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Laboratory Culture of the Pelagic Blue-Green Alga *Trichodesmium thiebautii*: Conditions for Unialgal Culture

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ABSTRACT: We investigated conditions for laboratory culture of the pelagic blue-green alga *Trichodesmium thiebautii* and conducted supplementary experiments with *Trichodesmium erythraeum*. *T. thiebautii* grew in the 'f' medium of Guillard and Ryther after careful removal of living contaminants, especially larvae of the copepod *Macrosetella gracilis*, from bundle colonies under the microscope. Spherical colonies were not suitable for the inoculum. *T thiebautii* appears to be auxotrophic, at least in terms of B_{12} requirement, under our culture conditions. *T erythraeum* survived up to 100 d in 'f' medium but failed to grow actively.

INTRODUCTION

Trichodesmium thiebautii and *T. erythraeum* are pelagic blue-green algae, distributed widely in tropical and subtropical seas. Although attention has been paid to the genus *Trichodesmium* as an important nitrogen fertilizer (Dugdale et al., 1961; Dugdale and Goering, 1964; Goering et al., 1966; Taylor et al., 1973; Carpenter and McCarthy, 1975; Saino and Hattori, 1978, 1979), many of their unique characteristics have remained unknown. Two examples: (1) Although *Trichodesmium* is a member of the Oscillatoriaceae and hence does not form heterocysts, it is able to fix atmospheric nitrogen under aerobic conditions as effectively as heterocystous algae (Goering et al., 1966; Taylor et al., 1973). (2) *Trichodesmium* is found mainly in open sea areas very poor in nutrients and even here sometimes forms dense blooms (Goering et al., 1966; Marumo and Asaoka, 1974a; Carpenter and McCarthy, 1975); such oligotrophy is unusual in marine blue-green algae, most of which live in littoral and coastal areas rich in nutrients. In order to investigate these physiological and biochemical peculiarities, stable laboratory cultures are of great importance. However, thus far attempts at laboratory culture of *Trichodesmium* have been rather unsuccessful. Ramamurthy was the first to report successful axenic culture of *T. erythraeum* in modified Erdschreiber medium (Ramamurthy, 1972). However, this medium contained antibiotics (which might cause genetic mutation of wild strains), and it was not possible to repeat his experiment. Carpenter and McCarthy (1975) succeeded in maintaining *T. thiebautii* alive in 'f' medium of Guillard and Ryther for over 100 d. However, they failed to obtain active growth.

As a first step towards a stable, axenic culture of *Trichodesmium*, the conditions for unialgal culture were surveyed with *T. thiebautii* and supplementarily with *T. erythraeum* collected from Kuroshio waters, Japan. Finally we succeeded in establishing conditions for continued unialgal culture of *T. thiebautii*, and in keeping *T. erythraeum* alive for up to 100 d, albeit without active growth.

MATERIALS AND METHODS

Trichodesmium thiebautii and *T. erythraeum* were collected from surface waters about 10 km south of Iroh-Saki, Izu-Peninsula on July 28 and 29, September 14 and October 6, 1980. In July, *T. thiebautii* was dominant, in September and October both *T. thiebautii* and *T. erythraeum* were present in large numbers.

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Samplings were made by hand-net surface tow (20 cm net diameter, 40 μm nylon mesh) of 1 to 2 min duration. The samples were diluted with sea water immediately after each tow. *Trichodesmium* colonies were separated with a Pasteur pipet and re-suspended in the surface water filtrated through Millipore HA filter. Separation was achieved within 2 h after sampling.

Most *Trichodesmium thiebautii* forms colonies, either spherical or bundle. Spherical colonies consisted of aged trichomes and contained numerous microorganisms, such as bacteria, fungi, ciliates and diatoms; hence they were not suitable for starting cultures. Since bundle colonies contained fewer microorganisms and seemed to consist of more active trichomes, we used these as inocula. However, the colonies often contained nauplius or early copepodid stages of *Macrosetella gracilis* which is known to feed on *Trichodesmium* (cf. Björnberg, 1965; Roman, 1978). Therefore, we first carefully removed *M. gracilis* from bundle colonies under a low-power binocular, and washed the colonies several times with Millipore-filtered sea water before inoculation. The "f" medium of Guillard and Ryther (1962), without addition of Na_2SiO_3, was used as basic culture medium: it was diluted 2 to 40 times with Millipore-filtered surface water of the Kuroshio area (34.1 ‰ S). Screw-capped test tubes made of hard glass were used as culture vessels. They were first soaked in dilute HCl, then rinsed well with de-ionized water to eliminate contaminating heavy metals.

For survival tests, 1 to 3 bundle colonies, consisting of 10 to 20 trichomes, were inoculated into 10 ml of sterilized medium. Seven to 20 batches were set up for each condition to be tested, and the number of batches in which cells survived was counted and listed in Tables as a function of incubation period. The cells were incubated at 23 °C ± 2 C° in fluorescent light (day-light type) and/or day light; light intensity ranged from 300 to 3000 lux. Fluorescent light appeared to be inhibitory; hence most experiments were done in day light. The light/dark regime September, 1980 to January, 1981) was 13.9:10.1 (light : dark) to 10.9:13.1. All colonies were transferred into fresh medium every 3 to 5 d in the initial phase of incubation, later every 20 to 30 d. For the growth test, several trichomes (3 to 8) of 150 to 250 cells were inoculated into each batch (10 ml), and the number of trichomes was counted up to the formation of bundle colonies.

RESULTS

Conditions for Long-Term Survival

Trichodesmium cells – both *T. thiebautii* and *T. erythraeum* – tend to die and disintegrate quickly, when incubated under artificial conditions. Hence we first analyzed the prerequisites for survival.

Table 1 shows the effect of nitrogen on survival for *Trichodesmium thiebautii* collected in September. NO_3^- or NH_3 was added to nitrogen-free "f" medium as indicated. Results indicate that (1) *T. thiebautii* can survive for over 100 d without combined nitrogen. (The same holds true for *T. erythraeum*); (2) ammonia is toxic, even at concentrations as low as 30 μM. Ammonia has been known as a nitrogen source assimilated with a very low Km value by this alga (Saino, 1978), and the effect observed here appears to be due to its toxicity generally observable in algae cultured in an alkaline medium.

Vitamin B_{12} is an essential growth factor for many phytoplankters; some of them can utilize not only cyanocobalamin but also its derivatives (Provasoli and Carlucii, 1974). As shown in Table 2, both *Trichodesmium erythraeum* and *T. thiebautii* survived for distinctly longer period in the medium containing hydroxo-cobalamin. Though *T. erythraeum* survived only for 76 d in this experiment, survival for more than 100 d was observed several times in other experiments. *Trichodesmium* is probably auxotrophic: it requires at least B_{12}; the specificity for B_{12} derivatives may be of the *Escherichia coli*- or *Lactobacillus leichmanii*-type (cf. Provasoli and Carlucii, 1974).

Table 1 *Trichodesmium thiebautii*. Effect of nitrogen on survival. Illumination: combined fluorescent and daylight. The culture medium for each batch was renewed every 3 to 5 d during the early period of incubation; later, every 20 to 30 d. f/10 and f/40: 1/10 diluted and 1/40 diluted 'f' medium, respectively

Medium	Number of batches tested	$NaNO_3$ (μM)	NH_4Cl (μM)	Incubation (d) 5	8	12	16	19	25	36	54	83	100
				(% of survival)									
	20	177	0	95	95	80	65	55	45	45	20	10	0
f/10	33	0	0	97	64	58	45	42	36	24	18	3	3
	7	0	30	86	57	43	14	0	0	0	0	0	0
	18	89	0	100	83	80	73	50	22	20	0	0	0
f/40	22	0	0	100	77	73	64	36	27	18	9	5	5
	9	0	30	89	56	44	22	11	0	0	0	0	0

Table 2. *Trichodesmium thiebautii* and *T erythraeum*. Effect of hydroxocobalamin on survival. NO_3-free f/40 medium, daylight. Medium of each batch was renewed every 3 to 5 d during the early period of incubation; later, every 20 to 30 d

Species	Hydroxo-cobalamin ($\mu g\ l^{-1}$)	Number of batches tested	Incubation (d) 2	5	7	12	17	25	35	61	76	81	98	100
			(% of survival)											
T. thiebautii	1.5	10	100	100	100	100	100	90	60	50	50	50	40	10
	0	14	100	86	86	86	86	57	36	14	14	7	7	0
T erythraeum	1.5	12	100	100	92	92	83	75	50	8	8	0	0	0
	0	9	100	89	89	78	33	0	0	0	0	0	0	0

Conditions for Active Growth of *Trichodesmium thiebautii*

A sample collected in October survived in hydroxocobalamin-enriched and NO_3^--free "f" medium of 1/40 strength (Table 2) and began to grow after a rest of 96 d. Once the alga started to grow, active growth was found to be reproducible even under conditions that earlier caused the long rest. The growth patterns obtained in "f" medium of various nutritional strength, with and without NO_3^- or hydroxocobalamin, are presented semiquantitatively in Table 3. When separate trichomes were inoculated, each trichome first increased in length up to 3 mm. Then, it split mechanically into 2 to 3 daughter trichomes. The general pattern was as follows: (1) Increase in trichome number starting after a long lag period (about 10 d), which was not shortened after adding vitamins; elongation of trichomes occurring at the end of this lag period. (2) The number of separate trichomes increased, without colony formation; bundle colonies appeared when the trichome population reached 15 to 20 per 10 ml. Though the size of trichomes in the cultures was variable, average size was almost constant throughout the culture period. Thus increase in trichome number (Table 3) suggests that the μ value under our culture conditions was as high as 0.1 to 0.3 d^{-1} and is not affected by added nitrogen or by added vitamin B_{12}; no significant difference was observable in media of 1/5 to 1/40 strength.

Trichomes were examined microscopically parallel to growth tests. In all cases, trichomes grown in test tubes showed the same morphological characteristics as those in the natural habitat. Average cell width ranged from 8 to 11 μm, and the ratio of cell width to cell height was almost unity or a little less; the tapered form at the end of trichome was also maintained (Fig. 1A *vs.* B, C, D). These characteristics were not altered by nutritional conditions (Fig. 1B *vs.* C). This provides proof that we observed the growth of *Trichodesmium thiebautii*, and not of any other, contaminating blue-green alga.

Table 3. *Trichodesmium thiebautii*. Growth under various nutritional conditions. Daylight illumination. C: some trichomes formed bundle colonies

Medium	$NaNO_3$ (μM)	Hydroxocobalamin ($\mu g\ l^{-1}$)	Incubation (d) 0	3	6	8	11	14	16	18	22	26
			(Number of trichomes)									
f/5	353	0	4	5	5	4	4	8	10	15	20	C
	0	0	3	5	2	6	5	12	15	C	C	C
f/10	177	0	8	8	10	10	10	15	20	20	C	C
	0	0	3	3	4	3	3	7	9	10	15	C
	177	1.5	3	4	7	5	4	7	8	8	15	20
	0	1.5	3	1	6	6	6	13	15	20	C	C
f/40	45	0	3	2	5	3	3	5	5	6	10	10
	0	0	6	5	10	7	5	15	20	C	C	C
	45	1.5	3	1	4	4	2	6	4	6	8	20
	0	1.5	7	5	5	4	5	10	15	C	C	C

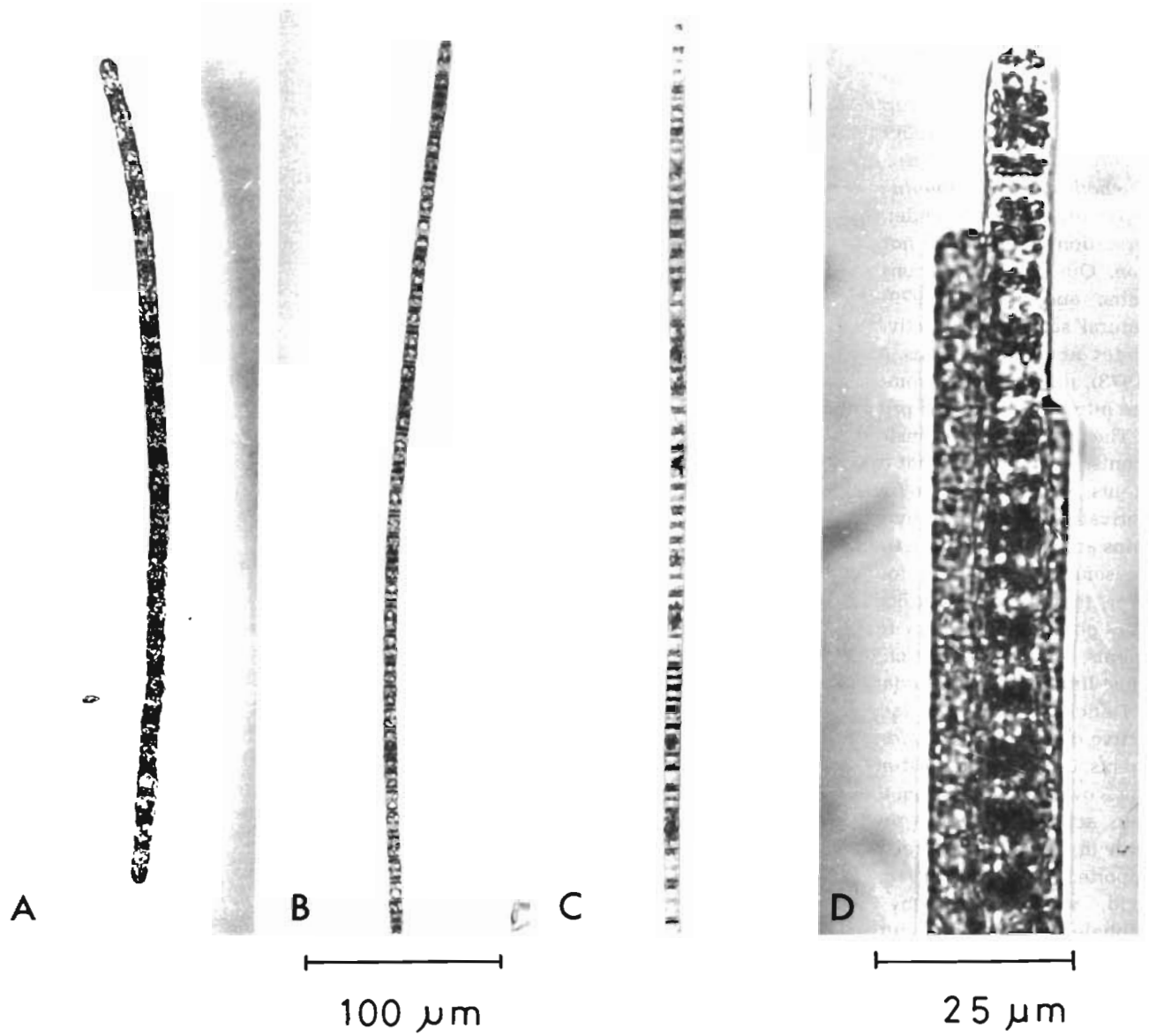

Fig. 1. *Trichodesmium thiebautii*. Microphotographs collected from natural habitats (A) and grown in laboratory culture (B, C and D). A: trichome collected in October, 1980; B: grown in f/40 medium free of NO_3but with hydroxocobalamin (1.5 μg^{-1}); C: grown in f/40 medium containing NO_3(45 µM) and hydroxocobalamin (1.5 µg l^{-1}); D: bundle colony formed in C conditions. Duration: 33 d

DISCUSSION

Of primary importance for the successful cultivation of *Trichodesmium* is the careful elimination of living contaminants from algal colonies to be inoculated. In our cultures, appreciable growth of microbial contaminants did not occur, and medium renewal was not required as often as suggested by Ramamurthy (1972). When the colonies or trichomes were carefully washed with sterilised sea water under the microscope, both *T. thiebautii* and *T. erythraeum* survived at least for 80 to 90 d in the same medium. The rapid disintegration occurring under artificial conditions is probably mainly due to living contaminants, especially the grazing of *Macrosetella gracilis*. Of secondary importance seems to be the protection from excessive heavy metals. Although we can use ordinary glassware for the culture vessels, washing with dilute HCl seems to be essential for successful *Trichodesmium* cultivation. The cultures tended to be more stable in media with lower nutrient concentrations (~ 1/40). Heavy metal contaminants in reagents used as nutrients are probably toxic and render the cultures unstable.

The growth rates suggested by our data were appreciably higher than those reported for natural populations (0.02 to 0.008 d^{-1}, Carpenter and McCar-

thy, 1975; Saino, 1978; McCarthy and Carpenter, 1979). Insignificant differences in growth rates with and without NO_3^- indicate that nitrogen supply from nitrogen fixation can support algal growth as efficiently as the NO_3^--reduction system. Since growth occurred in separated trichomes, nitrogen fixation of *Trichodesmium thiebautii* can be appreciable, even in separated trichomes under aerobic conditions; colony-formation is, therefore, not essential for nitrogen fixation. Our results are consistent with the finding by Saino and Hattori (1979) that single trichomes of natural samples are active in nitrogen fixation even under aerobic conditions. As suggested by Taylor et al. (1973), there must be some mechanism for protecting the nitrogenase system present in the cells.

The B_{12} effect was insignificant in growth experiments, in contrast to that observed in survival experiments. We also failed to find a stimulative effect of B_{12} derivatives other than hydroxocobalamin. Other vitamins and micrometals in the "f" medium were found to be somewhat effective for long-term survival. However, they were insignificant in growth experiments. Though *Trichodesmium thiebautii* requires micronutrients, some other factors – chemical or physical – must limit its growth under our conditions.

Deficiency in such factors may have prevented active growth of *Trichodesmium* in our earlier experiments. During a long resting period, *T. thiebautii* tends to recover gradually from the deficiency so as to facilitate active but slow growth; the factors still limit growth, even after recovery. Ramamurthy (1972) reported that the plant growth hormone, gibbelelic acid, was required by *T. erythraeum*. However, gibbelelic acids, aminopurine derivatives and kinetin were not stimulative but inhibitory. Search for the factors in question must be of primary importance for the establishment of axenic cultures; it is now in progress, using the unialgal cultures obtained.

The evidence that both *Trichodesmium thiebautii* and *T. erythraeum* can survive for a long period without active growth may explain the wide distribution of these algae in the tropical and subtropical sea areas. Because of their long survival (resting conditions), they can be transported by water currents to areas very distant from where they had grown actively; whenever the environmental conditions become advantageous, they will start to grow again. A dense *Trichodesmium* population often appears in the East China Sea and along the coast of Japan (Nagasawa and Marumo, 1967; Marumo and Asaoka, 1974 a, b). This may be the result of active growth of trichomes transported by the Kuroshio from tropical sea areas.

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