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Gracilaria and its epiphytes: 4. The response of two *Gracilaria* species to *Ulva lactuca* in a bacteria-limited environment

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Abstract

The responses of *Gracilaria lemaneiformis*, an easily epiphytized host, and the relatively resistant *G. cornea* mutant, to the green alga *Ulva lactuca* were studied using biculture experiments with and without antibiotics. Both *Gracilaria* species grown with and without *U. lactuca* showed different levels of growth rate, release of hydrogen peroxide and of halogenated hydrocarbons. These quantitative differences led to a successful response against *Ulva lactuca* in the case of *G. cornea* mutant and to a failure in response in the case of *G. lemaneiformis*. The response of each *Gracilaria* species to *U. lactuca* was qualitatively similar to its response to bacteria. This suggests the involvement of oligosaccharide elicitors produced in the presence of epiphytes and bacteria. A clear *Gracilaria* inhibition was demonstrated with extracts of the culture medium. It appears that hydrogen peroxide, halogenated hydrocarbons and oligosaccharides may be components of the inhibitory activity of the extracts. The responses of *Gracilaria* species to the presence of *U. lactuca* suggest the characterization of a defence response.

Introduction

We have shown in previous experiments that there is a significant inhibition in the growth of the red seaweed *Gracilaria conferta* during biculture with the green algal epiphyte *Ulva lactuca* under saturating irradiance, nutrients and carbon dioxide (Friedlander et al., 1996). This interaction between epiphyte and basiphyte was probably due to allelopathic effects, since ethyl acetate extracts of the media of *G. conferta* and *U. lactuca* resulted also in a major inhibition of growth in *G. conferta* (Friedlander et al., 1996). The molecular and physiological basis of similar allelopathic effects of epiphytic bacteria on *Gracilaria* species has recently been detected. *G. conferta* was hypersensitive when in contact with specific peptides that were excreted by certain microorganisms (Weinberger & Friedlander, 2000a) or when it was exposed to specific oligosaccharides that are products of bacterial enzymatic attacks on the algal agar cell wall matrix (Weinberger et al., 1999) or cellulose-like cell wall skeleton (Weinberger & Friedlander, 2000a). All these chemical signals elicited a fast tip bleaching response in *G. conferta* (Weinberger et al., 1997), which result directly from an autodestructive oxygen activation (Weinberger et al., 1999). Similar hypersensitive responses have been detected in *Chondrus crispus* gametophytes (Bouarab et al., 1999) and have been known for many years in higher plants as key mechanisms of defense (Doke et al., 1982; Callow, 1984; Dixon & Lamb, 1990; Kombirnk & Somssich, 1995). The oxidative burst after elicitation of *G. conferta* was correlated with increased algal respiration (Weinberger et al., 1999). Hydrogen peroxide is usually the limiting compound for algal haloperoxidases, which catalyze the synthesis of halogenated compounds from

halide ions and suitable acceptors (Mtolera et al., 1996; Pedersen et al., 1996). As a consequence, the oxidative burst in *G. conferta* was also correlated with an increased haloperoxidase activity potential (Weinberger et al., 1999). The algal generation of cytotoxic activated oxygen species and eventually halocarbons during the hypersensitive response resulted not only in the autodestructive tip bleaching effect, but also in the elimination of epiphytic microorganisms that were associated with *G. conferta* (Weinberger & Friedlander, 2000b). These same compounds could also affect the tissues and metabolism of epiphytic algae (Pedersen et al., 1996; Collen et al., 1995). Thus, a similar sequence is hypothesized for the responses of two *Gracilaria* species to the presence of *U. lactuca* in this study. A previous study supports this idea, showing that a biculture of *G. conferta* with *U. lactuca* resulted in the bleaching of growing tips, inhibition of growth, and increase in respiration of *G. conferta* (Svirski et al., 1993). Hence, growth, respiration rates, hydrogen peroxide release and haloperoxidase activity potential of *Gracilaria* species were studied, and the response of two *Gracilaria* species towards *U. lactuca* ascertained. One of the selected species was visually susceptible to epiphytes (*G. lemaneiformis*) and the other relatively resistant (*G. cornea* mutant). Both were chosen out of various cultured *Gracilaria* species, expecting a difference in their response to epiphytes.

Use of antibiotics should determine whether the responses of *Gracilaria* species to *Ulva lactuca* were influenced by bacterial growth. An antibiotic mix had previously been shown to cause almost total elimination of bacteria from culture within 3 days with less than 1 bacterium present per 6.75 mg fresh weight of *G. conferta* (Weinberger et al., 1997, 1999). As a consequence, the bacterial induction of hypersensitive responses in *G. conferta* was completely inhibited using the same procedures (Weinberger et al., 1997).

No previous interaction experiments have included comparative studies of physiological parameters using two *Gracilaria* species differing in their susceptibility to epiphytes. Thus, this study set out to compare the main responses of two different species of *Gracilaria* in biculture with *Ulva lactuca,* but without interference by bacteria. Additional interactions between species of *Gracilaria* and *U. lactuca* were also studied.

Materials and methods

The two *Gracilaria* species have been cultured in outdoor aerated tanks (40 L) during the whole year (Friedlander, 1992) at the Israel Oceanographic and Limnological Research facility in Haifa. *Gracilaria cornea* J. Agardh mutant strain (MUT), a spontaneous morphological mutation with a higher branching index was isolated from the wild type fronds originating from Jamaica (tropical climate) and has been cultured since 1991. *G. lemaneiformis* (Bory) Dawson, Acleto et Foldvik (LEM), a strain originating from Luderitz – Namibia (temperate climate), has been cultured since 1992 (Levy & Friedlander, 1994). *G. conferta* (Shousbhoe) J. & G. Feldmann was used as a test seaweed for extract growth experiments, and the epiphyte species, *Ulva lactuca*, was collected locally for the interaction experiments.

Growth experiments were usually carried out in 500-mL cylinders under 100 μmol photon m^{-2} s^{-1}, 25 °C, and pH 8 using CO_2 as the buffering agent with a pH controller. The biculture consisted of 1.5 g of the primary experimental species and 4.5 g of the accompanying species of *Gracilaria* or *U. lactuca*, being separated by a net screen. All primary species of *Gracilaria* or *U. lactuca* were grown for 7 days in biculture, after which they were weighed (growth), their respiration rates measured (oxygen electrode; Hansatech), and the media tested for presence of hydrogen peroxide (Miller & Kester, 1988). Further, the rate of algal bromination of phenol red was determined as an indication of the haloperoxidase activity potential (Weinberger et al., 1999). A mixture of antibiotics (Vancomycin 100 mg L^{-1} + Cefotaxim 100 mg L^{-1}) was used in a part of the treatments in order to eliminate bacteria in the seaweed cultures (Weinberger et al., 1997). One day before peroxide or bromination determination the seaweeds that were incubated with antibiotics were washed and transferred to an antibiotic free medium. Photosynthesis was also determined with an oxygen electrode (Hansatech).

The first fractionation of the culture media of species of *Gracilaria* and *Ulva lactuca* was operated with ethyl acetate following the procedures of Friedlander et al. (1996). In the second fractionation the ethyl acetate extract was separated using solvent partition (carbon tetrachloride, chloroform, aqueous methanol and hexane) after Kupchan et al. (1977). The third fractionation used a Sephadex LH-20 column, that was eluted with a petrol ether: chloroform: methanol = 2: 1: 1 mixture and collected into three frac-

Table 1. Weekly growth rate (%) of two *Gracilaria* species (LEM and MUT) and percentage of epiphytes (% total) in tank culture (mean ± SD; n = 26); different letters in same line express a significant difference (p <0.05)

Species	LEM	MUT
Gracilaria	B 32.0 ± 33.7	A 59.1±19.6
Epiphytes	b 55.6 ± 50.2	a 19 ± 23.8

tions. All fractions of the second and third separation phases were dried and dissolved in dimethyl sulfoxide (DMSO). Control extractions were carried out with sterile media as well. A sample of each dissolved fraction was diluted by 1: 10 with seawater (30 mL), and incubated with 50 mg *G. conferta* in a test tube at 25 °C and 100 μmol photon m^{-2} s^{-1} during 7 days to determine its effect on growth rate. Statistical analysis was carried out with Ln transformed data in order to meet the assumptions of analysis of variance. All significant differences are at the level of p <0.05.

Results

Biculture responses of two species of Gracilaria

The mean growth rate of two *Gracilaria* species and percent epiphytes (attached and floating) was measured in an outdoor annual experiment (Table 1). *G. lemaneiformis* (LEM) had a significantly lower growth rate and higher level of epiphytes when compared to the *G. cornea* mutant (MUT).

Both LEM and MUT showed significantly higher growth rates when cultured in glass cylinders without antibiotics than with the compounds (Figure 1A). In the presence of antibiotics, *Ulva lactuca* promoted the growth of LEM, although not significantly, while MUT was not affected by *U. lactuca* (Figure 1A). Hence, only the main growth-inhibiting effect of antibiotics was significant (Table 2).

The release of hydrogen peroxide was not significantly lower in LEM cultured without antibiotics compared with MUT (Figure 1B), but it was significantly higher in both species than with antibiotics (Table 2). The presence of *U. lactuca* with antibiotics increased the release of hydrogen peroxide by MUT, while decreasing it in LEM, although not significantly in either species (Figure 1B, Table 2). However, the presence of *U. lactuca* significantly inhibited the halogenation activity of the two *Gracilaria* species (Figure 1C, Table 2). The inhibition in the case of LEM was stronger as compared to MUT. Halogenation was not determined in the absence of antibiotics. Further, halogenation was significantly lower in a monoculture of *U. lactuca* (<20%) than in a monoculture of the two *Gracilaria* species (not presented). The rate of respir-

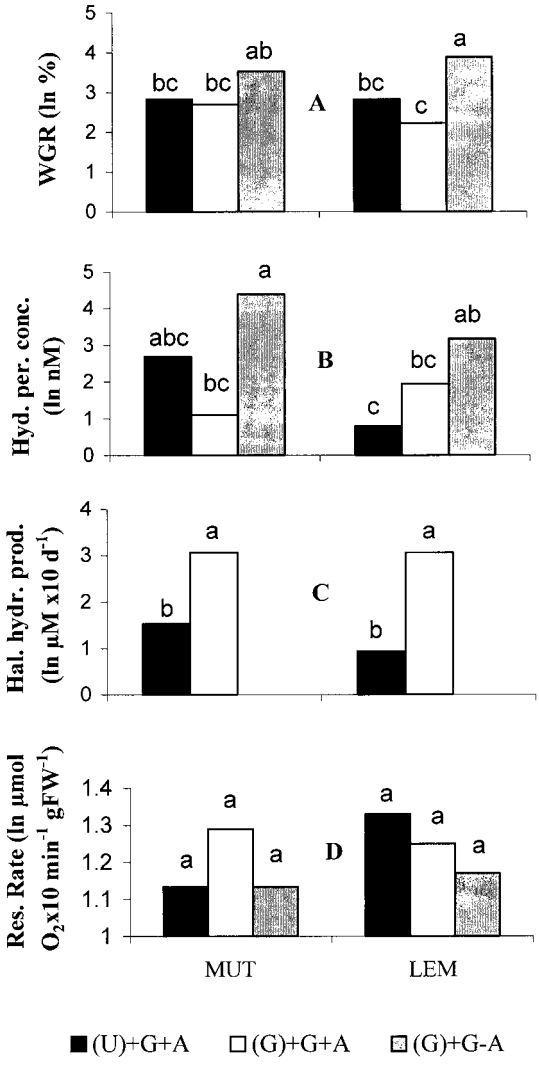

Figure 1. Effect of *Ulva lactuca* (U) presence in biculture with species of *Gracilaria* (G) on metabolic parameters of *G. cornea* mutant (MUT) and *G. lemaneiformis* (LEM) in 500-mL cylinders with or without antibiotics (A). A) Weekly growth rate (WGR; ln%; N = 6–15). B) Concentration of hydrogen peroxide released (ln nM; N = 6–12). C) Halogenated hydrocarbon production rate (ln μmol × 10 d^{-1}; N = 6–12). D) Rate of respiration (ln μmol O_2 × 10 min^{-1} gFW^{-1}; N = 6–15). The measured species appears in the legend without parentheses. Different letters express asignificant difference (p < 0.05).

Table 2. Three way ANOVA of activities by species of *Gracilaria*, using data of Figure 1: F value with probability levels; * p <0.05; ** p <0.01; *** p <0.001; n.s. non-significant

Source/Activity	Growth	Peroxide	Halogenation	Respiration
Antibiotics (A)	34.65***	46.81***	—-	0.16 n.s.
Gracilaria species (S)	0.03 n.s.	3.90 n.s.	0.57 n.s.	0.35 n.s.
Ulva pres. (U)	1.81 n.s.	1.57 n.s.	19.83***	0.34 n.s.
A × S	2.08 n.s.	0.21 n.s.	—	0.25 n.s.
A × U	0.05 n.s.	1.25 n.s.	—	1.33 n.s.
S × U	0.14 n.s.	1.25 n.s.	0.50 n.s.	0.50 n.s.
A × S × U	0.49 n.s.	4.14 *	—	0.99 n.s.
Number of observations	105	94	24	157

Table 3. Effect of *Gracilaria cornea* mutant density on production of hydrogen peroxide and phenol red bromination rate per g FW without antibiotics (n = 2)

Density ($g\ L^{-1}$)	Peroxide (nM)	Phenol red bromination (nM)
2.50	10.85	720
7.50	2.82	304
22.50	1.45	166

Table 4. Effect of hydrogen peroxide (300μM) on weekly growth rate (%) of *Ulva lactuca* and the *Gracilaria cornea* mutant (MUT) without antibiotics (mean± SD; n = 6–9)

Treatment	Peroxide added	Control
Ulva	a 66.02 ± 6.80	a 56.05 ± 23.80
Gracilaria	B 32.99 ± 4.38	A 54.65 ± 40.32

Table 5. Effect of *Gracilaria cornea* mutant (G) on rates of weekly growth (%) and photosynthesis (μmol O_2 g FW^{-1} min^{-1}) of *Ulva lactuca* (U) in biculture without antibiotics (n = 21)

Variable	U + G	U + U
Growth	98.8 B	72.3 A
Photosynthesis	58.9 a	68.3 a

ation of MUT and LEM did not change in the absence of antibiotics or with *U. lactuca* (Figure 1D, Table 2). Overall, there was a significant positive correlation for both *Gracilaria* species between peroxide release and growth rate (r = 0.394; p <0.001).

Other effects of biculture

Several supporting approaches to the responses of species of *Gracilaria* to *U. lactuca* were also examined. The density of MUT in the glass cylinders was optimal at 1.25 g FW 500 mL^{-1} regarding release of hydrogen peroxide and production of halogenated hydrocarbons based on the metabolite concentration in the fresh seaweed media without antibiotics (Table 3). Further, exposure to hydrogen peroxide without antibiotics significantly inhibited the growth rate of MUT, but did not significantly effect that of *U. lactuca* (Table 4). The presence of MUT without antibiotics resulted in a significant increase in the growth rate of *U. lactuca* (Table 5). By contrast, the rate of photosynthesis of *U. lactuca* did not significantly change in the presence of the mutant (Table 5).

The carbon tetrachloride (G1, U1) and chloroform (G2, U2) extracts (medium polarity) from the culture media of MUT and *U. lactuca* showed a strong inhibitory effect on the growth rate of *G. conferta* when compared with the controls (Figure 2a). Extracts using aqueous methanol (G3, U3) and hexane (G4, U4) showed less inhibition in the case of MUT and equal inhibition in the case of *U. lactuca* (Figure 2a). The chloroform fractions of *U. lactuca* (U2) and of MUT (G2) were further partitioned using a Sephadex LH-20 column. Three fractions were obtained from the elution corresponding to the early (high molecular weight), middle, and late (low molecular weight) fractions. The growth rates of *G. conferta* were most strongly inhibited by the high molecular weight extracts of the media from both the green and red sea-

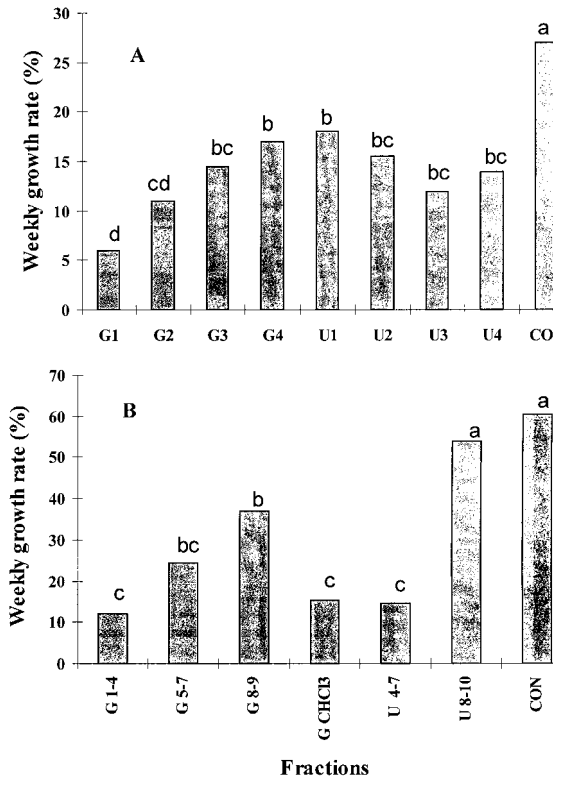

Figure 2. Effect of culture medium fractions on growth rate of *Gracilaria conferta*. The dried extracts were dissolved in DMSO and added to test tubes with seawater and *G. conferta* for incubation. Different letters express a significant difference ($p < 0.05$). A) Four extracts from the culture media of the *Gracilaria cornea* mutant (G1-4) and *Ulva lactuca* (U1-4) using: 1) carbon tetrachloride, 2) chloroform, 3) aqueous methanol, 4) hexane. B) The fractions of chloroform from the solvent partition of *Ulva lactuca* (U4-7, U8-10) and of *Gracilaria cornea* mutant (G1-4, G5-7, G8-9), which were partitioned on a Sephadex LH-20 column and eluted with a petrol ether: chloroform: methanol = 2: 1: 1 mixture and a full extract (G $CHCl_3$). The high MW fractions (1–4) were eluted first and the low MW fractions (8–10) later.

weeds (Figure 2b). Further separation failed to give fractions with higher activity. Addition of ethyl acetate extract of MUT to *U. lactuca* sporelings culture inhibited strongly the growth of the sporelings (results not presented).

Discussion

This study raises the question whether species of *Gracilaria* have defense mechanisms in response to epiphytes (*U. lactuca*) similar to *G. conferta* against bacteria (Weinberger et al., 1999), and *Chondrus crispus* against the endophyte *Acrochaete operculata* (Bouarab et al., 1999). Support comes from the first experiment in this study showing that *G. lemaneiformis* had a higher susceptibility to epiphytes while the *G. cornea* mutant was relatively resistant to epiphytization in outdoor cultures (Table 1). The difference in the spontaneous growth of epiphytes in the outdoor culture of the two *Gracilaria* species, suggests the involvement of a defense response against *U. lactuca* by the *G. cornea* mutant but less so for *G. lemaneiformis*.

The physiological parameters determined in a controlled cylinder culture with the two *Gracilaria* species were compared by determining separately their response in the presence of *U. lactuca* and bacteria. The epiphyte sensitive *G. lemaneiformis* showed increased growth in the presence of *U. lactuca*, a strong decrease in peroxide and halogenated hydrocarbon production, and no change in respiration; all corresponding with a weak defense against epiphytes (Figure 1A-D). However, the *G. cornea* mutant did not show a decreased growth in the presence of *U. lactuca*, increased its peroxide production, showed a moderate decrease in release of halogenated hydrocarbons with no effect on respiration; all corresponding to a strong defense against epiphytization (Figure 1A-D). The decrease in halogenated hydrocarbon production in the presence of *U. lactuca* is not a dilution effect, because the response of the *G. cornea* mutant was much higher than of *G. lemaneiformis* in the presence of *U. lactuca*. This quantitative difference suggests the involvement of peroxide and halogen production in the successful defense mechanism of the *G. cornea* mutant against *U. lactuca* without the presence of bacteria.

The responses of both *Gracilaria* species to the presence of bacteria were almost equal. Without application of antibiotics, they responded with increased growth, increased oxygen activation and unaffected oxygen consumption. It has been shown that the reduction of antibiotics on growth eventually results from a suppression of mutualistic microorganisms (Tait, 1991). Similarly, the reduced oxygen activation by both *Gracilaria* species after application of antibiotics probably resulted from the absence of defense response elicitors, due to the elimination of elicitor generating bacteria (Potin et al., 1999; Weinberger et al., 1999).

The hydrogen peroxide measurement indicates only its momentary absolute concentration. In contrast, the halogenation measurement gives us an indic-

ation of the carbohydrate bromination during a defined period of time (Weinberger et al., 1999). This is supported by the rate of halogenated products that were much higher (x50) than the concentration of peroxide. The difference in the two responses of *G. lemaneiformis* and *G. cornea* mutant toward *U. lactuca* is similar to the different responses of the sporophytic and gametophytic phases of *Chondrus crispus* towards an endophyte *Acrochaete operculata*. In this interaction, the susceptible *Chondrus crispus* sporophyte did not produce hydrogen peroxide like the resistant gametophyte, which responded after recognition of *A. operculata* with an oxidative burst (Bauarab et al., 1999).

The decrease in hydrogen peroxide and halogenated hydrocarbon production in higher culture densities by species of *Gracilaria* in the cylinder cultures may be correlated with lower growth rate. This suggests the control of different individuals of the same species (Table 3).

The growth inhibition response of the *G. cornea* mutant to hydrogen peroxide treatment points out that high concentrations (300 μm) of this metabolite are toxic (Table 4). Presence of *U. lactuca* in the medium resulted in an increase of the average hydrogen peroxide concentration by approximately 50 times in cultures of the resistant *G. cornea* mutant, but not in cultures of the susceptible *G. lemaneiformis.* The question may therefore be asked whether activated oxygen directly contributes to the resistance. Indications for such a role of hydrogen peroxide have already been reported for *G. conferta,* where presence of catalase prevents the elimination of epiphytic microorganisms after elicitation with oligoagars (Weinberger & Friedlander, 2000b), and a similar role has been suggested for the resistance towards algal epiphytes (Collen et al., 1995). In our experiments, *Gracilaria* had a promoting and not an inhibiting effect on the growth of mature *U. lactuca* in biculture. However, *U. lactuca* sporelings were inhibited in the presence of *G. cornea* mutant and are thus a more sensitive growth stage than mature plants (data not presented).

Removal of *Ulva lactuca* from species of *Gracilaria* is difficult or impossible (Friedlander, 1992; Buschmann & Kuschel, 1988). In most studies the common green algal epiphytic species of *Ulva* and *Enteromorpha* have been considered to be of the holoepiphyte type, which penetrates only the basiphyte's epidermal cell wall (Evans, 1981). More recent studies show distinctions between the outer wall and decklamellae construction in the two red algal agarophytes *G. tikvahiae* and *G. cornea* (Dawes et al., 2000). These structural differences may play a role in the ability of epiphytes to attach and penetrate the red algal cell wall and they explain why *G. tikvahiae* is more easily epiphytized than *G. cornea*. The cell wall structure of *G. cornea* is structurally distinct when compared to that of *G. tikvahiae*, which is also an epiphyte sensitive alga (Dawes et al., 2000). Thus epiphyte sensitivity may be also linked to wall structure.

The effect of extracts from culture media from species of *Gracilaria* and *Ulva lactuca* confirms that growth of *Gracilaria* species is self-inhibited when in high densities. Media extracts using carbon tetrachloride and chloroform inhibited the growth of *Gracilaria* species compared to more polar solvents. Based on fractionation with Sephadex columns, it appears that the active fraction consists of higher molecular weights. The growth-inhibiting factors may be cell wall compounds, such as oligocellulose, which acts as a defense response elicitor in species of *Gracilaria* (Weinberger & Friedlander, 2000a), or perhaps oligoulvan, which has yet to be tested (Potin et al., 1999). Judging by its high solubility, the inhibiting factor may also be distinct from oligosaccharides. Defenses by hosts against epiphytization can result in production of inhibiting chemicals or allelopathic agents (Davis et al., 1989), when basiphytes are responding to epiphytism.

In conclusion, the epiphyte-sensitive *G. lemaneiformis* and the relatively resistant *G. cornea* mutant showed different physiological responses to the presence of *U. lactuca*. The response of species of *Gracilaria* to the presence of *U. lactuca* and bacteria was not identical and may suggest the involvement of different oligosaccharide elicitors from the culture medium. The successful response of the *G. cornea* mutant against *U. lactuca* compared with *G. lemaneiformis* suggests that the higher production of hydrogen peroxide and halogenation of hydrocarbons by the *G. cornea* mutant limits the development of epiphytes in biculture and acts as a defense response. The question whether *U. lactuca* ultimately induces this defence response through its oligoulvan, oligocellulose or other compounds, must be studied.

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