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AID - CDR

Progress Report 1989 (January-June)

Title: Genetic improvement of *Gracilaria*,

an agar producing seaweed.

Israel Levy & Michael Friedlander

I.O.L.R. Report No. H9/89

I.O.L.R. REPORTS

Rec'd in SCI: AUG 1 1989

Reports to contracting agencies. Not for distribution.

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TITLE: Genetic improvement of *Gracilaria*, an agar producing seaweed

Israel Levy & Michael Friedlander

No. DPE-5544-G-SS-6045-00

with the participation of:

Dr. S. Beer, and Mr. A. Israel from the Dept. of Botany Tel Aviv University,

and Ms. C. Maxim, and Ms. H. Aramati from I.O.L.R.

SUMMARY

In this report we show progress in many objectives of the research project. Our algal stock has been expanded by new species and strains, obtaining for the first time superiority over corresponding W.T. strains through a selection procedure. More *Gracilaria* strains were characterized for their growth and other relevant biological properties. Photosynthesis performances and RuBPcase activity were measured in some *Gracilaria* strains. Several Jamaican *Gracilaria* species brought by our collaborator (Dr. Devi-Prasad) have been successfully acclimated in our local conditions and characterized. Preliminary inter- and intra-specific hybridization experiments, as well as tetrasporogenesis induction in additional species, have been operated and are under continuous study in our laboratory. Full results are presented about growth characterization of local strains with additional discussion and comments.

INTRODUCTION

In the last report we described the establishment of our algal stock cultures. A basic study of the life history of *Gracilaria* sp. was also reported. We characterized our local strains of *Gracilaria conferta* which have been used in the outdoor growth experiments. By performing well controlled, factorial (temp. vs. light) experiments, we could compare the following features of different strains: growth rate, carbohydrate content and quality, as well as galactose yield, and chlorophyll *a* content. From the results, we could conclude that growth rate had a major effect on the galactose (agar?) yield. We also found that the outdoor growth and agar features of these strains showed large variations. Some ideas about the preferred strains for the outdoor cultivation were discussed. An important strain selection procedure was described in detail by which we could expect improvement of the W.T strains.

In the present report we show significant progress in the major objectives: we show some promising results of the strain selection procedure and also photosynthesis and growth charaterization of some Jamaican *Gracilaria* species. This is another step towards the main goal of this project, namely the improvement of our local *Gracilaria* strains for agar production.

MATERIALS AND METHODS

ALGAL STRAINS: In addition to the local and foreign strains described in the previous report, several new strains have been grown clonally and used for the growth, photosynthetic, and RuBPcase activities. The new strains were obtained by a strain selection technique. The following strains have been used: GRT - *Gracilaria conferta*, W.T. female; GAR - *Gracilaria verrucosa*, W.T. female; A-18 - *G. verrucosa*, low temperature selected female strain; C-2 - *G. verrucosa*, high temperature selected female strain.

HYBRIDIZATION: Controlled crosses have been operated by transferring female and male gametophytic thalli of known strains into the same Ehrlenmeyers for

variable periods of time (1-7 days). Optimal growth conditions (25°C, 150 µE m^{-2} s^{-1}, enriched and aerated seawater medium, replenished once a week), have been provided throughout all the incubation period, after which the females were taken out and grown separately. Within one or two weeks, it was possible to observe whether fertilization did take place, by the appearance of early cystocarpic developments on the surface of the thallus.

PHOTOSYNTHESIS AND RESPIRATION: Net photosynthesis was measured as O_2 evolution in a closed glass system using a Clark type O_2 electrode (Beer et al. 1977, Beer and Levy 1983). About 50 mg algal fresh weight were transfered to a 10 ml measurement chamber which was kept and controlled at different temperatures, ranging from 15°C to 30°C. Light intensity of 250 µE m^{-2} s^{-1}, which was found to be saturating, was provided to all strains by an incandescent light bulb. Steady state rates of O_2 release were obtained within 5 min in the light, followed by a 5 min period of respiration rate measurements in the dark. After each measurement, the medium (aerated sea water) was replenished. Slopes of the O_2 response at steady state rates were monitored on a multichannel recorder. Rates of net photosynthesis, dark respiration, and a Diel Net Gas Exchange (DNGE - a 24 h net photosynthesis) were calculated (Lipkin et al. 1986), using a short day photoperiod regime in the growth rooms (10L:14D). Photosynthesis experiments for *Gracilaria* species from Jamaica were performed under different Photon Flux Density (P.F.D.) conditions (P-I curves) ranging from 0-200 µE m^{-2} s^{-1}. DNGE values were calculated for this experiment under a long day photoperiod regime (16L:8D). In addition, compensation (Co), and saturation (Ik) points and the slope of the linear portion of the P-I curves (α = Ps/P.F.D) were calculated.

PIGMENTS: Chlorophyll *a* content in the algal thalli was measured according to Moran's (1982) method, using N.N.dimethylformamide. Phycoerythrin was measured according to Beer and Levy (1983).

RuBPcase: The activity of this enzyme in different species and strains of *Gracilaria* was measured according to Beer et al. (1980) and Beer and Israel (1986). Fresh algal tissue (0.3 g) was homogenized in a glass homogenizer driven by a motor, with 1-2 ml of extraction buffer (100 mM Tris-SO_4 at pH 8.0; 1 mM mercaptoethanol; 0.25 nM $MgSO_4$; 20 mM ascorbic acid; 1.5% (w/v) PVP). The crude extract (0.1 ml) was assayed for RuBPcase activity in a reaction mixture (3.3 mM Tris-SO_4 at pH 8.0; 11 mM $MgSO_4$; 1.1 mM mercaptoethanol; 2.5 mM RuBP). After a preincubation period of 10 min, ^{14}C (2 mM $NaH^{14}CO_3$) was added, and after 30 seconds the reaction was terminated by adding 0.2 ml HCl 6M. Samples were dried and resuspended in 0.5 ml ethanol before adding the scintillation liquid for the radioactivity determinations.

STATISTICS: Results were analyzed with two way ANOVA and Duncan's multiple range test, making logarithmic transformations because of heterosedacsity (non homogeneity variance) and Log-Normality distribution of the data (Steel and Terrie 1960, Sokal and Rohlf 1981).

RESULTS AND DISCUSSION

ALGAL STOCK: Additional strains have been successfuly cleaned and grown in clonal unialgal cultures. All phenological types of *G. mammillaris* from Jamaica (female, male, tetrasporophyte) were obtained. Female and tetrasporophyte of the local *G. conferta* also have been clonally grown in the growth rooms. *Gracilaria verrucosa* strains which have been isolated in the course of an active strain selection procedure are also now being kept in the stock cultures. New *Gracilaria tikvahiae* as well as *G. verrucosa* type strains have also been transfered to IOLR from Tampa Bay (Florida, U.S.A) and are now under acclimation and cleaning processes. Our algal stock currently accounts for 40 different cultures.

REPRODUCTION: Successful induction of tetrasporogenesis was obtained with *G. mammillaris* from Jamaica and *G. verrucosa* from Argentina. In these species, we now have all the generation types which enable us to perform controlled

hybridization experiments. Studies concerning the local *G. conferta* species are still in progress regarding nutrient stress, combined with reciprocal transfer between 20°C and 25°C, and different light intensity conditions. Spore germination and young sporelings growth techniques are now under control. The only contamination problems with the unialgal cultures are bacterial ones. Most of these problems in the very young stages, whose media cannot be aerated, may be solved by limiting the nutrient load in the growth medium. In mature sporelings (>2 mm in length), a proper medium/thallus ratio and vigorous air bubbling can provide satisfactory solutions.

HYBRIDIZATION: Hybridization experiments have started with gametophytic clones of *G. verrucosa* (GAR), *G. mammillaris* (male and female), and *G. conferta* (GRT, SGY-2) strains. These experiments intend to verify the sexual compatibility between the different strains and species for transfer and combination of their features. This will permit the improvement of relevant characteristics such as growth rate, carbohydrate content and quality, herbicide and epiphyte resistance.

FURTHER DISCUSSION ABOUT GROWTH EXPERIMENTS OF THE LOCAL STRAINS: The full processed results of the factorial growth experiments (gradient table), which were described in the last report, are presented here (Figs. 1-4, Tables 1-4). In addition to the previous results, it is important to mention the difference between the local *Gracilaria conferta* strains (GSC-14, SGSC-6, SGY-2) and the foreign *Gracilaria verrucosa* strain (GFL-VR). It seems that the GFL-VR strain was much more eurythermic than the local ones in its growth response to the different temperature conditions (20-30°C), in all light intensities (Fig. 1). A most dramatic difference between the local and the foreign strains was shown under high temperature conditions. A moderate decrease in growth rate (13-40%) was demonstrated at 30°C by the GFL-VR strain as opposed to a growth rate decrease of 40-90% in the local strains. From this aspect, the GFL-VR strain is more suitable for

outdoor cultivation in Israel than the other ones, although higher growth rates were shown at 25°C by the local SGY-2 strain. However, the poor agar quality of the GFL-VR strain (previous report) eliminates it as a potential outdoor cultivation strain in spite of its high growth features.

When we applied the Duncan's Multiple Range Test for the chlorophyll data which were presented in the last report, we found a significant difference between the high growth rate strains (GFL-VR, SGY-2), which had higher levels of chlorophyll *a* as compared to the others (Table 5). This positive relationship between growth rates and chlorophyll *a* values suggests a possible selection method of high productivity strains by means of chlorophyll *a* measurements under different growth conditions.

STRAIN SELECTION: Using the previously mentioned selection technique with natural gametophytic populations of *G. verrucosa* (GAR), the first stage (5-10 mg) was selected for thallus length, and the following stages (10-20 mg) were selected for thallus length and higher branching manifestations. The strain selection procedure was separately performed under two temperature conditions of 18-20°C and 25-27°C. It began with two separate gametophytic populations of several hundred individuals weighing 20-40 mg each. One population grew at 18°C and the other at 25°C. Two different female gametophytic clones (0.8-1.0 g) which grew significantly faster than the others were isolated at the end of the procedure under different selection stages and temperature conditions. These two selected strains, A-18 (18°C) and C-2 (25°C), have been grown clonally and tested for their growth, photosynthesis and RuBPcase activity in comparison with the *G. conferta* (GRT) and *G. verrucosa* W.T. (GAR) strains.

GROWTH RATE AND CHLOROPHYLL *A* CONTENT: In order to assess the growth performances of the selected strains (A-18, C-2) we grew these strains with the W.T. female clones of *G. verrucosa* (GAR) and *G. conferta* (GRT) on the gradient

table under identical conditions. The temperature range was 10-32°C and the light intensity range was 40-350 μE m^{-2} s^{-1}. Since the experiment started with unialgal cultures of all the clones, almost no epiphyte contamination problems occurred. Bacterial contaminations were avoided or controlled by diluting the usual enriched sea water medium to half its usual concentration. The results (Fig. 5) showed higher growth performances of the *G. verrucosa* W.T. (GAR) as compared to the *G. conferta* W.T. (GRT) strains. A different response to light and temperature was demonstrated by these two species (Figs. 5, 6): *G. verrucosa* showed eurythermic growth activity between 16-28°C as compared to a normal Gaussian pattern by *G. conferta* (GRT) with a growth peak at 23°C. The growth rate of *G. conferta* ceased at 10°C and actually deteriorated and disappeared, while *G. verrucosa* showed a much higher tolerance to this low temperature (10% WGR). Both were similarly sensitive to high temperature (32°C). Average growth in different temperatures for each light intensity showed high standard deviation (Fig. 6b), while average growth under different light conditions for each temperature showed low S.D. (Fig. 6a). Hence, it may be concluded that growth activity was mainly influenced by the temperature fluctuations (ANOVA test will still be applied on these results). This confirms the general conclusion based on the statistics processing in the previous report. An inverse picture was observed when chlorophyll *a* content was measured under the different light and temperature combinations (Fig. 6). Light intensity demonstrated the major effect on pigment content as compared with temperature effects. Higher chlorophyll *a* values were demonstrated by the more productive strain, *G. verrucosa* (GAR), confirming the previous conclusion.

The only epiphytic contamination problems appeared under high PFD and temperature conditions where the growth rate values of the grown strains were very low (Fig. 5). Since light conditions in the outdoor installations are more manageable (by controlling the macrophyte density), it is possible to avoid some of the summer

growth inhibition by increasing the algal density in the ponds. The two selected strains are presently in the course of growth experiments.

PHOTOSYNTHESIS AND RuBPcase ACTIVITY: Photosynthetic and RuBPcase activity (Table 6) of the GRT and GAR strains showed a clear advantage of the *G. verrucosa* strains (GAR). Very low Pn (net photosynthesis) values caused negative DNGE (Diel Net Gas Exchange) values under both low (15°C) and high (30°C) temperature conditions. Photosynthestic activity (Pn) of *G. verrucosa* was positively related to temperature and remained very high at 30°C. The dark respiration which increased at that temperature caused a significant decrease of the DNGE values at 30°C, but nevertheless it remained high. The photosynthetic and the RuBPcase activities generally support the growth rate performances of the different strains, demonstrating the advantage of *G. verrucosa* growth characteristics over G. *conferta*. The higher photosynthetic activity of *G. verrucosa* under light saturation can be explained by the RuBPcase activity. The apparent similar high temperature sensitivity (significant decrease of the growth rate) of both species was not supported by the DNGE results. It is possible that under 32°C temperature, the photosynthetic apparatus suffers some damage and its accumulative effect is pronounced by the growth rate. We also observed high epiphytic contamination problems under such high temperature conditions which could influence the growth rate. Both possible effects could not be detected by short time photosynthetic measurements.

GRACILARIA SPECIES FROM JAMAICA: Several *Gracilaria* species: G. *cervicornis*, *G. domingensis*, *G. cornea*, *G. mammillaris* and *Gracilaria* sp. (*verrucosa* type) were brought to the I.O.L.R. laboratory by our collaborator, Dr. Devi-Prasad. in his visit to Israel. After a prolonged acclimation period of several months, we conducted growth rate and photosynthesis experiments. Growth rate under different temperature (11-30°C), and light intensity (40-280 $\mu E\ m^{-2}\ s^{-1}$) combinations was determined on the gradient table. The growth rate results (Fig. 7) show that all

Jamaican species suffered at low temperatures. All species degraded at 11°C and grew slowly at 16°C. Under most conditions, the growth response showed Gaussian distribution with a clear peak at 22°C. Only at low light intensity (40 μE m^{-2} s^{-1}) were growth rates at elevated temperatures higher in some cases (*Gracilaria* sp.). Under most temperature and light intensity combinations, the growth rate of *Gracilaria* sp. was higher as compared with other species, and those of *G. domingiensis* and particularly *G. cervicornis* were very low. They probably did not acclimate in the local conditions. The growth rate advantage of *Gracilaria* sp. was well supported by the photosynthetic activity (Fig. 8, Table 7), since net photosynthesis (Pn) of *Gracilaria* sp. was the highest under light saturation, and that of *G. cervicornis* was lowest. All Jamaican species showed light saturation between 115 and 135 μE m^{-2} s^{-1}, and compensation point at 30 μE m^{-2} s^{-1}. The DNGE values showed a positive relationship with Pn and α values (Table 7), and all these values are in good agreement with the growth manifestations of the different *Gracilaria* species. Inhibition processes of dark reaction due to low activity of RuBPcase may have caused low growth and photosynthetic activity of *G. cervicornis* in spite of its highest chlorophyll values. As mentioned before, *G. domingensis* and *G. cervicornis* had growth problems in our facilities and after 3-4 months they degraded and disappeared. The other species, *G. mammillaris, G. cornea* and *Gracilaria* sp., did better, and are growing in the stock cultures. *G. mammillaris* and *Gracilaria* sp. have been used successfully in the reproduction experiments, as mentioned before. After examining these Jamaican strains, we could not find economically promising strains which could now be adapted to the mariculture facilities.

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Table 1. Two way ANOVA of Weekly Growth Rate (WGR) in 4 *Gracilaria* clones grown under different temperature and P.F.D. conditions. A - temp., B - P.F.D., AXB - interaction; n = 3; * - P < 0.05, ** - P < 0.01, *** - P < 0.001.

Clone	Source of variation	F value	% of total variation explained
GSC-14	A	13.1 ***	25.3
	B	12.5 ***	32.1
	AXB	3.1 *	17.4
SGSC-6	A	10.4 ***	25.7
	B	6.2 **	18.8
	AXB	3.1 *	22.9
SGY-2	A	99.8 ***	61.1
	B	18.3 ***	14.2
	AXB	8.0 ***	17.3
GFL-VR	A	11.6 ***	18.1
	B	22.9 ***	49.5
	AXB	2.8 *	12.0

Table 2. Two way ANOVA of total carbohydrate (CRBH) in 4 *Gracilaria* clones grown under different temperature and P.F.D. conditions. A - temp., B - P.F.D., AXB - interaction; n = 3; * - P < 0.05, ** - P < 0.01, *** - P < 0.001.

Clone	Source of variation	F value	% of total variation explained
GSC-14	A	2.3 n.s.	0.9
	B	60.3 ***	58.6
	AXB	11.7 ***	31.6
SGSC-6	A	18.2 ***	28.3
	B	24.6 ***	51.9
	AXB	0.9 n.s.	0.0
SGY-2	A	14.2 ***	21.0
	B	6.6 **	12.0
	AXB	8.5 ***	47.8
GFL-VR	A	11.7 ***	5.9
	B	82.2 ***	59.7
	AXB	13.6 ***	27.7

Table 3. Two way ANOVA of GAL/GLU ratio in 4 *Gracilaria* clones grown under different temperature and P.F.D. conditions. A - temp., B - P.F.D., AXB - interaction; n = 3; * - P < 0.05, ** - P < 0.01, *** - P < 0.001.

Clone	Source of variation	F value	% of total variation explained
GSC-14	A	250.2 ***	5.9
	B	1984.4 ***	63.3
	AXB	318.6 ***	30.4
SGSC-6	A	345.1 ***	26.8
	B	467.7 ***	48.5
	AXB	77.3 ***	23.8
SGY-2	A	8.0 **	4.7
	B	72.7 ***	64.7
	AXB	9.3 ***	22.4
GFL-VR	A	12.2 ***	3.1
	B	62.8 ***	22.8
	AXB	64.1 ***	71.0

Table 4. Two way ANOVA of galactose yield content in 4 *Gracilaria* clones grown under different temperature and P.F.D. conditions. A - temp., B - P.F.D., AXB - interaction; n = 3; * - P < 0.05, ** - P < 0.01, *** - P < 0.001.

Clone	Source of variation	F value	% of total variation explained
GSC-14	A	81.1 ***	26.9
	B	77.8 ***	34.4
	AXB	26.9 ***	34.7
SGSC-6	A	16.9 ***	28.3
	B	8.4 ***	17.6
	AXB	5.6 ***	32.6
SGY-2	A	271.7 ***	56.2
	B	60.0 ***	16.4
	AXB	31.0 ***	24.9
GFL-VR	A	264.2 ***	19.1
	B	573.0 ***	55.4
	AXB	85.9 ***	24.7

Table 5. Duncan's Multiple Range Test for the chlorophyll *a* concentration in different strains (means with the same letter are nonsignificantly different in the level of $P < 0.05$).

Strains	N	Mean ± SD (µg mg DW-1)	Logarithmic transformation	Duncan's grouping
SGSC-6	11	0.832 ± 0.289	-0.247	B
SGYC-3	11	0.895 ± 0.154	-0.124	B
GSC-14	12	0.865 ± 0.221	-0.177	B
SGY-2	10	1.249 ± 0.295	0.196	A
GFL-VR	12	1.259 ± 0.452	0.175	A

Table 6. Photosynthesis (Pn), dark respiration (R), diel net gas exchange (DNGE, photoperiod regime of 10L-14D), and RuBPcase activity in *Gracilaria conferta* (GRT), and *G. verrucosa* (GAR) W.T. strains under different temperature conditions.

Strain	Temp. °C	(µmol O_2 g FW^{-1} h^{-1})		DNGE (µmol O_2 g FW^{-1})	RuBPcase (µmol CO_2 g FW^{-1} h^{-1})
		Pn	R		
GRT	15	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	-
GRT	20	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	-
GRT	25	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
GRT	30	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	-

Table 7. Photosynthetic characteristics and pigment content of several *Gracilaria* species from Jamaica. Co - compensation point, Ik - saturation point, α - linear slope value of the P-I curves, DNGE - diel net gas exchange, Pn - net photosynthesis at saturating light intensity, CHL - chlorophyll *a*, PE - phycoerythrin, and PC - phycocyanin.

Species	Pn (μmol O_2 g FW^{-1} h^{-1})	DNGE (μmol O_2 g FW^{-1})	(μE m^{-2} s^{-1})		α (Pn/PFD)	(μg g FW^{-1})		
			Co	Ik		CHL	PE	PC
G. cornea	22	27.9	30	115	0.26	78.2 ± 6.9	276 ± 5	27 ± 6
G. domingensis	22	28.2	30	115	0.26	115.7 ± 28.6	473 ± 79	64 ± 14
Gracilaria sp.	32	36.6	30	130	0.34	129.0 ± 12.5	963 ± 88	54 ± 13
G. cervicornis	16	16.9	30	135	0.2	209.6 ± 27.6	750 ± 58	121 ± 9

FIG. 1. Weekly growth rate (WGR) mesured in four *Gracilaria* strains under different PFD (40-280 µE m^{-2} s^{-1}) and temperature (20-30°C) conditions.

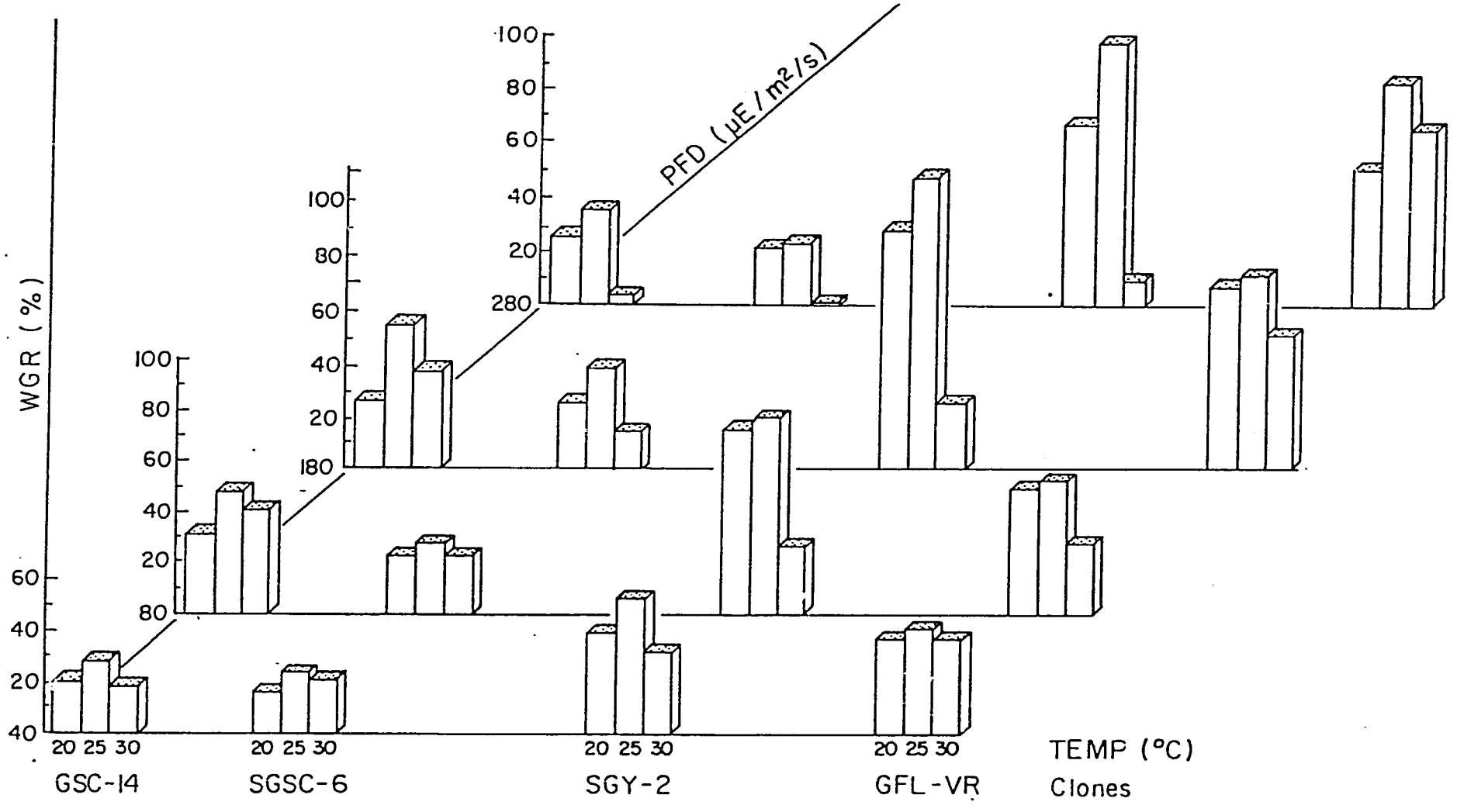

FIG. 2. Carbohydrate (CARB) content in four *Gracilaria* strains under different PFD and temperature conditions.

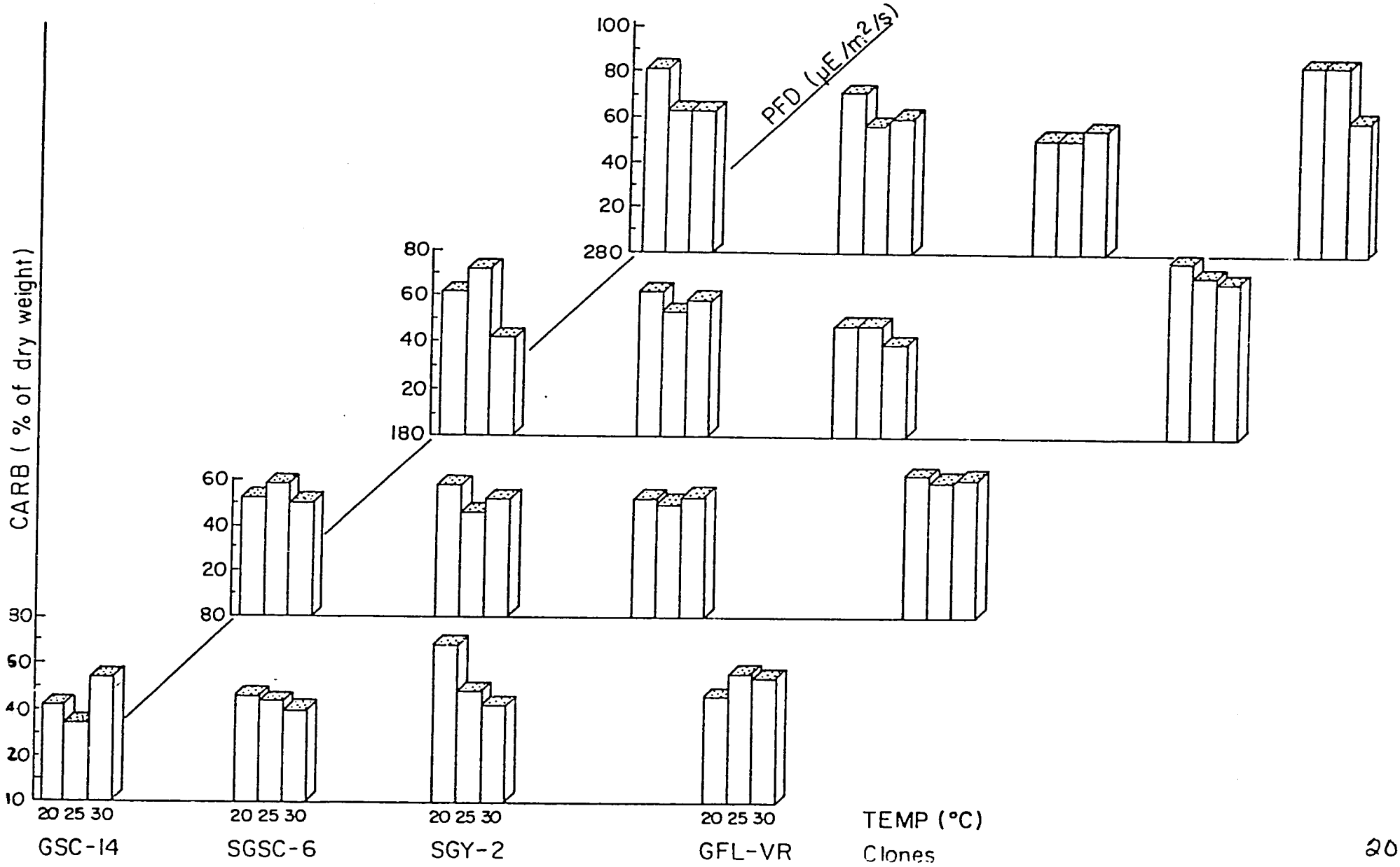

FIG. 3. Galactose/Glucose ratio (GAL/GLU) in four *Gracilaria* strains under different PFD and temperature conditions.

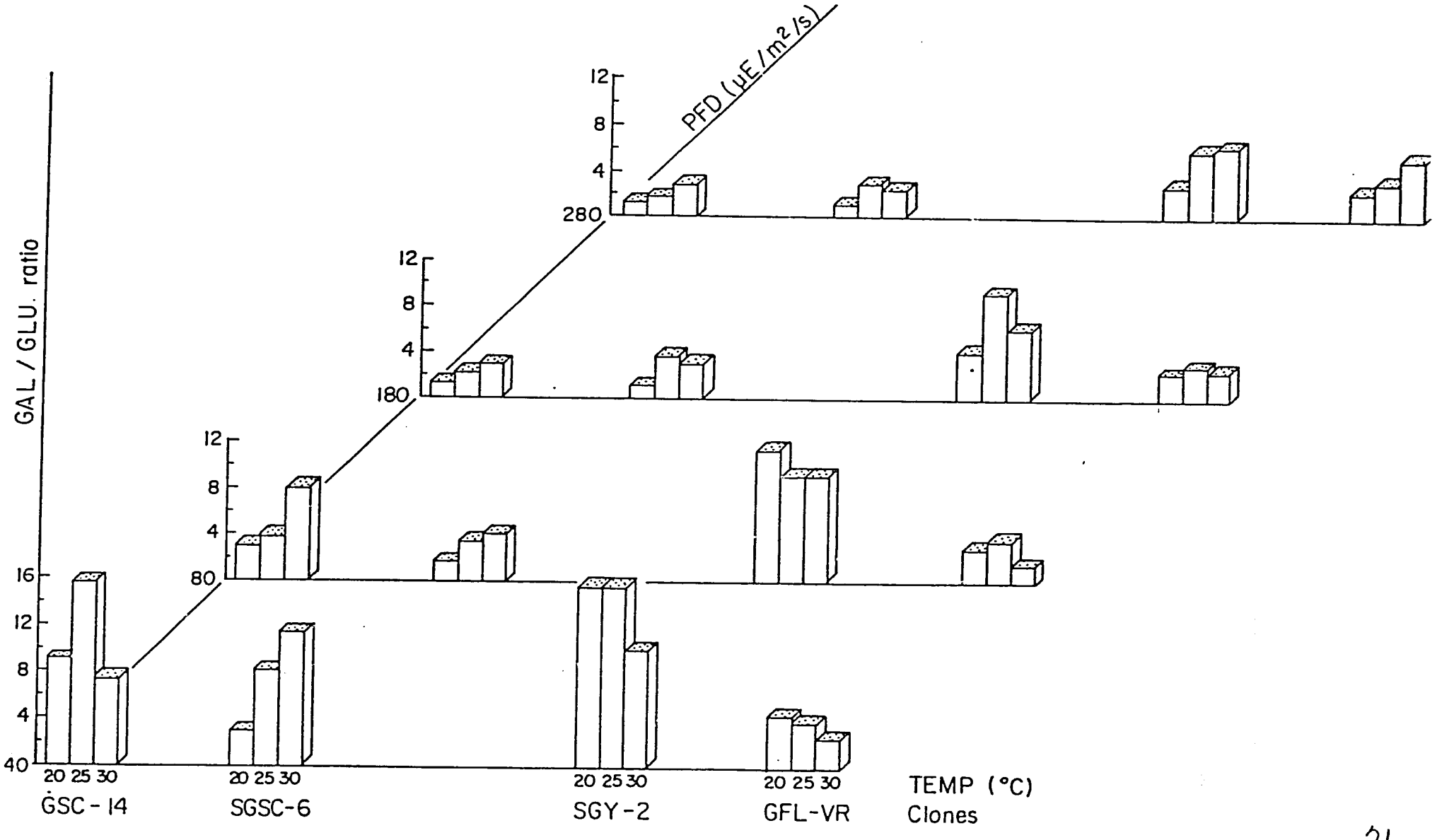

FIG. 4. Galactose yield (GAL YIELD) in four *Gracilaria* strains under different PFD and temperature conditions.

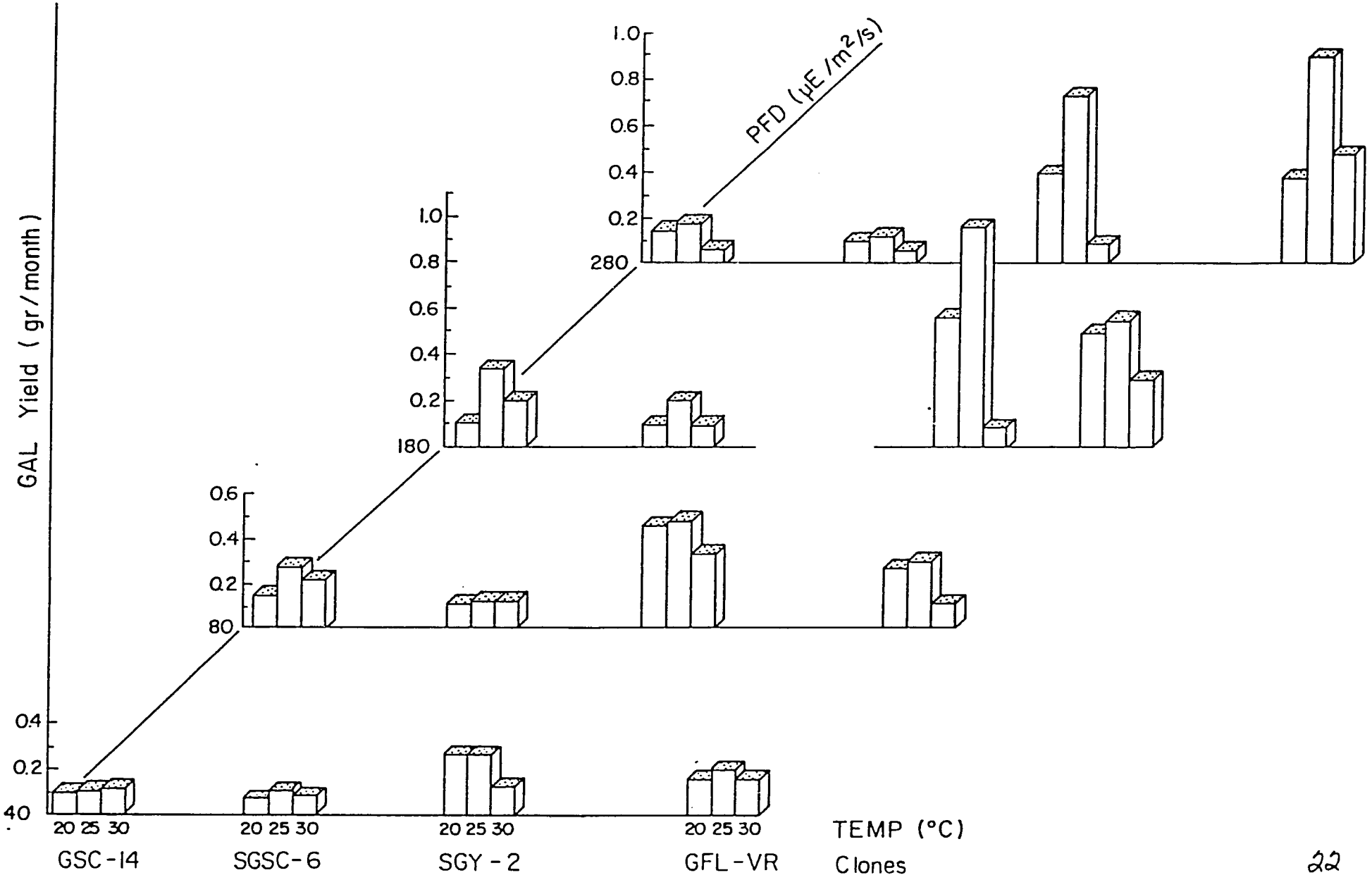

FIG. 5. Weekly growth rate (WGR) measured in two *Gracilaria* W.T. strains (GAR - *G. verrucosa*, GRT - *G. conferta*) under different PFD (40-350 µE m^{-2} s^{-1}) and temperature (10-32°C) conditions.

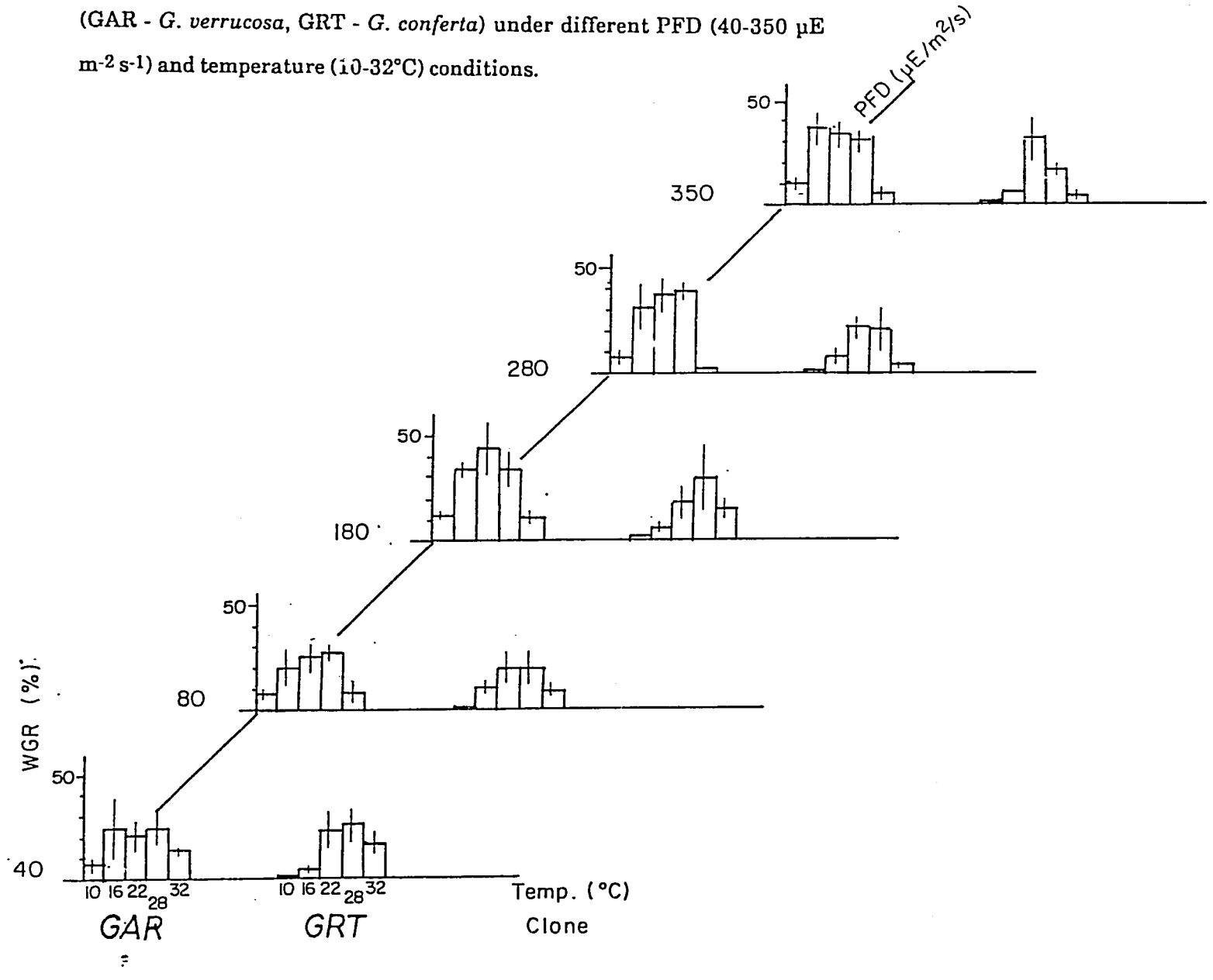

FIG. 6. Weekly growth rate (WGR %), and chlorophyll *a* content (CHL µg mg DW-1) measured in two *Gracilaria* W.T. strains (GAR - *G. verrucosa*, GRT - *G. conferta*). A. Each bar is an average of all PFD's under the same temperature B. Each bar is an average of all temperatures under the same PFD (temperature and PFD ranges are as in Fig. 5).

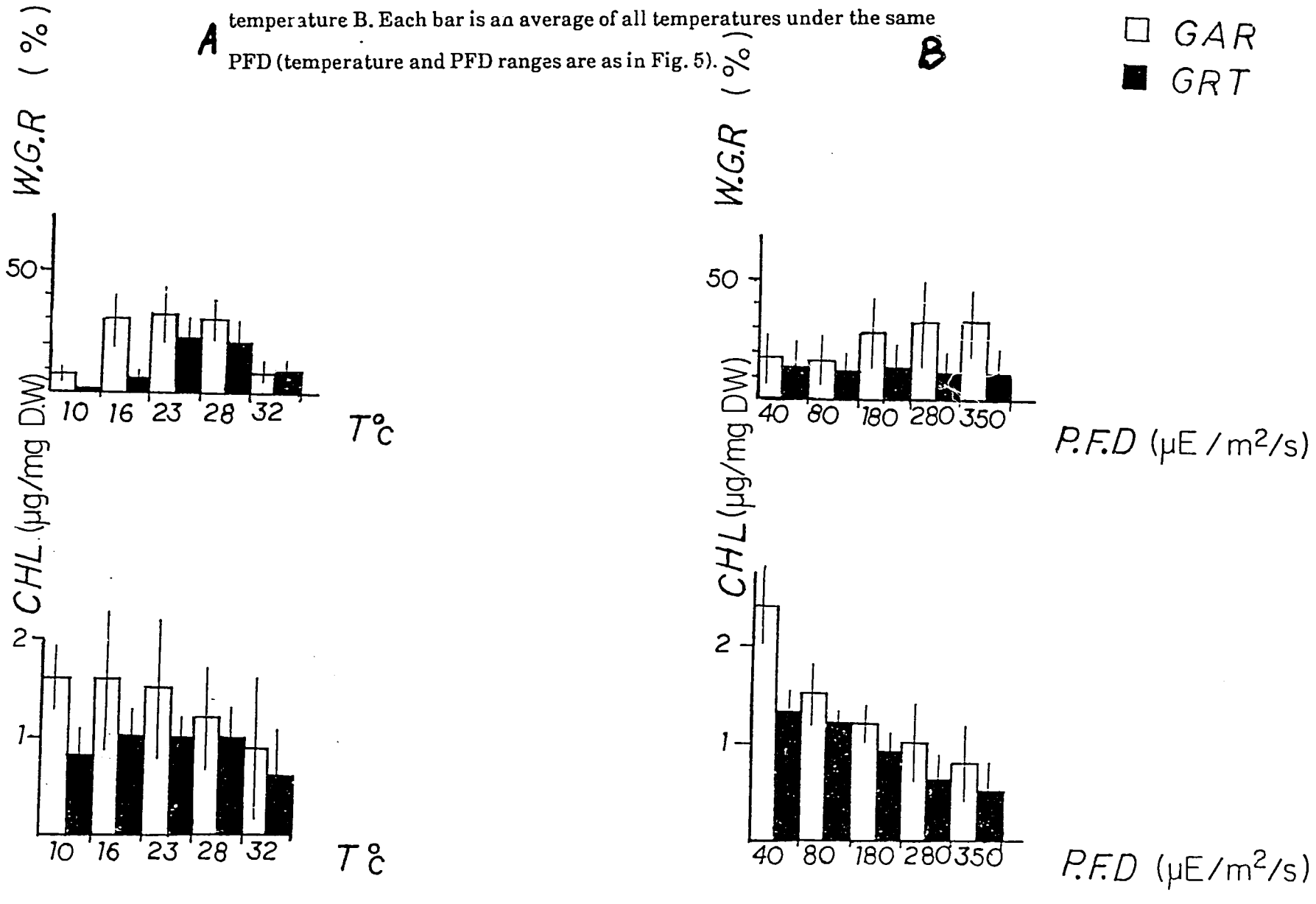

FIG. 7. Weekly growth rate (WGR) measured in four *Gracilaria* species from Jamaica (Sp - *Gracilaria* sp., Co - *G. cornea*, Ce - *G. cervicornis*, Do - *G. domingensis*) under different PFD (40-280 μE m^{-2} s^{-1}) and temperature (20-30°C) conditions.

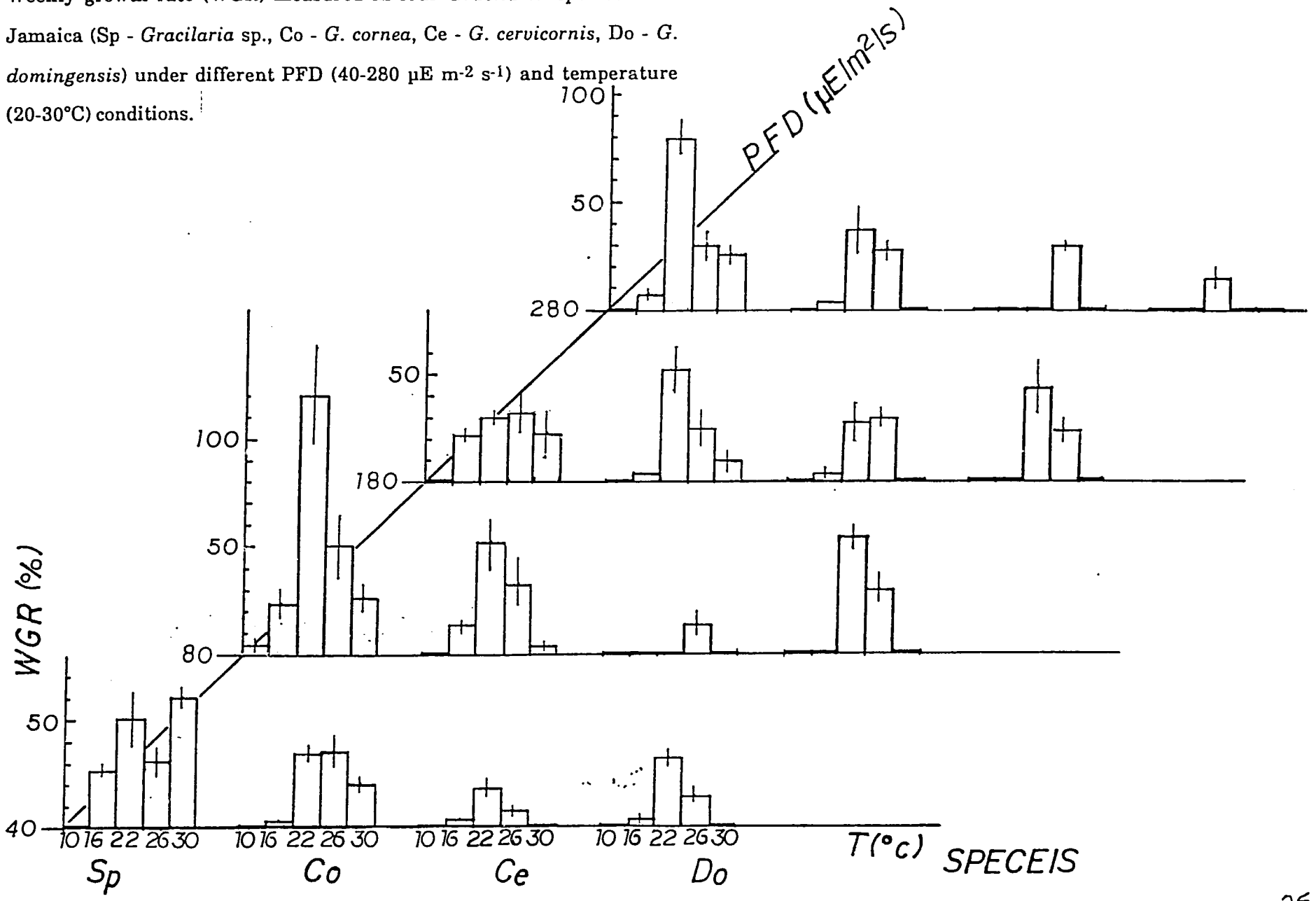
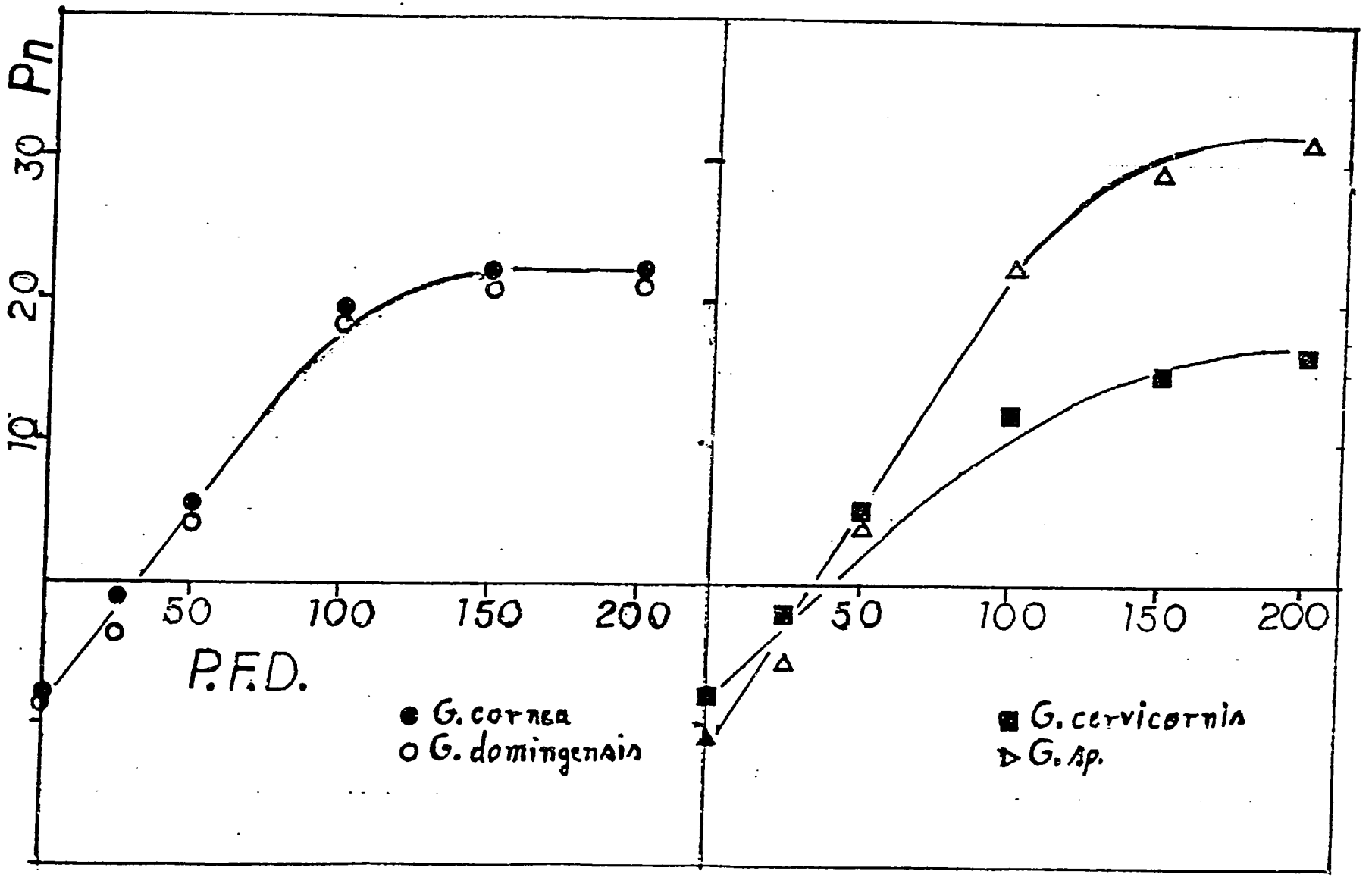
FIG. 8. Photosynthesis (Pn µmole O_2 g FW^{-1} h^{-1}) vs. PFD curves (P-I) of four *Gracilaria* species from Jamaica.