A Simplified Method to Isolate and Cultivate, Laurencia brongniartii J. Agardh (Rhodophyta, Ceramiales) from Kagoshima, Japan

A Simplified Method to Isolate and Cultivate, *Laurencia brongniartii* J. Agardh (Rhodophyta, Ceramiales) from Kagoshima, Japan

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Abstract: A simplified method to isolate and cultivate the red alga, *Laurencia brongniartii* J. Agardh was established to prepare it for indoor tank cultivation. This method was addressed so that relatively inexperienced personnel can start and maintain laboratory cultures of the organism. The method uses the apical tissue of branch tips which are relatively free of epiphytes and requires almost no specialized equipment or facilities. The effect of size and location of apical tissue on the relative growth rates (%RGR) and survival rates (%SR) were examined to determine the size and location of apical tissue that would promote the highest survivability and fastest growth rates. The larger sizes (5.0 mm) showed higher %SR (62.5%) and smaller sizes (1.0 mm) showed higher %RGR (4.43%/day). There was also a significant effect of tissue location, with material closest to the apex showing the highest %RGR (2.16%/day). Different concentrations of nitrate were not significant, however higher concentrations appeared to have a negative impact on %RGR. Temperature and light had a significant effect on %RGR. Optimal growth was observed at 24 and 28°C and at an irradiance of 20 μ mol photons/m^2/s.

Key words: *Laurencia brongniartii*; Growth rate; Laboratory culture; Survival rate

Antibiotic resistance is a serious economic and social issue (Horikawa et al. 1995, 1996, 1999; Kamei et al. 1995; Harada et al. 1996a, 1996b, 1997). In an era when the use of chemicals are discouraged and the number of cases of antibiotic-resistant micro-organisms increasing, alternatives are needed to control these micro-organisms. *Chlorella vulgaris* was one of the first algae which yielded extracts that expressed antimicrobial activity (Pratt et al. 1944). Thereafter, investigations into the application of algae as a source of pharmaceuticals have steadily increased with potential to become a major new source of antimicrobial compounds (Schwartz 1990). Surveys conducted on indigenous macroalgae of Japan found many species of algae that produce bioactive compounds capable of controlling antibiotic-resistant micro-organisms or exhibiting antitumor activity (Horikawa et al. 1995, 1996, 1999; Kamei et al. 1995; Harada et al. 1996a, 1996b, 1997). For example, Horikawa et al. (1995) reports that the brown alga *Dictyopteris undulata* and the red algae *Laurencia okamurae*, *Laurencia brongniartii*, and *Odonthalia corymbifera* have potent bactericidal activity. In particular, the red alga, *L. brongniartii* J. Agardh (Ceramiales, Rhodophyta) is reported to produce brominated compounds that are effective in controlling methicillin-resistant *Staphylococcus aureus*, *Aspergillus fumigatus*, *Trichophyton rubrum*, *Streptomyces acidiscabies*, and *Streptomyces scabies* (Horikawa et al. 1995, 1996a, 1999; Kamei et al. 1995).

Laurencia brongniartii J. Agardh is found throughout the Amami Oshima Island area of Kagoshima Prefecture, Japan, and there is great

interest by the local fisheries authorities to exploit this species as a new fisheries resource. However, Nishihara et al. (in press) suggests that sustainable harvesting of this species may be difficult. They note that *L. brongniartii* grows as widely spaced, small populations of individuals with an average coverage of 20% and population density of 16 individuals/m^2 (Nishihara et al. in press). Additionally, this area of Japan is under constant threat of high waves and poor weather during the typhoon season which coincides with the growing period of *L. brongniartii* (Nishihara et al. in press). This suggests that tank cultivation of this species may be a more appropriate means of commercialization of this species.
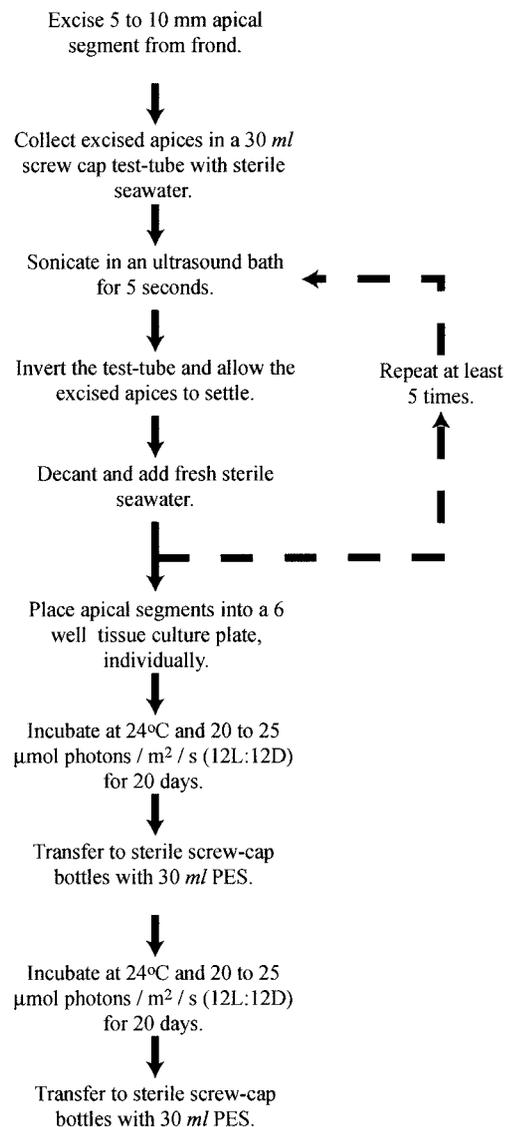
Therefore, to succeed in cultivating this species for commercialization, it is first necessary to establish a simple method to achieve a unialgal culture and to determine its fundamental cultivation requirements. Additionally, it is important to develop a method that is simple to conduct, so that cultivation of this species can be accomplished by personnel without specific technical expertise. Specifically, it is hoped that *L. brongniartii* would be developed into a new fisheries resource for Amami Oshima. Techniques commonly used in the isolation and cultivation of *Gracilaria* will be used as a template to develop an isolation and cultivation method for *L. brongniartii* (Lapointe et al. 1984; Garcia-Reina et al. 1987; Dawes et al. 1999; Yokoya 2000; Nishihara et al. in press). The primary objectives of this study are to develop a simplified method to isolate and cultivate unialgal explants of *L. brongniartii* by non-technical staff and to clarify its optimal cultivation requirements for laboratory cultivation and future tank culture.

Materials and Methods

Collection location and initial preparation

Laurencia brongniartii J. Agardh was collected during May through December 2001 from a coral reef located off the coast of Tsuchihama Beach, Amami Oshima Island, Kagoshima, Japan (31°15' N, 130°26' E). One to two whole plants were harvested and placed

in 500 *ml* polyethylene bottles containing seawater from the collection sites. The bottles were then placed into a cooler box and kept in the dark at the same water temperature of the collection sites. Collected algae were taken to the laboratory and removed of epiphytic animals by washing them in seawater diluted to 50% with fresh tap water. The algae were washed in the diluted seawater at least three times or until no animals were visible, then returned to full strength seawater. Finally, any visible epiphytes were removed by using tweezers and soft paint brushes. They were then washed at least five times in seawater and allowed to rest over night.

Excise 5 to 10 mm apical segment from frond.

↓

Collect excised apices in a 30 *ml* screw cap test-tube with sterile seawater.

↓

Sonicate in an ultrasound bath for 5 seconds.

↓

Invert the test-tube and allow the excised apices to settle.

↓

Decant and add fresh sterile seawater.

Repeat at least 5 times.

↓

Place apical segments into a 6 well tissue culture plate, individually.

↓

Incubate at 24°C and 20 to 25 μmol photons / m^2 / s (12L:12D) for 20 days.

↓

Transfer to sterile screw-cap bottles with 30 *ml* PES.

↓

Incubate at 24°C and 20 to 25 μmol photons / m^2 / s (12L:12D) for 20 days.

↓

Transfer to sterile screw-cap bottles with 30 *ml* PES.

Fig. 1. Outline of the protocol developed to isolate *Laurencia brongniartii* apical tissue from the branch tips for unialgal cultures.

Isolation procedure

The outline of the isolation procedure is shown in Fig. 1. The organisms that were allowed to rest overnight and which appeared healthy were chosen as candidates for isolation. Apical tissue from branch tips that were free from epiphytes and 5 to 10 mm in length, were excised and placed into 30 *ml* screw cap test-tubes with 20 to 30 *ml* of sterile seawater. Collected apical tissue were sonicated in an ultrasound bath (VC-1, As One, Inc.) for 5 s (Garcia-Reina et al. 1987). The test-tube was inverted to free small particles from the apical tissue and the liquid was decanted. This was repeated five times. After the fifth time, each apical tissue was introduced into individual wells of a 6 well tissue culture plate (Nunc Nalge International) with 3 *ml* of culture media and incubated at 24℃ and 25 μmol photons/m^2/s for 20 days under a 12 : 12 light-dark cycle (12L : 12D). Culture media was PES (Provasoli's Enriched Seawater) and 2 g/*ml* GeO$_2$ to control for diatoms at a salinity of 32 PSU (McDermid 1989). At the end of 20 days, uncontaminated healthy explants were transferred to 30 *ml* screw cap bottles and incubated under the same conditions for 20 days without GeO$_2$. After this stage, any healthy uncontaminated explants were transferred to fresh media and were considered unialgal after microscopic inspection. Isolated explants were then used in the culture media, salinity and light- temperature experiments. Media changes were done every other day for all experiments in this study.

Size and location

Preparation of apical tissue, from the branch tips, for experiments to explore the effect of size and location of the tissue were identical to that of the isolation procedure with a few modifications. Three size classes of 1.0, 2.5, and 5.0 mm were selected to determine the effects of initial apical tissue size on the growth rate (%RGR) and survival rates (%SR) of explants. The length of each apical tissue was measured with a millimeter scale using the apex as the origin. All material used were excised from a single immature plant. In addition to size, effects of tissue location on growth and survival rates of explants were also observed for three categories. The locations of the apical tissue were categorized as upper, middle, and lower of which all were 2.5 mm in size.

For both experiments, 24 apical tissues were individually cultured in a 24 well tissue culture plate (Nunc Nalge International). The tissue culture plates were incubated at 24℃ for 20 days at irradiances of 25 μmol photons/m^2/s under a light cycle of 12L : 12D. The medium used in this study was PES at 32 PSU. Growth rates and survival rates were computed after 20 days.

The effects of nutrient concentration on growth

The effects of medium concentration on the growth of explants were examined by varying the concentrations of nitrate (NO$_3$-N) and phosphate (PO$_4$-P) in PES. Phosphate was adjusted so that the N to P ratio was maintained at 17.8. No vitamins were added and the concentration of the other nutrients was constant for all treatments. Four concentrations of media and one control were set, for a total of 5 treatments with 6 replicates each. The control was unenriched sterilized seawater and the four concentrations of medium were PES enriched with 41.2 μmol NO$_3$-N/*l* and 2.3 μmol PO$_4$-P/*l* (0.5NP), 82.4 μmol NO$_3$-N/*l* and 4.6 μmol PO$_4$-P/*l* (1NP), 164.7 μmol NO$_3$-N/*l* and 9.3 μmol PO$_4$-P/*l* (2NP), and 329.4 μmol NO$_3$-N/*l* and 18.5 μmol PO$_4$-P/*l* (4NP). Explants were established from apical tissue excised from the branch tips. The explants were incubated for 20 days at a salinity of 32 PSU and at irradiances of 25 μmol photons/m^2/s under a light cycle of 12L : 12D. Incubation temperature was 24℃. Growth rates and survival rates were calculated after 20 days.

The effects of salinity on growth

The effects of 5 treatments of salinity at 17.2, 25.3, 33.4, 41.0, and 48.6 PSU were selected with 5 replicates per treatment. Fresh seawater was diluted with distilled water to create a stock solution of 17.2 PSU seawater. Higher salinities

were achieved by the addition of appropriate amounts of NaCl to reach the desired salinity. The seawater of various salinities was then autoclave sterilized. Explants from the apical tissue taken from branch tips were incubated for 20 days under the same physical conditions outlined for the previous experiment with the exception that the media used was PES.

The effects of light and temperature on growth

Light and temperature were varied over a combination of 3 levels each to examine the effect on the growth of explants. Explants were established from the apical tissue of the branch tips. Three temperatures of 20, 24, and 28℃ and three irradiances of 10, 20, and 50 μmol photons/m^2/s were chosen. The light cycle and medium were identical to that used for the isolation experiment. Growth rates were calculated after 20 days.

Growth rate calculation

Due to the characteristic compressed appearance of *L. brongniartii*, growth rates were calculated by measuring the change in the surface area of explants as they grew along the bottom of the culture vessel. The culture vessels along with a scale were scanned with a CCD flat bed scanner (Epson GT-8700F, Epson, Inc.) on day 0 and on day 20. The surface areas of the explants were then processed with the public domain software ImageJ (Rasband 2003) and the %RGR (%/day) was computed. The equation for %RGR is:

$$\%RGR = \frac{\ln(\sqrt{Area_{final}}) - \ln(\sqrt{Area_{initial}})}{\ln(\sqrt{Area_{initial}}) \times (day_{final} - day_{initial})} \times 100$$

Statistical analysis

The statistical analysis of %SR was analyzed by the chi-square test, with the null hypothesis that size and location does not affect the survival. Survival was defined as viable and uncontaminated explants.

ANOVA was used to determine if there were any significant differences between treatment levels. Differences in %RGR of size, location, PES concentration, and salinity were examined

by single factor ANOVA. The effect of light and temperature on %RGR was analyzed by two factor ANOVA. In all cases, Levene's test for homogeneity were applied to check the variability in the variances and Bonferroni's multiple comparisons test was used if ANOVA detected any significant difference between treatments at the 0.05 level.

Results

Size and location

Initial size of excised apical tissue significantly affected the %SR and %RGR of explants after a period of 20 days (Fig. 2). An increase in size showed significant positive correlation with %SR ($\chi^2_{(2)} = 7.200$, $P < 0.05$). Survival rates were 25.0, 37.5, and 62.5% for sizes of 1.0, 2.5, and 5.0 mm, respectively. Conversely, %RGR decreased as size increased with significant differences between treatments ($F_{(2,27)} = 21.820$, $P < 0.001$). However Levene's test for homogeneity was also significant ($F_{(2,27)} = 30.079$, $P < 0.001$)
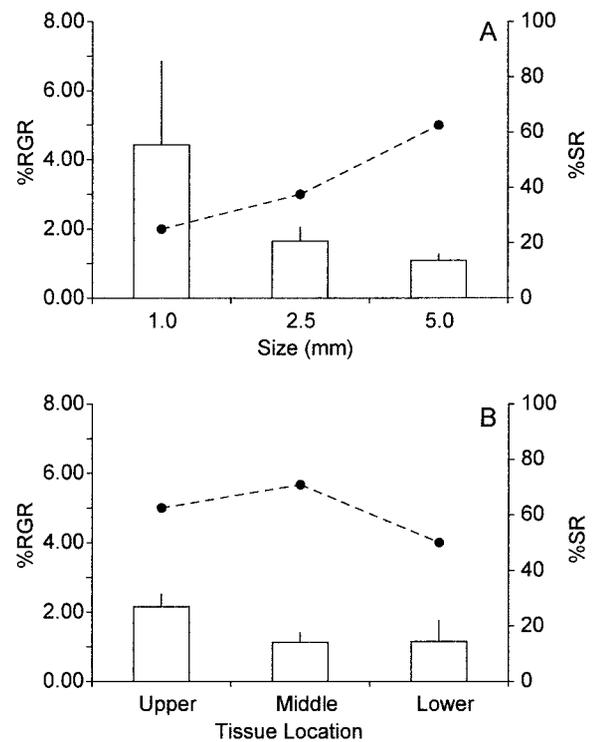
Fig. 2. The survival rates and the relative growth rates (%RGR) of excised *Laurencia brongniartii* apical tissue, from the branch tips, according to size of tissue (A) and location of tissue (B). Black dots indicate the survival rate and white bars indicate the %RGR of each treatment. Error bars indicate the 95% confidence interval.

indicating large variations in the variance of each treatment mean. This was most likely because of the unbalanced design. There were 6 replicates for the 1.0 mm tissue size, 9 replicates for the 2.5 mm tissue size, and 15 replicates for the 5.0 mm tissue size. Regardless, Bonferroni's multiple comparisons test showed that %RGR for the 1.0 mm tissue size was significantly larger than that of the 2.5 and 5.0 mm tissue sizes ($P < 0.001$). There were no significant differences between the 2.5 and 5.0 mm tissue sizes ($P < 0.677$). The %RGR and the 95% confidence interval (95%CI) for the 1.0 mm tissue size it was 4.43 ± 0.89%/day, for the 2.5 mm tissue size it was 1.65 ± 0.72%/day, and for the 5.0 mm tissue size it was 1.10 ± 0.56%/day.

No significant correlations were found between apical tissue location and %SR ($\chi^2_{(2)} = 5.512$, $P = 0.064$), however there was significant differences in %RGR ($F_{(2,37)} = 12.171$, $P < 0.001$) and variances were homogenous ($F_{(2,37)} = 1.109$, $P = 0.341$) (Fig. 2). The %RGR of the upper location was significantly larger than that of the middle and lower locations (upper vs. middle: $P < 0.001$ and upper vs. lower: $P = 0.017$). This analysis was also unbalanced, with 14 replicates for the upper location, 17 replicates for the middle location, and 9 replicates for the lower location. The %RGR and 95%CI for the upper, middle, and lower locations were 2.16 ± 0.34, 1.13 ± 0.31, and 1.16 ± 0.42%/day, respectively. Rates for %SR were 58.3, 70.8, and 55.6%, respectively.

The effects of nutrient concentration on growth

Variances were homogenous ($F_{(4,25)} = 2.120$, $P = 0.108$) and there was no significant differences in the treatment means ($F_{(4,25)} = 1.383$, $P = 0.268$) (Fig. 3). The %RGR $\pm$ 95%CI of 0.5NP was the highest of all treatments, at a rate of 3.57 ± 1.49%/day. All treatments including the control exhibited growth after 20 days. The control and 4NP treatments showed the lowest %RGR of 2.35 ± 1.27 and 2.53 ± 0.55%/day, respectively. At the 1NP and 2NP treatments, the %RGR was 3.41 ± 1.40 and 2.78 ± 0.94%/day, respectively.

The effects of salinity on growth

All explants bleached and died by the end of the 20 day period, at the lowest (17.2 PSU) and highest (48.6 PSU) salinities (Fig. 4). Therefore these two treatments were excluded from the ANOVA and only the remaining three treatments were analyzed. The results show significant effects of salinity on %RGR of explants ($F_{(2,12)} = 5.766$, $P = 0.018$) and homogeneous variances ($F_{(2,12)} = 0.736$, $P = 0.499$). Maximum %RGR $\pm$ 95%CI occurred at 33.4 PSU (3.62 ± 1.86%/day) and the minimum occurred at 41.0 PSU (0.84 ± 1.34%/day). At 25.3 PSU, the %RGR was 2.77 ± 1.71%/day. Bonferonni's multiple comparisons test showed significant differences between 33.4 and 41.0 PSU, with the former
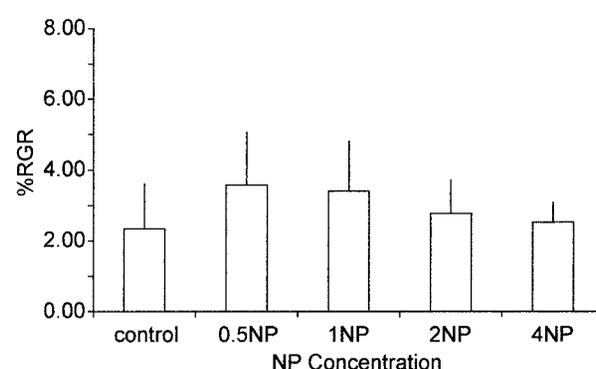
Fig. 3. The relative growth rates (%RGR) of *Laurencia brongniartii* explants, isolated from the apical tissue of the branch tips, grown in 4 treatments of PES (Provasoli's Enriched Seawater) and 1 treatment control (seawater). Error bars indicate the 95% confidence interval.
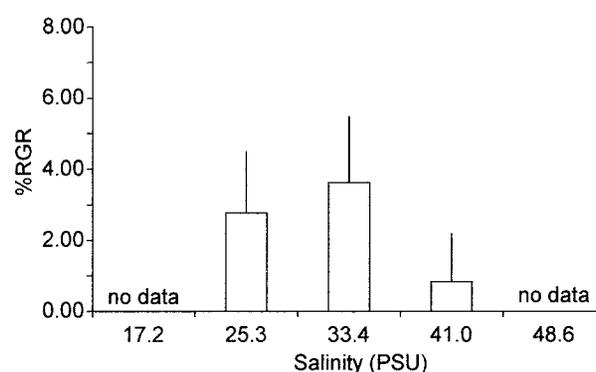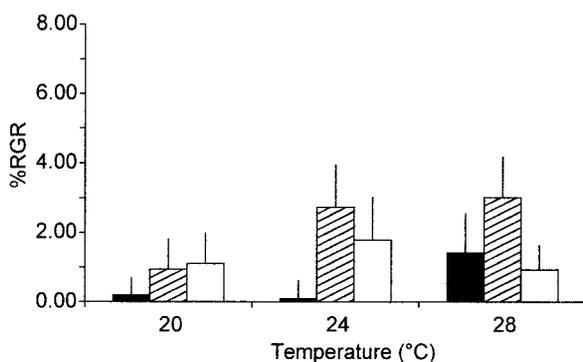

Fig. 4. The relative growth rates (%RGR) of *Laurencia brongniartii* explants, isolated from the apical tissue of the branch tips, grown in 5 treatments of salinity. Explants cultured in the 17.2 and 48.6 PSU treatments died at the conclusion of the 20 day trial. Error bars indicate the 95% confidence interval.

being significantly higher ($P = 0.019$). There were no significant differences between other combinations of treatments.

The effects of light and temperature on growth

Figure 5 shows the results of the light-temperature experiment. Growth was significantly affected by light ($F_{(2,36)} = 8.812$, $P = 0.001$) and temperature ($F_{(2,36)} = 3.793$, $P = 0.032$). Interactions between the two were not significant, however $P = 0.067$ ($F_{(4,36)} = 2.409$). Levene's test indicated homogeneous variance ($F_{(8,36)} = 0.674$, $P = 0.711$).

In general, %RGR was higher at $20\,\mu$mol photons/m^2/s and at $28°C$. Specifically, the highest %RGR $\pm$ 95%CI occurred at $20\,\mu$mol photons/m^2/s and $28°C$ (3.01 ± 1.21%/day) and the lowest %RGR occurred at $10\,\mu$mol photons/m^2/s and $24°C$ (0.11 ± 0.51%/day). Light appeared to limit %RGR at $20°C$, since growth rates increased from 0.21 ± 0.48 to 0.94 ± 0.89 to 1.10 ± 0.87%/day, over the three treatments. In the case of the higher temperatures of 24 and $28°C$, the temperature rather than light limited the growth rates. The %RGR of the $24°C$ treatment increased from 0.11 ± 0.51 to 2.73 ± 0.86%/day then decreased to 1.79 ± 1.23%/day across the light treatments. The $28°C$ treatment showed a similar trend as the %RGR increased from 1.43 ± 1.12 to 3.01 ± 1.21%/day then decreased to 0.91 ± 0.69%/day over the same light treatments.


Fig. 5. The relative growth rates (%RGR) of *Laurencia brongniartii* explants, isolated from the apical tissue of the branch tips, grown in 9 combinations of temperature and irradiance treatments. Black bars are $10\,\mu$mol photons/m^2/s, cross-hatched bars are $20\,\mu$mol photons/m^2/s, and white bars are $50\,\mu$mol photons/m^2/s. Error bars indicate the 95% confidence interval.

Growth observations

All explants that survived the cleaning protocol were observed to produce new branches from the remaining apical pits of each branch (Fig. 6). By day 20, a number of explants were found developing rhizoid-like organs along the excisions and where the explants were in prolonged contact with the culture vessel. Attachment was observed in less than half of the cultures. A number of explants were transferred to 200 *ml* of PES in 300 *ml* culture flasks and after 10 months grew to maximum size of 15 mm along the longest axis with periodic medium changes and no aeration. In the majority of the explants, new branches formed, with the original explant at the center of the cultured *L. brongniartii*. This growth pattern caused *L. brongniartii* to lose its compressed characteristic. Algae in flasks that were periodically swirled never attached to the culture vessel; however in flasks that were not disturbed, explants often attached. The majority of the branches that radiated out from the original explant were cylindrical in shape, however some compressed tissue could be observed. These branches also appeared to originate from the epidermal cells of the explant. After 18 months of culture, all explants that were grown in the culture flasks were roughly spherical in shape, and branches radiated out from the original explant. No explants in this shape were found attached to the culture vessel. Contamination by diatoms or blue-green algae was never observed in any of the cultures that survived the isolation and cleaning protocol.

Discussion

The heightened interest in the commercialization of this species of *Laurencia* has brought up a number of concerns that strongly suggest that harvesting of natural populations would not be sustainable. In particular, approximately 1.0 kg wet-weight of *L. brongniartii* are required to produce 18 to 317 mg of crude extract (Y. Kamei: Saga Univ., pers. comm.). With production targets in the
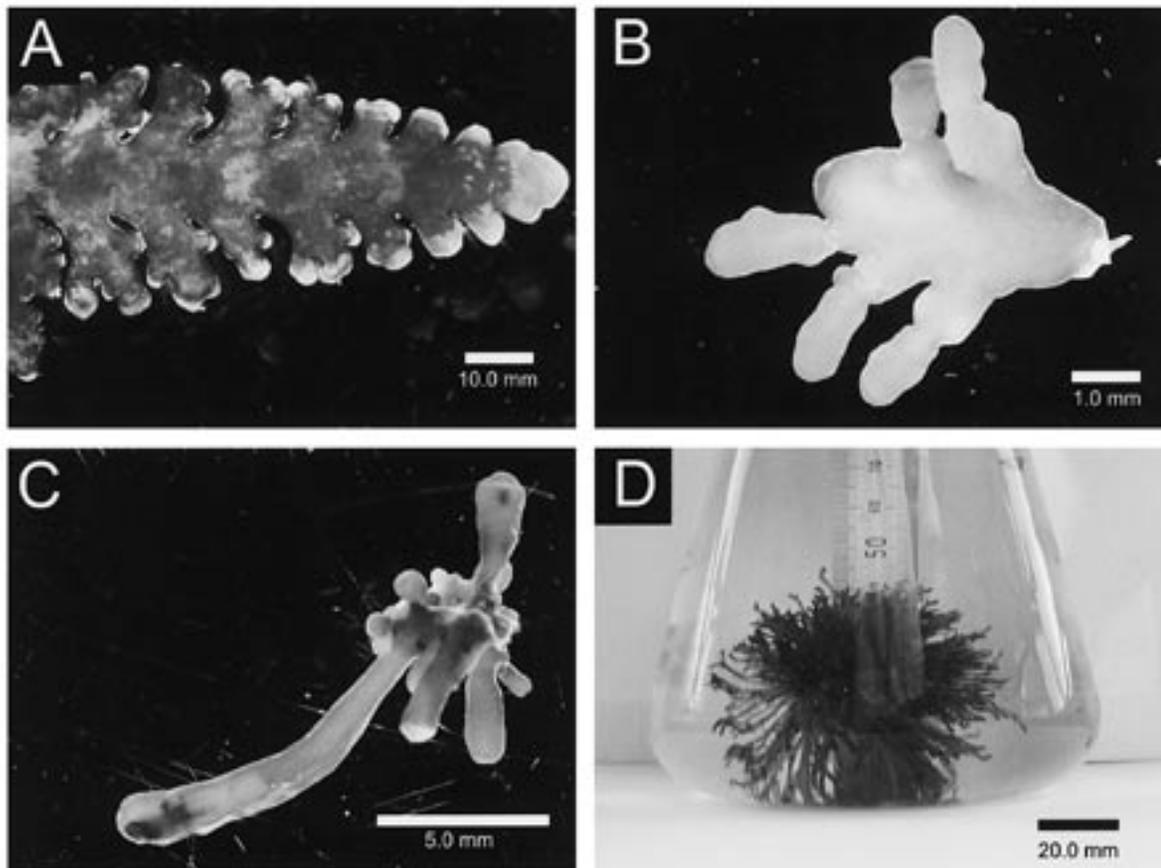
Fig. 6. Photographs A to D show the *in vitro* growth of *Laurencia brongniartii* explants, isolated from the branch tips of immature alga. A) The characteristic compressed structure of a frond from *L. brongniartii* prior to excision and treatment. B) After 20 days of culture in PES. C) At 10 months of culture, the approximate length of the longest axis is 15 mm. D) A free-living explant in a 300 *ml* culture flask after 18 months of cultivation.

range of 10 kg of extract a year, this suggests an annual harvest rate of 30 to 560 tons wet-weight of *L. brongniartii* a year. This harvest rate is believed to be unsustainable (Nishihara et al. in press). Therefore, the tank cultivation of *L. brongniartii* should be seriously considered as the primary method to develop this species as a new resource.

The largest concern of this study, was the need to develop a simple method of explant isolation and cultivation so that unskilled personnel could quickly learn and implement a *L. brongniartii* culture program. The need to develop an additional source of income for fishermen that were interested in the development of *L. brongniartii* as a new fisheries resource could not be ignored. Therefore, besides clarifying the environmental factors that affect the growth of *L. brongniartii*, it was important to develop a protocol to achieve

unialgal cultures of this species for commercial cultivation. There is considerable literature that suggests the importance of axenic cultures (Fries 1963; Gibor et al. 1981). However the establishment and maintenance of axenic culture requires careful handling and specialized equipment. Our method of isolation is simple to perform and easy to learn, which is ideal if this species is to be tank cultivated by fisheries personnel with little technical experience. We had little difficulty in maintaining the cultures.

The establishment of a "seed plant" can also be done by establishing unialgal cultures from spores. Mature tetrasporophytes and gametophytes can be harvested from August to November (Nishihara et al. in press) and it is not difficult to isolate spores by using established techniques. However, it is much faster and simpler to establish cultures from explants than from single spores. Additionally,

explants can be made whenever material is available, unlike spores and the required technical skill level is much lower.

The authors (Nishihara et al. in press) have shown that this species increases in size from May through October when water temperatures increase from 20 to 28℃ and are found in shaded areas of the reef which provides shelter from direct light. Our growth trials are consistent with these natural environmental conditions found at the collection site. In our culture studies, *L. brongniartii* grew best when temperatures were similar to the natural environment and when light was relatively low (Fig. 5). These low light levels are also commonly used to maintain cultures of other species of *Laurencia* (McDermid 1989; Kuwano et al. 1998). Figure 4 also suggests that *L. brongniartii* has a poor tolerance to large variations in salinity. The death of explants at the lowest salinity is likely due to the negative impact of large turgor pressures to the cell membrane and cell wall. On the other hand, plasmolysis is the likely cause of death at the highest salinity. This is well correlated to the location of natural populations. Natural populations of *L. brongniartii* are found in the subtidal areas of the coral reef where large variations of salinity are uncommon (Nishihara et al. in press). Therefore, to prevent large changes in media salinities of tank cultured *L. brongniartii*, care must be taken to prevent excessive evaporation and the influx of freshwater due to precipitation. Indoor tank culture maybe a more appropriate method of cultivation, considering the low light and stable salinity requirements.

The results of the tissue location experiment show no correlation with the survival rate and location of the excised tissue. However, the tissue from the upper location closest to the apex of the branch was found to have the highest %RGR. This was expected, since in general red algae increase in size by the division of the apical cells (Cole and Sheath 1990). The lack of survival rate and location correlation can be explained by the results of the size experiment, where we show that an

increase in size of the excised tissue increases the survival rate. Larger tissue sizes were probably much more able to tolerate the ultrasonic treatments than the smaller sizes. Also, it is likely that smaller tissue sizes were more susceptible to damage when handled with forceps. Conversely, %RGR of the small 1.0 mm tissue had the highest growth rates. This is an expected result and an artifact of the %RGR calculations, when ratio measurements are concerned. The ease in which excised tissue grew new branches suggests that fragmentation of the cultivated explants is a viable and simple method in which to split cultures.

Additionally, large amounts of nutrients appear unnecessary as shown in Fig. 3. Regardless of the lack of statistical significance in the data, growth was positively affected by the medium and that low concentrations of medium were just as effective in promoting growth as in high concentrations. The decrease in %RGR at higher concentrations may be due to the effects of high nutrient toxicity (Lobban and Harrison 1994).

Currently, trials are underway to scale up the cultivation of *L. brongniartii* from the laboratory scale to pilot scale. We suggest that, through the use of the cultivation factors clarified in this study and the isolation and cultivation protocol developed, *L. brongniartii* is a suitable candidate for tank cultivation by personnel with little technical experience. Through the modification and simplification of established culture protocols commonly used in the research environment, inexperienced workers may be provided the opportunity to increase their economic output through the establishment of cultures with novel species of economic interest. Finally, the cost of production of this species may be low due to the low light requirements, the unnecessary need for nutrient enrichment, and temperature requirements that are similar to the surface waters of the region. However, we recommend additional studies to clarify the running cost of a small scale operation and to maximize production.

Acknowledgment

References

Cole, K. M. and R. G. Sheath (1990) *Biology of the Red Algae*. Cambridge University Press, New York, 517 pp.

Dawes, C. J., J. Orduna-Rojas and D. R. Robledo (1999) Response of the tropical red seaweed *Gracilaria cornea* to temperature, salinity, and irradiance. *J. Appl. Phycol.*, **10**, 419-425.

Fries, L. (1963) On the cultivation of axenic red algae. *Physiol. Plant.*, **16**, 695-708.

Garcia-Reina, G., R. Robaina, M. Tejedor and A. Luque (1987) Attempts to establish axenic cultures and photoautotrophic growth of *Gelidium versicolor*, *Gracilaria ferox*, and *Laurencia* sp. cell cultures, in "Algal Biotechnology" (ed. by T. Stadler, J. Mollion, M. C. Verdus, Y. Karamanos, H. Morvan, and D. Christiaen), Elsevier Applied Science, Villeneuve, d'Ascq, France, pp. 111-118 .

Gibor, A., M. Polne, M. Biniaminov and M. Neushul (1981) Exploratory studies of vegetative propogation of marine algae: procedure for obtaining axenic tissues, in "10th International Seaweed Symposium Proceedings" (ed. by T. Levring), Walter de Gruyter and Co., pp. 587-593.

Harada, H., T. Noro and Y. Kamei (1996a) Effect of *in vitro* selective antitumor active extracts from marine algae on proliferations of murine tumor L1210 and normal NIH-3T3 cells. *Mar. High. Biosci. Cent. Rep., Saga Univ.*, **4**, 27-31 (in Japanese).

Harada, H., T. Noro and Y. Kamei (1996b) Cytotoxic spectra of selective antitumor extracts from marine algae extracts to several tumor cells lines. *Mar. High. Biosci. Cent. Rep., Saga Univ.*, **4**, 23-26 (in Japanese).

Harada, H., T. Noro and Y. Kamei (1997) Selective antitumor activity *in vitro* from marine algae from Japan coasts. *Biol. Pharm. Bull.*, **20**, 541-546.

Horikawa, M., T. Noro and Y. Kamei (1995) Screening of antibacterial activity from marine algae of Kyushu Island, Japan-II. *Mar. High. Biosci. Cent. Rep., Saga Univ.*, **2**, 43-48 (in Japanese).

Horikawa, M., T. Noro and Y. Kamei (1996) Comparison of antibacterial activity against causative agents of potato-scab between antibacterial substances purified from a red alga, *Laurencia okamurae* and commercial agricultural chemicals. *Mar. High. Biosci. Cent. Rep., Saga Univ.*, **4**, 17-21 (in Japanese).

Horikawa, M., T. Noro and Y. Kamei (1999) *In vitro* anti-methicillin-resistant *Staphylococcus aureus* activity found in extracts of marine algae indigenous to the coastline of Japan. *J. Antibiot.*, **52**, 186-189.

Kamei, Y., T. Noro and Y. Yamaguchi (1995) Screening of antiviral activity from marine algae-I. *Mar. High. Biosci. Cent. Rep., Saga Univ.*, **2**, 49-56 (in Japanese).

Kuwano, K., S. Matsuka, S. Kono, M. Ninomiya, J. Onishi and N. Saga (1998) Growth and the content of laurinterol and debromolaurinterol in *Laurencia okamurae* (Ceramiales, Rhodophyta). *J. Appl. Phycol.*, **10**, 9-14.

Lapointe, B. E., C. J. Dawes and K. R. Tenore (1984) Interactions between light and temperature on the physiological ecology of *Gracilaria tikvahiae* (Gigartinales: Rhodophyta). II. Nitrate uptake and levels of pigments and chemical constituents. *Mar. Biol.*, **80**, 171-178.

Lobban, C. S. and P. J. Harrison (1994) *Seaweed Ecology and Physiology*. Cambridge University Press, New York, 366 pp.

McDermid, K. J. (1989) *Laurencia crustiformans* sp. nov. (Ceramiales, Rhodophyta) from the Hawaiian Islands. *Phycologia*, **28**, 352-359.

Nishihara, G. N., Y. Mori, R. Terada and T. Noro (in press) Habitat characteristics and seasonal growth of *Laurencia brongniartii* (Ceramiales, Rhodophyta) in Kagoshima, Southern Japan. *Phycol. Res.*

Pratt, R., T. C. Daniels, J. J. Eiler, J. B. Gunnison, W. D. Kumler, J. F. Oneto and L. A. Strait (1944) Chlorellin, an antibacterial substance from *Chlorella*. *Science*, **99**, 351-352.

Rasband, W. S. (2003) *ImageJ*. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/.

Schwartz, R. E., C. F. Hirsch, D. F. Sesin, J. E. Flor, M. Chartrain, R. E. Fromtling, G. H. Harris, M. J. Salvatore, J. M. Liesch and K. Yudin (1990) Pharmaceuticals from cultured algae. *J. Indust. Microbiol.*, **5**, 113-124.

Yokoya, N. S. (2000) Apical callus formation and plant regeneration controlled by plant growth regulators on axenic culture of red alga *Gracilariopsis tenuifrons* (Gracilariales, Rhodophyta). *Phycol. Res.*, **48**, 133-142.

鹿児島産ソゾノハナ *Laurencia brongniartii* J. Agardh （紅藻綱，イギス目）の簡易な単離培養法

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　紅藻ソゾノハナ *Laurencia brongniartii* J. Agardh（イギス目）は，含有する生理活性物質の重要性から，有用海藻として資源の維持管理が試みられている。本研究では，本種の養殖用母藻を室内で大量に培養するための簡易な単離法と培養法を確立し，至適生育条件を検索した。最も良く生長する組織片を単離するため，藻体の上端部から様々な長さや部位の組織片を摘出し，生長率（%RGR）と生存率（%SR）を基に比較した。その結果，大形の組織片（5.0 mm）には高い生存率（62.5%）が見られ，小形の組織片（1.0 mm）には高い生長率（4.43%/day）が見られた。また，組織片は摘出部位によって生長率に差が見られ，先端に近い部位の生長率（2.16%/day）が最も高かった。組織片の生長は水温と光量に強く制限され，24℃および28℃で20μmol photons/m^2/s が至適な条件だった。さらに，組織片の生長は高濃度の硝酸態窒素濃度（329.4μmol NO$_3$-N/l）によっても制限された。本種の至適生育条件で単離培養法を用いると，容易に養殖母藻を養成・供給できることが明らかになった。