NITROGEN-FIXATION IN THE RHIZOSPHERE AND HABITAT OF WILD RICE, *ZIZANIA AQUATICA* (L).

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A C K N O W L E D G E M E N T S

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A B S T R A C T

ABSTRACT

Nitrogen-fixing anaerobes, facultative anaerobes and aerobic bacteria within the rhizosphere of wild rice, Zizania aquatica (L), were isolated and identified. Algal components of blooms occurring within the habitat were also identified and included several genera reported as nitrogen-fixing.

Experimental evidence showed bacterial nitrogenase activity to be higher on root surfaces than in the rhizosphere soils at lower depths. Algal nitrogenase activity was largely restricted to the rhizosphere soil surface.

Nitrogen-fixation potential as measured by acetylene reduction varied with different soil types. Higher acetylene reduction values were consistently obtained for rhizosphere soils than were noted in the water column of wild rice habitat.

Evidence suggests that nitrogen-fixation by bacteria may be of marked benefit to the nitrogen budget of wild rice habitat.

TO MY MOTHER, BETTY AND TO THE MEMORY OF OWEN.

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H I S T O R I C A L

HISTORICAL

Wild rice which is one of the two cereals native to North America presumably has been growing ever since the recession of the Wisconsin glaciation, a period of some 9000 years. It has provided and still provides the native peoples of northern hemispheric countries with a source of nutritious food. As a highly digestible cereal grain with a protein content of about 15%, it is low in fat and displays an average to high vitamin content except for vitamin A and is somewhat deficient in certain mineral elements (Steeves 1952). It provided a source of food for early explorers, traders and settlers in North America. Transportation of the grain along well-travelled trading routes probably, through spillage, accounted in part for its wide geographic distribution along the river and lake systems of Canada (Dore 1969).

The plant has been known by a number of local names: Indian rice, Canadian rice, water oats, river rice, tuscarora and manomin (Dore 1969), squaw rice, false oats, and blackbird oats (Edman 1969). By the middle of the 18th century, it had been recog-

nized by botanists and naturalists and had been given the generic name Zizania by Gronovius (Dore 1969). Carl von Linne (Linnaeus) began a taxonomic study of the plant in 1753 and retained the name Zizania which remains its proper generic name to this day. Four species have been described thus far, each with one or more varieties.

Today, wild rice grows in almost all continents of the world mostly in small stands. The largest areas of concentration are found in the Minnesota and Wisconsin areas of the United States and in some provinces of Canada (Edman 1969). In 1968 nearly 200,000 pounds of green wild rice were harvested from natural stands in Manitoba principally in the White-shell Provincial Forest area. In the same year, exports of this grain from Canada amounted to nearly one-half million dollars (Dore 1969) with Manitoba being the chief producer.

Although the mechanical harvesters are now coming into use, the traditional methods used by the native peoples is to beat the stalks of ripened grain with a stick to dislodge the grains into their canoes. Current estimates predict that wild rice harvesting could develop into a 10 million dollar annual revenue for the native peoples.

Despite the historical, sociological and economic interests generated by this crop plant, it is disheartening to realize that little is known about its growth requirements and physiology. What little information there is seems to have been accumulated mainly by the botanists and naturalists (Chambliss 1940; Rogosin 1951; Simpson 1966; Weber and Simpson 1967; Weir and Dale 1960; See Dore 1969 for these references; Thomas and Stewart 1969; Hawthorn and Stewart 1970). Recently, however, more researchers have taken interest in the biology of the wild rice plant. At the University of Manitoba, research is currently being carried out in the area of disease problems (Paul Gilbert; pers. comm.) genetics (Dr. Wood; pers. comm.) and general botany (Dr. Stewart; pers. comm.). No doubt in the next few years more and more information of biological interest will be available.

Contrary to the situation pertaining to the wild rice plants with regard to information, an enormous amount of information of biological as well as agricultural interest exists on the common cultivated rice-Oryza species. Most of the information has come from the extensive researches conducted in

the areas of the world where Oryza is the most important staple food, for example the Asiatic countries and Japan. Some information also comes from tropical and subtropical areas.* It is thus necessary for more intensive research on wild rice to be conducted in the countries where the plant grows in such abundance. Both the wild and the cultivated rice superficially have some identical features but some structures are different as pointed out by Dore (1969). Because of the general lack of information it is not known in what ways, if any, the ecology of the wild rice plants' rhizosphere is comparable to that of Oryza species.

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*See The Mineral Nutrition of the Rice Plant - Proceedings of a Symposium at the International Rice Research Institute IRRI-Laguns, Phillipines.

INTRODUCTION

For several centuries rice fields in China have been producing enough grains to sustain associated small populations with little if any mineral fertilization. Only recently have microorganisms been implicated in the nutrition cycle of this crop. De (1939) suggested the possible maintenance of this fertility of the paddyfields to some degree by the abundant green algae observed in the rice paddies of India and Asia. Since then several experiments have demonstrated convincingly that growth of rice plants (Oryza sativa) improved considerably in the presence of algae (Watanabe et al 1951; De and Mandal 1956; Watanabe 1956). Investigations have also shown that other microorganisms contribute to the fertility of the soil. Upall et al (1939) pointed out the importance of Azotobacter in the Nitrogen recuperations of paddy soils. Because of their widespread occurence, Clostridium species may also contribute significantly to the Nitrogen economy of the rice plants. Clostridium and Azotobacter however require a vast amount of carbon source in order to fix significant amounts of Nitrogen

(Takahashi 1964). Bhattacharya (1958) maintains that due to their low number in soils and the fact that they disappear from paddy soils when added, Azotobacter species are not likely to contribute much to the Nitrogen economy. It is probably because of this situation that the contribution of the bacterial nitrogen fixers to the paddy soils has received little attention (Rinaudo et al 1971).

Investigations on the contribution of microorganisms to the Nitrogen economy of rice plants have been carried out with plants growing in man-made rice farms - paddies. This is understandable, because there are no natural stands of Oryza sativa. In the second place, though Oryza sativa and Zizania look superficially alike, their rhizosphere and habitat ecology is likely to be different, and in that case their rhizosphere is likely to harbour different types of bacteria and/or different species of the same genera.

So far as the writer is aware, the ecology of the wild rice Zizania aquatica (L) has not been studied and apart from the general information that the Clostridium and Azotobacter species are the Nitrogen-fixing bacteria in the rhizosphere of Oryza sativa (Yoshida and Ancajas 1971; Rinaudo et al 1971)

I N T R O D U C T I O N

no attempts are made to classify the species. For the above reasons an attempt has been made in this investigation to isolate and classify the N-fixing bacteria in the rhizosphere of the wild rice plants growing in their natural habitat, to quantitatively assess their potential contribution within the rhizosphere and the contribution of Nitrogen-fixers - both heterotrophs and autotrophs - within the water in which the plants are growing, using the Acetylene reduction and Kjeldahl methods.

EXPERIMENTAL

I. Sampling Area

Zizania species and varieties are found mainly along the shorelines of lakes, rivers and streams in shallow water where they may form thick stands. In many streams, growth may extend almost to the stream centre leaving only a narrow mid-stream channel. Often this channel corresponds to where the water level is too deep or the current too swift to permit seed establishment and subsequent good growth.

Field experiments and laboratory samplings were done in two experimental areas; in the LaSalle

River and in Lone Island Lake (hereafter referred to as Locations A and B respectively) in the summer of 1972. Subsequent sampling and field studies were restricted to Location A.

The LaSalle River, Location A, is a small stream which meanders through the farming community around the village of LaSalle, Manitoba located about 16 miles southwest of the University of Manitoba. Location B is located within the Whiteshell Provincial Forest and is representative of the intricate network of shallow rivers, streams and lakes which have formed a suitable habitat for Zizania since the earliest days. Lone Island Lake is approximately 80 miles east and south of Winnipeg, Manitoba.

The soil types differ quite markedly between Locations A and B. Along the banks of the LaSalle River the seed beds are typical of the dark clayey soils of the Red River Valley while at Location B, they are located in loose unconsolidated organic debris over sand.

Sampling and field experiments were carried out from June to September 1972 and from May to early July 1973. In the latter period all studies were based on Location A for reasons of economy and accessibility.

II. Stages in the development of Zizania species and some general observations on the habitat.

Three distinct stages during the vegetative growth of Zizania species are discernible and since investigations of one form or another were carried out during these different stages, it is pertinent to review these developmental aspects.

Normally about mid-April, after the thaw, the dormant seeds which have over-wintered under the ice germinate to produce a long thin thread of white radicle which can be easily seen through the clear cold water (10 - 11°C). Between mid-April and mid-May, the first leaves are produced which are light brown in colour. Surrounding the young stems and covering the sediment are the remains of the previous year's algal bloom which has also over-wintered with the seeds. All the development up to this point is referred to as the first stage, the Submerged Leaf Stage.

Between May and June as the water temperature rises to 16 - 18°C, the young wiry stems elongate rapidly and turn a deep green colour. Stems and leaves at this stage, however, are weak and tend to bend over at the water surface in a floating mode to

provided stage two, the Floating Leaf Stage. Commensurate with this stage is, usually, the rapid development of two algal blooms. The first bloom consists of ribbon-like forms, probably Aphanizomenon species which persist for one to two weeks. These aphanizomenon-like algae are distributed more or less uniformly throughout the water column while the second dominant algal type is filamentous and quite localized in distribution along the water edge and around the floating rice leaves.

From mid July onwards the stems and leaves of Zizania strengthen and emerge into the atmosphere which is known as Aerial Leaf Stage. By now, water temperatures have reached as high as 28°C at a depth of one metre. The algal mass proliferates extensively forming quite thick mats surrounding the rice plants. Seed formation ensues later after the Zizania crops have reached full maturity by late August or early September.

III. Soil and Plant Sampling

Soil texture and consistency in wild rice habitats facilitate the sampling of the rhizosphere. The soil adheres to the root in such a way that a gentle pull on the stem is enough to dislodge the

whole root system with little if any damage. At the 'Submerged Leaf Stage', the dislodged root system carries with it little or no soil and it is therefore necessary to use a hand shovel to provide sufficient rhizosphere soil for study.

Because the sampling operation was carried out in water up to a metre in depth, two people were involved. One person held the uprooted plant over an opened plastic bag while the other removed the roots and adhering soil by sterile scissors. Thus, the root system with its adhering soil was allowed to drop into the plastic bag; the top of the bag was then folded over and quickly stapled. One or more rhizosphere samples from a given area were collected in each bag, the contents of which constituted an area sub-sample. Sampling within a location was random and all soil samples and whole plants (where necessary) were immediately transported back to the laboratory for examination and analyses as described subsequently. During the sampling period in September 1972, dissolved oxygen was measured 'in situ' in the rhizosphere with a YSI Model 54 oxygen meter.

IV. Soil Chemical Analyses

Soil samples returned to the laboratory were analysed for ammonium- and nitrate-nitrogen, for total organic matter and for moisture content.

(1) Ammonium- and nitrate-nitrogen

Ammonium- and nitrate-nitrogen values were determined in 20 sub-samples from each of Locations A and B according to the steam distillation methods of Bremner (1965). In brief, the method involves first extracting the exchangeable ammonium- and nitrate with 2N KCl (10 ml per g of soil), then steam distilling the extract in the presence of magnesium oxide and Devarda alloy, and titrating the distillate (ca 30 ml.) against 0.1N H_2SO_4. Ammonium- and nitrate nitrogen determinations are carried out on the same (30 ml.) aliquot of the distillate.

(2) Moisture Content

Soil samples were stored for one week at 4°C for equilibration before moisture content percentages were determined. Then, a 20 g sample was weighed from each area sub-sample (pooled) in a pre-weighed crucible. Each 20 g sample was then

dried at 80°C with its weight recorded hourly until a constant weight was obtained. Percent moisture content of soils from each location was based on the average of five such determinations.

(3) Organic matter content.

Total organic matter content of the soils under study was determined by the standard method of Walkley and Black as reported by Bremner (1965). These are expressed as the average of five determinations for each location.

V Demonstration of nitrogen-fixing microorganisms in rhizosphere soils and root surfaces of Zizania aquatica.

(1) Facultative and anaerobic nitrogen-fixers.

The presence of facultative and anaerobic nitrogen fixers in the rhizosphere soils and on washed root surfaces and their quantitative evaluation by MPN technique were determined by the method of Campbell and Evans (1969). For these determinations Hino and Wilson's medium (1958) was modified as follows: glucose was substituted for sucrose; $CaCl_2.6H_20$, 0.1

g/l replaced $CaCO_3$ and 0.1 ml/l of a trace element solution* was added to Part A of the medium before autoclaving. Parts A and B of the medium were autoclaved separately and then aseptically combined when cool. The medium had a final pH of 7.6

The method of Campbell and Evans (1969 was slightly modified in that the 'Suba' seals were separately autoclaved along with Pankhurst tubes plugged with cotton wool. After autoclaving, the culture medium and saturated alkaline pyrogallol were added aseptically by sterile pipette to the appropriate arms of the tube, cotton plugs were removed and sterile suba seals inserted aseptically.

For rhizosphere soils, MPN estimates and total counts were determined on 1:10 dilutions prepared from pooled sub-samples of each soil. Similar determinations were made on washed root samples which had been mascerated in the presence of PVP** in 0.1 M phosphate buffer prior to addition of the diluent.

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*In 100 ml distilled water: H_3BO_3, 93 mg; $ZnSO_4$, 100 mg; $MgSO_4.4H_2O$, 200 mg; Na_2MoO_4, 6 mg; $CuSO_4.7H_2O$, 5 mg and $CoCl_2.6H_2O$, 8 mg.

**PVP - acid-washed polyvinylpolypyrrolidone obtained from CalBiochem, La Jolla, California.

(2) Aerobic nitrogen-fixing bacteria

Total counts of aerobic nitrogen fixers were made on rhizosphere soils and on washed root surfaces in a manner similar to that described above except that the azotobacter medium of Brown et al (1962) was used.

VI Isolation of nitrogen-fixing bacteria

(1) Facultative and anaerobic N-fixers.

From the MPN series, culture tubes at the highest dilution showing positive acetylene reduction results were used as sources of inoculum for the enrichment and isolation of anaerobic and facultative nitrogen-fixing bacteria. In brief, 1.0 ml of inoculum from such a tube was aseptically transferred by syringe to a Pankhurst tube containing 10.0 ml of the modified Hino and Wilson's medium. Tubes thus inoculated were immediately flushed with sterile N_2 gas then incubated at 37°C for 10 days or until increasing turbidity was observed. This procedure was repeated at least twice more to provide assurance that growth (turbidity) was well established in the nitrogen-free medium. From these, pure culture were obtained by streak plating (0.1 ml inoculum) on plates of an agar medium of the same composition

incubated at 37°C for 5-7 days in a Gaspak anaerobic jar. After incubation, colonies were picked for plating using the same medium and cultural conditions. When uniformity of each isolate type was established by colony characteristic, microscopic examination, cultural characteristics and certain biochemical tests, isolates were routinely maintained on nitrogen-free agar slants.

(2) Aerobic N-fixing bacteria

Aerobic nitrogen-fixers were isolated from colonies that developed on nitrogen-free agar plates used for the plate count enumeration. After sub-culturing inoculum from each colony type several times to attain purity, biochemical tests were employed to positively identify the isolate as an aerobic nitrogen-fixer. A number of isolates were thus obtained.

Isolates, whether aerobes or facultative anaerobes, were tested for their ability to reduce acetylene to ethylene as a positive identification of their nitrogenase activity. Anaerobes or facultative anaerobes were tested with acetylene by the method of Campbell and Evans (1969). For aerobic

isolates, 0.1 ml of a dilute saline suspension of each culture was inoculated to 10 ml of the liquid azotobacter medium in a 50 ml Erlenmeyer flask plugged with cotton. After incubation at 37°C on a rotary shaker for sufficient time to produce moderate turbidity, flasks were capped with sterile 'Suba seals' and each received 0.5 ml of acetylene gas freshly prepared from calcium carbide and water. At intervals, gas samples were removed by syringe for chromatographic analysis using a Pye F.I. detector with a 6' x 1/8" stainless steel column packed with Porapak R. Column temperature was maintained at 23°C (ambient); nitrogen gas was used as the carrier at a flow rate of 20 ml/minute.

VII Identification of bacterial isolates

Isolates were identified on the basis of morphology, cultural characteristics and biochemical tests as are routinely used for studies of nitrogen-fixing bacteria. Details of the tests employed are presented in the section of Results and Observations. Only those isolates that clearly and consistently demonstrated the ability to reduce acetylene to ethylene when cultured in a nitrogen-free medium were

Fig. 1. Standard curve for ethylene at low concentration ranges.

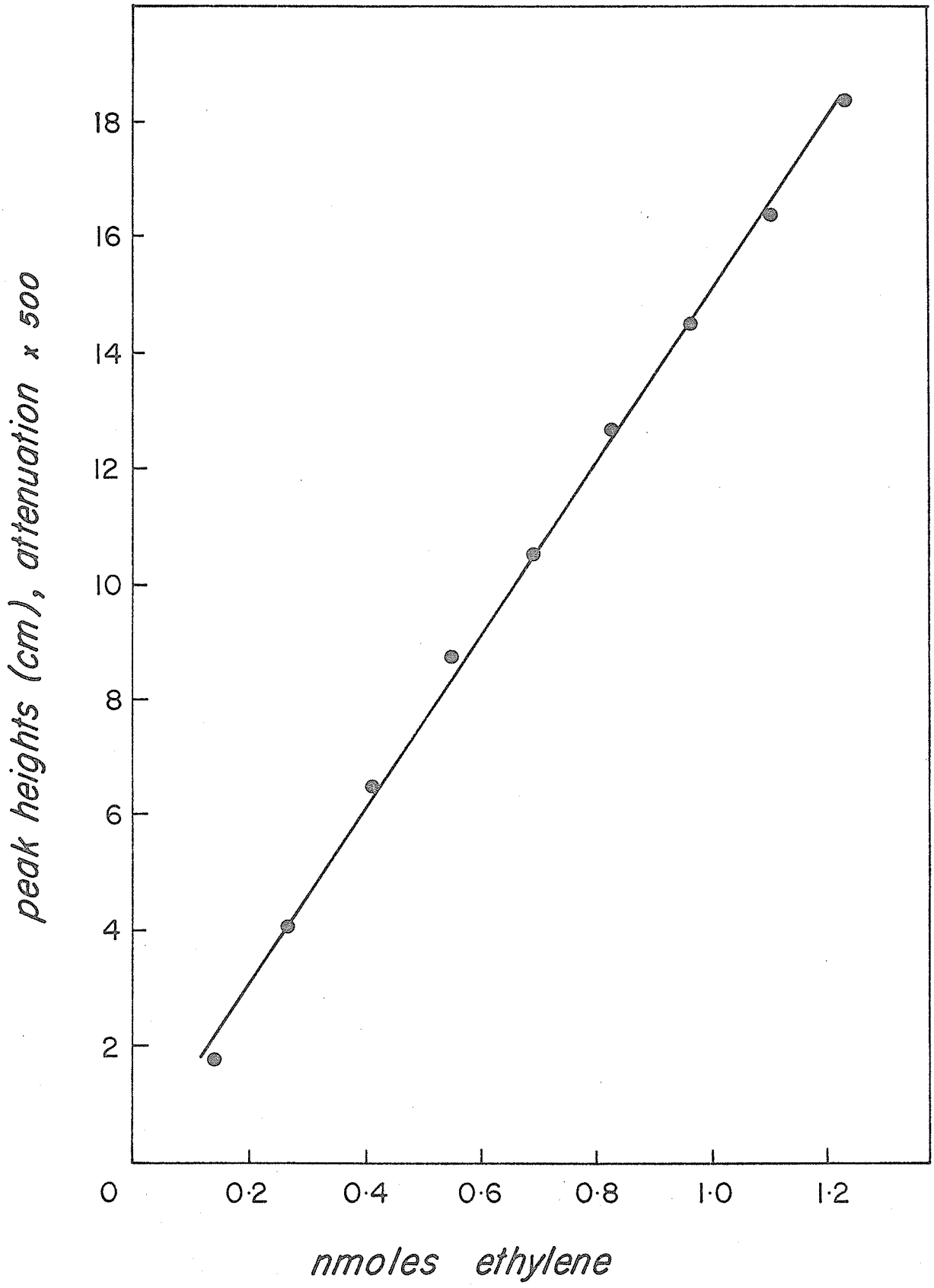

peak heights (cm), attenuation x 500
18
16
14
12
10
8
6
4
2
0
0·2
0·4
0·6
0·8
1·0
1·2
nmoles ethylene

Fig. 2. Standard curve for ethylene at medium concentration ranges.

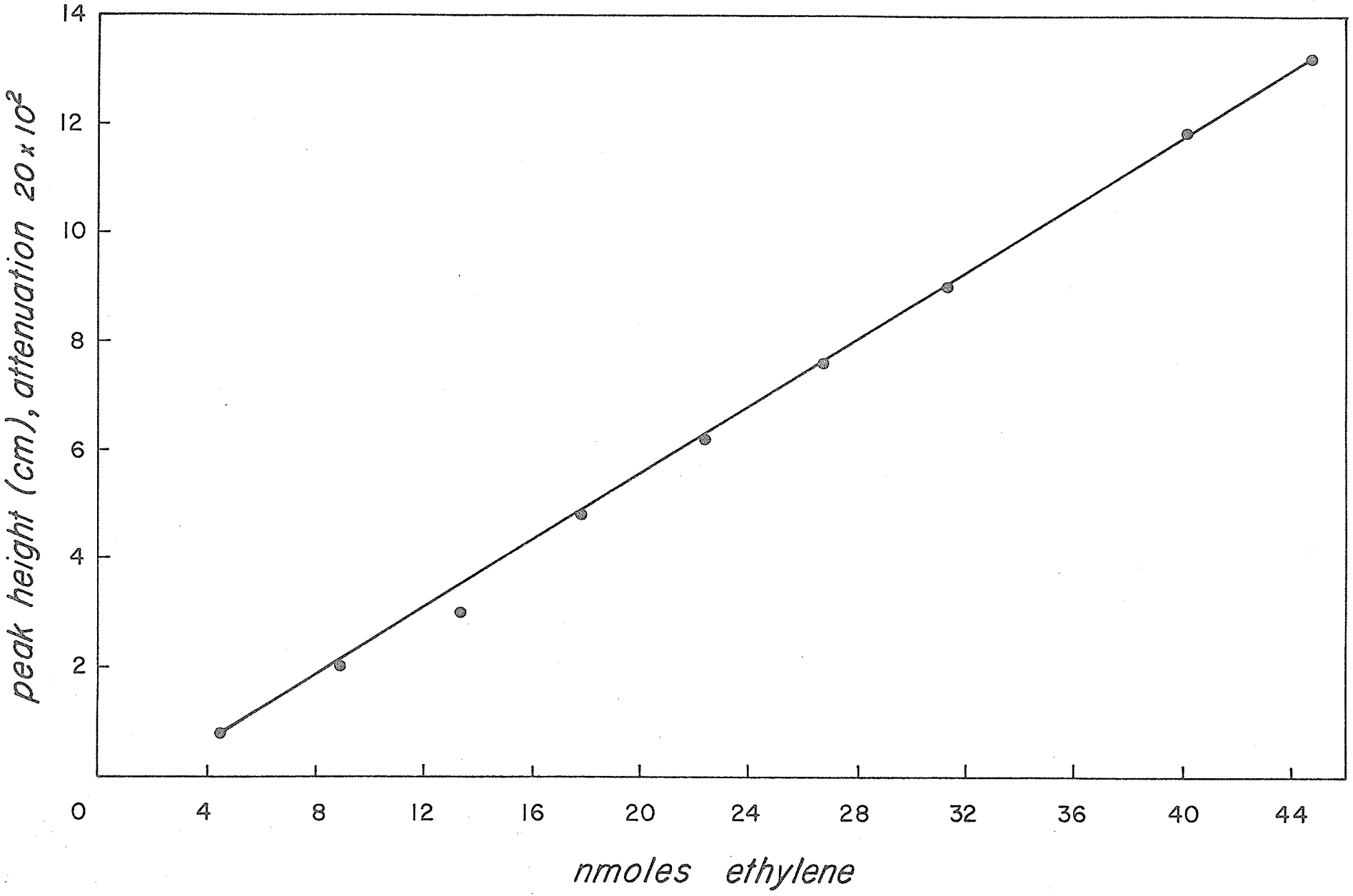

peak height (cm), attenuation 20 x 10²
14
12
10
8
6
4
2
0
4
8
12
16
20
24
28
32
36
40
44
nmoles ethylene

Fig. 3. Standard curve for ethylene at high concentration ranges.

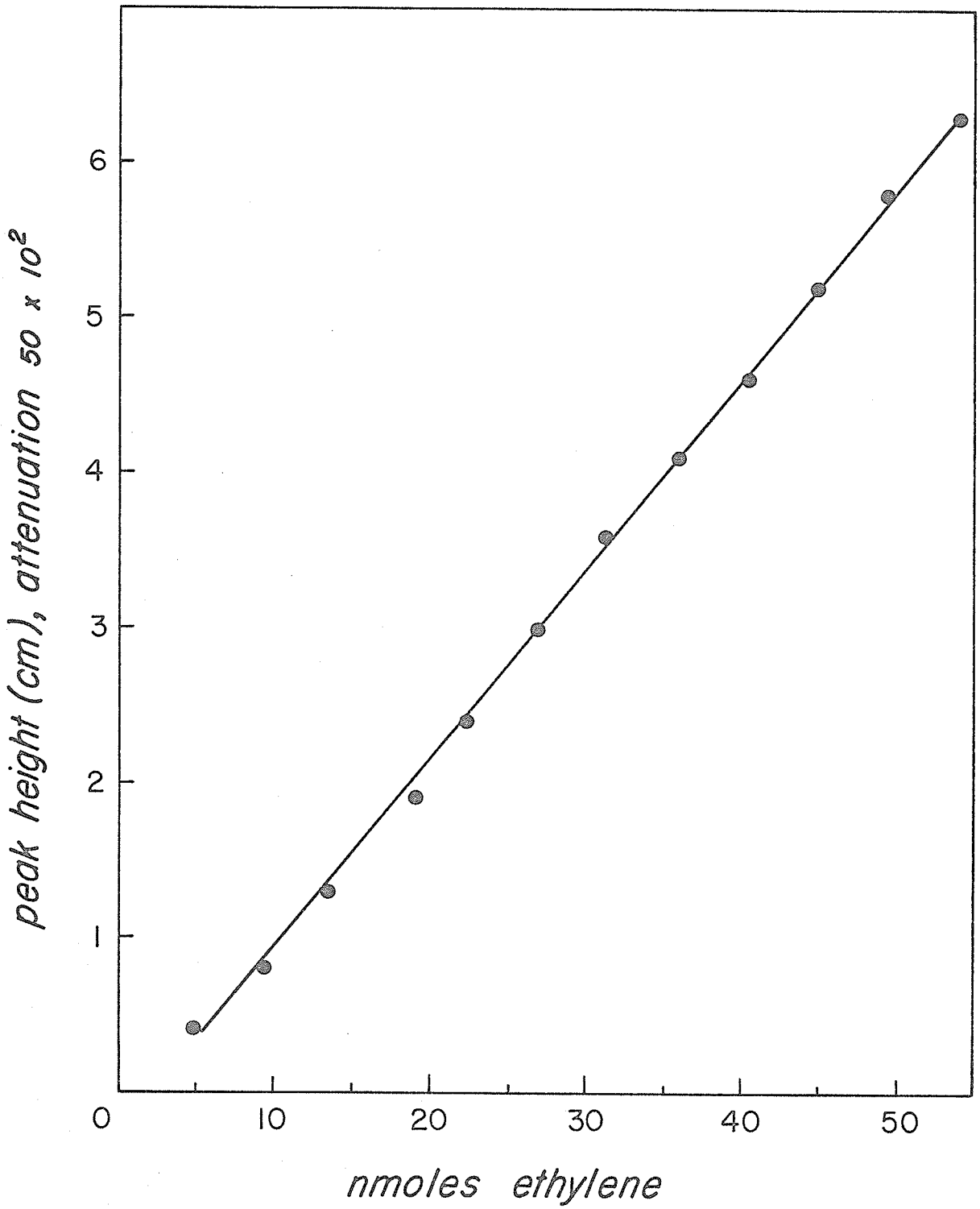
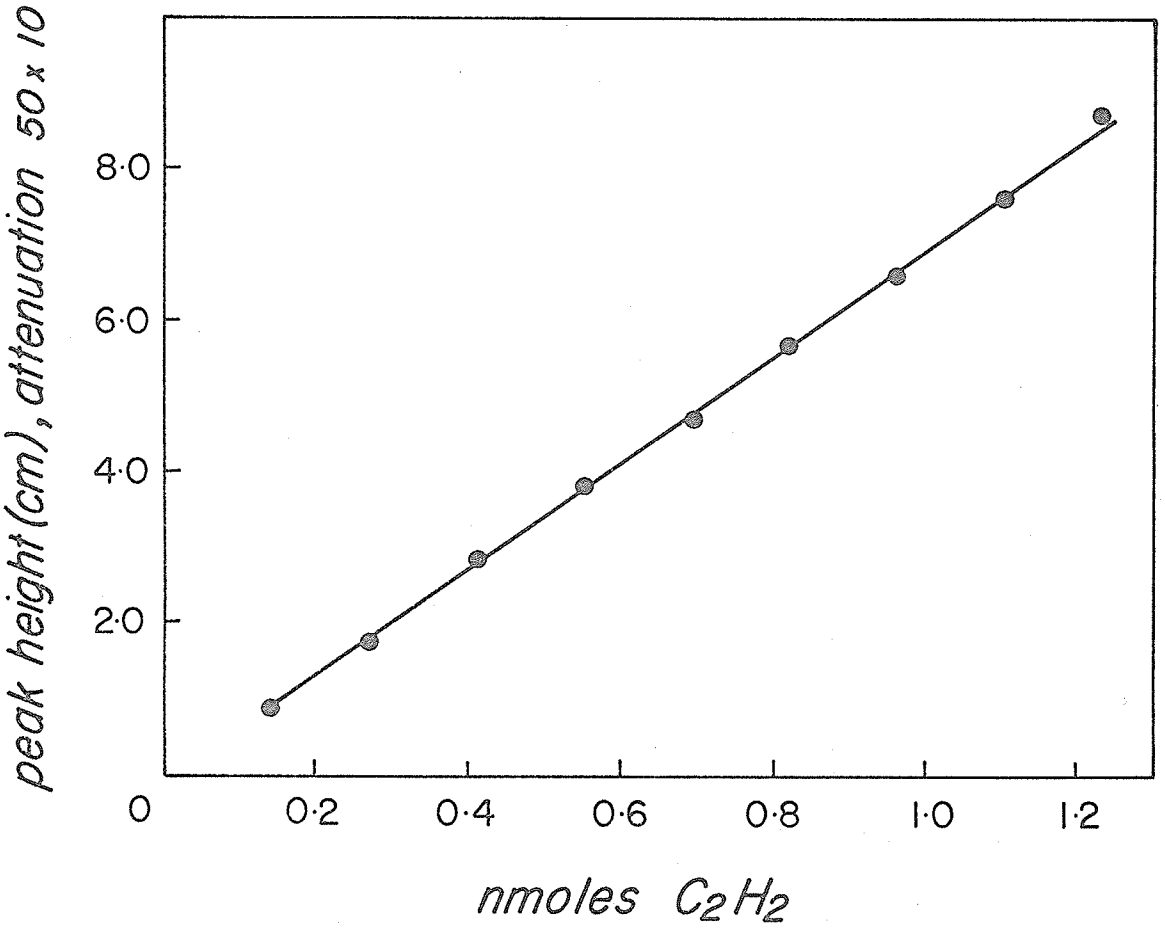

peak height (cm), attenuation 50 x 10²
6
5
4
3
2
1
0
10
20
30
40
50
nmoles ethylene

Fig. 4. Standard curve for acetylene at low concentration ranges.


peak height (cm), attenuation 50 x 10
8·0
6·0
4·0
2·0
0
0·2
0·4
0·6
0·8
1·0
1·2
nmoles C_2H_2

selected for further study and identification.

Morphology of each isolate was determined by phase contrast microscopy using wet mounts and by the Gram's stain procedure. Cultural studies mainly concerned the organisms growth response to various aerobic and anaerobic conditions. Biochemical tests to provide identification to the species level were as recommended in Standard Methods (Harrigan and McCance 1966; Norris and Chapman 1972; Breed et al 1957) . In the interests of brevity, details of these tests are not provided here but are noted briefly in the section on Results.

VIII Quantitative studies of nitrogen fixation as measured by acetylene reduction.

(1) In the rhizosphere soils

Excess soil was first shaken from the intact root systems of wild rice plants brought to the laboratory from locations A and B. Using a sterile spatula, most of the wet soil remaining on the root systems of several plants was removed to a sterile crucible and mixed to provide a uniform composite sample representative of each location. Four aliquots from the composite sample for each location

were then weighed into separate preweighed 50 ml flasks. Each was then reweighed to determine wet weight of soil present. After capping with 'Suba seals', flask atmospheres were evacuated by syringe then flushed with Helium gas. Freshly generated acetylene gas was then added by syringe to three of the flasks in each series to provide a final atmosphere composition of 99:1 as He: C_2H_2. Flask #4 in each series received no acetylene and served as a control. All flask samples were incubated at 27°C for several days in the dark. At intervals indicated in the legend to Figure 5, 0.25 ml of each flask atmosphere was removed by syringe for analysis. Flask atmosphere volumes were determined after correction for the volume of soil present in each case. Ethylene produced from acetylene (N-ase reaction) was determined by gas chromatography and is expressed as nmoles/g wet weight of soil.

(2) On root surfaces

Root systems remaining after Part I of the procedure were washed by gentle agitation in sterile water under aseptic conditions. After several washes, 10 to 15 pieces each about 1 to 2 cm long were cut using sterile forceps and scissors then

transferred to a sterile 50 ml flask containing filter paper saturated with 5.0 ml of sterile distilled water. Four such flasks were prepared from each pooled root sample. Flasks were capped with sterile 'Suba seals', evacuated by syringe and flushed with filtered helium as described previously. Again, flasks #1 to #3 received 0.01 atmosphere of acetylene; flask #4 was left untreated as a control. Incubation followed at 27°C in the dark for several days during which time 0.25 ml gas samples were removed at intervals as for experiments with rhizosphere soils. At the conclusion of incubation and after all ethylene determinations had been made, total root sample in each flask was weighed. Atmosphere volume in each flask was assumed to be approximately 40 ml; total volume of roots in each case was not calculated.

A similar though somewhat more restricted study was undertaken on roots of plants at the submerged leaf stage, location A, in early spring to include an aerobic gas phase. In this case, an aerobic gas phase consisting of a prepared mixture of oxygen: argon (22:78) was introduced to each flask in a manner identical to that used for helium. Prior

to incubation at 25°C, 0.5 ml of the aerobic atmosphere was removed by syringe and replaced with a similar volume of acetylene. Samples of the flask atmospheres were removed initially after six hours incubation; subsequently, to the end of the incubation period, samples were taken every 24 hours.

(3) Possible contributions by rhizosphere algae

As noted previously, algal masses were observed quite early in Spring at location A where they covered soil surfaces from which wild rice plants were growing. Since a number of genera within the Schizophyceae have been identified as nitrogen fixers (Stewart 1970), experiments were conducted to determine if any components in these algal masses were capable of reducing acetylene.

The procedure was first to remove the upper layer of soil over the developing root systems using a blunt knife. Several samples thus obtained were thoroughly mixed and from this composite sample 5.0 g portions were weighed out and transferred to each of seven 40 ml vials. Three of these vials were individually wrapped with aluminum foil to provide dark controls. All vials each received 2.0 ml sterile water, were plugged with sterile cotton wool, then

incubated in a controlled environment chamber. Incubation conditions were as follows: each 24 hour period provided 14 hours of light and a temperature of 25°C and 10 dark hours with a temperature of 23°C. Light intensity was maintained at 11,600 lux. After 17 days incubation, cotton plugs in the vials were replaced by sterile 'Suba seals' and each vial except one (light) then received 0.5 ml of sterile-filtered acetylene gas. Atmosphere samples were removed subsequently after 10 to 12 hours for chromatographic analysis.

IX Wild rice plants cultured in a nitrogen-free nutrient medium with and without nitrogen-fixing microorganisms.

A study was undertaken to determine if nitrogen fixed by rhizosphere microorganisms could sustain growth of wild rice seedlings cultured in a nitrogen-free nutrient medium. Experiments were restricted to plants collected at two stages of growth from location A.

(1) Young seedlings

During the last week of April 1973, about 100 young seedlings at the first leaf stage were collected

at location A and immediately returned to the laboratory. Ten to fifteen seedlings were then quickly transferred to each of four 2800 ml wide mouth pyrex flasks containing washed[1] silica sand (to a depth of 5 cm) and 1450 ml of a nutrient solution. Flasks were designated A, B_1, B_2 and C. The nutrient solution in flask A was the complete medium of Arnon and Hoagland (1940) which includes nitrate nitrogen adequate for liquid culture of plants. The nutrient solution in flasks B_1, B_2 and C was the nitrogen-free modification proposed by Steward (1965) which contains glucose. Details of the medium composition are given in the legend to Fig. 9a. Flasks B_1 and B_2 were then inoculated with a mixed 24 hour culture of bacteria consisting of equal numbers (11.5 - 11.8 x 10^8 cells) of the Azotobacter and Clostridium isolates. Population densities in the inoculum were determined by plate count.

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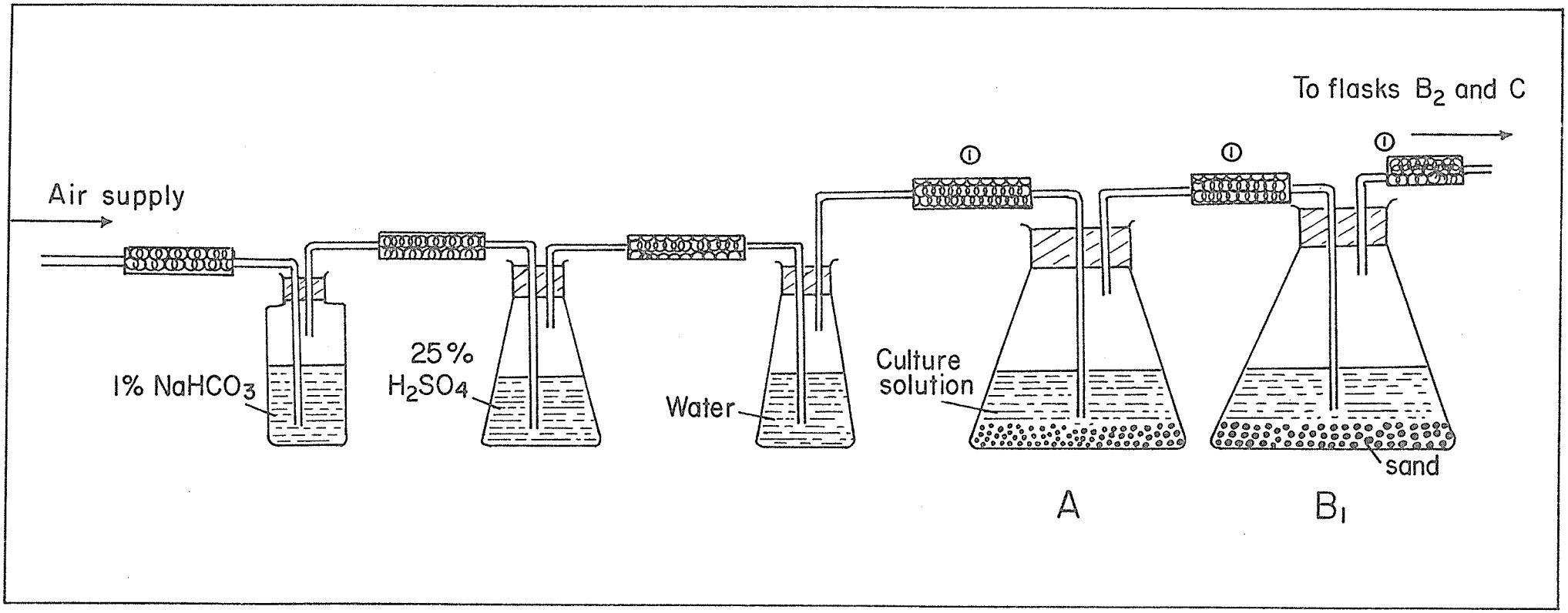
[1] Silica sand was first washed several times with deionized water, then soaked in 0.5 N KCl for 24 hours. Following this, the sand was again washed five times with deionized water before use. The final rinse water was tested for the presence of NH_4^+-and NO_3^- - nitrogen (Strickland and Parsons 1968).

Fig. 9a. Schematic diagram of the aeration train for seedling culture experiments.

Legend: symbol (1) represents sterile cotton wool filters. The culture solutions was made up as follows:- one litre of the 'complete' culture solution contained KNO_3 1.02 gm; $Ca(NO_3)_2$ 0.492 gm; $NH_4H_2PO_4$ 0.230 gm; H_3BO_3 2.86 mg; $MnCl_2.2H_20$ 1.81 mg; $CuSO_4.5H_20$ 0.08 mg; $ZnSO_4$ $7H_20$ 0.22 mg; $Na_2M_0O_4.2H_20$ 0.09 mg. In the nitrogen-free solution, KCl (1.02 gm/l) replaced KNO_3, $CaCl_2$ (0.492 gm/l) replaced $Ca(NO_3)_2$ and Na_2HPO_4 (0.460 gm/l) replaced $NH_4H_2PO_4$. Glucose 5 gm/l was added where necessary and 0.6 ml of a mixture of aquous solutions of $FeSO_4$ 0.5% and Tartaric acid 0.4% was added to each experimental flask 3 times a week.

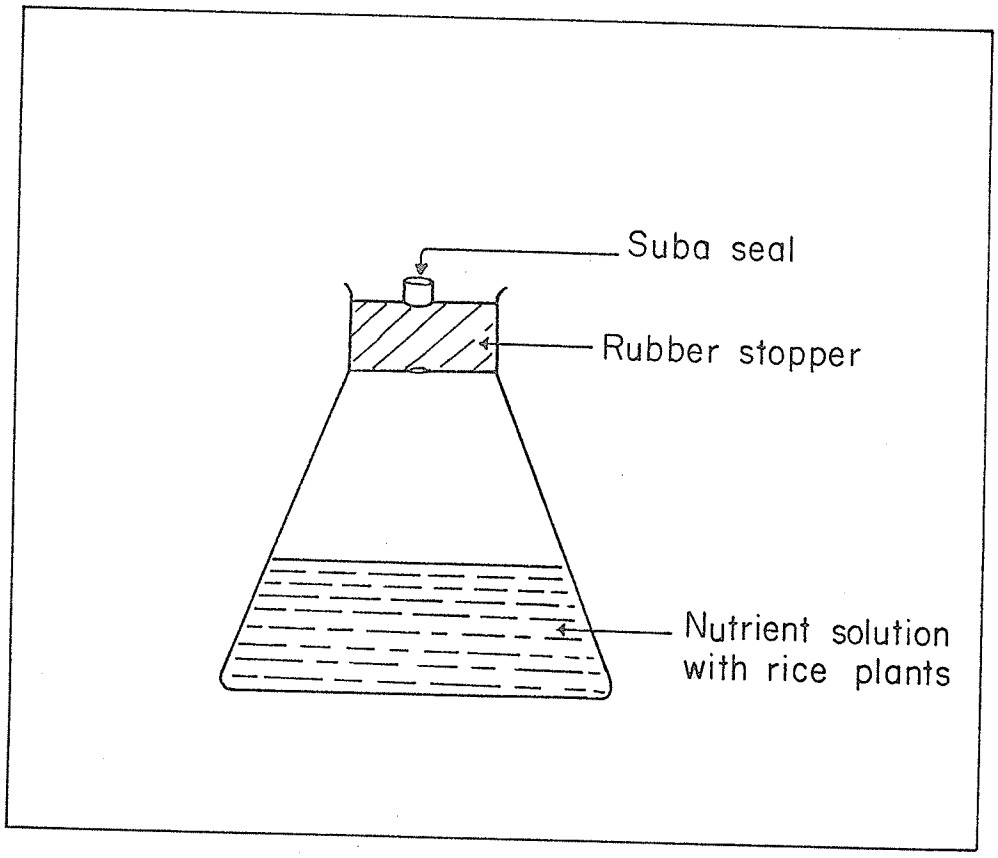

To flasks B_2 and C
Air supply
1% $NaHCO_3$
25 % H_2SO_4
Water
Culture solution
sand
A
B_1

Aeration for the seedlings during incubation was provided by a system as shown in Fig. 9a. Cotton filtered air was first bubbled through an aqeous solution (1%) of $NaHCO_3$ and then through a 25% solution of H_2SO_4 in water to remove oxides of nitrogen and ammonium respectively. This treated air was then bubbled through water and thence through a second cotton wool filter for sterilization before entering the first seedling flask in the series. Cotton wool filtration was also provided for the air supply between each flask in the series.

This seedling flask assembly was incubated in a controlled environment chamber with diurnal lighting and temperature cycles as previously described in Section 3 dealing with algal studies.

After 30 days incubation under these conditions, the air lines were temporarily disconnected, the flask stoppers bearing the air inlet connections were removed and the seedling flasks were recapped with sterile rubber stoppers each carrying a Suba seal port as noted in Fig. 9b. Each of the four flasks then received 50 ml of acetylene gas by syringe. After several hours further incubation, atmosphere samples were removed from each flask by syringe and

Fig. 9b. Schematic diagram of 'Suba' seal/ stopper arrangement used for acetylene gas introduction during seedling culture experiments.


Suba seal
Rubber stopper
Nutrient solution
with rice plants

analyzed for ethylene produced. This sampling and analysis procedure was repeated several times for each flask at intervals of several hours. Following this, the air supply system was reattached and all flasks were aerated during a further four days incubation to completely remove any residual ethylene or acetylene. When evidence for the complete removal of these gases was obtained by gas chromatography, the flasks were again capped with sterile rubber stoppers and each received 25 ml of freshly generated acetylene. Analyses for ethylene produced was again carried out at intervals of several hours.

During the 30 day incubation period, samples of the nutrient solution in flasks B_1 and B_2 were removed periodically by pipette for microscopic examination of the microbial population. Plate counts were made before and after the incubation period. Total nitrogen by micro Kjeldahl and nitrate-nitrogen determinations were also made before and after incubation.

(2) Seedlings at the floating leaf stage

These more mature plants were treated in much the same way as were the young seedlings except that

no sand substrate was used in the culture flasks. Representative plants from location A were gently uprooted with the soil still attached and immediately transported in plastic bags to the laboratory. Here, root soil was gently removed under running tap water and most of the older leaves were trimmed off. Five to six plants were then carefully transferred to each of three wide mouth flasks as before containing only the appropriate nutrient solutions; these were designated #1, #2a and #2b. Plant stems were loosely tied to a glass rod support which, at its upper end, passed through the rubber stopper enclosing the flask. The flask assembly was aerated as before. Flask #1 contained the complete nutrient solution while flasks #2a and #2b contained the nitrogen-free medium with glucose. The microflora on the individual plant roots surfaces provided the inoculum, and incubation continued for 17 days under the same controlled environment conditions previously described. After incubation, 50 ml of acetylene gas was introduced to flasks 1 and 2a by syringe followed by atmosphere sampling at intervals for ethylene analysis in all three flasks as before.

Microscopic examination was made in all the flasks at intervals. Because earlier analysis showed no detectable total nitrogen or nitrate in the nitrogen-free medium, no determinations for combined nitrogen in the medium were made in this experiment.

X Distribution of nitrogen-fixing bacteria along root surfaces

The objective of this experiment was to determine if any zonal distribution pattern of nitrogen-fixation microorganisms existed along the length of individual plant root systems.

Soil adhering to the roots of freshly collected plants from location A was removed by washing with sterile tap water under aseptic conditions. A number of well developed roots of approximately 6 - 10 cm total length were excised aseptically from each of several plants and transferred to sterile petri dishes. These roots were cut aseptically into three portions with sterile scissors and forceps as follows: root tips, 2-4 cm; mid-sections, 2-3 cm and root-stem sections including about 1-2 mm of stem base.

Ten to fifteen pieces representing each root region were transferred to sterile 50 ml flasks

containing moist filter paper. Flasks were capped with sterile Suba seals, evacuated by syringe and then flushed with sterile filtered helium gas. Acetylene (0.5 ml) was introduced in each case to replace a similar volume of flask atmosphere removed by syringe. Duplicate flasks for each root region were thus prepared and, in addition, one flask containing pieces of intact root without added acetylene served as a control. All flasks were incubated at 25°C and atmospheric analysis for ethylene was undertaken at intervals. Root pieces from each flask were weighed in aggregate at the end of the incubation period.

This experiment was performed on roots at two stages of plant development, the late floating-leaf stage and at the aerial leaf stage.

XI Assessment of an increase in numbers of nitrogen-fixing bacteria on root surfaces during the growing season.

Root samples were taken as previously described in Section V every 2 to 3 weeks over a period of two and one-half months during May, June and July 1973.

After removal of adhering soil material, roots

were rinsed several times in sterile distilled water and then mascerated under aseptic conditions by grinding in sterile moist sand. Numbers of nitrogen-fixing facultative and anaerobic bacteria were determined on the mascerate at the time intervals stated by a most probable number (MPN) method (Campbell and Evans 1969). Turbidity and ethylene production in the MPN series were used as indices of a positive result.

XII Nitrogen-fixation in the water column of wild rice habitat as a possible additional source of plant nitrogen supply.

As noted by Dore (1969) and by Thomas and Stewart (1969) the water regime of wild rice habitat in terms of its depth and chemistry plays a complex role in plant development. It is not inconceivable that nitrogen-fixation occurring therein could contribute to the nitrogen budget of these plants.

A series of three experiments were conducted during May, June and July 1973 at times coincident with the submerged, floating and aerial leaf stages of development. These experiments essentially consisted of measurements of nitrogen-fixation potential (by acetylene reduction) at a depth of one metre in the water column surrounding the developing plants.

Nine 250 ml pyrex flasks were used in each experiment. Four of these were tightly wrapped with aluminum foil which was then covered by waterproof masking tape. At the experimental site, all flasks were filled with water at a depth of approximately one metre. While still submerged, each flask was fitted with a rubber stopper through which two glass tubes had been inserted. One tube extended well down into the flask, the other tube protruded only slightly below the rubber stopper.

Eight of the nine flasks were then divided into two sets of four, each set consisting of two covered flasks (dark) and two flasks not covered (light); one flask was set aside for subsequent use as a control.

Four flasks in one set plus the control flask each received 50 ml of an atmosphere of oxygen/argon (22:78); each of the remaining four flasks in the second set was provided with a similar volume of helium. Atmospheres in each instance were introduced by a downward displacement of 50 ml of the water sample using tubing connections to appropriate gas cylinders. Displaced water samples were individually collected in 250 ml acid washed Nalgene screw-cap

bottles for analysis[1] on return to the laboratory.

All flasks except the control then received 25 ml of freshly generated acetylene after an equivalent volume of flask atmosphere had been withdrawn by syringe. Thus, duplicate flasks corresponding to each of the following incubation conditions were provided:

light-aerobic; light anaerobic; dark aerobic and dark-anaerobic

The flasks were then tethered to wooden stakes which were driven into the sediment such that the flasks were carried to a depth of about one metre.

After 24 hours incubation 'in situ' under these conditions, the flasks were recovered. Gas samples of the flask atmosphere were removed by syringe to water filled 7 ml vials capped with 'Suba' seals fitted with two hypodemic needles. As the gas sample was introduced by syringe through one of the needles, water in the vial was displaced through the needle open to the atmosphere. Both needles were then removed immediately. Vials filled with gas samples in this way remained air tight and were returned to the laboratory for immediate chromatogra-

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[1] Ammonium,-nitrate-and nitrite-nitrogen determined by the methods of Strickland and Parsons (1968).

phic analysis. After 48 hours further biological activity in the incubated flasks was then terminated by the addition to each flask of 5.0 ml of 50% trichloroacetic acid as recommended by Stewart (1970). Flasks were then returned to the laboratory for gas analysis. Microscopic examination was made of the water sample brought to the laboratory during the second algal bloom. Component genera of the bloom were subsequently identified.

R E S U L T S

RESULTS

General properties of the habitat soil. (Soil Chemical Analysis - Experimental IV).

Values for moisture content, total organic matter, ammonium-and nitrate-nitrogen of soils from Locations A and B are presented in Table I. The much higher values for organic matter content noted for soils at location B might be expected since, in this area, the shorelines have an overhanging canopy of trees and shrubs whose Fall leaf litter, branches and the like provide a large annual input of organic matter to the adjacent water system. Over a great many years, this would tend to accumulate to a greater degree in the relatively current-free bottom sediment. The greater density of plant species here might also account inpart for the lower combined nitrogen levels noted in terms of the flora demand on the soils' available nitrogen pool.

The LaSalle river, on the other hand, passes through farmlands from which much of the native vegetation has been removed. The river bank areas do not display the same density of foliage and plant species noted at location B. As well, the river

Table I. Soil moisture, organic matter, ammonium- and nitrate-nitrogen measurements in *Zizania* habitat at locations A and B Summer 1972.

Component measured	Location A(LaSalle R.)	Location B(Lone Island Lake)
Moisture content (percent)	42.0	84.0
Total Organic Matter (percent)	3.36	34.25
Ammonia-Nitrogen (ppm)[1]	3.87 (range, 0.53-11.73)	2.98 (range, 0.53-8.23)
Nitrate-nitrogen (ppm)[1]	3.69 (range, 1.75-6.30)	1.94 (range, 0.70-4.20)
Dissolved Oxygen, ppm (at depth of 1.0 mm)	nd	15.0

[1]Values given represent average of 20 sample determinations in each case.
n.d. not determined

receives runoff from adjacent farmlands where chemical fertilizers have been used and where animal populations have been reared. This input would tend to raise the combined nitrogen levels, particularly the ammonium- and nitrate-nitrogen, noted in Table I. Although it might be expected that the soils at location A with their higher clay content would show greater moisture holding capacities, soils from location B gave moisture content values of over eighty percent. This much higher moisture retention is attributable to the very large organic matter component with its associated colloidal properties.

Numbers of nitrogen-fixing bacteria in rhizosphere soils and on roots of Zizania as determined by the MPN method and by plate counts using nitrogen-free agar media are given in Table II. Total counts for each soil and for roots in both locations are quite similar; for nitrogen-fixing soil anaerobes, the area comparison numbers are essentially the same. It is also apparent from MPN data in Table II that in location A the anaerobic/facultative nitrogen fixers are evenly distributed between root surfaces and the soil though slightly higher in numbers on roots than in rhizosphere soils.

Table II. Most probable number estimates and plate counts[1] of bacteria in rhizosphere soils and on root surfaces of Zizania aquatica. Summer 1972.

ESTIMATED BY	NUMBERS/g AT LOCATIONS A	B
MPN (FACULTATIVE/ANAEROBIC)		
SOILS	7.90×10^3	4.90×10^3
ROOTS	2.30×10^5	2.30×10^3
PLATE COUNTS		
SOILS		
(AEROBES)	6.03×10^7	5.0×10^6
(FAC/ANAEROBES)	4.10×10^5	4.00×10^5
ROOTS		
(AEROBES)	1.90×10^6	1.06×10^6
(FAC/ANAEROBES)	2.09×10^6	1.20×10^6

[1]FOR AEROBES WITH AZOTOBACTER MEDIUM: FOR FACULTATIVE/ANAEROBIC ORGANISMS, A MODIFIED HINO AND WILSON'S MEDIUM.

Isolation and identification of nitrogen-fixing bacteria

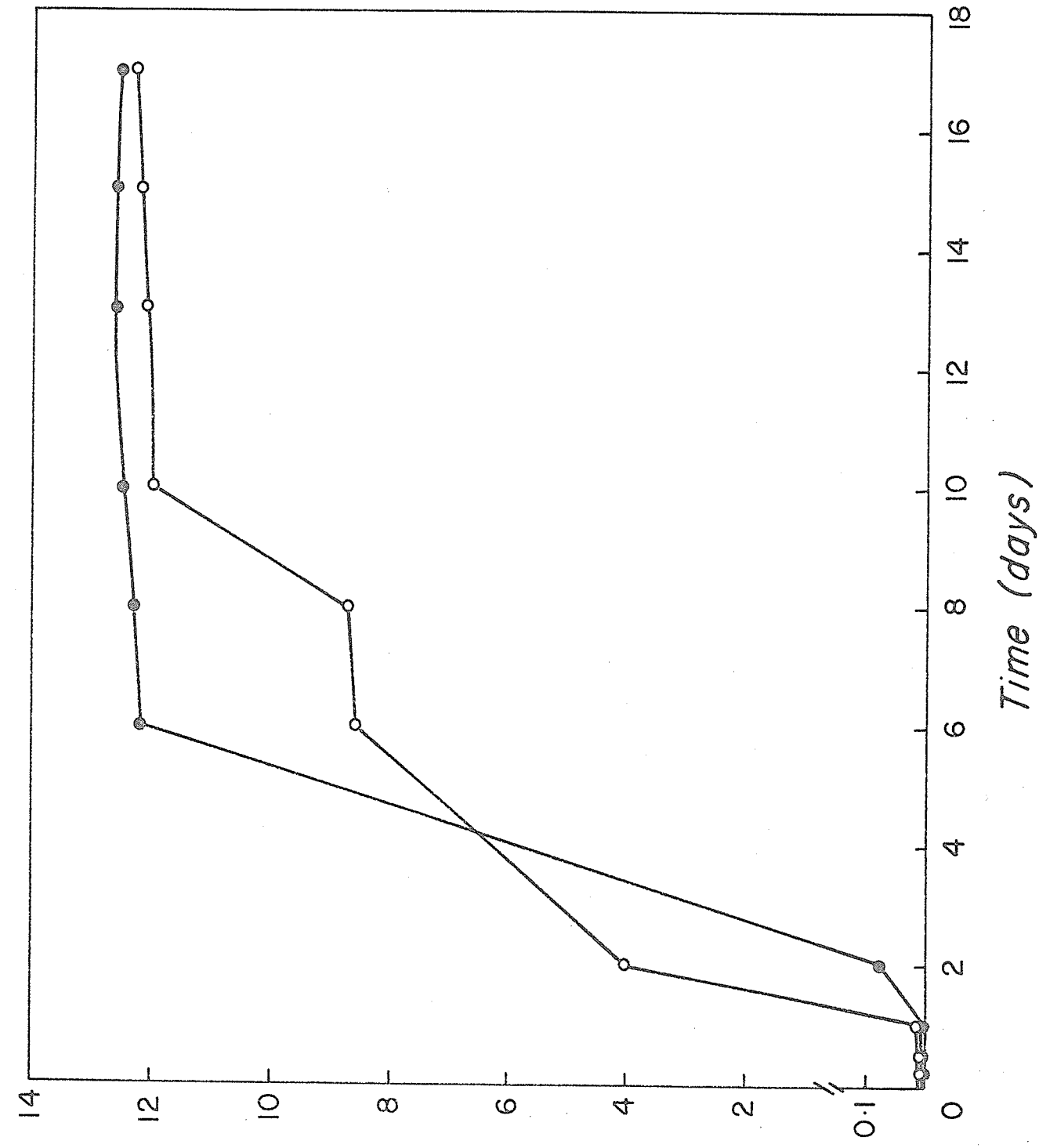
A total of seven isolates were obtained by nitrogen-free agar platings and MPN determinations on rhizosphere soils and roots of *Zizania* plants as described. These comprised two strict anaerobes, four aerobes and one facultative anaerobe. Of these, only three, designated F_1, F_2 and F_3 consistently demonstrated nitrogenase activity by reducing acetylene to ethylene although the remaining four gave weak colonial development on nitrogen-free agar media.

On the bases of the morphologic, cultural and biochemical characteristics as detailed in Appendix A, the anaerobic isolate F_1 was identified as *Clostridium butyricum* as described by Breed *et al* (1957) and by Prévot (1965); isolate F_2 similarly was identified as *Clostridium beijerinckii*. A review of characteristics displayed by isolate F_3 suggested it was a strain either of *Azotobacter chroococcum* or *Azotobacter beijerinckii*.

Acetylene reduction in rhizosphere soils

Acetylene reduction by rhizosphere soil samples from both locations is indicated in Fig. 5. Gas phases above the soils were anaerobic and incubation continued for more than two weeks. Although ethylene values

Fig. 5. Acetylene reduction by rhizosphere soils from locations A and B. Each value plotted represents an average of triplicate determinations. Legend: closed circles, location A; open circles, location B.


nmoles C_2H_4/gm wet weight rhizosphere soil
14
12
10
8
6
4
2
1·0
0
2
4
6
8
10
12
14
16
18
Time (days)

were low in both samples, material from location A (LaSalle) gave slightly higher ethylene production than that from location B at Lone Island Lake. This result parallels the MPN comparisons noted in Table 2. After an initial lag phase of about 24 hours duration, ethylene production was rapid over the next three days particularly for the soil sample from location A. In the LaSalle sample increases in ethylene values after this period were minimal but the soils from location B provided a further rapid acetylene reduction for an additional 48 hours. This phenomenon has been reported by other investigators, Rice and Paul (1971), but the reason for it has not been explained.

Since incubation conditions were anaerobic and dark, acetylene reduction could only occur through the activities of facultative or anaerobic bacteria for endogenous ethylene was not detected in the controls.

Acetylene reduction by populations on the root surfaces.

Acetylene reduction by microorganisms on wild rice roots from locations A and B are noted in Fig. 6a and 6b respectively. After a four day lag phase,

Fig. 6a. Acetylene reduction by roots of Zizania aquatica (L) collected from the LaSalle River area, October 1972. Each value plotted represents the average of duplicate determinations.

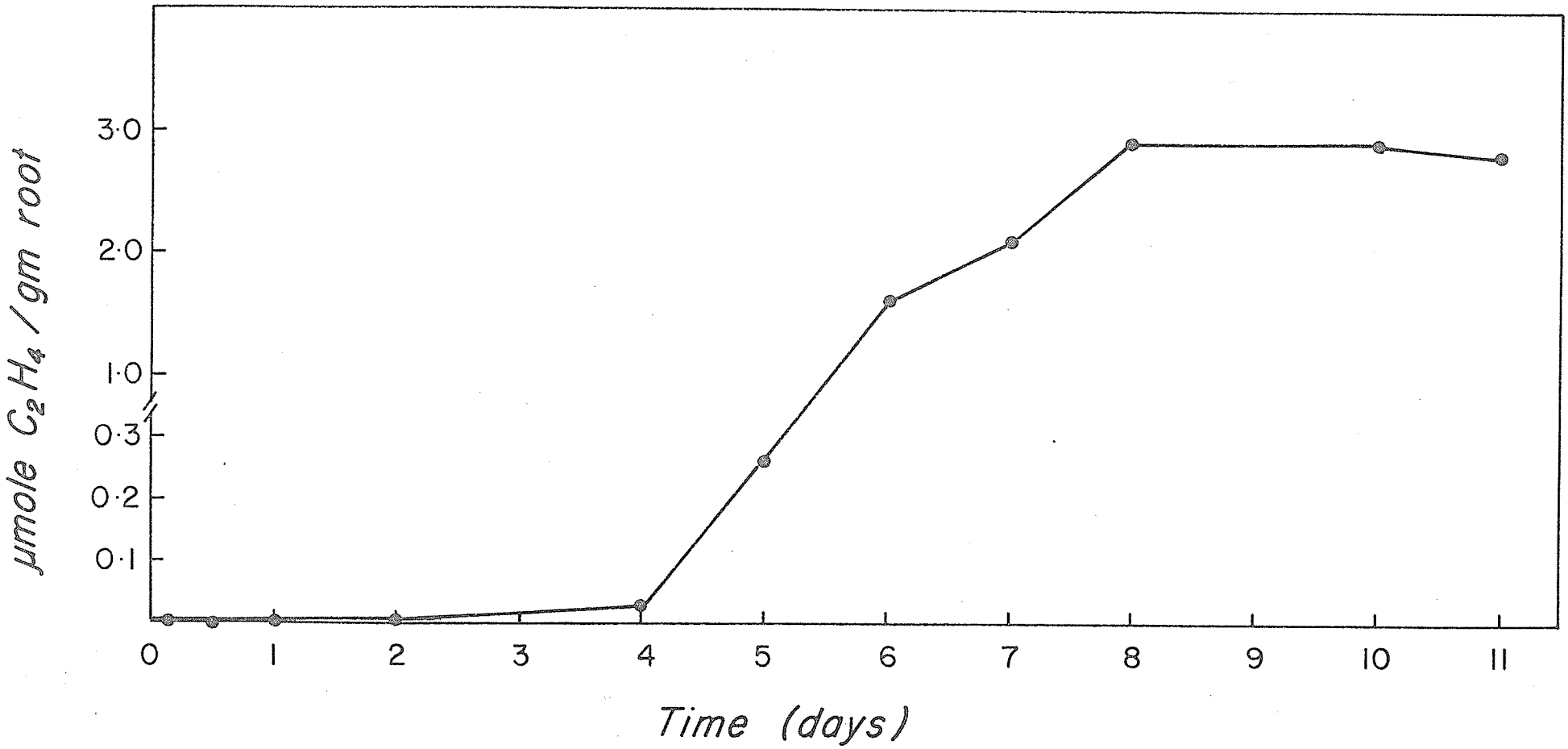
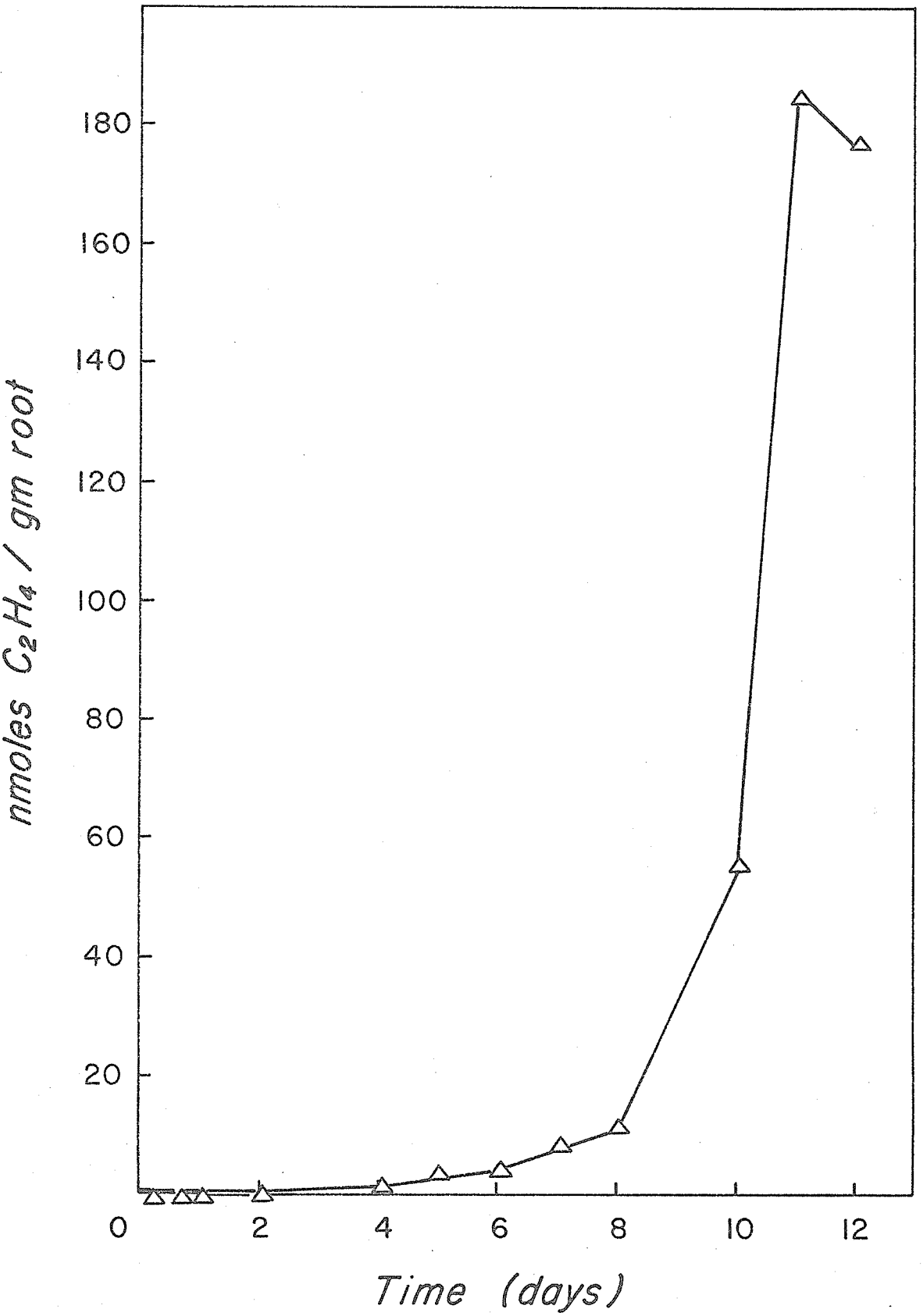

μmole C_2H_4 / gm root
3·0
2·0
1·0
0·3
0·2
0·1
0
1
2
3
4
5
6
7
8
9
10
11
Time (days)

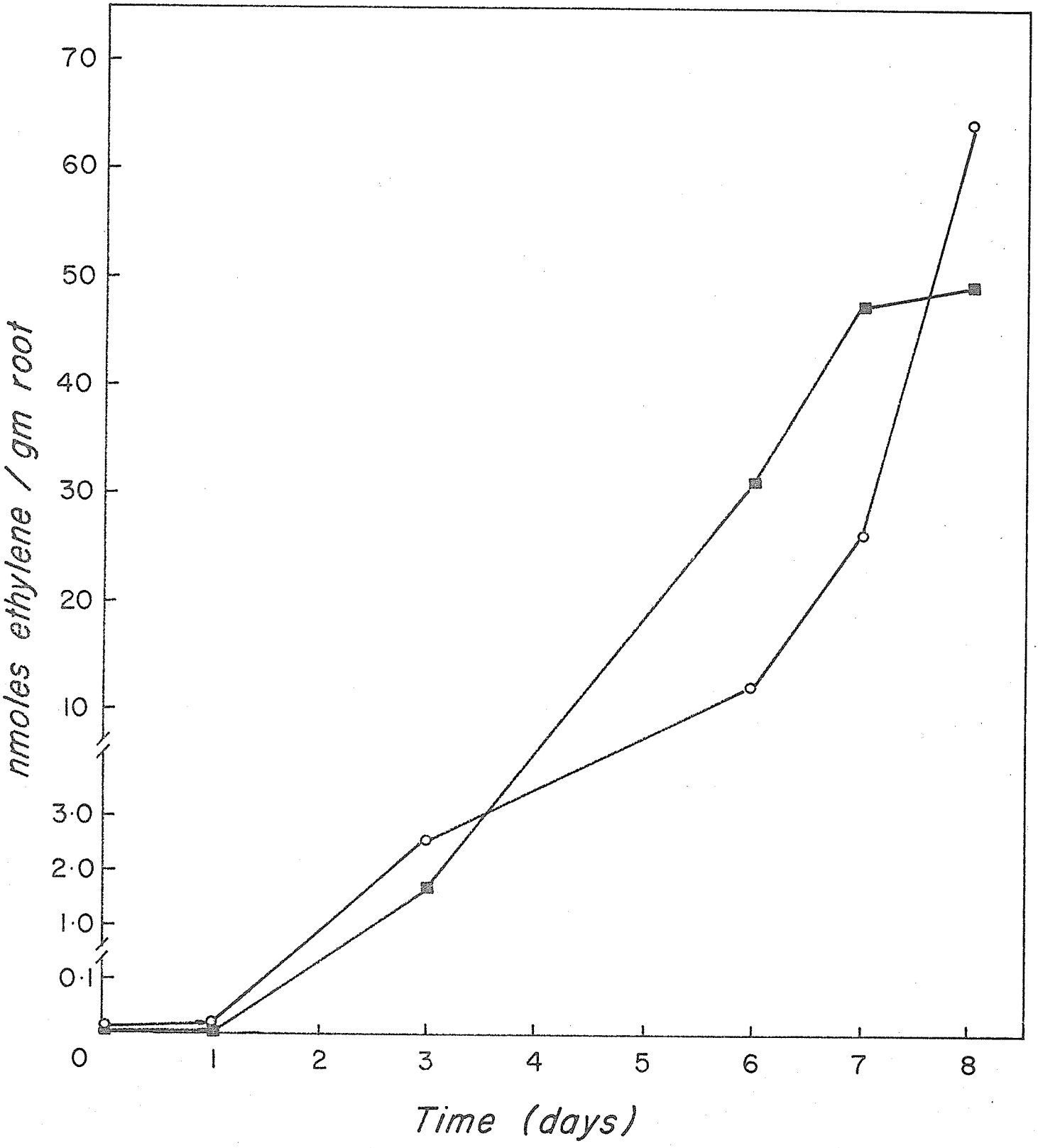
Fig. 6b. Acetylene reduction by roots of Zizania aquatica (L) collected from Lone Island Lake area, October 1972. Each value plotted is the average of duplicate determinations.


180
160
140
120
100
80
60
40
20
0
2
4
6
8
10
12
nmoles C_2H_4 / gm root
Time (days)

acetylene reduction by both root samples was observed though at a somewhat slower rate in the root samples from location B, Fig. 6b. For roots from location A, ethylene production was linear with time reaching a peak value of over 2500 n moles/g root sample by the eighth day of incubation. After an eight-day lag period, samples from location B showed a more rapid rate of acetylene reduction and reached a peak value of 185 n moles/g roots by the end of the eleventh day of incubation. In the next 24 hours incubation, however, a significant decrease in ethylene values was observed. Again, since incubation was in the dark and flask atmospheres were anaerobic, acetylene reduction could be ascribed only to facultative/anaerobic nitrogen-fixers.

Acetylene reduction in the presence of young roots harvested during the submerged-leaf stage at location A is shown in Fig. 6c. Since some of these thin wiry roots were sampled from the sediment surface as well as from below the sediment surface, it might be expected that the exposed roots would be in a more aerobic environment. If this was the case, they

Fig. 6c. Acetylene reduction by roots of Zizania aquatica (L) at the submerged leaf stage, early spring 1973. Incubation conditions represented by closed squares are aerobic; by open circles, anaerobic.


70
60
50
40
30
20
10
3·0
2·0
1·0
0·1
0
1
2
3
4
5
6
7
8
nmoles ethylene / gm root
Time (days)

would harbour, in part, an aerobic population of microorganisms. Roots incubated under anaerobic conditions gave somewhat higher ethylene values then those incubated aerobically. It should be noted however, that ethylene production was considerably lower in both cases than that observed for older roots, Fig. 6a. This no doubt reflects the considerable differences in numbers of nitrogen-fixing organisms that is seen when comparing root systems of varying degrees of maturity.

Possible N-fixing contributions by rhizosphere algae.

Time courses of nitrogenase activity in terms of ethylene production by rhizosphere soil incubated under light and dark conditions are presented in Fig. 7a and 7b. The ethylene values (in n moles) noted in Fig. 7a excludes endogenous ethylene production which is given in Fig. 7b where ethylene values derived under dark incubation are also noted. Endogenous ethylene production was considerable in this case being higher than that observed under dark incubation conditions up to the first 80 hour period. After 80 hours incubation, however, ethylene production in the dark environment first increased, then decreased

Fig. 7a. Acetylene reduction by upper layer rhizosphere soils from the LaSalle River area, 1973, incubated aerobically under light conditions. Each value plotted is an average of triplicate determinations.

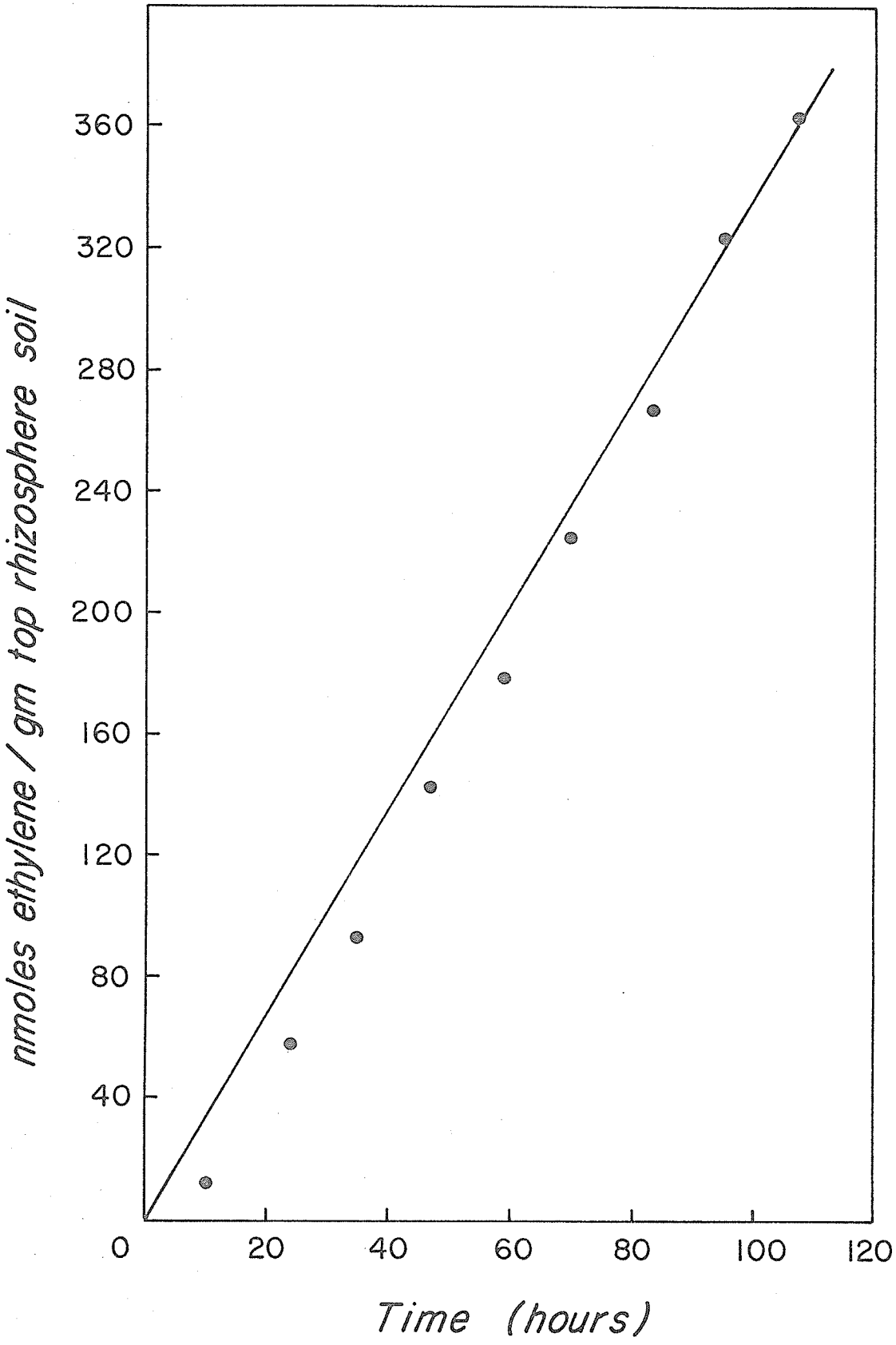
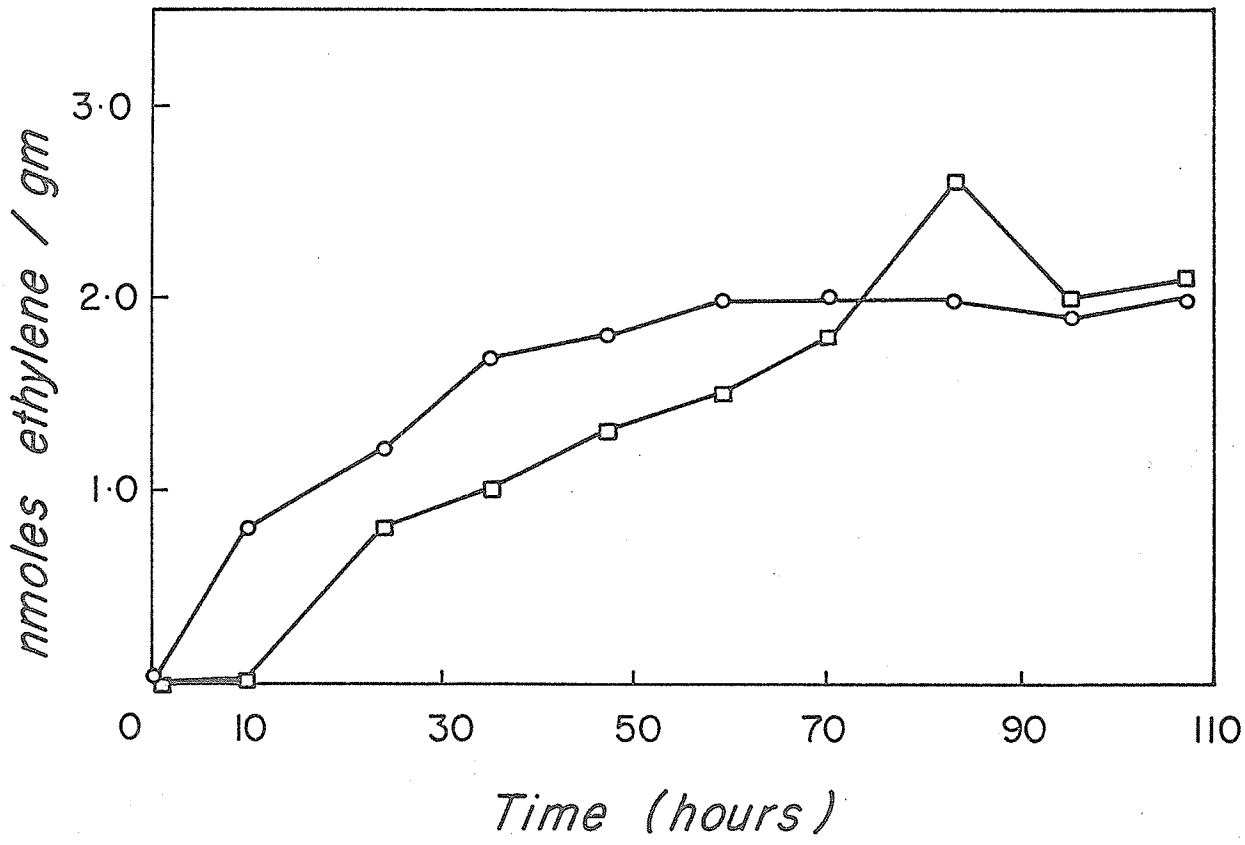

nmoles ethylene / gm top rhizosphere soil
360
320
280
240
200
160
120
80
40
0
20
40
60
80
100
120
Time (hours)

Fig. 7b. Ethylene production by rhizosphere soil samples incubated aerobically under light and dark conditions. Both sets of flasks were pre-incubated in the dark for 17 days before acetylene was added to soil flasks of the dark series only. Legend: open circles, no acetylene added and flasks incubated in the dark.


nmoles ethylene / gm
3·0
2·0
1·0
0
10
30
50
70
90
110
Time (hours)

and then increased again to provide the two peaks noted in this Figure. While there was a short lag phase before ethylene production in the dark bottles, endogenous ethylene evolution increased slowly with time for the first 30 hours incubation before levelling off. A similar endogenous production of ethylene has been noted by Rice and Paul (1972).

By comparison, nitrogenase activity in dark bottles was very much lower than under illuminated conditions where ethylene production was above 360 n moles/g wet weight of soil. Although Wolk (1973) indicated that limited algal fixation of nitrogen could occur in the dark, our results do not support this contention.

Wild rice plants cultured in a nitrogen-free medium in the presence and absence of nitrogen-fixing bacteria.

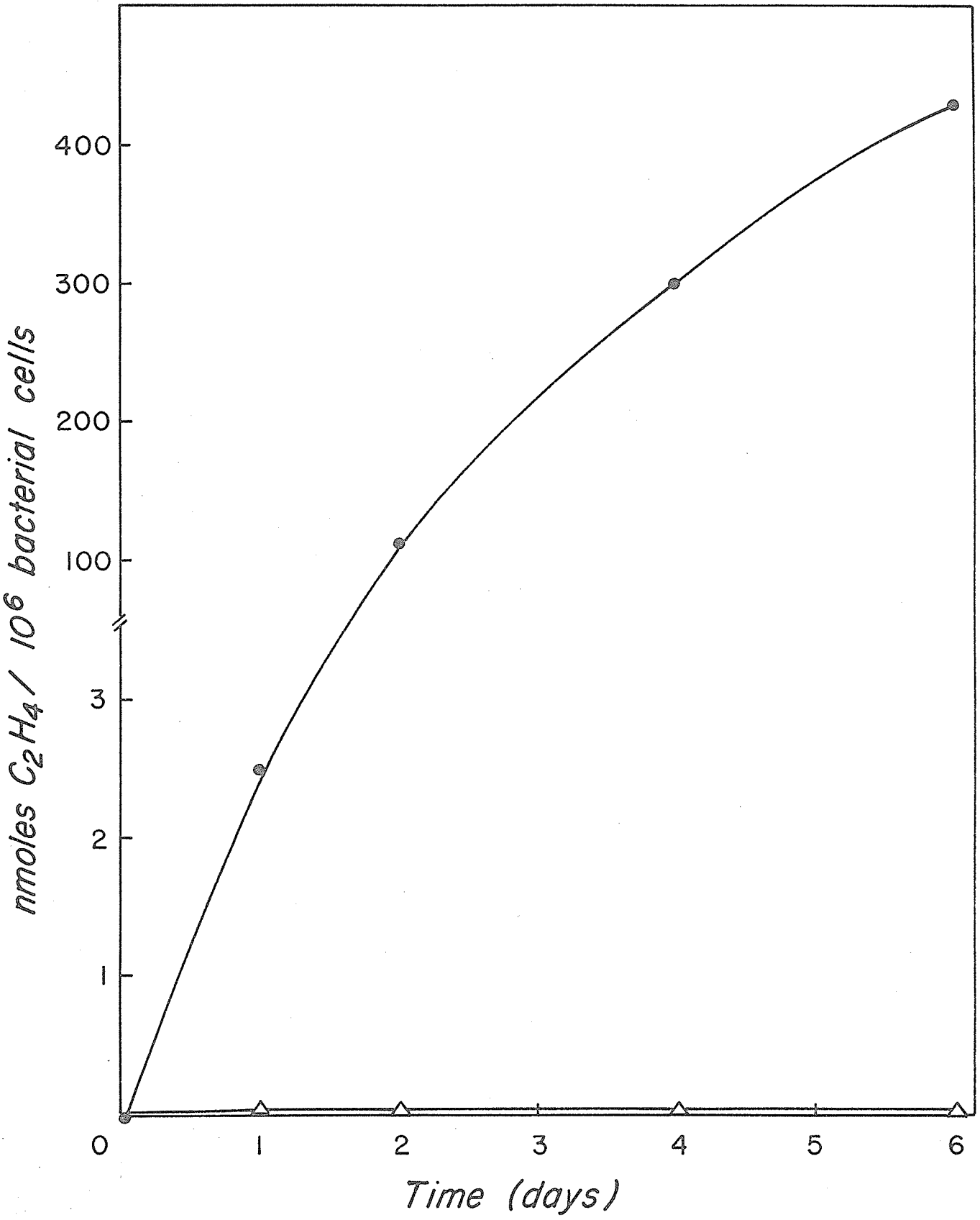
Experiments to determine if nitrogen fixed by rhizosphere microorganisms could sustain growth of wild rice seedlings cultured in a nitrogen-free but otherwise complete medium, were not conclusive.

In this experiment, young seedlings appear to develop normally for about 17 days after which symptoms of nitrogen deficiency develop. Symptoms were first observed in plants in Flask C which contained neither

combined nitrogen nor a bacterial inoculum. Plants grown in Flasks A, B_1 and B_2 showed no visible increase in size but retained their green color over a longer period before showing nitrogen deficiency symptoms. It was observed that during the course of this experiment, many of the young seedlings became covered by a fungal mycelium. These fungi were not identified but could have represented any of a number of fungal pathogens reported for Zizania species. This fungal mass was densest in Flask A which contained a complete culture medium.

Following the rapid proliferation of the bacterial inocula in Flasks B_1 and B_2 as observed by sharp increases in turbidity, a dark green color which increased with time developed in Flasks A, B_1, B_2 and C. Microscopic examination therein revealed large numbers of bacteria and algae. The latter were subsequently identified and are reported in Table III, page 58. Nitrogen fixation as indicated by acetylene reduction, Fig. 8, occurred only in those flasks inoculated with the Azotobacter and Clostridium isolates. Bacterial cells observed in Flask A were likely contaminants from the seedlings themselves. These were not isolated nor identified hence their nitrogen fixation potential is unknown. If they were nitrogen-

Fig. 8. Acetylene reduction in wild rice plant cultures grown in nitrogen-free nutrient media in the presence and absence of nitrogen-fixing microorganisms.
Legend: closed circles, average of values from Flasks B_1 and B_2; open triangles, values from Flask A (complete medium with combined nitrogen).


400
300
200
100
3
2
1
0
1
2
3
4
5
6
nmoles C_2H_4 / 10^6 bacterial cells
Time (days)

fixers, their nitrogenase activity was probably suppressed under the experimental conditions pertaining. Total bacterial counts in Flasks B_1 and B_2 at the end of the incubation period was markedly lower than could reasonably be accounted for by the numbers of Azotobacter and Clostridium cells introduced initially as inoculum. Reisolation experiments at the end of the incubation period gave indication of some small degree of contamination.

Kjeldahl analysis of the culture medium in Flasks B_1 and B_2 after incubation gave average values of 88.9 µg total N and 529 µg NO_3-N/l. Part of this combined nitrogen is derived, presumably, from dead and lysed cells and from exudate of the microflora present.

Seedlings at the floating-leaf stage

For the first ten days of this experiment, plants in all three flasks displayed a relatively healthy physiological condition as evidenced by a rapid vegetative development, the plants in the nitrogen-free flasks probably depending on their endogenous combined nitrogen for this initial growth. Microfloral proliferation in the culture solution, as indicated by turbidity, was observed first in Flasks 2a and 2b. Subsequently, in all three flasks, a dense algal crop developed. The algal populations

subsequently identified are also listed in Table III.

Considerable acetylene reduction (nitrogen fixation) occurred in nitrogen-free flasks. No acetylene reduction, however, occurred in Flask 1 where a complete medium (including combined nitrogen) was available. Although the microscopically-observed bacteria herein were not isolated nor identified, they were probably members of the genera Azotobacter and Clostridium since these have been shown to inhabit the rhizosphere of Zizania.

After the initial flush of new leaves and generally good vegetative growth, the seedlings were very rapidly damaged by fungal attack.

Distribution of nitrogen-fixing bacteria along root surfaces.

Results of experiments to determine the relative numbers of nitrogen-fixing bacteria along root surfaces at two stages of development of the wild rice plant are presented in Figs. 10a and 10b. At these two stages of development, floating-leaf and aerial-leaf stages, root development is under the sediment surface; accordingly, all incubations of root pieces were provided with anaerobic conditions.

Table III. Algal components observed in Zizania habitat.

(c.f. Expt. IX)

Seedling Stage	Family	Genera	Species
Young seedlings	Chrysophyceae	Ochromonas	granulata
Floating-Leaf Stage	Chrysophyceae	Ochromonas*	nd**
	Chrysophyceae	Mallomonas	nd
	Chlorophyceae	Chlorelle*	nd
	Chlorophyceae	Scenedesmus	2 spp
	Chlorophyceae	Oocystis	nd
	Chlorophyceae	Ankistrodesmus	braunii
	Cyanophyceae	Oscillatoria	nd
	Euglenophyta	Euglena	nd

*Most abundant Genera

**nd - not determined

Fig. 10a. Acetylene reduction by root sections of Zizania aquatica (L) at the floating leaf stage, June 1973. Legend: open circles, root-base pieces; open squares; root-tip pieces; closed circles, mid-root sections.

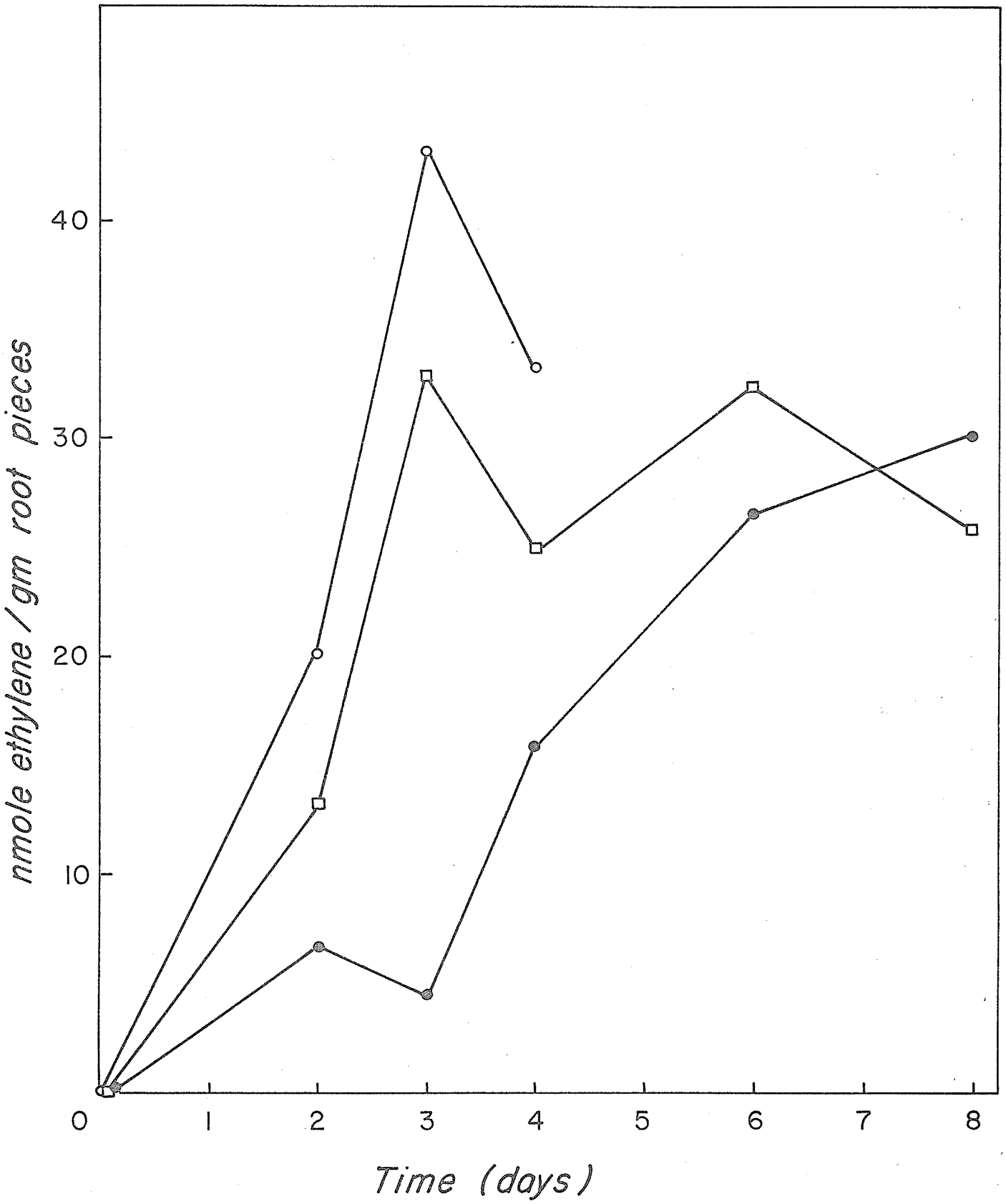
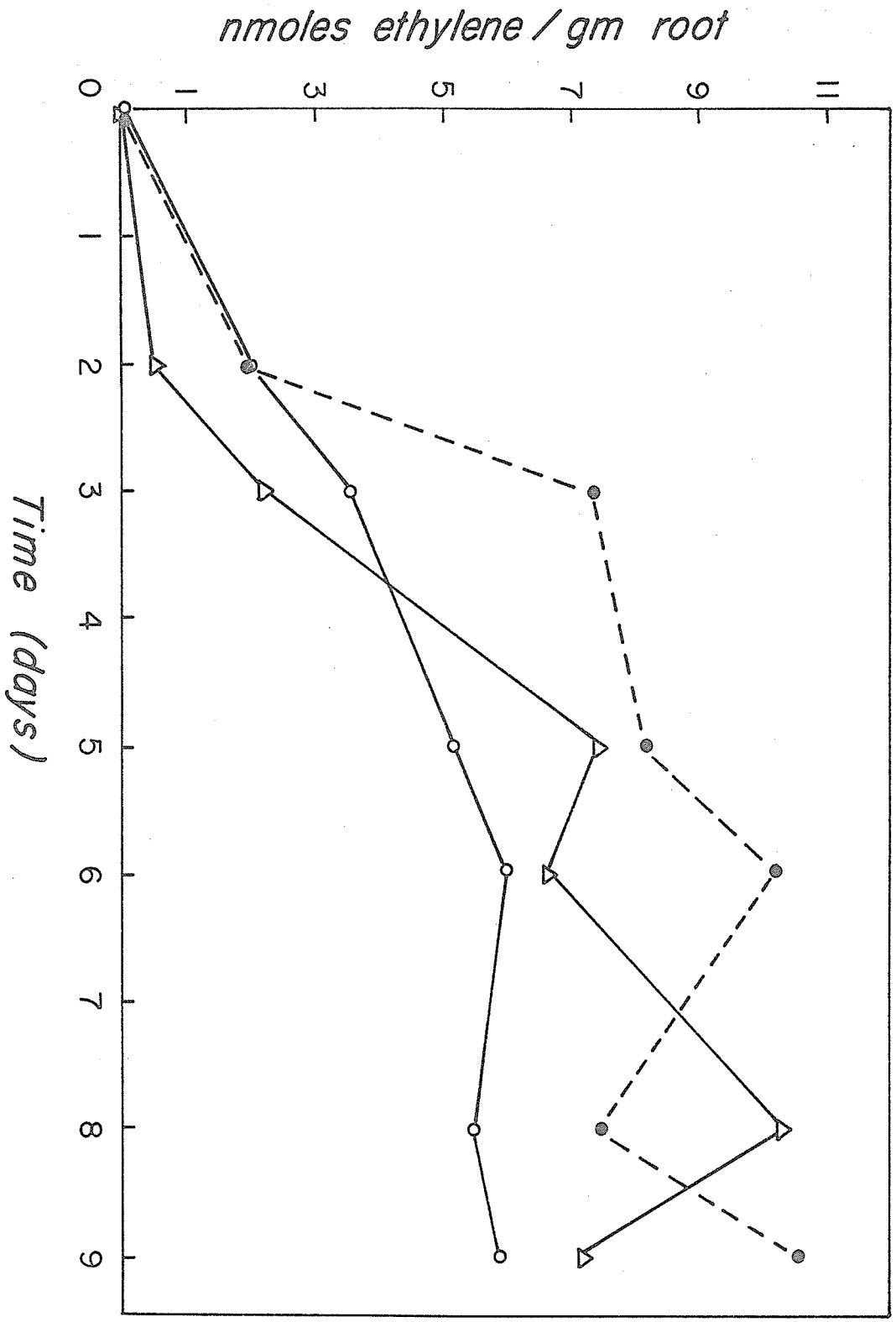

nmole ethylene / gm root pieces
40
30
20
10
0
1
2
3
4
5
6
7
8
Time (days)

Fig. 10b. Acetylene reduction by root sections of Zizania aquatica (L) at the early aerial-leaf stage, July 1973.
Legend: open circles, root base pieces; closed circles, root-tip pieces; open triangles, mid-root sections.


nmoles ethylene / gm root
1
3
5
7
9
11
0
1
2
3
4
5
6
7
8
9
Time (days)

As noted in the results presented in Fig. 10a (floating-leaf stage) a higher population of nitrogen fixers colonized the root base than in the other two regions; the root-tip harbours a larger population of nitrogen-fixers than does the middle region of the root.

The nitrogen-fixing population during the aerial-leaf stage is slightly different. Here, the lowest ethylene values were derived from the root-base region while acetylene reduction values for both the root-tip and the middle region are slightly higher, Fig. 10b.

At both stages of development, the overall population as reflected by ethylene production was not high. Sudden increases and decreases in nitrogenase activity were frequent.

Increases in numbers of nitrogen-fixing bacteria on root surfaces during the growing season.

The plant root-microorganism relationship is even more noticeable from the MPN values presented in Table IV. Here, results are presented of the increase in numbers of nitrogen-fixing bacteria on root surfaces during the growing season. Here, the abrupt rise and fall in numbers of nitrogen-fixing

Table IV Most probable number (MPN) estimates of facultative/anaerobic n-fixers on root surfaces.

	Date		
	30 May 73	16 June 73	15 July 73
MPN	1.50×10^5	4.90×10^7	2.30×10^4

bacteria on root tissue during five weeks of the growing season is particularly evident. The highest value was obtained at the floating-leaf stage (Table IV) and subsequent experiments indicate that this stage appears to be a period during which a considerable amount of nitrogen is introduced into the ecosystem.

Nitrogen-fixation in the water column of wild rice habitat.

As measured by acetylene reduction, nitrogen fixation in the water column at location A during May, June and July 1973 is indicated in Figs. 11a, 11b and 11c. Measurements were made on samples collected at 2-3 week intervals. Ethylene values were calculated as total ethylene in the 50 ml gas phase per 200 ml of water sample.

Acetylene reduction in the water column during the submerged-leaf stage (May) is noted in Fig. 11a. Here, the highest values occurred during the first 24 hours of incubation of sample under dark conditions. By 48 hours incubation, however, ethylene values decreased rapidly. Under illumination conditions, incubated samples showed a slow but steady acetylene reduction.

Fig. 11a. Acetylene reduction in water samples from location A, May 1973 incubated 'in situ' under light and dark conditions. Each value plotted is the average of duplicate samples.

Legend: ⊙------⊙, light, anaerobic

●————●, light, aerobic

o————o, dark, aerobic

▲————▲, dark, anaerobic

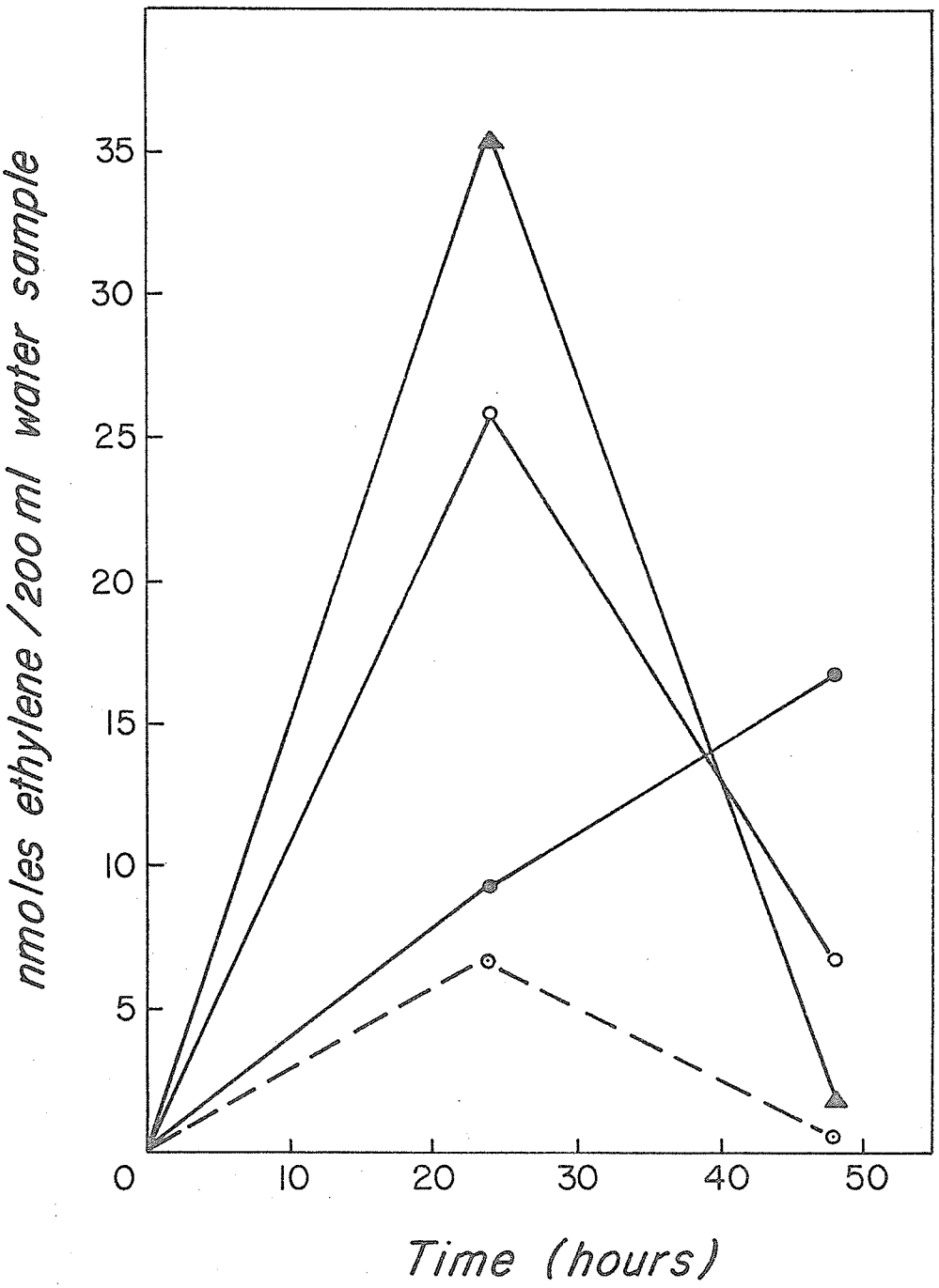

nmoles ethylene / 200 ml water sample
35
30
25
20
15
10
5
0
10
20
30
40
50
Time (hours)

Fig. 11b. Acetylene reduction in water samples from location A, June 1973, incubated 'in situ' under light and dark conditions. Each value plotted is the average of duplicate samples.

Legend: ●-·-·-·-·-·-●, Light, anaerobic

●————●, Dark, anaerobic

o— — —o, Light, aerobic

▲— — —▲, Dark, aerobic

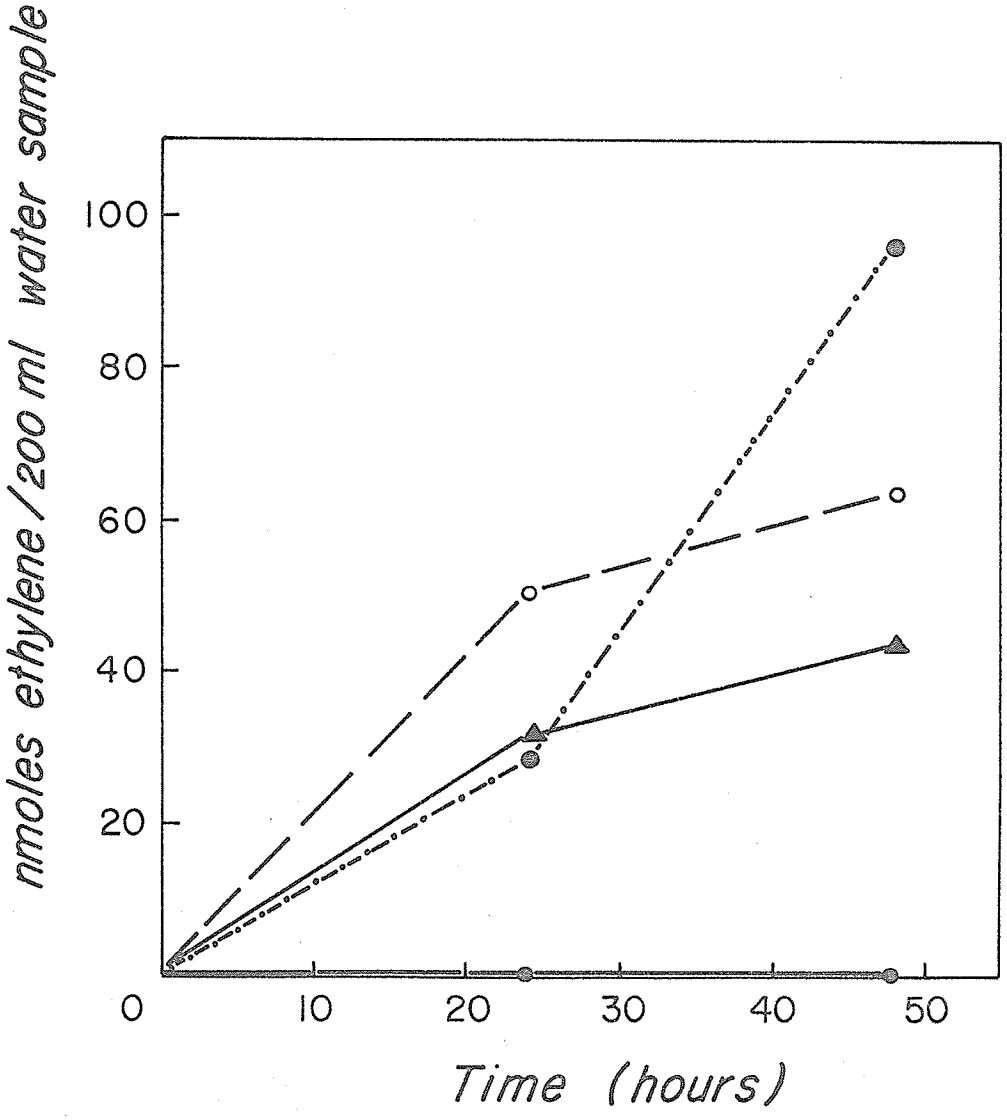

nmoles ethylene / 200 ml water sample
100
80
60
40
20
0
10
20
30
40
50
Time (hours)

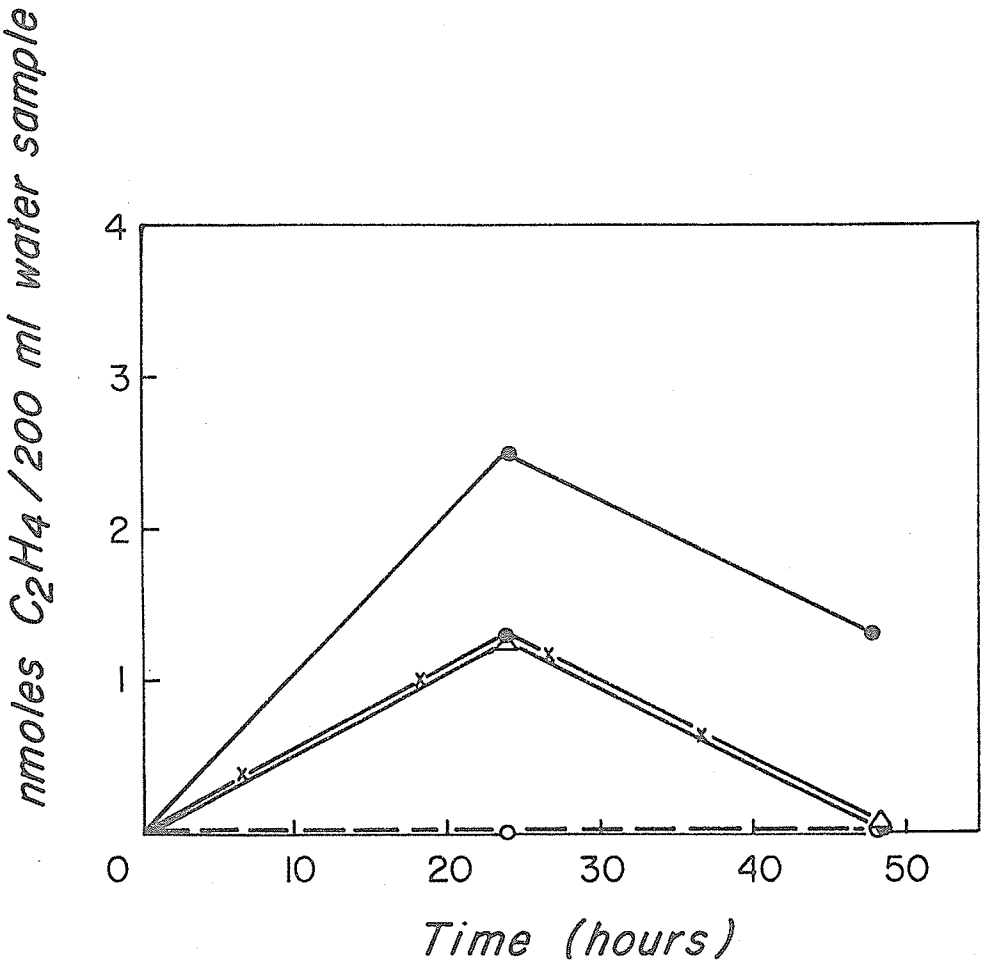
Fig. 11c. Acetylene reduction in water samples from Location A, July 1973, incubated 'in situ' under light and dark conditions. Each value plotted is the average of duplicate samples.

Legend: x——●——x, light, anaerobic

●————●, light, aerobic

Δ————Δ, dark, anaerobic

o--------o, dark, aerobic


nmoles C_2H_4 /200 ml water sample
4
3
2
1
0
10
20
30
40
50
Time (hours)

Acetylene reduction was greatest in samples collected in mid-June during the floating-leaf stage of plant development. This period of time coincided with a heavy bloom of aphanizomenon-like algae. Here, acetylene reduction in all sample bottles increased with time, Fig. 11b. As well, this time period coincides with the time when highest numbers of nitrogen-fixing bacteria occurred in root surfaces as noted in Table IV. Samples of water incubated anaerobically under light produced the highest ethylene values (approximately 95 n moles ethylene/ 200 ml water sample) found during the entire sampling period, Fig. 11b. Three weeks later, in July, ethylene values as noted in Fig. 11c were very low, less than 3 n moles/200 ml water sample, whether incubated aerobically or anaerobically.

During the sampling period May to July 1973, water samples returned to the laboratory for chemical analyses showed nitrate values that increased sharply in June but declined markedly by the July sample date, as indicated by Table V.

Component genera identified during the second algal bloom (July) are given in Table VI.

Table V. Total nitrogen and nitrate-nitrogen in water samples from location A, May to July 1973.

Sample Date	Sample Treatment	Total N (µg/l)	NO_3^--N (µg/l)
30 May	Filtered	2.00	0.44
	Unfiltered	2.04	
16 June	Filtered	15.00	7.01
	Unfiltered	16.50	
5 July	Filtered	0.55	1.81
	Unfiltered	0.87	

Table VI. Components in the second algal bloom at location A, July 1973.

FAMILY or DIVISION	GENUS
Chlorophyceae	*Rhizoclonium*
Bacillariophyceae	*Gomphonemia**
Euglenophyta	*Colaceum**

*Epiphytic on the *Rhizoclonium* species.

D I S C U S S I O N

DISCUSSION

Since 1901 when Beijerinck first isolated and identified the aerobic nitrogen-fixing Azotobacter species, the taxonomy of this group has been subject to considerable controversy. Breed et al (1957) recognized and described only three species while other authors, as noted by Rubenchick (1959), have placed as many as 17 species in this genus. Norris and Chapman (1968) have reviewed the systematics of this genus. Based on serology, cell size and flagellation, on pigment production (soluble, insoluble, fluorescent), fermentation of starch and on habitat, they recognized six species but conceded that further splitting of the group was probable. In this investigation, identification to the species level was made on data provided by Breed et al (1957), Tchan (1953), Norris and Chapman (1968), Rubenchick (1959), Krasil'nikov (1958) and Mishustin and Shilnikova (1971).

According to Norris and Chapman (1968), A. beijerinckii is non-motile and does not utilize starch while A. chroococcum either does not grow or grows poorly on media containing combined nitrogen. Isolate F_3 was weakly motile in young cultures,

utilized starch and grew in a nitrogen-containing medium. This places it, taxonomically, between A. beijerinckii and A. chroococcum and indicates, moreover, that the taxonomy of the genus Azotobacter is still not resolved. Difficulties in identification in this study were further complicated by the pleomorphism displayed by isolate F_3. In each of four different media, morphology varied markedly in terms of cell shape and size. It is interesting to note that Krasil'nikov (1958) has described nine somewhat different cell shapes for this species isolated from different soils in the U.S.S.R.

By comparison, the taxonomy of clostridial species is not so confusing. Rarely will any newly isolated species fail to fit species characteristics as described either by Prévot (1965) or Breed et al (1957). Of these authors, the descriptions by Prévot are more detailed and are particularly suited to classification of anaerobic bacteria in genera. Isolates F_1 and F_2 both appear to belong to the butyric acid group as characterized by Prévot (1965). Similar species of nitrogen-fixers have been reported previously by Rice and Paul (1972) in prairie soils.

The most common asymbiotic nitrogen-fixers are members of the genera Azotobacter and Clostridium.

They have been reported as present in the rhizosphere of many plant species by Mishustin and Shil'nikova (1971) and by Krasil'nikov (1958). Some Azotobacter species were found to be intimately associated with roots of certain grasses by Dobreiner et al (1972). Recently, Yoshida and Ancajas (1970) demonstrated that Azotobacter and Clostridium species were present in the rhizosphere of Oryza sativa. Our studies suggest that these bacterial nitrogen-fixers are restricted to the rhizosphere zone and that algal nitrogen-fixers are present only on the soil-sediment surface to any appreciable extent. It should be remembered, however, that cultural methods used in this study tend to be selective and possible further contributions by autotrophic nitrogen-fixers cannot be ignored.

Some authors, notably Takahashi (1964), Bhattacharya (1958) and Date (1973) maintain that heterotrophic nitrogen-fixation (asymbiotic) adds little to the soil's nitrogen economy. A similar view has been expressed by Mishustin and Shil'nikova (1971). These opinions are based mainly on the fact that heterotrophic nitrogen fixers require substantial amounts of carbon source (for energy)

and under natural conditions in soil, this demand is not usually met. The wild rice habitat, however, contains considerable amounts of organic matter which could be available to meet this requirement. More recently, studies by Dobreiner et al (1972), Dommergues et al (1973), Dobreiner and Campello (1971), Rinuado et al (1971), Abd-El-Malek (1971) and Paul et al (1971) indicate that heterotrophic nitrogen-fixers do contribute appreciable amounts of fixed nitrogen to the natural ecosystem. Our studies show that the root surfaces of Zizania aquatica display a marked degree of nitrogenase activity by indigenous microflora both under aerobic and anaerobic conditions.

The use of total plate counts of bacteria developing on a nitrogen-free medium as an estimate of the number of nitrogen-fixing bacteria is widespread although some investigators, Elwan and El-Naggar (1972) consider them to be of doubtful value. Most probable number estimates by Campbell and Evans (1969) and by Abd-El-Malek (1971) indicate that higher numbers of Azotobacter spp. in soils are provided by this method than by plate counts on the

same soils using nitrogen-free media. The major criticism of plate count enumeration using nitrogen-free media is that non-fixing bacterial species are able to produce weak colonial growth by being able to scavange trace amounts of combined nitrogen from the air. These are referred to as 'putative nitrogen-fixers' by Hill and Postgate (1969), and Okafor (1973) found aerobic bacteria isolates from Nigerian soil which although they developed colonies on nitrogen-free agar did not reduce acetylene to ethylene. It could also be said that tube culture methods for enumeration (MPN) of aerobic nitrogen-fixers is likewise suspect in that prolonged incubation periods could well lead to partial if not complete anaerobiosis in the lower portion of the medium even though cultural conditions were assumed to be aerobic. Thus, a positive result as indicated by turbidity or by acetylene reduction may be due to facultatively anaerobic populations that develop after the medium's oxygen content has been depleted by activities of non-fixing aerobes.

The relatively low counts of bacteria obtained on nitrogen-free agar represent only the population present at the particular time of sampling. This may not reflect the true picture. If counts were made at very short intervals, a better appreciation of

nitrogen-fixers in the rhizosphere during the plant growth season may be obtained. As reported by Meiklejohn (1965), rapid changes in microbial numbers occur in rhizosphere soils. Some evidence in support of this contention is seen in Table 3. Such rapid population changes are due, in part, to changes in moisture content, as reported by Taha et al (1967), to types and amounts of plant root excretions, Vancüra and Hovadik (1965), Bassioni and Ikonomova (1971); to microbial excretions, Brown and Walker (1970), Chopra and Dube (1971); and indirectly to photoperiodism, Srivastava (1971).

According to Meiklejohn (1965) citing Jensen, a population of 5 to 10 million Azotobacter cells per gm would be needed to add only one microgram of nitrogen to the soil. From the plate count figures, assuming the values to represent Azotobacter cells, the nitrogen input by aerobic nitrogen-fixers would then be very low. However, the ability of any group of nitrogen-fixing organisms to some degree is modified by the existing conditions. In some soils, the number of known nitrogen fixers may not reflect the potentiality for nitrogenase activity existing in that soil. Elwan and El-Naggar (1972) obtained

increases in nitrogen content in test soils and in controls at a time when Azotobacter cell numbers were decreasing. They postulate the existence of a cell-independent nitrogenase activity in the soils as one possible explanation of their results. Their observations appear relevant to the results obtained from the acetylene reduction assay of wild rice rhizosphere soils incubated with or without acetylene. When the soil samples were not pre-incubated before gassing with acetylene, significant ethylene values were obtained (Fig. 5), and ethylene was not detected in the control. Under complete darkness and in preincubated soil, negligible ethylene values were observed. Algal development was clearly visible in the light bottles at the end of the preincubation period. Some workers, Dommergues et al (1973) have obtained extremely high values of 2000 to 6000 n moles ethylene/g/hr in soils incubated under dark conditions. Such variations in results are not easily interpreted. In addition to microbial differences between these soils, one could expect that the relative soil chemistry or even physical structure could provide for observed differences in ethylene production, Dommergues et al (1973).

The presence of significant endogenous ethylene in acetylene reduction experiments and demonstration of more than one ethylene peak by soil samples under anaerobic gas phase has been noted by other workers. According to Rice and Paul (1971), Scott and Russel obtained 9-10.6 ppm endogenous ethylene in the gas phase of anaerobically incubated soil, but in their own experiments they did not detect any. Wild rice rhizosphere top soil incubated in the dark produced as much as 2 n moles/g wet weight. Rice and Paul (1971) have pointed out the impracticality of long term incubation of soils in acetylene reduction assays if such significant endogenous ethylene values are easily obtained. The cause or causes of the double ethylene peaks is not understood.

From experimental results, it seems that within wild rice rhizosphere, N-fixation in the top soil is mainly by algal species while heterotrophic bacteria contribute significant amounts on the root surfaces at lower depths. This algal fixation is probably by Oscillatoria species. Furthermore, the study indicates that potentiality for nitrogen-fixation varies from one rhizosphere soil type to

another under the same plant species which is in agreement with results obtained by Dommergues et al (1973), with rhizosphere soils of a number of grasses including rice plants. Also nitrogen fixing organisms in the wild rice rhizosphere occur more on the roots than in the soil.

Because of their ability to produce organic compounds, plants influence the microflora of the rhizosphere in terms of numbers and species, Krasil'nikov (1958). Rhizosphere microflora may also influence plant growth by producing growth promoting substances, Brown and Walker (1970). Therefore the observed differences in population of the nitrogen-fixing bacteria at different parts of the root is a result of a combined effect of microflora-root interaction on one hand and the soil on the other. The pattern of distribution of nitrogen-fixing bacteria is not clear cut but evidence shows that some pattern did exist. Roots at different stages of maturity presumably produced different amounts and kinds of exudates thereby affecting the nitrogen-fixing bacterial population differently at different regions on the root surface. While at one stage a higher population existed at the root base, at another time, the root apices

harboured greater numbers. Such changes may be very common in nature.

Evidence from the culture solution experiments was not conclusive. Plants in the nitrogen-free flask inoculated with bacteria showed nitrogen deficiency symptoms later than those in Flask C. This suggests that the nitrogen-fixing bacteria contributed enough combined nitrogen to sustain minimal growth in the plants. Nitrogen-fixation as indicated by acetylene reduction did not occur, in both parts 1 and 2 of this experiment, in flasks which contained combined nitrogen at the beginning of the experiments even in the presence of known nitrogen-fixing algae. This clearly suggests a possible suppression of the nitrogenase system by the combined nitrogen. The phenomenon has been demonstrated by laboratory experiments (Stewart 1970; Fogg 1971).

The average total counts of bacteria at the end of the experiments IX (1 and 2), gave lower values than the introduced number of Azotobacter and Clostridium cells. This suggests that there was an initial decrease in population of inoculum; whether the decrease would have been

followed by an increase with time was not investigated. The cells may have been demonstrating a temporary adverse reaction to a new environment. Although the nitrogen requirement of wild rice is not known, the combined nitrogen values from Kjeldahl analysis appear high enough to satisfy the requirements of plants for example, Oryza sativa (Takahashi 1964). In the presence of such amounts of combined nitrogen, the inability of the plants to show appreciable vegetative growth may be traced to the chemistry of the culture solution which resulted in the inability of the plants to absorb nitrogen. It is also possible that the physiology of the plants had been impaired by their removal from soil such that the plant roots became incapable of nitrogen absorption.

The identified alga which developed in the first part and subsequently in the second series of the culture solution experiment appears to be a soil alga because microscopic examination did not reveal its presence in water. Furthermore it is not known to fix nitrogen (Fogg 1971). Therefore the fixed nitrogen in the flasks in part (1) is derived from the nitrogenase activity of the inoculated Azotobacter and Clostridium. In the second

part of the experiments significant fixation occurred as indicated by acetylene reduction. This is attributable mainly to the Oscillatoria species, the bacterial component of the microflora also contributing to a lesser extent. The other non-nitrogen-fixing algae may well exist in a metabiotic association with the Oscillatoria species.

Experimental evidence does not point to any significant nitrogenase activity within the experimental water depth after the first few weeks of June. The highest ethylene values throughout the experimental period were obtained at the peak of the bloom of the aphanizomenon-like organisms. Fogg (1971) lists an Aphanizomenon species that fixes nitrogen. Of the algal genera in the second, no member has been reported to fix nitrogen. It appears from the experimental results and observations that an ecological succession affecting fixation rates in the water regime exists. After the thaw, bacteria and possibly soil algae carried upwards into the water by the elongating plant and also washed into the water by runoff, fix a very limited quantity of nitrogen. The nitrogen in turn encourages the development of the Aphanizomenon species, which become dominant in the water, fixing an appreciable quantity of nitrogen.

This nitrogen in the water, a slightly higher temperature and an increasingly sunny weather encourages the development of a fast growing filamentous algal bloom whose component genera do not fix nitrogen but are able to proliferate to become dominant, creating ecological conditions not favourable for bacteria proliferation. It is also possible that the filamentous algal matrix formed a localised environment, very rich in organic matter and exuded nitrogenous compounds mainly amino acids, Shtina (1968), where nitrogen-fixation becomes unnecessary, while the bacteria derive their nitrogen requirements from the algal exudates. Brock (1966) found many marine and freshwater algae, extensively colonized by epiphytic heterotrophic bacteria which seemed very likely to have subsisted on products excreted by the algae.

In addition nitrogen-fixation may be localized in the water on the phyllosphere of the actively growing plants only, particularly within the leaf sheath envelop. The phyllosphere is known to contain significant amounts of carbohydrates and amino acids, (Ruinen 1970).

The picture however may not be as simple as described; other factors no doubt will modify any existing successional sequence. The ecological succession in relation to nitrogen-fixation merits a more detailed study.

A sudden increase and decrease in the water total and nitrate-nitrogen content was observed; highest values occurred during the floating leaf stage. Much of the combined nitrogen existed in solution, as indicated by the very close figure of total nitrogen in the filtered and unfiltered samples. Higher value would probably be obtained in water samples from lower depths near the soil surface. Nitrite values were never detected at the depth where samples were obtained.

Validity of the Acetylene reduction assay as an estimate of nitrogen-fixation in natural ecosystem.

For the quantitative assessment of fixed nitrogen by the acetylene reduction assay (Hardy et al 1968) the ratio "C_2H_2:N_2"::3:1 is often used on the expectation that since 6 electrons are required for the reduction of a nitrogen molecule and 2 for an acetylene molecule, this ratio agrees with actual values. Hardy et al (1973) have made a comprehensive

review of this assay method as used by several workers in assessing nitrogen-fixation in ecosystems.

However as Bergersen (1970) has emphasised, a caliberation for this relationship for every system studied is necessary because in some of the natural systems studied (Paul et al 1971, Rice and Paul 1971), the ratio does not apply. Our knowledge of the ecology of nitrogen-fixing microorganisms, and of ecosystems where different types of nitrogen-fixing systems may be implicated, does not seem sufficient enough for an easy extrapolation of laboratory results to the field. For these reasons, acetylene reduced was not converted to nitrogen fixed.

In some of the experiments in this investigation, a more exact assessment of nitrogenase activity would have been obtained by using $^{15}N_2$ gas in the gas phases of incubated flasks, but the prohibitive costs of the $^{15}N_2$ gas sample which would have been sufficient for such experiments made its application impossible.

S U M M A R Y

SUMMARY

1. Experimental evidence clearly indicates a high nitrogen-fixation potential exists in the wild rice habitat.
2. *Azotobacter* spp. and *Clostridium* spp., as in the root-zone of *Oryza sativa*, are the heterotrophic nitrogen-fixing bacteria associated with *Zizania aquatica* (L) while certain algae (*Oscillatoria* spp) are the photoautotrophic nitrogen-fixers on the upper soil/sediment surface.
3. At certain periods during the growing season, aphanizomenon-like algae may fix nitrogen in the water column of *Zizania* habitat.
4. There appears to be a definite ecological succession within the water regime of the habitat involving both nitrogen-fixing algae and bacteria. The floating-leaf stage appears to be a period during which nitrogenase activity in the habitat is at its highest level. Subsequently, by the aerial-leaf stage, this activity has markedly decreased.

5. Yearly accumulations of wild rice straw in the natural habitat would adequately provide the carbon source (though indirectly) for the energy-dependent heterotrophic nitrogen-fixation. This situation is quite unlike that pertaining in cultivated rice paddies where straw is harvested along with the cereal grains. It could be assumed that energy potentials for heterotrophic nitrogen-fixation are much greater in the case of Zizania aquatica (L) than for Oryza sativa.
6. Given appropriate environmental conditions, nitrogen-fixation activities in wild rice habitat can only be assumed to benefit the nitrogen economy of the habitat.

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A P P E N D I X

APPENDIX A

Morphological, cultural and biochemical characteristics of the isolates F_1, F_2 and F_3.

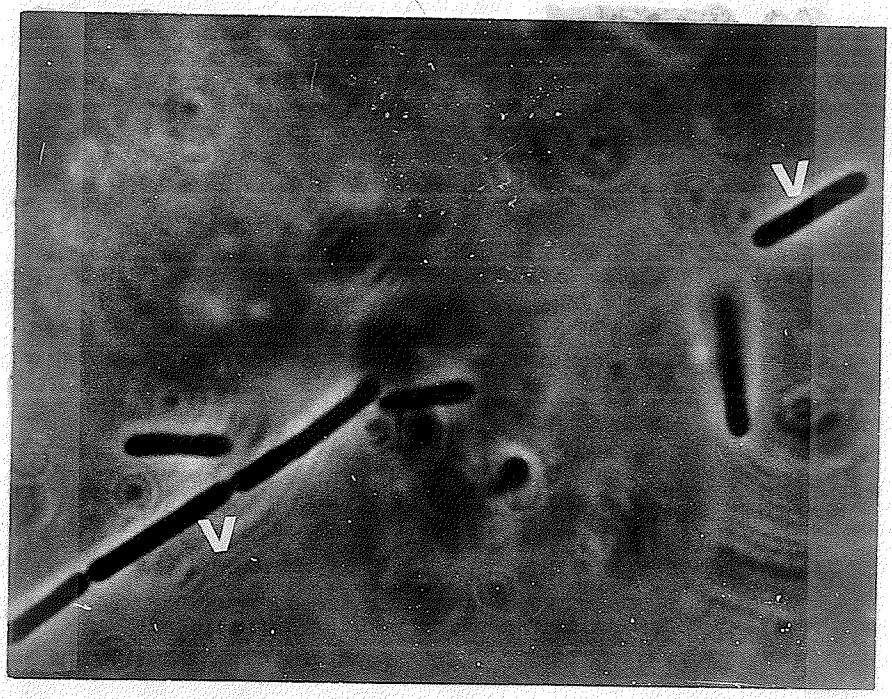
Isolate F_1

Gram positive rods, 2.4 to 7.0 μm by 0.6 to 1.2 μm, arranged singly, in pairs or in short chains. Motile with peritrichous flagellation. Spores are ovoid and sub-terminal in a swollen sporangium. Strictly anaerobic, catalase negative. Vegetative cells are small and thin when cultured in liquid nitrogen-free media but large when cultured in media containing nitrogen (Plate 1).

On glucose agar, colonies are circular to irregular, 2 to 5 mm in diameter, non-pigmented and smooth; on N-free glucose agar, colonies are small, greyish-white in color. In liquid media containing glucose, growth is moderate to heavy, uniformly turbid and accompanied by vigorous gas production.

When cultured in peptone broth, abundant acid and gas from glucose, sucrose, lactose, salicin, corn starch, potato starch, pectin, inulin, raffinose,

Plate 1. 1300X Phase contrast photomicrograph of isolate F_1 cultured in thioglycolate medium (Difco), showing vegetative cells (V). Single and short chained cells are clearly seen.


V
V

inositol and dulcitol. Glycerol, cellulose and adonitol are not fermented.

Gelatin was not liquefied, nitrates not reduced, indole and H_2S production are negative. Albumin was not liquefied nor was litmus milk reduced; calcium lactate was not utilized. No haemolysis was evident on blood agar while growth in Robertson's cooked meat medium produced a foul odour but no blackening or dissolution. Acetylene reduced to ethylene under anaerobic conditions.

Optimum temperature was 45° to 46°C while good growth was evident in temperatures from 27°C to 48°C.

Probable identification, Clostridium butyricum.

Isolate F_2

Gram variable rods with rounded ends, 2.4 µm to 7.2 µm by 1.6 µm, arranged as singles, pairs or in short chains. Weakly motile and sparingly sporulating. Spores where present spherical to oval, central to subterminal swelling the sporangium, Plates 2 and 3.

Plate 2. 4000X. Phase contrast photomicrograph of isolate F_2 cultured in Thioglycolate medium (Difco), showing vegetative cells (V). Single, paired and short-chained cells are clearly seen.

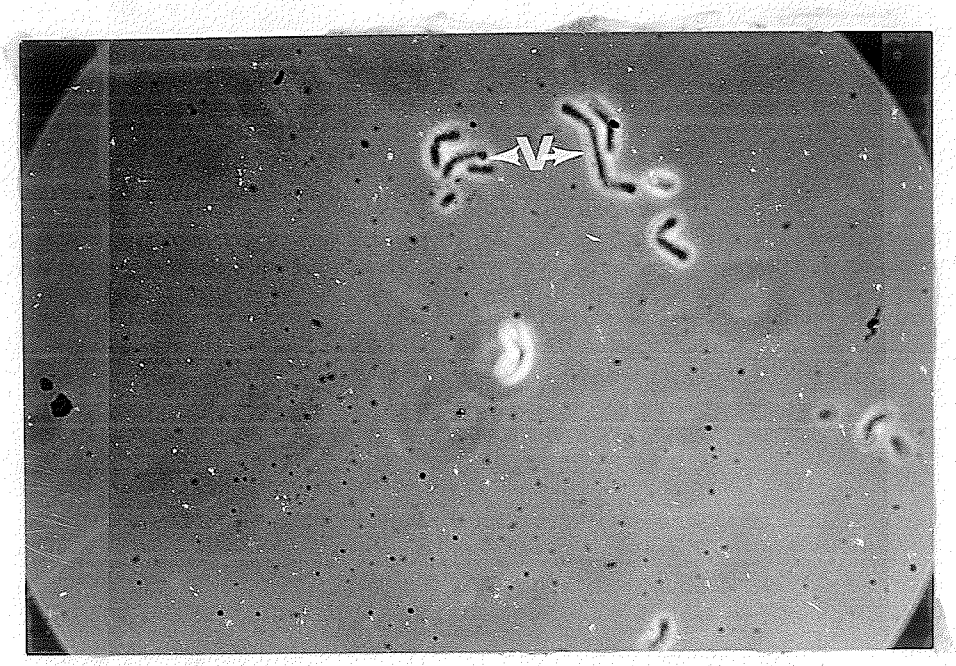
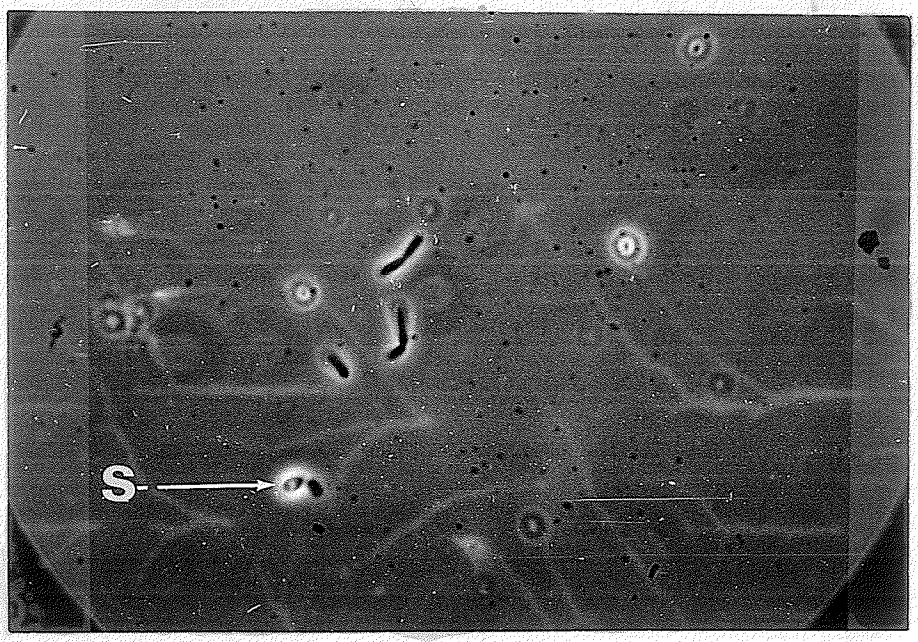

V

Plate 3. 4000X. Phase contrast photomicrograph of isolate F_2 cultured in Thioglycolate medium (Difco), showing spores (S). Some paired vegetative cells are also seen.


S

On glucose agar, colonies are circular, 1 to 2.5 mm in diameter, convex to umbonate, smooth with an entire edge. In glucose broth, growth is profuse and uniform. Anaerobic, catalase negative. Acid and gas from glucose, sucrose and raffinose. Lactose, mannitol, adonitol, sorbitol, dulcitol, glycerol, salicin, inulin, pectin, potato starch, corn starch and cellulose are not fermented. Gelatin was not liquefied, nitrates not reduced, indole not produced. Lactate was not utilized, litmus milk not reduced and albumin was not liquefied. No haemolysis was evident on blood agar. Acetylene reduced to ethylene under anaerobic conditions.

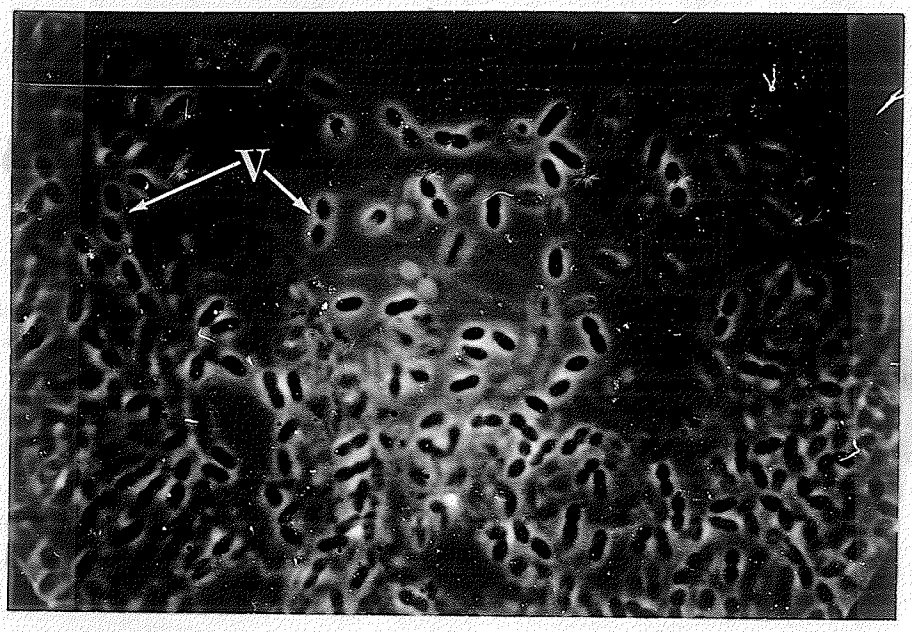
Optimum temperature was about 40°C while good growth was evident within the temperature range from 20°C to 45°C.

Probable identification, Clostridium beijerinckii.

Isolate F_3

Gram negative rods occurring singly, in pairs or in short chains, Plate 4, showing a high degree of pleomorphism when grown in various media. Weakly

Plate 4. 1400X. Phase contrast photomicrograph of isolate F_3, cultured in Azotobacter medium (Brown et al 1962), showing vegetative cells (V).


V
V

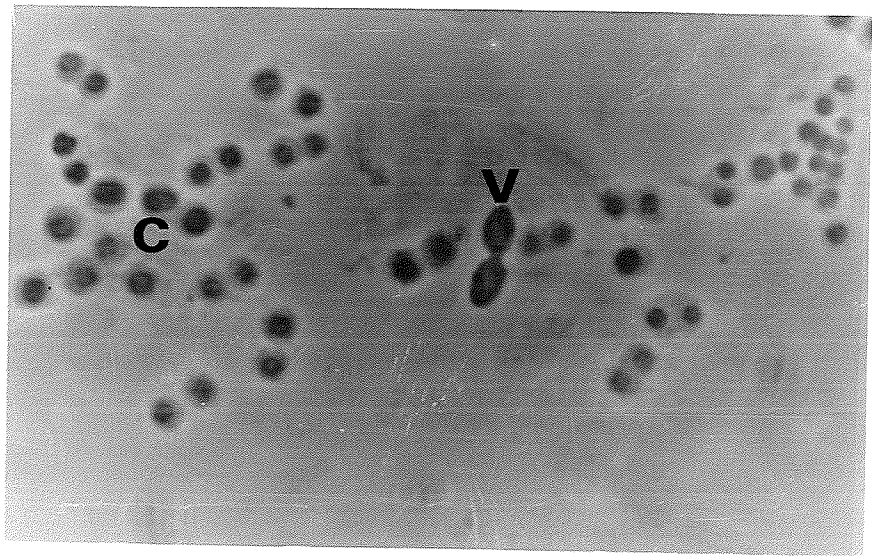
motile in young cultures, strictly aerobic, 2.4 to 5.4 μm by 1.2 to 1.8 μm when grown in liquid nitrogen-free medium. Forms cysts readily, Plate 5, within 48 hours of incubation.

On nitrogen-free glucose agar, colonies are circular, 2-6 mm in diameter after 48 hours. Initially colorless, colonies are transluscent later becoming opaque, dense and beige in colour. Young colonies are convex, glistening with entire margins. In liquid nitrogen-free media, growth is profuse with a thin pellicle formation, flocculent with a tendency for sediment formation.

Starch, rhamnose, glucose and mannitol are fermented. Acetylene is reduced to ethylene in the presence of oxygen. Optimum temperature is about 30°C while good growth is evident in the temperature range from 25°C to 35°C.

Probable identification, Azotobacter chroococcum or Azotobacter beijerinckii.

Plate 5. 13000X. Phase contrast photomicrograph of isolate F_3 cultured in Azotobacter medium (Brown *et al* 1962), showing cysts (C) and a single unusually large vegetative cell (V).


V
C