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Strain selection in *Gracilaria* spp. 2. Selection for high and low temperature resistance in *G. verrucosa* sporelings

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Abstract

A strain selection procedure using *Gracilaria verrucosa* gametophytic sporelings was found to be an efficient tool for the improvement of *Gracilaria* strains. Two strains, C-2 and A-18, which were isolated and grown clonally, showed higher growth rates under high and low temperature conditions, respectively, than the local *Gracilaria conferta*. Growth rate, photosynthesis and chlorophyll, which were measured under different temperature and photon flux densities, demonstrated an overall advantage of the selected strains over the wild type strains of both *G. verrucosa* and *G. conferta*. Growth rates were also generally in positive correlation with the carboxylase activity of Rubisco. The *G. verrucosa* wild type also had a 40% higher agar content than *G. conferta*. The selected strains thus showed higher potential for outdoor cultivation than local wild type populations.

Introduction

Strain selection for intensive seaweed cultivation has been deemed necessary in order to optimize yields (van der Meer, 1987). For example, the selection of the T-4 strain was an important step for the successful *Chondrus crispus* mariculture in Canada (Craigie & Shacklock, 1989). However, selection for such strains can be a frustrating and laborious process when based on growth trials only. Therefore, the identification of more efficient selection markers is in itself an important objective.

The red seaweed *Gracilaria* is a major source for agar production (Moss, 1977), and it has been estimated that between 25,000 and 30,000 t dry weight of this seaweed are harvested annually for the agar industry (Santelices & Doty, 1989). In spite of its economical importance, almost no breeding and strain selection efforts have been reported in cultivation and farming projects (Santelices & Doty, 1989), although their importance for successful *Gracilaria* mariculture has been stressed (Hansen 1983, 1984).

The Israel Oceanographic and Limnological Research institute is currently involved in a project of *Gracilaria* cultivation for the production of agar in outdoor systems (ponds and tanks). The local *G. conferta*, which is used at the facility, has a growth optimum at 24 °C. Growth data, monitored for several years (Friedlander *et al.*, 1987), show a significant reduction in growth both

during the winter (December–March) and peak summer (August). This reduction is mainly due to the seawater temperature at these seasons: 13 °C and 31 °C, respectively. Since one of our goals has been to reach stable agar yields during the whole year, we have tried to improve growth performances both during the summer and, especially, during the cold winter months. This was done by the use of solar heaters, which were found to be uneconomical, and by using the hot cooling waters of power stations during the winter, which proved futile because of heavy metal discharges from the cooling pipes contaminating the cultures (Friedlander, unpublished). Another possibility was to try to change the *Gracilaria* species or strains by either selecting local superior ones or introducing foreign forms. One of the local strains selected for (SGY-2) showed promising features of growth and agar quality, but like other local strains it had a very narrow temperature range for growth (Levy & Friedlander, 1990).

A tetrasporic wild type strain of *G. verrucosa*, originating in the cool Atlantic waters of soutern Argentina, has lately been used in our growth trials. This species has a very wide geographical distribution range (Bird *et al.*, 1982), and shows eurythermic and euryhaline growth responses (Jones, 1989). The goal of this study was to evaluate and compare some marker features of *G. verrucosa* and *G. conferta* strains under a range of temperature and photon flux density (PFD) conditions, and to assess the feasibility of using the strain selection procedure in order to improve *Gracilaria* mariculture in Israel. The features examined included growth rate, photosynthetic parameters and agar content.

Materials and methods

Algal strains

Gracilaria conferta (Schousboe) J. & G. Feldman local strains (tetrasporic and gametophytic cultures) have been grown clonally in unialgal cultures for 3 years in our laboratory. Tetrasporic thalli of *G. verrucosa* Hudson were recently brought to the laboratory from Argentina (Puerto Madryn). The algal culture was acclimated to local conditions for several months, and was made unialgal. Gametophytic populations of several thousand sporelings were grown out of tetraspores under two temperature conditions: 18 °C and 27 °C. Male gametophytes were removed from the sporeling populations as soon as spermatangial sori were observed. The strain selection was initiated with 2 cm long sporelings. Total sporeling length and number of branches were the selecting criteria during the first three weeks. In the second stage, about 100 sporelings (ranging from 40 mg to 2 g) of both populations were weighed weekly in order to select the fast growing ones. The clones C-2 and A-18 grew fastest under 27 °C and 18 °C, respectively. The female wild type strains GAR of *G. verrucosa* and GRT of *G. conferta* were used as references.

Growth rates

The different *Gracilaria* strains were grown on a growth gradient table (Levy & Friedlander, 1990) at temperatures ranging from 10–32 °C and a PFD range of 40–350 μmol photon m^{-2} s^{-1}. Since experiments started off with unialgal cultures, almost no epiphyte contamination occurred. Bacterial contamination was controlled by diluting to half its recommended concentration the enrichment medium (Provasoli, 1968) added to the seawater. Two fresh thalli (tips or lateral branches) of 40–80 mg were grown in each of 50 glass vials (5 × 4 cm) containing 50 ml sterilized enriched seawater. Growth was determined by weekly weighings, and the thalli were then cut back to their initial weight before being returned to the growth vials. The duration of these growth experiments was one month so as to give four weekly growth rate replicates.

Pigments

Chlorophyll *a* of the algal thalli was extracted in N.N. dimethyl formamide, and the amount was

measured spectrophotometrically according to Moran (1982).

Photosynthesis and respiration

Net photosynthesis and dark respiration rates were measured in a closed system using an O_2 electrode. Circa 50 mg algal material was enclosed into the 10 ml measuring chamber which was kept at controlled temperatures by circulating water through its double wall. A saturating PFD for photosynthesis of 250 μmol photon m^{-2} s^{-1} was applied in the light. Steady state rates of O_2 exchange were obtained within a few minutes of light or darkness. The diel net gas exchange (DNGE) was calculated as daily net photosynthesis (rates h^{-1} × 10 h of light for the present growth regime) less nightly dark respiration (rates h^{-1} × 14 h of darkness). It was previously found that DNGE was in close correlation with actual growth rates in *Gracilaria* (Lipkin *et al.*, 1986).

Rubisco

Rubisco was extracted from *Gracilaria* and its carboxylase activity assayed radiometrically, at pH 8.0 and 20 °C, as principally described elsewhere (Beer & Israel, 1986). The specific assay conditions included 2 mM ribulose-1,5-bisphosphate, 18 mM $NaH^{14}CO_3$ (0.5 mCi $mmol^{-1}$) yielding a saturating CO_2 concentration of 0.2 mM in the reaction mixture of the closed vial, a 10 min activation and a 0.5 min reaction time initiated by the addition of ribulose-1,5-bisphosphate.

Agar

Agar was extracted, after alkali treatment of the dried algae, according to the modified method of Craigie & Leigh (1978) described by Levy and Friedlander (1990). The recovery was about 50% using this method. A 1% agar solution, stabilized overnight at 25 °C, was used for triplicate gel strength determinations. The gel strength was measured with a plunger (1-cm diameter) descending at a constant speed of 3 mm s^{-1}.

Results

The growth results demonstrate significantly higher growth rates in *G. verrucosa* strains than in the *G. conferta* wild type (GRT) strain under most temperature conditions (Fig. 1; Table 2). The wild type *G. verrucosa* (GAR) also showed a more homogeneous growth pattern over the temperature range tested than did GRT, where a more normal Gaussian pattern, peaking at 23 °C, was obtained. At low temperatures (10 °C), all *G. verrucosa* strains grew significantly better than the GRT strain (Table 2). The most productive strain at 10 °C was A-18, which therefore selected under low temperature conditions. This strain was also very productive at 16 and 23 °C. At a higher temperature (28 °C) the strain C-2, which was selected under high temperature, was the most productive one. At the highest temperature (32 °C), there was a pronounced growth reduction for all strains, but particularly for those

Table 1. Two-way ANOVA of weekly growth rate (WGR-%) of 4 *Gracilaria* strains grown under different temperature (10–32 °C), and PFD (40–350 μmol photon m^{-2} s^{-1}) conditions. A, temperature; B, PFD; AXB, interaction ($n = 6$, $p < 0.001$).

Strain	Source of variation	*F* value	% of total variation explained
GRT	A	194.1	82.7
	B	5.6	2.4
	AXB	6.0	2.6
GAR	A	82.7	71.3
	B	6.4	5.5
	AXB	5.9	5.0
A-18	A	461.4	86.0
	B	34.5	6.4
	AXB	8.2	1.5
C-2	A	177.6	74.5
	B	22.5	9.4
	AXB	7.4	3.1

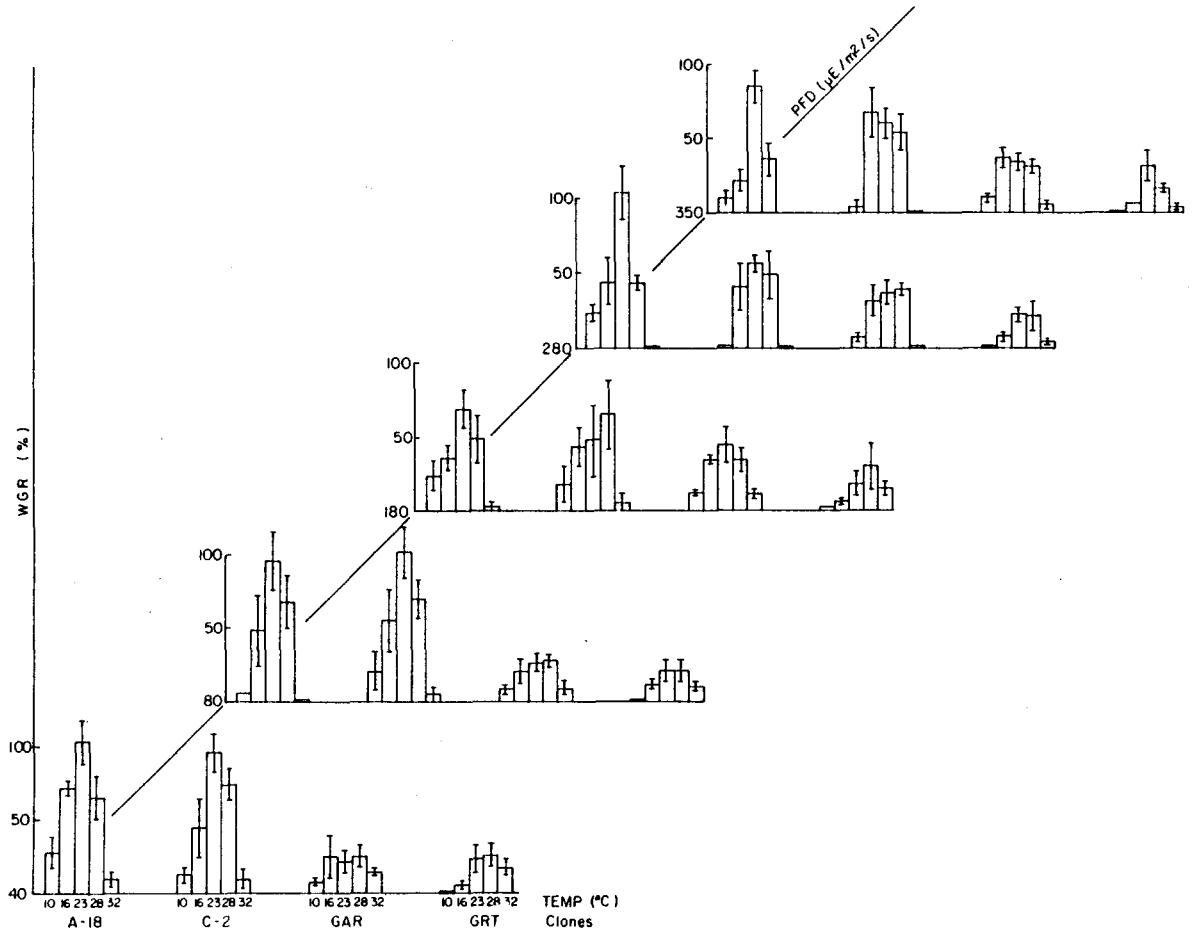

Fig. 1. Weekly growth rate (WGR-%) measured in four *Gracilaria* strains (GAR, *G. verrucosa* wild type; A-18, *G. verrucosa* low temperature selected strain; C-2, *G. verrucosa* high temperature selected strain; GRT, *G. conferta* wild type strain) grown under different PFD (40–350 µmol photon m^{-2} s^{-1}) and temperature (10–32 °C) conditions.

selected (A-18, C-2). Most growth rate variations could in all strains be explained by temperature effects (Table 1), while PFD was a less significant factor. The second most important factor determining growth rates was linked to internal features of the different strains and accounted for 8% of the growth rate variations (Table 3).

Results for chlorophyll *a* content of *Gracilaria*, grown under different light and temperature regimes, are presented in Table 4. The highest chlorophyll content in the *G. verrucosa* strains was obtained under 40 µmol photon m^{-2} s^{-1} and 16 °C, whereas in *G. conferta*, the highest value was found at the same PFD, but 32 °C. Photon flux rate generally had stronger effects on pigment content than did temperature (Table 5). Higher chlorophyll contents were found in the more productive strains of *G. verrucosa* (Table 6), confirming previous conclusions regarding local *G. conferta* strains (Levy & Friedlander, 1990).

Net photosynthesis and respiration of the different strains under saturating PFD (280 µmol photon m^{-2} s^{-1}) and different temperature conditions (Tables 7 and 8) generally showed the same trend as did the growth response. *Gracilaria verrucosa* strains thus had higher rates of photosynthesis than *G. conferta* at all temperatures. The most efficient strain was A-18, showing the highest net photosynthetic rates. It is likely that this is partly due to its low respiration rates under

Table 2. Weekly growth rates (WGR-%) of 4 *Gracilaria* strains under different temperature conditions. The mean growth response to variable PFD (40–350 μmol photon m^{-2} s^{-1}) was analyzed by Duncan's Multiple Range Test. Means with the same letter are not significantly different at $p < 0.001$.

Temp. °C	Strain	*N*	WGR (mean ± SD)	Duncan's grouping
10	GRT	30	0.9 ± 0.9	A
	GAR	25	8.9 ± 3.2	B
	A-18	30	15.8 ± 10.9	C
	C-2	30	12.1 ± 12.7	B, C
16	GRT	30	8.2 ± 7.5	A
	GAR	25	29.2 ± 12.1	B
	A-18	30	43.7 ± 22.3	C
	C-2	30	48.9 ± 21.3	C
23	GRT	30	21.7 ± 9.6	A
	GAR	25	32.5 ± 12.3	B
	A-18	30	91.4 ± 20.8	C
	C-2	30	70.6 ± 26.8	D
28	GRT	30	22.5 ± 10.5	A
	GAR	25	31.2 ± 8.4	B
	A-18	30	52.0 ± 18.7	C
	C-2	30	62.5 ± 18.9	D
32	GRT	30	9.4 ± 6.8	A
	GAR	25	7.8 ± 5.8	A
	A-18	30	2.8 ± 4.7	B
	C-2	30	6.1 ± 11.4	A, B

Table 3. Three-way ANOVA of weekly growth rates as affected by: temperature, A; PFD, B; different strains, C; and AXB, AXC, BXC, AXBXC-interactions ($n = 6$, $p < 0.001$).

Source of variation	*F* value	% of total variation explained
A	654.8	53.6
B	40.3	3.3
C	127.7	7.8
AXB	18.1	6.0
AXC	57.0	14.0
BXC	10.0	2.4
AXBXC	3.1	3.1

most temperatures (Table 8). The reduction of DNGE values under low temperatures was due to low photosynthetic rates whereas at high temperatures it was due to increased dark respiration (Tables 7 and 8). In spite of this reduction at 30 °C, the DNGE of *G. verrucosa* strains remained higher than that of *G. conferta*.

The apparent high-temperature sensitivity, demonstrated by a significant decrease of the growth rate in both species (Fig. 1; Table 2), was generally supported by the DNGE results. At the highest temperature tested (32 °C), growth rates of the wild type strains of both *Gracilaria* species was higher than in the selected strains. Photosynthetic activity, on the other hand, was inhibited at 30 °C, especially in the GRT strain. In this case there was no positive correlation between photosynthesis and growth. However, both photosynthetic and Rubisco activities generally concur with growth rate performances of the different strains, showing higher values in *G. verrucosa* than in *G. conferta*.

Agar content and quality (gel strength) were markedly (40%) higher in the *G. verrucosa* wild type strain than in the *G. conferta* one (Table 9). This increase was partly due to a 30% increase in relative dry weight.

Discussion

The production of suitable algal strains for mariculture should not rely only on naturally occurring seaweed strains, since it has been found that they are not necessarily the best for growth under controlled culture conditions (Craigie & Shacklock, 1989). Strain selection procedures could therefore be the answer to specific problems in the cultivation effort. Two main problems were identified in the local Israeli *Gracilaria* mariculture project: growth limitations by extreme temperatures and low agar quality. The introduction of *G. verrucosa*, which does not grow locally in the field but was brought to Israel from the temperate waters of Argentina, showed improved growth and agar characteristics in the laboratory. The high photosynthetic and growth rates could partly be attributed to the comparatively higher Rubisco activity. Such a correlation was observed also for other plants (Medina, 1969), including algae (Herron & Mauzerall, 1972). Another possible basis for higher growth rates could be the significantly

Table 4. Chlorophyll *a* content (μg mg DW^{-1}) in four *Gracilaria* strains subjected to different combinations of temperature (10–32 °C) and PFD (40–350 μmol photon m^{-2} s^{-1}).

PFD μmol photon m^{-2} s^{-1}	Strain	Temperature, °C				
		10	16	23	28	32
40	GRT	0.832	1.356	1.324	1.387	1.460
	GAR	2.092	3.020	2.722	2.428	2.353
	C-2	2.630	2.930	1.920	1.770	1.510
	A-18	2.373	2.593	1.579	1.400	1.687
80	GRT	1.205	1.285	1.238	1.339	0.935
	GAR	1.773	1.700	1.817	1.524	1.026
	C-2	1.860	1.730	1.540	1.210	0.000
	A-18	2.004	1.721	1.153	1.310	0.000
180	GRT	0.857	0.946	1.073	0.995	0.536
	GAR	1.491	1.483	1.910	1.107	0.940
	C-2	1.980	2.070	1.450	1.110	0.000
	A-18	1.349	1.336	0.829	0.642	0.000
280	GRT	0.734	0.745	0.780	0.774	0.120
	GAR	1.504	1.324	1.030	0.689	0.442
	C-2	0.600	1.660	1.290	1.280	0.000
	A-18	0.823	0.937	0.869	0.687	0.000
350	GRT	0.371	0.558	0.756	0.713	0.063
	GAR	1.190	0.905	0.848	0.767	0.065
	C-2	0.720	1.230	1.490	1.270	0.000
	A-18	0.704	0.686	0.665	0.932	0.000

Table 5. Two-way ANOVA of the average chlorophyll *a* concentration in the four strains as affected by temperature, A; PFD, B; and their interaction, AXB ($n = 24$; ***, $p < 0.001$; N.S., not significant).

Source of variation	*F* value	% of total variation explained
A	15.8***	24.5
B	29.9***	46.5
AXB	0.9 N.S.	1.3

Table 6. Duncan's Multiple Range Test of chlorophyll *a* (CHL) content in 4 *Gracilaria* strains averaged for different temperature and PFD conditions ($n = 25$, means with the same letter are not significantly different at $p < 0.05$).

Strain	CHL μg mg DW^{-1}	Duncan's grouping
GRT	0.895	B
GAR	1.417	A
A-18	1.051	A, B
C-2	1.33	A

higher chlorophyll content in *G. verrucosa* strains. This correlation has already been demonstrated for *G. conferta* (Levy & Friedlander, 1990).

The strain selection procedure exercised in this study resulted in improved growth performance of strains selected from the *G. verrucosa* wild type. This improvement was realized under most temperature and PFD regimes. Since growth rate was shown to be the major contributor to agar yield (Levy & Friedlander, 1990), we suggest that the present findings could be implemented in outdoor cultivation. The growth rate was shown to be affected mainly by temperature and indigenous strain characteristics.

Growth under different conditions was generally positively correlated with photosynthetic activity. Thus, as suggested by Hansen (1984), photosynthetic features can here be used as selection markers. However, some inconsistencies between growth rate and photosynthetic gas exchange un-

Table 7. Net photosynthesis, Pn; dark respiration, R; diel net gas exchange, DNGE (photoperiod regime of 10L–14D); and Rubisco activity in four *Gracilaria* strains under different temperatures (except for Rubisco activity), and saturating PFD conditions (280 μmol photon m^{-2} s^{-1}).

Temp. °C	Strain	Pn μmol O_2 g FW^{-1} h^{-1}	R μmol O_2 g FW^{-1} h^{-1}	DNGE μmol O_2 g FW^{-1}	Rubisco activity μmol O_2 g FW^{-1} h^{-1}
15	GRT	3.3 ± 1.0	6.3 ± 1.3	−55.2 ± 18.0	
	GAR	22.9 ± 3.0	7.1 ± 1.7	129.6 ± 17.1	
	A-18	20.5 ± 2.1	6.6 ± 0.7	112.6 ± 12.2	
	C-2	7.8 ± 1.3	4.6 ± 1.2	13.6 ± 2.3	
20	GRT	12.2 ± 2.7	6.0 ± 0.4	38.0 ± 8.4	
	GAR	36.2 ± 6.0	10.5 ± 2.2	215.0 ± 32.2	
	A-18	42.4 ± 2.1	9.6 ± 1.2	289.6 ± 14.5	
	C-2	14.7 ± 2.1	5.9 ± 2.2	64.4 ± 9.0	
25	GRT	16.1 ± 8.4	9.6 ± 0.8	26.6 ± 13.2	7.4 ± 1.4
	GAR	36.2 ± 6.0	10.5 ± 2.2	215.0 ± 32.2	14.1 ± 2.8
	A-18	51.3 ± 4.4	13.0 ± 1.4	331.0 ± 26.5	14.2 ± 1.8
	C-2	30.8 ± 7.5	8.9 ± 1.7	183.4 ± 36.5	13.8 ± 3.0
30	GRT	16.2 ± 2.2	17.7 ± 9.5	−85.8 ± 30.5	
	GAR	33.0 ± 4.5	13.4 ± 1.8	142.4 ± 20.0	
	A-18	55.1 ± 9.0	20.1 ± 9.8	269.6 ± 53.9	
	C-2	26.1 ± 7.6	12.3 ± 2.2	88.8 ± 26.6	

Table 8. Duncan's Multiple Range Test for gross photosynthesis and dark respiration rates (R% of Pg) of four *Gracilaria* strains measured under different temperature (15–30 °C) and saturting PFD (280 μmol photon m^{-2} s^{-1}) conditions ($n = 8$, means with the same letter are not significantly different in the level of $P < 0.05$).

Strain	Gross photosynthesis μmol O_2 g FW^{-1} h^{-1}		Dark respiration % of gross photosynthesis	
	Mean ± SD	Duncan grouping	Mean ± SD	Duncan grouping
GRT	24.10 ± 7.39	A	39.13 ± 8.91	A
C-2	27.77 ± 13.45	A	30.00 ± 6.13	A, B
GAR	42.45 ± 8.30	B	27.12 ± 8.28	B
A-18	54.65 ± 20.67	B	22.35 ± 3.50	B

Table 9. Agar content and quality in *G. conferta* and *G. verrucosa* (Bacto-Agar gel strength measurements under identical conditions resulted in 371 ± 18 g cm^{-2}).

Strain	Species	Origin	DW (% of FW)	Agar content		Gel strength (g cm^{-2})
				(% of DW)	(g kg^{-1} FW)	
GRT	*G. conferta*	Israel	11.7 ± 0.7	16.9 ± 1.4	19.7 ± 2.3	635 ± 50
GAR	*G. verrucosa*	Argentina	15.5 ± 0.5	17.8 ± 1.0	27.6 ± 2.5	869 ± 28

der high temperatures were also observed. For example, DNGE was negative for the *G. conferta* wild type strain at extreme temperatures, while the growth rate of this strain was higher than in other strains at 30 °C. There are thus apparently some differences between short-term gas exchange measurements and long term growth, and it is likely that acclimation processes caused these differences.

As reported earlier, peak summer conditions of high temperature and PFD caused reduced yields in the outdoor *Gracilaria* cultivation (Friedlander *et al.*, 1987). Similar conditions in the factorial experiment also caused very low growth rates. Under these conditions, epiphytic contamination was observed in the growth flasks. When PFD was reduced at 32 °C, the contamination problem was eased. This relationship between high temperature and PFD and increased epiphytic growth may thus explain some of the summer growth reduction. Since light conditions in the outdoor installations, unlike temperature, are easily manageable, it may be possible to avoid some of the summer decline by increasing the algal density in the ponds.

Variables which may affect the measured agar quality in general, and the gel strength in particular, include techniques such as the method of extraction, the measurement device and the concentration of the agar solution (Whyte *et al.*, 1984). Biological variables include growth conditions prior to agar extraction (Bird, 1988) and, since there is taxonomic confusion within the *G. verrucosa* group (Bird *et al.*, 1982), accurate classification of the different strains. Because of these constrains, it is almost impossible to compare agar characteristics among different reports. For example, reported gel strengths of *G. verrucosa* include a wide range of values: 33– (Craigie *et al.*, 1984), 266– (Hurtado-Ponce & Umezaki, 1988), 680– (Christiaen *et al.*, 1987) and 965 g cm^{-1} (Bird, 1988). It may therefore be more significant to compare agar quality in specific reports to a standard commercial agar such as Bacto-Agar (Difco). In this study, *G. verrucosa* gel strength was 40% higher than that of *G. conferta*, and twice that of Bacto-Agar.

In conclusion, our results show that *G. verrucosa* may be more suitable for cultivation purposes in Israel than the local *G. conferta*. The strain selection procedure was shown to be an efficient tool for improvement in the wild type strain of *G. verrucosa*, especially regards growth under low temperature conditions, and will be used in forthcoming attempts further to improve the selected strains. *Gracilaria verrucosa* was also found to contain more and higher quality agar. Only mass cultivation under outdoor conditions, however, will substantiate the prospect of using these newly introduced strains in large scale aquaculture. Such cultivation trials are presently in progress.

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