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The interaction between epiphytes and seaweeds

Final report for BARD (1995-1999)

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Abstract

Two Israeli laboratories (IOLR and TAU) cooperated with one American laboratory (USF) in the research of the interaction between epiphytes (*Ulva* sp.) and the cultivated seaweed (*Gracilaria* sp.). The main objectives included the following aspects: Structural aspects, effects of different irradiances on growth, sensitivity studies, allelopathic excretions, selective chemicals and integration of studies on epiphytization. The studies were operated in outdoor tanks, indoor growth chambers, and in the lab. The main conclusions and their relevance for mariculture are as following: 1. The green algal epiphyte, does penetrate its red algal host. 2. *Gracilaria* spp. in monoculture released more halogenated hydrocarbons than in biculture with *U. lactuca*, whereas other metabolic parameters did not show a discriminating effect in biculture. 3. Hydrogen peroxide and halogenated hydrocarbons could be a part of the effective excretion compounds in biculture. 4. The presence of mature *Gracilaria* inhibited the growth of *U. lactuca* sporelings. 5. *G. conferta* is most sensitive to epiphytes among *Gracilaria* species tested. 6. The use of green light can enhance growth in basiphytes but inhibit epiphytes. 7. Effective selectivity has been defined by the use of hydrogen hypochlorite. 8. It may be more profitable in seaweed mariculture to select for epiphyte resistant strains than to search for inhibitors of epiphytization. 9. It is important as well to examine how the basiphyte may be able to prevent penetration. 10. Definition of the effective excretions in biculture has still to be done.

1. Introduction and original Objectives

This Final Report presents the major results and discussion of the entire BARD-supported study dealing with the interaction between epiphytes and seaweeds. The Report includes our general conclusions in relation to the objectives of the research. During the last four years, detailed studies focused on six different aspects of interaction between epiphytes and seaweeds: Structural aspects, Effects of different irradiances on growth, Sensitivity studies, Allelopathic excretions, Selective chemicals and Integration of studies on epiphytization. Because of operational reasons the six aspects were drawn out of the following five original objectives:

1. Sensitivity and structural relationship between *Gracilaria* sp. as a basiphyte and *Ulva* sp. as an epiphyte.
2. The effect of allelopathic excretions of *Gracilaria* sp. and *Ulva* sp.
3. The growth response of *Gracilaria* sp. and *Ulva* sp. to light spectrum components.
4. Epiphytization control of *Gracilaria* sp. to selective chemical agents.
5. Integration of the results regarding basiphyte-epiphyte relationship for seaweed cultivation.

2. Materials and methods

2.1. Structural studies

Tissues were prepared for light microscopy and also for transmission and scanning electron microscopy as described by Dawes (1988). Details on handling, fixation, embedding, sectioning, and staining appear in the text as well as in the previous reports.

2.2. Effects of irradiance on growth

Detailed methods appear in the second report.

2.3. Sensitivity studies

These experiments were primarily carried out in 500 mL cylinders under a controlled light intensity ($100 \mu\text{mol photon min}^{-1} \text{m}^{-2}$), temperature (25°C), and pH 8 (using CO_2 as the controlling agent). The biculture consisted of 1.5 g of *Gracilaria* spp. and 4.5 g of the accompanying species, being separated by a net screen, and tested after 7 days for growth (weight), photosynthesis and respiration (oxygen electrode; Hansatech), hydrogen peroxide release, and hydrocarbon halogenation (Weinberger et al, 1999).

Statistical analysis has been performed with the Ln transformed data in order to meet the assumptions of analysis of variance. All described differences from control in the text are in the level of $P < 0.05$.

2.4. Allelopathic excretions

Detailed methods appear in the second report.

2.5. Selective chemicals

Methods described in the text.

3. Results and discussion

3.1. Structural aspects of epiphyte attachment

A. Introduction.

The study on *Ulva lactuca* and two species of *Gracilaria*, *G. tikvahiae* and *G. cornea*, was initiated in order to determine the structural interaction between the epiphyte and its basiphyte. Studies have shown that removal of the epiphyte *U. lactuca* from species of *Gracilaria* is difficult to impossible (Friedlander 1992, Bushmann and Kuschel, 1988). This study hypothesized that the green algal epiphyte penetrates and damages the cell wall of the red algal basiphyte and forms a firm, highly resistant attachment site.

Using light, transmission and scanning electron microscopes, the epidermal wall of the basiphyte was found to consist of a bi-layered wall that is covered by a decklamelle or "cuticle". Induction of zoids in *U. lactuca* was successful and they were studied during initial stages of attachment and penetration into the basiphyte. As reported in earlier studies, the green algal epiphyte initially produced an adhesion pad that was evident on glass slides as well as on the basiphytes. What was most critical were the findings that *U. lactuca* germlings penetrated the outer wall layers of *G. tikvahiae* and *G. cornea* within a few days of attachment. Further, the epidermal cells of both species produced an electron-dense concentration of material near the site of penetration of the epiphyte, apparently in response to the penetration. The differences in wall construction between the two basiphytes can be correlated with the ability of zoospores of *U. lactuca* to attach and penetrate the decklamelle and outer wall of the basiphytes. The data thus far suggest that it may be possible to select

epiphyte-resistant strains of *Gracilaria* for mariculture. A manuscript is now in ready presenting in more detail that which is given below.

B. The cell wall of *Gracilaria*.

The branches of *G. cornea* (Fig 1A, 1B) and *G. tikvahiae* (Fig 2A, 2B) have a continuous covering that varies in terms of shrinkage, with the former species showing a higher degree of surface folding than the latter. Tips of *G. cornea* (Fig 1A) showed significant levels of folding (rugoseness) probably reflecting some shrinkage of the meristemic epidermal cells during critical point drying due to surface tension. On the other hand, the tips of *G. tikvahiae* (Fig 2A) did not display such severe folding and this may reflect the more mucilaginous texture of this species branches that prevented wrinkling of the surface covering. There is little folding of the surface covering when mature regions of the branches were examined (Fig 1B, 2B).

Frozen sections of *G. cornea* (Fig 3A) and *G. tikvahiae* (Fig 3B) showed that the decklamelle is continuous when seen under the light microscope. The outer wall is also continuous and covers the individual inner epidermal cell walls. Ultrathin sections of the epidermal cells of both species (Fig 4A, 4B) showed the decklamelle covering a continuous outer wall. The decklamelle is dense and granular, 0.1 to 0.2 microns thick in older branches, and distinct from the outer wall in *G. cornea* (Fig 4A). Because field collected plants were used, the branches of *G. tikvahiae* had epiphytes and a deposit of what appears to be sediment (Fig 4B) that was not visible on surfaces of cultured plants. In contrast to *G. tikvahiae*, the outer coverings of *G. cornea* had a more diffuse decklamelle that appeared fibrous on the surface (Fig 4A). In addition, the surface of the cultured branches was devoid of epiphytes or sediment with only occasional bacteria. Further, the decklamelle of *G. cornea* appeared to grade into the electron dense, granular outer wall (Fig 4A) lacking a distinct boundary.

C. Epiphyte attachment.

The attachment of young *U. lactuca* germlings on glass slides (Fig 5A) or on *G. cornea* (Fig 5B) initiates with an adhesive pad as described in the literature when viewed with the scanning electron microscope. The rim is probably the result of unequal shrinkage of the *U. lactuca* cells compared to the decklamelle of *G. tikvahiae* during critical point drying. Further, when young epiphyte germlings are removed,

the adhesion pad may remain or cause the loss of the decklamelle at the site of attachment (Fig 5C) as seen on the surface of *G. cornea*. A more extensive adhesive pad of the epiphyte is evident with older sporelings (15+ celled filaments) as viewed on *G. tikvahiae* (Fig 5D). If the germlings remain attached, their bases penetrate the decklamelle in both *G. tikvahiae* (Fig 5D) and *G. cornea* (Fig 5B, E). A raised edge or rim is formed around the attachment site as viewed with the scanning electron microscope (Fig 5B, 5C).

Ultrathin sections of *U. lactuca* on *G. tikvahiae* show penetration of the epiphyte through the decklamelle (Fig 6A, 6B), and the formation of a rim around the site of penetration. In the initial stages of attachment (Fig 6A) the basal cell of the filament of *U. lactuca* has penetrated the decklamelle and is partially into the outer wall layer. This is the phase seen in the scanning electron micrograph shown in Figure 5D. Penetration continues through the outer wall covering and into the cell walls of the individual epidermal cell walls of *G. tikvahiae* (Fig 6C). At this stage, a concentration of electron dense material is evident in the adjacent epidermal cells of the basiphyte, which increases over time (Fig 6C). Further, the cell membrane in the penetrating cells of the epiphyte become convoluted adjacent to the epidermal cells of the basiphyte. Penetration of the epiphyte into *G. cornea* also occurs (Fig 7A) and by the 15 celled filament stage rhizoids will have penetrated into the outer wall (Fig 7B).

D. Conclusions.

In a review of algal epiphytes, Ducker and Knox (1984) point out the variety of relationships that are known to occur with basiphytes. They recognized the distinction between holo-epiphytes or ones that are attached to the outer layers of the basiphyte and amphi-epiphytes that are anchored deeply in the tissues of the basiphyte. In most studies, the ubiquitous green algal epiphyte species of *Ulva* and *Enteromorpha* have been considered to be of the holo-epiphyte type (Evans, 1981). However, species of these green algal genera are also cited as difficult to impossible to remove in mariculture of certain species of *Gracilaria* (Friedlander *et al.*, 1996; Buschmann and Gomez, 1993), while other species (e.g. *G. cornea*, Friedlander, unpublished), seem to avoid or have a reduced level of epiphytism. The present study demonstrates that *U. lactuca* penetrated the outer wall of *G. tikvahiae* and *G. cornea* and that there are distinctions between the outer wall and decklamelle

construction in the two red algal agarophytes. This evidence also helps explain the difficulty in removing the green algal epiphyte from the former but not the latter species. Further, the study suggests that whether a species is of the holo- or amphiepiphytic type depends on its host.

Germlings of the green algal epiphyte produced an adhesion pad during the initial phase of attachment on both species of *Gracilaria* as described in studies following attachment of zoospores on glass slides for *Ulva* (Braten, 1974) and *Enteromorpha* (Evans and Christie, 1970; Leonardi and Ciceres, 1991). Detailed studies of the initial stages of attachment on glass slides by zoids of *Enteromorpha* spp. show that the sequence of events, not followed in the present study, are highly precise (Callow *et al.*, 1997). There is an initial, temporary attachment phase via the apical papilla of the zoid followed by a discharge of adhesive material (as also described by Evans and Christie, 1970) and the absorption of the flagella (Callow *et al.*, 1997). In this study, within a few days to a week, the basal cell of young *U. lactuca* sporelings, had begun to penetrate the decklamelle and into the outer wall of both species and there is the appearance of digestion of the outer layers of the host. Penetration appears to result from the dissolving of the decklamelle and outer wall by the epiphyte whose plasmalemma in the basal cell became highly convoluted. Rhizoid penetration into paint surfaces by a related genus, *Enteromorpha* (Moss and Woodhead, 1970) demonstrates how effective these green algae are in attachment.

Are metabolites being transferred between *U. lactuca* and its *Gracilaria* host?

Detailed studies on another red alga *Harveyella mirabilis* that parasites *Odonthalia floccosa* have shown that metabolites will move from the host to the parasite by diffusion through the cell wall (Goff, 1979) and this has been shown for the *Polysiphonia lanosa*-*Ascophyllum nodosum* relationship as well (Harlin and Craigie, 1975). The epidermal cells of *G. tikvahiae* respond to the epiphyte's penetration with an accumulation of electron dense material outside the plasmalemma. There are no similar responses reported for either epiphytic or parasitic relationships and thus it is difficult to determine what the epidermal cells of *G. tikvahiae* are producing. The ability of *U. lactuca* to penetrate the outer walls of *G. tikvahiae* and the buildup of material in the host's epidermal cells adjacent to penetration suggests that a defensive reaction is occurring. Defenses by hosts against epiphytization can result in

production of inhibiting chemicals or alleopathic agents (Davis, 1989) although there is no firm evidence in this chapter of the study. This suggests that basiphytes respond to epiphytism. Perhaps selection of epiphyte resistant strains of economically important seaweeds is a reasonable approach in mariculture venture.

E. Figure descriptions for structural studies.

Fig. 1. Scanning electron micrographs of a young branch of *Gracilaria cornea*. Fig. 1A. The tip of the branch. Fig. 1B. Mature region 3 to 4 cm behind the tip and lack bacterial or algal epiphytes reflecting long term culturing.

Fig. 2. Scanning electron micrographs of a young branch of field collected *Gracilaria tikvahiae*. Fig. 2A. The branch tip. Fig. 2B. Mature region 3 to 4 cm behind tip of branch shows the individual epidermal cells seen beneath the covering layer. Bacterial cells and diatoms are also evident in the field collected samples.

Fig. 3. Light micrographs of branch sections showing the wall construction of the epidermal cells consisting of a continuous decklamelle and outer wall covering of the epidermal cells. Fig. 3A. *Gracilaria cornea*. Fig. 3B. *G. tikvahiae*.

Fig. 4. Ultrathin sections showing the construction of the outer epidermal wall. Each epidermal cell has distinct individual cell wall layers that are then covered by the outer wall and the decklamelle. A layer of debris is visible on the surface of the field-collected branches. Fig. 4A. *Gracilaria cornea*. Fig. 4B. *G. tikvahiae*.

Fig. 5. Cross sections showing the attachment of *Ulva lactuca* germlings. Fig. 5a. Attached to glass slides showing adhesion pad from initial attachment. Fig. 5b. Attached to *Gracilaria cornea*. Fig. 5c. Attachment sites on *Gracilaria cornea*. Fig. 5d. Attached to *Gracilaria tikvahiae* showing adhesion pad of a 3-celled germling. Fig. 5e. Attachment site on *Gracilaria tikvahiae* (older germlings, penetrating).

Fig. 6. Ultrathin sections showing the penetration of the basal cell of a young filament of *Ulva lactuca* through the decklamelle and outer wall. Fig. 6a. Initial penetration into the outer wall of *Gracilaria tikvahiae*. In the early phase of attachment the decklamelle is pushed up forming a rim around the adhesion pad of the sporeling. Fig. 6b. During the later stages, electron dense material is concentrated outside the protoplast of the basiphyte epidermal cells of *G. tikvahiae*, and the plasma membrane of the epiphytes is convoluted. Fig. 6c. Increased electron dense material

around the protoplast of the basiphyte epidermal cells, and increased convolution of the plasma membrane of the epiphyte.

Fig. 7. Ultrathin sections showing the penetration of the basal cell of young filaments of *Ulva lactuca* through the decklamelle and outer wall of *Gracilaria cornea*. Fig. 7a. Attachment by the epiphyte is superficial and the basal cell is not penetrating the outer cell wall. Fig. 7b. Two rhizoids of the epiphyte have penetrated the outer wall layers and have grown toward the middle lamellar region of *G. cornea* epidermal cells.

3.2. Effect of different irradiances on growth

3.2.1. Use of UV-B light

Although *Ulva* was affected by exposure to UV-B, it was apparent that all the three agarophytes presently in culture at the IOLR were more sensitive. Even 1 hour exposures to UV-B resulted in bleaching after 1 week and stop of growth. Thus, use of UV-B radiation does not appear to be useful to control epiphytization by *Ulva*.

3.2.2. Use of different irradiances

A. Culture experiments.

A total of 10 experiments were run over the grant period using chambers made from red (680 nm), green (510 nm), blue (430 nm) and clear (normal PAR) plexiglass (perspex). Irradiance levels were balanced and intensity within each pair of chambers ranged from 120 (red) to 140 (blue) to 150 (green and white) $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. All light quality measurements were made using an International Radiometer (Model I55A). All culture experiments ran for 6 to 8 weeks using four 5 cm long branches of each species placed in deep-well petri dishes that were placed in the four light regimes within the plexiglass chambers. Thus, there were 4 replicate branches of each species with two replicate dishes ($n = 4 \times 2$ replicates), one in each of two identical plexiglass chambers.

Pigment levels. Levels of chlorophyll a and phycoerythrin varied without a distinct pattern for *G. tikvahiae*, *G. cornea*, and *U. lactuca* (chl a only) as shown in Figures 8, 9, and 10a. Significant differences were evident in levels of chlorophyll a for the three species as well as with phycoerythrin for the two species of *Gracilaria* but there was no pattern (Tables 1 to 4).

Growth rates. *U. lactuca* grew significantly slower under green light (Fig. 10b, Table 6) in a 8 week experiment in October-November 1997. By contrast, both species of *Gracilaria* showed high growth rates under green light (Fig. 11a, 11b) with growth being significantly higher in an 8 week experiment ending in January 1997 (Fig. 11a; Table 5). Similar results have been achieved in tank experiments (Svirski et al, 1993)

B. Photosynthetic rates.

Photosynthetic responses of 8 replicates each of *G. tikvahiae*, *G. cornea*, and *Ulva lactuca* were measured under white (control), green, blue and red plexiglass filters in the dark (respiration) and at 50 and 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in order to compare with growth responses. Regardless of light quality, the green alga *U. lactuca* showed the highest response at 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, including under green light (Fig. 12). In summary, although green light results supported higher growth response in the red seaweeds compared with the green algal epiphyte, photosynthetic responses did not demonstrate this.

C. Conclusions

The use of UV-B radiation does not appear to be useful to control epiphytization by *Ulva*. Levels of chlorophyll a and phycoerythrin varied under different light qualities without a distinct pattern for *G. tikvahiae*, *G. cornea*, and *U. lactuca*, showing also species differences. Green light inhibited *Ulva* growth and could therefore be used for selective *Ulva* inhibition in a *Gracilaria* culture. However, photosynthesis did not present this differentiation.

D. Figure description

Fig. 8. Effect of light quality on pigment conc. of *G. cornea* in four different experiments (n=8). a. Chlorophyll a. b. Phycoerythrin.

Fig. 9. Effect of light quality on pigment conc. of *G. tikvahiae* in two different experiments (n=8). a. Chlorophyll a. b. Phycoerythrin.

Fig. 10. Effect of light quality on (a) chlorophyll a conc. and (b) daily growth rate of *Ulva lactuca* in 2-4 different experiments (n=8).

Fig. 11. Effect of light quality on daily growth rate of (a) *G. cornea* in four different experiments, and of (b) *G. tikvahiae* in one experiment (n=8).

Fig. 12. Effect of light quality on daily growth rate of *G. cornea*, *G. tikvahiae* and *Ulva lactuca* (n=8).

3.3. Sensitivity studies

A. Introduction

The major objective of the sensitivity studies was to isolate the main responses of *Gracilaria* spp. to the presence of *Ulva lactuca* in biculture (hereafter named *Gracilaria* and *Ulva*). Previous experiments suggested in some species of *Gracilaria* an inconsistent inhibition in growth. However, in the previous studies there was no integrated study of five parameters using five *Gracilaria* species concurrently. The working hypothesis was that under light, CO₂ and nutrient saturation, an interaction between epiphyte and basiphyte will be expressed by changes in growth, photosynthesis and respiration, because of effective excretions. Algal excretions like hydrogen peroxide and halogenated hydrocarbons, which have been identified in the response of *Gracilaria* to bacteria, did effect the algal tissues and metabolism (Pederson et al, 1996; Collen et al, 1995). Such effects were hypothesized in the response of different *Gracilaria* species to *Ulva*. In order to define whether the *Gracilaria* response is related to the presence of bacteria, a set of antibiotic experiments was carried out. These experiments demonstrated an almost total elimination of bacteria from the culture. Three other questions have been tested under this sensitivity objective. First, optimal conditions of hydrogen peroxide release and halogenation were defined, being a crucial component of the *Gracilaria* - *Ulva* relationship. Second, assuming a peroxide release by the biculture partners the response to external treatment by peroxide was tested with *Gracilaria* and *Ulva* separately. Third, an inverse effect of *Gracilaria* on *Ulva* was questioned using the main parameters of interaction.

B. *Gracilaria* response in biculture

Five parameters of sensitivity studies have been tested in the biculture of *Ulva* with five *Gracilaria* species in a number of different cylinder experiments. No significantly consistent inhibition of *Gracilaria* species growth rate in the presence of *Ulva* could be seen in biculture. The only significant effect was the increase of *Gracilaria* H-6 growth rate in the presence of *Ulva* (Fig 13). The only significant inhibiting effect of *Ulva* on the rate of photosynthesis of *Gracilaria* appeared in *G. conferta* (Fig 14). The only significant inhibiting effect of *Ulva* on the rate of respiration of *Gracilaria* species was shown in *G. conferta* and *G. cornea* (Fig 15). The effect of *Ulva* on

hydrogen peroxide production of *Gracilaria* was an inhibition of three rapid growth species of *Gracilaria* (COR, LEM, H-6), and a promotion in two slow growing species (MUT, CON, Fig 16). The effect of *Ulva* on halogenated hydrocarbon production by *Gracilaria* species consistently showed a significant inhibition (Fig 17). Halogen production in a monoculture *Ulva* cylinder was higher than in the *Gracilaria* monoculture cylinder only in the H-6 *Gracilaria* experiment (not presented).

A highly positive relationship could be defined in *Gracilaria* between peroxide release and growth rate ($r=0.377$), peroxide release and rate of respiration ($r=0.659$) and between halogen release and rate of photosynthesis ($r=0.855$; Tab 7).

The primary effect of the *Ulva* and *Gracilaria* biculture was on the metabolism of *Gracilaria*, which consistently showed a significant inhibition of the release of halogenated hydrocarbons. Halogen release resulted in a clear inhibition of *Gracilaria* metabolism by *Ulva* as in the case of species H-6, and not just a dilution effect of the *Ulva* presence. The halogenation process showed a very high correlation with the rate of photosynthesis suggesting the consumption of photosynthetic products for the halogen release, which is probably also conditioned by peroxide release. The other effects of hydrogen peroxide release, and rates of growth, photosynthesis and respiration are species dependent. *G. conferta* showed the highest sensitivity to the presence of *Ulva* regarding inhibition of photosynthesis and respiration.

C. Effect of bacteria on biculture

Four parameters of sensitivity have been studied in biculture in the presence of antibiotics (Vancomycin 100mg/L + Cefotaxim 100mg/L). Growth rate of all *Gracilaria* species was significantly inhibited in the presence of antibiotics. *Ulva* inhibited the growth rate of two *Gracilaria* species in the presence of antibiotics (MUT, COR), and in two species *Ulva* promoted *Gracilaria* growth rate in the presence of antibiotics (LEM, CON, Fig 18). The rate of photosynthesis generally increased in the presence of antibiotics. *Ulva* increased the rate of *Gracilaria* photosynthesis in the presence of antibiotics in three species (MUT, COR, LEM), and in one species *Ulva* decreased the rate of *Gracilaria* photosynthesis in the presence of antibiotics (CON, Fig 19). The rate of respiration was generally lower in the presence of antibiotics. *Ulva* increased the rate of *Gracilaria* respiration in the presence of

antibiotics (COR, CON, LEM), and in one species *Ulva* decreased the rate of *Gracilaria* respiration in the presence of antibiotics (MUT, Fig 20). The release of hydrogen peroxide was generally lower with antibiotics. In one case *Ulva* increased the release of peroxide by *Gracilaria* in the presence of antibiotics (MUT), and in one case *Ulva* decreased the release of peroxide by *Gracilaria* in the presence of antibiotics (COR, Fig 21).

The general inhibiting effect of antibiotics on *Gracilaria* growth, respiration and peroxide release suggests that these physiological parameters are partially promoted by bacteria, whereas the rate of photosynthesis is partially inhibited by bacteria. The different *Gracilaria* species responded to *Ulva* in presence of antibiotics in a species dependent way in all four physiological parameters. *G. cornea*, which is an epiphyte resistant species, responds differently to the presence of antibiotics and *Ulva* than *G. conferta*, an epiphyte sensitive species.

D. Hydrogen peroxide and halogenation.

The production of hydrogen peroxide by *G. cornea* showed a highly positive relationship with light intensity, whereas the halogenated hydrocarbon production showed a low positive relationship with light intensity (Fig 22). The optimal light exposure of *G. cornea* before determination of these metabolites showed that 2-3 hours was optimal for peroxide release whereas almost any photoperiod was optimal for halogenation (Fig 23). The density of *G. cornea* in the cylinders was optimal at 1.25g/500mL for peroxide release and halogenation after calculating the metabolite concentration per gr. FW of the seaweed (Fig 24).

Regarding growth rates, seaweed treatment with hydrogen peroxide dramatically inhibited the *G. cornea* mutant, moderately inhibited *Ulva*, and dramatically increased that of *Enteromorpha* (Fig 25).

The optimization of peroxide and halogen release by *Gracilaria* showed different light duration, and light intensity conditions for both processes. This suggests that in spite of the assumption that halogenation follows peroxide release they have independent limiting factors.

There is a variety of sensitivity responses by different seaweed species to hydrogen peroxide treatment. This suggests that if the main peroxide producer in a biculture would be *Ulva* then *Gracilaria* would be strongly inhibited, which is not the usual

case in biculture. But if *Gracilaria* would be the main producer than *Ulva* would probably be promoted under the suitable concentrations. It may be suggested that the major source of hydrogen peroxide in biculture is *Gracilaria*.

E. The inverse effect

The inverse effect of *Gracilaria* on growth rate of *Ulva* showed a significant positive effect under high light intensity. The rate of photosynthesis and respiration of *Ulva* decreased in the presence of *Gracilaria* under high light intensity (Fig 26).

The clear increase in growth of *Ulva* in presence of *Gracilaria*, which is partially supported by the decrease in respiration, suggests a positive effect of *Gracilaria* excretions. This might include defined concentrations of hydrogen peroxide and other promoters. However, the growth of *Ulva* sporelings was inhibited by the presence of the *G. cornea* mutant, suggesting that the sporelings are different in sensitivity from mature plants (Rep.2).

F. Conclusions

The general response of *Gracilaria* spp. to the presence of *Ulva lactuca* in a biculture is characterized by halogenated hydrocarbon release, whereas the response of growth, respiration and peroxide release are species dependent. Growth, respiration and peroxide release by *Gracilaria* are partially controlled by the presence of bacteria in the medium, and are clearly species dependent. *Gracilaria conferta* is the most sensitive of the *Gracilaria* species tested regarding its physiological responses to biculture with *Ulva* and its culture appearance. The two released metabolites, hydrogen peroxide and halogenated hydrocarbon are probably controlled by two different processes although they are interconnected. *Gracilaria* growth proved to be much more sensitive to hydrogen peroxide inhibition than *Ulva*. On the other hand *Gracilaria* is probably the major source of peroxide in the biculture. The inverse effect of *Gracilaria* on mature *Ulva* in biculture is basically promotive. However, *Ulva* sporelings have been inhibited by the presence of *Gracilaria*, probably through being more sensitive than mature *Ulva*.

G. Figure and table description

Fig 13. Effect of *Ulva* on the growth rate of *Gracilaria* in biculture. The experiment was operated in controlled cylinders with and without *Ulva* (U) with five different

Gracilaria (G) species: *G. cornea* (COR), *G. cornea* mutant (MUT), *G. conferta* (CON), *G. lemaneiformis* (LEM), and the hybrid (H-6). The weight ratio was *Ulva:Gracilaria* = 3:1 (n=3-21).

Fig 14. Effect of *Ulva* on the rate of photosynthesis of *Gracilaria* in biculture. Details as in Fig 13 (n=6-12).

Fig 15. Effect of *Ulva* on the rate of respiration of *Gracilaria* in biculture (n=6-12). Details as in Fig 13.

Fig 16. Effect of *Ulva* on the hydrogen peroxide release of *Gracilaria* in biculture (n=6-21). Details as in Fig 13.

Fig 17. Effect of *Ulva* on the halogenated hydrocarbon release of *Gracilaria* in biculture (n=3-15). Details as in Fig 13.

Fig 18. Effect of *Ulva* (U) on *Gracilaria* (G) growth rate in biculture in the presence of antibiotics (A) (n=3-15). The experiment was operated in controlled cylinders with and without *Ulva* in the presence and absence of antibiotics with four different *Gracilaria* species: *G. cornea* mutant (MUT), *G. conferta* (CON), *G. lemaneiformis* (LEM), *G. cornea* (COR) and their mean (All).

Fig 19. Effect of *Ulva* (U) on *Gracilaria* (G) rate of photosynthesis in biculture in the presence of antibiotics (A) (n=3-15). Details as in Fig. 18.

Fig 20. Effect of *Ulva* (U) on *Gracilaria* (G) rate of respiration in biculture in the presence of antibiotics (A) (n=3-15). Details as in Fig. 18.

Fig 21. Effect of *Ulva* (U) on *Gracilaria* (G) peroxide release in biculture in the presence of antibiotics (A) (n=3-12). Details as in Fig. 18.

Fig 22. Effect of light intensity in culture on *G. cornea* release of hydrogen peroxide and halogenated hydrocarbon (n=3). The experiment was operated in deep petri dishes on a growth gradient table.

Fig 23. Effect of light duration before sampling on *G. cornea* hydrogen peroxide and halogenated hydrocarbon release (n=6).

Fig 24. Effect of algal density in culture dishes on *G. cornea* hydrogen peroxide and halogenated hydrocarbon release (n=2).

Fig 25. Effect of hydrogen peroxide treatment (10 ppm) on the growth rate of *Ulva*, *Enteromorpha* (Enter), and *Gracilaria* (Grac) as compared to control (n=6-9).

Fig 26. Effect of *G. cornea* mutant (G) on rates of growth, photosynthesis (Pho), and respiration (Res) of *Ulva* (U) in the presence of antibiotics (n=21).

Tab 7. Correlation coefficients of Ln transformed results of *Gracilaria* parameters in the main interaction experiments with *Ulva*: Growth rate, photosynthesis rate, respiration rate, hydrogen peroxide conc., and halogenated hydrocarbon conc.

3.4. Allelopathic excretions

A. Introduction

Previous experiments showed that under controlled conditions and saturated light, nutrients and carbon dioxide there still is an inhibition of *Gracilaria* growth in biculture with *Ulva* (Friedlander et al, 1996). Therefore the following experiments tested the possible excretion of inhibiting compounds in the biculture system. Recent studies about the effect of bacteria on *Gracilaria* suggest a model for such an interaction (Weinberger et al, 1997, 1999).

B. Organic extracts

The effect of ethyl acetate extract of *Gracilaria* and *Ulva* medium on the *Gracilaria* growth showed overall a slight inhibition of *Gracilaria* extract as compared to control and to other extracts (Fig 27). A comparison of species showed that *G. lemaneiformis* and the *G. cornea* mutant had the strongest inhibition. An inverse effect of *Ulva* inhibition by *Gracilaria* extract has also been identified (Rep. 1).

The effect of the ethyl acetate extract of *Gracilaria*, which inhibits the growth of *Gracilaria* more than the other extracts, suggests that its growth is self controlled, mainly under high densities. In previous experiments two medium extracts (carbon tetra chloride and chloroform) were more inhibiting *Gracilaria* growth than other solvents (Rep. 2), and their effectiveness was relatively short.

C. Absorbency of excretions

Experiments described in the Second year report showed the importance of a selected group of excretions on the growth inhibition of *Gracilaria* (Rep. 2). Absorbents like Amberlite and Perhydrol were expected to neutralize inhibiting excretions, however, they did not show this effect (Rep. 1). There is, however, a slight inhibition of *Gracilaria* growth in the presence of Amberlite, suggesting the absorption of growth promoting substances (Fig. 28).

D. Conclusions

Absorbents of organic compounds did not prevent the interaction effects in the biculture. However, extracted compounds from the growth medium of *Ulva* and *Gracilaria* did clearly inhibit the growth of the latter species. The inhibiting compounds are characterized by being soluble in ethyl acetate, chloroform, and carbon tetra chloride. The compounds themselves have not been isolated. The excretion might, however, involve hydrogen peroxide or halogenated hydrocarbons as shown in the sensitivity chapter.

E. Figure description

Fig. 27. Effect of ethyl acetate extracts of seaweed culture medium on *Gracilaria* (G) species growth rate. The extracts were prepared from growth medium of *Ulva* (U ex), *Ulva* and *Gracilaria* in biculture (U+G ex), *Gracilaria* (G ex), and of seawater (SW ex). Natural seawater served as general control (SW con). The tested species were: *G. cornea* (COR), *G. lemaneiformis* (LEM), *G. conferta* (CON), *G. cornea mutant* (MUT), and a mean of all together (All).

Fig 28. Effect of Amberlite on *G. conferta* (G) growth rate (n=6). The treatments included: Seawater control (Con), Amberlite (Amb), *Gracilaria* extract control (Gext Con), *Gracilaria* extract with Amberlite (Gext Amb), and seawater extract (SW ext).

3.5. Chemical control of epiphytization

A. Selectivity treatments

Chemical control of epiphytization has not been tested in this study by wide scale experiments. However, the growing experience in *Gracilaria* cultivation during this study showed that at least one selective chemical which degrades filamentous and folios seaweeds (*Ulva*, *Enteromorpha*, *Ectocarpus* etc.) and which does not appear to damage *Gracilaria* is hydrogen hypochlorite (Fig 29). The treatment does, however, inhibit the growth rate of *Gracilaria*, and causes total disintegration to the epiphytes. The risk in using this selective chemical is related to determination of concentration and temperature on which its effectiveness is dependent. However, it is a powerful tool for *Gracilaria* cultivation. The selectivity of hydrogen hypochlorite is probably a result of its external surface oxidative activity.

B. Figure description

Fig. 29. Effect of hypochlorite treatment on *G. conferta* growth rate during 3-15 days from treatment to harvest. Growth rate is expressed as mean daily growth rate during the time from treatment to harvest.

3.6. Integration of studies on epiphytization

In addition to parallel studies carried out in Haifa, Tel Aviv and Tampa, C. Dawes joined M. Friedlander at the IOLR during March through early June 1997. During this period a series of experiments were designed to study the effect of UV-B light on the epiphyte and basiphyte. Further, light microscopic studies were carried out to examine older examples of epiphytization by *U. lactuca* on species of *Gracilaria* that were presently in culture at the IOLR (e.g. *G. conferta*, *G. cornea*, *G. lemaneiformis*). The former studies have been presented (section 3.3.1) while the latter studies were used to better explain the attachment and penetration of the green alga on the red seaweed. Throughout this study the investigators have corresponded by E-mail and letter.

The outcome of this integration may suggest several aspects.

First, the limited penetration of *Ulva lactuca* into *G. cornea* is supported by the fact that it is the most resistant species in this study.

Second, *G. cornea* differs from *G. conferta* in sensitivity responses of excretions, growth and photosynthesis.

Third, both species produce halogenated hydrocarbons.

Fourth, *Ulva lactuca* responds differently to the light field than *Gracilaria*.

4. Evaluation of the research achievement

4.1. The first objective deals with the relationship of species of *Gracilaria* to *Ulva lactuca* in terms of sensitivity and structural relationships.

One interesting finding in this study is the direct evidence that the green algal epiphyte, common to many species of seaweeds, does penetrate its red algal host. The penetration by first the basal cell and then rhizoids of *U. lactuca* is similar to that described for the red algal hemiparasite, *Polysiphonia lanosa* on its obligate brown algal host *Ascophyllum nodosum*. The information is new to our understanding of the

relationship between "typical" algal epiphytes and their attachment to basiphytes. This is an area that should be explored further, particularly the possibility of reactions between the invading and host cells. The data further suggest that once epiphytized by *U. lactuca*, complete removal from the host is almost impossible. Thus, it may be more profitable in seaweed mariculture to select for epiphyte resistant strains more than search for inhibitors of epiphytization.

Another main conclusion of the sensitivity study of *Gracilaria* in biculture with *Ulva* showed the decrease in release of halogenated hydrocarbons in biculture as compared to monoculture of *Gracilaria* which is its main source. Whereas other processes like hydrogen peroxide release, and performance of respiration, photosynthesis and growth did not show a total discriminating effect. However, inhibitory effects of growth in biculture were manifested by *G. conferta* which turned out to be the most sensitive *Gracilaria* species, as compared to the most resistant one *G. cornea*. All these responses were different under antibiotic treatment, suggesting bacteria involvement in the interaction processes. These responses showed also strong species dependence. On the other hand the presence of mature *Gracilaria* inhibited the growth of *Ulva* sporelings and promoted the growth of mature *Ulva*.

4.2. The second objective is concerned with the allelopathic excretions by *Gracilaria* and *U. lactuca* and how they affect one another. The interaction of the two species in the biculture medium did clearly show an inhibitory effect of *Gracilaria* growth using several solvents for medium extraction. Hydrogen peroxide and halogenated hydrocarbons could be a part of the effective excretion compounds.

4.3. The third objective is involved in comparing growth responses of the green epiphyte and its red algal basiphyte under different light fields. The three year study has shown that use of green light can enhance growth in the latter but not in the former, but that the differences are limited. Both pigment and photosynthetic data do not support the growth data but do show why the green alga is so responsive in mariculture.

4.4. The fourth objective is the testing of selected chemical agents as a method to control epiphytization of *Gracilaria*. This objective is probably not fully attainable when the intimate interaction between the epiphyte on the red algal basiphyte are

considered. However, a relative effective selectivity has been defined by the use of hydrogen hypochlorite.

4.5. The final objective is an integration of the results of the first four objectives regarding basiphyte-epiphyte relationships for seaweed cultivation. It appears, based on the ability of *U. lactuca* to penetrate both *G. cornea* and *G. tikvahiae*, that a potent direction of farming is to select for epiphyte resistant strains as well as examine how the basiphyte may be able to prevent penetration (e.g. decklamelle structure, production of peroxide).

5. Description of the cooperation

5.1. The first objective has been studied at USF and the IOLR. In addition, C. Dawes spent 3.5 months working with M. Friedlander at the IOLR in the spring-summer of 1997.

5.2. The second objective was studied at the IOLR and TAU.

5.3. The third objective was studied at USF and IOLR and tests using green plexiglass have been carried out to determine if epiphytization by the green alga can be reduced in tank culture.

5.4. The fourth objective was questioned due to the ability of the epiphyte to penetrate *Gracilaria*. However, tank experiments in IOLR did give a relatively reliable answer.

6. General conclusions and plans

A. Conclusions

6.1. The green algal epiphyte, common to many species of seaweeds, does penetrate its red algal host.

6.2. *Gracilaria* spp. in monoculture released more halogenated hydrocarbons than in biculture with *Ulva lactuca*, whereas hydrogen peroxide release, and performances of respiration, photosynthesis and growth did not show an overall discriminating effect in biculture versus monoculture.

6.3. *Gracilaria conferta* is most sensitive to epiphytes among *Gracilaria* species tested in this study.

6.4. The presence of mature *Gracilaria* inhibited the growth of *Ulva lactuca* sporelings.

6.5. Hydrogen peroxide and halogenated hydrocarbons could be a part of the effective excretion compounds in biculture.

6.6. The use of green light can enhance growth in basiphytes but not in epiphytes.

6.7. Effective selectivity has been defined by the use of hydrogen hypochlorite.

B. Plans

6.8. It may be more profitable in seaweed mariculture to select for epiphyte resistant strains than to search for inhibitors of epiphytization.

6.9. It is important as well to examine how the basiphyte may be able to prevent penetration (e.g. decklamelle structure, production of peroxide).

6.10. The definition of the effective excretions in biculture

7. Changes in direction

No changes in direction of the original research program have been made.

8. List of publications

A paper dealing with the structure and penetration of the cell wall of *Gracilaria* spp. by *Ulva lactuca* is in final preparative stages. Another paper dealing with the sensitivity studies is under preparation. Both drafts are attached to this proposal.

9. Report on any patents

No patents are involved in this study.

10. References

Braten, T. 1975. Observations on mechanisms of attachment in the green alga *Ulva mutabilis* Foyn. An ultrastructural and light microscopical study of zygotes and rhizoids. *Protoplasma* 84: 161-173.

Buschmann, A.H. and Gomez, P. 1993. Interaction mechanisms between *Gracilaria chilensis* (Rhodophyta) and epiphytes. *Hydrobiologia* 260/261: 345-351.

Buschmann, A.H. and Kuschel, F.A. 1988. Cultivo intermareal de *Gracilaria* sp: Colonizacion de esporas e interacciones con *Ulva lactuca*. *Biota (Chile)* 4: 107-113.

- Callow, M.E., Callow, J.A., Pickett-Heaps, J.D. and Wetherbee, R. 1997. Primary adhesion of *Enteromorpha* (Chlorophyta, Ulvales) propagules: Quantitative settlement studies and video microscopy. *Journal of Phycology* 33: 938-947.
- Collen, J., Del Rio, M.J., Garcia-Reina, G. and Pedersen, M. 1995. Photosynthetic production of hydrogen peroxide by *Ulva ridgida*. *Planta* 196: 225-230.
- Dawes, C.J. 1988. *Introduction to Biological Electron Microscopy: Theory and Techniques*. Ladd Research Industries, Publisher, Burlington VT.
- Ducker, S.C. and Knox, R.B. 1984. Epiphytism at the cellular level with special references to algal epiphytes. pp. 113-133. In H.-F. Linskens and J. Heslop-Harrison (eds.), *Encyclopedia of Plant Physiology* 17. Springer, Berlin.
- Evans, L.V. 1981. Marine algae and fouling: A review, with particular reference to ship-fouling. *Botanica Marina* 24: 167-171.
- Evans, L.V. and Christie, A.O. 1970. Studies on the ship-fouling alga *Enteromorpha*. I. Aspects of the fine-structure and biochemistry of swimming and newly settled zoospores. *Annals of Botany* 34: 451-466.
- Friedlander, M. 1992. *Gracilaria conferta* and its epiphytes: the effect of culture conditions on growth. *Botanica Marina* 35: 423-428.
- Friedlander, M. Gonen, Y., Kashman, Y. And Beer, S. 1996. *Gracilaria conferta* and its epiphytes: 3. Allelopathic inhibition of the red seaweed by *Ulva cf. lactuca*. *Journal of Applied Phycology* 8:21-25.
- Goff, L.J. 1979. The biology of *Harveyella mirabilis* (Cryptonemiales, Rhodophyceae). VII. Structure and proposed function of host-penetrating cells. *Journal of Phycology* 15: 87-100.
- Harlin, M.M. and Craigie, J.S. 1975. The export of organic carbon from *Ascophyllum nodosum* as it relates to the epiphyte *Polysiphonia lanosa*. *Proceedings of the International Seaweed Symposium* 8: 193.
- Leonardi, P.I. and Ciceris, E.J. 1991. Ultrastructure of the fresh-water alga *Enteromorpha flexuosa* spp. *pilifera*. I. Germination of biflagellate zoospores (Ulvophyceae, Chlorophyta). *Nova Hedwigia* 53: 115-126.
- Moss, B. And Woodhead, P. 1970. The breakdown of paint surfaces by *Enteromorpha* sp. *New Phytologist* 69: 1025-1027.

- Pedersen, M., Collen, J., Abrahamsson, K. and Ekdahl, A. 1996. Production of halocarbons from seaweeds: an oxidative stress reaction? *Sci. Mar. Barc.* 60: 257-263.
- Svirski, E., Beer, S. and Friedlander, M. 1993. *Gracilaria conferta* and its epiphytes: (2) Interrelationship between the red seaweed and *Ulva* cf. *lactuca*. *Hydrobiologia* 260;261: 391-396.
- Weinberger, F., Hoppe, H.-G. and Friedlander, M. 1997. Bacterial induction and inhibition of a fast necrotic response in *Gracilaria conferta*. *J. Applied Phycol.* 9: 77-285.
- Weinberger, F., Friedlander, M. and Hoppe, H-G. 1999. Oligoagars elicit a physiological response in *Gracilaria conferta*. *J of Phycol.* (accepted).

3A

3B

4A

4B

6a

7a

6b

7b

6c

Gracilaria cornea Chl a

Gracilaria cornea Phycoerythrin (PE)

Gracilaria tikvahiae Chl a

Gracilaria tikvahiae Phycoerythrin (PE)

Ulva lactuca Chl a

10a

Ulva lactuca growth (30 days) (50 μ mol photons)

10b

***Gracilaria cornea* growth (30 days)
(50 μ mol photons)**

***Gracilaria tikvahiae* growth (30 days)
(50 μ mol photons)**

Fig. 12

Fig 13

Fig 13

Fig 14

Fig 15

Fig 16

Fig 17

Fig 18

Fig 19 Chart 1

Fig 19

Fig 20

Fig 21

Fig 22

Fig 23

Fig 24

Fig 24

Fig 25

Fig 26

Fig 27

Fig 28

Fig 29 Chart 1

Fig 29

Table 1: *Gracilaria cornea* Chl a

July 1996	Significant differences	Green vs. all other treatments	P=.013390
November 1996	Significant differences	Blue vs. Green, Red vs. Green	P=.0024272
January 1997	No significant difference	-	-
October 1997	Significant differences	Green vs. all other treatments	P=5.9345E-009

Table 2: *Gracilaria cornea* Phycoerythrin (PE)

July 1996	No significant differences	-	-
November 1996	No significant differences	-	-
January 1997	Significant differences	Blue vs. control Blue vs. Green	P=.00002487
October 1997	Significant differences	Control vs. Green Control vs. Blue Red vs. Blue Red vs. Green	P=.00011084

Table 3: *Gracilaria tikvahiae* Chl a

April 1997	Significant differences	Red vs. Control Red vs. Blue Red vs. Green	P=.00017176
July 1997	Significant differences	Red vs. All treatments Control vs. All treatments	P=.00077793

Table 4: *Ulva lactuca* Chl a

November 1996	No Sig. Differences	-	-
April 1997	No Sig. Differences	-	-
July 1997	Significant differences	Green vs. Control	P=.0123
October 1997	Significant differences	Control vs. all treatments Green vs. Red	P=.00000106

Table 5: *Gracilaria cornea* growth (30 days @ 50 umol photons)

July 1996	No significant differences	-	-
November 1996	No significant differences	-	-
January 1997	Significant differences	Green vs. all treatments	P=.0165
October 1997	No significant differences	-	-

Table 6: *Ulva lactuca* growth (30 days @ 50 umol photons)

November 1996	No Sig. Differences	-	-
April 1997	No Sig. Differences	-	-
July 1997	No Sig. Differences	-	-
October 1997	Significant differences	Control vs. Red Control vs. Green	P=.00000106

Tab 7

Tab 7						
Ln	Growth	Photos	Res	Peroxide	Halogen	
Growth	1	*-0.275	0.198	**0.377	-0.128	
Photos		1	**0.313	-0.036	**0.855	
Res			1	**0.659	0.081	
Peroxide				1	-0.123	
Halogen					1	