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Recent advances in the understanding of the biological basis for *Gigartina skottsbergii* (Rhodophyta) cultivation in Chile

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Abstract

The demand in Chile for carrageenophytic algae has increased strongly during the last 3 years, with emphasis on *Gigartina skottsbergii*, a species representing landings of 32 438 t (wet) during 1996. Various sources of information indicate that this species is being over-exploited and therefore the development of cultivation technologies is needed to support the local carrageenan industry. In this study we summarize currently available information on laboratory, outdoor tank and open sea culture of *G. skottsbergii*. The results indicate that viable spores of *G. skottsbergii* can be obtained, mainly during winter, with germination rates of both tetraspores and carpospores, up to 40%. Germlings of *G. skottsbergii* were succesfully transplanted from the laboratory to outdoor tanks, where they displayed survival values higher than 80% during spring. Experimental trials in the field indicate that *G. skottsbergii* can be cultivated on rope systems, with tissue fragments used as inoculum. This last result suggests that regeneration from fragments is an alternative method for propagation and massive cultivation of *G. skottsbergii* in Chile.

Introduction

In Chile, the demand for carrageenophytic algae has increased steadily in the past 3 years. The most valuable species is *Gigartina skottsbergii* Setchell et Gardner, with landings of 32 438 wet t during 1996 (Buschmann et al., 1998). *G. skottsbergii* shows a seasonal variation in biomass, with a maximum recorded during summer, on both the Atlantic and Pacific coasts of southern South America (Piriz, 1996; Zamorano & Westermeier, 1996). Available information indicates that this species is being overexploited and therefore the development of cultivation technologies is urgently needed to support the local carrageenan industry. In this study we summarize the current status of a long-term research program, initiated in 1997, whose main objective is to provide the basic biological knowledge needed to allow the profitable farming of this species. In this context we concentrate on aspects related to spore availability and viability, germling suvivorship in the laboratory and in outdoor tanks, as well as alternative propagation methods in the field.

Materials and methods

All algal material used in this study was obtained from a subtidal population of *G. skottsbergii* located in Ancud (41° 51′ S, 73° 49′ W) in southern Chile (Figure 1). Samples for transmission electron microscopy (TEM) were fixed in 1.5% glutaraldehyde in 0.45 μm filtered seawater for 2 h at room temperature. Post-fixation was done in 1% osmium tetroxide. Dehydration in ethanol was followed by embedding in Spurr's resin. Thin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963). Photographs were taken in a Siemens ELMISKOP IA TEM operated at 60 kV.

Germination experiments were done using carposporophytic and tetrasporophytic fronds collected

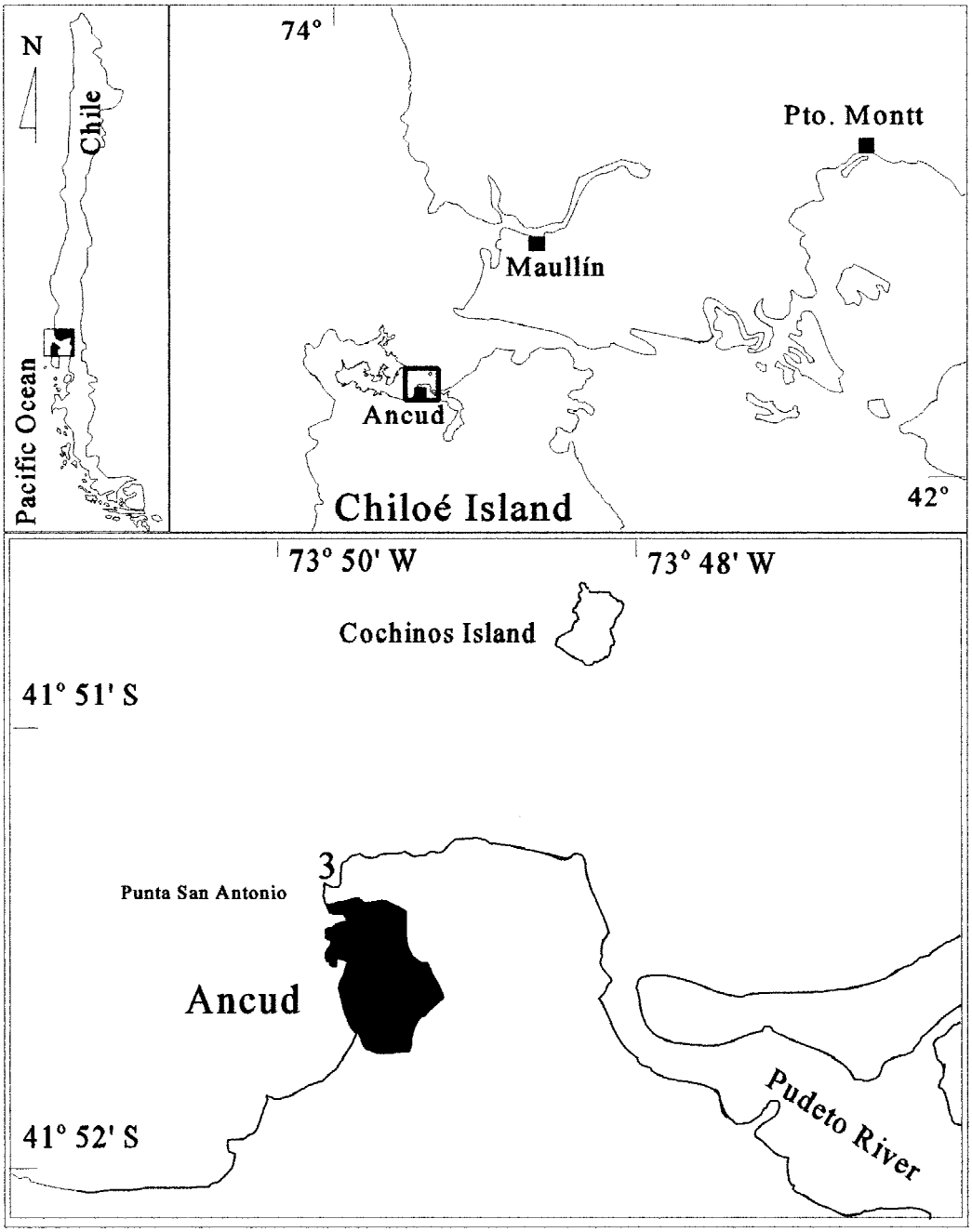

Figure 1. Map of the study area and location of the collection site (3) of *Gigartina skottsbergii* in southern Chile.

in Ancud (Figure 1). These fronds were brought to the laboratory, rinsed with sterile seawater and air dried for 10 min at room temperature. Subsequently, fragments of fertile tissues were transferred to Petri dishes with Provasoli culture medium and incubated in growth chambers at 5, 10 and 15 °C, a neutral photoperiod of 12:12 h light:dark and a photon irradiance of 48 μmol m^{-2} s^{-1}. A total of five Petri dishes were used for each temperature and karyological phase. After six days, the percentage of germination was determined by counting the number of germinated spores and the total number of spores, in three microscopic

fields per dish. Positive germination was scored when a cell presented clear evidence of a complete cell division. This experimental protocol was repeated at monthly intervals during a full year to determine the existence of seasonality.

To assess the feasibiity of using laboratory-raised germlings as inoculum for an outdoor culture system, we transplanted plastic Petri dishes, 6 cm in diameter, from the laboratory to 40 l plastic outdoor containers with a water regime of a full volume replacement every 3 days. These containers were placed in 500 l tanks (see Buschmann et al., 1994, for further details) to maintain a constant temperature and were covered with black shadowing mesh to reduce to 1% and 10% the levels of irradinace reaching the germlings. At mid-day and with high solar irradiance, these treatments produce very different light environments in the culture tanks, which mimic the conditions in the field. Preliminary observations demonstrated that full irradiance levels in outdoor tanks rapidly bleached the germlings. The experiment began in November with a mean temperature of 12–14 °C, salinity of 29–31‰ and pH of 8.2–8.4. Survival of the germlings was monitored twice a month by photographic recording of fixed 1 cm^2 quadrats (n = 6). The final size of the plantlets was estimated by haphazardly removing a minimum of ten individuals per Petri dish ($n > 30$) and measuring individual heights. Survival and final size of the plantlets in the two light conditions were compared by one-way ANOVA after ensuring normality and homocedasticity of variances.

For assessing the wound healing responses and regeneration, vegetative plants were brought to the laboratory and rinsed with sterile seawater. Fragments of tissue, 2 × 2 cm each, were excised from the borders of the fronds. A total of ten fragments were transferred to 500-ml culture flasks containing 250 ml of sterile seawater. The culture conditions were 5, 10 and 15 °C at 5 μmol m^{-2} s^{-1} and neutral photoperiod. Three replicate flasks were used in each experimental condition and the culture medium was changed every 3 days. This experiment lasted 32 days, after which the number of healed fragments was recorded. Data were analyzed by a two-way ANOVA with time as a repeated measurement, as data are time dependent (Wilkinson et al., 1992). Normality and homocedasticity of variances were tested before running the standard analyses.

To test the ability of *G. skottsbergii* to regenerate in the field, 16 fragments excised from immature fronds were fastened to an 8-m nylon cord by entangling the pieces of tissue between the nylon fibers. The nylon cord was maintained at 20 cm above the bottom by fastening it to concrete blocks. The initial size of the fragments was 5 × 30 cm and the meristems at the edges of each portion were left untouched. Changes in size were recorded on a monthly basis by SCUBA divers.

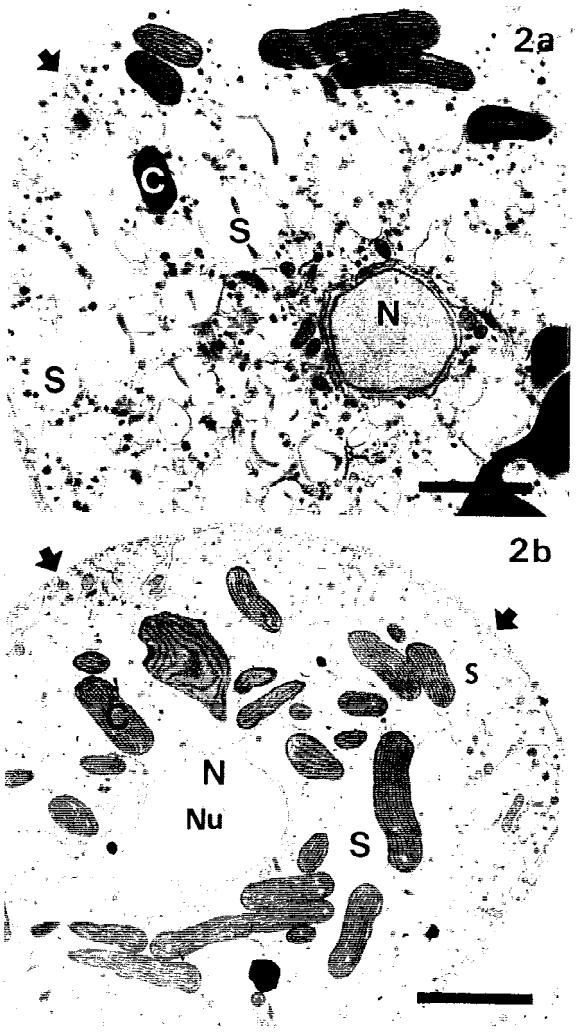

Figure 2. Ultrastructure of carpospore (a) and tetraspore (b) of *G. skottsbergii*. N, nucleus; C, chloroplast; S, starch; Nu, nucleolus. Arrows show plasmalemma without cell wall. The bar represents 5 μm.

Results

From October to April the number of spores available was very low and the germination success was zero. Carpospores and tetraspores were available in autumn to early spring, with a maximum in June. Ultrastructural information indicates that spores lack a cell wall at the moment of release (Figure 2a, b). Newly released spores are characterized by a central nucleus with a clearly distinguishable nucleolus. Abundant starch granules, plastids scattered around the nucleus, and large numbers of vesicles containing electron dense fibrillar material, likely to be the precursor of the early stages of cell wall, were present. No differences could be detected at the TEM level between carpospores and tetraspores (Figure 2a, b).

Tetraspore germination varied according to the month of frond collection in the field, with a maximum value of ca 40% observed in August (Figure 3A). Temperature modified the intensity of germination, with significantly higher values (F = 10.086; $p<0.001$) recorded at 5 °C as compared to 10 and 15 °C (Figure 3A). Carpospores showed similar responses to those displayed by tetraspores (Figure 3B). Both types of spores were successfully cultivated under laboratory conditions up to the stage of juvenile fronds of ca 10 mm characterized by an umbrella-like frond shape (Figure 4a–d). This level of development was achieved in 7 months under an irradiance of 25 μmol m^{-2} s^{-1}, a temperature of 10–15 °C and neutral photoperiod.

Germlings of *G. skottsbergii*, with an initial size of 0.017 mm, were transplanted from the laboratory to outdoor tanks. After 1 month of culture and regardless of the irradiance conditions, survivorship was high, ranging from 68 to 85% (Figure 5A). During this period the plantlets reached 0.012 mm in height, with no significant differences induced by the two levels of irradiance (Figure 5B). In 3 months, plantlets of both karyological phases reached 1–2 mm, with a morphology similar to that displayed in the laboratory (Figure 4d).

Laboratory wound healing responses were significantly enhanced (F = 33.74; $p<0.001$) by temperature, reaching mean values close to 100% at 10 and 15 °C (Figure 6). Wound healing responses also varied significantly through the year (F = 6.125; $p < 0.015$), although did not significantly interact (F = 2.375; $P > 0.11$) with temperature. At 10 and 15 °C, the maximum wound healing was reached at day 20. The histological events occuring during wound healing are by a new cortex that seals the cut and provides meristematic cells, which eventually issue new uprights (Figure 7a–c). Following the success of the above experiments, a first trial was done to test the possible use of tissue fragments for regeneration in the field (Figure 8). The fragments showed a significant increase (F = 4.899; $p < 0.03$) in length, with an average increase of 44.6% for a 6-month period. The width of the fragments also increased significantly (F = 44.394; $p < 0.0001$) with a mean value of 48.6%. At the end of the study, the fragments of *G. skottsbergii* did not show

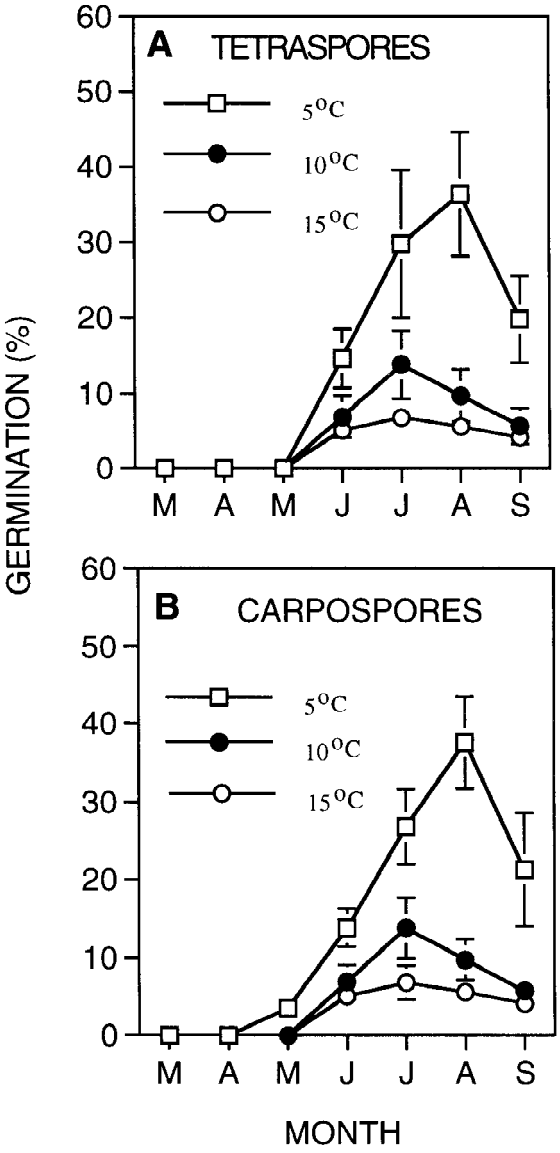

Figure 3. Monthly variation (mean ±1 S.E.) of the germination responses (%) of *G. skottsbergii* at different temperatures. A, Tetraspores and B, carpospores.

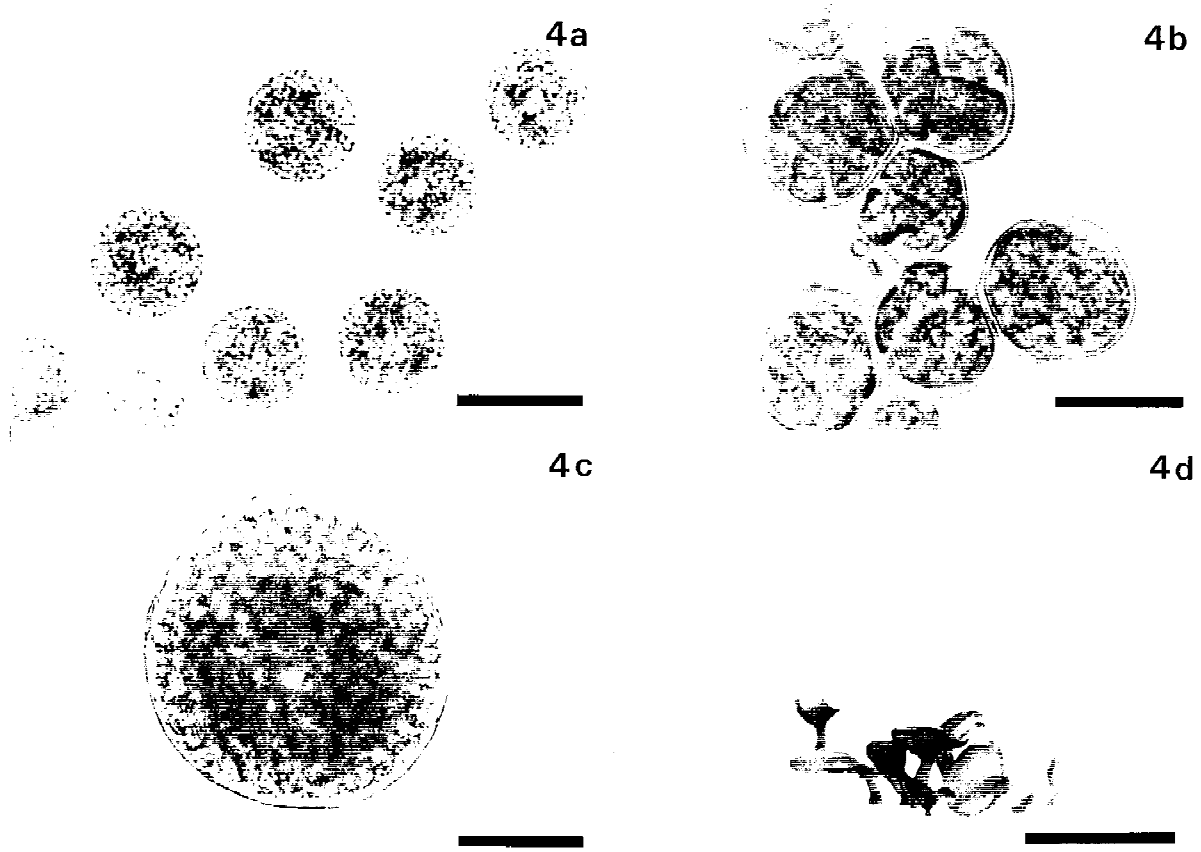

Figure 4. Development stages of gametophytes of *G. skottsbergii* in laboratory culture conditions. a, Tetraspores at time zero; bar = 30 μm; b, tetraspore germination after 4–6 days of cultivation, bar = 30 μm; c, gametophyte discs after 30 days of cultivation, bar = 40 μm; and d, gametophytic plantlets after 7 months of cultivation in the laboratory, bar = 5 mm.

any macroscopic indication of epiphytic or endophytic infections.

Discussion

Our results indicate that spores of *G. skottsbergii* do not have cell walls at the time of release. This seems to be a common feature in members of the Gigartinales and Gracilariales, as indicated by the reports on *Gracilaria* (Santelices et al., 1996), and on *Mazzaella*, *Sarcothalia*, *Chondrus*, *Ahnfeltia* and *Ahnfeltiopsis* (Santelices et al., 1997). At early stages of development (e.g. 2–4 h after release), fine structure appears normal with no indication of disruption or abnormal spatial distribution of the organelles within the cell, allowing us to individualize which spores were mature (e.g. adequate for germination). However, preliminary experiments in our laboratory testing spore viability using vital stains suggest that most spores (>90%) released under laboratory conditions are physiologically viable. This is an important finding because one of the major bottle necks preventing massive production of juveniles from spores in *G. skottsbergii* is the high mortality rates of spores before germination. It seems apparent now that spore mortality is the result of a factor, operating after the release of the spores, which triggers premature spore death. This factor remains to be found, and efforts are being invested to assess some potential candidates. One of them is temperature. It is known that *G. skottsbergii* is a cold water species, with a thermal limit reported to be 5 °C (Bischoff-Bäsmann & Wiencke, 1996). It is likely, however, that this limit is different for populations occurring in the northern extreme of the species distribution along the Pacific coast. This is supported by our results on germination, where the best responses were obtained at temperatures of 5 °C or higher. Without

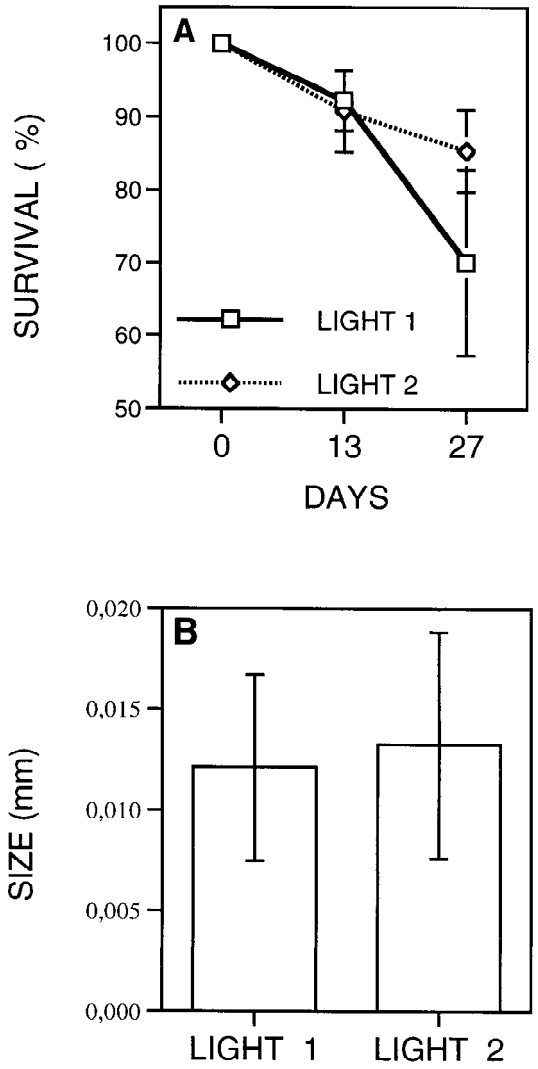

Figure 5. Mean (±1 S.D.) survivorship (A) and final size (B) of plantlets of *G. skottsbergii* transplanted to outdoor culture conditions under two light irradiances: Light 1 = 10% and Light 2 = 1% of the natural irradiance.

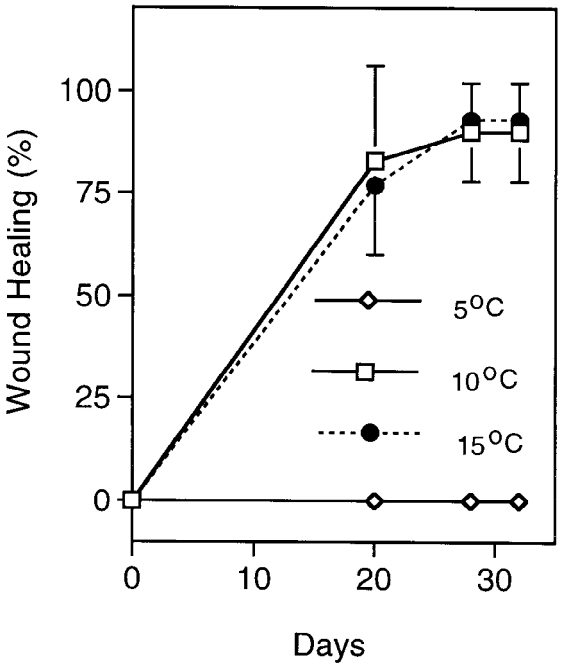

Figure 6. Wound healing responses (mean ±1 S.D.) of *G. skottsbergii* as a function of temperature under a low irradiance regime.

ruling out rapid adaptive responses in the laboratory as an explanation for the better responses at higher temperatures, the effect of this factor needs to be fully addressed. The importance of this issue is based on the fact that the major exploitation of *G. skottsbergii* is currently taking place in the northern extreme of its distribution, and because of logistic constraints, it is expected that farming operations will be established in the same geographic area.

Gigartina skottsbergii produces carrageenans of the kappa family in the gametophytic phase and carrageenans of the lambda family in the sporophytic phase (Piriz & Cerezo, 1991). Regardless of the methodology used to obtain spores in the laboratory, tetraspores were always more rapidly available and handled than carpospores. Post-germination development also showed better performances (e.g. growth and survivorship) in gametophytic than in sporophytic germlings (Correa et al., unpubl.). These differences may explain, at least partially, the higher abundance of gametophytic individuals found in both the Atlantic and Pacific coasts of South America (Piriz, 1996; Zamorano & Westermeier, 1996). Cultivating only plants derived from tetraspores (i.e. gametophytes) would result in a production of raw material only suitable for the extraction of carrageenans of the kappa family. As this may be an undesired feature in a farming context, alternatives must be found to allow cultivation of the two phases of *G. skottsbergii*.

Tissue culture and protoplast production have been attempted in other algae, with various degrees of success (Dawes & Koch, 1991; Kloareg et al., 1991; LeGall et al., 1990; Polne-Fuller & Gibor, 1986; Saga et al., 1986). These approaches are expensive and in most cases have been considered as unrealistic alternatives for large scale seaweed aquaculture.

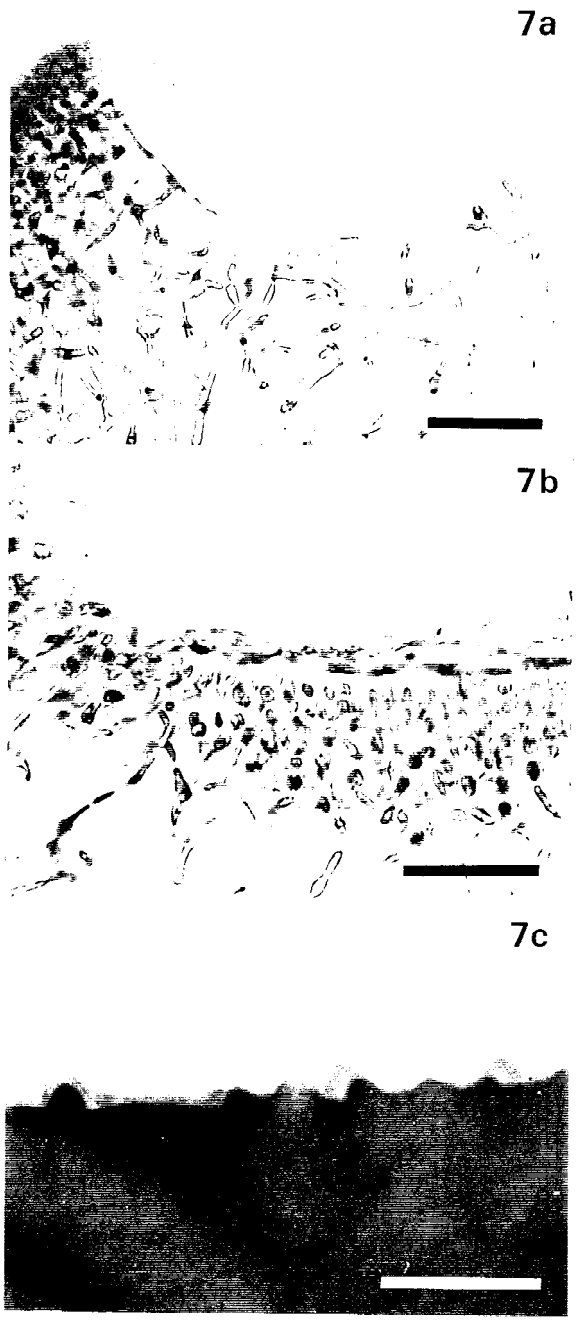

Figure 7. Photographs showing the wound healing and regeneration responses of *G. skottsbergii* in the laboratory. a, Tissue at time zero, bar = 100 μm; b, tissue showing a wound healing response after 30 days of cultivation, bar = 50 μm; and c, regeneration tissues after 3 month in culture conditions, bar = 1.5 mm.

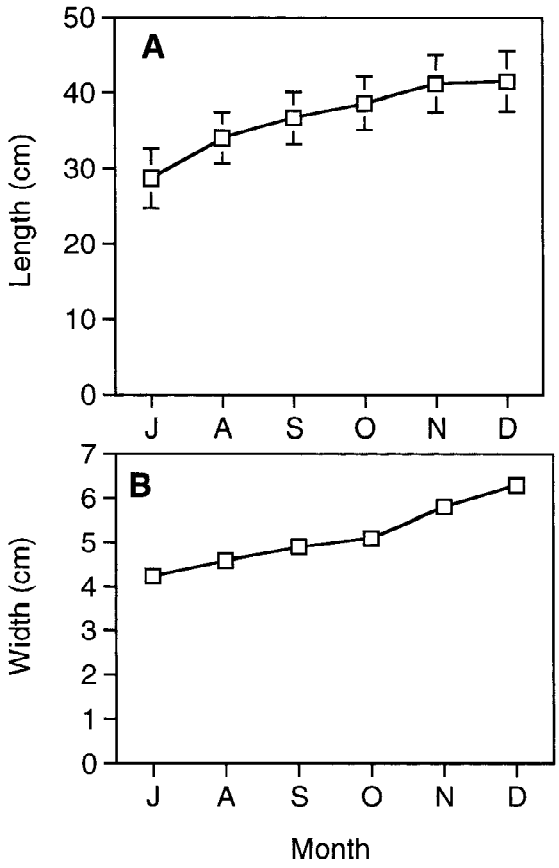

Figure 8. Growth responses of *G. skottsbergii* of tissue fragments. Data show average (±1 S.E.) of the length (A) and width (B) of the fragments.

Still, they should not be fully ruled out for *G. skottsbergii*. A simpler approach, which has been one of the main reasons for the farming success of *Gracilaria* (Buschmann et al., 1995; Santelices & Doty, 1986), *Eucheuma/Kappaphycus* (Azanza-Corrales et al., 1996), *Grateloupia* (Iima et al., 1995) and *Chondrus* (Craigie, 1990), is to rely upon the regeneration capacity of the algal thallus. This seems to be a promising aspect in *G. skottsbergii*, which showed wound healing responses in both laboratory and in the field.

The demand for *G. skottsbergii* is likely to remain high in the near future and natural stocks appear to be quickly declining (Buschmann et al., 1998; Westermeier et al., 1997). For this reason, gaining basic biological knowledge on this resource is required, and its application is considered the only way to allow long-term sustained supply of raw material for the carrageenan industry.

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