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A new approach to kelp mariculture in Chile: production of free-floating sporophyte seedlings from gametophyte cultures of *Lessonia trabeculata* and *Macrocystis pyrifera*

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Abstract

Substantial amounts of *Macrocystis* and *Lessonia* are traditionally harvested and exported from Chile as raw material for alginate. Because of intense mari culture of abalone (*Haliotis* ssp.), herbivorous mol luscs that feed on brown kelps, pressure on local populations of *Macrocystis* and *Lessonia* has in creased to critical levels within the past 5 years, strongly supporting efforts to produce algae maricul tured biomass. Here, we present our results on the de velopment of new techniques for large scale kelp mariculture in Chile. We have abandoned the tradi tional technique of direct spore seeding onto inocula tion lines. Instead, we used gametophyte cultures that were manipulated to enter gametogenesis and to produce synchronous batches of 10^4 10^5 embryos. Juvenile sporophytes were cultured under perma nent aeration and agitation, floating unattached in contamination free glass bottles up to 10 L, plexiglass cylinders and 800 L greenhouse tanks. When hold fast initials were formed at a size of 8 cm, the sporo phytes were spliced into Nylon rope fragments and transferred to the sea. Twelve months after initiation of gametogenesis in the laboratory, *Macrocystis pyri-fera* attained 14 m length and 80 kg fresh weight m^{-1} line in the sea. For *Lessonia trabeculata* 6 months after gametogenesis initiation, 0.25 kg fresh weight m^{-1} was attained in the sea.

Keywords: Chile, gametophyte, kelp, *Lessonia*, *Macrocystis*, mariculture

Introduction

Starting in 1989, mariculture of red and green aba lone (*Haliotis* ssp.) has been introduced in Chile, and has since grown to significant economic scale (Serna pesca 2003). *Haliotis* is a herbivorous mollusc, which feeds preferentially on the local brown kelps *Macro-cystis pyrifera* (L.) C. Agardh, *M. integrifolia* Bory, *Lessonia trabeculata* Villouta et Santelices and *L. nigrescens* Bory. Further expansion of the abalone production will increase the demand for forage bio mass, and various consequences of overexploitation of natural kelp beds in Chile are presently discussed (Vásquez 1999). Abalone producers are facing in creasing problems with the acquisition of forage sup ply, because transport distances and costs are rising. Furthermore, governmental actions are expected to impose conservation and protection schemes for marine resources, which will include restrictions in kelp harvesting (Vásquez & Westermeier 1993; Vásquez 1999).

In anticipation of these problems, we started to de velop laboratory techniques for the establishment of kelp mariculture in Chile. Traditional kelp maricul ture uses direct seeding of spores onto inoculation lines (Kain 1991). This technique, however, causes biofouling by propagules, which are co inoculated with the spores and compete with the kelp embryos from the beginning (Devinny & Leventhal 1979). Ed ding and Tala (2003) found biofouling to be a major problem in spore derived pre cultured sporophytes of *L. trabeculata* transferred to the sea.

Cultures of laminarialean gametophytes can be propagated vegetatively and manipulated by simple ambiental changes to enter gametogenesis (Lüning 1980). Because our laboratory routinely maintained clonal gametophyte cultures of *L. trabeculata* game tophytes, we decided to develop methods to produce and grow contamination free sporophytes up to a size ready for transfer to marine culture. In a later stage of our study, we added *M. pyrifera*.

Materials and methods

A fresh specimen of *L. trabeculata* was collected at Mar Brava (Chiloé, X Region, southern Chile) in Jan uary 1985 (Fig. 1). Fragments of mature sorus tissue (1×1 mm) were cut out with a razor blade and introduced into 4 mL polypropylene tubes (Fig. 2). Culture medium was autoclaved natural sea water, supplemented with 20 mL L^{-1} enrichment of Provasoli medium (PES medium; Starr & Zeikus 1993). In the laboratory, the culture tubes were exposed to low irradiation with white fluores cent light (4 5.3 µmol m^{-2} s^{-1}) for 16 h day^{-1} at 14 15 °C. After 8 weeks, several thousand filamen tous gametophytes had developed from the germinat ing spores, and were transferred to plastic Petri dishes with 10 mL culture medium. Under low light intensity (4 5.3 µmol m^{-2} s^{-1}), they remained ster ile, and male and female gametophytes could be clearly distinguished under a stereo microscope at $\times$ 40 magnification. Individual gametophytes were collected with Pasteur pipettes and distributed into a 96 well microtitre plate. The isolates continued to grow, and 8 weeks later individual gametophytes were screened for the absence of contaminants at $\times$ 40 60 magnification. One clean male and one fe male gametophyte were selected to initiate a pair of clonal stock cultures. They were maintained as part of a culture collection with one transfer to fresh cul ture medium per year. In 1998, we began to expand their biomass. In 2 3 month intervals, gameto phytes were gently fragmented using a Teflon pestle glass homogenizer (Glas Col, Terre Haute, IN, USA). The material was re suspended in fresh culture med ium in screw cap glass bottles with increasing volumes from 100, 200 to 500 mL in size (Fig. 3). With this treatment, under a low light intensity (4 5.3 µmol m^{-2} s^{-1}), *L. trabeculata* gametophyte biomass approximatly doubled in about 2 months. Routinely, we maintained a stock supply of several 500 mL flasks with 200 400 mg fresh weight per flask for each gametophyte strain. This system pro vided sufficient material for new sporophyte produc tion series every few weeks.

In the same manner, one pair of clonal female and male gametophyte culture of *M. pyrifera* was estab

Figure 1 Mature specimen in January 1985, which gave rise to the gametophyte cultures used for the present study.
Figure 2 Polypropylene sample tubes for spore inoculations and maintenance of gametophyte stock cultures.
Figure 3 Expanded gametophyte culture in a 500 mL bottle.
Figure 4 Teflon pestle glass homogenizer for fragmentation of gametophytes.
Figure 5 Gametogenesis unit: male and female gametophyte fragments mixed in a sealable household polyethylene bag.
Figure 6 Advanced gametogenesis: female gametophyte cells have developed to oogonia (below), and male gametophyte cells transformed into anheridia (above). Scale bar 50 µm.
Figure 7 Liberated egg cell adhering to oogonium aperture. Scale bar: 50 µm.
Figure 8 Few celled zygote germlings and residual gametophytes. Scale bar: 50 µm.
Figure 9 Group of young sporophytes at the end of gametogenesis treatment. Scale bar: 50 µm.
Figure 10 Juvenile sporophyte showing differentiation in rhizoids, stipe and blade. Scale bar: 100 µm.
Figure 11 Same stage, detail showing unicellular rhizoids. Scale bar: 50 µm.
Figure 12 First expansion step: sporophytes in a 1 L bottle with magnetic stirrer and aeration. Entry tube (left) with ster ile air filter. Entry and exit ducts with cotton plugged glass olives.
Figure 13 Later expansion stage in a 10 L bottle.
Figure 14 Close up of aeration culture shortly before transfer to tanks.
Figure 15 Greenhouse tank culture. Turbulence created by aeration from perforated pipe along the bottom circumference.
Figure 16 Advanced sporophyte with well developed multicellular haptera at the base of the stipe.
Figure 17 Insertion of sporophyte base into primary polypropylene rope fragments.
Figure 18 Continuation of tank phase to promote fixation of haptera.
Figure 19 Fixation of sporophyte units to carrier line.
Figure 20 Interim storage of carrier lines on plastic frames before explantation.
Figure 21 Sporophytes removed from carrier line after a 5 month exposure in the sea.

lished from a sporophyll collected in December 2001 at Teupa, Island of Chiloé, X Region.

We used the following treatment to initiate gametogenesis in vegetative gametophytes of *Lessonia* and *Macrocystis*: 10 15 mg fresh weight, corresponding to 1 mL of a dense suspension from a female gametophyte stock culture, plus 1 mL of the corresponding male were mixed in a teflon pestle glass homogenizer, and culture medium was added up to 30 mL (Fig. 4). After mild homogenization, more culture

medium was added up to 100 mL and the material was allowed to sediment in a 100 mL glass bottle. The supernatant was discarded, and the sedimented gametophyte fragments were introduced with 400 mL PES medium into a sealable household polyethylene bag (Fig. 5). This culture was subjected to white fluorescent light with an irradiance of 20–25 µmol m^{-2} s^{-1} at 8–10 °C for 16 h day^{-1}. Four days later, irradiance was increased further to 50–54 µmol m^{-2} s^{-1}. Under this treatment, oogonia and antheridia were formed (Fig. 6), and the first eggs, spermatozoids and zygotes appeared after 18 days (Fig. 7). Numerous zygotes, embryos and first rhizoids appeared during the following days (Figs 8–11). We terminated this gametogenesis phase 25 days after initiation by introducing the material into a 500 mL glass bottle. The supernatant was discarded, and the total number of sporophytes was estimated by counts in 25 µL aliquots and extrapolation to the total volume. The size of the juvenile sporophytes was determined with a calibrated ocular grid at × 100 magnification.

The sporophytes were suspended in 800 mL PES medium and introduced into a 1 L glass bottle with a screw on gas washing device (System Drechsel, Karlsruhe, Germany, Fig. 12). A membrane pump supplied an air flow of 200 mL min^{-1}. The air inlets of our culture bottles were equipped with sterile air filters (Millipore 0.2 µm multiple use autoclavable PTFE membrane filters, Millipore, Bedford, MA, USA). In addition, inlet and outlet ducts contained a cotton plugged glass olive in order to protect the sterile filters and to prevent the entrance of airborne contaminants through the exit vent (Figs 12 and 13). The parts were connected with 10 mm i.d. silicone tubing. Completely assembled aeration units were autoclaved before use. Expansion steps with our gas washing bottle type units ranged from 1, 2 to 5 L volume. The next step included the use of 10 L wide neck glass bottles, which were sealed with a thermo stable household plastic bag (Fig. 13). This unit was aerated by a straight glass tube supplied with a cotton olive and sterile air filter. Glass bottles of all sizes were placed on magnetic stirrers for agitation.

Aeration units were exposed to lateral white light from fluorescent tubes with an irradiance of 24–26 µmol m^{-2} s^{-1} for 16 h day^{-1} at 13–15 °C. Culture medium was exchanged in weekly intervals, numbers and size of sporophytes determined, and the material was transferred to the next expansion step if the appropriate size was attained (2–3 cm) (Fig. 14). In the protocol for our *Macrocystis* production, we included an additional expansion step with Plexiglass cylinders of 20 L volume. At a size of 3–4 cm, the juvenile sporophytes from our laboratory batch cultures were transferred to greenhouse tanks (Fig. 15) containing 800 L filtered and UV sterilized natural sea water without additional nutrients, which was exchanged over 3–4 day intervals. Natural daylight was reduced by black Nylon screens to levels ranging from 4 to 20 µmol m^{-2} s^{-1}. Multiple air inlets in our tanks were arranged to create turbulence to maintain the sporophytes in permanent flotation.

When *Lessonia* and *Macrocystis* sporophytes reached an average size of 8 cm in the tanks, their basal holdfast initials were well developed (Fig. 16). Individuals were spliced singly into 10 cm length fragments of 4 mm polypropylene rope (Fig. 17). These units remained in the tank for 2 more days (Fig. 18). Then, they were fixed 10–30 cm apart with two plastic clips onto 10 m length of a 12 mm polypropylene carrier rope (Fig. 19). The line with thalli attached was then wrapped around a plastic frame (50 × 50 cm), which remained immersed in seawater until the explants were transported to the field station in mobile tanks (Fig. 20).

The cultivation sites were located in the Interior Sea of Chile (X Region) at Calbuco (41°43′ South 73°05′ West Province Llanquihue), and Teupa Curanué (42°24′S 73°38′W Province Chiloé). We used the supporting structures of abalone farming. The carrier lines described above were either fixed directly to supporting structures for horizontal exposition at various depths, or exposed vertically by attaching a weight to one end. The lines were hauled in for growth measurements at monthly intervals.

Results

Figures 6–9 illustrate the efficiency of our gametogenesis treatment for *L. trabeculata*, and a similar result was obtained with *M. pyrifera*; nearly the entire biomass of gametophytes was converted into oogonia and antheridia. Tables 1 and 2 summarize our results for laboratory based mass production. Synchronous batches of embryos numbering from 10^4 with *Lessonia* to 10^5 with *Macrocystis* originated in our gametogenesis bags. Within 70–80 days, sporelings reached 3–4 cm in size, and were exposed to semi natural conditions in greenhouse tanks. Up to this stage, they retained their unicellular rhizoids, characteristic for the embryo stage (Figs 10 and 11).

Because the number of seedlings considerably exceeded the capacity of our infrastructure, we had to

Table 1 *Lessonia trabeculata*

Time scale			Culture units			Sporophyte			
						Size			
Month	Days	Year	Type	Number	Volume (L)	mm	SD	Number per unit	Remarks
November	15	2002	PE bag	1	0.4			18000	Gametogenesis 25 days
December	10		Bottle	1	1	0.06		10000	Discarded 8000
	17			2	1	0.26	0.04	5000	
	23			2	2	0.7	0.18		
	30			2	2	1.1	0.32		
January	7	2003		2	5	2.0	0.59		
	14			2	5	3.3	0.82		
	21			2	10	4.5	1.16		
	28			2	10	6.6	1.76		
February	4			4	10	7.9	1.83	2500	
	11			4	10	12.9	2.77		
	18			4	10	10.2	3.19		
	25			4	10	26.7	5.61		
March	3			4	10	37.3	6.17		
	10		Tank	2	800	42.4	7.6	5000	
	18			4	800	51.3	6.65	2500	
	25			4	800	55.9	8.49		
April	15			4	800	56.3	9.29		
May	20			4	800	77.1	13.3		
June	25		Open sea			82	27	300	Surplus material discarded
July	30					117	31		
August	27					151	63		
September	30					284	42		
October	30					292	35		

Time course and key parameters of mariculture experiment from initiation of gametogenesis to explantation and growth performance in the sea. Volume expansion was done by introducing parallel units or upscaling into larger containments. Sporophyte size determined as average from 20 individuals. Sporophyte numbers are estimations, determined by counting random samples and extrapolation to total volume. Exposure in the sea with 6 thalli m^{-1} on a horizontal line at 5 m depth.
SD, standard deviation; discard, batch sizes reduced for lack of capacity.

reduce part of our growing crops at certain intervals, marked 'discard' in Tables 1 and 2. We tried to use these manipulations to reduce the number of abnormal individuals with slow growth, deformations, or lack of polarity, which occurred at a frequency of 10% and 20% in the sporophyte crops.

At a size of about 7–8 cm, sporophytes showed a significant morphogenetic change. Even in the absence of substrate contact, they began to form their characteristic multicellular haptera (Fig. 16) and were then ready to be twisted with their bases into 10 cm fragments of 4 mm polypropylene rope. Subsequently, the haptera intensified their growth and fixed themselves to the rope fragment. Thallus growth of laboratory produced *L. trabeculata* seedlings after transfer at sea is documented in Table 1 and Fig. 21, while Fig. 22 shows an increase in biomass and thallus length for our *M. pyrifera* seedlings in the sea.

Except for occasional turbidity caused by commensalic bacteria, no evidence of contamination, fouling or diseases occurred in laboratory installations, including the greenhouse tank stage.

Discussion

Our study shows that it is possible to produce seedling biomass of *Macrocystis* and *Lessonia* from gametophyte cultures at a scale that can easily fill commercial demands. Our study further demonstrates that gametophyte cultures offer important advantages over the traditional direct seeding of spores.

Gametophyte cultures can be established and maintained under unialgal condition, which means that except for commensalic bacteria, all biotic contaminants are strongly deminished. This protected status can be maintained routinely with standard laboratory methods and equipment using the aseptic techniques described above. Seedlings are 3–4 cm in size when they confront potentially unknown germs for the first time in the greenhouse tanks, and about

Table 2 *Macrocystis pyrifera*

	Culture units			Sporophyte			
				Size			
Time scale: weeks	Number	Type	Volume (L)	mm	SD	Total number	Remarks
1 3		PE bag	0.4	0.07	0.01	100 000	Gametogenesis
4	1	Bottle	1	0.11	0.04	80 000	Discard 20 000
5	1		1	0.29	0.13	80 000	
6	1		2	0.48	0.13	60 000	Discard 20 000
7	1		2	0.94	0.42	60 000	
8	2		5	1.53	0.41	20 000	Discard 40 000
9	2		5	4.29	1.48	20 000	
10	2		10	6.24	1.48	10 000	Discard 10 000
11	2		10	8.02	2.15	10 000	
12	2	Cylinder	20	10.08	2.17	10 000	
13	4		20	22.88	3.87	10 000	
14	4	Tank	800	32.33	5.64	10 000	
15	4		800	64.12	11.82	10 000	
16	4		800	78.42	16.35	10 000	

Time course and key parameters from initiation of gametogenesis in December 2002 to end of greenhouse tank stage. Measurements as in Table 1. Transfer took place in March 2003. Continuation of experiment with growth performance in the sea is shown in Fig. 22.

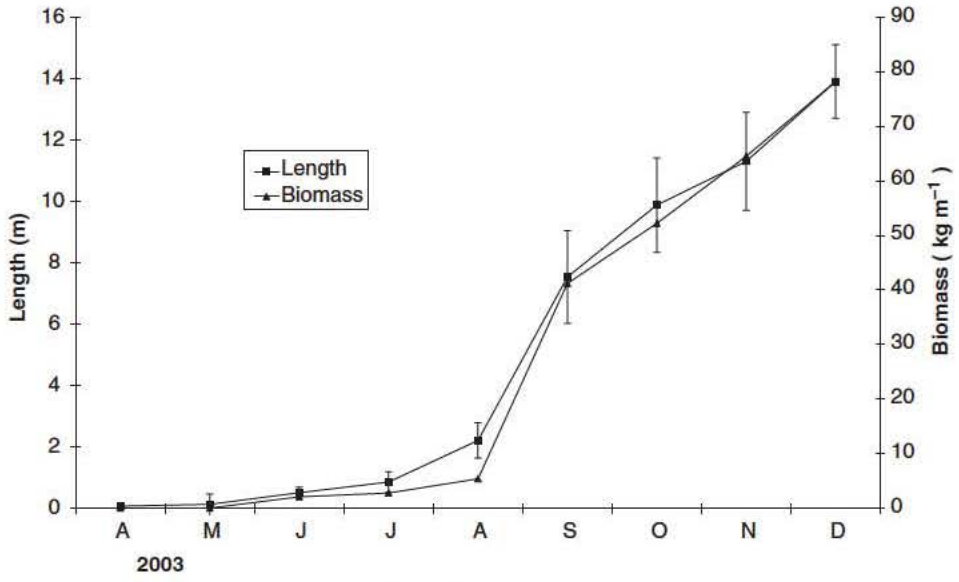

Figure 22 *Macrocystis pyrifera.* Growth performance of laboratory produced sporophyte seedlings (continuation of Table 2) after explantation in the sea with six individuals m ^{1} on a horizontal rope at 5 m depth. Abscissa, time scale with monthly measurements in 2003. Ordinate; left scale, thallus length (m) with standard deviation; right, biomass (kg m ^{1}).

8 cm in size when they encounter the fouling germs of their future habitat. This offers excellent opportunity for the kelp inoculants to outgrow their potential epiflora and epifauna. Our study clearly showed that transferred kelp specimens were not subject to substantial biofouling or diseases up to 14 m harvest size in *M. pyrifera*.

Gametophyte cultures can be manipulated to initiate sporophyte seedling crops at any time of the year, independent of the availability of natural spores. Natural recruitment in *M. pyrifera* begins in around September in southern Chile (Westermeier & Möller 1990). Transfer was performed in March, and produced a substantial biomass by October December,

i.e. several months ahead of the natural population climax. This suggests that it will be possible to work out a year round M*acrocystis* production scheme.

The intense growth activity in our sporophyte cul tures increases the demand for carbon dioxide, and limitation of photosynthesis is likely to occur under stagnant conditions. Our experiments demonstrate that this deficit can be compensated by permanent aeration and turbulence. With this technique, we were able to maintain densities as high as 10^4 individuals of 1 cm size in 40 L of culture medium (Tables 1 and 2). This is likely to keep costs for space, energy, seawater supply and fertilizer chemicals low, although there are no data available to compare our method with traditional line inoculation techni ques directly.

Crop improvement projects in commercial mari culture of *Laminaria* in Japan and China have used the selection of superior parent sporophytes as spore producers. However, meiosis as the basic process of spore formation as well as multiple individuals as spore suppliers contributed to the degeneration of se lected strains. To overcome this dilemma, Li, Zhou, Liu and Wu (1999) described the use of gametophyte clones from selected parent sporophytes of *Laminaria japonica* Aresch. as a basis for the maintenance of a stable genetic stock for commercial strains. Our stu dies with *L. trabeculata* and *M. pyrifera* extend this principle by producing large batches of seedlings directly from gametophytes.

The culture methods described here provide the basis to initiate systematic kelp breeding programmes similar to those in terrestric agronomy, including es tablishment of genetically defined and stable culti vars, selection of fast growing and highly fertile gametophytes, sexual crosses of parents with favour able characteristics and the generation of high per formance hybrids. Furthermore, our study confirms the longevity and stability of gametophyte cultures in *L. trabeculata* over 18 years, and similar character istics are likely to apply for other kelps such as *Macro-cystis*.

As Table 1 documents, the linear growth rate of *L. trabeculata* sporophytes varied considerably. The overall growth from 2 to 292 mm in 296 days gives a value close to 1 mm day^{-1}. The highest value with 3.9 mm day^{-1} was obtained in September in the sea. In northern Chile, Edding, Venegas, Orrego and Fonck (1990) observed lowest values of 1 mm day^{-1} for transferred *L. trabeculata* juveniles in October, and values of 6 mm day^{-1} in March. In a more recent study, Edding and Tala (2003) reported values be tween 3 and 6 mm day^{-1}. These data suggest that *L. trabeculata* is a slow growing kelp, requiring years to reach adult size. This is supported by comparison with other kelps. *Laminaria digitata* (Hudson) La mour. and *L. longicruris* Pylaie reached 10 mm day^{-1} in summer (cited in Edding *et al.* 1990). Chilean *M. pyrifera* showed values up to 13 mm day^{-1} in Octo ber November, and summer averages between 7 and 8 mm day^{-1} (Westermeier & Moeller 1990), while 5 m fronds of Californian *M. pyrifera* were re ported to show a daily length increase up to 20 cm (North 1971). The performance of *M. pyrifera* com pares favourably with these values: 42 mm day^{-1} from zygote to 14 m frond (Table 2, Fig. 22). Clearly, *Macrocystis* is a far more promising candidate for kelp biomass production than *Lessonia*. We have therefore discontinued our studies on *L. trabeculata*, and are now focusing our present efforts towards further im proving mariculture techniques for *M. pyrifera*.

Nevertheless, our results on *Lessonia* seedling pro duction may be useful in another context. Because of overexploitation (Sernapesca 2003) and El Niño ef fects (Vásquez 1999) *L. trabeculata* has disappeared from a number of localities in northern Chile. Rope fragments with inoculants produced using our tech niques could be fixed to natural or artificial sub strates in such areas to re introduce the species. In such cases, a broader genetic basis of the seedlings would be desirable, which can be easily achieved by using a multi individual mixture of gametophytes for seedling production.

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