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Genetically Altered Cyanobacteria (Blue-Green Algae) as Nitrogen Fertilizer Supplier for Growth of Rice Plants

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EXECUTIVE SUMMARY

Availability of nitrogen is a major factor limiting plant productivity. Many plants have evolved to overcome this limitation by establishing symbiotic associations with dinitrogen-fixing bacteria. However, major food crops such as rice, wheat, etc. lack this ability to develop such associations and depends on the soil and rhizosphere bacteria for the supply of dinitrogen. These free-living bacteria assimilate the ammonia produced by nitrogenase for their own growth and the plant benefits only after the death and decay of the bacteria. It would be beneficial to the plant if the ammonia produced by these bacteria is made available to the standing crop by genetic alteration. Towards this goal we have isolated mutant strains of cyanobacterium *Anabaena variabilis* which excrete the ammonia produced by nitrogenase into the environment. The main objective of this study is to characterize and evaluate one such mutant, strain SA1.

Strain SA1 excreted the ammonia produced by nitrogenase into the medium and this property was determined to be associated with a mutation in the glutamine synthetase gene (*glnA*). The effect of the mutation was manifested on the biochemical properties of the enzyme. The glutamine synthetase from strain SA1 had Km values for ammonia and glutamate which were 300 and 6 times greater, respectively, than the values obtained with the enzyme from the parent, strain SA0. This alteration in the Km values are responsible for the observed phenotype of the mutant strain.

The mutant strain supported rice plant growth in greenhouse experiments and the ammonia produced from $^{15}N_2$ was incorporated into the rice plants. Under North Florida soil conditions, strain SA1 also supported plant growth in pot experiments, under field conditions. Significant increases in both the shoot and grain weight can be detected along with increased N-content of root, shoot and grain fractions of rice plants grown with strain SA1. However, studies conducted at the International Rice Research Institute in Philippines showed that strain SA1 failed to establish in soil in significant numbers to contribute to the fertility of different tested soils from traditional rice-growing areas.

These studies show that ammonia-excreting cyanobacterium has very limited utility, if any, in supplying fertilizer nitrogen in the rice growing regions of the developing world mainly due to the organism's inability to survive in the soil in large numbers. Significant advances need to be made in establishing introduced organisms in soil before genetically-altered microorganisms like *A. variabilis* strain SA1 can meet their agronomic potential.

RESEARCH OBJECTIVES

Introduction: Symbiotic nitrogen-fixing organisms, such as rhizobia, or the symbiotic cyanobacterium *Anabaena* from *Azolla* are leaky and a portion of the NH_4^+ produced by nitrogenase is released into the external environment and presumably becomes available to the host. O'Gara and Shanmugam (1) showed that the leakiness of rhizobia persists *ex planta*. The export of NH_4^+ is enhanced by glutamate in *Bradyrhizobium japonicum* and Shanmugam *et al.* (2) proposed that glutamate regulates the export of fixed nitrogen to the plant in the legume nodule. On the other hand, free-living dinitrogen-fixing organisms utilize the ammonia produced by nitrogenase for their own growth and the fixed-N becomes available to plants only after death and decay of the organism.

Among the dinitrogen-fixing bacteria, cyanobacteria are the only organisms which utilize sunlight and water for nitrogen fixation (3-5). They are ubiquitous in nature and contribute significantly to the fertility of soil, especially in rice paddies (6). Although dinitrogen-fixing symbiotic associations with cyanobacteria are found in nature, such a symbiosis does not occur with Rice and other cereal crops. Free-living cyanobacteria found in rice paddies assimilate the ammonia for their own growth and the standing rice crop benefits very little from their presence in the paddies. Mutant strains of filamentous, heterocystous cyanobacteria *A. variabilis* and *Nostoc muscorum* which excrete NH_4^+ into the medium have been described (7-9). Many of these mutants are conditional glutamine auxotrophs, requiring glutamine only under nitrogen-fixing conditions. In heterocystous cyanobacteria, the heterocysts lack glutamate synthase but possess glutamine synthetase and the fixed nitrogen is exported to the vegetative cells as glutamine and is then mobilized by glutamate synthase for biosynthesis. During this grant period, our initial studies (7) of *A. variabilis* mutants have been extended.

Objectives: The project had two major objectives. The first objective was to determine the genetic and biochemical changes in the mutant strain that led to the ammonia-excretion phenotype. The second was to evaluate the role of *A. variabilis* mutant strain SA1 as a nitrogen fertilizer supplier for the growth of rice plants under defined culture conditions and in natural soil and optimize the conditions needed for maximum ammonia production and yield of rice plants.

METHODS AND RESULTS

1. Excretion of NH_4^+ by mutant strain SA1: Strain SA1 was isolated as an MSX-resistant derivative of *A. variabilis* strain SA0 after EMS mutagenesis (7). Although the mutant strain produced nitrogenase activity, this strain failed to grow with N_2 as sole N-source. The NH_4^+ produced by nitrogenase is excreted into the medium. The rate of NH_4^+ production by this culture in a medium containing ambient CO_2 concentration, at 25°C was 0.43 μmoles/hr mg. dry wt. of cells. When the concentration of NH_4^+ in the medium was greater than 0.5mM, the organism utilized this NH_4^+ and growth of the filaments was detected. The parent culture produced no detectable free NH_4^+ in the medium, under similar experimental conditions. The production and consumption of NH_4^+ was obviously tightly coupled in the parent.

The fact that strain SA1 was excreting the NH_4^+ fixed from N_2 and not releasing organic nitrogen compounds was confirmed by bioassay. *Klebsiella pneumoniae* strain SK-24 lacks the NH_4^+ assimilation enzymes glutamate synthase and glutamate dehydrogenase and is a glutamate auxotroph (10). This strain utilizes amino acids such as glutamate, glutamine, aspartate etc. for growth but not NH_4^+. When *K. pneumoniae* strain SK-24 was cocultured with *A. variabilis* strain SA1 in a sucrose-supplemented medium, *K. pneumoniae* strain SK-24 did not grow. If *A. variabilis* strain SA1 excreted organic nitrogen compounds, such as amino acids, growth of *K. pneumoniae* strain SK-24 would have been detected.

These results indicated that strain SA1 is a conditional auxotroph requiring preformed organic nitrogen compounds, like L-glutamine, for growth when the NH_4^+ concentration was low and independent of glutamine when the NH_4^+ concentration exceeded 0.5 mM. The defect in the assimilation of NH_4^+, when present at low concentrations, suggested that one or both of the "ammonia-assimilation" enzymes are altered. Since the strain SA1 was isolated as a MSX-resistant mutant, properties of glutamine synthetase were evaluated. Glutamine synthetase from the mutant was resistant to MSX and showed only 10% inhibition even at an MSX concentration of 0.2 mM. The enzyme from the parent was completely inhibited by about 25-30 μM.

2. Kinetic Properties of the Mutant GS: To determine the kinetic properties of glutamine synthetase, the enzyme from the parent (strain SA0) and mutant (strain SA1) were purified to homogeneity. The apparent Km values for different substrates were higher for the mutant enzyme as compared with the enzyme from the parent (Table 1). At pH 7.5, the optimum pH for the parent enzyme, the apparent Km values for the mutant enzyme were higher by 90-fold for ammonia, 9-fold for glutamate, and 2-fold for ATP. Although the apparent Km values were higher for the mutant enzyme, the rate of substrate turnover by the two enzymes were similar (3,000 and 2,300 moles/min. per mole of enzyme). These results suggest that strain SA1's inability to utilize low concentrations of ammonia is a consequence of the change in the affinity of the enzyme for its substrates. However, the kinetic properties measured *in vitro*, may not reflect the actual kinetic characteristics of the enzyme *in vivo* where various metabolites can serve as

Table 1. Kinetic properties of glutamine synthetase from *A. variabilis* strains SA0 and SA1

	Strain SA0		Strain SA1	
	pH 6.8	pH 7.5	pH 6.8	pH 7.5
Apparent Km (mM)				
Glutamate	1.45	1.74	9.2	16.78
Ammonia	0.04	0.015	12.4	1.40
ATP	0.10	0.13	0.17	0.21
Molecular Activity (moles/min. mole of enzyme)	2,450	2,985	3,533	2,263

Kinetic characteristics of the enzyme were determined using the biosynthetic activity.

Table 2. Apparent Km values for glutamine synthetase isolated from *K. pneumoniae gln* mutant strain SK25 carrying *A. variabilis gln* genes

	pH	Apparent Km (mM) Glutamate	Ammonia
SK25/pSA100 (SA0)	6.8	0.88	0.04
	7.5	1.00	0.003
SK25/pSA111 (SA1)	6.8	4.55	5.50
	7.5	11.10	2.50

Plasmid pSA100 and pSA111 carry the *gln* genes from *A. variabilis* strain SA0 and SA1, respectively.

Table 3. Levels of nitrogenase and glutamine synthetase in *K. pneumoniae* strain SK25 carrying *A. variabilis gln* genes

Strain	Nitrogenase (umoles/hr. mg cell protein)	Glutamine synthetase (nmoles/min. mg cell protein) LB	Glutamate
M5A1	4.1	168	473
SK25/pLC28-36	ND	195	725
SK25/pSA100 (SA0)	7.5	711	3,750
SK25/pSA111 (SA1)	12.5	769	3,380

Nitrogenase activity was determined using whole cells. GS activity of extracts was determined using transferase activity. For glutamine synthetase determination, cells were grown in either in LB or sucrose-minimal medium with glutamate as sole N-source. Plasmid pLC28-36, pSA100 and pSA111 carry the *gln* genes from *E. coli K12*, *Anabaena variabilis* strains SA0 and SA1, respectively.

effectors in regulating the enzyme activity. In preliminary experiments, the kinetic properties of the enzyme from the mutant were not changed by preincubating the enzyme with thioredoxin and appropriate electron donors both in the light and in dark.

Other experiments revealed that the pH optimum for catalysis was also different in the two strains. Strain SA0 had a pH optimum of about 7.5 while strain SA1 had an optimum of about 6.8 (Fig. 1). When the apparent Km for different substrates were determined at these two pH values, the GS from strain SA1 still exhibited higher apparent Km for both ammonia and glutamate (Table 1). At the optimal pH values (pH 7.5 for GS from strain SA0 and 6.8 for GS from strain SA1) glutamine synthetase from strain SA1 had an apparent Km for ammonia which was 830 times higher than the values observed with GS from the parent, strain SA0. The wild type enzyme from strain SA0 was inhibited by higher glutamate concentrations while the enzyme from strain SA1 required higher concentration of glutamate for activity. The wild type enzyme required Mg^{++} for activity while the enzyme from the mutant required Mn^{++} for optimal activity (Fig. 2). Mn^{++} inhibited the activity of the wild type enzyme. These results clearly show that the ammonia-assimilation enzyme, GS, is biochemically altered in the mutant strain and the mutation leading to the growth defect in strain SA1 is located in the *gln* gene. Peptide analysis after digestion of the proteins with trypsin, chymotrypsin and papain followed by reverse-phase HPLC analysis did not identify any unique peptide present in one and not in the other GS.

The specific activity of the enzyme in the crude-extracts of the mutant strain was about 2-5%, as compared with the parent strain. It is possible that the mutant strain failed to derepress glutamine synthetase, under conditions of N-starvation enforced before harvesting the cells for enzyme analysis and purification. Haselkorn and his coworkers (11) reported that *Anabaena* 7120 *glnA* gene has two promoters, one used during N-excess and the second under N-limitation conditions. The second promoter allowed the derepression of glutamine synthetase. It is likely that the *glnA* gene in the mutant strain may have a defect in the second promoter. The extensive kinetic changes observed with the GS suggests that the mutation is in the structural gene for GS and not in the promoter region. However, the possibility that a second mutation in the promoter region of the *gln* gene in strain SA1 could not be ruled out at this time. Additional experiments with the *glnA* gene and pure glutamine synthetase from the parent and the mutant strain are needed before the physiological properties of the mutant strain can be related to the genetic change leading to biochemical alteration of glutamine synthetase.

3. Transfer of the *gln* Genes: In order to establish that the ammonia-assimilation defect observed with strain SA1 is due to the kinetic differences of the GS, the appropriate genes were cloned as 2.8 kb restriction endonuclease HindIII fragments into vector plasmid pBR322. These plasmids, pSA100 and pSA111, carrying the *gln* genes from the parent, strain SA0 and mutant, strain SA1, respectively, were transformed into an *E. coli gln* deletion strain E220 and into *K. pneumoniae* strain SK25. Strain SK25 produced normal levels of nitrogenase and did not produce glutamine synthetase including the GS antigen (10). The

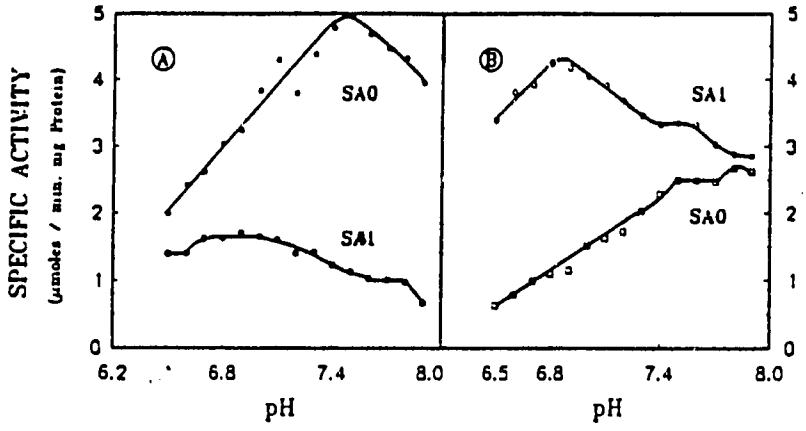


Fig. 1. pH profile of glutamine synthetase from strains SA0 and SA1. The data presented in panel A were obtained with 10 mM glutamte, 50 mM ammonium chloride and 3 mM ATP in the assay mixture. The concentrations of the substrates in the assay mixture for the experiment presented in panel B are, glutamte (40 mM), ammonia (0.2 M) and ATP (3 mM).

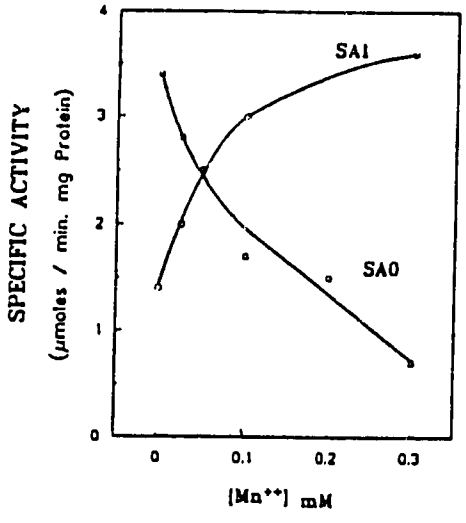


Fig. 2. Divalent cation requirement for activity of glutamine synthetase from strains SA0 and SA1. The concentration of substrates in the assay mixture at pH 7.5 were glutamate (10 mM), ammonia (50 mM) and ATP (3 mM).

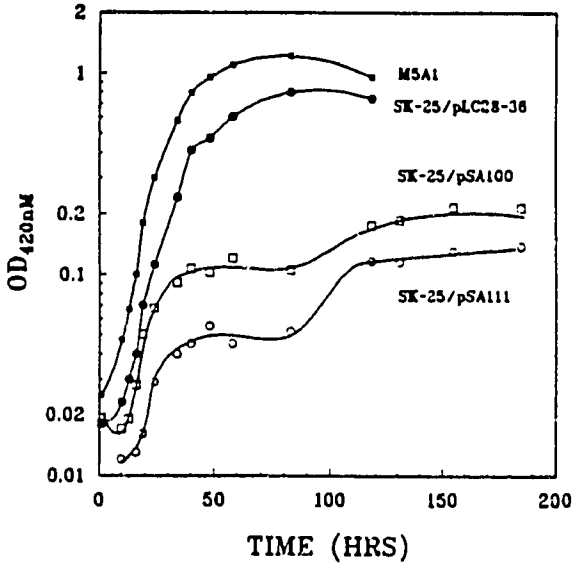


Fig. 3. Growth of *K. pneumoniae* strain M5A1 and strain SK25 with plasmids containing *gln* genes from different organisms. Plasmids pLC28-36, pSA100 and pSA111 carry the *gln* genes from *E. coli*, *A. variabilis* strain SA0 and strain SA1, respectively.

glutamine synthetase from the cultures carrying the *A. variabilis gln* genes were purified to homogeneity and the kinetic properties were determined at both pH values. The results from the *K. pneumoniae* hybrids are presented in Table 2. The apparent Km for ammonia, at the optimal pH, was over 1,800 times greater with GS from strain SK25/pSA111 as compared to the GS from SK25/pSA100. This ratio in Km values is comparable to that obtained with the enzymes purified from the *Anabaena* strains. Similar results were also obtained with the GS from the *E. coli* hybrids. These results show that the altered kinetic properties of the GS from strain SA1 resides in the enzyme and is not associated with unique enzyme activation and/or modification associated with cyanobacterial physiology. The *K. pneumoniae* hybrids also produced nitrogenase activity (Table 3). The levels of GS activity in the extracts were elevated when the cells were cultured in N-limiting medium (glutamate as N-source). In LB medium, which is rich in organic nitrogen, the levels of GS in the hybrids were significantly lower. These results also show that the *A. variabilis gln* genes are expressed in enteric bacteria and the levels of the enzyme responds to the nitrogen levels of the medium.

Since the kinetic properties of the enzyme from the mutant is altered, strain SK25/pSA111 would also be defective in assimilating the ammonia produced by nitrogenase. In experiments where dinitrogen was the sole N-source, strain SK25/pSA111 grew very poorly (Fig. 3) and excreted the ammonia produced from nitrogenase into the medium. The ammonia concentration in the medium reached as high as 10 mM under these conditions. After about 48 hours of growth, the cell yield of strain SK25/pSA111 was only about 5% of the wild type, strain M5A1. Slight increase observed after about 80 hours could be the result of assimilation of ammonia from the medium. Rapid growth at the expense of the ammonia present in the medium (excreted by the cell) did not occur because of the exhaustion of the carbon source and presence of metabolic end products. The final cell yield after about 180 hours was less than 15 % of the wild type, strain M5A1.

Surprisingly, strain SK25/pSA100 which carries the *gln* genes from the wild type *A. variabilis* also showed similar growth defect when dinitrogen was the only source of nitrogen (Fig. 3). These cells which contained nitrogenase also excreted the ammonia into the medium. Active GS which was sensitive to MSX can be detected in these cells. In contrast, *K. pneumoniae* strain SK25 carrying the *gln* genes from *E. coli*, (plasmid pLC28-36) grew well in dinitrogen-containing medium ruling out the possibility that the observed ammonia-assimilation defect is not due to the presence of *gln* genes in a multicopy plasmid. These results suggest that a fundamental difference exists between the *A. variabilis* and *K. pneumoniae* cytoplasms. This difference is responsible for the observed defect in strain SK25/pSA100. This is observed only in media in which GS is expected to be the main ammonia assimilation enzyme and the concentration of ammonia is considerably lower. The GS is expressed *in vivo* and is active can be seen by the ability of the plasmids to complement the *gln* mutation in both enteric bacteria in glucose-minimal medium containing 15 mM ammonia. In N-limiting conditions (glutamate as N-source), strain SK25 with either plasmid produced GS which can be detected readily in extracts (Table 3). Thus, the ammonia-assimilation defect could not be attributed to the absence of GS protein. Another possibility that the GS is part of a futile cycle which is wasting energy (assimilation of ammonia

to glutamine followed by glutaminase action to release glutamate and ammonia) could not be ruled out although it is not likely.

4. Reductant supply for ammonia production: The yield of ammonia produced by nitrogenase depends on the rate of ATP and reductant supply to nitrogenase. In *Anabaena*, cyclic photophosphorylation supplies the needed ATP. Since the heterocysts lack photosystem II, they are defective in the production of reductant for nitrogenase activity (12-14). Nitrogenase, thus depends on the adjoining vegetative cells for supply of reductant. In this process, nitrogenase is indirectly connected to photosystem II and provides ample room for several rate-limiting steps in coupling water-splitting system to ammonia production. Any attempts to enhance the overall efficiency of ammonia production needs to identify these rate-limiting steps.

Since the parent, strain SA0, assimilates the ammonia for its own growth, it is difficult to evaluate and compare the rate of reductant supply to nitrogenase in strains SA0 and SA1 for ammonia production. In the absence of dinitrogen, nitrogenase produces dihydrogen as an exclusive product. Our studies took advantage of this property of nitrogenase in evaluating any potential difference between the two strains in the rate of nitrogenase activity.

i) CO_2-dependent dihydrogen production: Dinitrogen-fixing cyanobacterium *A. variabilis*, grown with air, produced very little detectable dihydrogen. This can be increased by supplementing the medium with bicarbonate or CO_2 and shifting the gas phase to argon. Under the conditions employed in our experiments (argon atmosphere and light), the rate of net dihydrogen produced in the presence of 10 mM bicarbonate was about 1μmole / day. mg dry weight. This rate was maintained only for a short period and the total net dihydrogen produced by these cultures never exceeded 4.0 μmoles / mg dry weight. Increasing the bicarbonate concentration to 20 mM or adding an additional 10 mM after 72 hours did not alter the rate or total dihydrogen produced by the culture appreciably.

If cultures grown with air-CO_2 (sparging) were used in the dihydrogen production experiments, about 20 μmoles of H_2 / mg dry weight can be readily detected over a 72 hour period without any addition of CO_2 or bicarbonate. This was in contrast to the air or fructose grown cultures which lacked the ability to support nitrogenase activity using endogenous reserves. Addition of CO_2 or bicarbonate increased the net dihydrogen production by only about 4 μmoles/ mg dry wt., irrespective of the previous growth characteristics. These results showed that CO_2-grown cultures could not be used to distinguish between the two strains with respect to their reductant supply to nitrogenase.

ii) Fructose-dependent dihydrogen production: Addition of fructose, a compound used by *A. variabilis* for photoheterotrophic growth, led to significant increase in dihydrogen production. Under these experimental conditions, growth of the culture was insignificant amounting to less than 10% of the starting material due to nitrogen starvation conditions. Cultures maintained in the dark produced very little, if any, dihydrogen in the gas phase.

The net amount of dihydrogen produced by the cultures was dependent on the concentration of fructose added to the medium (Table 4). At low concentration of fructose (1.25 mM), the rate and total amount of dihydrogen produced was very low. This amounted to only a 5-fold increase over the levels observed in the presence of bicarbonate. Increasing the fructose concentration also enhanced the rate and amount of dihydrogen produced by the filaments. Net dihydrogen detected in the gas phase increased linearly with increasing fructose concentration beyond an initial concentration of 1 mM. This concentration of fructose may represent the amount used by the culture for biosynthesis at the expense of phycocyanin as the N-source. Once the need for biosynthesis is satisfied, fructose was used mainly to support nitrogenase activity and dihydrogen evolution.

iii) Stoichiometry of fructose and dihydrogen production: Since a linear relationship between fructose added and net dihydrogen produced by the culture was observed (Fig. 4), the ratio of fructose to dihydrogen was determined. In these experiments, 10 mM fructose was used since this concentration led to production of dihydrogen at a constant rate for longer periods of time. This concentration also brought forth the differences between strains SA0 and SA1 in the total amount and rate of dihydrogen production (Fig. 4). The rate of fructose consumption from the medium reached maximum levels by the second day for strain SA0 and by the fifth day for strain SA1 after commencement of the experiment (1.1 and 1.8 μmoles/ day. mg dry wt. for strains SA0 and SA1, respectively). There after a constant rate was maintained up to 22 days at which time the experiment was terminated. The rate of dihydrogen production followed fructose consumption until about 18 days into the experiment. Between the 4th and 18th days for stain SA0 and 7th and 18th days for strain SA1, the rate of dihydrogen production was constant (14 and 22 μmoles/ day. mg. dry wt. for strains SA0 and SA1, respectively). After 18 days the rate of dihydrogen production declined, probably a consequence of senescence of the filaments. Transferring the filaments at this stage to fresh medium did not restore the initial rate of dihydrogen production.

These results revealed that strain SA1 consumed fructose at a higher rate than strain SA0 and this fructose is utilized by the nitrogenase for dihydrogen production. In the presence of dinitrogen this higher nitrogenase activity can be translated into more ammonia produced and excreted by strain SA1.

5. Field Experiments with Rice: In greenhouse studies, strain SA1 was found to support growth of rice plants (15). These initial studies were repeated with rice in both controlled environment chambers and field conditions. The controlled environment chamber experiments also included variations in the atmospheric CO_2 levels.

For the field experiments, eight treatments replicated eight times were applied. Pots were filled with local soil (Millhopper fine sand, loamy hyperthermic family of grossarenic paleudults) and arranged in 8x8 rows and columns on an area approximately 100 m^2. The distance between pots within a row was 0.7 m^2. The 64 pots were surrounded by a row of border pots on all sides and were also planted with rice. A water column of 5 cm was maintained above the

Table 4. Effect of Fructose Concentration on H_2 Production by *A. variabilis*

[Fructose] mM	Rate SA0	Rate SA1	Net Evolution SA0	Net Evolution SA1
0.00	UD	UD	UD	UD
1.25	5.5	5.6	7.0	5.7
2.50	50.0	47.0	85.0	104.0
5.00	40.0	33.0	183.0	178.0

Filaments grown with fructose (10 mM) were harvested after 5 days and resuspended in fresh medium and used in these experiments. The cultures with the indicated amounts of fructose were incubated in light under argon gas phase. The rate of H_2 produced is expressed as µmoles/ day. mg dry weight of cells. The net H_2 evolution is in µmoles. UD - undetectable.

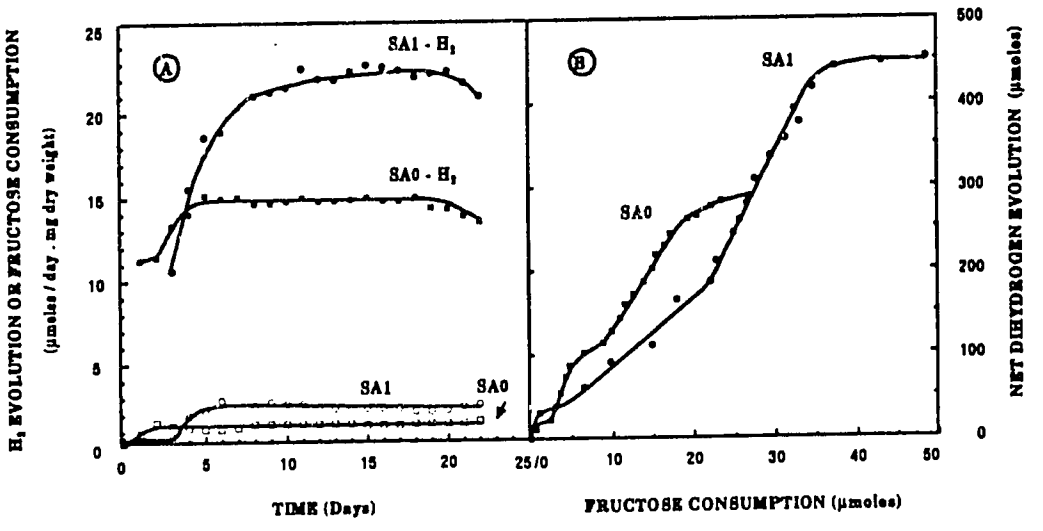


Fig. 4. (A) Rates of fructose consumption and net H_2 evolution by *A. variabilis* strains SA0 and SA1. (B) Relationship between fructose consumption and H_2 evolution by strains SA0 and SA1. Experimental conditions are as described in Table 4.

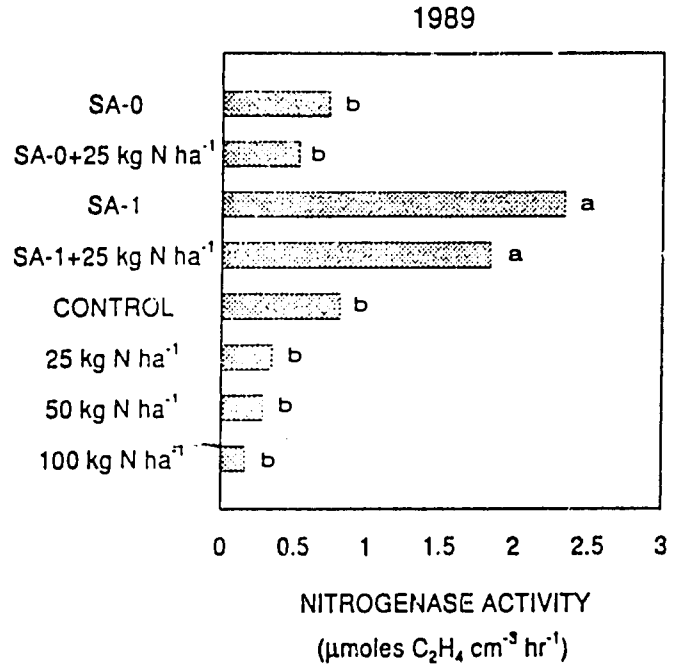
soil level. The distance between the top of the water column and the top of the pot was also 5 cm. Each pot held about 6 L of water which was maintained through the duration of the study. N-free Hoagland's solution was added (100 ml/pot) twice during the study both at the time of cyanobacteria addition.

Rice plants were inoculated with either the parent or mutant strain of *A. variabilis*, and inoculated plants were also fertilized with different levels of nitrogen. Fertilizer nitrogen was added as ammonium sulphate at the rate of 0.73, 1.46 and 2.92 g/ pot, corresponding to 25, 50 and 100 kg N/ ha. The control plants were neither inoculated nor fertilized. In these experiments, the soil water nitrogen and pH were determined every week and nitrogenase activity was determined once in every two weeks. At maturity, root, shoot and grain weight as well as the nitrogen content of these fractions were determined. The experiment was run in two seasons (1989 and 1990).

In these experiments, nitrogenase activity (acetylene reduction) was highest immediately after inoculation and steadily declined with time (Fig. 5). Pots inoculated with the parent strain of the cyanobacterium had only 32% of the nitrogenase activity measured in the pots inoculated with the mutant strain. Although nitrogenase activity was reduced in both strains by addition of 25 kg N/ ha, pots inoculated with the mutant cyanobacterium in the presence of combined nitrogen (ammonium sulphate) had 2.24 times more activity than those that had the parent strain under the same condition. Uninoculated pots generally had low nitrogenase activity, with the activity declining with increasing amounts of ammonium sulphate. The uninoculated control pots had over three fold the activity of pots that had 100 kg N/ ha in both years. In agreement with the higher nitrogenase activity, the pots with the mutant strain SA1 also had higher average concentration of ammonia (Fig. 6).

Root dry matter of the plants was smallest in comparison to shoot and grain dry matter. Inoculation with the mutant strain resulted in a significantly greater ($p>0.05$) root dry matter than inoculation with the parent strain in both years (Fig. 7). The highest root dry matter was obtained from plants fertilized with 100 kg N/ ha (29.1 g/ pot in 1989 and 32.5 g/ pot in 1990), while the lowest root dry matter occurred in the uninoculated control plants (12.4 g/ pot in 1989 and 18.3 g/ pot in 1990). The root dry matter of plants inoculated with the parent cyanobacterium was not significantly different ($p>0.05$) from the dry matter obtained from the uninoculated control plants.

Shoot dry matter comprised 37% of the total biomass of the plants inoculated with the mutant cyanobacterium in 1989 and 1990. In the plants inoculated with the parent strain, the shoot dry matter represented 36% of the biomass in 1989 and 40% in 1990 (Fig. 8). Addition of 25 kg N/ ha together with the mutant cyanobacterium did not change the shoot biomass of the plants inoculated with the mutant strain in 1989, but in 1990, addition of fertilizer nitrogen decreased the shoot dry matter below the value obtained by inoculation with the mutant cyanobacterium only. Plants that were inoculated with the parent strain had more shoot biomass when 25 kg N/ ha was added than inoculation with the cyanobacterium only. In 1990, there was no difference in the shoot dry matter of the plants inoculated with the parent strain with or without added



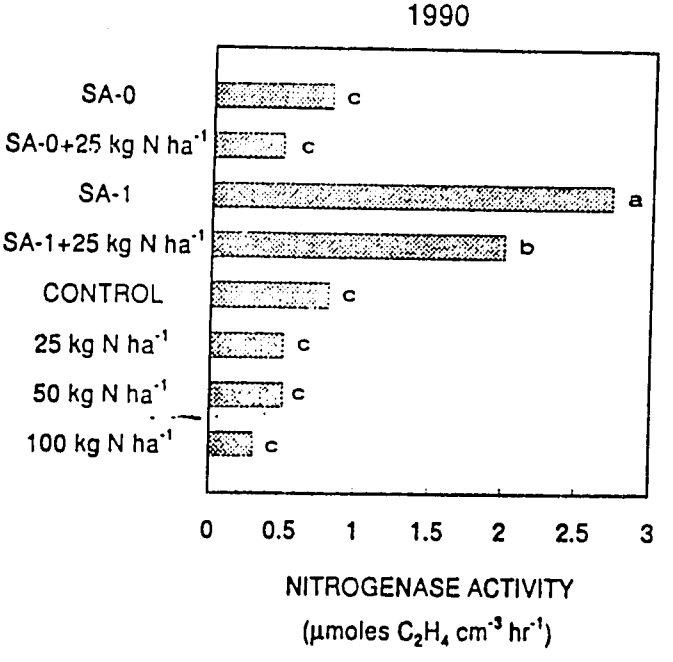
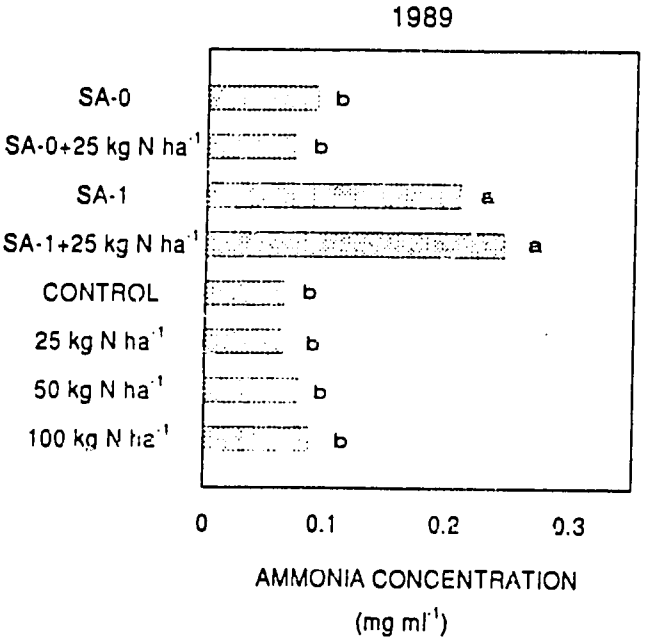


Fig. 5. Average nitrogenase activity in the pots of rice plants grown with different sources of nitrogen in 1989 and 1990. Letters to the right of the bars indicate mean separation using the Duncan's multiple range test. Bars with the same letter are not significantly different at the 5% level. Strains SA0 and SA1 indicate inoculation with the parent and mutant cyanobacterium, respectively. The control plants were neither inoculated nor fertilized.



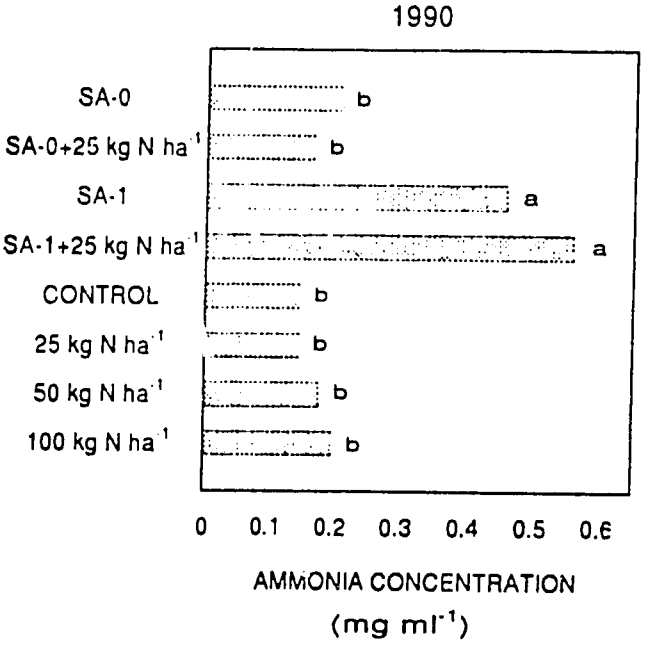
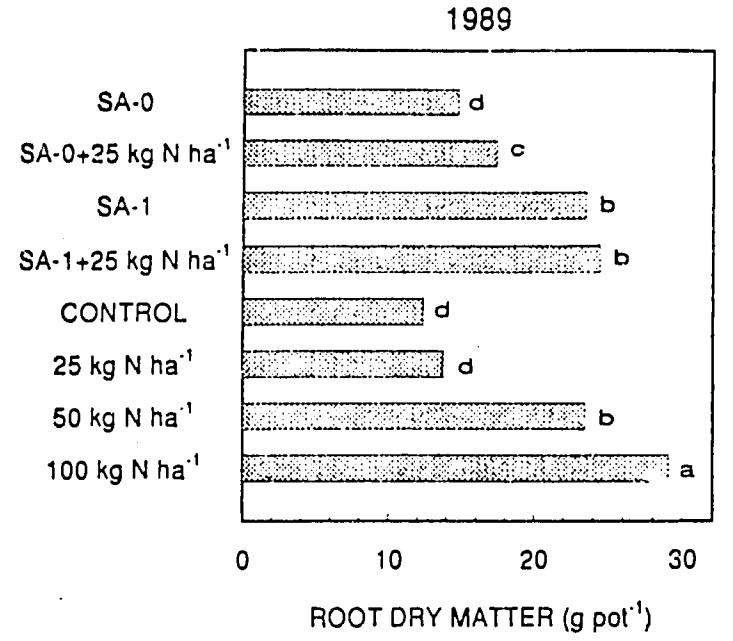


Fig. 6. Average ammonia concentration in the flood water of pots containing rice plants grown with different sources of nitrogen in 1989 and 1990. Letters to the right of the bars indicate mean separation using the Duncan's multiple range test. Bars with the same letter are not significantly different at the 5% level. Strains SA0 and SA1 indicate inoculation with the parent and mutant cyanobacterium, respectively. The control plants were neither inoculated nor fertilized.



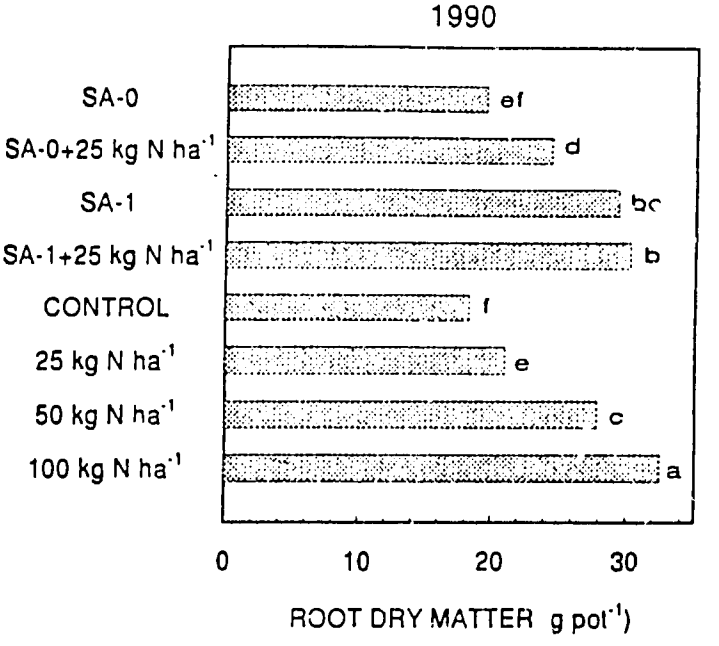
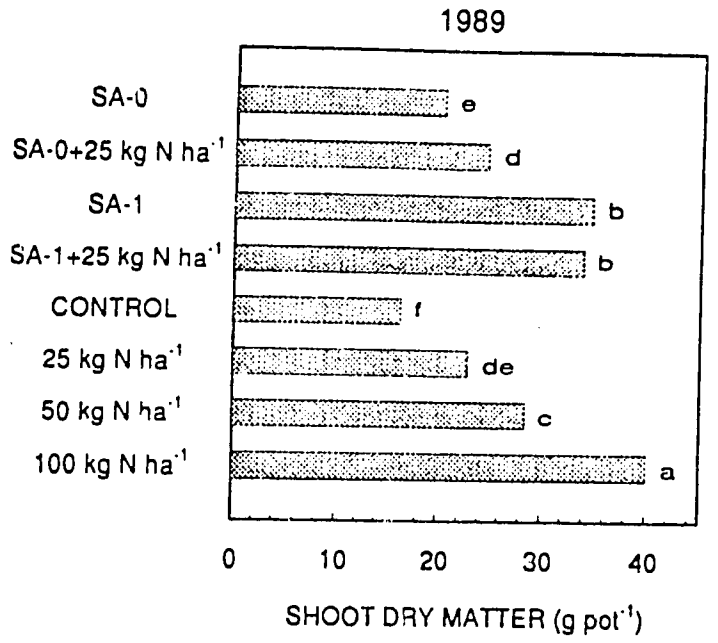


Fig. 7 Average root dry matter of rice plants grown to maturity with different sources of nitrogen in 1989 and 1990. Letters to the right of the bars indicate mean separation using the Duncan's multiple range test. Bars with the same letter are not significantly different at the 5% level. Strains SA0 and SA1 indicate inoculation with the parent and mutant cyanobacterium, respectively. The control plants were neither inoculated nor fertilized.



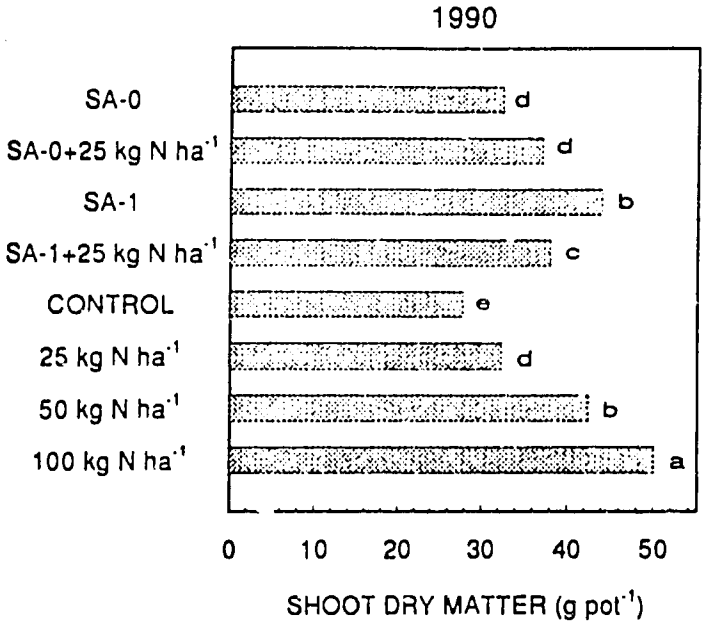


Fig. 8. Average shoot dry matter of rice plants grown to maturity with different sources of nitrogen in 1989 and 1990. Letters to the right of the bars indicate mean separation using the Duncan's multiple range test. Bars with the same letter are not significantly different at the 5% level. Strains SA0 and SA1 indicate inoculation with the parent and mutant cyanobacterium, respectively. The control plants were neither inoculated nor fertilized.

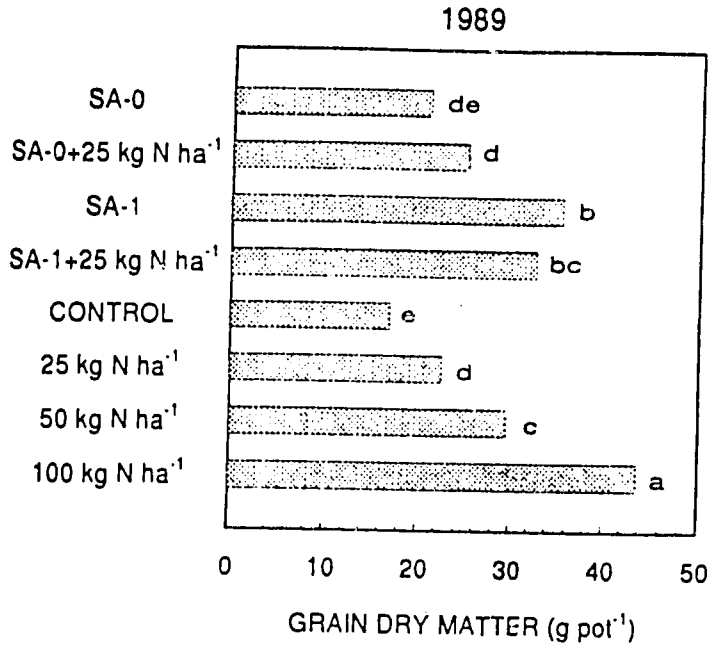
fertilizer nitrogen. The greatest shoot dry matter was obtained by application of 100 kg N/ ha (40.1 and 50.1 g/ pot in 1989 and 1990, respectively), followed by inoculation with the mutant strain (34.8 g/ pot in 1989 and 44.0 g/ pot in 1990) and the mutant combined with 25 kg N/ ha (34.0 g/ pot and 38.0 g/ pot in 1990).

The grain dry matter of rice plants inoculated with the mutant strain of the cyanobacterium represented 38% of the total biomass in both years. Plants inoculated with the parent strain had 37% and 36% of their biomass in the grains in 1989 and 1990, respectively (Fig. 9). Addition of 100 kg N/ ha resulted in the most grain dry matter (43.7 g/ pot in 1989 and 46.0 g/ pot in 1990), while the least grain dry matter was obtained from the uninoculated control plants (17.0 g/ pot in 1989 and 23.4 g/ pot in 1990). Plants inoculated with the mutant cyanobacterium produced a grain dry matter that was equivalent to or greater than the dry matter obtained by addition of 50 kg N/ ha. Inoculation with the parent strain resulted in a grain dry matter that was equivalent to that obtained by fertilization with 25 kg N/ ha in 1989, but was slightly less in 1990.

Greater amounts of nitrogen occurred in the above ground fractions than below. The root total nitrogen varied with source of nitrogen. Plants fertilized with 100 kg N/ ha accumulated the highest amount of nitrogen (272.8 and 302.8 mg N/ pot in 1989 and 1990, respectively), while the uninoculated control plants had a root nitrogen content of 59.0 mg N/ pot in 1989 and 166.4 mg N/ pot in 1990. Plants inoculated with the mutant cyanobacterium had 52% and 38% more nitrogen in the root biomass than was present in the plants inoculated with the parent strain in 1989 and 1990, respectively. The mean root total nitrogen of the plants inoculated with the mutant cyanobacterium was 244.3 mg N/ pot, while plants inoculated with the parent strain had 150.4 mg N/ pot in 1990. In 1989, the mean root nitrogen content was 87.3 mg N/ pot and 184.0 mg N/ pot for the plants inoculated with the parent and mutant strains, respectively. In both years, the nitrogen content of the roots of fertilized plants increased with the amount of fertilizer nitrogen applied.

The shoot total nitrogen of plants fertilized with 100 kg N/ ha was highest (201.9 mg N/ pot in 1989 and 302.3 mg N/ pot in 1990), while the uninoculated control plants had the least amount of nitrogen in the shoot (63.1 mg N/ pot in 1989 and 109.6 mg N/ pot in 1990). Plants inoculated with the mutant cyanobacterium had 184.3 mg N/ pot in the shoots compared to 84.1 mg N/ pot for plants that were inoculated with the parent strain in 1989. In 1990, these values were 131.4 mg N/ pot for plants inoculated with the parent strain and 254.8 mg N/ pot for the plants with the mutant strain.

The grain had at least 50% of the total nitrogen in the biomass of the inoculated rice plants. In 1990, the percentage of nitrogen accumulated in the grain of the inoculated plants was 52 and 47 for the parent and mutant strain, respectively. Plants fertilized with 100 kg N/ ha accumulated the most nitrogen in the grain (494.5 mg N/ pot in 1989 and 530.0 mg N/ pot in 1990), while the uninoculated control plants had 260.6 mg N/ pot and 355.0 mg N/ pot during the same period. Plants inoculated with the mutant cyanobacterium had 370.5 mg N/ pot and 440.3 mg N/ pot, while those inoculated with the parent strain had 218.6



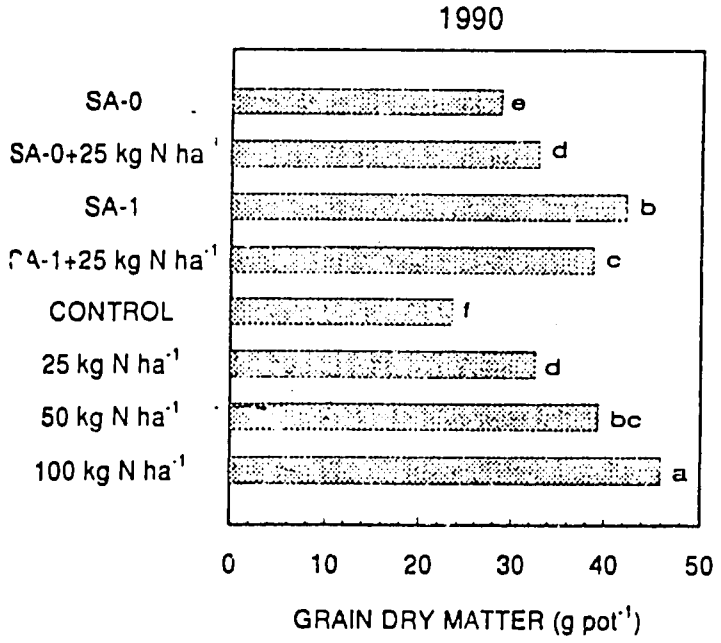


Fig. 9. Average grain dry matter of rice plants grown to maturity with different sources of nitrogen in 1989 and 1990. Letters to the right of the bars indicate mean separation using the Duncan's multiple range test. Bars with the same letter are not significantly different at the 5% level. Strains SA0 and SA1 indicate inoculation with the parent and mutant cyanobacterium, respectively. The control plants were neither inoculated nor fertilized.

mg N/ pot and 304.4 mg N/ pot in 1989 and 1990, respectively. The nitrogen content in the grain of the fertilized plants increased with the amount of fertilizer nitrogen added.

6. $^{15}N_2$ FIXATION AND UPTAKE BY RICE PLANTS: The objective of this part of the study was to determine the amount of biologically fixed nitrogen from the ammonia-excreting mutant strain of the cyanobacterium (*A. variabilis*, SA1) taken up by rice plants during the growth of rice plants. A comparison of the performance of the mutant strain was made with the wild type (parent strain). For these experiments, $^{15}N_2$ was used as the source of dinitrogen and the amount of ^{15}N in the plants was determined. The data obtained from the study are presented below.

The experimental design was similar to the one used for greenhouse experiments (15). There were a total of 7 treatments replicated 7 times in this study: rice plants inoculated with either strain of *A. variabilis* and exposed to $^{15}N_2$, inoculated plants not exposed to $^{15}N_2$, two sets of uninoculated plants one exposed to $^{15}N_2$ and the other not exposed to the gas, and plants fertilized with 50 kg N/ ha applied as ammonium sulphate (16.9 mg/ fleaker) not exposed to $^{15}N_2$.

For exposing plants to $^{15}N_2$, the rice plants in the fleakers were gently pushed down and a rubber stopper was used to cover the fleakers to make them air tight. Rice plants were exposed to 1.96% $^{15}N_2$ by injecting 12 ml of $^{15}N_2$ (98% atom excess) in a syringe with a hypodermic needle through a rubber stopper into the fleaker which had a gaseous volume of 600 cm^3 above the water. The plants were exposed to the gas for 4 hours in the second and fifth week of study. Exposure to $^{15}N_2$ was carried out from 10:00 to 14:00 hours on both days. Plant samples were collected for analysis every week after week 2 until 8 weeks when the experiment was terminated.

The results presented in Fig. 10 and Fig. 11 show that the rice plants accumulated $^{15}N_2$ into plant material when inoculated with strain SA1. The amount of nitrogen accumulated by the plants inoculated with the parent strain was slightly higher than the control plants during the eight week period and these values were significantly less as compared to the plants cocultured with strain SA1. These results also show that the ammonia produced and excreted by strain SA! is immediately available to the standing rice crop.

7. Effect of increased $[CO_2]$ on rice-cyanobacteria association: Carbon dioxide concentration ($[CO_2]$) in the atmosphere has been increasing steadily since the industrial revolution from about 265-285 μmol/ mol (16) to the present level of more than 355 μmol/ mol (17). As global population increases and industrialization expands, the $[CO_2]$ of the atmosphere is expected to double in the next century (18). High $[CO_2]$, together with other climatic factors, will affect plant growth and development and are the focus of substantial research worldwide.

There is little information on the response of both the cyanobacteria and

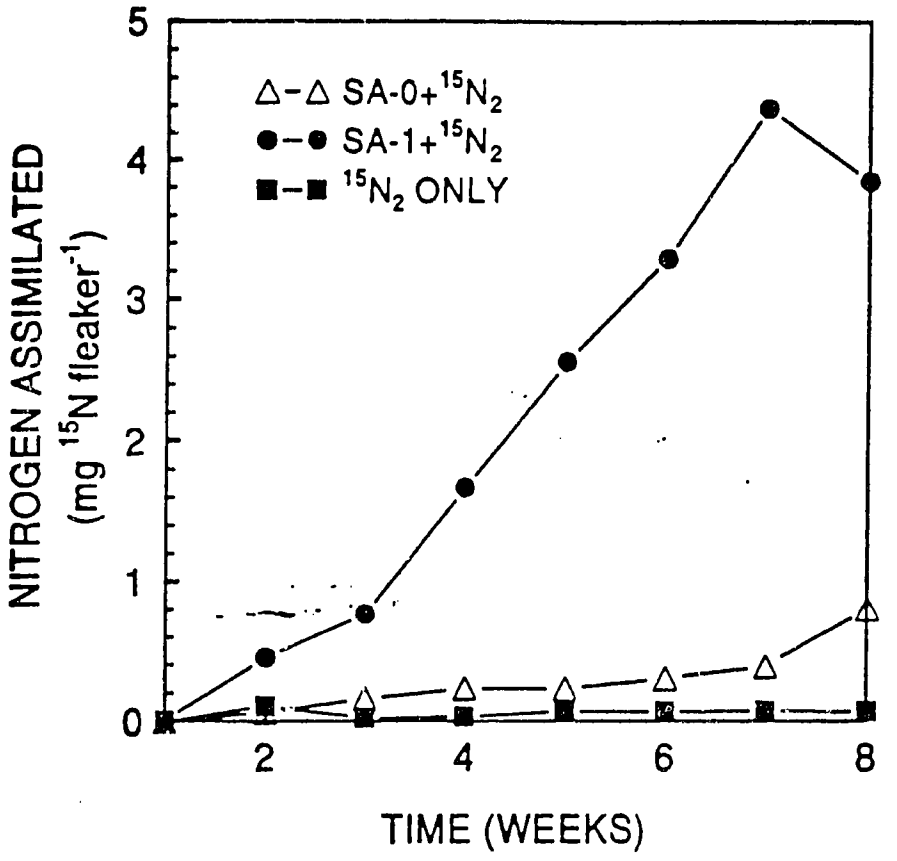


Fig. 10. Average amount of nitrogen assimilated by inoculated and uninoculated rice plants exposed to 1.96% $^{15}N_2$ at second and fifth week after planting. Plants were harvested at the end of the eighth week. Strains SA0 and SA1 indicate inoculation with the parent and mutant cyanobacterium, respectively.

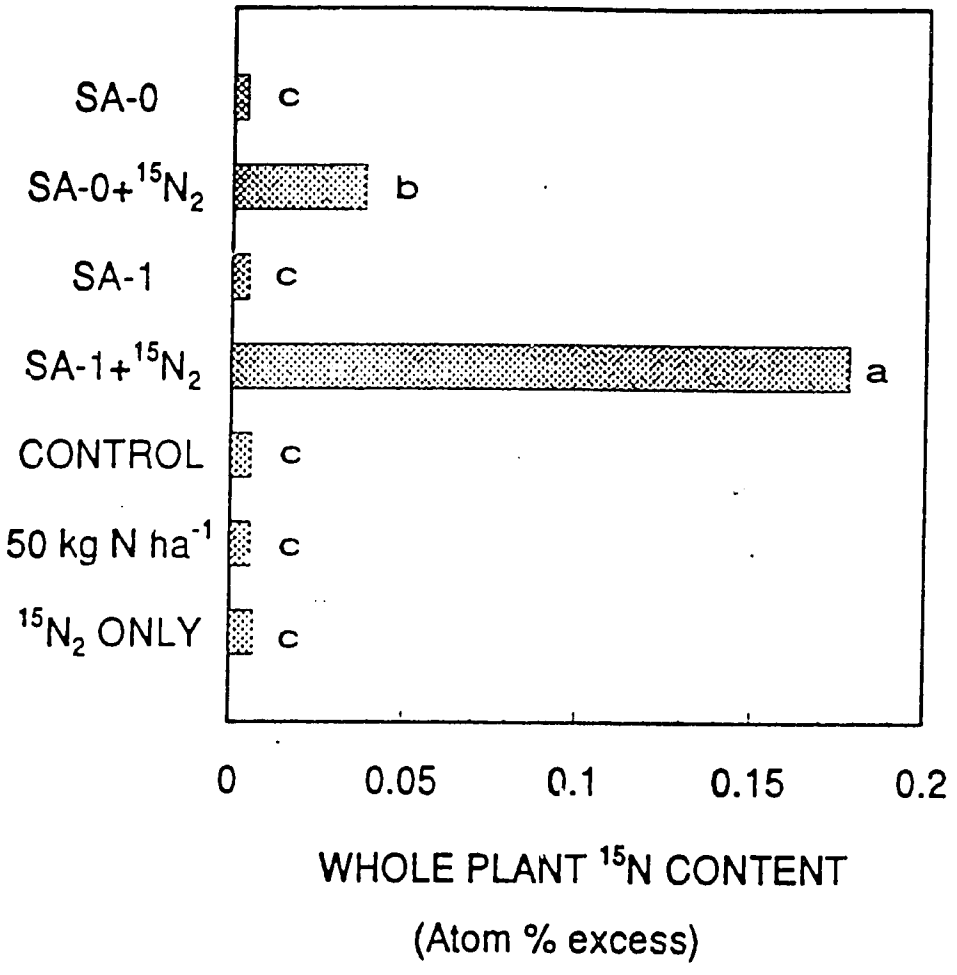


Fig. 11. Average ^{15}N-content of rice plants grown with different sources of nitrogen and exposed to 1.96% $^{15}N_2$ at second and fifth week after planting. Plants were harvested at the end of the eighth week. Letters to the right of the bars indicate mean separation using the Duncan's multiple range test. Bars with the same letter are not significantly different at the 5% level. Strains SA0 and SA1 indicate inoculation with the parent and mutant cyanobacterium, respectively.

rice plants grown together at elevated [CO_2]. Studying the response of the cyanobacteria-rice interaction to superambient [CO_2] may help us predict how paddy rice production in low input systems may be affected by the projected high [CO_2] in the next century.

The objectives of these experiments were to evaluate the effects of four different N treatments on dry matter and total N accumulation in rice plants grown under two levels of CO_2 (330 and 660 µmol/ mol). More specifically, the subobjectives of the N treatments were to compare the effects of a paddy culture of cv. IR30 inoculated with *A. variabilis* strains SA0 and SA1.

Rice performance with N supplied by the mutant cyanobacterium was compared with that of three other N treatments: rice plants inoculated with the parent strain (strain SA0), plants fertilized with 75 kg N/ ha as urea, or control plants (uninoculated, with no nitrogen fertilization).

Plants at elevated [CO_2] had a higher dry matter content and accumulated more nitrogen than at near ambient CO_2. At either CO_2 concentration, the mutant strain again enhanced the shoot and grain dry matter accumulation more than did the parent strain. Total N concentration of the shoot and grain fractions was significantly greater ($P>0.05$) in plants inoculated with the mutant than with the parent strain of the cyanobacterium. The benefit of inoculation with the ammonia-excreting mutant, strain SA1 was equivalent to or greater than the response observed in plants receiving 75 kg N/ ha. A combined response of both the rice plants and the cyanobacterium to high CO_2 levels probably accounted for the higher dry matter and N at the elevated than at the ambient CO_2 concentration. The potential of the ammonia-excreting mutant cyanobacterium as a N source for sustainable, low-input rice production in the predicted high CO_2 atmosphere of the next century is promising.

8. Survival and establishment of strain SA1 in soil: This part of the grant was performed by Dr. P. Roger at the International Rice Research Institute, Manila, under a subcontract from the University of Florida with a specific objective to study in microplot experiments the possibility of establishing strain SA1 in different traditional paddy soils under various fertilizer levels.

Pot experiments with rice and strain SA1 have shown that under gnotobiotic conditions (rice and strain SA1 grown on artificial medium) strain SA1 could supply enough N to the plant to support its growth in a N-free medium. Under the various experimental conditions tested - five soils, three levels of inoculum, N and P fertilizer application and insecticide application to control grazers - strain SA1 never dominated the algal population or even multiplied to any detectable extent.

Plating experiments conducted during this study showed that strain SA1 has a very low competitiveness. In fact its ability to excrete ammonia might be one of the reasons for it. Nitrogen excreted by the colonies of strain SA1 might favor the growth of other algae around the strain SA1 and competition for light and other nutrients. This was observed to some extent with mixed cultures of

strain SA1 and diatoms growing on plates. Another reason might be its susceptibility to grazing, a characteristic shared with other *Anabaena* strains. The low competitiveness of strain SA1 observed on plates was confirmed in the inoculation experiments where it never developed a bloom of agronomic significance nor became dominant.

Strain SA1 although suitable for ammonia production in the laboratory and in gnotobiotic conditions is not a suitable strain for field inoculation in rice-growing regions because of very low competitiveness that does not allow the strain to establish in soil. These results also confirm that inoculation of foreign strains has a limited potential in rice culture. Phosphorous application and the control of grazer populations allowed development of blooms of indigenous cyanobacteria and algae leading to the accumulation of 4.5-40 kg N/ ha in 3-4 weeks. However the low ratio of P applied to N accumulated indicates that the cost of P-fertilizer is an important factor to be considered in agronomic practices aiming at promoting nitrogen fixation in rice fields.

CONCLUSION

The significant differences obtained in this study in the root, shoot and grain dry matter and their total nitrogen content under florida soil conditions further support the relative superiority of the ammonia-excreting mutant strain over the parent strain as a source of nitrogen for growth of rice plants. Mutant cyanobacteria like *A. variabilis* strain SA1 used in this study have the potential of serving as suppliers nitrogen in crop production, especially in cereals, which unlike the legumes, have not evolved the ability to reduce dinitrogen in symbiosis with N_2-fixing procaryotes.

A further study on the manipulation of the paddy rice agroecosystem for increasing the survival and biological nitrogen fixation by cyanobacteria holds the key to increasing the potential agronomic benefit of the ammonia-excreting mutant cyanobacterium in rice production. Millions of low income farmers with insufficient capital to purchase fertilizer nitrogen would benefit from a sustainable, low-input fertilizer technology that can be developed from N_2-fixing procaryotes. In addition, the problem of environmental pollution from agricultural fields due to the heavy use of inorganic fertilizers may be minimized.

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

This study evaluated the potential agronomic utility of a cyanobacterial mutant of *A. variabilis* strain SA1 as N-fertilizer supplier for the growth of rice plants. This strain supported plant growth under N-limiting conditions in greenhouse, and to a limited extent in the field. However due to the ammonia-excretion phenotype of the mutant, the organism failed to establish in the soil from the rice growing regions of Philippines. This failure precluded the use of this organism as a N-supplier in the developing world for rice cultivation.

PROJECT ACTIVITIES/OUTPUTS

Meetings in which this work was presented:

Annual Meeting of the American Society for Microbiology, 1989, 1990 and 1991
American Society of Agronomy, 1992
Annual Meeting of Southern Regional Project S187, 1989, 1990
Eighth International Congress on Nitrogen Fixation, Knoxville, TN, 1990
Fifth International Symposium on Nitrogen Fixation with Non-legumes, Florence, Italy, 1990
USAID/PSTC sponsored biotechnology conference, Washington, DC, 1988
Biological Nitrogen fixation meeting (BOSTID and USAID/PSTC), Ames, Iowa, 1989
Rice Technical Working Group (24th Meeting), Little rock, Arkansas, 1992

Students Trained:

Mr. Freeman Kamaru, a Nigerian Ph. D. student in Agronomy was supported with funds from this grant. He completed and received his Ph. D. degree in 1992. His dissertation title was "Ammonia Production by a Mutant Strain of *Anabaena variabilis* and its Contribution to Growth and Yield of Rice (*Oryza sativa* L.)"

Dr. P. M. Reddy was trained as a Post-doctoral Research Associate. Dr. Reddy is from India and currently holds a faculty position in Goa University, India.

Manuscripts

1. Albrecht, S.L., C. Latorre, J.T. Baker and K.T. Shanmugam. 1992. Geentically altered cyanobacteria as nitrogen fertilizer supplier for growth of rice. In: Biological nitrogen fixation associated with rice production (Ed. S.K. Dutta and C. Sloger). Oxford &IBH Publishing Co. Pvt. Ltd., New Delhi, India. pp. 163-175.

2. Kamaru, F., S.L. Albrecht, J.T. Baker, L.H. Allen, Jr. and K.T. Shanmugam. Growth responses of paddy rice to an ammonia-excreting mutant cyanobacteriaum at elevated CO_2 concentration. Submitted for publication to Agriculture, Ecosystems and Environment.

3. Kamaru, f. and s.L. albrecht. Dry matter and nitrogen accumulation in rice plants grown with ammonia produced by a nitrogenase derepressed mutant strain of the cyanobacterium *Anabaena variabilis*. in preparation.

4. Latorre, C., P,M. Reddy, S.L. Albrecht and K.T. Shanmugam. Structure/function rel;ationship of glutamine synthetase from an ammoni-excreting mutant strain of *Anabaena variabilis*. in preparation.

5. Reddy, P.M., S.L. Albrecht and K.T. Shanmugam. Photodissimilation of fructose by *Anabaena variabilis* to dihydrogen and carbon dioxide. in preparation.

PROJECT PRODUCTIVITY

This project accomplished all of the proposed objectives.

FUTURE WORK

This project can lead to future work in isolating mutants from cyanobacteria which are native inhabitants of rice fields. These mutants should have mutations in glutamine synthetase leading to decreased rate of assimilation while the Km for substrates are not altered. These mutations can be introduced into the cloned *glnA* gene of *A. variabilis* and studied in *E. coli*. Upon identification of appropriate mutation, the altered gene can be introduced into cyanobacteria from the rice fields and replace the native gene. Such organisms will have higher rate of survival in the field and can support plant growth in N-free or N-limited conditions.

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