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Growth of the Dinoflagellate *Alexandrium tamarense* Isolated from Jinhae Bay, Korea in Axenic Cultures

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Abstract - We examined effects of water temperature, salinity, irradiance, and different media on the growth of the toxic dinoflagellate *Alexandrium tamarense* (HYM9704), which was isolated from Jinhae Bay, Korea. The ranges of temperature and salinity in which the strain was able to grow were 10~20°C and 20~34 psu, respectively. These values were in accordance with those observed *in situ*. The maximum growth rates of axenic *A. tamarense* (HYM9704) was 0.25 d^{-1} at 15°C, 30 psu, and 100 μE m^{-2} s^{-1}. The temperature affected the growth rates of axenic *A. tamarense* more significantly than the salinity. The type of culture media did not affect the growth rates of axenic *A. tamarense*. The strain in N-limited and P-limited media went into the stationary phase faster than that in T1 and T1/2 medium.

Key words : *Alexandrium tamarense*, axenic, growth, irradiance, salinity, temperature, different media

INTRODUCTION

Alexandrium tamarense (Lebour) Balech is present in temperate areas and is known as a causative agent of paralytic shellfish poisoning (PSP) (Ichimi *et al.* 2001; Wang and Hsieh 2002; Lee *et al.* 2003). The physiological characteristics of *A. tamarense* in batch culture have been reported in many previous studies (Watras *et al.* 1982; Achiha and Iwasaki 1990; Su *et al.* 1993; Yamamoto *et al.* 1995; Yamamoto and Tarutani 1997). In Korea, Kim *et al.* (1996) reported the physiological characteristics of *A. tamarense* isolated from Jinhae Bay.

It has been suggested that bacteria associated with dinoflagellates either in the natural environment or in laboratory cultures, may influence the production of PST (Paralytic shellfish toxins) by dinoflagellates (Doucette 1995; Gallacher *et al.* 1997). Some authors report higher toxicity in bacteria-free cultures (Singh *et al.* 1982; Dantzer and Levin 1997) while others show the reverse results (Doucette and Powell 1998). The feeding by *A. tamarense* on marine bacteria showed by Jeong *et al.* (2005). However, there is no evidence for a species-specific difference. Therefore, although there are several difficulties in the culturing of dinoflagellates, the axenic algal culture is useful to understand the characteristics of PSP (Paralytic Shellfish Poisoning) toxins and physiology in algae themselves.

The aim of the present study is to investigate the growth of *A. tamarense* (HYM9704) in axenic cultures under the different conditions of temperature, salinity, light intensity, and different media.

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MATERIALS AND METHODS

1. Isolation and culture of *A. tamarense* (HYM9704) strain

A single vegetative cell of *A. tamarense* was isolated from Jinhae Bay, Korea when the bloom of *A. tamarense* occurred. A stock culture of *A. tamarense* strain was grown in a modified T1 medium (Ogata *et al.* 1987), which contained 1,000 μM $NaNO_3$, 100 μM NaH_2PO_4, 5 μM Fe-EDTA, 1 μM $ZnSO_4$, 0.1 μM $MnCl_2$, 0.5 μM $NaMoO_4$, 2 nM $CoCl_2$, 0.01 μM $CuSO_4$, 0.24 μM Na_2-EDTA, 593 nM Thiamine HCl, 4.1 nM Biotin, 0.738 nM Cyanocobalamin, Tris-HCl (pH 8.0) and maintained at 15°C and 100 μE m^{-2} s^{-1} with a cool-white fluorescent light under a 12 : 12 L : D cycle. The sea water was filtered by a 0.45 μm millipore filter (whatman cellulose nitrate). Aged seawater from Sagami Bay was adjusted to 30 psu by adding deionized Milli Q water. All equipments and glassware were autoclaved.

2. Antibiotics treatment and verification of axenic culture

A stock solution of antibiotics was prepared in the following ratios: gentamycin 100 mg (50 mg), streptomycin sulfate 50 mg (25 mg), and distilled water 10 mL (5 mL) and filtered with a membrane filter (0.2 μm). Each flask containing different concentrations of antibiotics (0, 0.03, 0.04, 0.05, 0.1 and 0.2 mL per 2 mL) was inoculated with freshly concentrated algal cells, and incubated for 12 to 48 hr. The active algal cells were aseptically removed from the antibiotic-treated medium and re-inoculated into fresh media.

Both the yeast-bacto extract and direct epifluorescence microscopic observation were used to verify the axenic cultures. The constituents of the media were as follows; yeast extract 10 g, bacto-peptone 10 g, and seawater (30 psu) 1,000 mL. After the verification of axenic culture with the yeast-bacto extract, the cells was stained with DAPI, and filtered by black 0.22 μm filters for direct counts of the contaminating bacteria by the epifluorescence microscopy.

3. Treatment of axenic *A. tamarense* (HYM9704) strain in various environmental conditions

The axenic *A. tamarense* strain was incubated at 15°C in T1 medium (30 psu) with an irradiance of 100 μE m^{-2} s^{-1} under a 12 : 12 L : D cycle. Each experiment was conducted in triplicates. Cells in the exponential phase of growth from the stock culture were transferred into the screw-top glass tubes in all experiments.

To find the optimal temperature of the growth for axenic *A. tamarense*, each glass tube was incubated under the four different temperature conditions (at 10, 15, 20 and 25°C). For determining the optimal salinity of axenic *A. tamarense*, the filtered seawater was diluted with deionized water (Milli Q) to get the four desired salinity levels (20, 25, 30 and 34 psu). A series of light experiments created 4 different light intensities (34, 75, 100, and 150 μE m^{-2} s^{-1}). Four types of media, control (T1 medium), 1/2 T1medium, N-limited and P-limited medium were employed to reveal the nutrient dependent growth of axenic *A. tamarense*. Nitrogen-limited medium contained 50% (50 μM) of the normal T1 medium phosphate and 5% (50 μM) of the normal T1 medium nitrate. Phosphate-limited cultures contained 50% (500 μM) of the normal T1 medium nitrate with 1% (1 μM) of the normal phosphate. No effort was made to remove N or P from the natural seawater used to make the media.

4. Relationship between the density of *A. tamarense* (HYM9704) in axenic cultures and *In vivo* Chlorophyll *a* fluorescence

The various densities of axenic *A. tamarense* from the stock cultures were established by adding fresh T1 medium to verify the relationship between the density of axenic *A. tamarense* and *in vivo* chlorophyll *a* contents. *In vivo* chlorophyll *a* fluorescence was measured with a fluorometer (Turner Designs: Model 10AU). Cell density was determined by a direct cell count on the Sedgwick chamber (1 mL) being preserved in Lugol's iodine solution, under an inverted light microscope (Nikon Diaphot).

5. Measurement of growth rate

A growth rate of *A. tamarense* (HYM9704) in axenic culture was extrapolated from the *in vivo* chlorophyll *a*, which was measured daily by the fluorometer (Turner Designs: Model 10AU). The growth rate (d^{-1}) was calculated by the following formula;

$$\mu_2 = \ln(F_1/F_0)/((t_1 - t_0)\ln_2)$$

where μ_2 is the specific growth rate, or relative growth constant (d^{-1}), and F_0 and F_1 represent *in vivo* chlorophyll *a* fluorescence values at time t_0 and t_1, respectively.

RESULTS AND DISCUSSION

1. Relationship between cell density of axenic *A. tamarense* (HYM9704) and *in vivo* Chlorophyll *a* fluorescence

Cell density was positively correlated with *in vivo* chlorophyll *a* fluorescence of axenic *A. tamarense* (Fig. 1). The equation of the curve obtained by the regression linear was as follows;

$$CD=483.5\times F-60$$

Where CD is cell density and F is values of *in vivo* chlorophyll *a* fluorescence.

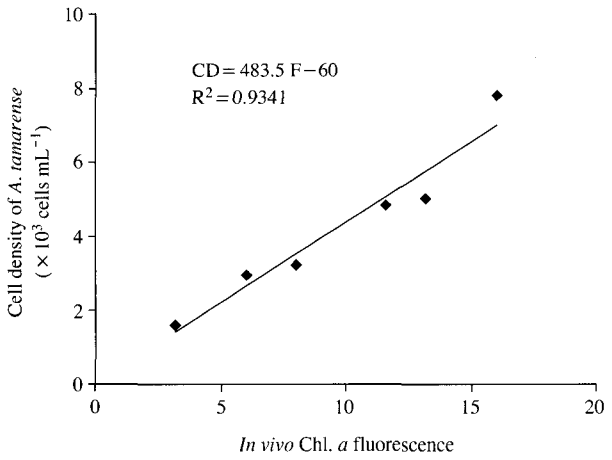

Fig. 1. Relationship between cell density of axenic *A. tamarense* ($\times 10^3$ cells mL^{-1}) and *in vivo* chlorophyll *a* fluorescence.

2. Determination of optimal light intensity of axenic *A. tamarense* (HYM9704)

The growth curve and the growth rate of axenic *A. tamarense* at 34 μE m^{-2} s^{-1} was low and the growth rates of the stain at 75 μE m^{-2} s^{-1} and 100 μE m^{-2} s^{-1} increased and the strains was saturated at 150 μE m^{-2} s^{-1} (Fig. 2). The maximum growth rate (0.25 d^{-1}) of the stain was obtained at 100 μE m^{-2} s^{-1}. Yamamoto and Tarutani (1997) reported that the irradiance-growth curve of *A. tamarense* isolated from Hiroshima Bay, Japan showed the maximum growth rate of 0.23 d^{-1} at 124 μE m^{-2} s^{-1}, while that isolated from Mikawa Bay was reported to show the maximum growth rate of 0.16 d^{-1} at 170 μE m^{-2} s^{-1} (Yamamoto *et al.* 1995). On the other hand, Anderson *et al.* (1984) reported that the maximum growth rate of *A. tamarensis* from a Cape Cod strain was from 150 to 200 μE m^{-2} s^{-1} in laboratory. The maximum growth rate of *A. tamarense* strain from the northeastern Canada was 0.41 day^{-1} at 230 μE m^{-2} s^{-1}

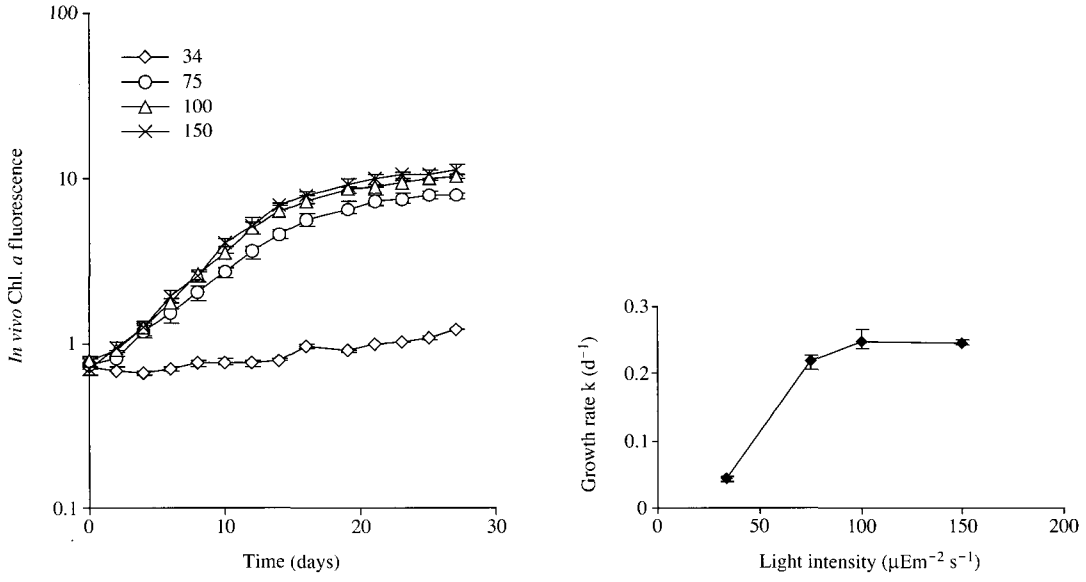

Fig. 2. Growth of axenic *A. tamarense* (HYM9704) in different light intensities at 15°C in 30 psu. Each symbol represents the average of triplicates.

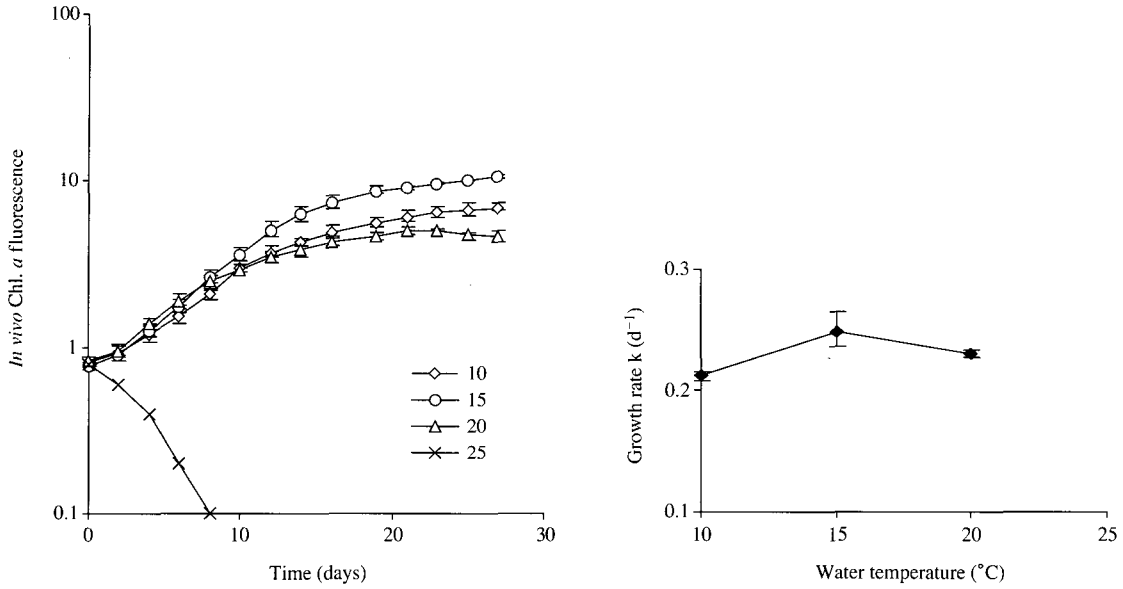

Fig. 3. Growth of axenic *A. tamarense* (HYM9704) in different temperature condition in 30 psu with 100 μE m^{-2} s^{-1}. Each symbol represents the average of triplicates.

(Parkhill and Cembella 1999). Axenic *A. tamarense* than strains from other region and country showed higher growth rate in low light intensity. But all strains except this strain did not axenic *A. tamarense*, and then we could not be compared accurately.

3. Determination of optimal temperature and optimal salinity of axenic *A. tamarense* (HYM9704)

Axenic *A. tamarense* grew from 10°C to 20°C, but the strain did not grow at 25°C. The optimal growth temperature of axenic *A. tamarense* was obtained at 15°C (Fig. 3). In the field, the highest cell densities of *A. tamarense* collected from Jinhae Bay were observed at 10~11°C. The temperature at which *A. tamarense* grows in the field is sometimes different from the temperature at which the maximum growth rate occurs in culture (Eppley 1977). Yamamoto *et al.* (1995) and Yamamoto and Tarutani (1997) showed that the optimum temperature for the growth of *A. tamarense* isolated in Mikawa Bay and Hiroshima Bay, Japan was at around 15°C. The temperate *A. tamarense* strain found in North America could grow at 5 to 20°C, with an optimum at 15~20°C (Yentsch *et al.*, 1975). This implies that axenic *A. tamarense* strain and strains from North America and Japan have the similar response to temperature.

This axenic *A. tamarense* (HYM9704) strain grew well at salinity of 20 psu to 34 psu under all temperature conditions. Strains in all salinities at 15°C grew faster than those in all salinities at 10°C and 20°C (Fig. 4). The ranges of temperature and salinity in which *A. tamarense* was able to grow were 10~20°C and 15~34 psu in accordance with those observed *in situ*. The maximum growth rate of salinity showed at 30 psu in each temperature. This study supposes that temperature affected the growth of axenic *A. tamarense* more significantly than the salinity. On the other hand, the optimal growth of this strain in 30 psu was slightly higher than that reported in Kim *et al.* (1996), who reported the optimal growth of *A. tamarense* at 25 psu. Prakash (1967) reported the optimal salinity of *A. tamarense* strain was at 19~20 psu. On the other hand, the optimum salinity for *A. tamarense* strains from Mikawa Bay and Hiroshima Bay was 32 psu and 30 psu, respectively. This shows that growth chracteristics of the same species sometimes differs between localities.

4. Growth of axenic *A. tamarense* (HYM9704) under four different media

Nutrient-limited cultures were incubated at 15°C in 30 psu with an irradiance of 100 μE m^{-2} s^{-1} under a 12 : 12 L : D cycle. Growth curve of the strain in other media except T1 media reduced in the later stages of stationary phase.

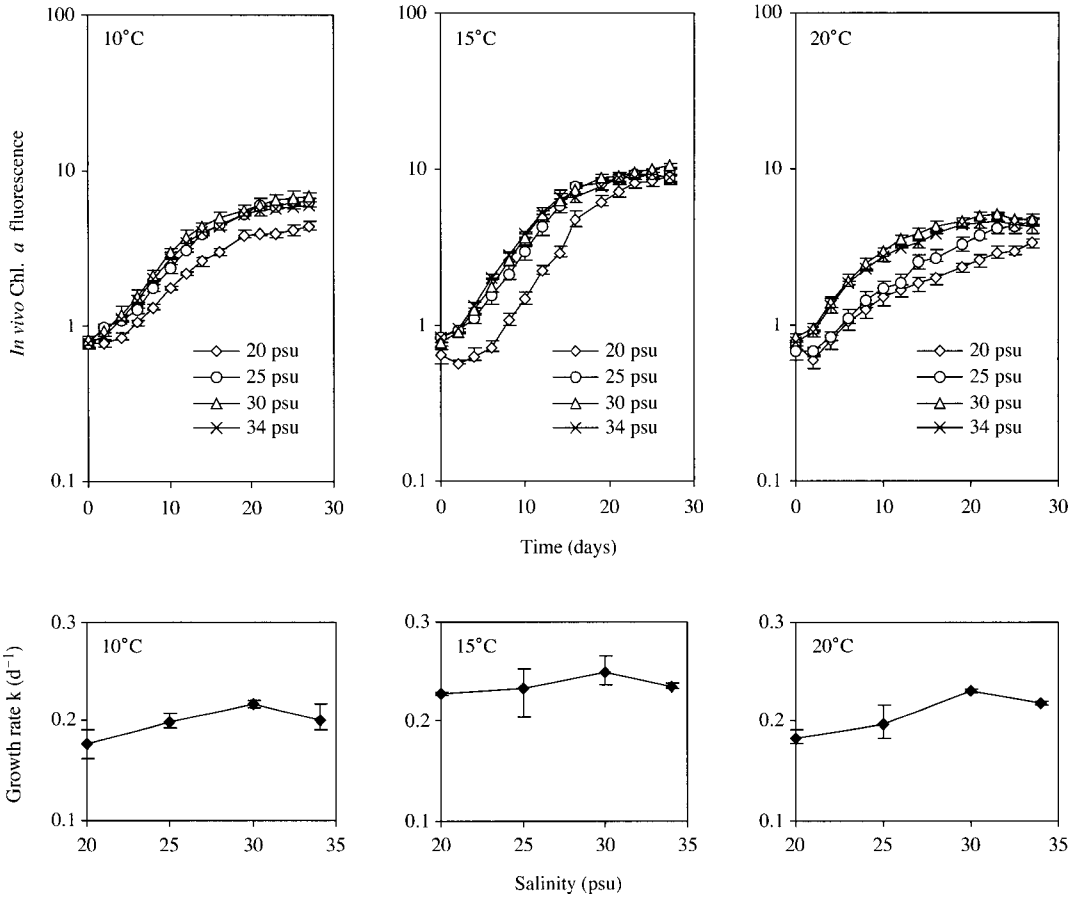

Fig. 4. Growth of axenic *A. tamarense* (HYM9704) in various salinity at 10°C, 15°C and 20°C with 100 μE m^{-2} s^{-1}. Each symbol represents the average of triplicates.

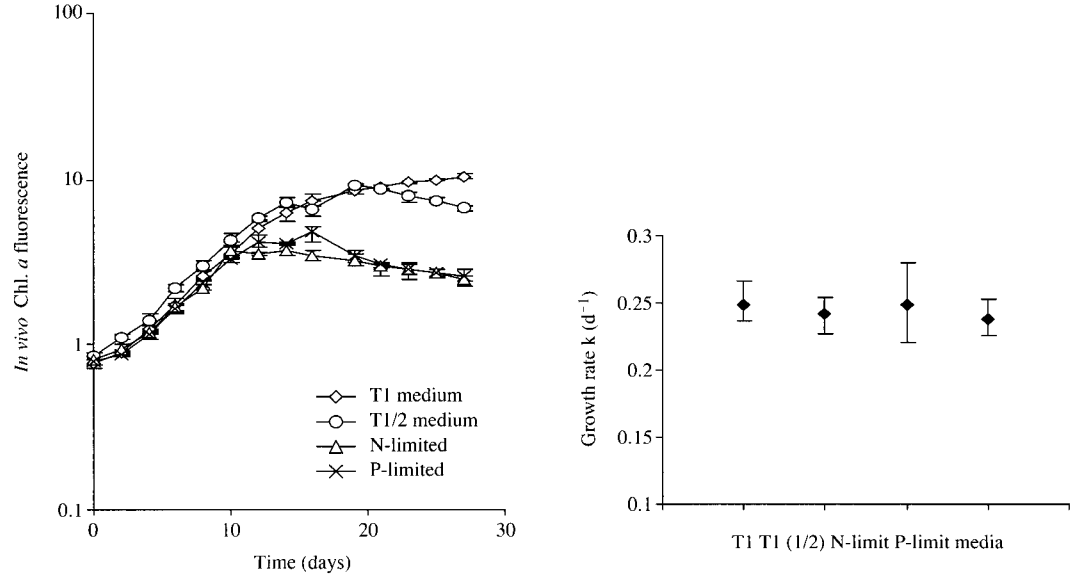

Fig. 5. Growth of axenic *A. tamarense* (HYM9704) in different mediain various salinity at 15°C in 30 psu with 100 μE m^{-2} s^{-1}. Each symbol represents the average of triplicates.

This strain cultured in different media conditions showed [Hanms1]similar growth rates (Fig. 5). The strain in N-limited and P-limited media went into the stationary phase faster than that in T1 and T1/2 medium. This result also was seen in Boyer *et al.* (1987). We suppose that the entry of the cells into stationary growth phase is due to specific nutrient limitation.

Alexandrium species are not known for rapid or explosive growth rates. The growth rate in exponential phase of the axenic strain was 0.25 d^{-1}, which is lower than xenic *A. tamarense* (0.6 d^{-1} to 0.82 d^{-1})observed in Jinhae Bay (Kim *et al.* 1996) and other strain isolated from Korea (Han *et al.* 1993). Also the maximum growth rate of *A. tamarense* strain from northeastern Canada was 0.50 d^{-1} (Parkhill and Cembella 1999). These results probably indicate that the growth rate in the axenic culture was slower than that in the unialgal culture as showed by Singh *et al.* (1982), who suggested that the retarded growth and the low maximum cell density in the axenic culture was due to the deficiency of some nutrients in the medium and the possible compensation for such substances by bacteria in the unialgal culture.

It is concluded that the temperature affected the growth of axenic *A. tamarense* more significantly than the salinity and the maximum growth rate of salinity showed at 30 psu in each temperature condition and that the strain showed a higher growth rate under low light intensity. It is now clear that the maximum growth rates of axenic *A. tamarense* (HYM9704) was determined 0.25 d^{-1} at 15°C, 30 psu and 100 $\mu E\ m^{-2}\ s^{-1}$.

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