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Research Article

Secondary attachment discs: a new alternative for restoring populations of *Chondracanthus chamissoi* (Gigartinales, Rhodophyta)

Felipe Sáez[1] & Juan Macchiavello[1]
[1]Departamento de Biología Marina, Facultad Ciencias del Mar
Universidad Católica del Norte, Coquimbo, Chile
Corresponding author: Juan Macchiavello (jmacchia@ucn.cl)

ABSTRACT. When facing the growing, international demand for the species *Chondracanthus chamissoi* as a source of carrageenan and for human food consumption, it is very important to generate new aquaculture techniques that allow for its repopulation, maximizing biomass yield whilst reducing the pressures on existing small, natural populations. This paper describes the use of secondary attachment discs (SAD) as a new vegetative propagation technique. It involves the following two stages: i) Tying the tetrasporophytic and cystocarpic fronds to natural substrates (shells and rocks), the conditions generated in the tanks allowed for quick SAD formation (12 days of experimentation), and ii) The maintenance of SAD under normal conditions (at sea for 83 days). The results indicated a quick formation of SAD, a high capacity of persistence (40%), the growth of new apices (4% d^{-1}), quick substrate colonization through colonization discs (SADc), and the formation of reproductive structures. As a result, SAD is therefore proposed as both an innovative way to achieve the recovery of the now reduced natural beds of *C. chamissoi* and as an alternative to traditional methods of vegetative culturing for a species of high commercial interest.

Keywords: *Chondracanthus chamissoi*, carrageenan, vegetative propagation, stocking, growth, colonization, seaweed.

INTRODUCTION

Marine algae have been part of the human diet since ancient times, using algae as an integral part of everyday meals (Dillehay *et al*., 2008). These algae form part of the decoration for Asiatic dishes, offering not only a variety of colors and textures but also a series of nutritional components that benefit human health (Cornish & Garbary, 2010). *Chondracanthus chamissoi* (C. Agardh) Kützing is a Rhodophyta alga endemic to Pacific coasts from south of Paita in Peru (5ºS) to Ancud, Chile (42ºS) (Ramírez & Santelices, 1991), it has been exploited by both countries, principally for the production of carrageenan. More recently, Chile began exporting algae to Asiatic markets as a consumable commodity (Bulboa *et al*., 2005; Bulboa & Macchiavello, 2006). The economic value of *C. chamissoi* in Chile has increased. This caused an overexploitation of this resource, decreasing the volume of biomass harvested at a national level: 25,000 ton were harvested in year 2000, while during 2010 only 977 ton were recollected (SERNAPESCA, 2011). Recently, some studies have focused finding solutions to the decreased yield of *C. chamissoi* through different cultivation strategies (Bulboa & Macchiavello, 2001, 2006; Bulboa *et al.*, 2005) Also, the conservation of this resource through management plans and repopulation of natural beds has been studied (Vásquez & Vega, 2001; Macchiavello *et al*., 2003). These strategies, however, have not been enough to restore historic production in Chile to meet the growing international demand (SERNAPESCA, 2011). The poor recuperation of *C. chamissoi* algal beds means that the overexploitation may lead to the progressive disappearance of this resource (C. Bulboa *pers. comm.*). The reattachment of fronds as a growth strategy for some marine algae is considered fundamental as part of a propagation technique that has the capacity to repopulate and recover historic harvesting volumes for *C. chamissoi* in Chile. The fronds reattachment can be carried out using various methods, such as the formation of rhizoids (Santelices & Varela, 1994; Smith & Walters, 1999; Hernández-González *et al*., 2007), or secondary attachment discs (SADs) from the

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contact between the apical regions of the lateral branches with the substrate (Perrone & Cecere, 1997; Bulboa *et al*., 2005; Pacheco-Ruiz *et al*., 2005; Fonck *et al*., 2008; Sáez *et al*., 2008). The use of SADs, as a reattachment strategy, is common for diverse species of red. It has been utilized for vegetative propagation in the aquaculture industry, and for the repopulation of species that have a high economic value like *Gracilaria* spp. (Goldstein, 1973; Santelices & Fonck, 1979; Pizarro & Santelices, 1993; Westermeier *et al*., 1999), *Chondrus crispus* (Neish & Fox, 1971), *Kappaphycus alvarezii* (Bulboa & Paula, 2005), and *Chondracanthus chamissoi* (Bulboa *et al*., 2005; Bulboa & Macchiavello, 2006). For *C. chamissoi*, the reattachment of drifting fronds occurs when pieces of algae are trapped on hard substrates (Macchiavello *et al*., 2003), followed by the rapid formation of SADs. However, studies performed *in vitro* by Fonck *et al*. (2008) and Sáez *et al*. (2008) demonstrates that SAD function as colonization units that have the capacity to persist, even under the circumstance of being isolated from the original fronds, they are capable of generating new apices with quick growth (5 ± 1% d^{-1}). Various controlled propagation techniques have been developed (Bulboa & Macchiavello, 2001; Bulboa *et al.*, 2013) using this capacity to develop secondary attachments discs, allowing it to adhere to any kind of substrate.

The current study produced *C. chamissoi* seedlings, generated from fronds that were reattached by SADs, connected to natural substrates, which were later introduced into the sea in order to evaluate the effectiveness of these as clonal propagation units. These methods allowed the recuperation of depressed algal beds of *C. chamissoi.*

MATERIALS AND METHODS

Frond collection and selection

We collected 4 kg of *C. chamissoi* fronds through free diving at Puerto Aldea, Chile (30º17'31"S, 71º36' 32"W) to a depth of approximately 4-5 m. The algae were transported in thermic containers to the Marine Botanical Laboratory of the Universidad Católica del Norte (UCN). Afterwards, 150 tetrasporophytic fronds and 150 female gametophytic fronds were separated.

Substrate preparation

Vegetative fronds with many branches and 25 cm in length were tied with a cotton string to *Argopecten purpuratus* shell with an available surface of 100 ± 6 cm and rocky with a surface of 150 ± 20 cm^2 of the variable surface for both of the life cycle phases of *C. chamissoi* (gametophytic and tetrasporophytic). Fifty seaweeds units were prepared and marked for each life phase type. These units were placed in a 2,000 L outdoor tank with continuous seawater flux and air. The 2,000 L was divided into four compartments of 120×124×100 cm, made with plastic mesh (8 mm Ø). In each of these compartments, 50 substrates units (25 shells and 25 rocks) were randomly placed. All substrates were maintained for 27 days (January 22 to 17 February) under natural summer temperature conditions 19ºC (SST) (IFOP, 2008) and PAR light 11,981.5 ± 2,323.0 (Rotha-Üsler *et al*., 2011). The light impinging over the cultivation tanks reached 40 to 60 µmoL photons m^{-2} s^{-1}. The seawater was supplied at a flow rate of 3 L min^{-1} during the cultivation period.

After 12 days of cultivation, the number of reattached fronds was registered as a percentage based on the branches initially found and frond reattached to the substrate, for each life cycle phase and kind of substrate used. After 20 days of cultivation, the number of formed SADs was once again registered for each treated frond, this was considered the starting value for the separation of new fronds and later, cultivation. After this registry, the SADs were separated from the mother and returned to the fronds using a transversal cut, taking care not to dislocate them from the substrate, and returned to the cultivation tanks until day 27, when they were moved to the sea.

Transportation of substrates to the sea

The substrates with SADs already separated from mother fronds for both life cycle phases of *C. chamissoi* were introduced to the sea in four isolation boxes composed of a red net (8 mm ø). Both the shell and rock substrates used were randomly dispersed along the inferior side of each box (25 shells and 25 rocks), but only the calcareous substrate was fixed with plastic cords. The boxes were fixed to the bottom of the Bahía de la Herradura (29º58'S, 71º22'W) in the concession allotted to the UCN by metallic stakes to a depth of 4 m. The samples were maintained in this location for 83 days (18 February to 11 May), under natural summer-autumn conditions of temperature 19ºC (SST) (IFOP, 2008) and PAR light 11,981.5 ± 2,323.0 (Rotha-Üsler *et al*., 2011).

The survival rate of the SAD on the substrates was registered after 83 days in the sea, and this was calculated as a percentage based on the initial number of existing SADs prior to introduction to the sea (20 days in hatchery stage). In parallel, photographs were taken to measure the growth, in length, of new fronds, for which the SADs formed for each substrate were photographed at the moment of introduction to the sea and at the end of the study. The growth rate of the

forming apices was calculated with the following formula:

$$GR = [(Lt/Lo)\,1/t - 1] \times 100$$

where: GR: Growth rate; L_0: Initial length (20 days); L_t: Final length (83 days); and t: 83 days. After completion of the sea cultivation stage, the number of fronds carrying reproductive structures (tetrasporophytic and cystocarpic) generated in plants formed from initial SADs was registered for each substrate and for both life cycle phases of *C. chamissoi*.

Statistical analysis

The data obtained on the reattachment of fronds, number of SADs, survival, and growth rates were submitted separately by a two-way ANOVA to detect possible differences between substrates (calcareous and rocky) and the distinct life phases of *C. chamissoi* (Zar, 1984). A Tukey test was used when registering significant differences. However, before this analysis, the normality and homoscedasticity of the variance were evaluated for all data obtained in both experiments through Levene and Kolmogorov-Smirnov tests, respectively (Sokal & Rohlf, 1981). The data obtained as percentages in different experiments were transformed via the application of Arcsine, which permitted subsequent analysis of this data (Zar, 1984). The analyses were carried out using the SPSS software (IBM version 22).

RESULTS

Reattachment of fronds and the formation of SADs

Reattachment of fronds to the different substrates cultivated in the tanks was observed after 12 days through the mucilaginous union of the fronds to both substrates (calcareous and rocky). Rates of reattachment to the calcareous substrate were 20 ± 5% and 17 ± 6% for tetrasporophytic and female gametophytic fronds, respectively. On the rocky substrate, rates of reattachment were 23 ± 4% and 26 ± 3% for tetrasporophytic and female gametophytic fronds, respectively (Fig. 1).

Between the two life cycle phases, non-significant differences were found for rates of reattachment ($P > 0.05$), however, there were significant differences ($P < 0.05$) between the substrates used for reattachment, with a higher rate for the rocky substrate ($P < 0.05$) (Table 1). After 20 days, the number of SADs formed by fronds on the calcareous substrate was 10 ± 4 and 7 ± 1 for tetrasporophytic and female gametophytic fronds, respectively. For the rocky substrate, values of formed SADs were 15 ± 4 and 12 ± 3 for tetrasporophytic and female gametophytic fronds, respectively (Fig. 2). Significant differences ($P < 0.05$) were found for the number of SADs between both phases and both substrates used (Table 1).

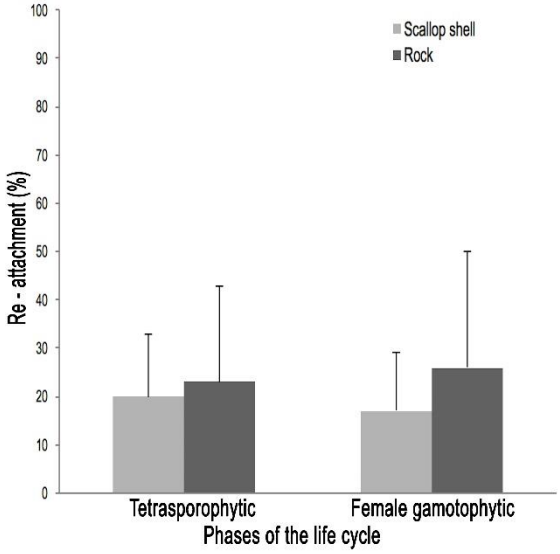

Figure 1. Reattachment percentages of tetrasporophytic and female gametophytic fronds of *C. chamissoi* after 12 days of culturing in outdoor tanks on two natural substrates (scallop shell and rock) (means ± SD).

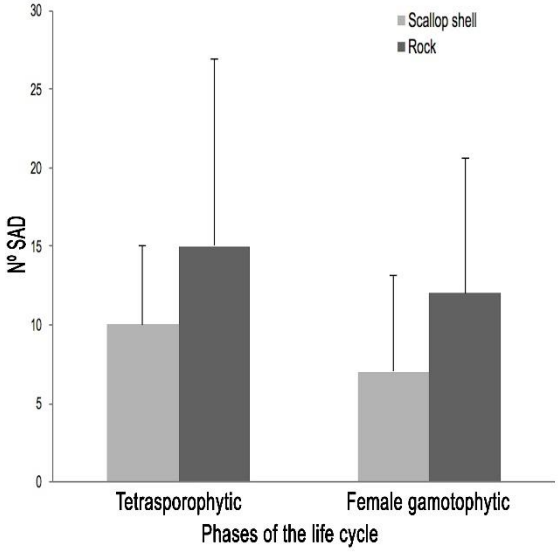

Figure 2. The average number of secondary attachment disks (SAD) developed by each frond on both substrates (scallop shell and rock), for the different phases of *C. chamissoi* life cycle after 20 days of culturing in outdoor tanks (means ± SD).

Survival of SADs in the sea and the growth of new apices

At the finalization of the experiment, the SADs initially generated by both types of fronds and in both substrates

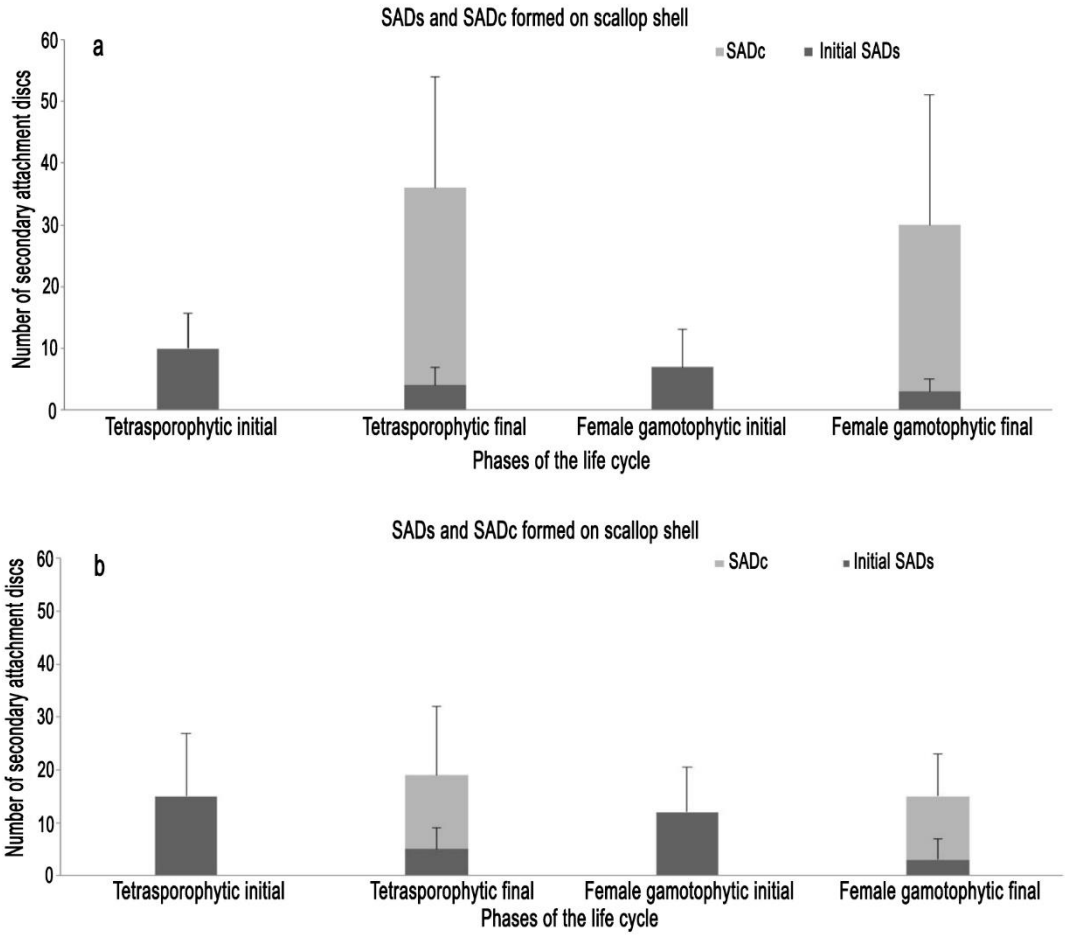

Figure 3. The total number of attachment disks formed at the end of 83 days of sea culturing on both hard substrates. Secondary attachment discs (SADs) and colonization disks (SADc) registered at the moment of sea immersion and at the end of the experiment for the scallop shell a) and rock b) substrates for both phases of *C. chamissoi*. The final number of attachment discs is the sum of the initial SADs survivors and the SADc (means ± SD).

Table 1. Two-way ANOVA was used to evaluate the reattachment of algae (%), number of secondary attachment discs (SADs), survival in the sea of colonization disks (SADc), growth rate of new apices in the sea and number of colonization disks: on two substrates (A) and during two stages of the life cycle for *C. chamissoi* (B). *Significant differences ($P < 0.05$).

	Effects	SS	df	MS	F	*P*
% Re-attachment	Substratum (A)	0.202	1	0.202	4.5	0.035*
	Phases (B)	0.013	1	0.013	0.3	0.585
	A×B	0.038	1	0.038	0.847	0.359
Number of secondary attachment disc (SADs)	Substratum (A)	3.89E+04	1	3.89E+04	12.47	0.001*
	Phases (B)	1.25E+04	1	1.25E+04	4.03	0.046*
	A×B	2665	1	2665	0.85	0.356
Survival SAD (%)	Substratum (A)	1.36E+00	1	1.36E+00	8.285	0.004*
	Phases (B)	0.336	1	0.336	2.055	0.153
	A×B	0.481	1	0.481	2.939	0.088
Growth rate new exes	Substratum (A)	0.4865	1	0.4865	2.544	0.116364
	Phases (B)	0.2216	1	0.2216	1.158	0.286423
	A×B	0.3188	1	0.3188	1.667	0.201968
Number of colonization disks (SADc)	Substratum (A)	164107	1	164107	67.7240	0.000*
	Phases (B)	17242	1	17242	7.1156	0.008*
	A×B	3890	1	3890	1.6052	0.206675

showed a decrease in number in relation to the initial number of SADs transferred to the sea (Fig. 3). Thus, it was possible to record the survival on the calcareous substrate at 50 ± 17 and 44 ± 13% for tetrasporophytic and female gametophytic fronds, respectively, while the survival on the rocky substrate reached 40 ± 9 and 26 ± 3% for tetrasporophytic and female gametophytic fronds, respectively. The statistical analysis only detected significant differences between the substrates used ($P < 0.05$) (Table I), with the calcareous substrate obtaining higher survival rates. The surviving SADs generated new fronds which registered growth rates of 3.7 ± 0.2 and 3.4 ± 0.7% d^{-1} on the calcareous substrate for tetrasporophytic and female gametophytic phases, respectively, while the rocky substrate had growth rates of 4.0 ± 0.2 and 3.7 ± 0.1% d^{-1} for tetrasporophytic and female gametophytic phases, respectively.

The phases did not present significant differences ($P > 0.05$) nor did the substrate used (Table 1).

Colonization discs

During the cultivation of the SADs in the sea, the formation of new discs was observed from the apices of new plants, which curved and developed contact with a substrate (Fig. 3). These new discs are called colonization discs (SADc) (Fig. 4). On the calcareous substrate 32 ± 15 and 27 ± 7 SADc were generated for the tetrasporophytic and female gametophytic phases, respectively, while for the rocky substrate, measurements were 14 ± 1 and 12 ± 4 SADc for the tetrasporophytic and female gametophytic phases, respectively. Significant differences ($P < 0.05$) in the number of SADc were registered for the substrates used in the cultivation (Table 1), where the sporophyte phase obtained its greatest values on the calcareous substrate.

Generation of reproductive structures

After a period of sea cultivation, SADs were assessed to quantify the presence of reproductive structures on fronds. Six SADs among the rocky treatment were carrying fronds with tetrasporangial spores. On the calcareous substrate, treatment was found two SAD's carrying fronds with tetrasporangial sori and eight-SADs with cystocarpic fronds.

DISCUSSION

The rate at which *C. chamissoi* formed SADs, which in turn allowed for the quick reattachment of fronds from both life phases of *C. chamissoi* (12 days), coincided with the data described by Sáez *et al*. (2008) for cultivation in tanks. This situation has also been previously documented for other species of Rhodophyta like *Gelidium sesquipedale* (Juanes & Puente, 1993). The reattachment of fronds from *C. chamissoi* allowed for the generation of a large quantity of SADs on each substrate used. These new cloned units, when cultivated under natural conditions, presented a survival rate of 40% and the development of new apices with a growth rate close to 4% d^{-1}. The SADs introduced into the sea, although presenting a decrease from their initial number, showed the potential for colonization through the generation of new fronds, new colonization discs from the fronds and the development of reproductive structures (tetrasporophytic and cystocarpic) in a period of 83 days. The SADc allows for the fixation of new plants to the substrate and favors the rapid colonization of the available substrate (greater colonization of the substrate). The strategy of using its branches to adhere to the substrate, without needing to break itself, has been previously documented, especially in brown and green algae like *Dictyota* spp., *Bryopsis* spp., *Sphacelaria* spp. and *Nereocystis* spp. (Sarabhai & Arora, 2002). The present work describes the formation of numerous SADc on both calcareous and rocky substrates, with a maximum of 37 SADc on the calcareous substrate, which is a measurement that confers this species with a high capacity for persistence and colonization of the provided substrate (Fig. 4).

When looking at this study's data as a whole, it is possible to highlight the differences found between both life phases for the experiments performed under natural conditions. The sporophyte phase had the highest values in the number of SADs formed and for the colonization of the substrate via the generation of SADc, reaching 32 ± 15 discs on the calcareous substrate. Just as with previous studies of this species (González & Meneses, 1996; González *et al*., 1997; Bulboa & Macchiavello, 2001; Ávila *et al*., 2011; Vega & Meneses, 2001), the results presented here show significant differences between the phases studied, with higher values in the number of SADs and SADc, which would give advantages to one phase over another. This is a highly important factor to consider for this species due to its growing economic importance and because the type of carrageenan produced varies according to phase. A strong understanding of this could open the possibility of aquaculture development with differential management according to each life phase of *C. chamissoi* (Ávila *et al*., 2011). Moreover, the SADc are also highly important to consider as they successfully compensate for the mortality of initial SAD.

The rapid formation of SADs, their high capacity for persistence, the successful colonization of substrate by SADc formation and the low biomass required all represent arguments for the viability of developing alternative techniques of repopulation. Alternative tech-

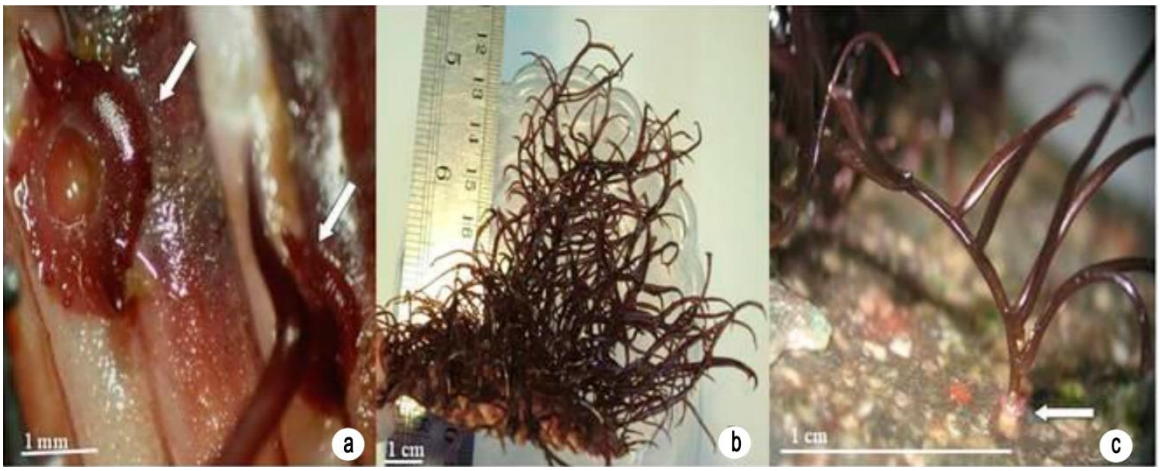

Figure 4. Photographs of SAD, of new apices proliferation, and SADc. a) SAD once detached from the originating fronds (white arrow), b) Growth of new apices from the periphery of the SAD, after 83 days of sea cultivation, and c) SADc formed on the calcareous substrate from the new apex, white arrow indicates the SADc.

niques could offer greater comparative benefits over classical methods of seedling propagation generated by spores, or through the macropropagation of fragments. Both of these methods are limited by different problems like restricted availability, low survival, long-term growth, elevated cost (in the case of spore propagation), and the high biomass required in the case of macropropagation of fragments (Alveal *et al*., 1995; Bulboa *et al*., 2005). The results of this paper allow for the development of a low complexity and low-cost repopulation technology, constituting a technique transferable to fishermen's organizations, which would allow the recovery of the natural algal bed of northern Chile.

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