

The fate of overgrown germlings in coalescing Rhodophyta

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The overgrowth of young sporelings by older and larger thalli is often assumed to result in death of the overgrown individual. However, if sporelings and larger conspecifics are able to coalesce, the fate of the overgrown sporeling may be different to the physical compression, starvation and mortality often assumed for them. Using cytological studies on laboratory-cultured individuals, as well as observations of recruitment in the field, we found that sporelings of the red alga *Mazzaella laminarioides* can be incorporated within the tissue of larger, overgrowing germlings or they may coalesce side-by-side with it, depending on sporeling size before contact. Thus, young sporelings do not necessarily die when overgrown by their older, conspecific competitors. Field observations suggest that these processes are common during natural recruitment; therefore, coalescence might be considered an alternative mechanism to intraspecific competition. Coalescing seaweeds should be included among the groups of sessile organisms that might be formed by genetically polymorphic thalli (chimeras).

INTRODUCTION

Red algal germlings confront a variety of dangers in the field, including abiotic extremes, inter- and intraspecific competition and grazing (Santelices 1990; Amsler *et al.* 1992; Brawley & Johnson 1992; Fletcher & Callow 1992). Sporeling overgrowth by older and larger individuals of similar or different species is considered a common example of competitive exclusion because it often results in death of the overgrown sporeling due to tissue compression or due to drastic light or nutrient reduction by the overgrowing partner (Lobban & Harrison 1994; Worm & Chapman 1996; Figueiredo *et al.* 2000). However, individuals of species in about half the number of orders of the Rhodophyta may coalesce with similar-sized conspecific partners, forming composite entities (Santelices *et al.* 1996, 1999, 2003a). Whether coalescence can occur between conspecific partners of varying sizes is not known, but it raises the question of whether the fate of the overgrown sporeling might be different from the physical compression and mortality generally assumed for them. Such a possibility would suggest that coalescence might be an alternative mechanism to intraspecific competition, a condition recently documented for adult clumps in field populations of coalescing seaweeds (Santelices *et al.* 2003a). In this study, we report the fate of young (5–30 days old) tetrasporophytes and gametophytes of *Mazzaella laminarioides* (Bory) Fredericq & Hommersand, overgrown by older (3 months old) conspecific tetrasporophytes under controlled laboratory conditions. We also searched for evidence of equivalent processes in the field, studying spores and germlings that naturally settled on experimental substrata placed in rocky intertidal habitats along the wave-exposed coast of central Chile.

MATERIAL AND METHODS

Laboratory cultures

Laboratory-grown, 3 month old unispore tetrasporophytes of *M. laminarioides* were used as overgrowing crusts in all the experiments. These were cultivated in isolated plastic cover slips (EMS 72260; Electron Microscopy Sciences, Washington, DC, USA) under constant conditions of temperature [$14 \pm 2^\circ\text{C}$ ($\bar{x} \pm s_{\bar{x}}$ here and elsewhere)], irradiance ($45 \pm 10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and photoperiod (12:12 light–dark). After being inoculated with one spore, all slides were incubated in individual Petri dishes ($50 \times 10 \text{ mm}$; Corning Labware & Equipment, Washington, DC, USA) filled with SFC culture medium (Correa *et al.* 1988) replaced every 2 days. A 3 month old tetrasporophyte is generally formed by rounded or ovate basal crusts and one to four erect axes of up to 0.5 mm long.

To control sporeling size at the time of initial contact with the tetrasporophytes, one to four recently released gametophytes or sporophytes were placed at four distances from the meristematic border of the older sporophyte. These distances were $113 \pm 30 \mu\text{m}$, $181 \pm 90 \mu\text{m}$, $220 \pm 80 \mu\text{m}$ and $420 \pm 100 \mu\text{m}$ (Table 1).

Sporeling growth was monitored every 3 days until reaching physical contact with the older sporophyte. Under the above growth conditions, contact between the older and the younger sporelings occurred after about 6 ± 1.2 days, 7.9 ± 4.1 days, 14.2 ± 3 days, 20.1 ± 4.6 days and 30.2 ± 3.8 days of growth (Table 1). In order to study the fate of the overgrown crust, monitoring continued for 5, 10, 20 and 30 days after contact. At each one of these times, the overgrowing thallus with the overgrown sporeling was fixed, embedded and cut, along with the plastic cover slip, so as to insure the integrity of the basalmost portion of the germlings.

In total we used seventy 3 month old overgrowing plants

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Table 1. Life-history phase, number of spores used (*n*), initial spore diameter and distance, precontact age and precontact diameter of germlings overgrown by the larger and older crusts (data are $\bar{x} \pm s_x$). Precontact refers to the condition measured in the last control before contact between the overgrowing and overgrown germlings.

| | <i>n</i> | Initial distance (μm) | Initial spore diameter (μm) | Precontact age (days) | Precontact diameter (μm) |
|-------------|----------|---------------------------------------|---|--------------------------|--|
| Gametophyte | 39 | 113 \pm 30 | 24 \pm 2.0 | 6 \pm 1.2 | 37.8 \pm 4.2 |
| Sporophyte | 125 | 181 \pm 90 | 23.7 \pm 1.4 | 7.9 \pm 4.1 | 61.1 \pm 12.3 |
| Sporophyte | 35 | 220 \pm 80 | 24.9 \pm 3.5 | 14.2 \pm 3 | 103.2 \pm 47.6 |
| Sporophyte | 38 | 420 \pm 90 | 21.8 \pm 1.5 | 20.1 \pm 4.6 | 179.4 \pm 50.3 |
| Gametophyte | 40 | 420 \pm 110 | 23.8 \pm 1.5 | 30.2 \pm 3.8 | 210 \pm 83.3 |

(sporophytes), 79 young (up to 30 days old) gametophytes and 198 young (up to 30 days old) sporophytes. The difference in numbers between young sporophytes and gametophytes reflects the higher abundance and longer fertile period of cystocarpic thalli relative to tetrasporophytes in central Chile.

Cytological studies

Fixation for cytological analysis followed the protocol described elsewhere (Santelices *et al.* 1996, 1999, 2003b). Sporelings were fixed at room temperature in 5% glutaraldehyde and 3% acrolein in SWM-3 culture medium (McLachlan 1973) for 3 hours under vacuum. Samples then underwent postfixation in an osmium–potassium ferricyanide mixture, followed by dehydration and embedding. Thin sections (500 nm) were later stained with 0.25% toluidine blue in 0.25% boric acid.

A total of forty-three 3 month old germlings overgrowing young sporophytes and 27 overgrowing young gametophytes were fixed at various times (5, 10, 20 and 30 days) after contact. The position of the overgrown sporeling relative to the older germling was recorded photographically and used to orientate the sectioning of the older crust.

Field studies

Recruitment of spores and sporelings relative to larger crusts in the field was measured using circular, 6.0 cm diameter, 0.2 cm thick plates composed of previously detoxified (Brawley & Johnson 1991) epoxy resin (Sea Goin Poxxy Putty; Peumalite Plastic, Newport Beach, CA, USA). Ten plates were placed at each of two sites at Caleta Maitencillo (32°39'S, 71°29'W), approximately 100 km north of Valparaiso, Chile, where other field studies on coalescence are being conducted (see Santelices *et al.* 2003a for detailed description of the study sites). At each site, plates were bolted to the rock surface at the middle and lower vertical limits of a *c.* 1 m wide middle intertidal belt of *M. laminarioides*. After 72 hours in the field, plates were removed, placed in individual Petri dishes with filtered sea water maintained at 15 \pm 2°C in refrigerated coolers and transported to the laboratory (2–3 hours).

In the laboratory, plates were checked for presence of new recruits by analysing 25 randomly selected subsections (1 mm²) of each plate with the aid of a microscope. Because massive recruitment occurred on most plates, they were then incubated for 30 days in a marine aquarium with circulating sea water, set at 14 \pm 2°C, 60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and 12:12 hour light–dark of daily light. Afterwards, plates were returned to the field, exposed for 5 days and brought back to the laboratory, where we searched for evidence of spores and

young crusts coalescing with larger crusts. Images were captured using a Cool Snap-Pro Camera (Media Cybernetics, Silver Spring, MD, USA) mounted on a stereomicroscope (Nikon SMZ-10A; Nikon, Melville, NY, USA). Image analysis was done using ImagePro Plus 4.5 (Media Cybernetics).

RESULTS

Laboratory cultures

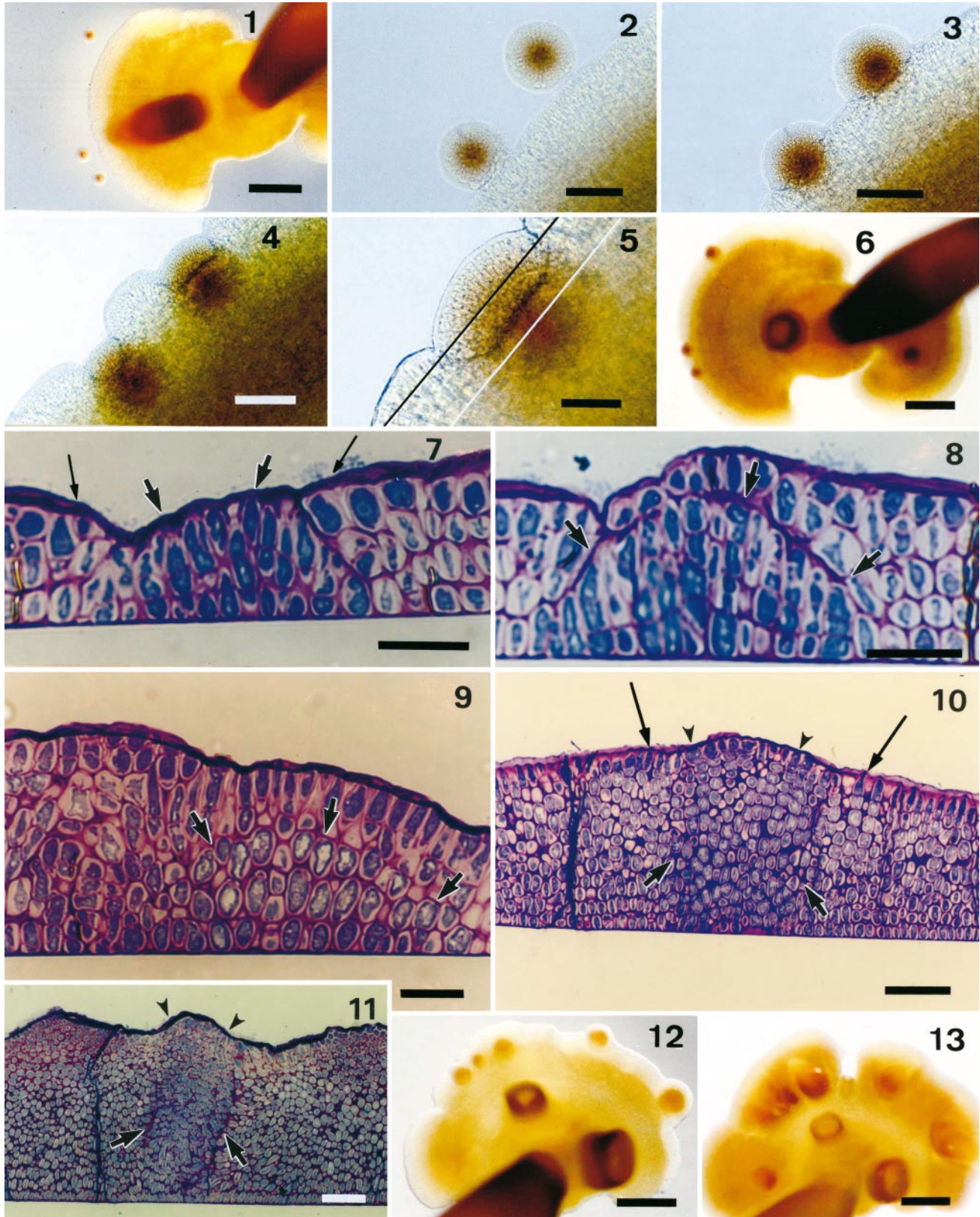
All spores placed around individual 3 month old germlings germinated within 36 hours after release and grew to form a small crust (Fig. 1). Simultaneously, the meristematic border of the older germlings continued to grow radially, until both partners came in contact with each other (Fig. 2).

The after-contact process was different for young sporelings smaller and larger than 100–150 μm of precontact diameter. The small ones were overgrown by the larger germling, whereas those sporelings larger than 150 μm diameter coalesced side-by-side with it. Young sporelings of less than 100–150 μm diameter were overgrown by the 3 month old germling within 3–5 days after germination (Figs 3–5), appearing thereafter as small, dark, rounded nodules inside the larger germling (Fig. 6). Perpendicular sections of the larger germling overgrowing these young sporelings show that the larger thallus gradually covers the small sporeling completely (Figs 7, 8). The small crust then remains inside the larger germling as a discrete cell assemblage contacting the substratum, but is surrounded on all other sides by the tissue of the larger germling (Figs 9–11).

Within this small size group, the fate of the overgrown sporeling now within the holdfast of the larger germling also varied with size. After 15–20 days of overgrowth, the cells of the smallest sporelings studied (30–40 μm in diameter, 5–6 days old at contact) could no longer be distinguished from the cells of the larger overgrowing germling (Fig. 9). No evidence of cell or tissue destruction of the small sporelings was found in any of the larger crusts examined. Seemingly, the tissue of the young sporeling became an undifferentiated part of the holdfast of the larger crust.

Overgrown germlings with larger precontact diameter (60–150 μm ; 6–14 days of precontact age) remained as discrete cell assemblages within the holdfast of the overgrowing germling for longer periods (e.g. 30 days, Figs 10, 11). These continue to grow while inside the overgrowing crust, eventually reaching the germling surface (Figs 10, 11). The cells of the young sporelings often appeared slightly darker and smaller than those cells of the overgrowing germling (Fig. 11),

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Figs 1–13. Coalescence of 3 month old germling of *Mazzaella laminarioides* with smaller (less than 150 µm of diameter) sporelings of the same species.

Fig. 1. Three 6 day old sporelings placed around a 3 month old germling with two erect axes. Younger and older thalli are sporophytes. Scale bar = 500 µm.

Fig. 2. Forty-eight hours after Fig. 1. Details of the young sporelings approaching and contacting the meristematic border of the larger germling. Scale bar = 100 µm.

and we often observed evidence of destroyed cell walls around the young sporeling (arrows in Fig. 11). Moreover, the tissue of the young sporeling often appeared to protrude beyond the older crust surface (arrowheads in Figs 10, 11). In all these cases, new erect axes originated, within 20–30 days after contact, from the young sporelings growing within the holdfast of the larger germling (Figs 12, 13).

In contrast, young sporelings of precontact diameters larger than 100–150 μm were only partially overgrown by the larger and older germling (Figs 14, 15). The large and small crusts coalesced side-by-side, similar to that described for germlings (Santelices *et al.* 1996, 1999) and established plants in the field (Santelices *et al.* 2003a). Depending on the number of coalescing sporelings, the resulting holdfast adopted an irregular rather than a rounded shape, thereafter exhibiting several centres of expansion and growth (Fig. 16).

The only exception to the two major response patterns described above was observed with three 6 day old crusts (40–50 μm in diameter at contact). As the small sporeling and the large germling approached each other, the border of the old germling opposite the young sporeling exhibited a slightly larger marginal outgrowth (Fig. 17). In turn, the young sporeling exhibited excentric growth, also with slightly larger marginal outgrowth at the point directly opposite the overgrowing crust (Fig. 17). A cellular bridge was then established between the small and large crusts (Figs 18, 19). The young sporeling was then incorporated within the structure of the larger crust (Figs 20, 21). However, 5 days after contact, the young crusts started growing, later becoming centres of radial growth in the now irregularly shaped, larger holdfast (Figs 21, 22).

No differences between sporophytes and gametophyte sporelings were found in the above responses.

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Field studies

Spores naturally settled onto plates in the field and different sizes of spores and sporelings were observed near larger crusts (Fig. 23). For example, a subsection of a plate presented at least six sporelings close to the marginal border of the larger crusts (black arrows in Fig. 23), matching our laboratory images of young crusts being overgrown or coalescing side-by-side with the larger crust (Figs 2, 3, 20). At least four sporelings appear as darker nodules within the larger crust (white arrows in Fig. 23), which could correspond to sporelings already overgrown by the larger crust, as shown in our laboratory experiments, or to spores and sporelings settled on top of the larger crust. In addition, we observed evidence of cytoplasmic bridges (the pale tissue) extending between the young sporelings and the larger crust.

Images similar to Fig. 23 were found in 16 of the 20 experimental plates placed at Maitencillo (80%). The number of subsections within a plate depicting such images varied from 1 to 7 out of 25 subsections. Average within-plate frequency was 10.01% at site 1 and 18.06% at site 2.

DISCUSSION

Field and laboratory evidence gathered in this study indicates that young sporelings of *M. laminarioides* do not necessarily die when contacted by the holdfast of a larger conspecific competitor. Depending on the sporeling size at the time of contact, sporelings are overgrown and incorporated into the larger, overgrowing holdfast or they can coalesce border-with-border with the larger germling. Border-with-border coalescence also occurs between similarly aged sporelings and

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Fig. 3. Forty-eight hours after Fig. 2. Young sporelings being partially covered by the border of the larger germling. Scale bar = 500 μm .

Fig. 4. Twenty-four hours after Fig. 3. Young sporelings are almost completely covered by the growing border of the larger germling. Scale bar = 100 μm .

Fig. 5. Detail of Fig. 4. The black line illustrates the direction and position of the section in Fig. 7; the white line shows the direction and position of the section in Fig. 8. Scale bar = 50 μm .

Fig. 6. Older germling overgrowing the three younger sporelings. One of the erect axes has been sectioned to facilitate the view of the young sporelings that now appear as dark nodules within the holdfast of the 3 month old germling. Fig. 6 is 2 days older than Figs 4 & 5 and 5 days older than Fig. 2. Scale bar = 500 μm .

Fig. 7. Vertical section of the border of the larger germling partially covering a young sporeling. The direction and position of the section are similar to those indicated by the black line in Fig. 5. Short arrows indicate the body of the young germling being overgrown. Long arrows indicate the advancing borders of the overgrowing crusts. Note the differences in cell size between the sporeling and the germling. Scale bar = 20 μm .

Fig. 8. Vertical section of the border of the holdfast of the larger germling in an area that has covered completely a young sporeling. The direction and position of the section is similar to the white line illustrated in Fig. 5. Arrows indicate the limits of the young sporeling within the holdfast of the larger germling. Scale bar = 20 μm .

Fig. 9. Six day old (precontact age) sporophyte, 10 days after contact. The body of the young sporeling is surrounded completely by the cells of the larger germlings. There is no obvious histological differences between both types of tissues. The presence of the young sporeling is detected by the presence of the dark nodule (as in Fig. 6) within the germling. Scale bar = 20 μm .

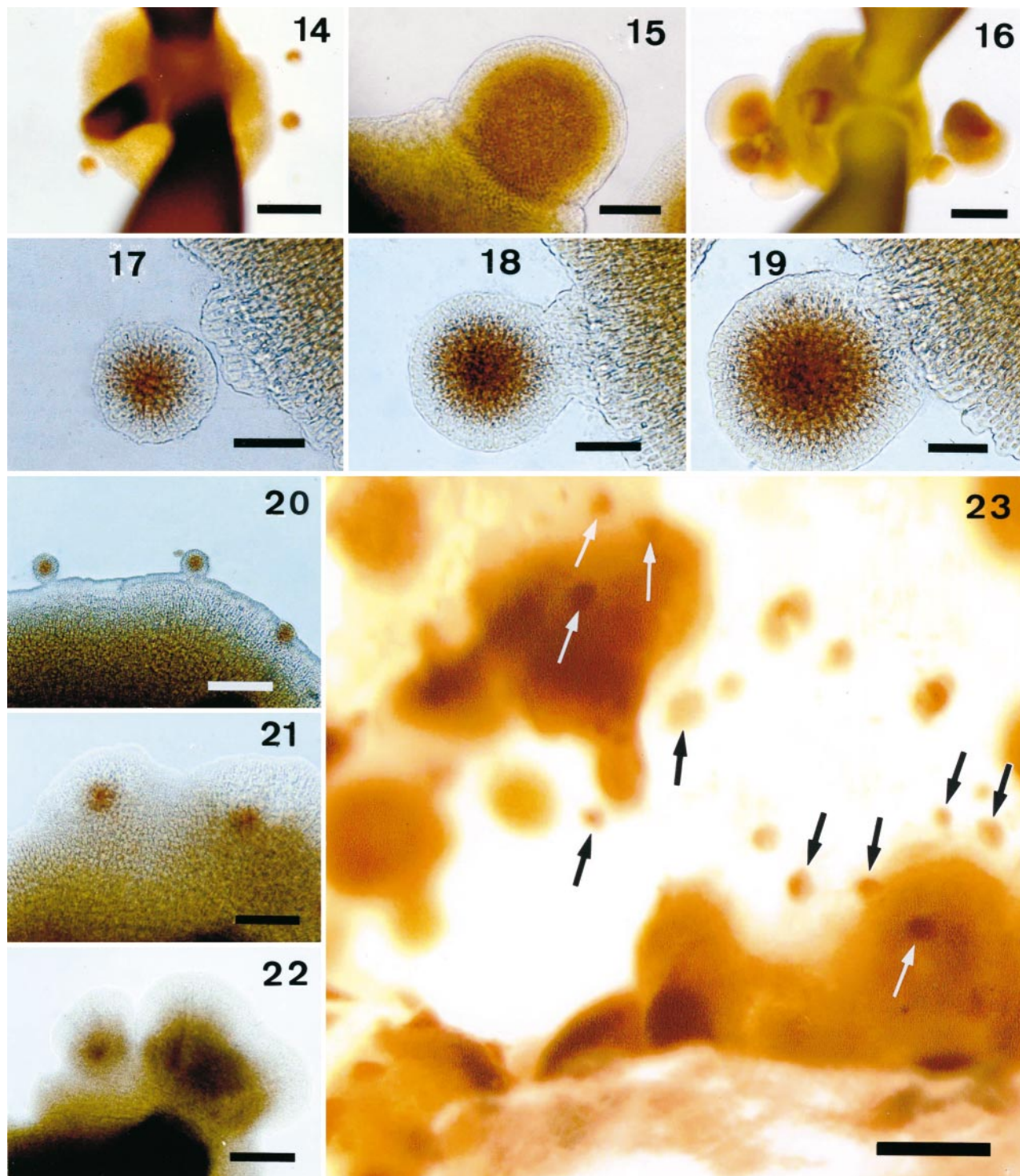
Fig. 10. Ten day old sporophyte (precontact age) inside the holdfast of a 3 month old sporophyte 20 days after contact. The younger sporeling remains as a relatively homogeneous tissue with clear boundaries (short arrows). Note the meristematic activity of the surface cells in the younger sporeling (arrowheads). They appear shorter and more protruding than those in the rest of the holdfast (long arrows). Scale bar = 70 μm .

Fig. 11. Ten day old gametophyte (precontact age) inside the holdfast of a 3 month old sporophyte 30 days after contact. Note the homogeneous tissue of the younger sporeling (short arrows) and its protruding surface cells (arrowheads). Scale bar = 90 μm .

Fig. 12. Five 10 day old sporophytes (precontact age) overgrown by a 3 month old sporophyte 15 days after overgrowing. Two erect axes of the older sporophyte have been sectioned to facilitate the view of the young sporelings. Note the external appearance of the young sporophytes as darker nodules at some distance from the border of the holdfast of the larger germling. Scale bar = 500 μm .

Fig. 13. Same as in Fig. 12, but 15 days later. Note uprights arising from the places where young sporelings were located. Scale bar = 400 μm .

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Figs 14–16. Coalescence of 3 month old germlings of *Mazzaella laminarioides* with young sporophytes larger than 150 μm in diameter.
Fig. 14. Three 15 day old sporophytes placed around a 3 month old sporophyte. Scale bar = 500 μm .
Fig. 15. Partial overgrowth of the smaller sporophyte by the border of the older one. Picture taken 3 days after Fig. 14. Scale bar = 100 μm .
Fig. 16. Same sporophytes and germling as in Fig. 14, 10 days later. The left erect axes of the germling was sectioned to facilitate the view of the young sporophyte coalescing side-by-side with the larger germling. Note the now irregular shape of the holdfast by coalescence and proliferation of the young sporophytes. Scale bar = 500 μm .
Figs 17–22. Coalescence of young sporophytes of *Mazzaella laminarioides* with 3 month old germlings by the establishment of a cellular bridge.
Fig. 17. Six day old sporophyte approaching a larger sporophyte. Note the excentric shape of the young sporophyte with outgrowth on the side approaching the larger germling. Note also the outgrowth in the meristem of the larger crust. Scale bar = 50 μm .
Fig. 18. One day later than Fig. 17, a cellular bridge is observed between the young sporophyte and the 3 month old germling. Scale bar = 50 μm .
Fig. 19. One day later than Fig. 18. The border of the 3 month old germling starts overgrowing the young sporophyte. Scale bar = 50 μm .

germlings (Santelices *et al.* 1996, 1999) and between adult holdfasts of naturally established clumps in the field (Santelices *et al.* 2003a). Incorporation of a young sporeling into the holdfast of a larger thallus is a completely new, previously undescribed process. Future research with other coalescing species should help to determine whether this response is widespread among other coalescing Rhodophyta and if it is also restricted to the smallest coalescing sporelings.

The fate of an overgrown sporeling seems strongly dependent on the size of the sporeling at the time of contact. The smallest sporelings we observed were incorporated within the larger holdfast and eventually lost individuality, seemingly becoming an otherwise undifferentiated part of the larger holdfast. In contrast, larger overgrown sporelings remained differentiated within the holdfast tissue of the larger germlings, and were able to grow, reaching the holdfast surface and eventually issuing new erect axes. The capacity of clusters of spore derivatives, sporelings and germlings to generate their own uprights after coalescence has been documented in several species (Santelices *et al.* 1996, 1999, 2003a) and seems to be a property of all coalescing Rhodophyta. The present results indicate, however, that a minimum size seems necessary to maintain sporeling integrity and growth capacity within a larger conspecific holdfast.

The images of spores that naturally settled in the field indicate that coalescence among sporelings and larger crusts occurs naturally and frequently during the main recruitment season. Spores settling and growing near larger, conspecific crusts reproduce several of the responses we observed in our laboratory cultures, supporting our interpretation of the overgrowing and coalescing processes. The field results also indicate an additional possibility of spores recruiting and developing on top of larger crusts. Such conditions would correspond to a case of zytagmatic spore germination and growth (see Santelices 1990 for review), which often leads to successful recruitment in coalescing and noncoalescing seaweeds.

Our results suggest the need to evaluate more critically the long-assumed idea that sporelings are killed when overgrown by larger entities (Santelices 1990; Amsler *et al.* 1992; Worm & Chapman 1996). It is probably true that species with different growth patterns kill the overgrown germlings. However, coalescing conspecifics, even of different life-history phases, allow for the survival and growth of the overgrown younger germling. Coalescence, therefore, might be considered an alternative mechanism to intraspecific competition. Middle intertidal and shallow subtidal rocky habitats are often characterized as highly competitive environments for sedentary organisms, including seaweeds (Menge & Branch 2001; Witman & Dayton 2001). In these habitats, intraspecific competition has been documented to cause heavy postrecruitment mortality. Coalescence, on the other hand, allows for the survival

and growth of the small germling either by side-by-side coalescence or within the holdfast of the larger crust.

It is comparatively more difficult to understand the adaptive value of coalescence for the larger, older crust. Perhaps it lies in size and variability. Each coalescence process helps to increase the crust area. Larger sizes in these intertidal and shallow subtidal taxa may result in lower mortality due to herbivory or abiotic extremes (Maggs & Cheney 1990; Santelices *et al.* 2003a). In addition, coalescence with genetically different germlings will increase the reservoir of genetic variability of the larger holdfast, which is expected to confer wider ranges of physiological plasticity to the whole clump.

Considering the frequency of naturally occurring coalescence, it is very likely that adult clumps of *M. laminarioides* are composed of genetically heterogeneous holdfasts. In addition, and given the capacity of the overgrown sporelings and of the coalescing sporelings to ensue their own uprights, genetic heterogeneity among erect axes of a single clump should also be expected. Results thus support the suggestion by Santelices *et al.* (1999, 2003a) that coalescing red algae should be included among the groups of sessile organisms able to produce genetically composite entities (chimeras *sensu* Sommerfeldt & Bishop 1999). Aided by molecular techniques, the presence and frequency of such composite groups in natural habitats could be quantified in the future.

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Fig. 20. Three days later than Fig. 19, the young sporeling in different degrees of overgrowth by the larger germling. Scale bar = 100 µm.
Fig. 21. Three days later than Fig. 20, the young sporelings appear as dark nodes inside the larger holdfast. Scale bar = 100 µm.
Fig. 22. Five days after Fig. 21, the young sporelings within the larger holdfast become centres of radial growth. Scale bar = 100 µm.
Fig. 23. Recruitment of *Mazzaella laminarioides* on field-exposed experimental plates. The dark, massive crusts are 35 days old. Young sporelings are up to 5 days old. Note young sporelings contacting and coalescing (black arrows) with the larger crust, as previously seen in our laboratory studies. Note also sporelings that appear as darker nodules in the larger crust (white arrows) that might correspond to overgrown sporelings or sporelings recruiting on top of the larger crust. Scale bar = 200 µm.

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