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Genetic Improvment for the Low Salinity-Tolerant *Porphyra* Sp. by Cell Culture Techinque

I. Tissue Culture of *Porphyra yezoensis* foma *narawaensis*

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세포배양기술에 의한 김의 내저염성 품종개량

I. 큰방사무늬김의 조직배양

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ABSTRACT

Axenic tissue culture of a marine red algae *Porphyra yezoensis* foma *narawaensis* was established for the vegetative propagation of tissues as a seed stock and for the development of a low salinity-tolerant cell line. Callus tissues have been induced from the vegetative area of blade away from the holdfast when grown on PES-agar medium. The brownish red fragile callus was maintained under fluorescent light of ca. 2000 lux with 12 : 12 hr L : D at 16℃. Amounts of carbohydrate and protein was determined against the weight of callus. Optimum temperature of the callus growth was 14℃～18℃. Optimun concentration of sodium chloride was 2.0% for the callus growth in PES-agar medium.

요 약

양식 해조류인 큰방사무늬김에 대한 조직배양을 종자 세포 보존 및 장차 세포수준의 내저염성 품종개량을 목적으로 시도하였다. 김 엽체의 영양 성장부위를 무균적으로 PES-한천배지 상에 배양함으로서 callus의 형성을 유도하였으며 이 적갈색의 연약한 callus를 16℃에서 12시간 주기로 2000 lux의 형광등 하에서 1개월마다 이식하면서 배양하였다. 총 탄수화물 및 단백질 함량을 측정하였으며, 최적 배양온도는 14℃～18℃였다. PES-한천배지 상에서 2.0%의 NaCl 농도가 callus의 성장에 가장 우수하였다.

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INTRODUCTION

Most seaweeds are being harvested from naturally existing seaweed beds, and thus resulted in over harvested populations and slow regeneration which does not meet the demand (Moss 1978). In the Far East, however, there is a developing industry of algal cultivation involving some level of selecting seed stock, seeding artificial substrates and supplying the seeded farms with fertilizers (Tseng 1981). *Porphyra* sp. is one of the aquacultural seaweeds having the highest production cost in Korea. Tissue and cell culture will serve as a meterial for genetic improvements of marine algae. But seaweed tissue culture involves lots of problems which are quite different from higher plant. Seaweed meristematic cells are located on the surface and will be damaged upon application of chemical agents to obtain axenic tissues. Little is known about specific hormones controlling seaweed growth and differentiation. Seaweeds do not have seed stock which could serve the physiological equivalent of the resistant seed of higher plants (Polne−Fuller and Gibor 1986a). Gibor *et al.* (1981) reported on the development of a relatively simple procedure for obtaining clean, bacteria free algal fronds from material collected from the field. Tissues of *Porphyra perforata* and *P. yezoensis* were cultured, and calluses and isolated single cells were regenerated into new plants(Polne−Fuller *et al.* 1984; Polne−Fuller *et al.* 1986 ; Polne−Fuller and Gibor 1986b; Polne−Fuller and Gibor 1987). Several authors have addressed protoplast isolation and development in *Porphyra* sp.(Fujita and Migita 1985; Chen, 1986 ; Chen 1987 ; Liu and Gorbon 1987 ; Song and Chung 1988). This study is intended to establish procedures for simplifying the propagation and seed stock maintenance to improve the genetic qualities of the most valuable aquacultural seaweed, *P. yezoensis* foma *narawaensis.*

MATERIALS AND METHODS

Aquacultural seaweed of *P. yezoensis* foma *narawaensis* was collected at the *Porphyra* sp. farm near the mouth of the Nakdong River, Pusan, Korea. The vegetative area of blade served as an initial material for obtaining callus. The procedure for obtaining clean tissues was modified from the methods of Chen (1986), Polne−Fuller and Gibor (1986a). The blade was brushed with cotton plug and vortexed 4 times for every 30 seconds in sterile seawater to remove epiphytes. One of 3cm×3 cm sized blade was successively submerged into 1% Betadine solution containing 2%.of Triton X-100 for 2 min, washed 4 times thoroughly with sterile seawater, and submerged again into a mixed antibiotics solution for 2 days at 10℃. The antibiotics solution contained 300 μg of ampicillin, 50 μg of chloramphenicol, 200 μg of kanamycin, 100 μg of streptomycin, and 0.8 mg of nutrient broth per ml of seawater. The blade was finally washed thoroughly. Sections of the axenic fragments were prepared as 2mm×2mm in size. The axenic tissues were placed on PES medium (Provasoli 1968a) containing 1.5% agar, and incubated at 16℃ under 2000 Lux of cool white fluorescent lamps with 12 hr light and 12 hr dark cycle. Explants were regarded as axenic if the pressence of infection could not be revealed by microscopic examination after 3 months of incubation on the PES-agar medium. Axenic calluses were transplanted on fresh media every 1 month. The amount of soluble protein in callus was estimated according to the method of Lowry *et al.* (1951) after heating the crushed callus suspension at 90℃ in 1 N NaOH for 10 min. to obtain complete solubilization of protein. Bovine serum albumin was used as a standard expressed for the protein amount. Total carbohydrate was estimated according to Debois *et al.* (1956) after crushing the callus suspension. Glucose was used as a standard expressed for the carbohydrate amount. At least 10 calluses were inoculated on

PES-agar medium for the callus growth in response to temperature and sodium chloride. Increase of callus weight was corrected by subtracting 0.25g fresh weight for the inoculum. All the seawater used in this work was the artificial sea water (Pawson *et al.* 1986), of which pH was adjusted to 7.8 with 50 mM Tris-HCl buffer.

RESULTS AND DISCUSSIONS

Blades of *Porphyra yezoensis* foma *narawaensis* were collected from the aquacultural seaweed farm and cleaned up according to the above procedures. *Porphyra* sp. blade contains generally five major types of cells arranged in four distinct areas on the thallus. Polne－Fuller *et al.* (1984) presented that the rates of tissue dissociation and success of regeneration of the isolated cells or protoplasts were dependent on which area of the blade the cells were isolated from. Cells isolated from vegetative area had the highest regeneration potential. Therefore, the vegetative area of blade was selected to induce callus. Cell morphology of the area is shown in Fig.1. Size of the vegetative and dividing cells was about 11 μm×12 μm. By the application of Betadine and antibiotics as sterilizing agents, axenic tissues were obtained as 60% to 92% according to the experimental conditions. The axenic tissues were incubated on PES-agar medium for 1 month. We observed the formation of brownish red fragile callus on the surface or along the edge of the sections(Fig.2). Large callus had diameter of about 2mm and height of 1.5mm. The callus was very brittle to release free cells or small clumps under a slight pressure with cover glass (Fig.3).

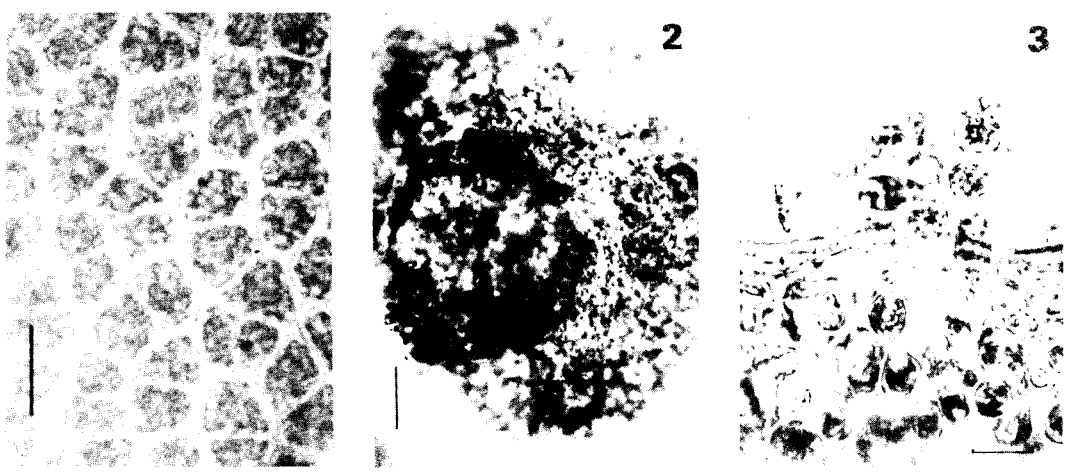

Fig.1. Vegetative cells of *Porphyra* sp. blade. The cells represent small size with diameter of about 11 to 12 μm and dividing morphology at the middle area of blade. Bar＝20 μm.

Fig.2. Callus induction on the surface of blade. The axenic vegetative tissue was grown on PES-agar medium for 1 month. bar＝200 μm.

Fig.3. Fres cells and small clumps released from the fragile callus which was slightly pressed with cover glass. Bar＝20 μm.

Cell size of the callus was increased with average diameter of 26 μm, and the size variations were from 15 μm to 50 μm. A typical callus was selected, dissected and transplanted onto the PES-agar medium, and incubated to propagate new calluses at 16℃ under 2000 Lux of fluorescence light. The growth of callus structures was a few millimeters after 3 months (Fig.4). It had a rough surface and consisted of little parts of white dead cells and most parts of brownish red alive cells. The young callus was observed to form a leafy thallus in the PES-liquid medium after 2 months (Fig.5). Such plantlets attached by rhizoids on the bottom of the culture flask. But the old callus in culture over 1 year does not regenerated into plantlet. Some calluses developed into a filamentous growth such as conchocelius. The powdery callus was regenerated into sprout to make plantlet and the filamentous conchocelius was elongated in PES-liquid medium after 10 days culture (Fig.6)

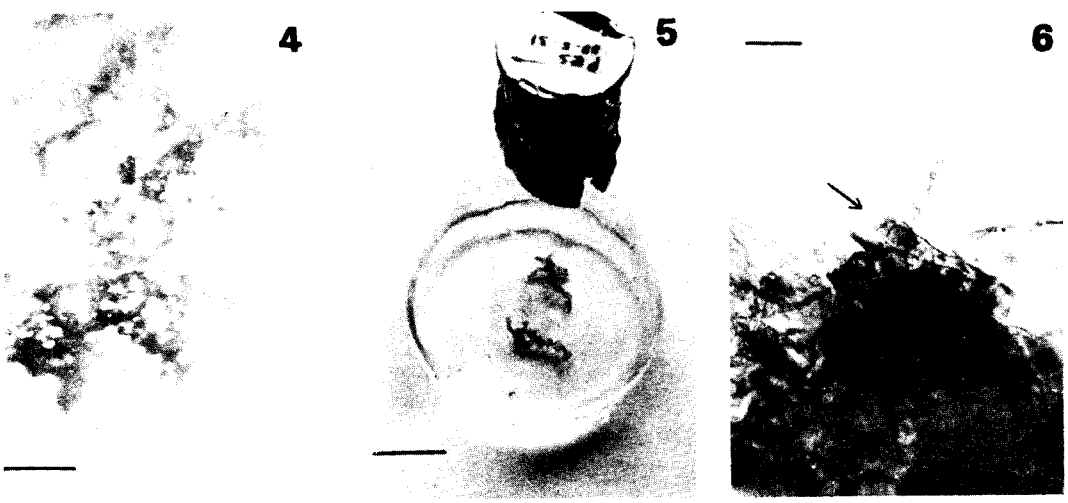

Fig.4. Callus grown on PES-agar medium for 3 months from small dissected callus particle. Bar=50 μm.

Fig.5. Regeneration of *Porphyra* sp. plantlet from callus. Plantlet was developed from callus in PES-liquid medium after 2 months culture. Bar=16 cm.

Fig.6. Sprout and conchocelius developed from callus in PES-liquid medium. 4 months old callus with powdery conchocelius was grown in PES-liquid mdeium for 10 days. Arrow indicates the development of sprout. Bar=30 μm.

We are now seeking the reason why some calluses only developed into filamentous conchocelius on the agar medium under the same culture conditions. It appeared that protoplast could be released from the callus by the action of abalone digestive enzyme. About 90 protoplasts were produced from 1 mg of callus clump in the 30 mg/ml of enzyme solution at 20℃, pH 6.0 for 18 hrs. The alive cells excluded 0.1% Evans blue dye in seawater containing 0.6 M sorbitol stabilizer. The amounts of total carbohydrate and soluble protein were estimated to know the relationship against the wet weight of fresh callus (Fig.7).

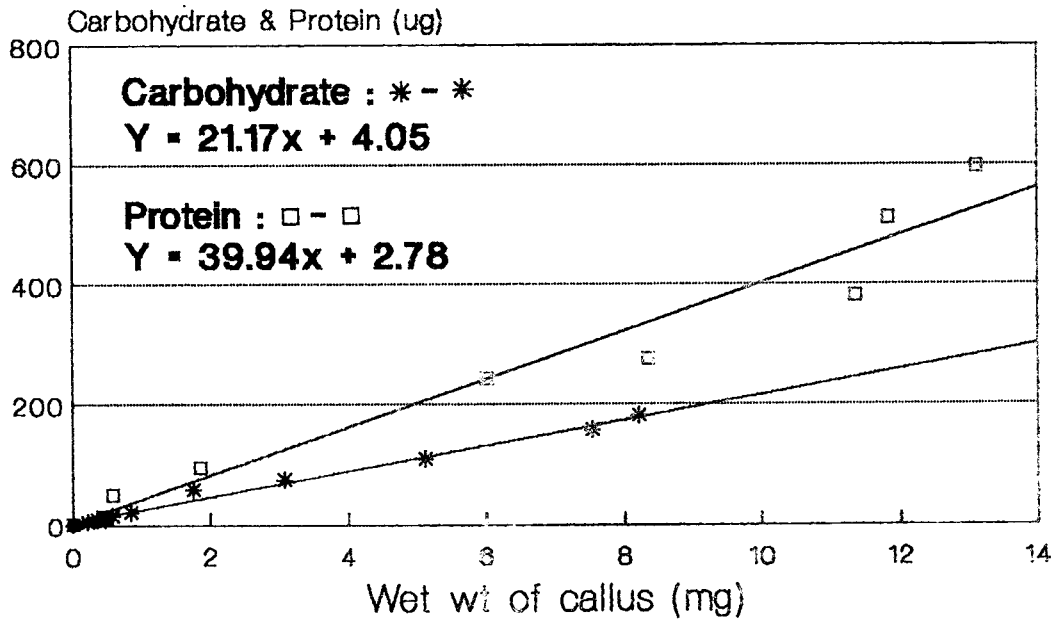

Fig.7. Relationships of total carbohydrate and soluble protein against wet weight of fresh callus. One month grown calluses were used for the materials.

The relationship was calculated by the computer systat program. The result was that total carbohydrete (μg) = 21.17 × callus (mg) + 4.05 as expressed by the glucose standard. Soluble protein (μg) = 39.94 × callus (mg) + 2.78 as expressed by the bovine serum albumin standard. Optimun temperature

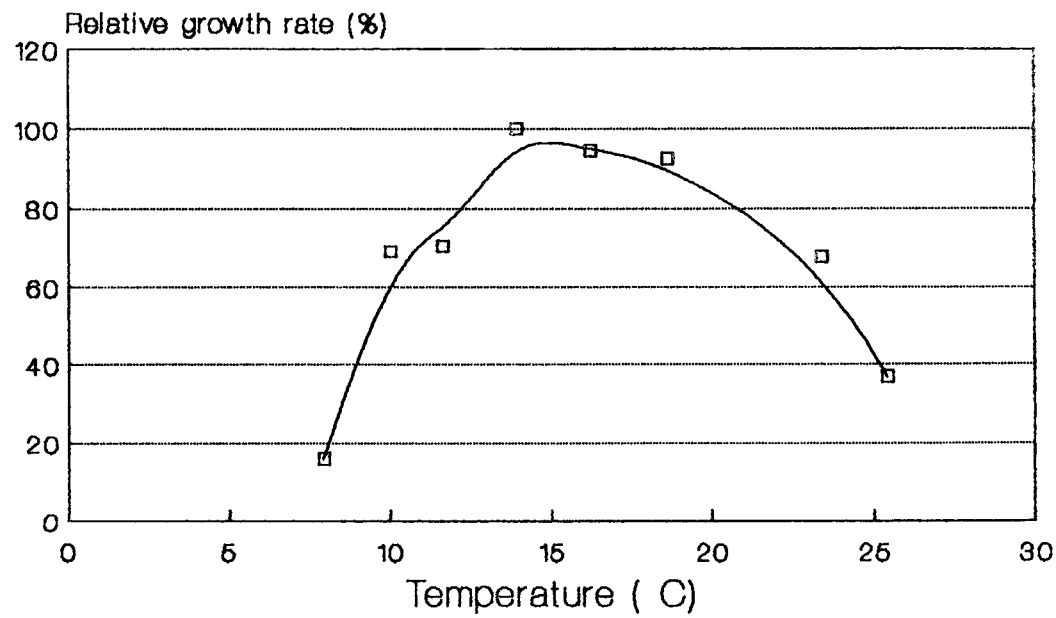

Fig.8. Optimum temperature for the callus growth under 2000 Lux light at 16℃.

for the callus formation and maintenance in most marine algae occured at 18℃ to 20℃ (Polne－Fuller and Gibor 1987 ; Gusev *et al.* 1987). Our studies revealed that the callus growth occured at 14℃ to 18℃ as shown in Fig.8. The effect of sodium chloride was examined to know the salinity tolerance for the callus cell growth. Optimum concentration of sodium chloride was 2.0% for the callus growth in PES-agar medium (Fig.9).

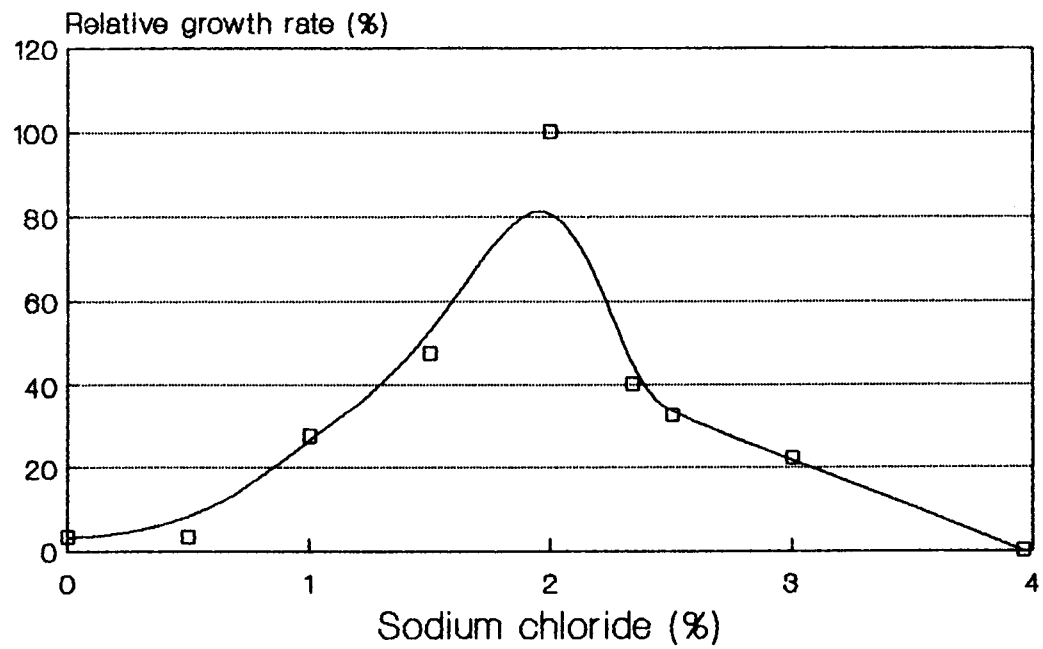

Fig.9. Optimum concentration of sodium chloride for the callus grown in PES-agar mdeium.

Therefore, this aquacultural *Porphyra* sp. appeared to be adepted to the lower salinity in seaweed farm than the sea salinity of 2.3% sodium chloride in artificial seawater. All callus inoculum at 0% sodium chloride was discolored and swelled within 3 weeks. Twenty % of inoculum at 0.5% sodium chloride had the same result. Thd callus inoculum was not attached on agar medium over 4% or sodium chloride. All inoculum was shrinked at 4.5% of sodium chloride. This paper is a preliminary step in developing a strain improvement against low salinity for the aquaculture farm near the river mouth.

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