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Suppression of the nitrogen fixing activity of cyanobacterium *Anabaena* HA101 by ethylene

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Abstract. Ethylene at concentrations 0.1, 0.2, and 0.3 μl per 25 ml of gas phase in incubation bottle was used to treat *Anabaena* HA101 for 24 hours and six days. Ethylene reduced the chlorophyll concentration, heterocyst frequency, hydrogen evolution and nitrogen fixation rate. Ethylene also enhanced the degeneration of existing heterocysts. The inhibitory effect of the highest ethylene concentration during 12 hours on nitrogen fixation could be overcome within 12 hours by the removal of the ethylene. However, after the long-term (6 days) treatment of *Anabaena* with ethylene, the algae appeared yellowish, and no recovery of nitrogenase activity was found within 24 hours.

Key words: *Anabaena*; Ethylene; Heterocyst; Nitrogenase.

Introduction

The gaseous hormone ethylene inhibits cell division and DNA synthesis in *Pisum sativum* (Apelbaum and Burg, 1972), regulates gene expression in carrot root (Christoffersen and Laties, 1982; Nichols and Laties, 1985), affects enzyme activity in pea seedlings (Apelbaum *et al.*, 1985), retards growth of mungbean (Colclasure and Yopp, 1976) and rice seedlings (Ishizawa and Esashi, 1983; Satler and Keide, 1985). It also inhibits photosynthesis (Kays and Pallas, 1980; Pallas and Kays, 1982), but enhances respiration (Burg, 1968; Pratt and Goesch, 1969; Aharonic and Lieberman, 1979), fruit-ripening (Tucker and Grierson, 1982; Pressey, 1983), and senescence (Abeles, 1973; Salisbury and Ross, 1985). In 1971, Grobbelaar *et al.*, found that ethylene at concentrations of 0.4 ppm and above extensively inhibits the nodulation of bean plants, and so, decreases the activity of nitrogen fixation. Drennan and Norton (1973) demonstrated that application of 2 ppm ethrel, which releases ethylene, to roots of pea plants inhibits root growth and so inhibits nodulation. In 1978, David *et al.*, suggested that ethylene might alter the steric conformation of the nitrogen fixing enzyme systems of the cyanobacterium *Anabaena cylindrica* and increase the transfer of electrons to reducible substrates.

The acetylene-ethylene assay is a widely-used technique to determine the nitrogen-fixing activity of diazotrophs (Masterson and Murphy, 1980). Ethylene is a product of acetylene reduction catalysed by nitrogenase, but little is known about the effects of ethylene on the nitrogenase activity of cyanobacteria. In this study, attempts were made to study the effect of ethylene on the nitrogen-fixing activity of the filamentous blue-green alga, *Anabaena* HA101, growing under laboratory conditions.

Materials and Methods

Culture of ***Anabaena*** *HA101*

A pure strain of *Anabaena* HA101 was cultured on the agar plates containing SM medium (Huang and Wu, 1982). The *Anabaena* stock culture was grown under the following conditions: light intensity 200 lux; photoperiod 16 hrs; day/night temperature 28/24°C. For the experiments, aliquots of the culture were evenly distributed into 500 ml flasks. Each flask contained

300 ml of SM medium that was free from combined nitrogen. The *Anabaena* cultures were then kept under the following conditions: light intensity 6000 lux; photoperiod 16 hrs; day/night temperature 30/24°C and aerated continuously with air at a rate of 250 ml per min. The organism was harvested for measurements or treatment when the optical density of the *Anabaena* culture at 660 nm was about 0.6.

The filaments in 300 ml of *Anabaena* culture were harvested by the centrifugation at 500 g for 10 min and resuspended in 10 ml of fresh medium. One ml of concentrated *Anabaena* suspension was placed in each of 24 serum bottle of 26 ml capacity. The serum bottles were divided into eight groups (a,b,c,d,e,f,g,h). Each group of bottles was placed in a 5 litre vacuum desiccator. Group a was not treated with ethylene but rest of groups were incubated with ethylene at concentrations of 0.1, 0.2 and 0.3 μl per 25 ml gas phase in bottle respectively, for 24 hours (b,c,d) or six days (e,f,g,h). After a treatment, the ethylene in a desiccator was removed under hypobaric condition. The gas phase in the desiccator was evacuated continuously at about 250 ml per minute with a vented exhaust oil pump for 20 minutes. The pressure within the desiccator was then brought to normal atmospheric condition by admitting fresh air to the desiccator. The *Anabaena* samples in the serum bottles were then removed from the desiccator, sealed with serum stoppers and were ready for the measurements.

Determination of Nitrogen Fixation

The nitrogen fixing activity of the *Anabaena* samples was determined by the acetylene reduction technique (Masterson and Murphy, 1980). Acetylene was injected into the serum bottle to give a partial pressure of 0.1 atm. For each measurement a 0.05 ml gas sample was used. The gas samples were withdrawn at 30 minute or 4 hour intervals by hypodermic syringe, and analysed immediately for ethylene by measns of a gas chromatograph equipped with a hydrogen-flame ionization detector and a glass column 0.8 m long and an i. d. of 3 mm packed with Porapak Q. The flow rate of carrier gas, nitrogen, was 60 ml per minute. The oven temperature was 60°C.

Determination of Hydrogen Production

Hydrogen production was measured by gas chromatograph using a thermal conductivity detector. The stainless steel column, 3 mm i.d., 2 m long, was packed with a molecular sieve 5 A (80/100). The operation conditions were: detector current 60 mamp; flow rate of carrier gas, argon, 50 ml per minute; column and injection port temperatures 65 and 100°C respectively. The hydrogen concentrations were calculated as described previously (Huang, 1988).

*Determination of Chlorophyll **a** Concentration*

Three hundred ml of *Anabaena* culture was centrifuged and the chlorophyll of the sedimented material was extracted with 10 ml of 80% acetone. The chlorophyll concentration was determined spectrophotometrically by the method described by Vernon (1960).

Induction of Heterocyst Formation and Degeneration

Samples of the concentrated *Anabaena* culture were re-cultured in SM medium containing 5 mM ammonium nitrate. This combined nitrogen inhibits the differentiation of vegetative cells into heterocysts (Huang, 1988). Non-heterocystous filaments were obtained about eight days after the treatment was initiated. Aliquots of the heterocyst-free *Anabaena* cultures were harvested and washed twice with fresh ammonium nitrate-free SM medium. The cyanobacteria were then re-cultured in 300 ml fresh ammonium nitrate-free SM medium in 500 ml flasks. After six days when the heterocyst frequency reached its maximum, the nitrogen fixing activity of the cultures was measured. Samples of the heterocystous *Anabaena* cultures were the treated with ethylene at a concentration of 0.3 μl per 25 ml gas phase in incubation bottle, and after the six days' treatment, the degenerative effect of ethylene on the existing heterocysts was determined. The heterocyst frequencies were measured using a light microscope at a magnification of 100 X.

Recovery of Nitrogen Fixing Activity

The heterocystous *Anabaena* samples in unstoppered serum bottles were distributed into two desiccators a and b. There were nine bottles in each desiccator. Samples in desiccator a, and b were incubated with ethylene at 0.3 μl per bottle of 25 ml gas phase. After 12 hours the bottles were removed from desiccator a. After sealing the bottles, the rate at which the enclosed cultures reduced acetylene was determined. Meanwhile the gas phase of desiccator b was

replaced with ethylene-free fresh air. Twelve hours late, the cultures were used to determine their nitrogenase activities.

Results

The gaseous hormone, ethylene, inhibited the nitrogen fixing and hydrogen evolving activities of the cyanobacterium *Anabaena* HA101 (Fig. 1). The nitrogen fixation and hydrogen evolution activities decreased from 100% [specific activity 24.00 μmol C_2H_4 (mg chlorophyll)$^{-1}$ hr^{-1}, and 6.91 μmol H_2 evolved (mg chlorophyll)$^{-1}$ hr^{-1} respectively] to 90.4% and 88.5% respectively, during the 24 hours that the *Anabaena* was exposed to a concentration of 0.3 μl per 25 ml gas phase in incubation bottle. The higher the ethylene concentration used. The greater the depression of the observed nitrogenase activity (Figs. 1 and 2).

The levels of nitrogen fixing and hydrogen evolving activities of nitrogenase were changed with the length of exposure of the cyanobacterium to ethylene. The deleterious effect of the 0.3 μl ethylene per 25 ml gas phase on the enzyme activities increased drastically after eight hours of treatment. On the other hand, the 0.1 μl ethylene per 25 ml gas phase treatment reduced the nitrogenase activities gradually and at a more or less steady rate throughout the experiment (Fig. 2).

The chlorophyll concentration, dry mass and the heterocyst frequency were also reduced by ethylene. The chlorophyll concentration decreased by 6.5% within 24-hours (Fig. 3), the dry mass by 85% (Table 1) and the heterocyst frequency by about 82% (Table 2) during six days of treatment with a concentration of 0.3 μl per 25 ml gas phase in incubation bottle. The remaining 18% of the original heterocysts did however not exhibit any nitrogen fixing activity at the end of the experiment. (Table 2). The heterocysts of *Anabaena* culture that grown under the normal condition started to autolyze or degenerate spontaneously after a maximum number of heterocyst had already formed on the filaments. As the data in Table 2 show the heterocysts were reduced from the maximum frequency of 6.4% to 2.3% within six days. However, The heterocysts were enhanced to autolyze or degenerate from 6.4% to 0.6% by ethylene treatment (Table 2).

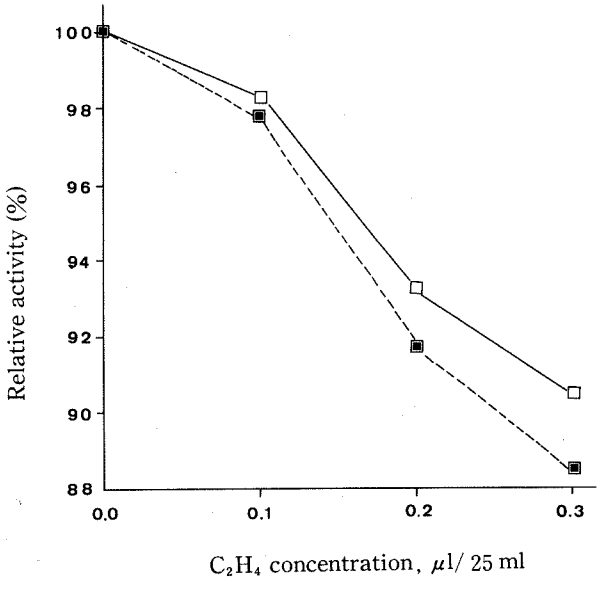

Fig. 1. Effect of exposing *Anabaena* HA101 to different ethylene concentrations for 24 hours on it nitrogen fixing and hydrogen evolving activities. Nitrogen fixation (□ —— □); hydrogen evolution (■·····■).

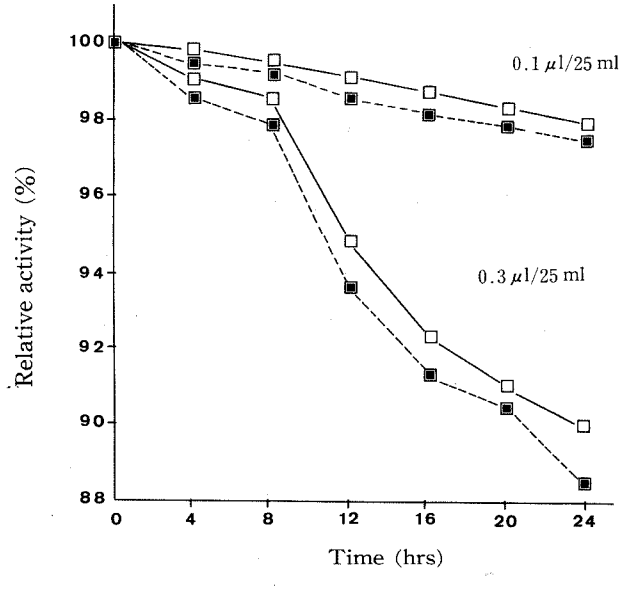

Fig. 2. Effect of the length of exposures of *Anabaena* HA101 to ethylene at concentrations of 0.1 and 0.3 μl per 25 ml gas phase in incubation bottle on the rate of its nitrogen fixation (□ —— □) and hydrogen evolution (■·····■). The activities were measured at an 4-hour interval.

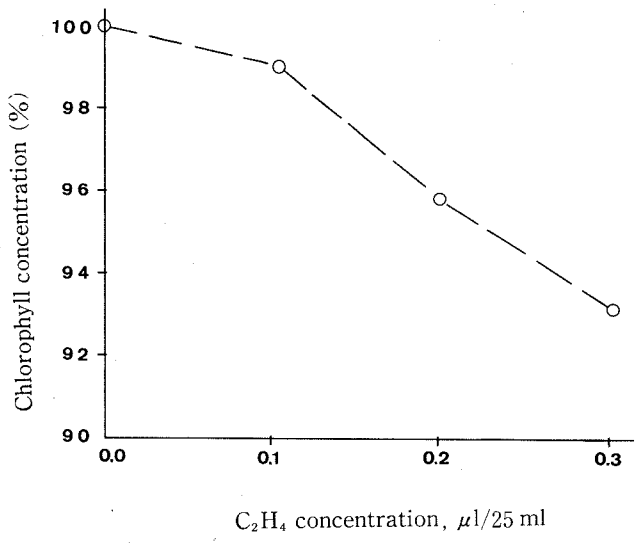

Fig. 3. Reduction of the chlorophyll concentration of *Anabaena* HA101 by exposure to 0.3 μl ethylene per 25 ml gas phase in incubation bottle for 24 hours.

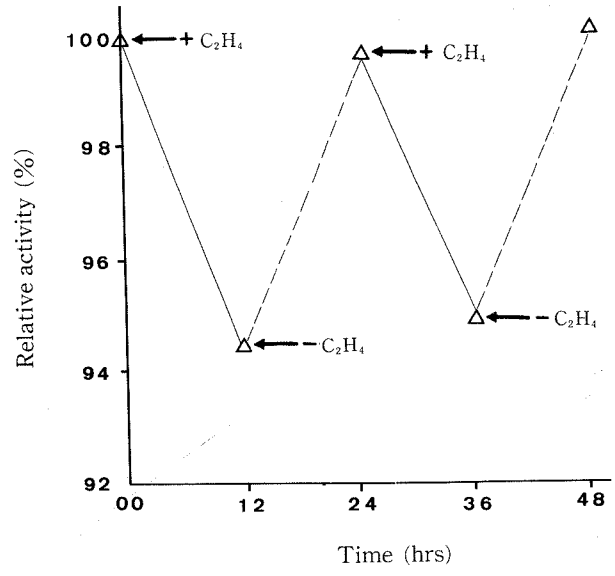

Fig. 4. Reversible inhibition of nitrogenase activity of *Anabaena* HA101 by exposure to 0.3 μl ethylene per 25 ml gas phase in incubation bottle for 12 hours.

Table 1. *Effect of ethylene on the dry mass of* ***Anabaena*** *HA101*

Treatments	dry mass (gm/300 ml)	relative mass (%)
a. Control	0.13	100
b. C_2H_4 (0.3 μl/25 ml)	0.02	15

Each figure is the mean of three values.

The inhibitory effect of ethylene on the nitrogenase activity was reversible. The nitrogen fixation activity which decreased from 100% to 94.3% within 12 hours of ethylene treatment, almost completely regained its original activity within the next 12 hours after the removal of ethylene and these reversible phenomena were repeatable (Fig. 4).

Table 2. *Effects of ethylene on the formation and autolysis of* ***Anabaena*** *HA101 heterocysts*

Treatments	Heterocyst frequency (%) A	Nitrogenase activity [μmol C_2H_4 (mg chl)$^{-1}$hr^{-1}] B	Relative (%) A	Relative (%) B
1. Heterocyst formation				
a. Control	6.4	23.59	100	100
b. +C_2H_4 (0.3 μl/25 ml)	1.1	0	17.2	0
2. Heterocyst autolysis				
a. −C_2H_4	2.3	7.86	100	100
b. +C_2H_4 (0.3 μl/25 ml)	0.6	0.05	26.0	0.64

Discussion

Ethylene inhibited the nitrogenase activity of *Anabaena* HA101 both in nitrogen fixation and hydrogen evolution. The reduction of two nitrogenase activities depended on the ethylene concentration and the duration of the ethylene treatment (Figs. 1 and 2).

The heterocysts of *Anabaena* are the sites of nitrogenase activity in nitrogen fixation and hydrogen evolution (Fay, 1980). The effect of ethylene on heterocyst formation is very similar to its effect on the nodulation of legumes (Grobbelaar *et al.*, 1971). As the data in Table 2 show, ethylene inhibits heterocyst formation. In 1971, Grobbelaar *et al.*, reported that ethylene restricted the infection of legume roots by *Rhizobium* and inhibited the initiation of legume root nodules.

The reduction of the chlorophyll concentration of *Anabaena* filaments was detected within 24 hours of ethylene treatment (Fig. 3). It has been also reported (Burg, 1968; Pratt and Goesch, 1969; Aharonic and Lieberman, 1979) that ethylene enhances chlorophyll degradation in leaf blades. The external appearance of the *Anabaena* filaments changed from greenish to yellowish when the cyanobacterium was exposed to ethylene at a concentration of 0.3 μl per 25 ml gas phase in incubation bottle for six days. Ethylene also reduced the dry mass of the *Anabaena* cultures (Table 1). This might be due to a synergistic effect of ethylene on the reduction of photosynthesis and nitrogen fixation. However, it has been reported that ethylene promotes the respiration of intact leaves or leaf discs (Aharonic and Lieberman, 1979).

The mechanism of the repressive effect of ethylene on nitrogenase activities in nitrogen fixation and hydrogen evolution is still unresolved. It is possible that ethylene exerts it effect on the reduction of chlorophyll, and consequently inhibits heterocyst initiation. It is known that the C/N ratio of blue-green algae regulates the heterocyst initiation and development (Fay, 1980). The heterocyst is the site of nitrogenase synthesis (Fleming and Haselkorn, 1973; Fay, 1980; Huang, 1988; Huang *et al.*, 1988), therefore the levels of heterocyst formation will affect the biosynthesis of nitrogenase and so the activities of the enzyme. In addition, photosynthate is required in the reactions catalysed by nitrogenase in cyanobacteria (Fay, 1980), and soybean plants (Huang *et al.*, 1975). Therefore, it is conceivable that the reduction of chlorophyll concentration by ethylene is the primary factor that limits nitrogenase activities.

Ethylene promoted the autolysis or senescence of existing heterocysts. This may be due to a shortage of a suitable C and N source for the cyanobacterium. However, ethylene may have a direct effect on heterocyst degeneration, because it is known that ethylene regulates many aspects of plant senescence (Abeles, 1973; Salisbury and Ross, 1985).

Over-all, the data presented in this study show that ethylene exerts inhibitory effects on the photosynthesis, heterocyst formation, and nitrogen fixing activity of *Anabaena*. Therefore, the results may suggest that rice plants growing with *Anabaena* in paddy fields may not benefit maximally from the nitrogen fixing activity of the cyanobacteria because it is known that the higher concentration of ethylene are released in un-aerated or flooded soils (Yang and Hoffman, 1984).

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乙烯對藍綠藻 *Anabaena* HA101 固氮作用之抑制現象

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使用三種濃度之乙烯，即 0.1, 0.2, 0.3 μl/25 ml 處理藍綠藻 *Anabaena* HA101 24 小時或是 6 天，乙烯可減少葉綠素含量，異形細胞形成，氫氣的產生及固氮作用，乙烯不僅抑制異形細胞之合成，亦可加速其老化。使用 0.3 μl/25 ml 濃度之乙烯處理藍綠藻 *Anabaena* HA101 12 小時，然後把乙烯去除，被抑制之固氮作用於 12 小時內即可恢復正常。但是處理 6 天，其已被抑制之固氮作用於 24 小時內仍然不能恢復並且該藍綠藻已有黃化之現象。