Bot. Bull. Academia Sinica **19**: 41-52 (1978)

EFFECTS OF NITROGEN FIXING ACTIVITY OF BLUE-GREEN ALGAE ON THE YIELD OF RICE PLANTS[1]

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(Received November 3, 1977; Accepted December 1, 1977)

Abstract

The efficiency of nitrogen fixation of blue green algae grown in pots and in paddy fields were compared. In the nitrogen poor soil (pot experiments), the nitrogen fixing activity of the blue green algae increased grain productivity of rice plants by 34% or 41%, depending upon the rice cultivar. This was higher than when plants were supplied with chemical N fertilizers. This increment of grain yield was due to the nitrogen fixation by blue green algae providing bio-N fertilizers which caused an increase in the number of tillers, panicles, and grains on the rice plant. However, no enhancement effect of nitrogen fixation by blue green algae on the yields of rice cultivated in paddy fields (field experiments) was observed. Both of the rice cultivars, Tainan 6 and Chia Hsin 11, grown with blue green algae like the control plants gave a grain yield of about 20% lower than those that were supplied with chemical N ferilizers. This indicates that there was little or on nitrogen fixing activity by blue green algae in the paddy fields which had previously been cultivated with other crops. This was probably because the residual chemical N fertilizer left in the soil had an inhibitroy effect on the nitrogen fixation activity of blue green algae. Besides this, the nitrogen fixing activity of the blue green algae was also suppressed by fungicides or insecticides. However, blue green algae showed different sensitivities to different kinds of fungicides or insecticides. The blue green algae treated with insecticides having the side group, $CONHCH_3$, such as Sevin and Lannate, were able to regain some of their nitrogen fixation ability.

Introduction

Blue-green algae which posses both photosynthetic-and nitrogen fixing-activities are generally inconspicuous organisms. It has been noted that the filamentous blue-green algae possessing heterocysts have vigorous nitrogen fixing activity. They make a valuable contribution to the fertility of many soils, and so to the yield of plants. It had been estimated that free-living

(1) This research was supported by the National Science Council of the Republic of Cihna.

filamentous-heterocystous blue-green algae added from 12.7 to 70 pounds of nitrogen to rice paddy fields per acre (De and Sulaiman, 1950; Singh 1961; Watanabe *et al.*, 1951; Watanabe *et al.*, 1971). It has also been reported that blue-green algae, associated with *Azolla*, growing in water in rice paddies gave yields of 50 to 100% greater than those obtained from adjoining paddy fields where *Azolla* was absent (Galston, 1975). This is a valuable character, for the nitrogen fixing potential of a particular habitat, can be assessed simply by determining the abundance of the blue-green algae. In the present study, the nitrogen fixing activity of blue-green algae has been studied because there is relatively little information on the magnitude of nitrogen fixation brought about by the blue-green algae in rice paddy fields in this country. Furthermore, the effects of fungicides and insecticides on the nitrogen fixing activity of blue-green algae were were also investigated.

Materials and Methods

Algal culture

A pure strain of filamentous-heterocystous blue-green algae, *Anabaena cylindrica*, was grown in a controlled enviroment room (day/night temperature, 28°C/24°C; daily irradiance, 0.13 cal/cm^2-min, cool white fluorescent; photo-period, 16 hours). The algae were cultured with continuous aeration in 5 liter flasks, which contained 2 liters of nitrogen-free nutrient medium, and 500 ml of sterilized fresh medium was added to the culture once a week after the equivalent amount of used medium had been removed. This was to keep the algae growing continuously and vigorously.

Treatment of fungicide and insecticide

One hundred milliliter of algae were withdrawn from the stock cultures and poured into 250 ml flasks, in which the selective fungicides such as, Sevin Lannate, Endrin, or Asozin, were added to make the following desired concentrations: Benlate, Sevin, and Endrin in 0 ppm. 10 ppm, 20 ppm, 40 ppm, and 80 ppm, respectively; and Asozin, and Lannate in 0 ppm, 20 ppm, 40 ppm, 80 ppm, 160 ppm, respectively.

The algae treated with a fungicide or insecticide were kept growing by aeration. After 4 days of treatment, the algal suspensions were filtered. The algal filaments of each treatment remaining on the filter paper were carefuly rinsed several times with a small amount of distilled water and then removed and transferred into a serum vial having a volume of 27 ml to determine the algal nitrogen fixing activity by the acetylene reduction method (Huang *et al.*, 1975). After having measured the nitrogen fixing activity of the algae, the algal filaments were dried overnight in an oven at a temperature of 75°C, the algal dry weight was then recorded.

Acetylene reduction

Acetylene reduction technique was used to assay the nitrogen fixing activity of *Anabaena cylindrica.* Algal samples were placed in serum bottles with a volume of 27 ml. Acetylene was injected through the stopper into each bottle to leave an acetylene partial pressure of 0.1 atm. The reaction of acetylene reduction in the bottle was terminated after 60 minutes by injecting 5 ml of 5N H_2SO_4 into the vial. Samples of gas phase were withdrawn. The acetylene and ethylene were separated with gas chromatography having a hydrogen ionization detector at an oven temperature of 65°C. The glass column 0.8 m long and 3 mm i. d. packed with Porapak R was used for separation. The carrier gas was N_2 flowing at 30 ml per minute.

Total nitrogen determination

The total nitrogen of nitrogenous compounds in algal filaments and those extracellular nitrogenous compounds liberated into the culture medium were determined by the Micro-Kjeldahl method (Yoshida *et al.*, 1973).

Algal inoculation

(a) Field experiments; Three day-old rice seedlings (*Oryza sativa* var. *japonica* Tainan 6) were transplanted in a paddy field having an area of 1.8×25.6 m^2. This field was divided into eight sections (1.8×3.2 m^2/section). There were four treatments with two replicates in these 8 sections. i. e.

Section I, and V; Rice plots were inoculated with *Anabaen cylindrica* but not supplied with chemical N fertilizers.

Section II, and VI; Rice plots were not inoculated with *Anabaena cylindrica* but supplied with chemical N fertilizers.

Section III, and VII; Rice plots were both inoculated with *Anabaena cylindrica* and supplied with chemical N fertilizers.

Section IV, and VIII; Check, neither the algae nor the chemical N fertilizers were added to the rice plots.

Another variety of rice seedlings (*Oryza sativa* var. *indica* Chia Hsin 11) was used and the previous experiment was repeated.

The 1000 ml of cultured algae, which had an average N fixing activity of 1.65 mg N_2 fixed/day, was removed from the stock culture and inoculated into the rice plots in each section. The first inoculation was started one week after transplanting. The second and third were carried out on the 30th and 60th day after being transplanted, respectively.

(b) Pot experiments: The experimental designs and varieties of rice seedlings planted in these experiments were similar to those used in the previous field experiments except that these seedlings were planted in pots. In each pot a single seedling was transplanted. There were four treatments as described above as in the field experiments. Each treatment had six replicates. The algal inoculated seedlings in each pot had the average N_2 fixing activity of 0.028 mg N_2 fixed/day. In order to remove those residual chemical N fertilizers left in the soil, those soils used in this experiments were flushed with tape water and drained several times.

Harvest

The mature heads of rice were harvested on the 1st of July. The grains in each panicle were counted and weighed. The number of tillerings of each plant was also recorded.

Results

The quantity of N fixed by *Anabaena cylindrica* is adequate to support the growth and development of the rice plants. As the data on Table 1-1 shows the yields of both cultivars of rice were enhanced by the nitrogen fixing activity of algae. The relative grain yield of both those rice cultivars, Tainan #6 and Chia Hsin #11, increased from 100% to 141% and from 100% to 134% respectively, when grown with blue-green algae as compared with supplied with chemical N fertilizer. This increase in grain productivity was due to the nitrogen fixation of algae which caused the increments in the number of tillers, panicles, and grains of plant. However, the nitrogen fixing activity of algae did not have any positive effect on increasing the grain weight. It has been estimated that plants provided with bio-N fertilizer (product of nitrogen fixation of algae), on the individual plant basis, the number of tillers, panicles, and grains of plant were 28%-57%, 7%-60%, and 43%-74%, greater than those supplied with chemical N fertilizer, depending on the cultivar of rice. However the beneficial effects of nitrogen fixation of algae on the yield of rice was not synchronized with the presence of chemical N fertilizer. On the contrary, the relative productivity of rice grown with blue green algae decreased from 141% to 119% and from 134% to 106% after being supplied with chemical N fertilizer.

The control plants gave the lowest yield because of the deficiency of both bio-and chemical-N fertilizer.

Some contradictory results were obtained from plants cultivated in paddy fields (Table 1-2). Plants grown with blue green algae in the field had a yield of about 20% lower than those supplied with chemical N fertilizer. Apparently, nitrogen fixation of blue green algae did not have any enhance-

Table 1. *The comparative effects of the nitrogen fixation of* ***Anabaena cylindrica****, of supplying chemical N fertilizer, and both on the yields of rice plants*

All of the treatments, CK, A, N, and A+N, were supplied with chemical P (60 kg/acre) and K (60 kg/acre) fertilizers. No chemical N fertilizer was supplied to treatment CK and A, but to treatment N and A+N. Tainan 6 is a cultivar of the *japonica* type, and Chia Hsin 11 is a cultivar of the *indica* type.

Observation	1. Pot experiments(3)								2. Field experiments(3)							
	Tainan 6				Chia Hsin 11				Tainan 6				Chia Hsin 11			
	CK	A	N	A+N	CK	A	N	A+N	CK	A	N	A+N	CK	A	N	A+N
# of plants/section(1)	6	6	6	6	6	6	6	6	58	58	58	58	64	63	64	64
# of tillers/plant	20	23	18	20	23	33	21	32	20	18	22	26	25	22	25	20
# of panicles/plant	11	15	14	13	16	24	15	24	16	20	21	26	22	20	22	21
# of grains/plant(2)	790	1,182	892	937	624	1,145	658	986	729	673	804	760	760	796	983	751
# of grains/panicle	72	79	59	72	39	48	44	41	45	34	38	29	44	40	45	36
grain weight mg/grain	22.69	22.15	22.41	23.66	24.86	22.05	28.62	20.29	19.81	20.92	21.86	21.49	19.76	19.47	19.40	26.65
grain weight g/panicle	1.63	1.75	1.32	1.71	0.97	1.05	1.25	0.83	0.88	0.70	0.83	0.63	0.68	0.78	0.87	0.88
grain weight g/plant	17.93	26.18	18.58	22.17	15.51	25.25	18.83	20.01	14.40	14.08	17.58	16.33	15.06	15.52	19.07	18.51
relative grain yield (%)	96	141	100	119	82	134	100	106	81	80	100	93	79	81	100	97

(1) There were six pots in each section, one plant in each pot. In the field experiments, the area of each section was $1.8 \times 3.2\ m^2$.
(2) The empty grains were not counted and recorded.
(3) The abbreviations of the treatments in experiments are:
CK: No algae and no chemical N fretilizers were added as control.
A: *Anabaena cylindrica.* the algal nitrogen fixing activity was 0.028 mg N_2 fixed/day-pot, and 1.65 mg N_2 fixed/day-section.
N: chemical N fertilizer (80 kg N/acre).
A+N: Anabaena cylindrica +chemical N fertilizer.

Table 2. *The effect of fungicides and insecticides on the nitrogen fixing activity and total N content of* ***Anabaena cylindrica***

Treatment (ppm)	Algal d. wt. (g)	Nitrogen fixation			μg total N	% of N in algae on d. wt. basis
		μ mole C_2H_4	μg N_2 fixed	μg N_2 fixed released		
	flask (100 ml)	g. d. wt.[1]-30 min	g. d. wt.[1]-30 min	flask (100 ml)	g. d. wt.[1]%	
Benlate						
0	0.0599	44.45	414.73	1.57	7954	13.28
10	0.0428	22.97	214.30	0.96	3321	7.76
20	0.0282	11.28	105.21	0.62	0998	3.54
40	0.0249	11.57	107.99	0.52	0896	3.60
80	0.0224	10.72	100.01	0.51	0676	3.02
Endrin						
0	0.0509	44.13	411.70	1.50	6994	13.74
10	0.0387	15.34	143.12	0.79	1118	2.89
20	0.0284	8.96	83.63	0.66	0812	2.86
40	0.0277	8.95	83.65	0.53	0781	2.82
80	0.0230	8.51	79,39	0.51	0357	1.55
Sevin						
0	0.0518	44.68	416.90	1.60	7122	13.75
10	0.0296	5.16	48.12	0.46	0441	1.49
20	0.0348	8.04	74.12	0.60	0911	2.62
40	0.0386	17.07	159.29	1.53	1860	4.82
80	0.0420	19.00	177.24	1.60	2259	5.38
Asozin						
0	0.0501	44.02	410.71	1.46	6988	13.95
20	0.0392	21.64	201.87	0.92	2544	6.49
40	0.0357	21.04	196.33	0.80	2191	6.14
80	0.0128	3.15	29.39	0.11	0117	0.92
160	0.0107	3.02	28.15	0.03	0093	0.87
Lannate						
0	0.0532	43.02	401.35	1.58	7277	13.68
20	0.0327	31.14	290.50	0.76	3230	9.88
40	0.0244	6.09	56.85	0.33	0478	1.96
80	0.0304	12.53	116.89	1.02	1237	4.07
160	0.0320	15.88	148.11	1.46	1696	5.30

[1] g. d. wt.=gram dry weight.

ment effect on the productivity of rice plants grown in the field. This may have been because of the large amount of the residues of the chemical N fertilizer in the paddy fields which had an inhibitory effect on the nitrogen fixing ability of the blue green algae.

In Taiwan, fungicides and insecticides are commonly used by farmers wherever economic plants are cultivated. Both fungicides and insecticides have been found to have a certain inhibitory effect on the nitrogen activity of the blue green algae (Table 2, Fig. 1, and 2). However, algae showed different sensitivities to the treatment of the fungicides or insecticides, and

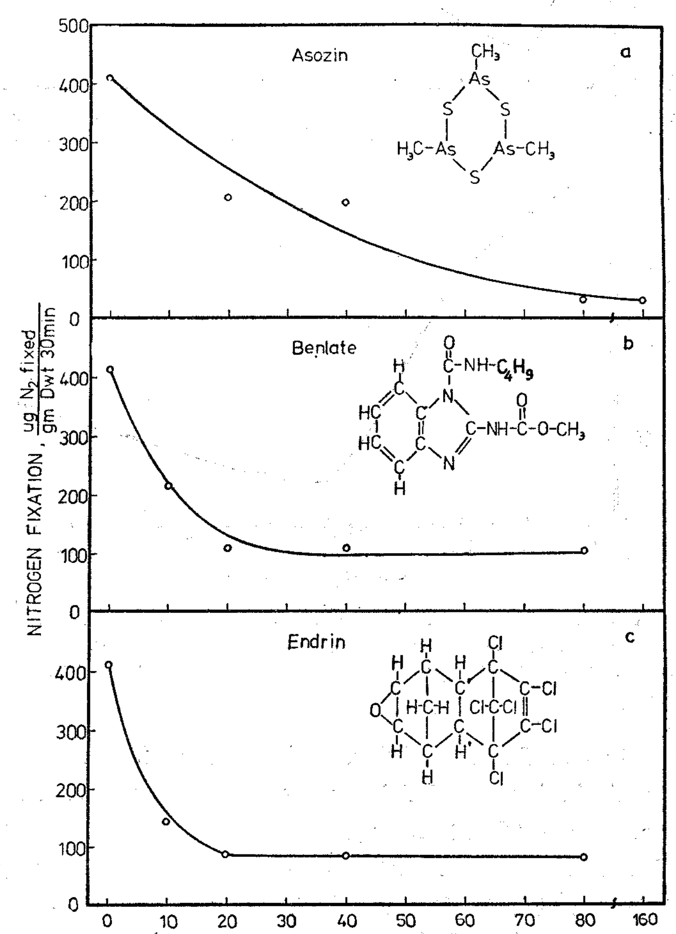

Fig. 1. Effect of fungicide and insecticide of the nitrogen fixing activity of ***Anabaena cylindrica***, Asozin, methylarsine sulfied, and Endrin, 1, 2, 3, 4, 10, 10-hexa chloro-6,7-epoxy 1, 4, 4a, 5, 6, 7, 8, 8a-octahydro-1, 4-endo-5, 8-dimethanonaphthalene, are insecticides. Benlate, 1-(butylcarbamonyl)-2-benzimidazole carbamic acid, methyl ester, is one of the fungicides. The chemical was added directly to the algal culture (100 ml) to make a desired concentration as indicated in the figure,

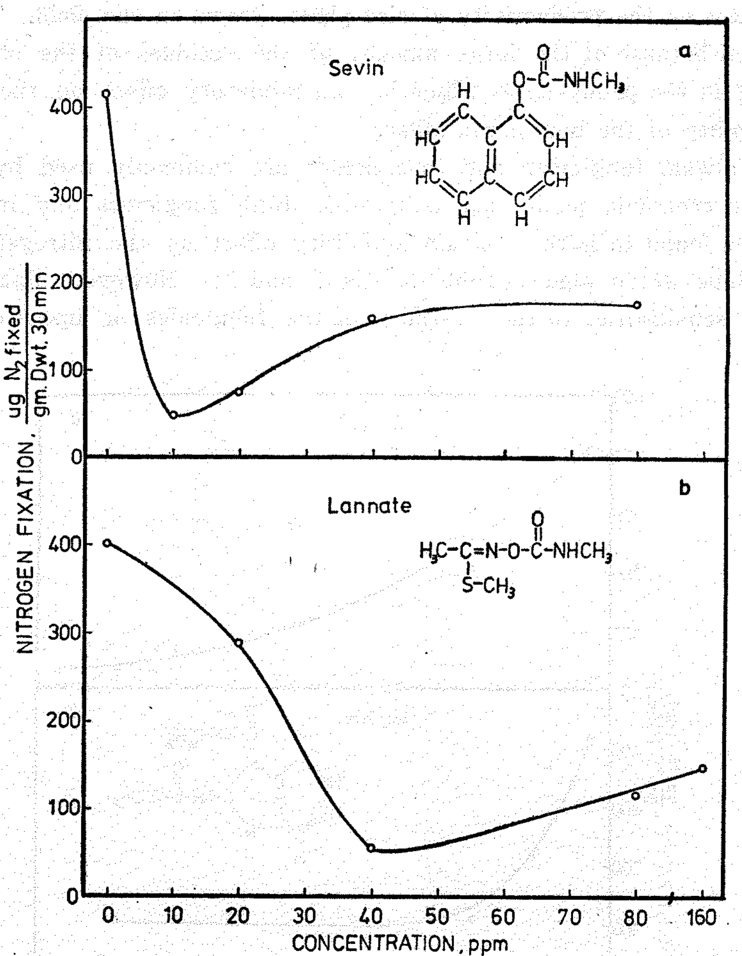

Fig. 2. Effect of insecticide on the nitrogen fixing activity of *Anabaena cylindrica.* Sevin, 1-naphthy-N-methylcarbamate, and Lannate, s-methyl-N-[(methylcarbamonyl)-oxy]-thio-acetimidate, are the insecticides having thioacetimide group, $CONHCH_3$.

also to their nitrogen fixing activity. There were 3 patterns of nitrogen fixing activity of *Anabaena cylindrica* in response to the treatments of the fungicide or insecticide (Fig. 1 and 2).

Asozin type The nitrogen fixing activity of the algae decreased gradually as the concentration of Asozin increased. At the concentration of 20–40 ppm, the nitrogen fixing activity of algae decreased to 50% of the original activity. As the concentration increased further to 80–100 ppm, there was less thar 1% of the original activity of the nitrogen fixing left [Fig. 1-(a)].

Benlate-Endrin type The nitrogen fixing activity of the algae sharply diminished to one-fourth of the original activity by the treatment of Benlate or Endrin at the concentration of 20 ppm. However, the activity did not decrease further when the concentration of Benlate or Endrin were increased from 20 to 80 ppm (Fig. 1–b and –c). At the same concentration of 80 ppm algae treated with Asozin, Benlate or Endrin showed that the latter two chemicals caused the algae to lose about three-fourth of their nitrgen fixing activity, but Asozin caused an inhibition of more than 94% of its activity (Table 2 and Fig. 1). The algae were more tolerant to treatments of Benlate or Endrin than to Asozin.

Sevin-Lannate type The algae treated with Sevin or Lannate obviously had different patterns of nitrogen fixing activity than from those treated with Asozin, Benlate, or Endrin. Sevin or Lannate could inhibit about 90% of the nitrogen fixing activity at a concentration of 10 ppm or 40 ppm, respectively (Table 2 and Fig. 2). Sevin gave a more detrimental effect on the nitrogen fixing activity of algae than Lannate did. Although both Sevin and Lannate had inhibitory effects on the activity of the algae, yet unlike those treated with Asozin, Benlate or Endrin, they showed about 40% of recovery even when Sevin or Lannate concentration was increased from 10 ppm to 80 ppm or 40 ppm to 160 ppm, respectively. However, algae treated with Asozin, Benlate, or Endrin did not recover after treatments.

Table 2 shows that both the total N of nitrogenous compounds and the dry weight of algae decreased and then recovered in a similar way corresponding to the differential sensitivities of the nitrogen fixing activity to the treatments of the fungicide or insecticide.

Discussion

By estimation, there were 101.10 kg of N fixed per acre based on nitrogen fixing activity of blue green algae in stock culture, 1.6 mg per day, 16 hours in a day, the activity was measured just before the algae were inoculated into the rice plots or pots, by *Anabaena cylindrica* during the growth period of the rice plants in paddy field or in pots. This quantity of bio-N fertilizer is greater than the amount of chemical N fertilizer supplied to the rice in paddy field or pots, consequently, the yield of both cultivars of rice were 34%–41% higher than those supplied with chemical N fertilizer in the pots. However, the quantity of N fixed by algae in this study may have been overestimated because the weather conditions outside the room were not so fine or as constant as that in the controlled environment room, so the nitrogen fixing activity of algae in paddy field or pots may not have been so vigorous as that in the room. Besides, the algae themselves must consume a part of N fixed to maintain their own growth and development. In addition to the large quantity of N enriched by blue green alge in pots, there were three other possibiles which might have increased the production of rice in the pots;

Oxygen evolved in water by photosynthetic activity of the algae provide an aerobic condition for the growth of the root system of rice plants. It has been noted that, under the aerobic coditions, the root system produces more numerous root hairs, and grows more intensively and extensively, so that the root systems proportionally increased their absorptive surface and consequently the growth and development of the plants are greatly improved (Kramer, 1969).

The dead algae embedded in rice pots may become humus and so the physical properties of the soils will be improved, and the growth of the root system would thus be promoted, therefore, the growth and development of the rice plants were augmented.

Photosynthetic activity of blue green algae could be enhanced by the CO_2 released by the respiratory activity of root systems or rice plants. It has been well demonstrated that the nitrogen fixing ability of plants is paralleled by their photosynthetic activity (Huang *et al.*, 1975). Therefore, there would be much more N_2 fixed by algae growing in association with rice plants than by algae growing in stock culture.

From the above observations, it is easy to account for the rice plants growing with blue green algae producing a greater number of tillers, panicles, grains, and so a greater yield of grain (Table 1–1).

However, rice plants cultivated in the field plots (field experiments), inoculated with blue green algae, did not have the beneficial effects of nitrogen fixation that those did that were cultivated in the pots with blue green algae. Their yields were lowered. Table 1–2 shows that in the field the rice plants cultivated in controlled sections and in algal inoculated section had the same yield. This indicated that there was little or no of algal nitrogen fixation in these sections by the blue green algae. This may have been because the nitrogen fixing activity of blue green algae was inhibited by the residues of chemical N fertilizers, which were inherited from the utilization of chemical N fertilizers to previous crops, in paddy soil.

Many reports have shown that nitrogen fixation ability of all N_2 fixers is sensitive to the presence of chemical N fertilizers, such as nitrate salts or ammoninum salts, either in a cultural medium or in the soil, because the nitrate or ammonium can block the process of RNA transcription in the steps of enzyme synthesis, consequently, the synthesis of nitrogen fixing enzyme nitrogenase, is repressed (Daesch and Mortenson 1972; Davis *et al.*, 1972; Streicher and Valentine 1973). In this case these would contain little or no bio-N fertilizer in the field plots inoculated with blue green algae, therefore, unless the plants were supplied with exogenous chemical N fertilizer, the plants would be N starved. Although the external appearence of the rice plants in these sections did not show any visual sympton of N deficiency, yet their vegetative or reproductive growth may have been retarded. It has been known that extra amounts of N fertilizer are needed by rice plants during the tillering and panicle formation periods (personal communication with Dr. Chi-Tzu Wang).

Besides the possibility of inhibitory effect of the residual chemical N fertilizers, the fungicides and insecticides which are commonly used on rice plants by farmers is one of the restricting factors to the practical application of bio-N fertilizer by blue green algae to replace the chemical N fertilizer to rice plants. Although these fungicides and insecticides did not kill the blue green algae, at least in the range of concentrations used in these experiments, these used on rice plants have the side effect of suppressing the nitrogen fixing activity of algae (Fig. 1, 2, and Table 2). However, blue green algae treated with Sevin or Lannate, unlike those treated with Asozin, Benlate, or Endrin, could regain about 40% of their initial nitrogen fixation activity (Table 2, and Fig. 2). It is supposed that by the treatment with Sevin or Lannate, the algae were able to induce adaptative enzymes to decompose these two chemicals, consequently, the toxic effect of the chemicales was eliminated and the nitrogen fixing activity of the algae regained. However, the after-effects of chemical toxicity prevented the nitrogen fixing activity from returning to their original levels. Therefore, it may be concluded that although the blue green algae have a very good potential of producing the bio-N fertilizer, this potential is annulled by uses of chemical N fertilizers, and fungicides or insecticides, on the rice plants in this country.

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藍綠藻的固氮作用對水稻產量的影響

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本實驗對藍綠藻在水稻田裏及在盆栽水稻的盆裏的固氮作用效率作一比較。在缺乏氮素的土壤中(盆栽實驗)，藍綠藻的固氮作用可使水稻稻谷產量比施用化學含氮肥料者增加34%(嘉興11號)或41%(臺南6號)。此稻谷產量的增加是由於藍綠藻固氮作用的產物，生物氮肥，使每株水稻的分蘗數、穗數、和穀粒數增加的結果。然而，在田間實驗中，藍綠藻的固氮作用却並未導致稻谷產量的增加。上述兩種水稻品種在田間裏其生產量並不因爲有藍綠藻的存在而加强，相反的其產量與對照組的產量幾乎相等，而都比供給化學含氮肥料的產量低20%左右。這顯示藍綠藻在水田中沒有固氮作用或是僅有微乎其微的固氮作用。這也許因爲前作殘留在土壤中的化學氮肥抑制了藍綠藻的固氮作用。此外藍綠藻的固氮功能也受殺蟲劑或殺菌劑的抑制。惟藍綠藻對不同種類的殺菌劑或殺蟲劑的反應互不相同。經治蟲寶和萬靈處理的藍綠藻，其被抑制的固氮作用能够再恢復一部分。