated soybean plants in the presence of 1mM ammonium. Malik et al., (1987) reported that exposure of soybean plants to 0.5mM nitrate resulted in no inhibition of nodule formation while exposure to 5mM nitrate inhibited nodulation by 5 to 20 per cent when inoculated with USDA-I-110 ARS.

Some of the strains like P-132, UP1M$_1$ UP8M$_1$, TAL-1000 and AT2M$_3$ although formed more number of nodules in the presence of higher (5mM) level of nitrate but these nodules were small, round and lacked leghaemoglobin indicating that they were ineffective (Fix$^-$). Similar nodule morphology of ineffective nodules was reported by Maier and Brill (1976).

nifA mutants of *Bradyrhizobium japonicum* are known to induce an increased number of small nodules with altered morphology and ultrastructure in addition to their inability to fix nitrogen (Fischer et al., 1986) and leghaemoglobin is present only at very low levels in such nodules.

Reduction in nodule number for some mutant inoculated plants in the presence of high nitrate concentrate could be due to inhibition in the attachment of rhizobia on root (Dazzo and Brill, 1973), by inhibiting formation of new root hairs (Thornton, 1936 and Munns, 1968), by preventing induction of root hair curling by bacteria (Thornton 1936) or by blocking the initiation of infection threads (Darbyshire, 1966).

Weight of nodules per plant and unit weight of nodules are also important parameters for judging the effectiveness of symbiosis.

Both the *Bradyrhizobium* parent strains exhibited greater weight of nodules per plant when plants were grown in nutrient solution supplemented with 0.5mM nitrate but decreased in the presence of higher concentrations of nitrate.

Plants inoculated with mutants AP2M$_3$, AP5M$_2$, AP20M$_2$, AP50M$_1$, UP1M$_1$, UP2M$_2$ and UP4M$_1$ produced greater weight of nodules per plant in the presence of 1.5mM nitrate than when grown in lower or higher concentrations of nitrate.

In general, the two parent strains and all 20 mutants showed a reduction in weight of nodules per plant in the presence of 5mM nitrate. In the presence of 5mM nitrate, AP20M$_2$ and UP10M$_2$ inoculated plants produced maximum weight of nodules per plant (15.63 and 15.73 mg/plant respectively), which were significantly higher than weight of nodules produced by all other mutants and their parent strain P-132 (Table 17a and Fig 2). Among the TAL-1000 mutants, AT5M$_2$ inoculated plants produced maximum weight of nodules per plant (17.83 mg/plant) in the presence of 5mM nitrate and was comparable to its nitrate free control condition indicating its tolerance to high NO$_3$. It was significantly greater than all the other 19 mutants and parent strains grown in the presence of 5 mM nitrate. (Table 17b and Fig 3).

Plants inoculated with mutants AT5M$_2$, AT50M$_2$, UTIM1, UT4M$_3$, UT6M$_2$ and UT10M$_3$ exhibited greater weight of nodules per plant when the plant nutrient solution was supplemented with 1.5mM nitrate than when grown in nitrate free medium or in the presence of 5mM nitrate.

Mutant AT2M$_3$ inoculated cowpea plants produced maximum weight of nodules per plant (29.67 mg/plant) and unit weight of nodule (1.54 mg / nodule) in nitrate free rooting medium which was significantly greater than all other 19 mutants and two parent strains inoculated plant but was sensitive to the presence of nitrate causing a decrease in weight of nodules. (Table 17b and 18b and plates 7 and 8).

McNeil (1982) while investigating the difference in sensitivity to nitrate of soybean symbiosis with 16 different *Bradyrhizobium japonicum* strains reported that in low nitrate levels (0.5mM), symbiosis with CB : 1809 increased total nodule mass by 30% relative to nitrate free control, however in the presence of 2mM nitrate CB : 1809 maintained total nodule mass, for remaining 6 strains tested total nodule mass decreased below the levels of nitrate free controls. Streeter (1982) observed that moderate level of nitrate (2.13mM) had little effect on nodule weight per plant while high nitrate concentrations (7.1 mM) depressed nodule weight per plant by 70 to 80% in soybean plants.

In general the two parent strains of *Bradyrihzobium* spp and mutants derived from them exhibited reduction in unit weight of nodule in the presence of

5mM nitrate except mutants AP5M$_2$, AP50M$_1$ and AT5M$_2$.

Plants inoculated with parent strain P-132 showed an increase in unit weight of nodule in the presence of 0.5mM nitrate compared to nitrate free control like the mutants AP2M$_3$ and UP8M$_1$ inoculated plants and decreased at higher concentration. But plants inoculated with mutant AP20M$_2$ and UP4M$_1$ maintained unit weight of nodule even in the presence of 1.5mM nitrate, while mutant AP5M$_2$ and AP50M$_1$ inoculated plants produced higher unit weight of nodule in the presence of 5 mM nitrate than other nitrate levels tested.

Mutants AP5M$_2$ and AP50M$_1$ inoculated plants produced maximum unit weight of nodule in the presence of 5 mM nitrate (0.56 mg / nodule and 0.58 mg / nodule respectively) compared to other P-132 mutants followed by AP20M$_2$ and UP10M$_2$ (0.42 mg/nodule and 0.41 mg/nodule respectively), which were significantly higher than P-132 inoculated plants (0.29 mg/nodule) (Table 18a).

Among parent strain TAL-1000 and its mutants, AT5M$_2$ and UT10M$_3$ inoculated plants produced greater unit weight of nodule in the presence of 1.5mM nitrate (1.35 mg per nodule and 1.30 mg per nodule respectively) compared to when grown in nitrate free medium, however there was a decrease in unit weight per nodule in the presence of 5mM nitrate but it was significantly greater than weight of nodule produced at nitrate free condition and 0.5mM nitrate.

AT5M$_2$ inoculated plants produced maximum unit weight of nodule compared to all 19 mutants and their two parent strains tested in the presence of 5mM nitrate (1.27mg per nodule) followed by UT10M$_3$ (0.87 mg per nodule) (Tables 18a and 18b).

Streeter (1982) observed a positive effect of moderate nitrate supply (2.13mm nitrate) on nodule growth rate in soybean plants inoculated with *Bradyrhizobium japonicum* strain 61A76 which was reflected in the increase in plant vigor in response to nitrate.

Gibson and Harper (1985) were of opinion that concentration of nitrate surrounding the root appeared to be of most importance in inhibition of nodule initiation while the rate of uptake and metabolism of nitrate appeared to have a greater effect on nodule development and nitrogenase activity.

The overall results support the earlier observations that the depression of legume nodule growth occurs at higher concentration of nitrate for most of the mutants studied. One of the simplest explanation for the effect of nitrate on nodule growth and nitrogenase activity could be that some product of nitrate metabolism is inhibitory. Nitrite is a logical candidate for such an inhibitory role because (a) it is a potent inhibitor of nitrogenase activity when mixed with purified enzyme (Trinchant and Rigaud, 1980) or with bacteriods *in vitro* (Rigaud and Puppo, 1977) or supplied to intact nodules (Kamberger, 1977) (b) it deoxygenates and oxidizes leghemoglobin *in vitro* resulting in the formation of ferric leghemoglobin (Rigaud and Puppo, 1977).

Manhart and Wong (1980) reported high nitrite concentration in lupine and cowpea nodules supplied with very high nitrate (15mM).

Higher plant height, greater leaf number, leaf length and leaf width are also the indicators of more efficient biomass accumulation. Due to this significance , performance of rhizobial mutants in the presence of nitrate on these parameters were also studied.

In general there was an increase in plant height for all the mutant inoculated plants in the presence of 1.5mM or 5.0mM nitrate compared to their nitrate free controls except mutants UP2M$_2$, UP8M$_1$, TAL-1000, AT2M$_2$ and AT2M$_3$ inoculated plants which showed a significant decrease in plant height in the presence of 5mM nitrate because this concentration inhibited effective symbiosis in the later mutants.

Among the plants inoculated with mutants derived from strain P-132 and grown in the presence of 5mM nitrate, AP20M$_2$ inoculated plants were tallest (29.67 cm) which was significantly taller than P-132 (20.77cm) inoculated plants (Table 20a and plate 9).

Among the mutants derived from TAL-1000, AT2M$_3$ inoculated plants were the tallest (27.30 cm) and significantly taller than parent TAL-1000 inoculated plants (21.10cm) when grown in nitrate free medium. But their height decreased significantly when grown in higher concentrations of nitrate (plate 8).

Mutant AT5M$_2$ inoculated plants were tallest (26.53 cm) among the mutants studied in the presence of 5mM nitrate and was also significantly taller than parent TAL-1000 inoculated plants (20.63cm) (Table 20b and plate 10).

In general most of the strains did not show any significant variation in the number of trifoliate leaves produced either in the presence or absence of nitrate, except AP50M$_1$, UP4M$_1$, UP10M$_2$, AT50M$_2$ AND UT10M$_3$ inoculated plants which showed an increase in leaf number in the presence of nitrate.

In the presence of 0.5, 1.5 and 5.0mM nitrate there was no significant difference in the number of leaves produced among parent strains and their respective mutants, however some variation was observed among the mutants under nitrate free conditions.

Mutant AT2M$_3$ inoculated plants produced maximum number of trifoliate leaves (5 leaves per plant) which were dark green with maximum length and width of leaflets in nitrate free condition (6.67 cm and 4.17 cm respectively) (Table 21b, 22b and 23b) (plate 8) but this mutant could not tolerate higher concentrations of nitrate.

Among the mutants derived from strain P-132, AP20M$_2$ inoculated plants produced bigger leaves which had significantly longer (5.63 cm) and wider leaflets (4.50cm) than P-132 and all other mutants derived from it in the presence of 5mM nitrate (Table 22a and 23a).

Among the mutants derived from strains TAL-1000, AT5M$_2$ inoculated plants produced maximum number of leaves (4.67 leaves/plant) with greater length and width of leaflets (5.47 cm and 3.30 cm respectively) in the presence of 5mM nitrate in the rooting medium which was significantly higher than parent strain TAL-1000 and all other mutants derived from it (Table 21b, 22b and 23b).

These increases in plant growth parameters in the presence of 5mM nitrate for the mutant AP20M_2 and AT5M_2 inoculated plants is due to their greater tolerance of symbiosis to high nitrate concentration (5mM) than the other mutants or their parent strains tested. However Gibson and Harper (1985) observed that soybean plants inoculated with strain USDA 110 and USDA 123 gave significantly greater shoot dry weight in the presence of much lower (0.5 and 1.5mM) nitrate concentration than for their non nitrate control.

The entire experiment to screen for mutants tolerant to normally inhibitory concentrations of nitrate revealed variation in the efficiency of different rhizobial mutants with respect to nodule initiation, nodule growth and plant growth.

Reports on variation among strains of rhizobia in their tolerance to the inhibitory effects of nitrate on nodulation and subsequent nitrogen fixation have been found in different legume species. (Gibson and Pagan, 1977 ; Manhart and Wong, 1980; McNeil 1982; Harper and Gibson, 1984 ; Gibson and Harper, 1985).

However with respect to all the above parameters most of the rhizobial mutants inoculated plants gave better results at low (0.5mM) to medium (1.5mM) concentration of nitrate than the nitrate free control plants. This augments several earlier observations that plants receiving low nitrate may actually fix more nitrogen than plants entirely dependent on atmospheric nitrogen (0 - NO_3).

Enhanced nitrogen fixation coupled with nitrate utilization results in greater size and vigor of nitrate supplied plants. (Harper, 1974 ; McNeil, 1982 ; Streeter, 1982). The results of this study show that mutant AP20M_2 among the P-132 mutants and AT5M_2, among mutants derived from TAL-1000 are capable of better nodulation and nitrogen fixation at high concentration of nitrate (5 mM NO_3) compared to their parents.

These results indicate that mutations can be used as an important tool to improve the effectiveness of *Bradyrhizobium* inoculants.

Future line of work would be to check the performance of these mutants under field conditions.

SUMMARY

VI. SUMMARY

Mutants from two strains of *Bradyrhizobium* spp were obtained using acridine orange (chemical mutagen) and ultra violet radiation (physical mutagen). The morphological, cultural and physiological characters of mutants were studied. The mutants were also tested for their cross-inoculation properties and nitrate tolerant symbiosis.

Cowpea strain P-132 and groundnut strain TAL-1000 were mutagenized with various concentrations (0 to 50µg/ml) of acridine orange and exposed to different time lengths of UV radiation (0 to 60 sec) and mutants were isolated on yeast extract mannitol agar medium (YEMA) containing 30µg/ml streptomycin. Only those mutants which effectively nodulated *Macroptilium atropurpureum* (siratro) were used for the study.

The mutants varied in colony size between 0.5mm to 2.0mm on YEMA medium containing 30µg/ml streptomycin. Acridine orange mutants $AP5M_2$, $AP20M_2$, $AP50M_3$, $AT2M_2$, $AT2M_3$, $AT50M_1$, $AT50M_2$ and UV mutants $UP6M_2$, $UP10M_2$, $UT1M_3$, $UT2M_1$, $UT4M_3$ and $UT10M_3$ formed big colonies (2.0 mm). Only two UV mutants $UP6M_2$ and $UT1M_1$ showed growth on glucose peptone agar medium. Six acridine orange mutants and two UV mutants exhibited faint growth in Hofers alkaline medium. Although all mutants were slow growers like parent strains, 11 mutants were alkali producers like parent strains and remaining 23 mutants were acid producers.

Carbohydrate utilization studies, showed that mutants did not show any significant variation from parent strains in their utilization of glycerol, arabinose, mannitol, glucose and galactose. Mutants (AO) $AP5M_4$, $AP20M_2$ exhibited faint growth on xylose while $AP20M_3$ showed faint growth on both pentoses tested unlike the parent strains which showed good growth. Two AO mutants $AT50M_1$ and $AT50M_2$ showed moderate growth on maltose and two UV mutants $UT1M_3$ and $UT2M_1$ showed moderate growth on sucrose, both of which were not utilized by parent strains.

All the acridine orange mutants showed growth at pH 4.0 which was inhibitory to parent strains and their UV mutants. Most of the AO and UV mutants were tolerant to pH 10, which inhibited the parent strains. Salt tolerance studies showed that $AP50M_1$, $UP1M_1$ and $UP6M_2$ showed growth at 2.5% NaCl and $AP2M_1$, $AP5M_4$, $UP6M_4$, $AT2M_2$, $AT50M_1$, $AT50M_2$ and $UT10M_4$ could tolerate 2% NaCl which were inhibitory to parent strains P-132 and TAL-1000.

Host range studies of selected AO and UV mutants and their two wild strains of *Bradyrhizobium* sp on *Cicer arietinum* (L.) (chickpea) and *Glycine max* (L.) Merr. (soybean) revealed that none of them nodulated chickpea. Strain P-132 and all mutants derived from it nodulated soybean but strain TAL-1000 and one of its UV mutant $UT6M_2$ could not nodulate soybean however all other mutants of TAL-1000 nodulated soybean.

Among the mutants of strain P-132, UV mutant $UP10M_2$ nodulated soybean significantly earlier and showed increased nodulation followed by AO mutant $AP20M_2$ which were significantly superior than P-132 inoculated plants. $AT2M_3$ and $UT8M_1$ derived from strain TAL-1000 nodulated soybean significantly earlier and formed more number of nodules and $UT8M_1$ inoculated plants formed bigger nodules.

Finally AO and UV mutants were screened for nitrate tolerant symbiosis on *Vigna unquiculata* (L.) (cowpea) in the presence of 0, 0.5, 1.5 and 5.0 mM nitrate (corresponding to 0, 16, 47 and 157 kg N/ha) in growth pouches. Some of the mutants like parent strains of *Bradyrhizobium* sp performed better with respect to nodule size and number, weight of nodules per plant, unit weight of nodules, height of plants and leaflet size in the presence of low (0.5mM) or medium (1.5mM) level of nitrate but decreased in the presence of high (5mM) nitrate concentration.

In general most of the mutants like parent strains exhibited delay in nodule initiation in the presence of high (5mM) concentration of nitrate however mutants

AP20M$_2$, UP1M$_1$, UP2M$_2$ and AT50M$_2$ initiated early nodulation in the presence of 5mM nitrate than when grown in nitrate free medium.

Among the mutants derived from strain P-132 grown in the presence of 5mM nitrate, mutants AP20M$_2$ and AP50M$_1$ formed bigger nodules and mutants AP2M$_3$, AP20M$_2$ and UP10M$_2$ formed more nodules. Mutant AP20M$_2$ and UP10M$_2$ inoculated plants produced higher weight of nodules per plant and AP20M$_2$ inoculated plants were tallest with maximum sized leaflets which were all significantly higher than parent strain P-132.

Among the mutants derived from strain TAL-1000 grown in the presence of 5mM nitrate, mutant UT4M$_3$ produced bigger nodules and mutant UT1M$_1$ formed more nodules but they were small in size and ineffective. Although mutants AT5M$_2$ and UT10M$_3$ inoculated plants formed fewer nodules they were big sized. AT5M$_2$ inoculated plants produced maximum weight of nodules per plant, unit weight of nodules, height of plants, number of leaves and leaflet size which were all significantly higher than their parent strain TAL-1000 inoculated plants.

These results indicate that acridine orange mutants AP20M$_2$ derived from strain P-132 and AT5M$_2$ derived from strain TAL-1000 were more tolerant to 5mM nitrate than the other mutants and their parent strains.

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VII. REFERENCES

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* Original not seen .

APPENDIX

APPENDIX

COMPOSITION OF MEDIA

YEAST EXTRACT MANNITOL AGAR MEDIUM (YEMA)

Mannitol	10.0g
$K_2 HPO_4$	0.5g
$MgSO_4.7H_2O$	0.2g
NaCl	0.1g
Yeast extract	0.5g
Distilled water	1000ml
pH	6.8
Agar	15.0g

Congo red: 10ml of 0.25% solution per litre of YEMA.

GLUCOSE PEPTONE AGAR MEDIUM (GPA)

Glucose	5.0g
Peptone	10.0g
Agar	15.0g
Distilled water	1000.0 ml
Bromocresol purple (1% in ethanol)	10.0 ml

KETOLACTOSE MEDIUM

$CaCl_2$	0.20g
$FeCl_3$	0.10g
K_2HPO_4	0.50g
Lactose	10.00g

NaCl	0.20g
$MgSO_4.7H_2O$	0.10g
Yeast extract	15.00g
Distilled water	1000ml
pH	6.8

Benedicts reagent

a. Sodium citrate	173.00g
Sodium carbonate	100.00g
Distilled water	66.00ml
b.Copper sulphate	17.30g
Distilled water	100.00ml

Solution (b) was added to solution (a) with a constant stirring. Volume of the filtrate was made upto 100ml with distilled water

JENSEN'S SEEDLING AGAR MEDIUM

$CaHPO_4$	1.0g
K_2HPO_4	0.2g
$MgSO_4.7H_2O$	0.2g
NaCl	0.2g
$FeCl_3$	0.1g
Distilled water	1000ml
pH	6.8
Agar	15.0g

N-FREE NUTRIENT SOLUTION

Stock Solutions	Compounds	g/l
1.	$CaCl_2.2H_2O$	294.1
2.	KH_2PO_4	136.1
3.	Fe-citrate	6.7
	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4.H_2O$	0.338
4.	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$Na_2MoO_2.2H_2O$	0.048

Five ml each of solutions 1 to 4 was added to 5.0 litres of distilled water, then diluted to 10.0 litres. pH was adjusted to 6.6-6.8.