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THREE ALGAL PROPAGATION METHODS ASSESSED TO CREATE A RHODOPHYTA DIET FOR JUVENILE GREENLIP ABALONE (*HALIOTIS LAEVIGATA*) IN THE LATER NURSERY PHASE

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ABSTRACT A variety of red algal species have been identified as potential food sources for juvenile Greenlip Abalone, *Haliotis laevigata* (>5 mm shell length). To provide the red algal species in a diet suitable for juvenile abalone three propagation methods; spore production, protoplast isolation, and fragment culture were investigated. The potential algae requirements and consumable costs for each propagation method were determined, using experimental data and values from the literature, to assess the viability of utilizing each of these propagation methods in a commercial abalone nursery. The use of red algal spores required 592–52,000 kg of algae, depending on the level of spore release and the percentage of fertile algal thalli collected. Protoplast isolation reduced the amount of algal biomass to 8.55–910 kg but was affected by the efficiency of the isolation procedure. Even at an efficient production of 1×10^8 protoplasts·g^{-1} wet weight alga the cost of consumables (enzymes) was $US 13,576. A feeding trial utilizing *Laurencia* sp. fragments adhered to the plates using agar, produced juvenile abalone growth rates comparable to those obtained with the current commercial nursery diet of the green alga *Ulvella lens* plus the diatom *Navicula* cf. *jeffreyi*. The *Laurencia* fragments did not regenerate on the plates so it was reapplied weekly, which is not feasible on a commercial scale because it would require 10.6 t of *Laurencia* and 443 kg of agar at a cost of $US 34,899. *Gracilaria* sp. fragments were able to regenerate with a growth rate of 4.42%·day^{-1} and therefore the algal fragments would only need to be applied to the PVC plates once, at the start of the later nursery phase (5 mm SL), reducing the amount of algal biomass to 432 kg. We therefore conclude that regenerating fragment culture (fragments <1 mm) is the only method that could successfully produce red algal diets for juvenile abalone on a commercial scale in the later nursery phase.

KEY WORDS: juvenile abalone, *Haliotis laevigata*, red algae, spore formation, protoplast isolation, fragment culture, algal biomass

INTRODUCTION

In Australian abalone nurseries, juvenile abalone currently feed on the crustose green alga *Ulvella lens* Crouch, the diatom *Navicula* cf. *jeffreyi* and/or any naturally occurring diatoms (Daume 2006). This diet provides adequate nutrition and achieves growth rates of 70 μm·day^{-1} for juvenile *Haliotis laevigata* Donovan (3–10 mm SL) (Daume & Ryan 2004, Strain et al. 2006). However, as juveniles reach 6–8 mm in shell length, depending on stocking density, food availability may become inadequate, jeopardizing growth rates and consequently the later growth and survival of the animals.

Two alternatives to overcome this inadequacy in feed availability are: firstly to move stock into growout systems and wean the juveniles onto formulated feed, or secondly, to maintain a suitable food supply throughout the later nursery phase by utilizing different algal species.

Weaning onto formulated feeds can be done at anytime during the nursery stage and is dependent on growth rates, food availability and tank space. Many studies have examined the advantages or disadvantages of formulated feeds with varying results. Some studies have observed greater growth rates of juvenile abalone consuming various algae species (Daume & Ryan 2004, Naidoo et al. 2006), whereas others show a significant increase in growth rates utilizing formulated feed (Corazani & Illanes 1998, Viana et al. 1993), while no difference between formulated feeds and natural diets has also been recorded (Boarder & Shpigel 2001, Serviere-Zaragoza et al. 2001).

Incorporating different algal diets into nursery systems may allow juveniles to remain in the system for a longer period of time, maintain higher growth rates, and reduce husbandry stress. Australian abalone tend to prefer red algae, unlike their counterparts elsewhere that feed predominately on brown algae (Fleming 1995, Poore 1972, Shepherd & Cannon 1988, Shepherd & Steinberg 1992, Wells & Keesing 1989). This preference could be due to the high abundance of red algae in the natural environment, as well as the presence of high concentrations of unpalatable polyphenolic compounds in Australian brown algae (Steinberg 1988, 1989, Steinberg & van Altena 1992).

To allow juvenile abalone access to a variety of red algae species in the nursery system, techniques need to be developed to seed the vertical plates with red algae. Three propagation methods; seeding the plates with algal spores, protoplasts, or fragments have been identified.

Red algae produce two types of nonmotile spores that can settle on plates, attach and grow into small plants: carpospores (zygospores) and tetraspores (meiospores) (Destombe et al. 1989, 1992). However, spore production in red algae is highly variable (Azanza & Ask 2003) and would directly affect the plantlet density on the plates and the quantity of fertile red algal biomass required to release adequate spores.

The use of thallus fragments to produce plantlets by vegetative growth (asexual reproduction) removes the need

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for fertile material and spore production. This method of propagation has been successfully used in the agarophyte industry in the commercial cultivation of many species including *Gelidium*, *Gracilaria,* and *Gigartina* sp. (Ajisaka & Chiang 1993, Buschmann et al. 1995, 2001, Hurtado-Ponce 1990). To present the fragments to juvenile abalone in the nursery system they need to be adhered to the plates. This is possible by the use of a gelling agent such as agar, through a procedure similar to the agar/formulated feed mixture method of Stott et al. (2004a, 2004b).

The production of protoplasts creates single cells from pieces of algal thallus and these protoplasts subsequently regenerate into whole plants. Protoplasts have been isolated from several species of red algae including *Gracilaria*, *Grateloupia*, and *Bangia* sp. (Araki et al. 1994, Chen & Chiang 1994, Polne-Fuller & Gibor 1984, Xing-Hong & Wang 1993). Protoplasts could greatly reduce the red algal biomass required by the abalone farm but methods to facilitate adhesion of the protoplasts onto the PVC plates would still be required.

This study therefore examines the three propagation methods regarding their capacity to produce a red algal diet for juvenile abalone in the later nursery phase.

MATERIALS AND METHODS

Small-scale laboratory experiments were conducted to determine the potential algal requirements and consumable costs of each propagation method for a commercial abalone farm. These potential requirements and costs were compared with theoretical minimum values determined using optimal results attained from the literature. Each of the three propagation methods can be applied to a variety of red algal species therefore several algal species have been used as examples of the methodology. Only the fragment feeding trial experiment incorporates an abalone growth trial.

Spore Production

The spore production experiment utilized *Plocamium mertensii* (Greville) Harvey, collected at 12–20 m depth from King George Sound, Albany, Western Australia. Only fertile tetrasporophyte thalli of *P. mertensii* were selected and a cold treatment of 4°C to 6°C in darkness for 24 h was then applied. Ten grams blotted wet weight (bww) was placed in 200 mL of sterilized seawater at 16°C ± 2°C on a 12 h:12 h, light:dark cycle at 100 μmol photons·m^{-2}·s^{-1} and spore release occurred over a 4 h period.

Protoplast Isolation

The red alga *Gracilaria* sp. was collected from the Swan Estuary, Perth, Western Australia and maintained in an aerated culture system at 18°C ± 2°C on a 12 h:12 h, light:dark cycle at 35 μmol photons·m^{-2}·s^{-1}. After being cleaned of epiphytes with sterile seawater, deionized water and cotton wool, the young branches were placed in f/2 medium (Guillard & Ryther 1962) at 18°C ± 2°C on a 12 h:12 h, light:dark cycle at 50 μmol photons·m^{-2}·s^{-1} one day prior to protoplast isolation.

The method for protoplast isolation was modified from Araki et al. (1998). The enzymes Cellulase Onozuka RS, Macerozyme R-10 (Yakult Pharmaceutical IND.CO, LTD), agarase, papain and mannitol (Sigma-Aldrich) were dissolved separately in two treatments, sterile seawater and an osmotic shock of deionized water, then filter-sterilized through a 0.2 μm membrane filter.

Young *Gracilaria* sp. branches (3–5 cm) were pretreated with 30 mL papain solution (20 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer, pH 6.5–7.5, containing 5% (w/v) papain and 0.7 M mannitol) and incubated at 25°C for 30 min with agitation on a reciprocal shaker (30 rev·min^{-1}). Treated branches were then washed three times with f/2 medium containing 0.7 M mannitol and cut into 1–2 mm segments. Segments of 0.5 g were treated with 10 mL of the enzyme mixture at 25°C and agitated in the dark for 150 min (30 rev·min^{-1}). The enzyme mixture contained 4 units agarase, 4% (w/v) Cellulase Onozuka RS, 2% (w/v) Macerozyme R-10, 0.7 M mannitol, and 20 mM MES buffer (pH 6.5). The reaction mixture was then filtered through 0.45 μm nylon mesh and the protoplasts collected by centrifugation at 190 *g* for 5 min. The pellet was gently resuspended in 2 mL f/2 medium containing 0.7 M mannitol and the number of released protoplasts counted using a hemocytometer.

Fragment Feeding Trial

The fragment feeding trial utilized a diet consisting of *Laurencia* sp. fragments adhered to the vertical PVC plates with agar. The *Laurencia* fragment/agar diet was compared with the current commercial nursery diet of *Ulvella lens* and *Navicula* cf. *jeffreyi* and a formulated feed in a weaner system (round, shallow tanks).

Laurencia sp. was collected at 8–12 m depth in King George Sound, Albany weekly and placed in an outdoor tank under natural irradiance with strong aeration. To prepare the diet, the thalli were cut into 2–3 mm fragments, with 1.8 kg wet weight of thallus needed to produce 2 L of fragments.

Agar was used as the adhesion substance and 75 g was dissolved in 4 L of 1 μm filtered seawater. Once the agar had cooled to approximately 40°C, 2 L of fragmented *Laurencia* sp. was added (2:1, agar:algae) resulting in a final mixture concentration of 1.25% agar. The mixture was then sprayed onto 20, 60 × 26 cm PVC plates using a compressed air undercoat spray gun. The plates were allowed to set for 1 h and then distributed into the tank with the aeration off overnight. The process was performed for 3 replicate tanks and the *Laurencia* fragment/agar diet reapplied weekly to maintain sufficient algal biomass on the plates.

The *Ulvella lens* and *Navicula* cf. *jeffreyi* diet was produced according to the methods in Daume & Ryan (2004).

For both of the *Laurencia* fragment/agar and *U. lens*/*N. jeffreyi* diets, three replicate 400 L tanks were each stocked with three baskets of 20 plates (60 × 26 cm). The tanks received 5 μm filtered seawater via a spray bar and were aerated by three airlines spaced evenly along the bottom. Each of the 6 nursery tanks was stocked with 2,400 juvenile greenlip abalone (*Haliotis laevigata*) of 8.13 ± 0.036 mm shell length, averaging 40 juveniles per 60 × 26 cm plate (128 abalone·m^{-2}).

Three 160 L shallow weaner tanks were each stocked with 6,000, 8.52 ± 0.092 mm juvenile abalone (1,910 abalone·m^{-2}). This system utilized commercially produced formulated feeds (50% coarse crumb and 50% noodle, Adam and Amos, South Australia) at a rate of 2% body weight per day (dry food, live abalone).

Abalone were measured by shell length fortnightly for the duration of the trial via a subsample of 50 juveniles per tank in the nursery tank system and 100 juveniles per tank in the weaner tank system. The *Laurencia* fragments were counted as the number of fragments per cm^2 at the beginning and end of each application (weekly) during the trial to determine the consumption rate by the juvenile abalone.

Fragment Culture

Gracilaria sp. was collected at 8–12 m depth in King George Sound, Albany and transported to the laboratory in Perth (4 h) between moist newspapers in coolers. *Gracilaria* sp. was used for fragment culture as *Laurencia* sp. fragments did not regenerate, other macroalage were only seasonally available and a limit on the supply of fresh material because of the closure of the abalone farm facilities in Albany. The algae were maintained in an aerated culture system for 2 wk at 18°C to 20°C on a 12 h:12 h, light:dark cycle at 35 µmol photons·m^{-2}·s^{-1}. Fragments of approximately 5 cm in length were cleaned of epiphytes with sterile cotton wool, surface sterilized using a 1% (v/v) Betadine solution and then rinsed with sterile seawater. These fragments were then cut into 5–7 mm fragments and placed in 150 mL, f/2 medium. The growth of the *Gracilaria* sp. fragments was examined over 2 wk at important temperatures (15°C ± 2°C and 18°C ± 2°C) in the tolerance range of juvenile greenlip abalone. The light attenuation found in nursery tanks was also tested by using the two irradiances, 75 and 350 µmol photons·m^{-2}·s^{-1} on 12 h: 12h, light:dark cycle.

Data Analysis

Protoplast yield and survival for the two treatments, sterile seawater, and the osmotic shock (deionized water) were both compared by analysis of variance (1-way ANOVA). Abalone growth rates throughout (start to week 8) the fragment feeding trial were compared using a repeated measures analysis of variance, whereas the abalone shell length and growth rates at the end of the trial (only week 8) were compared by analysis of variance (1-way ANOVA). A multiple regression carried out comparisons of the algal fragments and agar removed from the plates.

RESULTS

Spore Production

Only 8.69% of the fresh *Plocamium mertensii* thallus collected from the field had fertile fronds. A spore yield of 4.05×10^3 spores·g^{-1} blotted wet weight (bww) of thallus was recorded after 2 h, increasing to 4.75×10^3 spores·g^{-1} bww algae after 4 h.

Protoplast Isolation

A yield of $9.4 \pm 1.03 \times 10^5$ protoplasts was obtained from 1 g bww of *Gracilaria* sp. segments digested using the enzyme mixture in the osmotic treatment, deionized water (Table 1). This was significantly greater than the number of protoplasts isolated utilizing the enzyme mixture dissolved in sterile seawater ($F_{(df\ 1,4)} = 29.99$, $P < 0.05$). The proportion of protoplasts intact after 24 h obtained by the osmotic treatment (95%) was not significantly greater than for the sterile seawater treatment ($F_{(df\ 1,4)} = 2.34$, $P = 0.2$) (Table 1). However after eight days in culture no protoplasts survived as cell wall regeneration was inadequate.

TABLE 1.

Protoplast yield ($\times 10^5$ protoplasts g^{-1} bww algae) and percentage of protoplasts intact after 24 h for the two treatments, sterile seawater and the osmotic shock (deionized water) enzyme mixtures. Mean ± std. error ($n = 3$).

Enzyme Base	Protoplast Yield (10^5)	Intact Protoplast (%)
Seawater	3.3 ± 0.44	76.7 ± 5.74
Deionised Water	9.4 ± 1.03	95.2 ± 10.62

Fragment Feeding Trial

The shell length of the abalone increased on all three diets (start 8.13 ± 0.04 mm SL) with the *U. lens*/*N. jeffreyi* diet producing abalone of 10.79 ± 0.13 mm by week eight compared with 10.26 ± 0.23 mm for the *Laurencia* fragment/agar diet and 10.54 ± 0.15 mm for the formulated feed diet. At week 8 there was no significant difference in shell length among the three treatments ($F_{(df\ 2,\ 6)} = 2.25$, $P = 0.19$).

Growth rates on all three treatments increased steadily during the eight-week trial with no significant difference among the three treatments over time ($F_{(df\ 2,\ 6)} = 2.67$, $P = 0.15$). The formulated feed treatment had a very low initial growth rate but the rapid increase between weeks 4 and 8 resulted in a significantly higher ($F_{(df\ 2,\ 6)} = 5.42$, $P < 0.05$) growth rate than that achieved on the *Laurencia* fragment/agar diet at week 8 (Table 2).

The number of *Laurencia* fragments removed was positively correlated with the percentage cover of agar removed throughout the experiment ($R = 0.968$; $P < 0.05$). At the beginning of the trial the diet "peeled" of the plates, lasting only a week and there was no regeneration of algae fragments, therefore weekly application was required. After week 3 the amount of both algae and agar removed from the plates decreased dramatically because the diet was no longer "peeling" off the plates but being consumed by the juvenile abalone. Grazing resistant was lower than *Ulvella lens* so weekly application was still required to maintain sufficient algal biomass.

Fragment Culture

During the first week of culture the fragments of *Gracilaria* sp. decreased in biomass, whereas in the second week the biomass increased in all treatments (Table 3). The highest specific growth rate of *Gracilaria* sp. fragments over the 2 wk period was 4.42%·day^{-1} in the 15°C, 75 µmol photons·m^{-2}·s^{-1} treatment.

Potential Algal Biomass Requirements and Consumable Costs

To assess the potential algal biomass requirements and costs of consumables required for the three propagation methods on a commercial nursery scale various assumptions have been made. For a commercial abalone farm to grow ≈0.6 million juvenile greenlip abalone from 5–15 mm shell length the nursery

TABLE 2.

Mean growth rates (μm·day^{-1}) for juvenile greenlip abalone on the *Laurencia* fragment/agar, the *Ulvella lens*/*Navicula* cf. *jeffreyi* and the formulated feed diets. Mean ± std. error ($n = 3$).

	Week 0–2		Week 2–4		Week 4–6		Week 6–8	
Diet	Mean GR (μm·day^{-1})	SE (μm·day^{-1})	Mean GR (μm·day^{-1})	SE (μm·day^{-1})	Mean GR (μm·day^{-1})	SE (μm·day^{-1})	Mean GR (μm·day^{-1})	SE (μm·day^{-1})
Laurencia/agar	12.88	5.18	35.09	12.45	45.2	4.78	58.19	5.57
U. lens/*N. jeffreyi*	28.02	15.43	42.81	14.89	52.53	4.18	67.76	0.24
Formulated feed	5.91	4.01	29.64	3.92	48.12	6.26	81.79	6.85

requires 40, 3,000 L nursery tanks each containing 360, 60 × 30 cm vertical, flat PVC plates at a stocking density of 40 juvenile abalone per plate.

Spore Production

The production of tetraspores and carpospores by red algae is able to produce macroalgae germlings that are suitable for juvenile abalone (5–15 mm SL) consumption. However, spore production is extremely variable and is dependent on numerous factors including species, temperature, irradiance, day length, desiccation and osmotic pressure (Friedlander & Dawes 1984, Garza-Sanchez et al. 2000, Guzman-Uriostegui & Robledo 1999, Orduna-Rojas & Robledo 1999). The number of carpospores released from a gram of fertile female gametophyte can be up to 8×10^4 spores·g^{-1} (*Gracilaria arcuata*, *G. asiatica*, *G. corticata* and *G. pacifica*), whereas tetraspore release of up to 1×10^5 spores·g^{-1} (*G. arcuata*, *G. corticata*, *G. pacifica,* and *Palmaria palmata*) has been reported (Gall et al. 2004, Garza-Sanchez et al. 2000, Kaliaperumal et al. 1986, Umamaheswara Rao 1976, Xiuliang & Meizhen 1989).

Spore survival and attachment success will influence the number of tetraspores germinating and ranges from 0% to 85% (Buschmann et al. 1999, Gall et al. 2004, Garza-Sanchez et al. 2000, Oza & Tewari 1994). Forty percent germination (survival) is adequate for the germlings to be grown for approximately 1–2 mo to a sufficient length (0.5 cm) and density of 165 germling·cm^{-2} (Gall et al. 2004, Strain et al. 2006). Strain et al. (2006) utilized the green alga *Ulva* sp. and the germling density of 165 germling·cm^{-2} was found to be appropriate for juvenile *Haliotis laevigata* of 3.5–10 mm shell length.

From the *Plocamium mertensii* spore production experiment 4.75×10^3 spores·g^{-1} were released. The area covered by germlings, if 40% of the spores germinate, can be calculated using Eq. 1:

$$G_{area} = [P \times G]/G_{density} \quad (1)$$

where G_{area} is the area (cm^2) covered by germlings, P is the number of propagules produced per gram of wet weight thallus (propagules·g^{-1}), G is the germination (%) and $G_{density}$ is the density of germlings required (germlings·cm^{-2}).

Therefore 1 g of fertile *P. mertensii* thallus produces enough spores to cover 11.5 cm^2 of a PVC plate with germlings. Then the amount of fertile *P. mertensii* required by the commercial nursery can be calculated by:

$$AB = S_{area}/G_{area} \quad (2)$$

where AB is the algal biomass required (kg) and S_{area} is the total surface area of the PVC plates (cm^2).

The nursery would require 4.5 t of fertile *P. mertensii* thalli. However, the amount of fresh material actually required would be considerably greater (52 t), as the fertile fronds are only a small percentage (8.69%) of the algal thalli collected in the wild.

If an optimum tetraspore release of 1.7×10^5 spores·g^{-1} and survival (85%) was achieved (Oza & Tewari 1994, Umamaheswara Rao 1976), only 59 kg of fertile tetrasporic thalli would be required. Again the total amount of red algae collected to provide the fertile thalli (≈10%) would be much greater, 592 kg (Table 4).

TABLE 3.

The specific growth rate (%·day^{-1}) of *Gracilaria* sp. fragments (5–7 mm) grown at 15 ± 2°C and 18 ± 2°C, under two irradiances of 75 and 350 μmol photons·m^{-2}s^{-1}.

		Specific Growth Rate (% day^{-1})		
Temperature (°C)	Irradiance (μmol photons·m^{-2}s^{-1})	Wk 1	Wk 2	2 Wks
15	75	–4.42	19.2	4.42
	350	–6.56	14.41	0.06
18	75	–4.09	8.18	1.44
	350	–7.48	6.59	–1.5

Protoplast Isolation

After cell wall regeneration, protoplasts of *Gracilaria* sp. can form sporophytes on the PVC plates used in commercial abalone farms. However only a small percentage (<1%) of protoplasts isolated from various algae species form whole sporophytes (Chen & Chiang 1994, Cheney et al. 1986, Matsumura et al. 2000). If 1% of the 9.4×10^5 protoplasts·g^{-1} isolated *Gracilaria* sp. (Table 1) germinated, then the area covered by plantlets (sporophytes) can be calculated using Eq. 1.

Plantlets would cover 57 cm^2 of a PVC plate from the protoplasts isolated from 1 g of *Gracilaria* sp. thalli. Therefore the algal biomass required calculated by Eq. 2 to seed the 40 commercial tanks is 910 kg. To produce protoplasts from the 910 kg of *Gracilaria* sp. a large quantity of enzymes would be required. Based on the protoplast isolation experiment, the cost for the enzymes to treat the 910 kg would be $US 5,672,160 (Table 4). If the protoplast yield could be increased to 1×10^8 protoplasts·g^{-1} of *Gracilaria* sp. thallus (Araki et al. 1998) only 8.55 kg of *Gracilaria* sp. thallus would be required and the cost would be reduced to $US 13,876 (Table 4).

TABLE 4.

The algal biomass requirements and the cost of consumables for the three red algae propagation methods used to produce juvenile greenlip abalone (5–15 mm SL) diets in the nursery system (plates) of a commercial farm. Consumables cost includes enzymes and agar, while optimum values are sourced from the literature.

Method	Algal Biomass (kg)	Consumable Cost $US
Spore Production		
Results (Exp)		
Fertile Thalli	4,500	0
Total Thalli	52,000	0
Optimum		
Fertile Thalli	59	0
Total Thalli	592	0
Protoplast Isolation		
Results (Exp)	910	5,672,160
Optimum	8.55	13,876
Fragment Feeding Trial (Exp)	10,600	34,899
Fragment Growth Trial (Exp)	432	1,418

Fragment Feeding Trial

In the feeding trial 5.4 kg of *Laurencia* sp. was used to inoculate 180 plates (0.03 kg·plate^{-1}) each week because the fragments did not regenerate. To utilize this diet on a commercial scale and grow juveniles from 5–15 mm SL the amount of *Laurencia* sp. required will be considerably greater than if it was just an initial application. The time needed to achieve the 10 mm SL of juvenile growth will alter the number of applications required. If the juveniles continue at a growth rate of 58 μm·day^{-1} (Table 2) the number of *Laurencia* sp. agar applications can be calculated by:

$$A = [GI/GR]/7 \quad (3)$$

where A is the number of applications, GI is the growth interval (μm) and GR is the growth rate (μm·day^{-1}).

Based on Eq. 3, *Laurencia* sp. would need to be reapplied 24.6 times to produce juveniles of 15 mm SL. If the 40 commercial tanks' plates (360 plates.tank^{-1}) are applied with 0.03 kg·plate^{-1}, the biomass of *Laurencia* sp. requirement by the abalone nursery can be calculated using Eq. 4.

$$AB = A_{plate} \times Pl \times A \quad (4)$$

where A_{plate} is the amount of algae/agar per plate (kg·plate^{-1}) and Pl is the number of plates used. Nearly 10.6 t of *Laurencia* sp. would be needed to produce the 15 mm SL juvenile abalone (Table 4). To adhere the 10.6 t of algae over 6 mo 443 kg of agar is required at a cost of $US 34,899 (Eq. 4, Table 4).

Fragment Culture

The ability of *Gracilaria* sp. fragments to heal and attach to the PVC plates means only one application at the start of the juvenile nursery period would be required. If the same amount of *Gracilaria* sp. and agar are required to seed a PVC plate as the *Laurencia* sp. (30 g·plate^{-1}) and agar (1.25 g·plate^{-1}) in the fragment feeding trial then the amount of *Gracilaria* sp. and agar required to seed 40 commercial tanks (360 plates·tank^{-1}) can be calculated by Eq. 4. Therefore 432 kg of *Gracilaria* sp. and 18 kg of agar at a cost of $US 1,418 would be required by the commercial nursery (Table 4).

DISCUSSION

All three propagation methods; spore production, protoplast isolation, and fragment culture can be used in the creation of red algal diets for juvenile abalone on a small scale. However, in examining the algae biomass requirements and cost of consumables the use of the three propagation methods in the later nursery phase of a commercial abalone farm is more complex (Table 4).

Utilizing algal spores to seed PVC plates allows a diet of juvenile red macroalgae to be created at very low cost to a commercial nursery, because no consumables are required. However, it would be very difficult to obtain maximum spore release (Umamaheswara Rao 1976) and germination (Oza & Tewari 1994) every time the procedure was carried out. The achievement of consistently high yields would require a significant increase in algal culture facilities and staff skills, as the high spore release values given in the literature were produced under strict laboratory conditions. Red algal propagules are nonmotile and the majority of germination experiments are conducted on horizontal surfaces (Buschmann et al. 1999, Fletcher & Callow 1992, Orduna-Rojas & Robledo 1999). This produces significantly higher settlement and subsequent germination rates compared with settling on vertical plates, whereby either considerably larger tank area or greater spore production is needed to produce the seeded plates.

Overall, the algal biomass required to seed the PVC plates in a commercial nursery system with red algal spores is too great and it is not a viable option. Gall et al. (2004) indicated that platelets seeded with *Palmaria palmata* at a density of 200 germlings·cm^{-2} seemed to be a suitable method for supplying fresh macroalgae germlings in an abalone hatchery. However, based on their conclusion that 3 kg of fresh algae was required to seed 1 m^2, 15.5 t of *P. palmata* would be needed to seed the 40 commercial nursery tanks used in this commercial nursery model.

Protoplast isolation reduces the amount of red algal biomass required to produce a red algal plantlet diet (Table 4) and removes the problems associated with seasonality, because fertile thallus is not required to produce the protoplasts. If cell wall regeneration and cell differentiation occurs, then plantlets of red algae could be seeded on PVC plates; however, only a very small percentage of protoplasts survive to this stage (Chen & Chiang 1994, Cheney et al. 1986, Matsumura et al. 2000).

Isolation of protoplasts can be enhanced by the use of an osmotic treatment but to produce a red algal diet the method comes at considerable cost, because the enzymes are very expensive. Even if very high protoplast yields were attained on a regular basis, the cost of the enzymes is still $US 13,876 (Table 4). As for the spore production procedure, specialized culture equipment, laboratory facilities and skilled technicians would be required, increasing the cost to the farm and making

this method an unrealistic approach to red algal diet production for a commercial abalone nursery.

The production of fragment cultures, also removes the need for fertile material because whole thallus can be utilized. The ability of some red algae (*Gelidium*, *Gracilaria*, and *Gigartina* sp. etc) to regenerate from small fragments (Ajisaka & Chiang 1993, Buschmann et al. 1995, 2001, Hurtado-Ponce 1990) allows the algae diet to grow once inoculated into the nursery system and this considerably reduces the biomass of algae required. However, the weekly reapplication of the *Laurencia* fragment/agar diet to the plates in the fragment feeding trial because of the fragments inability to regenerate and the weak grazing resistance is not sustainable, both in terms of the algal biomass required and the cost of the agar (Table 4).

The juvenile abalone were taken off an *Ulvella lens* and *Navicula* cf. *jeffreyi* diet, subsequently producing a higher growth rate on the *U. lens*/*N. jeffreyi* diet in the first few weeks of the trial (Table 2). The first two weeks of growth rates were low and can be accounted for by a weaning period as the diets efficiency may be masked by the abalones predisposed condition (Fleming et al. 1996). The *Laurencia* fragment/agar diet, even though the algae did not regenerate (fragments died), was still able to produce abalone growth rates comparable to the current commercial nursery diet of *U. lens*/*N. jeffreyi* (Table 2).

If the *Laurencia* sp. could be kept alive and regenerate on the plates, as with the *Gracilaria* sp. fragment culture, it could prove to be an excellent diet. The results presented here indicate that the use of fragment culture is a viable method of producing a red algal diet for juvenile abalone in the later nursery phase of a commercial abalone farm.

In comparison with *U. lens*/*N. jeffreyi* diet the *Laurencia* fragment/agar diet provided substantially greater algal biomass. The *U. lens*/*N. jeffreyi* diet grows two dimensionally on the plates and subsequently has quite low biomass compared with the 430 kg of *Laurencia* sp. fragments. However the labor (1 person) required to produce the *U. lens*/*N. jeffreyi* diet is approximately six days (48 h, 8 h·day^{-1}) over a 6 wk period before the tanks are stocked with juvenile abalone. This labor requirement has been determined by extrapolating the time the diet development took in the fragment feeding trial. The *U. lens*/*N. jeffreyi* diet is fairly self-sufficient, because the *U. lens* can be induced to spore regularly via temperature, light, and nutrient addition (Daume & Ryan 2004). Therefore it only requires nutrients (Abasol) to be added once a week and the plates rotated every few weeks.

The *Laurencia* fragment/agar requires considerably more time (labor), because the fragments need to be adhered to the plates at the start of the later nursery phase. Based on the fragment feeding trial procedure it would take 30 d (240 h, 8 h·day^{-1}) to create the diet. If the juvenile abalone (5 mm SL) are moved into a weaner system utilizing formulated feed there are different time allocations and costs associated. Instead of requiring labor to create the diet the labor is needed every second day for half a day to feed and clean throughout the 6-mo season (5–15 mm SL) and therefore requires a total of 45.625 d (365 h, 8 h·day^{-1}). However labor requirements for the weaner system are highly variable and depend on growth rates, stocking densities, feeding rate, and weather season consequently the number of hours could be greatly reduced.

To produce a sustainable red algal diet in a commercial nursery via fragment culture, more work needs to be done on fragment regeneration, algae growth rates, and plate adherence. In the growth experiments with *Gracilaria* sp., growth was negative in the first week while wound healing occurred. A similar decrease in biomass was also recorded by Hernandez-Gonalez et al. (2007) for *Gigartina skottsbergii* fragments, where the excised margin of the fragments degraded during the first 2 wk, before specific growth rates of up to 1%·day^{-1} were attained.

An important benefit of fragment regeneration and growth is that substantially less algal biomass needs to be collected form the wild, because the algae can be cultured on site. This would reduce the reliance on wild populations of red algae making the nursery self-sufficient, as well as allowing the biochemical composition of the algae to be manipulated, potentially providing juvenile abalone with a consistently nutritionally superior red algae diet. However countries that have access to large quantities of algae such as Chile and South Africa would not need to culture on site and also be able to run the fragment culture at a lower efficiency.

Vegetative propagation using fragments is currently being used successfully by the agarophyte aquaculture industry, which farms a range of *Gracilaria* species on vertical ropes and in pond/tank culture. Hurtado-Ponce (1990) reported growth rates of up to 10.5%·day^{-1} for 10 cm fragments at a low stocking density (20 cm intervals) using a vertical rope cultivation system and maximum growth rates ranging from 0.86–4.45%·day^{-1} have been attained for 5–10 cm fragments for a variety of *Gracilaria* species (Chaoyuan et al. 1993, Chirapart & Ohno 1993). However, smaller fragments (<1 mm) are required for the juvenile abalone (5 mm SL) as well as to perform the adhesion methods (spraying) utilizing agar (fragment feeding trial). In the fragment culture experiment growth rates of 4.42%·day^{-1} were achieved over a 2 wk period for 5–7 mm fragments of *Gracilaria* sp. (Table 3). Such growth rates now need to be attained while the fragments are adhered to the PVC plates, thus providing greater biomass and a self-sustaining red algal diet.

Of the three methods studied, spore production and protoplast isolation have been shown to be unsuitable as a red algal diet on a commercial nursery scale. Spore production requires enormous amounts of algal biomass, and because of the variable spore production and seasonality of fertile material the algal diet biomass would be difficult to obtain. Although protoplast production requires very little algal biomass, the difficult procedure along with the high costs of the enzymes, adhesion methods and infrastructure required means that this method is not commercially viable. Regenerating fragment culture appears to be a suitable method to produce red algal diets. Substantial work still needs to be done to optimize fragment culture and growth rates before red algal fragments can provide juvenile abalone with a suitable diet in the later nursery phase.

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