EFFECTS OF AMITROL ON *CYANOBACTERIUM* *NOSTOC LINCKIA*

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The effect of 3-amino-1,2,4-triazole (amitrol), an uncoupler of the Q of photosystem II, on growth, heterocyst frequency, H₂ production, acetylene reduction (nitrogenase activity) and photosynthetic O₂ evolution was studied in *Nostoc linckia*. Whereas amitrol inhibited growth, acetylene reduction and O₂ evolution, it increased heterocyst frequency and H₂ production. Exposure of *Nostoc linckia* to higher concentrations of amitrol caused pronounced fragmentation of filaments into 5- to 10-celled trichomes. The observed effects on growth and other physiological processes may be due to the reduction in reductant and ATP pool(s).

In recent years, the heterocyst of filamentous nitrogen-fixing cyanobacteria has become an attractive model system for studying the process of dinitrogen fixation (1). Heterocystous cyanobacteria can evolve hydrogen in a nitrogenase catalysed, ATP-dependent reduction of protons. Interest in the H₂ metabolism of cyanobacteria has increased recently because of the relationship of hydrogen metabolism to nitrogen fixation and biological solar energy conversion. Like many other herbicides, the effects of amitrol on plants are varied and many of them are not clear. Amitrol, a histidine analogue, an uncoupler of Q and an inhibitor of imidazole phosphatase dehydrase (2,3) affects the growth and riboflavin (4) and the chlorophyll of higher plants (5). FREDRICK (2) has studied the effect on phosphorylases of different algae and KUMAR (6) the pigmentation of *Anacystis nidulans*. Perusal of literature showed that there has been no previous report on the effect of amitrol on nitrogen fixation, H₂ production and O₂ evolution by cyanobacteria. Here I report the effect of amitrol on the cyanobacterium *Nostoc linckia*.

MATERIALS AND METHODS

The test organism, *Nostoc linckia*(Roth) Born. et Flah., was isolated from

Table 1. Effect of amitrol concentrations on heterocyst frequency, acetylene reduction, aerobic photo-production of H_2 , and photosynthetic O_2 evolution in *Nostoc linckia*. Growth and measuring conditions were as described in the text. Heterocyst frequency was determined after 72 hr of incubation. The measuring light intensity was $240 \mu E m^{-2} sec^{-1}$. Activities are means of three replicates.

Supple- mentation (μM)	Heterocyst frequency (%)	Acetylene reducing activity ($\mu mol C_2H_4 mg dw^{-1} hr^{-1}$)	Hydrogen production ($\mu l H_2 mg dw^{-1} hr^{-1}$)	Photosynthetic O_2 evolution ($\mu l O_2 mg dw^{-1} hr^{-1}$)
None	5.6	1.0	18.5	200
5	5.8	1.0	19.0	170
10	7.5	0.9	19.5	130
20	8.8	0.6	22.0	70
50	10.3	0.4	21.5	None
100	fragmentation and lysis	0.3	21.4	None

paddy fields of Banaras Hindu University, Varanasi, India and was grown axenically in ALLEN's and ARNON's (7) medium adjusted to pH 7.5 after autoclaving. Other growth conditions were the same as described previously (8). The cultures were illuminated with cool-white fluorescent tube lights at $240 \mu E m^{-2} s^{-1}$ for 16 hr daily. Growth experiments were carried out in culture tubes by measuring the absorbance in a BAUSCH and LOMB Spectronic-20 colorimeter at 660 nm. Heterocyst frequency was determined as a percentage of total cells by counting 1,000–1,200 cells. Acetylene reduction was measured as described earlier (9) by the method of STEWART et al. (10). Ethylene was measured by injecting 0.2 ml of the gas phase into a CIS gas chromatograph (Baroda, India), fitted with a porapak R column and hydrogen flame ionization detector. N_2 at $40 ml min^{-1}$ was used as a carrier gas.

Hydrogen production was determined by injecting 0.2 ml of the gas phase from 7.0 ml vials into a model 8,500 gas chromatograph (Earle Inst, Inc., Anaheim, CA, USA.). N_2 was the carrier gas at $15 ml min^{-1}$. The H_2 content was calculated from the recorder signal by reference to the calibration curve.

Oxygen evolution was monitored with a Clark-type platinum electrode; The data were expressed as μl of O_2 evolved $mg dry weight^{-1} hr^{-1}$. The electrode was incorporated into a 1.8 ml water-jacketed electrode chamber. The measuring temperature was $26^\circ C$. The contents of the chamber was mixed with a small magnetic stirring bar. Other conditions were the same as described by ZHANG et al. (11). Dry weight was measured as performed by BOTTOMLEY and VAN BAALEN (12). All biochemicals used were of reagent grade. Amitrol was purchased from Sigma Chem. Co., St. Louis, Mo, USA. Ethylene was from Matheson Gas Co., Lyndhurst, USA. Other gases were from Indian Oxygen Ltd., Bombay, India.

RESULTS AND DISCUSSION

Figure 1 shows that the addition of amitrol at a concentration as low as $10\ \mu\text{M}$ results in inhibition of the algal growth. Growth was restricted at a concentration of $100\ \mu\text{M}$. There was a linear relationship between the growth inhibition and amitrol concentrations over the range of 10 to $100\ \mu\text{M}$ ($r = -0.9654$, $p > 0.01$). Amitrol significantly altered the lag phase of growth from 2.0 to 6.0 days and the generation time from 54 hr to 80 hr at $50\ \mu\text{M}$. At $100\ \mu\text{M}$, growth was not resumed even 15 days after the treatment ended. Percent survival was determined from the absorbance of the cultures of the growth experiment after 15 days of inoculation. Here only percent survival is plotted in Fig. 1. Cell lysis was determined microscopically after 24 hr of incubation in the presence of $100\ \mu\text{M}$ of amitrol. About 80% of the vegetative cells of *N. linckia* was lysed by amitrol. I have not observed any lysed heterocysts in the culture. The filaments were fragmented into 5- to 10-celled trichomes after 15 hr of incubation with $100\ \mu\text{M}$ of amitrol. Amitrol-treated filaments were narrower as compared to the control. This thinning of the filaments is probably related to the growth inhibition.

The heterocyst frequency of *Nostoc linckia* was significantly increased from 5.6% in the control to 10.3% in $50\ \mu\text{M}$ amitrol (Table 1). Higher concentrations of amitrol caused fragmentation of the filaments. The increase in heterocyst frequency was related to the partial nitrogen-starvation due to inhibition of nitrogenase (acetylene reduction) by amitrol-supplementation.

Amitrol-supplementation caused a drastic decrease in the acetylene reducing activity of *N. linckia*. Amitrol as low as $10\ \mu\text{M}$ inhibited slightly but higher concentrations reduced the activity to the level of about 70% (Table 1). However, amitrol-supplementation increased the nitrogenase catalysed H_2 production by about 30% at $20\ \mu\text{M}$ but this increase in production was the same even at higher

Fig. 1. Percent survival of *Nostoc linckia* at different concentrations of amitrol. Growth was measured after 15 days of incubation of *N. linckia* with different concentrations of amitrol. Percent survival was calculated by keeping control as 100%.

Table 2. Effect of pretreatment of MSX (20 μ M) on acetylene reduction and H₂ production by *Nostoc linckia*. All conditions were as described in the text. After pretreatment, the cultures were suspended in the fresh medium for the measurement of activities.

Time (hr)	Acetylene reducing activity (μ mol C ₂ H ₄ mg dw ⁻¹ hr ⁻¹)	H ₂ production (μ l H ₂ mg dw ⁻¹ hr ⁻¹)
None	1.0	18.5
24	1.0	18.4
48	0.95	18.3
72	0.94	18.4

concentrations of amitrol (Table 1). Hydrogen production in *N. linckia* was strictly nitrogenase catalyzed since there was no H₂ production in cultures supplemented with 5 mM nitrate. The increase in H₂ production by amitrol was probably related to the reduced energy state of the cells. This favours high electron allocation to H₂ over N₂ (C₂H₂ reduction, 13). Like DCMU, amitrol at 50 μ M completely inhibited photosynthetic O₂ evolution in *N. linckia* (Table 1).

L-methionine-DL-sulfoximine (MSX) was used to see if there is any effect of nitrogen-starvation on the H₂ production. Because it is known that under argon-CO₂ incubation (nitrogen-starvation), H₂ production is greatly increased, but not under N₂-CO₂ incubation (11). Amitrol also caused partial nitrogen-starvation (inhibition in nitrogenase activity). So it was desirable to use MSX as another means of bringing about nitrogenstarvation. Air-grown, nitrogen-fixing cultures were treated with 20 μ M of MSX for 24 to 72 hr and then the activities were determined. There was almost no effect of MSX on H₂ production and acetylene reduction (Table 2).

In conclusion, my results show that H₂ production in *N. linckia* is the nitrogenase catalysed, ATP-dependent reduction of protons. This assumption is based on the fact that there was no nitrogenase or H₂ production activities in the nitrate-supplemented (heterocyst free) cultures. Amitrol inhibited growth and other physiological processes except heterocyst frequency and H₂ production. This increase in heterocyst frequency was not associated with nitrogenase activity (Table 1). The inhibition of acetylene reduction may be related to the inhibition in reductant pool(s). The sensitivity of photosynthetic O₂ evolution to amitrol demonstrates the effect of amitrol on PS II as reported for DCMU in *Anabaena* sp. strain 7120 (14).

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