SOME STUDIES ON THE DIAZOTROPHIC BIOCOENOSIS IN KALLAR GRASS (*LEPTOCHLOA FUSCA* (L) KUNTH).

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ABBREVIATIONS

ARA	Acetylene (C_2H_2) reduction assay.
at%	Relative amounts of ^{14}N and ^{15}N of sample.
at% ex	Atom percent excess difference between the relative amount of ^{14}N and ^{15}N in given material and that of natural abundance (0.366% ^{15}N at.).
BNF	Biological nitrogen fixation.
°C	Degree centigrade.
CCM	Combined carbon medium.
cm	Centimeter, 10^{-2}m.
cl	Culture of bacteria.
DAP	Days after plantation.
DNA	Deoxyribonucleic acid.
FID	Flame ionization detector.
Fig.	Figure
fs	Nitrogen fixing system.
g,	Gram
(G+C)	Guanosine + Cytosine.
h	Hour, hours
ha	Hectare
HP	Histoplane
Kg	Kilogram, 10^3 g.
L	Liter.
L.S.D.	Least significant difference
M	Molar, mol.wt. in g, L^{-1}.
mg	Milligram, 10^{-3} g
min	Minutes
ml	Milliliter, 10^{-3} L
mm	Millimeter, 10^{-3} m.
mM	Millimolar, 10^{-3} M.
MPN	Most probable number.
N	Nitrogen element.
N_2	Dinitrogen gas.
N_2-ase	Nitrogenase activity (= ARA).
^{15}N	Isotope of nitrogen with mass number of 15.
nfs	Non fixing system.

nmoles	Nanomoles, 10^{-9} moles.
P	Probability.
pH	Negative log concentration of H^+.
PO_2	Pascal of O_2.
PGS	Plant growth substances.
RNA	Ribonucleic acid.
RP	Rhizoplane
rpm	Revolution per minute.
RS	Rhizosphere
sp.	Specie
spp.	Species
Temp.	Temperature °C.
TTC	2,3,5, triphenyl tetrazolium chloride.
TTF	2,3,5, triphenyl tetrazolium formazons.
u	Micron 10^{-6} m.
ug	Microgram, 10^{-6} g.
umoles	Micromoles 10^{-6} moles.
v/v	Volume by volume.
wt.	Weight
wt/wt	Weight by weight.

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C O N T E N T S

CHAPTER-I INTRODUCTION AND LITERATURE SURVEY

1.1 INTRODUCTION: Nitrogen is an essential element for all living systems. The atmospheric source of N is inexhaustable but plants and animals can only use this element in combined form i.e. in oxide ($NO^{'}_{3}$) or reduced (NH^{+}_{4}) form and not in free state (N_2). Biological nitrogen fixation (BNF), the conversion of atmospheric nitrogen gas to ammonium ion, a form of nitrogen fertilizer, is restricted to few prokaryotes. No higher organisms have been shown to contain this capability (Postgate, 1980). The N_2-fixing microorganisms fix atmospheric nitrogen either in free living or in symbiotic state. A wide variety of nitrogen fixing systems have been observed in nature. The most familiar example is the root nodules of leguminous plants (Allen & Allen, 1981 and Halliday, 1984). Comparable to legumes are actinorhizal plants in forestry (Gordon & Wheeler, 1983). Azolla-Anabena system is another example of such symbiotic system. Free living diazotrophs consist of bacteria and blue green algae (Cyanobacteria). Nearly 81 genera of the latter have been described and nitrogen fixing species are found in 25 of these genera i.e. 31% (Akkermans & Houwers, 1983). In Bergey's Manual (1974), 245 genera of bacteria are distinguished including 39 genera with uncertain affiliation. Nitrogen fixing bacteria are classified in 26 genera, i.e. ca 10% of all genera. Based on this classification, it can be concluded that the occurrence of the N_2-fixing enzyme nitrogenase is rather exceptional in nature.

Free living nitrogen fixing bacteria are present in most soils as well as in plant rhizosphere. Their role in the nitrogen economy of the soil, either in natural habitats or where the bacteria has been artificially introduced, usually in the form of so-called "bacterial fertilizers" has been a subject of great intrest for the scientists, since long time. (van Berkum & Bohlool, 1980).

The development of the simple, sensitive and rapid acetylene reduction assay and world wide efforts to enhance biological production of nitrogen fertilizers, a process which uses solar energy (a renewable energy form) rather than

ever shrinking fossil fuel supply, resulted in the renewed interest in several systems other than legumes. The occurrence of associative symbiosis between nitrogen-fixing bacteria and the roots of grasses has been pursued more vigorously since last ten years. Evidence for this was provided by the number of scientific papers published during this period on the root associated bacteria-grass systems. The fact that a scientific meeting devoted exclusively to dinitrogen-fixing associative systems which held at Piracicaba, Brazil (1979) is perhaps the best testament of how far this field of research has advanced. Since then two similar biannual meetings were held at Banff, Canada (1982) and at Helsinki, Finland (1984) respectively.

Many advances have been made during these five years in understanding of the true nature of the diazotrophic biocoenœsis. Emphasis has presently been laid on the question of invasion, colonization and symbiotic (?) development of N_2-fixing bacteria on the roots of grasses. Primary among the areas of our ignorance is the exchange of carbon and nitrogen substrates between bacteria and the host. Undoubtedly part of the difficultly is due to a lack of proper methodology to study the associative grass-bacteria N_2-fixing system. If research efforts in the next few years proceed as enthusiastically and vigorously as in the past, large gain in our knowledge of the diazotrophic biocoenosis will be realized.

There are many natural systems i.e. salt marshes, wastelands etc where amounts of associative fixed nitrogen may reach to an important proportion. One of the such systems is saline-sodic soils of Pakistan which constitute 33% of the total irrigated lands of the country. A salt tolerant grass, Kallar grass (Leptochloa fusca (L) Kunth Syn. Diplachne fusca) is recommended as primary colonizer for such soils (Sandhu & Malik, 1975). It is a C-4 grass (Zafar & Malik, 1984) which can grow on soils having pH 9-10 and electrical conductivity 30-40 $mScm^{-1}$. L. fusca yield 30-40 tons of biomass $ha^{-1}, year^{-1}$ without application of any

fertilizer. Preliminary investigations (Malik et al.,1980,1981) indicated N_2-fixation in the excised roots as demonstrated by acetylene reduction assays. Present studies are primarily concerned with N_2-fixation in the rhizosphere of L. fusca which seems to be responsible for maintenance and production of the biomass on saline lands.

1.2 REVIEW OF LITERATURE: The literature on associative N_2-fixation is quite extensive. Comprehensive reviews on this subject have been made by Van Berkum & Bohlool (1980), Van Berkum & Sloger (1984), Brown (1982), Dart & Day (1975), Hubbel & Gaskins (1984); Neyra & Dobereiner (1977); Schank & Smith (1984) and Vose (1983).

1.2.1 HISTORY: Nitrogen fixation in grasses is not a new concept because in 1922, Lipman & Taylor reported N_2-fixation in the wheat plant. Truffant & Bezssonoff (1923) reported the conditions which permits the association between N_2-fixing bacteria and maize plants. Asymbiotic nitrogen fixation in the rice field was reported as early as 1929 by Sen. The reported data on nitrogen accumulation in soils are difficult to interpret, nevertheless, it has been concluded that non-symbiotic nitrogen fixation occurs at magnitudes of agronomic significance (Moore, 1966). Parker (1957) observed by direct balance studies that there was N_2-fixation in rye grass field and reported 185 kg ha^{-1} N-gain in 3 years study. N_2-fixation in the rhizosphere have earlier been reported by Brown (1933) and in the rhizosphere of peas (without nodules) by Bukatsch & Heitzer (1952). The long term nitrogen balance studies at Rothamsted, England have produced convincing evidence for nitrogen accumulation due to non-symbiotic nitrogen fixation. Part of the continuous wheat experiment was fenced off in 1883, and the natural vegetation was allowed to return. This area, the wilderness has gained nitrogen. It has been estimated that the BNF has contributed 34 kg-N ha^{-1} $year^{-1}$ (Day et al., 1975; Jenkinson, 1977). Nitrogenase activity in the rhizosphere of plants common to the site has been reported by Harris & Dart (1973). With the advent of acetylene reduction test (Dilworth, 1966; Hardy et

al. 1968) search for N_2-fixation has taken a new turn. The discovery of nitrogen fixing association between bacteria and graminaceous roots by Dobereiner & Campelo (1971) and a detailed study on Paspalum notatum - Azotobacter paspali association in Brazil by Dobereiner et al (1972) stimulated the concernted efforts in this field. At the first international symposium on N_2-fixation at Pullman (Washington, USA) in 1974, Dobereiner and Day reported a comprehensive study on N_2-fixation in Digitaria decumbens(var. batatis) with Spirillum lipoferum (Azospirillum brasilense). This paper attracted lot of attention. Recent energy crisis of 1973 and the discovery that nitrogenase is also able to reduce acetylene to ethylene, a compound which can rapidly and sensitively determined by gas chromatography technique and potential benefits of this system in agricultural world ushered in the explosion of search of nitrogen fixation in grasses that exist at the present time.

1.2.2 ASSOCIATIVE N_2-FIXING SYSTEMS: Numerous reports are available on nitrogen fixation in grasses and cereals. Using the acetylene reduction test, significant nitrogenase activities of grass roots were observed. In most cases either no information on the responsible bacteria is available or isolation and enumeration of certain groups of bacteria from the rhizosphere are given without reference to specific plant-bacteria interactions. Some of the systems in which associative N_2-fixation have been reported are described. Attempt has been made to include only those papers which described the association in natural environments. Other N_2-fixing studies related to these crops are described under different subtitles.

Maize: Nitrogenase activity in maize field was thoroughly investigated by Von Bulow & Dobereiner (1975). This paper elicited much publicity. Earlier Dommergues et al (1973) along with other plants reported N_2-fixation in maize. Further work on this association was carried out by Burris et al., (1978); De-Polli et al (1982); Helvecio et al. (1982); Lakshimi Kumari et al. (1976); Patriquin & Dobereiner, (1978) & Raju et al (1972).

Wheat: Dinitrogen fixation associated with common wheat in the field has been reported by several investigators. Unfertilized wheat on the Rothamsted Broadbalk plots gained upto 34 kg N $year^{-1}$ of which 2-3 kg N ha^{-1} was attributable to bacterial fixation. Larson & Neal (1976) described a highly specific association of a facultative Bacillus sp. with a disomic chromosome substitution line of wheat. Considerable studies have been reported on this specific system(Lethbridge & Davidson,1983;Nelson et al., 1976; Neal & Larson, 1976 and Rennie & Larson, 1979).

Sugar Cane: In many parts of the tropics, sugar cane (Saccharum officinarum) has been grown for centuries without addition of nitrogen fertilizers. Moreover this crop is poor in response to N-fertilization. Association of N_2-fixing microorganisms with sugar cane roots was demonstrated by Dobereiner (1959). In latter studies on sugar cane Dobereiner (1961) has found higher proportion of Beijerinckia sp. in rhizosphere soil than in soil between rows. This was subsequently confirmed by acetylene reduction technique (Dobereiner et al., 1972 & Ruschel et al., 1978). The recent studies using ^{15}N showed direct evidence for dinitrogen fixation in sugar cane roots presumably originating from microorganisms inhabiting the root and also confirming translocation of the fixed nitrogen to the plant tissues (Hegazi et al., 1979; Jadhav & Andhal, 1976; Patriquin et al., 1980; Purchase, 1980 and Ruschel & Vose, 1977).

Rice: Like sugar cane, paddy rice has been grown for centuries in the Far-East without application of nitrogen fertilizers. A long term N-balance study at IRRI, Phillipines has revealed constant yield of rice without input of N-fertilizers (Watanabe & Lee, 1977). Rice fields are inhabited by other N_2-fixing systems like blue green algae and Azolla-Anabena system however Yoshida & Ancajas (1971) observed that roots of wetland rice had the ability to fix N_2 due to heterotrophic bacteria that live on and in the rice roots. Later on Balandreau et al (1975) in Senegal; Lee et al (1977); Watanabe (1984);

and Ladha et al, (1984) in Phillipine; Rao et al. (1983) in India and Qui Yuanshag et al (1984) in China reported nitrogenase activity in the rhizosphere of rice.

Sorghum & Millet: These two crops are mainly grown on dryland and semi-arid tropical regions. Dart & Wani (1982) have emphasized that these regions produce 95% of the world's millet crop and 60% of the world's sorghum on a total cropped area of some 70 million ha . Nitrogen fertilizer use is small and virtually all production is dependent on mineralized - N from soil organic matter including biological N_2-fixation. Other studies on these crops have been reported by Bouton et al, (1979); Klucas et al .1981; Pedersen et al.,(1978);Suba Rao & Dart (1981) and Wani et al; (1983).

Forage Grass: As grasses cover the major part of arable land the presence and estimates of any nitrogen fixation in them will be of great intrest. Grass-bacterial association for N_2-fixing system was first described for Paspalum notatum, a C-4 tropical grass (Dobereiner, 1966). This grass shows a very specific association with Azotobacter paspali (Dobereiner & Campelo, 1971). It was estimated that the nitrogen fixation in this grass amounted to 340 g N ha^{-1} day^{-1} (Dobereiner & Day, 1974). The other thoroughly studied association is of Digitaria decumbens . It was estimated from ARA by Dobereiner & Day (1975) that nitrogenase activity equivalent to 880, 480 and 970 g ha^{-1}, day^{-1} was present in intact soil core in the summer 1975 of three different cultivars of this grass. Spirillum lipoferum was found to be major organism responsible for nitrogenase activity (Dobereiner & Day, 1976).

Acetylene reduction assays of excised roots have shown that several other grasses, are able to fix N_2. Most of the studies have been performed with tropical grasses in Brazil, Nigeria and Ivory Cost. The commonly studied grasses were Andropogen spp (Balandreau et al, 1973), Brachiaria spp (Dobereiner & Day, 1975), Cynodon spp. (Dobereiner & Day, 1975), Cyperus spp. (Balandreau et al., 1973 & Dobereiner et al., 1975). Panicum maximum (Dobereiner & Day,

1975) and Pennisetum purpureum (Dobereiner & Day, 1975). A survey of forage grasses was carried out in the subtropical region of Texas (USA) by Weaver et al (1980) for estimating biological N_2-fixation. The most active rhizosphere samples were extrapolated to fix N at rates of 33, 26, 20 and 20 kg ha^{-1} 100 $days^{-1}$ for Cynodon dactylon, Paspalum urvillei, Brachuaria sp and Andropon gerardi respectively. Klucas et al., (1981) reported considerable progress in the study of associated nitrogen fixation in Kentucky blue grass (Poa pratensis L.) growing in Nebraska. Earlier Nelson et al (1976) studied some grasses growing in Oregon State for associative N_2-fixation. Several tropical grasses belonging to the genera Brachiaria, Cenchrus, Chloris, Cymbopogon Dicanthium, Euchlaena, Panicum, Pennisetum & Setaria were examined by Suba Rao & Dart (1981) in an observation graden. Thirty four out of 48 entries were found to be active in stimulating N_2-fixation. Nitrogenase activity of nine forage grass cultivars growing in Sweden were screened by Lindberg & Granhall (1984).

Other Systems: Salt marshes are among the most productive ecosystem in the world. Biological nitrogen fixation associated with roots is considered to be the major source of nitrogen for the salt marsh grass Spartina alterniflora (Patriquin, 1978). The distribution and magnitude of N_2-fixation associated with Thalassia testudinum, a sea grass was measured by Capone & Taylor (1980) while Smith & Hayasaka (1982) examined the seasonal nitrogen fixation rate associated with another sea grass, Halodule wrightii. Considerable progress has been achieved in understanding the associative system of S. alterniflora (Patriquin et al, 1979).

Root samples of 11 non cultivated monocotyledonous and 7 dicotyledonus species growing during wet summer in W. Germany were examined by Jagnow (1982) for associated N_2-fixation. Nitrogen fixation by bacteria in associative symbiosis with washed roots of 13 Poaceae and 8 other non cultivated plant speices in Finland was demonstrated by ARA by Haahtela et al (1981). Cichorum intybus, a very common cultivated crop in Belgium and Taraxacum officinale a

common wild plant were examined by Vlassak & Jain (1976) for associated N_2-fixation. Suba Rao (1983) analysed rhizosphere soil of several plantation and orchard plants to enumerate the associated nitrogen-fixing bacteria. TTC reducing bacteria were found to be more abundant in xylem cells than in cortical cells of roots of these plants. Nitrogenase activity has also been found associated with roots of sweet potato (Ipomoea batatas) by Hill et al (1982). The nitrogenase activity associated with the roots of several forage grasses native to Indian deserts was examined by Rao & Venkateswarlu (1982). Nitrogen fixation in saline-sodic soils of Pakistan associated with Leptochloa fusca and different weeds have been reported by Malik et al (1980) and Bilal & Malik (1984) respectively.

The examination of naturally occurring root-associated nitrogen fixation in the soil environment is continued although major research emphasis has presently been laid on understanding the true nature of diazotrophic biocoenosis.

1.2.3 MICROBIOLOGY OF THE ASSOCIATIVE SYSTEM: Nitrogen-fixing bacteria are probably among the most extensively studied soil microorganisms. Despite this, very little is known about their biology in nature and their contribution to the structure and function of the habitat that they occupy. There have been several recent review about N_2-fixing bacterial genera and species (Knowles 1977, 1978, LaRue 1977; Mulder & Brotonegoro, 1974 and Postgate, 1981).

It is well recognized that numerous soil bacteria that are capable of fixing nitrogen are known to proliferate in the rhizosphere of different plants, from which they are readily isolated. Dobereiner (1961) showed that N_2-fixing bacteria of the genus Beijerinckia are selectively stimulated in the rhizosphere of sugar cane. Subsequently, Dobereiner (1970) showed a specific association of Azotobacter paspali with certain ecotypes of Paspalum notatum. Research on the general phenomenon of associative N_2-fixation in grasses received great impetus due to this work. Dobereiner & Day (1976) later reported a third system made up of tropical forage grass Digitaria decumbens with the N_2-fixing bacteria Spirillum.

The group of gram-negative spirilla has been reclassified into new genus Azospirillum (Tarrand et al, 1978). This bacterium has been intensively studied since that time and therefore serves as a representative example of associative N_2-fixing systems (Dobereiner & Baladani, 1979; Hubbell & Gaskins, 1984 and Klingmuller, 1982). The Azospirillum was suggested to associate preferentially with tropical grasses (Dobereiner, 1977) however recent research indicates its promiscuous nature. The organism is generally found whereever it is sought. Numerous reports now show that many other species of rhizosphere bacteria other than Azospirillum achieve high rates of N_2-fixation under optimum conditions. The list of associated microorganisms grows longer and includes genera of Bacillus, Enterobacter, Klebsiella, Erwinia, Azotobacter, Beijerinckia, Pseudomonas Campylobacter and Xanthobacter. (Balandreau, 1983).

The foremost problem in associative nitrogen fixation deals with the predominant fixer(s) in the rhizosphere of a particular plant. Counts of N_2-fixing bacteria are usually done on a selective medium by characterizing the presence of nitrogenase in dilutions of the initial sample. Isolations are then routinely be made using the highest dilutions exhibiting N_2-ase activity. In many instancs N_2-fixing bacteria have been isolated from actively C_2H_2-reducing root pieces incubated at low oxygen tension. This method first described by Dobereiner & Day (1976) is very efficient for isolating Azospirillum from active roots. Many problems are encountered using the above two methods. The starting material and the expression of results, the selectivity of the medium employed for enrichment and isolation and proliferation of N_2-fixation bacteria due to low redox potentials and root fermentation in isolation from excised roots are few of them. The nature of the carbon source used in N-free medium encourages the growth of different groups of bacteria. Rennie (1981) recommended the use of media containing a mixture of carbon sources. As far as rhizosphere bacteria are concerned, it would seem advisable to use C sources more similar to those used in situ by bacteria i.e., the root exudates. Based on these facts a spermosphere

model was proposed by Thomas-Bauzon et al (1982) in which source of energy and C for N_2-fixing bacteria is constituted by the exudates. This spermosphere model has been used with rice and maize with equal success. Moreover, identification of N_2-fixer is also troublesome as features of most of the diazotrophs are not yet fully defined. Many reports are available concerning the isolation and identification of dominant N_2-fixer in a particular rhizosphere yet few associations are reasonably defined (Dobereiner & De-Polli, 1980). These diazotrophic associations are the sugar cane Beijerinckia biocoenosis (Dobereiner, 1961); the Paspalum notatum - Azotobacter paspali rhizocoenosis (Dobereiner, 1970); the rhizocoenosis of certain wheat lines with Bacillus sp. (Neal & Larson, 1976), the biocoenosis of rice with Achromobacter like (now Pseudomonas) organisms (Watanabe & Barraquio, 1979); the various Azospirillum rhizocoenosis (Baladani & Dobereiner, 1979) and Campylobacter- Spartina alterniflora biocoenosis (McClung & Patriquin, 1980). The available methods are unable to sufficiently define the dominant N_2-fixer(s) in a particular rhizosphere. Immunofluorescence is perhaps the only direct method for studying specific groups (even strains) of microorganisms in nature (Bohlool & Schmidt, 1980). Preliminary attempts using immunofluorescence for autoecological studies of asymbiotic nitrogen fixers directly in soil and rhizosphere have been promising (De Ville & Tchan, 1970; Diem et al; 1977 and Schank et al; 1979).

Moreover, presence of N_2-fixers in the rhizosphere does not signify its importance to the plant. The extent to which nitrogen fixation by associated organisms benefits the plants has not been determined (Van Berkum & Bohlool, 1980). Major obstacle to study this problem is the absence of any specialized structure like that of nodule in the symbiotic association. Capacity to survive and multiply in the rhizosphere is a much more important performance characteristics than is the relative capability for N_2-fixation by pure culture. Hubbell & Gaskin (1984) described three characteristics namely survival capability, metabolic

capability and competative capability which determine the capacity to grow in the rhizosphere.

Intense intrest in associative N_2-fixing bacteria developed after some early investigations indicated that they grew and fixed nitrogen inside living plant cells. Evidence for the presence of asymbiotic nitrogen fixing bacteria inside the roots of the host has been indirect. The isolation of Azospirillum from the surface-sterilized roots of field grown grasses by Dobereiner & Day,(1976) was suggested as an indication of the presence of bacteria in the histoplane. Diem et al. (1978) reported that N_2-fixing bacteria could be isolated from surface-sterilized rice roots, but only if the roots were crushed to release the bacteria. Dobereiner & Day (1976) observed that root pieces of Digitaria exhibiting high nitrogenase activity after the pre-incubation period had cells which were packed with tetrazolium-reducing like particles. Likewise, Patriquin & Dobereiner (1978) using the vital stain TTC demonstrated that even the inner cortex, xylem and stele of maize roots were colonized by A. brasilense. The authors presumed that these infections occurred initially in the branched roots and then spread into main roots because high nitrogenase activity was often detected in highly branched roots with intact cortex. TTC-reducing bacteria inside the roots have also been observed in maize (Magalhaes et al., 1979); sugar cane (Patriquin et al. 1980) and in the roots of orchard plants (Subba Rao, 1983).

It is not clear whether these bacteria fix N_2 in-situ or contribute in any manner towards plant growth. Moreover, it is yet to be shown that those bacteria which inhabit the cortical cells, xylem vessel and stelar portions are identical to the ones isolated from roots on N-free selective media in the field grown plants.

There is no clear evidence that Azospirillum infect living plant cells (Hubbell & Gaskin, 1984). This also holds true for other associated N_2-fixing bacteria. Little information is available about the site and process of association between

diazotrophs and grasses. This subject has recently been reviewed by Patriquin et al., (1983). There are compelling evidence which accumulated during recent years have indicated that the bacterial cells infect moribund or dead cells of the root cortex (Umali-Gracia et al., 1978). This implies that N_2-fixer may not fix appreciable nitrogen within the root. The mucigel layer found on growing root tips is colonized by Azospirillum in gnotobiotic culture (Umali-Gracia et al., 1980). It has not been demonstrated in natural conditions but it probably occurs. Thus in mucigel, a suitable carbon substrate is at hand in an environment in which the levels of organic nitrogen and oxygen are low, and nitrogenase activity should not be repressed.

Numerous approaches have been used to study conditions that support growth of Azospirillum in the rhizosphere. As plants differ in the composition of their root exudates (Rovira, 1964 and Vancura & Hovadic, 1965) and since Azospirillum shows chemotaxis to oxidizable substrates (Barak et al., 1983), it can be postulated that chemotaxis may play a role in the adaption of these bacteria to their host. Chemotaxis of Enterobacter cloacae and Azospirillum lipoferum towards maize mucigel has been studied by Mandimba et al., (1984). Similar studies with 2 strains of A. brasilense and one isolate (= A. lipoferum ER15) from Kallar grass were carried out by Reinhold et al., (1985). These authors observed that chemotaxis was strain-specific in the genus Azospirillum. These studies will help in understanding the degree of specificity in the establishment of plant-bacteria association.

Another important question which has not yet been satisfactorily answered relates with the availability of enough energy for the N_2-fixers to sustain the energy intensive process of biological N_2-fixation. Tracer experiments with ^{14}C-labelled compounds have been used to determine rates at which plant metabolites become available to root-associated microorganisms (Bouton et al., 1984). Legume-Rhizobium system is an ideal association. Since associative N_2-fixation is not closely coupled to the plant, both energy transfer from the

plant to the bacteria and transfer of fixed nitrogen from the bacteria to the plant would be inefficient. Pulse-labelling experiments show that as much as 10 to 20% of the carbon fixed by leaves may be exported in one or another form from the root but only very small portion of it was metabolized by Azospirillum or similar bacteria (Hubbell & Gaskin, 1984). Nitrogen-fixing bacteria have not been shown to "leak" fixed nitrogen, rather they turn over nitrogen when cells die. The fixed nitrogen is released into the soil where it is available to the flora and fauna of the soil as well as to the plants. Use of ^{15}N-labelled Klebsiella and Azospirillum bacteria in mineralization studies have revealed that 60-70% of the applied bacterial nitrogen was incorporated into the first corn crop (Milam et al., 1984). Further studies on metabolic coupling that occurrs between the bacteria and roots are badly needed especially with in-vivo system.

Extensive studies are being made on the physiology of nitrogen fixing bacteria that associated with roots of grasses and substantial amount of information has been collected. However, most of these studies were made with pure cultures, and, therefore, the relationship between growth and efficient nitrogen fixation by Azospirillum or similar other N_2-fixers in roots of grasses is unknown. Among N_2-fixing bacteria, strain to strain differences exist in a number of characters such as tolerance to O_2 (Bergersen, 1984); production of plant growth substances (Brown, 1972; Gaskin et al., 1977 and Reynders & Vlassak, 1979); effect of combined nitrogen (Burris et al., 1978; Neyra & Van Berkum, 1977); denitrification and resistance to antibiotics (Nelson & Knowles, 1978; Neyra et al., 1977; Dobereiner & Baladni, 1979 and Zuberer & Roth (1982); chemolithotrophic growth (Malik & Schlegel (1981); use of different substrates e.g. simple sugars (Tarrand et al., 1978 and Martinez-Drets et al., 1984), pectic compounds (Tien et al., 1981), phenolic compounds (Chan, 1984 and Hussein et al., 1976); antigenic properties (DePolli et al., 1980) and lysogenic phages (Elmerich & Franche, 1982). Moreover, presence of plasmids have also been shown in N_2-fixing bacteria (Singh & Wenzel, 1982) and recent report of

Singh & Klingmuller (1984) present evidence that at least the nif-structural genes are located on these plasmids. These studies have contributed in better understanding the mechanism of plant-microbe co-adaptations and will provide a better matching of the pro-and-eu-karyotic partners in N_2-fixing associations.

METHODOLOGY AND PROBLEMS ASSOCIATED WITH MEASUREMENT OF NITROGEN FIXATION IN GRASSES

Sound methodology allows objective interpretation of data and also allows intelligent, rational hypothesis to be formulated. Evidence of associative symbiosis was earlier derived from studies based on increase in total fixed nitrogen in certain land areas (Day et al., 1975). These long term N-balance studies which ranged from 2-50 years are cumbersome and less senstive. With the advent of acetylene reduction assay (ARA) there was proliferation of data regarding nitrogen fixation in grasses and cereals. Almost all of the existing literature deals with the measurement of nitrogenase activity by the indirect acetylene reduction assay procedure. Excellent reviews on the methodology have been published (Rennie & Rennie, 1983; Turner & Gibson,1980;Van Berkum & Bohlool, 1980; Van Berkum et al., 1981 and Van Berkum & Sloger, 1984). Two principal procedures have been devised to determine nitrogen fixation in grasses. The methods consist of measurement with intact plants in soil or with disrupted samples.

Non-disruptive methods: In this method measurement are made with intact plants in soil without any disturbance during the determinations. Estimates of nitrogen fixation have been derived either from the change in total soil and plant nitrogen mediated by growth of fallow vegetation (Greenland, 1977) or by assiduous measurements of nitrogen input and loss in well designed N-balance experiments (Jenkinson, 1977). The limitation of this approach is that the method is indirect, inaccurate, and lacks senstivity (Van Berkum & Sloger, 1984). Nitrogen gain mediated by N_2-fixation might be more conveniently indentified using ^{15}N. This subject will be disscussed separately.

Disruptive method: Majority of the experiments on associative N_2-fixation have made use of this technique. Disruptive methods are techniques in which the soil surrounding plants is disturbed either by placing of cylinders which encloses the roots of the plants or in which plant tissue is cut for sub samples to be used for measurements of nitrogen fixation.

Soil core assay: Soil cores of grasses have been used to measure nitrogenase activity with acetylene reduction (Van Berkum & Bohlool, 1980). ARA has been performed in-situ in the field (Balandreau et al., 1977) or removed from the rhizosphere (Day et al., 1975 and Tjepkema & Van Berkum, 1977) or in pots containing greenhouse-grown plants (Harris & Dart, 1973 and Hirota et al., 1978). Factors affecting the nitrogenase activity associated with sorghum and millet in soil core assay have been studied in detail by Wani et al (1983). Limitations associated with soil core assay includes slow or poor penetration of C_2H_2 and C_2H_4 through soil (Van Berkum & Day, 1980); endogenous ethylene accumulation due to inhibition of ethylene oxidation by acetylene (Lethbridge et al., 1982 and Witty, 1979) and superinduction of nitrogenase activity due to inhibition of nitrogen fixation by acetylene (Van Berkum & Bohlool, 1980). A critical drawback common to all the techniques is the limitation that information about the precise location of nitrogen fixation could not be obtained although Dobereiner et al (1972) observed that measured nitrogenase activity of soil cores was mostly associated with the roots and rhizomes of the plants as exhibited by higher ARA values of component parts of the cores.

Excised root assay: It is most widely used assay in the study of associative symbiosis. Measurements of acetylene reduction with excised grass roots were also made to identify root tissues exhibiting higher rates of nitrogenase activity in order to obtain enrichment cultures in which the bacteria responsible for associative nitrogen fixation predominated (Dobereiner & Day, 1976). This is the only assay in N_2-fixing systems where nitrogenase activity could not be detected immediately (Van Berkum, 1980). A 8-18 h lag period has been reported

before nitrogenase activity by excised root commences. Thus overnight preincubation of roots without acetylene at a low partial pressure of oxygen has been adopted as a routine method to prepare samples for the measurement of nitrogen fixation by grasses (Abrantes et al., 1975). It was suggested by Dobereiner et al., (1972) that exposure of O_2 when roots were excised from the plants caused the observed lag. However, prepration of excised roots of P. notatum for nitrogenase activity in nitrogen did not reduce acetylene for the initial 12 h of assay. On the other hand excised roots of sorghum, after prolonged incubation were exposed to air for 1 h by Van Berkum (1980) did not exhibit a prolonged period of inactivity. In several other systems nitrogenase activity have been detected without long delay and even in the presence of air (Lethbridge et al., 1982; Van Berkum & Sloger, 1981 and Van Berkum et al., 1981).

The most serious criticism on the excised root assay with preincubation is the proliferation of the nitrogen fixing bacteria during this period. These observations have been made by several authors (Barber & Martin, 1976; Eskew & Ting, 1977; Okon et al., 1977b and Van Berkum, 1980). It was concluded from these studies that the prolonged incubation of washed excised roots of grasses at room temperature induces the proliferation of the nitrogen fixing bacteria leading to the development of nitrogenase activity in tissue which ordinarily does not support nitrogen fixation. However, Dobereiner (1977) has argued that proliferation of diazotrophs as a sole reason for higher activities of excised roots is not justified as higher numbers of Azospirillum was observed in young plants which did not fix nitrogen. Moreover, when nitrogen fertilizer was applied in the field higher counts were available although no nitrogenase activity in excised roots or the soil core was observed thus indicating the complexity of the system. Nevertheless it is suggested that preincubation period must be avoided during measurements of nitrogenase activity of excised roots of grasses.

Immediate detection of nitrogenase activity in excised roots of rice, wild rice and Spartina alterniflora have been reported (Van Berkum & Sloger, 1981 and Van Berkum et al., 1981). These observations prompted Van Berkum & Sloger (1984) to conclude that presence of root-associated nitrogen fixation in grasses is characterized by immediately detectable excised root acetylene reduction and inability of excised roots to reduce acetylene immediately upon collection would indicate that nitrogen fixation is not associated with them. Therefore this procedure can be recommended as a method to screen for nitrogen fixing plants.

Evidence for the presence of N_2-fixing bacteria inside of the roots of grasses has routinely been inferred by showing nitrogenase activity in the surface-sterilized excised roots. It has been recommended by Dobereiner (1980) that roots of grasses be surface sterilized with 1% chloramine T for 30 seconds to study the internally located bacteria. However McClung et al. (1983) reported that 1% chloramine- T for 1 h was not effective in preventing the recovery of nitrogen fixing bacteria from the root surfaces of S. alterniflora, maize and sorghum. They showed that treatment of 5% NaOCl for 1 h nearly eliminated the recovery of N_2-fixing bacteria from the rhizoplane of S. alterniflora. The same treatment, however, was less effective in reducing the recovery of nitrogen fixing bacteria from the rhizoplane of maize and sorghum. Use of ethanol and $HgCl_2$ have also been made in surface sterilizing of the roots. These studies indicate that no set procedure exist for surface sterilizing the roots and this technique must be evaluated for each plant before being used in studies of microbial ecology (Van Berkum & Sloger, 1984).

^{15}N-isotope techniques: ^{15}N isotope has traditionally been used primarily as a definitve test to prove that N_2-fixation has occurred. The $^{15}N_2$ atmosphere has been used successfully to prove N_2-fixation only in association with tropical grasses (De-Polli et al., 1977); sugar cane (Matsui et al., 1981; and Ruschel et al., 1978, 1981), sorghum and millet (Giller et al., 1984) and rice (Bin

Othman, 1984, Eskew et al.,1981, Ito et al., 1980 and Yoshida & Yoneyama, 1980). These studies showed that levels of N_2-fixation in these crops were low relative to legumes. Estimates for heterotrophic N_2-fixation associated with rice averaged 10 kg N fixed ha^{-1} (Eskew et al., 1981 & Yoshida & Yoneyama, 1980). Studies employing relatively long-term incubations (= 7 days) by Ito et al., 1980 and Yoshida & Yoneyama, (1980) may not differentiate between root associated heterotrophic N_2-fixation with subsequent transfer of a portion of this fixed N to the plant and more direct transfer. However, studies with sugar cane (Ruschel et al., 1975, 1978) and rice (Eskew et al., 1981) had incubation times less than 3 days. De-Polli et al(1977) detected ^{15}N enrichment only hours after initial exposure. In these cases, a more intimate association approaching a symbiosis may exist. Most of the fixed N_2 is concentrated in the roots, although with time translocation to the shoots does occur. As most of the studies on associative N_2-fixation have made use of ARA, there is an urgent need to experimentally establish N_2: C_2H_2 ratio. This have not yet been done with non legumes despite the fact that the theoratical 3:1 ratio of N_2:C_2H_2 is not acceptable under field conditions. The use of $^{15}N_2$ is a short term kinetic measurement like ARA. It is relatively expensive, difficult to handle for a longer period and as such is no more applicable to field quantification of atmospheric fixed-N.

The ^{15}N isotope dilution technique (Rennie & Rennie, 1983) has been applied to several natural ecosystems and the amount of plant nitrogen derived from nitrogen fixation (%Ndfa) was estimated. The technique of labelling the soil ^{15}N has been used by Owens (1977) to measure fixed N by isotopic dilution in field grown maize and sorghum. His results indicated that no associated N_2-fixation occurred with these crops. In contrast, Rennie (1980b) used isotope dilution in-vitro to determine that, when sufficient C substrate was available to bacterium, upto 38% of the maize plant N was derived from associated N_2-fixation by A. brasilense. Subsequently several investigators carried out ^{15}N isotopic dilution

experiments under defined laboratory conditions. Ndfa values for rice can reach between 20-35% (Ventura & Watanabe, 1982) and a value of 17% was reported for sugar cane (Boddey & Dobereiner, 1982). In case of P. notatum, Boddey et al., (1983) found Ndfa values ranging from 8-25%. For wheat, high values ranging between 15 and 30% were obtained for some genotypes inoculated with A. brasilense or a Bacillus sp. (Rennie et al., 1983). However, Okon, (1984) reported that no more than 5% of the nitrogen fixed by A. brasilense Cd inoculated to Setaria italica was incorporated in the plant host. Vose (1983) has described two major problems: the difficulty of applying the technique to obtain an integrated value for N_2 fixed by a crop like grass which is harvested several times during a growing season, and secondly, selecting a suitable non fixing control test crop with similar growing season and rooting habits. However, uptill now it is the best available technique to evaluate the associative nitrogen fixation. It could especially be used in screeing different varieties or cultivars of host plant for supporting nitrogen fixation.

The use of natural ^{15}N abundance data for the estimation of nitrogen fixation in natural (Rennie et al., 1976) and associative systems like sugar cane (Ruschel & Vose, 1977) has been achieving greater recognition. However, in addition to the problems encountered in isotopic dilution technique this method has many other disadvantages. Quantifying nitrogen fixation from ^{15}N abundance data is tricky because of isotope discrimination between roots and shoots, denitrification which increase the ^{15}N and very minute differences between fixing and non-fixing system. Present day very stable instrumentation, with automatic sample changers permits handling relatively large numbers of sample and it is hoped that in future more research will be counducted to establish this technique.

It appears that much more information about N_2-fixation rates of soil-grown plants is needed and none of the available methods could truly measure N_2-fixation in the field (Beringer, 1984). This is more true for diazotrophic associations

and refining of methodology for studying such associations with improved nitrogenase (acetylene reduction) procedures and greater use of ^{15}N methods still remains a priority area.

INOCULATION EXPERIMENTS: The inoculation of crop plants with bacterial preparations is generally necessary because many soils lack specific microorganisms shown to or believed to stimulate productivity. Legume crops are frequently inoculated with Rhizobium to promote effective nodulation and efficient nitrogen fixation. Free living nitrogen fixing bacteria have also been inoculated into the rhizosphere of crop plants to increase the productivity by increasing the levels of available N in the soil. Earlier studies were carried out in Russia with Azotobacter chroococcum and this work has been reviewed by Brown (1974). Recent studies on associative system raised the hope that nitrogen fixing bacteria which grow in association with crop plant roots have the potential to reduce nitrogen fertilizer requirements in many agricultural areas. Such observations stimulated the inoculation experiments on cereals and forage grasses. Many workers in various part of the world have reported favourable responses to inoculation with Azospirillum and other N_2-fixing bacteria, however, not all experiments have been successful. Inoculation studies were reviewed by Boddey & Dobereiner (1982) Brown (1982)and Okon (1984).

Extensive experimentation in the phytotron and field over the last 8 years, carried out under diverse environmental and soil conditions in Belgium (Reynders & Vlassak, 1982), Brazil (Boddy & Dobereiner, 1982), Egypt (Hegazi et al., 1981), India (Subba-Rao, 1981), Israel (Kapulnik et al., 1983) and USA (Albrecht et al., 1981, Smith et al., 1976, 1984), has demonstrated that significant increases (5-30%) over controls in yields of sorghum, maize, wheat and several forage grasses, could be obtained following inoculation with Azospirillum. Increase in dry matter and total nitrogen content was observed

in some instances however results were found to be highly variable. Initial inoculation experiments were carried out by Smith et al., (1976, 1977). Their results indicated that 2 out of 40 genotypes responded to an inoculation of Azospirillum. One genotype of D. decumbens and one of P. maximum yielded at 163 and 153% respectively, of uninoculated controls in the presence of these bacteria. No response to inoculation was reported with other 38 genotypes tested. Barber et al (1979) and Sloger & Owens (1978) studied millet and cereal crops respectively in which yields were not stimulated by inoculation. Albrecht et al., (1981) studied maize crop and their data was mixed; some genotypes responded whereas others did not. Reports from Bahamas (Taylor, 1979), India (Subba-Rao, 1981), Egypt (Hegazi et al., 1981) and Belgium (Reynders & Vlassak, 1982) showed yield responses in various grass crops and forages. Consistent yield responses in maize and Setaria italica (Cohen et al., 1980) wheat, sorghum and panicum (Kapulnik et al., 1981) were observed in Israel when inoculated with Azospirillum.

Inoculation experiments were mostly carried out by using Azospirillum as an inoculant. It was observed by Boddy & Dobereiner (1982) that most of the inoculations were performed with A. brasilense nir^+ strains such as Sp 7 or Cd on C_4 plants (= maize, sorghum etc.) which, under natural conditions (Baladani & Dobereiner, 1980), share more specific association with A. lipoferum. De-Polli & Dobereiner (1980) reported that 58% of the maize isolates were A. lipoferum and 100% of the isolates of wheat and 96% of the isolates from rice were A. brasilense nir^-. A. brasilense nir^+ was not found to predominate in any plant roots. During last decade very few inoculation experiments were performed by using bacteria, other than Azospirillum (Okon, 1984). Klebsiella pneumoniae have recently been used to inoculate the plants (Lethbridge & Davidson, 1983; Wood et al., 1981).

The primary problem in any inoculation experiment deals with the establishment of the introduced bacteria in a competative environment of the rhizosphere. Earlier studies have not dealt this aspect. In the absence of any specialized structure (= nodule) this task is not easy, however, several markers like that of streptomycin resistance have been used with inoculant bacteria to study the colonization. Dobereiner & De-Polli (1980) reported use of streptomycin resistant Azospirillum to inoculate field grown maize and wheat. Reisolation studies revealed that more than 80% of the soil and rhizosphere isolates belonged to inoculated bacteria. Smith et al (1984) observed a rapid decline of A. brasilense population. Inoculated bacteria dropped down from $5x10^3$ to 10^2 within 5 weeks which showed that these bacteria did not become established in the soil in high numbers. Schank et al., (1983) carried out series of experiments in which monoxenic root-bacteria associations were first studied in test tubes containing agar. Later on active ARA associations were transferred to mason jar assemblies and lastly to the field. The degree of association was evaluated by visually rating the amount and location of bacterial growth and by assaying for ARA. Their results suggest that the complex natural systems are better able to persist in competitive situations, than the inoculated associations established from selected N_2-fixing strains. The data which have gathered uptill now indicate that bacterial inoculation is not yet as effective as some highly active natural systems due to the non-competative ability of inoculant to survive in sufficient number in the root zone of the plant.

Root colonization, which results in an efficient association between the plant and the bacteria, appears to involve a degree of specificity which may also involve the plant (= host). Enough data are available demonstrating that plant genotypes could be selected for an increased nitrogen fixation. It was pointed out some time ago that response to inoculation varies greatly with plant cultivar and genetic line (Boddy & Dobereiner, 1982; Subba-Rao & Dart,

1981, Van Berkum & Bohlool, 1980; Von Bulow & Dobereiner, 1975 and Vose, 1983). For spring wheat (Triticum aestivum) it was observed that varietal lines with chromosome 5 substitution between the Cadet and Rescue varieties gave a good response to inoculation with A. brasilense Sp 7 and a Bacillus sp (Rennie & Larson, 1979). This was also confirmed by ^{15}N isotopic dilution measurement (Rennie & Larson, 1979 and Rennie et al., 1983). For maize (Ela et al., 1982 and Von Bulow & Dobereiner, 1975) and rice (Hirota et al., 1978 and Sano et al., 1981), the screeing of a large number of genotypes led to the selection of plants which developed higher rates of acetylene reduction. In addition, after genetic crosses in maize and analysis of the F1 progeny, lines were selected for increased nitrogen fixation in association with Azotobacter vinelandii OP (Ela et al, 1982). For rice, genetic analysis of plant genotypes was in agreement with the existence of several genes involved in the process, some characters likely being recessive (Iyama et al., 1983). Rennie & Rennie (1983) have suggested "nis" as the designation of the plant genotype involved in the nitrogen fixation efficiency for nitrogen fixation supportive trait. Still more evidence for varietal differences comes from the work of Ruschel & Ruschel (1979) on sugar cane and on pearl millet by Bouton et al., (1979). Thus like Rhizobium-legume symbiosis, bacterial and plant genes are likely involved in the association and that the genetic modification of one of the partners would improve the association. Plant breeding should allow the selection of lines on the basis of nitrogen fixing efficiency which possibly enhance colonization and N_2-fixation by root-associated bacteria (Hubbell & Gaskin, 1984).

The most important question regarding the yield response due to inoculation is that beneficial effect on plant growth was due to N_2-fixation and/or some other factor(s) were also involved. Brown (1972) while reviewing the Russian work on soil inoculation with Azotobacter and other bacteria observed that plant growth hormones produced by the microorganisms could be responsible

for the observed plant response. In later experiments Brown (1976) confirmed the role of plant growth regulators on the various grasses and other crops when inoculated with Azotobacter. Tien et al., (1979) demonstrated production by an Azospirillum strain of auxin (IAA), cytokinins, and substances tentatively identified as gibberellins. Reynders & Vlassak (1979) found that IAA was formed in cultures of A. brasilense after adding tryptophan to the medium and they suggested that auxins were responsible for the increased growth of plants with many A. brasilense cells on or inside their roots. These studies were with pure cultures, and there is as yet no definite proof that the bacterium produces these substances when growing in the rhizosphere. Hubbell & Gaskin (1984) have found that Azospirillum inoculation increased the cytokinin content of xylem exudate from decapitated plants. In several studies growth responses were also observed when plants were supplied with only growth substances, bacterial filtrate or heat killed bacterial suspension (Brown, 1982). A growth response in maize by the inoculation of a nif^- mutant of Azospirillum has also been reported (O'Hara et al., 1981). Van Berkum & Sloger (1984) urges closer examination of the mechanism(s) by which plant productivity was enhanced when Azospirillum was inoculated in the presence of applied fertilizer nitrogen. According to Brown (1982) the production of plant growth substances (PGS) is one of several mechanisms by which inoculation of nitrogen fixing bacteria may cause growth responses. Therefore, it is quite possible that several factors other than microbial production of PGS by Azospirillum contributed to the observed inoculation responses.

It was observed by Tien et al., (1979) that Azospirillum inoculated seedlings of Pennisetum developed more roots and root hairs. Kapulnik et al., (1981) also observed a marked development of roots of Setaria italica. Okon (1984) reported the same phenomenon in wheat growing in pots, hydroponic systems and by sampling roots at different times during growth from field experiments. A recent report by Lin et al., (1983) indicates that plant nutrient

uptake could be stimulated by inoculation with A. brasilense. They observed that inocualtion of corn seeds with A. brasilense significantly enhanced (30-50% over controls) the uptake of NO_3^-, K^+ and $H_2PO_4^-$ into 3-4 days old and 2 weeks old root segments.

Following inocualtion Azospirillum colonizes and proliferates "on and in" the roots, and it causes an enhanced root development, and mineral and water uptake by the roots although the mechanisms through which it happens have not yet been demonstrated. This results in faster accumulation of dry matter in the plant and in many instances produces higher crop yield. As a result of inoculation BNF apparently contributes small amounts of combined N to the plant whereas its contribution to the soil might be more significant. The available data indicates that the lack of consistent inoculation response is the major obstacle to practical use of bacterial inoculation of grass crops. It is also evident that N_2 fixation is probably not the only mechanism producing the increased yields. The major question still remains as to what extent the nitrogen has been fixed by the inoculated roots is incorporated and contributes to the yield. Some recent studies have made use of $^{15}N_2$ (De-Polli et al., 1977; Eskew et.al.,1981 and Rennie & Rennie, 1983). Still much remains to be done in order to elucidate the colonization dynamics, the mechanism of action, the methods of application and the optimal bacteria-plant-soil type combination. The recent advances discussed in this chapter show clearly the need for better understanding of host-strain interactions before it will be known which characteristics of Azospirillum, Bacillus, or other diazotrophic bacteria are of advantage in the various biocoenoses.

The foregoing survey of literature clearly reveals the importance of the association between N_2-fixing bacteria and non-leguminous plants which do not form nodules. There is no longer any doubt that N_2-fixation actually occurs with grasses as numerous $^{15}N_2$ reduction studies have clearly proved its existence. In view of the obvious importance of Leptochloa fusca (L.)

Kunth (kallar grass) as primary colonizer of salt affected soils of Pakistan, some studies on its diazotrophic biocoenosis have been carried out which are being reported in this thesis.

CHAPTER-2. MATERIALS AND METHODS

2.1 PLANT MATERIAL:

Leptochloa fusca (L.) Kunth (Syn. Diplachne fusca (L.) belongs to family Gramineae. Its classification and botanical description is described by Ahmed (1954). Locally this grass is known as Kallar grass. Plant material alongwith large soil cores of 30 cm diameter upto depth of 20 cm were transported to the laboratory. Samples were analysed on the same day.

2.2 LOCATIONS:

Kallar grass samples were collected from two sites.

2.2.1 Site-I Biosaline Research Sub-Station (BSRS), Lahore:

The place is situated at 73.8° E longitude and 31.5° N latitude. Average maximum summer temperature is 41.1° C and average minimum winter temperature is 4.4° C. Average rainfall is 250 mm which mostly occurs in July and August (monsoon months). Soil of this location belongs to Kurrianwala series and silt loam in texture, strongly saline sodic with pH ranges from 8-9.6 and $E.C_e$ from 1.65-33 m mhos Cm^{-1}.

2.2.2 Site-2 Shahkot(Faisalabad):

Kallar grass was found growing along Faisalabad-Lahore road (48 km.) The site is located at 73.5° E longitude and 31.8° N latitude. Average rainfall is 250 mm which also mostly occurs in July and August (monsoon months).Mean maximum summer temperature is 41.1° C and average minimum temperature is 4.4° C.Soil of this location belongs to Gajiana series and loam to silt loam in texture, slightly sticky and slightly plastic. Average pH ranges from 8-10 and $E.C_e$ from 3-40 m mhos Cm^{1}.

2.3 ECOLOGICAL STUDIES:

Plant samples were collected from two sites at every month during year 1983-84. Large soil cores of 30 cm diameter having a plant of Kallar grass was dug to depth of 21 cm and the whole core was brought to laboratory. Atleast 3 plant samples were brought and were analysed on the same day.

2.3 1 Soil Core Studies:

Soil cores of 23 mm x 70 mm upto depth of 20 cm were taken by cork borer as shown in Fig. 1 . Each core was placed in a plastic container

(250 ml, 3.5 x 10 cm). The lid of each container was fitted with a serum stopper in the centre and rubber sheath was placed inside the lid to make them gas tight. Each jar was evacuated by using rotary pump (Disto pump, USA) and reflushed with nitrogen gas (Pak Oxygen Limited, 99.9% pure). After repeating this process thrice, 12% (V/V) acetylene (C_2H_2) and 1% air (V/V) were injected replacing the same volume of N_2-gas. Soil cores were incubated at 30°C ± 1 in an incubator (Memmert, W.Germany) for 18 h. Samples were analysed for the production of nitrogenase mediated ethylene (C_2H_4). Two controls, one bottle with C_2H_2 but without soil core and other with soil core but without addition of acetylene were also included during each assay. Results are described in nanomoles C_2H_4, h^{-1}, soil $core^{-1}$.

2.3.2 Excised Root Assays:

Acetylene reduction assays on excised roots of Kallar grass as described by Dobereiner & Day (1976) were done every month. Following fractions were analysed.

2.3.2.1 Unwashed Roots:

The soil adhereing to the roots was gently removed. A portion of healthy roots was cut into small pieces by sterilized scissors and transferred into wide mouth McCartney vial (30 ml) fitted with a silicone septum. The gas phase was replaced by N_2 by repeated evacuation and reflushing with N_2-gas. Ten percent air (=2% O_2) was injected into vials and left overnight in an incubator set at 30°C. In case of dry roots, one drop of sterilized distilled H_2O was also added. After overnight pre-incubation, samples were again evacuated and refilled with N_2 gas. Acetylene (12% V/V) and air (1%, V/V) were introduced by replacing same volume of N_2-gas . All vials were incubated at 30°C for 18 h. 100 ul gas samples were analysed on a gas chromatograph (GC) for the reduction of acetylene into ethylene. Two control samples as described for soil core study were also maintained.

2.3.2.2 Washed Roots:

Another portion of roots was thoroughly washed with tap water till the supernatant was clear of soil particles. Roots were finally washed with sterilized distilled water and small pieces were transferred to 30 ml McCartney vials. Rest of the procedure was same as described in section 2.3.2.1. for unwashed roots.

2.3.2.3 Surface Sterilized Roots:

Some portion of washed roots was dipped in absolute ethanol (C_2H_5OH, E. Merck) for one minute and repeatedly washed with sterilized distilled water. Atleast seven washings were performed and roots were transferred to McCartney vials. Rest of the procedure was same as described in section 2.3.2.1.

2.3.2.4 Direct Root Assay:

Acetylene reduction assay of excised roots without preincubation was performed as described by Van Berkum (1980). Root system of 3 plants were made free of adhering soil and small pieces of roots were transferred to McCartney vials (30 ml). Remaining procedure was same as described in section 2.3.2.1 except that C_2H_2 and air were introduced just after flushing with N_2 gas.

2.4 ENUMERATION OF N_2-FIXING BACTERIA:

Quantitative estimation of N_2-fixing microorganisms was made by two methods namely (MPN) most probable number (Alexander, 1965) and plate count. MPN was estimated by positive acetylene reduction assay (ARA) and appearance of pellicle or bacterial growth in the vials. Estimates from two sites (section 2.2) were repeated every month. For enumeration, root system of Kallar grass was divided into rhizosphere (RS), rhizoplane (RP) and histoplane (HP) as described by Dommergues (1978). Large soil cores of 30 cm diameter having a plant of Kallar grass was dug upto depth of 20 cm and the whole core was brought to the laboratory. The fractions of RS, RP and HP were obtained as shown in flow diagram (Fig.2). The detailed procedure is as follows:

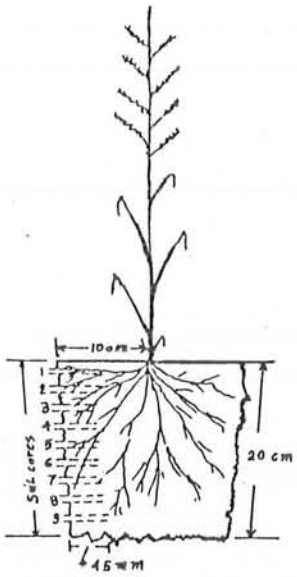


Fig.1. Diagramatic presentation of rhizosphere of Leptochloa fusca showing soil core sampling positions

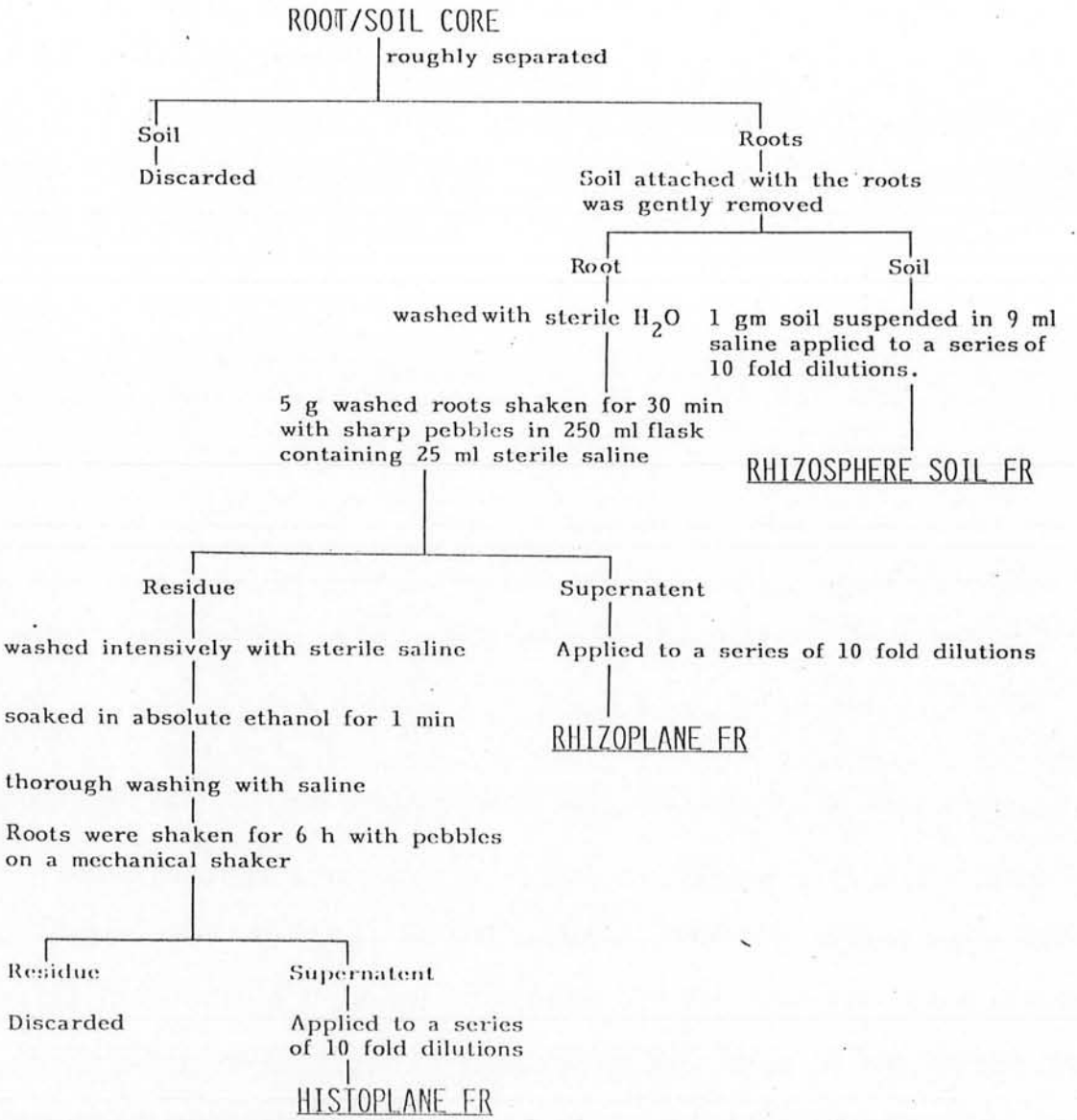


Fig.2. Procedure for fractioning root/soil core sampling for enumeration of N_2-fixers.

2.4.1 Rhizosphere (RS):

For rhizosphere (RS) enumeration, 1 g of soil adhering to roots was suspended in 9 ml saline (8.85% NaCl aqueous solution) and dilution series were made. Inoculation from 10 fold dilution series were made in 5 replicates of each dilution on modified combined carbon medium (CCM) of Rennie (1981). CCM was supplemented with 0.2% Agar (Difco) and contained in 17 ml McCartney vials for MPN. After 24 h of incubation at 30°C, pellicle formation or turbidity was observed as a sign of growth and recorded. To the same vials, serum stoppers (13 x 18 mm) were placed after removing the cotton plugs in a laminar flow (Tekno Labo; Italy). Acetylene 12% was injected after replacing same amount of air and acetylene reduction assay (ARA) was performed after 24 h of incubation at 30°C. One positive control and a blank sample were also included as a check.

The most probable number of organisms in the original samples were estimated as described by Alexander (1965) and table of Cochran (1950) for use with 10 fold dilutions and 5 tubes per dilution was consulted.

2.4.2 Plate Counts:

Plate counts for each dilution were also made and pour plate technique was employed. Plates of CCM medium supplemented with 1.5% agar were made. Tubes containing 5 ml of CCM with 0.7% agar were kept in water bath (50°C) and 0.2 ml of inoculum from each dilution was added. After thorough shaking, contents were poured on to the plates and incubated at 30°C for 48-96 h. Colonies were counted on colony counter (Tekno, Italy). Four plates of each dilution were used.

2.4.3 Rhizoplane (RP):

For rhizoplane fraction, the roots were washed with sterile distilled water till the supernatant was clear of soil particles. The 5 g washed roots were then shaken for 30 minutes with sharp pebbles in 250 ml Erlenmeyer flask containing 45 ml sterile saline and the turbid supernatant was taken as RP fraction. Inoculation from 10 fold dilution series and plate counts were made as described in section 2.4.1 and 2.4.2.

2.4.4 Histoplane (HP):

For obtaining histoplane fraction, the residual roots of RP were again washed intensively with 0.85% sterile saline and then soaked in absolute ethanol for 1 minute. After thorough washing with saline, roots were shaken for 6 h with pebbles on a mechanical shaker which macerated the roots completely. Supernatant was taken for making HP dilution series. Rest of the procedure was same as described in section 2.4.1 & 2.4.2.

2.5 MICROBIOLOGICAL STUDIES:

Isolation of N_2-fixing bacteria was made and some of the isolates were thoroughly characterized by common morphological, biochemical and nutritive tests. Mol % (G+C) was also determined. Confirmation of N_2-fixing ability of some isolates was done by using ^{15}N-enrichment method. Effects of various physiological factors viz combined nitrogen(NO_3^-, NH_4^+ and yeast extract), pH and NaCl + pH, on nitrogenase activity of three isolates were also determined.

2.5.1 Isolation of N_2-fixing Bacteria:

Nitrogen fixing bacteria were obtained by enrichment method during the enumeration in RS, RP and HP fractions. Isolations were made by streaking the vials on to nutrient agar (Difco) plates. Single colonies were picked up and rechecked by acetylene reduction assay.

Isolations were also attempted from positive ARA vials containing roots as described by Von Bulow & Dobereiner (1975). Small root pieces (2 cm long) were inoculated on to N_2-free semi-solid medium (5 ml in 17 ml McCartney vials) of combined carbon medium (Appendix 1) and incubated for 2 days at 30°C. ARA was performed after incubation period. Microorganisms responsible for N_2-fixation were isolated from positive ARA vials by streaking a loopful on nutrient agar plates.(Appendix 2 Single colonies were rechecked for nitrogenase activity by acetylene reduction test. Cultures were given code numbers and maintained on nutrient agar slants at 5°C and recultured after every 3 months.

.5.2 Characterization of Isolated Bacteria:

Isolates exhibiting higher activity were selected for further studies. Bacteria were subcultured from the maintenance medium, grown for 24-48 h in nutrient broth and inoculum from this was used for common identification tests. Standard morphological, biochemical and nutritive tests as described in Manual of Methods for General Bacteriology (1981) and Practical Manual for training course, Brono, Czeckoslavikia, (13-25 July, 1979).

2.5.3 Commercial Identification Kits:

Ready and easy to use commercial identification kit of api-20 E (api-System, France) containing 20 test of various biochemical characters and api 50CH for testing acidification of 49 carbohydrate substrates were also used. For api-20 E, one day old culture was suspended in the api-20 suspension medium. For inoculating api-50CH gallaries, 24-48 h old colonies were suspended in api-50 medium (Appendix 3) and change in the colour of indicator (Phenol red) was observed after 4,8,12 and 24 h.

2.5.4 Mol % (G+C) of Bacterial DNA:

Isolation and purification of bacterial DNA for mol % (G+C) determination was carried out by the method of Marmur (1961). Base composition of DNA sample was measured by thermal denaturation (T_m) method of Marmur & Doty (1962) and Mandel & Marmur (1968). Hyperchromic shift ws observed by a UV-VIS microprocessor controlled Gilford spectrophotometer (System 2600) fitted with a fixed block of four cuvetts (0.3 ml each). Instrument was attached with Gilford Thermoprogrammer 2527 and Hewlett & Packard 7225 A recoder. Mol % (G+C) contents were calculated by equation of Mandel & Marmur (1968)

$$(G+C) = 2.44\ (T_m - 53.9)$$

Escherichia coli K 12, with known (G+C) content of 51.7 was used as a reference strain. Base composition of three isoltes, NIAB-I, C-2 and isolate-2 were determined.

2.5.5 ^{15}N-Enrichment Studies of Bacterial Isolates:

Cultures of NIAB-I, C-2, ISO-2 and Azospirillum brasilense sp 7 (a positive check) were grown in semi-solid CCM. 20 ml medium was distributed in 50 ml flask with narrow mouth for placing of serum stoppers (13 x 18 mm). Parallel samples were maintained to observe the onset of acetylene reducing activity. After 36 h of growth, cotton plugs of one set of conical flasks were replaced with serum stoppers. Flasks were evacuated and filled with argon gas. After repeating this process for seven times following mixture was added (V/V):

98 % at.ex. ^{15}N = 30%
(Prochem Ltd. England)

O_2 = 1%

Ar = 69%

Flasks were incubated at 30 $\pm$ 2 °C for two days. At the end of incubation period the N-analysis was carried out by Kjeldahl method (Bremner, 1965). Distillates were collected in 0.02 N HCl and after back titration with 0.02 N NaOH on a E-526 Titrator (Metrohm, Switzerland) samples were concentrated on a rotary vaporator (Buchi, Switzerland) for ^{15}N analysis. Dumas method (Fielder & Porksch, 1975) was used for the production of N_2-gas in the discharge tube. $^{28}N/29_N$ ratios were determined on ^{15}N emission spectrometer (^{15}N-Analysator NOI Statron, E.Germany).

2.6 NITROGEN DETERMINATIONS:

2.6.1 Total N: For the determination of total N in solid or liquid samples the microkjeldhal method (Bremner, 1965) was used which is briefly described.

A known quantity of the test sample was weighted into the digestion flask (Standard Microkjeldahl digestion System of Tecator, Switzerland) to which was added 5 ml of concentrated H_2SO_4 and 1 g of the disgestion mixture (mixture of finely ground 100 g K_2SO_4, 10 g $CuSO_4$ and 1 g selenium). Digestion was carried out for 30 minutes after clearing of the digest. The digested material was steam distilled

after addition of 40% NaOH solution. The assembly was attached with steam generator. The NH_3 released was collected in 5 ml of boric acid indicator solution until the final distillate was 35 ml. The distillate was titrated against N/100 H_2SO_4. Boric acid indicator solution was prepared by dissolving 20 g boric acid powder in about 900 ml distilled water and 20 ml of mixed indicator solution (0.099 g bromocresol green and 0.066 g methyl red in 100 ml ethanol) was added to it. The pH of the solution was adjusted to about 5.0 by adding 2.5 ml of 0.1 N NaOH solution. The solution thus prepared attained a reddish purple colour. It was made to 1 liter volume by adding distilled water and the solution was thoroughly mixed before use. In some analysis, automatic titrator (E.526, Metrohm, Switzerland) was used and only boric acid solution (without indicator) was taken in the collecting flask.

2.6.2. Calculations for Nitrogen:

Nitrogen content of the distillate was calculated by using the formula:

1 ml of N/100 acid = 0.00014 g of N as NH_3 or
= 0.14 mg of N as NH_3 or
= 140 ug of N as NH_3.

2.7 ^{15}N DETERMINATIONS:

Normally the $^{14}N/^{15}N$ ratio of a system is determined by means of mass spectrometric or emission spectrometric techniques using nitrogen gas generated from the sample. The number of ^{15}N atoms in the total amount of nitrogen atoms in percent is called atom % or % abundance. Both mass and emission spectrometry determine the $^{14}N/^{15}N$ ratio in one operation. The ^{14}N and ^{15}N atoms in the gas are paired to form the nitrogen molecules $^{14}N_2$, $^{14}N\ ^{15}N$ and $^{15}N_2$. Both mass and emission spectrometric methods provide output signals which are proportional to the number of the three types of molecules. In the present studies mass spectrometer Varian GD 150 and NO1-5 optical ^{15}N analyser (VEB Statron) were used for ^{15}N determinations.

2.7.1 Sample Prepration for Emission Spectrometry:

The titrated solution (obtained as above) was acidified with hydrochloric acid and evaporated to small volume (2-3 ml) using a water bath. Optical emission spectrometric analysis requires 1-10 ug of N in the sample.

A modified Dumas method (Fiedler & Proksch, 1975) was used for generating N_2 gas. The appropriate amount of sample in the prepared solution as described above was placed in a quartz capillary (1.5 mm o.d. and 0.8-0.9 mm i.d.), dried at 40-50 $^{\circ}$C and inserted into discharge tube. A 7-10 mm long copper oxide wire (1-2 mg) and a calcium oxide briquette (3 mg CaO) which was activated by pre-heating to 1000 $^{\circ}$C were also added. The discharge tube was cleaned by heating with hot air from hair dryer and evacuated to 10^{-2} torr. The tube was then sealed off with a hand torch and checked for any leaking by Tesla coil. The contents of the tubes were carefully mixed and tubes were placed in a muffle furnace (Gallenkamp, England) for 4-5 h at 550 $\pm$ 5 $^{\circ}$C in a horizontal position. The $^{14}N/^{15}N$ ratio was determined by NO1-5 optical ^{15}N analyser (VEB Statron) and the % abundance was then calculated from the equation as described in section 2.7.3.

2.7.2 Sample Prepration for Mass Spectrometry:

The titrated solution was acidified with hydrochloric acid and evaporated to small volume (2-3 ml) using a water bath. Mass spectrometric analysis requires 1-2 mg of N in the sample. In samples with lesser amounts of total N, ammonium sulphate was added to bring the N content to the desired level.

The sample prepared as above, was placed in one leg of the Rittenberg tube and in the other 1 ml sodium hypobromite solution was added. Sodium hypobromite solution was prepared as follows:

200 g of NaOH was dissolved in 300 ml of water and the solution was cooled in the ice. Half of the solution was transferred to a 500 ml wide-mouth Erlenmeyer flask immersed in crushed ice and 60 ml of bromine added over a period of 30 minutes

with vigorous stirring; the temperature of the solution was kept at 5°C. The remainder of NaOH solution was added, stirred the contents for a few minutes, stoppered and kept in the refrigeration for 4-6 days. The precipitate was removed by suction filtering through a sintered funnel, an equal amount of 0.2% solution of pottasium iodide in water added and the solution stored in a tightly stoppered bottle in the refrigerator.

The tube was attached to the evacuation assembly, its contents frozen by immersing the liquid containing portion in liquid nitrogen. The tube was detached from the evacuation assembly after evacuation with its tap closed. It was then attached to the inlet system of the mass spectrometer and the contents liquified by warming the tube with the hands. The sample and reagent were then mixed by turning the tube up and down. The reaction yielded N_2 gas. The contents of the tubes were again frozen and the gas allowed to enter the spectrometer by opening the valve.

2.7.3 Calculations for ^{15}N:

^{15}N abundance in the samples was calculated by using the formula:

$$h_f = \left(\frac{m_s}{m_f} + 1\right) h_m - \left(\frac{m_s}{mf}\right) hAS$$

where

ms = mg N in the added ammonium sulphate
mf = mg N in the sample
hm = measured abundance of the sample
hAS= abundance of the added ammonium sulphate

% abundance can be derived from the formula

$$\frac{^{30}N + \frac{1}{2}\,^{29}N}{(^{28}N + {}^{29}N + {}^{30}N)} \times 100$$

which represents the ratio of the ^{15}N atoms to the total number of nitrogen atoms expressed in %. For practical purposes, however, only the ^{28}N and ^{29}N signals are measured. The % abundance can then be calculated from the equation:

$$^{15}N \text{ abundance} = \frac{100}{2R + 1}$$

where R is the ratio of peak heights found for ^{28}N and ^{29}N.

When ^{15}N abundance in the sample is known, atom % ^{15}N excess can be determined by substracting the natural abundance of ^{15}N i.e., 0.366 at % from that of the sample.

2.8 ACETYLENE REDUCTION ASSAY:

Acetylene reduction assay (ARA) was performed as described by Hardy et al (1968).

Ethylene (C_2H_4) and acetylene (C_2H_2) gases were measured on a gas chromatograph (Carlo-Erba Fractovap Series 2150) fitted with 0.75 m x 2 mm stainless steel column packed with Porapak-N (80-100 mesh, Water Associates Inc. USA) using flame ionization detector (FID). Column temperature was maintained at 50 °C and temperature of injection port was set at 100 °C. Nitrogen was used as a carrier gas at a flow rate of 30 ml min^{-1} while for FID, hydrogen gas at a flow rate of 28 ml min^{-1} and air at the rate of 300 ml min^{-1} were used. Hydrogen gas (99.99% pure) was obtained from hydrogen generator (Teledyne HG 501) while nitrogen and air were procured from Pakistan Oxygen Limited (POL). Oxygen trap (Phase Sepration Ltd., UK) was used for nitrogen gas. Filters packed with silica gel were used for all three gases to avoid water vapours and other contaminants. Perkin-Elmer-56 recoder set at 1 mV current and chart speed of 5 mm min^{-1} was used. Gas sample (100 ul) was injected by gas tight syringe (Hamilton, USA). C_2H_4 levels in the samples were quantified by measuring peak heights relative to standards. Standard ethylene gas (1% C_2H_4) in nitrogen (V/V) was obtained from Linde Technische Gase (W.Germany). Concentration in nano (10^{-9}) or micro (10^{-6}) moles was determined by using the common gas law

$$PV = nRT$$

where P = pressure in atmosphere, V = volume in liters, n = number of moles,

R = gas constant (0.08206 lit atm deg^{-1} $mole^{-1}$) and T = absolute temperature ($t\ ^{\circ}C + 273$).

Rate of acetylene reduction to ethylene was converted into rate of nitrogen reduction by the following formula.

$$mg\ N = \frac{mM\ C_2H_4 \times 28}{3}$$

2.9 CHEMICALS:

All chemicals were of analytical grade and obtained from E. Merck, Difco and Sigma Chemical Company. ^{15}N gas (98% ^{15}N) and 50% at.ex. $^{15}NH_4{}^{15}NO_3$ were procured from Prochem Ltd, BOC, Deer Park Road, London, U.K. Nitrogen and acetylene gases were obtained from Pakistan Oxygen Limited (POL) while ethylene and argon gases were purchased from Linde A. G. Unterschleisheim, W. Germany.

CHAPTER-3. ECOLOGY OF N_2-FIXING SYSTEM OF *LEPTOCHLOA FUSCA* (L) KUNTH

The rhizosphere is one of the natural habitats (or micro-habitats) which is influenced by various plant, microbial, climatic and edaphic factors (Blandreau & Knowel, 1978). The term rhizosphere was first introduced by Hiltner in 1904 to designate the region of soil influenced by plant roots. It is a well known fact that root exude organic material. Barber & Martin (1976) and Martin (1977) by using ^{14}C reported that carbon released from roots growing in soil amounted to some 20% of the total plant dry matter. Rovira et al (1979) divided the exuding organic material of the plant into five different categories. Root exudates can be used by microorganisms and as a consequence the microbial population close to the root is higher than at some distance from the root. The R/S ratio, which is the ratio of numbers of organisms per gram of rhizosphere soil to the numbers of organisms per gram of control soil has been widely used to express the extent to which the plant roots affect the numbers of microorganisms. The R/S ratio, is commonly present in the range of 5-20 and in some extreme cases it was reported as high as 100. There is evidence that N_2-fixation is enhanced in the rhizosphere of some plants due to enrichment of nitrogen fixing bacteria. Balandreau et al (1975) and Balandreau & Fares-Hamad (1975) examined the rhizosphere of rice and maize and observed R/S ratio of the N_2-fixing bacteria in the range of 1.6-19. It is not yet known what plant/bacterial factors are responsible for the specificity. Moreover, R/S ratio is not an absolute figure, being dependent upon the soil type, root system, and the manipulation of the sample (Rovira, 1969).

The biology of soil-root interface is complex and difficult to study due to the limitation of methods that will allow adequate quantification of the plant-microbial system. Dilution plate count used for R/S measurement although used widely, has some serious draw backs. Acetylene reduction assay is one of the important indices of nitrogen fixing activity. This technique has been used with great success for the detection of nitrogen-fixing competence in bacteria and the detection of nitrogen fixing associations (Dommergues et al., 1978). The present study was initiated to assess the role of soil and the plant itself in affecting the acetylene reducing activity (ARA) of the rhizosphere of Kallar grass. ARA was observed at two sites over a period of one year.

Rhizosphere association is loose and unstable in which variations in the composition of microbial population are occurring continously. Therefore different areas of rhizosphere should be regarded as a single microbial milieu with no sharp demarcation between them (Old & Nicolson, 1976). According to Balandreau (1983) there is no fundamental definition of the rhizosphere soil. He argued that it is most often defined as the soil adhering to the roots after "gentle" shaking but what considered to be "gentle" is questionable. For the present study different portions of rhizosphere of Kallar grass were made by following the classification of Dommergues (1978). He divided the rhizosphere into three zones:

1. The rhizosphere sensu strictu (= outer rhizosphere) comprising the region of soil immediately surrounding the plant roots and the microbial population inhabiting this.
2. The rhizoplane (= root surface) formed the root surface and the microorganisms living on it.
3. The endorhizosphere (= inner rhizosphere) formed by the root cortical tissue invaded and colonized by saprophytic soil microorganism (non-pathogenic host infection). It is also called as histoplane.

In order to clarify the relationship between field-grown grass and N_2-fixing bacteria, various parameters were employed as none of the single method is reported to be sufficient. These are described under appropriate sub-titles.

3.1 Soil Core Studies:

Soil cores containing plant roots, removed from the field have been used to measure C_2H_2-reduction activity (ARA) of both grasses and grain crops (Day et al., 1975; Van Berkum & Day, 1980; Wani et al, 1983). In-Situ assays have been used by Balandreau & Dommergues, (1973); Tjepkema & Van Berkum, (1977) but these are cumbersome and the measurements are difficult to interpret. Green house-grown, potted plants have also been assayed for nitrogenase activity (Harris & Dart, 1973; Hirota et al, 1978 and Lee et al. 1977) but special precautions must be taken to control growth of blue gree algae if one is measuring heterotrophic

N_2-fixation. Studies were therefore conducted to measure the N_2ase activity of the soil cores containing Kallar grass roots. Field grown plants were sampled for one year since March' 83. The surface layer of soil around the plant to be sampled was first scraped to remove any algal growth, and the plant top was cut off. Six soil cores were removed from each of the three plants and placed in plastic containers. Acetylene reduction assay of soil cores was performed as described in Ch.2.

Nitrogenase activity of soil cores collected from two locations is presented in Table 1 . The frequency of positive samples was higher in soil cores obtained during March' 83; 100% at BSRS and 83% at Shahkot. This tendency remained till the month of August and after wards it declined. The frequencies of positive samples in the month of October at Shahkot and BSRS Lahore were 35% and 67% respectively. No activity was observed in the later samples till the end of experiment.

The nitrogenase activity of soil core is presented in mean as well as in ranges because large plant-to-plant variability has been reported using this method. The maximum mean activities 24 and 25 nmoles, soil core^{-1} h^{-1}were found to be present in the samples collected from BSRS, Lahore during the months of April and May. At the other site (Shahkot), higher activities18, 25, 11 nmoles, soil core^{-1} h^{-1}were observed in the months of May, June and July. Although the frequency of positive samples declined in the month of October but activity was found to be little higher; 8 and 2 nmoles, soil core^{-1} h^{-1} at Shahkot and BSRS, Lahore respectively. No nitrogenase activity was observed in the latter samples, collected till the end of the experiment. Kallar grass is a prennial grass but during winter season it is dry and photosynthetically inactive. Presence of nitrogenase activity during active growth period (March-August) and less or no activity during inactive growth period of Kallar grass clearly demonstrate that the nitrogenase activity is a plant-related phenomenon. The rhizosphere effect has also been observed by other workers. Van Berkum (1980) found highest activity in sorghum at flowering stage while Wani et al (1983) observed peak activity in

Table 1 : Nitrogenase activity of soil cores (23 x 70 mm) taken from the root zone of Kallar grass at two locations. 18 soil cores were taken from 3 plants on every month and incubated for 24 h with acetylene. Readings are expressed as nmoles C_2H_4 soil $core^{-1}$ h^{-1}. Average value is calculated from the positive samples.

Months	Shahkot			BSRS, Lahore		
	% Frequency	nmoles soil $core^{-1}$ h^{-1} Average	Range	% Frequency	nmoles soil $core^{-1}$ h^{-1} Average	Range
March '83	83	3	1-11	100	5	2-7
April	50	8	2-25	100	24	3-45
May	100	18	11-39	100	25	15-50
June	100	25	13-42	50	7	2-25
July	77	11	3-42	77	12	3-29
August	61	8	3-23	83	6	1-22
September	83	3	1-8	56	3	1-10
October	39	8	4-21	50	2	1-5
November	0	-	-	0	-	-
December	0	-	-	0	-	-
January '84	0	-	-	0	-	-
February	0	-	-	0	-	-

Sorghum and Millet when these were in late flowering-early grain filling stage. Large variations in the nitrogenase activity between the plant samples and even in the soil cores from same plants as found in this study was also observed by various other workers (Dart and Wani, 1982; Van Berkum & Day,1980). Balandreau et al (1978) listed 8 factors acting on nitrogenase activity in the rhizosphere of grasses and among those soil moisture was found to be important. Results reported by Day et al.,(1975);Subba Rao & Dart (1981);Tjepkema & Burris (1976) and Vlassak et al., (1973) indicate that plant associated nitrogenase activity increased as the soil moisture content was increased. Wani et al (1983) studied effect of mechanical disturbance, delay between cutting off the plant top and injecting C_2H_2 gas, soil moisture and seasonal variations in an attempt to understand the reasons for the variability. All these factors have significant relation with the nitrogenase activity of soil core. Weier (1980) measured the nitrogenase activity of 3 tropical grasses by using soil core assays and observed strong co-relation of temperature and soil moisture with the N_2-ase activity. Moreover, lag phase was not been observed and immediate linear rates were found in his studies. Interference with the measurement of nitrogenase activity in soil cores may also be caused by poor penetration of acetylene into soil and thus results in delay for saturating the entire samples (Van Berkum & Day, 1980).Although no attempts have been made to study these factors seperately however they seems to be operative in the present study. A long dry winter season (Oct-Jan.' 84) resulted in less or complete absence of nitrogenase activity of soil core samples taken during the said period. In addition to inactive growth time of Kallar grass, soil moisture also seems to be affecting the nitrogenase activity of soil core.

3.2. EXCISED ROOT ASSAYS:

Most of the studies on associative dinitrogen fixation by grasses have made use of excised root assays (Dobereiner et al.,1972 & Neyra & Dobereiner, 1977). All nitrogen fixing systems show immediate and linear C_2H_2 reduction except

excised root assays of grasses. Initial 8-18 hours delay was reported before C_2H_2 reduction begins. Dobereiner et al., (1972) modified the excised root assay to include over night preincubation period. Later on it was adopted as a routine method to prepare excised roots for the measurement of nitrogen fixation in grasses. This technique has been criticised because it was shown that N_2-fixing bacteria proliferated and induced N_2-ase activity during the period of incubation and thus over estimates rates of N_2-ase activity (Barber et al., 1976 Van Berkum, 1980; Van Berkum & Bohlool, 1980). Van Berkum & Sloger (1981) have reported 15-1800 fold increase in the nitrogenase activity of preincubated excised roots. According to Van Berkum et al., (1981) the excised root assay is useful in identifying N_2-fixation associated with grasses when C_2H_2 reduction is detected immediately and no preincubation period is used. Van Berkum & Sloger (1981) measured reduction of C_2H_2 by roots of aquatic grasses excised in the presence and absence of air. C_2H_4 accumulation was found to be non-linear in the former case while immediate linear rates were observed in the latter experiments.

In the present study excised roots of Kallar grass were assayed with and without preincubation and reported here seprately.

3.3 NITROGENASE ACTIVITY OF EXCISED ROOTS OF KALLAR GRASS WITH PREINCUBATION:

Excised roots of Kallar grass were divided into three portions and were subjected to different treatments as described in Ch.2. Preincubation in nitrogen atmosphere was done for overnight and after reflushing with appropriate gases, samples were analysed for the production of C_2H_4 gas after 24 h of incubation at 30 $\pm$ 2 $^{\circ}$C.

Nitrogenase activities of excised roots of Kallar grass obtained from Shahkot and Lahore are presented in Tables 2&3 respectively . The frequencies of positive samples were lower during the month of October-December' 83 which is the inactive

growth period of the Kallar grass. Frequency of positive samples was especially lower in the unwashed roots samples. No activity was observed in the surface sterilized root samples taken in the month of November. In all other samples frequency of positive samples was found to be fairly high (78-100 %).

The nitrogenase activity of excised roots is described in mean as well as in ranges due to the reason described for soil core studies. At Shahkot site mean nitrogenase activities of 8-18 nmoles, g dry root^{-1}, h^{-1} were observed for unwashed roots; 9-31 nmoles g dry root^{-1} h^{-1} for washed roots except sample of August and 2-18 nmoles, g dry root^{-1} h^{-1} for surface-sterilized roots during the period of March to September. The mean nitrogenase activity of root samples in the month of October, November and December was higher although less number of roots were found to exhibit nitrogenase activity druing the said period. In subsequent months activity reached maximum in all the samples of Kallar grass roots. At the other site (BSRS, Lahore), the frequency of positive samples was also found to be lower during the period of October-December' 83. The number of root segments exhibiting nitrogenase activity were always found to be lower in the surface-sterilized treated roots. The average mean nitrogenase activities of positive samples during the months of March-September were observed to be 9-104 nmoles, g dry root^{-1} h^{-1} for unwashed roots, 3-109 nmoles, g dry root^{-1} h^{-1} for washed (except August sample, 621 nmoles,g dry root^{-1} h^{-1}) and 6-50 nmoles g dry root^{-1} h^{-1} for surface-sterilized roots. The number of root samples exhibiting nitrogenase activity although decreased in the subsequent samples but nitrogenase activity was found to be fairly high.

It is clear from the data that maximum mean activities of positive samples were observed in washed roots at both the locations while lowest activities were observed in the surface-sterilized roots. Unwashed roots exhibited nitrogenase activities which were found to be similar for direct root assays (see next portion) and also closer to the values obtained for soil core assays. This study indicates

Table 2 : Nitrogenase activity of excised roots of Kallar grass. Roots were pre-incubated overnight in N_2-atmosphere (2% O_2) and then incubated with acetylene for 24 h. Readings are expressed as nmoles C_2H_4 g dry root wt^{-1} h^{-1}. Average value is calculated from the positive samples.

Site: Shahkot

Months	Unwashed			Washed			Sterilized		
	% Frequency	nmoles g dry wt^{-1} h^{-1} Mean	Range	% Frequency	nmoles g dry wt^{-1} h^{-1} Mean	Range	% Frequency	nmoles g dry wt^{-1} h^{-1} Mean	Range
March'83	100	12	8-17	83	31	1-137	0	-	-
April	78	9	1-26	89	9	2-25	78	6	1-13
May	100	19	3-39	100	22	13-33	100	18	14-21
June	100	13	1-36	100	27	8-40	89	4	1-16
July	89	9	1-43	100	30	8-64	78	2	1-4
August	100	8	1-45	100	187	1-630	89	2	1-4
September	100	18	4-35	100	19	4-45	89	2	1-4
October	22	159	100-219	89	189	37-373	100	322	69-594
November	67	20	4-43	100	48	5-113	0	-	-
December	67	62	23-127	33	170	91-303	89	710	58-24[illegible]
January'84	100	171	75-282	100	205	125-280	22	154	131-178
February	100	99	11-320	100	113	45-213	89	65	10-137

Table 3 : Nitrogenase activity of excised roots of Kallar grass. Roots were pre-incubated overnight in N_2-atmosphere (2% O_2) and then incubated with acetylene for 24 h. Readings are expressed as nmoles g dry root wt^{-1} h^{-1}. Average value is calculated from positive samples.

Site; BSRS, Lahore

Months	Unwashed			Washed			Sterilized		
	% Frequency	nmoles g dry wt^{-1} h^{-1} Mean	Range	% Frequency	nmoles g dry wt^{-1} h^{-1} Mean	Range	% Frequency	nmoles g dry wt^{-1} h^{-1} Mean	Range
March'83	100	13	1-78	100	55	13-178	100	33	25-50
April	100	61	23-90	100	40	7-54	89	17	13-20
May	100	9	2-27	100	3	1-6	100	4	1-5
June	78	14	1-35	89	26	2-64	56	8	2-16
July	89	30	1-105	78	38	6-95	56	6	2-15
August	33	104	53-143	56	621	134-2413	11	50	0-51
September	83	97	13-396	100	109	39-239	17	329	0-329
October	56	225	58-380	67	205	26-450	67	21	9-65
November	44	586	205-1095	67	2521	164-4929	0	-	-
December	33	52	6-114	89	144	55-285	67	227	52-476
January'84	56	30	7-41	78	92	35-176	67	47	2-145
February	100	46	9-118	100	30	13-51	0	-	-

that nitrogenase activity associated with Leptochloa fusca roots was seasonally dependent with temperature and moisture being major factors affecting the root associated N_2-ase activity. Maximum number of positive samples exhibiting nitrogenase activity occurred during active growth period of L. fusca (March-September). Similar results have also been obtained by (Capone & Taylor,1980; Patriquin et al,1981 and Smith & Hayasak, 1982). However, nitrogenase activity in extreme summer months (May-August) was found to be lower. Maximum temperature during this period mostly remains in the range of 40-45 $^{\circ}$C and there is little or no rainfall (Figs.6 & 7). On the other hand the N_2ase activity was found to be higher in the root samples obtained during winter months (October-January). Mean maximum temperature during this particular winter season remained exceptionally higher. It is intresting to note that no nitrogenase activity was observed in the soil core studies during this period while excised washed and surface-sterilized roots showed maximum N_2-ase activities. These observations indicate that at lower in-situ soil temperatures N_2-ase activities might results from bacteria which are closely associated with roots and are not killed by surface-sterilization. At the higher temperatures (38-45 $^{\circ}$C), most nitrogenase activity may results from rhizoplane bacteria that are less closely associatd with roots and killed by surface-sterili ation. Smith & Hayasaka (1982) while studying the nitrogenase activity in Holodule wrightii roots also observed the differential N_2-ase activities in surface-sterilized and untreated roots with the variation of in-situ soil temperatures. Another explaination may lies in the distribution of nutrients from the roots of Kallar grass. During the active growth period plenty of root exudates are available and thus association of N_2-fixation is loose. On the other hand, in winter months the growth of grass is poor and bacteria have to be tightly associated with the roots in order to obtain little available nutrient (exudate) supply. This may be the reason why higher nitrogenase activity have been observed in washed and surface-sterilized roots during in-active growth period of grass.

Another important aspect to be noted is consistent higher nitrogenase activity in the washed excised roots. Abrantes et al (1975) explained this phenomenon due to prevention of oxygen to inactivate nitrogenase as dissolution of O_2 is very low in water however they also observed a delay of 8-18 h before initiation of nitrogenase activity by roots treated in this way. This delay does not support their own view. Van Berkum (1980) examined the nitrogenase activity of sorghum roots by not washing or incubating them at 4°C. He was unable to detect any N_2-ase activity as proliferation of nitrogen fixing bacteria was prevented. On the other hand washing the roots resulted in the proliferation of N_2-fixing bacteria and substantial rates of acetylene reduction were associated even with the wash water which remained in the assay bottles. Okon et al (1977b) observed that vigorous growth of nitrogen-fixing bacteria was supported by the production of organic acids through anaerobic metabolism during preincubation of excised maize roots. Van Berkum & Bohlool (1980) concluded that water from the washing procedure which adheres to the root samples enables anaerobic metabolism and the reported production of organic acids to take place. The liberation of these substrates into the water component is responsible for the reported proliferation of the microflora and the induction of nitrogenase activity. Although production of organic acid have not been measured in the present study but higher N_2-ase activity in all the samples of washed roots may indicate that this phenomenon is also taking place in this grass.

Presence of nitrogenase activity in the surface-sterilized root portions is significant. The evidence that association is not only situated on the outside of the root but also the inner parts of the root can participate in the dinitrogen fixation is routinely inferred from the measurement of nitrogenase activity and isolation of N_2-fixing bacteria from surface-sterilized roots (Dobereiner & Day 1976; Patriquin & Dobereiner, 1978 and Von Bulow & Dobereiner, 1975). In the present study absolute ethanol was used for one minute while others have

also used NaOCl, chloramine T or 0.1 $HgCl_2$ for various time intervals. In surface-sterilised root samples, obtained during the months of September and December at BSRS, Lahore and in the months of October and December at Shahkot, nitrogenase activities were found to be higher than washed or unwashed root samples. These observations agree very well with the studies of Vlassak & Reynder (1981) on enrichment cultures from washed maize roots. The presence of N_2-fixing bacteria has also been shown by using 2, 3, 5-triphenyltetrazolium chloride (TTC) (Patriquin & Dobereiner, 1978) or by using (TEM) transmission electron microscopy (Umali Gracia et al 1981). In this part of the study, we have not used TTC but in inoculation studies (Ch.5) formation of dark red colour by formazan was observed by light microscopy at the surfaces of the cells lining the air spaces, within the xylem and between pith cells in the stele in only inoculated treatments clearly demonstrates the tight association of N_2-fixing bacteria with the roots of Kallar grass.

The applied significance of excised root nitrogenase activity is uncertain, however, because of the unexplained "lag" that precedes it (Patriquin, 1978).

3.4 NITROGENASE ACTIVITY OF EXCISED ROOTS WITHOUT PREINCUBATION:

Van Berkum & Sloger (1979) have reported immediate reduction of acetylene by excised roots of several grasses. No preincubation period was found to be necessary to induce nitrogenase activity, no proliferation of the microflora during the incubation time was detected, and assays were done at atmospheric pO_2. According to Van Berkum et al (1981) the excised root assay is useful in identifying N_2-fixation associated with grasses when C_2H_2-reduction is detected immediately and no preincubation period is used. Therefore, in addition to convential excised root assay, roots were also analyzed by ARA without preincubation in reduced O_2 atmosphere. Root system of Kallar grass was seprated from adhering soil and roots were placed in a container containing running tap water. Roots were cut off by scissors and small pieces of roots were placed in 20 ml McCartney vials.

After flushing with N_2-gas, 1% Air and 12% C_2H_2 was added by replacing the same amount of N_2-gas. Samples were analyzed after 24 h of incubation and results are summarized in Table 4. The frequencies of positive samples were found to be lower in the months of November and December at the both locations. The nitrogenase activit of excised roots measured during the period of April-September' 1983 co-related well the N_2ase activities of excised unwashed roots which were subjected to preincubation. This co-relation is obvious as it has been reported by Van Berkum (1980) that proliferation of microorganisms was prevented by not washing the excised roots. In the above study samples were analysed after 24 h of incubation and time period required for initiation of N_2-ase activity was not followed.

A time course study was also carried out to assess the time of initiation of nitrogenase activity of excised roots. The roots collected from 3 plants were replicated six times. After removing the adhering soil, roots were immediately dipped in the tap water in order to have minimum contact of air. Roots were cut into small pieces and few roots were placed in the 20 ml McCartney vials. After flushing with nitrogen, 1% Air and 12% C_2H_2 was added. C_2H_4 in the samples was measured at various time intervals and shown in Fig. 3 . Nitrogenase activity in the excised roots was observed not earlier than 5-7 h. Initial non linear rates of C_2H_2 reduction have been observed but in the latter assays, activit found to be increasing linearly. Fairly high nitrogenase activities 2.2, 3.3 & 5.8 umoles C_2H_4 g dry root^{-1} were observed at 11, 13 and 15 h of incubation respectively. Van Berkum & Sloger (1979) measured the N_2-ase activity of aquatic grasses by exposing the plant tops to C_2H_2 and obtained rates of N_2-fixation that were not immediately linear. The same authors (Van Berkum & Sloger, 1981) were able to get immediate linear rates of C_2H_2reduction when roots were protected from exposure to air during sampling. In the present study roots were cut into small pieces while they were dipped in water to avoid direct contact of air and incubated in 1% air. No activity was observed before 5-6 h of incubation. Samejima (1981)

Table 4 : Nitrogenase activity of excised roots of Kallar grass. Roots were incubated directly with acetylene and samples were analysed after 24 hours. Readings are expressed as nmoles C_2H_4 g. dry root^{-1} h^{-1}. Average value is calculated from the positive samples.

Months	Shahkot			BSRS, Lahore		
	% Frequency	nmoles g dry wt^{-1} h^{-1} Average	Range	% Frequency	nmoles g dry root^{-1} h^{-1} Average	Range
April '83	44	9	2-24	60	9	3-16
May	33	36	0-36	72	11	3-16
June	83	7	1-22	67	10	2-22
July	72	6	3-15	89	41	11-100
August	94	5	1-13	56	109	100-130
September	94	14	1-44	83	198	85-353
October	100	392	8-989	89	111	4-367
November	33	63	34-121	44	41	15-70
December	89	889	21-3392	33	80	66-100
January '84	100	151	73-333	56	29	3-79
February	100	359	24-1437	67	762	52-5668
March	67	101	4-838	72	753	21-2694

examined the roots of Pennisetum purpureum and observed nitrogenase activity after 6 h of incubation in 0.02 atm O_2 while Van Berkum & Sloger (1982) were able to detect the nitrogenase activity in excised roots of rice within 30 minutes. They employed special measures to avoid air exposure and obtained immediate linear rates of acetylene reduction. Van Berkum et al (1981) has concluded that excised root assays were useful in identifying N_2-fixation associated with grasses when C_2H_2 reduction is detected immediately and no preincubation is used. In the present study ARA activity was observed without preincubation but only after 5-6 h of incubation in 1% air.

3.4.1 Effect of O_2 on excised root assays

Nitrogenase activity is known to be sensitive to O_2 (Burns & Hardy, 1975) and exposure of roots to O_2 during their prepration could cause an initial but temporary inactivation leading to the non linear profiles of C_2H_2 reduction (Van Berkum et al., 1981). Earlier Dobereiner et al (1972) suspected that inactivation of nitrogenase by entering O_2 when roots were excised from the plants caused the long delay before the detection of acetylene reduction. However, several reports are available which are contradictory to this hypothesis. Paspalum notatum roots which were excised and prepared for measurement of nitrogenase activity in nitrogen did not reduce acetylene for the initial 12 h of assay (Dobereiner et al., 1972). Furthermore, Sorghum roots exposed to air after the preincubation period reduced acetylene within 2 h of this treatment upon assay (Van Berkum, 1980).

The effect of different concentrations of oxygen on nitrogenase activity of excised roots of L. fusca was studied in an attempt to know the optimum O_2 concentration for maximum AR activity. Roots were detached and cut into small pieces (2-4 cm) under water and desired concentration of gas mixture was introduced. The results of this study are shown in Fig.4 . It is evident that increase in the O_2 concentration resulted in reducing the AR activity of the excised roots. Assays were also performed at 3%, 4% and 5% air but activity was found to be low and

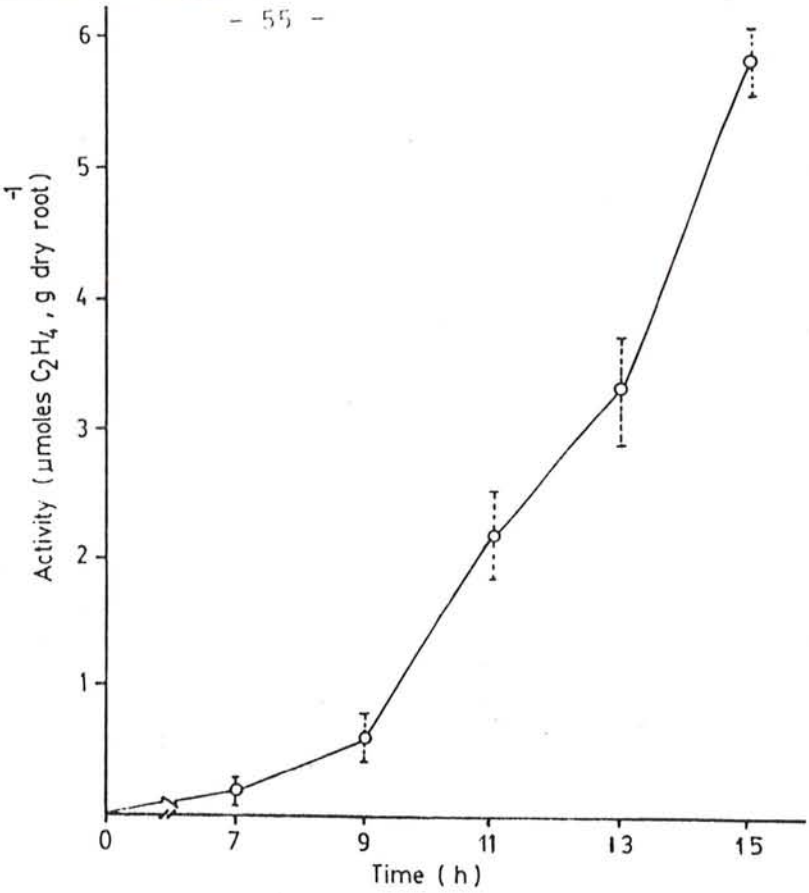


Fig. 3 : Time course study of excised roots of Leptochloa fusca. Roots were incubated in N_2-atmosphere (1% air) and 12% acetylene. Vertical bar is standard deviation.

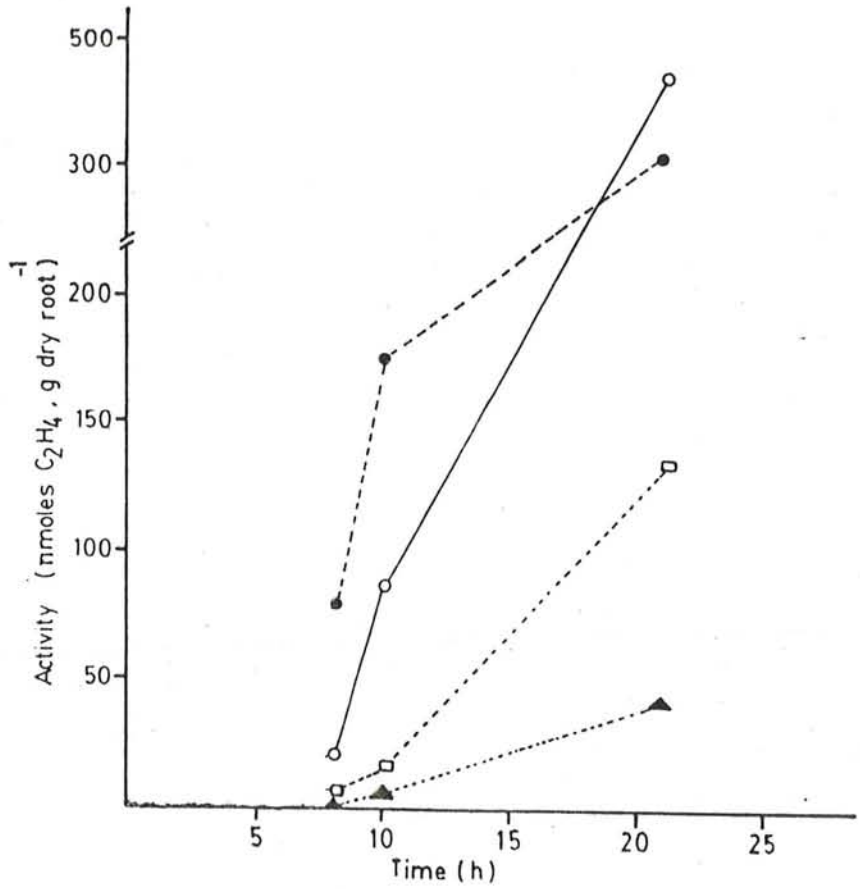


Fig. 4 : The rate of C_2H_2-reduction by the roots of L. fusca exposed to different concentrations of O_2 in the assay vial. ▲--▲ aerobe; □--□ 2% air; o—o 1% air and ●····● anaerobe.

therefore values are not shown in the figure. At the time of first assay (7h), maximum activity was observed in roots incubated under anaerobic conditions while no activity was observed in vials having aerobic atmosphere. After 10 h of incubation, activity was still higher in anaerobic vials but ARA values of excised roots placed in 1% air were close to the anaerobic ones. At this time of assay, activity was also observed in the excised roots incubated under aerobic condition. This might be due to the lowering of O_2-concentration in the assay vial due to the respiration of microflora and thus initiation of nitrogenase activity. At the time of final assay, AR activity of excised roots incubated in 1% air was found to be higher. For the present study, assays were rotuinely performed by incubating the roots in 1% air as maximum activity was observed at this level.

3 4 2 Nitrogen Activity VS Proliferation of N_2-fixers:

It is now well known that preincubated excised root assay results in higher estimates of nitrogen fixation (Barber et al., 1976; Okon et al, 1977b; Tjepkema & Van Berkum, 1977 and Van Berkum, 1980). The major factor responsible for this over estimation is reported to be the proliferation of nitrogen-fixing bacteria during preincubation. The multiplication of N_2-fixing bacteria during preincubation of sorghum roots was prevented by not washing the samples or by incubation at low temperature ($4^{\circ}C$). Moreover, nitrogenase activity was detected with the wash water which remained in the assay bottles (Van Berkum, 1980). Production of organic acids through anaerobic metabolism during preincubation is reported to be a carbon (energy) source for the proliferation of microorganisms (Okon et al, 1977b) They concluded that the bacteria multiplied and fixed nitrogen during preincubation, which led to artificially high estimates of fixation.

A study was thus carried out to estimate the microbial population at different time intervals of excised root assays. Whole root system of L. fusca was washed thoroughly with tap water to remove the adhering soil. Six root samples

from each of the 3 plants were obtained. During this process the roots remained dipped in water to avoid the direct contact of air. Vials were flushed with nitrogen and 1% air and 12% acetylene (v/v) were added. Nitrogenase activity of excised roots was measured at 0, 8 and 21 h of incubation. At each assay time excised roots from 3 vials were pooled together, homogenized and serial dilutions were prepared. MPN counts based on ARA and plate counts were made from this suspension as described earlier. Results are presented in Table 5 . At both sites, nitrogenase activity of excised roots was found to be increasing with time. On the other hand population of diazotrophs as estimated from MPN based on ARA showed insignificant change. The colony counts however showed an increase of 3 to 15 folds. Dilution plate technique has some serious draw backs as not true N_2-fixers (N-Scavenger) are also able to grow on N-free media. In contrast acetylene reduction assay is a more reliable technique for the detection of nitrogen fixation and counts based on this technique are thus more authentic. A two fold increase was observed in the population of N_2-fixers during first 8 h of incubation of roots collected from Shahkot. In the latter estimates (21 h) no further increase was observed. At the other site (BSRS, Lahore), initial load of N_2-fixers (measured at 0 h) was found to be higher and no proliferation of N_2-fixers was observed in the latter assays for MPN counts based on ARA.

These results thus indicate that increase in nitrogenase activity with the time is not only due to the proliferation of microorganisms but other factors are also responsible for the observed lag. Oxygen senstivity might be the major factor in case of excised root assays of Kallar grass. As reported in the previous section that increase in O_2-concentrations resulted in the reduction of nitrogenase activity of excised roots of L. fusca.

Table 5 : Time course study of nitrogenase activity, MPN and plate counts on excised roots of Kallar grass collected from two locations (Shahkot and BSRS, Lahore). Six portions of roots were taken from each of the 3 plants. After acetylene reduction at 0, 8 and 21 h, excised roots from 3 vials were collected. Roots were macerated and serial dilutions were made for MPN (based on ARA) and colony counts

Time (h)	Shahkot			BSRS, Lahore		
	ARA of excised roots nmoles C_2H_4 g dry root^{-1}	MPN (ARA) based 10^3	Plate counts 10^6	ARA of excised roots nmoles C_2H_4 g dry root^{-1}	MPN (ARA) based 10^4	Plate counts 10^8
0	-	8.4	12	-	17	3
8	117 (55-193)	14	57	306 (58-691)	14	11
21	1183 (415-1500)	12	52	2201 (658-4413)	17	50

Readings in parentheses are the ranges of ARA activity of excised roots.

3.5 QUANTITATIVE ESTIMATION OF N_2-FIXING ORGANISMS BY MPN-METHOD :

To investigate the seasonal population of N_2-fixing microorganisms in root soil system most probable number method as well as plate counts were made over a period of one year at two sites. Samples were obtained from the plants which were at the same time also analysed for nitrogenase activity in its different fractions for excised root assays.

a. MPN-METHOD:

Most probable number (MPN) technique was used for determining the distribution and population of nitrogen-fixing bacteria in the rhizosphere of L. fusca. Procedures used for fractionating a root soil core samples into three fractions i.e. Rhizosphere, Rhizoplane and Histoplane are described earlier in 2nd chapter. Two crieteria namely turbidity as a sign of growth on nitrogen free media (visual) and reduction of acetylene (ARA-positive) were employed as none of the single method is sufficinet to describe the complex nature of rhizosphere. The results are shown in Figs. 5,6 & 7 . The seasonal pattern was obtained at both sites. The MPN counts based on visual observations were found to be higher during the period of April - June'83 but declined in the samples taken afterwards. MPN counts based on visual observation were again found to be rising after the month of October. In this study bacterial counts were always higher in rhizosphere fraction than the histoplane fraction except in the samples of November and January. During these samplings, histoplane fraction yielded higher number of N_2-fixing bacteria. MPN counts based on ARA have similar pattern as of counts based on growth on N-free media however counts were lower in the former case. Histoplane always had lower counts while rhizoplane exhibited higher number of N_2-fixing microorganisms. At the other site (BSRS, Lahore), the counts based on visual observation and ARA exhibited nearly the same pattern as of Shahkot. Enumeration of N_2-fixing organisms based on visual

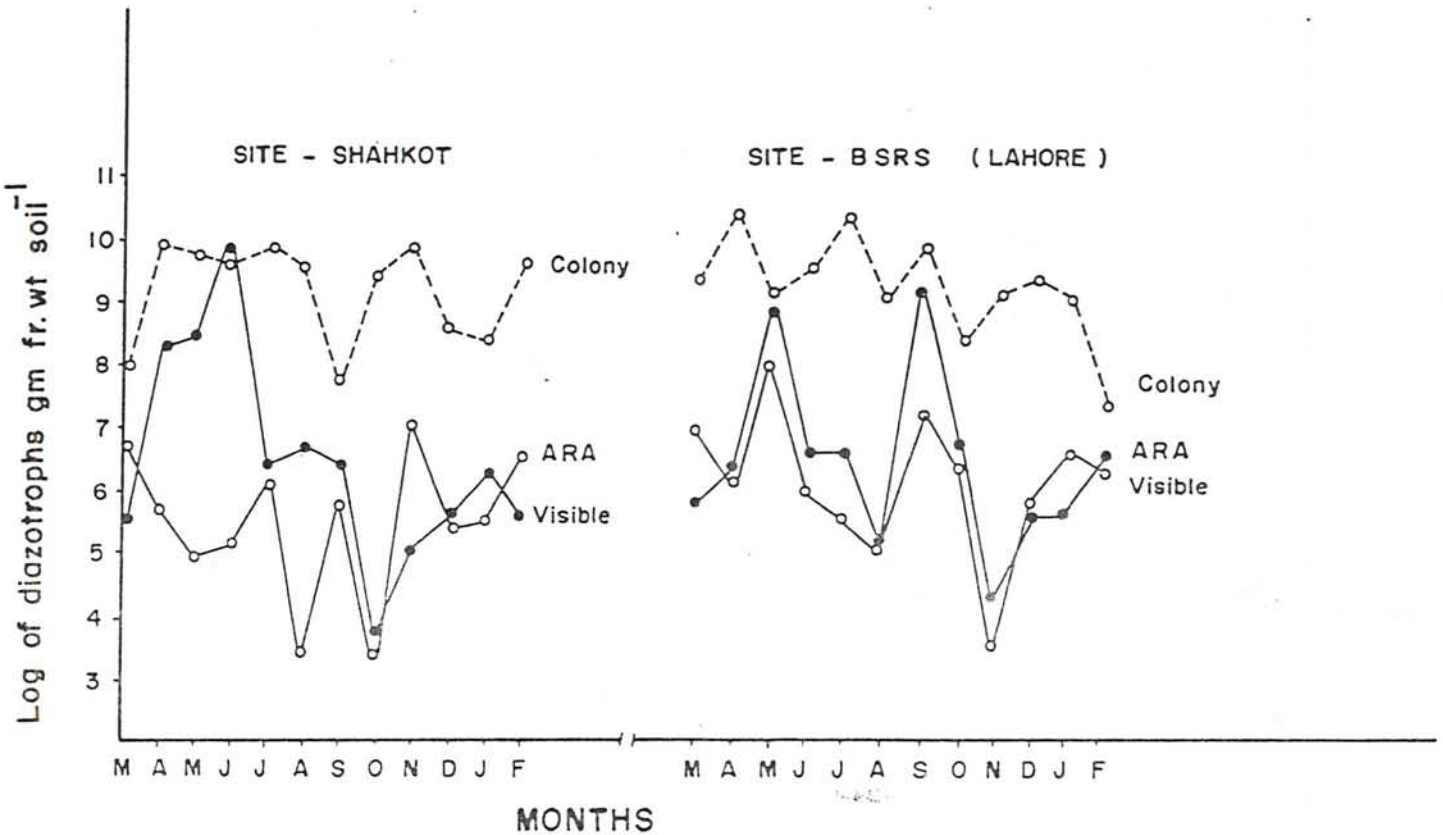


Fig. 5 : Population of diazotrophs as estimated by MPN, visual (●—●); MPN, ARA (○—○) and colony counts (○----○) of the rhizosphere of L. fusca collected from two locations.

observation gave variable results. However two peaks of rhizosphere and histoplane fractions were observed in the months of April-May and second one in the month of September. Histoplane fractions gave higher counts than Rhizoplane and Rhizosphere portions in the months of April and September. Population of N_2-fixing bacteria as estimated by ARA positive samples also exhibited large variations as it was found in visual observation but pattern of histoplane fraction was found to be similar to the latter. Except in the month of April, HP-fraction counts remained low.

b. COLONY COUNTS:

Enumeration of N_2-fixing bacteria in all the three fractions of soil/root system of Kallar grass was also carried out by using classical plate counting procedure on N-free medium. Results of both the locations are presented in Figs.5,6 & 7 . Except the counts of March at Shahkot, less variation was observed during the whole sampling period. At Shahkot, low number of N_2-fixing microbes was observed in the month of March (Fig.5,6) Correspondingly similar data was obtained for MPN counts by using two indices. Like MPN counts, highest population of diazotrophs was observed in the month of May. Among three fractions, HP-fraction gave less counts except in the months of May, September and January. Similar pattern for HP-fraction was obtained from MPN-counts for these 3 months.

At the other location (BSRS, Lahore) the colony counts of HP-fraction except in the month of October, remained lower than the other two fractions namely RS & RP (Figs 5 & 7).

The counting of the bacteria in different months of the year has been done with a view to understand the distribution and population of N_2-fixing organisms in the rhizosphere of Kallar grass. Little work has been done concerning the possible seasonal occurrence of heterotrophic N_2-fixing bacteria in natural habitats. Dicker & Smith (1980) enumerated acetylen-reducing bacteria in a

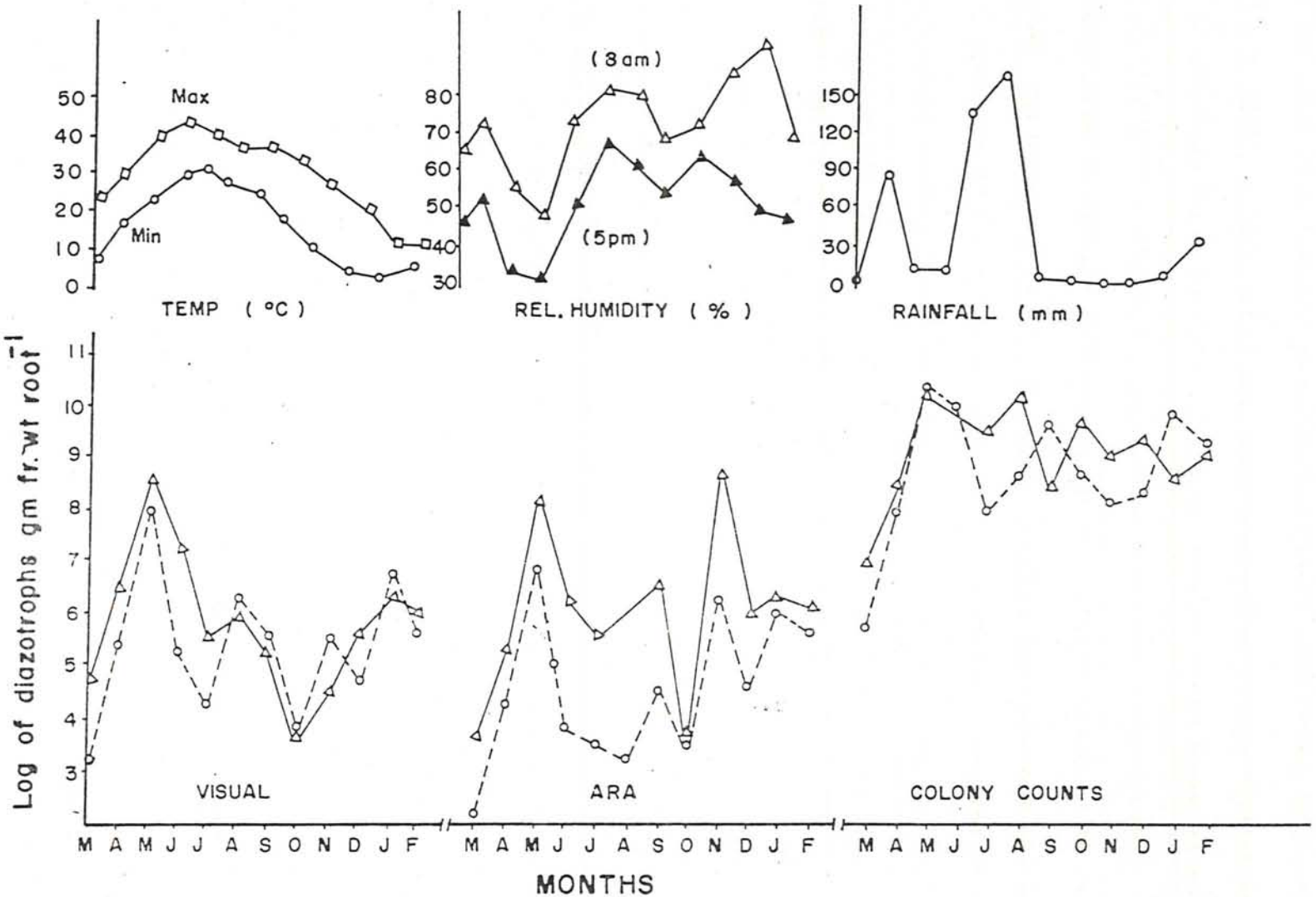


Fig. 6 : Population of diazotrophs in the rhizoplane (△—△) and histoplane (○·····○) of Kallar grass (L. fusca) roots as estimated by MPN method and colony counts from Shahkot area.

Delaware Salt Marsh (USA) for a period of 9 months. Their results showed no discernible seasonal patterns for any of the 3 groups of N_2-fixing bacteria (Azotobacter sp., Clostridium sp.and Desulfovibrio sp.).They were also unable to observe any difference in population of N_2-fixing organisms among the different vegetational areas, and concluded that there was a heterogenous population of N_2-fixers present. Barraquio et al (1982) measured the nitrogen fixing activity and populations of nitrogen fixing bacteria associated with two varieties of rice grown in dry land and wet land conditions at various growth stages during the dry season. The population of N_2-fixing heterotrophic bacteria associated with rhizosphere soil, root and basal shoots was determined by MPN method. They observed higher number of N_2-fixing bacteria in wet land conditions than in dry land conditions. Wright & Weaver (1981) measured the population density of nitrogen fixing bacteria of forage grasses growing in a subtropical region of Texas (USA). Earlier, heterotrophic N_2-fixing bacterial population in corn was estimated by MPN methods at different growth stages by Barber et al (1976) and Okon et al (1977b)and in rice by Ishac et al.(1981)and Watanabe et al., (1979).

The studies on the cereals and forage crops for measuring the population density of N_2-fixing heterotrops are either performed at only one stage or at the most 3-4 growth stages. As L. fusca is a prennial grass, thus in addition to measuring nitrogenase activity associated with plants, N_2-fixing microbial population was also determined for one whole year in order to see the seasonal fluctuations or to co-relate them with the nitrogenase activity of roots. Three parameters were employed to measure the N_2-fixing bacterial population of soil/root system of Kallar grass. Among them, the colony counting procedure is the oldest (Beijerinck, 1901& Winogradsky, 1926) but major difficulty with this type of technique was that it gave a high proportion of colonies which were not true N_2-fixers but N-scavengers. In the present study higher number of N_2-fixing microbes were consistently observed in all the samples by using this technique.

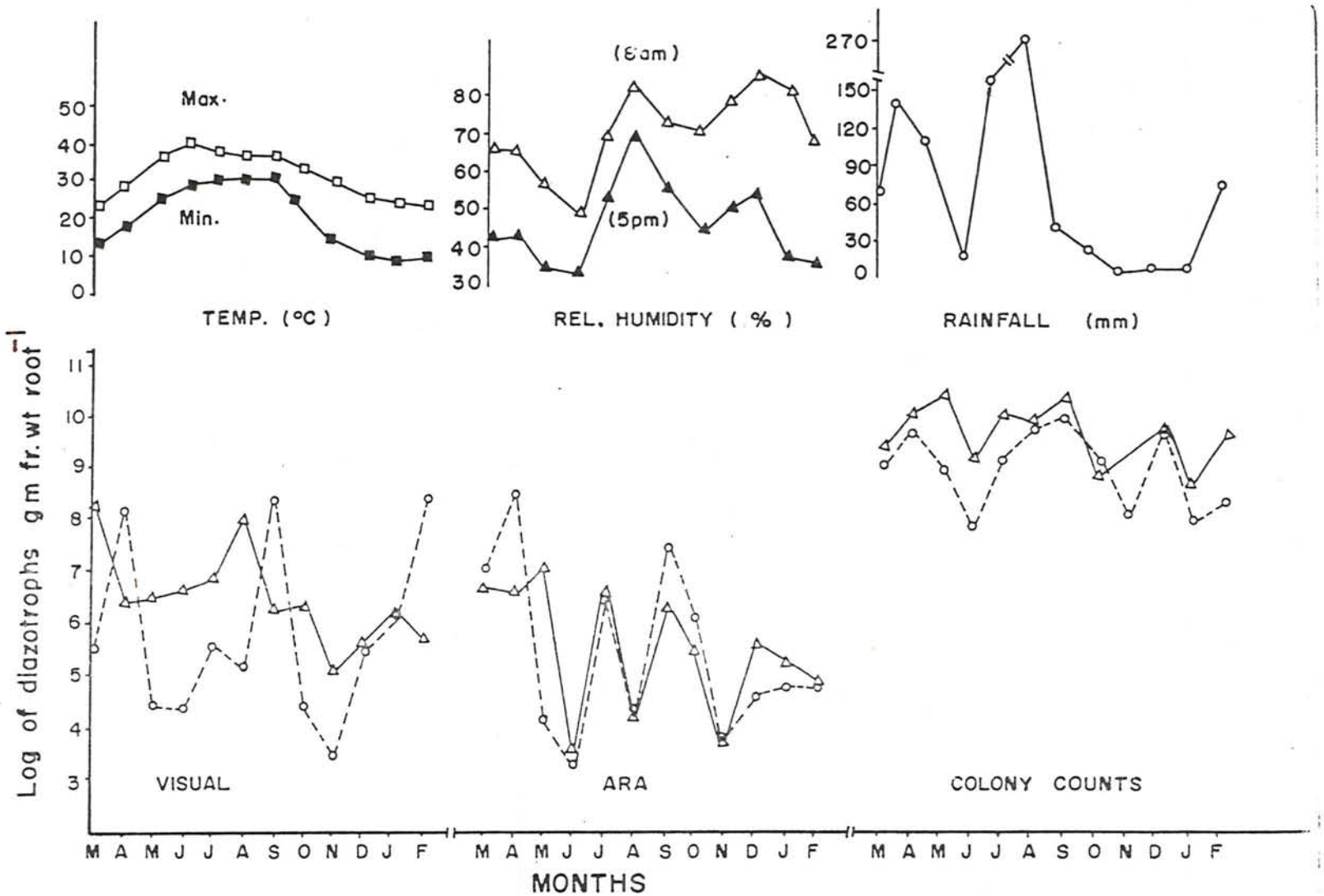


Fig.7 : Population of diazotrophs in the rhizoplane (△——△) and histoplane (○····○) of Kallar grass (L. fusca) roots as estimate by MPN method and colony counts from BSRS (Lahore) area.

The reduction of acetylene to ethylene is specific tool for detecting microorganisms possessing nitrogenase. N_2-fixing bacteria in soils can now be estimated by inoculating soil dilution into tubes of . N-free medium incubated under a partial pressure of acetylene, low enough to allow N_2-fixation and multiplication of bacteria, and high enough to allow detectable levels of ethylene evolution (Villemin, 1974). In the present study ARA gave always less bacterial counts which seems to be more reliable. The drawback in dealing with N_2-fixing bacteria is that the C-sources used in N-free media encourage the growth of different groups of bacteria. Thus, the nature of the C-sources affect the nature and the numbers of N_2-fixing bacteria which grow on N-free media, and this is a very serious impediment to any ecological survey of the true diazotrophic microflora of soil (Thomas-Bauzon et al, 1982). For the present study, Combined Carbon Medium (Rennie, 1981) was employed containing standard basal salts and three common carbon sources (Sucrose, Mannitol and Sod. Lactate). Rennie (1981) proposed that on this single, simple medium most dinitrogen fixing bacteria would exhibit good growth as the carbon source, does not necessarily result in selectivity. Accordingly during a survey of the bacteria from three Southern Alberta soils (Canada), CCM yielded the highest count of putative dinitrogen-fixing bacteria. We have routinely used used semi solid CCM for ARA and visible growth and CCM agar for colony counts. However, such counts provided the information regarding the population densities of N_2-fixing microorganisms without reference to specific plant-bacteria interactions. Moreover, mere occurrence of asymbiotic N_2-fixer could not inform about the extent to which nitrogen fixation by the associated organisms benefits the plants (Van Berkum & Bohlool, 1980). The study of this problem requires a direct approach, whereby the organism of intrest is identified in-situ. Immunofluorescence technique in autoecological stuides in soil and rhizosphere by Diem et al,(1977)and Schank et al (1979) is a promising technique. Reisolation of Streptomycin resistant Azospirillum by Baladani & Dobereiner,(1979)is yet another tool for studying the specific association

Such detailed and quantitative studies are needed to understand the population ecology of nitrogen fixers in rhizospheres (Bohlool & Schmidt, 1980).

3.6 CONCLUSIONS:

Results of this chapter illustrate the ubiquitous association between diazotrophic bacteria and Kallar grass roots in a saline subtropical region. Manipulation of any biological system is only possible after obtaining basic information about it, which is absolutely essential. In the first instance, leaves of Kallar grass were examined for ascertaining whether it has a C-3 or C-4 pathway of photosynthesis. After the essential techniques have been used in this regard it was found that L. fusca has a C-4 pathway (Zafar & Malik, 1984). It is known that grasses possessing this pathway utilize their available nitrogen more efficiently in producing biomass than grasses having C-3 pathway (Black et al., 1978). Four carbon plants may also be ideal for arid lands because they grow better than C-3 plants at higher temperatures and would require less irrigation water.

After obtaining preliminary information about the plant, a detailed study was thus initiated to look for N_2-fixing activity in different regions of soil/root system. The acetylene reducing activities reported for L. fusca vary considerably due to the difficulty in obtaining a representative root sample and due to the natural biological variability inherent in the plant. Although higher number of roots have been observed to exhibit nitrogenase activity in the summer months (Temp. 40-45°C), maximum nitrogenase activity was observed in the months of September-November when the temperature is not too high and photosynthetic activity is also reasonably good. The excised root assay has been criticised because of proliferation of N_2-fixing bacteria during the prolonged incubations (Barber et al., 1976; Okon et al., 1977b). Excised root assays were performed without preincubation and AR activity was observed after 5-6 h. No significant

change in N_2-fixers as observed by MPN counts based on ARA was detected during the incubation period although 3-18 fold increase in microbial population was observed as estimated by conventional plate counts. It is however, known that in the latter method N-Scavengers also grow and result in higher number. These observations are contradictory to the reported results of Eskew & Ting, (1977);Tjepkema & Van Berkum, (1977) and Van Berkum (1980). Present results confirmed the earlier hypothesis of Dobereiner et al., (1972) that O_2 senstivity might be responsible for the reported lag. In a seprate study to look for the effect of oxygen concentrations on the nitrogenase activity of excised roots of Kallar grass it was observed that incubation under 1% air gave maximum AR activity. Moreover, with the increase in O_2-concentrations, reduction in nitrogenase activity was observed. Earlier Trolldenier (1977) measured the nitrogenase activity of excised roots of rice at 21% & 3% O_2 after 3 h of incubation. No activity was observed at 21% O_2. However after 14 h of incubation, nitrogenase activity was detected even in 21% O_2. Maximum activity was still present in vials incubated in 3% O_2. Roots incubated under anaerobic conditions also showed fairly high nitrogenase activity. Watanabe & Barraquio (1979) also studied the effect of O_2 on rice roots and observed maximum activity at pO_2 of 0.01 atm as compared to pO_2 0.05 and 0.2. Samejima (1981) examined the nitrogenase activity in Pennisetum roots as affected by pO_2. The pO_2 of 0.02 maximized the acetylene reduction while 20% of O_2 severely inhibited the rate to about one-fourth. Results on the effect of O_2 on nitrogenase activity of excised roots of Kallar grass followed the similar pattern and maximum activity was observed in roots incubated under 1% air.

The difficulties of root sampling combined with diurnal and seasonal variations in AR activities plus the imprecision of the arbitrary 3:1 nitrogen acetylene ratio, make extrapolation to field conditions in terms of Kg-N ha^{-1} highly questionable. Moreover, acetylene redution is a short term kinetic assay and

thus no attempt has been made to extrapolate these activities into field conditions. Earlier Bors et al., (1983) extrapolated a value of 127 Kg-N_2 ha^{-1}, 100 $days^{-1}$ for Kallar grass by making few studies on soil cores.

Population of heterotrophic N_2-fixing bacteria in the different regions of soil/root system of Kallar grass was estimated by MPN-technique as well as by classical plate count method. The latter method although gave higher bacterial counts yet all of them may not be true N_2-fixers as counts were observed from roots which showed no AR activity. The MPN-counts based on ARA gave reliable counts. The same observation has been made by Wright & Weaver (1981). They observed a significant Spearman rank corelation co-efficient (P 0.01) between AR activity of soil-root cores and the population of N_2-fixing bacteria on roots but the relationship was not linear. Such a co-relation does not exist with our system. These studies are unable to define specific bacterial association and future studies should be directed to the enumeration of specific bacteria by immunoflourescence technique.

Presence of nitrogenase activity in the surface sterilized roots and presence of high number of N_2-fixing heterotrophs in the histoplane fraction is an indirect evidence for the occurrence of these bacteria inside the roots. Van Berkum et al., (1981) while reviewing the procedures for surface sterilization concluded that washing the roots with Chloramine T, NaOCl, alcohol or $HgCl_2$ do not unequivocally result in surface sterilizing the roots of grasses. McClung et al., (1983) tried 1% or 5% Chloramine T for 1 h or 1% NaOCl for 1 or 2 h for obtaining rhizoplane microorganisms of Spartina alterniflora. They found that treatment in 5% NaOCl for 1 h was more effective in distinguishing the bacteria present inside the roots. However, this treatment was less effective with roots of maize and sorghum. They concluded that techniques to surface sterilize the roots should be evaluated for different plants. For the studies on histoplane fraction of Kallar grass for MPN and colony counts, root

residue after obtaining rhizoplane fraction was soaked in 95% ethanol for 1 min. Earlier 0.1% $HgCl_2$ was employed for 1 minute but no, nitrogenase activity in the surface-sterilized roots or bacterial counts were obtained in histoplane fraction. Nitrogenase activity in histoplane fraction is an indirect evidence of the localisation of N_2-fixing bacteria inside the roots. This association is more tight when energy supply is not abundant (during winter months) and consequently higher nitrogenase activity was observed during this duration. In a seprate study (Ch.4) bacterial presence was observed inside the root tissues by TTC staining and light microscopy. Still it is an indirect evidence and the site (s) of fixation is still unknown.

Numerous problems still exist in ascertaining the quantitative importance of associative symbiosis in grasses. ^{15}N reduction techniques are the only absolute proof of dinitrogn fixation but use of this gas is hampered due to the soil/root system. ^{15}N isotope dilution technique provides the true quantitative means of estimating dinitrogen under field conditions. These isotopic studies have been carried out and reported in Ch.5.

The predominant N_2-fixer (s) in any grass association is difficult to ascertain and mostly presumptive because of the difficulties in identifying them. Some of the efficient strains collected from the rhizosphere of L. fusca were identified and various physiological experiments were performed which are reported in the next chapter.

CHAPTER-4. MICROBIOLOGY OF ASSOCIATIVE SYSTEM OF *LEPTOCHLOA FUSCA* (L) KUNTH

4.1 ENRICHMENT AND ISOLATION OF N_2-FIXING BACTERIA:

Soil cores and roots of Kallar grass were examined for the presence of nitrogenase activity. This study along with enumeration of N_2-fixers by MPN method resulted in a collection of several bacterial isolate. These bacteria were normally obtained from decimal dilution vials of MPN counts or from enrichment cultures of different portions of roots. Combined carbon medium (CCM) by Rennie (1981) was employed for enrichment and isolation as all commonly isolated genera of dinitrogen fixing bacteria show growth on this medium. In the present study semi-solid CCM was used which resulted in the isolation of aerobic or micro-aerophilic genera of diazotrophs. No attempt was made to look for the presence of strict anaerobes.

Most of the reports concerning the associative symbiosis are either limited to ARA (Hanson,1977 and Jain & Vlassak,1975) or isolation and enumeration of certain groups of bacteria from the rhizosphere without given reference to specific plant-bacteria interactions (Barber et al, 1976, Nelson et al, 1976 Watanabe et al, 1979). Evidences for the presence of specific associations in an environment are restricted to few types namely Azotobacter paspali- Paspalum natotaum ; Beijerinckia-Sugar cane; Bacillus sp.- Wheat; Achromobacter-like organisms (now Pseudomonas)- rice and Azospirillum with several grasses (Dobereiner & De-Polli, 1980). After the rediscovery of Azospirillum (previously Spirillum lipoferum) search for this microbe resulted in detailed analysis of the microbial part of the association. Except N_2-fixing ability, no other common characteristics exist among diazotrophs to aid in their identification (La Rue,1977) Complete identification of N_2-fixing organism is a difficult and cumbersome ta and many soil microbiologists are not very keen on taxonomy (Balandreau, The studies reported in this chapter are based on the identification ar gical properties of some selected, efficient and most occurring isol the rhizosphere of Kallar grass.

4.2 IDENTIFICATION & CHARACTERIZATION OF ISOLATES:

Dinitrogen fixing (acetylene-reducing) bacteria may be readily isolated from soils or rhizosphere but extensive biochemical or immunological, or both are required to identify them absolutely (Rennie,1980a). N_2-fixing bacteria are ubiqitous in nature (Knowles, 1977), however N_2-fixing ability has probably very little phylogenetic meaning (Balandreau, 1983). A series of morphological, biochemical and nutritional tests were performed on three isolates namely NIAB-I, C-2 and Iso-2 for identifying them.

4.2.1 Morphological Tests:

Three isolates were examined by Microlux-11 phase-contrast microscope and following data was obtained.

NIAB-I: A small rod, cell size range from 2-5 μ and non-motile (Fig. 8). Cells were gram-negative under all conditions. On nutrient agar the colonies were off white, circular, convex, entire and intermediate to large in size. On RBA medium (Appendix - 4) incubated under 5% air and 95% N_2, colonies were gummy, opaque, raised, entire and large in size. Same colony morphology was observed on N_3 medium (Appendix 5). Neither slime nor pigment was formed on either media. No resting stage or spore was observed however capsule was found to be present. Organism is capable of aerobic growth with combined nitrogen.

C-2: Intermediate to long rods, (Fig. 9) range of cell size 1.5- 4.2 μ and motile. Polar or peritrichous flagella as stained by the method of Mayfield and Inniss (1977) and spirillum-like motility. Cells gave gram-negative reaction. On nutrient agar, colonies were circular to filamentous, raised, convex, pale, small to intermediate and consistent. On RBA medium incubated with 5% ai and 95% N_2 for 5 days, abundant growth occurred. Colonies were diff large in size and gummy with gum spreading all over the plate. S morphology was observed on N_3 medium. No pigment was produce

Fig. 8 : Two days old culture of NIAB-I grown in N-free medium. Phase-contrast microscopy was performed with a Microlux-II microscope (x 1000).

Fig. 9 : One day old culture of C-2 grown in N-free medium. Phase-contrast microscopy was performed with a Microlux-II microsocpe (x 1000).

Fig. 10 : One day old culture of Iso-2 grown in N-free medium. Phase-contrast microscopy was performed with a Microlux-II microscope (x 1000).

Plate – 1

Fig. 8

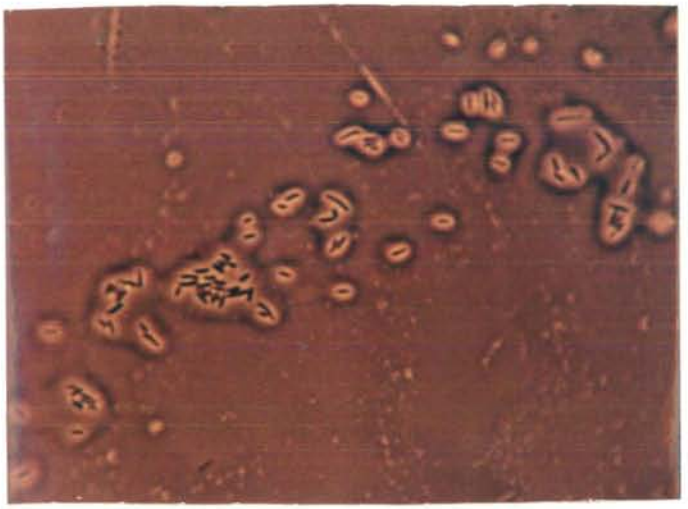


Fig. 9

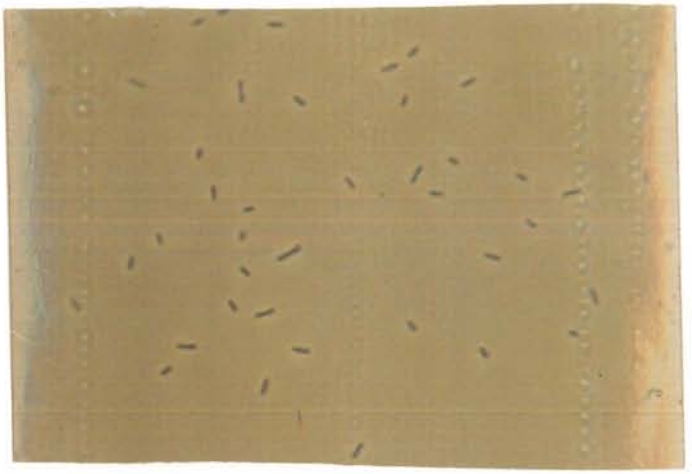
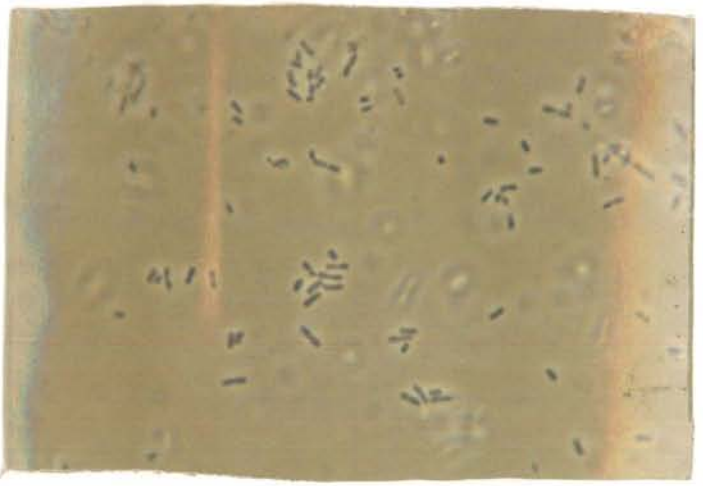


Fig. 10



agar, RBA or N_3 however abundant slime was formed on the last two media. Sporulation was found to be absent. Capable of aerobic growth with combined nitrogen.

Iso-2: Small rods, cell size range from 2.1-3.7 μ and motile.(Fig.10).Backward and forward movement but flagella lost easily on staining. Cells were gram-negative under all growth conditions and mostly occurring singly. On nutrient agar, colonies were circular, convex, entire thick and gummy. The slime usually is extremely tough and tenacious even elastic and therefore it is difficult to remove a part of colony on solid medium with a wire loop . Colonies become flat and less gummy on long incubation on nutrient agar. On nutrient broth whole medium becomes homogenously viscous by slime. On RBA medium incubated under 5% Air and 95% N_2, small, gummy, raised and entire colonies were observed. Same colony morphology was observed on N_3 medium. Pigment was not formed however colonies were pale or yellowish in colour and this pigment is more pronounced in broth. No resting stage or spore was observed. Capable of aerobic growth with combined nitrogen.

4.2.2 Biochemical Characteristics of Isolates:

Diagnostic biochemical tests were conducted according to methods described by Claus (1979) in Practical Manual for training course on running and management of culture collection, Brno, Czeckoslavikai, (13-25 July 1979).

The results are summarized in Table 6 . All isolates were unable to hydrolyse starch, pHB, gelatin and Tween (20,60 and 80). All isolates have assimilatory nitrate reductase but denitrification was found to be absent. Microbial isolates were catalase positive but oxidase reaction was observed very weak only with C-2. Citrate utilizing ability was observed in all the isolates. They gave positive V.P. reaction while indole reaction was found to be negative. The bacterial isolates NIAB-I and Iso-2 were able to hydrolyse urea.

Table 6 : Biochemical and physiological characteristics of 3 bacterial isolates from the rhizosphere of Leptochloa fusca.

Characteristics	Reaction		
	NIAB-I	C-2	Isolate-2
Catalase production	+	+	+
Oxidase production	-	(+)w	-
Indole production	-	-	-
Voges-Proskauer reaction	+	+	+
Citrate use	+	+	+
Urea hydrolysis	+	-	+
$NO_3^{'} \rightarrow NO_2^{'}$	+	+	+
Denitrification	-	-	-
Gelatin hydrolysis	-	-	-
Starch hydrolysis	-	-	-
Tween hydrolysis	-	-	-
20	-	-	-
60	-	-	-
80	-	-	-
pHB hydrolysis	-	-	-
Arginine dihydrolase	-	+	-
Lysine decarboxylase	-	(+)w	
Ornithine decarboxylase	-	+	
β-galactosidase	+	+	+
H_2S-production	-	-	-
Pigment production	-	-	-

+, positive; (+)w, Weak; -, negative.

Activity of B galactosidase was found to be present in all the isolated bacteria from kallar grass. Activity of Arginine dihydrolase was observed only in isolate C-2. The enzymatic activities of ornithine decarboxylase and lysine decarboxylase were found to be absent in NIAB-I while they were present in C-2.

4.2.3 Nutritional Tests on Isolates:

Utilization, acidification and fermentation of six carbohydrates (Glucose, Sucrose, Mannitol, Xylose, Arabinose & Trehalose) was observed for NIAB-I, C-2 and Iso-2 as described in Training Manual for running and management of culture collection (1979). O-F basal medium of Hugh & Leifson (E.merck) was used. The acidification (change of green dye into yellow colour) and formation of gas (removal of paraffin plug) was observed after 24 h,48 h and five days of growth at 30°C.

Results of this experiment are presented in Table 7 . All isolates were able to utilize these sugars oxidatively and end reaction was found to be acidic. Moreover, these isolates were also able to ferment these sugars and gas was formed. Utilization of 17 substrates as sole source of carbon were analysed on petri plates by using multidisc technique as described by Malik (1980). This technique helps to make maximum use of the petri plate to accommodate 9-19 bacterial strains. Bacterial suspension was dropped gently on each disc by pasture pipette Results of these experiments are described in Table 8 . All isolates were unable to utilize sodium propionate and m-hydroxy benzoate while growth on other 15 substrates was observed.

4.2.4 Use of Commercial Identification Kits:

The API 20E system designed for the identification of Enterobacteriaceae., contain 20 tests for various biochemical characteristics of bacteria. These tests are (1) presence of β-galactosidase, arginine dihydrolase, lysine decarboxylase

Table 7: Oxidation - Fermentation (O - F) test with 6 sugars on 3 bacterial isolates from the rhizosphere of Kallar grass. Observations were made after 48 h of incubation on Hugh & Leifson medium (E.merck).

Isolate	Glucose		Sucrose		Trehalose		Arabinose		Mannitol		Xylose	
	O	F	O	F	O	F	O	F	O	F	O	F
C-2	A	A/G	A	A/G	A	A/G	A	A/G	A	A/G	A	A/G
Iso-2	A	A/G	A	A/G	A	A/G	A	A/G	A	A/G	A	A/G
NIAB-I	A	A/G	A	A/G	A	A/G	A	A/G	A	A/G	A	A/G

A = Acid reaction G = Gas formation.

Table 8 : Results of carbon utilization test as sole carbon source performed on 3-bacterial isolates from the rhizosphere of Leptochloa fusca.

Isolates	C-2	Iso-2	NIAB-I
L (+) Arabinose	++	++	++
D (+) Glucose	++	++	++
Saccharose	++	+++	++
D (+) Xylose	+	++	++
D (-) Trehalose	++	++	++
D (+) Galactose	+M?	++	++
Na. Acetate	+M?	+	++
Na. Propionate	-	-	-
DL. Sod. Lactate	+	-	+
Na.Benzoate	-	++	++
Na. Glutamate	+?	++	+M
Na Succinate	+M	++	++
Na Pyruvate	+	++	+
Ethanol	+	++	++
p Hydroxy benzoate	+	++	++
Mannitol	++	++	++
m-Hydroxy benzoate	-	+?	-

-, negative; +, Weak positive; +, positive; ++, strong positive,
M = Mutational growth.

ornithine decarboxylase, tryptophane deaminase and urease(2) Citrate utilization (Simmons), hydrogen sulphide production, gelatin liquefication and Kovac's inodole and Voges- Proskauer tests; and (3) fermentation or oxidation of glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, and L(+) arabinose. Six supplemental API 2-E tests for oxidase, reduction of nitrate to nitrite or denitrification, catalase, motility and growth on MacConkey's bile salt medium could also be performed on same kit. The results obtained from these easy to use kits were found to be similar to conventional tests as described earlier. Use of this kit for rotuine identification of N_2-fixing bacteria have been recommended by Rennie (1980[a]).

API-50 CH:

Bacterial classification is too complex and to extend the data base, additional tests for the utilization of 49 carbohydrates were also performed by making use of commercial API- 50 CH test kit. The inoculum was prepared by suspending the 24-48 h old colonies in 50 CH suspension medium. 10 ml of inoculum suspension is required to fill the 50 tubes. Results were recorded after 3,6, 24 h. The results obtained from these kits were used for the identification of the bacterial isolates.

4.3 DNA-BASE COMPOSITION:

Bacterial taxonomy depends primarily on gathering a lot of characteristics about a given isolate. In addition to observing morphological features, the nutritional, physiological and biochemical characteristics, the analysis of bacterial genome as mol % (G+C) have now been a useful feature in species description.

In this study overall DNA base composition of three N_2-fixing bacteria isolated from Kallar grass was determined by thermal denaturation technique as described in Ch 2. DNA of *E coli* K 12 withknown (G+C) content of 51.7 served as a reference strain. The results based on the melting

points (T_m) and mol % guanine + cytosine (G+C) values are presented in Table 9 . As indicated, the G+C values were in the range of 53.4-63.7. Significant differences occurred in DNA base composition and according to Kreig & Tarrand (1978) this difference alone would be a strong evidence for the existance of more than one species.

The mol % G+C values of NIAB-I was found to be 56.99. In the Bergey's Manual (1974) the range for DNA base composition for different species of Klebsiella is given from 52-56 mol % G+C. Earlier Gillis et al (1970) measured the mol % G+C content of K. penumoniae and observed value of 58.6. Recently Seidler (1981) has reported the overall mean DNA base composition of some 40 isolates identified as K. pneumoniae of both medical and environmental origins and values ranged from 53.9 to 59.2. Our value (56.99) along with other characteristics (non-motility, acidification of glucose, indole negative, VP negative) indicate clearly that NIAB I belongs to genus Klebsiella .

The mol % G+C values for C-2 and Iso-2 were found to be 63.7 and 53.4 respectively. A comparison of DNA base composition of various species of diazotrophs with the two isolated bacteria revealed that both are related to family Azobacteriaceae. The mol % G+C value for genus Azospirillum, is in the range of 69-70(Kreig & Doberiner,1984)and thus the remaining two isolates do not belong to this genus. The range for genus Beijerinckia is given by Johnstone (1974) in the range of 53.2-60.7 and Iso-2 seems to belong to this genus. The taxonomic position of isolate C-2 could not be resolved and will be discussed in more detail in section 4.6

4.4 ^{15}N-INCORPORATION BY BACTERIAL ISOLATES:

Incorporation of stable and heavier isotope of nitrogen (^{15}N) provides a direct and absolute proof of N_2-fixing ability. Studies were therefore conducted with the 3 selected bacterial isolates from the roots of Kallar grass to look for the ^{15}N enrichment as described in Ch. 2

Results of these experiments are presented in Table 10 . ^{15}N incorporation was observed in all the bacterial isolates. The labelling of Iso-2 was higher (1.18 at % excess) while C-2 and Azospirillum brasilense sp 7(a positive check) exhibited lowest enrichment, 0.17at % ex and 0.15 at % excess respectively. N_2-fixation by N-determination through Kjeldahl also followed the similar pattern. Becking (1962, 1963) by using 43 at % ^{15}N and 65 at % ^{15}N on Spirillum lipoferum observed that ^{15}N enrichment could be increased from 0.43 at % ^{15}N to 1.04 at % ^{15}N. Nayak et al (1981) studied ^{15}N incorporation in Azospirillum isolated from different rice cultivars by using 72.5 at % ^{15}N and observed the labelling in range of 0.205 to 11.8 atom % ^{15}N after four days of incubation. In the present study low values of enrichment as compared to reported values of bacterial isolates from rice field by Nayak et al (1981) in addition to low nitrogenase activity were due to low quantity of medium (20 ml instead of 30 ml) and less incubation period (2 days instead of 4 days). Marked variation even within the strains of genus Azospirillum have been observed by Burris et al (1978) ; Nayak et al (1981) and Kreig & Dobereiner (1984).

The first report concerning the use of ^{15}N with Klebsiella was made by Hamilton & Wilson (1955). Later Mahl et al (1965) measured the incorporation of ^{15}N into bacterial cells and screened 31 strains of K. pneumoniae. After 5 h of incubation of 3 ml culture medium in 33 at % ^{15}N, they measured the enrichment in the range of 0.01-0.1 at% excess in 13 strains.Watanabe and Barraquio (1979a) while studying the free living N_2-fixing organisms from rice roots observed ^{15}N enrichment of 0.18 and 0.25 at % excess in two isolated strains which were like- Achromobacter. They incubated 4 days old static culture in 98 at % ^{15}N for 24 h. The positive check strain Azospirillum lipoferum sp. Br 17 exhibited 1.16 at % excess ^{15}N enrichment.

Table 9 : Melting points (T_m values) and mol % (G+C) values for the DNA of 3 bacterial isolates from the rhizosphere of Kallar grass (Leptochloa fusca).

Isolate	Average* T_m °C	Mol % G+C
C-2	76.10	53.4
Iso-2	80.30	63.8
NIAB-I	77.5	56.9

* Normalized to 0.1 x SSC values relative to E. coli K.12 with known G+C content of 51.7

Table 10: ^{15}N incorporation by bacterial isolates from Kallar grass

Isolate	Nitrogen fixed* mg/20 ml medium	Atom % excess** ^{15}N at the end of incubation
Azospirillum brasilense	0.56 c	0.15 ± 0.01
C-2	0.67 c	0.17 ± 0.03
NIAB-I	1.43 b	0.72 ± 0.13
Isolate-2	1.71 a	1.18 ± 0.16

* Calculated from total N determination by Kjeldahl assay. Data followed by same letter are not significantly different at 1% level.

** Values are the mean, ± standard deviaion of the mean (triplicate samples for ^{15}N analysis).

In the present study an enrichment of 0.72 at % ^{15}N excess was observed for NIAB-I which belongs to genus Klebsiella when it was incubated under 98 at % ex.^{15}N atmosphere. The enrichment of other two isolates C-2 and Iso-2 was found to be 0.17 and 1.18 at % ex respectively. These values provided absolute proof of N_2-fixing ability in all the three isolates.

4.5 PHYSIOLOGICAL EXPERIMENTS ON N_2-FIXING ISOLATES:

Effect of combined nitrogen (NO_3^-, NH_4^+ and yeast extract), pH and salt (NaCl) on nitrogenase activity of three isolates, NIAB-I, C-2 and Iso-2 were examined. Conditions were similar for all the three isolates if not mentioned otherwise.

A loopful of bacteria was taken from slant and suspended in 30 ml nutrient broth (Difco) in 150 ml Erlenmeyer flask. After 24 h of growth, 1 ml of this suspension was added to 30 ml phosphate buffer (0.1 M, pH 7.0) and mixed thoroughly. This suspension was used as inoculum for further studies. Modified CCM (Appendix 1) was used and pH of solution I was adjusted to 7.0 before adding 0.2% agar. Solution I (180 ml) was distributed in 500 ml Erlenmeyer flasks and kept at 50°C in a water bath. Twenty ml of solution II was mixed aseptically, 3 ml of bacterial inoculum was added and swirled thoroughly. Twenty ml of inoculated CCM was distributed in 50 ml Erlenmeyer flasks with narrow mouth and incubated at 35°C. Duplicate samples were taken after 24 hrs of growth, cotton plugs were replaced with serum stoppers and 12% C_2H_2 was injected after replacing same amount of air. Gas samples were analysed after 1, and 3 hours of incubation at 35°C and mean values of acetylene reduction per hour was calculated. For each assay, 100 ul gas sample was injected into gas chromatograph. Acetylene reduction assays were also performed after 48, 72 and 96 hours of growth.

4.5.1 NO_3^- and NH_4^+ Treatments:

Effects of ammonia and nitrate on nitrogenase activity of free living bacteria and legume systems are being studied by many worker (Ahmad,1978; (Dobereiner & De-Polli,1980; Nelson & Knowles,1978, Rao & Venkateswarlu,1982 and Strandberg & Wilson, 1968) . There is renewed intrest in the use of nitrogen fixing bacteria as inoculant for cereal and grasses. Thus, it is important to carry out some cultural and physiological studies with free living diazotrophs. In agronomic practice, combined nitrogen i.e. NH_4^+ and NO_3^- are very vital for crop productivity. Striking effects of manipulating diazotrophic biocoenosis in increased crop yields or even in the fertilizer economy can only be expected, if ever, after the nature of various systems is better understood. The effect of ammonia on the nitrogenase activity of whole cells of Azotobacter vinelandii was studied by Klugkist & Haaker(1984).They observed that different effect of ammonia may be ascribed to difference in growth and test conditions and they found O_2, pH and stage of growth at which cells were harvested are important to measure the effect of inhibition.

In the present study attempt has been made to study the effect of NH_4^+ and NO_3^- on nitrogenase activity of selected bacterial isolates. Ammonium chloride (NH_4Cl) was used to study the effect of ammonium ion (NH_4^+). For I mM NH_4^+ treatment, 53.49 mg NH_4Cl L^{-1}; 2 mM NH_4^+, 106.98 mg NH_4Cl L^{-1} and for 5 mM NH_4^+, 267.45 mg NH_4Cl L^{-1} was taken.Control was without any N-source. For supply of NO_3^-, sodium nitrate (E.merck) was used. Three treatments of 1mM (85 mg $NaNO_3$ L^{-1}), 2mM (170 mg $NaNO_3$ L^{-1}) and 5 mM (425 mg $NaNO_3$ L^{-1}) were employed. Control was without any N-source. Duplicate samples were analysed for N_2-ase activity after 24, 48, 72 and 96 h of growth as described in section 4.5.

Results on the effects of ammonium and nitrate on nitrogenase activity of 3 isolates from the roots of Kallar grass are shown in Figs.11 a,b,c. Control samples (in N-free medium) of all the three isolates exhibited low nitrogenase activity as compared to the other treatments. This clearly shows that combined nitrogen was required for growth and N_2-fixing activity of these isolates. Such observations have also been made by Watanabe & Barraquio (1979) while isolating N_2-fixing organisms from rice roots. None of their isolates could grow on nitrogen free basal medium (glucose + mineral salt) but majority (31/40 grew on basal + ammonium medium (NH_4Cl 61 mg L^{-1}). Rennie (1980a) has also recommended 100 mg yeast extract L^{-1} as starter combined nitrogen dose for isolating the N_2-fixing bacteria on combined carbon medium. Isolates from the rhizosphere of Kallar grass also seems to require low level of combined-N for their growth.

After 24 h of growth on modified combined carbon semi-solid medium supplemented with NH_4Cl and $NaNO_3$, 3 isolates showed substantial differences. In all three isolates, nitrogenase activity was found to be less in NH_4^+-treatments as compared to NO_3^-. In the latter treatment, maximum activity (799 nmoles C_2H_4, cl^{-1}, h^{-1}) for C-2 bacteria was observd in medium supplemented with 2 mM NO_3^-. Nitrogenase activity (787 nmole C_2H_4 cl^{-1} h^{-1}) was also higher in the same treatment for Iso-2 while NIAB-I exhibited maximum activity in 5 mM NO_3^--treatment. In media, supplemented with NH_4^+, maximum activity in all the three isolates were in the order of 2 mM > 5mM > 1 mM except for C-2 in which . medium · activity was observed in 1mM NH_4^+ instead of 5mM.

Nitrogenase activity measured after 48 h showed decreasing trend in all the treatments(NO_3^- as well as NH_4^+) of 3 isolates except in 5mM NH_4^+ treatment for C-2 and 2mM NH_4^+ treatment of Iso-2. In these two isolates, nitrogenase activity was found to be higher than the 24 h old cultures. Seventy two and 96 h grown cultures again showed lower activity. No nitrogenase activity was

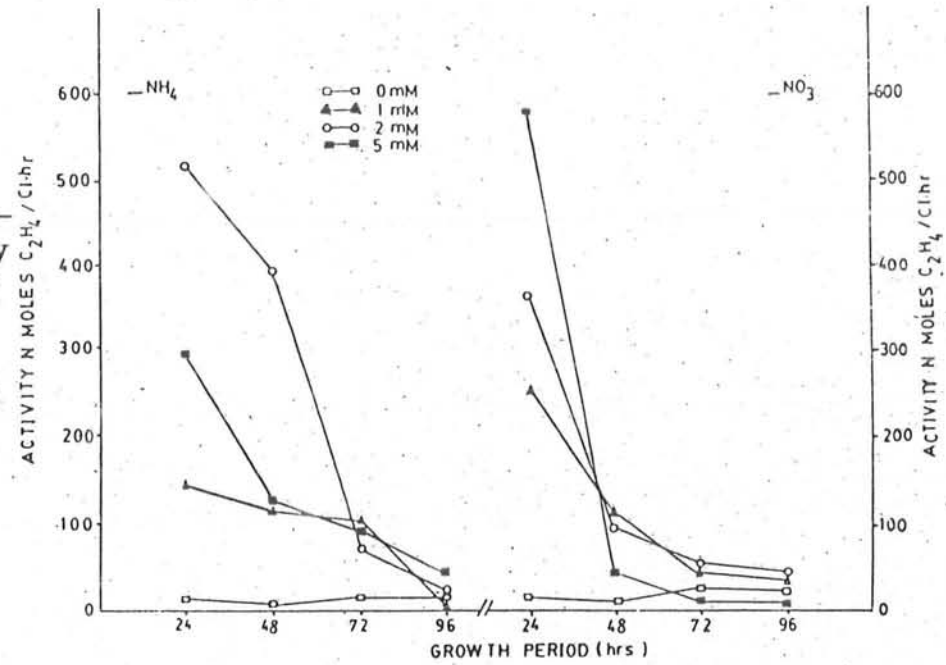


Fig. 11a: Effect of combined-N (NH_4^+ or NO_3^-) supply on nitrogenase activity of bacterial isolate, NIAB-I.

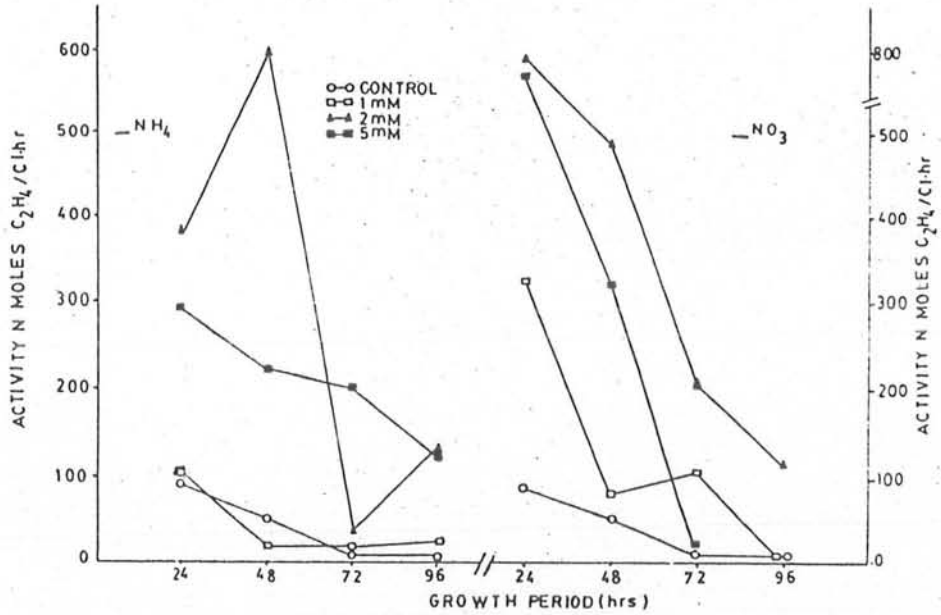


Fig. 11b: Effect of combined-N (NH_4^+ or NO_3^-) supply on the nitrogenase activity of bacterial isolate, Iso-2

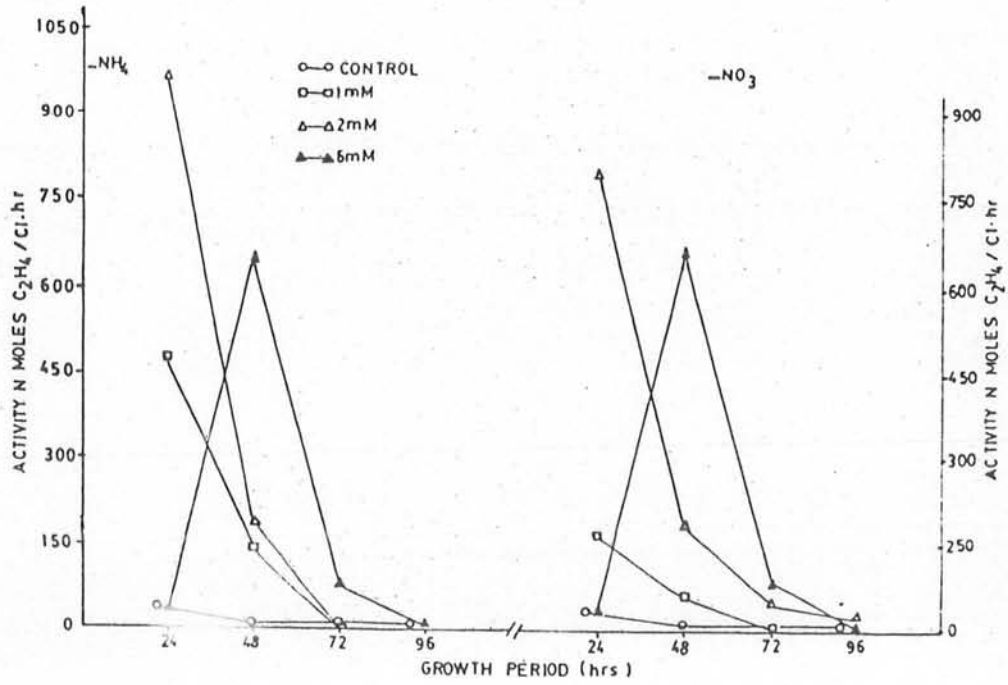


Fig. 11c: Effect of combined N (NH_4^+ or NO_3^-) supply on the nitrogenase activity of bacterial isolate, C-2.

observed in C-2 cultures grown with 1mM NO_3^- on NH_4^+ as well as in N-free medium in 3rd and 4th assay. In an attempt to study the effect of NH_4^+ and NO_3^- on three tropical N_2-fixing bacteria, Pena & Dobereiner (1974) observed substantial differences between species. In Drexia gummosa and Azotobacter vinelandii, nitrogenase activity stopped completely 3 h after application of 10 mM nitrate. In Beijerinckia indica nitrogenase activity continued at a slower rate in the presence of 10 mM NO_3^- and B. fluminensis nitrogenase activity was not affected over a six h period. Dobereiner & Day (1975) studied the effect of nitrate and ammonia on Azotobacter paspali in pure cultures as well as in the presence of Paspalum notatum roots. They observed that A. paspali nitrogenase remained unaffected by nitrate concentration of upto 80 mM. The nitrogenase activity of 20 mM NO_3^--grown cells was similar to the values of control while activity was lower in 20 mM NH_4^+ grown cells. For further study on nitrogenase activity of A. paspali, cells were grown by these authors in pure culture on N_2, ammonia or nitrate (10mM). Washed cells were mixed with either 20 mM of $(NH_4)_2SO_4$ or KNO_3 or without any N-source. It was shown that 10 mM NO_3^- did not affect nitrogenase activity of cells previously grown on N_2 or nitrate. Moreover, nitrogenase in NH_4^+ grown cells was derepressed as rapidly in the presence as in the absence of NO_3^-. They attributed the ability to fix N_2 in the presence of even 80 mM NO_3^- due to lack of nitrate reductase. Day & Dobereiner (1976) observed that both species of Azospirillum are able to assimilate NO_3^- and NH_4^+ and there is no N_2-fixation with more than 1 mM NO_3^- or NH_4^+ under aerobic conditions. However, Magalhaes et al (1978) isolated several mutants by selection of ClO_3^- resistance for both species of Azospirillum which in the presence of 10 mM NO_3^- fix as much N_2 as in its absence. Shanmugam et al (1978) also isolated several strains of Klebsiella which produce nitrogenase activity even in the presence of 15 mM NH_4^+.

In the present study, experiments were carried out in semi-solid static cultures and first assay for nitrogenase activity was performed after 24 h of growth in the presence of 1 mM, 2 mM and 5 mM NO_3^- and NH_4^+. Presence of nitrogenase activity, even higher than the control signifies the importance of these characteristics as a basis for studies on complementation of N_2-fixation with mineral fertilizers.

4.5.2 Effect of Yeast Extract on Nitrogenase Activity of Bacterial Isolates:

Growth factors are required for the optimum growth of bacteria including N_2-fixing microorganisms. Several authors have included yeast extract in the isolation medium as a starter N-dose and also as a mean to provide growth factors to N_2-fixing microbes (Rennie, 1980a and Watanabe & Barriquo, 1979).

Five levels of yeast extract (Difco); 10, 50, 100 200 and 300 mg yeast extract L^{-1} were supplemented to combined carbon medium. Control samples were without any N-source. All other parameters were same as described for NO_3^- and NH_4^+ treatments. Samples were analysed after 24, 48, 72 and 96 h.

Control samples measured at different time intervals showed less activity than all other treatments (Figs.12a,b,c). Except in one treatmnt (300 mg Y.E,L^{-1}) nitrogenase activity declined sharply after growth of more than 24 h in static cultures. At the end of the experiment, the activity in all the treatments were nearly the same. After 24 h of growth in the semi-solid media supplemented with different amount of yeast extract, nitrogenase activity was found to be maximum in media supplemented with 50 mg Y.E,L^{-1} in all the isolates selected for the assay. Nitrogenase activity in the medium supplemented with 300 mg Y.E,L^{-1} followed the highest value. In all other treatments values were found to be low.

Nitrogenase activity measured after 48 h of growth showed a steep decline in nitrogenase activity as the organisms had exhausted the C-source and also due to the adverse changes in the growth conditions. However in one treatment (300 mg Yeat Extract, L^{-1}) with Iso-2 and C-2, nitrogenase activity showed an increase, Initially (after 24 h) low activity in this treatment may be due to the repression of nitrogenase activity by combined-N present in the yeast extract. Once the bacteria had used up some part of the available fixed N, activity started rising up. Nitrogenase activity measured after 72 and 96 h of growth again found to be low. No activity of Iso-2 was detected in control as well as in medium supplemented with 10 mg Y.E, L^{-1}.

Earlier, effect of yeast extract (0.1 g L^{-1}) on nitrogenase activity of a strain (SSt 22) isolated from Kallar grass was compared with control in a semi-solid medium by Bors et al (1982). They observed that addition of yeast extract considerably shortened the lag phase of bacteria and significant difference in nitrogenase activity existed between the two treatments upto 3 days. After this period the activity was levelled up. Watanabe & Barriquo (1979a) while isolating free living N_2-fixing organisms from rice roots observed that mineral or organic-N was required for the successful isolations. They supplied 100 mg Yeast Extract, L^{-1} to the basal semi-solid glucose mineral medium. These authors also compared nitrogenase activity of 29 inner rhizoplane isolates on several media based on different supplements of organic-N (Yeast Extract), vitamin, casamino acids or mineral-N (NH_4Cl). Maximum nitrogenase activity was obtained in the medium supplemented with 100 mg yeast extract, L^{-1} and in Basal + vitamin mixture + casamino acid medium. No activity was observed in N-free medium. O'Gara and Shanmugam (1976) also recommended addition of fixed nitrogen for growing free living N_2-fixing rhizobia as they can not assimilate fixed nitrogen. Earlier Pagan et al (1975) and Kurz & La Rue (1975) have observed requirement of organic nitrogen for the detection of nitrogen fixation in N_2-fixing culture of

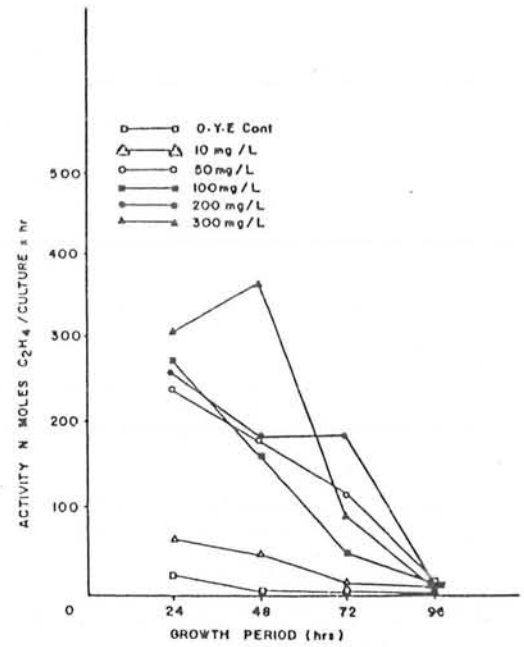


Fig. 12 a: Effect of yeast extract supply on the nitrogenase activity of bacterial isolate, NIAB-I.

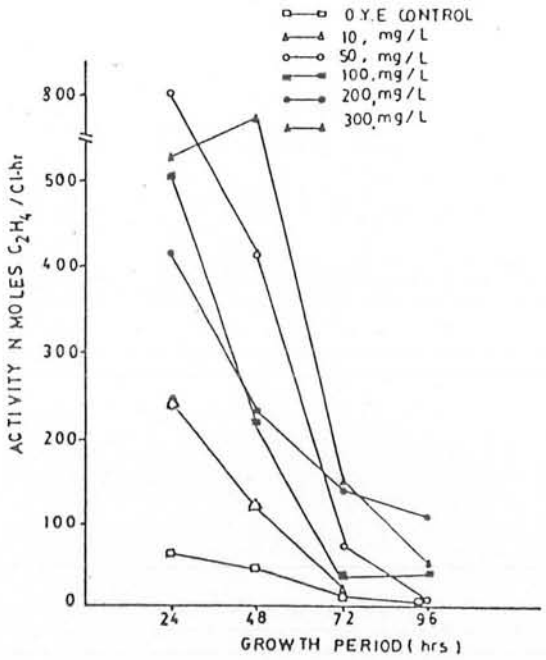


Fig. 12 b: Effect of yeast extract supply on the nitrogenase activity of bacterial isolate, Iso-2.

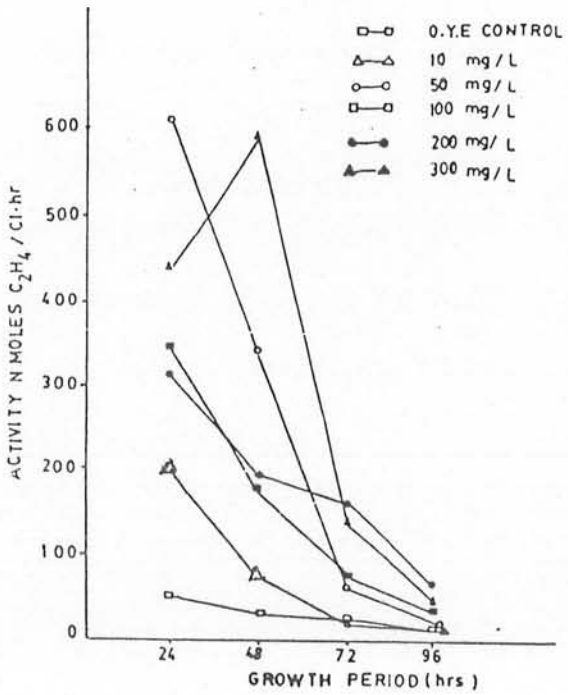


Fig. 12 c: Effect of yeast extract supply on the nitrogenase activity of bacterial isolate, C-2.

free living rhizobia. In the present study, low growth and activity was in isolates grown on completely N-free medium which may be due to the that nitrogenase activity is too low. In the presence of organic-N, gre cell mass was produced which resulted in lowering the oxygen concent and increasing the nitrogenase activity. No excretion of N was observe sample which showed that unlike free living rhizobia these isolates are assimilate the fixed nitrogen.

4.5.3 Effect of pH on Nitrogenase Activity:

A physiological pH value is a prerequisite for the growth of fr living nitrogen fixing bacteria, however, range of pH tolerated is wide (Postgate, 1981). It is a major physio-chemical factor affecting the gro diazotrophs.

The mean value for the pH of saline soils is 8-10 indicating the alkalinity of a great part of the soils. Azotobacters are generally report to grow in neutral or alkaline soils. A survey of 264 Danish soils of wi different pH recorded by Jensen (1965) revealed that practically 100% o soils above pH 7.5 contained Azotobacters in numbers varying between 10^4. Below pH 6.5 only a small fraction of soils tested contained a few Azotobacter cells however alkaline substance(s) responsible has not yet identified (La Rue, 1977). This indicated the existance of some relatio and occurrence of specific group of bacteria. Therefore, nitrogenase ac of three isolates were measured at 8 different pH (5.5-9.0). Bacterial n was same as described in Appendix-1 but with lower concentration of ph (K_2HPO_4 = 0.4 gm L^{-1} KH_2PO_4 = 0.1 gm L^{-1}) and yeast extract (10 was also added. pH was adjusted with NaOH or HCl and rechecked aft Duplicate samples were analysed after 24, 48, 72 and 96 hrs of growth described for NO_3^- and NH_4^+ treatments.

These experiments revealed that bacterial isolates when grown in pure culture under laboratory conditions, tolerated a wide range of pH values (Figs.13a,b,c). Maximum nitrogenase activity was observed in the pH range 7.0-8.5 and lower activity at acidic pH. However, each bacterial isolate behaved differentially. After 24 h of growth in modified medium of combined carbon, Iso-2 produced maximum nitrogenase activity (1011 nmoles culture^{-1} h^{-1}) at pH 8. The other isolate NIAB-I showed nearly same values at pH 7, 8 and 9. The third isolate, C-2 have relatively narrow range and maximum mean activity (135 nmoles culture^{-1} h^{-1}) was observed at pH 7 and at both extremes of pH low values of nitrogenase activities were observed.

The nitrogenase activity measured after 48 h of growth were found to have similar pattern however the differences between various pH treatments more pronounced in Iso-2. After 72 h of growth, nitrogenase activity dropped down sharply in the same isolate but in NIAB-I and C-2 isolates marked differences between different pH treatments were still clear. Nitrogenase activity measured after 96 h of growth was levelled down for NIAB-I and Iso-2 while activity of C-2 at pH 7 and 8 was still higher than the remaining treatments.

Most bacteria fix dinitrogen optimally at a pH near neutrality (La Rue, 1977) however various exceptions exist. Becking (1961) measured the nitrogen fixing ability of Beijerinckia at alkaline pH and observed the growth in a medium with initial pH of 10. Azotobacter could grow on combined nitrogen at pH 5 but it can not fix nitrogen below pH 6.0 (Becking, 1961). The range for Drexia gummosa to grow and fix nitrogen under laboratory conditions was estim. by Jensen et al (1960) at the pH range of 5-9 with optimum values from pH 5.6-6.2. The optimum pH for Mycobacterium flavum was reported by La Rue (1977) to be 4.

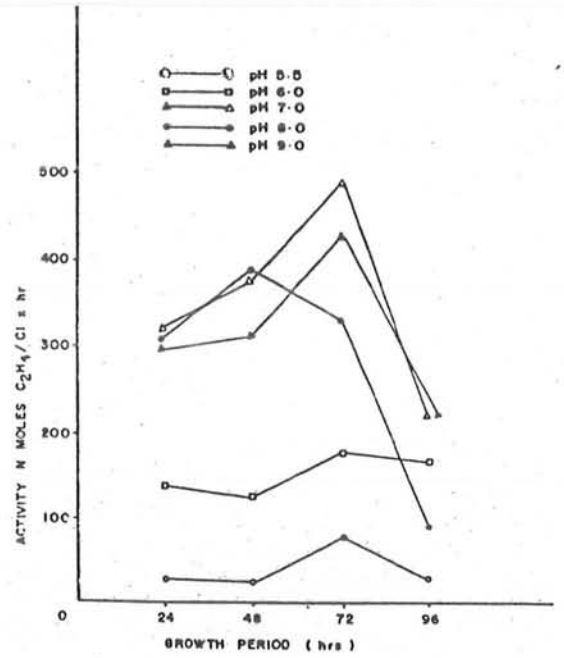


Fig. 13 a: Nitrogenase activity of bacterial isolate NIAB-I measured at different pH levels.

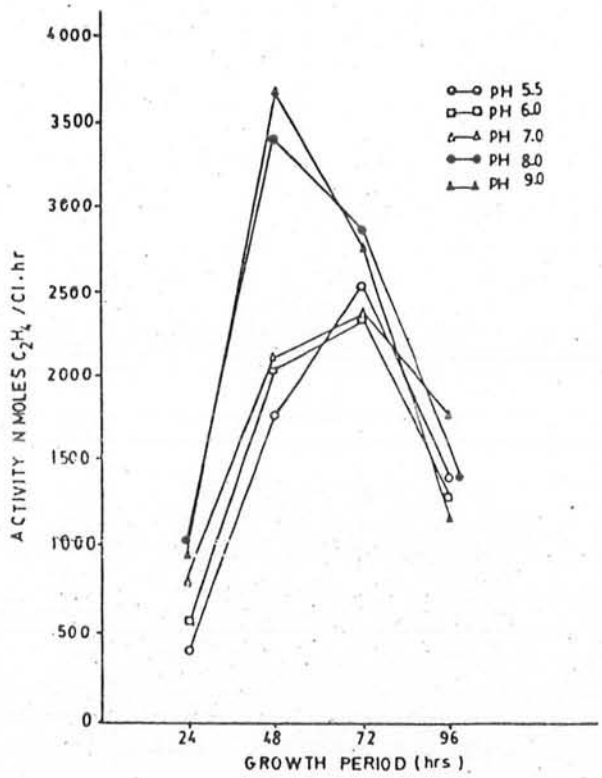


Fig. 13 b: Nitrogenase activity of bacterial isolate Iso-2 measured at different pH levels.

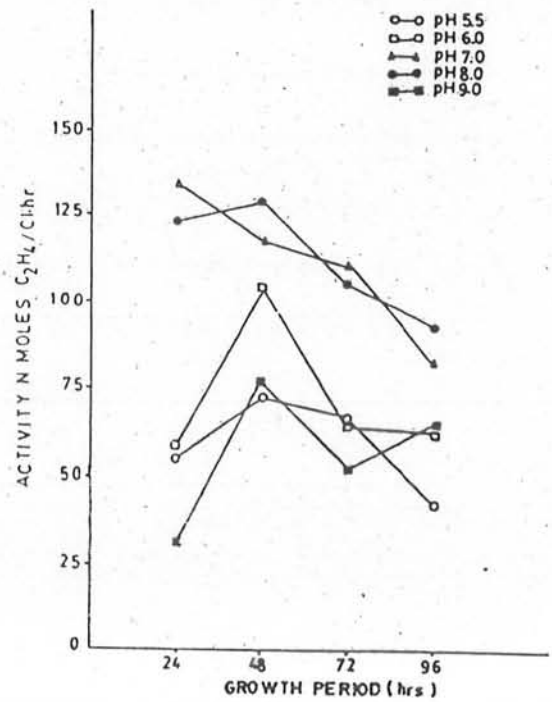


Fig. 13 c: Nitrogenase activity of bacterial isolate C-2 measured at different pH levels.

Although different tolerant ranges exist for N_2-fixing bacteria however it is not known what are the factor(s) which are responsible for such behaviour. On the other hand the isolation of such bacteria is extremely useful enabling its potential use in extreme environments like salt marsh, saline soils and tropical acid soils. Our studies on pure cultures of bacterial isolates under laboratory conditions showed that all isolates have wide range of pH for N_2-fixing ability with a optimum pH range of 7.0-8.5. The range is more narrow for C-2 (7-8) while NIAB-I and Iso-2 have much wider range. The pH of the medium was measured after every estimation in each treatment and it was observed that pH dropped down to 5-6 and at this acidic pH, nitrogenase activity was found to be low in all the isolates.

4.5.4 Combined Effect of pH and Salt on Nitrogenase Activity of Isolates:

Salinity along with drought involve changes in water potential. The situation is further complicated by the type of salinity (ionic species) and whether it is coupled to pH changes (Sprent, 1983). The sites descriptions from where the L. fusca was collected are described in Chapter- 2 . These soils have alkaline pH (8-10) and predominant cation is Na^+ (135 mmol L^{-1}) thus making these soils saline-sodic. Free heterotrophic bacteria present in saline environments (Water, Soil) have been listed recently by Sprent (1983). According to this author, N_2-fixing bacteria in such regions tend to be carbon limited and fix little nitrogen. Some may survive in the saline conditions without growth, others tolerate salinity and still others are halophytic. Studies conducted at different pH (5.5-9.0) with the N_2-fixing isolates from Kallar grass are already reported in the previous section but this problem (high pH) in our soil is linked with the preponderance of sodium salts (Na^+).

Experiments were therefore conducted to measure the effec nitrogenase activity by addition of different amount of sodium salt at two pH levels (7.5 and 9.5). Three isolates, NIAB-I, C-2 and I were selected to study the effects of 5-levels of salt (NaCl) at two pH. The isolates were inoculated on to semi-solid CCM as described experiment except that 1,2,3 ,4 and 5% NaCl (E. merck) was added. was adjusted to 7.5 and 9.5 with NaOH and 0.2% agar was added. S milliliter medium was distributed in 30 ml McCartney vials. A control having 0.01 % NaCl at each pH (7.5 & 9.5) was also included. After of growth, duplicate samples were analysed for nitrogenase activity 1,2 and 3 h of incubation with acetylene. Similar observations were after 48, 72 and 96 h. Details of assay conditions are given in Ch.

Results of these experiments are presented in Figs. 14 a,b All isolates were completely unable to grow and fix nitrogen at 5% level at pH 7.5 as well as at pH 9.5. The nitrogen fixing activities the isolates at 4% NaCl level were also found to be extremely low an at the same level at four measuring intervals. Moreover, with the of Iso-2 other bacterial isolates produced higher activity at 1% Na Iso-2 exhibited higher activity at 0.01 % NaCl (control).

After 24 h of growth nitrogenase activity was measured in samples. NIAB-I exhibited higher activity (871 nmoles culture^{-1} h grown in medium supplemented with 1% NaCl and pH adjusted to 7 activities of control samples (0.01 % NaCl) at both pH levels were f less than the nitrogenase activity at 2% salt level. In isolate C-2 activity (464 nmoles culture^{-1} h^{-1}) was obtained at 1% NaCl level At the other pH (7.5), ARA value (420 nmoles culture^{-1} h^{-1}) wa close. The nitrogenase activities at 3% and 1% level of NaCl were al

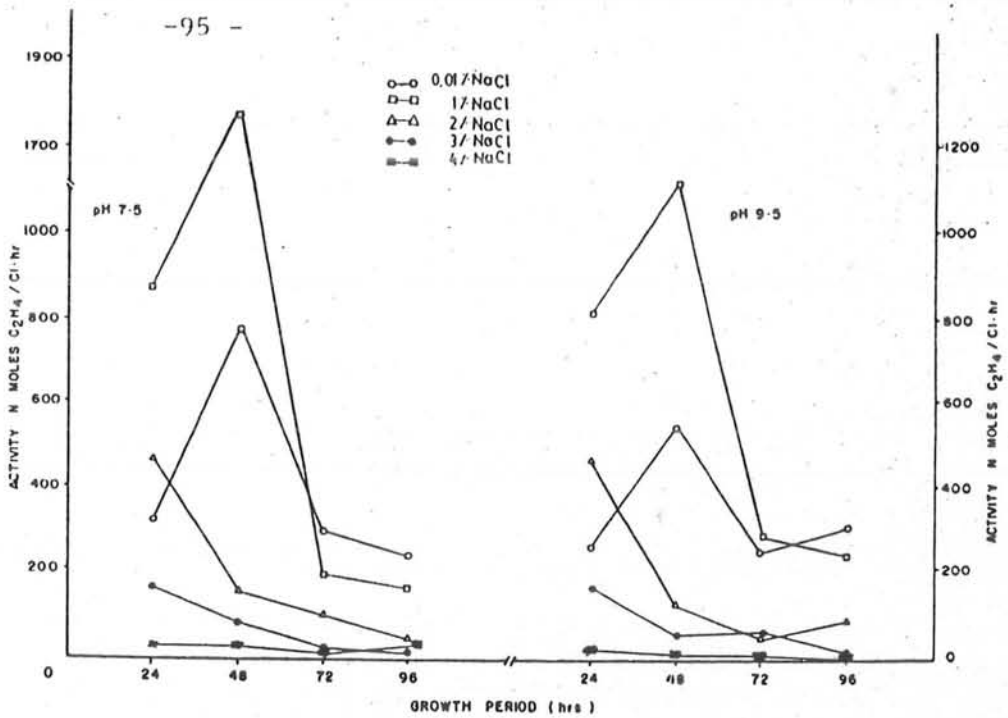


Fig. 14 a: Effect of different salt concentrations (NaCl) and pH on the nitrogenase activity of bacterial isolate, NIAB-I.

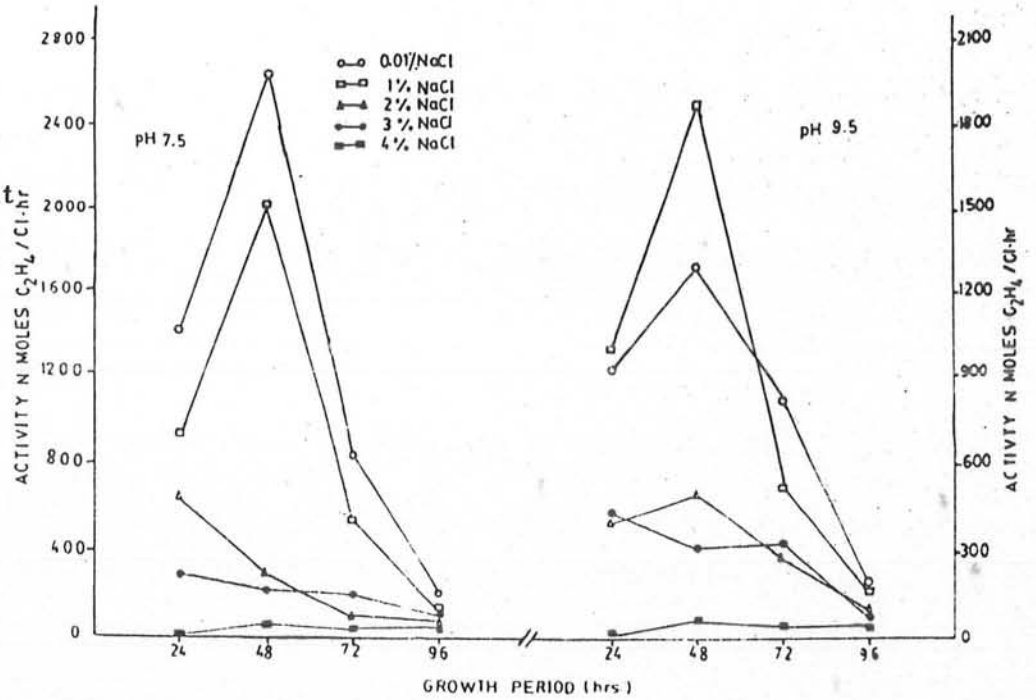


Fig. 14 b: Effect of different salt concentrations (NaCl) and pH on the nitrogenase activity of bacterial isolate, Iso-2.

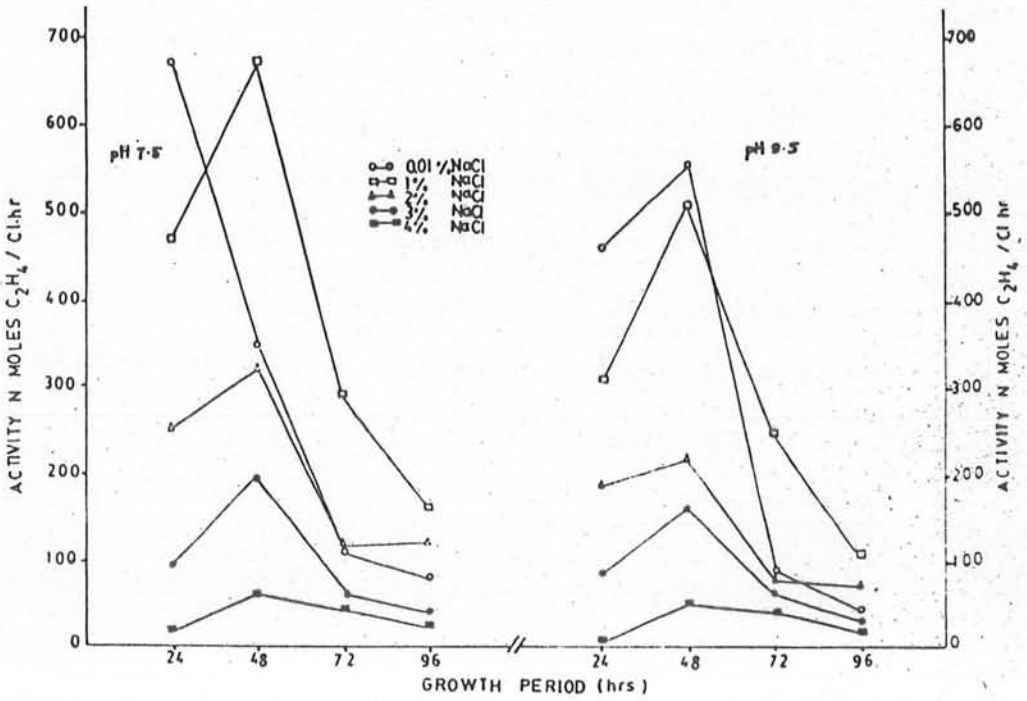


Fig. 14 c: Effect of different salt concentrations (NaCl) and pH on the nitrogenase activity of bacterial isolate, C-2.

be higher than the control (0.01 % NaCl). Iso-2 was the only bacteria among all the isolates which showed maximum activity at 0.01 % NaCl and at pH 7.5. The activity declined as the salt levels were increased.

Nitrogenase activities measured after 48 h of growth showed same pattern in all isolates except in C-2 where maximum nitrogenase activity was observed at 1% NaCl level (pH 7.5) instead of 2% NaCl (pH 9.5). After 72 h and 96 h of growth period, nitrogenase activities in all treatments declined sharply. Two isolates, NIAB-I and Iso-2 exhibited maximum activity at 0.01 % level (control) while mean maximum activity in C-2 was consistently measured in medium supplemented with 1% NaCl (pH 7.5).

It is inferred from this study that N_2-fixing bacteria isolated from the rhizosphere of Kallar grass are salt tolerant. The extent of tolerance varies with different isolates as well as with the age of culture and hydrogen ion concentration (pH). The exact mechanism(s) of salinity tolerance is not known (Sprent, 1983). The physiological basis of tolerance from extreme halophytes has been extensively reviewed by Dundes (1977). Most of the studies on the effect of salinity on diazotrophs deals with the occurrence of such bacteria in these environment (Abd-el-Malik, 1971 & Mishustin & Shil nikova, 1971). Jensen(1981) while reviewing the heterotrophic N_2-fixing microorganisms stated that Azotobacter strain isolated from a strongly saline soil could grow with sodium chloride concentrations upto 0.75 M, but showed maximum nitrogen fixation with 0.14 M sodium chloride. Werner et al (1974) isolated a strain of Klebsiella pneumoniae and a strain of Enterobacter aerogenes, which could grow and fix nitrogen in the presence of sea water. Jensen (1981) reported that five strains of nitrogen fixing Clostridia were completely unaffected by sodium chloride in the medium upto 0.5 M and that 12 strains of Desulfovibrio had an obligate salt concentrations

between 0.2-0.4 M NaCl. Patriquin (1978) isolated from roots of Spartina growing in salt marshes a Spirillum-like nitrogen fixing organism which showed good growth over the range of 0.0 to 0.5 M NaCl and a facultative anaerobe which could not grow without added salt, but grew well in media containing 0.03 to 1.20 M NaCl. Nayak et al (1981) also isolated some strains of Azospirillum from unfavourable alkaline rice soils (pH 8.0, C.E.C, 42.0 meq 100 g^{-1}). They observed considerable N_2-fixing activity in cultures from such soil but the authors pointed out that physiological activity of these isolates under such adverse conditions needs to be established under natural conditions.

The occurrence and capability of fixing atmospheric nitrogen under higher salt and hydrogen ion (pH) concentrations open up some possibilities for selection of the most efficient strains, even though the question remains whether such strains will be as efficient in the soil under natural conditions as they were active in laboratory conditions.

4.6 CONCLUSIONS

Enrichment and isolation of roots of Kallar grass resulted in collection of large number of acetylene reducing bacteria. Three of the most occurring isolates namely NIAB-I, Iso-2 and C-2 were studied extensively for characterization.

C_2H_2-reduction is rapid, easy and senstive technique but still it is an indirect method (Turner & Gibson, 1980). Therefore ^{15}N incorporation studies were carried out on these isolates. Enrichment was observed in all the isolates which clearly indicates that these isolates are true N_2 fixers.

If significant differences were to occurr in DNA base composition among a number of strains, this alone would be a strong evidence for the existence of more than one species (Kreig & Tarrand, 1978). The mol % G+C of NIAB-I, C-2 and Iso-2 were found to be 56.9, 63.6 and 53.2 respectively.

The possibility of Azospirillum could not arise as the mol % G+C of this genus is present in the range of 69-70 (Tarrand et al, 1978). In another study on Kallar grass roots, Bors et al (1982) were able to identify one of their strains (SSt 22) as A. lipoferum but it is known that type of organisms isolated from rhizosphere depends on type of media and conditions used for the enrichment and isolation (Balandreau, 1983).

Battery of tests were performed to identify these isolates but comparison with known N_2 fixers is difficult as complete biochemical characterization of N_2-fixing bacteria is not common (Buchanan & Gibbons, 1974) since they are usually not of medical intrest. The characteristics of these isolates were compared with the known N_2-fixer; Azotobacter and Azotomonas (Johnstone,1974 and Becking, 1981) Beijerinckia (Becking, 1974, 1981) and Klebsiella (Seidler, 1981).

The summary of major similarities between NIAB-I and Klebsiella pneumoniae and K. oxytoca is presented in Table 11 . It is evident that NIAB-I could be identified as K. pneumoniae although one deviation do exists (NIAB-I is catalase +ve while K. penumoniae is negative). This isolate could not be placed in K. oxytoca as all of the strains of this species are indole positive (Jain et al, 1974). The occurrence of this genus has recently been reviewed by Seidler (1981). Recently the presence of this organism has also been reported by Lethbridge & Davidson, 1983 , Lutfi et al (1981), Raju et al (1972) and Wood et al.(1981).

Comparison of different characteristics of remaining two isolates (Iso-2 and C-2) with the possible closer genera is presented in Table 12 . With one or two exceptions, Iso-2 could be safely placed in the genus Beijrenckia. It is know that some B. indica strains do not produce any pigment (var. alba). The actual position of C-2 isolate is uncertain and although it appears to be related to the Azotobacteriaceae, there are significant differences between

Table 11: Comparison of NIAB-I isolate with Klebsiella species. Data of K. pneumoniae and K. oxytoca is taken from Bergeys Determinative Bacteriology (1974) and from Siedler (1981).

Characteristics	NIAB-I	K pneumoniae	K. oxytoca
Mole % G + C	56.9	53.9-59.2	53.9-59.2
Motility	-	-	-
Capsule	+	+	+
Spore	-	-	-
Oxidase	-	-	-
Catalase	+	-	-
Indole	-	-	+
VP*	+	+	+
ADH**	-	-	-
ODC***	-	-	-
Ureas	+	d mostly +	d mostly +
$NO_3' \rightarrow NO_2'$	+	+	+
Citrate utilization	+	+	+
Starch hydrolysis	-	-	-
Acidification of:			
Glucose, Sucrose, Mannitol, Xylose, Trehalose	+	+	+

* Voges- Proskaur reaction; ** Arginine dihydrolase ; *** Ornithine decarboxylase; d- differential; +, Positive; -, negative.

Table 12: Comparison of the bacterial isolates with other Gram-negative aerobic N_2-fixing bacteria. Data for all bacteria except C-2 and Iso-2 is taken from Bergey's Determinative Bacteriology (1974) and from Becking (1981).

Characteristics	C-2	Iso-2	Azotobacter	Azomonas	Beijernckia	Mycobacterium flavum 301	Drexia
DNA (G+C mole %)	63.7	53.4	63-66	53-59	54-59	69	70
Cell size	Large	Small	Large	Large	Small	Small	Large
Motility	+	+	±	+	±	-	+
Pigment (non-white)	Off-while yellowish	Off-white	+(Also fluoresces)	(+ with age)	± (+ with age)	+	± (+ with age)
Slime	+ (less)	+	±	+	+	±	++
Catalase	+	+	+	+	+		-
Growth on Peptone	+	+	NA	-	+	+	NA
Malate	+	+	NA	NA	±	NA	NA
Glucose	+	+	+	+	+	-	+
Starch Hydrolysis	-	-	±	-	NA	NA	-

NA = Not available.

the isolates and each of the species in the family of Azotobacteriace . Recently some new N_2-fixing microorganisms have been reported i.e. Campylobacter from Spartina alterniflora(Patriquin, 1978) Xanthobacter (Malik & Claus, 1979), Pseudomonas spp from rice (Barraiquo et.al, 1983, Thomas Bauzon et al., 1982) and from grass, Deschampsia caespitosa (Haahtela, 1983) and Lignobacter strain K.17 (Salkinoja-Salonen et al,(1979) and non-motile Azospirillum (Bally et al, 1983). The precise description of a bacteria species now even involves DNA/RNA hybridization (de Smedt et al. 1980) and immunological reactions. We have deposited the cultures to DSM (German collection of Microorganisms), Gottingen, F.R. Germany for their complete identification.

Commercial identification kits, API 20E & API 50 CH were also employed but their utility is only of some advantage if computure linked analysis of the results is possible as described by Rennie (1980a).

Physiological experiments revealed that all these isolates have relatively high tolerance limits of pH and sodium chloride. Activity was found to be higher in the medium supplemented with 50 mg yeast extract L^{-1}. All bacterial isolates were able to exhibit nitrogenase activities in the semi-solid static cultures amended with as higher as 5 mM nitrate or ammonium.

This study and related studies by several other workers (reviewed by Vose, 1983) on a variety of N_2-fixing bacteria from rhizosphere will help to increase our understanding of the biology of N_2-fixation in the soil and to better define the importance of a particular bacterial strain. From this study it is not possible to estimate the frequency of occurrence of these bacterial strains in the rhizosphere of Kallar grass. Use of fluorescent antibody technique would be useful in quantification of these particular isolates in the presence of similar N_2-fixing bacteria in the soil and near the roots.

Contribution by these bacterial strains to N_2-fixation in association with Kallar grass was examined by performing inoculation studies which are reported in the next chapter.

CHAPTER-5. INOCULATION STUDIES

Inoculation of free living bacteria to cereals and grasses is not a new concept. By 1958 about 10^7 heacters in the USSR were treated with the bacterial fertilizers mainly prepared from cultures of Azotobacter chroococcum. This work was reviewed by Brown (1982) who concluded that inoculation of seeds or roots led to changes in plant growth and some times to yield increase but most often effect was found to be insignificant. Scientists from other countries had dissmissed the value of these inoculant. Due to recent increase in cost of N-fertilizers there is a renewed interest especially in developing countries to increase and exploit biological N_2-fixation by grasses - N_2-fixing bacterial associations. Present research efforts are mainly concentrated on the use of Azospirillum as bacterial inoculant (Albrecht et. al, 1977; Bouton et al.. 1979; Cohen et al. 1980 and Smith et al, 1977). Only few attempts to inoculate plants with other N_2-fixing bacteria like Klebsiella pneumoniae (Klucas et al. 1981; Lethbridge & Davidson, 1983; Wood et al. 1981 and Wright & Weaver, 1982) and Bacillus polymyxa (Lethbridge & Davidson, 1983 and Rennie & Larson., 1979). have been made.

Measurement of responses to inoculation of grass roots with selected diazotrophic bacteria is important in evaluating associative N_2-fixation. The rhizosphere of kallar grass in saline areas are found to be active in AR. Diazotrophic Klebsiella (NIAB-I) and two other isolates C-2 and Iso-2 were isolated from roots of some of these plants. In the present chapter, inoculation studies on kallar grass are reported. Inoculation experiments were carried out to study the effect of N_2-fixing bacteria on the growth of L. fusca. Studies were performed by using

a) Sterile glass assembly
b) Pot cultures containing sterile washed sand with 6 levels of NO_3^- and NH_4^+ .
c) Gravel culture experiments in culture tubes for quantitative estimation of BNF in L. fusca by using N-15 isotopic dilution technique.

In all the above experiments bacterial isolates from kallar grass or Azospirillum spp. were used and heat killed bacterial suspension was also employed to look for the effects other than the N_2-fixing activity. These experiments are described under appropriate sub-titles.

5.1 INOCULATION STUDIES OF HYDROPONICALLY GROWN KALLAR GRASS

For this experiment, seeds of kallar grass (Leptochloa fusca (L.) Kunth) were surface sterilised according to modified method of Nielson (1952). The seeds were placed in 2% streptomycin sulphate (w/v) solution containing 0.5 ml detergent. After 2 h seeds were soaked in 0.2% HgCl (w/v) for one hour. Several washing with sterile distilled water was performed and seeds were transferred to 1% agar plates. Plates were placed in a controlled climate chamber set at 30°C day and 26°C night temperature, day length of 16 h and light intensity of 20,000 Lux.

After 4 days, seedlings were aseptically transferred to a PVC disc (dia 2.9 cm) hanging in a long culture tube (33x3 cm) with a help of thread (Fig 15). Tubes were covered with perforated autoclavable silicone stoppers (Shinetsu Polymer Inc., Chu-O-Ku, Tokyo, Japan). To each tube, 40 ml of half strength Hoagland nutrient solution (pH 6.8) with N adjusted to 5mM NO_3^- was added and two seedlings were transferred to each culture tube. The tubes were placed in a controlled climate chamber as described above.

After 7 days of growth, each disc was aseptically transferred to a special glass assembly for growing plants under strict sterile conditions (Figs. 16 a & b). Half strength N-free Hoagland nutrient solution with pH adjusted to 6.8 (Appendix 6) was supplied to each flask. The assemblies were placed in a climate chamber having 25-28°C day (16 h), 23-25°C night (8 h) temperature; Rel. humidity 70% and light intensity (reaching at the assembly) of 14,000 Lux. Bacterial inoculations were made 3 days after transplanting to large assembly.

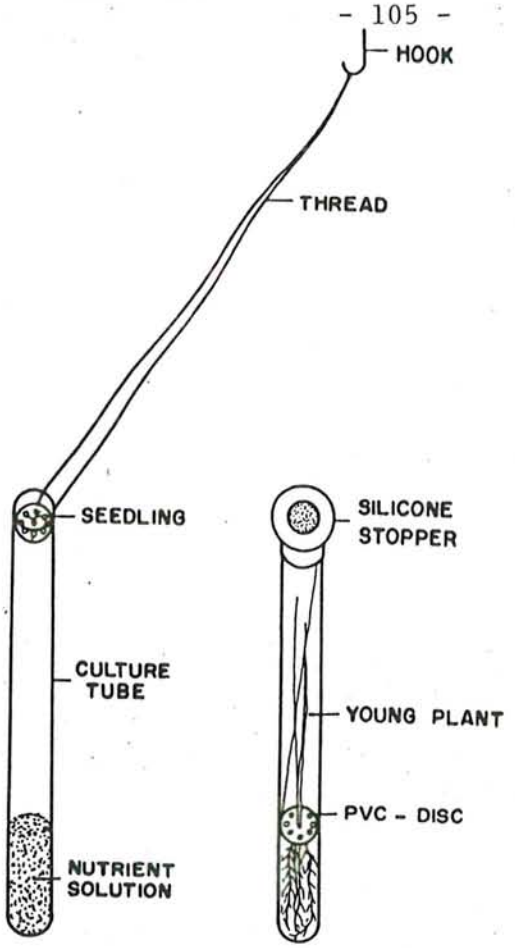


Fig. 15: L. fusca seedlings growing in long culture tube. Hoagland's solution wih 5 mM NO_3 was supplied as nutrient solution.

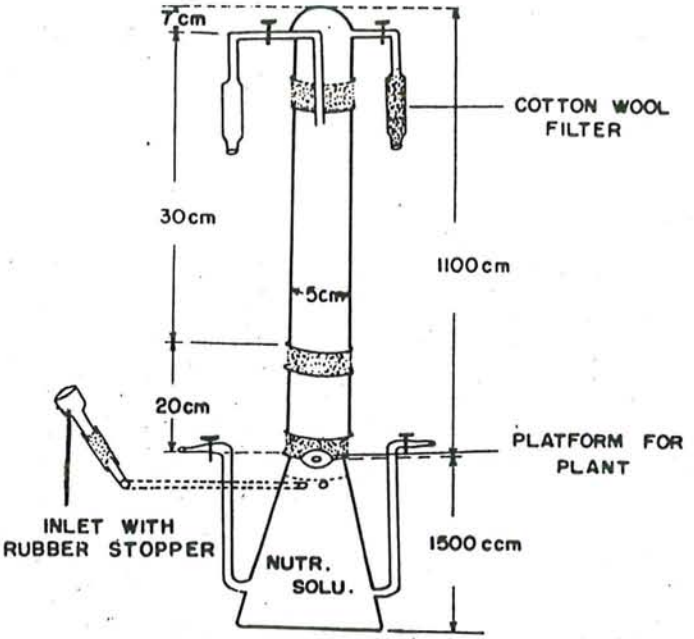


Fig.16 a: Diagramatic presentation of Special plant-defined glass assembly for studying bacteria interactions under conditions.

Fig.16 b: Glass assembly for studying plant bacteria interactions under defined conditions.

Bacterial inoculations of NIAB-I and Azospirillum sp (supplied by Dr Vlassak, Belgium) were made. For the production of inoculum, bacteria were grown seprately in CCM with 100 mg yeast extract and 1 gm NH_4Cl L^{-1}. Culture suspensions containing 100 mg bacterial protein were supplied to each assembly containing two plants of L. fusca. Another portion of the suspension was autoclaved to provide same amount of protein (bacteria killed) to other set of plants. Other control was without any treatment.

Three days after inoculation, ARA was performed on 6, 8 and 10th day of growth in assembly. All inlet ports were closed with serum stoppers (13x18 mm) and 12% acetylene was injected after replacing same amount of air. Gas samples were collected in Vacutainer (Becton-Dickinson, NJ, USA) after every 4 h using two way sterile needle. Gas samples were analysed on a gas chromatograph as described in Ch.2.

Plants were harvested after 27 days of growth and root/shoot length and fresh weights were taken. Dry weights were determined after keeping the plants at 70°C in a forced air oven for two days. Total-N was measured by Kjeldahl method as described in Ch.2. Protein was determined according to the method of Lowry et al (1951).

The results of the effect of inoculation by a N_2-fixing strain NIAB-I isolated from rhizosphere of kallar grass and Azospirillum sp. on the growth of L. fusca are summarized in Table 13. It is now well known that N_2-fixing microorganisms produce phytohormones (Brown & Burlingham,1974 and Tien et al., 1979) which in part may be responsible for higher yield (Hubbell et al.,1981). Heat killed bacterial suspensions were supplied to cultural solutions to look for such effects. The effect on root and shoot length by both live and dead bacteria was not so pronounced. However, in case of live inoculation, abundant root hairs

Table 13 : Effect of inoculation on the growth of L. fusca grown in sterile nutrient solution. Readings are averages of 3 replicates. Figures followed by same letter are not significantly different at 1% level as determined by DMR test.

Sr.No.	Treatment	Length (cm)		Fresh wt mg.plant^{-1}		Total fresh wt. mg,plant^{-1}	Dry wt. mg,plant^{-1}		Total dry wt. mg,plant^{-1}
		Root	Shoot	Root	Shoot		Root	Shoot	
1.	Control	14 b	36 a	19 c	68 c	87	7 c	21 d	28
2.	Azospirillum (Heat Killed)	17 a	30 a	36 b	81 c	117	10 bc	31 bc	41
3.	NIAB-I (Heat Killed)	11 c	34 a	38 b	112 b	150	8 c	26 cd	34
4.	Azospirillum sp.	14 b	33 a	5 aa	139 ab	189	12 ab	48 a	60
5.	NIAB-I	12 bc	37 a	168 a	211	14 a	41 a	41 ab	55

were observed which did not contribute to the root length but were quite apparent from the fresh weights of the roots. Maximum fresh weight of roots (42.5 mg) was obtained in case of Azospirillum sp while NIAB-I gave the maximum shoot fresh weight (167.5 mg). An increase in dry matter was observed in all treatments. Inoculation with Azospirillum sp increased the root and shoot dry weights by one fold. Comparable results were obtained by the inoculation of NIAB-I. Several other workers have also found that plant inoculated with N_2-fixing microorganisms produced yield increase (Kapulnik et al, 1981; Nur et al. 1980; Okon. 1984 and Smith et al. 1976).

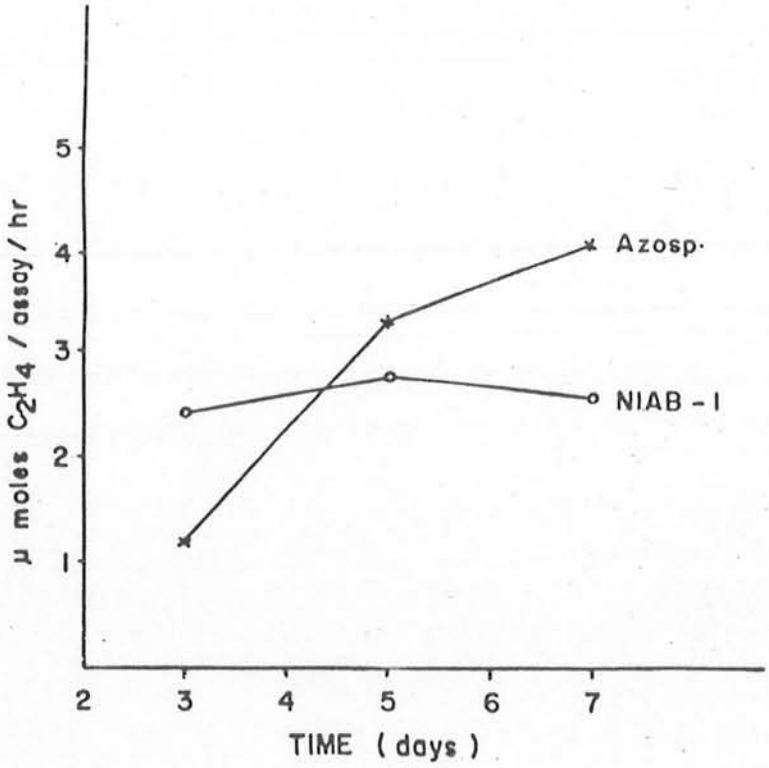
Inoculation of bacterial isolates resulted in higher total-N content of the plants (Table-14) Maximum nitrogen was estimated in case of NIAB-I which was 538 µg, plant^{-1} as compared to 438 µg in case of Azospirillum sp and 382 µg in uninoculated control. On the other hand, % N of treated plants was found to be equal or less than the control plants. Rennie (1980) has also made this observation and concluded that % N is a poor parameter and real important effect is the increase in the total-N. Schank et al., (1981) speculated that inoculated plants not only grew faster but also matured earlier and this resulted in lower percentage of N-content of the inoculated plants but a higher dry matter.

Acetylene reduction assays were performed 3 days after inoculation and these results are presented in Fig. 17 . In inoculated assemblies nitrogenase activity was observed after 4 h of incubation and remained linear when samples were collected at 4 h intervals upto 24 h. In case of NIAB-I initial acetylene reduction rate was higher than Azospirillum sp but after 5 days, Azospirillum sp was able to reduce acetylene at a higher rate. It was already observed that NIAB-I isolate has a short doubling time and higher population of this organism may be responsible of its higher activity in the first assay. After 7 days, a rate of 4.1 µ moles C_2H_4 assembly was detected in case of Azospirillum sp as compared to 2.6 µ moles assem $^{-1}$ for NIAB-I.

Table 14 : Effect of inoculation on the nitrogen contents of Leptochloa fusca. Samples from each treatment were pooled together and digested material were titrated thrice.

Treatments	Total Nitrogen μg plant^{-1}	% N
Control	382	1.36
Azospirillum sp (Heat Killed)	408	0.99
NIAB-I (Heat Killed)	473	1.39
Azospirillum sp	438	0.73
NIAB-I	538	0.97

Fig. 17 : Nitrogenase activity of Azospirillum sp. and NIAB-I inoculated to aseptically growing Kallar grass in nutrient solution.



By the utilization of special glass assembly for growing plants under strict sterile conditions the beneficial effects of N_2-fixing microorganisms inoculation on kallar grass were demonstrated. Higher values of acetylene reduction clearly indicates the establishment of N_2-fixing microorganisms and enhanced yield and total-N is partly due to the supply of newly fixed atmospheric nitrogen by the diazotrophs.

5.2 SAND CULTURE EXPERIMENTS:

After performing experiment in strict sterile conditions it was established that inoculation has a significant effect on yield and total-N of kallar grass. In order to further understand the process and for determining some optimal levels of combined-N at which growth and N_2-fixing activity could complement, pot experiments with six levels of combined-N (NO_3^- & NH_4^+) were carried out.

Seeds were surface-sterilised and germinated on 1% agar plate as described in Section 5.1. After 4 days seedlings were transferred to each plastic pots (13x11 cm) containing acid-washed sand. Five seedlings were transferred to each pot which were latter thinned to 3 plants. Seedlings were irrigated with half strength Hoagland nutrient solution with N-adjusted to desired NO_3^- or NH_4^+ levels. The pH was adjusted to 6.8. Nutrient solution was provided twice a week while sterile water was supplied according to requirements. Following N-treatments were provided.

NO_3^-		NH_4^+
$KNO_3 + Ca(NO_3)_2$		NH_4Cl
	Low Level	
1. 0.1 mM		0.1 mM
2. 0.5 mM		0.5 mM
3. 1.0 mM		1.0 mM
	High Le	
4. 2.5 mM		2.5 mM
5. 5.0 mM		5.0 mM
6. 10.0 mM		10.0 mM

Pots were placed in a controlled climate chamber set at 25-28°C day (16 h) and 22-24°C (8 h) night temperature, light intensity of 14,000 Lux and Rel.humidity at 70%. Each treatment was replicated thrice and placed in a complete randomized fashion. After two weeks of growth, inoculum was provided.

Azospirillum brasilense sp 107 (obtained from Dr. Lethbridge, Aberdeen UK, originally from wheat field in Brazil) was grown in sodium malate medium with 1 gm NH_4 Cl L^{-1} as described by Okon et al (1977b). After 3 days of growth at 30°C on a rotary shaker, bacterial cells were harvested by centrifugation on a L 265 B Beckman centrifuge at 18,000 rpm for 30 minutes. Pellet was washed with phosphate buffer (0.1 M, pH 6.8) and resuspended in the same buffer. One ml of concentrated bacterial suspension (0.5 mg Protein ml^{-1}) was injected by sterile syringe near the roots of each plant. In all experiments one set of control plants was treated with an equal volume of an autoclaved cell suspension.

Samples were analysed on 30, 45 and 60th day after transplantation (DAP). Three pots from each treatment were taken and fresh weights were recorded for root and shoot. Dry matter was taken after keeping the samples at 70°C in a forced air oven for 5 days. Total nitrogen of each part was determined by Kjeldahl method as described in Chapter 2. Protein was estimated by Lowry method (Lowry et al., 1951).

At the time of each sampling, ARA of detached roots was also performed. Roots were washed in a gentle pale of tap water and placed in 30 ml McCartney vials, evacuated and flushed with argon gas. After repeating the process for 4 times, 12% (v/v) acetylene and 1% (v/v) air was added to vials after replacing the same amount of gas. Gas samples were analysed on a gas chromatograph as described in Chapter 2.

5.2.1 Effect of Low Level of Combined-N on Yield, Total-N and Nitrogenase activity:

The purpose of these experiments was to find out the optimal concentrations of NO_3^- & NH_4^+ needed to give maximum C_2H_2 reduction and yield in kallar grass when inoculated with Azospirillum brasilense sp 107 in sterilized sand system. A two factor factorial experiment in randomized complete block design with 3 replicates was used for statistical analysis. The data was subjected to analysis of variance and least significant difference test.

It was observed that with the increase of N-supply, fresh and dry weights also increased and this increment was more pronounced during 2nd and 3rd harvest. At lower levels of NO_3^- and NH_4^+, fresh and dry weights were consistently higher in treatments supplied with heat killed and live bacteria however difference between these two treatments was found to be significant at 0.5 and 1 mM NO_3^- or NH_4^+ . No difference in average yield was observed when plants were supplied with NH_4^+ or NO_3^-. It is now well established that most plants can effectively utilize either ammonium or nitrate ions and no prefrence exists for any of the ions (Beevers & Hageman, 1980).

Fresh and dry weights were taken after 30, 45 and 60 days but statistical analysis was performed only for the dry matter. Fresh weights of control plants at the three NH_4^+-levels at DAP 60 were found to be (2415, 4186 and 6683 mg pot^{-1}) while plants supplied with heat killed bacteria had fresh weights 3402, 4775 and 7021 mg pot^{-1}. Maximum fresh weights at all the 3-N levels at DAP 60 were observed in inoculated treatments (4123, 4853 and 7318 mg pot^{-1}). Similar results were obtained when plants were irrigated with NO_3^--solution.

Dry weights of roots and shoots were obtained seprately. Marked effect of inoculation was observed on dry matter of roots and after statistical analysis data are presented in Tables 15 and 16 . One to five fold increase from control plants was observed in treated plants. The difference between heat killed bacteria

Table 15 : Effect of low NO_3^- supply on root weight of kallar grass with Azospirillum inoculation. Readings are expressed as mg, pot^{-1}

Treatment	Levels of N ($mM NO_3^-$)	Total roots weight mg, pot^{-1}		
		30 DAP	45 DAP	60 DAP
Control	0.1	38	101	157
	0.5	132	207	302
	1.0	139	283	440
Heat Killed	0.1	57	117	207
	0.5	146	235	358
	1.0	166	305	482
Inoculated	0.1	60	130	253
	0.5	167	294	398
	1.0	198	370	489
LSD ($P < 0.05$)		13	18	25

LSD for interaction (level x treatment) at DAP 30, 45 and 60 were estimated to be 18, 26 and 35 respectively.

Table 16 : Total dry weight of roots of Leptochloa fusca supplied with low levels of NH_4^+ with (Azospirillum brasilense sp 107) or without inoculation

Treatment	Levels of N ($mM NH_4^+$)	Total roots weight mg, pot^{-1}		
		30 DAP	45 DAP	60 DAP
Control	0.1	41	78	163
	0.5	143	203	342
	1.0	160	316	465
Heat Killed	0.1	54	116	263
	0.5	154	239	390
	1.0	161	356	496
Live	0.1	67	173	315
	0.5	169	256	376
	1.0	0	380	534
LSD ($P < 0.05$)		18	17	27

L.S.D. for interactions (level x treatment) at DAP 30,45 and 60 were estimated to be NS, 25 and 38 respctively.

Table 17 : Effect of low NO_3 supply on yield of Leptochloa fusca with inoculation (Azospirillum brasilense sp 107) and without inoculation. Readings are expressed as mg, pot^{-1}

Treatment	Levels of N ($mMNO_3$)	Yield (mg, pot^{-1})		
		30 DAP	45 DAP	60 DAP
Control	0.1	92	194	406
	0.5	288	569	803
	1.0	354	607	1125
Heat Killed	0.1	113	244	445
	0.5	308	598	829
	1.0	365	661	1307
Inoculated	0.1	143	280	465
	0.5	334	608	857
	1.0	370	686	1383
LSD ($P < 0.05$)		22	20	107

LSD for interactions (level x treatment) at all measuring intervals (30,45,60) were estimated to be non-significant.

Table 18 : Effect of low NH_4^+ supply on yield of Leptochloa fusca with inoculation (Azospirillum brasilense sp 107) and without inoculation. Readings are expressed as mg, pot^{-1}

Treatment	Level of N ($mMNO_3$)	Yield (mg, pot^{-1})		
		30 DAP	45 DAP	60 DAP
Control	0.1	135	228	410
	0.5	314	548	641
	1.0	326	634	1048
Heat Killed	0.1	167	284	453
	0.5	342	576	715
	1.0	367	707	1240
Inoculated	0.1	168	312	481
	0.5	363	594	768
	1.0	400	705	1288
LSD ($P < 0.05$)		21	22	44

LSD for interactions (level x treatment) at DAP 30,45 and 60 were estimated NS, 31 and 63 respectively

and live inoculum treatments was found to be significant ($P < 0.05$) at 0.5 and 1 mM NO_3^- at each successive harvest. When plants were supplied with solution containing ammonium as combined-N source, similar results were obtained. Treated plant (heat killed bacterial suspension and live bacteria) were significantly different from control as well as from each other at 0.5 and 1 mM NH_4^+ at DAP 30 and 45. At final harvest (DAP 60) effect was more clear and all treatments at each level of N were significantly different ($P < 0.05$, 25) from each other.

The yield of upper parts was found to be higher when levels of N (NO_3^- or NH_4^+) were increased (Tables 17 and 18). Effects were more pronounced when plants were supplied with either autoclaved suspension of bacterial solution or live inoculum. At lower levels of nitrate the difference between the latter two treatments were significantly different ($P < 0.05$, 22 and 19) at DAP 30 and 45 respectively. At the time of last analysis (DAP 60), though the differences between each N-levels were markedly evident however non significant differences were observed between treatments supplied with heat killed bacterial suspension and live inoculum except at 1 mM NO_3^- level. At this level both treatments were significantly different ($P < 0.05$, 107). Similar results were obtained when plants were supplied with solution containing ammonium as N-source except at 1 mM NH_4^+ . At this level non significant difference was observed between treatments supplied with heat killed bacterial suspension and live inoculum at DAP 60. These two treatments were however significantly higher ($P < 0.05$, 107) then the control.

The % N of the root when supplied with only solution containing NO_3^- was in the range of 0.68-1.47. The percent-N of treated plants (heat killed bacterial suspension and live inoculum) were found to be in the ranges of 0.69-1.49 and 0.57-1.21. The % N of plants supplied with NH_4^+ instead of NO_3^- was less than the latter treatment but difference was not significant. The % N of root declined as plants attained maturity. The % N of shoot was found to be higher.

The percent-N of shoot of control plants was present in the range of 0.74-2.27%. The % N of treated plants (Heat killed bacterial suspension and live inoculum) were found to be in the ranges of 0.74-2.33% and 0.63-2.21%. The percent N of shoot declined as plants attained maturity. Same observations were earlier made in case of roots. The total-N of root as well as of shoots were analysed. The total N of roots are presented in Tables 19&20. It is clear from the data that with the increase in the supply of combined-N (NH_4^+ or NO_3^-) the total-N was also found to be higher. Total-N of root was found to be higher in NO_3^--grown plants. At each level of NO_3^- (0.1, 0.5 and 1.0 mM) the total-N of inoculated plant was higher than the control except at first harvest (DAP 30) of 0.5 mM NO_3^- level. The total-N of roots in NH_4^+-growth plants was found to be less than NO_3^--treated plants however difference was estimated to be non significant.

The total-N of tops was analysed and presented in Tables 21 and 22. Total-N of shoot was found to be higher in NO_3^--grown plants at all N-levels except at 0.1 mM NO_3^- treatment. At this level total-N was higher in NH_4^+ treated plants. At lower level of NO_3^- only in 1 mM NO_3^- level, total-N of inoculated plants was higher than the control. In other two levels (0.1 and 0.5 mM NO_3^-), N-contents of inoculated plants were found to be lower than the plants treated with heat killed bacterial suspension or control plants. In NH_4^+- grown plants, total-N was higher in all inoculated treatments than the control however the difference between the treatments supplied with heat killed bacteria and live inoculum were non-significant except at 0.1 mM NH_4^+ at DAP 30 and at 0.5 mM NH_4^+ level at DAP 60.

Nitrogenase activity of detached roots was determined at each time of harvest (DAP 30, 45 and 60). Nitrogenase activity was found to be present only in inoculated plants which indicate that non-inoculated plants remained free of contamination by N_2-fixing microorganisms. ARA values for plants supplied with NH_4^+ was fairly lower than the NO_3^--treatments (Table 23). Maximum nitrogenase activity

Table 19: Total-N (mg-N, pot^{-1}) of roots of L. fusca as affected by inoculation of Azospirillum brasilense sp 107 at lower levels of combined-N (NO_3^-)

Treatments	Levels (mM NO_3)	Total-N mg, pot^{-1}		
		30 DAP	45 DAP	60 DAP
Control	0.1	0.29	0.72	1.14
	0.5	1.92	2.14	2.53
	1.0	1.64	2.74	3.20
Heat Killed	0.1	0.77	2.16	1.22
	0.5	1.52	2.11	2.44
	1.0	2.52	3.98	4.15
Live	0.1	0.73	0.90	1.58
	0.5	1.66	2.23	2.60
	1.0	2.14	3.98	4.15
LSD (P 0.05)		0.19	0.21	0.27

LSD for interactions (level x treatment) at DAP 30, 45 and 60 were estimated to be 0.27, 0.21 and 0.38 respectively.

Table 20: Total-N (mg-N, pot^{-1}) of roots of L. fusca as affected by inoculation of Azospirillum brasilense sp 107 at higher levels of combined-N (NO_3^-)

Treatments	Level of N (mM NH_4^+)	Total-N (mg, pot^{-1})		
		30 DAP	45 DAP	60 DAP
Control	0.1	0.41	0.79	1.19
	0.5	1.02	1.46	2.17
	1.0	1.50	1.86	2.65
Heat Killed	0.1	0.56	1.05	2.15
	0.5	1.45	1.43	2.25
	1.0	1.54	2.80	2.56
Live	0.1	0.63	1.36	2.13
	0.5	1.52	1.64	2.28
	1.0	1.42	3.01	3.56
LSD (0.05)		0.18	0.18	0.17

LSD for interactions (level x treatment) at DAP 30, 45 and 60 were estimated to be 0.28, 0.18 and 0.24.

Table 21 : Total-N (mg-N pot^{-1}) of upper parts of L. fusca affected by inoculation of Azospirillum brasilense sp 107 at various levels of nitrate (NO_3^-)

Treatments	Levels of -N(mM NO_3^-)	Total-N (mg N, pot^{-1})		
		30 DAP	45 DAP	60 DAP
Control	0.1	1.46	1.98	2.94
	0.5	5.76	7.24	7.56
	1.0	7.82	8.06	10.08
Heat Killed Bacteria	0.1	2.27	3.39	3.06
	0.5	4.62	7.17	7.45
	1.0	8.45	9.50	12.04
Inoculated	0.1	2.07	2.76	2.84
	0.5	5.41	6.22	8.21
	1.0	8.58	10.03	13.81
LSD (P 0.05)		0.37	0.56	0.30

LSD for interaction (level x treatment) at DAP 30, was 0.53; at DAP 45, 0.79 and at DAP 60 it was 0.43

Table 22: Total-N (mg-N 3 plots) of upper parts of Leptochloa fusca as effected by inoculation of Azospirillum brasilense sp 107 at various level of ammonium (NH_4^+)

Treatments	Levels of -N (mM NH_4^+)	Total-N, (mg, pot^{-1})		
		30 DAP	45 DAP	60 DAP
Control	0.1	1.57	2.17	3.11
	0.5	3.21	5.09	5.20
	1.0	4.97	7.07	7.23
Heat Killed Bacteria	0.1	1.73	2.46	3.63
	0.5	3.73	4.99	5.19
	1.0	4.97	7.04	7.23
Inoculated	0.1	2.46	2.55	3.82
	0.5	3.40	5.13	5.25
	1.0	5.10	7.19	8.76
LSD (P 0.05)		0.20	0.19	0.21

LSD for interactions (level x treatment) at DAP 30 was 0.27; at DAP 45, 0.26 and at DAP 60 it was 0.30

Table 23 : Nitrogenase activity (C_2H_2-reduction) of detached roots of Leptochloa fusca inoculated with Azospirillum brasilense sp 107 at various levels of combined-N (NO_3^- & NH_4^+). Readings are averages of 3 replicates and ± is the standard deviation from the mean. Values are expressed as nmoles C_2H_4, g dry weight $root^{-1}$, h^{-1}

Treatment		Activity nmoles C_2H_4, g dry wt. $root^{-1}$, h^{-1}		
		30 DAP	45 DAP	60 DAP
0.1 mM	NO_3^-	35 ± 18	371 ± 63	1493 ± 520
	NH_4^+	2. ± 0.2	42 ± 11	138 ± 65
0.5 mM	NO_3^-	6 ± 3	255 ± 47	337 ± 65
	NH_4	-	15 ± 6	77 ± 12
1.0 mM	NO_3^-	4 ± 1	72 ± 12	117 ± 10
	NH_4^+	-	4 ± 1	51 ± 10

(35, 371 and 1493 nmoles gm dry wt^{-1} h^{-1}) was found to be present at DAP 30, 45 and 60 respectively. In NH_4^+ grown plants, no activity was observed at 0.5 and 1 mM NH_4^+ level at the time of first analysis however N_2-ase activity was observed in latter assays.

5.2.2 Effect of High Level of Combined-N on Yield, Total-N and Nitrogenase Activity:

In the first set of experiments low levelsof combined-N (0.1, 0.5 and 1.0 mM NO_3^- or NH_4^+) were supplied to plants and the effect of inoculation was measured. Another series of experiments with the similar design but with higher levels of N (2.5, 5 and 10 mM NO_3^- or NH_4^+) were carried out to elucidate the maximum tolerance limit of combined-N for kallar grass at which inoculation could proceed.

With the increase of N-supply, fresh and dry weights had also increased and increment was much rapid during 2nd and 3rd harvest interval. Fresh weights were not subjected to statistical analysis. The control samples when supplied with only 2.5, 5.0 and 10 mM NH_4Cl had fresh weights 13.21, 26.70 and 28.73 gm ,pot^{-1} respectively at DAP 60. The fresh weights of plants supplied with heat killed bacterial suspension were found to be 13.66, 25.55 and 30.18 gm pot^{-1} while inoculated plants had fresh weights 15.07, 26.58 & 30.88 g, pot^{-1} for each level (2.5, 5.0 and 10 mM NH_4Cl) at DAP 60. It is evident that there was significant effect of N-supply but effect of inoculation was completely eliminated at all the N-levels. Similar results were obtained when plants were supplied with solution containing NO_3^-.

The dry weights of root and shoot were obtained separately. The dry weights of roots after statistical analysis are presented in Tables 25 and 26. It is clear from the results that yield of roots were higher when supplied with NO_3^-. With the increment of combined-N levels yield also increased significantly ($P < 0.05$) but inoculation or heat killed bacterial suspension had no effect. Similar pattern was

Table 24 : Effect of Azospirillum brasilense sp 107 inoculation on dry weight (gm, pot^{-1}) of roots of L. fusca at higher levels of combined-N (NO_3^-)

Treatments	Level mM (NO_3^-)	Total roots weight gm, pot^{-1}		
		30 DAP	45 DAP	60 DAP
Control	2.5	0.35	0.58	0.82
	5.0	0.49	0.59	1.21
	10.0	0.67	0.68	1.36
Heat Killed	2.5	0.44	0.56	0.80
	5.0	0.56	0.60	1.23
	10.0	0.57	0.59	1.37
Live	2.5	0.44	0.56	0.82
	5.0	0.60	0.57	1.03
	10.0	0.56	0.60	1.31
LSD ($P < 0.05$)		0.15	0.06	0.20

LSD for interactions *level x treatment) at each successive harvest was estimated to be non significant.

Table 25 : Effect of Azospirillum brasilense sp 107 inoculation on dry weight (gm, pot^{-1}) of roots of L. fusca at higher levels of combined-N (NH_4^+)

Treatments	Level mM (NH_4^+)	Total roots weight gm, pot^{-1}		
		30 DAP	45 DAP	60 DAP
Control	2.5	0.21	0.48	0.79
	5.0	0.26	0.63	1.13
	10.0	0.29	0.74	1.09]
Heat Killed	2.5	0.26	0.44	0.77
	5.0	0.28	0.60	1.02
	10.0	0.28	0.69	1.06
Live	2.5	0.21	0.43	0.78
	5.0	0.20	0.66	1.10
	10.0	0.32	0.63	1.04
LSD ($P < 0.05$)		0.09	0.12	0.17

LSD for interactions (level x treatment) at each successive stage were estimated to be non significant.

Table 26 : Effect of Azospirillum brasilense sp 107 inoculation on dry weight (gm,pot $^{-1}$) of aerial parts of Leptochloa fusca at higher levels of combined N (NH_4^+)

Treatments	Level mM (NH_4^+)	Yield (gm, pot^{-1}) 30 DAP	45 DAP	60 DAP
Control	2.5	0.55	0.98	2.31
	5.0	0.83	3.68	5.88
	10.0	1.21	4.69	6.79
Heat Killed Bacteria	2.5	0.48	1.13	2.51
	5.0	0.83	3.59	5.73
	10.0	0.95	4.78	7.09
Inoculated	2.5	0.49	1.37	2.78
	5.0	0.92	3.91	5.73
	10.0	0.94	4.83	7.38
LSD ($P < 0.05$)		0.15	0.16	0.56

LSD for interactions (treatment x level) at DAP 30, 45 and 60 was found to be non-significant.

Table 27 : Effect of Azospirillum brasilense sp 107 inoculation on dry weight (gm,pot^{-1}) aerial parts of Leptochloa fusca at higher levels of combined-N (NO_3^-)

Treatments	Level mM (NO_3^-)	Yield(gm, pot^{-1}) 30 DAP	45 DAP	60DAP
Control	2.5	0.54	1.93	3.29
	5.0	1.10	3.94	5.86
	10.0	1.23	4.28	7.04
Heat Killed Bacteria	2.5	0.65	2.05	3.40
	5.0	0.83	3.97	5.83
	10.0	1.24	4.20	7.43
Inoculated	2.5	0.57	1.85	3.42
	5.0	1.02	3.90	6.03
	10.0	1.24	4.21	7.42
LSD ($P < 0.05$)		0.15	0.22	0.33

LSD for interactions (treatment x level) at DAP 30 was 0.21; at DAP 45 and 60 it was non significant.

observed for shoots of kallar grass when supplied with higher levels of combined-N (Tables 26 and 27). Yield of upper parts was higher when supplied with solution containing NO_3^- as compared to NH_4^+-treated plants but difference was not pronounced. A one fold increase in the yield was observed when combined-N (NO_3^- or NH_4^+) level was increased from 2.5 mM to 5.0 mM however when level was further increased upto 10 mM the effect was not to the extent of the previous treatment.

The % N of the root were found to be in the ranges of 0.85-1.42, 0.68-1 27 and 0.55-1.26 at successive stage of harvest (DAP 30, 45 and 60) when supplied with NH_4^+ containing nutrient solution. Percent-N declined as the plants attained maturity. The % N of shoot for the same plants were higher and ranged from 1.62-2.41, 0.81-1.57 and 1.02-1.73 for each successive harvest. When plants were supplied with nutrient solution containing NO_3^-, the % N of the root found to be in the ranges of 0.99-1.35, 0.80-1.20 and 0.81-0.98 at DAP 30, 45 and 60 respectively. The percent-N for shoots were found to be in the ranges of 1.30-2.13, 1.08-1.43 and 1.05-1.35 for each successive harvest. The % N was found to be less in the NO_3^--grown plants but difference between this and NH_4^+-treated plants was found out to be non significant. As explained earlier, the % N was reported to be not a good parameter. The real estimate for the increase due to inoculation is total nitrogen. The total-N was measured by Kjeldahl method and reported in Tables 28, 29, 30 and 31 . It is clear from the results that with the increase in the supply of combined-N, the total-N was also found to be higher. However, the plants which were supplied with heat killed bacterial suspension or live inoculation, total-N was either same or in some cases even less than the control.

Nitrogenase activity of detached roots was measured as described in Section 5.2.1. The ARA was performed but no activity was observed in any of the samples.

Table 28 : Total-N (mg-N, pot^{-1}) of roots of L. fusca as affected by inoculation of Azospirillum brasilense sp 107 at higher levels of combined-N (NO_3^-)

Treatments	Levels of N (mM NO_3^-)	Total-N (mg, pot^{-1})		
		30 DAP	45 DAP	60 DAP
Control	2.5	3.63	5.09	6.12
	5.0	5.89	6.95	11.6
	10.0	5.63	6.07	12.29
Heat Killed	2.5	4.10	4.54	7.72
	5.0	6.07	6.25	1.14
	10.0	5.49	6.19	11.76
Live	2.5	3.92	4.94	5.66
	5.0	5.93	6.21	9.76
	10.0	5.36	5.48	12.45
LSD ($P < 0.05$)		0.37	0.40	0.60

LSD for interactions (treatment x level) at DAP 30, 45 and 60 were estimated to be NS, 0.57 and 0.87.

Table 29: Total-N (mg-N, pot^{-1}) of roots of L. fusca as affected by inoculation of Azospirillum brasilense sp 107 at higher levels of combined-N (NH_4^+)

Treatments	Level of N (mM NH_4^+)	Total-N mg, pot^{-1}		
		30 DAP	45 DAP	60 DAP
Control	2.5	1.91	3.14	4.36
	5.0	2.47	5.40	10.94
	10.0	3.28	8.06	14.06
Heat Killed	2.5	2.03	3.18	10.02
	5.0	2.47	5.39	10.02
	10.0	3.38	8.13	12.70
Live	2.5	1.97	3.29	3.92
	5.0	2.24	5.6	10.89
	10.0	3.34	7.96	12.01
LSD ($P < 0.05$)		0.28	0.35	1.19

LSD for interactions (level x treatment) at each successive stage were estimated to be NS, NS and 1.68.

Table 30 : Total N (mg N, pot^{-1}) of upper parts of Leptochloa fusca as affected by inoculation of Azospirillum brasilense sp 107 at higher levels of combined-N (NO_3)

Treatments	Levels mM (NO_3)	Total-N (mg-N, pot^{-1})		
		30 DAP	45 DAP	60 DAP
Control	2.5	7.46	19.47	37.20
	5.0	20.61	47.25	79.08
	10.0	23.44	61.08	83.78
Heat Killed Bacteria	2.5	8.11	21.78	40.21
	5.0	18.85	53.02	78.82
	10.0	22.84	57.07	75.47
Inoculated	2.5	7.54	20.94	35.54
	5.0	20.19	50.59	80.64
	10.0	22.80	57.81	79.90
LSD ($P < 0.05$)		0.54	2.83	1.94

LSD for interactions (treatment x level) at DAP 30 was 0.76; at DAP 45, 4.01 and at DAP 60 it was estimated to be 2.75.

Table 31: Total-N (mg-N, pot^{-1}) of upper parts of Leptochloa fusca as affected by inoculation of Azospirillum brasilense sp 107 at higher levels of combined-N (NH_4^+)

Treatments	Levels mM (NH_4^+)	Total-N (mg-N, pot^{-1}		
		30 DAP	45 DAP	60 DAP
Control	2.5	8.86	10.80	27.77
	5.0	17.91	47.69	83.27
	10.0	28.90	70.53	111.79
Heat Killed Bacteria	2.5	9.11	11.82	27.78
	5.0	18.35	47.55	83.05
	10.0	27.71	70.15	112.37
Inoculated	2.5	9.1	11.46	27.91
	5.0	17.86	48.08	81.89
	10.0	27.07	69.17	113.28
LSD ($P < 0.05$)		0.92	0.53	2.63

LSD for interactions (treatment x level) at DAP 30 was 1.31; at DAP 45 0.75 and at DAP 60 it was non significant.

5.3 INOCULATION STUDIES BY ^{15}N-ISOTOPIC DILUTION TECHNIQUE

^{15}N reduction is the only absolute proof of dinitrogen fixation. The use of ^{15}N isotope dilution techniques provide only truly quantitative means of estimating dinitrogen fixation under field conditions. Use of ^{15}N stable isotope for detection of dinitrogen fixation in grasses and cereals is increasing. ^{15}N isotope dilution method was used by Owens (1977) in field grown maize and sorghum. Rennie (1980) employed this method for measuring the contribution of Azospirillum brasilense inoculation in maize which were grown under controlled environmetnal conditions. Ventura & Watanabe (1983) used ^{15}N dilution techniques for assessing contribution of nitrogen fixation to rice plants. In wheat plants contribution of N_2-fixing organisms was estimated by Lethbridge & Davidson (1983) and Rennie (1983) by using ^{15}N isotopic dilution technique. Inoculation studies were carried out on L. fusca as described by Rennie (1980b) for maize with ^{15}N labelled fertilizer.

Seeds of kallar grass were surface sterilized by dipping in 50% commercial sodium hypochlorite for 30 minutes. Several washings were done with sterile distilled H_2O. Seeds were then transferred to 1% agar plates. Plates were kept in a controlled temperature growth room with 16 h day and 8 h night. Day temperature was 30 $\pm$ 2°C while night temperature was kept at 28 $\pm$ 2°C. Light intensity was 20,000 Lux.

After 4 days, seedlings were transferred to long tubes (20x3 cm) fitted with perforated silicone stoppers (Shinetsu Polymer Inc. Japan). Each tube was filled with gravel (small size, black colour) and 30 ml of N-free Hoagland nutrient solution was provided to each tube. Plants were transplanted onto silicone stoppers in such a way that shoots were present in free environment outside the tube(Fig.18a&b) After establishment of plants in culture tubes (ca 3-5 days), 15% at.ex. $^{15}(NH_4)_2SO_4$ solution (500 µg-N $tube^{-1}$) was given which was filter sterilized (0.2 micron, Sartorius, W. Germany). Two weeks after germination, inoculation was provided

Fig. 18 a: Experimental set up of isotopic dilution study. Plant of L. fusca are growing in the long culture tube under controlled envirnmental conditions.
(C) control and (I) inoculated.

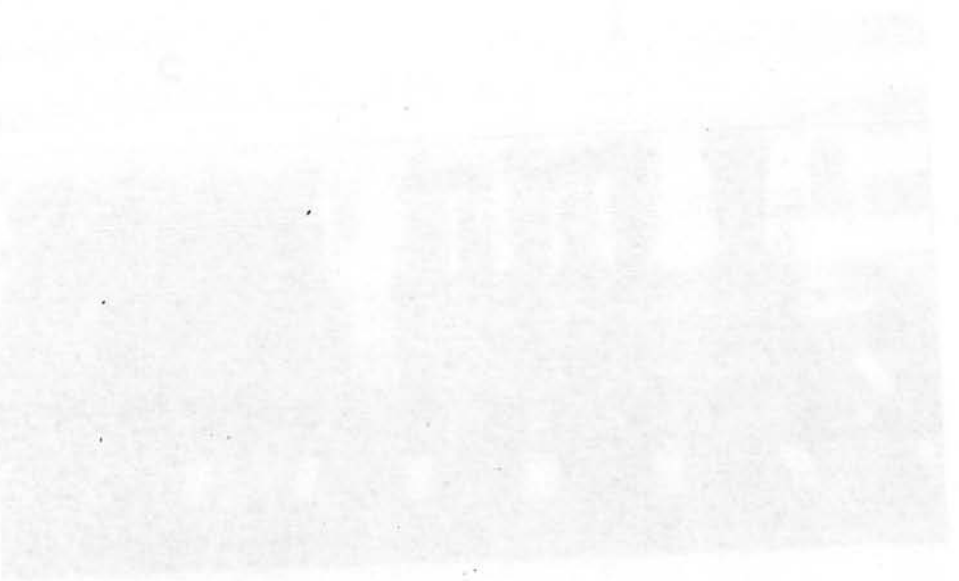
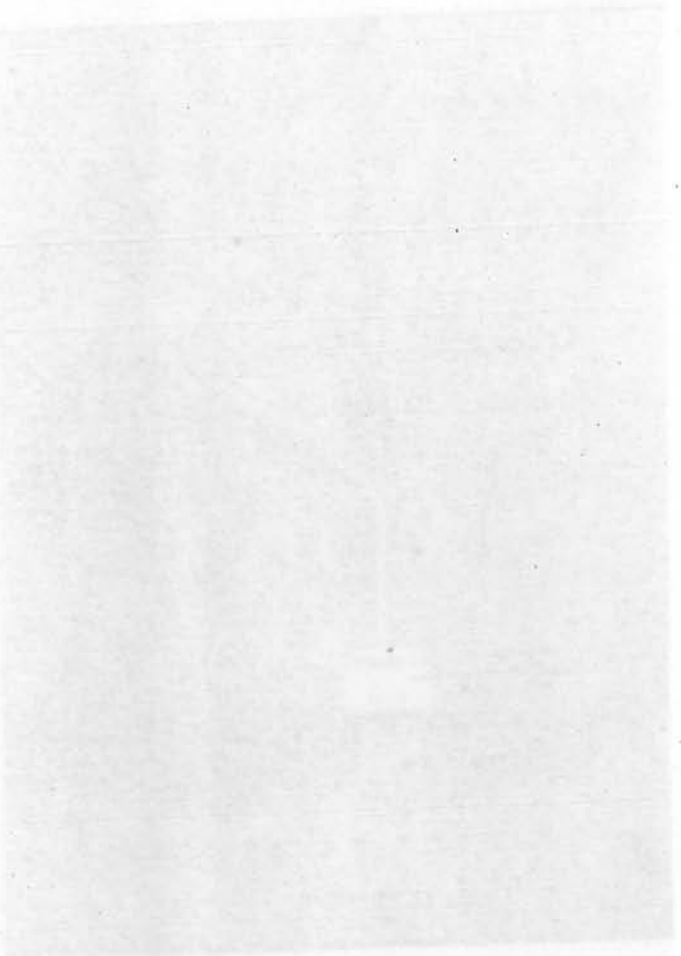


Fig. 18 b: A close up of culture tube with Kallar grass. (C) culture glass tube; (G) gravel with N-free Hoagland's solution; (P) Plant of L. fusca and (S) Silicone stopper.



to half of the tubes. Each treatment was replicated 6-10 times and placed in the controlled temperature growth room as described above.

Bacteria (NIAB-I, Iso-2 and Azospirillum brasilense sp 107) were grown in 500 ml nutrient broth in one liter Erlenmeyer flasks on a rotary shaker and after 48 h of growth, bacterial cells were harvested by centrifugation (Kokusan Model H-251, Japan) at 10,000 rpm for 30 minutes. Pellet was washed thrice with phosphate buffer (0.1 M, pH 6.8) and resuspended in the same buffer to obtain concentrated bacterial suspension. One ml of inoculum was provided to one set of tubes while to other set (control) no inoculum was provided. Total-N in the bacterial suspension was determined by Kjeldahl method.

Plants were harvested after 5 weeks of growth. Fresh and dry weights were taken. Inoculation resulted in higher yield in all treatments (Table 32). Maximum dry weights were obtained when kallar grass was inoculated with bacterial isolates of its own rhizosphere. Inoculation with Azospirillum brasilense sp 107 gave lowest yield (103 mg plant^{-1}) among inoculated treatments. Yields were significantly higher ($P < 0.01$) in inoculated treatments as compared to control in all the three experiments. Maximum dry weight (153 mg plant^{-1}) was observed in plants inoculated with bacterial isolate Iso-2. One to two fold increase in yield was obtained when plants were supplied with N_2-fixing bacteria.

Acetylene reduction assays were performed at the end of experiments to confirm the presence of N_2-fixing microorganisms. Whole root system from each culture tubes was placed in 30 ml McCartney vial and flushed with N_2 gas. Further procedures were similar as described in Section 5.2 . No activity was observed in non-inoculated plants which indicated the absence of any N_2-fixing contaminant. Nitrogenase activity was found to be low in inoculated treatments (Table 33). Highest ARA value was observed in plants inoculated with bacterial isolate Iso-2. Maximum yield was also observed in the same treatment.

Table 32: Fresh and dry matter yield and N-contents of upper parts of Leptochloa fusca. Readings are averages of 6 replicates. Figures followed by same letter are not significantly different at 1% level as determined by DMR test.

S.No.	Treatment	Fresh wt $mg, plant^{-1}$	Dry wt $mg, plant^{-1}$	Total-N $mg, plant^{-1}$	% N
1.	Control	130 C	62 C	0.63 C	1.03 B
2.	Azospirillum brasilense	223 B	103 A	1.31 A	1.34 A
3.	NIAB-I	355 A	128 A	1.01 B	0.80 C
4.	Iso-2	337 A	153 A	1.53 A	1.00 B

Table 33: Nitrogenase activity of roots of L. fusca at the end of the experiments. Roots from each plant were placed in McCartney vials, flushed with N_2 gas and 1% air (v/v) and 12% (v/v) acetylene was added after replacing same amount of nitrogen gas. Vials were incubated at 35°C for 24 hours. Gas samples were analysed on a Carlo-Erba gas chromatograph Model 180. Readings are averages of 6 replicates and ± is the standard deviation from the mean

S.No.	Inoculum	Nitrogenase activity nmoles $C_2H_4, g\ wt^{-1}\ 24\ h^{-1}$
1.	Azospirillum brasilense	190 ± 101
2.	NIAB-I	81 ± 32
3.	Iso-2	291 ± 115

The tetrazolium salts are low redox potential indicators which on reduction forms insoluble, coloured formazans. Triphenyl tetrazolium chloride (TTC) reduction test of roots was performed as described by Patriquin & Dobereiner (1978). Washed root segments were incubated over night in tubes containing sterili ed tetrazolium buffer solution (Appendix- 7). Whole root segments from the solution or hand cut sections were examined and photographed on a Microlux-11 microscope equipped with Pentax ME camera. Inoculated roots turned intense red (Fig .19). Hand cut sections showed the crystals of formazan in the cortical as well as in the stelar regions (Fig.20) Uninoculated roots were unable to reduce the compound.

Total nitrogen content of plant material was determined by Kjeldahl method. Higher total-N was present in inoculated plants as compared to control (Table 32). Percent N was found to be in the range of 0.84-1.34. Percent N was found to be same or even less than the control except of plants inoculated with A.brasilense.

^{15}N abundance was measured on a mass spectrometer as described in Ch.2. ^{15}N abundance was found to be higher in non-inoculated plants (at % ^{15}N = 9.84 $\pm$ 0.87) as compared to inoculated plants where ^{15}N abundance ranged from 5.28 $\pm$ 1.36 for A. brasilense to 2.65 $\pm$ 1.13 in Iso-2 inoculated plants (Table 34).

Biologically fixed nitrogen in the inoculated treatments was measured by the following formulae (Rennie, 1980).

$$(a)\quad \%\ N_2\text{-fixed} = \frac{\text{Yield of } N_{(fs)} - \text{Yield of } N_{(nfs)}}{\text{Yield of } N_{(fs)}} \times 100$$

and

$$(b)\quad \%\ N_2\text{-fixed} = 1 - \frac{(\text{at \% N-15 inoc})}{(\text{at \% N-15 Uninoc})} \times 100$$

Table 34 : ^{15}N abundance (at % ^{15}N) in the aerial parts of L. fusca. Samples were analysed by Rittenburgh method after spiking with known amount(2 mg-N) of unenriched $(NH_4)_2SO_4$ on a varian MAT GD 150 mass spectrometer.

Replicates	Control	Azospirillum brasilense	NIAB-I	Iso-2
1.	9.251	3.855	4.721	1.095
2.	10.040	3.647	3.494	4.094
3.	10.072	6.621	6.578	2.535
4.	10.287	5.530	2.888	3.723
5.	8.451	4.960	5.997	2.753
6.	10.973	5.148	3.719	1.748
Average	9.845 a	4.960 b	4.566 b	2.658 c

Readings followed by same letter are not significantly different at 1% level as determined by DMR test.

Table 35 : Estimates of biologically fixed nitrogen by analysing yield data and ^{15}N abundance in aerial parts of L. fusca.

S.No.	Treatments	% N fixed (yield based)	% N fixed based on ^{15}N dilution
1.	Azospirillum brasilense	51.91 a	49.62 b
2.	NIAB-I	37.62 b	53.62b
3.	Iso-2	58.22 a	73.00 a

Readings are averages of six replicates. Figures followed by same letter are not significantly different at 1% level as determined by DMR test.

Fig. 19 : A portion of inoculated (I) and non-inoculated (NI) root segment of L. fusca after staining with TTC.

Fig. 20 : Transverse section of inoculated roots of L. fusca showing dark red zones of TTF (x 25).

Plate – III

Fig. 19

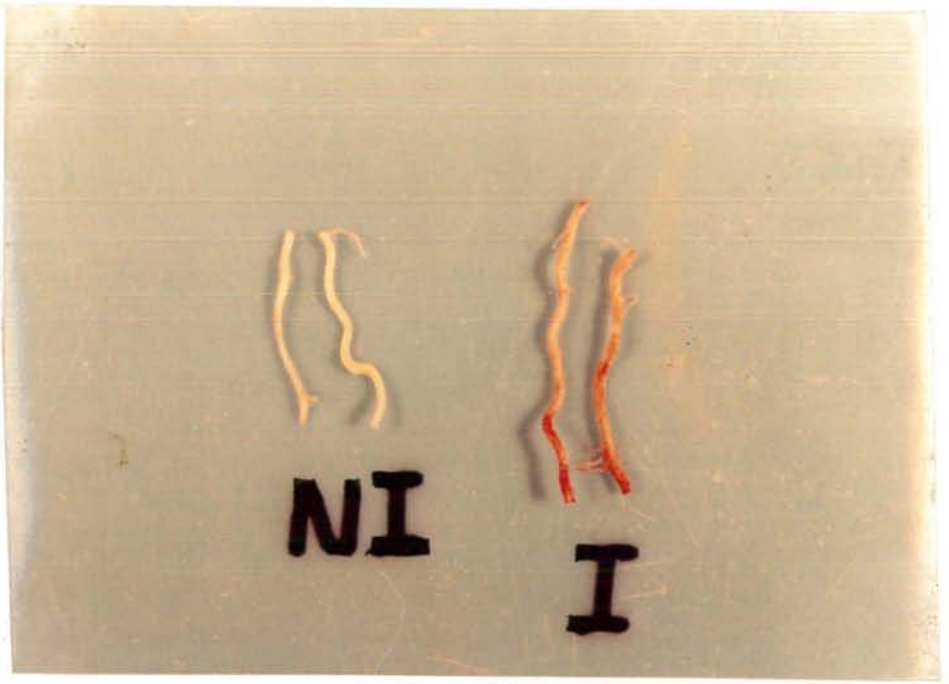
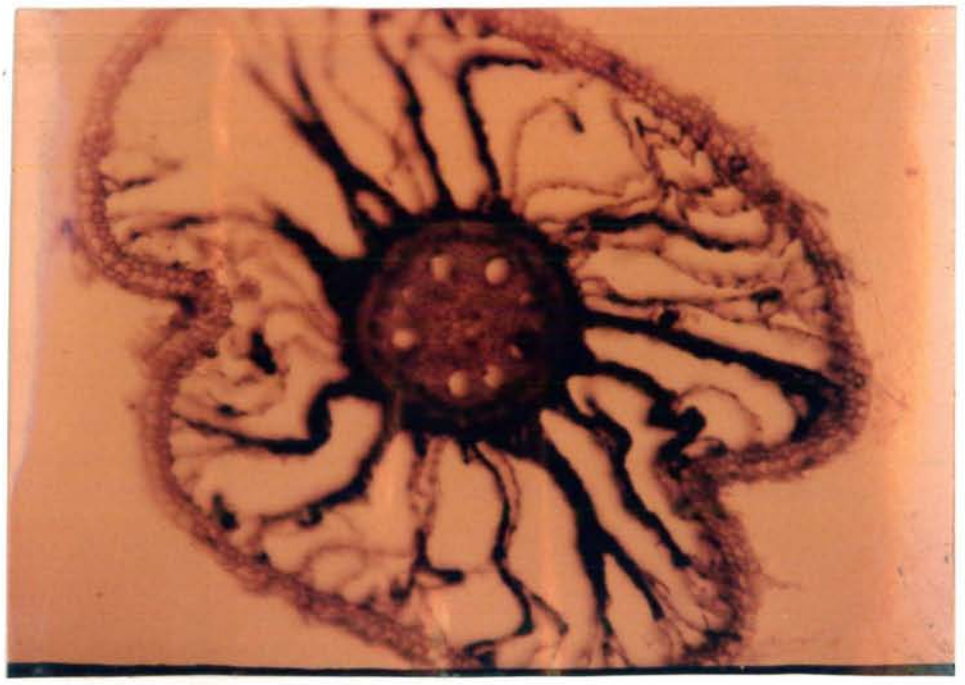


Fig. 20



Estimates based on the above equations are presented in Table 35 . The yield dependent values were found to be lower than the values estimated by ^{15}N abundance. The highest values obtained were for plants inoculated with Iso-2. It was estimated that 58% and 73% N in the plants was obtained through biological fixation as measured by N-balance and ^{15}N -abundance data respectively. This trend was also observed in all other measured parameters (Fresh & Dry Wts, ARA) for this treatment (Iso-2.).

Measurement of all inputs of N (seed 7.07 µg, inoculum 148 µg and $^{15}(NH_4)_2SO_4$ 500 µg) clearly indicates that higher total-N in the inoculated treatments have possibly come through the N_2-fixing bacteria.

5.4 CONCLUSIONS: In the present investigations, inoculation responses of known N_2-fixing bacteria, Azospirillum brasilense and of two bacterial isolates from L. fusca were determined on total-N, fresh & dry wt and nitrogenase activity of kallar grass.

Experiments were first carried out in special glass assembly for growing plants axenically, with selected inoculant bacteria under conditions which permit control of environmental conditions and monitoring of plant and bacterial growth. Such apparatus has earlier been used by Hubbell et al.(1981) for studying inoculation effects on plants with selected Azospirillum sp, Azotobacter sp and various other bacterial species. The use of complicated apparatus which permits experiments under more controlled conditions has recommended by Wright & Weaver (1981) as more fruitful. Inoculation resulted in one fold increase in yield. Several other workers have also found that plants inoculated with N_2-fixing microorganisms produced yield increase (Cohen et al., 1980; Kapulnik et al.1981 and Nur et al. 1980). Unlike many other studies (Hubbell et al, 1981 and Rennie 1980b) no external carbon source was added in the growth medium of this experiment and it was observed that nutrient secretions from roots of L. fusca were probably enough

to sustain microbial growth in this short term experiment. Kloss et al (1984) has carried out a detailed study of organic acids in the root exudates of L. fusca in sterile and inoculated conditions. They observed that 95% of the acids can be directly used by the diazotroph and found co-relation in increase of biomass of the inoculated bacteria with a decrease of exudates in the nutrient solution.

It is now well established that diazotrophs also produce plant growth substances (PGS) in the free living state and in association with grasses. The PGS results in improved root growth (Hubbell et al.1981; Tien et al, 1979, 1981 and Umali Gracia et al., 1980) resulting in increased access to soil-borne nutrients. This latter possibility have recently been examined by Lin et al (1983). They used labelled material and observed enhanced (30-50% over control) uptake of NO_3^-, K^+ and $H_2PO_4^-$ into 3-4 days and 2 weeks old root segments. In our inoculation experiments, heat killed bacterial solution was supplied to one set of plants to look for such effects. Stimulatory effects were observed in these treatments but increase in fresh and dry weights was less than the live bacterial inoculant. It was observed by Okon (1984) that yield response to Azospirillum spp may result from several factors. High acetylene reduction values in our experiment confirmed the presence of N_2-fixing microorganisms in association with L. fusca as an agent responsible for nitrogen fixation. Similar observations were also made by Albrecth et al (1981) in maize plants grown under axenic conditions. Thus, enhancement of plant growth by N_2-fixing bacteria may be due to either dinitrogen fixation and growth hormone production or both is not clear as data are not yet available to indicate the relative importance of both the factors (Tien et al; 1979).

Although complicated apparatus provides many advantages for studying the interactions of plant and bacteria, it is desirable to use standard procedures for plant growth. Therefore, inoculation experiments in pots were also carried out in

sand culture. The mineral nitrogen supply is pointed out to be most critical one in inoculation experiment (Hubbell et al. 1981). Six levels (3 lower and 3 upper) of NH_4^+ and NO_3^- were employed to look for the optimum concentration of combined N (NO_3^- or NH_4^+) at which maximum yield and nitrogenase activity could take place. Nitrate and ammonium were equally taken up well by L. fusca and no significant difference was observed between these two treatments. Beevers & Hageman (1980) in a recent review also advocated this theory of assimilation for most of the plants. L. fusca thus belongs to this category of higher plants which are not specific for either of the available combined-N (NO_3^- or NH_4^+). At lower levels of combined-N (0.1, 0.5 and 1.0 mM NO_3^- or NH_4^+) nitrogenase activity was detected in detached root however activity was found to be totally absent at higher levels of combined-N (2.5, 5 and 10 mM NO_3^- or NH_4^+). It is well established that high levels of combined-N repressed the nitrogenase activity of N_2-fixing bacteria (Okon, 1984). 1.0 mM (NO_3^- or NH_4^+) level was found to be optimum for suitable growth and for sustaining nitrogenase activity. Activity was found to be low in the lowest N-level (0.1 mM) as the overall growth of plants was found to be poor. Hubbell et al.(1981) has pointed out that under restricted mineral N-supply, carbon leakage from the plant is severly restricted. It is possible that at medium level of N-fertilization (1.0 mM NO_3^- or NH_4^+) the simultaneous utilization of biologically fixed nitrogen and mineral nitrogen fertilizer may take place. Similar results were obtained by Kapulnik et al., (1981) with Setaria italica. They used five different levels of NH_4NO_3 (0,0.01, 0.04, 0.1 and 0.2 g, L^{-1}) in the mineral solution that was used to irrigate the plants. They observed that inoculation increased the plant growth and yield mainly at lower medium level of combined nitrogen (0.04 g,L^{-1}) whereas the effect was less marked at 0 or at high level of N-fertilization.

Increased levels of combined-N resulted in better plant growth but differences were non-significant between 5mM and 10 mM. Moreover, at higher levels no differences were observed between all the three treatments. Our results are consistent

with the observation of Albrecth et al (1981). They conducted a green house experiment on maize which indicated that when levels of combined-N were adequate for plant growth, there was little if any difference between inoculated and the control plants. Pal & Malik (1981) inferred from their studies on the contribution of Azospirillum brasilense to nitrogen needs of sorghum that higher contribution to the N-uptake was observed when inoculation was supplemented with farm yard manure (10 tons ha^{-1}). Moreover, yields of plants treated with higher levels of fertilizer were above all the other inoculated treatments.

Extensive data is available on the inoculation effects which resulted in yield increase although many other attempts failed. Inoculation experiments under more controlled conditions may be more fruitful as specific requirements of the association and many of the characteristics of the microorganisms are unknown. Moreover, in order to exploit the system it is essential to know the amounts of N_2 potentially fixed in various system (Vose, 1983). For this purpose, isotopic dilution studies were carried out in controlled environmental conditions. ^{15}N-isotopic dilution technique was advocated by Rennie (1980b) as the absolute proof for measuring the amount of N_2-fixed in the field conditions. He used maize as the test crop and observed that 12.6% - 38% of N in the plants was contributed by the atmospheric N_2-fixation. In our studies values estimated by ^{15}N abundance data were ranged from 50-80%. The values are high when compared to ones reported in the literature for other plants (Lethbridge & Davidson 1983; Owens, 1977; Rennie, 1980b and Ventura & Watanabe, 1983). This is mainly because the seeds of L. fusca are very small (148 µg-N $seed^{-1}$) and there is very little dilution of ^{15}N-isotope. In ^{15}N isotopic experiment, comparable values of N_2-fixed were obtained by classical difference method and ^{15}N abundance method. However, the values of classical method were always found to be less. Rennie (1980b) also observed the difference between these two methods however these were found to be insignificant.

Most of the inoculation studies reported in the literature have employed known N_2-fixer, Azospirillum and few studies on other N_2-fixing bacteria (Wright & Weaver, 1982). In the present experiments in addition to known N_2-fixing bacteria, isolates from the rhizosphere of L. fusca (NIAB-I & Iso-2) were also used as inoculant. These isolates gave better performance than the standard N_2-fixing bacteria. It has been stressed by Dobereiner & De-Polli (1980), Rennie (1980b)and Vose (1983) that interaction of specific strains with different genotypes of the host is a crucial aspect to be understood for better use of the associative system.

The data presented in this chapter suggest considerable potential benefit from the N_2-fixing bacteria - plant association. Such fixation would be an important source of combined-N especially in the waste land conditions where L. fusca is recommended for cultivation. Data is not yet available to indicate the relative importance of dinitrogen fixation and growth hormones production in the enhancement of plant growth by N_2-fixing bacterial inoculant under field conditions. However,at the present stage inoculation experiments under more controlled conditions may be more fruitful. Future research should be directed towards determining the factors and conditions needed for colonization of grass roots by nitrogen fixing bacteria (Wright & Weaver, 1982).

CHAPTER-6. SUMMARY

The study in this thesis deals with the associative symbiosis of N_2-fixing bacteria and Leptochloa fusca (L.) Kunth. This grass has been locally known as "Kallar ghas" (salt grass) because of its tolerance to salt to a high percentage of exchangeable sodium and to waterlogging conditions. L. fusca is recommended as primary colonizer of saline sodic soils which constitute the 33% of irrigated soils of Pakistan. Under such conditions this grass grows luxurinatly yielding about 40 tonnes ha^{-1} biomass annually. Such a yield in terms of nitrogen, amounts to about 180 kg N, ha^{-1}, $year^{-1}$. The soils which sustain such fields are very low in fertility and thus it was worthwhile to investigate the rhizosphere of Kallar grass for any possible N_2-fixation.

A detailed examination of naturally occurring root associated non-symbiotic nitrogen fixation in the soil environment of kallar grass was made (Ch.3). Two saline-sodic sites (Shahkot and BSRS, Lahore) were selected and samples were obtained every month over a period of one year. Soil cores, unwashed, washed and surface-sterilized roots were subjected to acetylene reduction assay (ARA). ARA values upto 50 nmoles h^{-1} for soil cores, 1095 n moles gm dry $root^{-1}$, h^{-1} for unwashed roots, 4292 nmoles gm dry $root^{-1}$, h^{-1} for washed roots and 2494 n moles gm dry $root^{-1}$, h^{-1} for surface-sterilized roots were observed but majority of samples exhibited a range of 1-200 n moles gm dry $root^{-1}$, h^{-1}. It is difficult to draw out a set pattern for this grass system as it exist for legumes however several important observations were made. The frequency of positive samples in all the sub-systems of the rhizosphere of L. fusca was higher during the active growth period of grass (March-September) while highest nitrogenase activities in excised roots with preincubation were observed in washed roots. Surface-sterilized roots exhibited lowest N_2-ase activities. It is interesting to note that no nitrogenase activity was observed in the soil core studies during the winter months while maximum activity was observed during this period in washed and surface-sterilized

roots. Lower in-situ soil temperatures and limited nutrient supply during inactive growth period might be responsible for the tight association of N_2-fixing bacteria with the roots during the winter season.

Excised root assay with preincubation is much criticised because of bacterial proliferation during the incuabtion period which over estimates the rate of N_2-ase activity. Therefore excised roots of Kallar grass were also analysed without preincubation by ARA. The nitrogenase activities of this assay correlated well with the ARA values of soil cores as well as of excised unwashed roots which were subjected to preincubation in N_2-atmosphere.

Time course study revealed the lag period of 7-10 h before the onset of N_2-fixing activity and O_2-levels had no effect on this lag. Moreover, activities were found to be higher when roots of L. fusca were incubated with 1% air.

An important aspect pursued in the present study deals with the parallel comparison which was made between the nitrogenase activity of the excised roots of L. fusca and numbers of N_2-fixing bacteria at different time intervals. No significant change in the population of N_2-fixers as observed by MPN counts based on ARA was detected during the incubation period. These results suggest that mere proliferation of diazotrophs was not a sole reason for the higher nitrogenase activity of the excised roots and thus indicated the complexity of the system which needs to be pursued further.

Population of heterotrophic N_2-fixing bacteria in the different regions of soil/root system of Kallar grass was estimated by MPN-technique as well as by "classical" plate count method for a period of one year. Enumeration based on latter method always gave higher counts. Quantitative estimates based on ARA seems to be more reliable as these take into account only active N_2-fixing microorganisms associated with the roots. Consistently higher numbers of N_2-fixers in the rhizoplane and histoplane fraction indicate towards the localisation of N_2-fixing bacteria on and inside of the roots. These studies are unable to

estimate the frequency of predominant N_2-fixer(s) in the rhizosphere of Kallar grass. Use of fluorescent antibody technique in the future studies of quantification of predominant N_2-fixer(s) in the presence of similar N_2-fixing bacteria in the rhizosphere of Kallar grass is suggested.

Broad spectrum of N_2-fixing bacteria was found to be associated with roots of L. fusca. The systematic position of the three isolates, NIAB-I, C-2 and Iso-2 was determined by morphological, biochemical tests and mol % (G+C) DNA contents (Ch.4). Two isolates were identified as Klebsiella pneumoniae (NIAB-I) and Beijerinckia sp. (Iso-2). The identification of the third isolate, C-2 could not be ascertained. ^{15}N enrichment studies confirmed the nitrogen fixing ability of these isolates. Some physiological studies were also carried out. Effect of different levels of combined nitrogen (NO'_3, NH^+_4 and yeast extract), pH (5.5---9.0) and salt (NaCl) on nitrogenase activity of the isolates was determined at various time intervals. All isolates exhibited nitrogenase activity even in the presence of 5 mM NO'_3 or NH^+_4 in a semi-solid medium after 24 h of growth. Maximum nitrogenase activity was observed at alkaline pH and all the isolates were able to tolerate 3-4% NaCl in the medium.

The role of N_2-fixing bacteria on the growth and nitrogenase activity of Kallar grass under defined conditions was determined by carrying out inoculation experiments in special glass assembly (Ch.5). Such apparatus permits axenic growth of plants with selected inoculant bacteria. Inoculation resulted in one fold increase in yield. Stimulatory effects by heat killed bacterial suspension were also observed thus yield response by N_2-fixing bacteria may result from several factors.

The mineral nitrogen supply is pointed out to be most critical one in inoculation experiments. Six levels (3 lower & 3 upper) of NH^+_4 and NO'_3 were employed in sand culture to look for optimum concentration of combined-N at which maximum yield and nitrogenase activity could take place. It was

observed that $NO^{'}_{3}$ and NH^{+}_{4} were equally taken up well by L. fusca. Thus this grass belongs to the category of higher plants which are not specific for either of the available combined-N. The level of 1.0 mM ($NO^{'}_{3}$ or NH^{+}_{4}) was found to be optimum. No nitrogenase activity was observed at upper levels (2.5, 5 & 10 mM) of combined-N although yield was found to be maximum. At lower levels (0.1 & 0.5 m M) associated nitrogenase activity was low as the overall growth of plant was found to be poor.

Isotopic dilution studies with ^{15}N-labelled fertilizer were carried out in controlled environmental conditions to estimate the amounts of N_2 potentially fixed in Kallar grass system. Inoculation resulted in higher yield and ARA values along with TTC staining of the roots confirmed the establishment of N_2-fixer around the roots. In addition to known N_2-fixing bacteria, isolates from the rhizosphere of L. fusca were also used as inoculant. These isolates gave better performance than the standard N_2-fixing bacteria. The estimates based on isotope dilution indicated that 50-70% N in the plant was derived from BNF in case of inoculated treatment. The results based on N-balance gave relatively lower values of 40-60% of total N derived from fixation. This data has indicated that in Kallar grass a substantial amount of plant N was derived from BNF. The inoculation experiments suggest considerable potential benefit from the N_2-fixing bacteria-plant association. Such fixation would be an important source of combined N especially in the wasteland conditions where L. fusca is recommended for cultivation.

This study and several other studies resulted in accumulation of enough data on naturally occurring root-associated nitrogen fixation in the grasses and cereals. It is urgently needed that future research should be directed towards detecting the factors and conditions needed for colonization of grass roots by N_2-fixing bacteria. Above all, the methods should be devised which could demonstrate the direct exchange of carbon and nitrogen substrates between grass roots and associated N_2-fixing bacteria.

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APPENDIX

1. COMBINED CARBON MEDIUM (Rennie, 1981)

Sol.1 (g, L^{-1})	
Sucrose	5.0
Mannitol	5.0
Sod.Lactate (60% v/v)	0.5 ml
K_2HPO_4	0.80
KH_2PO_4	0.20
Yeast extract	100 mg
$Na_2MoO_4.2H_2O$	25 mg
NaFe EDTA	28 mg
Distl.H_2O	900 ml
Sol.II ($g\ L^{-1}$)	
$MgSO_4.7H_2O$	0.20
$CaCl_2$	0.06
Distl.H_2O	100 ml.

In modified CCM, 5g, L^{-1} Sodium malate (E. merck) was added instead of sod. Lactate. Moreover 0.2% agar (Difco) was also added to make the medium Semi-Solid. For plates, 1.5% agar was added.

2. NUTRIENT BROTH

Peptone	5.0 g
Meat extract	3.0 g
Distl.H_2O	1000 ml

For nutrient agar 1.5% agar (Difco) was added to the medium.

3. API-50 CH MEDIUM

KCl	0.20 g
$MgSO_4.7H_2O$	0.20 g
Yeast extract	0.20 g
HEPES buffer	0.60 g
Phenol red	0.18 g
pH	7.5
Dist.H_2O	1,000 ml

4. RBA MEDIUM

For N_2-fixing bacteria Rhizobium Bacillus polymyxa, Azospirillum & Azotobacter.

a)

$CaCl_2.2H_2O$	100 mg
$MgSO_4.7H_2O$	100 mg
$Na_2MoO_4.2H_2O$	5 mg
$MnSO_4.H_2O$	5 mg
KH_2PO_4	100 mg
K_2HPO_4	900 mg
$FeSO_4.7H_2O$	10 mg
SL-6*	3 ml
Yeast extract	50 mg
H_2O	950 ml
Agar	15 g
pH	7.3

b) After sterilization of medium a) add 5 ml filter sterilized vitamin** solution.

c) Also add following carbon solution after filter sterilization.

Glucose	2 g
Mannitol	2 g
Sod.Malate	2 g
Sod.Succinate	1 g
Distl.H_2O	50 ml

* SL-6

$ZnSO_4.7H_2O$	0.1 g
$MnCl_2.4H_2O$	0.03 g
H_3BO_3	0.3 g
$CoCl_2.6H_2O$	0.2 g
$CuCl_2.2H_2O$	0.01 g
$NiCl_2.6H_2O$	0.02 g
$Na_2oO_4.2H_2O$	0.03 g
Distl.H_2O	1000 ml

** VITAMINS SOLUTION

Biotin	2.0 mg
Folic acid	2.0 mg
Pyridoxin-HCl	10.0 mg
Thiamine-HCl	5.0 mg
Ribof[illegible]e	5.0 mg
N[illegible]id	5.0 mg

Ca. Panthothenate	5.0 mg
p-Aminobenzoic acid	1.0 mg
Cyanocobalamine	0.01 mg
Distl. H_2O	1000 ml

5. N_3 (AZOTOBACTER) MEDIUM

Glucose	10.0 g
$CaCl_2.2H_2O$	0.1 g
$MgSO_4.7H_2O$	0.1 g
$Na_2MoO_4.2H_2O$	5.0 mg
K_2HPO_4	0.9 g
KH_2PO_4	0.1 g
$FeSO_4.7H_2O$	10.0 mg
$CaCO_3$	5.0 g
Agar	15.0 g
Distl. H_2O	950 ml

* 10 g glucose in 50 ml H_2O. After filter sterilization add in the remaining solu.

6. HOAGLAND NUTRIENT SOLUTION

KNO_3	0.20 g
$Ca(NO_3)_2$	0.47 g
KH_2PO_4	0.054 g
$MgSO_4.7H_2O$	0.19 g
Na Fe EDTA	3.5 ml
*Micronutrients	2.0 ml
Distl. H_2O	1000 ml

MICRONUTRIENTS STOCK SOLUTION L^{-1}

H_3BO_3	2.85
$MnCl_2.4H_2O$	1.81
$ZnCl_2$	0.11
$CuCl_2.2H_2O$	0.50
$Na_2MoO_4.2H_2O$	0.025

N-FREE HOAGLAND NUTRIENT SOLUTION

KCl	0.149 g
$CaCl_2$	0.294 g
KH_2PO_4	0.054 g
$MgSO_4.7H_2O$	0.19 g
Na [illegible]	3.5 ml
[illegible] nts	2.0 ml
	1000 ml
	6.8