Biol Fert Soils (1985) 1:61–72

Biology and Fertility of Soils

Effect of grazer regulation and algal inoculation on photodependent nitrogen fixation in a wetland rice field

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Summary. A dry season field experiment conducted for two consecutive years highlighted problems of achieving increased populations of N_2-fixing blue-green algae (BGA) in wetland rice fields. Inoculation of non-indigenous BGA strains, either dried or as fresh viable inocula even at high levels of application, was unsuccessful. A limiting effect of grazing invertebrate populations on BGA establishment was evident, but other factors were involved. Reducing grazer pressure did not permit establishment of inoculated BGA; interspecific competition and environmental factors may explain the inoculation failure. Grazer regulation permitted the establishment of a fast-growing indigenous N_2-fixing *Anabaena* and the doubling of N_2-fixing activity over a control. Neither inoculation nor grazer control affected grain yields significantly.

Key words: Cyanophyceae – Ostracoda – Ricefields – Grazing – N_2-fixation

Over the past 2 decades several reports have appeared concerning the use of blue-green algal inoculants to enhance biological nitrogen fixation in wetland rice fields. These reports conflict both in the success of inoculation (algalisation) and in the inoculation's effect upon rice yields.

Reviewing field experiments, Roger and Kulasooriya (1980) calculated that algalisation, when effective, caused an average increase in grain yield of about 14% over the control, corresponding to about 450 kg grain/ha per crop. They also showed the importance of the experimental design in such experiments. Pot experiments invariably produced greater increases in yield than did field trials. In most experiments only grain yield was measured. Very little information is available on qualitative and quantitative succession of the N_2-fixing algal flora and on changes of the photosynthetic N_2-fixing activity in an inoculated paddy field. Many results are therefore open to interpretation.

Failures of algalisation have been reported under the widely different agroclimatic conditions of China (Chi Ying Huang 1978), India (All India Coordinated Project on Algae 1979), Japan (Watanabe 1973; Yamaguchi 1980), Pakistan (NIAB 1977), and the Philippines (Alimagno and Yoshida 1975). A recent study in Tamil Nadu State (India) showed that whereas algalisation increased yield in the experimental plots of an agricultural research station, no effect was observed in the farmers' fields in the same area (Agricultural Economics Research Centre 1982).

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Possible reasons for failure of algalisation include unfavourable soil properties (low pH, low available P), climatic factors (heavy rains, too low or too high light intensities), biotic factors (competition, grazing) and the quality of the inoculum (Roger and Kulasooriya 1980). When soil and climatic conditions are favourable for blue-green algal growth, grazing can prevent establishment of N_2-fixing populations (Wilson et al. 1980; Osa-Afiana and Alexander 1981). Conventional pesticides can control invertebrate grazers (Grant et al. 1983a), but their cost is prohibitive and, furthermore, acquired reisistance to them is evident. Plant pesticides such as neem (*Azadirachta indica*) seeds offer a cheaper alternative (Grant et al. 1983b).

A field experiment was conducted to study simultaneously the effects of algal inoculation and grazer control on the N_2-fixing algal biomass, the photodependent N_2-fixing activity and the rice yield.

Methods

Plot design

The experiment was conducted for two consecutive dry seasons at the International Rice Research Institute's experimental farm in a field where no nitrogen fertilizer had been applied for 10 years. After ploughing and harrowing the field, levees were built to form 4 × 4-m^2 plots, which were then levelled and puddled. To keep the experimental area undisturbed, a 1-m^2 metal frame was pushed into the centre of each plot to isolate an area into which operators could step. In the first dry season (1982), 2-week-old seedlings of IR36 were transplanted at a 20 × 20 cm spacing, 1 month after puddling. In the second dry season, IR42 was similarly transplanted, 3 days after puddling.

Treatments

Four treatments were assigned within a latin square design of 16 plots: (1) neem, (2) inoculated, (3) inoculated + neem and (4) control.

Neem. Microcrustaceans of the subclass Ostracoda, and pulmonate and prosobranch molluscs are intensive grazers of BGA in rice fields. Reduction of ostracod density through the use of neem (*Azadirachta indica*) has been successful (Grant et al. 1983b). For the first crop, chopped neem nuts were applied 1 day after transplanting (DAT) at the rate of 46 kg ha^{-1}, and 29 DAT, at 100 kg ha^{-1}.

As large populations of grazing snails were recorded early in the cultivation cycle of the first crop, 1 kg a.i. ha^{-1} of the molluscicide Bayluscide (Bayer) (5, 2′-dichloro-4′ nitrosalicylic-anilide-ethanolamine) in conjunction with neem (100 kg ha^{-1}) was applied in the second crop at 50 DAT to control them.

Inoculated. Plots were inoculated with algae 10 days after transplanting the first crop and 8 days after transplanting the second crop. This allowed 9 days in the first crop and 3 days in the second for neem to reduce grazing populations of ostracods. For the first crop, two N_2-fixing BGA strains *Nostoc* sp. *S.L.* and *Tolypothrix tenuis* were broadcast as a mixed dry powder at a combined rate (four-fifths *Nostoc* and one-fifth *Tolypothrix tenuis* w/w) of 2.5 kg ha^{-1} or 3.75 g dry wt. $plot^{-1}$. Nitrogen-fixing strains produced as described below inoculated in the second crop were *Aulosira* sp., *T. tenuis*, *Nostoc* sp., *Anabaena variabilis*, *Fischerella* sp. and *Scytonema* sp., and these were broadcast as composite "soil-based" fresh inoculum at 1 l $plot^{-1}$ (equivalent to 176 kg dry matter ha^{-1}). *T. tenuis* 1482/3a came from the Cambridge Centre for Algae and Protozoa, England; *Aulosira* from G.S. Venkataraman, Indian Agricultural Research Institute, New Delhi; *Nostoc* sp. from S.A. Kulasooriya, University of Peradeniya, Sri Lanka; *A. variabilis* from M. Martinez, University of the Philippines, Los Baños, Philippines; and *Fischerella* and *Scytonema* were isolated from Philippine rice fields.

Inoculated + neem. A combination of treatments (1) and (2).

Control. None of the above amendments.

Algal inoculum production

Cyanophycean strains were grown separately, under fluorescent light, in 20-l carboys on GO medium (Rippka et al. 1971)), continuously stirred and supplied with 0.5% CO_2-enriched air. Cultures were harvested by decantation shortly after the logarith-

Table 1. Characteristics of the monostrain soil-based inocula and their mixture

CFU	N_2-fixing CFU (/ml fresh inoculum)	N_2-fixing CFU (/g (dry wt.) dried inoculum)	Non-N_2-fixing CFU (/ml fresh inoculum)	N_2-fixing CFU (/ml multistrain inoculum) Calculated[a]	Observed
Anabaena variabilis	5.3×10^6	2.1×10^7	3.5×10^6 (Ps)	8.2×10^5 (33)	1.0×10^5 (6)
Aulosira fertilissima	2.5×10^6	8.5×10^6	3.0×10^6 (Ps)	5.0×10^5 (21)	7.0×10^5 (41)
Fischerella sp.	7.0×10^4[b]	3.6×10^5[b]	2.0×10^6 (Ps, OS, LPP)	1.2×10^4 (0)	10^4 (0)
Nostoc sp.	1.1×10^6	3.3×10^6	8.0×10^6 (Ps)	1.4×10^5 (6)	2.7×10^5 (16)
Scytonema sp.	10^4	10^4	1.1×10^7 (Ps, OS)	3×10^2 (0)	10^4 (0)
Tolypothrix tenuis	6.0×10^6	2.2×10^7	1.7×10^6 (Ps)	9.8×10^5 (41)	6.7×10^5 (39)
Sum of the six inocula	1.7×10^6	9.8×10^6	6.3×10^6 (Ps)	2.4×10^6 (100)	1.7×10^6 (100)

Ps, *Pseudanabaena*; OS, *Oscillatoria;* LPP, *Lyngbya, Plectonema, Phormidium* group

[a] Figures in parentheses are percentages of the totals;
[b] Dominat N_2-fixing strain was *Calothrix* sp.

mic phase of growth. Inoculum for the first crop was prepared by drying harvested material at 40°C, powdering it in a mortar and mixing the strains. The mixture had about 4.6×10^7 N_2-fixing colony-forming units (CFU) g^{-1} dry matter. Quantity applied in the field corresponded to about 1.2×10^5 N_2-fixing CFU cm^{-2} soil. Inoculum for the second crop was prepared by further multiplying laboratory-produced material in shallow 1-m^2 trays in a greenhouse. Trays contained 5 kg clay soil, 10 g 18% superphosphate, 2 g NaCl and 4 cm deionised floodwater into which the monostrains were inoculated. Perthane (1,1-dichloro-2,2,-bis (4-ethylphenyl) ethande) was added at a rate of 2.2 kg a.i. ha^{-1} to prevent development of grazing invertebrates. After 2 weeks, algal mats were visible on the soil surface or were detached and floating. These mats were harvested, but prior to mixing and formulation of the fresh inoculation they were sampled for CFU enumeration on solid GO medium with and without nitrogen. Of the six strains inoculated into trays, *Fischerella* and *Scytonema* grew poorly. Dominant algae observed growing in trays inoculated with these strains were actually homocystous species, among which *Pseudanabaena* was dominant. CFU of the four other strains ranged from 3.3×10^6 to 2.2×10^7 g^{-1} dry inoculum (Table 1). In all the inocula, the presence of non-fixing strains, mainly *Pseudanabaena*, was recorded at densities ranging from 10^6 to 10^7 g^{-1} dry inoculum.

The composition of the monostrain mixture was evaluated: (a) using results of separate enumerations on the monostrain inocula (fourth column in Table 1) and (b) through plating suspension dilutions of the mixture (fifth column in Table 1). Results did not significantly differ except for *A. variabilis*, where CFU measured in the mixture was 8 times less than the CFU calculated. This may indicate competition between strains when growing together on a Petri dish; such competition can also be expected in situ. The composite inoculum had about 2×10^6 N_2-fixing CFU ml^{-1} and 6×10^6 non-N_2-fixing CFU ml^{-1}. This corresponded to 8×10^6 N_2-fixing CFU/g^{-1} dry wt. and 2.4×10^7 non-N_2-fixing CFU g^{-1} dry wt. respectively. The dominant N_2-fixing strains were *Aulosira* (41%), *T. tenuis* (39%) and *Nostoc* sp. (16%). The dominant non-N_2-fixing strain was *Pseudanabaena* sp. The inoculum contained 0.55% N, 0.17% P, 4.11% C, and 79% ash (dry weight). Applied material was equivalent to 0.97 kg N_2 ha^{-1} and 0.29 kg P ha^{-1}.

Amendments and cultural practice

During the first crop, 5 kg P ha^{-1} as KH_2PO_4 was broadcast on all plots at 1, and at 29 DAT. The second crop received 8.2 kg P ha^{-1} as supersphosphate 9 DAT or 1 day after inoculation (DAI). Cultural practices included two hand weedings with minimum disturbance of the soil surface, maintenance of floodwater until 2 weeks before harvesting and rat control at maturity. Carbofuran was applied twice to rice plants as Furadan (12% sprayable, 2 kg a.i. ha^{-1}) to control pests. During fallow, levees were kept open to avoid standing water and multiplication of grazers.

Measurements

Enumeration of nitrogen-fixing BGA. Sixteen glass tubes (2.5 × 25 cm) were pushed into the soil surface of each plot to an approximate depth of 10 cm, then removed and stoppered from below to give soil cores containing floodwater. Cores were taken at random, but floating colonies of BGA were avoided; thus the sample for enumeration was of soil surface and suspended algae. When floating colonies were abundant, their biomass was estimated separately. The tubes sampled a total area of 85 cm^2. In the laboratory the floodwater was removed by suction, leaving only 1-2 mm water covering the soil surface. The floodwaters from the 16 tubes were combined, and after acetylene reduction activity (ARA) assays of the soil cores, the top 2 mm of soil was cut from each core and stirred with its respective floodwater to give a 10^{-1} dilution (adjusted to volume with distilled water). After 1/2 h stirring, serial soil dilutions were made and 1 ml 10^{-3}, 10^{-4} and 10^{-5} dilutions plated onto solid GO medium without N. Petri plates were incubated at laboratory temperatures (26°C day, 30°C night) under constant white fluorescent lamps for 3 weeks before colonies were counted and identified. Counts were reported as CFU cm^{-2}. The first sampling for algal enumeration was made just before transplanting, to record the existing flora at the beginning of the experiment and to test the heterogeneity of the distribution of BGA in each plot. A similar plating method was used for enumerating N_2-fixing CFU in the inoculum.

Estimation of floating algal biomass. Later in the rice cultivation cycle, floating, mucilaginous colonies comprising *Nostoc* spp., *Gloeotrichia* sp. and *Aphanothece* sp. were collected by hand-held nets, weighed in the laboratory after draining the water and roughly sorting the contents (mainly removing weeds), and returned to their respective plots within 24 h. Floating algae were harvested on seven occasions during the first crop and on two occasions (32 and 72 DAT) during the second crop. Logarithmic transformation of fresh weight of floating biomass data was necessary before statistical analysis.

Acetylene reduction activity. After the floodwater was removed from the soil cores as previously described, the sample (16 cores) was enclosed in a plastic bag containing a gas sampling port. The bag was heat sealed and the air within slowly evacuated through a needle inserted into the sampling port. A known volume of air was then introduced followed by a 10% v/v injection of C_2H_2. Throughout the first crop, bags were incubated under natural light and were cooled to 35°C in an ice-cooled water-bath. Gas samples were withdrawn from each bag at 0.5, 1.5 and 3 h and stored in Vacutainers until 0.5 ml of each was injected into a gas liquid chromatograph column at 22°C. ARA was calculated from the ratio of C_2H_2 : C_2H_4 peak heights and integrated over the sampling period to estimate mol C_2H_4 formed $m^{-1}h^{-1}$. The first ARA measurement was made 9 days after transplanting, i.e. 1 day before algal inoculation.

During the second rice crop, ARA was determined under laboratory conditions, 23°C and 2 klux. Laboratory determination was found to reduce the variation caused by fluctuations in temperature and light during exposure to sunlight. The first determination was made 3 days after algal inoculation. ARA measurements on a composite representation of the floating mucilaginous strains were made under soft white fluorescent light at 23°C. The wet weight of algae was determined and and approximation of ARA per unit weight of floating algae was then used to calculate the theoretical ARA for all estimates of floating algae. Distribution of ARA approximates a log-normal model (Roger et al. 1977) and logarithmic transformations of the data were made prior to statistical analysis.

Enumeration of Ostracoda. Plastic cylinders, enclosing an area of 28 cm^2, were pushed at random into the soil surface to contain the floodwater and the animals living within. The floodwater and surface

2–4 mm of soil was sucked from each cylinder and stored separately in bottles. The contents of each bottle were transferred onto a 100-μm sieve, washed with tapwater and backwashed onto a white tray divided into equal counting squares. Five such cylinders from each plot constituted a sample with a standard error within 20% of the mean. Ostracods from each bottle were counted and the average-of-sample means were expressed as a population mean ± standard error m^{-2}. The first sampling was made 5 DAT. A χ^2 test (variance to mean ratio) rejected agreement of their distribution with a Poisson series ($P < 0.05$) at each sampling interval, and the negative binomial distribution was accepted as a suitable model. The statistic b (1.7) of Taylor's power law (Taylor 1961) indicated a contagious population distribution over the series of sampling intervals. Logarithmic transformation was applied to the data before statistical analysis.

Only two population estimates were made during the second crop. These were to check the efficacy of neem in reducing ostracod population size.

Enumeration of Mollusca. Quadrats (0.25 m^2) were used until algal growth obscured direct counting of snails on the soil surface. A contagious distribution was apparent during the early part of the rice cultivation cycle. Later, when algal mats disappeared, the distribution of snail populations was regular. This was probably related to a change in feeding behaviour, when later in the season grazing by *Lymnaea viridis* of stem epiphytes on regularly spaced rice plants was observed. When direct counting was obscured by algal or rice canopy growth, snails were counted in the soil cores taken for BGA enumerations after the cores were washed in a 280-μm sieve. However, this method biased the sample in favour of *Thiara riquetti*, which, unlike *L. viridis*, did not move to the rice stem to feed epiphytically. Numbers of both the above species were combined before statistical analysis and graphing of data. Contagiously distributed data were transformed (log x + 1). When dispersion was random or regular, no transformation was made. Only two population estimates were made during the second crop to check the efficacy of the molluscide.

Grain yield

A 6-m^2 area from each plot was harvested for determination of grain yield. Rice growing adjacent to levees was not harvested. Grain was air dried before measurement of moisture and correction of grain weight to 14% moisture.

Results

Five days before algal inoculation, the ostracod populations of all treatments did not significantly differ

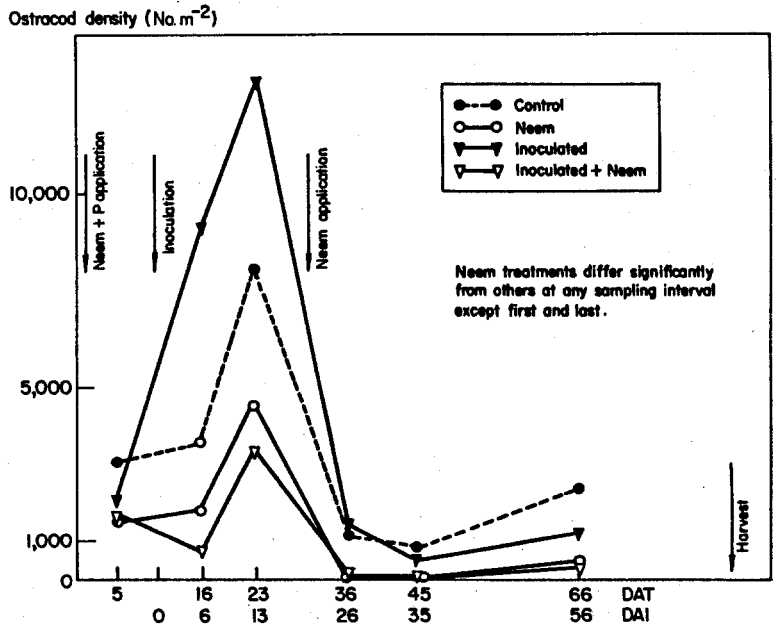

Fig. 1. Population density of Ostracoda during the crop cycle

Table 2. Density of grazers at two intervals after neem and Bayluscide application on the second crop

	Density (No. m^2 ± SE)			
	Ostracoda		Mollusca	
	10 DAI	18 DAI	9 DAI	17 DAI
Control	2826 ± 1016	3560 ± 890	60 ± 20	76 ± 14
Neem (+ Bayluscide)	72 ± 11*	113 ± 41*	2 ± 1	44 ± 13
Inoculated	3917 ± 997	4718 ± 1147	275 ± 30**	200 ± 44*
Neem (+ Bayluscide) + Inoculated	121 ± 20*	512 ± 107*	4 ± 1	12 ± 4

* Significantly different at 5% from "Control" and "Inoculated" treatments
** Significantly different at 1% from other treatments

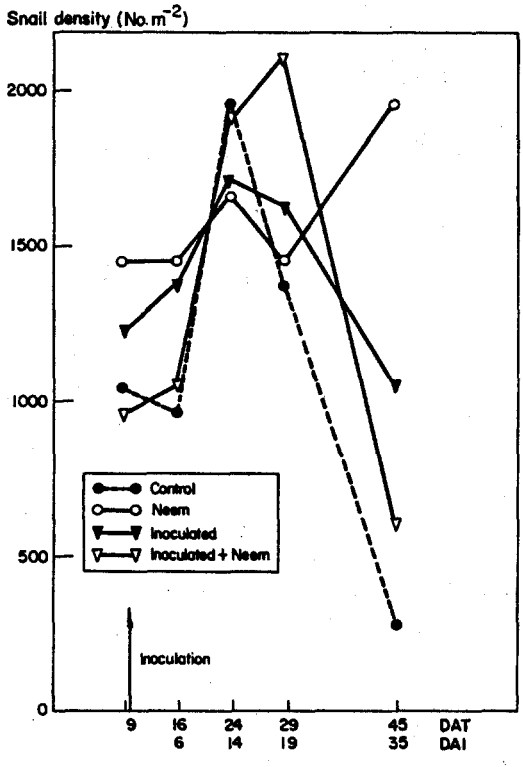

Fig. 2. Population density of Mollusca during the first half of the crop cycle

(Fig. 1). Ostracod densities were 1500-1700 m^{-2} in plots treated with neem nuts 4 days earlier and 2000–3000 cm^{-2} in untreated plots. Six days after algal inoculation (DAI), significant differences ($P < 0.05$) appeared between populations in neem-treated plots and untreated ones. Neem and inoculated + neem plots maintained lower densities of ostracods than control plots, and particularly lower than inoculated plots, where density jumped sharply. By 13 DAI, populations had reached 8000 m^{-2} in the control and 13 000 m^{-2} in the inoculated plots. Neem-treated plots showed minor increases of ostracods. Re-application of neem was made at a higher rate, 28 days after initial administration. This effectively reduced the ostracod populations to 100 m^{-2}. These densities were virtually maintained until 20 days before harvesting of rice, when sampling was abated. Grazer populations in the control and inoculated plots had also crashed by 25 DAI, but reached significantly higher populaion densities at 34 DAI.

In the second crop, ostracod densities were measured 10 and 18 days after neem application. Densities in neem and inoculated + neem plots at the above times were significantly lower than in control and inoculated plots (Table 2). Compared with the same period of the previous year (first crop; 16 and 23 DAT), ostracod densities in neem-treated plots were significantly (5% level) reduced.

Lymnaea and *Thiara* were the predominant snails encountered in both crops. Their combined densities for the first crop from inoculation to DAI are shown in Fig. 2. Population fluctuations were marked; yet significant differences between snail densities in the treatments at each sampling time were not evident. Inoculated + neem plots sustained the highest densities. Neem-treated plots and inoculated plots exhibited relatively stable numbers of molluscs until 34 DAI, when densities in the former increased and those in the latter rapidly decreased.

Bayluscide, applied together with neem treatments of the second crop, controlled mollusc populations for 9 days, after which population size increased slowly, but remained significantly different from populations of the control and inoculated plots (5% level) at 17 days (Table 2). Populations of molluscs in control and inoculated plots at the same sample intervals were much smaller than those recorded at the same stage during the first crop.

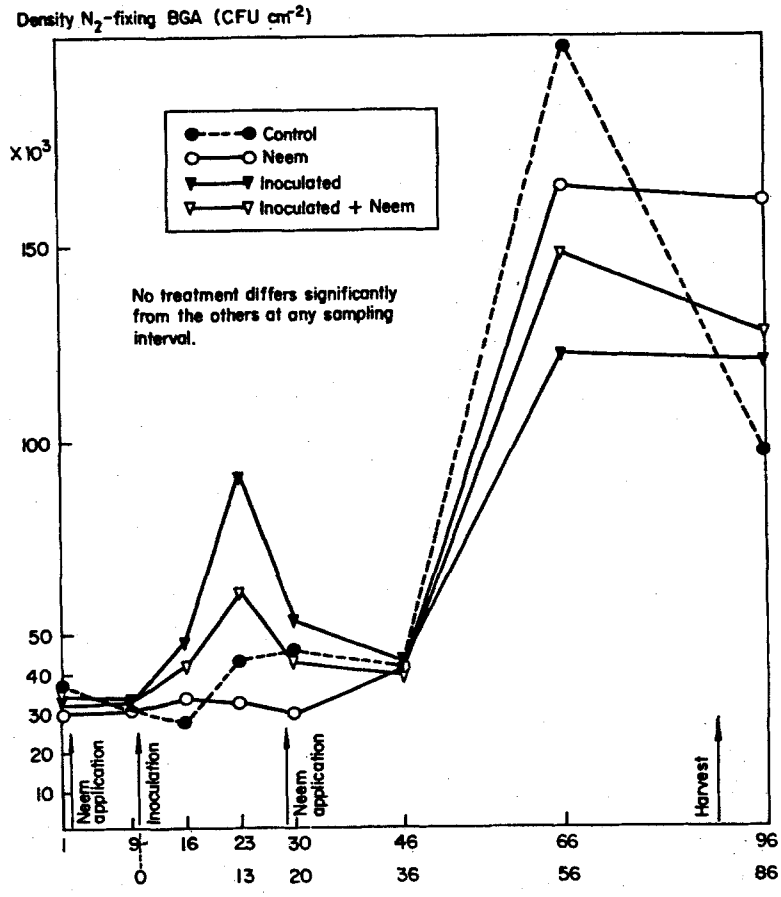

Fig. 3. Density of soil N_2-fixing blue-green algae during the crop cycle

Background levels of N-fixing BGA in the first crop, i.e. before inoculation, were within a close range of 30–37 × 10^3 cm^{-2} (Fig. 3). Ten days after P application, non-mucilaginous *Nostoc* sp. and *T. tenuis* inoculants were administered. No statistically significant ($P > 0.05$) change in number of N_2-fixing BGA was recorded in the inoculated and inoculated + neem plots during the 2 weeks following inoculation. Microscopic observations and immunofloures-cence techniques indicated that inoculated strains of *Nostoc* and the *Nostoc* strains growing on the plates were different. *Tolypothrix* sp. did not appear on BGA plates at this time either. This indicated that apparent increases in BGA in both inoculated treatments were not the result of algal inoculation. BGA densities in control and neem treatments were stable until 36 DAI and were not significantly different from inoculated treatments. Up until 36 DAI, the BGA recorded were dominated by three *Nostoc* spp., *Anabaena* and *Calothrix*. The peaks of algal density at 13 DAI were caused by mucilaginous and non-mucilaginous *Nostoc* spp. By 56 DAI, all treatments showed marked increases in density due, primarily, to growth of mucilaginous *Nostoc* spp. and *Aphanothece* sp. Differences between inoculated and non-inoculated or neem and control plots were not statistically different at the 5% level. The fresh weight of floating algae in the first crop (Fig. 4) increased exponentially from 20 to 50 DAI. Doubling time was approximately 7 days and the predominant species were *Nostoc*, *Gloeotrichia* and *Aphanothece*. After a stationary phase of 3 weeks, the algae went into decline. Twelve tonnes fresh weight of algae per hectare was recorded in neem, inoculated + neem, and control plots. A value of 15 tonnes in inoculated plots was not significantly different from the biomass in the other treatments and, because of a high water content, was only equivalent to 2.7 kg N ha^{-1}.

The qualitative and quantitative evolution of the N_2-fixing CFU during the first month of the second crop is presented in Table 3. Within the accuracy of BGA enumeration by plating, no significant change in the number of N_2-fixing CFU was observed except in neem-treated plots 16 DAT, where a high density

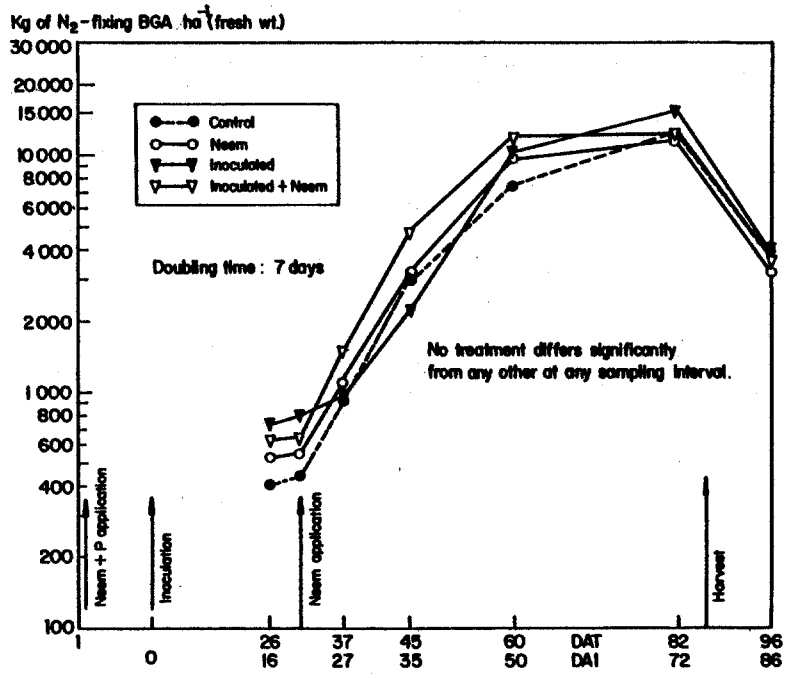

Fig. 4. Floating biomass of N_2-fixing algae during the crop cycle

Table 3. Quantitative and qualitative evolution of the BGA population during the second crop

	BGA/CFU		9 DAT	11 DAT	16 DAT	22 DAT	29 DAT
Control	N_2-fixing	No. cm^{-2}	19×10^4	15×10^4	18×10^4	9×10^4	31×10^4
		Dominant[a]	*Nostoc*	*Nostoc/ Anabaena*	*Anabaena*	*Anabaena*	*Aphanothece*
Neem	N_2-fixing	No. cm^{-2}	19×10^4	14×10^4	60×10^4	17×10^4	29×10^4
		Dominant	*Nostoc*	*Anabaena/ Nostoc*	*Anabaena*	*Anabaena*	*Nostoc*
Inoculated	N_2-fixing	No. cm^{-2}	25×10^4	19×10^4	23×10^4	15×10^4	11×10^4
		Dominant	*Nostoc*	*Nostoc*	*Anabaena/ Nostoc*	*Anabaena*	*Nostoc*
	Inoculated	No. cm^{-2}	3×10^3	10×10^3	13×10^3	23×10^3	3×10^3
		Dominant	*Aulosira/ Anabaena*	*Tolypothrix*	*Tolypothrix/ Aulosira/ Anabaena*	*Tolypothrix Aulosira/ Anabaena*	*Aulosira*
Inoculated + neem	N_2-fixing	No. cm^{-2}	25×10^4	13×10^4	27×10^4	33×10^4	15×10^4
		Dominant	*Nostoc*	*Nostoc*	*Anabaena*	*Anabaena*	*Nostoc*
	Inoculated	No. cm^{-2}	3×10^3	6×10^3	10×10^3	7×10^3	10^3
		Dominant	*Aulosira/ Anabaena*	*Tolypothrix/ Aulosira / Anabaena*	*Tolypothrix/ Aulosira / Anabaena*	*Tolypothrix/ Aulosira*	None

[a] When several dominant genera are recorded they are classified by decreasing abundance

of *Anabaena* sp. CFU was recorded. The succession of dominant strains was similar in all treatments. For the first 10 DAT, dominant CFU were *Nostoc* strains. However, direct observation of the algal mats showed that *Anabaena* sp. filaments were the major component of the N_2-fixing algal flora. Therefore *Nostoc* colonies on the plates must have arisen mainly from spores present in the soil at a high concentration (2×10^5 cm^{-2}). Plating methods do not permit differentiation between spores and living cells.

After 10 days *Anabaena* sp. became a, or the, dominant strain in the four treatments, thus confirming visual observations. At 29 DAT *Anabaena* sp. was replaced by mucilaginous strains of *Nostoc* sp., *Gloeotrichia* sp. and unicellular N_2-fixing *Aphanothece* sp.

Inoculated strains were applied at 1.1×10^4 CFU cm^{-2}. Among the four major inoculated strains, *T. tenuis*, *Aulosira* sp. and *A. variabilis* were easily recognizable. They were observed on the Petri dishes, but their density never became significantly higher than the initial level, and this indicated failure of inoculated algal growth. Floating biomass of the second crop was 10 times greater at 32 DAT and up to twice as much at 72 DAT than that measured for the equivalent period during the first crop. Although biomass was highest in the control plots 32 DAT, there were no significant differences in fresh weight between treatments at either 32 or 72 DAT (Table 4).

In the first crop, ARA of the soil surface (Fig. 5) in all plots peaked around 16 days after transplanting. Control plots had the highest activities (255 μmol C_2H_4 $m^{-1}h^{-1}$), but these were not significantly different from the inoculated treatment which had the lowest activity (110 μmol C_2H_4 $m^{-2}h^{-1}$). The alga inoculated at 10 days after transplanting was of no measured additional value to the N_2-fixing potential of the plots. *Anabaena* populations, abundant early in the rice cultivation cycle, were probably responsible for the peak of ARA. Twenty DAI, soil surface ARA in all treatments was weak and remained so throughout the rice cultivation cycle. Slightly higher activities towards the end of the cycles were obtained but probably reflect the increased difficulty of avoiding floating algae within the sample.

Extrapolated ARA estimates of floating algae were obtained from the fresh weight of algae collected and the ARA of averaged subsamples then measured in the laboratory. The graph of extrapolated ARA mirrors the growth curve of the floating algae (Fig. 5). Relatively high values for ARA (> 200 μmol $m^{-2}h^{-1}$) were obtained from this method when algal biomass was greatest.

In the second crop, as in the first, a peak of ARA occurred about 14 DAT (Fig. 6) in all plots, but the activities were maintained over a longer period. In treatments controlling grazing by ostracods and molluscs, activities > 700 μmol C_2H_4 $m^{-2}h^{-1}$ were recorded. Control and inoculated plots had half this activity; yet theirs exceeded that of the first crop by a factor of 1.5–3. By 29 DAT, ARA was weak in all treatments but 3 times higher than for the same period during the first crop. It is possible that the greater ARA in the second crop reflects, in part, the different assay conditions that eliminated the extremes of light and temperature encountered during first crop measurements. A debatable extrapolation of the

Table 4. Fresh weight of floating BGA in second crop

	Fresh weight (kg ha^{-1} ± SD)	
	32 DAT	72 DAT
Control	10 810 ± 5 403	22 400 ± 2 958
Neem	4 639 ± 3 847	28 600 ± 4 855
Inoculated	5 075 ± 1 514	29 800[a]
Neem + inoculated	4 706 ± 1 423	21 900[a]

[a] Mean of two replicates only

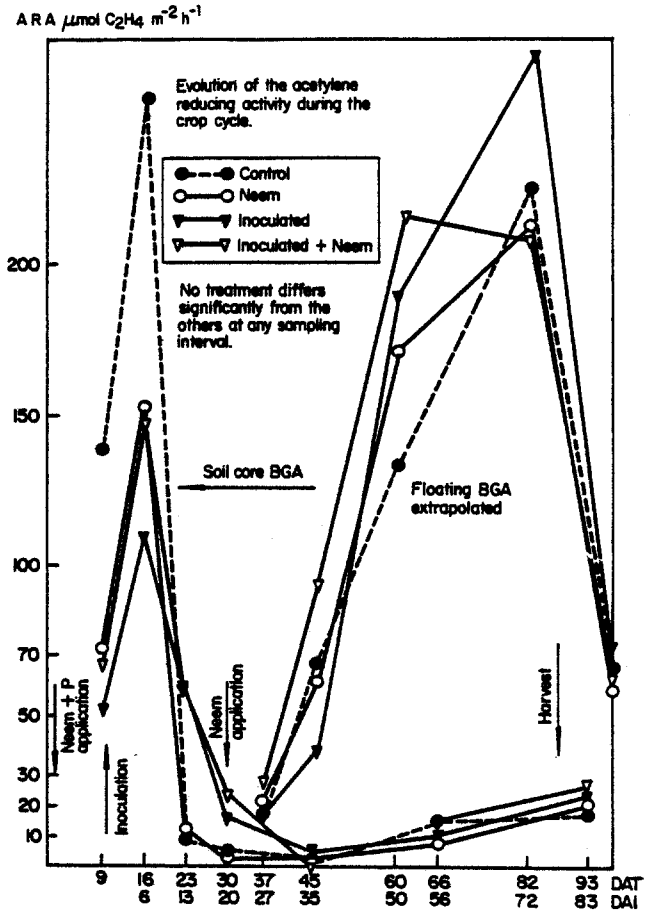

Fig. 5. Acetylene-reducing activity during the first crop cycle. Soil core BGA = soil cores minus floodwater or soil surface BGA. Floating BGA = ARA per unit weight of composite samples of mucilaginous colonial BGA used to estimate ARA from wet weight of samples removed at the intervals shown. See text

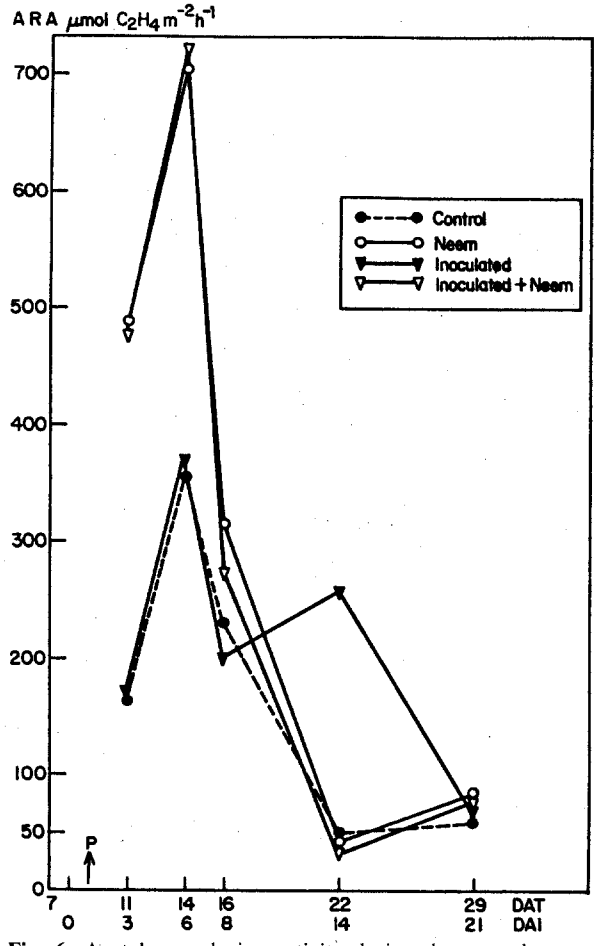

Fig. 6. Acetylene-reducing activity during the second crop cycle

Table 5. Filled grain yields

	Filled grain yield (g 15 m^{-2} ± SD)	
	First crop	second crop
Control	2760 ± 420	2875 ± 935
Neem	2540 ± 310	2644 ± 479
Inoculated	2458 ± 360	2595 ± 1297
Neem + Inoculated	2543 ± 305	2133 ± 1253

ARA measurements to N fixed, on the basis of a C_2H_2:N ratio of 3.1 and 12 h N_2 fixation day^{-1} indicated, on average, N_2-fixing activity of about 3 kg N ha^{-1} by soil algae and 10 kg N ha^{-1} by the floating algae during the first crop.

During the second crop, the activity of soil algae was estimated to be about 3 kg N ha^{-1} in the plots with no grazer control and 6 kg N ha^{-1} in the plots treated with neem and molluscide. Assuming level of N_2-fixing activity follows from amount of biomass, the average contribution of floating algae was estimated to be about 20 kg N ha^{-1}.

Grain yields were generally low for both crops and ranged between 1.4 and 1.9 tonnes ha^{-1} (Table 5). There was no significant difference in yield between treatments or crops. The second crop was badly affected by tungro virus, which reduced grain yield and increased the statistical variance.

Discussion

Fate of inoculated algae

During the first crop, BGA inoculum was applied at a rate corresponding to 1.2×10^3 CFU cm^{-2} soil, whereas indigenous N_2-fixing CFU density was 3.5×10^4 cm^{-2}. That inoculated strains were not recorded, even immediately after inoculation, may be due to the plating method, in which strains comprising only a few per cent of the total flora are frequently lost. It did indicate that no sustained growth of inoculated strains occurred. During the second crop, BGA inoculum was applied at a rate of about 1.3×10^4 N_2-fixing CFU cm^{-2}, whereas indigenous N_2-fixing CFU density was 1.9×10^5 cm^{-2} (indicating a build-up of BGA spores or propagules after the first crop). Inoculated strains were recorded at densities lower or not significantly higher than the inoculation density (3×10^3 to 2.3×10^4 cm^{-2}). indicating a failure of the inoculum to grow.

The BGA inoculated into plots were chosen according to the literature that recommends algalisation as a rice farming practice (Venkataraman 1981). All species inoculated are readily consumed by the ostracod *Heterocypris luzonensis* Neale (Grant et al. 1983a, and unpublished), which completely dominated the ostracod populations established during both rice crops.

That the greatest densities of ostracods were consistently recorded in the inoculated plots indicates they were sustained, at least partly, by inoculum ingestion. Other explanations must, however, be sought for the lack of inoculant success in inoculated + neem plots. Consumption by molluscs, at least in the first crop when populations were initially large, must have contributed to inocula failure.

The inocula may not have been compatible with the floodwater environment. Algal inoculants were, and usually are, grown in high nutrient concentra-

tions, and sudden adjustment to floodwater constitutes a physiological shock, which may put them at a competitive disadvantage to adapted native strains. The apparent increase of indigenous BGA in both the inoculated treatments suggests that nutrients released either from the inocula upon degradation or from grazers after consumption may be responsible for the increase. Yet, the micronutrients remaining in the soil of soil-based inocula must also contribute to the growth of indigenous algae.

The failure of algal inoculation was clear and no increase in the grain yield of inoculated plots occurred. Neither the viability of the inoculum, tested by plating, nor its rate of application, which was about 10–30 times higher than that recommended (Venkataraman 1981), was questionable. The causes of failure are difficult to assess and no single factor can be held responsible.

The results address the problem of algal inoculation in soils where indigenous N_2-fixing BGA are already present. The International Rice Research Institute (IRRI) fields are fertile and suitable for algal growth. In the non-nitrogen-fertilised plots, growth of N_2-fixing indigenous species is generally high in the dry season. Thirteen N_2-fixing species have been recorded, among which mucilaginous *Nostoc*, *Gloeotrichia* and *Aphanothece* dominate over much of the dry season. Such strains, because of their high water (95%) and ash (55% dry wt.) contents, develop very impressive blooms that contribute little nitrogen to the ecosystem. Ten tonnes of such material contain less than 3 kg N. Replacing it by fast-growing and efficient N_2-fixing BGA would benefit the nitrogen economy of the ecosystem. However, the failure of inoculated strains to grow even in plots where grazers were controlled shows that many factors are involved in the establishment of inoculated BGA.

Grazer populations

Grazing of BGA by ostracods is arrested by biologically active components of the neem seed (Grant et al. 1984). The yield and biological efficacy of these components are known to vary with origin, age and storage conditions of the seed (Schmutterer and Zebitz 1984). During the first crop, satisfactory control of grazing was not achieved until a second (and larger) application of a different batch of neem seeds.

In both neem-treated and untreated plots, the grazer biomass within the first 2 weeks of the first crop was much greater than that of the second crop. During this period, when instantaneous N_2-fixing activities of BGA can reach a maximum, ostracod and mollusc populations were very large in all plots because of the reproduction possible in the elapsed time (1 month) between ploughing and planting with P application, and also because no molluscicide was applied. With irrigated rice, grazers are introduced into rice fields primarily with floodwater from irrigation canals, but even in rainfed conditions ostracod development from resistant eggs harboured in the soil is rapid, i.e. a few days. In any event, cultural practices that minimise the time between flooding and transplanting or P input can reduce grazer pressure, as demonstrated in the second crop (3 days lapse), where initial grazer densities in control and inoculated plots were much lower than in the first. A result of such a practise, corroborated by effective neem and molluscicide use, was increased nitrogen fixation by indigenous BGA in the early part of the cultivation cycle. Population growth and decline of grazers were rapid, as available food sources, which apparently included N_2-fixing BGA, were exploited and exhausted. The change of algal community structure, around 30 DAT, from non-mucilaginous to floating mucilaginous colonial forms of BGA, appeared the key factor responsible for the greatest change in the grazer population from growth to decline. The coincidence of major change in grazer populations with change from non-mucilaginous to colonial mucilaginous BGA agrees with the observation that ostracods readily consume non-mucilaginous BGA (Grant and Alexander 1981) as does the mollusc *Lymnaea*, but neither group successfully eats mucilaginous colonies of *Gloeotrichia*, *Nostoc* or *Aphanothece* (Grant, unpublished).

During the first crop, grazer control was incomplete and no significant difference in the number of BGA or in ARA was observed among treatments. During the second crop, a bloom of indigenous *Anabaena* developed around 16 DAT. This strain, which is susceptible to grazing, probably became dominant because of grazer control, which led to a doubling of ARA in the treated plots. Assuming a C_2H_2:N ratio of 3:1 and 12 h N_2 fixation day^{-1}, the peak activity in neem-treated plots was roughly equivalent to 800 g N ha^{-1} day^{-1}. However, such a high activity was sustained for a very short time only and

the corresponding increase in ARA (estimated at 3 kg N ha^{-1}) was around 10% of the total ARA along the cycle. Such a small change in both absolute and relative value was not enough to induce a significant difference in yield among the treated and untreated plots. The positve effect on N_2 fixation of reducing grazer pressure on BGA might be prolonged by additional use of indigenous plant insecticide. However, under the agroecological conditions of this experiment, an insecticide treatment to increase biological nitrogen fixation has no economic justification. Under different conditions, such as a soil poor in indigenous N_2-fixing BGA or with no mucilaginous strains resistant to grazing, inoculation and grazer control with pesticides of indigenous plant origin may be of value. In addition, control of grazers with such materials could be an economically feasible integrated management. Neem, besides controlling microcrustaceans in the floodwater (Grant et al. 1984), has an insect repellent action that also benefits the rice plant (Saxena et al. 1981). Also, reports indicate that neem reduces the loss of N fertilizers by inhibiting nitrifier populations, which cause N losses through nitrification denitrification processes (Misra and Chhonkar 1978; Ketkar 1974). Certain molluscs that inhabit rice fields are vectors of schistosomiasis; therefore their control may be important in contaminated areas.

Acknowledgements. Neem nuts of Indian origin were kindly supplied by C.M. Ketkar, Directorate of Non-Edible Oils and Soap Industry, Khadi, and Village Industries Commission, Ganeshkind Road, Shirvajinagar, Pune, India. Experimental results reported in this paper have required the collection of about 3600 core samples and the plating on about 3000 petri dishes. We gratefully acknowledge the technical assistance of G. Buenaventura, V. Lantican, S. Santiago-Ardales and A. Tirol in this tedious and difficult part of the work. This research was conducted under a scientific agreement between IRRI, Boyce Thompson Institute for Plant Research (USA) and ORSTOM (France) and was supported by the United Nations Development Programme.

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Received January 23, 1985