CHEMOLITHOTROPHY AND H_2-SUPPORTED DINITROGEN FIXATION IN AEROBIC HETEROTROPHIC N_2-FIXING BACTERIA ASSOCIATED WITH WETLAND RICE

A TERMINAL REPORT SUBMITTED TO
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BY

T. K. SIDDARAME GOWDA
POSTDOCTORAL FELLOW
SOIL MICROBIOLOGY DEPARTMENT

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Chapter 1

Rationale

Nitrogen balance studies (App et al. 1980) and long term soil fertility and crop response studies (Watanabe et al. 1981) have confirmed nitrogen gains in wetland rice.

Dinitrogen fixation associated with wetland rice has been reported by several workers (Yoshida and Rinaudo 1982; Watanabe 1981) which includes N_2-fixation that occurs on or in the leafsheaths, and basal portion of shoot, and in the root zone (rhizosphere soil, rhizoplane, and endorhizosphere). A large percentage of the aerobic heterotrophic populations, particularly in the wetland rice root, is N_2-fixing (Watanabe et al. 1979). Different genus of bacteria were isolated as the N_2-fixing population from wetland rice. Enterobacter (Watanabe et al. 1979), Azospirillum (Watanabe et al. 1979; Ladha et al. 1982), and Pseudomonas, which was dominant (Watanabe and Barraquio 1981; Watanabe et al. 1982; Barraquio 1981; Watanabe et al. 1982; Barraquio et al. 1983).

H_2-oxidizing chemolithotrophic ability is reported for heterotrophic aerobic N_2-fixing bacteria: Rhizobium japonicum (Hanus et al. 1979) and Derxia spp. (Pedrosa et al. 1980), and Azospirillum lipoferum (Malik and Schlegel 1981). H_2-supported N_2-fixation has been reported for H_2 oxidizers: Azotobacter chroococcum (Walker and Yates 1978) Azospirillum brasilense (Pedrosa et al. 1982) and Rhizobium japonicum (Emerich et al. 1979).

H_2 is known to evolve in wetland rice soils (Yamane and Sato 1964; Harrison and Aiyer 1919) from wetland rice root (Kimura 1981).

Preliminary studies of Watanabe et al. (1982) suggested that the dominant aerobic heterotrophic N_2-fixing bacteria associated with endorhizosphere of wetland rice were able to show improved growth under H_2 atmosphere and also

showed H_2-supported C_2H_4 reduction.

Therefore, detail studies were carried out to confirm the chemolithotrophic capabilities and H_2-supported N_2-fixation in the bacteria associated with wetland rice. By employing radioisotope technique and acetylene reduction technique followed by $^{15}N_2$ technique, chemolithotrophic ability and H_2-supported N_2-fixation is confirmed in pure cultures. A ^{14}C-autoradiographic technique was developed and used to study the population in endorhizosphere of rice grown under field conditions in different soils and water regimes. In situ studies with wetland rice were carried out and confirmed the H_2-supported N_2-fixation.

PART I CHEMOLITHOTROPHY

Chapter 2

Autoradiographic technique for screening chemolithotrophic hydrogen-utilizing bacteria

Abstract

A simple autoradiographic method for screening aerobic H_2-dependent chemolithotrophs is described. Bacteria were grown on agar plates in ambient air at 32°C. Colonies from the plates were inoculated to H_2-uptake agar plates by replica plating or streaking, and incubated for 14 days in a desiccator under 20% H_2, 2% O_2, 5% CO_2 and balance N_2 or 2% O_2, 5% CO_2 and balance N_2. The plates were further incubated for 9 days with $^{14}CO_2$ in the above gas mixtures. The colonies were transferred to muslin cloth by replica method and the cloth was exposed to x-ray film for autoradiography.

H_2 was necessary for CO_2 fixation under autotrophic conditions. By this technique, the predominance of H_2-utilizing facultative aerobic chemolithotrophs among wetland rice root isolates was confirmed.

The strains of Azospirillum lipoferum tested were found to fix $^{14}CO_2$ but the A. brasilense strains tested were unable to fix $^{14}CO_2$.

Introduction

Aerobic hydrogen-oxidizing bacteria presently comprise 28 species belonging to 15 genera (Bowien and Schlegel 1981). Facultative autotrophic bacteria which use H_2 to provide energy and reducing equivalents for growth and CO_2 fixation include various Pseudomonas, Alcaligenes (Hydrogenomonas) (Buchanan and Gibbons 1974), Xanthobacter (Wiegel et al. 1978; Malik and Klaus 1979), the nitrate-reducing Paracoccus species (Rittenberg 1969), Azospirillum, (Sampaio et al. 1981), and Rhizobium japonicum (Hanus et al. 1979). Many other obligate aerobic N_2-fixing bacteria have H_2-uptake hydrogenase, which can utilize H_2 to provide reducing equivalents and energy for metabolic processes, but H_2-supported growth was not described. However, Pedrosa et al. (1980) recently described H_2-supported chemolithotrophic growth for Derxia spp. The aerobic hydrogen bacteria are taxonomically the most diverse group of the aerobic chemolithotrophic bacteria. They share with almost all the phototrophic organisms a common mechanism for CO_2 reduction by Calvin-Bassham cycle (Schlegel 1981).

Watanabe et al. (1982) found a high proportion of H_2 uptake positive nitrogen-fixing bacteria among heterotrophic bacteria in the wetland rice root. Their chemolithotrophy was suggested only from the capability of the growth under $H_2 + CO_2 + O_2$ gas mixture.

In this paper we report a simple autoradiographic technique for screening CO_2- and H_2-dependent chemolithotrophic capability of various N_2-fixing and non-N_2-fixing bacteria and its application to

demonstrate predominance of H_2-dependent chemolithotrophs among bacteria isolated from wetland rice root and chemolithotrophy of some bacteria.

Materials and methods

Source of bacteria strains

N_2-fixing Azospirillum (Ladha et al. 1982), Pseudomonas sp. (Watanabe and Barraquio 1979 and Barraquio et al. 1983), Enterobacter cloacae, and Klebsiella pneumoniae (unpublished) were isolated from wetland rice root and shoot (Watanabe et al. 1979), identified, and maintained at the International Rice Research Institute. Azospirillum lipoferum USA Sp5 and Azospirillum brasilense Sp7 were obtained from J. Döbereiner, EMBRAPA/SNLCS-CNPq, Brazil. Klebsiella pneumoniae M5a1 was obtained from D. Valentine, University of California. Alcaligenes latus DSM 1124 and Renobacter vaculatum DSM 1277 were obtained from German Collection of Microorganisms (DSM) (Malik et al. 1981, Malik and Schlegel 1981). Agrobacterium radiobacter ATCC 19358, Derxia gummosa ATCC 15994, Enterobacter cloacae ATCC 13047, Pseudomonas cepacia ATCC 25416, and P. facilis ATCC 17695 were obtained from the American Type Culture Collection.

Rice plant sampling and bacteria isolation

The methods and media used were those used by Watanabe et al. (1979). Four rice plants from wetland at heading stage (IR26) were dug from the soil and soil adhering to roots was

gently removed with water. The roots (10 g) were cut into 3-cm pieces and washed with sterile water six times, then macerated with pestle and mortar. The macerated sample (histosphere) was serially diluted with sterile distilled water. The dilutions (10^{-5}, 10^{-6}) were spread (0.1 mL) on predried 0.1% Difco tryptic soy agar (TSA), or H_2-uptake agar (Watanabe et al. 1982) plus triphenyl tetrazolium chloride (TTC) (0.1 g/L) as used by Maier et al. (1978). The plates were incubated at 32°C in an incubator for a week under ambient air.

H_2-supported growth and $^{14}CO_2$ incorporated studies

To demonstrate $^{14}CO_2$ uptake by bacteria and its dependence on H_2 under autotrophic conditions, the following four methods were used.

Bacteria isolated from the histosphere of wetland rice root and reference strains were tested quantitatively for CO_2 fixation and H_2-uptake activity. Bacteria were grown on tryptone yeast extract agar (TYEA) slants for 48 h at 32°C in air. Bacteria from the slant were suspended in 3.4 mL H_2-uptake medium (Watanabe et al. 1982) and 0.1 mL of this suspension was inoculated into 3.4 ml of H_2-uptake medium in a 13-mL volume tube with cotton plug. Tubes were incubated for 8 days in desiccators under 20% H_2, 2% O_2, 5% CO_2, and balance N_2 or under 2% O_2, 5% CO_2 and balance N_2. The tubes were further incubated for 16 days under the same gas mixtures after adding $^{14}CO_2$. In this case, ^{14}C-enriched CO_2

(7.76 μCi/mmole) generated by adding 10 mL conc HCl to 1 g $NaHCO_3$ + NaH $^{14}CO_3$ was provided to a desiccator of 10 L volume. The tubes were taken out of the desiccator and evacuated after replacing cotton plugs with serum stoppers, refilled with 1% H_2, 2% O_2, 5% CO_2 and balance N_2 gas mixture, and incubated for 24 h at 32°C, then, H_2-uptake activity was measured. By keeping the tubes at 4°C for four days and then acidifying with 6N HCl (1 mL), all unused $^{14}CO_2$ in solution was removed. All contents of the tubes were transferred to scintillation vials with two washings of 2 mL distilled water. Ten mL scintillation cocktail (Packard-Instagel) was added and mixed. Radioactivity (dpm) was recorded by Packard Tri-Carb 460 CD scintillation counter (Method A).

One-week-old bacterial colonies of wetland rice histosphere on TSA or H_2-uptake agar plates were directly transferred to H_2-uptake agar plates by replica method (Scheme 1) (Method B).

Twelve representative colonies (Method B) were also picked off from TSA and H_2-uptake agar plates supplemented with TTC. They were purified and grown on TYEA for 48 h at 32°C in an incubator under air; bacterial growth on the slants was suspended in 3 mL H_2-uptake broth. The suspension was streaked (1 cm) on predried H_2-uptake agar plates. Six isolates were inoculated onto each plate (Method C).

Seventeen bacterial isolates including N_2-fixing, non-N_2-fixing, H_2 uptake positive, and H_2 uptake negative isolates obtained from various sources were screened for their chemolithotrophic capabilities as described in Method C (Method D).

The inoculated plates by Methods B, C, and D were incubated in dessicators (10 L volumes) under 20% H_2, 2% O_2, 5% CO_2 and balance N_2 or under 2% O_2, 5% CO_2 and balance N_2. Pseudomonas facilis ATCC 17695 and P. cepacia ATCC 25416 were used as reference strains for positive and negative growth, respectively. After 14 days at 32°C growth under the gas mixture with and without H_2 was compared.

The plates were incubated for an additional 9 days under the same gas mixtures containing $^{14}CO_2$ prepared as mentioned in Method D.

Autoradiography

The bacterial colonies on the agar plates (Methods B, C, and D) were transferred to muslin cloth as replica (2 layers of muslin cloth, one side pasted with broad tape). The cloth was fixed to a cardboard after drying it in ambient air for 1 h, and exposed to x-ray film (Fuji IX Industrial) in an x-ray cassette for 4 days. The film was developed for 4 min (Fuji Rendol) and fixed for 4 min (Fuji Renfix). The dark spots observed on the x-ray film were proof of $^{14}CO_2$ fixation.

H_2-uptake activity

H_2-uptake activity for all bacteria tested, except Azospirillum brasilense strains, was measured by gas chromatography as described by Watanabe et al. (1982). For Azospirillum brasilense strains the following method was used: forty eight-hour-old bacterial cultures grown in TYEA were inoculated to 100 mL of N-free broth containing Na-malate (5 g/L) and biotin (100 µg/L) (Okon et al., 1977) in 250-mL conical flask and incubated at 30°C with 2% O_2 and 98% N_2 for 5 days. The suspension was centrifuged at 6000 x G under N_2 atmosphere for 30 min. The pellet was resuspended in 30 mL of fresh sterile medium in a 50-mL flask saturated with N_2. The flasks were evacuated and filled with 1% H_2, 2% O_2, and balance N_2. H_2 content in the flask was measured by gas chromatography with a ceramic sensor detector (Sensortech Inc. Ltd., Shiga, Japan).

Results and Discussion

$^{14}CO_2$ fixation and H_2-uptake activity of wetland rice root bacteria and reference bacteria are shown in Table 1. The results clearly showed that all N_2-fixing wetland rice root isolates (KLH76, H8, and 34H) have H_2-uptake activity and fixed $^{14}CO_2$ when incubated on H_2-uptake broth with H_2, but $^{14}CO_2$ fixation was not observed in the absence of H_2. Therefore, these bacteria were chemo-autotrophs. Similarly, among reference bacteria, only those showing H_2-uptake

activity fixed $^{14}CO_2$. *P. cepacia*, an H_2-uptake negative pseudomonad, did not show $^{14}CO_2$-fixing activity. The results thus showed that uptake hydrogenase activity and H_2 gas are necessary for $^{14}CO_2$ fixation.

CO_2 fixation studies by autoradiographic technique

Carbon dioxide fixation by colonies was not identified by exposing the agar plates by Methods B, C, and D directly to the x-ray film for autoradiography because of uniform darkness against an entire plate. The problem was overcome by transferring the colonies to a muslin cloth.

H_2-dependent CO_2 fixation was confirmed quantitatively by the data in Table 1, and also confirmed by autoradiography (Fig. 1). It is possible, to screen bacteria for their H_2-dependent chemolithotrophic activities by this technique.

$^{14}CO_2$ fixation by several colonies of wetland rice histosphere bacteria in the presence and absence of hydrogen is shown in the autoradiographs (Figs. 2 and 3). The autoradiographs showed that CO_2 fixation occurred in all the inoculated plates only when incubated with H_2. Six of 7 colonies at 10^{-6} dilution and 36 of 40 colonies at 10^{-5} dilutions, which appeared on TSA plates, showed $^{14}CO_2$ fixation. The growth of these bacterial colonies, which was examined before preparing for autoradiography, was significantly more in the plates incubated with H_2 than in plates incubated

without H_2 (Fig. 2). Growth on H_2-uptake agar under H_2 containing gas and CO_2 fixation show most of these histosphere bacteria are chemolithotrophs.

When replica from initial growth in air on H_2-uptake agar plus TTC was inoculated to H_2-uptake agar, only one of 50 colonies at 10^{-6} dilution showed $^{14}CO_2$ fixation in the presence of H_2 and none without H_2 (Fig. 3). This indicated that only few colonies are chemolithotrophs. It appears, therefore, that replica from TSA is better for detecting chemolithotrophic growth of rice root bacteria and for isolating and counting chemolithotrophs than replica from H_2-uptake media.

Several representative colonies of rice root histosphere bacteria were picked off from TSA plates (7 colonies) and H_2-uptake agar plus TTC (2 pink and 3 colourless), and purified. They were tested for $^{14}CO_2$ fixation. The colonies that were big on TSA plates and consisted of more than 90% of colonies on TSA plates grew well on H_2 uptake agar incubated with H_2, and fixed $^{14}CO_2$, whereas the same isolates incubated under similar situation without H_2 showed poor growth and did not fix $^{14}CO_2$ (Fig. 4).

Among rice root bacterial colonies grown on H_2-uptake agar plus TTC in ambient air, colonies that were pink due to TTC reduction showed good growth and $^{14}CO_2$ fixation when incubated on H_2-uptake agar with H_2. But colonies incubated under similar conditions without H_2 showed poor growth and did not fix $^{14}CO_2$.

None of the colourless colony isolates grew or fixed $^{14}CO_2$ either in the presence or in the absence of H_2. The results, indicated that the chemolithotrophic bacteria from rice root reduce TTC. TTC reduction test has also been used for screening H_2-uptake positive Rhizobium strains (Maier et al. 1978; Pahwa and Dogra 1981).

Several genera of bacterial isolates comprising N_2-fixers, non-N_2-fixers, and H_2-uptake positive and negative strains were tested for their chemolithotrophic capability by autoradiography (Table 2).

From the above results it is confirmed that under autotrophic conditions, H_2 is necessary for CO_2 fixation. Organisms that do not have H_2-uptake hydrogenase activity cannot fix CO_2. However, Azospirillum brasilense Sp7, a known H_2-uptake positive strain (Pedrosa et al. 1982), and two other strains, 36H2 and 26H2, isolated from wetland rice root histosphere (Ladha et al. 1982) did not grow and fix $^{14}CO_2$ autotrophically. But strains of Azospirillum lipoferum Sp5 and 34H (wetland rice root isolate) grew well and fixed $^{14}CO_2$ under similar conditions. Therefore, the technique also is useful for differentiating Azospirillum. Watanabe et al. (1982) reported that higher proportion of bacteria from wetland rice plant roots showed positive growth under $CO_2 + H_2$ and uptake H_2ase activity, than from the soil or dryland rice roots. Our results clearly confirmed their earlier

findings that most of the bacteria associated with wetland rice root histosphere are H_2-dependent chemolithotrophs.

The autoradiographic technique we have described is simple and precise. It can also be adopted for counting, isolating, and screening autotrophic cultures.

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Table 1. $^{14}CO_2$ fixation and H_2-uptake activity in different bacteria incubated on H_2-uptake broth with and without hydrogen gas mixture.

Bacteria	$^{14}CO_2$ fixation x 100 dpm		No. of times increased $^{14}CO_2$-fixation over $-H_2$	H_2-uptake, µL H_2/tube/24 h
	$-H_2$	$+H_2$		$+H_2$
Renobacter vaculatum DSM 1277	0.166 (± 0.03)**	69.0 (± 37.2)	416	46.7
Alcaligenes latus DSM 1124	0.716 (± 1.5)	5.59 (± 1.1)	7.8	16.7
Pseudomonas cepacia ATCC 25416	0.213 (0.01)	0.20 (± 0.015)	0.0	0.00
Pseudomonas facilis ATCC 17695	0.098 (0.06)	152.7 (± 104.9)	1560	100.0
Pseudomonas sp. KLH76*	0.066 (0.07)	33.6 (± 6.2)	510	56.7
Pseudomonas sp. H8*	0.094 (± 0.01)	29.2 (± 1.9)	311	33.3
Azospirillum lipoferum 34H*	0.075 (± 0.03)	22.3 (± 1.9)	297	20.0
Uninoculated	0.023 (± 0.01)	0.017 (± .005)	0.0	0.00

*Isolated from wetland rice.
**Figure in parenthesis is the standard deviation of 3 replicates.

Table 2. Uptake-hydrogenase and $^{14}CO_2$ fixation in various bacteria used for testing chemolithotrophy.

	Uptake H_2-ase	$^{14}CO_2$-fixation (on autoradiography)	
		$-H_2$	$+H_2$
Wetland rice root bacteria			
Pseudomonas sp. H8	+	-	+
Pseudomonas sp. KLH76	+	-	+
Pseudomonas sp. LH4	+	-	+
Azospirillum lipoferum 34H	+	-	+
Azospirillum brasilense 36H2	+	-	-
Azospirillum brasilense 26H2	+	-	-
Enterobacter cloacae Euf. 29-3	-	-	-
Bacteria from different sources			
Alcaligenes latus DSM 1124	+	-	+
Azospirillum brasilense Sp7	+	-	-
Azospirillum lipoferum USA Sp5	+	-	+
Agrobacterium radiobacter ATCC 19358*	-	-	-
Derxia gummosa ATCC 15994	+	-	+
Enterobacter cloacae ATCC 13047*	-	-	-
Klebsiella pneumoniae M5a1	-	-	-
Klebsiella pneumoniae LL.4	-	-	-
Pseudomonas cepacia ATCC 25416*	-	-	-
Pseudomonas facilis ATCC 17695*	+	-	+
Renobacter vaculatum DSM 1277	+	-	+

*Non-N_2-fixing bacteria.

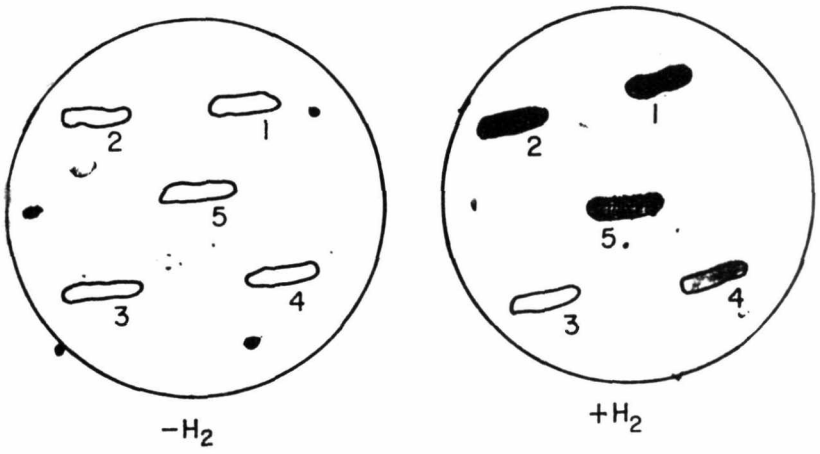


Fig. 1. Autoradiograph showing $^{14}CO_2$ fixation, 1. Renobacter vaculatum; 2. Alcaligenes latus DSM 1124; 3. Pseudomonas cepacia ATCC # 17695; 4. Pseudomonas facilis ATCC # 25416; 5. Pseudomonas sp. H8.

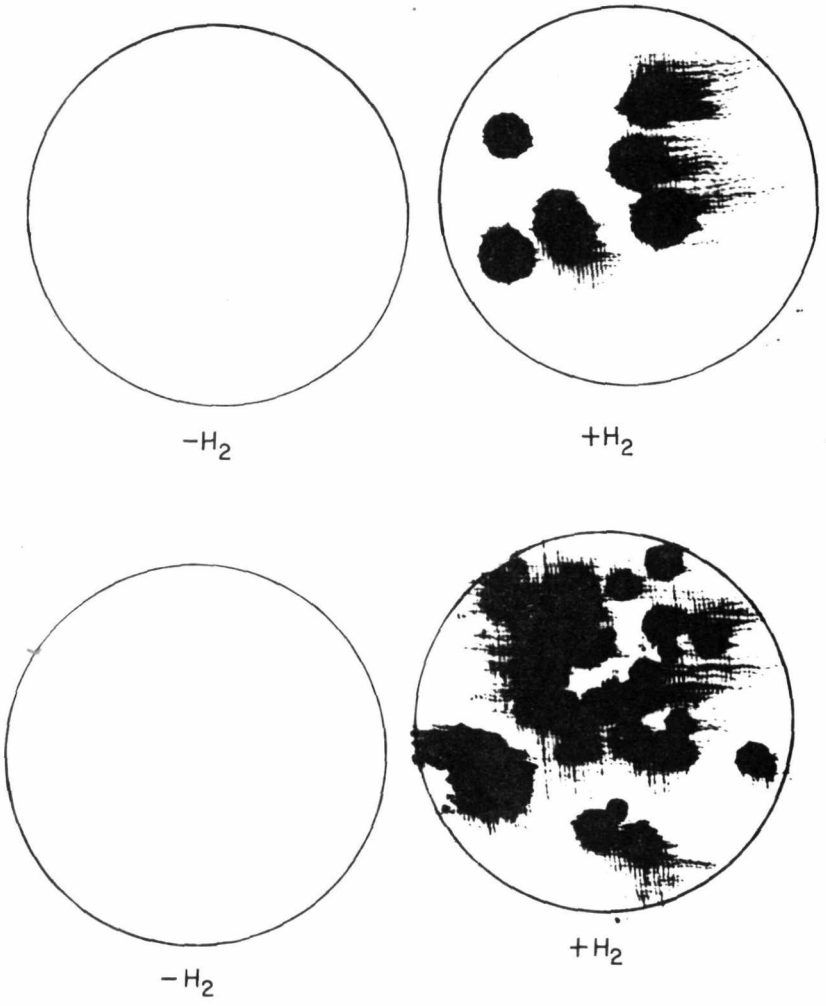


Fig. 2. Autoradiograph showing $^{14}CO_2$ fixation by histosphere bacterial colonies of wetland rice on H_2-uptake agar (inoculated by replica method from TSA plates, top 10^{-6}, bottom 10^{-5} dilutions).

Rice root macerated, dilutions prepared, plated on TSA, grown for 1 week under air, colonies inoculated by replica method to H_2-uptake agar, incubated with and without H_2 for a total of 23 days.

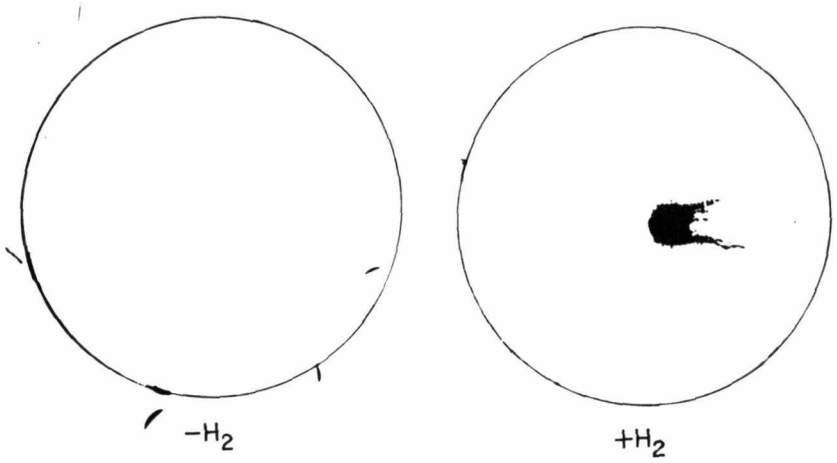


Fig. 3. Autoradiograph showing $^{14}CO_2$ fixation by histosphere bacterial colonies of wetland rice on H_2-uptake agar (inoculated by replica method from H_2-uptake agar with TTC, 10^{-6} dilution). Experimental details as in Fig. 2.

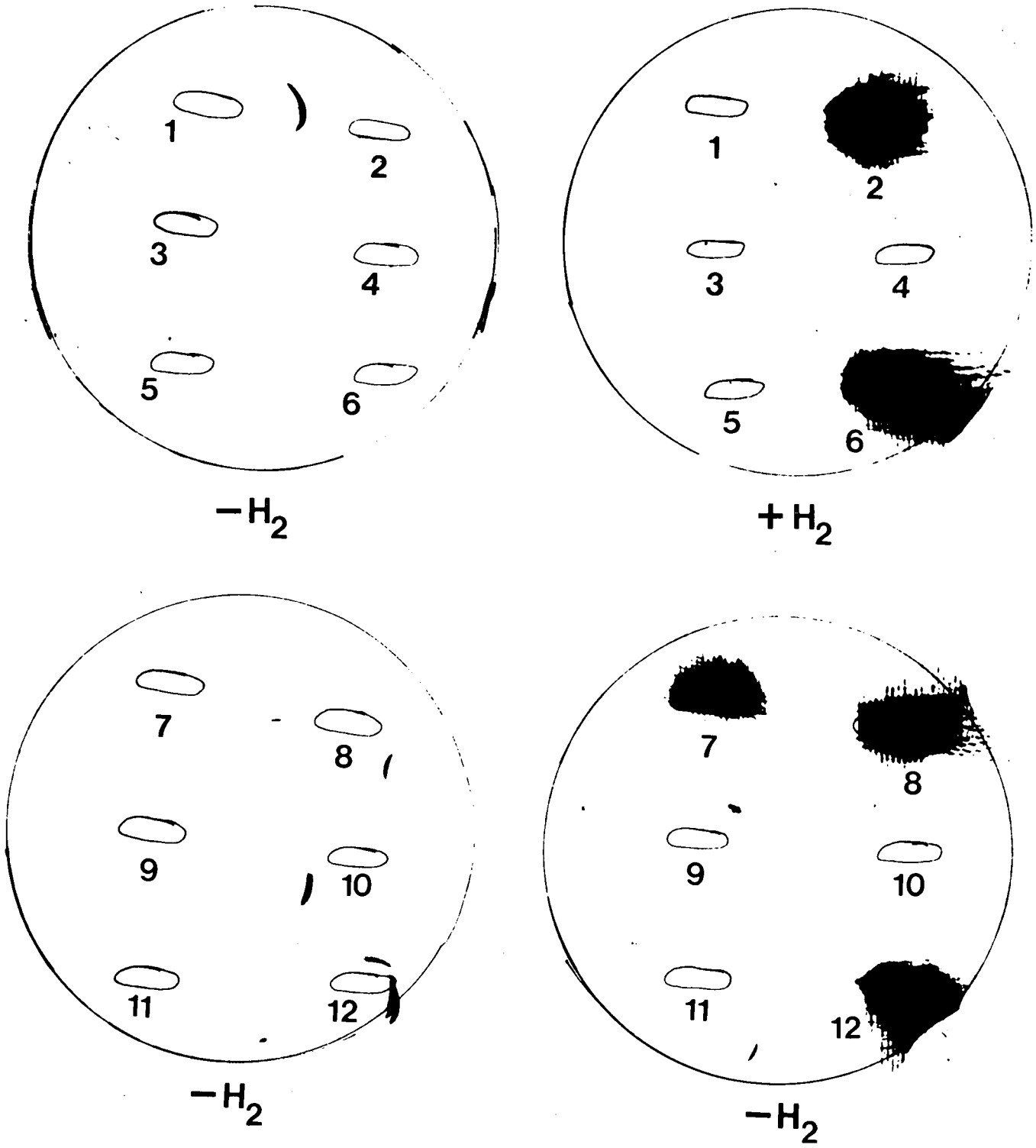
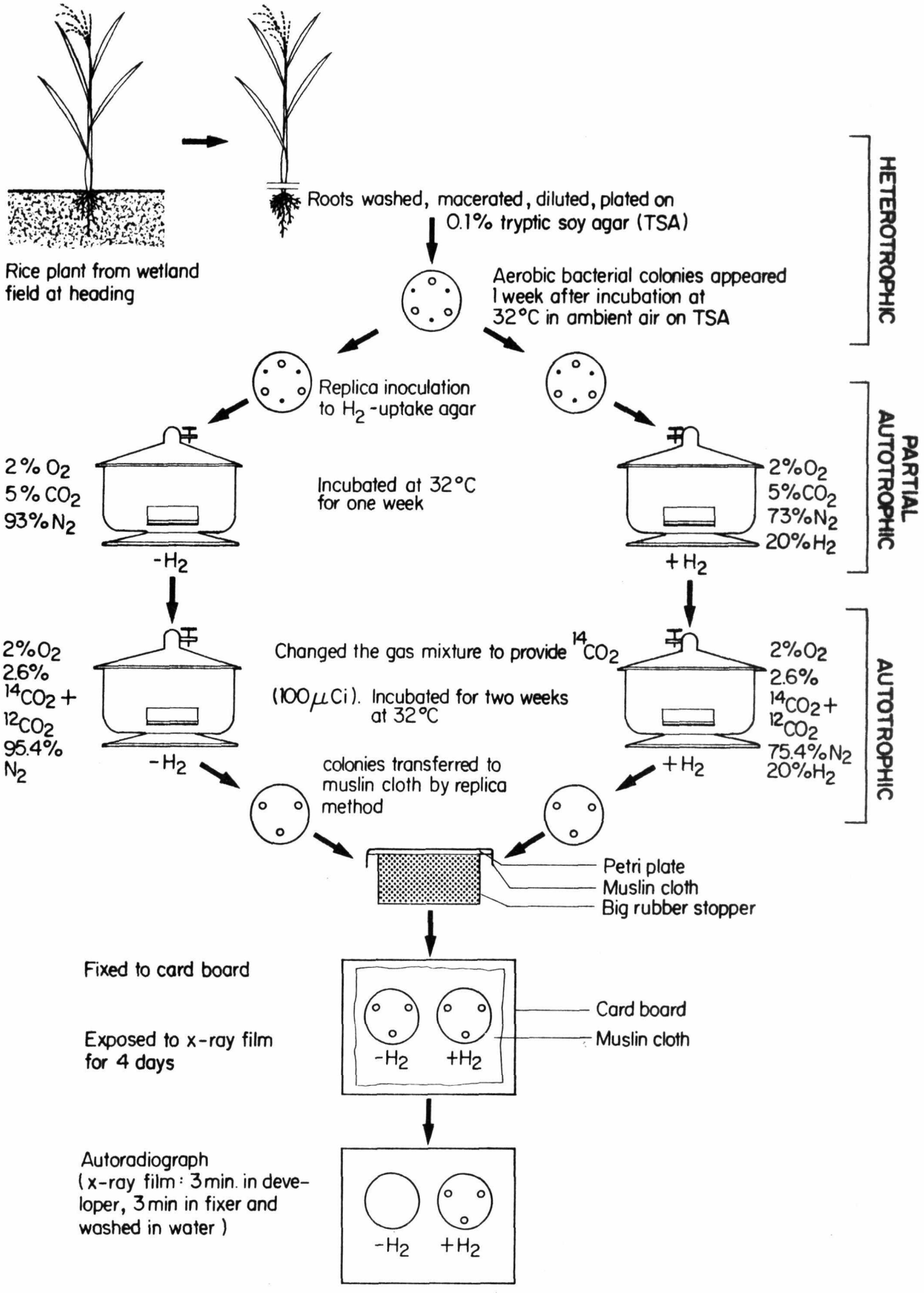


Fig. 4. Autoradiograph showing $^{14}CO_2$ fixation by histosphere bacteria of wetland rice on H_2 uptake agar. Isolate 1 to 7 picked from TSA plates and 8 to 12 from H_2-uptake agar + TTC plates.



Scheme **1.** Autoradiographic technique for studying chemolithotrophy of rice histosphere bacteria.

Chapter 3

Variation in the incidence of H_2-oxidizing chemolithotrophic bacteria in rice grown under different cultivation conditions

Abstract

The incidence of H_2-oxidizing chemolithotrophic bacteria associated with rice grown under continuous wetland, upland, and rainfed wetland conditions was studied by ^{14}C-autoradiographic technique in a neutral soil at IRRI (Maahas) and an acid rainfed wetland soil (Luisiana).

In Maahas soil, H_2-oxidizing chemolithotrophic bacteria were not detected in the endorhizosphere, rhizosphere, and nonrhizosphere soil of rice grown under upland conditions. Under continuously flooded conditions a very large population of these bacteria were found in the endorhizosphere but not in the oxidized and reduced soil.

A very low population of these bacteria were found in the endorhizosphere and basal culm of rice grown under rainfed wetland conditions at Luisiana. Bacteria isolated from Maahas wetland rice and inoculated to rice seedling planted in Luisiana soil failed to establish.

Both Maahas and Luisiana soils consumed externally supplied H_2 and produced H_2 and CH_4 almost at the same rate when they were amended with rice straw or sucrose. This paper discusses possible causes of variation in the number of these bacteria and their distribution in rice grown under different cultural and soil conditions.

Introduction

Rice is grown under different water regimes, namely, upland, continuously flooded, rainfed wetland, and deep water. Yoshida and Ancajas[25] measured a higher acetylene reduction activity (ARA) in wetland rice compared with upland rice. Barraquio et al.[4] reported that both ARA measured in situ and under water culture conditions and the percentage of aerobic N_2-fixing bacteria associated with root and basal shoot were higher in wetland rice than in upland rice. Barraquio and Watanabe[3] also found that the incidence of aerobic N_2-fixing bacteria among aerobic heterotrophic bacteria in the washed roots was higher in other wetland monocots than in upland rice and other upland crops.

Some aerobic heterotrophic bacteria are H_2-oxidizing chemolithotrophs[6]. Watanabe et al.[23] reported a very large number of H_2-oxidizing N_2-fixing bacterial association in association with wetland rice and a negligible population of these bacteria in association with upland rice. Recently Gowda and Watanabe[9] confirmed the presence of a large number of H_2-oxidizing chemolithotrophic N_2-fixing bacteria in the endorhizosphere of wetland rice by employing a ^{14}C-autoradiographic technique.

Daroy and Watanabe[7] reported low ARA activity and a negligible number of aerobic N_2-fixing bacteria associated with rice in a rainfed wetland soil of Luisiana as compared with rice grown in continuously

flooded Maahas soil. Application of lime and phosphorus (20 g CaO plus 6 g P_2O_5/m^2) in Luisiana soil did not increase their abundance.

Since wetland soils[24,10] and wetland rice roots[12] are known to produce H_2 the observed variations in ARA and N_2-fixing bacterial population were suspected to be due to H_2-production and utilization in these soils.

^{14}C-autoradiographic technique was used to measure the distribution of H_2-oxidizing chemolithotrophic bacteria associated with rice grown under different water regimes and soil conditions.

Materials and methods

Growth conditions of rice and other plants

IR26, IR42, IR52, and IR56 rice were grown at the International Rice Research Institute (IRRI) in a continuously flooded Maahas field where nitrogen fertilizers have not been applied for the past 9 years.

IR52 and maize were grown in an upland field at IRRI. IR42 was grown in a rainfed acid soil (Ultisol) at Luisiana during the wet season after a 6-month dry period. The chemical properties of Maahas soil (IRRI field, wetland) and Luisiana soil are shown in Table 1.

Of the rice transplanted in Luisiana soil, some rice seedlings were inoculated with bacteria. Two predominant H_2-oxidizing chemolithotrophic N_2-fixing bacteria, namely, Pseudomonas sp. H8[5] and Azospirillum lipoferum 34H[13], isolated from the endorhizosphere of rice grown in flooded Maahas soil, were used for inoculation. The bacteria were grown on nutrient agar slants in flat bottles for 48 h and then harvested by washing with 0.1 M phosphate saline buffer (pH 7.0). The

roots of the rice seedlings were dipped separately in each bacterial suspension (10^8 cells/ml) for 24 h and then planted. Uninoculated plants were treated similarly in buffer without bacteria.

Sample preparation

Oxidized and reduced soils were collected from an unplanted, continuously flooded plot adjacent to the planted plot at IRRI by inserting a 30-cm-diameter metal ring into the undisturbed soil. Water and algae were siphoned out with a syringe. The oxidized layer of soil was scraped out. The pooled samples collected from five places were used as oxidized soil. The reduced soil was collected using a metal cylinder (5 cm in diameter), inserted into the soil up to a depth of 20 cm, from 5 places after the aerobic soil was scraped off. The samples were then pooled and mixed thoroughly, and a subsample was immediately used for enumeration of bacterial population. The endorhizosphere samples were prepared by the method described by Watanabe et al.[22]. After the roots and leaf sheath were removed, two to three cm of the basal (submerged) portion of the culm was cut, washed several times by sterile water, and macerated.

Soil, macerated root, or culm samples were diluted and an appropriate dilution was spread on 0.1% tryptic soybroth agar (TSA), and incubated for a week at 30°C. The colonies on the plates were counted and transferred by replica method to the H_2 uptake agar plate. CO_2-uptake active chemolithotrophs were counted by the method described by Gowda and Watanabe[9].

Hydrogen production, utilization, and CH_4 production

Soils collected from wetland (Maahas and Luisiana) were air-dried and passed through a 2-mm sieve. Twenty grams of soil with 20 ml water was incubated for 24 h in 20 mm x 200 mm tubes for equilibration. The tubes were plugged with rubber stopper to which had a small glass tube (Fig. 2) with serum cap attached. The tubes were evacuated, flushed two times with N_2 free of O_2, and finally filled with N_2. Different quantities of H_2 were injected to each set of tubes (0, 2.5 ml, 5.0 ml) after removing the same volume of N_2. Similarly, separate sets of tubes were incubated after mixing soil with 0.5 % ground rice straw (200 mesh) or sucrose (2 M).

To determine H_2 consumption, and the production of H_2 and methane, H_2 content was measured at periodical intervals by gas chromatograph (GC) with a sensor detector (Sensortech Inc. Ltd. Shiga, Japan) and CH_4 content was measured by GC with flame ionization detector (FID).

Results

A large proportion (62-94%) of the aerobic heterotrophic bacteria associated with the endorhizosphere of rice grown in continuously flooded Maahas soil were H_2-oxidizing chemolithotrophic bacteria (Table 2 & Fig. 1). H_2-dependent chemolithotrophs were not detected in the endorhizosphere of upland rice and in the rhizosphere and nonrhizosphere soil in upland conditions. The same was the case with maize grown in the same upland conditions (Table 3). The population of aerobic heterotrophic bacteria in the endorhizosphere of wetland and upland rice were almost similar.

The incidence of chemolithotrophic bacteria did not differ between rice varieties grown in different seasons of the year in flooded conditions of Maahas soil (Table 2). Although the incidence of chemolithotrophic H_2-oxidizing bacteria in the endorhizosphere of rice grown in the flooded Maahas soil was high, this was almost negligible in oxidized and reduced soils collected from the unplanted flooded field.

The number and percentage of occurrence of chemolithotrophic H_2-oxidizing bacteria in the endorhizosphere and basal culm of rice grown in acidic rainfed wetland soil of Luisiana (Table 4) were almost 20 times lower than those in rice grown in Maahas wetland conditions (Table 4). The data also showed that the inoculated N_2-fixing H_2-oxidizing chemolithotrophic bacteria (which were predominantly present in the endorhizosphere of rice grown in wetland Maahas) failed to establish in rice grown in Luisiana soil under wetland conditions by heading time (Table 4). The number of chemolithotrophic bacteria was almost the same in the endorhizosphere and basal culm of both inoculated and uninoculated plants. The colony type of the chemolithotrophic bacteria observed in rice grown in Luisiana soil was different from that observed in the endorhizosphere of rice grown in continuous wetland conditions of Maahas soil (small and colored) and pure culture of Pseudomonas sp. H8.

Both Luisiana and Maahas soils were almost similarly capable of consuming externally supplied H_2 and of producing H_2 (Tables 5 and 6). H_2 production was more pronounced after the addition of rice straw or sucrose.

Discussion

The data obtained from Maahas soil confirmed the preliminary observation of Watanabe et al.[23] that a large proportion of the aerobic heterotrophic bacteria associated with the endorhizosphere of rice grown in continuously flooded Maahas soil are H_2-oxidizing chemolithotrophs.

In our earlier studies[9] with pure culture of H_2-oxidizing N_2-fixing chemolithotrophic bacteria isolated from the endorhizosphere of rice grown in flooded Maahas soil, the chemolithotrophic ability of bacteria was expressed only when the oxygen level was low (2%). Similarly, several other H_2-oxidizing bacteria were reported to oxidize H_2 only when the pO_2 level was low; many of these bacteria were isolated from mud, sewage, lake bottom sediments, etc. where pO_2 is low[6]. Uptake hydrogenase has been found to be sensitive to O_2 in H_2- oxidizing Azospirillum brasilense[20] and was reported to be more sensitive to O_2 than the nitrogenase itself[17]. N_2-fixing aerobic heterotrophic bacteria associated with wetland rice in Maahas soil were capable of chemolithotrophic growth only when H_2 was provided[9]. Watanabe and Yates (unpublished) found that uptake hydrogenase of Pseudomonas sp. H8 was formed in heterotrophically grown cells only when O_2 was low (2%). H_2 is not necessary for hydrogenase induction in heterotrophically grown cells. Therefore, it can be postulated that the undetectable population of these bacteria in upland rice endorhizosphere and rhizosphere (unlike in flooded Maahas soil) must be due to the highly aerobic condition and to the nonavailability of H_2, and probably to competition from other aerobic microorganisms such as fungi and actinomycetes which are not dominant in wetland conditions.

Rice root was found necessary for these bacteria in the flooded Maahas soil, in oxidized and reduced soil under similar conditions, these were not found. The required O_2 level and probably the other growth factors needed for derepression of hydrogenase and protection mechanism from O_2 may be provided by the wetland rice root. This hypothesis is not yet proven. Chemolithotrophic H_2-oxidizing bacteria isolated from rice grown in flooded conditions of Maahas soil could fix nitrogen only under microaerobic conditions[5]. Therefore, this suggests that unlike Azotobacter, these bacteria do not have the mechanism to protect nitrogenase from oxygen damage. Since they are associated with the wetland rice root, the rice plants must be providing the protection from O_2 damage to nitrogenase. This must be the reason for their predominance in the endorhizosphere of the rice grown in Maahas soil but not in oxidized soil.

However, the low population in rice grown in wetland conditions of Luisiana soil in contrast to rice grown in continuous wetland of Maahas soil is not due to lack of H_2, evolution, and its utilization, because Luisiana soil can produce and consume H_2 to the same extent as Maahas soil. The absence of these bacteria in reduced soil is probably due to lack of O_2. Since the chemolithotrophic H_2-oxidizing bacteria are sensitive to a high O_2 level during the drying cycle of soil (during dry season in Luisiana), the population of these bacteria might have reached a low level because of lack of moisture and high O_2 level. But during the crop period (rainy season) the soil was under flooded conditions and the cultivar grown in both soils was the same (IR42). If there were no other soil factor involved, at least the inoculated chemolithotrophic bacteria should have established. Because the

inoculated bacteria failed to establish, the reason for the low population of H_2-oxidizing chemolithotrophs and reported low ARA associated with low N_2-fixing bacterial populations in Luisiana soil[7] is most probably related to some factors associated with the soil which is not found in Maahas soil.

Under flooded conditions large quantities of ferrous iron are known to be formed. This process is known to be further enhanced due to the yearly cycle of dryland to wetland every year. Luisiana soil contains more free Fe oxides (4.3%) than Maahas (2.9%) (Table 1). Ferrous iron formation in Luisiana soil under wetland conditions was found significantly higher than in Maahas soil[21]. Further, the rice plant is known to have different mechanism to tolerate or avoid ferrous iron toxicity[18,19]. One of these mechanisms is the oxidation of ferrous iron to ferric form inside or on the root surface, enzymatically or by the O_2 excreted from the roots. O_2 transport from shoot to root in wetland rice is well documented[11,2, 14,1]. Electron microscopic studies have clearly revealed that under excess ferrous iron in the growing medium of rice, the roots were found to accumulate large deposits of ferric iron precipitate in the intercellular spaces of the root[8]. Electron micrographic studies have recently confirmed the distribution of associative N_2-fixing bacteria in the intercellular spaces of cortical cells in rice and also in other aquatic plant roots[16,15]. Therefore, it is probable that the oxygen-mediated ferric iron deposition inside the roots of rice grown in ferrous iron-rich Luisiana soil (unlike Maahas grown rice) was not favorable for these chemolithotrophic H_2-oxidizing N_2-fixing bacteria for their

activity, which may be due to competition for space, higher Eh, or other ferric iron-associated factors inside the root.

Therefore, oxygen must be directly and indirectly responsible for the ecological distribution of chemolithotrophic H_2-oxidizing N_2-fixing bacteria and their activity in different soils and cultivation practices of rice.

Further studies are needed to confirm this hypothesis. The nitrogen fixing activity and nitrogen-fixing bacterial population reported earlier[3,4,23,9,7] are found closely parallel with the H_2-oxidizing chemolithotrophic bacterial populations observed in the present study.

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Table 1. Characteristics of Luisiana and Maahas soil.

Soil type	pH (water)	Carbon (%)	Total-N (%)	Free iron oxides (%)	CEC (meq/100 g)	Soil texture
Luisiana soil	4.7	1.33	0.17	4.3	23.2	Heavy clay
Maahas soil	6.6	1.24	0.15	2.9	33.5	Silty clay

Table 2. Aerobic heterotrophic and H_2-oxidizing chemolithotrophic bacterial populations in the root histosphere of wetland rice grown in continuously flooded Maahas soil.

Variety	Population (no. per g dry wt of root)	
	Aerobic heterotrophic bacteria on TSA ($\times 10^7$)	H_2-oxidizing chemolithotrops ($\times 10^7$)
IR26	40	36 (90%)
IR52	53	33 (62%)
IR56	120	133 (94%)
IR42	36	32 (86%)

Table 3. Aerobic heterotrophic and H_2-oxidizing chemolithotrophic bacterial population in upland and in continuous wetland conditions of Maahas soil.

	Population (no. per g dry wt)	
	Aerobic heterotrophic bacteria on TSA ($\times 10^7$)	H_2-oxidizing chemolithotrophs ($\times 10^7$)
Upland		
IR52 rice endorhizosphere	19.7	none*
Non rhizosphere soil	1.65	none
Corn endorhizosphere	11.3	none
Non rhizosphere soil	3.87	none
Wetland		
IR42 rice endorhizosphere	23.20	18.2
Oxidized soil	0.19	0.01
Reduced soil	1.88	0.01

*No colony was positive at the dilution of 10^{-6} or 10^{-5}.

Table 4. Aerobic heterotrophic and H_2- oxidizing chemolithotrophic bacterial population in rainfed wetland rice in Luisiana soil inoculated with N_2- fixing bacteria at heading (IR42).

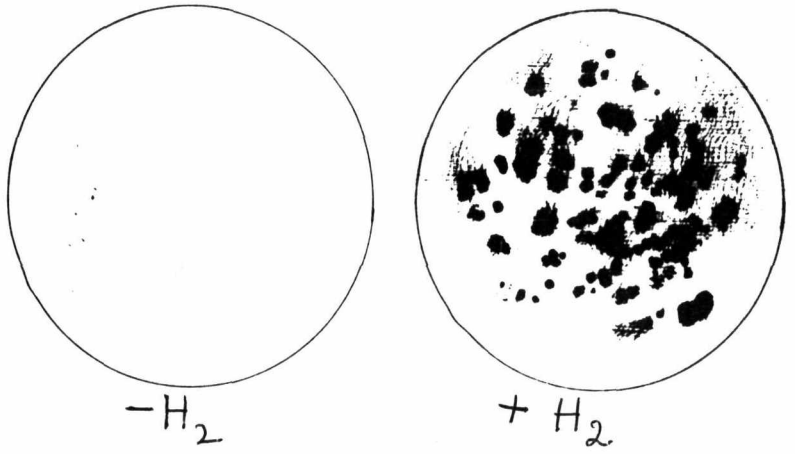
	Population (no. per g dry wt)	
	Aerobic heterotrophic bacteria on TSA ($\times 10^6$)	H_2-oxidizing chemolithotrophs ($\times 10^6$)
Inoculated		
Pseudomonas sp. (H8)		
endorhizosphere	25	8 (31%)
basal culm histosphere	28	3 (10%)
Azospirillum lipoferum (34H)		
endorhizosphere	36	2 (5%)
basal culm histosphere	33	7 (21%)
Uninoculated		
endorhizosphere	46	11 (24%)
basal culm histosphere	36	8 (23%)

Table 5. Hydrogen gas utilization and/or hydrogen gas production in Maahas soil (µl H_2/20 g air dried soil, mean of four replicates).

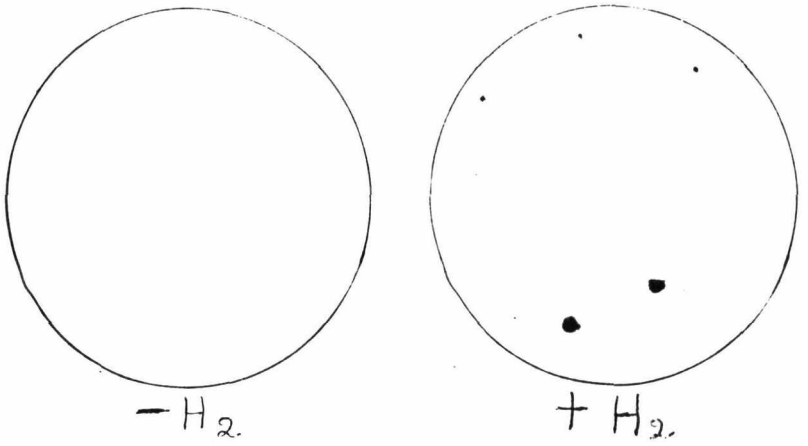
	Incubation period						
	0 h	14 h	36 h	84 h	11 days	17 days	43 days
Unamended	x 1						
+ 0 ml H_2	0	0	25	0	0.4	0.3	0
+ 0.5 ml H_2	393	284	300	0	0.4	0.3	0
+ 1.0 ml H_2	697	459	476	0	0.4	0.3	0
+ 2.0 ml H_2	1612	738	469	77	0.4	0.2	0
Rice straw amended	x 1						
+ 0. ml H_2	0	0	0	0	4.5	6.7	22
+ 0.5 ml H_2	430	266	76	0	4.5	9.9	24
+ 1.0 ml H_2	729	390	156	0	8.2	10.1	33
+ 2.0 ml H_2	1726	998	840	5	6.4	9.0	31
Sucrose amended	x 100						
+ 0 ml H_2	0	0	130	240	123	117	0.50
+ 0.5 ml H_2	4	3	106	369	125	111	0.72
+ 2.0 ml H_2	17	12	121	311	101	75	0.59

Table 6. Hydrogen gas utilization and/or hydrogen gas production in Luisiana soil (µl H_2/20 g air dried soil, mean of four replicates).

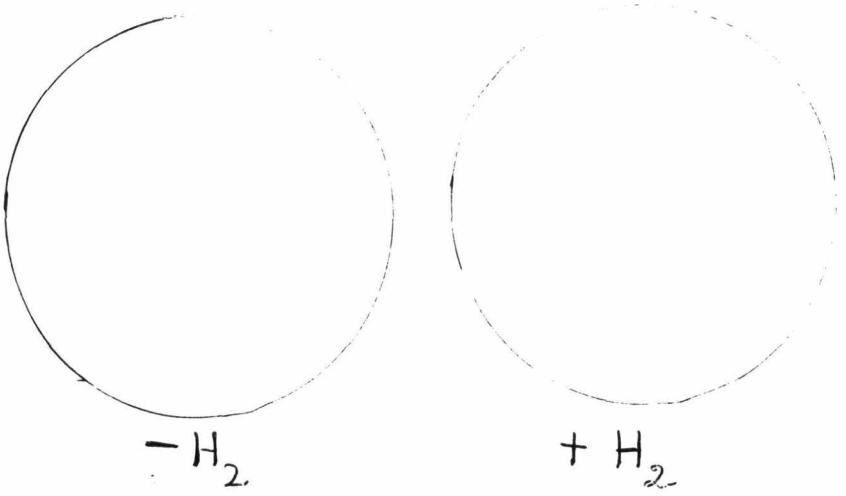
	Incubation period						
	0 h	14 h	36 h	84 h	11 days	17 days	43 days
Unamended	x 1						
+ 0 ml H_2	0	0	4	0	0.1	0.2	0
+ 0.5 ml H_2	365	200	177	53	0.1	0.2	0
+ 1.0 ml H_2	612	290	199	259	0.5	0.2	0
+ 2.0 ml H_2	1470	1100	1430	599	0.3	0.2	0
Rice straw amended	x 1						
+ 0 ml H_2	0	0	0	0	2	6	29
+ 0.5 ml H_2	402	278	286	0	2	3	23
+ 1.0 ml H_2	784	551	359	178	2	4	33
+ 2.0 ml H_2	1470	862	950	169	4	8	27
Sucrose amended	x 100						
+ 0 ml H_2	0	0	69	240	122	108	0.69
+ 0.5 ml H_2	4	3	96	226	117	24	0.69
+ 1.0 ml H_2	7	5	91	305	123	122	0.80
+ 2.0 ml H_2	15	12	113	340	115	123	0.85



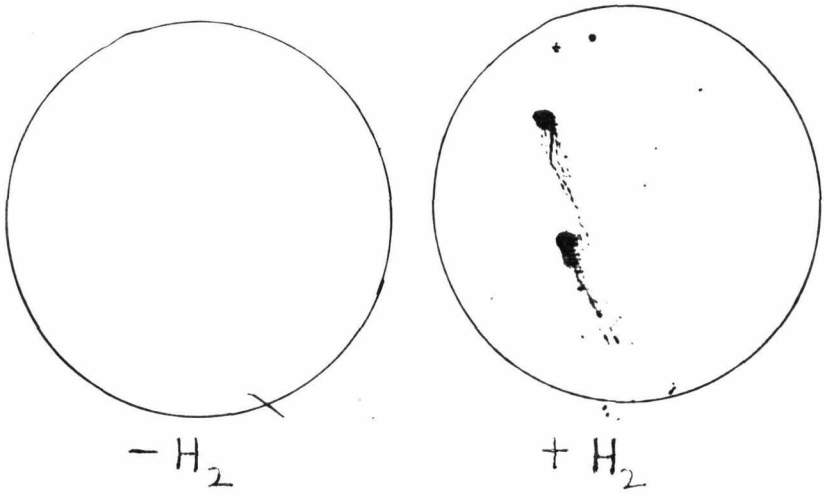
A. Endorhizosphere - Maahas wetland rice, dilution 10^{-6}



B. Oxidized soil - Maahas wetland dilution 10^{-5}



C. Endorhizosphere - Maahas upland rice dilution 10^{-5}



D. Endorhizosphere - Luisiana wetland rice, dilution 10^{-5} (uninoculated)

Fig. 1. Autoradiographs showing population of H_2-oxidizing chemolithotrophic bacterial population.

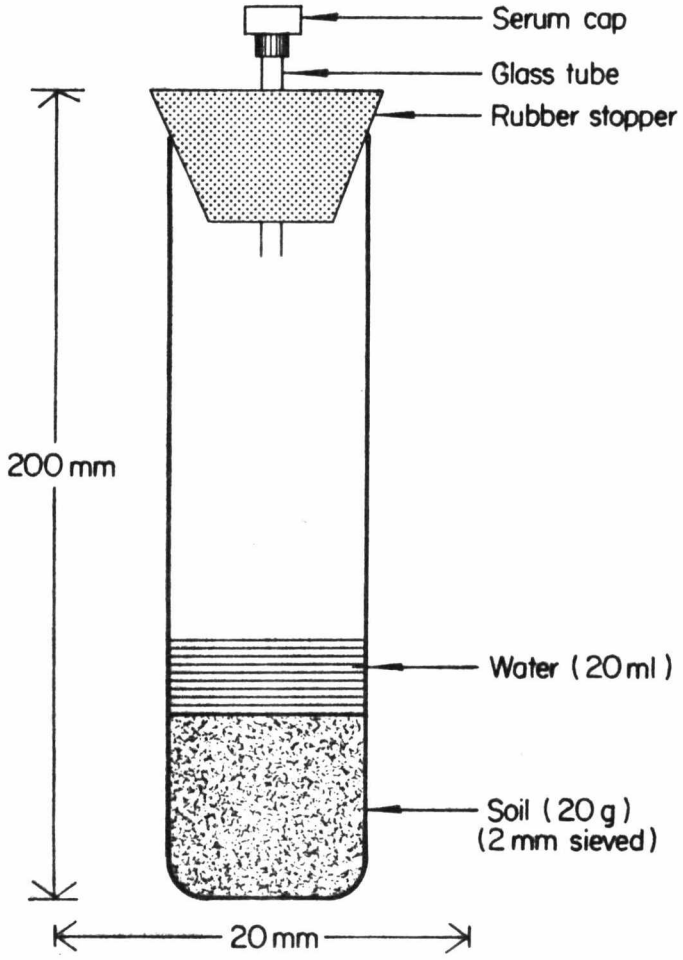


Fig. 2. Diagram showing the apparatus used for studying H_2-consumption and H_2-production.

Chapter 4

H_2-dependent chemolithotrophy in the genus *Azospirillum*

Abstract

H_2-dependent chemolithotrophy was studied in 33 strains of *Azospirillum* spp. obtained from different sources by employing ^{14}C-autoradiographic technique. All the fourteen strains of *A. brasilense* were unable to show chemolithotrophy. But only six out of nineteen strains of *A. lipoferum* tested showed chemolithotrophic ability. These differences in chemolithotrophy were found among the strains of both the species of *Azospirillum* which were isolated from rice and other crops obtained from different countries. The chemolithotrophic ability of different strains of *A. lipoferum* was found to be immunologically related. From the above observation it is suggested that all the *A. brasilense* are non chemolithotrophs, and there are two groups among the *A. lipoferum* strains of which some of them have chemolithotrophic ability. The non chemolithotrophic strains of *A. lipoferum* are seemed to be the intermediary group of *A. brasilense* and *A. lipoferum*. There seems to be a possible enzymatic and evolutionary relationship between their ecological and adaptation and chemolithotrophic activity.

Introduction

The genus Azospirillum has recently attracted the attention of microbiologist, agronomists and ecologists.

Azospirillum had been found to be associated with rhizosphere soil and roots of many forage grasses; in maize, millets, sorghum, rice, wheat and many other plants and they are dinitrogen fixers (Döbereiner, 1983). A wealth of information about its taxonomy, physiology and ecology has accumulated (Krieg, 1976; Sampaio et al., 1978; Krieg and Hylemon, 1976; Burris et al., 1977; Tarrand et al., 1978; Döbereiner and De Polli, 1980; Ladha et al., 1982). Based on morphological physiological and DNA homology studies, Tarrand et al. (1978) described them into two separate species, Azospirillum brasilense and Azospirillum lipoferum. The major differential characteristics are absolute biotin requirement and use of glucose and α-ketoglutarate as sole carbon sources and typical large involution forms in older, especially alkaline cultures in A. lipoferum.

Both species contains two groups, one which denitrifiers (nir^{+}) and another which does not (nir^{-1}). Differentiation between the two species and between the nir groups was also possible by fluorescent antibody technique (De Polli et al., 1980), and they also showed the clear cut differentiation between the strains of A. lipoferum and A. brasilense belong to different host plant specificity groups.

Association of Azospirillum in wetland rice has been reported by several workers (Kumari et al., 1976; Nayak and Rajaramamohan Rao, 1977; Silva and Döbereiner, 1978; Watanabe et al., 1979).

Watanabe et al. (1979) reported that these are present in large numbers in endorhizosphere of wetland rice.

Several strains of N_2-fixing Azospirillum strains were isolated from the wetland rice root, stem and soil of paddy fields at the International Rice Research Institute by Watanabe et al. (1979); Watanabe and Barraquio (1979), and they were grouped into two species, A. brasilense and A. lipoferum, by immunodiffusion and immunofluorescence technique (Ladha et al. 1982).

In our earlier studies (Gowda and Watanabe, 1983) we observed that all the three strains of A. brasilense tested were failed to show H_2-dependent chemolithotrophy but two strains of A. lipoferum were capable. Therefore, we examined more numbers of strains of both species obtained from different sources to confirm these differences between species.

Materials and Method

Source of bacteria

Twenty Azospirillum strains isolated from rice root, stem and soil of paddy fields at the International Rice Research Institute (IRRI) (Watanabe et al. 1979; Watanabe and Barraquio, 1979) identified by immunological techniques by Ladha et al. (1982) were used. Azospirillum lipoferum strains sp. USA5, Sp. 17 and Sp. 59 and A. brasilense strains Sp. 7, Sp. 13, Sp. 34 and Sp. 107 were obtained from Döbereiner, EMBRAPA/SNLCS-CNPq, Brazil. Azospirillum lipoferum strain RO7, S-28, Da-43, K-67, KP-77 and S-25 were obtained from Dommergues, ORSTOM, Dakar, Senegal. Some of the characteristics of all the above strains are listed in Table 1, 2, and 3.

Chemolithotrophic ability test

H_2-oxidizing chemolithotrophic property of all the strains of Azospirillum brasilense and Azospirillum lipoferum were tested by

^{14}C-autoradiographic technique described by Gowda and Watanabe (1983) as follows: The bacteria were grown on Tryptone yeast extract agar slants for 48 h at 32°C in ambient air. Three loops of the growth was suspended in 3.4 ml of H_2-uptake mineral medium. Using long tooth picks the suspension of bacteria was inoculated to predried agar plates of H_2-uptakes mineral medium containing 0.05 percent yeast extract. The plates were incubated in 10 liter desiccators providing 2% O_2, 5% CO_2 and balance N_2 ($-H_2$) or with 2% O_2, 5% CO_2, 20% H_2 and balance N_2 ($+H_2$) for 9 days at 32°C. The gas contents were evacuated and filled with $^{14}CO_2$ (100 uCi) plus the corresponding gas mixture provided previously ($-H_2$ or $+H_2$) and incubated for 11 days at 32°C. The growth of the bacteria with H_2 was compound with those grown without H_2. Then the bacterial growth was transferred to muslin cloth by replica method and exposed to x-ray film, and autoradiograph was obtained as described earlier. The dark spots correspond to the area of bacteria growth was the positive proof for $^{14}CO_2$-fixation. Since the bacteria were pregrown for 9 days on the H_2-uptake agar containing small quantity of yeast extract (as growth factor supplement) and then was exposed to $^{14}CO_2$, the $^{14}CO_2$ fixation observed was considered as autotrophic $^{14}CO_2$ fixation (chemolithotrophy).

Results

^{14}C-autoradiographs clearly revealed that, all the strains of Azospirillum brasilense and also Azospirillum lipoferum (both rice and non rice strains) were unable to fix $^{14}CO_2$ under autotrophic condition in the absence of H_2 (Table 2). In presence of H_2 also, all the strains of Azospirillum brasilense were unable to fix $^{14}CO_2$. However interestingly, some strains of Azospirillum lipoferum (both rice and non rice strains) showed $^{14}CO_2$ fixation in presence of H_2 and some strains of Azospirillum lipoferum (both rice and non rice strains) showed $^{14}CO_2$ fixation in presence of H_2 and some strains did not (Table 3 and Fig. 1).

Therefore, the results clearly suggested that all A. brasilense strains do not have H_2-dependent chemolithotrophic ability, but there are two groups in Azospirillum lipoferum.

Recognizable growth was observed in all the bacteria tested on H_2-uptake agar plate incubated with or without H_2 because of yeast extract. But only those strains of A. lipoferum showed distinguisable growth in presence of H_2 as compared with their growth observed under similar conditions without H_2 were able to show H_2-dependent $^{14}CO_2$ fixation.

Because some of the non H_2-oxidizing bacteria were able to fix CO_2 in the presence of organic substrate (our unpublished data) a strain of A. brasilense (Sp. 107) was also tested under heterotrophic conditions on Tryptone yeasts extract (TYE) and also under mixotrophic conditions (TYE + H_2) to know if there is expression of $^{14}CO_2$ fixation (RuBP case). Even under these conditions the above strain was unable to fix $^{14}CO_2$.

Discussion

The results clearly demonstrated that under the experimental conditions employed by us, the species of Azospirillum differed in their chemolithotrophic ability. Irrespective of their origin and host, all the Azospirillum brasilense strains were found to be non chemolithotrohps, whereas, among the strains of Azospirillum lipoferum, there were two groups of which one of them is chemolithotroph and other is not.

In their preliminary observation while studying the chemolithotrophy of Derxia spp. Pedrosa et al. (1980) observed that strains of A. brasilense (Sp. 7 and Sp. 197 and three non-nitrogen fixing mutants of Sp. 197) were unable to grow under autotrophic conditions (H_2 + CO_2 atmosphere) whereas some of the A. lipoferum strains (RS 20 and Sp. 208) were found to show

improved growth but not in strain Sp. 17. Malik and Schlegel (1981) reported that almost all the strains of A. lipoferum (Sp. 59b, A3a, RG-20a, RG6 xx, Br. 17) showed good growth under autotrophic condition (H_2 + CO_2). In contrast, none of the strains of A. brasilense were able to grow under similar autotrophic condition. Therefore, they suggested that these differences may probably add another differentiative character to the description of both species. The present studies with ^{14}C-autoradiographic technique also confirmed their observations which was mostly done with (non-radioactive studies) for strain 59b, Br. 17, Sp. 7 which were also studied by us in the present investigations (Table 1).

The immunological characteristics for all the Azospirillum isolates of IRRI used in the present study have been reported by Ladha et al. (1982). The results of cross reactions of 20 Azospirillum isolates with antisera and fluorescent antibodies showed at least one heat labile agar diffusable antigen in all strains (Table 4), except in few strains of A. lipoferum (36H8, $26S_3$ and $36R_4$) which failed to produce any band with any of the antisera tested and, also had lower FA reactions (1 + to 3 +) than other strains (Table 4 and 2). Interestingly, our studies also showed that these strains of A. lipoferum are also lacking H_2-dependent chemolithotrophic ability. In contrast, all the strains of A. lipoferum which showed H_2 dependent chemolithotrophy in the present study had more than one heat stable and had higher FA reaction (3 + to 4 +) (Table 4 and 2). The data therefore clearly revealed the presence of immunological relationship of chemolithotrophic ability among strains of A. lipoferum.

The variations in number of immunodiffusion band (heat treated and not treated antigen) among the strains of A. lipoferum with different antibodies (cross reaction test) and their relationship with chemolithotrophic ability

may be useful for genetic analysis to understand their autotrophic carbon metabolism.

Sampaio et al. (1978) reported two groups among A. brasilense based on the reduction of NO_2^- and sensitivity to tetracycline (Nir^+ and Nir^-). We did not find any relationship with nitrate reductase and chemolithotrophy among strains of Nir^- and Nir^+ activity of both A. brasilense and A. lipoferum (Table 1, 2 and 3).

Recently, enzyme studies with A. brasilense, (Sp. 7) have showed that this bacterium lacks a catabolic but has an anabolic Embden-Meyerhof-Parnashexose phosphate pathway and for N_2 fixation it uses Entners-Doudoroff pathway for gluconate dessimination (Westby et al., 1983). A. lipoferum (Sp. 59b), forms F-6-P via hexokinase, an enzyme which was found to be lacking in Sp. 7 (A. brasilense). However, both species lack oxidative Hexose monophosphates pathway (Maddalana et al., 1983).

The regulatory properties of Fructose-biphosphatase from Alcaligenes eutrophs H16, and H_2 oxidizing chemolithotrophs, have been more thoroughly studied (Abdelal and Schlegel, 1974) and it was reported as one of the important regulatory enzyme involved in the calvin cycle. Involvement of the RuBP carboxylase in autotrophic CO_2 fixation of A. lipoferum (Sp. 59b) have been shown by Malik and Schlegel (1981). The absence of Hexokinase (6-phospho-fructokinase) in A. brasilense (Sp. 7) seems to be mainly responsible for lacking H_2-dependent chemolithotrophy.

A. brasilense sp. 7 and $26H_2$ and A. lipoferum 34H have uptake hydrogenase, H_2-serves as energy source for CO_2 fixation under autotrophic conditions for chemolithotrophic strains of A. lipoferum (Gowda and Watanabe, 1983). We have also observed that some of the bacteria (i.e. Pseudomonas cepacia) which have no H_2-oxidizing ability but they are also capable of CO_2 fixation

under heterotrophic and also under mixotrophic conditions suggesting RuBP carboxylase operates if the suitable energy source is available (data not shown). Therefore, in a preliminary studies was carried out with Sp. 107 (A. brasilense). We did not observe $^{14}CO_2$ fixation even under heterotrophic or under mixotrophic conditions in this strain. It appears therefore the non chemolithotrophic properties of all A. brasilense strains and A. lipoferum strains may be due to lack of RuBP carboxylase itself and or due to the enzyme related with calvin cycle. It needs further confirmation.

Dübereiner (1983) reviewed the reported host specificity of Azospirillum, in which C_3 plants are associated with A. brasilense and C_4 plants with A. lipoferum reported that there were also exceptions i.e. sugarcane. Wetland rice (C_3) plant has large number of A. lipoferum (Watanabe et al. 1979). All the strains of A. lipoferum isolated from rice which were obtained from Dr. Dommergues were found to be non chemolithotrophs. It appears therefore the non chemolithotrophic strains of A. lipoferum are probably the intermediary group between A. brasilense and A. lipoferum and there seems to be an evolutionary relationship between the two species. The lack of certain regulatory enzyme in A. brasilense also supports this hypothesis. Our recent results (Gowda and Watanabe, 1985) showed that large number of H_2-oxidizing chemolithotrophs are associated mainly in endorhizosphere of wetland rice but not in oxidized and reduced soil or in the endorhizosphere of upland rice and its rhizosphere. The differences observed in the genus Azospirillum in their autotrophic carbon metabolism seems to have an ecological and evolutionary relationship further studies may lead to classify the intermediary group observed in this study to a separate species.

Table 1. Chemolithotrophy and some associated characteristics of Azospirillum lipoferum and Azospirillum brasilense strains obtained from different sources.

Strains	Source	Host plant	DNA homology Ab	DNA homology Al	$^{14}CO_2$ fixation (Autoradiograph)	Growth with H_2	Nitrate reductase activity
Sp 7 Ab Nir$^+$	Döbereiner	Digitaria	100	34*	-	+	+
Sp34 Ab Nir$^-$	"	"	81	43*	-	+	-
Sp 13 Ab Nir$^+$		"	94	32*	-	+	+
Sp107 Ab	"		-	-	-	+	ND
RO7 AL	Dommergues	rice	ND	ND	-	+	ND
S-28 AL	"	"	ND	ND	-	+	ND
Da-43 AL	"	"	ND	ND	-	+	ND
K67 AL	"	"	ND	ND	-	+	ND
KR 77 AL	"	"	ND	ND	-	+	ND
S25 AL	"	"	ND	ND	-	+	ND
Sp17 AL Nir$^+$	Döbereiner	maize	30	73*	-	+	+
SpUSA5 AL Nir$^+$	"	"	36	70*	+	++	+
Sp59 AL	ATCC		31	100*	+	++	ND

ND = not determined.

*Data from Tarrand et al., 1978.

Table 2. Chemolithotrophy and FA cross-reaction of Azospirillum lipoferum strains isolated from wetland rice.

Strain no. and source	FA cross reaction					$^{14}CO_2$ fixation (Autoradiography)	Growth with H_2	Nitrate reductase activity
	34H	$36H_9$	$26H_1$	FS	$36H_2$*			
26H1 Histosphere	4 +**	4 +	4 +	3 +	-	+	++	-
$26H_3$ "	4 +	4 +	4 +	4 +	-	+	++	-
34H "	4 +	3 +	3 +	3 +	-	+	++	-
$36R_1$ Rhizoplane	4 +	4 +	4 +	4 +	-	+	++	-
$36R_2$ Rhizosphere soil	4 +	4 +	4 +	4 +	-	+	++	-
DWR Deep water rice	4 +	3 +	3 +	4 +	-	+	++	+
FS Field soil	2 +	2 +	2 +	4 +	-	-	+	+
$26S_2$ Stem	2 +	2 +	3 +	3 +	-	-	+	-
$36R_5$ xx Rhizoplane	3 +	2 +	-	2 +	-	-	+	-
36H8 Histosphere	3 +	1 +	-	-	-	-	+	-

Data on FA cross reactions is adopted from Ladha et al., 1982.

*Azospirillum brasilense isolated from wetland rice.

**1 + to 4 + indicates maximum to minimum fluoresence.

Underscored values indicate homologous reaction.

Table 3. Chemolithotrophy and FA cross reaction of Azospirillum brasilense strains isolated from wetland rice.

Strain no. and source	FA cross reaction $36H_2$	$^{14}CO_2$ fixation (Autoradiograph)	Growth with H_2	Nitrate reductase activity
$26H_2$ Rotoon root histosphere	4 +*	-	+	+
$36H_4$ Root histosphere	4 +	-	+	+
$36H_6$ Root histosphere	4 +	-	+	+
$36H_7$ Root histosphere	4 +	-	+	+
36R5 YY Rhizosphere soil	4 +	-	+	+
$36H_2$ Root histosphere	4 +**		+	+
$36H_3$	ND	-	+	ND
$36H_5$	ND	-	+	ND
$26H_5$	ND	-	+	ND
CH-1 (Chara) (Aquatic weed)	ND	-	+	ND

FA cross reaction data is adopted from Ladha et al., 1982.

*4 + means maximum fluorescence.

**Underscored indicate homologous reaction.

ND = not determined.

Table 4. Chemolithotrophy and gel immunodiffusion bands (cross reaction) of Azospirillum lipoferum strains isolated from wetland rice.

	Non-treated					Treated				
	34H	FS	36H9	26H1	36H2*	34H	FS	36H9	26H1	36H2
$26H_1$	3	3	1	4	-	1	2	1	2	-
$26H_3$	2	2	1	1	-	1	1	-	-	-
34H	4	2	1	2	-	3	1	-	-	-
$36R_1$	2	1	1	2	-	1	1	-	1	-
$36R_2$	2	2	1	2	-	1	1	-	1	-
DWR	2	2	1	2	-	-	-	-	-	-
FS	1	4	1	-	-	-	3	-	-	-
$26S_2$	1	1	-	1	-	-	-	-	-	-
$36R_5XX$	1	1	-	-	-	-	-	-	-	-
36H8	-	-	-	-	-	-	-	-	-	-

Data on gel immunodiffusion is adopted from Ladha et al., 1982.

*Azospirillum brasilense isolated from wetland rice.

Values indicate number of precipitation bands.

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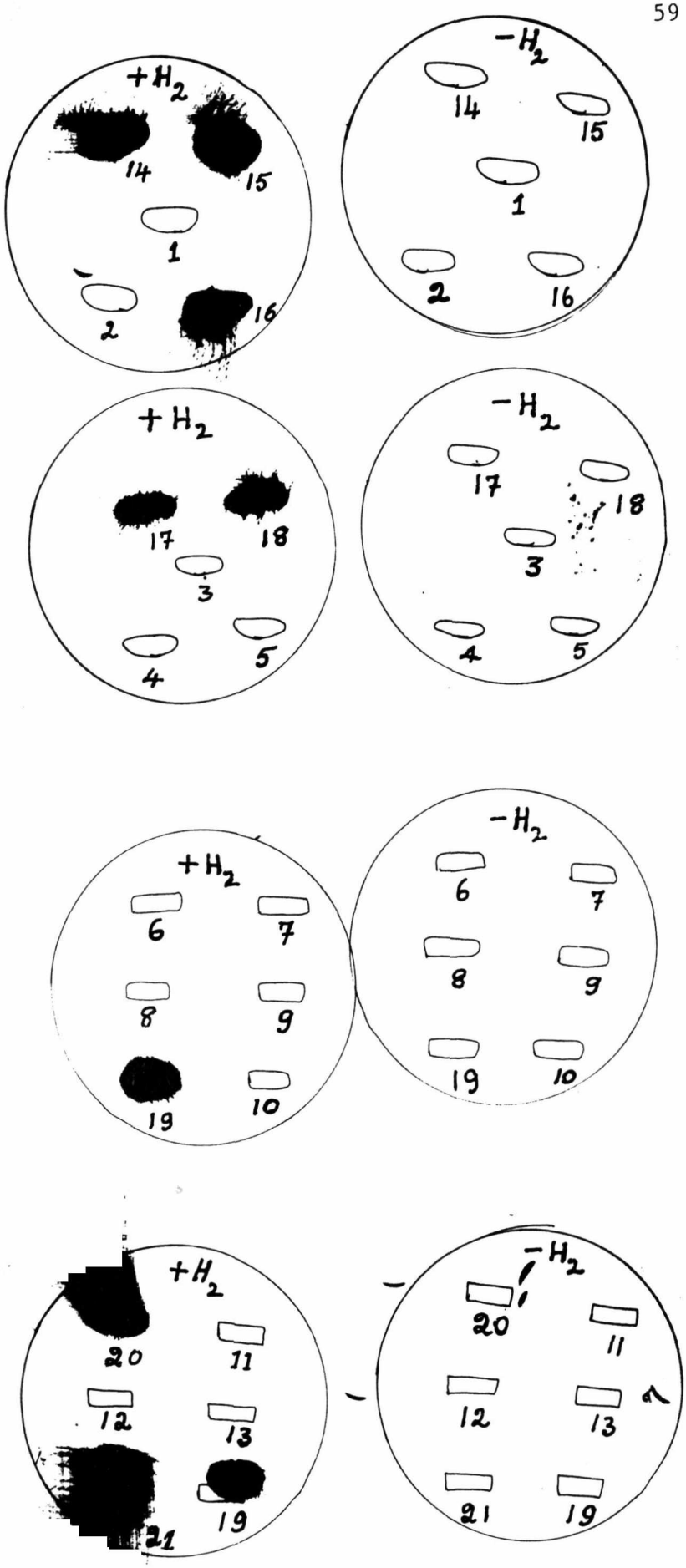

Azospirillum brasilense strains

1 Sp-7

2 Sp-34

3 Sp-13

4 Sp-107

5 $26H_2$

6 $36H_4$

7 $36H_6$

8 $36H_7$

9 36R5 YY

Azospirillum lipoferum strains

10 FS

11 $26S_2$

12 $36R_5$ XX

13 36 H8

14 Sp USA-5

15 Sp USA-59

16 $26H_1$

17 $26H_3$

18 34H

19 $36R_1$

20 $36R_2$

21 DWR

Fig. 1. ^{14}C-autoradiographs showing differences in H_2-oxidizing chemolithotrophic activity in strains of Azospirillum brasilense and Azospirillum lipoferum.

PART II HYDROGEN SUPPORTED DINITROGEN FIXATION

Chapter 5

Hydrogen supported N_2-fixation of *Pseudomonas* sp. and *Azospirillum lipoferum* in free-living and monoaxenic conditions of rice

Abstract

Hydrogen dependent dinitrogen fixation under chemolithotrophic condition of *Pseudomonas* strains H8, KLH76 and *Azospirillum lipoferum* strain 34H, which are dominant in association with wetland rice root, was confirmed by acetylene reduction assay (ARA) and $^{15}N_2$ incorporation. Glucose lower than 2 mM did not repress H_2-dependent acetylene reduction of chemolithotrophically grown *Pseudomonas* sp. H8 and *Azospirillum lipoferum*. ARA of bacteria associated with etiolated seedlings of rice was not stimulated by H_2 (10%), but ARA associated with the root of 4 wk old rice seedling was stimulated by H_2. In the absence of H_2, difference in ARA was found among rice varieties, but in the preence of H_2, the varietal difference was not apparent.

Introduction

Nitrogen balance studies (App et al. 1980) and long term soil fertility and crop response studies (Watanabe et al. 1981) have confirmed nitrogen gains in wetland rice.

Dinitrogen fixation associated with wetland rice has been reported by several workers (Yoshida and Rinaudo 1982; Watanabe 1981) which includes N_2-fixation that occurs on or in the leafsheaths, and basal portion of shoot, and in the root zone (rhizosphere soil, rhizoplane, and endorhizosphere). A large percentage of the aerobic heterotrophic bacteria in the wetland rice root is N_2-fixing (Watanabe et al. 1979). Different genus of bacteria were isolated as the N_2-fixing bacteria from wetland rice root and basal parts of shoots Enterobacter (Watanabe et al. 1979), Azospirillum (Watanabe et al. 1979; Ladha et al. 1982), and Pseudomonas (Watanabe and Barraquio 1981; Watanabe et al. 1982; Barraquio et al. 1983).

H_2-oxidizing chemolithotrophic ability is reported for heterotrophic aerobic N_2-fixing bacteria: Rhizobium japonicum (Hanus et al. 1979) and Derxia spp. (Pedrosa et al. 1980), and Azospirillum lipoferum (Malik and Schlegel 1981). H_2-supported N_2-fixation was reported for H_2 oxidizers: Azotobacter chroococcum (Walker and Yates 1978) Azospirillum brasilense (Pedrosa et al. 1982) and Rhizobium japonicum (Emerich et al. 1979). H_2 dependent acetylene reduction activity (ARA) and

chemolithotrophy were also found in Pseudomonas sp. H8 and Azospirillum isolated from wetland rice (Watanabe et al. 1982; Gowda and Watanabe 1983).

Because Smith et al. (1976) reported the inhibition of H_2-uptake activity by C_2H_2 in N_2-fixing bacteria, and Dixon (1978) suggested the possibility of inhibitory effect of H_2 to N_2-reduction, but not C_2H_2 reduction, it was necessary to confirm dinitrogen reduction of bacteria grown under chemolithotrophic condition. Because the used bacteria were isolated from wetland rice root, it is interesting to know if they show H_2 dependent ARA in association with rice.

The sterile rice seedlings were used to demonstrate H_2-supported ARA in association with rice plant.

Materials and methods

Bacteria strains

N_2-fixing Pseudomonas sp. H8 and KLH76 (Watanabe and Barraquio 1979; Barraquio et al. 1983) and Azospirillum lipoferum 34H (Ladha et al. 1982), which were isolated from endorhizosphere of wetland rice, were used.

H_2-dependent N_2-fixation under free living conditions

Bacteria were grown in tryptone yeast extract agar (TYEA) slants for 48 h at 32°C, in ambient air. Two loops of growth from the slant was suspended in 3.4 ml of NH_4 Cl free H_2-uptake broth (Watanabe et al. 1982). This suspension (0.1

mL) was inoculated into the 13 mL tubes, containing 3.4 mL of NH_4Cl free H_2-uptake semisolid agar medium per tube. Tubes with cotton plugs were incubated for 25 days in dessicators (10 L capacity), containing 20% H_2, 2% O_2, 5% CO_2 and N_2 as normalizing gas (or under the same gas mixture without H_2). Cotton plugs of the tubes showing visible pellicles on the top of agar were replaced with U.V. sterilized serum stopper, then evacuated, flushed twice with N_2, and finally filled with different gas mixtures consisting 5 or 10% C_2H_2, 2% O_2, 5% CO_2 plus different levels of H_2 (0, 1%, or 10%), N_2 was normalizing gas. The tubes were incubated at 32°C and C_2H_4 formation was measured at periodical intervals.

For $^{15}N_2$ fixation studies, bacteria was grown in 25 mL NH_4Cl free H_2 uptake medium in 50 mL flask under 20% H_2, 2% O_2, 5% CO_2 and balance N_2 for 30 days, as described above. Then, cotton plugs were changed with rubber stoppers, evacuated and filled with 73% $^{15}N_2$ (96% ^{15}N) 2% O_2, 5% CO_2, 20% H_2 or Ar. $^{15}N_2$ gas was purchased from Monsanto Research Corp. U.S. Dept. of Energy (98% ^{15}N). The gas was cleaned before use as described by Ohyama and Kumazawa (1978). After 5 days of incubation at 32°C, bacteria were digested by Kjeldahl method, and ^{15}N content of this distillate was measured by emission spectroscopy after micro Dumas combustion. ^{15}N content of the gas was measured by emission spectroscopy.

To study the effect of organic compound on H_2-dependent ARA, bacteria were grown on 3.4 mL of NH_4Cl free H_2-uptake liquid medium in 13 mL tubes under chemolithotrophic conditions for 25 days under 20% H_2, 2% O_2, 5% CO_2 and balance N_2 in dessicator. Cotton plug was replaced with U.V. sterilized serum stopper, evacuated and filled with gas mixture providing 20% H_2, 2% O_2, 5% CO_2, 10% C_2H_2 or the same gas mixture without H_2. Ar was the normalizing gas. A filter sterilized glucose solution (0.1 mL) was injected to each tube separately to give 0.0, 0.02, 0.2 and 2.0 mM at final concentration. The tubes were incubated at 32°C and ARA was measured at periodical intervals.

Etiolated rice seedlings with bacteria

Spermosphere model developed by Bauzon et al. (1982) was used with some modifications as follows. Dehusked rice seed was sterilized by dipping 5 min in 70% ethyl alcohol, washing twice with sterile water, dipping in 20 min in 5% chlorox, washing twice in sterile water, dipping 2 min in 0.1% acidified HgCl, and final washing 20 times with sterile water. Seeds were germinated on plain agar in petri plates for 3 days at 30°C in dark. The contaminant free uniformly germinated seeds were transferred aseptically to test tubes (150 x 16 mm) containing N-free plant nutrient semisolid agar (0.3% agar) (Yoshida 1976) and inoculated with (0.2 mL) washed bacterial suspension (10^8 cells/mL). The

tubes were filled with 5% C_2H_2 either with or without H_2 (10%) after removing the same quantity of air from each tube, and incubated under dark for 6 days. At periodical intervals C_2H_4 formed in the tubes were measured.

Rice seedlings with bacteria

Bacteria were grown on shaker for 24 h at about 25-28°C in ambient air in tubes containing 5 mL of TYE broth plus 0.25% glucose. The entire bacterial growth in the tube was suspended to 50 mL of the same medium and grown for another 18 h under similar conditions. The cells were washed twice with sterile 0.1 M Na phosphate buffer with saline (pH 7.1) by centrifugation at 6,000 rpm for 10 min at 4°C. The cell suspension was diluted with the same buffer, and O.D. was adjusted to 0.2 at 450 nm and used for inoculation.

Three sterilized uniformly germinated seeds were transferred to each tube (300 mm x 34 mm), Fig. 1 containing 30 mL plant nutrient agar (Yoshida 1976; modified to contain 20 ppm N as NH_4 NO_3, iron as Fe-EDTA, and Noble agar 0.8%). After 2 days of growth and establishment of the seedlings in tube, 0.2 mL of bacterial suspension of approximately 10^8 cells/mL was inoculated to each tube.

The inoculated seedlings were grown under light in KG cabinet (at 29/21°, 30 klux 12 h photoperiod) for 4 weeks. The plants were removed from tubes and only the excised root of all

three plants of each tube was placed in 10 mL plastic syringe with serum cap, and the gas mixture consisting of 2% O_2, 5% CO_2, 10% C_2H_2 with and without 10% H_2 and Ar as the normalizing gas was filled in the tube. Ethylene produced was measured at periodical intervals by FID gas chromatography.

Results

A. H_2-dependent nitrogenase activity in pure bacteria

At 25 days of incubation, N_2-fixing H_2-oxidizing bacteria tested could show visible pellicles on the surface of semisolid agar only in presence of externally supplied H_2, suggesting chemolithotrophic growth and H_2-dependent N_2-fixation.

H_2-dependent ARA. ARA activity was observed in the chemolithotrophically growing bacteria only when 10 percent H_2 was provided in the gas phase. But no ARA was observed when H_2 was zero or only one per cent H_2 was provided. No ARA was observed in a non-N_2-fixing but H_2-oxidizing bacteria, Pseudomonas facilis (Table 1). The present results confirmed that the tested bacteria have H_2-dependent N_2-fixing ability under chemolithotrophic conditions. However, for detectable ARA, more than 1 percent H_2-supply was needed. Because, C_2H_2 is known to inhibit O_2-dependent H_2-uptake at low H_2-concentrations in A. brasilense (Chan et al. 1980), two

levels of C_2H_2 were used. ARA activity observed with 5 and 10 per cent C_2H_2 was almost the same. Therefore, 10% C_2H_2 concentration was used in further studies.

H_2-dependent $^{15}N_2$-fixation

Pseudomonas sp. H8 grown under chemolithotrophic condition were further incubated with $^{15}N_2$ in presence and absence of H_2. The ^{15}N atom per cent excess measured was 0.758 ± 0.08 in the presence of H_2 and 0.098 ± 0.02 in the absence of H_2. This result clearly showed H_2-dependent chemolithotrophic dinitrogen reduction.

H_2-dependent ARA in chemolithotrophically grown bacteria in the presence of different levels of glucose

Lag of ARA was observed in most cases.Chemolithotrophically grown Pseudomonas sp. H8 continued to show H_2-supported ARA until 72 h in the presence of glucose. With 2 mM of glucose, bacteria could switch to glucose supported ARA no earlier than 72 h (Table 2). In Azospirillum lipoferum, at 24 h and 72 h, ARA was negligible. At 5 day incubation, 2 mM glucose could switch from H_2-supported ARA to glucose supported ARA similarly as Pseudomonas.

B. H_2-supported ARA of the N_2-fixing bacteria in association with rice plant under monoaxenic condition

ARA in association with etiolated seedlings

ARA was found by bacteria inoculated to etiolated seedlings both

in the presence and the absence of H_2, but no significant difference in ARA was found in both conditions (Table 4). ARA was not detected in uninoculated plants and also in bacteria alone without plant.

H_2-supported ARA in the inoculated rice seedlings grown under light

Root of 4 wk old IR42 rice seedlings grown under light showed ARA from all tubes inoculated with A. lipoferum. Significantly higher ARA was found in presence of H_2 than without H_2 (Table 5). The ethylene formation of uninoculated plants was negligible. When Pseudomonas sp. H8 was inoculated into various rice varieties, difference in ARA were found among varieties in the absence of H_2. IR58, Mahsuri, and IR60 showed lower ARA and more stimulated ARA by H_2 than others. But in the presence of H_2, varietal difference almost disappeared (Table 6). In IR42 and OS4, H_2 stimulated ARA.

Discussion

In our earlier studies with ^{14}C-autoradiographic technique it was found that the large number of H_2 oxidizing N_2-fixing chemolithotrophs are associated with wetland rice root and the predominant N_2-fixing bacteria isolated from endorhizosphere of rice roots, namely Pseudomonas sp. and Azospirillum lipoferum 34H were H_2-oxidizing chemolithotrophs (Gowda and Watanabe 1983). Since the bacteria were grown in chemolithotrophic condition and

ARA was observed with 10 percent H_2, the result definitely confirmed the dependency of these bacteria on H_2 for energy necessary for ARA.

H_2-dependent ARA has been reported for various aerobic H_2-oxidizing N_2-fixing bacteria: Azotobacter chroococcum (Walker and Yates 1978), Azospirillum brasilense (Pedrosa et al. 1982), and Rhizobium japonicum (Emerich et al. 1979), Renobacter vaculatum, and Alcaligenes latus (Malik and Schlegel 1981). The present report add the genus Pseudomonas also into the above list.

Our results suggested that at 2 mM of glucose or lower, no immediate repression of H_2 supported ARA took place and at 2 mM glucose chemolithotrophic ARA was switched to heterotrophic one after a lag period.

Because after longer incubation with glucose (2 days and longer), glucose was also found to support ARA activity both in the presence or the absence of H_2, suggesting the synthesis of enzymes needed for glucose utilization which might have repressed in chemolithotrophic condition. It is quite possible that mechanisms differ from that of glucose supported ARA in the H_2-oxidizing N_2 fixing bacteria of H_2-dependent N_2-fixation.

Absence of enzymes of EDP pathway due to H_2 repression in H_2 oxidizing Alcaligenes eutrophs (Schink and Schlegel, 1978); a branched electron transport pathway in R. japonicum (O'Brian

and Maier, 1982); part of the respiratory chain involvement in catalysis of oxyhydrogen reaction in R. japonicum (Emerich et al., 1980); different terminal oxidase of high affinity to O_2 in A brasilense (Tibelius and Knowles, 1983); involvement of flavoprotein, b and c type cytochromes (O'Brian and Maier, 1983), ubiquinone, b and c type cytochromes (Eisbrenner and Evans, 1982) have been reported to have involved in oxyhydrogen reaction of R. japonicum. It is quite possible that these deviated mechanisms are also involved in the H_2-oxidizing N_2 fixing bacteria associated with wetland rice for H_2-dependent N_2-fixation.

Maier et al. (1979) reported that all usable carbon substrate (15 mM) tested with H_2-oxidizing R. japonicum were found to repress hydrogenase formations but none of them were found to inhibit hydrogenase activity. It is reported that the uptake hydrogenase in A. lipoferum and Azotobacter chroococcum is enhanced under carbon limited condition (Volpan et al. 1981. Walker and Yates 1978). Hydrogenase activity is apparently dependent on the availability of reducing equalents (Friedrich 1982).

In our recent studies (unpublished) we have also observed H_2-supported ARA in these bacteria (Pseudomonas sp. H8 and A. lipoferum) even under heterotrophic conditions with glucose in semisolid agar incubated in ambient air. The H_2-supported ARA observed under the condition with glucose were significant only at low levels of glucose (up to 2 mM) but not at higher levels of

glucose. This is similar to the other bacteria which have been reported to show H_2 supported N_2 fixation on different organic carbon sources: A. brasilense (Tibelius and Knowles 1983), and A. chroococcum (Walker and Yates 1978). From these findings it appears that hydrogen dependent ARA is active in carbon limited condition.

H_2-supported ARA under monoaxenic conditions

Although H_2-supported N_2-fixation has been reported in pure cultures of a few aerobic N_2-fixing bacteria mentioned above, no report was available to show this in association with plants. Also, no studies were available with bacteria associated with wetland rice. Our present studies gave the evidence for the presence of H_2-supported ARA in association with rice plant under monoaxenic condition.

We did not observe significant H_2-supported ARA under spermosphere condition, where inoculated sterile germinated seeds were grown under dark for a short period (6 day) under etiolated conditions. But we observed H_2-supported ARA in the rice plants grown under light for 4 weeks in mineral plant nutrient agar (under photosynthetic conditions). The absence of H_2-supported ARA in the etiolated seedlings may be attributed to high availability of readily available carbon from the germinating seeds. Whereas, in 4 wk seedling, as the plants were grown in presence of light for longer period in carbon free mineral agar,

the reserved carbon and energy sources of the root are unlikely so high as to show catabolite repression to H_2-dependent ARA. But it is note-worthy that varietal difference was observed in ARA in the absence of H_2. This finding suggests that lower ARA associated with IR58, Mahsuri and IR60 was due to the limiting concentration of organic substrates supply from the plant root to support heterotrophic N_2-fixation. The external supply of H_2 eliminates, at least partially, limitation of energy supply to the bacteria. This result suggests H_2 may be used in root-bacteria association where energy supply from root is limited.

As ATP-dependent evolution of hydrogen is an inherent property of nitrogen fixing bacteria they must contain an uptake hydrogenase, enabling them to recycle the hydrogen. It is possible that possession of hydrogen dependent and hydrogen supported nitrogen fixation and H_2-dependent CO_2 fixation (Gowda and Watanabe 1983) and probably mixotrophic growth must provide them further advantages of survival and increased biochemical activity in nature, especially in organic carbon limited soils and endo and ectorhizosphere.

It was observed that the rice varieties which showed lower ARA (Table 6) in the absence of H_2 (Mahsuri and IR60) had lower dry weight or root (Table 6b). Interestingly, in these varieties methane content (in the tubes incubated for ARA) was significantly lower (Table 6a). However, this was not observed when the same

was incubated with H_2 under similar condition. In those varieties which showed higher ARA in the absence of H_2 (IR42, OS4) there was no change in methane content when incubated either with or without H_2.

It is possible that those varieties (Mahsuri and IR60) showed lower ARA (Table 6) are oxidizing methane when incubated without H_2. But this oxidation is inhibited in the presence of H_2. Since methane oxidation is known to be inhibited by C_2H_2 it is not clear whether the total disappearance of methane in Mahsuri variety or lower methane content IR60 is due to oxidation or due to some other mechanism. But it is worthy to note that there is a relationship with lower ARA activity and disappearance of methane, and it is different among the varieties tested. Further studies may prove to be beneficial in studying the varietal difference in rice in relation to methane metabolism and N_2-fixation.

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Table 1. H_2-dependent N_2-fixation (ARA) under chemolithotrophic conditions by the bacteria isolated from wetland rice (n mol C_2H_4 evolved/tube).

	% H_2	36 h after incubation						5-days after incubation					
		0		1		10		0		1		10	
	% C_2H_2	5	10	5	10	5	10	5	10	5	10	5	10
Pseudomonas sp. (H8)		0	0	0	0	149** ± 36	142 ± 21	0	0	0	0	225 ± 56	197 ± 41
Pseudomonas sp. (KLH 76)		0	0	0	0	101 ± 10	121 ± 13	0	0	0	0	184 ± 42	117 ± 41
Azospirillum lipoferum (34H)		0	0	0	0	405 ±115	410 125	0	0	0	0	728 277	1056 135
Pseudomonas facilis*		0	0	0	0	0	0	0	0	0	0	0	0

*A non N_2-fixing, H_2-oxidizing chemolithotrophic bacteria.

**Mean and SEM of 4 replications.

Table 2. Effect of glucose addition on H_2-dependent ARA in chemolithotrophically grown Pseudomonas sp. H8.

mM glucose added/tube	Incubation period (n mol C_2H_4/tube)					
	24 h		72 h		5 days	
	+ H_2	- H_2	+ H_2	- H_2	+ H_2	- H_2
0.0	112* ± 47	1 ± 0.0	113 ± 29	1.3 ± 0.57	113 ± 45	9 ± 6
0.02	88 ± 28	1.6 ± 1	176 ± 49	2.3 ± 1.5	197 ± 36	4.3 ± 2
0.2	89 ± 9	11 ± 4	160 ± 98	18 ± 6	196 ± 125	24 ± 6.6
2.0	54 ± 10	5 ± 1	85 ± 20	68.7 ±34	348 ± 200	369 ±159

*Mean and SEM of three replicates.

Table 3. Effects of glucose addition on H_2-dependent/H_2-supported ARA in chemolithotrophically grown Azospirillum lipoferum 34H.

n mol glucose added/tube	After 5 days incubation	
	+ H_2	- H_2
0.0	217* ± 147	3 ± 1
0.02	111 ± 97	1 ± 0.0
0.20	145 ± 254	2 ± 1
2.0	1450 ± 387	1400 ±112

Negligible ARA was observed at 24 h.

*Mean and SEM of three replicates.

Table 4. H_2-supported N_2-fixation (ARA) in spermosphere of IR42 rice seeds inoculated with bacteria isolated from wetland rice.

		Incubation period (n mol C_2H_4 tube)		
		36 h	60 h	5 days
Pseudomonas sp. (H8)				
	+ H_2	540 ± 206*	1885 ± 307	4316 ± 1761
	- H_2	367 ± 161	1733 ± 232	3796 ± 1596
Azospirillum lipoferum (34H)				
	+ H_2	465 ± 139	1515 ± 626	2340 ± 983
	- H_2	208 ± 30	1482 ± 534	3536 ± 1220

*Mean and SEM of five replicates.
No detectable ethylene was found both in uninoculated control and no-plant (bacteria alone) control.

Table 5. ARA in the roots of rice plants grown in light for four weeks under monoaxenic conditions with Azospirillum lipoferum 34H inoculated to sterile germinated seeds. (IR42)

Incubation period	n mol C_2H_4 roots of 3 plants	
	+ H_2	- H_2
16 h	57.8* ± 26.7	9.50 ± 2.5
24 h	195.6 ± 99	19.7 ± 4.9
36 h	1095 ± 146	153.1 ± 68

*Mean of five replicates and SEM.

Table 6. ARA in the roots of rice plants grown in light under monoaxenic conditions for four weeks with Pseudomonas sp. H8 inoculated to sterile germinated seeds.

	Average hourly and n mol C_2H_4/tube*	
	$- H_2$	$+ H_2$
IR42	6.9 ± 2.7	13.7 ± 4.7
OS4	3.6 ± 0.7	14.1 ± 3.2
BPI 76	9.3 ± 4.6	5.1 ± 1.4
IR58	0.3 ± 0.18	11.9 ± 2.1
Mahsuri	0.1 ± 0.05	6.5 ± 3.5
IR60	0.2 ± 0.06	6.2 ± 0.3
STE	3.00**	2.9

*Measurements were made at 24, 43, and 70 hrs of incubation.

$$\text{Hourly ARA} = 1/3 \left[\frac{(x_1)}{24} + \frac{(x_2 - x_1)}{19} + \frac{(x_3 - x_2)}{27} \right]$$

where x_1, x_2, and x_3 are C_2H_4 found at 24, 43, and 70 hrs.

**Average ± standard errors of three replicates, only valid for IR42, OS4 and BPI 76.

Table 6a. ARA in the roots of rice plants grown in light under monoaxenic conditions for four weeks with Pseudomonas sp. H8, inoculated to sterile germinated seeds. (Mean of three replications)

Rice variety	Incubation period (n mol C_2H_4/roots of 3 plants)					
	24 h		43 h		70 h	
	- H_2	+ H_2	- H_2	+ H_2	- H_2	+ H_2
IR42	60.4 a	70.4 a	139.2 a	494.0 ab	524.0 ab	924.0 ab
OS4	22.2 abcde	174.9 a	116.5 ab	684.0 a	276.0 abc	914.0 a
BPI-76	66.8 ab	32.0 abcd	73.1 abc	262.6 abc	774.0 a	318.0 abcde
IR58	28.3 abc	48.2 ab	26.5 abcd	344.6 abcde	28.6 d	838.0 abc
Mahsuri	0 f	32.5 abcde	4.1 e	204.0 abcdef	6.6 e	447.3 abcde
IR60	13.4 abcd	25.5 bcdef	17.8 bcde	236.0 abcd	13.3 de	414.0 abcd
Effect of H_2		*		*		*
Varietal difference		*		*		*
V x H_2		*		ns		*
CV (%)		29.38		19.74		12.49

*Statistically significant.

Statistical analysis was done from transformed values, i.e. log (x + 1).

Table 6b. Dry weight of seeds and seedlings of different varieties of rice which were inoculated with Pseudomonas sp. H8 and grown for four weeks in KG cabinet.

Rice varieties	Dry wt of 3 seeds (mg)	Dry wt of 3 plants (mg)	% increase in plant wt (over seed wt)	Dry wt of root (mg) (of 3 plants)	Shoot/root
IR42	42.7	75.52	76.86	19.82	3.81
OS4	78.0	90.99	16.65	33.62	2.70
BPI 76	43.5	72.47	66.59	20.96	3.45
IR58	54.0	84.20	55.90	21.14	3.98
Mahsuri	43.3	66.49	106.49	13.68	4.86
IR60	50.8	84.05	65.00	18.33	4.58

Values are average of three replicates, each replicate had three seeds or plants.

Table 6c. Methane content in the syringes incubated with roots of rice plants inoculated with Pseudomonas sp. H8 for ARA (n moles/tube).

	- H_2			+ H_2		
	24 h	43 h	70 h	24 h	43 h	70 h
IR42	37*	28	33	37	28	37
OS4	32	25	30	37	31	37
BPI 76	22	17	19	35	36	36
IR58	32	22	28	34	35	47
Mahsuri	0	0	0	39	36	41
IR60	10	8	8	42	38	37

*Mean of three replicates.

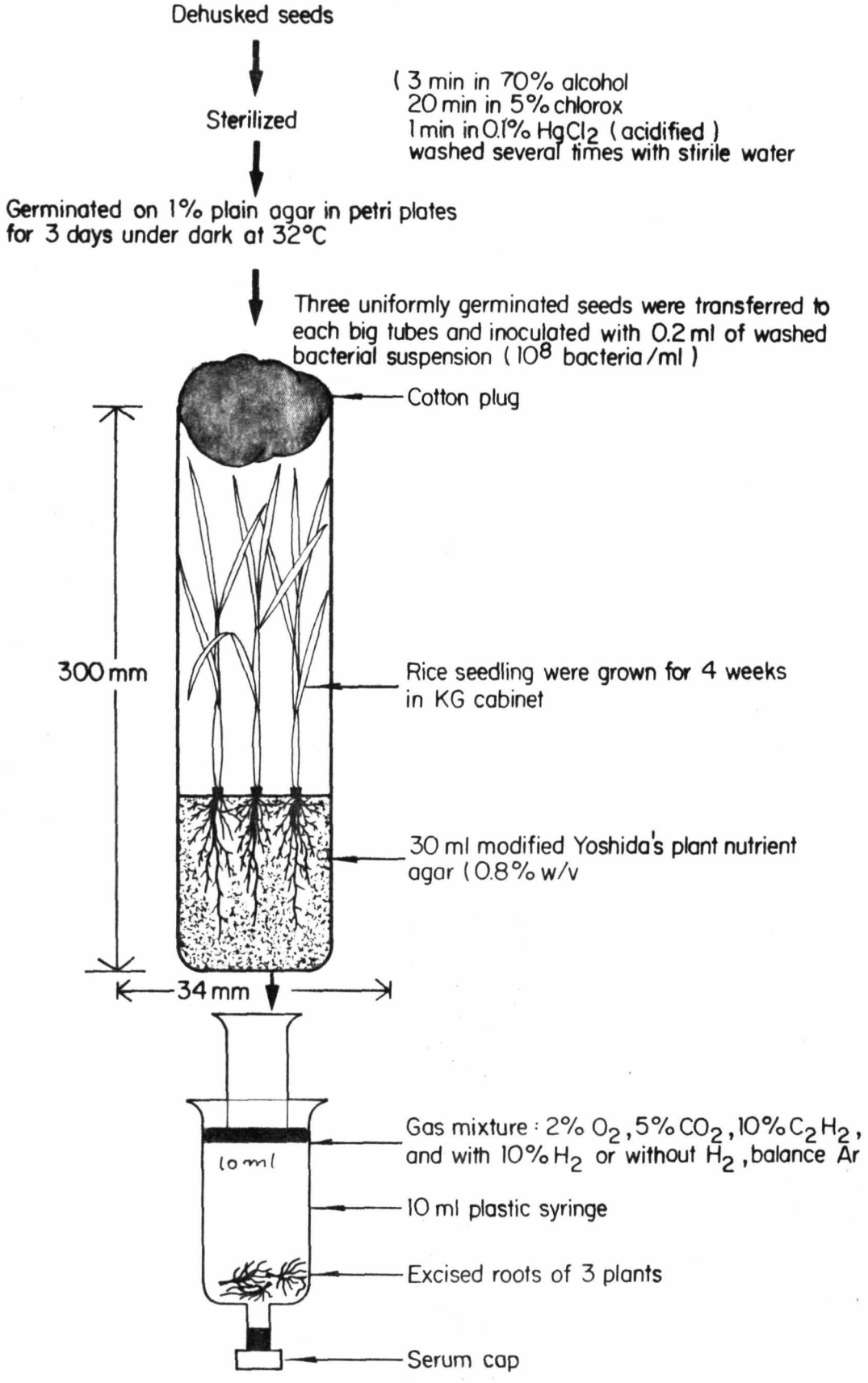

Fig. 1. Schematic diagram showing the monoaxenic culture technique of rice used for studying H_2-supported acetylene reduction and differences among varieties.

Chapter 6

Hydrogen supported dinitrogen fixation in bacteria associated with field grown wetland rice

Abstract

The stimulation of acetylene reduction by the externally added H_2 (10% in volume) was found in association with the excised root, the plant from which the aerial shoots were removed and the intact plant of the rice grown in the continuously flooded field. The excised root had H_2-uptake activity. The stimulation of N_2-fixation by H_2 in association with the rice plant from which aerial parts of shoot were removed under field conditions was confirmed by $^{15}N_2$ incorporation.

Introduction

Watanabe et al. (1982) reported that large percentage of aerobic heterotrophic bacteria associated with wetland rice are H_2 oxidizing N_2-fixers, and these bacteria are H_2-oxidizing chemolithotrophs (Gowda and Watanabe 1983). In previous paper, Gowda and Watanabe (1985) confirmed chemolithotrophic H_2-dependent N_2-fixation of these bacteria by ^{15}N. H_2-supported N_2-fixation (acetylene reduction) was also found, when these bacteria were grown in association with sterile 4 wk old rice seedlings.

H_2 is known to evolve in wetland rice soils4 (Yamane and Sato 1964; Harrison and Aiyer 1916) and from wetland rice root (Kimura 1981). The facts of H_2 evolution in flooded rice soils, predominance of H_2-dependent chemolithotrophic N_2-fixing bacteria in association with wetland rice root, and H_2-supported acetylene reduction by bacteria in association with sterile energy-supply-limited rice seedling lead the hypothesis that N_2-fixation associated with field grown rice may be stimulated by H_2. To examine this hypothesis, the effect of externally added H_2 on N_2 fixation associated with wetland rice was studied by acetylene reduction and $^{15}N_2$ incorporation. The results are reported in this paper.

Materials and methods

H_2-uptake and ARA of washed roots

Rice plants were grown in Maahas soil of the International Rice Research Institute (IRRI's) field which is kept under continuously flooded condition with continuous planting with no fertilizer application for past 9 years. Rice plants at heading, some after acetylene reduction assay (ARA), were collected. The soil from the root was washed while bubbling N_2 gas in water to minimize the exposure of roots to air. All the washed roots of each plant were placed in a 500 mL flask filled with water and stoppered with rubber plug (Fig. 1). The water was displaced introducing N_2 gas through rubber stopper using hyperdermic needles. The flasks containing roots were evacuated and flushed with N_2 and finally incubated with a gas mixture consisting 0.5 or 1% H_2, 2% O_2, 5% CO_2 and balance Ar. H_2 content in the incubated flasks were measured at intervals by Sensor gas chromatography to record the H_2-uptake activity.

Similarly, the roots were incubated in 500 mL flask with 10% H_2 or Ar, with 2% O_2, 5% CO_2, 10% C_2H_2 and balance Ar. C_2H_4 formed was determined at intervals to measure ARA activity.

ARA of intact plants in the field

The field ARA assay method was modified from the method described by Lee and Watanabe (1977) (Fig. 2).

Plants with the same number of tillers were selected randomly. A metal ring (30 cm dia.) was inserted around each selected plant. To avoid blue green algae (BGA) activity, the water, weeds, BGA and top aerobic soil layer which were inside the ring was removed and the dead leaves of plants were also removed. Then the soil surface and submerged portion of shoot was covered with aluminum foil. Two liter deionized distilled water was added. The whole plant was enclosed by the rigid, transparent plastic cylinder of 12 L capacity (75 cm high x 20 cm diameter) by pushing it into the soil up to 15 cm deep. One liter of C_2H_2 or 1 liter C_2H_2 + 1 liter H_2 was introduced into the cylinder through an opening at the top and simultaneously displaced the same amount of the inside air through the side opening of the cylinder. After closing the side opening the gas inside was mixed with serum stopper and finally top opening was also closed. At periodical intervals, C_2H_4 evolved was measured in the gas sample obtained from the cylinder. At heading, or maximum tillering stage, assays were made as follows.

H_2 supported ARA of the field grown rice from which aerial shoots were removed

In order to minimize the quantity of $^{15}N_2$ because of its high cost and technical difficulty in enclosing big plants in field condition, the possibility of using cut plant was examined.

The top of the rice plant was cut at 20 cm above soil. The procedures were followed to avoid BGA activity as mentioned above. The cut rice hill was covered by a glass jar of 10 liter capacity (20 cm dia x 40 cm high, by pushing it into the soil up to 15 cm deep) and, then, the bottle with rice hill was filled with water (Fig. 4). The mouth of the jar was tightly plugged using rubber stopper and scotch tape. One liter of gas mixture prepared in plastic bag, consisting of 10% C_2H_2, 20% O_2, and balance He or the same gas mixture with 10% H_2, displaced the same volume of water in the glass jar. The glass tubes were provided with the rubber plug for this purpose. ARA was measured at periodical intervals and compared with ARA observed with intact plants.

$^{15}N_2$ feeding to rice grown in field.

The cut rice hill from which aerial shoots were removed was enclosed in air tight glass jar as shown in Fig. 4 as described above. One liter of the gas mixture prepared in plastic bag, consisting of 70% $^{15}N_2$ gas with 95 atom % ^{15}N, 20% O_2, 10% H_2 or He, displaced the same volume of water in the glass jar. $^{15}N_2$ gas was purchased from Monsanto Research Corp., U.S. Department of Energy (98% ^{15}N), was cleaned as described by Ohyama and Kumazawa (1978). The glass tubes in the rubber plug were sealed with serum stoppers. One of two adjacent hills was exposed to H_2 and there were five such replications.

After the introduction of gas mixture with $^{15}N_2$ the rice hills were exposed for $^{15}N_2$ for 60 h. Then the rice hill was uprooted, washed carefully with water and separated into roots (all roots of rice hill, including aerial roots), submerged shoots (4-6 cm lower portion of shoot which was under water), upper shoot (the shoot which was above the water level, including freshly grown shoot during the period of enclosure with $^{15}N_2$). The ^{15}N content in the samples were measured by mass spectrometer after Rittenberg conversion. $^{15}N_2$ gas content from each jar was measured at periodical intervals by emission spectrometer. Methane content in the gas samples was measured by GC with FID, and H_2 content by Sensor gas chromatography.

Results

H_2-supported ARA and H_2 uptake by washed roots

ARA activity was measured at heading stage in the presence or the absence of externally supplied H_2 in the washed roots of rice plants collected from wetland field. The ARA was observed both in presence and absence of H_2 (Table 1). ARA increased by 43 - 112% in presence of H_2. The data, therefore, show the H_2-supported ARA activity in washed roots of rice plants grown in wetland conditions of Maahas soil.

The consumption of externally supplied H_2 in washed roots of rice plants grown in the continuously flooded Maahas soil that

were collected at heading was measured. The data clearly show that (Table 2) within 24 h, the incubated roots consumed almost all the externally provided H_2 (2.5 to 5 mL H_2 per roots of each plant hill). The data, therefore, substantiate the ability of consuming externally supplied H_2 by the roots of rice grown in the flooded Maahas soil. But, H_2 uptake activity was much higher than needed for the stimulated acetylene reduction. Previous exposure of plants to acetylene or acetylene with H_2 did not change H_2 uptake activity.

H_2-supported ARA of the whole plant in field condition

The effect of H_2 on ARA of the field grown rice was studied. Because the relationship between ARA and total dry wt of plant was observed, the data are presented per gram dry wt of total plant to compare between experiments. All data obtained from three experiments (Table 3) showed a significant increase of ARA by H_2. Therefore, H_2-supported ARA was quite evident in the plants grown in field condition.

H_2-supported ARA of plants from which aerial shoot was removed under field conditions

Data obtained from this experiment (Table 4) showed that ARA in the excised plants without shoot was not different from that of (Table 3) intact plants, a significant increase in ARA with H_2 than without external H_2 supply was observed.

H_2-supported $^{15}N_2$-fixation in the rice plants from which aerial shoot was removed under field condition

To minimize the quantity of $^{15}N_2$ only the basal portion of the field grown rice hill was enclosed tightly and the shoots were exposed to ambient air as shown in Fig. 3. $^{15}N_2$ of the gas within the enclosed portion was rapidly diluted, probably by N_2 transfer to the enclosed portion through plant shoots. Therefore, this method was not practicable. Hence, $^{15}N_2$ fixation studies were carried out in the plants from which the part of aerial shoot was detached. The $^{15}N_2$ inside the glass jar was diluted to a small extent (Table 6). Therefore, the average of ^{15}N content during the exposure was used to calculate N_2 fixation rate.

Methane content was found to increase gradually in all the jars (Table 5) and, higher amount of CH_4 was observed in the plants provided with H_2. This suggested that part of the H_2 provided from external source was utilized for the production of CH_4. The increased formation of methane by H_2 was about 15 mL per hill, corresponding to 60 mL H_2 consumption. Almost all the externally supplied H_2 was consumed within 60 h and a small quantity of H_2 also produced without H_2 (Table 5). A large fraction of H_2 seemed, therefore, to have consumed for methane production.

Data on $^{15}N_2$-fixation by the rice plants without aerial shoot showed the enrichment of ^{15}N detectable by mass

spectroscopy after 60 h of exposure to $^{15}N_2$ (Table 6). Further, the plants exposed to $^{15}N_2$ with externally supplied H_2 consistently had higher ^{15}N content than those without H_2. In particular, ^{15}N contents in the root was increased by H_2 supply. Therefore, the data confirmed the H_2-supported N_2-fixation in wetland rice under field conditions.

Discussion

In our earlier studies with ^{14}C-autoradiographic technique we have confirmed that a large number of H_2-oxidizing chemolithotrophs are associated with wetland rice grown in continuously flooded Maahas soil. The predominant N_2-fixing bacteria isolated from histosphere (endorhizosphre) of rice roots, Pseudomonas sp. and Azospirillum lipoferum, were H_2-oxidizing chemolithotrophs (Gowda and Watanabe 1983). In addition, these bacteria were found to have H_2-dependent N_2-fixing ability under chemolithotrophic condition, and H_2-supported N_2-fixation in associations with young rice seedling under monoaxenic conditions (Gowda and Watanabe, 1985).

In the present study the evidence was obtained on H_2-supported ARA associated with intact rice (Table 3) and the excised root (Table 1), and utilization of externally supplied H_2 in the washed roots (Table 2).

$^{15}N_2$-isotopic studies showed (Table 6) the higher ^{15}N atom % excess, and total fixed N content in most of the plant

parts when H_2 was provided externally. Therefore, data definitely confirmed that the ARA stimulation by H_2 was due to H_2-supported N_2-fixation.

From ^{15}N atom % excess in different parts of the plant within 60 h of exposure to $^{15}N_2$, (Table 6) root and submerged shoot appeared to be primary sites of N_2-fixation as shown by intact plant (Ito et al. 1980). But significant amount of ^{15}N was found in upper shoot which includes the newly grown shoot during the exposure period.

A new flush of vegetative growth occurred from the cut end of all the plants, suggesting that the excised plants were metabolically active. Therefore, the ^{15}N observed in the parts above submerged parts could be partly due to transportation of fixed ^{15}N from roots and submerged portion of plant.

Because H_2 is used by many microearobic and strict anaerobic bacteria, the stimulation of ARA by H_2-does not necessarily mean the use of H_2 by microaerobic N_2-fixing bacteria.

The root of all plants studied with or without H_2 were free of black colouration due to sulphide deposition after enclosure of excised plant. The roots also remained reddish brown. This visual observation after H_2 exposure suggests that rhizosphere remained oxidized. This may indicate the minimum influence of anaerobic N_2-fixing bacteria, particularly sulphate reducing bacteria. Because H_2 stimulated CH_4

formation during the exposure, strict anaerobic methagenic process might have occurred. So, the contribution to N_2-fixation by strict anaerobic bacteria under this experimental condition cannot be ruled out. Further experiments are needed to know what kinds of N_2-fixing bacteria in reduced soil and rhizosphere and phyllosphere of wetland are involved in H_2-supported N_2-fixation.

The plant uptake of $^{15}N_2$ fixed by non associative N_2-fixing bacteria from outside the rhizosphere may not be significant, because the duration of the experiment seems too short to be absorbed from soil to plant.

Our earlier reports on chemolithotrophy (Gowda and Watanabe 1983); and H_2-dependent N_2-fixation in the chemolithotrophically grown bacteria both in free-living and in association with wetland rice (Gowda and Watanabe 1985) have clearly shown the possibility of production of reducing power and ATP needed for N_2-fixation from H_2. The present studies provides evidences for the possibility of the energy availability through H_2-oxidation to bacteria associated with field grown wetland rice as an additional energy source for N_2-fixation.

Malik and Schlegel (1981) reported that chemolithotrophic ability of H_2-oxidizing and N_2-fixing bacteria can not be considered marginal. It is likely that under natural conditions the carbon supply is actually limiting N_2-fixation by bacteria. Having hydrogenase in these bacteria may be beneficial for

N_2-fixation. Saving of photosynthates which are otherwise utilized for N_2-fixation (Lespinat and Berlier 1981) is an advantage and this may reflect on the increased dry matter production of legume plant with H_2-uptake positive Rhizobium.

Few studies were made on the effect of H_2 on higher plants. Banerjie and Shukla (1977) have showed the increased shoot weight in the rice varieties which were grown in culture solutions aerated with H_2 and attributed to micro nutrient nutrition.

It has been reported that, 0.31 to 2.58 percent of soil gas was H_2 in the Maahas soil at different period of rice crop growth under flooded wetland conditions (Yoshida et al. 1975). Similarly, 0 to 10 percent H_2 was observed in the evolved gas from an unplanted Indian soil under flooded condition (Harrison and Aiyer 1916).

Therefore, it may be said that concentration of H_2 supplied externally in our experiments was higher than that generally found in soil air, and, consequently, the stimulation of N_2-fixation by endogenous H_2 may be lower than observed one.

Because the stimulation of N_2-fixation by H_2 was more pronounced when organic substrates supply is limited in H_2-oxidizing N_2 fixing bacteria (Walker and Yates 1978; Tibelius and Knowles 1983; Volpon et al. 1981), stimulation of N_2-fixation by H_2 in association with field grown wetland

rice suggest that organic matter supply may be limited in the field condition. However, this hypothesis needs further verification.

Acknowledgment

We thank Mr. W. Ventura for his assistance during the field experiments and particularly during $^{15}N_2$-fixation studies.

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Table 1. H_2-supported ARA in the washed roots of rice collected from field at heading stage (IR42).

Incubation period	mol C_2H_4 evolved/g dry wt of roots	
	+ H_2	- H_2
24 h	9.43 ± 0.01*	4.4 ± 0.24
48 h	26.51 ± 1.65	18.54 ± 1.47

*Average of five replicates and SEM.
Average dry weight of root/flask 1±36 g.

Table 2. H_2-uptake activity of washed rice roots collected from field at heading (IR42).

	ν mole H_2 consumed/g dry wt of roots/day	
	Experiment 1 (with 1.0% H_2)	Experiment 2 (with 0.5% H_2)
0[a]	125 ± 17*	88 ± 9
+ C_2H_2[b]	123 ± 15	99 ± 9
+ C_2H_2 + H_2[c]	138 ± 10	90 ± 14

*Average of five replicates and SEM.

[a]Normal plants at heading (not enclosed).

[b]Plants were enclosed for 5 days in plastic cylinder with 10% C_2H_2.

[c]Plants were enclosed for 5 days in plastic cylinders with 10% C_2H_2 plus 10% H_2.

Table 3. H_2-supported ARA in wetland rice under field conditions.

	n mol C_2H_4 evolved/g total dry wt of plant					
	24 h after			48 h after		
	+ H_2	- H_2	% increase due to H_2	+ H_2	- H_2	% increase due to H_2
Expt. I	810	580	39.65	2910	1910	52.32
IR42 at heading % CV	16.3			21.9		
Nov. 1982 LSD at .05	165		*	770		*
Expt. II	880	320	177	1710	1020	67.64
IR42 at heading % CV	43.5			18.0		
Jan. 1983 LSD at .05	379		*	359		*
Expt. III	1591	957	66.24	5220	2740	90.51
IR52 at maximum % CV	11.5			14.4		
Tillering LSD at .05	215		*	835		*

*Significant at 5% level.

Average dry wt. of plant Expt. I - 20.48 g
Expt. II - 43.25 g
Expt. III - 16.84 g

Table 4. H_2-supported ARA in rice hill from which aerial shoots were removed under field conditions at heading (IR42).

	n mole C_2H_4 evolved/g dry wt of plant		
	Incubation period in hours		
	24	48	60
+ H_2	1190 ± 85*	4530 ± 235	5620 ± 350
- H_2	627 ± 60	2780 ± 490	4100 ± 345
% increase due to H_2	89.8	62.9	37.1

*Mean of four replicates and SEM.

Average total dry wt of plant 20.85 g.

Table 5. $^{15}N_2$, CH_4 and H_2 content in the gas sample inside the glass jar (ml/jar) enclosing excised plant.

	Period of enclosure with glass jar			
	0 h	24 h	48 h	60 h
$^{15}N_2$ atom % excess				
- H_2	82.9 ± 1.58*	ND	ND	62.1 ± 0.95
+ H_2	84.0 ± 0.98	67.50	ND	62.9 ± 1.44
CH_4 (mL/jar)				
- H_2	0.60 ± 0.01	18.03 1 0.81	45.27 ± 1.24	64.99 ± 1.99
+ H_2	0.46 ± 0.16	23.95 ± 3.09	63.04 ± 5.70	81.61 ± 10.31
H_2 content (mL/jar)				
- H_2	0.07 ± 0.01	0.887 ± 0.05	1.229 ± 0.35	2.10 ± 0.35
+ H_2	82.42 ± 1.79	37.84 ± 5.29	17.45 ± 5.54	3.242 ± 0.71

*Mean and SEM of five replicates.

ND: not determined.

Table 6. Data showing H_2-supported N_2-fixation under field conditions.

		Excised plant parts exposed to $^{15}N_2$			
		Root	Submerged shoot	Upper shoot	Total
^{15}N atom % excess	- H_2	0.119 ± 0.028*	0.145 ± 0.014	0.069 ± 0.009	-
	+ H_2	0.179 ± 0.021	0.173 ± 0.028	0.095 ± 0.023	
^{15}N content (µg)/plant	+ H_2	32.8 ± 7.6	42.0 ± 4.1	41.43 ± 6.1	116 ± 12
	- H_2	54.4 ± 5.5	49.1 ± 4.8	52.20 ± 11.2	155 ± 11
% increase due to H_2		65.9	16.9	26.9	34.0
N fixed (µg)**	- H_2	45.2 ± 10.5	57.9 ± 5.78	57.15 ± 8.48	160 ± 16.9
	+ H_2	74.2 ± 7.52	66.8 ± 6.61	71.03 ± 15.3	212 ± 14.9

*Figures are mean of five rice hills from which aerial shoots were removed and exposed to $^{15}N_2$ with or without H_2 and standard error of mean.

**Atom % excess of $^{15}N_2$ was 72.5 to 73.45 (Table 5). Because ^{15}N found in each portion may include ^{15}N transported from elsewhere, the measured values are apparent N_2-fixing rate.

Table 6a. Some characteristics of the plants exposed to $^{15}N_2$ (mean and SD of five plants). (Experiment 2).

	(a) Exposed to $^{15}N_2$ gas in glass jar				(b) Not exposed to $^{15}N_2$	
	Root	Shoot (submerged)	Upper shoot	Total	Top shoot detached	Total (a + b)
Dry wt (g)						
$-H_2$	3.83 ± 0.27	6.55 ± 1.29	8.53 ± 1.39	18.92 ± 2.52	13.52 ± 3.82	32.45 ± 6.22
$+H_2$	4.46 ± 0.51	6.48 ± 0.41	8.37 ± 0.55	19.31 ± 0.44	14.72 ± 2.03	34.04 ± 2.27
% nitrogen						
$-H_2$	0.718 ± 0.036	0.449 ± 0.027	0.716 ± 0.023	- -	- 1.625	- -
$+H_2$	0.723 ± 0.021	0.449 ± 0.028	0.669 ± 0.047	-	1.611 ± 0.137	-
Total -N (mg)						
$-H_2$	27.45 ± 1.33	29.54 ± 6.43	59.76 ± 8.99	116.76 ±14.28	218.93 ± 65.13	335.69 ± 78.70
$+H_2$	32.28 ± 4.19	29.13 ± 2.84	56.11 ± 6.34	117.53 ± 6.06	236.04 25.12	353.57 ± 29.34

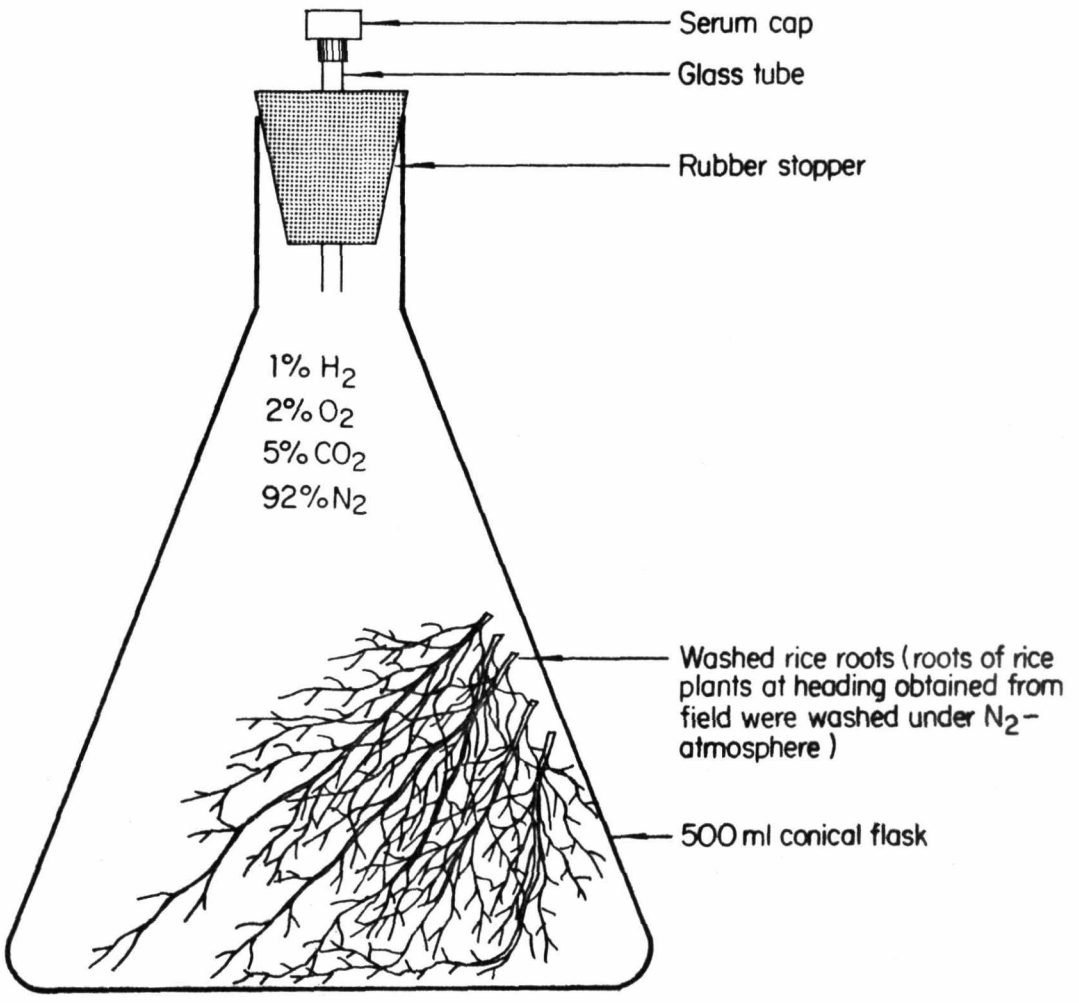


Fig. 1. Diagram showing the technique used to study the H_2-uptake activity of excised roots of rice.

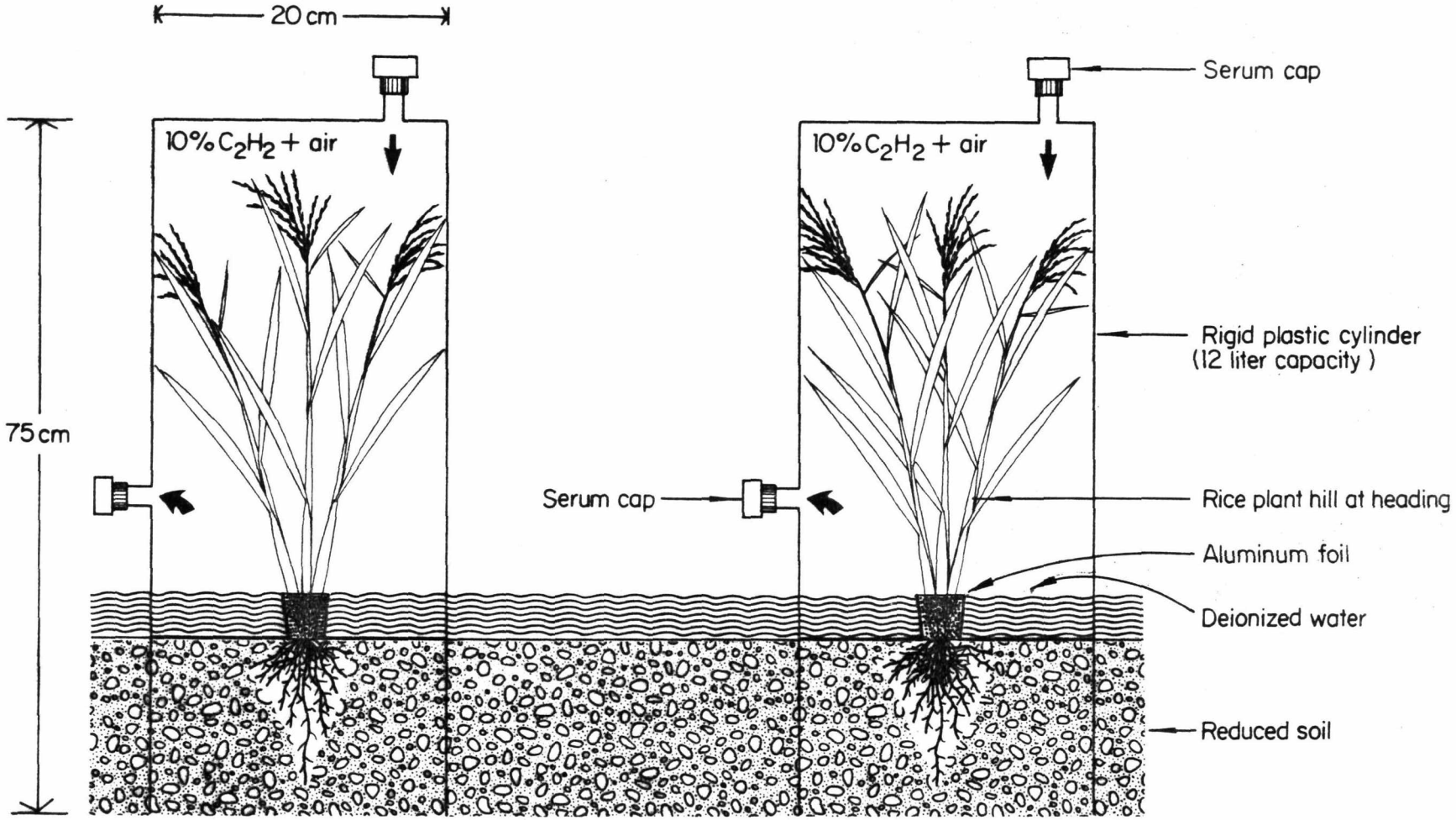


Fig. 2. Diagram showing the technique used to study H_2-supported acetylene reduction under wetland field conditions.

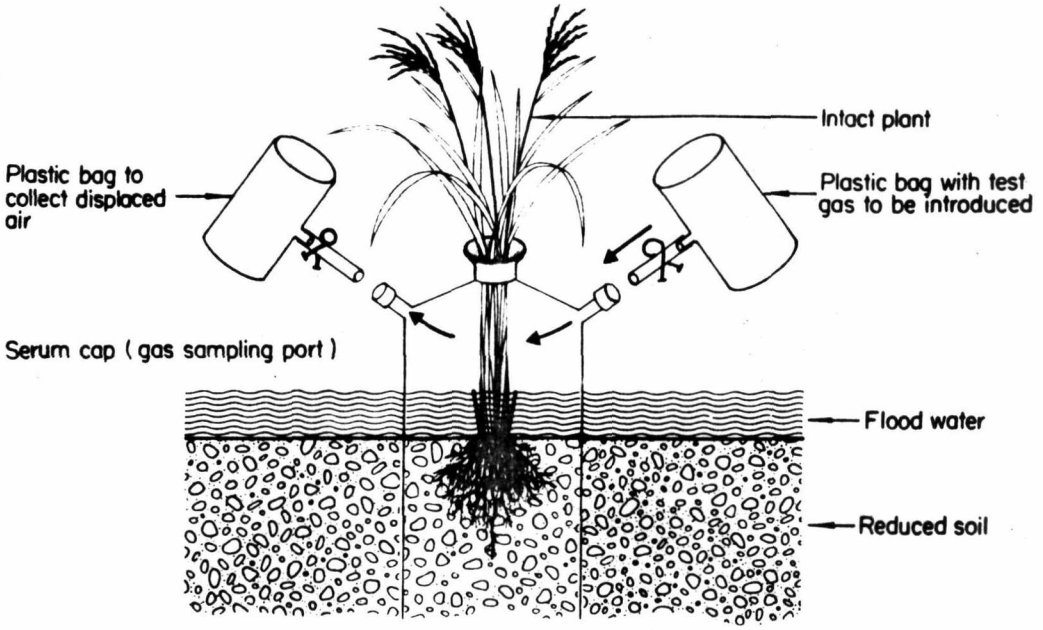


Fig. 3. Apparatus used to study loss of introduced gases through rice plant from glass jar to air.

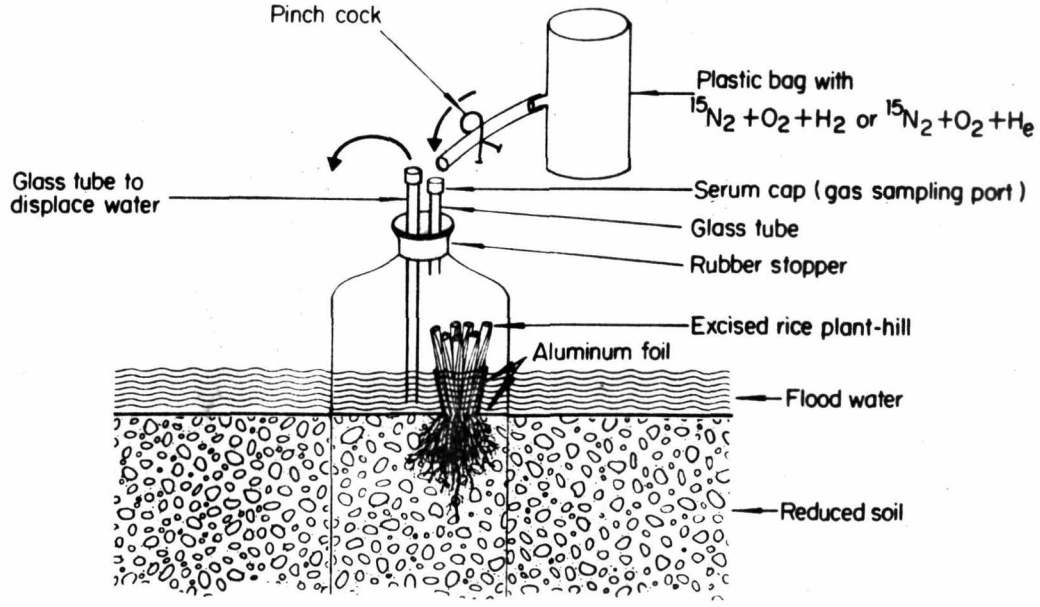


Fig. 4 $^{15}N_2$ - Isotope technique for In Situ N_2 - fixation studies in wetland rice.

PART III EFFECT OF ORGANIC SUBSTRATES ON H_2-SUPPORTED CHEMOLITHOTROPHY AND H_2-SUPPORTED N_2-FIXATION

Chapter 7

Effect of organic compounds on H_2-supported chemolithotrophy in aerobic heterotrophic H_2-oxidizing N_2-fixing bacteria with particular reference to wetland rice

Abstract

For the dominant bacteria which are associated with endorhizosphere of wetland rice, the routinely used yeast extract (0.005%) as growth factor was found to be not essential for their H_2-oxidizing chemolithotrophic activity. Organic substrates could substitute H_2 as energy source for both H_2-oxidizing and non H_2-oxidizing bacteria for fixing CO_2. Some organic substrates were found to repress H_2-supported CO_2 fixation but some of them were found to stimulate Pseudomonas sp. H8. The dominant H_2-oxidizing, N_2-fixing endorhizosphere bacteria associated with wetland rice was found to stimulate H_2-supported CO_2 fixation when grown with H_2 plus Na Pyruvate. Bacterial strains isolated from endorhizosphere were found to differ in their ability of CO_2-fixation on different organic carbon sources suggesting the possibility of existence of varietal interactions with H_2-oxidizing N_2-fixing bacteria.

Effect of organic compounds on H_2-supported chemolithotrophy in aerobic heterotrophic H_2-oxidizing N_2-fixing bacteria with particular reference to wetland rice

Watanabe et al. (1982) reported that large percentage of aerobic heterotrophic bacteria associated with endorhizosphere of wetland rice grown in Maahas soil are H_2-oxidizers. By employing ^{14}C-autoradiographic technique, Gowda and Watanabe (1983) confirmed that, these bacteria are H_2-oxidizing chemolithotrophs. Our further studies showed that their populations were negligible in oxidized and reduced soil of wetland and not detectable in the endorhizosphere and rhizosphere of upland rice grown in Maahas soil. Therefore, it was convinced that, both rice root and wetland conditions are necessary for these bacteria (Gowda and Watanabe, 1984).

Since wetland soils (Yamane and Sato, 1964; Harrison and Aiyer, 1916) and rice root (Kimura, 1981) produced H_2, it was suspected that the availability of H_2 as energy substrate might be the cause for their predominance in endorhizosphere of wetland rice. But the rice grown in Luisiana wetland soil, despite it had the capacity to produce and also consume H_2 similar to Maahas, the populations of these bacteria in the endorhizosphere of rice grown in this soil were significantly lower than the endorhizosphere of rice grown in Maahas (Gowda and Watanabe, 1984).

Because the bacteria are associated with the root, we suspected that the wetland rice root itself might be providing some favourable factors. The root environment is heterotrophic due to its organic cellular metabolism associated with the excretions of organic compounds which might be eventually available to these bacteria. The predominant chemolithotrophic bacteria associated with wetland rice root are N_2-fixers (Watanabe et al., 1982)

therefore, ATP dependent H_2-production catalyzed by nitrogenase is also expected in the root. Since both organic and inorganic (H_2) energy substrate are likely to be available in the root environment, the effect of organic compounds on H_2-dependent CO_2 fixation in these bacteria has been examined by using ^{14}C-autoradiographic techniques. Four organic compounds which are suspected to be present in rice root as energy source and or as growth factors, which are also reported to show mixotrophy in some H_2-oxidizing chemolithotrophs (Bowien and Schlegel, 1980) are studied. These were studied individually or in combinations i.e. heterotrophic and mixotrophic conditions. Non-rice aerobic heterotrophic H_2-oxidizing N_2-fixing bacteria obtained from different sources were also studied for the purpose of comparison with the bacteria associated with wetland rice. The results of these studies are presented in this paper.

Materials and methods

Sources of bacteria strains

N_2-fixing *Pseudomonas* spp. H8, KLH 76, LH-4 and *Azospirillum lipoferum* 34H and *Enterobacter cloacae* were isolated from endorhizosphere of rice grown in wetland of Maahas soil (Watanabe and Barraquio, 1979; Barraquio *et al.*, 1983), *Alcaligenes latus* DSM 1124 and *Renobacter vaculatum* DSM 1277 were obtained from the German Collection of Microorganisms (DSM) (Malik *et al.*, 1981; Malik and Schlegel, 1981). *Derxia gummosa* ATCC 15994, *Pseudomonas cepacia* ATCC 25416 and *P. facilis* ATCC 17695 were obtained from the American Type Culture Collection.

$^{14}CO_2$ incorporation studies

To demonstrate H_2-supported and organic compound supported $^{14}CO_2$ incorporation, the method and media were followed as described by Gowda and

Watanabe (1983). The deviations from the method is described wherever necessary. Fructose, glycerol and Na pyruvate were filter sterilized and used at 0.25%. Yeast extract (0.005%) and Tryptone yeast extract were sterilized by autoclaving together with mineral components of the medium used.

Rice plant sampling and bacteria isolation

Methods and media used were those used by Watanabe et al. (1979) and Gowda and Watanabe (1983).

H_2-uptake or H_2 production under mixotrophic conditions

Forty eight hour old bacteria grown in TYEA slants at 32°C were suspended (3 loops of growth) in 3 ml of TYE broth 0.2 ml of this suspension was inoculated to 25 ml of TYE broth in 50 ml flask. The flasks were stoppered with sterilized serum caps evacuated and filled with 1% H_2, 2% O_2, 5% CO_2 and balance N_2. Uninoculated flasks were treated similarly. At periodical intervals H_2 content in the flask was measured by gas chromatography with thermal conductivity detector. A set of flasks inoculated with bacteria were incubated with cotton plug in ambient air to compare the growth.

Results

A. $^{14}CO_2$ fixation in pure cultures

a) H_2-dependent and heterotrophic CO_2 fixation in liquid cultures:

The bacteria pregrown for 8 days on H_2-uptake broth with 0.005% yeast extract and then exposed to $^{14}CO_2$ for 15 days showed that bacteria having H_2 uptake activity fixed $^{14}CO_2$ in the presence of H_2, but $^{14}CO_2$ fixation was negligible in all these bacteria in the absence of H_2 (Table 1, Expt. 1). Whereas, bacteria exposed to $^{14}CO_2$ for 9 days directly (without pregrowing) showed considerable quantity of $^{14}CO_2$ fixation in all bacteria even in the absence of H_2 (Table 1, Expt. 2). However, $^{14}CO_2$ fixation was several times

higher in the presence of H_2 in all those bacteria had H_2-uptake activity, but not in those lacking uptake H_2-ase (Table 1, Expt. 2).

Bacteria grown on H_2-uptake liquid medium containing yeast extract (0.005% as growth factor) supplemented with 0.25 percent of fructose or glycerol or Na pyruvate incubated for 9 days with $^{14}CO_2$ (without pregrowing) showed that all the bacteria tested were able to fix large quantity of $^{14}CO_2$ in the absence and also in the presence of H_2. However, $^{14}CO_2$ fixation was comparatively higher in presence of H_2 (Table 2 and Fig. 1a, b, c and d). The growth of the bacteria was also relatively higher with H_2 (Mixotrophic conditions) (Table 3) than those grown without H_2 (Heterotrophic conditions). Among the carbon sources, in general, pyruvate was found to show higher fixation of $^{14}CO_2$ in most of the bacteria both in presence or absence of H_2 (Fig. 1d). Fixation of $^{14}CO_2$ among the carbon sources and among the bacteria were found to be variable both in presence and also in the absence of H_2.

b. Effect of organic compounds on H_2-supported $^{14}CO_2$ fixation: To find out the effect of different organic carbon sources on H_2-supported $^{14}CO_2$ fixation calculations were made by subtracting the $^{14}CO_2$ fixed in the absence of H_2 (heterotrophic) from that of $^{14}CO_2$-fixed in presence of H_2 plus organic compounds (Mixotrophic condition) (Table 4). The values obtained were compared with $^{14}CO_2$ fixed in the corresponding control /i.e. (H_2-uptake broth with yeast extract + H_2) - (H_2-uptake broth with yeast extract - H_2)/. Control represent the H_2-supported $^{14}CO_2$ fixation in the corresponding bacteria (Table 4).

Fructose: R. vaculatum, A. lipoferum 34H, Pseudomonas H8, Derxia gummosa, H_2-supported $^{14}CO_2$ fixation found to repress by fructose. Whereas, Pseudomonas cepacia, P. facilis, and Pseudomonas sp. KLH 76 were found to be stimulated. However there was no effect in case of Pseudomonas sp. LH-4 and Alcaligenes latus (Table 5).

Glycerol. A. lipoferum (34H), Pseudomonas sp. KLH-76, Derxia gummosa, and R. vaculatum showed repression of H_2-supported $^{14}CO_2$ fixation. Whereas, P. cepacia, P. facilis, Pseudomonas H8 and LH4 were found stimulated (Table 5).

Na-pyruvate. Pseudomonas Sp. KLH-76, A. lipoferum and Pseudomonas Sp. LH4 were repressed the H_2-supported $^{14}CO_2$ fixation, whereas P. cepacia, P. facilis, R. vaculatum, A. latus, D. gummosa and Pseudomonas Sp. H8 were found stimulated.

B. Effect of organic compounds on H_2-supported and organic substrate supported CO_2 fixation in Agar medium (^{14}C-autoradiographic technique studies.)

a) Fructose: Bacteria inoculated to H_2-uptake agar with yeast extract (0.005%) supplemented with 0.25% fructose were pregrown for 9 days in presence of H_2 and then exposed to $^{14}CO_2$ for 11 days in presence of H_2. The $^{14}CO_2$ fixation was compared with the $^{14}CO_2$ fixation observed in the absence of fructose under similar conditions (Fig. 2).

R. vaculatum, Pseudomonas Sp. KLH-76, Pseudomonas Sp. LH4, showed $^{14}CO_2$ fixation in presence of fructose (Mixotrophic condition) and also in the absence of fructose (autotrophic condition). However, P. cepacia, Klebsiella pneumoniae ℓℓ-4, were able to fix $^{14}CO_2$ only in the presence of fructose. But P. facilis, Pseudomonas Sp. H8, A. lipoferum 34H, D. gummosa, were unable to fix $^{14}CO_2$ in presence of fructose but they were able to fix in the absence of fructose (+ H_2). Since A. latus did not grow, the effect was not confirmed.

b) Tryptone yeast extract (TYEA): The bacteria inoculated to TYEA which were pregrown for 9 days in presence or absence of H_2 and then exposed to $^{14}CO_2$ in presence or absence of H_2 for 11 days, showed $^{14}CO_2$ fixation in all the bacteria, not only in presence of H_2 (Mixotrophic conditions) but also in the absence of H_2 (heterotrophic). The intensity of dark spots due to

$^{14}CO_2$ fixation observed on the autoradiograph in the bacteria grown in presence of H_2 was relatively more (Table 6).

C. Endorhizosphere sample (^{14}C-autoradiographic technique Studies)

The samples from endorhizosphere of rice grown in continuous flooded condition of Maahas soil was plated on Tryptic Soya Agar (TSA). The colonies appeared on TSA were inoculated by replica method to H_2-uptake mineral medium not containing yeast extract (-YE) or with 0.005 percent yeast extract (+YE); or to TYEA. These were pregrown for 9 days in presence or absence of H_2, and then exposed to $^{14}CO_2$ for 11 days in presence or absence of H_2. On H_2-uptake agar containing with and without yeast extract, when incubated in the absence of H_2, no $^{14}CO_2$ fixing colonies were detected (Fig. 3a and b). But in presence of H_2, large number of $^{14}CO_2$ fixing colonies appeared on H_2-uptake agar with and without yeast extract (Fig. 3a and b). Few more $^{14}CO_2$ fixing small colonies appeared in presence of yeast, extract but they were absent without yeast extract. In contrast to above, on TYEA (Fig. 3c) more colonies of $^{14}CO_2$ fixing bacterial colonies were detected both in presence and absence of H_2. Among the $^{14}CO_2$ fixing colonies detected on TYEA, some of them were more darker (on autoradiograph) in presence of H_2 than those were grown without H_2. The $^{14}CO_2$ fixing colonies which were appeared on H_2-uptake agar with and without yeast extract were also found to fix $^{14}CO_2$ on TYEA.

Requirements of yeast extract for $^{14}CO_2$ fixation

Six representative colonies of endorhizosphere bacteria which appeared on TSA (under aerobic conditions) were pregrown for 9 days on H_2-uptake agar either with or without yeast extract (0.005%) and then grown for 11 days

with $^{14}CO_2$ in presence or absence of H_2. The dominant type of colonies (Pseudomonas type) which were more than 90% on TSA were able to fix $^{14}CO_2$ only in presence of H_2 but both in presence and absence of yeast extract (Fig. 4, No. 2 and 6). But the non pseudomonas type (Small colonies type) which were few in number on TSA were able to fix $^{14}CO_2$ only with yeast extract in presence of H_2 (Fig. 4, No. 1). But none of the bacterial isolate from TSA were able fix $^{14}CO_2$ in the absence of H_2 either with or without yeast extract.

D. H_2-uptake and Production of H_2 under Mixotrophic conditions

Bacteria inoculated to TYE broth incubated with 1% H_2 showed that R. vaculatum, Pseudomonas Spp. KLH 76, LH-4, H8, and D. gummosa, consumed all the H_2 provided from external source. Whereas, P. facilis, P. cepacia, A. lipoferum, and Enterobacter cloacae Euf-26-9 had more H_2 than the H_2 provided from external source (Table 6) suggesting the production of H_2 from TYE itself by these bacteria. Among the bacteria tested, only P. facilis was found to accumulate large quantity of NO_2 in the medium suggesting the possibility of ammonia oxidation i.e. heterotrophic nitrification. Visual observations of the growth of all the bacteria showed that there was relatively less growth in presence of H_2 (1% H_2, 2% O_2, 5% Co_2 balance N_2) than those grown under air.

Discussion

The purpose of the present investigation was mainly to find out the possible causes for the predominance of H_2-oxidizing chemolithotrophic bacteria associated in the endorhizosphere of wetland rice. The data obtained from Experiment 1 and 2 (Table 1) suggested that yeast extract which is routinely used at low concentration as a growth factor (0.005%) in H_2-uptake medium, could also support $^{14}CO_2$-fixation in the presence and also in

the absence of H_2 only when the bacteria were grown directly with $^{14}CO_2$. But the bacteria pregrown for 8 days and then exposed to $^{14}CO_2$, no fixation of $^{14}CO_2$ was observed. This is attribulable to non availability of YE may be because of consumption during pregrowing period itself (Expt. 1). The predominant bacteria associated with endorhizosphere of wetland rice (which we are mainly concerned) showed that they do not require growth factors from external source because it was clearly evidenced by their ability to fix the $^{14}CO_2$ in presence and also in the absence of yeast extract under H_2 atmosphere (Fig. 3a and b; and Fig. 5). It appears therefore the predominance of these bacteria in endorhizosphere of wetland rice is not related to the availability of growth factors.

The observed $^{14}CO_2$-fixation with fructose, glycerol, and Na pyruvate under heterotrophic condition in all the rice and non rice bacteria clearly suggest that all the bacteria associated with wetland rice and also non rice bacteria are capable of fixing CO_2 utilizing organic substrates as energy source. However, differences among the bacteria and organic carbon sources in the quantity of CO_2 fixed was probably due to the selectivity of organic carbon sources for their growth, and $^{14}CO_2$ fixing activity. The $^{14}CO_2$ fixation was also observed even in the bacterium which is known to have no H_2-uptake activity P. cepacia. Therefore the data suggested that under heterotrophic conditions H_2-uptake activity is not necessary for CO_2 fixation (Fig. 1 and 2). Therefore the data suggest that the energy needed for CO_2 fixation can be substituted with suitable organic compounds for both H_2-oxidizing and non H_2-oxidizing bacteria.

Among the rice bacteria, significantly higher $^{14}CO_2$ fixation was observed with A. lipoferum in the presence of fructose (29.30 x 1000 Dpm) and comparatively low $^{14}CO_2$ fixation was observed on all Pseudomonas Spp. H8,

KLH-76 and LH-4 (0.67 to 3.99 x 1000 Dpm). Whereas, on Na pyruvate although higher $^{14}CO_2$ fixation was observed than fructose but they were almost the same (22.32 to 26.76 x 1000 Dpm). However, $^{14}CO_2$ fixation was comparatively lower in all the rice bacteria with glycerol (0.58 to 4.23 x 1000 Dpm) and also with non rice bacteria. Therefore, with reference to $^{14}CO_2$ fixation under heterotrophic conditions, it appears there is an organic substrate specity among the bacteria associated with endorhizosphere of wetland rice (A. lipoferum and Pseudomonas Spp.).

Under mixotrophic conditions (presence of both organic compounds and H_2), all the rice bacteria tested, (Pseudomonas Sp. H8, KLH-76 and LH-4 and A. lipoferum) showed great variation in $^{14}CO_2$ fixation. In general, all the rice bacteria showed higher $^{14}CO_2$ fixation under mixotrophic condition than the $^{14}CO_2$-fixed under heterotrophic condition. This suggested that most of the bacteria studied were benefited by the presence of H_2 under mixotrophic conditions. It was worthy to note that Pseudomonas H8, the most dominant N_2-fixing endorhizosphere bacteria of wetland rice was highly stimulated the $^{14}CO_2$ fixation (115 x 1000 Dpm) in the presence of Na-Pyruvate but not with fructose and glycerol (1.5 to 6.96 x 1000 Dpm). The stimulation with Na pyruvate was also found in non rice bacteria R. vaculatum and A. latus. The H_2-dependent $^{14}CO_2$ fixation of the control (Table 4) was also low in all those bacteria showed stimulated $^{14}CO_2$ fixation with Na pyruvate as compared to the other H_2-oxidizing bacteria associated with wetland rice. The latter (A. lipoferum, Pseudomonas KLH-76) did not show the stimulation (Table 4) of H_2-dependent CO_2 fixation.

The effect of organic carbon sources on H_2-dependent CO_2-fixation calculated from the $^{14}CO_2$ fixation under mixotrophic conditions (Fig. 1e) showed that, fructose represses H_2 dependent CO_2-fixation in all the rice

bacteria except Pseudomonas Sp. KLH 76. In latter it was stimulated (1.2 times). Glycerol was found to repress autotrophic $^{14}CO_2$ fixation in all the rice bacteria except in Pseudomonas Sp. H-8. The latter was found to be stimulated (3.2 times).

Pyruvate also found to repress H_2 dependent $^{14}CO_2$ fixation in all the rice bacteria except Pseudomonas H8. In the latter it was highly stimulated (66.4 times).

In general, presence of organic compound represses the H_2-dependent $^{14}CO_2$ fixation in almost all the N_2 fixing bacteria studied. But there are also exceptions. Pseudomonas Sp. KLH-76 was the only bacteria stimulated with fructose among the N_2-fixing bacteria (1.2 times), similarly Pseudomonas H8 was the one stimulated with glycerol. However, pyruvate was highly stimulatory to Pseudomonas H8 and all other bacteria except the three rice bacteria (A. lipoferum 36H, Pseudomonas Sp. KLH 76, LH04). But it was stimulatory to non rice bacteria also R. vaculatum, A. latus and P. facilis. In general, Pyruvate seemed to be the best organic substrate under mixotrophic condition as well as heterotrophic condition for all the N_2-fixing H_2-oxidizing bacteria studied. Fixation of $^{14}CO_2$ under Mixotrophic condition using fructose in presence of H_2 is also confirmed by autoradiographic technique. All those rice bacteria showed repression of H_2 dependent $^{14}CO_2$ fixation in the earlier experiment studied (without preincubation) also unable to show $^{14}CO_2$ fixation with fructose. The complete absence of $^{14}CO_2$ fixation in these bacteria as compare to $^{14}CO_2$ fixation observed under liquid culture conditions (Table 2) may be due to the insensitivity of the autoradiographic technique in detecting very low ^{14}C activity. However, under similar conditions on TYEA, all the bacteria including the above were able show $^{14}CO_2$ (autoradiograph) both under heterotrophic and mixotrophic condition.

The intensity of $^{14}CO_2$ fixed was detectably higher in presence of H_2. Since TYEA contains large quantity of organic carbon sources, exhaution of organic carbon source during the bacterial growth is not likely. These results therefore suggest that mixotrophy is operating even with the complex organic carbon substrates.

The replica inoculation of the endorhizosphere bacteria which were grown on TSA to TYEA also confirmed that the predominant endorhizosphere bacteria are able to fix $^{14}CO_2$ both under heterotrophic and mixotrophic conditions.

H_2-provided from external source was consumed by most of the H_2 oxidizing bacteria on TYE. However, some of the H_2-oxidizing bacteria A. lipoferum, P. facilis, and also non H_2-oxidizing bacteria P. cepacia and Enterobacter cloacae produced H_2 from TYE broth. Therefore, it was difficult to know if there was simultaneous H_2-uptake and H_2 production. In these bacteria, they are known to have H_2-uptake system (A. lipoferum and P. facilis). However, all the strains of Pseudomonas of rice (H8, KLH-76 and LH-4) consumed all the H_2 provided from external source. The growth of the bacteria was significantly lower under mixotrophic conditions than the bacteria grown under aerobic conditions. Therefore exhaution of organic carbon source from TYE is unlikely. Hence possibility of simultaneous utilization of organic compounds and H_2 as energy source for CO_2 fixation (Mixotrophy) can not be ruled out.

From the above results it can be said that the bacteria associated with wetland rice endorhizosphere are definitely capable of heterotrophic and also H_2-dependent CO_2 fixation. Although the H_2-dependent $^{14}CO_2$ fixation was repressed with organic compounds with some bacteria, the total $^{14}CO_2$-fixation under mixotrophic conditions was higher than heterotrophic

conditions. But it was interesting to know that among the predominant endorhizosphere bacteria of rice, there are strain differences in $^{14}CO_2$ fixation. Pyruvate was found to stimulate H_2-dependent CO_2-fixation with Pseudomonas H8 but not with strain KLH-76 and LH-4. Whereas, fructose stimulates only KLH-76 but not other Pseudomonas strains. Since these strains are isolated from different varieties of wetland rice, it appears that the strain differences observed in $^{14}CO_2$ fixation may be due to their accosication of such varieties which providing favourable organic compound for their activity. In fact in our recent studies (Gowda and Watanabe, 1985) we did find the varietal differences among six cultivars tested for H_2-supported ARA under monoaxenic culture conditions (rice seedlings in association with Pseudomonas H8).

The ability of the endorhizosphere bacteria to fix $^{14}CO_2$ autotrophically (H_2-dependent), heterotrophically and also H_2-supported CO_2 fixation under mixotrophic conditions seems to have greater ecological relationship with wetland rice. There appears a cultivar microbial interaction with respect to $^{14}CO_2$ fixation both under heterotrophic and mixotrophic conditions. More studies are needed to exploit this advantage of $^{14}CO_2$ fixation to utilize (if possible) for increased N_2-fixation through selective breeding for compatibility between varieties and H_2-oxidizing chemolithotrophic N_2-fixing bacteria. By $^{15}N_2$-isotopic studies we have observed both under pure culture as well as under field conditions, externally supplied H_2 (10%) was found to support N_2-fixation (Gowda and Watanabe, 1985). In the present study showed that H_2 had no repressive effect on heterotrophic $^{14}CO_2$-fixation but this needs to be confirmed with short term experiments. Since some of the bacteria were able to produce H_2 from TYE, it is possible that there is interaction between the N_2-producing bacteria associated with root and H_2-consuming N_2-fixing bacteria in the endorhizosphere.

In the present investigation all the experiments were carried out with longer incubation period and we did not measure the organic substrate consumed during the experiments and also, the growth of the bacteria was not quantitatively measured. Therefore simultaneous utilization both the substrates (organic energy substrate and H_2) for supporting CO_2 fixation is not conclusive. It is possible that the increased CO_2 fixation under mixotrophic conditions on some organic compounds observed could be due to higher number of bacterial cells attained due to initial growth on organic carbon substrate provided, and when organic substrate was no more available they might have utilized H_2. Further studies are needed to confirm mixotrophy (simultaneous use of both substrates) and effect of organic substrates on H_2-supported CO_2 fixation with short term experiments.

Table 1. H_2 and yeast extract (0.005%) supported $^{14}CO_2$ fixation in bacteria on H_2-uptake liquid medium ($^{14}CO_2$ fixation x 1000 dpm)

		Expt. 1 (Pregrown)*		Expt. 2 (Not Pregrown)**		H_2-uptake activity
		$-H_2$	$+H_2$	$-H_2$	$+H_2$	
1.	Renobacter vaculatum	0.166	69.0	1.15	24.50	+
2.	Alcaligenes latus	0.716	5.59	0.76	15.80	+
3.	Pseudomonas cepacia	0.213	0.20	2.27	2.40	-
4.	Pseudomonas facilis	0.098	152.7	2.16	2.50	+
5.	Pseudomonas Sp. KLH-76***	0.066	33.6	5.53	91.76	+
6.	Pseudomonas Sp. H-8***	0.094	29.2	0.63	2.59	+
7.	Pseudomonas Sp. LH-4***	ND	ND	2.31	18.00	+
8.	Azospirillum lipoferum 34H***	0.075	22.3	2.24	81.15	+
9.	Derxia gummosa	ND	ND	0.81	56.64	+
10.	Uninoculated	0.023	0.017	0.01	0.02	-

*Bacteria were pregrown with and without H_2 for 8 day then grown in presence of $^{14}CO_2$ with or without H_2

**Bacteria were grown in presence of $^{14}CO_2$ with or without H_2 for 9 days (No pregrowing)

***Bacteria from endorhizosphere of wetland rice

ND - not determined.

Table 2. $^{14}CO_2$ fixation in H_2-uptake mineral medium supplemented with different organic carbon sources. (x 1000 dpm) (Not pregrown exposed to $^{14}CO_2$ for 9 days)

	+ Hydrogen				- Hydrogen			
	C	F	P	G	C	F	P	G
Renobacter vaculatum	24.50* (± 9.05)	5.59 (± 1.04	219.64 (± 35.85)	23.67 (± 4.86)	1.15 (± 0.04	3.59 (± 1.91	9.33 (± 0.30)	2.18 (± 0.38)
Alcaligenes latus	15.80 (± 6.78)	15.55 (± 1.82)	141.30 (± 43.81)	3.51 (± 0.08)	0.76 (± 0.03)	1.29 (± 0.14)	21.02 (± 5.74)	1.25 (± 0.08)
Pseudomonas cepacia	2.40 (± 0.27)	23.80 (± 0.43)	38.50 (± 32.78)	22.26 (± 0.14)	2.27 (± 0.10)	13.37 (± 0.36)	30.81 (± 0.91)	14.31 (± 0.26)
Pseudomonas facilis	2.50 (± 0.22)	29.57 (± 1.77)	37.58 (± 0.31)	22.23 (± 0.98)	2.16 (± 0.17)	9.37 (± 1.66)	29.38 (± 0.76)	11.63 (± 0.04)
KLH-76	91.79 (± 19.22)	115.18 (± 19.29)	46.51 (± 3.86)	60.06 (± 3.51)	5.53 (± 0.05)	3.58 (± 0.20)	22.38 (± 1.92)	4.23 (± 3.48)
H-8	2.59 (± 0.79)	1.50 (± 0.01)	155.08 (± 1.85)	6.96 (± 5.40)	0.63 (± 0.05)	0.67 (± 0.02)	24.88 (± 1.34)	0.58 (± 0.02)
34-H	81.15 (± 48.27)	33.21 (± 12.93)	64.73 (± 7.37)	7.53 (± 2.11)	2.24 (± 0.79)	29.30 (± 0.19)	22.32 (± 0.10)	3.73 (± 0.92)
Derxia gummosa	56.64 (± 1.80)	24.46 (± 18.53)	113.04 (± 20.18)	38.55 (± 3.74)	0.81 (± 0.04)	2.66 (± 0.23)	13.96 (± 0.19)	1.20 (± 0.31)
LH-4	18.00 (± 2.36)	19.13 (± 4.77)	49.00 (± 7.01)	20.80 (± 4.34)	2.31 (± 0.90)	3.99 (± 1.91)	26.76 (± 2.90)	2.58 (± 0.41)
Uninoculated	0.01 -	0.01 -	0.02 -	0.01 -	0.02 -	0.02 -	0.01 -	0.01 -

C - Yeast extract (0.005%); F - Fructose (0.25%); P - Na Pyruvate (0.25%); G - Glycerol (0.25%).

* - Mean and SD of three replicates.

Table 3. Growth of bacteria in different organic carbon sources.

		+ Hydrogen				- Hydrogen			
		C	F	P	G	C	F	P	G
1.	Renobacter vaculatum	++	++	+++	+++	+	+	+++	+
2.	Alcaligenes latus	+	+	+++	-	-	-	+	-
3.	Pseudomonas cepacia	+	+++	+++	+++	+	++	++	++
4.	Pseudomonas facilis	+	+++	++++	++++	+	++	+++	++
5.	Pseudomonas Sp. KLH-76	++	++++	+++	++++	+	+	++	+
6.	Pseudomonas Sp. H-8	+	+	++++	+	-	-	++	-
7.	Azospirillum lipoferum 34-H	+	+++	++	++	+	+	+	++
8.	Dergia gummosa	+	+++	++++	++++	-	+	+	-
9.	Pseudomonas Sp. LH-4	++	++	++	++++	+	+	+	-
10.	Uninoculated	-	-	-	-	-	-	-	-

\+ Indicates the intensity of growth (visual)

\- No visible turbidity

C = H_2 uptake broth + 0.005% Yeast extract

F = C + 0.25% Fructose

P = C + 0.25% Pyruvate

G = C + 0.25% Glycerol

Table 4. Effect of hydrogen on $^{14}CO_2$ fixation under mixotrophic conditions (difference over -H) dpm x 1000

	C	F	G	P
Renobacter vaculatum	23.35	2.0	21.49	210.31
Alcaligenes latus	15.04	14.26	2.32	120.28
Pseudomonas cepacia	0.13	10.43	7.95	7.69
Pseudomonas facilis	0.34	20.2	10.60	8.20
KLH-76	88.26	111.6	55.83	24.13
H-8	1.96	0.83	6.38	130.20
34-H	78.91	3.91	3.80	42.41
Derxia gummosa	55.83	21.8	37.35	99.08
LH-4	15.69	15.14	18.22	12.6
Uninoculated	0	0	0	0

C - Control; F - Fructose; G - Glucose; P - Na Pyruvate

Table 5. Effect of different organic carbon sources on H_2-supported $^{14}CO_2$ fixation.

	C-F	C-G	C-P
	dpm x 1000		
Renobacter vaculatum	- 21.3	- 1.86	+ 186.96
Alcaligenes latus	- 0.77	- 12.72	+ 105.24
Pseudomonas cepacia	+ 10.3	+ 7.82	+ 7.56
Pseudomonas facilis	+ 19.86	+ 10.26	+ 7.86
KLH-76	+ 23.34	- 32.43	- 64.13
H-8	- 1.13	+ 4.42	+ 128.24
LH-4	- 34.03	- 18.48	+ 24.77
Derxia gummosa	- 0.55	+ 2.53	- 3.09
Uninoculated	-	-	-

\- indicates repression due to organic carbon source added

\+ indicates increased activity due to H_2

'able 6. $^{14}CO_2$ fixation and H_2 uptake activity in Tryptone yeast extract medium.

	$^{14}CO_2$ Fixation (Autoradiography)		9 days incubation			
			H_2 consumed	H_2 produced	Growth	
	+ H_2	+ H_2	ul H_2/flask	ul/flask	1% H_2	air
:enobacter vaculatum	+	+	250	-	+	++
.lcaligenes latus	+	+	nd	nd	+	++
'seudomonas cepacia	+	+	0.0	+ 265	+	++
seudomonas facilis*	+	+	0.0	+ 40	+	++
.LH-76	+	+	250	-	+	++
-8	+	+	250	-	+	++
.4-H	+	+	0.0	+ 378	+	++
)erxia gummosa	+	+	250	-	+	++
H-4	+	+	250	-	+	++
.uf. 26-9	+	+	0.0	+ 412	+	++

*Significant amount of NO_2 accumulation was found throughout the period on TYE No NO_2 was found in other organisms.

nd - not determined

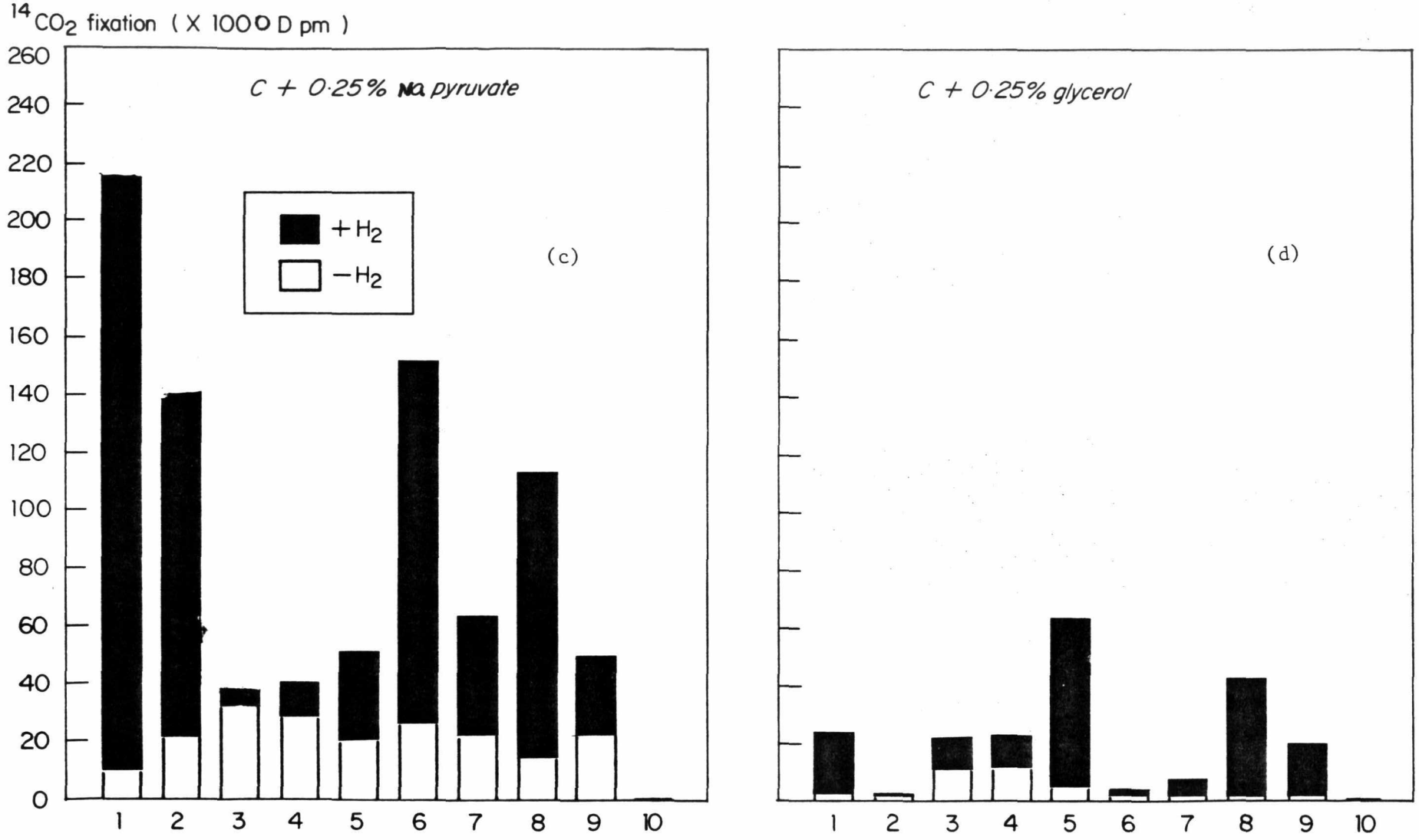


Fig. 1 (c and d). $^{14}CO_2$ fixation in H_2-uptake mineral liquid medium supplemented with different organic carbon sources.

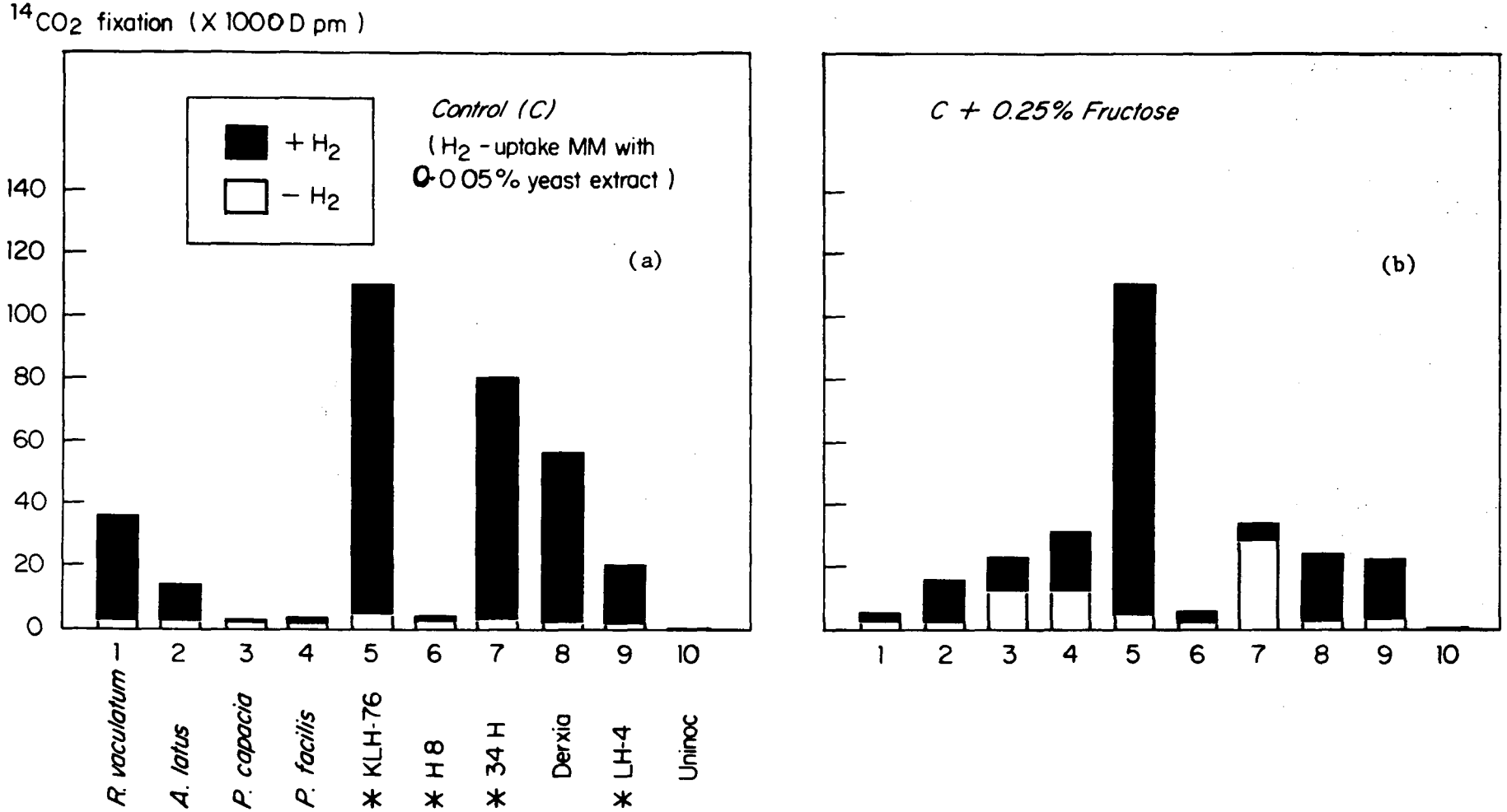
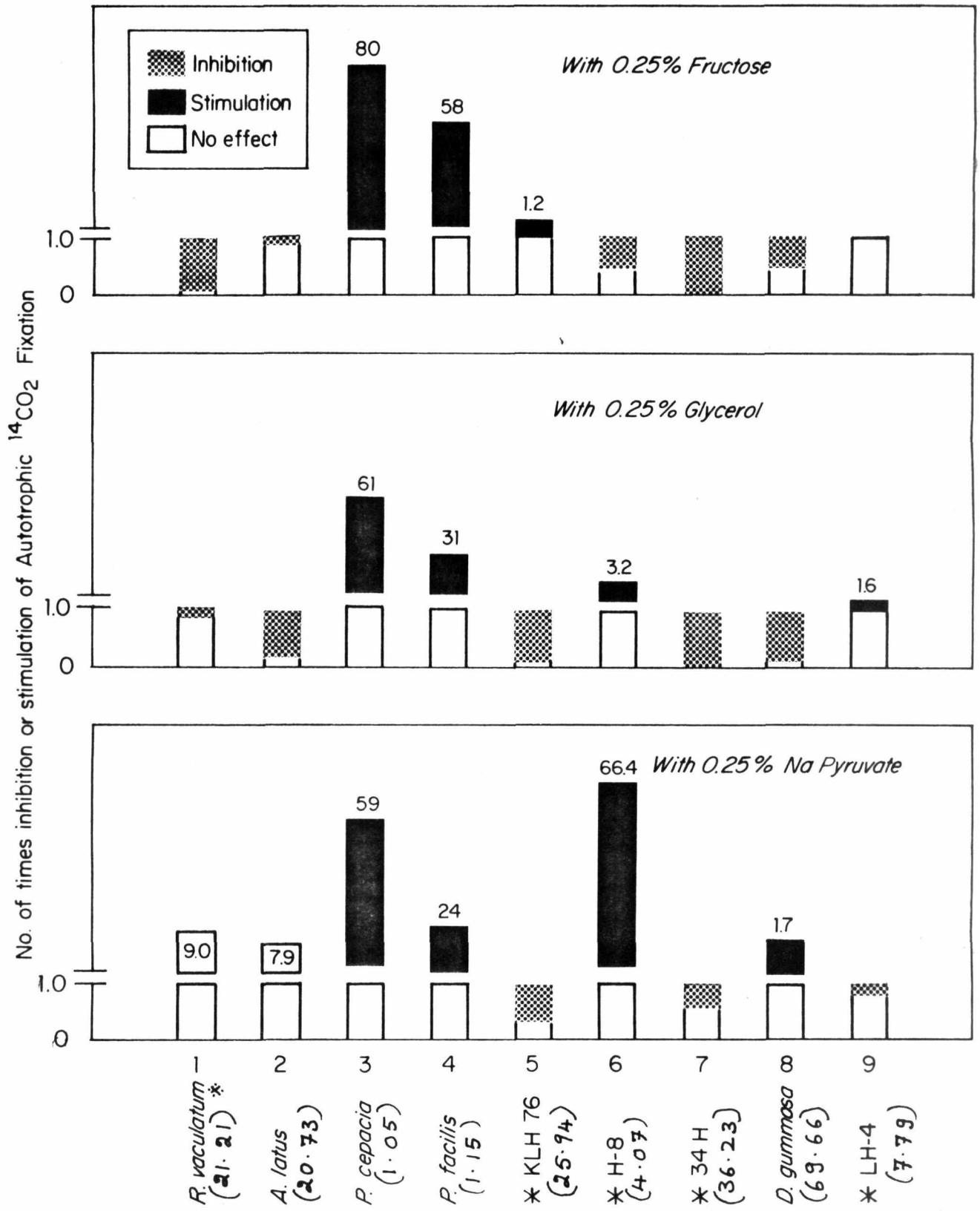
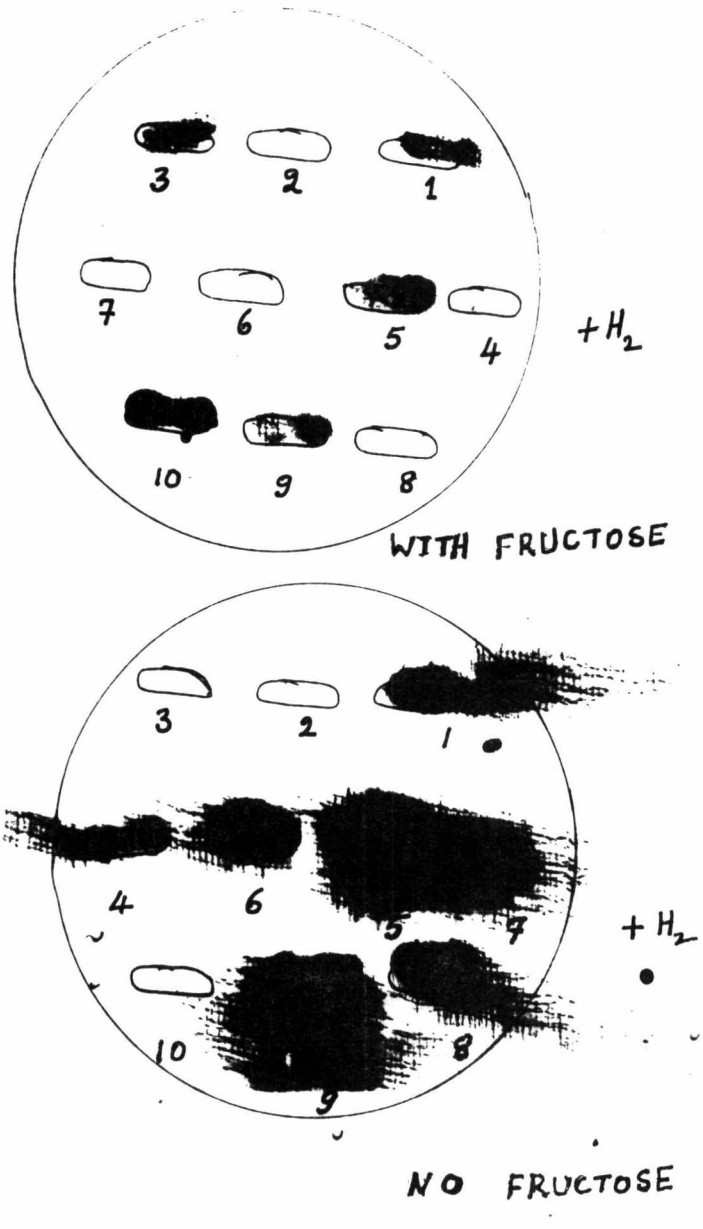


Fig. 1 (a and b). $^{14}CO_2$ fixation in H_2-uptake mineral liquid medium supplemented with different organic carbon sources.



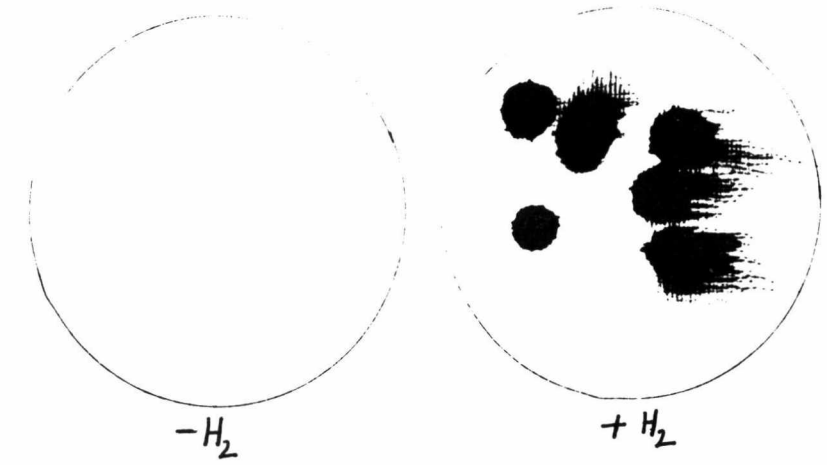
*Figures in parenthesis represents the H_2-supported $^{14}CO_2$ fixation (x 1000 dpm) of correspoding organism in the control [i.e. H_2-uptake agar with YE + H_2) - (H_2-uptake agar with yeast extract - H_2)] which represents autotrophic $^{14}CO_2$ fixation.

Fig. 16. Effect of organic substrate on H_2- supported $^{14}CO_2$ fixation.

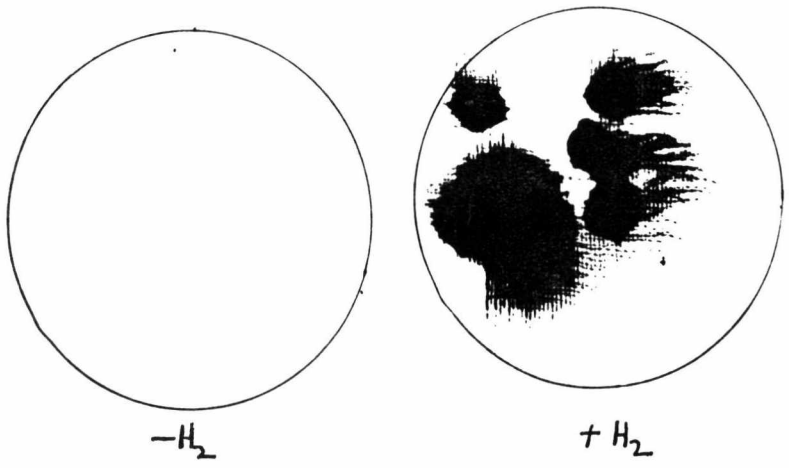


1. R. vaculatum
2. A. latus
3. P. cepacia
4. P. facilis
5. Pseudomonas sp. KLH-76
6. Pseudomonas sp. H8
7. Az. lipoferum 34H
8. D. gummosa
9. Pseudomonas sp. LH-4
10. E. cloacae Euf. 29-6

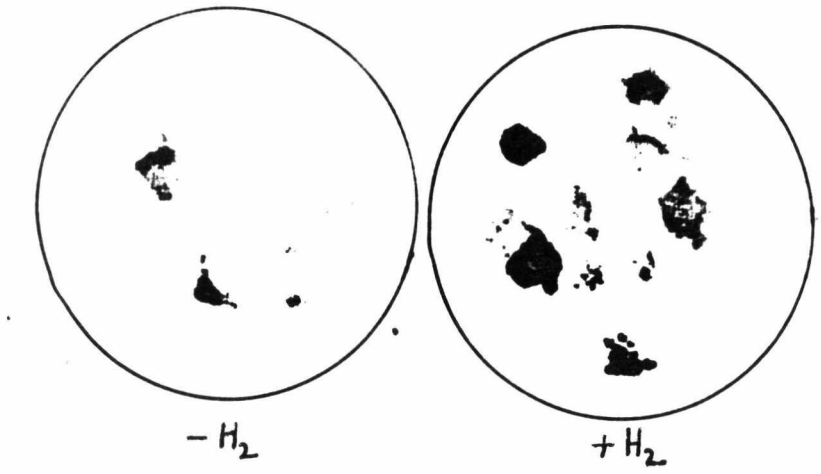
Fig. 2. $^{14}CO_2$ fixation of bacteria in presence and absence of fructose in H_2-uptake agar under H_2 atmosphere.



(a)
H_2-uptake agar without yeast extract



(b)
H_2-uptake agar with yeast extract



(c)
Tryptone yeast extract agar

Fig. 3. $^{14}CO_2$ fixing bacterial population from endorhizosphere of wetland rice under autotrophic and mixotrophic conditions.

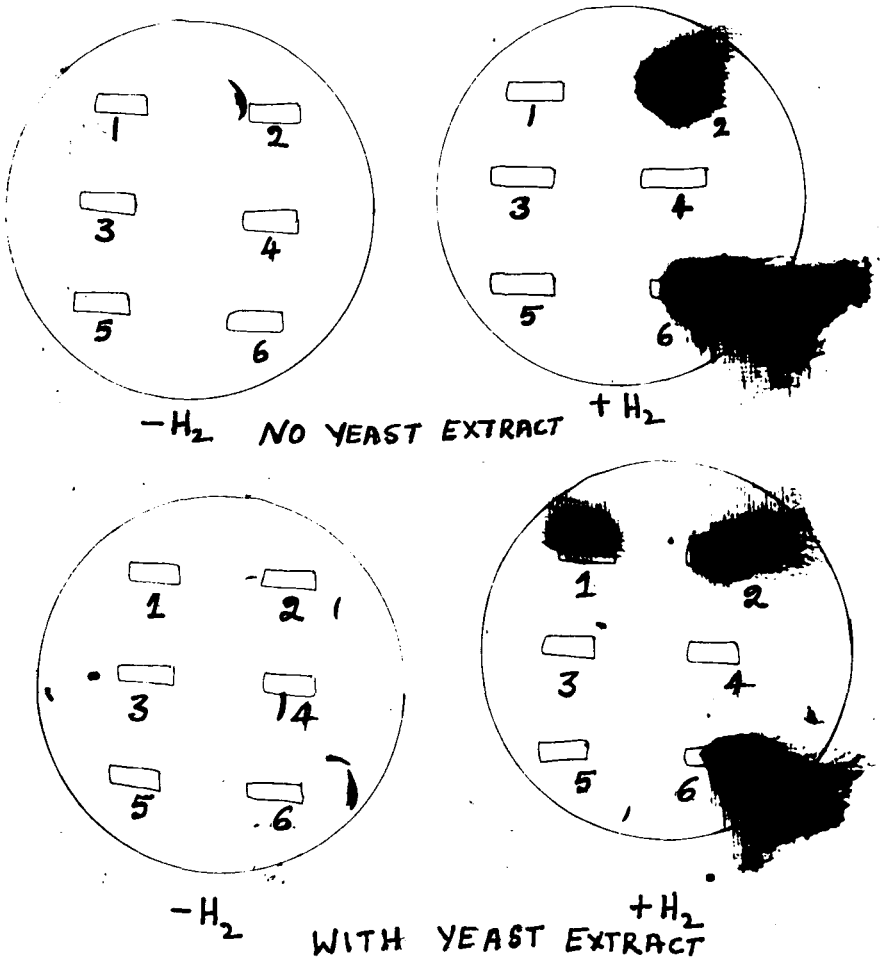


Fig. 4. $^{14}CO_2$ fixation of endorhizosphere isolates of wetland rice in presence and absence of yeast extract in H_2-uptake agar medium.

(Nos. 2 and 6 were the Pseudomonas type, dominant colonies on TSA)

Chapter 8

Effect of organic substrates on H_2-supported N_2-fixation

Objectives:

1) To find out H_2-supported N_2-fixation in the presence of organic compounds.

2) To find out the optimum concentration of organic compounds for H_2-supported N_2-fixation.

3) To find out the best media and best organic compound for H_2-supported N_2-fixation.

4) To find out if there is a selective preference to organic substrates among N_2 fixing organisms - varietal preference.

5) To explain why H8 (Pseudomonas) population is predominant than 34H (Azospirillum lipoferum)in the endorhizosphere of wetland rice.

I. Effect of different concentrations of organic compounds on H_2 supported N_2 fixation in the organisms grown heterotrophically in ambient air.

a) GLUCOSE

Experimental conditions. H8 and 34H were grown for 4 days in ambient air at 32°C on semi solid glucose yeast extract agar consisting different levels of glucose 0, (only YE as 'C' source), 0.1, 0.2, 0.3, 0.4 and 0.5% glucose plus (filter sterilized) the yeast extract added to the medium (0.01%) as carbon source. The cotton plugs were replaced with serum caps. After removing 2 ml air from each tube one set of tubes were injected with 1 ml C_2H_2 + 1 ml Ar and another set with 1 ml C_2H_2 + 1 ml H_2. The tubes were incubated at 32°C. At periodical intervals ARA was measured.

Results and Discussion

Growth of H8 and 34H were observed at all concentrations of glucose when incubated in ambient air. ARA was observed in all these tubes with and without H_2 (Table 1). All concentrations of glucose showed increased ARA in presence of H_2 indicating the H_2-supported N_2-fixation in heterotrophically grown Pseudomonas sp. (H8) in ambient air (in semisolid agar medium). The interesting observation is that the Pseudomonas sp. (H8) did not respond for addition of glucose at any level. The yeast extract (100 mg/l) added to GYE was enough for the growth and ARA activity. Higher level of organic carbon sources although not inhibitory to H8 but was not necessary. Therefore, it appears that the organism (H8) is an oligotroph i.e. requires less carbon for their growth and activity. This may be one of the reasons for its predominance in the roots of rice than other N_2-fixing organisms which may require more organic compounds. H8 must be multiplying faster with limited carbon source which may be available in the roots than other organisms.

In contrast, 34H also grew well at all concentrations of glucose. But it showed significantly higher ARA activity at all concentrations of glucose both with and without H_2 than those observed with H8 (Table 2). This shows 34H is much more efficient than H8 under similar conditions and needs more organic carbon source for higher ARA.

The important observation from this study was that unlike H8, 34H has responded to glucose addition. However, proportional increase in ARA was observed up to 0.2 percent glucose only. More than 0.3% glucose showed the decrease in ARA. Similarly, H_2 supported N_2-fixation was observed only up to 0.2 percent glucose only. Further increase in glucose concentration there was not H_2-supported N_2-fixation. Therefore, higher amount of glucose

(i.e. more than 0.2%) inhibits ARA activity and also H_2-supported N_2-fixation.

The data suggests that H8 and 34H although associated in the same rice root they must be differing in their organic carbon requirement. The optimum concentration of glucose for H_2-supported N_2 fixation is less than 0.3% for 34H, and still lesser amount for H8. Such low concentrations of glucose may be available in the rice roots. Therefore, it may be possible that the varieties differing in the concentration of organic compounds in their roots (which may be available to these organisms) may differ also in N_2-fixation due to selective N_2-fixing activity of these organisms. Availability of organic compounds at different stages of the crop may also influence the activity of these bacteria. Availability of these organic compounds must be more at tillering to heading stage.

b) Na-MALATE

Experimental conditions

Experimental procedure followed were same as described above. But the medium used was malate yeast extract semisolid agar instead of GYE. Organism tested was 34H only.

Results and Discussion

The bacteria grew well at all concentrations of Na-malate in ambient air. ARA was observed at all concentraitons with and without H_2 (Table 3).

The interesting observations from this data was that there was a proportional increase in ARA with increased levels of Na-malate even at 0.5% unlike the glucose. In the latter increase in ARA was observed only up to 0.2%. But there was no H_2-supported ARA at any level of malate. Some negative interactions with yeast extract and low levels of malate was observed. It appears that H_2-supported N_2-fixation differs with the kind

of organic compounds available during growth.

c) DIFFERENT ORGANIC COMPOUNDS

Experimental conditions

All the experimental conditions are same as described above except that, different organic compounds at 0.1% (filter sterilized) were added to GYEA. H8, KLH76, 34H, and $26H_2$ were studied.

Results and Discussion

ARA was observed on all the organic carbon sources tested in all the N_2-fixing bacteria except with Na-formate (H8, KLH76, and 34H) (Table 4). Except Na-formate, all the carbon sources are used for ARA as energy source. But in case of $26H_2$ (Azospirillum brasilense) no ARA was observed even with glucose and fructose in addition to Na-formate. H_2-supported ARA was not found with some of organic carbon sources although they could show ARA in the absence of H_2. Glucose, pyruvate and succinate were found to show H_2 supported ARA in H8 whereas H_2 supported ARA with KLH76 was with glucose and pyruvate only, but not with succinate.

34H showed H_2-supported ARA with glucose, pyruvate and succinate. Whereas $26H_2$ showed ARA with pyruvate and succinate only. No H_2 supported ARA was observed with fructose with KLH76 and also with 34H.

From the above results it may be concluded that H_2-supported N_2-fixation varies among the carbon sources and also between the H_2-oxidizing N_2-fixing bacteria when they were grown heterotrophically in ambient air on semisolid agar medium.

II. H_2-supported N_2-fixation in mixotrophically grown bacteria at 2% O_2

Experimental conditions

Organisms grown on semisolid N-free H_2-uptake agar supplemented with different organic carbon sources (0.1% filter sterilized), in desiccator

providing 2% O_2, 5% CO_2, 20% H_2 and 73% N_2. After 30 days growth at 32°C, the tubes were removed from the desiccator, the cotton plugs were replaced with serum caps, evacuated and flushed with Ar and finally filled with 2% O_2, 5% CO_2, 10% C_2H_2, 10% H_2 and balance Ar. or the same gas mixture without H_2. ARA was measured at periodical intervals.

Results and Discussion

All the organic compounds were found to show H_2-supported ARA in H8, KLH76, and 34H (Table 5). But not in the reference strains Pseudomonas facilis which is a non N_2-fixer. It is interesting to know that no ARA was observed in tubes not provided with H_2. Hence, all the ARA observed is purely H_2-supported. In case of H8 all the organic compounds except Na-formate were found to show high H_2-supported ARA than the control (no organic carbon source). However, succinate and glucose were found to be the best.

In case of KLH76, succinate and pyruvate found to be the best sources for H_2-supported ARA. Glucose, pyruvate, Na-malate and formate were found to show reduced activity than the control.

However, in 34H, only pyruvate was the best. But all the other organic carbon compounds were found to show lesser ARA than the control.

Therefore the data indicates that all the ARA which was recorded, was due to H_2-supported ARA because there was no activity in the tubes not provided without H_2. Therefore, all the organic carbon sources must have been utilized for their multiplication during the first few days of incubation heterotrophically and/or mixotrophically for growth. It is possible that, those organic compounds showed lesser ARA than the control must be inhibiting the H_2 supported growth during their multiplication with organic compounds in H_2 atmosphere. The ARA activity which was observed with H_2

may be due to adoption and/or synthesis of uptake H_2-ase during this period of incubation.

But significant H_2-supported ARA was observed after 5 days of incubation. This delay may have some relevance to synthesis or the activitation of uptake H_2-ase which must have taken place during the lag period.

Na-formate and Na-malate seem to be more inhibitory than the other compounds for H_2-supported ARA.

Table 1. Effect of different concentrations of glucose on ARA of H8 (Pseudomonas sp.) in semisolid glucose yeast extract agar medium (after 4-days growth in air) (n moles C_2H_4/tube).

% glucose	24 h		36 h	
	$-H_2$	$+H_2$	-H2	$+H_2$
0	233 ± 112	320 ± 40	420 ± 94	800 ± 520
0.05	280 ± 240	405 ± 306	765 ± 286	650 ± 532
0.1	194 ± 52	395 ± 133	300 ± 115	750 ± 100
0.2	195 ± 44	465 ± 156	345 ± 132	715 ± 366
0.3	325 ± 270	435 ± 195	500 ± 206	530 ± 199
0.4	270 ± 26	410 ± 171	460 ± 95	595 ± 240
0.5	290 ± 136	750 ± 240	525 ± 24	920 ± 326

Table 2. Effect of different concentrations of glucose on ARA of 34H (Azospirillum lipoferum) in semisolid glucose yeast extract agar (afer 4-days growth in air) (n moles C_2H_4/tube).

% glucose	24 h		36 h	
	$-H_2$	$+H_2$	$-H_2$	$+H_2$
0	900 ± 140	1600 ± 364	700 ± 424	1600 ± 800
0.05	1066 ± 100	1850 ± 300	1200 ± 340	2332 ± 100
0.01	2400 ± 520	3500 ± 1310	2800 ± 1112	4050 ± 2762
0.2	4132 ± 230	4400 ± 1058	5600 ± 692	6866 ± 230
0.3	4266 ± 1250	3450 ± 640	6732 ± 3406	5250 ± 1180
0.4	3200 ± 230	3350 ± 340	5500 ± 1732	5150 ± 596
0.5	2200 ± 1366	2400 ± 782	1950 ± 1600	2800 ± 1232

Table 3. Effect of different concentrations of Na-malate on ARA of 34H (*A. lipoferum*) in semisolid malate yeast extract agar incubated air for 4 days (n moles C_2H_4/tube).

% malate	24 h		36 h	
	$-H_2$	$+H_2$	$-H_2$	$+H_2$
0	1595 ± 446	1386 ± 369	2500 ± 840	2050 ± 596
0.05	606 ± 75	500 ± 227	1060 ± 100	800 ± 346
0.1	895 ± 236	845 ± 427	1400 ± 346	1050 ± 580
0.2	1965 ± 672	1995 ± 454	3450 ± 190	2850 ± 100
0.3	2430 ± 408	2540 ± 206	3800 ± 160	3600 ± 200
0.4	2855 ± 388	2790 ± 286	4200 ± 258	4200 ± 282
0.5	3086 ± 186	3155 ± 578	4600 ± 340	4700 ± 380

Table 4. H_2-supported N_2-fixation in heterotrophically grown bacteria in air on different organic compounds (on N-free GYE semisolid agar). (n mol C_2H_4/tube/day).

0.1% filter sterilzed	*Pseudomonas* sp (H8)		*Pseudomonas* sp. (KLH76)		*Azospirillum lipoferum* (34H)		*Azospirillum brasilense* ($26H_2$)	
	+ H_2	- H_2	+ H_2	- H_2	+ H_2	- H_2	+ H_2	- H_2
Glucose	975 ± 302*	638 ± 347	172 ± 75	119 ± 22	982 ± 264	385 ± 32	0	0
Fructose	nd	nd	128 ± 25	137 ± 60	721 ± 60	750 ± 84	0	0
Na-Pyruvate	1101 ± 143	928 ± 121	90 ± 41	28 ± 23	129 ± 68	49 ± 22	1565 ± 336	1172 ± 294
Na-Succinate	1087 ± 159	470 ± 52	8 ± 4	11 ± 4	105 ± 46	42 ± 12	617 ± 195	333 ± 35
Na- Malate	64 ± 52	55 ± 42	23 ± 13	8 ± 0.8	161 ± 24	172 ± 6	133 ± 16	135 ± 29
Na-Formate	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0

*Average of three replications and SD.
After 4-days incubation in air at 32°C, ARA with 10% C_2H_2 with and without 10% H_2.
ND = not determined.

Table 5. H_2-supported N_2-fixation (ARA) in heterotrophically grown bacteria with 2 per cent O_2 in presence of H_2 and different organic compounds.

(0.1%)	*Pseudomonas* sp. (H8)		*Pseudomonas* sp. (KLH76)		*Azospirillum lipoferum* (34H)		*Pseudomonas facilis***	
	+ H_2	- H_2	+ H_2	- H_2	+ H_2	- H_2	+ H_2	- H_2
Glucose	989*	0	336	0	25	0	0	0
Fructose	294	0	820	0	75	0	0	0
Na-Pyruvate	463	0	242	0	1052	0	0	0
Na-Succinate	884	0	1073	0	nd	0	0	0
Na-malate	357	0	294	0	252	0	0	0
Na-formate	33	0	147	0	27	0	0	0
Control	58	0	673	0	547	0	0	0

No ARA was observed at 24 after incubation in all the tubes.
Values expressed as n moles ethylene evolved/tube after 5-days incubation.
*Average of two replicates; ** a non N_2-fixer used as a reference strain.
N-free H_2-uptake semisolid agar with YE organic compounds added at 0.1% and incubated with 20% H_2, 2% O_2, 5% CO_2 balance N_2. Incubated in desiccators for 1 month at 32° C, evacuated, flushed with Ar and incubated with 10% C_2H_2, 2% O_2, 5% CO_2, with and without 10% H_2 balance Ar.

Effect of different concentration of oxygen on H_2 supported N_2-fixation

Objectives:

1) To find out the H_2-uptake hydrogenase sensitivity to higher lvels of O_2.

2) To find out the reasons for inconsistency in H_2-supported ARA and $^{15}N_2$ fixation between replicates which is observed repeatedly in all the earlier experiments.

Experimental conditions

The organisms was grown under chemolithotrophic condition on N-free H_2-uptake semisolid agar tubes (3-4 ml in Venoject tubes) in desiccator consisting 10% H_2, 2% O_2, 5% CO_2 and 83% H_2 for 30 days. Very good chemolithotrophic growth of the bacterium on the surface of the agar was found than those tubes incubated without H_2. This once again confirms the H_2-supported N_2-fixation under chemolithotrophic conditions.

The tubes were removed from the desiccators and replaced the cotton plug with serum stoppers. The tubes were evacuated and flushed with Ar. and finally filled with Ar. After removing the required quantity of Ar all the tubes were injected with 1 ml H_2, 1.0 ml CO_2, 1.0 ml C_2H_2 (to provide 10% H_2, 10% CO_2, 10% C_2H_2 and different amounts of O_2 (0 to 20% O_2). ARA was measured at periodical intervals.

Results and Discussion

H_2-supported ARA was observed in all the tubes at all concentrations of O_2 (even at O_2 concentration of ambient air, 20% O_2) (Table 1). There was no significant difference in ARA between O_2 levels. H_2-supported ARA is therefore protected in semisolid agar from O_2 damage in the chemolithotrophically grown N_2-fixing bacteria. However, large variations between

replications is observed at all concentrations of O_2. The reason for this variation which is observed in all the several previous experiments also under similar conditions (which was thought to be due to O_2-damage) is not clearly confirmed. O_2 is necessary for H_2-supported N_2-fixation.

No ARA was observed in all those tubes incubated under similar conditions without H_2.

Table 7. Effect of different oxygen levels on H_2-supported N_2-fixation in chemolithotrophically grown bacteria.

% O_2	n moles C_2H_4/tube Incubation period					
	24 h			72 h		
	R1	R2	R3	R1	R2	R3
0	0	0	0	2	2	5
2	68	10	0	267	20	8
4	28	5	10	15	10	14
8	10	13	5	8	30	25
12	25	12	18	5	4	5
16	6	10	15	29	21	13
20	5	5	3	4	8	11

No activity was observed in all the tubes without H_2.

Conclusions

H8 and 34H are oligotrophs - optimum glucose concentration for these organism is less than 0.2%. The organic compounds used routinely in GYE is too high. H_2-supported ARA was observed in both heterotrophically and chemolithotrophically grown organisms. Malate does not show H_2-supported N_2-fixation. Higher concentration of malate is more beneficial (0.5%) for ARA. Na-formate is inhibitory to ARA for all N_2-fixing organisms tested. N_2-fixing bacteria isolated from rice root differ in their organic compound requirement for ARA as well as H_2-supported ARA. Chemolithotrophically grown organisms show delay in oxidation of glucose. Mixotrophy is possible with some organic compounds but not in all. H_2-supported N_2-fixation is beyond doubt in 34H, H8 and also in $26H_2$. The variations in H_2-supported N_2-fixation with different organic compounds and with different N_2-fixing bacteria suggests that there must be rice varietal interactions (because of the differences in the quantity and quality of organic substances may be available in the roots of different rice varieties). H_2-supported ARA may be due to CO_2 fixation. (Due to the availability of increased amount of carbon and energy for chemolithotrophic N_2-fixers, i.e. H8, KLH76, and 34H). H_2-supported N_2-fixation is also possible in non chemolithotrophic N_2-fixers i.e. $26H_2$ (Azospirillum brasilense) where energy must be available directly to N_2-fixation due to H_2-oxidation. Predominance of H8 in the endorhizosphere must be also related to its oligotrophic growth, and less competition with other rhizospheric bacteria due to low organic compounds availability.

SUMMARY

A simple autoradiographic method for screening aerobic H_2-dependent chemolithotrophs is described. Bacteria were grown on agar plates in ambient air at 32°C. Colonies from the plates were inoculated to H_2-uptake agar plates by replica plating or streaking and incubated for 14 days in a desiccator under 20% H_2, 2% O_2, 5% CO_2, and the balance N_2, or 2% O_2, 5% CO_2, and the balance N_2. The plates were further incubated for 9 days with $^{14}CO_2$ in the above gas mixtures. The colonies were transferred to muslin cloth by replica method and the cloth was exposed to X-ray film for autoradiography. H_2 was necessary for CO_2 fixation under autotrophic conditions. By this technique, the predominance of H_2-utilizing facultative aerobic chemolithotrophs among wetland rice root isolates was confirmed. The strains of Azospirillum lipoferum tested were found to fix $^{14}CO_2$, but the A. brasilense strains tested were unable to fix $^{14}CO_2$.

The incidence of H_2-oxidizing chemolithotrophic bacteria associated with rice grown under continuous wetland, upland, and rainfed wetland conditions was studied by ^{14}C-autoradiographic technique in a neutral soil at IRRI (Maahas) and an acid rainfed wetland soil (Luisiana).

In Maahas soil, H_2-oxidizing chemolithotrophic bacteria were not detected in the endorhizosphere, rhizosphere, and nonrhizosphere soil of rice grown under upland conditions. Under continuously flooded conditions a very large population of these bacteria were found in the endorhizosphere but not in the oxidized and reduced soil.

A very low population of these bacteria were found in the endorhizosphere and basal culm of rice grown under rainfed wetland conditions at Luisiana. Bacteria isolated from Maahas wetland rice and

inoculated to rice seedling planted in Luisiana soil failed to establish.

Both Maahas and Luisiana soils consumed externally supplied H_2 and produced H_2 and CH_4 almost at the same rate when they were amended with rice straw or sucrose.

H_2-dependent chemolithotrophy was studied in 33 strains of Azospirillum spp. obtained from different sources by employing ^{14}C-autoradiographic technique. All the fourteen strains of A. brasilense were unable to show chemolithotrophy. But only six out of nineteen strains of A. lipoferum tested showed chemolithotrophic ability. These differences in chemolithotrophy were found among the strains of both the species of Azospirillum which were isolated from rice and other crops obtained from different countries. The chemolithotrophic ability of different strains of A. lipoferum was found to be immunologically related. From the above observation it is suggested that all the A. brasilense are non chemolithotrophs, and there are two groups among the A. lipoferum strains of which some of them have chemolithotrophic ability. The non chemolithotrophic strains of A. lipoferum are seemed to be the intermediary group of A. brasilense and A. lipoferum. There seems to be a possible enzymatic and evolutionary relationship between their ecological and adaptation and chemolithotrophic activity.

Hydrogen dependent dinitrogen fixation under chemolithotrophic condition of Pseudomonas strains H8, KLH76 and Azospirillum lipoferum strain 34H, which are dominant in association with wetland rice root, was confirmed by acetylene reduction assay (ARA) and $^{15}N_2$ incorporation. Glucose lower than 2 mM did not repress H_2-dependent acetylene reduction of chemolithotrophically grown Pseudomonas sp. H8 and Azospirillum lipoferum. ARA of bacteria associated with etiolated seedlings of rice was not stimulated by H_2 (10%), but ARA association with etiolated seedlings of rice was not stimulated by H_2 (10%), but ARA associated with the root of 4 wk old rice seedlings was stimulated by H_2. In the absence of H_2, difference in ARA was found among rice varieties, but in the presence of H_2, the varietal difference was not apparent.

The stimulation of acetylene reduction by the externally added H_2 (10% in volume) was found in association with the excised root, the plant from which the aerial shoots were removed, and the intact plant of the rice grown in the continuously flooded field. The excised root had H_2-uptake activity. The stimulation of N_2-fixation by H_2 in association with the rice plant from which aerial parts of shoot were removed under field conditions was confirmed by $^{15}N_2$ incorporation.

For the dominant bacteria which are associated with endorhizosphere of wetland rice, the routinely used yeast extract (0.005%) as growth factor was found to be not essential for their H_2-oxidizing chemolithotrophic activity. Organic substrates could substitute H_2 as energy source for both H_2-oxidizing and non H_2-oxidizing bacteria for fixing CO_2. Some organic substrates were found to repress H_2-supported CO_2 fixation but some

of them were found to stimulate. Pseudomonas sp. H8 the dominant H_2-oxidizing, N_2-fixing endorhizosphere bacteria associated with wetland rice was found to stimulate H_2-supported CO_2 fixation when grown with H_2 plus Na Pyruvate. Bacterial strains isolated from endorhizosphere were found to differ in their ability of CO_2-fixation on different organic carbon sources suggesting the possibility of existence of varietal interactions with H_2-oxidizing N_2-fixing bacteria.

H8 and 34H are oligotrophs - optimum glucose concentration for these organism is less than 0.2%. The organic compounds used routinely in GYE is too high. H_2-supported ARA was observed in both heterotrophically and chemolithotrophically grown organisms. Malate does not show H_2-supported N_2-fixation. Higher concentration of malate is more beneficial (0.5%) for ARA. Na-formate is inhibitory to ARA for all N_2-fixing organisms tested. N_2-fixing bacteria isolated from rice root differ in their organic compound requirement for ARA as well as H_2-supported ARA. Chemolithotrophically grown organisms show delay in oxidation of glucose. Mixotrophy is possible with some organic compounds but not in all. H_2-supported N_2-fixation is beyond doubt in 34H, H8 and also in $26H_2$. The variations in N_2-supported N_2-fixation with different organic compounds and with different N_2-fixing bacteria suggests that there must be rice varietal interactions (because of the differences in the quantity and quality of organic substances may be available in the roots of different rice varieties). H_2-supported ARA may be due to CO_2 fixation. (Due to the availability of increased amount of carbon and energy for chemolithotrophic N_2-fixers, i.e. H8, KLH76, and 34H). H_2-supported N_2-fixation is also possible in non chemolithotrophic N_2-fixers i.e. $26H_2$ (Azospirillum brasilense) where energy must be available directly

to N_2-fixation due to H_2-oxidation. Predominance of H8 in the endorhizosphere must be also related to its oligotrophic growth, and less competition with other rhizospheric bacteria due to low organic compounds availability.

Appendix 1

Bacteriological medium and buffer used

1. Tryptone yeast extract agar

	g/L
Tryptone difco	5.0
Yeast extract	3.0
$CaCl_2$	0.9
Noble agar	15.0
Deionized distilled water	1000 ml

2. H_2-uptake agar (modified, De Bont and Leijten, 1976)

NH_4Cl	1.0 g
K_2HPO_4	0.5 g
KH_2PO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
$CaCl_2$	0.015 g
$FeSO_4.7H_2O$	0.001 g
$CuSO_4.5H_2O$	5 µg
H_3BO_3	10 µg
$ZnSO_4.7H_2O$	70 µg
$MnSO_4.5H_2O$	10 µg
$NaMoO_4$	100 µg
Yeast extract	50 mg
Deionized distilled water	1000 ml

3. Tryptic soya agar (maintenance medium)

	g/L
Tryptic soya agar	1.0 g
Agar	15.0 g

4. Glucose yeast extract (semi solid agar)

Glucose	5.0
Yeast extract	0.1
K_2HPO_4	0.5
$FeSO_4.7H_2O$	0.04
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.1986
$NaMoO_4.2H_2O$	0.0059
$CaCO_3$	1.0
BTB (0.5% alcoholic BTB)	5.0 ml
Trace elments*	5.0 ml
Deionized distilled water	1000 ml
Noble agar	1.75 ml

Adjust the pH to 7.0 using 1N H_2SO_4

*Stock solution of trace elements

	mg/L
H_3BO_3	0.15
$ZnSO_4.7H_2O$	0.11
$CoSO_4.7H_2O$	0.07
$CuSO_4.7H_2O$	0.005
$MnCl_2.4H_2O$	0.004

5. Malate yeast extract (Dobereiner's medium)

	g/L
Na-malate	5.0
Yeast extract	0.1
KH_2PO_4	0.4
K_2HPO_4	0.1
$MgSO_4.7H_2$	0.2
NaCl	0.1
$CaCl_2$	0.02
$FeCl_3$	0.01
$NaMoO_4.2H_2O$	0.002
Noble agar	1.75
Distilled water	1000 ml
BTB (0.5% alcoholic solution)	5.0 ml

Adjust the pH to 7.0

6. Medium for Azospirillum (Okon et al., 1978)

	g/L
K_2HPO_4	6.0
KH_2PO_4	4.0
$MgSO_4\ 7H_2O$	0.2
NaCl	0.1
$CaCl_2$	0.02
$FeCl_3$	0.01
$NaMoO_4\ 2H_2O$	0.002
Distilled water (deionized)	100 ml
pH (Adjusted with 0.1N NaOH)	6.8
Na malate	5.0
Biotin (filter sterilized)	100 µg (added to autoclaved medium)

7. Rice plant nutrients agar medium (Yoshida et al., 1976, modified) used for growing rice seedlings of different varieties under mono-axenic conditions.

Nutrient	Concentration used (ppm)	Chemical used	mg/L
N	20	NH_4NO_3	57.14
P	10	$Na\ H_2\ PO_4.H_2O$	44.19
K	40	K_2SO_4	89.16
Ca	40	$CaCl_2.2H_2O$	147.0
Mg	40	$MgSO_4.7H_2O$	405.6
Fe	2.00	Fe-EDTA ($C_{10}H_{12}N_2O_8Na\ Fe\ H_2O$)	13.16
Trace elements mixture*			1 ml
Mn	0.5		
Mo	0.05		
B	0.2		
Zn	0.01		
Cu	0.01		

*Trace element mixture (stock solution)

$MnCl_2.4H_2O$	1800
$(NH_4)_6Mo_7O_{24}.4H_2O$	92
H_3BO_3	1140
$ZnSO_4$	44
$CuSO_4.5H_2$	39

8. Sodium phosphate buffer (0.1M, pH 7.1)

Stock solutions

A. 0.2M solution of monobasic sodium phosphate ($NaH_2PO_4.H_2O$)

27.6 g in 1000 ml water

B. 0.2 M solution dibasic - sodium phosphate

53.65 g of $Na_2HPO_4.7H_2O$ or

71.7 g of Na_2HPO_4 $12H_2O$

Mix 33 ml of A with 76 ml B and make up the volume to 200 ml.

The resulting buffer is 0.1M of pH 7.1

9. Phosphate buffer-saline (PBS)

By adding 0.8 percent NaCl for the above buffer 0.1M PBS of pH 7.1 can be obtained.

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