NITROGEN FIXATION IN GRASSES AND SEDGES

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CERTIFICATE

The research work embodied in this dissertation has been carried out in School of Environmental Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any University.

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KAILASH MANDHAN

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INTRODUCTION

Nitrogen is a major, essential macroelement required by all plants and animals. It often limits biological productivity in the biosphere. Though, elemental nitrogen is abundant, representing about two third of the atmosphere, it can not be utilized by most of the living systems as they could use it only when available in the "fixed" form, i.e., combined with other elements such as carbon, hydrogen and oxygen. In nature, elemental nitrogen is fixed by abiotic processes such as electric discharge and lightening and by biotic processes through nitrogen fixing organisms living freely or in symbiotic associations with higher plants. Recently, man has acquired the capability of augmenting the supply of fixed nitrogen through industrial nitrogen fixation to meet the agricultural requirements. With growing human population, dwindling supply of fossil fuels and rapidly escalating costs of chemical nitrogen fertilizers, increasing attention is being paid to biological nitrogen fixation which is independent of the constraints imposed by the energy crisis syndrome. A recently discovered nitrogen fixing system involving loose association of bacteria with grass roots, (Dobereiner, 1979) has opened up a new avenue of research in this area.

This thesis is concerned with this newly discovered nitrogen fixing association : In Chapter I, an attempt has

been made to collate and systematically present the available information on associative symbiosis. Chapter II describes the methodology used for studying the nitrogen fixing potential of local grasses using acetylene reduction bioassay test of the undisturbed soil root cores. Chapter III gives the results of the survey of the nitrogen fixation potential of local grasses and sedges. The soil root cores of sixteen plant species were found to possess positive acetylene reduction activity. Of these fourteen species have been reported to possess acetylene reduction ability for the first time, according to the information available in the literature.

The results of this survey clearly indicate the widespread occurance of associative symbiosis in the local ecosystems. The information gathered during the course of these investigations would help in exploring the possibility of exploiting the potential of this newly discovered loose symbiotic association.

CHAPTER I

ASSOCIATIVE SYMBIOSIS : A REVIEW

Living world is exposed to an atmosphere containing over 70 per cent nitrogen but one of the greatest anomalies of nature is that with the exception of only a few micro-organisms, most of the living organisms are incapable of using this vast reservoir of nitrogen which is essential for growth and development. In general, most of the plants and animals are incapable of using the atmospheric nitrogen. Therefore, availability of sufficient quantities of fixed nitrogen is a crucial factor limiting biological productivity including primary and secondary production and human health. Nitrogen needs of modern agroecosystems which are largely met by chemical fertilizers are growing rapidly over the years. Nitrogen fixation is an energy intensive process, and requires about 104 k joules per mole of nitrogen fixed. Modern agroecosystems require heavy energy subsidy in the form of fertilizer nitrogen. The energy crunch has greatly complicated the problem and has led to an unprecedented escalation in the cost of nitrogen fertilizers. These developments have brought biological nitrogen fixation into sharp focus for moderating the crippling effect of spiralling energy costs on agriculture, particularly in the developing world.

Availability of sufficient supply of dietary nitrogen requires that ways must be found to enhance biological nitrogen

fixation. This natural process of biological nitrogen fixation has generated considerable excitement because it carries a great promise as a cheap source of fixed nitrogen.

Studies on biological nitrogen fixation until recently, have remained largely confined to the agronomically important legume-bacterial associations. However, the discovery of nitrogen fixation by associative symbiosis in ~~the~~ Digitaris decumbens and Paspalum notatum (Dobereiner, 1972) has triggered considerable interest in the potential of non leguminous nitrogen fixers which form loose symbiotic associations (Silver and Jump, 1975; Eskew and Ting, 1978; and Ogan, 1979). Thereafter, many laboratories from different parts of the world have reported nitrogen fixation by grasses and sedges. So far, thirty eight genera of Gramineae have been shown to possess nitrogen fixing potential by employing acetylene reduction (AR) bioassay test (Table 1). Most of the grasses reported to fix nitrogen belong to subtribe Panicoideae of tribe Paniceae. However, it is only after the thorough survey and systematic evaluation of a large number of grasses that a proper distribution of nitrogen fixing potential in different subtribes of family Graminese can be determined. In addition to grasses seven genera of sedges belonging to family

Table 1.1: List of Grasses shown to Possess Acetylene Reduction Activity

Name (A)	C_3/C_4 (B)	Tribe/ Subtribe (C)	ARA (D)	Country (E)	Reference (F)
ANDROPOGON GAYANUS	C_4	Panicoidaeae Andropogoneae	110	Brazil	Dobereiner et al. (1975)
ALOPECURUS GENICULATUS	C_3	Pooideae Agrostideae	37.7-76.6	Nova-Scotia	Smith and Patriquin (1978)
AGROPYRON DASTACHYUM	C_3	Pooideae Triticeae	3.6	Oregon	Wullstein et al. (1979)
AGROSTRIS ALBA	C_3	Pooideae Agrostideae	4.4-65.5	Nova-Scotia	Smith and Patriquin (1978)
ANTHOXANTHUM ODORATUM	NA	Pooideae Aveneae	0.1-50	Nova-Scotia	Smith and Patriquin (1978)
AMMOPHILA ARENARIA	NA	Pooideae Poaceae	+	Philli-pines	Hassouna and Wareing (1964)
ARISTIDA PURPURBA	NA	Pooideae Aristideae	4.9	Oregon	Wullstein et al. (1979)
BRACHIARIA REGULOBA	C_4	Panicoideae Paniceae	59	Brasil	Dobereiner and Day (1973)
AXONOPUS COMPRESSUS	NA	Panicoideae Paniceae	+	Australia	Weier (1980)

Contd.../-

Table 1.1 (Contd.)

(A)	(B)	(C)	(D)	(E)	(F)
GENCHRUS CILIARIS C. REGULOSA	C_4	Panicoideae Paniceae	6.5	Brazil	Day and Dart (1976)
CALAMAGROSTRIS CANADENSIS	C_3	Pooideae Agrostideae	*	Nova-Scotia	Smith and Patriquin (1978)
CYMBOPOGON GIGANTEUS	C_4	Panicoideae Andropogoneae	34.7	Brazil	Day and Dart (1976)
CYNODON DACTYLON	C_4	Pooideae Chloridene	110	Cali-fornia	Eskew and Ting (1976)
DIGITARIA DECUMBENS	C_4	Panicoideae Paniceae	588	Brazil	Neyra and Dobereiner (1977)
DISTICHILIS STRICTA	NA	Pooideae Poaceae	0.8± 1.1	California	Eskew and Ting (1977)
ELUSINE CARACANA	C_4	Pooideae Eragrosteae	31	Brazil	Dobereiner et al. (1975)
ERAGROSTRIS SP.	C_4	Pooideae Eragosteae	*	Wisconsin	Tjepkema and Burris (1976)
FESTUCA CAPITATA	C_3	Pooideae Festuceae	0.8± 54.8	Nova-Stocia	Smith and Patriquin (1978)
GLYCERIA BOREALIS	C_3	Pooideae	60	Ontario Canada	Bristow (1974)

Contd.../-

Table 1.1 (Contd.)

(A)	(B)	(C)	(D)	(E)	(F)
GLYCERIA GRANDIS	C_3	Pooideae Glyceriaae	82-174	Nova-Scotia	Smith and Patriquin (1978)
HYPARRHENIA RUFA	C_4	Panicoideae Andropogoneae	12	Brazil	Dobereiner and Day (1973)
LOUDETIA SIMPLEX	NA	Pooideae Poaceae	22	Brazil	Day and Dart (1976)
MELINIS MINUTIFLORA	C_4	Panicoideae Paniceae	16	Brazil	Dobereiner and Day (1973)
ORYZA SATIVA	C_4	Pooideae Aveneae	120	Philli-pines	Watanabe and Kukki-Lee (1975)
ORYZOPSIS HYMENOIDES	NA	Pooideae Stipeae	5.9	Oregon	Wullstein et al. (1979)
PANICUM MAXIMUM	C_4	Panicoideae Paniceae	1222	Brazil	Dobereiner and Day (1976)
PASPALUM NOTATUM	C_4	Panicoideae Paniceae	124	Brazil	Neyra and Dobereiner (1977)
PENNISETUM PURPUREUM	C_4	Panicoideae Paniceae	365	Brazil	Dobereiner et al. (1975)
PHLEUM PRATENSE	C_3	Pooideae Agrostideae	0.7-243	Nova-Scotia	Smith and Patriquin (1978)
SACCHARRUM OFFICINALIS	C_4	Panicoideae Andropogoneae	8	Brazil	Dobereiner (1961)
SETARIA ANCEPS	C_4	Panicoideae Paniceae	49	Wisconsin	Tjepkema and Burris (1976)

Contd.../-

Table 1.1 (Contd.)

(A)	(B)	(C)	(D)	(E)	(F)
SPARTINIA ALTERNIFLORA	C_4	Pooideae Poaceae	75 - 150	Nova-Scotia	Patriquin (1976)
S. PECTINATA	C_4	"	+	"	Smith and Patriquin (1978)
SPOROBOLUS HETEROLEPIS	C_4	Pooideae Sporoboleae	1	Wisconsin	Tjepkema and Burris (1976)
SORGHUM BICOLOR	C_4	Panicoideae Andropogoneae	63	Brazil	Dobereiner et al. (1976)
S. VULGARE	C_4	"	+	"	van Berkum and Day (1980)
STENOTAPHURIM SECUNDATUM	C_3	Panicoideae Paniceae	6.5 ± 11.3	Califor-nia	Eskew and Ting (1976)
STIPA COMATA	NA	Pooideae Stipeae	6.4	Oregon	Wulletein et al. (1979)
TRITICUM VULGARE	C_3	Pooideae Triticeae	3.0	Brazil	Neyra and Dobereiner, (1977)
ZEA MAYS	C_4	Panicoideae Maydeae	730	Brazil	Dobereiner et al. (1975)

Contd.../-

Table 1.1 (Contd.)

(A)	(B)	(C)	(D)	(E)	(F)
ZIZANIA AQUATICA	C_3	Pooideae Oryzeae	144	Nova-Scotia	Smith and Patriquin (1978)
ZOYSIA JAPONICA	C_3	Pooideae Zoysieae	3.3	Californ-ria	Eckew and Ting (1976)

ARA - acetylene reduction activity in n mole g^{-1} dry root h^{-1}.

\+ - indicates that ARA was observed but it was not possible to convert the values in present form.

C_3/C_4 - indicates the carbon fixation cycle prevalent in the species (Krenzer et al. (1975).

N.A. - The information on the photosynthetic pathway is not available.

Adapted from Gahalian (1978).

Typhaceae and Cyperaceae have also been found to fix substantial amounts of nitrogen (Table 1.2).

The loose symbiotic association found in the rhizosphere of grasses and sedges is becoming increasingly important on account of the following factors :

1. Grain crops are mostly members of the family Gramineae
2. Nitrogen deficient ecosystems are generally in the tropical belt.
3. In many cases grasses are primary colonizers appearing first in the newly created habitats.
4. Tropical grasses possess C_4 type of photosynthetic pathway and have obvious advantage over other plants with C_3 type of photosynthesis, in view of the greater availability of photosynthates for supporting nitrogen fixation by associative microsymbiont.
5. The association between grass roots and nitrogen fixing microsymbiont is relatively simple, (Berg et al., 1980).The associative symbiosis facilitates much greater manoeuvrability as compared to nodule symbiosis.

NATURE OF THE LOOSE SYMBIOTIC ASSOCIATION

The term associative symbiosis refers to a loose association of a nitrogen fixing microorganism with the plant parts of an angiosperm : No visible structures

Scapter

Table 1.2: List of Sedges Shown to Possess Acetylene Reduction Activity

Name	ARA	Country	References
TYPHACEAE:			
TYPHA ANGUSTIFOLIA	2.0	Ontario, Canada	Bristow (1974)
CYPERACEAE:			
BULBOSTYLIS APHYLLANTHOIDES	30	Brazil	Day and Dart (1975)
CYPERUS OBTUSIFLORUS	253	France	Balandreau et al. (1973)
FIMBRISTYLIS SP.	77	France	Balandreau et al. (1973)
JUNCUS BALTICUS J. TENUIS	+	Oregon USA	Barber et al. (1976).
CAREX NIGRA C. LURIDA	12.7-144 4-125	Nova-Scotia	Smith and Patriquin (1978)
SCIRPUS ATROVIRENS	0-66.0	Nova-Scotia	Smith and Patriquin (1978)
S. ATROCINCTUS	6-60.5	"	
S. POLYPHYLLUS	100-16	Madison	Kamna and Tjepkema (1978)

ARA = acetylene reduction activity in n mole g^{-1} dry root h^{-1}.

+ = indicates that ARA was observed but it was not possible to convert the values in present form.

e.g., nodules, pouches, coralloid outgrowths etc. are produced to protect the microsymbiont from competition with other microbes. Such nitrogen fixing systems are termed as associative symbiotic systems (Dobereiner and Day, 1976) or nitrogen fixing associations (Brill, 1979). The sloughing off of the superficial cortical layers of roots (Dobereiner and Campelo, 1971) and their decay appears to promote multiplication of the microsymbiont (Berg et al., 1980). It may also be found in deeper layers of cortex and in vascular bundle; endorhizosphere, (Patriquin and Dobereiner, 1978). It has been postulated that bacteria multiply in the 'mucigel' secreted by the lateral roots (Umali-Garcia et al., 1980). These workers have shown the presence of an unknown proteinaceous factor secreted by the host roots, which binds microsymbiont cells to root surface.

The association of bacterium with angiosperm roots is highly fragile, and is prone to external perturbations such as pO_2, injury and washing etc. This indicates that the associative microsymbiont is an intermediate between completely independent forms such as Azotobacter, Derxia, Beijerinckia etc., and the nodule symbionts such as Rhizobium and Frankia.

METHODOLOGY FOR DETECTING NITROGEN FIXATION : ACETYLENE REDUCTION BIOASSAY

The discovery of acetylene as a serogate substrate of nitrogenase helped in evolving an inexpensive and rapid procedure of accurately assaying the nitrogenase activity. The technique developed by Scholhorn and Burris (1967) and by Hardy et al. (1973) is simple, adequately sensitive and highly suitable for field investigations. Essentially the acetylene reduction (AR) bioassay involves incubation of plant parts in a chamber, followed by introduction of ten per cent (v/v) acetylene and the gas chromatographic analysis of ethylene in the samples drawn from incubation chamber, at regular intervals.

The observations made by different workers on the potential of non legume flowering systems, entering into loose associative symbiosis, vary considerably. This is partly due to the differences in the details of the incubation procedure employed for assessing AR activity. Therefore, the discrepancy in estimation may result even when the same plant is studied using the variants of the assay procedure.

Variants of Incubation Procedure :

Variants of the assay procedure as described by different workers can be categorised as follows :

a. *In situ* assay.
b. assay of green house grown plants in special incubation chambers;
c. soil root cores removed from field and assayed under laboratory conditions; and
d. excised root assay.

The *in situ* incubation involves inverting a transparent incubation chamber over the shoots. This procedure entails several difficulties such as long incubation period required for the assay, the inhibition of acetylene reduction by requisite pC_2H_2 and the chances of ethylene leakage from the incubation chamber. These problems have been systematically enumerated by Patriquin and Denike (1978). The assay of green house grown plants incubated in special chambers also involves similar problems such as the requirement for a long incubation period and a high pC_2H_2 for maintaining stable levels of acetylene reduction activity. Under these conditions the reduction of acetylene is limited because of the dilution of ethylene formed, which undermines the sensitivity of the bioassay.

The soil root cores of nitrogen fixing plants have been incubated for determining their nitrogen fixing potential but the values obtained following this procedure are low (Burris, 1977) as compared to the activity of excised

washed roots *in vitro* (Dobereiner, 1978b). It seems that the excised washed roots failed to reduce acetylene immediately after their incubation in 10 per cent acetylene. Since, the *Azospirillum* system is sensitive to molecular oxygen and when roots are excised, it tends to loose the ability to fix nitrogen (Day, 1977). The period required for initiating nitrogen fixation by harvested roots depends upon the stage of development of the plant, lasting 8-18 h. Therefore, Dobereiner (1972) introduced a preincubation period of 14 h prior to the incubation in the excised root assay. However, the rates of acetylene reduction by soil root cores are reportedly 3 to 30 times less (Eskew and Ting, 1976; van Berkum and Bohlool, 1980) than those obtained by excised root assay. The apparent variation observed in the bioassay of excised washed roots and of soil root cores has been the subject of active controversy. Burris (1977), hypothesised that incubation of excised roots in 0.02 atm oxygen results in depletion of oxygen and a fermentative metabolism of root cells sets in. The acids produced in this process promote the proliferation of the nitrogen fixing microsymbiont (Gaskins and Carter, 1975; Barber *et al*., 1976). Based on MPN counts Okon *et al*. (1977) and van Berkum and Day (1980) have shown an 8 to 665

fold multiplication in the number of bacteria following the preincubation period. Dobereiner (1978) contended that mere counting of MPN of bacteria is not a sufficient evidence to indicate the proliferation of the microsymbiont because other organisms than the nitrogen fixing bacteria were present in the MPN counts.

However, evidence is accumulating that multiplication in the population of microsymbionts takes place during the preincubation, for example, there is no conclusive evidence to show that preincubation should continue for 14 h, to restore the natural ability to fix nitrogen by roots, because if preincubation is prolonged beyond this period, higher rates of acetylene reduction are observed. Van Berkum (1980) has shown that (a) preincubated roots which were not washed developed only 4-11 per cent of the nitrogenase activity as compared with replicate washed samples; (b) control bottles containing soil only failed to reduce acetylene; (c) after an overnight preincubation the roots were separated from the water washings collected at the bottom of the serum vials where the former gave only 50 per cent of the original activity. Rest 50 per cent was found to be associated with the water washings of the roots; (d) a 50 to 1000 fold increase in the number of nitrogen fixing bacteria during 14 h preincubation at 30 C was observed, and (e) the addition of increasing amounts of

combined N to the roots prior to the incubation resulted in a progressive increase in the lag phase. This shows that although rapid increase in the population of nitrogen fixing bacteria occurs, nitrogenase is not synthesized until the available amount of combined N drops beyond certain critical level.

In view of the above discussion, there is a need for reassessment of nitrogen fixation rates reported for grass-bacterial system. It has also been recommended that assays should be made on excised roots without washing and only such samples which show immediate acetylene reduction should be considered to possess natural nitrogen fixing ability (van Berkum and Bohlool, 1980).

The use of soil root cores has also been recommended by several workers, if the criticism on excised root assay has to be overcome (Barber et al., 1976; Eskew and Ting, 1976; Burris, 1977). The assay of soil root cores is most reliable because it involves minimum shock injury to the nitrogen fixing grass root bacterial system, it requires minimum lag period to achieve linear rates of nitrogen fixation and it does not involve the use of costly gas mixtures. Hence, this procedure of incubation appears to eliminate the drawbacks of in situ as well as excised root assays and provides nearest natural rates of nitrogen fixation in terms of acetylene reduction (Table 1.3).

Table 1.3: Matrix showing Advantages and Disadvantages of the Various Incubation Procedures for Estimation of Their Acetylene Reduction by Underground Parts of Plant Materials

Method	1*	2	3	4	5	6	7	8
In situ incubation	-	+	-	+	-	+	-	+
Greenhouse grown plants	-	+	-	+	-	+	-	+
Soil root cores	+	+	+	+	+	+	+	+
Excised root assay	-	-	+	-	+	-	-	-

*the numbers indicate the feature of the incubation procedures as:

1. Length of the incubation period,
2. Preincubation period required,
3. Lag period required to achieve stable levels of activity,
4. Involves any shock to the nitrogenase system of microbes,
5. Chances of diffusion of ethylene out of the chamber,
6. Makes use of costly gas mixture,
7. Variability observed within subsamples;
8. Chances of introduction of artefacts like multiplication of microsymbionts leading to false estimation.

+ indicates advantage, - indicates disadvantage.

Associative symbiosis is found to be affected by various physico-chemical factors, any one of which could be limiting under a given set of environmental conditions.

Temperature - Temperature plays a key role in governing the nitrogenase activity. Effect of temperature on nitrogenase activity was shown by Dobereiner (1978a) on roots of Zea mays. She correlated rhizospheric nitrogenase activity with that of two strains of Azospirillum sp. showing a maxima at 35 C. The only drawback of this study is that rhizospheric nitrogenase activity was estimated using the excised roots for AR assay. The higher incidence of Azospirillum strains in tropical areas has been attributed to the high temperature requirement of these bacteria (Neyra and Dobereiner, 1977).

Light - Rhizospheric nitrogenase activity is mainly limited by the supply of energy substrates from the plants as exudates or dead material. The quantum of exudation is species specific and depends on rate of photosynthesis. Balandreau (1979) has demonstrated the determining role of light on nitrogenase activity of maize plants grown in growth cabinets as well as in field. Promotory effects of increasing light intensity on nitrogenase are preceded by a time lag of 1-2 h (Balandreau et al., 1978). The observed time lag has been ascribed to the time required for

transportation of photosynthates down the roots and their subsequent exudation from roots finally to be used by the nitrogen fixing microsymbiont.

In view of the above, it can be envisaged that C_4 plants may make better associations with nitrogen fixing microsymbiont as compared to C_3 plants. However, no direct evidence to this effect has been furnished.

Soil moisture - Soil moisture seems to play a vital role in determining the nitrogenase activity of rhizosphere by influencing the *in situ* partial pressure (pO_2) of oxygen (Balandreau, 1979). Tjepkema and Evans (1976) hypothesised that wetland habitats show higher rates of nitrogen fixation because of depletion of molecular oxygen resulting from water logging. During the vegetative period of maize, Balandreau *et al.* (1976) found a significant positive correlation between rainfall, soil moisture, temperature and the *in situ* nitrogenase activity of the rhizosphere. The high rates of nitrogen fixation with low-land rice and the much lower rates in the rhizosphere of upland rice, found by Yoshida and Ancjas (1977) also demonstrate the strong effects of soil aeration and oxygen accessibility on rhizospheric nitrogenase activity. Various workers have noticed high nitrogenase activity

with soil root cores in wetlands and moist habitats (Day et al., 1975; Kanna and Tjepkema, 1978). The availability of adequate amounts of water may also enhance nitrogenase activity by washing the soil of its excessive fixed nitrogen content. If this is true, dry soil species can be used to advantage by proper irrigation and drainage of anticipated habitats (van Berkum and Bohlool, 1980).

pO_2 - Exposure to oxygen causes irreversible damage to the Mo-Fe protein of the nitrogenase complex. Therefore, the process of nitrogen fixation is highly dependent on pO_2 of the nitrogen fixing sites. The majority of obligate aerobic diazotrophs behave as obligate microaerophiles (Beijerinck, aero, air; philous, I love; needing but little free oxygen), when they grow on N-free medium (Yates and Eady, 1979). These organisms, apparently, have very feeble oxygen protection mechanisms, e.g.,

a. Protection by conformational changes in enzyme structure,
b. Secretion of gummy substances around the colony,
c. Consumption of excessive oxygen by hydrogen evolution,
d. Protection of the central cells of colony by sacrificing the peripheral cells,
e. Protection by development of association with a non diazotroph which can produce gummy substances ; such an association might help the diazotroph in maintaining requisite concentrations of oxygen.

Azospirillum cells under free living conditions as well as in association with grass rhizosphere requires an optimum pO_2 of 0.015 to 0.02 atm for optimal nitrogenase activities. Oxygen is needed for nitrogen fixation to provide ATP but it also acts as an inhibitor of nitrogenase activity at natural concentration of 0.2 atm. These two opposite requirements of nitrogenase, create a paradoxical situation (Postgate, 1978). In semisolid agar cultures Azospirillum develops a pellicle a few mm below the surface (Dobereiner and Day, 1976). As the oxygen demand of the culture increases the whole colony migrates upwards (Day, 1977). It may be possible that under rhizospheric association the microsymbiont cells migrate in the endorhizosphere to an optimal pO_2 site for nitrogen fixation. However, no specific oxygen protection mechanism, in this case has been identified. It is likely that outer cells in a mass protect the cells at the core of the colony.

Redox potential (Eh) - Pronounced effect of soil redox potential on Azospirillum nitrogenase activity associated to rhizosphere has been shown recently. Decreasing Eh was related to an increase in nitrogenase activity (+ 100 mv) but further fall in Eh was inhibitory (Trolldenier, 1977). The amendment of flooded soil with

rice straw favourably reduced the Eh and increased nitrogenase activity as well as MPN counts of Azospirillum (Charyulu and Rao, 1980). It may be possible to realize much higher yields by monitoring the Eh through addition of regulated amounts of organic matter (standardized for a particular soil) to maintain persistent high rates of nitrogenase activity of the microsymbiont populations.

pH - The nitrogenase activity is specific to the pH of the medium. Dobereiner (1978a) observed significant correlation between soil pH and nitrogenase activity of the enrichment cultures of A. lipoferum grown in N-free semisolid malate medium after 40 h incubation at 33 C. Most actively acetylene reducing cultures were obtained from those roots of Panicum maximum which had a soil pH of rhizosphere ranging from 6.8 to 7.8. Even in acid soils with pH 4.6, the enrichment cultures of Azospirillum have been reported to reduce acetylene (Dobereiner, 1978a). Probably, the nitrogen fixation by bacteria occurs in the rhizosphere at a site where the specific pH requirement can be met (Dobereiner, 1978).

Combined nitrogen - Availability of ammonium (NH_4^+) is known to inhibit and repress nitrogenase of Azospirillum lipoferum (Okon et al., 1976a) and it also suppresses the rhizospheric AR activity of cereal roots (Balandreau and

Dommergues, 1973) and grasses (Vaughn and Jones, 1976) under the field conditions. Application of low initial level of combined nitrogen (NH_4^+) in large semisolid cultures improves growth and nitrogen fixation by Azospirillum (Watanabe and Barraquio, 1979). Similar effects of application of NH_4^+ on associative symbiosis have been observed by Abrantes et al. (1975) under field conditions.

Low level of nitrate (NO_3^-) application to soil promotes while the presence of high level of NO_3^- inhibits nitrogen fixation in rhizospheric systems. That NO_3^- was active only via nitrite (NO_2^-) formation under anaerobic conditions, was shown by Magalaes et al. (1978) who found the presence of dissimilatory nitrate reductase system in Azospirillum cultures : NO_3^- supplied to the NR^- mutants did not inhibit nitrogenase activity. Therefore, this species of combined nitrogen (NO_3^-) is not an inhibitor but it is the NO_2^- which is inhibitory to nitrogenase activity. In light of this study, the knowledge of nitrogen content, pH, porosity and aeration level of soils are important determinants for achieving significant yields through artificial inoculation with bacteria.

Molybdenum - Among the micronutrients molybdenum plays a key role in nitrogen fixation because it is required for activation of component I apoprotein of nitrogenase. It has been shown that each atom of Mo together with eight atoms of Fe and six labile sulphides, forms active site of component I protein of nitrogenase (Brill, 1980). Mo also acts as cofactor for another enzyme nitrate reductase but the cofactors for nitrogenase and nitrate reductase are different from each other. Probably they share a common molybdoenzyme permease for processing $MoO_4^{=}$. Plants growing in molybdenum deficient soils show all symptoms of nitrogen starvation. Addition of molybdenum in such soils could even improve rhizospheric nitrogenase activity (Diem and Dommergues, 1979). Molybdenum is active in regulation of nitrogenase synthesis (Nagatani and Haselkorn, 1978) in algal systems but similar attempts to relate Mo with regulation of enzyme synthesis in associative symbiotic systems have not been made.

Biocides - Various workers have shown positive effects of low levels of pesticides and herbicides on nitrogenase activity in Azospirillum. Dobereiner (1978a) showed that a herbicide Gesaprin used in concentrations applied commonly to maize crops (3.5 kg ha^{-1}) did not

only increase numbers of A. lipoferum in soil but also stimulated its growth in semisolid malate medium. Charyulu and Rao (1978) reported beneficial effects of 10, 20 and 100 ppm benomyl application to flooded rice soils in terms of population of Azospirillum sp. and their nitrogen fixing efficiency which was highest when 100 ppm benomyl was applied but they could not detect any appreciable increase in nitrogenase activity when the biocide was applied to pure cultures (Charyulu and Rao, 1978). Various insecticides obtained from natural sources (e.g. Pyrethrum, Azadiracta) namely, pyrethrum, neem oil, and allitin proved beneficial when applied at 0.005 per cent, 1 per cent and 0.5 per cent levels respectively, with the increasing vegetative period. Detailed information is needed to formulate a relationship between various biocides and nitrogenase activity of Azospirillum (Nayak et al., 1980).

Root exudates - The amount and composition of exudates may affect microbial growth and influence the rhizospheric nitrogenase activity. For instance, it has been shown that rate of rhizodeposition is 300 per cent of the root weight of mature wheat plants (Sauerbeck et al., 1976). Barber and Martin (1976) found that sterile plants released 5 to 10 per cent of their photosynthates through their roots whereas under unsterile

conditions roots excreted double the amount. The observed differences in nitrogenase activity of the roots between different maize cultivars may be due to the differences in amounts and composition of their root exudates (von Bulow and Dobereiner, 1975). However, this hypothesis needs to be checked through further experimentation. It appears that physical factors such as light and temperature exercise their influence at secondary level, through regulation of root exudation on nitrogenase activity.

Biotic factors - Weier (1980) has shown that soil root cores detopped prior to the incubation leads to increased rates of nitrogen fixation. Empirical data on this aspect is needed to relate the effect of grazing with nitrogenase activity of grasses.

THE ASSOCIATIVE MICROSYMBIONT

Studies on associative symbioses have recently begun, but interesting information has been gathered as an outcome of past ten years of work. The proper understanding and appreciation of the nature of associative symbiosis calls for a clear comprehension of the associative microsymbiont entering into loose symbiosis responsible for the observed rhizospheric nitrogen fixing activity of grasses and sedges.

Several bacterial systems entering into loose symbiotic association with grasses have been recorded. Larson and Neal (1978) have described the occurance of Bacillus sp. in one wheat line specific to this bacterium. Nioh (1979) reported an Azotobacter sp. associated with roots of Eragrostris ferruginea. Wullstein et al. (1979) have isolated Bacillus polymyxa - like organisms from rhizosheaths of certain xeric grasses. Bhide and Purandare (1979) and Tikhe et al. (1980) reported the occurcance of Azotobacter sp. on roots of about 30 non-leguminous angiosperms. McClung and Patriquin (1980) have described the occurance of a bacterium Campylobacter sp. in the rhizosphere of an aquatic macrophyte Spartinia alterniflora. Studies on isolation, purification and characterization of associative microsymbiont have shown that a microaerophilcic bacterium Azospirillum is most frequently involved in loose symbiotic nitrogen fixing systems. Dobereiner and Day (1973) and Tyler et al.,(1979) have described Azospirillum as the only major organism associated with the rhizosphere of grasses and sedges that forms efficient association with a large number of hosts and is distributed over a wide range of geographic regions.

History of discovery - This organism was first isolated in 1925 from soils by Beijerinck in impure cultures of nitrogen fixing bacteria and he named it as Spirillum

lipoferum. Schroder (1932) was able to demonstrate low levels of nitrogen fixation by pure culture of A. lipoferum from German and Austrian soils. Becking (1963) used $^{15}N_2$ as a tracer to produce evidence of its ability to fix nitrogen under reduced the oxygen concentration. When Schroder incubated pure cultures at normal concentration of oxygen, apparently its nitrogen fixing activity was suppressed. However, this organism did not attract much attention until Day and Dobereiner (1976) isolated this bacterium from Digitaria decumbens roots. Thereafter, nitrogen fixing species of Spirillum have been reported from over fourty plant species which are now included under the generic name Azospirillum (Kreig et al., 1977).

Taxonomy - At present more than 150 nitrogen fixing isolates of this genus have been isolated (Dobereiner et al., 1977) The diversity shown by these organisms makes it difficult to contain all these strains within one species. Based on colony features, ability to utilize sugars and other physiological considerations (Table 1.4) the isolates of Azospirillum have been divided into three groups (Okon et al., 1976b; Sampio et al., 1976 and Neyra et al., 1977). Kreig et al. (1977a,b) studied the DNA homology of the three groups. They confirmed the observations of earlier

Table 1.4: Characteristics of Various Isolates of *Spirillum Lipoferum*

Isolate	Growth on enrichment medium (0.005% agar)	Growth on solid enrichment medium	Growth on nutrient agar
GROUP I: *Azospirillum brasilense*			
Sp4,7 and 13 from Digitaria decumbens (km 47) Sp81,82,80, 75 from maize (km 47) Sp60,51e from wheat (km 47)	1. Typical pellicle below surface 2. Good growth and acetylene reduction on malate succinate, lactate. 3. Slow growth on galactose. 4. No growth or acetylene reduction on gglucose 5. No need for yeast extract as starter 6. Catalase positive 7. Resistent to antibiotics.	1. Round or irregular wet, hard colonies 2. A pink pigment development after two weeks (51e has a strong pink pigment)	1. Round or irregular dry translucent colonies 2. Develop pink pigment after 1 week of incubation
GROUP III: Sp107, 107st 106,109 from wheat	1-7 As Group I, except it is unable to reduce NO_3^- further and shows higher sensitivity to tetracycline.	As Group I	As Group I
GROUP II: *Azospirillum lipoferum*			
Sp59 from wheat (Km 47) SpRG from wheat (Brazil) SpUSA5 from grasses (Washington)	1-3 As Group I 4. Growth and acetylene reduction on glucose 5. Need for yeast extract as starter 6. Catalase negative 7. Sensitive to antibiotics	1. Growth only when yeast extract is added in the medium.	1. Dry colonies 2. Develop pigment only five week after incubation.

(Adopted from Okon *et al.* 1976; Sampio *et al.* 1976).

workers and found that based on DNA homology experiments, group II strains could be seperated from groups I and III strains. Both the later had DNA homologous with representative strain Sp7 and DNA of group II strains was homologous with representetive strain Sp59B.

The nitrogen fixing species of Spirillum are morphologically distinct from non nitrogen fixing spirilla, they have a sickle shaped body with single flagellum and only half to one turn per cell. But after a careful study, Tarrand and Kreig (1978) concluded that nitrogen fixing strains form a coherent group within themselves; and are distinct from members of Pseudomonadaceae in terms of their DNA homology values which are less than ca. 20 per cent. It has been suggested to retain nitrogen fixing spirilla in family Spirellaceae because like spirilla, the nitrogen fixing strains of S. lipoferum possess poly-β-hydroxy granules, show respiratory type of metabolism, and grow well on salts of organic acids. However, it would be desirable to place them at a generic position at par with other spirilla. Accordingly, a new genus Azospirillum was created for the nitrogen fixing group of spirilli represented by type strains Sp7 and Sp59B (Kreig et al., 1977).

Presently, the genus Azospirillum consists of two species namely; A. lipoferum (Beijerinck) comb. nov. and A. brasilense sp. nov.. The species A. lipoferum is type

Table 1.5: Characteristics of a Bacterium of the Genus *Azospirillum* from Cellulolytic Nitrogen Fixing mixed Cultures (Mc-2s)

Characteristics	Mc-2s	*A. lipoferum*	*A. brasilense*
DNA base composition (mol% G + C)	71.6	70	70
Biotin requirement	yes	yes	no
Glucose used as sole carbon for N-deficient semisolid medium	0	+	0
Changes in cell morphology in N-deficient semisolid medium	yes	yes	no
Acidification of glucose media	0	+	0
Yeast Growth in glutamate medium	+	+	+(weak)
Anaerobic growth in nitrate	+(weak)	+	+
Growth in presence of bile	0	+(weak)	+
Growth in 3% NaCl	0	0	+
Oxidase, esculin hydrolysis	+	+	+
Starch hydrolysis	0	0	0
Catalase	+(strong)	+(moderate)	+(moderate)
Dissimilation of nitrate to nitrite	+	+	+
Dissimilation of nitrate to gas	0	+	+

Contd.../-

Table 1.5 (Contd.)

Characteristics	Mo-2s	A.lipoferum	A.brasilense
Sole carbon source :			
Cellulose	0	NT	NT
Mannitol	+	+	0
Sorbitol	+	+	0
Ribose	0	+	0
Glucose	0	+	0
Fructose	+	+	+
α-ketoglutarate	+(weak)	+	0
C_4 acids	+	+	+
β-hydroxybutyrate	0	+	+

NT - Not tested.

Adopted from Weng et al. (1979).

species of the genus with type strain Sp59B named as A. lipoferum as representative of strains grouped under group II of Sampio et al. (1976) and type strain Sp7 named as A. brasilense as representative of group I and group II organisms.

There are exceptions to this systematic indentification as certain strains have been isolated which cannot be contained by either of the two species for example, Wong et al. (1979) isolated an Azospirillum from cellulolytic N_2 fixing mixed cultures, which shares characteristics of both the species namely, A. lipoferum, and A. brasilense. The features of this new strain, named as Mc-2s are given in Table 1.5. Similarly, Nur et al. (1980a) have reported an Azospirillum isolate which utilises glucose as C-source without requiring biotin. The colony characteristics and pigmentation of this new strain are different from both the species. Detailed work on DNA homology and base composition will determine whether this new strain from Israel is a new species of Azospirillum or not.

Geographical distribution - After its first discovery in 1925 from Germany, Azospirillum has been reported from many parts of the world indicating its wide geographical range. The reports of this bacterium from Austria, Germany and Europe by Beijerinck (1925) and Schroder (1932) represent the only

Table 1.6. Host Range and Geographical Distribution of Genus *Azospirillum*

Name (A)	Tribe/ Subtribe (B)	Isolate No. (C)	Country (D)	Reference (E)
BRACHIARIA	Panicoideae Paniceae	SA31, Sp Col 3	Columbia	Dobereiner et al. (1977)
CENCHRUS CILIARIS	"		India	Lakshmikumari et al. (1976)
DIGITARIA DECUMBENS	"	Sp4, Sp7, Sp34, Sp35 SpRG19a, Sp13r, Sp13	South America	Tyler et al. (1979)
HYPARRHENIA RUFA	Panicoideae Andropogoneae	SpR90, SpCol5	South Africa	Tyler et al. (1979)
PANICUM MAXIMUM	Panicoideae Paniceae	JM82A1, JM51B1, JM75A1, SA526 SpA8	S. Africa S. America Nigeria	Tyler et al. (1979) Dobereiner et al. (1977)
PENNISETUM TYPHOIDEUM	"	JM119A4, FL8NB JM125A2, FL42 302A5, 302A6	Florida USA	Tyler et al. (1979)
RHYNCHELYTRIUM	"	SA4, SA40	S. Africa	Tyler et al. (1979)
SETARIA ANCEPS	"	SAR41	S. Africa	Tyler et al. (1979)
SORGHUM VULGARE	Panicoideae Andropogoneae	Sp52, Sp88, Sp90, SpF4, SpP6	S. America India Gainsville	Dobereiner et al. (1977) Lakshmikumari et al. (1976) Schank (1977)

Contd.../-35

Table 1.6 (Contd.)

(A)	(B)	(C)	(D)	(E)
ZEA MAYS	Panicoideae Maydeae	Sp63, Sp67, Sp75, Sp76, Sp80, Sp82, Sp84	Brazil	Dobereiner et al. (1977)
		JM6A2, JM6B2, JM24B4, JM28A2	Ecuador	Tyler et al. (1979)
		JM73B3, JM73C2B, JM73 C3, JM51B1	Venezuela	Tyler et al. (1979)
		SpA2	Nigeria	Dobereiner et al. (1977)
			India	Lakshmikumari et al.(1976)
		SpCol2b	Columbia	Dobereiner et al. (1977)
		SpBr11, SpBr16, SpBr16y, SpBr17y, SpBr18	Barzilia	Neyra et al. (1977)
CHLORIS SP	Pooideae Chlorideae	SR90	S. Africa	Tyler et al. (1979)
CYNODON DACTYLON	"	SA29, SAS29, SA91, SAS94,	S. Africa	Tyler et al. (1979)
			India	Lakshmikumari et al.(1976)
ERAGROSTRIS SP	Pooideae-Eragrosteae	SA29, SA 91	S. Africa	Tyler et al. (1979)
KOELERIA SP.	Pooideae Aveneae	SAS32	S. Africa	Tyler et al. (1979)
LOLIUM SP.	Pooideae Festuceae	SpRG16a	S. Africa	Dobereiner et al. (1977)

Contd.../-

Table 1.6 (Contd.)

(A)	(B)	(C)	(D)	(E)
ORYZA SATIVA	Poideae Oryzeae	SpPH1, SpA7	Phillipines Nigeria India	Watanabe et al. (1979) Neyra et al. (1977) Nayak et al. (1977)
TRITICUM AESTIVUM	Poideae Triticeae	SpBr11, SpBr14, SpBr15, SpBr19, SpBr20	Brazil	Dobereiner et al. (1977)
		SO51, SP59y, Sp60, SpRG20a, SpRG6xx, SpRC8c, SpRC9c	Columbia	Neyra et al. (1977)
		SpL60, SpL62, SpL69	Londaris	Neyra et al. (1977)
			India	Kavimandan et al. (1976)
*CYPERUS SP.	Cyperaceae	SAR19	India S. Africa	Tyler et al. (1979)
*GRASS	Gramineae	SpA3y	Senegal	Neyra et al. (1977)
*LEGUME	Leguminosae	SpA9	Liberio	Neyra et al. (1977)
*MUSA SP.	Musaceae	JM24B4, JM28A2	Ecuador Brazil	Tyler et al. (1979) Dobereiner (1978)

*- indicates that Azospirillum was isolated from the rhizospheric soil of this plant species.

Contd..../-

Table 1.6 (Contd.)

Name	Family	Country	Reference
AMARANTHUS SP.	Amaranthaceae	India	Lakshmikumari et al.(1977)
BOERHAAVIA RAPENS	Nyctaginaceae	India	Nayak et al. (1979)
CLERODENDRON VISCOSUM	Verbenaceae	India	Nayak et al. (1978)
COLOCASIA ANTICORUM	Araceae	India	Nayak et al. (1979)
COMMELINA BENGHALENSIS	Commeliaceae	India	Nayak et al. (1979)
CYPERUS SP.	Cyperaceae	India	Nayak et al. (1979)
ECLIPTA ALBA	Compositae	India	Nayak et al. (1979)
EUPHORBIA HIRCTA	Euphorbiaceae	India	Nayak et al. (1979)
FICUS SP.	Moraceae	India	Lakshmikumari et al. (1976)
EICHHORNIA CRASSIPES	Pontederiaceae	India	Nayak et al. (1979)
IPOMOEA BATATA	Convolvulaceae	Brazil	Doberainer (1978)
I.REPTANS	"	India	Nayak et al. (1979)
LANTANA CAMARA	Verbenaceae	India	Lakshmikumari et al. (1976)
LUCUS ASPERA	Labiateae	India	Nayak et al. (1979)

Contd.../-

Table 1.6 (Contd)

Name	Family	Country	Reference
MANIHOT ESCULENTA	Convoluvlaceae	Brazil	Dobereiner et al. (1977)
MARDANIA SPIRATA	Commelinaceae	India	Nayak et al. (1979)
MARSILEA QUADRIFOLIA	Marsiliaceae	India	Nayak et al. (1979)
MIMOSA PUDICA	Mimoseae	India	Nayak et al. (1979)
PHYLLANTHUS NIRURI	Euphorbiaceae	India	Lakshmikumari et al.(1976)
PISTIA STRATIATES	Araceae	India	Nayak et al. (1979)
POTAMOGETON FILIFORMIS	Potamogetonaceae	Brazil	Sylvester-Bradley (1976)

reports of its occurance in temperate regions. Tyler et al. (1979) reported its occurance from various stations in subtemperate and tropical regions. Various workers reporting this organism from tropical regions include Dobereiner and Day (1976), Lakshmi-kumari et al. (1976), Lakshmi et al. (1977), Nayak et al. (1977), Reynders and Vlassak, (1977), Sylvester-Bradley (1977), Dobereiner (1978), Kavimandan et al. (1978), Tyler et al. (1979), Watanabe et al. (1979) and Nur et al. (1980). The occurance of this organism has been reported from Austria, Belgium, Columbia, Germany, India, Nigeria, Pakistan, Phillipines, S. America and S. Africa.

Host range - Azospirillum has been shown to possess a wide host range including many species of grasses, sedges, grain and forage crops, economically important plants and various dicotyledonous weeds, (Table 1.6). It has been isolated from roots of 40 plant species representing 16 families of angiosperms namely, Amaranthaceae, Araceae, Commelinaceae, Compositae, Convolvulaceae, Cyperaceae, Euphorbiaceae, Gramineae, Labiatae, Leguminosae, Moraceae, Musaceae, Nyctaginaceae, Potamogetonaceae, Potenderiaceae, Verbenaceae. Seventeen of the above mentioned plant species are members of family Gramineae of these 12 possess C_4 photosynthetic pathway. Azospirillum has been also isolated from soils under free living conditions. The

wide host range of Azospirillum has been demonstrated by Child and Kurz (1977), Vasil et al. (1979) and Berg et al. (1980) on the basis of the colonization of callus tissue from the large number of angiosperms by the bacterial cells, some of which are not included in the above mentioned 40 plant species.

Localization - The exact seat of occurance of the bacterium Azospirillum in the host rhizosphere is not certain. Dobereiner and Day (1976) and Lakshmikumari (1977) have shown the existence of this bacterium within the roots of various grasses using vital staining with tetrazolium chloride. Azospirillum differs from most other rhizosphere microbes in its ability to colonize the intercellular and intracellular spaces of root cortical cells. Partiquin and Dobereiner (1978) found that the bacteria colonized the inner cortex and stele of maize roots without significant colonization or decay of the outer cortex tissue. The bacterium remained viable inside the roots after a 6 hour treatment with sterilizing agents indicating that the endodermis was intact. According to them, Azospirillum infection initially takes place in the cortex of lateral roots, and then spread into main roots where it occupies stele and inner cortex. Berg et al. (1980) explained the intracellular occurance of the bacterium

to be due to the ruptured cell walls rather than to the cell wall degrading properties of the bacterial cells. Recently, the studies of Umali-Garcia *et al.* (1980) have furnished evidence for pectolytic enzyme activity in cells of *Azospirillum*, suggesting thereby the definite cell wall degrading ability of this bacterium. They have also proved the existence of a low molecular weight protein factor which helps in establishment of the association through binding *Azospirilla* to the root cortical cells.

Morphology - This organism forms flagellate, curved cells of 1-5 um length when grown in liquid media along with a source of combined nitrogen under normal oxygen pressure. During N-limited growth, however, nitrogenase is expressed only under 0.1 to 0.05 atm of oxygen. This typical microaerophillic growth is expressed in semisolid N-free agar media by a pellicle formed a few mm below the surface where, low pO_2 is maintained due to poor diffusion of oxygen through the medium lying above the pellicle. In fermenter cultures, optimal nitrogenase activity is obtained by aeration with nitrogen gas containing 0.005 to 0.007 atm of oxygen (Okon *et al.*, 1977). On the solid media with combined nitrogen the typical colonies grow slowly and are composed of cells that have atypical morphology. Therefore, they are normally detected on agar plates inoculated with suspension without previous

enrichment in N-free semisolid media. The pH increases as the colony ages. This is evident from the colour reactions of Azospirillum with vital strain (Lakshmikumari et al., 1980). On nutrient agar the aging colonies of A. brasilense tend to develop pink pigment. This pink pigment is not due to leghaemoglobin and its exact nature is not known. Eskew et al. (1977) have reported the presence of a yellow pigment in 5 week old cultures. The nature of this pigment also is not known. Azospirillum shows negative reaction to gram staining. Under the phase contrast microscope the cells show presence of poly β-hydroxybutyrate granules. Pleomorphic forms of Azospirillum have been reported by Dobereiner and Day (1976), Eskew et al. (1977), Tarrand et al. (1978) and Berg et al. (1980), in old cultures grown on nutrient broth.

Using phase contrast and electron microscopic preparations, isolates of Azospirillum have been classified into two groups by Hegazi and Vlassak (1980) :

1. Thick curved rods (sized 2.0-3.0 x 1.5-3.0 um) with one flagellum, forming a pellicle in semisolid medium.
2. Spirally twisted cells (sized 3.5-5.0 x 1-1.5 um) with single sinous flagellum and moving back and forth along a central axis in cork screw fashion, also forms pellicles.

Physiology - It can be grown on a simple medium described by Dobereiner and Day (1976) at a pH of 6.8 using malate as a source of carbon. The malate is utilized fast and with it the pH of the medium rises. The organisms becomes inactive at a pH of about 7.8. Okon et al. (1976b) modified this medium by addition of 10 g phosphate salts to increase the buffering capacity of the medium, without any toxic effects on Azospirillum. The pH requirements for growth of this organism are manifestations of its enzyme nitrogenase. Azospirillum is a microaerophilic organism requiring an optimal pO_2 of 0.005 to 0.007 atm for nitrogen fixation. It is also capable of utilizing ammonia, requiring a pO_2 of 0.2 atm. The rate of growth is very fast under such conditions.

Azospirillum uses organic acids derived from fermention. Malate is readily utilized and therefore, it is most commonly used for isolation of this bacterium. Succinate, lactate, pyruvate and citrate can also be used as source of carbon with equal efficiency. The efficiency of the C-source utilization is expressed in terms of mg of N fixed per gram of C-source. The efficiencies of six nitrogen fixing bacteria occuring in humid tropics have been compared in Table 1.7. The specific activity of Azospirillum lies in the range of

Table 1.7: Comparison of Nitrogen Fixing Efficiency of Some Bacteria found in Humid Tropics

Genus	Efficiency
Eubacteriales	
AZOTOBACTERIACEAE	
AZOTOBACTER SP.	10-25 mg N g^{-1} C-source
BEIJERINCKIA SP.	10-20 mg N g^{-1} C-source
DERXIA SP.	25,30 mg N g^{-1} C-source
BACILLIACEAE	
CLOSTRIDIUM SP.	2-27 mg N g^{-1} C-source
RHIZOBIACEAE	
RHIZOBIUM LEGUMINOSARUM	40-60 n mole C_2H_4/h/ mg dry wt.
Pseudomonadales	
SPIRILLACEAE	
AZOSPIRILLUM LIPOFERUM	1200-5000 n mole C_2H_4/h/ mg protein
	6.16 ± 0.73 mg N g^{-1} C-source

1200-1500 n moles of ethylene per milligarm protein per hour which is as high as the rates reported for free living rhizobia under *in vitro* condition (Kurz and LaRue, 1975; McComb *et al.*, 1975; Pagan *et al.* 1975).

The nitrogenase from *A. lipoferum* was first isolated by Okon *et al.* (1977a) using cell free extracts. Nitrogenase requires Mg^{++} and Mn^{++} ions and has an optimal pH range of 7.1 to 7.4. Like other diazotrophs the nitrogenase is highly sensitive to molecular oxygen - requiring pO_2 of 0.005 to 0.007 atm. The apparent km of its nitrogenase for acetylene is about 0.0036 atm. Nitrogenase extracts of *A. lipoferum* loose their activity on storage at -18 C. However, the activity can be restored by adding active Fe-protein obtained from other nitrogen fixing bacteria with varying degrees of success (Okon *et al.*, 1977a). The Fe protein itself required an activating factor. This activating factor can be substituted by that obtained from the extracts of *Rhodospirillum rubrum*. These are the only two activating factors known to replace each other for Fe-protein of nitrogenase. These property of Fe-protein can be made use of in repressing and derepressing nitrogenase activity at will, under natural conditions.

Nitrate reduction - Studies on actual contribution of the nitrogen fixed by rhizosphere microorganisms (mainly Azospirillum) revealed that only 25-50 per cent of the nitrogen fixed by the symbiont could be incorporated in the host (De-Polli et al., 1976). The cause of leakage of rest of the 50 to 75 per cent of the fixed nitrogen led to the discovery of the dissimilatory nitrate reduction by Neyra and van Berkum (1977). Thus, Azospirillum not only fixes atmospheric nitrogen but also catalyses denitrification under oxygen limiting conditions. Scott and Scott (1978) observed simultaneous nitrogen fixation and reduction in anaerobic cultures. They observed that those mutants which did not have nitrate reductase system also did not fix sufficient nitrogen in presence of NO_3^-, confirming that NO_3^- reduction is necessary for the NO_3^- enhanced nitrogenase activity. It has been suggested that nitrate respiration provides the ATP necessary for nitrogenase activity. The observation that, at slightly higher than primer levels (10 mM NO_3^-) NO_3^- becomes inhibitory to nitrogenase activity in nir^- mutants lacking nitrite reductase system; led to the realization that dissimilatory nitrate reductase can reduce nitrate to nitrite, and nitrite if unreduced inactivates the enzyme nitrogenase. Most of the mutants which are lacking dissimilatory nitrate reductase (nr^-) are also lacking

nitrite reductase (nir^-). The occurance of nr^- mutants is not a fresh observation (Motohara et al., 1976). By using chlorate resistance method (Piechaud et al., 1967), Magalhaes et al. (1978) described the occurance of nr^+ mutants which were nir^- and vice-versa. Accordingly, four types of mutants the nr^+nir^+, nr^-nir^+, nir^-nr^+ and nir^-nr^- mutants occur in the natural populations of Azospirillum.

In light of this knowledge, the basic similarity between organisms of Group I and III (Sampio et al., 1978) was established confirming that the group III strains, now under A. brasilense do not denitrify but accumulate nitrite formed from reduction of nitrate, which lack dissimilatory nitrite reductase are otherwise identical with group I in all characteristics including DNA homology (Kreig, 1977).

Host specificity - De-Polli et al. (1980) have further divided the group III organisms into two categories, one isolated from well sterilised roots of grain crops (such as Sp107, 107st, 106 and 109st) and second from root surface and rhizosphere soils (such as Sp28). Category one forms precipitate with fluorescent antibody obtained from reference strain Sp107 but not with that from another group. Organisms of the second category react with flouresent antibodies from Sp28 and

not with that from group one organisms. This represents some kind of serological differences between host plant specific groups within the populations of this nitrogen fixing associative symbiont. Whether the serological difference enable the organism to establish association with specific host or conversely it is the association of the organism with host that induced the observed serological differences; is a question which could only be answered through further studies.

Hydrogen evolution - A characteristic property of diazotrophs is their ability to evolve hydrogen along with nitrogen fixation. Hydrogen evolution in diazotrophs can occur through two independent pathways. The first pathway is hydrogenase catalysed, reversible, ATP independent and CO inhibited (Kleiner and Burris, 1970; Nakos and Mortenson, 1977) as :

$$\text{Ferredoxin}_{(red)} + 2H^{+} \xleftarrow[CO]{\text{Hydrogenase}} \text{Ferredoxin}_{(ox)} + H_2$$

The second pathway is nitrogenase-catalysed, irreversible, ATP dependent and unhibited by CO (Winter and Burris, 1968), as :

$$\text{Ferredoxin}_{(red)} + 2H^{+} \xrightarrow[ATP,\ Mg^{++}]{\text{Nitrogenase}} \text{Ferredoxin}_{(ox)} + H_2$$

The characteristics that distinguish the nitrogenase-from hydrogenase - catalysed H_2 evolution have been listed in Table 1.8 .

Table 1.8 : Characteristics of Nitrogenase and Hydrogenase Catalysed Hydrogen Evolution

Hydrogenase-dependent	Nitrogenase-dependent
Do not require ATP	ATP dependent
Unaffected by N_2	Inhibited by N_2 and C_2H_2
Sensitive to CO	Uninhibited by CO
Reversible hydrogen evolution.	Irreversible hydrogen evolution.

The evidence for multiplicity of the types of reactions catalysed by enzyme nitrogenase existed prior to its discovery. It is known to acquire various oxidation reduction levels ranging from -1 to -3 depending upon the clustering of the 4Fe4S ferredoxin protein. The enzyme nitrogenase also exists at two other levels namely 0 and -4 in addition to these three redox levels. On the basis of knowledge gained from EPR and Mossebaur spectroscopic studies, Thorneley et al. (1978) suggested that a proton is converted into hydrogen atom at the site

of hydrogen evolution in Klebsiella pneumoniae when redox potential of nitrogenase protein drops from -2 to -3. The reaction takes place in presence of reductant and is ATP dependent. The original state of the enzyme is then restored by another protein (Kp_2) coupled with a further investment of ATP. Evolution of hydrogen can also occur through hydride formation (Thorneley et al., 1978).

It is known that CO inhibits the nitrogenase dependent substrate reduction except hydrogen evolution, it was, therefore, suggested that nitrogenase possesses two binding sites namely (a) a high affinity CO site and (b) a site with comparatively low CO affinity. When CO is bound at its high affinity site, the electron flow to the N_2 reduction site is interrupted in such a way that an Fe-S centre becomes oxidized to -1 level, whereas when second CO molecule binds to low affinity site, the centre is reduced to -3 level. This explaination is in line with the changes observed in the EPR spectrum of the FeS protein at characteristic 'g' values. Lowe et al. (1978) explained the same phenomenon in terms of differential CO binding to the 4Fe4S ferredoxin species, affecting the overall distribution of the charge on the active sites, ensuing the characteristic changes in the FeS protein.

The enzymes which catalyse the reversible reaction $H_2 \longrightarrow 2H^+ + 2e^-$ are present in a wide variety of microorganisms, in autotrophic and heterotrophic, aerobic and anaerobic bacteria, in procaryotic as well as eucaryotic algae. Smith *et al.* (1976) showed uptake of the hydrogen evolved in the nitrogenase activity by a specialized system of hydrogenases, which later came to be known as hydrogen uptake (Hup) mechanism which catalyses the reverse reaction, as :

$$H_2 \text{ -------- } H_2O + ATP$$

The possible role of this hydrogenase catalysed H_2 reutilisation in various nitrogen fixers (Hyndman et al., 1953; Dixon, 1972, 1976; Schubert and Evans, 1976; Smith *et al.*, 1976; Bothe *et al.*, 1977; Peterson and Burris, 1978; Tel-Or *et al.*, 1978; Fay, 1979), appears (a) to restore ATP and the reductant that would otherwise be lost in hydrogen evolution by nitrogenase; (b) to remove O_2 from site of activity and allow normal functioning of nitrogenase and (c) to prevent H_2 accumulation near nitrogenase which is known to inhibit nitrogen fixation (Bothe *et al.*, 1977, 1978; Peterson and Burris, 1978; Tel-Or *et al.*, 1978; Fay, 1979).

This process occurs at substantial rates ranging from 0.001 to 7.16 μ moles per h per g of nodule, in

various strains of Rhizobium leguminosarum, at a relative efficiency of 0.20 to 0.99 (ARA- 2.1 to 17.37 μ mole per h per g nodule). The hydrogen evolution in Azolla ranges between 0.09 to 0.5 μ moles per h per g fresh weight at a relative efficiency of 0.46 (ARA- 0.34 to 0.9 n mole per h per g fresh weight). The hydrogen evolution rate of K. pneumoniae varies between 1.0 to 3.3 μ mole per h per mg cell protein and ARA between 1.5 to 4.5 μ mole per h per mg cell protein at a relative efficiency of 0.25 to 0.99. The hydrogen evolution, acetylene reduction and their relative efficiencies have been tabulated in Table 1.9.

Table 1.9: Hydrogen Evolution, ARA and Relative Efficiencies in Various Nitrogen Fixing Microorganisms

System	H_2 Evolution	ARA	R.E.*	References
RHIZOBIUM LEGUMINOSARUM	0.001-7.16 μmole/g/h fresh wt.	2.1-17.37 μmole/h/g fresh wt.	0.58-0.99	Schubert and Evans (1979)
ANABAENA AZOLLAE	0.09-0.5 μmole/h/g fresh wt.	0.34-0.9 μmole	0.44 0.99	Schubert and Evans (1976)
KLEBSIELLA PNEUMONIAE	1.0-3.3 μmole/h/g	1.5-4.5 μmole/h/g	0.26-0.99	Fay (1979)
ANABAENA	1.3 μmole/ μg Chl a/h	-	-	Peterson and Burris (1976)

* $R.E. = 1 - \frac{H_2 \text{ evolution}}{ARA}$

Using continuous mass-spectrometric detectors, Berlier and Lespinat (1980) have shown that A. brasilense possesses an aerobic uptake hydrogenase activity which recycles all the hydrogen produced by the nitrogenase and that under anerobic conditions it exhibits a bi-directional activity. In microaerophilic fixers like A. brasilense which have obligate dependence on oxygen for their metabolism but their nitrogenase gets inactivated by fairly low partial pressure of oxygen, the uptake hydrogenase could be expected to provide the necessary protection for nitrogenase complex, and also an increased ATP yield through energy gain resulting from this process.

As emphasised by Mortenson (1978) the hydrogenase concentration always increases with derepression of the nitrogenase. Is the coexistence of the these engyme complexes a mere coincidence or the two are metabolically complementary - remains to be solved.

Inoculation studies - As compared with Rhizobium not enough is known about the invasion and propagation of A. lipoferum in root systems, both in sterile and insterile soils. Inoculation with the organism did not always increase plant yields. While a very successful field inoculation experiment with high microbial fixation rates

corresponding to 40 kg N ha^{-1} in the presence of 40-80 kg N ha^{-1} of fertilizer N with elephant grass (Pennisetum purpureum) and Guinea grass (Panicum maximum), cultivars selected after extensive screening has been reported from Florida by Smith et al. (1976a, b, c), rhizospheric nitrogen fixation of 120 sorghum and 25 inbred maize cultivars inoculated with A. lipoferum in the field conditions in Oregon was negligible (Barber et al., 1976). Also, inoculation experiments with A. lipoferum and maize under green house conditions carried out by Albrecht et al. (1977) did not show any positive effects at various combinations of illumination and ambient temperature.

An intensive screening is required for suitable host plant cultivars. As not all varieties show similar response to inoculation with Azospirillum. The work of Smith et al. (1976) has shown that only 2 out of 4 varieties responded favourably to inoculation under experimental conditions. Work of Baldani and Dobereiner (1980) also shows that Azospirillum strains which exclusively associated with maize were A. lipoferum, while A. brasilense nir$^-$ is specific to the roots of rice plants.

The nggative reports of extensive field studies by Barber et al. (1976) and Albrecht et al. (1976) in pot experiments might have been the result of insufficient establishment of A. lipoferum due to microbial competition and/or insufficient adaption of the inoculum strains the prospective host plants. Recently significant increase in dry weight and total N-content have been shown by Nur et al.(1980) and Subba Rao et al. (1980) in inoculated plants.

The specific differences of nitrogenase activity of maize cultivars reported by von Bulow and Dobereiner (1975). Cohen et al. (1980) and Nur et al. (1980) clearly indicated the possibility of establishing effective endorhizosphere associations through careful screening of cereal cultivars. There is a need to study the inherent mechanism of compatibility between the microsymbiont and the roots of host plants for exploiting the nitrogen fixing potential of the endorhizospheric associations.

A realistic assessment of the relative contribution of associative symbiotic systems to overall nitrogen cycling of biosphere is difficult on account of pausity of quantitative data and a lack of systematic survey for identifying the various grasses, sedges and other non leguminous plants possessing loose symbitoic associations (Table 1.10).

Table 1.10: Estimates of Nitrogen Fixation Rates Associated with Grasslands, Forests, Cereal crop Legumes, Salt Marsh and Marine Ecosystems

Vegetation	Location/ Climate	Rate (kg. ha^{-1} $year^{-1}$)	Reference
1. Established Grasslands:			
Prairie	Oklahoma	2.5-3.7	Kapustaka and Rice (1978)
Prairie	Saskatchewan	1-2	Paul et al.(1971) Vlassak et al(1973)
Prairie	Wisconsin	1-3	Tjepkema and Burris (1976)
Softchess	California	ca. 16	Vaughan and Jones (1976)
Savanna	Ivory Coast	ca. ?	Balandreau (1976)
2. Cereal Crops:			
Cyon	France	2.5	Balandreau et al. (1976)
Corn Sorghum	Oregon	0.2-0.3	Barber et all1976)
Corn	Brazil	0.4	Tjepkema and van Berkum (1977)
Sorghum	Nebraska	0.3	Pedersan et al.(1978
3. Legumes:			
Grain Legumes	Temperate	7-128	Nutman (1972)
Grain Legumes	Tropical	17-270	"
Forage legumes	Temperate	23-620	"
Forage legumes	Tropical	30-700	"
Peas and Beans	Japan	50-60	Nishustin (1971)
Alfalfa and Clover	Japan	125-300	"

Contd.../-

Table 1.10 (Contd.)

Vegetation	Location	Rate ($kg.ha^{-1}year^{-1}$)	Reference
4. Nodulated nonlegumes (Forests)			
Cercocarpus ledifolium stands	Berlin	6.86	Lepper (1977)
Ceanothus stands	San Diego California	0.1	Kummerow et al. (1978)
Alnus sitchensis	Alaska	62	Crocker and Major (1955)
Alnus rugosa	Alaska	150 $lb/acre^{-1}$ $year^{-1}$	Daley (1966)
Alnus Incana	Norway	58 43	Akkermans (1978) Johnsrud (1978)
5. Salt Marsh:			
Menyanthes trifoliata	Novascotia	14	Patriquin and Keddy (1978)
Carex mackenziei	Novascotia	1.35	"
6. Marine Ecosystems:			
Thalassia testudium	Temperate	5	Capone and Taylor (1977)

The importance of associative symbiotic systems in the nitrogen economy of the biosphere is evident from the results of inoculation experiments which show a 40-60 kg of N gain under various ecosystems (Subba Rao *et al.* 1979; Doberein er, 1979). The ability of grasses and sedges known to enter into nitrogen fixing association with bacteria to partially replace the need for chemical nitrogen fertilizers has been proved beyond doubt in extensive trials made in India and elsewhere (Subba Rao, 1980). Improvement in efficiency of such associations through genetic engineering, breeding programmes and agronomic practices offer attractive opportunities for future work.

CHAPTER II

MATERIALS AND METHODS

DESCRIPTION OF THE STUDY SITE

The study sites were located in the Union Territory of Delhi, particularly around the JNU campus and the New Delhi Ridge which comprises of an uneven hilly area with ramifying ravines and shouldering ribs of rocks. The soil texture varies from gravel sand to sandy loam. Soil moisture, except during the winter months is low for most part of the year. The soil pH varies from 6.5-7 and organic carbon from 0.05-0.5 per cent w/w.

In addition to the above sites marsh plants occuring on the margins of temporary and permanent wetlands in the neighbourhood of the JNU campus, across river Jamuna, near Hindan river, Badarpur Thermal Power Station and the Indraprastha Thermal Power Station were also studied. Populations of marsh plants grow in half to one meter deep water on the wet margins of aquatic habitats. The soils of these habitats are silty or sandy loam. In general the surface soil has a pH of 7 though in the subsurface layers it approaches 8.9-9. The organic carbon varies between 3.4-5.7 per cent w/w.

The climate of the Union Territory of Delhi is characterised by extremes of temperature varying between 6 C to 46 C, during the winter (December, January and

February) and the summer (May and June) seasons. The average annual rainfall of the area is 716 mm, 80 per cent of which is restricted to the monsoon period (August and September).

A total of 16 non leguminous flowering plant species; 12 belonging to family Gramineae, 3 to Cyperaceae and 1 to Typhaceae were examined for evaluating their nitrogen fixing ability. Of these 7 plant species namely, Alopecurus nepalensis, Cyperus rotundus , Dactyloctaenium aegyptium, Digitaria adscendens, Fimbristylis bisumbellata, Paspalidium flavidum, and Sporobolus marginatus were annual and other 10 were perennial. The marsh plants sampled from aquatic habitats include Cyperus rotundus, Fimbristylis bisumbell ata Paspalidium flavidum, Scirpus tuberosus and Typha angustata.

Seven plant species namely, Bothriochloa pertusa, Cenchrus ciliaris, Cyperus rotundus, Fimbristylis bisumbellata, Heteropogon contortus, Sporobolus marginatus and Typha angustata, were sampled for seasonality in AR activity.

ACETYLENE REDUCTION BIOASSAY

Nitrogenase activity was evaluated using acetylene reduction bioassay (Burris and Scholhorn, 1973). The bioassay comprises of the following main four steps:

1. Sampling of soil-root cores
2. Acetylene generation
3. Incubation of soil-root cores in 10 per cent acetylene.
4. Gas chromatographic analysis of ethylene production.

Sampling of soil root cores - A soil coring device was designed and fabricated indigenously for the extraction of intact undisturbed soil cores of uniform size for the evaluation of their acetylene reduction activity. The device consists of an internally threaded metallic piece of pipe of size 5.8 cm diameter and 12 cm length, which has been designated as socket (A) in Figure 2.1. The socket was welded to a pair of angled irons of equal length. The angled iron bars supported a horizontal bar welded in such a way that the margins of horizontal bar served as handles for the corer. The socket accomodated an externally threaded, 20 cm long pipe which was split into two equal halves. The unthreaded end of the split pipe was serrated to act as cutting edge of the soil corer.

The corer was driven manually into the soil to a depth of 20 cm and the corer was lifted along with the soil corer. The socket was unscrewed to remove the soil root core lying between two halves of the split iron pipe.

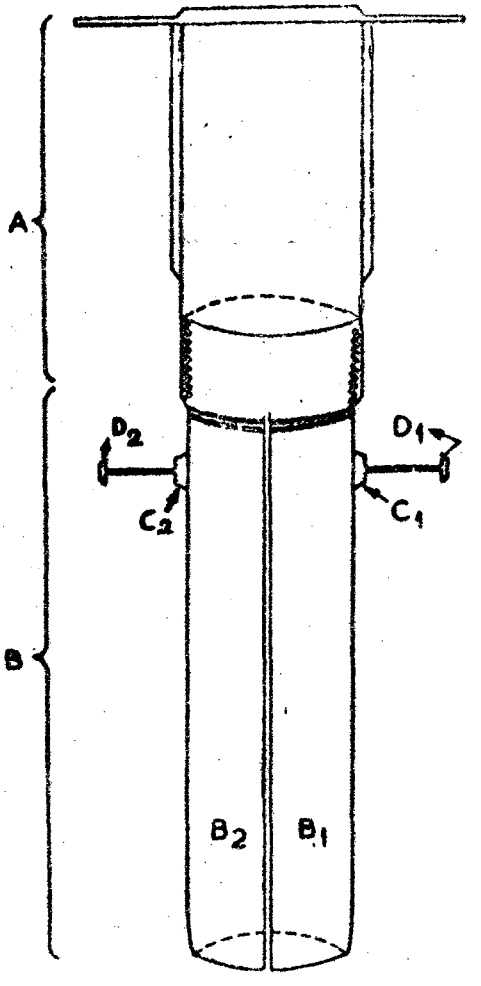


Fig. 2.1: The soil corer.

A - socket; B_1 and B_2 - two halves of the split iron pipe; C_1 and C_2 - nuts; D_1 and D_2 - screws.

The two halves of the pipe were separated to obtain uncompressed core of standard size (20 x 5.8 cm).

Acetylene generation - Acetylene was generated by reacting commerical grade calcium carbide with water in a special reaction chamber described by Burris (1972).

Incubation of soil root cores - Mature and healthy plants of grasses and sedges were selected and soil root cores were sampled in the late afternoon and brought to the laboratory without delay. Each soil root core was placed in a steel incubation chamber with a throded steel cap having a subbaseal vent port. The lid was securely tightened over the chamber and sealed. The chamber was checked for leakage by evacuating upto 0.2 atm. The chamber was then refilled with air. The soil root cores were incubated following the method described by Tjepkema and Burris (1976). The head space of each chamber containing intact soil root core was replaced with 10 per cent acetylene using an air tight syringe. Soil cores without root system were also incubated in the similar manner, to serve as controls. The incubation chambers were maintained at ambient temperature.

Gas chromatographic analysis of ethylene production - Subsequent to the incubation of soil root core in acetylene

1 ml of the head space of the incubation chamber was sampled at regular intervals and the gas samples were analysed in a gas chromatograph equipped with Porapak 'R' column of 32 cm length and 2 mm diameter having mesh size 80-100 at a carrier gas flow of 40 ml/min and fuel at 30 ml/min, using a hydrogen flame ionization detector. The peak heights of ethylene were compared with a standard curve obtained using dilutions of pure ethylene gas obtained from M/s Matheson Ltd, USA (Fig. 2.2). The ethylene peaks were recorded on a 10 mv Toshniwal recorder at a chart speed 10 mm/min.

ESTIMATION OF ROOT BIOMASS

Following the suspension of assay the soil root cores were washed free of soil. The plant roots were dried in an electric oven at 80 C for 24 h for estimating dry weight. Head space was determined separately in each case, after terminating the bioassay.

CALCULATION OF RESULTS

Results were calculated following the procedure given by Dart et al. (1972).

The value of C_2H_4 produced, in uM C_2H_4/h was calculated using the following procedure :

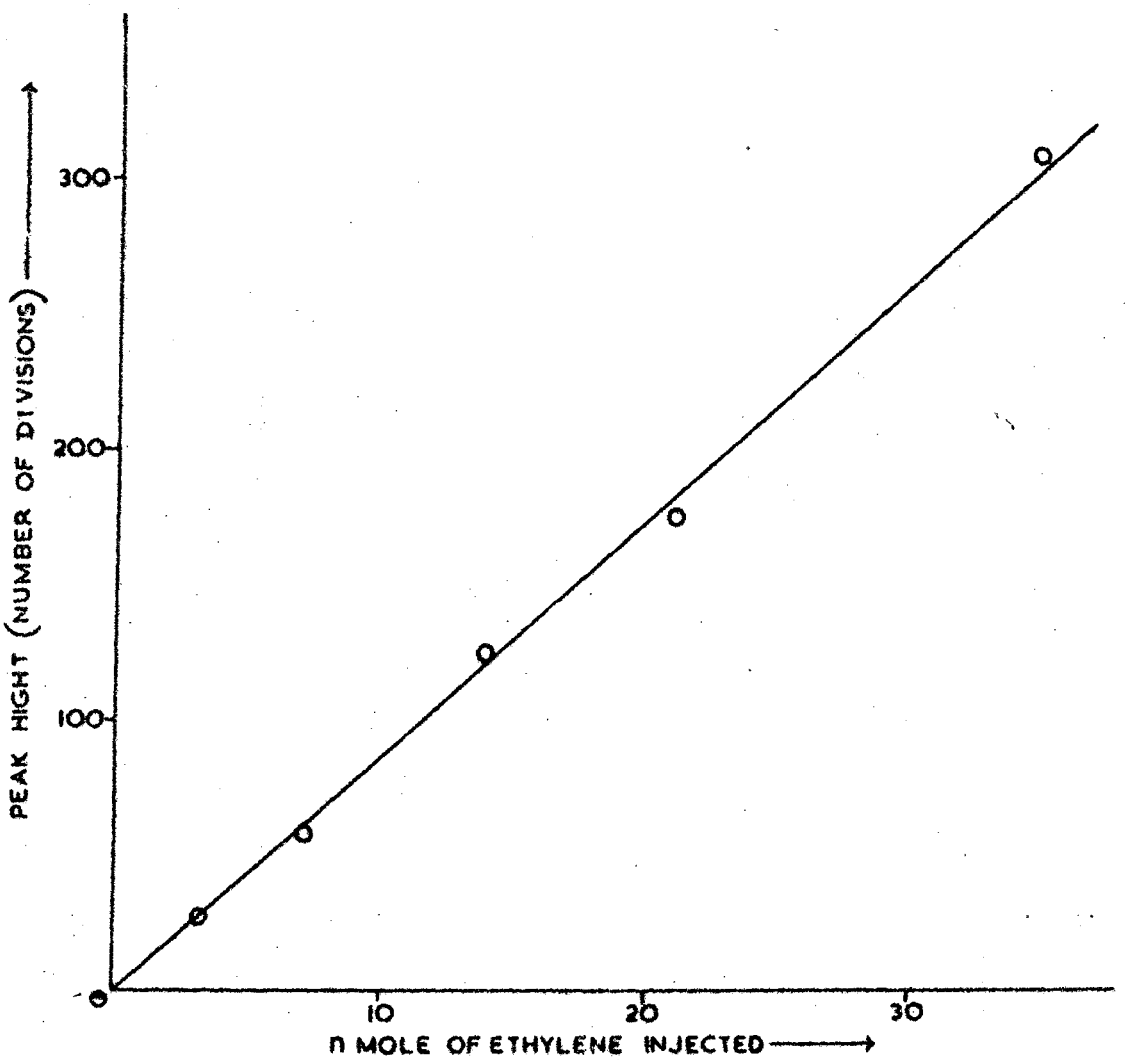


FIG.2.2 STANDARD CURVE FOR CALIBERATION OF AIMIL NUCON GAS CHROMATOGRAPH (SERIES 5500) ATTACHED TO A 10mv RECORDER

C_2H_4 produced, in uM C_2H_4/h =

$$C_2H_4 \text{ sample C.U.} \times \frac{\text{Vol. of head space incubation chamber}}{\text{Vol. injected into GLC}} \times K$$

$$\text{minus } C_2H_4 \text{ blank C.U.} \times \frac{\text{Vol. of head space in blank}}{\text{Vol. of injected into GLC}} \times K$$

where, C.U. = Chart Units (number of divisions) used for measuring peak height.

K = Conversion factor obtained using a standard mixture to calibrate the chromatograph.

The value of K was calculated using the procedure given by Dart et al. (1972).

1 ml of 100 ppm of C_2H_4 containing 100×10^{-6} ml = X C.U.

22.4 l C_2H_4 at STP = 1 mole C_2H_4

$$1 \text{ ml } 100 \text{ ppm } C_2H_4 = \frac{100 \times 10^{-6}}{22.4 \times 10^3} \text{ mole } C_2H_4$$

$$= 0.00446 \text{ } \mu \text{ mole } C_2H_4 = X \text{ C.U.}$$

$$\text{then, K or 1 C.U.} = \frac{0.00446}{X} \text{ } \mu \text{ moles } C_2H_4$$

1 ml of 100 ppm of C_2H_4 containing 100×10^{-6} ml = 44 C.U.

$$\text{Then, } K = \frac{0.00446}{44} = 0.1 \text{ n mole } C_2H_4$$

The blank is a chamber containing 10 per cent acetylene.

CALCULATION OF ACTIVITY

The rate of acetylene reduction was determined using straight part of the curve, using the formula :

$$\frac{(n_2 - n_1)}{(t_2 - t_1)\,a} = \mu \text{ mole ethylene } h^{-1} m^{-2}$$

where, n_1 and n_2 are the number of n moles of ethylene at time t_1 and t_2 (in hours) and a is the area of the soil root core in square metres. In cases, where it was not possible to plot curve because of lack of frequency of observations, the rates was calculated as follows :

$$\frac{\text{total } \mu \text{ moles produced}}{\text{total h after zero time x area}} = \mu \text{ mole } h^{-1}\ m^{-2}$$

A mean of at least three such calculations was used to represent the activity of a particular core.

CHAPTER III

RESULTS AND DISCUSSION

A survey of common grasses and sedges was undertaken to identify the nitrogen fixing species in the local ecosystems. About 26 species of grasses and sedges were screened for determining their nitrogen fixing potential. Preliminary observations have revealed that the following 16 species of grasses and sedges possess positive acetylene reduction (AR) ability,(Table 3.1).

GRASSES

Alopecurus nepalensis Trin. ex steud., Bothriochloa pertusa (Linn.) A. Camus., Cenchrus ciliaris Linn., Chloris barbata (Linn.) Sw., Cynodon dactylon (Linn.) Pers., Digitaria adscendens (H.B. & K.) Henr., Dactyloctaenium aegyptium (Linn.) Beauv., Heteropogon contortus (Linn.), Roem and Schult., Paspalidum flavidum (Retz.) A., Saccharrum munja Linn., Saccharrum spontaneum. Linn. and Sporobolus marginatus Hochst. ex A.

SEDGES

Typha angustata Bory et. Chaub.,
Cyperus rotundus Linn., Fimbristylis bisumbellata (Forsk.) Bub., Scirpus tuberosus Desf.

A brief account of each species along with their acetylene reduction activity is given below :(Table 3.4).

Table 3.1: Acetylene Reduction Ability of Grasses and Sedges studied in the Present Investigation

Sr. No.	Name	ARA: μ mole m^{-2} h^{-1}	ARA: n mole g^{-1} dry root day^{-1}
G	GRAMINEAE		
1.	ALOPECURUS NEPALENSIS	10.5	52.6
2.	BOTHRIOCHOA PERTUSA	7-100	301-744
3.	CENCHRUS* CILIARIS	150-200	750-4776
4.	CHLORIS BARBATA	20-23	188
5.	CYNODON* DACTYLON	38.8-45.9	52.7
6.	DACTYLOCTAENIUM AEGYPTIUM	5	120
7.	DIGITARIA ADSCENDENS	32	684
8.	HETEROPOGON CONTORTUS	0-288	864-1200
9.	PASPALIDIUM FLAVIDUM	50-191	2,952
10.	SACCHARRUM SPONTANEUM	87-114	ND
11.	SACCHARRUM MUNJA	20	ND
12.	SPOROBOLUS MARGINATUS	80-127	249-3024

Contd.../-

Table 3.1 (Contd.)

Sr. No.	Name	ARA	
		μ mole m^{-2} h^{-1}	n mole g^{-1} dry root day^{-1}
	TYPHACEAE		
13.	TYPHA ANGUSTATA	15-520	615-792
	CYPERACEAE		
14.	CYPERUS ROTUNDUS	3-90	14.4-86.4
15.	FEMBRISTYLIS BISUMBELLATA	13.3-178	30-400
16.	SCIRPUS TUBEROSUS	21.7-29	55

*- Grasses have been reported: C. ciliaris by Day and Dart (1976) and C. dactylon Eskew and Ting (1976). Rest fourteen grasses are first reports of acetylene reduction in literature.

ND - not determined.

GRASSES

Alopecurus nepalensis Trin. ex Steud - It is a winter season annual grass with small densely tufted leaves and laterally compressed grains, growing in ditches and shallow depression. It flowers and fruits in the months of July to September. The acetylene reduction rate of this plant (sampled only once) was 10.5 μ mole m^{-2} h^{-1} or 52.6 n mole g^{-1} dry root day^{-1}.

Bothriochloa pertusa (Linn.) A. Camus. - It is a slender erect perennial grass with bearded nodes and linear leaves, common in grazing lands and waste places, pastures, stony crevices and on very dry soils. This grass flowers and fruits in the months of July to October. It is a primary colonizer in newly formed habitats. Acetylene reduction by soil root cores varied between 7-100 μ mole m^{-2} h^{-1} or 301-744 n mole g^{-1} dry root day^{-1}. A 20 h time lag was observed in some soil root cores of this plant (Fig. 3.4).

Cenchrus ciliaris Linn. - It is a tufted erect or decumbent C_4 grass occuring in a wide range of habitats. This plant forms a dense mat on the ridge among the bushes, in open fields and similar habitats as an early colonizer. Once established it is not easily killed out. It is considered to be a good pasture grass. The acetylene reduction of the soil root cores of this species ranged

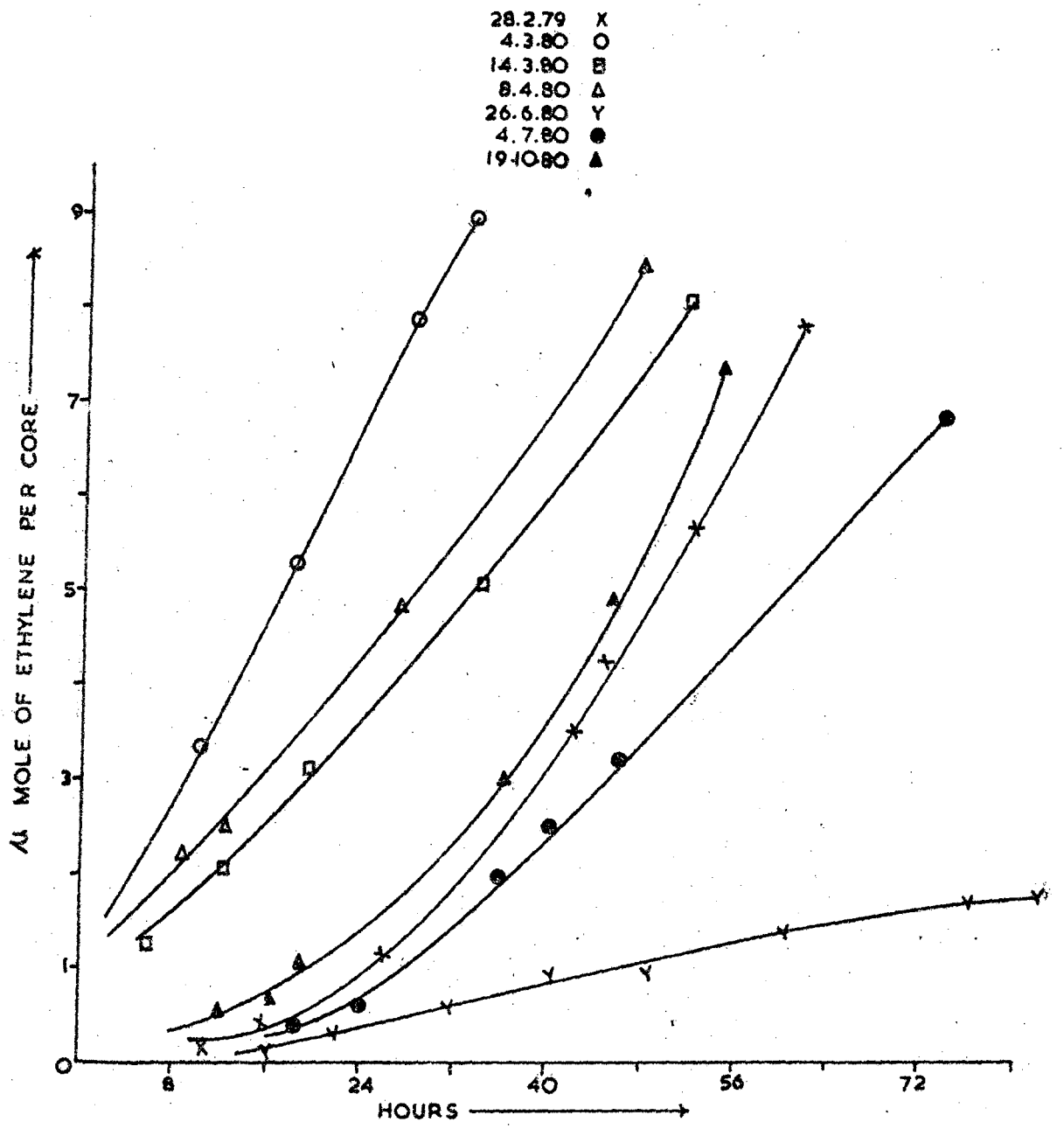


FIG.3.4 TIME COURSE OF ACETYLENE REDUCTION BY SOIL ROOT CORES OF BOTHRIOCHLOA PERTUSA (LINN.) A.CAMUS. IN DIFFERENT SEASONS

between 150-200 μ mole m^{-2} h^{-1}, an equivalent to 750-4776 n mole g^{-1} dry root day^{-1}. A lag phase of 16-30 h was observed (Fig. 3.5).

Chloris barbata (Linn.) Sw. - It is a tufted perennial grass with creeping base, profusely growing on pasture grounds and in cultivated fields especially on sandy soils. The soil root cores of this species reduced acetylene at the rate of 20-23 μ mole m^{-2} h^{-1} equivalent to 188 n mole g^{-1} dry root day^{-1}.

Cynodon dactylon (Linn.) Pers. - It is a common perennial grass with an extensively creeping base. It is used as lawn grass and as cattle feed. Acetylene reduction rates of this grass were found to vary between 38.8-45.9 μ mole m^{-2} h^{-1} or 52.7 n mole g^{-1} dry root day^{-1}.

Dactyloctaenium aegyptium (Linn.) Beauv. - It is an annual grass which appears during the monsoon period on the Delhi ridge in cultivated fields and in open places often becoming abundant to form a thick tuft of plants matted with the soil. This plant flowers during May to October. Its acetylene reduction rate was 5 μ mole m^{-2} h^{-1}, an equivalent of 120 n mole g^{-1} dry root day^{-1}.

Digitaria adscendens (H.B. & K) Henr. - It is an annual grass which appears during monsoon from July through October. This C_4 grass grows on soils with a

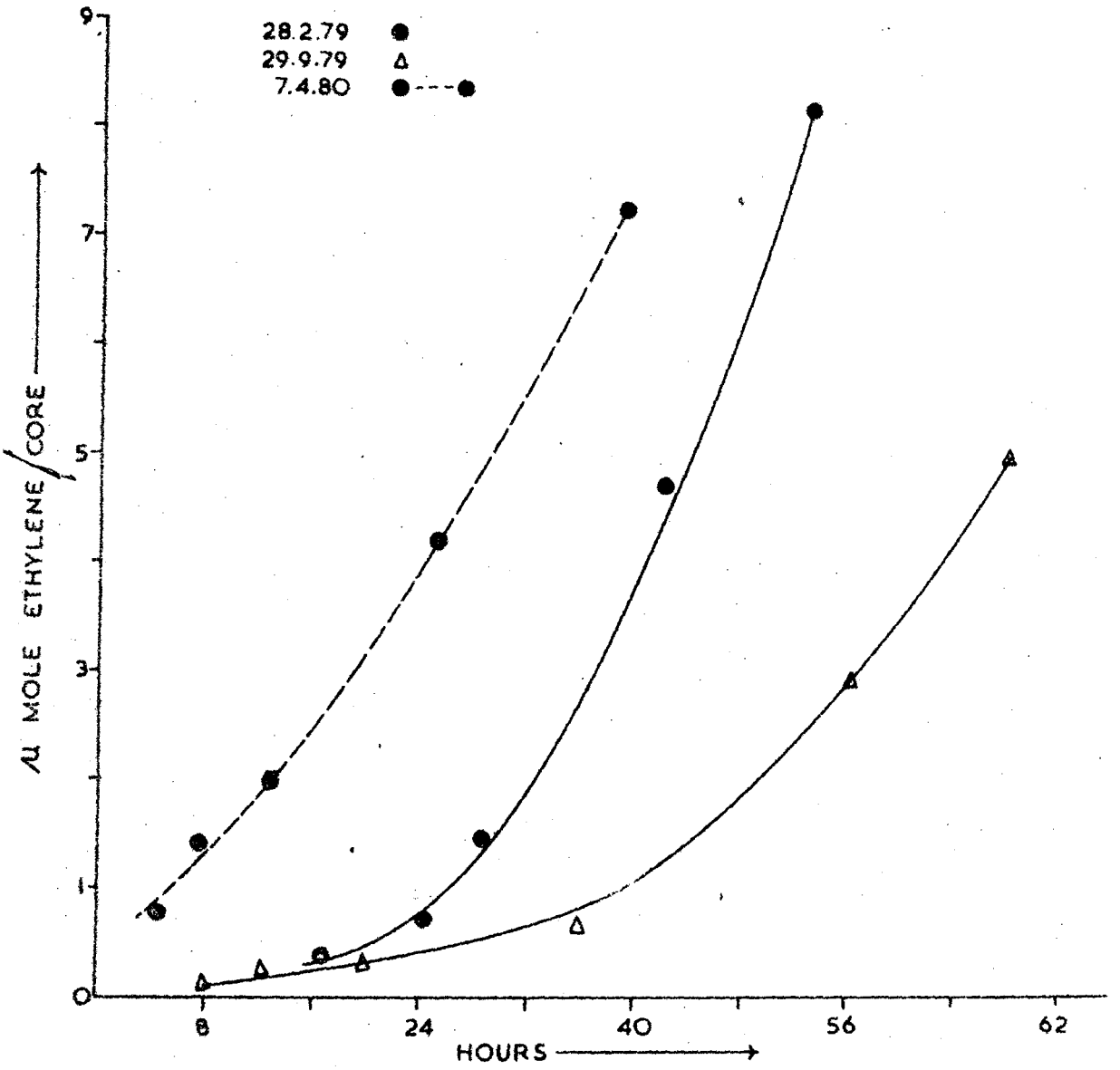


FIG.3.5 GRAPH SHOWING CUMULATIVE AMOUNTS OF ETHYLENE PRODUCED IN SOIL CORES OF CENCHRUS CILIARIS LINN. IN DIFFERENT SEASONS

wide range of moisture content and is easily recognizable by its conspicuously bearded spikelets. It is an indicator of disturbance and commonly occure in areas which suffer from various types of human activities. The acetylene reduction in soil root cores was 32 μ mole m^{-2} h^{-1}, equivalent to 684 n mole g^{-1} dry root day^{-1}.

Heteropogon contortus (Linn.) Roem. and Schult. - It is an erect tufted slender perennial grass attaining a height of 1 m, commonly found on the ridge and adjacent hilly tracts near Mehrauli and the JNU Campus. This C_4 plant serves as a good fodder if used before flowering. The acetylene reduction by the soil root cores of Heteropogon was recorded upto 288 μ mole m^{-2} h^{-1}. On dry weight basis, it works out between 864 to 1200 n mole g^{-1} dry root day^{-1}. A time lag of <6 h lapsed before maximum stable levels of acetylene reduction could be achieved (Fig. 3.6).

Paspalidium flavidum (Retz.) A. Camus. - It is a tufted annual grass which grows along canal banks and ponds on wet sandy soils. This plant flowers during May to October and is easily identified from its distinct inflorescence. Its acetylene reduction ability was found to range between 50-191 μ mole m^{-2} h^{-1}. In terms of dry

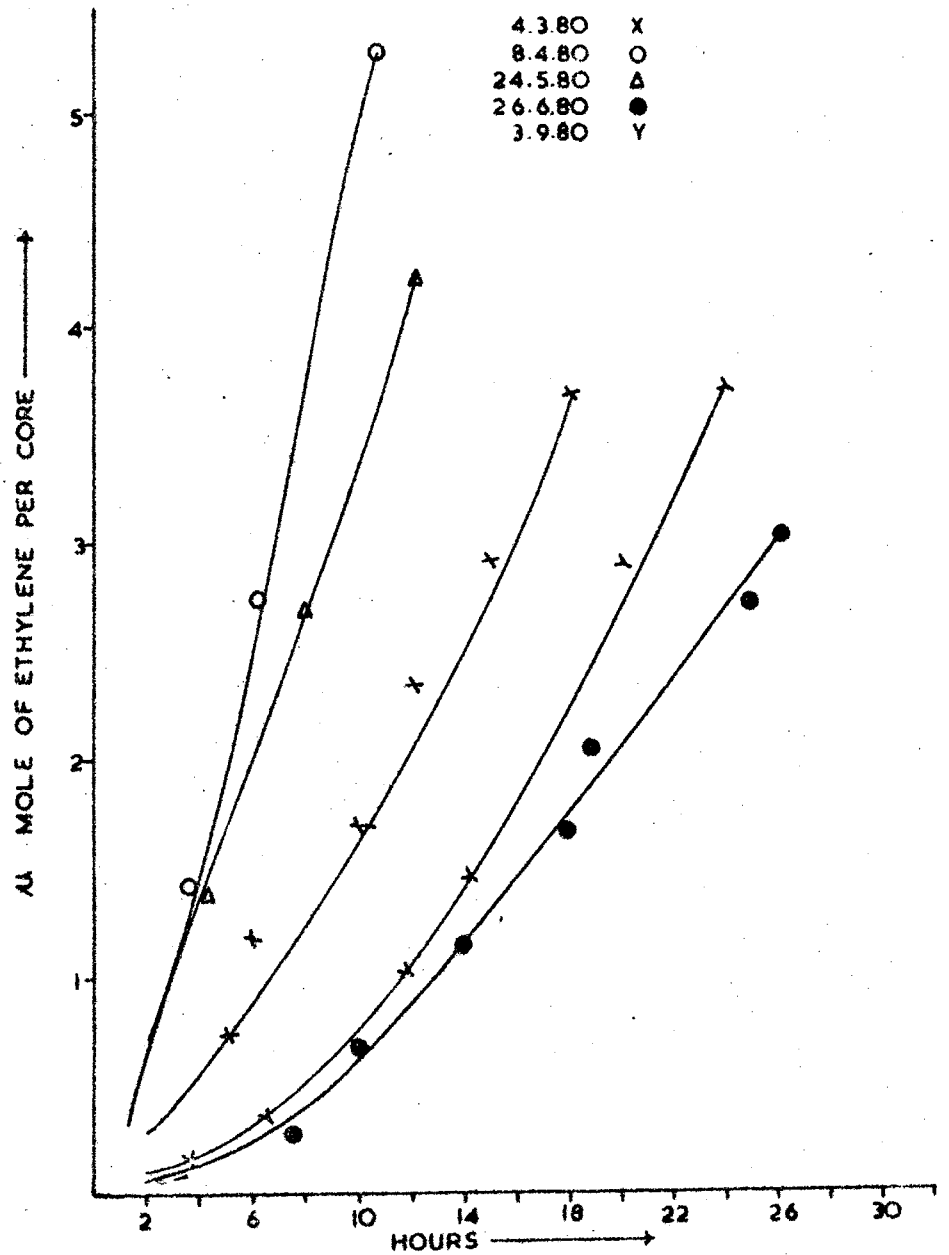


FIG.3.6 TIME COURSE OF ACETYLENE REDUCTION IN HETEROPOGON CONTORTUS (LINN.) ROEM. AND SCHULT INCUBATED IN DIFFERENT SEASONS

root weight it is estimated to be 2,952 n mole g^{-1} dry root day^{-1}. A time lag of 16 h was recorded before acetylene reduction achieved stable levels.

Saccharrum spontaneum Linn. - It is a pernicious grass common in unused grounds, fallow fields, dried ponds and along both sides of railway lines. It is a perennial plant that flowers and fruits in September through December. This species is a primary coloniser of freshly exposed habitats. The acetylene reduction rate was found to range between 87-114 μ mole m^{-2} h^{-1}. A time lag of 12 h was observed.

Saccharrum munja Linn. A variable tall perennial densely tufted grass which commonly grows on dry as well as wet habitats. This plant is used for making brooms. The acetylene reduction rate was 20 μ mole m^{-2} h^{-1} (Fig. 37). There was a lag phase of 12 h.

Sporobolus marginatus Hochst. ex A. Rich. - This species is one of the dominant elements in the vegetation of dry habitats and occurs along roadsides and usar or saline soils. It is often associated with plant like Suaeda sp. and Salsola sp. common to salt affected soils. Its acetylene reduction varied from 80-127 μ mole m^{-2} h^{-1}, or 249-3024 n mole g^{-1} dry root day^{-1}. A lag period of 16 -20 h was observed (Fig. 3.8).

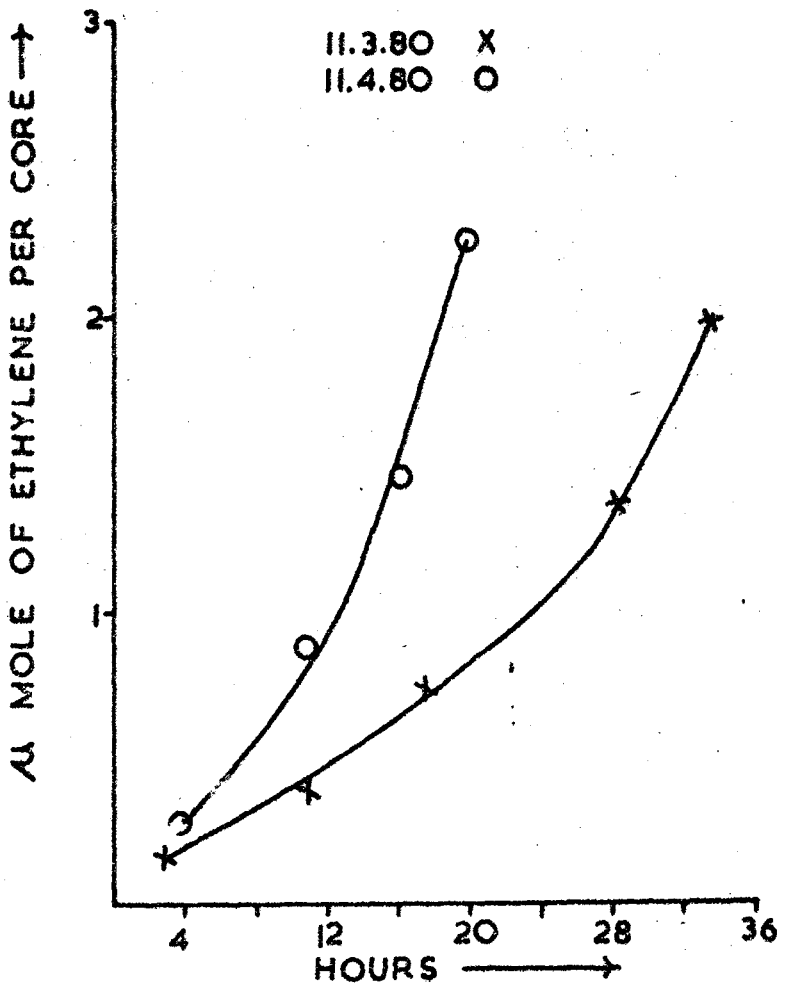


FIG.3.7 TIME COURSE OF ACETYLENE REDUCTION BY SOIL ROOT CORES OF SACCHARRUM MUNJA LINN.

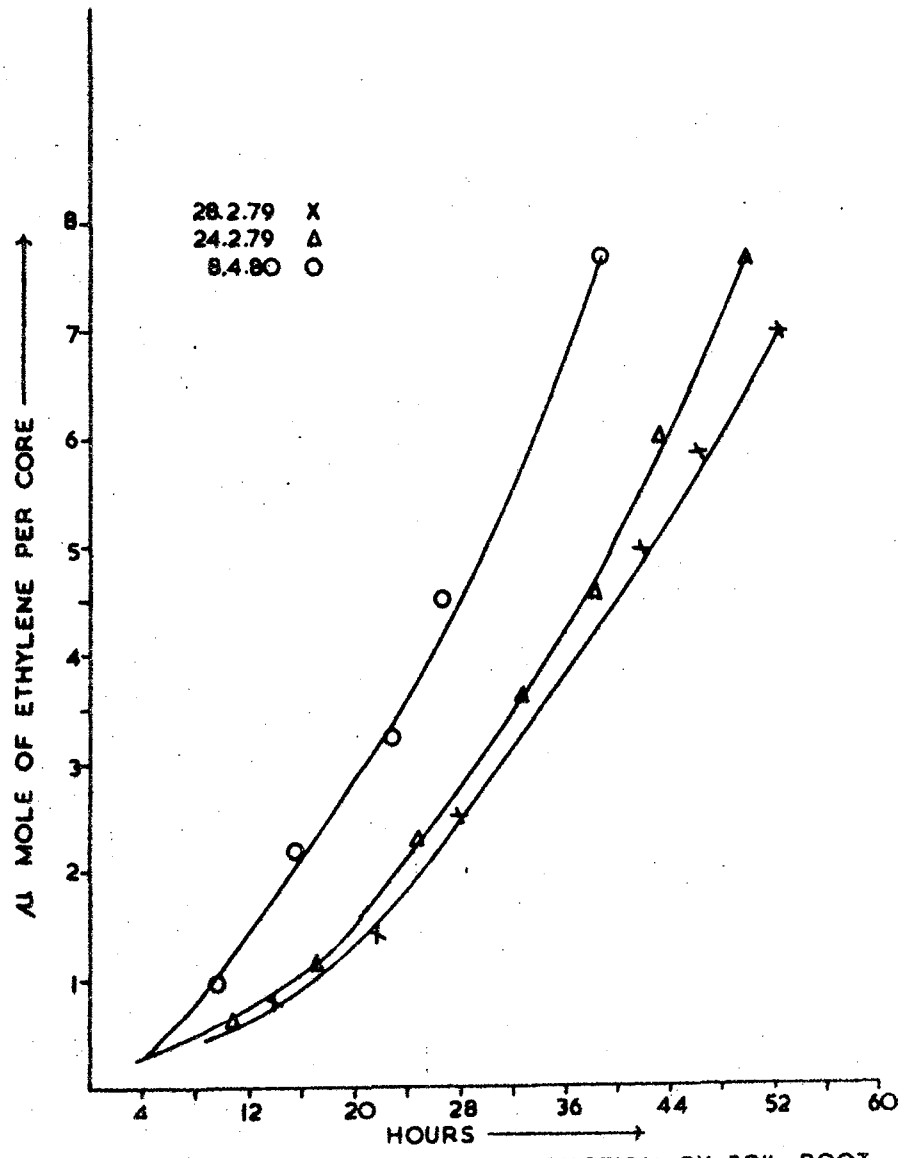


FIG. 3.8 TIME COURSE OF ACETYLENE REDUCTION BY SOIL ROOT CORES OF SPOROBOLUS MARGINATUS HOCHST. ex. A. RICH. IN DIFFERENT SEASONS

SEDGES

Typha angustata Bory et Chaub. - A tall perennial, marshy amphibious shrub occuring at pond margins upto one meter depth of water. It perpetuates by means of its underground rhizome. It forms the sedge grass stage in hydrosere. The time course of acetylene reduction in soil root cores sampled from four different localities namely, JNU, Hindan river across Jamuna, Badarpur and Indraprastha Thermal Power Stations, follow the typical sigmoid curve with a lag phase of about 4-8 h (Fig. 3.1). The rate of acetylene reduction was found to vary between 15-520 μ mole m^{-2} h^{-1} with a mean at 128 μ mole m^{-2} h^{-1}.

Cyperus rotundus Linn. - It is an erect glabrous herb with a triangular stem. A variable weed growing in different types of habitats such as unused grounds, agricultural fields, lawns and parks. It is often abundant and is a dominant element of the grassland vegetation, particularly during the rainy season. In comparison to Fimbristylis bisumbellata this sedge occupies comparatively drier habitats. This species

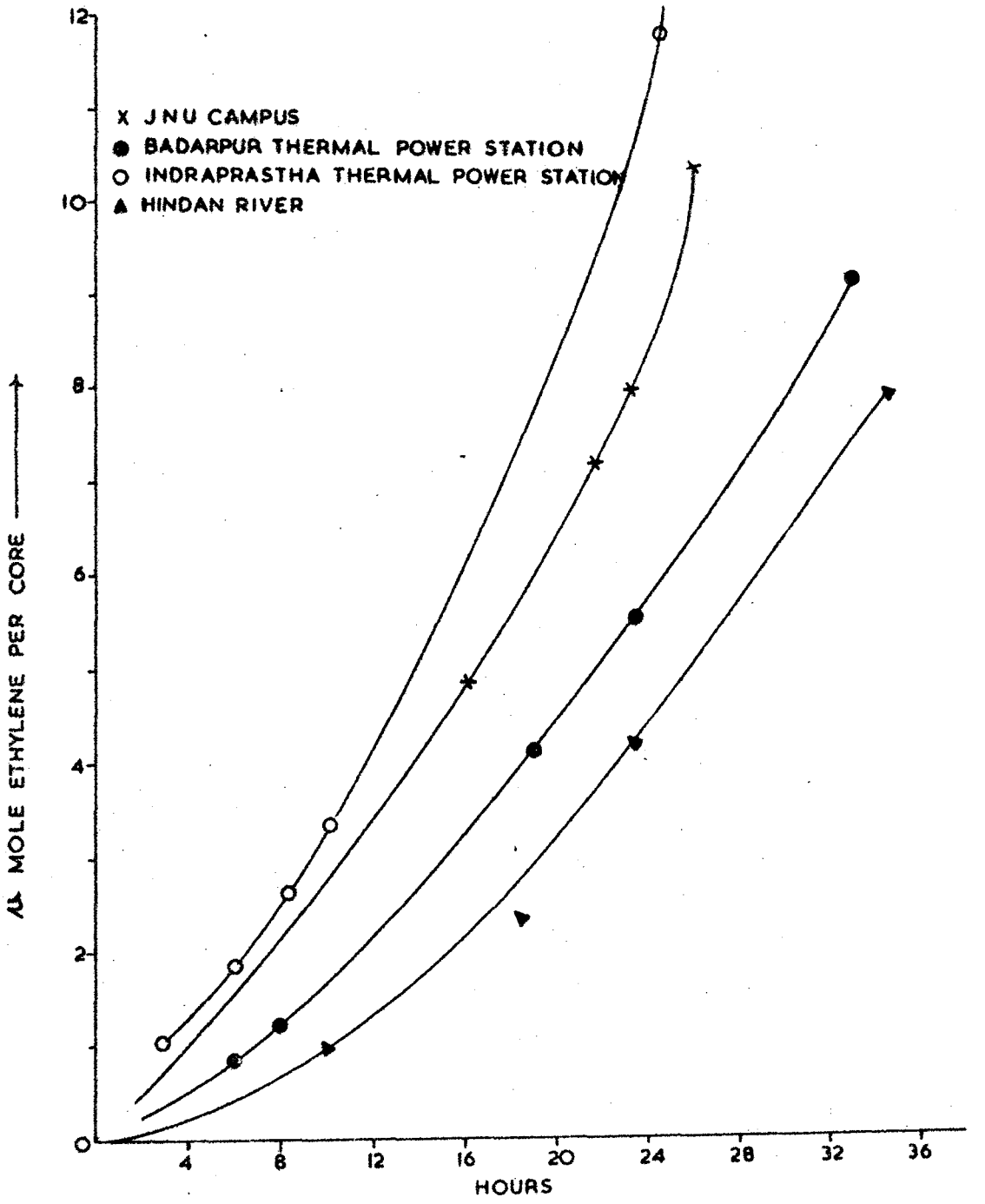


FIG. 3.1 TIME COURSE OF ACETYLENE REDUCTION BY SOIL ROOT CORES OF TYPHA ANGUSTATA BORY. et. CHAUB. SAMPLED FROM FOUR DIFFERENT POPULATIONS

is an indicator of human activities causing soil disturbances. The acetylene reduction activity this species varied between 3-90 μ mole m^{-2} h^{-1}. The time course of acetylene reduction (Fig. 3.2) shows a lag phase of 4-6 h. The activity keeps on increasing till 72 h when the experiment was suspended.

Fimbristylis bisumbellata (Forsk.) Bub. - It is a tufted fibrous rooted sedge with acaulous leaves, found in moist silty and sandy soils along ravines, marshes and pond margins. It flowers and fruits in the months of September and October. It is a primary colonizer of the margin of aquatic systems. The roots of this plant reduce acetylene at rates varying between 13.3-178 μ mole m^{-2} h^{-1}. The activity could be recognized only 4-10 h after incubation in almost all cores (Fig. 3.3), but indications of acetylene reduction were observed even earlier. The rates of acetylene reduction continued to increase till about 24 h.

Scirpus tuberosus Desf. - It is an amphibious plant with creeping rhizomes and stout stems. It is an erect variable sedge which flowers and fruits during March through July. It is common in marshes near Hindan river in the drying beds of ponds and canals. The acetylene reduction by the plant was found to vary between 21.7 - 29 μ mole m^{-2} h^{-1}.

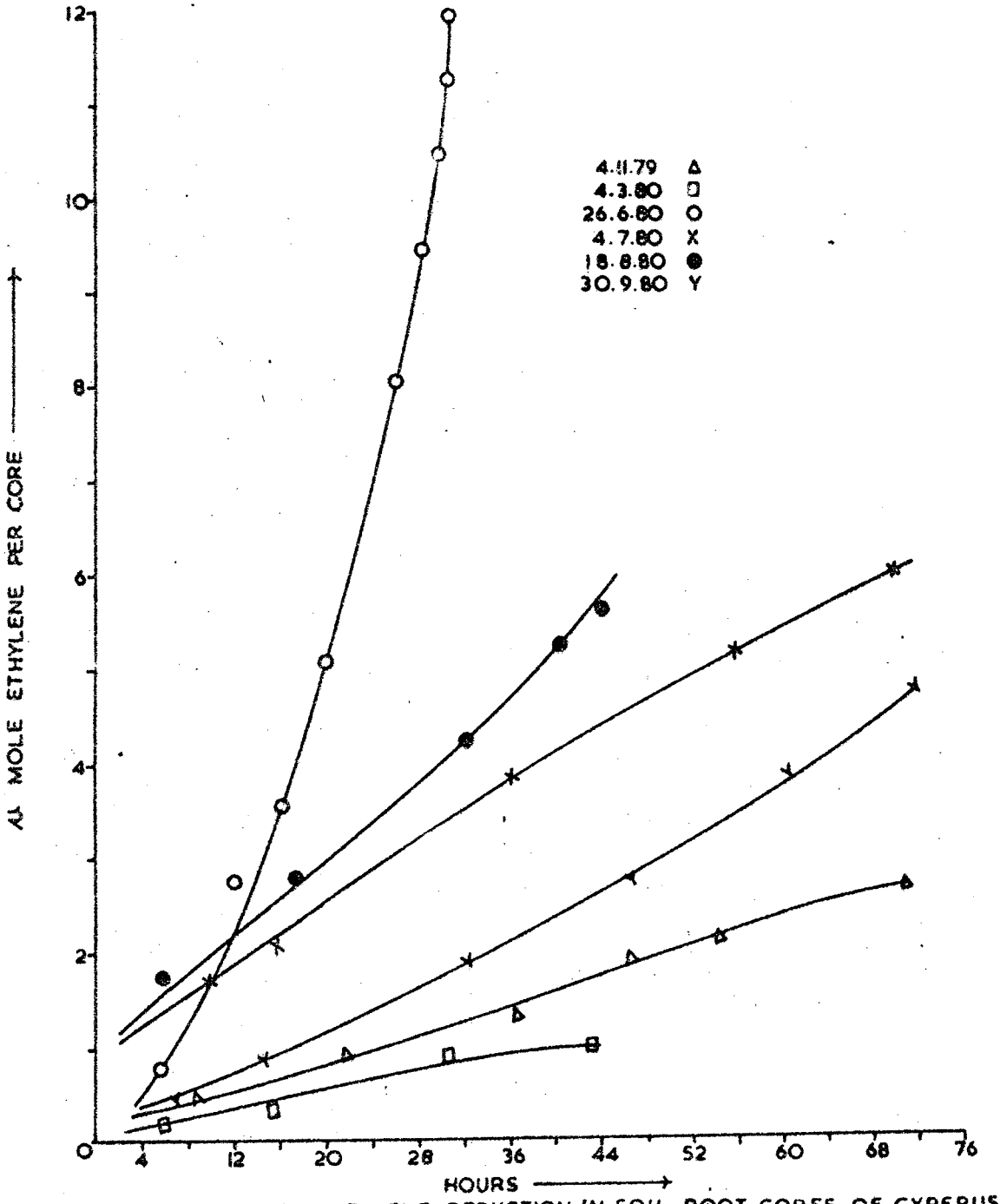


FIG.3.2 TIME COURSE OF ACETYLENE REDUCTION IN SOIL ROOT CORES OF CYPERUS ROTUNDUS LINN. IN DIFFERENT SEASONS

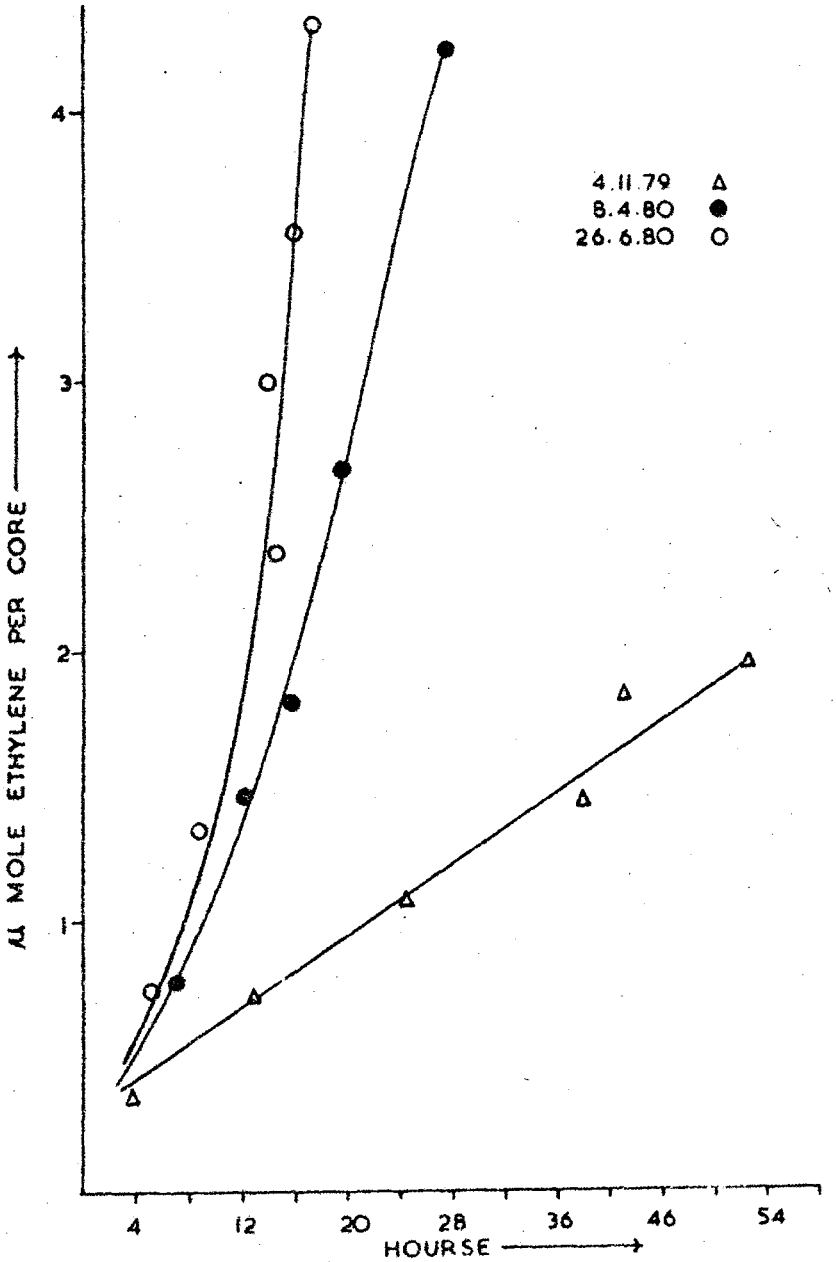


FIG.33 TIME COURSE OF ACETYLENE REDUCTION BY SOIL ROOT CORES OF FIMBRISTYLIS BISUMBELLATA (FORSK.) BUB IN DIFFERENT SEASONS

The rates of acetylene reduction in the above mentioned plant species ranged between 7-288 μ mole m^{-2} h^{-1} in grasses, 14-147 μ mole m^{-2} h^{-1} in cyperaceous sedges and 21-520 μ mole m^{-2} h^{-1}, in Typha. There was great variation in the acetylene reduction ability of different grasses and sedges. The acetylene reduction potential of Heteropogon contortus and Cenchrus ciliaris was higher among perennial grasses as compared with Saccharrum munja and Saccharrum spontaneum. In annual grasses Paspalidium flavidum gave the highest value while those of Alopecurus nepalensis and Dactyloctaenium aegyptium were lowest. In the case of sedges acetylene reduction was highest in Typha angustata and lowest in Scirpus tuberosus.

On the basis of their acetylene reduction ability the plant species studied could be divided into two groups : those possessing high (> 30μ mole m^{-2} h^{-1}) acetylene reduction activity and those possessing low (< 30μ mole m^{-2} h^{-1}) acetylene reduction activity. High activity species are those grasses found near marshes or other wetland habitats viz., Typha angustata, Scirpus tuberosus, Fimbristylis bisumbellata, Cyperus rotundus and Paspalidium flavidum. Species occuring in comparatively drier habitats were less vigorous nitrogen

fixers viz., Alopecurus nepalensis and Dactyloctaenium aegyptium. The high activity species exhibited a shorter lag phase varying between 4-8 h whereas in the case of low activity species a lag phase 8-24 h long was observed. Certain species did not exhibit a lag phase. It appears that their lag phase merged into log phase. These observations are comparable with those of Kanna and Tjepkema (19 78).

Acetylene reduction rates varied greatly within species. The following two factors appear to contribute to the observed variation : (1) Variation from one sampling site to another and the (2) Genetic, racial or ecotypic differences within plants of same species. More work is needed to understand the causes of such variations. Various workers have made similar observations (Bergersen, 1970; Eskew and Ting, 1976; Witty, 1979).

The high rate of acetylene reduction upto 90 kg N ha^{-1} $year^{-1}$ has been reported by many workers in the case of tropical grain and forage grasses (Abrantes et al. 1975; Patriquin and Keddy, 1978; Smith and Patriquin, 1978; Nioh, 1979; Ogan, 1979; Nur, 1980). The acetylene reduction rates of soil root cores of B. pertusa, C. ciliaris, H. contortus P. flavidum and S. marginatus are comparable with the values reported for soil root cores of other grasses (Table 3.2).

Table 3.2: A Comparison of Acetylene Reduction by Soil Root Cores of Grasses

Species	Investigator(s)	Rate
AXONOPUS COMPRESSUS	Weier (1980)	6-40 μ mole core^{-1} day^{-1}
DIGITARIA DECUMBENSI	Weier (1980)	4-24 μ mole core^{-1} day^{-1}
PASPALUM NOTATUM	Weier (1980)	3-12 μ mole core^{-1} day^{-1}
PASPALUM NOTATUM	Van Berkum and Day (1980)	293±66 n mole core^{-1} h^{-1}
BRACHIARIA MUTICA	"	339±45 n mole core^{-1} h^{-1}
DIGITARIA DECUMBENS	"	61±10 n mole core^{-1} h^{-1}
SORGHUM VULGARE	"	86 ±11 n mole core^{-1} h^{-1}
BERMUDA GRASS	Schank and Day (1977)	500 gN fixed ha^{-1} day^{-1}
BOTHRIOCHLOA PERTUSA	Present study	0.3-47 μ mole core^{-1} day^{-1}
CENCHRUS CILIARIS	"	7-9.4 μ mole core^{-1} day^{-1}
HETEROPOGON CONTORTUS	"	0-12 μ mole core^{-1} day^{-1}
PASPALIDIUM FLAVIDUM	"	2.35-9.0 μmole core^{-1} day^{-1}
SPOROBOLUS MARGINATUS	"	4.& 3.7 -6.0 μ mole core^{-1} day^{-1}

In addition to grasses, certain sedges have been also shown to fix atmospheric nitrogen (Bristow, 1974; Patriquin and Denike, 1978; Varshney and Mandhan, 1981). A comparison of acetylene reduction by various sedges is given in Table 3.3.

Table 3.3: Comparison of Acetylene Reduction of Sedges Shown to Fix Atmospheric Nitrogen

Species	Investigator	Procedure	Acetylene reduction
TYPHA LATIFOLIA	Patriquin and Keddy (1978)	Abrantes et al. (1975)	46.7 - 226 μ mole g^{-1} h^{-1}
TYPHA ANGUSTIFOLIA	Bristow (1974)	Abrantes et al. (1975)	3500 n mole g^{-1} dry root day^{-1}
SPARTINIA ALTERNIFLORA	Patriquin and Denike (1977)	Balandreau and Dommergues (1973)	39.6 μ mole m^{-2} h^{-2} (edge stands) 67.4 μ mole m^{-2} h^{-1} (inner stands)
TYPHA LATIFOLIA	Kanne and Tjepkema (1978)	Tjepmeka and Burris (1976)	180 g N ha^{-1} day^{-1}
SCIRPUS ATROVIRENS	"	"	0.66 gN ha^{-1} day^{-1}
FIMBRISTYLIS BISUMBELLATA	Varshney and Mandhan (1981)	"	13.3 - 178 μ mole m^{-2} h^{-1}

The acetylene reduction rates of Indian species of family Cyperaceae and Typhaceae identified during the course of present investigations are comparable with the values reported in literature for corresponding members of these two families.

Table 3.4: Table showing Growth Form, habitat and Flowering Period of Grasses and Sedges Studied in the Present Investigation

Species (A)	Habitat (B)	Flowering period (C)	C_3/C_4 (D)	Lag Phase (E)	Growth Form (F)
GRAMINEAE :					
ALOPECURUS NEPALENSIS	Found in dirty water ditches	February	C_3	Very long	Annual
BOTHRIOCHLOA PERTUSA	Stony crevices, in lawns and pastures, on very dry habitats	July - October	C_4	20 h	Perennial
CENCHRUS CILIARIS	On almost all habitats during and after rains; grows even on very dry habitats	July - October	C_4	4-24 h	Perennial
CHLORIS BARBATA	During rainy season especially on sandy soils, pasture grounds	August - October	C_4	Very long	Perennial
CYNODON DACTYLON	Common and abundant, forming carpet in lawns	During major part of the year	C_4	10 h	Perennial
DIGITARIA ADSCENDENS	A common grass occuring on all types of soils.	July - October	C_4	8 h	Annual
DACTYLOCTAENIUM AEGYPTIUM	Common during monsoon period on sandy soils.	July - October	C_3	Very long	Annual

Contd..../-

Table 34 (Contd.)

(1)	(B)	(C)	(D)	(E)	(F)
HETEROPOGON CONTORTUS	Common on ridge and hilly tracts	October - December	C_4	8 h	Perennial
PASPALIDIUM FLAVIDUM	Commonalong canal banks and ponds on wet, sandy soil	May - October	C_3	16 h	Annual
SACCHARRUM MUNJA	Occurs on both sides of railway tracts, near canal etc.	August - October	C_4	12 h	Perennial
SACCHARRUM SPONTANEUM	A pernicious grass , common in unused grounds fallow fields	Sepetember- December	C_4	12 h	Perennial
SPOROBOLUS MARGINATUS	Common on ridge in gravellary soils	May - October	C_4	16-20 h	Annual
CYPERACEAE :					
CYPERUS ROTUNDUS	Common during monsoon period on the ridge, on moist silty or sandy soils	July - October	NA	4-10 h	Annual
FIMBRISTYLIS BISUMBELLATA	Common in marshes of canals along moist edges of follow fields; hygrophilous.	February- June	NA	4-10 h	Annual

Contd..../-

Table 3.4 (Contd.)

(A)	(B)	(C)	(D)	(E)	(F)
SCIRPUS TUBEROSUS	Common in marshes in beds of drying ponds, in canals as well as pond margins	September-November	NA	Not recorded	Perennial
TYPHACEAE:					
TYPHA ANGUSTATA	An amphibious sedge occuring along banks of ponds and in shallow waters forming dense patches at some places.	October - May	C_3	4-8 h	Perennial

NA - The information on the photosynthetic pathway is not available.

Information on growth form habitat and flowering period has been obtained from 'Flora of Delhi', J.K. Maheshwari.

SEASONAL VARIATION IN NITROGEN FIXATION BY GRASSES AND SEDGES

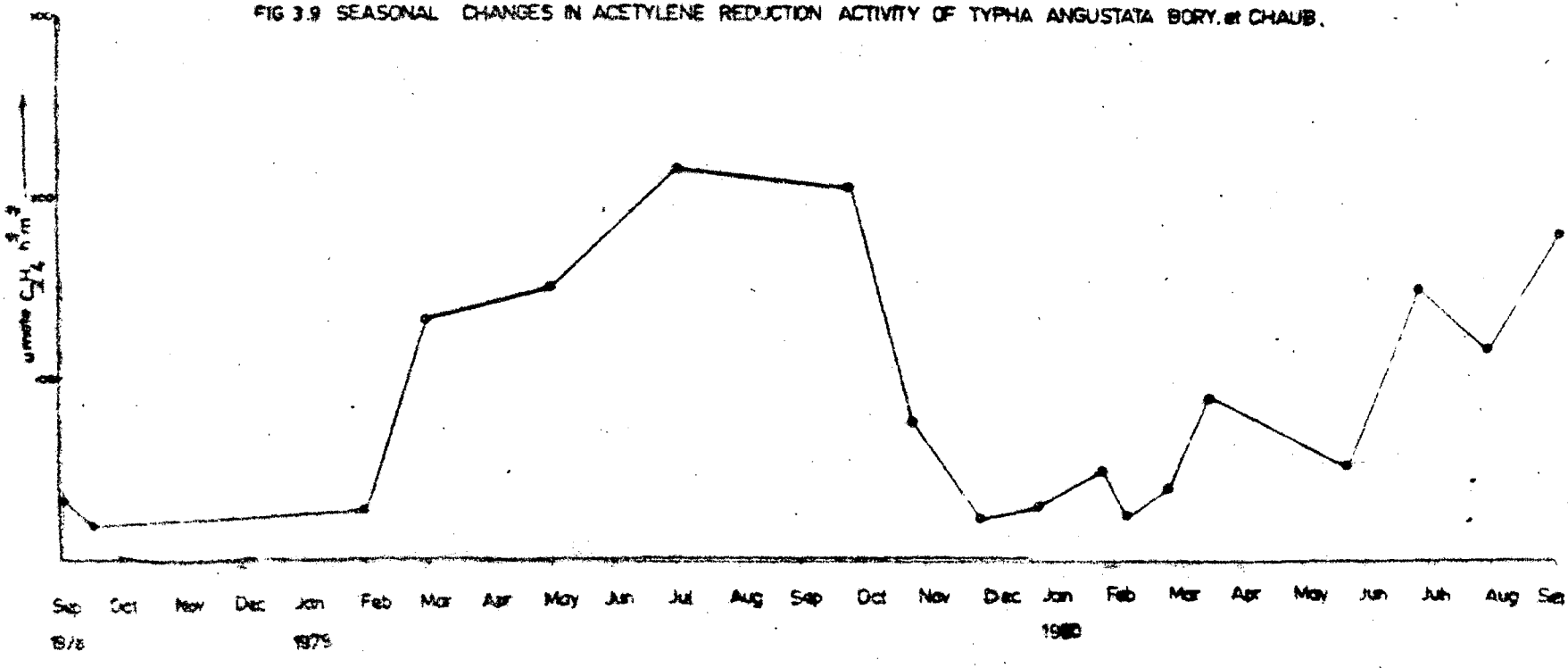
The seasonal variation in nitrogen fixing ability of the following seven grasses and sedges was studied. Typha angustata, Cyperus rotundus, Fimbristylis bisumbellata, Bothriochloa pertusa, Cenchrus ciliaris, Heteropogon contortus, and Sporobolus marginatus.

Typha angustata - Seasonality in nitrogen fixation by T. angustata is depicted in Fig. 3.9. A substantial part of the year ranging from June till September seems favourable for acetylene reduction by roots and rhizomes of this species. The rates gradually decline during December, January and February, but they never go below 16 μ mole m^{-2} h^{-1}.

Figure 3.10. shows the seasonal variation in nitrogen fixing ability of the following six grasses and sedges.

Cyperus rotundus - There was a gradual increase in acetylene reduction rates in January through March till a peak in April. This was followed by a steep decline in May and June. Another small peak was observed in July which was followed by periods of gradual decline till November.

FIG 3.9 SEASONAL CHANGES IN ACETYLENE REDUCTION ACTIVITY OF TYPHA ANGUSTATA BORY. et CHAUB.



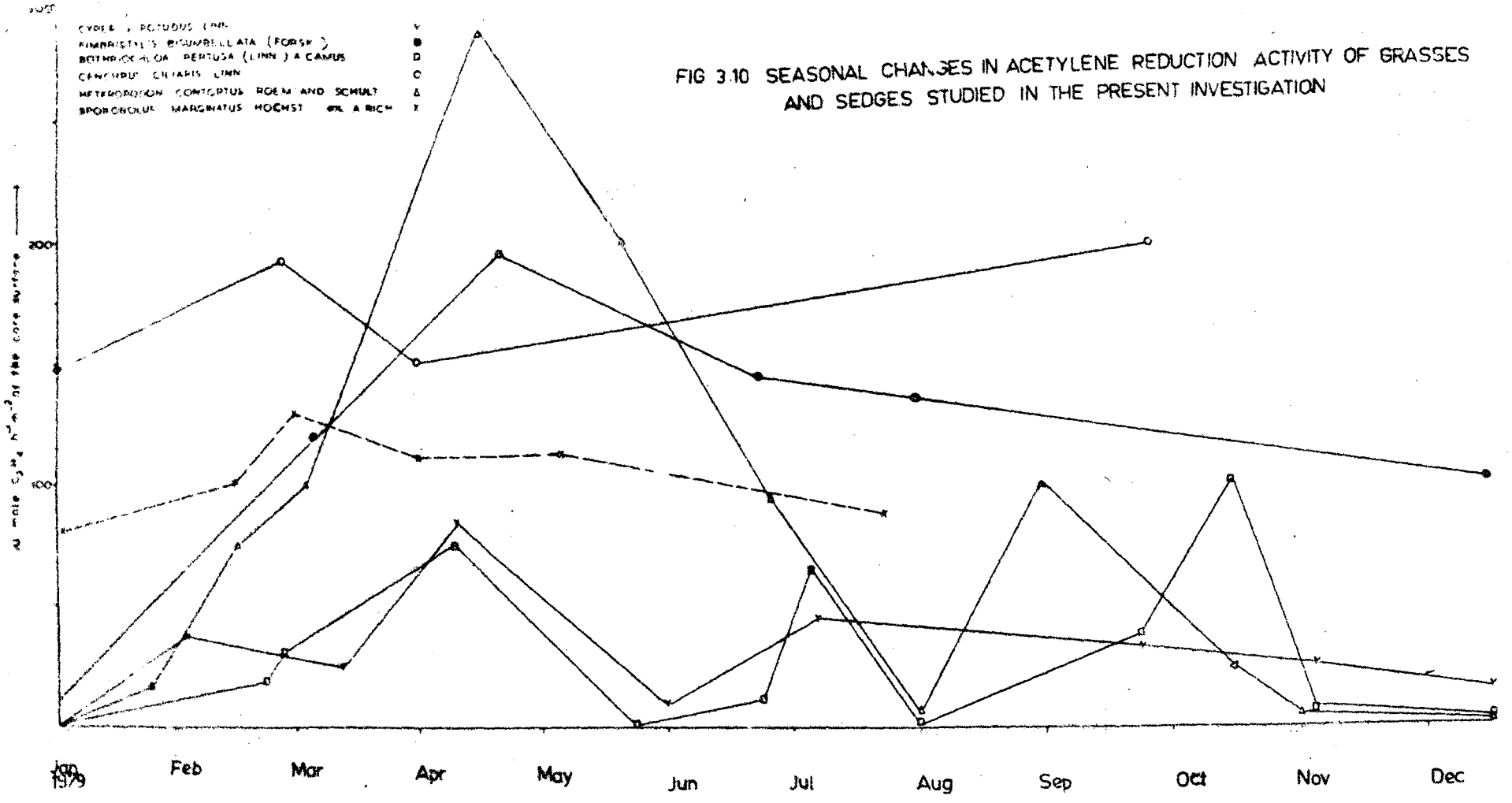


FIG 3.10 SEASONAL CHANGES IN ACETYLENE REDUCTION ACTIVITY OF GRASSES AND SEDGES STUDIED IN THE PRESENT INVESTIGATION

Fimbristylis bisumbellata - Starting from 13.3 μ mole m^{-2} h^{-1} in January the activity rose to a peak in April-May, at 178 μ mole m^{-2} h^{-1}. The rates declined gradually till December.

Bothriochloa pertusa - This grass species is characterized by three periods of active acetylene reduction in a year; first in April, second in July and the third in October-November at 100 μ mole m^{-2} h^{-2}, alternated by three lean periods in May, August and November-December respectively.

Cenchrus ciliaris - The rates of acetylene reduction in the soil root cores of this plant remained consistently high in almost all parts of the year, varying between 150-200 μ mole m^{-2} h^{-1}.

Heteropogan contortus - There was a peak acetylene reduction rate of 2.88 u mole m^{-2} h^{-1} in the period April-May. Another shoulder of peak at 99 μ mole m^{-2} h^{-1} was observed in September. This was followed by a sharp decline to 50 μ mole m^{-2} h^{-1} in October. Then, there was a gradual decrease to zero by the month of December.

Sporobolus marginatus - The acetylene reduction varied within a narrow range of 80 to 127 μ mole m^{-2} h^{-1}. There was a peak at 127 μ mole m^{-2} h^{-1} in the month of March.

Seasonal profile of the various nitrogen fixing plant system has been studied by various workers. Sloger et al. (1977) studied seasonal variation in $N_2(C_2H_2)$ fixing activity in field soybeans. Ruegg and Alston (1978) studied seasonal variation of nitrogenase activity in Medicago truncata Watanabe (1978) worked out seasonal changes of the nitrogen accumulation in Azolla grown in the paddy fields. But seasonality in nitrogen fixation in members of family Typhaceae and Cyperaceae has not been studied. Carpenter et al. (1978) worked out seasonal changes in Spartinia alterniflora growing in marsh habitats of Massachusettes ($71^{o}02'$W longitude, $42^{o}22'$ N latitude). Maximum nitrogen fixation was observed during mid summer i.e. June-July with little nitrogen fixation during winter season.

In the present investigation, the changes in acetylene reduction rates corresponded with changes in the ambient temperature, light intensity, soil moisture and the season. Maximum acetylene reduction was found in the period between September-October, which remained only 10-20 per cent in the following months. This shows that nitrogen fixation by Typha and other sedges varies seasonally. This difference is because the ambient temperature rises to 46 C in June and becomes harsh for plant growth. High rates of N-fixation was

were observed in post-monsoon periods.

Seasonality of nitrogen fixation in 30 tropical forage grasses was studied by Schank and Day (1977) at Brazil, South America (10°S latitude, 55°W longitude). The periods of lowest activity were the dry winter months. Seasonal profile of nitrogenase activity of grasses studied in the present investigations also followed the similar pattern. November, December and January were the lean periods. The activity started rising slowly. March to May is a favourable period for N_2 fixation which attains a peak in April, however, June was characteristically severe. Another period of appreciable acetylene reduction varied between July and October. This indicates a clear seasonality in the nitrogen fixing ability of the tropical grasses and sedges.

SUMMARY

Eversince the discovery of nitrogenase activity in tropical grasses namely Paspalum notatum and Dicitaria decumbense, by the Brazilian workers using the acetylene reduction technique, the phenomenon of rhizospheric nitrogen fixation by associative symbiosis is receiving increasing attention. In most of the cases Azospirillum has been shown to be the associated microsymbiont responsible for nitrogen fixation in the rhizosphere of grasses and sedges. The thesis presents a comprehensive review on the associative symbiosis including geographic distribution, morphology, physiology and the biochemical peculiarities of this newly discovered nitrogen fixing system. Results of inoculations of grass roots with Azospirillum carried out in India and elsewhere, have been discussed.

The review clearly shows an information gap about the nitrogen fixing potential of Indian wild grasses. In view of this, twenty six species of local grasses and sedges were surveyed with the help of acetylene reduction (AR) bioassay to determine their nitrogen fixing capacity. Intact soil root cores were used to determine the AR activity. For this purpose, a split metallic soil corer was designed and fabricated to obtain intact and undisturbed soil root cores of uniform size. As a result of the survey, twelve grass

species Alopecurus nepalensis, Bothriochloa pertusa, Cenchrus ciliaris, Cynodon dactylon, Chloris barbata, Dactyloctaenium aegyptium, Dicitaria adscendens, Heteropogon contortus, Paspalidium flavidum, Sporobolus marginatus, Saccharrum munja and Scharrum spontaneum and four sedges Cyperus rotundus, Fimbristylis bisumbellata, Scirpus tuberosus and Typha angustata, were found to possess positive AR activity. This includes fourteen plant species which have been reported to possess AR activity for the first time according to information available in the literature.

Field studies were undertaken to determine seasonal variation in the AR activity of seven grasses and sedges for a period of one year. The results show two peak periods in March-May and July-October.

The results of this investigation suggest that rhizospheric nitrogen fixing potential of the local grasses and sedges ranges from 7-520 u mole m^{-2} h^{-1} and is comparable with the activity of other grasses and sedges shown to possess rhizospheric nitrogen fixing potential. Studies on isolation, characterization and identification of the microsymbiont and inoculationn experiments using local wild grasses are urgently needed to acquire a better understanding of the nitrogen fixing grass-root-bacterial associations in the local ecosystems.

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