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Culture of *Kappaphycus alvarezii* in Deep Seawater and Nitrogen enriched medium

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Abstract: *Kappaphycus alvarezii* (Doty)Doty ex .Silva , red seaweed, was cultured in deep seawater and in different concentration of NO_3-N at the laboratory. Higher daily growth rate (DGR)of the thallii were observed as 1.17% in deep seawater. The materials in 1mM NO_3-N, lower concentration nitrogen enrichment to normal surface seawater produced daily growth rate (DGR) of 0.97%, while a higher NO_3 content suppressed the growth of the thallus and showed DGR of 0.51%. The growth of new branches, wound healing and regeneration capacity of the thallus is much faster in the deep seawater medium with a DGR of 0.94%, which is nearly double than the normal seawater (DGR:0. 48 %). The results suggest that deep seawater can be effectively used as a cheap medium for culture and mass cultivation of *Kappaphycus* as well as many other marine algal species and low NO_3 enrichment can enhance the growth rate.

Key words: *Kappaphycus* culture, deep seawater.

INTRODUCTION

During the last three decades species of *Eucheuma* and *Kappaphycus* have been successfully farmed as raw materials for carrageenan production (Doty, 1986; Sahoo, 2000). First mariculture of *Eucheuma* started in early 1967 in the Philippines and became commercially viable in 1974 (Parker, 1974). Since then *Kappaphycus* has been successfully introduced to 19 other countries, where as *Eucheuma* has been introduced to 13countries. Culture studies on *Kappaphycus* have been undertaken both in the laboratory and field under different environmental and nutrient conditions by various workers. Dawes et al. (1991 and 1993) studied the tissue culture and clonal propagation in *E. denticulatum* and *K.alverzii* using different media. Dawes et al.(1994) also measured the growth rates of the above genera in the field and laboratory. Ohno et al.(1994) studied the growth rate of *K. alvarezii* in the subtropical waters of southern Japan. Gerung and Ohno (1997) measured the growth rate of *E.denticulatum* and *K.striatum* under different environmental conditions. Rui et al. (1990) studied the effect of Ammonium on the growth of *K.alvarezii*. More recently Paula et al.(2002) studied the growth rate of *K.alvarezii*, which was introduced in subtropical waters of Brazil.

Although the literature reveals a large number of studies on *Kappaphycus*, but the effect of nutrients on the growth of the genus is not well studied. Such the objective of our study was two fold. Since previous studies demonstrated that nitrogen supply has significant effect on the growth

rate of various agarophytes and carrageenophytes (Rui et al.1990;Chopin and Wagey ,1999;Ryder et al.,1999). We decided to study the effect of nitrogen on the growth of the thallus. Recently deep seawater has found several applications (Sahoo and Ohno, 2001; Sahoo ,2002). Sahoo et al.(2002) found that *Eucheuma serra* can be cultured in deep seawater and produce high growth rate. So our second objective was to find out whether *Kappaphycus* can grow in deep seawater with high growth rate or not?

Our study suggests that a lower dose of nitrogen can accelerate the growth rate and deep seawater can be effectively used as a cheap medium for culture and cultivation of *Kappaphycus* and various other algae.

MATERIAL AND METHODS

Deep seawater was transported to Usa Marine Biological Laboratory, Kochi in clean plastic containers from Kochi Prefecture Deep seawater Laboratory, Muroto City, Kochi Prefecture, Southern Japan. (Deep seawater was pumped from a depth of 320 meter at this laboratory).

The analysis of deep seawater revealed some interesting data. The water is uncontaminated by bacteria. Phytoplankton was absent due to the lack of sunlight at such a high depth. The concentration of nutrients especially nitrate, phosphate and silicate was very high usually 5-10 times higher than the surface seawater (Sahoo et al. 2002). Trace metals and vitamins were found in reasonable quantity. The temperature of deep seawater remained between 13-15°C through out the year whereas the temperature of surface seawater varied between 17-31°C. The salinity and pH of deep seawater was similar to surface seawater during this experiment period i.e. 34 ppt and 8.3 respectively. The water was stored in the cold room at 5°C till further use.

Kappaphycus alvarezii were originally transplanted from Philippines in June 1991 and cultured both in the tank and field in Uranouchi inlet of Tosa Bay Southern Japan(Ohno et al. 1994). The plants were collected from the culture station at Tosa Bay and brought to the laboratory (Figure 1A).The thalli were cleaned up of all the visible epiphytes and cultured in an Aquatron(Ohno,1977) for two weeks at 25°C and 125 µmol photon m^{-2} s^{-1} irradiance under 12 :12 light :dark cycle. After the acclimatization period, the thalli were again cleaned up of epiphytes with a fine brush under a Nikon stereomicroscope. For the deep seawater experiment, young and healthy thallus measuring up to 4-5 cm weighing 3 g each were taken and cleaned several times in autoclaved seawater. Five explants were transferred into culture jars containing 2 L of deep seawater at 25°C. Same number of replicates was also placed into culture jars containing 2 L of plankton net filtered normal seawater, which was used as control. For the Nitrate uptake experiment, young and healthy thalli were inoculated into 5mM, 1mM and 2mM of $NaNO_3$ enriched seawater. All the jars were covered with cellophane and incubated at 25°C. Light was supplied from cool white fluorescent tubes at 125 µmol photon m^{-2} s^{-1} irradiance under 12:12 light: dark cycle. The increase or decrease in weight of plants was measured at weekly interval and media were replaced. Daily growth rate (DGR mean±s. d) were calculated using a formula $G=[(Wt/Wo)^{1/t}-1]$, where the G=% increase in fresh weight per day, Wo=initial weight, Wt=weight after t days. Regeneration at the cut ends and the growth of the thalli were observed under a Nikon stereomicroscope. Black and white pictures were taken using a Fuji Neopan film. The experiment was carried out for a period of 8 weeks. Seawater was analyzed in the laboratory by the methods of Japanese Oceanographic Society (1985).

RESULTS

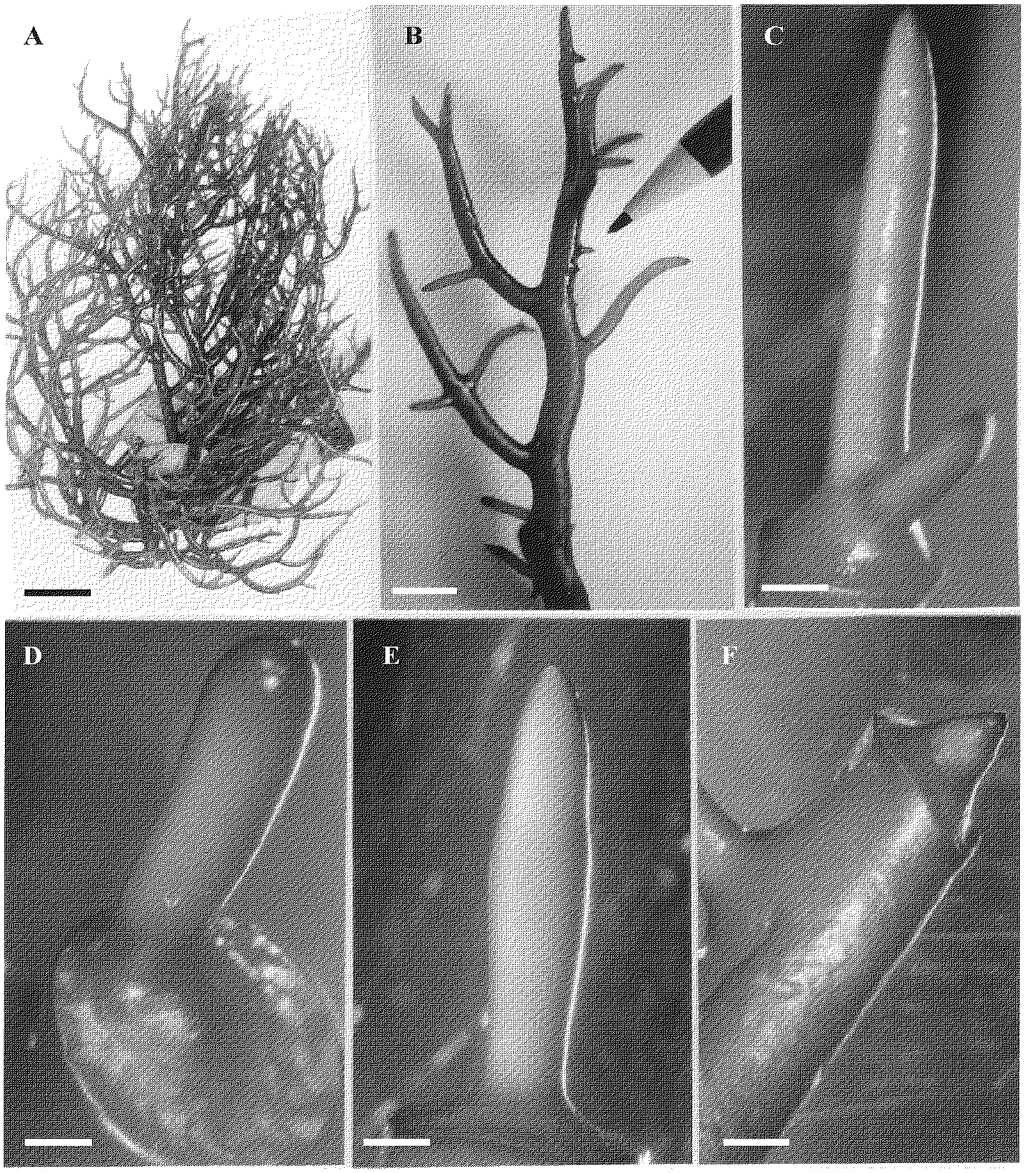

Figure 1. A-F. Growth of *Kappaphycus alvarezii* (Doty) Doty ex. Silva

A. Thallus of *Kappaphycus alvarezii* cultured in the field. (Scale bar=1.5cm.)
B. Part of the thallus showing new protrubances. (Scale bar=0.1cm)
C-E Growth of new branches at cut ends of the thallus from cortical and medullary regions after 3 weeks of culture in deep seawater. (Scale bars=0.1cm)
F. A small protrubance at cut end of the thallus after 3 weeks in culture in normal Sea water. (Scale bar=25 mm)

When the wounded or cut portion of the thalli were observed under a stereo-microscope, whitening of cortical and medullary tissues was observed after 3-4 days on the cut end of thallus which was cultured in deep seawater. Subsequently, new protrubances grew at these cut ends which measured nearly 1 cm after three weeks of culture in deep seawater (Fig.1C-E) whereas the thalli cultured in the normal seawater showed a much delayed activity of wound healing and the new outgrowth at the cut ends measured only. Two cm after three weeks of culture (Fig.1F). A large number of small protrubances were also observed on the thallus surface (Fig. 1B) in the deep seawater material, which subsequently formed new branches. The analysis of deep seawater and surface seawater before the inoculation of thallus showed high availability of nutrients. But after one week of culture, a depletion in the nutrients level was found in both the media (Table 1).

It has been found that the uptake of nitrogen (NH_3, NO_2, NO_3) and phosphate from the deep seawater was much faster compared to them of normal seawater .

The thalli cultured in deep seawater and normal seawater did not show any significant differences in their growth rate during the first week. However, during the subsequent weeks the thalli cultured in deep seawater showed an increasing rate of growth compared to the thalli cultured in normal seawater (Table 2). The growth rate of thalli in deep seawater increased substantially reaching a peak during the second week with DGR of 1.17% and then declining to 1.1% in the third week. But during the fourth week the growth rate substantially decreased to 0.65% then slightly increased in the fifth week to 0.75% and stabilized subsequently. Similar pattern of increase and decrease in growth rate was also observed in the thalli cultured in the normal seawater (Table2). However, the average DGR of the thalli cultured in deep seawater was 0.94% and the average DGR of the thalli cultured in normal seawater was0. 48%. Interestingly the thalli cultured in deep seawater remained very healthy compared to the thalli in normal seawater.

In the Nitrogen uptake experiment, when the thalli were cultured in 2mM enriched seawater,

Table 1. Water analysis data showing uptake of various nutrients by *K. alvarezii* in the first week. A comparison of nutrients between deep seawater and normal seawater showed PO_4, NH_4, NO_3 level are 4, 2 and 7.8 times higher respectively in deep seawater where as NO_2 is 0.75 time lower.

Nutrients	Fresh Deep seawater µg at./L	After one week of culture µg at./L	Normal seawater µg at./L	After one week of culture µg at./L
PO_4	1.24	0.16	0.31	0.05
NH_4	1.26	0.47	0.63	0.31
NO_2	0.11	0.09	0.14	0.04
NO_3	1.64	0.19	0.21	0.19

Table 2. Daily Growth Rate (mean% s. d) of *Kappaphycus alvarezii* in deep seawater and normal seawater after weekly interval

	1st week	2nd week	3rd week	4th week	Average DGR
Deep seawater	0.57 ± 0.22%	1.17+0.22%	1.1+0.2%	0.65+0.18%	0.94+0.03%
Normal seawater	0.46%	0.54+0.19%	0.62+0.41%	0.27+0.39%	0.48+0.05%

the colour of the thalli changed to dark green and dark brown in both the strains. The colour remains very dark throughout the culture and the growth rate decreased gradually in the following weeks and then, the DGR became 0% in the 4th week. However, in 0.5mM enriched medium the growth rate decreased to DGR of 0% in the 2nd week, but subsequently increased during 3rd and 4th week. But in 1mM enriched medium the growth rate was maintained and never reached 0% (Table 3). The average DGR of thalli cultured in normal seawater was 0.48%, whereas it is 0.65% in 0.5mM enriched medium. Interestingly during the entire period of experiment in both deep seawater and N enriched medium, no difference in the growth rate was observed in green and brown strain of *K. alvarezii.*

Table 3. Daily Growth Rate (mean% s. d) of *Kappaphycus alvarezii* in different concentration of NO_3 after weekly interval

	1st week	2nd week	3rd week	4th week	Average DGR
Control	1.37%	0.24+0.55%	0%	0.73+0.67%	0.48+0.15%
0.5mM	1.34 ± 0.93%	0%	0.46+0.63%	0.71+0.65%	0.65+0.23%
1mM	1.34+0.93%	1.09+0.23%	0.90+0.5%	0.62+0.56%	0.97+0.13%
2mM	1.34+1.07%	0.58+0.67%	0.27+0.55%	0%	0.51+0.43%

DISCUSSIONS

One of the major problems in the farming of *Eucheuma* is the slow and decline in the productivity of different strains of *Kappaphycus* and *Eucheuma*. It is important to develop standard inexpensive culture procedures that will permit not only the maintenance of seed stocks with optimal growth rate, but also can produce high quality carrageenan. In order to achieve this goal, Dawes and Koch (1991) used several media to culture *K.alvarezii*. They reported DGR of 1.52 % after 52 days of culture in enriched seawater medium which was supplemented with various plant growth regulators. Dawes et al;(1993) reported a DGR of 0.65% for *E. denticulatum* and 0.26% for *K. alvarezii* when the thalli were cultured in ESS medium enriched with kinetin and IBA. The growth rate of *K.alvarezii* did not show any significant difference in ESS media which was enriched with low concentration of coconut water and plant growth regulators. DGR between 0.24%-.0.34% were observed in various combinations of plant growth regulators in the media.

At the present study, we reported a high DGR of mainly 0.94% in deep seawater and a low DGR of 0.48% in normal seawater for *K. alvarezii*. This high growth rate of thallus in deep seawater is due to simultaneous and rapid uptake of nutrients like NO_3-N, NH_4-N and PO_4-P which was available in higher quantity in the medium compared to their availability in normal seawater. Our observation is fully supported by the water analysis data both before and after culture, which showed that the nutrients level significantly dropped in deep seawater after one week of culture.

It has been found that seaweeds grow faster of NO_3-N and PO_4-P are added to the seawater. The effect of NO_3-N and PO_4-P has been well studied in several species of seaweeds. Chemical factors such as the nutrient concentration and the form of the limiting nutrient e.g. NO_3 N and NH_4-N may influence uptake rates (Harrison and Hurd, 2001). It has been found that some seaweeds (especially kelp) are able to take up NO_3-N and NH_4-N simultaneously at the same rate and able to grow faster (Bird, 1976; Harrison et. al, 1986). However, Navarro-Angula and Robledo (1999) observed that growth rate remains unchanged in *Gracilaria cornea* even though when

nitrogen is supplied as NH_4, NO_3 and NO_3+NH_4 (and urea). So we think, in *K. alvarezii* nutrient uptake mechanism is more similar to kelp than *Gracilaria*.

Matsuyama et al (2002) studied the macroalgal growth response in deep seawater. They found that growth and uptake of nutrients by *Ulva sp.* were highest in deep seawater and lowest in surface seawater. Our studies in *K. alvarezii* agree with their results. They found that growth rate is not proportional to nutrient uptake rates. Sahoo et al (2002) cultured *Eucheuma serra* in the tank supplied with continuous circulation of deep seawater and reported the highest DGR of 8.2%. When Yamaguchi et al. (1994) cultured *Laminari, Ecklonia* and *Undaria* in deep seawater, they did not report high growth rate in these plants but found their growth was healthy. Recently, Ohno and Hiraoka(2002) cultured *Porphyra dentata, Meristotheca papulosa, Eucheuma serra, Enteromorpha prolifera* and *Scytosiphon lomentaria* in deep seawater. They reported an extremely high DGR of 60% in *Enteromorpha prolifera* for the month of April and between 30%-46% for the other months. They reported a DGR of 39% in *Porphyra dentata* in the month of January and found that the thalli produced high-grade commercial products. The DGR for *Scytosiphon lomentaria* ranged between 25%-30% in winter season and that for *Meristotheca papulosa* between 1.5%-2.0% and for *Eucheuma serra* between 2.0%-2.5%. Oka et al (2002) reported a DGR of 15%-20% in *Undaria pinnatifida* when cultured in deep seawater.

It has been found that seaweeds with a high surface to volume ratio generally have a higher nutrient uptake thus having a high growth rate (Wallentinus, 1984; Hein et al. 1995, Taylor et al., 1998). Since, *Porphyra, Enteromorpha, Ulva* and *Scystosiphon* are membranous with more surface area, they have a high growth rate compared to *K. alvarezii*, which is having cylindrical and compressed thallus with less surface area.

Secondly in the present study, the growth rate of thalli during the second week of culture in deep seawater showed a very high DGR of 1.17% and subsequently slowed down to 1.1% during third week and to 0.65% during the fourth week and then stabilized. This high growth rate during the second week was attributed to the high nutrient absorption from the medium during the first week of culture. Paula et al. (2002) have also reported similar variations in the growth rate in *K. alvarezii* in subtropical waters of Brazil.

Nitrogen is the nutrient most frequently reported to limit the growth of seaweeds in natural ecosystem (Hanisak, 1983). Previous studies demonstrated that nitrogen supply have a significant effect on the growth rate, content and quality of phycololloids in agarophytes and carragenophytes (See Rui et al. 1990; Chopin and Wagey, 1999). In the present study when plants were inoculated into 2mM NO_3-N enriched seawater the colour of the thalli changed from green to dark green and from brown to dark brown in both the strains. However in 0.5mM and 1mM nitrogen enrichment such colour changes were not noticed. Earlier studies showed that nitrogen enrichment leads to an increase in the growth rate and changes in the thallus colour has been observed in several species of seaweeds (Wu, 1962; Neish et al., 1977, Lapointe and Ryther, 1979). Rui et al (1990) also found that the colour of the thallus in *K. alvarezii* changed from yellowish brown to dark brown when the seawater medium was enriched with higher concentration of NH_4-N. Interestingly Chopin and Wagey (1999) also noticed similar changes in colour in *Chondrus crispus* when the thalli were cultured in N enriched medium. So our study suggests that change in the colour of the thallus seems to be an important response to high dose of nitrogen.

In the present study, the lowest growth of 0.48% was observed in the normal seawater. However when the medium was enriched with 0.5mM of NO_3-N, the DGR slightly increased to 0.65% and to 0.97% at 1mM Nitrogen enrichment, and then declining to DGR of 0.51% at 2mM

enrichment. This suggests, that while a moderate level of Nitrogen enrichment can produce best growth in *K. alvarezii* a higher level of nitrogen enrichment will not only reduce the growth rate but can be detrimental to the plants. Interestingly, DeBoer (1979) reported that growth rate and carrageenan content in *Agardhiella subulata* (as *Neoagardhiella baileyi*) was the highest in 0.5-1.0μM ammonium enrichment and lower both at higher ammonium concentrations and unenriched seawater. Rui et al (1990) found that *K. alvarezii* can grow optimally when nitrogen (NH_4-N) applied in a concentration between 5-25mM. However, an increase in the concentration of NH_4-N between 35-50mM, not only decreased the growth rate but causes break down of the thalli. Our studies on *K. alvarezii* agree with their results.

In conclusion, our study suggests that since deep seawater is clean, non-contaminated and rich in nutrients *K. alvarezii* can be cultivated in this medium at desired temperature. Besides, high quality seedlings can be maintained as seed stocks in the culture tanks in deep seawater. Secondly, moderate level of Nitrogen enrichment can be done to the seawater to achieve high growth rate of the thallus. Third, since deep seawater is much colder (13-15°C) compared to surface seawater (18-31°C), it can be mixed in different proportions to get desired temperature to culture seaweeds. Similarly, nutrient concentrations can be manipulated by the mixing of different proportions of both surface seawater and deep seawater. Such manipulation of water temperature and nutrient conditions can be created to manipulate algal growth and reproduction at any time of the year.

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