Hydrobiologia **260/261**: 327–333, 1993.
A. R. O. Chapman, M. T. Brown & M. Lahaye (eds), Fourteenth International Seaweed Symposium.

Exudates from *Gracilaria chilensis* stimulate settlement of epiphytic ulvoids

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Key words: exudate from *Gracilaria*; stimulation of settlement, epiphytes, ulvoids, polysaccharides, water soluble agar

Abstract

Differences in susceptibility to epiphytes among algal species have been explained traditionally on the basis of various defense and escape mechanisms. *Gracilaria chilensis* exhibits inter-strain differences to susceptibility to epiphytes but such differences seem more related to stimulation of propagule attachment rather than to defense or escape mechanisms. Culture medium previously used to grow *Gracilaria* stimulates recruitment of *Ulva* and *Enteromorpha* compared to settlement (recruitment) in non-used growth medium. Elimination of bacteria from the culture media does not reduce the stimulatory effect. Chemical analysis of the culture medium indicates the presence of a mixture of polysaccharides consisting mainly of sulphated galactans, similar to those present in the water soluble fraction of agar produced by *Gracilaria*. Additions of various concentrations of soluble fraction of agar extracted from dry thalli of *Gracilaria chilensis* to SWM-3 culture medium results in statistically significant increases in the density of settled spores of *E. compressa* and *U. rigida*.

Introduction

Epiphytes may compete with hosts for nutrients (Enright, 1978), interfere with their carbon uptake and light capture (Sand-Jensen *et al.*, 1985) and negatively affect production of a desired crop (Shacklock & Doyle, 1983; Craigie, 1980). Therefore, epiphytes have been a common concern to many seaweed cultivation efforts and several procedures have been suggested to remove them, including the use of selective grazers (Shacklock & Doyle, 1983; Brawley & Fei, 1987), manual removal (Tseng, 1981), pesticides or other chemical compounds (Craigie, 1990) and the search for epiphyte-resistent strains of agronomically interesting crops (Patwary & van der Meer, 1983a, 1983b; Levy & Friedlander, 1990).

The biological basis for inter-individual or inter-population differences in susceptibility to epiphytes seem important when searching for epiphyte-resistant strains. Essentially two types of protections: defense and escape mechanisms, have been described for the seaweeds. Defense mechanisms include exudation of epiphyte deterrents (Sieburth & Conover, 1965; Russell & Veltkamp, 1984), sloughing-off of heavily fouled epidermis (Moss, 1982; Filion-Myklebust & Norton, 1981) and possession of thallus structures that are able to clean themselves of epiphytic propagules while in moving water (Smith *et al.*, 1984). Fast growth seems to be the commonest escape mechanism and several authors (e.g.: Neish *et al.*, 1977; Patwary & van der Meer, 1983a; Levy *et al.*, 1990) have stressed that whenever their crops achieved fast growth rates, they were markedly free of epiphytes.

The possibility of host exudates increasing settlement or attachment capabilities of epiphytes seemingly has not been tested yet. However, several sources of evidence point to this possibility. While characterizing populations of *Gracilaria chilensis*, Santelices & Ugarte (1990) noted not only significant inter-strain differences in susceptibility to epiphytes (*Ulva, Enteromorpha* and *Ectocarpus*) but also that the most susceptible strains were colonized by significantly more epiphytes than glass rods used as controls. This was interpreted to represent stimulation of epiphyte attachment by the thalli of *G. chilensis*. Stimulatory growth effects on *Ulva* germlings by mucilaginous exudates from diatoms had already been reported by Huang & Boney (1984). Furthermore, research on agar composition of species of *Gracilaria* (Lahaye *et al.*, 1986) has identified a water soluble fraction of agar that in *G. chilensis* has a yield of 1.4%, a galactose content of 55.4% and a sulphate content of 15.8% (Matsuhiro & Urzúa, 1990). The yield of the water soluble fraction of agar of some species of *Gracilaria,* changes according to their nutritional status and the photon fluence rate used (Ekman & Pedersen, 1990). Interestingly, nutrient, salinity and irradiance balance can affect the degree of epiphytism of some red algal species (Enright, 1978; Harlin *et al.*, 1985; Craigie, 1990). In this study we first evaluate the effects that the culture medium used by *G. chilensis* has on the settlement and germination of two common epiphytes, *Enteromorpha compressa* and *Ulva rigida*. Then, we searched for the presence of polysaccharides in such a medium. Later, we tested whether the effects of the used culture medium on the settlement of *U. rigida* and *E. compressa* could be replaced by various concentrations of the water soluble fraction of agar from *G. chilensis*.

Materials and methods

Differential settlement of ulvoids

A series of experiments was designed to test whether the culture medium used to incubate *Gracilaria chilensis* could stimulate settlement and recruitment of propagules of *Enteromorpha compressa* and/or *Ulva rigida*. Used culture medium was prepared by incubating 50 g of *G. chilensis* for 15 days in a 1000 ml beaker with filtered seawater, no water exchange and controlled conditions of light (45 mol photon m^{-2} s^{-1}), temperature (14 °C) and photoperiod (12:12 LD). The seawater was then filtered (0.45 μm) and enriched with nitrates, phosphates, vitamins and metals, in the concentrations used in the SWM-3 culture medium (McLachlan, 1973). Control culture medium was prepared maintaining filtered seawater for similar length of time and no macroscopic algae under similar culture conditions, then filtering and enriching as described above.

All the materials of *Gracilaria chilensis* used in the experiments had been acclimatized to controlled laboratory conditions (60 μmol photon m^{-2} s^{-1}; 14 °C; 12:12 LD) for at least a month prior to any experiment. The different strains were collected either in Niebla beach, near Valdivia (39° 48′ S; 79° 24′ W) or in Maullin, near Puerto Montt, 41° 36′ S; 73° 36′ W. Propagules of ulvoids were obtained from newly collected specimens of *Ulva rigida* and *Enteromorpha compressa*. After collecting, the thalli were kept in wet paper towels and darkness overnight. The next morning they were dried, then reimmersed in seawater and illuminated to stimulate sporulation. The spore solution was then used to compare settlement behaviour of ulvoids under different treatments.

Eight replicate small dishes (60 mm × 15 mm) per treatment were used for each experiment. Each dish received 10 ml of the respective culture medium and 5 ml of the newly prepared spore suspension of ulvoids. Comparison of the total number of spores settled in each dish was done 24, 48 and 96 h after the beginning of the experiment, counting 5 microscopic fields in an inverted (Leitz) microscope. Significance of results were compared using ANOVA, followed by Tukey's test, whenever appropiate.

The first experiment in this series compared propagule recruitment of *Ulva rigida* after 24 h in used and control culture medium. The thalli of *Gracilaria* used were vegetative female thalli collected in Niebla.

The second experiment was essentially similar to the first one but incorporated used culture medium previously filtered through a Sartorious filter with smaller mesh size (0.2 μm) to eliminate bacteria. Three samples of each culture medium used were then incubated in bacto agar plates to check for bacterial contamination.

The third and fourth experiments compared the stimulatory effects induced by several strains and health appearance of *Gracilaria chilensis*. Besides the thalli collected in Niebla, a green mutant collected in Puerto Montt and wild-type specimens collected in Niebla were used to compare strains. Healthy, bleaching and deteriorating thalli collected in Maullin were used to compare health states. The counting of settled propagules of *Enteromorpha compressa* was done 48 and 96 h after seeding these culture dishes.

Initial search for organic compounds in the culture medium used by G. chilensis

Search for organic compounds in the culture medium previously used to incubate *G. chilensis* was done by Drs B. Matsuhiro and C. Urzúa, at the Chemistry Laboratory of the Universidad de Santiago. A total of 500 ml of used seawater was liophylized and frozen, yielding 2.54 g of a white solid, mainly represented by inorganic salts. The solute was dissolved in distilled water and then dialyzed against distilled water for 72 h. The solution was then concentrated, frozen and liophylized again. The 5 mg of the white solid obtained reacted positively to phenol-sulphuric acid. The sample was then split into two subsamples. One subsample of 2 mg was used to quantitatively determine the sulphate content of the sample, using the turbidimetric method of barium-gelatine. The second subsample (3 mg) was hydrolyzed with 2M trifluoroacetic acid. The resulting solution was reduced with $NaBH_4$ and acetylated with acetic anhydride in dry pyridine. The mixture was analyzed by gas liquid chromatography coupled to mass spectroscopy using a Hewlett-Packard GC-MS 5890.

Effects of the water soluble fraction of agar from Gracilaria chilensis *on the settlement of ulvoids*

A total of 50 g of dry *Gracilaria chilensis* originally collected in Maullin was used to extract the water soluble fraction of agar, as described by Matsuhiro & Urzúa (1990). The dried sample was milled, stirred with 500 ml of distilled water at 25 °C for 3 h and filtered through muslin. The residue was extracted twice. The filtrates were pooled, dialyzed against distilled water, concentrated and freeze-dried. Then the material was dissolved in 50 ml and quantities of 2, 4, 8, 16, 32 and 64 ml of the soluble agar solution were added to 1 l of control culture medium. Each concentration was taken to be a treatment. All other experimental procedures were similar to those described above. Spore solutions of *Enteromorpha compressa* and *Ulva rigida* were used. Counting of settled spores in each treatment was done 48 h after seeding.

Results

Differential settlement of ulvoids

Spores of *Ulva rigida* settled in significantly higher numbers in the culture medium previously used to incubate *Gracilaria chilensis* (Fig. 1) than in the seawater used as control. The factors stimulating settlement require some accumulation time under the culture conditions used in our study. Empirically, we have determined that 15 days of residence of *G. chilensis* in the culture medium is the minimum time required to ensure a response by the ulvoids propagules.

Bacterial counts in the dishes with bacto-agar indicated abundances of 3300 ± 556 colonies per ml of used experimental medium and 9660 ± 630 per ml of control culture medium whenever filtered through a 0.45 μm size mesh. No bacterial colony grew in the bacto-agar plates filtered through the 0.2 μm filter. Yet, the factor stimulating spore settlement persists in the used culture medium (Fig. 2) after bacterial removal.

The abundance of spores settled in culture

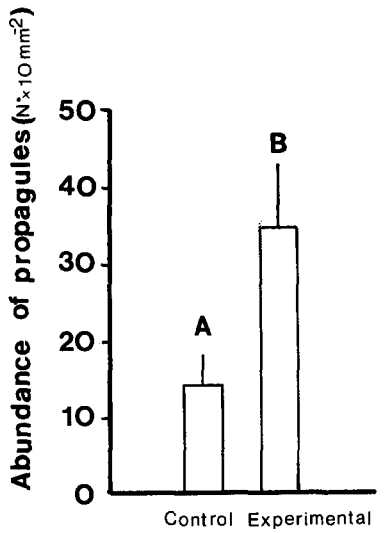

Fig. 1. Abundance of propagules of *Ulva rigida* settled in experimental and control culture medium. Different letters indicate significant differences ($p<0.05$) among treatments.

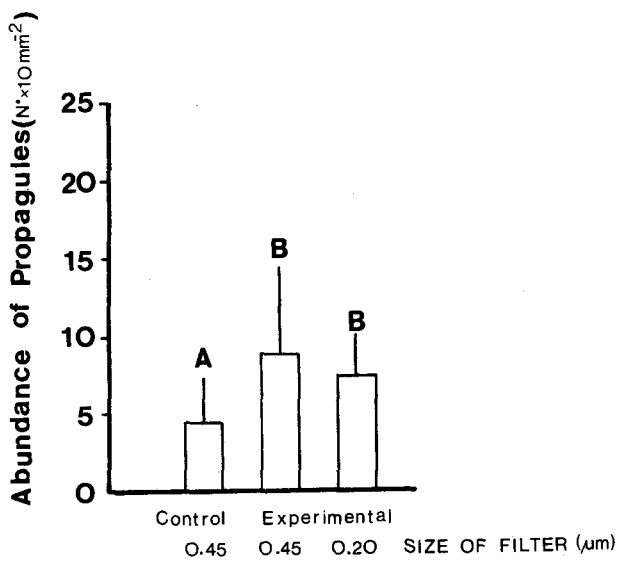

Fig. 2. Abundance of propagules of *Ulva rigida* settled in used and control culture media and filtered through different filter sizes. Different letters indicate significant differences ($p<0.05$) among treatments.

media previously used to incubate *Gracilaria chilensis* may change. depending on the strain used and the time of counting (Fig. 3). Forty eight hours after settlement, the density of spores of

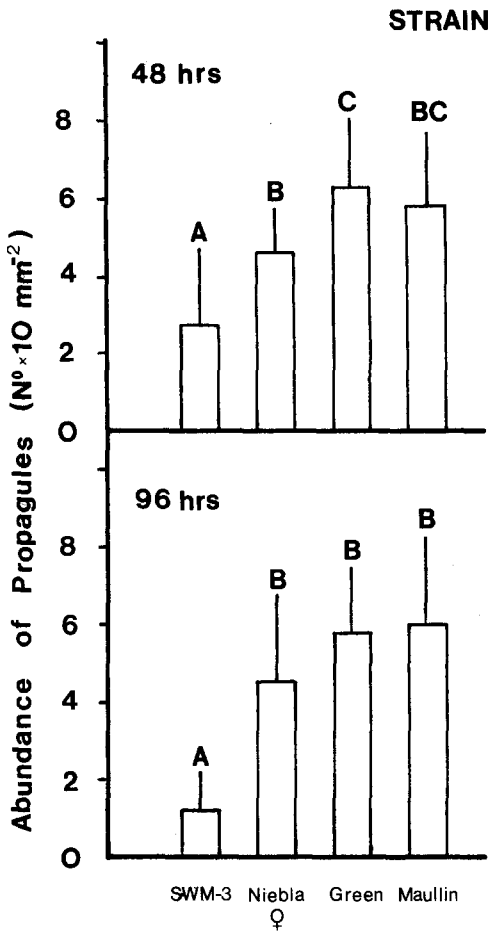

Fig. 3. Abundance of propagules of *Ulva rigida* settled in culture media previously used to incubate various strain of *Gracilaria chilensis*. Different letters indicate significant differences ($p<0.05$) among treatments.

Ulva rigida settled in the culture medium used by a healthy green mutant collected in Puerto Montt was significantly higher than the number of spores settled in the culture medium used by healthy, wild-coloured, vegetative female thalli collected at Niebla. The abundance of spores settled in culture medium used by the Maullin population was intermediate between the other two used media. This pattern of settlement changed after 96 h, when the abundance of spores in the culture media used by all three strains was similar, and the only significant difference ($p<0.05$) found was between used and control culture medium.

No significant ($p>0.05$) differences in abun-

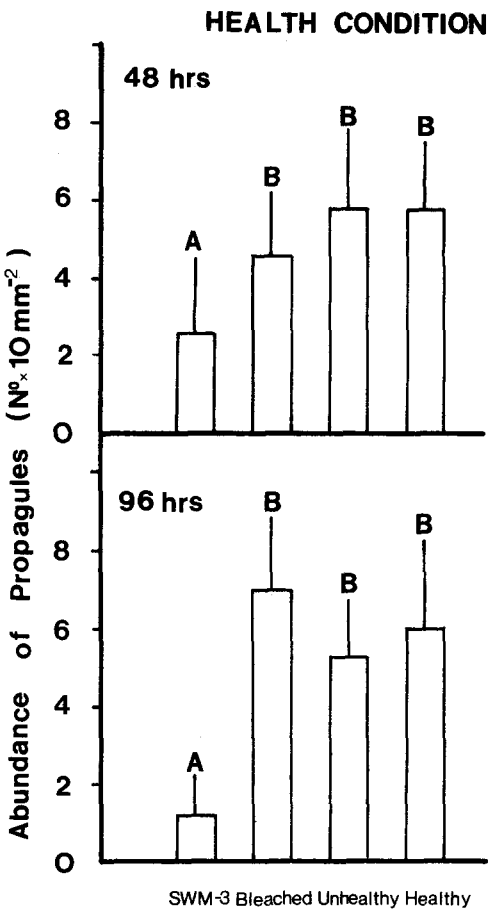

Fig. 4. Abundance of propagules of *Ulva rigida* settled in culture media previously used to incubate thalli of *Gracilaria chilensis* in various conditions.

dance of spore of *Ulva rigida* settled were found when comparing culture media used to incubate *Gracilaria chilensis* of different health states for 48 and 96 h. after incubation (Fig. 4). Used versus control culture medium was the only significant difference found in this experiment.

Initial search for organic compounds in culture medium used by G. chilensis

A 2 mg extract of culture medium used by healthy *Gracilaria chilensis* treated with barium-gelatine indicated a sulphate content of 28%.

A study of another 3 mg of the extract indicated presence of hexa-O-acetil galactitol as the principal component, with several other minor components.

Therefore, the extract of culture medium used by *Gracilaria chilensis* contains a mixture of polysaccharides whose principal components are sulphated galactans (Matsuhiro & Urzúa, personal communication, May 26, 1992).

Effects of the water soluble fraction of agar from Gracilaria chilensis *on settlement of ulvoids.*

The stimulation effect of the culture medium used by *Gracilaria chilensis* on the settlement of spores of *Enteromorpha compressa* and *Ulva rigida* can be replaced by the addition of various concentrations of the water soluble fraction of agar to the SWM-3 medium (Fig. 5). Additions of 2–32 ml of soluble fraction of agar to the SWM-3 resulted in statistically significant ($p < 0.05$) increments in the density of settled spores of *Enteromorpha com-*

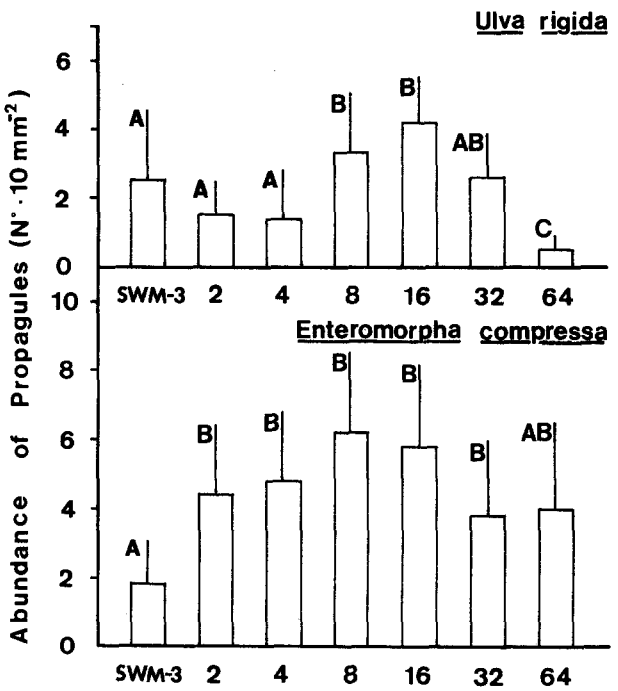

Fig. 5. Abundance of propagules of *Enteromorpha compressa* and *Ulva rigida* settled in SWM-3 with various concentrations of soluble agar added to the culture media.

pressa, as compared to SWM-3 culture medium without the addition of soluble agar. The highest concentration of dissolved agar used in this experiment (64 ml of soluble fraction of agar) did not exhibit significant differences ($p > 0.05$) from the SWM-3 culture medium, suggesting that the stimulatory effect is likely to be lost at increasing concentrations.

Additions of the water soluble fraction of agar to culture media with spores of *Ulva rigida* also exhibited stimulation of settlement, but the range of action with this species is more restricted (Fig. 5). Low concentrations did not show any effect, while the highest concentration used showed inhibitory effect on the settlement of propagules of *U. rigida*.

Discussion

The experimental data gathered in this study suggest that factors or substances associated with the presence of *Gracilaria chilensis* and remaining in the culture medium after thallus removal, are able to modify the settlement rate of propagules of ulvoids such as *Enteromorpha compressa* or *Ulva rigida*. Furthermore, the evidence suggests that sulphated polysaccharides could be involved in the stimulatory effect. Preliminary chemical analysis of the used culture media indicates a presence of a mixture of polysaccharides in which highly sulphated galactans are the predominant compounds. Highly sulphated galactans are also found in the water soluble fraction of agar from *Gracilaria chilensis* (Matsuhiro & Urzúa, 1990). Interestingly, the addition of various concentrations of the water soluble fraction of agar to the SWM-3 culture medium was also able to stimulate settlement of propagules of ulvoids, replacing the effect of the factor associated with the presence of *G. chilensis*. The galactans found in the culture media, however, exhibited higher sulphate contents compared to the water soluble fraction of agar from *G. chilensis*, suggesting that if one compound originates the other, it is only after some chemical modification of the water soluble fraction of agar.

Increased epiphyte recruitment is often observed in seaweed cultures, especially when incubated under high photon fluence and/or low nutrient enrichment. Competition for nutrients coupled with fastest growth by the epiphyte has been the commonest explanation for increased epiphyte abundance (Enright, 1978). The finding that a highly sulphated polysaccharide exudated by *Gracilaria chilensis* may stimulate settlement of such epiphytes adds a new perspective to the problem and is consistent with the findings (Ekman & Pedersen, 1990) that stressful cultivation conditions may stimulate carbon excretion, increasing the yield of the water soluble fraction of agar. However, the rate of settlement of propagules of ulvoids did not change with the health conditions of the seaweed. Perhaps, the settlement of propagules depends not only on the stimulatory effects of substances contained in exudates but also on the physiological state of the propagules. Alternatively, increased growth of microorganisms may perhaps consume increased carbon exudate under stressful conditions. Additional experimental studies seem necessary to evaluate these alternatives.

Overall, the data gathered in this study suggest the need to consider stimulatory effects as one of many possible mechanisms determining epiphyte abundance. This type of interaction can be as important as the traditionally described defense and escape mechanisms and should be incorporated as potential explanation for inter-individual or inter-population differences in susceptibility to epiphytes.

Acknowledgements

We thank E. Stangher for help with laboratory work, Dr G. Mora for allowing us to use his laboratory and C. Asenjo for helping to incubate and quantify bacteria. Our gratitude to Drs B. Matsuhiro and C. Urzúa for the search of sulphated galactans in the used culture medium.

This study was supported by FONDECYT-CHILE, Grant 803/90.

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