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Settlement of *Gracilaria gracilis* carpospores (Gracilariales, Rhodophyta) on natural substrates from the southwestern Atlantic coast (Chubut, Argentina)

Abstract: *Gracilaria gracilis* is an important agarophyte that is exploited in Argentina by the harvest of detached thalli cast on the shore. Therefore, the study of carpospores is relevant because such spores can be used as inoculum for commercial aquaculture. In this study, the potential of two natural substrates from the Patagonian coast, for the settlement and growth of *G. gracilis* carpospores, was evaluated in the laboratory. Highly significant differences were found between substrates both in the proportion of substrates with settled spores and in carpospore density, shells being more suitable than pebbles. Regarding carposporeling growth, differences in sporeling length between substrates could not be detected by the end of the study, although basal discs reached a significantly higher diameter on pebbles than on shells. Results indicate that shells would be a good substrate to start spore culture because they offer a suitable surface for spore settlement. In addition, if the substrate is inoculated with sufficient seedling material to avoid competition among individuals and, if epiphytism is successfully reduced, sporelings should reach the required size for transport to the natural environment.

Keywords: carpospores; *Gracilaria gracilis*; spore culture; substrates.

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Abbreviation

SGR, Specific growth rate (% day^{-1}).

Introduction

Gracilaria (Gracilariales, Rhodophyta) is a genus of red algae with great economic importance because of its agar content, providing at present more than half the supply of raw material for this phycocolloid (Bixler and Porse 2011). Over the last decades, the demand for these algae has significantly increased, leading in many cases to the overexploitation of natural resources, a problem that resulted in the promotion of controlled cultivation (Oliveira et al. 2000, Jayasankar and Varghese 2002).

Due to the high regeneration capacity of *Gracilaria*, cultivation by means of vegetative propagation has been successful (Oliveira et al. 2000, Buschmann et al. 2001). Nevertheless, productivity drops after 2 or 3 years because of thalli aging (Buschmann et al. 2001). Besides, vegetative propagation is a method that requires a great amount of algal material to start and revitalize cultures (Glenn et al. 1996, Jayasankar and Varghese 2002). An alternative to this technique is spore culture, whose principal advantage is that it requires a small amount of reproductive material for substrate inoculation and the production of a large number of thalli (Halling et al. 2005, Mantri et al. 2009). The use of carpospores, instead of tetraspores, has been preferred because they are profusely released from cystocarps, which are structures easily distinguishable at glance on the female gametophyte (Glenn et al. 1996). Moreover, carpospores produce diploid tetrasporophytes, which are more vigorous than the haploid gametophytes produced from tetraspores (Mantri et al. 2009).

In Argentina, the genus is represented by only one species: *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine and Farnham, which forms beds in Bahía Bustamante, Bahía Melo and Bahía Arredondo, in the north of Golfo San Jorge, and in Bahía Nueva in Golfo Nuevo (Province of Chubut) (Boraso de Zaixso 1989). The most productive

of these beds is the one in Bahía Bustamante, which maintains itself by sexual and vegetative reproduction (Martín et al. 2011). Considered by the market as an excellent source of agar (Macchiavello et al. 1998), this species has been used for over 40 years for phycocolloid extraction by a private company; however, in the last few years there has been a considerable drop in biomass (Boraso et al. 2006) that resulted in the import of raw material. Recent studies on a biannual cycle of Bahía Bustamante's bed indicate some recovery in biomass with the presence of all reproductive stages (Martín et al. 2011). In addition, Michetti et al. (2013) have reported on carposporeling development, as well as sporulation periodicity, induction methods and factors affecting carpospore release. However, it is still necessary to evaluate the potential of different substrates for sporeling development. On this aspect, the most frequently employed substrates at a commercial scale are artificial ones (Alveal et al. 1997, Glenn et al. 1998) and there are very few references on the utilization of natural substrates (Infante and Candia 1988, Glenn et al. 1996).

The aim of this study was to evaluate and compare carpospore settlement, and the resulting tetrasporophyte growth, on two natural substrates from the southwestern Atlantic coast.

Materials and methods

Gracilaria gracilis thalli were collected in Bahía Bustamante (66° 32′ W/45° 07′ S), Chubut province, Argentina (Figure 1) in autumn (April and May 2010), winter (June 2010) and spring (September 2010). Thalli were drained and refrigerated for transport to the laboratory. Healthy cystocarpic thalli (without bleached segments and with low epiphyte loads) with mature cystocarps (1–2 mm in diameter), were selected. When epiphytes were present, they were removed manually with tweezers and by brushing, and thalli were washed with filtered (0.45 μm) and sterilized seawater.

Two substrates were used: small pebbles from Playa Unión (65° 01′ W/43° 18′ S), of 2.61 mm±0.08 (mean±SE), and bivalve shells from Bahía Bustamante (66° 30′ W/45° 08′ S), of 4.74 mm±0.27 (mean±SE) (Figures 2 and 3). Substrates were rinsed with tap water, submerged for 12 h in 10% sodium hypochlorite solution and then sterilized.

Culture

For each experiment, the bottoms of sterile Petri dishes, which were 10 cm in diameter, were completely covered with either pebbles (between 1088 and 1301 per dish) or shells (between 291 and 523 per dish). A 15-mesh plastic-net disc was placed above the substrate, and fragments of the selected cystocarpic thalli were placed on this disc, so that each Petri dish contained 300 mature cystocarps. Finally, thalli were covered with Seawater Filter Medium C (SFC) culture medium (Correa 1990) with the addition of germanium dioxide (GeO_2) to avoid diatom proliferation. Petri dishes were incubated in a culture chamber at 15±1°C, 12:12 h (light:dark) and 30 μmol m^{-2} s^{-1}, and the culture medium was renewed every week.

Cystocarpic thalli were left for 4 weeks to allow the spontaneous release of carpospores (Michetti et al. 2013). During this period, the thalli were brushed and washed with sterile seawater, bleached segments were discarded, and the plastic discs were brushed with 70% ethyl alcohol.

Carpospore settlement

Five weeks after starting culture, settlement success for each type of substrate was evaluated as the proportion of substrates with settled spores, i.e., number of substrates (shells or pebbles) with spores/total number of substrates (n_{shells}=7; $n_{pebbles}$=6, where n is the number of replicate Petri dishes for each type of substrate). Substrates with spores were counted through a Leica EZ4 stereo microscope.

Carpospore density

To calculate maximum carpospore density per substrate, random photographs of substrates with settled spores were taken through a stereo microscope. All spores inside an area of 3 mm^2, chosen on a high density region on a shell/pebble, were counted, and differences among substrates were evaluated in the fifth (n_{shells}=24; $n_{pebbles}$=14, where n is the total number of shells/pebbles with settled spores in all Petri dishes) and tenth (n_{shells}=40; $n_{pebbles}$=39) weeks of culture. The reason for choosing high density regions is that, since spores settle in clumps, methods that assume a homogeneous distribution would be inappropriate (Glenn et al. 1998).

Sporeling growth

Basal disc diameter and sporeling length were measured for all sporelings found in micrographs of random 40X fields in a stereo microscope. These measurements were made starting on cultures of 10 weeks (from this moment it was possible to obtain precise measurements) until cultures were 28 weeks old.

Figure 1 Sampling sites for *Gracilaria gracilis* and substrates on the coast of Chubut province, Argentina.

Basal disc diameters were compared between substrates on weeks 10 (n_{shells}=190; $n_{pebbles}$=122, where n is the total number of basal discs measured in all Petri dishes) and 22 of culture (n_{shells}=60; $n_{pebbles}$=43). The same procedure was performed to compare carposporeling length on weeks 10 and 22 (for both, n_{shells}=60; $n_{pebbles}$=62, where n is the total number of carposporelings measured in all Petri dishes).

Specific growth rate (SGR) was calculated from week 7 until week 28 according to the following equation:

$$SGR(\%\ day^{-1}) = 100 \ln(L_t / L_0) / t.$$

L_0 is the sporeling initial length, L_t is the sporeling final length, and t is the number of days between L_0 and L_t (Rueness and Tanger 1984).

Statistical analysis

Results were expressed in terms of mean±standard error. Because of their unequal variances, settlement success data were transformed with arcsine (square-root [X]). Student's t-test was applied to evaluate differences between

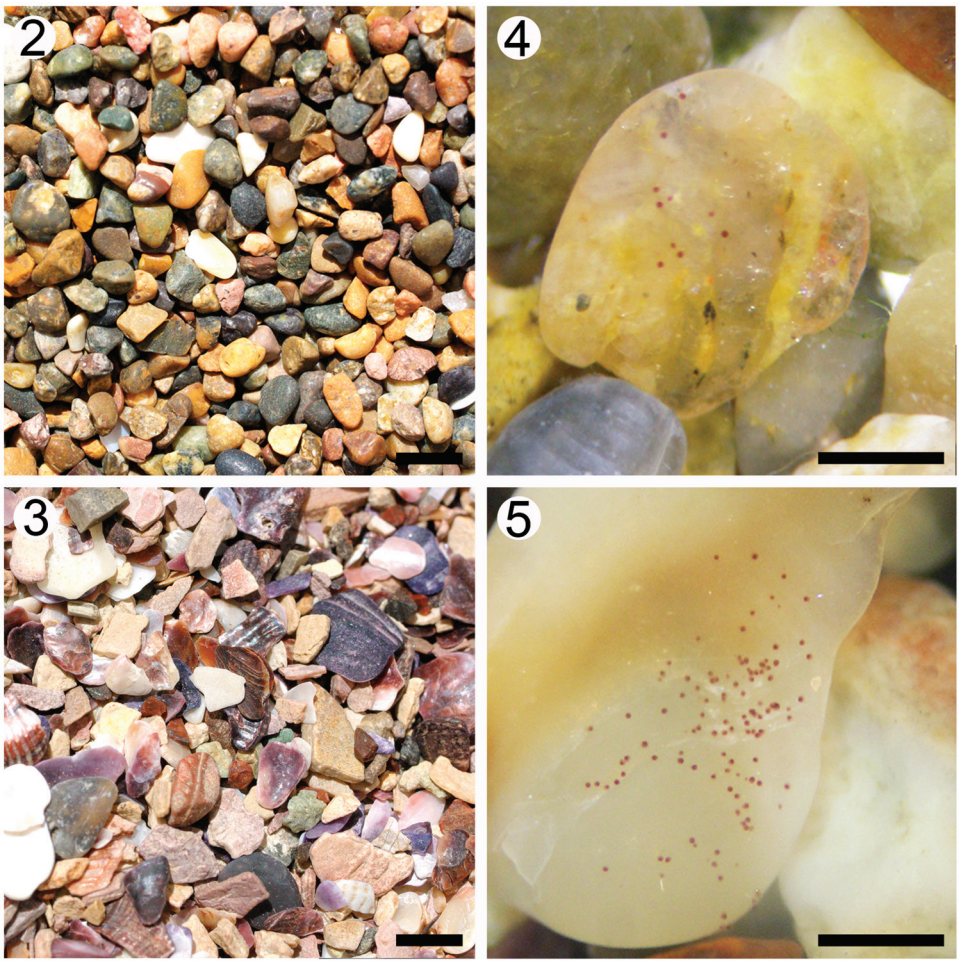

Figure 2–5 *Gracilaria gracilis*: evaluated substrates and settled carpospores on shells and pebbles. (2) Pebbles. (3) Bivalve shells. Scale bars=5 mm. (4) Basal discs of 5 weeks growing on pebbles. (5) Basal discs of 5 weeks growing on shells. Scale bars=1 mm.

substrates. Density data were transformed with square root before applying two-way analysis of variance where the factors were substrate (shells, pebbles) and culture time (5 and 10 weeks). Basal disc diameters and erect frond length were transformed with Ln X before applying Student's t-test to compare diameters and lengths between substrates. Statistical analyses were carried out using InfoStat software, Córdoba, Argentina (Di Rienzo et al. 2011).

Results

Carpospore settlement

Carpospore settlement was more successful on shells (8.55%) than on pebbles (0.48%; t_{11}=5.56; p=0.0002). For all the assays performed, the percentage of colonized substrates did not exceed 15% for shells and 1% for pebbles.

Carpospore density

Spore density was approximately ten times higher on shells than on pebbles ($F_{1, 113}$=33.48; p<0.0001) after 5 weeks (Figures 4 and 5), and decreased at a similar rate with increasing culture time on both types of substrate ($F_{1, 113}$=6.69; p=0.011; Figure 6). No interaction was found between substrates and culture time ($F_{1, 113}$=2.46, p=0.12).

Survival percentages on week 10 were 43.5% on shells and 49.7% on pebbles, and these had decreased to 25.7% on shells and 29.7% on pebbles by week 22.

Sporeling growth

After 10 weeks of culture, basal disc diameters on both substrates were similar (t_{310}=0.41; p=0.68), but after week 22 started to show differences (t_{101}=4.49; p<0.0001), with larger basal disc diameters on pebbles than on shells

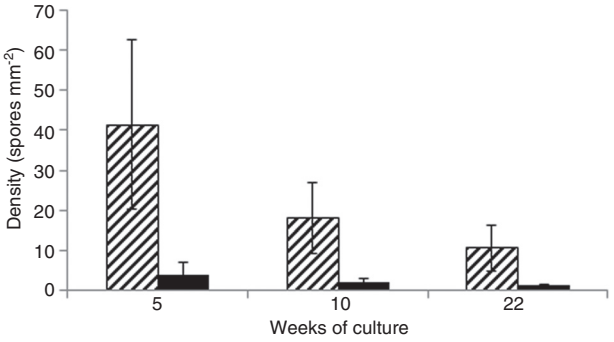

Figure 6 *Gracilaria gracilis*: sporeling density on shells (hatched bars) and on pebbles (solid bars) on weeks 5, 10 and 22 of culture. Error bars represent 95% confidence intervals.

(Figure 7). Initial basal disc diameter was considered to be 0.035 mm, which is the mean diameter of a carpospore (Michetti et al. 2013).

The mean length of sporelings grown on shells and pebbles did not differ significantly after either 10 (t_{120}=-1.89; p=0.062) or 22 weeks of culture (t_{120}=-1.66; p=0.099; Figure 8).

To calculate SGR, the smallest measurement for sporelings growing on slides in their third week of development (0.05 mm) was considered as the initial length. From week 7 to week 10, SGR was 5.67% day^{-1} on shells and 7.59% day^{-1} on pebbles, considerably decreasing to 0.24% day^{-1} on shells and 0.55% day^{-1} on pebbles between weeks 16 and 22 (Figure 9).

Discussion and conclusions

In this study, it was demonstrated that shells offer a better surface than pebbles for the settlement of *Gracilaria*

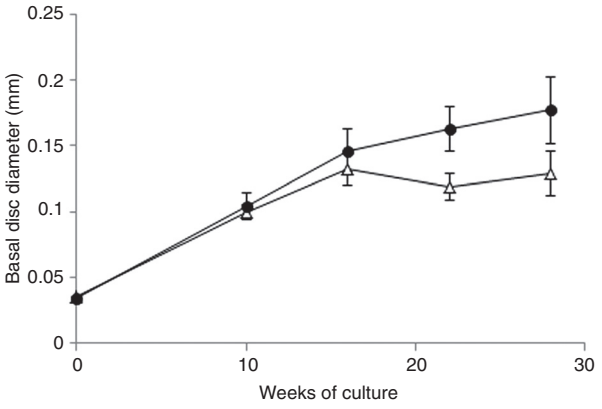

Figure 7 *Gracilaria gracilis*: basal disc expansion on shells (Δ) and on pebbles (•). Error bars represent 95% confidence intervals.

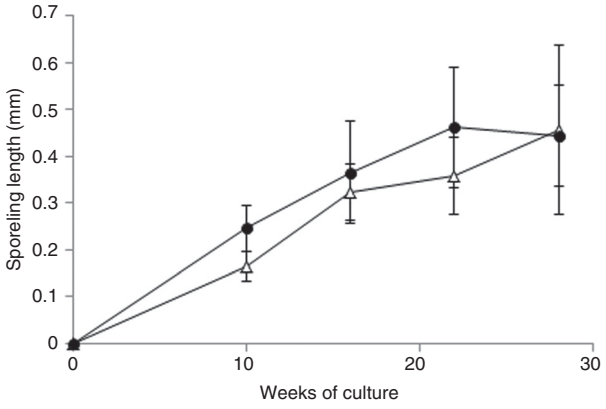

Figure 8 *Gracilaria gracilis*: sporeling growth in length on shells (Δ) and on pebbles (•). Error bars represent 95% confidence intervals.

gracilis carpospores, in terms of both the percentage of colonized substrates and carpospore density. This may be due to a more suitable surface for spore fixation on shells, as they have depressions where spores can settle and grow, less exposed to water movement and predators than on the smooth and convex surface of pebbles. In a similar way, a tendency to a higher settlement on coral fragments, rather than on pebbles, was observed in *G. parvispora* carpospores, which was attributed to the presence of holes on the fragments where carposporelings proliferated protected from predators (Glenn et al. 1996). In the red alga *Iridaea*, it has also been proposed that cracks and roughness in a rocky substratum would benefit spore adhesion (Romo and Alveal 1995).

Maximum carpospore density was estimated in this study because it is a good indicator of settlement success in *Gracilaria* (Glenn et al. 1998). As a matter of fact, in this

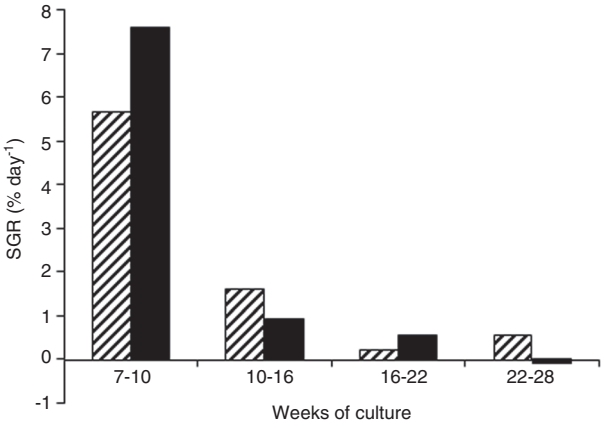

Figure 9 *Gracilaria gracilis*: sporeling specific growth rate (% day^{-1}) on shells (hatched bars) and on pebbles (solid bars) in different culture periods.

genus spores tend to aggregate and viable thalli frequently grow from areas with high initial spore density by coalescence of basal discs (Santelices 1990), a process that was observed during the experiments carried out.

Although a decrease in thallus density with culture age was observed, survival percentages after 22 weeks in culture for both substrates (25.7% in shells and 29.7% in pebbles) were much higher than that indicated in *Gracilaria parvispora* (<1%) for the same culture period (Glenn et al. 1996). These authors attributed this low survival to the coalescence of sporelings, as well as to competition and predation, which are relevant factors considering that these experiences were carried out in a natural environment. In the present study, as cultures were carried out in the laboratory, the decrease in spore density was attributed to the coalescence of individual thalli and to both intra- and interspecific competition. The latter was due to the rapid proliferation of other algal species, such as brown filamentous algae, green algae and cyanobacteria, a frequent problem in this type of study (Buschmann and Gómez 1993, Alveal et al. 1997).

Despite the fact that pebbles were not as good as shells for spore fixation, sporelings grew well on pebbles, reaching similar sporeling length and larger basal discs, probably because their smooth surface facilitated disc growth. In addition, an SGR of 5.67% day^{-1} on shells and of 7.59% day^{-1} on pebbles was obtained between weeks 7 and 10 of culture. Despite the fact that no significant differences were found in sporeling length between substrates, competition for space, nutrients and light might be expected among sporelings as densities on shells were much higher than on pebbles. This could be due to the fact that conditions for their development were not optimum, because of possible nutrient depletion in the small volume of medium in the Petri dish. This negative interaction among sporelings in high density areas has been observed in *Gracilaria chilensis* (Alveal et al. 1997), as well as in other red algae (Santelices et al. 2004).

To establish spore cultures on a commercial scale, it is necessary to have information on the initial spore density needed to generate viable thalli that are able to adapt to the marine environment. As was mentioned earlier, even though viable thalli usually grow in high density areas, competition is likely to appear between sporelings if densities are too high. In *Gracilaria chilensis*, a density of 200 spores cm^{-2} is considered sufficient to avoid competition for light and nutrients among sporelings (Alveal et al. 1997). In addition, initial densities of *G. parvispora* higher than 200 spores cm^{-2} are normally enough to generate one or two adult thalli every 30 cm^2 (Glenn et al. 1998). According to this, in the present study, initial spore densities were more suitable on pebbles (392 spores cm^{-2}) than on shells (4153 spores cm^{-2}). However, in other red algae such as *Palmaria palmata*, densities of 500 spores cm^{-2} are considered adequate (Le Gall et al. 2004).

On average, carposporelings did not exceed 0.5 mm in length after 5 months of culture on either of the two substrates. This size is much smaller than that reported for other species. Lengths of 0.7–1.5 mm were obtained in *Gracilaria chilensis* carposporelings after 2 months in culture in enriched filtered seawater (Alveal et al. 1997), while carposporelings of *G. dura* reached 1–2 mm in the same culture period but in sterile seawater (Mantri et al. 2009). In another study on *Gracilaria* spp. from Japan, Malaysia and India, longer sporelings were obtained (4–5 mm) in a shorter period of time (30–40 days) in Provasoli's Enriched Seawater (PES) medium (Raikar et al. 2001). It is important to note that, in a previous study carried out on slides with *G. gracilis* from Bahía Bustamante, sporelings with lengths of 1.4 mm were obtained in the second month of culture. Besides, fronds reached 14.2 mm in length with apical branches after 6 months (Michetti et al. 2013), indicating that this strain has the potential to grow better.

In accordance with the information given above, improved growth rates are expected to be obtained by inoculating shells with an adequate amount of seed material, so as to get a suitable initial density of sporelings that should reach the required size for transport to the sea. Besides, survival rates of sporelings on shells should also be high because spores are better attached than on pebbles.

Although artificial substrates have been proved to support growth of thalli, natural substrates offer some significant benefits, such as the facts that they are available in the natural habitat of *Gracilaria gracilis* and have no acquisition cost. In addition, as they are native materials, they do not have an impact on the marine ecosystem. Another advantage of using substrates of this shape and size in a natural environment is that, because of the movement in the sea bottom, they may be partially covered with sediment whose abrasive effect could reduce colonization by other algae without affecting the viability of *G. gracilis* thalli, which are resistant to burial (Boraso de Zaixso 1989).

Finally, it is expected that these results at laboratory scale will provide baseline information to perform experiments in the sea, in order to incorporate environmental variables.

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