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Spore culture of the edible red seaweed, *Gracilaria parvispora* (Rhodophyta)

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Abstract

A hatchery was established for the inoculation of coral chips, pebbles and lines with carpospores of *Gracilaria parvispora*, an edible market seaweed in Hawaii. Cystocarpic thalli were placed over various substrates in tanks of aerated seawater. Carpospores attached readily to substrates and after 72 h in hatchery tanks, mean spore density on slides placed in hatch tanks was 1800 cm^{-2}. Inoculated coral chips and pebbles were placed out in a seawater pond. After 18–22 weeks spore density declined to 4 cm^{-2} but 61% of substrates still had plants. Only 36% of inoculated lines developed good growth, but growth was more rapid on lines than on pebble or chips. Lines yielded two crops per year, each approximately 800 g m^{-2} (fresh weight), whereas chips and pebbles required 50 weeks growth for an equivalent harvest. Tetrasporophytes were the dominant adult stage but cystocarpic plants accounted for approximately 10% of the culture products, demonstrating that the life cycle of this species was completed within the culture system. Spore culture of *Gracilaria* allowed mass production of plants on a variety of artificial substrates but the disadvantages included the long lag period and the lower reliability compared with vegetative production methods.

Keywords: *Gracilaria*; Carpospores; Tetraspores; Culture method

1. Introduction

Gracilaria seaweeds (Rhodophyta) are raw material for agar extraction and are marketed as edible sea-vegetables in some parts of Asia and the Pacific (Santelices and

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Doty, 1989). Most of the *Gracilaria* harvest still comes from wild stocks but by 1991 approximately one-third of the harvest was from cultured sources (McHugh, 1991). At present the culture methods rely on vegetative fragments, rather than spores, as the propagating units to obtain new plants (LaPointe et al., 1976; Santelices and Doty, 1989; Hurtado-Ponce, 1990; Trono, 1990; Friedlander, 1992; Bravo et al., 1992; Hurtado-Ponce et al., 1992). However, thalli of many species are too small and delicate to be efficiently grown by hand-planting, and even for the larger species, vegetative propagation is inefficient because it requires large amounts of propagating material to start or revitalize mass plantings. In pond culture, for example, 20–30% of the harvest may be used as seed material (Hurtado-Ponce et al., 1992). Several research groups have pointed out the need for mass-production, spore-culture methods for *Gracilaria* similar to those used for other seaweeds (Lin et al., 1979; Romo-Donoso, 1988; Buschmann and Kuschel, 1988; Santelices and Doty, 1989; Levy et al., 1990).

Gracilaria spp. typically have two diploid life stages in addition to a gametophyte generation (Gargiulo et al., 1992; Destombe et al., 1993). The first diploid tetrasporophyte stage and the male and female gametophytes are similar in appearance and grow into harvest plants. The second diploid stage forms spore-releasing nodules called cystocarps on the surface of the female gametophytes. Variations on the life cycle exist: some species have mixed stages on the same thallus (Prieto et al., 1991) and others can form cystocarps without fertilization (Van der Meer, 1981). Completing the life cycle in culture will require the ability to produce all the life stages of a given species.

Small-scale culture studies have demonstrated the feasibility of growing *Gracilaria* from either carpospores or tetraspores (Van der Meer, 1981; Friedlander and Dawes, 1984; Levine, 1986; Levy et al., 1990; Prieto et al., 1991; Destombe et al., 1993), but ocean-based aquaculture attempts have used carpospores as the starting material, since they are released in profusion from cystocarps which are visible to the naked eye on female gametophytes (Lin et al., 1979; Doty et al., 1986; Doty and Fisher, 1987; Buschmann and Kuschel, 1988; Santelices and Doty, 1989; Li and Li, 1990). These studies have established the technical feasibility of spore culture, yet none of the methods has been adopted in commercial production.

We have explored spore culture methods for *G. parvispora*, marketed as the edible seaweed 'long ogo' in Hawaii. This species was classified as *Gracilaria bursapastoris* before 1985 and is probably the same as the species still classified as *G. bursapastoris* in Asia (Abbott, 1985), where it is harvested as an agar source, but it is apparently distinct from the Mediterranean species classified as *G. bursapastoris* (Abbott, 1985; Gargiulo et al., 1992). Our culture methods were based on experiments in Hawaii by Doty et al. (1986) and Levine (1986) which showed that cystocarpic thalli of *G. parvispora* release spores prolifically onto rocks and other substrates, that can be placed into the ocean to grow into adult plants, and on preliminary results of Doty and Fisher from a pilot hatchery and line farm for *G. tenuistipitata* in Malaysia (Doty and Fisher, 1987; Santelices and Doty, 1989). Those experiments demonstrated the potential for mass spore culture of *Gracilaria*.

Our objectives were to scale up the methods into a production system and establish the overall reliability of the methodology. We established a hatchery for the production of sporelings and planted inoculated substrates into the ocean to evaluate the survival

rate, length of the crop cycle and yields of tetrasporophytes. We demonstrated that cultured tetrasporophytes released viable tetraspores and observed that the alternation of generations was completed in the culture system, through the production of cystocarpic gametophytes.

2. Materials and methods

2.1. Location of experiments and source of seaweed

Culture experiments were carried out at Puko'o Pond, on the southeast reef of Molokai, Hawaii. The pond was originally a shallow, rock-walled Hawaiian fishpond, but was modified by dredging to support a deep-water boat inlet leading to several shallow mooring bays. The pond is now operated as a demonstration aquaculture facility by Ke Kua'aina, a non-profit organization developing small-scale economic activities for coastal residents. Seaweed grown during the project was harvested and test-marketed by Ke Kua'aina personnel.

The pond bottom is sand and silt. Water was generally murky, Secchi disk visibility 0.5 m or less, owing to silt in the water column. Water motion was lower than on the open reef, since the location was protected from waves. Experiments were carried out in shallow water, 0.5–1.5 m at high tide, along the shores of the lagoon.

G. parvispora is not native to Molokai. It was deliberately introduced to the island from inoculated rocks brought from Oahu by others in 1984 (Doty et al., 1986). Those rocks were distributed at known locations along the south reef. Isolated, naturalized populations of *G. parvispora* developed from the rocks at several of the locations and were the source of starting material for the present experiments. Before the start of the experiments in 1992, we surveyed Puko'o pond and did not find any *G. parvispora* or other *Gracilaria* spp. The nearest *G. parvispora* population was approximately 10 km from the pond on the reef.

2.2. Hatchery methods

Carpospore hatchery experiments were carried out in 1.5 m diameter, 0.6 m depth tanks equipped with aeration. Seawater was pumped from the pond through a sand filter into the tanks. Spores were settled onto pebbles and coral chips (2–5 cm) and braided nylon line, 0.2 or 0.5 cm diameter.

Coral chips were rounded, fill material available on site. Their surfaces had numerous small cavities into which spores settled and grew. Pebbles were collected from a stream bed and had smooth surfaces. Approximately 150 pebbles or chips were placed in a single layer in 24 shallow plastic nursery trays (40 cm × 40 cm) with open mesh bottoms. Twelve trays were placed in a single layer in the bottom of a tank filled with seawater. Trays were overlain with approximately 2 kg per tank of cystocarpic thalli (100–200 g per tray), collected from the nearest wild population outside the pond. Tanks were shaded to admit approximately 25% sunlight during a hatch. Hatches were conducted using aeration but without water exchange. The aeration level was adjusted so

that the water in the tank was kept moving but the fertile thalli laid over the substrates remained in place.

Lines were treated similarly to rocks and chips, using loose coils of line (up to 60 m per hatch tank) arrayed in the bottom of the tank. The coils were turned daily to encourage uniform spore settling.

Substrates were removed from tanks after 72 h. The success of spore settling during a hatch was evaluated by placing ten microscope slides in each hatch tank. These were recovered and counted for spores under a light microscope using ×400 or ×100 magnification. One area on each slide was randomly selected by moving the slide on the stage without looking through the eyepiece, and all the spores within the selected field of view were counted. If there were more than 100 spores in the field of view under ×100, the slide was counted under ×400. Cystocarpic material was used for three hatches and then replaced with fresh material. Hatchery tanks were drained, cleaned with bleach solution, and refilled with fresh seawater between hatches.

2.3. Growth of sporelings

From August 1993 to February 1994, 27 hatches were conducted at approximately weekly intervals. Coral chips (14 hatches) or pebbles (eight hatches) in the hatchery trays were placed on the lagoon bottom in water 0.5–1 m deep. The trays could be relocated and sampled to follow the progress of individual hatches. Inoculated lines (five hatches) were stretched between wooden posts or concrete blocks on the pond bottom near the trays. Sporeling survival and density on substrates was evaluated by counting sporelings under a dissecting microscope. Ten pebbles or coral chips were selected at random from trays from a hatch. The entire surface of each substrate was scanned under low power (×10) to determine if any sporelings were present. The success percentage of a hatch was calculated from the number of substrates that had visible sporelings. If spores were found, the density of spores per square centimeter of substrate surface was estimated by counting all spores in a ×30 field of view similar to the procedure for counting sporelings on slides, except the field of view was not chosen randomly, but was chosen in an area of high sporeling density, since most of the surface of substrates did not have any sporelings present. After a hatch was sampled, the substrates were broadcast onto the pond bottom and the trays were returned to the hatchery for reuse after cleaning.

After 20–25 weeks, plants were visible on substrates without magnification. The further growth of plants was measured by the increase in length of thalli and, at maturity, by the fresh weight of algae harvested from samples of pebbles, coral chips and line segments. Growth experiments were conducted by fastening eight coral chips or pebbles containing sporelings into trays (40 cm × 40 cm) and placing trays on the pond bottom at a spacing of one tray per square meter. Growth of plants on lines was measured on marked 0.3-m segments on inoculated lines.

After the first 27 hatches, the hatchery operation continued, but inoculated substrates were broadcast directly onto the pond bottom to save the expense of placing trays in the ocean. The latter hatches used mainly large, smooth river rocks (5–15 cm diameter), rather than pebbles or chips, as substrates, because these were easier to locate on the

pond bottom and were more stationary during storms. The latter hatches were broadcast into areas protected by pens made of 1-cm-mesh, plastic fence material (Vexar) to keep out fish and turtles. The long term survival of plants on pebbles and chips was estimated by collecting broadcast substrates from the bottom. In each location where substrates had been broadcast, 100 coral chips, pebbles or rocks were randomly sampled from the bottom, by reaching through the plants and into the sand and retrieving the first substrate encountered. The substrate was scored for the presence or absence of *Gracilaria* plants, and the next sample was drawn at least two paces from the previous sample.

2.4. Other methods

The percentages of cystocarpic plants in cultured and wild populations were compared by collecting approximately 10 kg (300–500 plants) from each population on the same day. The samples from the cultured population were drawn throughout the pond. Plants were placed in vigorously aerated tanks, such that thalli were moving in the water, and 100 plants were withdrawn from each population and scored as cystocarpic or not cystocarpic. The frequency of cystocarps on thalli was determined by sampling one side branch, 8–12 cm length, from the first ten cystocarpic plants sampled from the tanks. Spore release by tetrasporophytes and carposporophytes was compared in small-scale experiments in which 50 g of either smooth thalli (presumed to be tetrasporophytes) or thalli with cystocarps were placed over microscope slides for 48 h in 20 l containers of aerated seawater. Spore diameters were measured on spores settled onto microscope slides using a calibrated ocular micrometer in a paired aquarium experiment comparing tetrasporangial to cystocarpic thalli.

Analysis of variance of measurements among treatments was conducted using the t-test for comparison of two means and the F-test for comparison of more than two means, after testing for homogeneity of variances. When variances were significantly different among treatment groups, tests assuming unequal variances were used. Percentages of cystocarpic plants in wild and cultured populations and percentage of substrates with sporelings in penned and unpenned locations were compared using the χ^2 test. Statistical tests were from Li (1964).

3. Results

3.1. Carpospore release and development

Carpospores appeared on slides as dark red, spherical cells, approximately 20 μm in diameter with some undergoing cell division after 72 h (Fig. 1). The slides contained numerous organisms in addition to *Gracilaria* sporelings, including diatoms, protozoans, nematodes, fungi and other algal spores. Hence, the hatchery procedure did not produce unialgal cultures. However, spores and sporelings were easily distinguished from contaminating organisms by their color and shape. Slides could be held under running water without dislodging the sporelings, and this removed many of the contaminating organisms and facilitated counting the sporelings.

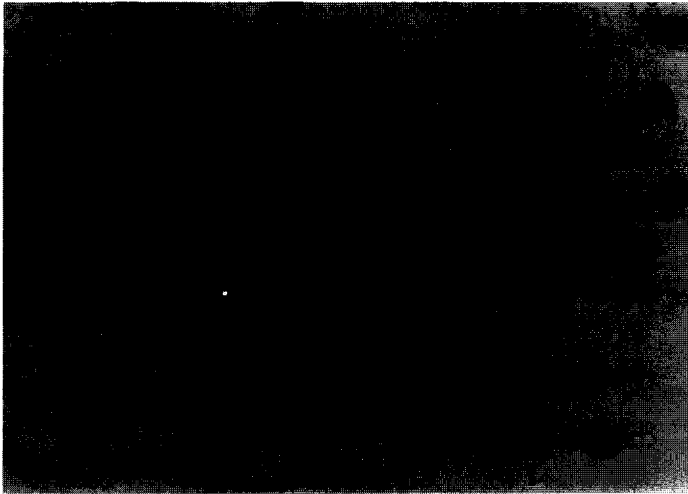

Fig. 1. Carpospores of *Gracilaria parvispora* adhering to a glass slide retrieved from a hatchery tank after 72 h exposure to cystocarpic thalli. The bar in the lower right hand corner is 100 μm.

By 14 days after inoculation, sporelings had formed a basal cell mass and had initiated thallus growth. By 21 days, sporelings were visible on substrates under a dissecting microscope as raised, unbranched growths and reached several millimeters length by 10 weeks (Fig. 2).

Test slides from 20 of the first 22 pebble and coral chip hatches were counted. Slides had a mean sporeling density of 1810 cm^{-2} but with considerable variability among

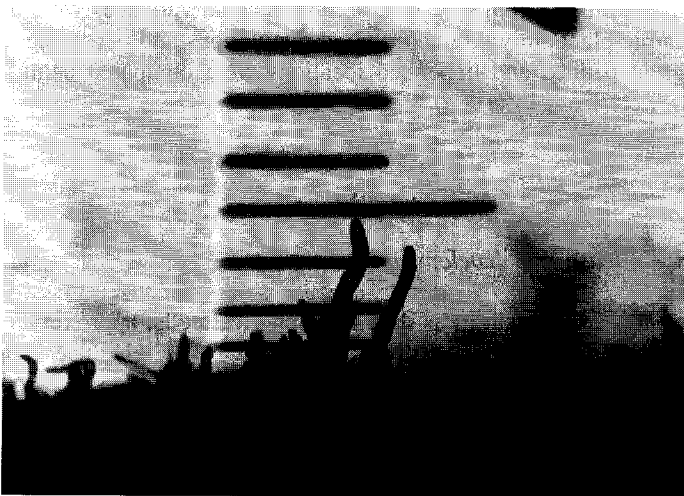

Fig. 2. Sporelings of *Gracilaria parvispora* growing on a pebble, approximately 18 weeks after spore release. Each division on the background ruler is 1 mm.

Table 1
Percentage of inoculated pebbles and coral chips having one or more sporelings of *Gracilaria parvispora*, and density of sporelings on the substrate, after 18–22 weeks in a fishpond. Table entries are mean values across hatches ($n = 14$ for coral chips, $n = 8$ for pebbles). Standard deviations of means are in parentheses. Differences between means were tested using the t-test for unequal sample size and variances; coral chips did not differ from pebbles at $P < 0.05$. Means are weighted for unequal sample sizes

Substrate type	Success (%)	Sporeling density (cm^{-2})
Coral chips	66.4 (35.9)	4.74 (4.42)
Pebbles	51.3 (38.3)	2.91 (2.31)
Mean	60.9	4.08

hatches (SD = 1610). Only one of the hatches was judged to be a failure; mean sporeling density on the test slides from that hatch was only 20 cm^{-2} compared with a range of 200–6950 cm^{-2} for the other hatches. However, even this hatch had spores visible on nine of the ten test slides. The cause of low spore release in this hatch is not known, but the same substrates were successfully inoculated by replacing the cystocarpic material in the hatch tanks with freshly collected material.

Sporeling survival and growth were evaluated on coral chips and pebbles retrieved from the first 22 hatches after 18–22 weeks in the pond. Coral chips produced denser plantings and a higher success percentage than pebbles, but differences were not significant at $P < 0.05$ (Table 1). Combining data for chips and pebbles, 61% of substrates had visible sporelings after 18–22 weeks. The success percentage of individual hatches varied from 0% (four hatches) to 100% (four hatches). Mean sporeling density on substrates was 4.08 cm^{-2}, much lower than the density on slides, indicating a survival rate of less than 1% of sporelings in the pond environment. Sporelings on pebbles and chips were small at 18–22 weeks, ranging from 0.02 to 0.6 cm with no branching evident.

Other seaweeds also became established on the substrates, notably the red alga *Acanthophora spinifera* and the green alga *Enteromorpha intestinalis*, which were the major epiphytes in the culture system. The substrates were also colonized by sponges, worms, small crabs and other fauna. The small size of the sporelings may have been due to fish grazing on the substrates and the fact that they were covered with a fine layer of sand and silt, or dormancy as noted by Levine (1986).

Spore settling and development on lines was not monitored as closely as on coral chips and pebbles, since it was necessary to destructively sample the line segments to examine them under the microscope. However, lines developed plants up to 4–5 cm with branching evident by 10 weeks (Fig. 3), much earlier than on coral chips or pebbles. In contrast to the coral chips and pebbles, the lines were not stationary in the water, but moved with the waves and tides, and this may have offered partial protection against bottom-feeding predators, and helped keep the sporelings from being covered by sand and silt.

The success rate of line hatches was evaluated by inspecting lines for visible plants after 10 weeks. Of five hatches, three produced lines that were nearly continuously covered with *Gracilaria* thalli that matured to harvest size by 23–25 weeks. One hatch

Fig. 3. Young plants of *Gracilaria parvispora* growing on a nylon rope, approximately 10 weeks after spore release.

did not produce any *Gracilaria* plants and one produced only occasional thalli along the lines. However, the numbers of lines planted out from each hatch were unequal. Of 19 total lines placed out, seven produced successful crops, for a 36% success rate.

While *Gracilaria* established faster on lines than on pebbles or chips, competing organisms also established quickly on lines. The lines with poor *Gracilaria* growth became overgrown with *A. spinifera*, *E. intestinalis*, sponges, oyster spat and blue-green, filamentous algae. The rapid growth of competing organisms, rather than lack of initial spore settling, appeared to be the cause of the lower success rate of line hatches compared to pebble or chip hatches.

We attempted to control the growth of competing organisms on coral chips and pebbles by two methods. In the first method, we held trays in tanks of aerated seawater with daily water exchange for up to 8 weeks. Trays were cleaned of epiphytes under running seawater at least weekly, and larger epiphytes and other organisms were removed from substrates by hand as they appeared. In the second method, trays were placed in the pond but were retrieved for weekly cleaning. A similar attempt was made to remove growths from lines through weekly cleaning. These attempts were very labor intensive and did not result in greater growth or survival of sporelings; competing organisms became established despite the cleaning efforts.

3.2. Growth and yield of carpospore cultures

The first plants to reach harvest size were from a line hatch conducted in August 1993 (Fig. 4). The hatch consisted of 14 individual, 0.2-cm-diameter lines, which were

Fig. 4. Line planting of *Gracilaria parvispora* 15 weeks after spore settling (the planting is actually 14 separate lines twisted together into a single planting).

twisted together into one 13.7 m length planting between two stakes. The lines produced *Gracilaria* thalli up to 50 cm in length by 15 February 1995 (15 weeks). The line was partially harvested on 15 February and cut back to 5 cm along its entire length on 3 March 1995, yielding a total of 863 g m^{-1} over 23 weeks. This line continued to produce *Gracilaria*, yielding approximately 1650 g m^{-1} over 12 months of harvest, but with an increasing amount of epiphytic growth appearing on the line over time.

A replicated growth experiment was conducted comparing sporelings settled onto line, coral chips and pebbles. Six inoculated lines, 0.5 cm diameter and 30 m in length (three each from two separate hatches 1 week apart in February, 1994) were spaced 1 m apart between stakes. Three sampling sections were marked off on each line for repetitive sampling of number and length of developing plants, and final harvest weight of plants. Twenty-four pebbles and chips, strapped in trays, from hatches conducted in September 1994 were placed in a penned area in the lagoon simultaneously with planting the lines in February. Two additional trays (one of coral chips and one of pebbles) were placed outside the enclosure to determine the effect of predation on *Gracilaria* growth. The substrates all had visible sporelings (0.2–0.4 cm) at the start of the experiment. Plantings were allowed to establish further growth, and were measured for 7 weeks from 17 June to 4 August 1994, at which point plants were cut back to 5 cm length to determine the yield of harvestable material.

In the penned area, plants on coral chips were longer initially, elongated faster during the 7 week experiment, and produced more biomass per plant at harvest than plants on pebbles ($P < 0.05$) (Table 2). Plants on coral chips grew into large plants, up to 50 cm in length, by harvest (Fig. 5). The number of plants per substrate at harvest did not differ significantly ($P < 0.05$) between chips and pebbles, ranging from five to eight per

Table 2
Gracilaria parvispora growth on coral chips, pebbles and lines. Length increase was measured over the final 7 weeks before harvest. Weeks to harvest is the total time from hatchery to harvest. Yields per square meter are based on eight chips or pebbles or one line per square meter. Standard deviations of means are in parentheses; means followed by different letters within a row are different at $P < 0.05$ by the F-test for unequal sample size ($n = 24$ for chips and pebbles, 18 for lines). The number of plants at harvest on chips and pebbles was not compared with that on lines because measurement units were different

Substrate	Coral Chips	Pebbles	Lines
No. plants [a]	8.0 (6.5)a	5.2 (3.8)a	21.3 (12.7)
Initial length (cm)	4.52 (2.46)a	3.17(2.31)b	11.4 (5.07)c
Length increase (cm week^{-1})	3.29 (2.47)a	1.22 (0.86)b	4.52 (1.78)a
Yield per plant (g fresh wt.)	16.0 (17.4)a	5.3 (12.2)b	16.3 (12.2)a
Yield (g m^{-2})	974 (1216)a	126 (130)b	830 (381)a
Time to harvest (weeks)	50	50	25

[a] Units are: per chip for coral chips, per pebble for pebbles, and per 0.3 m for lines.

substrate (approximately one to two plants cm^{-2}), but was lower than the density of sporelings measured after 18–22 weeks (Table 2). Plants on lines produced the same amount of biomass per plant as plants on coral chips (Table 2), both significantly greater than plant biomass on pebbles ($P < 0.05$). When yields were expressed on a unit area basis, using eight chips or pebbles per square meter and one line per square meter as the planting densities, coral chips and lines yielded 974 g m^{-2} and 830 g m^{-2}, respectively, whereas pebbles yielded only 126 g m^{-2} in this experiment. Although the coral chips and lines produced similar yields on harvest, the line plantings required only 25 weeks to reach harvest size whereas the coral chips and pebbles required 50 weeks, due to the long lag period before visible growth appeared on coral chips and pebbles.

Pebbles and chips in trays outside the penned area showed no net growth in length increase or biomass over the experiment, but final plant densities were similar to those inside the penned area (five to eight per substrate). The plants outside the pens had short, blunted thalli, indicating that they were being grazed upon.

3.3. Survival and yield of penned plants

Several thousand inoculated pebbles, chips and rocks from the hatchery were broadcast into three penned sections of pond (total area 690 m^2) and compared with substrates broadcast into unpenned locations between February and December 1994. The areas were sampled twice in 1995, at which time plants ranged in age from 5 to 18 months. Approximately 45% of substrates in penned areas had plants compared with 14–30% in unpenned areas (Table 3) ($P < 0.01$). Ke Kua'aina personnel harvested the pond weekly, by wading through the planted areas and collecting growth greater than 5 cm length on substrates. Plants in pens ranged in size from 1 to 25 cm or larger, and many had been harvested at least once, whereas plants in unpenned areas were nearly all small. The yield of plants from the penned areas ranged from approximately 5 to 30 g m^{-2} week^{-1}, with an estimated annual harvest of approximately 520 g m^{-2} from June 1994 to June 1995. Yields were highest from August through October, and lowest from

Fig. 5. Tray of *Gracilaria parvispora* plants 50 weeks after spore release onto coral chips. The plants are growing on eight chips fastened to the bottom of the tray. The tray was inside a pen to keep out larger predators.

December through March. The yields from the penned areas were close to what was expected on a unit area basis based on data in Table 2 but plants in unpenned areas generally did not produce a harvestable yield.

Table 3
Percentage of substrates with visible *G. parvispora* plants in penned ($n = 300$ per sample date) and unpenned ($n = 200$ per sample date) pond locations on two dates. *P* gives significance level of differences between success percentages at each sample date by χ^2 test (d.f. = 1)

Sample date	Success percentage		*P*
	Penned	Unpenned	
2 May 1995	47.3	29.5	< 0.001
21 July 1995	45.7	14.3	< 0.001
Mean	46.5	21.9	

Table 4
Percentage of cystocarpic plants and frequency of cystocarps along branches in wild and cultured populations of *G. parvispora*. Percentages determined at each sampling date were compared with a model system in which all plants are reproductive and 25% are cystocarpic. *P* gives significance level of difference between the sample compared with the model by χ^2 test ($n = 100$, d.f. = 1). Frequency of cystocarps was determined for ten branches from wild and cultured cystocarpic plants at each date; *P* gives significance level for difference between means by *t*-test assuming equal variance (d.f. = 18). Standard deviation is in parentheses

Sample date	% cystocarpic		No. of cystocarps (cm^{-1})	
	Wild	Cultured	Wild	Cultured
6 June 1995	24 NS	11 $P < 0.01$	4.46 (1.54)	1.92 (0.70) $P < 0.01$
24 July 1995	19 NS	9 $P < 0.01$	2.93 (0.89)	1.69 (0.80) $P < 0.01$
7 Aug. 1995	25 NS	14 $P < 0.05$	3.57 (1.05)	2.20 (0.62) $P < 0.01$
Mean	23	11	3.65	1.94

3.4. Production of cystocarpic gametophytes in the culture system

During 1993–1994, nearly all the plants produced in the culture system were smooth (without cystocarps). Starting in late 1994, however, cystocarpic material began to appear among the culture products. This suggested that the alternation of the generations had been completed in the culture system, through the release of tetraspores that attached to substrates and grew into male and female gametophytes, which were fertilized and produced cystocarps. We quantified the production of cystocarpic thalli in the culture system and tested the spore release ability of cultured tetrasporophytes.

The percentage of cystocarpic thalli was determined for cultured and wild plants on three different sampling dates (Table 4). Percentages were compared with a model system in which 50% of plants are tetrasporophytes, 25% are male gametophytes and 25% are female gametophytes bearing cystocarps. This model assumes that there is no separate vegetative stage, and that all female gametophytes become fertilized and exhibit cystocarps. 23% of wild plants were cystocarpic, a percentage which did not differ significantly from the model, but only 11% of cultured plants were cystocarpic. Cultured, cystocarpic plants were compared with wild cystocarpic plants for the frequency of cystocarps along the branches, and on each sampling date there was a lower density of cystocarps on cultured plants than wild plants (Table 4). Cultured plants had only half as many cystocarps per length of thallus as wild plants, and were readily distinguishable from wild, cystocarpic plants on this basis.

Tetrasporophytes from the culture system were compared with wild cystocarpic thalli for the ability to release spores. Tetrasporangia were visible on tetrasporophytes as dark-pigmented, cruciately dividing cells scattered on the thallus surface, but they were difficult to quantify due to the presence of numerous other pigmented, dividing cells in the cortex. Tetraspore hatches were initiated with smooth plants from the culture system, which were presumed to be mainly tetrasporophytes but which also could have included male gametophytes. The rates of spore release onto slides from tetrasporophytes and cystocarpic plants were compared in three experiments using freshly collected plants (Table 5). The density of starting material was approximately ten times greater than in a

Table 5
Spore release by wild cystocarpic (carpospores) and cultured tetrasporic (tetraspores) *G. parvispora* plants on three dates.The table entries give mean spore densities on slides placed under 50 g of thalli of each plant type. *P* gives significance level of the difference between means by *t*-test assuming unequal variances

Sample date	Spore density (cm^{-2})		*n*	*P*
	Carpospores	Tetraspores		
21 Dec. 1994	30400 (21850)	6950 (7950)	9	< 0.05
6 Feb. 1995	9000 (2400)	600 (590)	8	< 0.01
5 May 1995	16450 (10950)	1550 (400)	5	< 0.01
Mean	18600	3050		
Ratio	6.1	1		

normal hatch, in order to detect the expected low release of spores from tetrasporophytes. In each experiment cystocarpic plants released more spores than tetrasporophytes, but the tetrasporophytes still showed significant spore release, with slides having from 600 to 7000 sporelings cm^{-2} after 72 h. The ratio of spores released from tetrasporophytes compared to cystocarpic thalli was approximately 1:6 over all sampling dates. The (presumed) tetraspores were similar in appearance to carpospores and underwent normal cell division. A comparison of spore diameters 48 h after release showed that on average tetraspores were slightly smaller than carpospores, although the range of values overlapped (mean $\pm$ SD tetraspore diameter 18.7 ± 18.3 μm, $n = 36$; mean carpospore diameter 21.7 ± 16.4 μm, $n = 98$; significant difference between means at $P < 0.001$).

4. Discussion

Carpospore culture of *G. parvispora* proved to be a feasible method to generate large numbers of inoculated substrates at low cost. The method was much more efficient than attaching individual vegetative fragments to substrates by hand, as is the current practice in some culture systems (Santelices and Doty, 1989). The principal difficulty was in growing the sporelings out to harvestable size. Sporelings underwent an apparent high attrition rate in our culture system. Starting densities of 1800 cm^{-2} were reduced to 2 cm^{-2} or less by harvest, for a survival rate of approximately 0.1%. This loss in numbers may have been partly due to coalescence of individual sporelings to form fewer plants (Muñoz and Santelices, 1994), but the numerous predatory and competing organisms that were observed on the substrates at all stages of growth must have accounted for most of the loss of sporelings. The sporelings also had a long lag period before harvest-size plants appeared.

Despite the attrition rate of individual sporelings, a significant percentage of substrates eventually produced plants. The 45% success percentage of pebbles and chips in penned areas may be adequate, given the low cost of production of these substrates and the lack of maintenance requirements after hatchery. Plants grown on lines produced potentially two crops per year rather than one, yet the line method did not appear to be

sufficiently reliable in our system to justify large scale plantings. Two of every three lines placed out were not productive, and lines required more labor and expense to set out than coral chips or pebbles.

Given the more rapid development of sporelings on lines compared with chips or pebbles, a protected nursery phase, in which lines are held in filtered seawater to prevent growth of competing organisms, may be justified. Our attempts to develop a nursery system for pebbles and chips were not promising, owing to the high labor required and our lack of results in actually subduing competing organisms through weekly maintenance.

Although coral chips supported better plant growth than pebbles in this experiment, pebbles also produced large plants over time. The reason for the better performance of plants on coral chips is not known. The chips had numerous holes, which we observed to be the first sites where sporelings proliferated, presumably due to protection from predators. By contrast, the smooth pebble surface was completely accessible to feeding animals. Conceivably, sporelings were able to establish faster and form sturdier basal structures on coral chips than on pebbles. The shorter lag period of sporelings on lines compared to chips or pebbles is understandable since pebbles and chips on the pond bottom were covered by sand and silt, which must have slowed the initial growth of sporelings, whereas lines were kept clear of silt by the movement of the line in the water from wind and tides. The decision on what type of planting system to employ will be based on local site considerations, substrate availability and on whether the success percentage of line plantings can be improved; spores settle readily on almost any type of material.

G. parvispora does not appear to have a strictly vegetative stage, since the percentage of cystocarpic plants in wild populations was that expected if all plants were reproductive at all times. The lower percentage of cystocarpic plants among culture plants compared to wild plants was expected, since the cultured population was skewed towards tetrasporophytes due to the continual introduction of new tetrasporophytes from the hatchery. The lower frequency of cystocarps along branches of cultured compared with wild plants indicates that fertilization of eggs was not as efficient in the culture system as in the wild, perhaps owing to the lower frequency of gametophytes in the cultured population. The slightly smaller size of haploid than diploid spores is similar to reports from other species (Destombe et al., 1993).

The release rate of tetraspores from cultured tetrasporophytes indicated that the same hatchery procedures that were used for carpospores can be used for tetraspores, but using six times more fertile material per hatch, to compensate for the lower spore release rate by tetrasporophytes. The deliberate cultivation of tetraspores as well as carpospores would augment the supply of fertile material available to start hatches, which can be a limiting step in the culture system.

The biomass yields of approximately 500–1600 g m^{-2} $year^{-1}$, depending upon culture method, were low compared with tank culture (LaPointe et al., 1976; Ugarte and Santelices, 1992) that can yield 100 times higher. On a per-crop basis, our yields were higher than Taiwan pond yields (300 g m^2 per crop), but the ponds yield six to eight crops per year, compared with only one to two for the spore method. The fewer crops reflect the long lag period required for sporelings to reach first harvest. The substrates

continued to yield material after the first harvest and it would be advantageous to continue to harvest substrates for as long as they are productive.

In conclusion, spore culture of *G. parvispora* and perhaps other *Gracilaria* species appears to be a simple method to initiate mass plantings, which can be cultured on lines or hard substrates. The low cost of the coral chip method makes it attractive as a method for enhancing wild stocks of *Gracilaria* that have been diminished by overharvesting, as well as for producing *Gracilaria* for direct harvest. In the case of *G. parvispora*, all the life stages can be produced in aquaculture, making the system independent of wild stocks. The chief disadvantages of spore culture are the long lag period and therefore lower yield potential due to the small starting size of spores compared with vegetative fragments, the negative effects of predatory and competing organisms on growth and survival of sporelings, and the unpredictability of results, especially of line cultures.

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