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Inorganic nutrition in pond cultivated *Gracilaria conferta* (Rhodophyta): nitrogen, phosphate and sulfate**

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Abstract

The experience gained in the outdoor cultivation system of *Gracilaria conferta* in Israel suggests three main inorganic variables: inorganic carbon, macronutrients and micronutrients. The effects of the macronutrients nitrogen, phosphate and sulfate on growth rate, dry weight yield, and agar yield and quality were assessed in this research. The starvation period between ammonium pulse feedings affected the agar yield and its quality as a function of the water temperature. Phosphate was the second macronutrient in the usual 1:10 proportion. Dark starvation as well as sulfate starvation decreased agar yield. The results of nitrogen or sulfate starvation suggest a biphasic process of agar biosynthesis. Starting with an overall growth stage richer with sulfated agarose, and turning into an agar accumulation stage with more neutral agarose. The elucidation of the integrated effects of starvation and seasonality on agar production of *Gracilaria* is the major contribution of this research.

Introduction

The technology of *Gracilaria* outdoor cultivation includes usually a nitrogen and phosphate pulse feeding, with optimization of the frequency and concentration for high yields (Friedlander & Levy, 1995; Capo et al., 1999). It may be assumed that except of the continuous growth due to nitrogen and phosphate feedings, there is also a unique change in agar yield and quality between the feedings, which might be crucial for harvest timing (Craigie & Shacklock, 1989).

In *Dunaliella* cultivation, a clear second phase of β-carotene accumulation was identified after a first phase of biomass accumulation (Ben-Amotz, 1995). The 'Neish effect' (Neish & Shacklock, 1971), which was extended to the 'P effect' suggests that *Chondrus crispus* grown under limited nitrogen or phosphate increased the production of carrageenan (Chopin et al., 1990; Chopin & Gallant, 1995). No difference was found between the effect of ammonium and nitrate feeding on growth of *Gracilaria* sp. (Lapointe & Ryther, 1978), whereas a more efficient uptake of ammonium by *Gracilaria* sp. was pointed out (De'Elia & DeBoer, 1978; Friedlander et al., 1991; Haglund & Pederson, 1993). Bidwell et al. (1985) presented the advantage of pulse feeding for *Gracilaria* to avoid epiphytism. Nitrogen limitation of *Gracilaria gracilis* decreased significantly the specific growth rate after two weeks of growth, but increased the carbohydrate content (Bird et al., 1981, 1982; Smit et al., 1997). Limiting concentrations of phosphate under high nitrogen feeding of *Gracilaria* G-16S showed higher native agar concentrations than under high nitrogen and high phosphate concentrations. Gel strength showed the opposite response to these conditions (Lewis & Hanisak, 1996). Agar isolated from *Gracilaria verrucosa* G-

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16 cultured under nitrogen limited conditions showed large non-polar components (ethanol soluble), minor amounts of methylation and presence of Floridian starch, whereas nitrogen supplemented cultures produced more polar agar (hot water soluble) with little starch (Chiles et al., 1989). Agar from *Gracilaria tikvahiae* mutant MP-40 responded to nitrogen depletion with lower levels of 3,6-anhydro-L-galactose and lower gel strengths (Craigie et al., 1984). Agar with maximum gel strength and 3,6-anhydro-L-galactose content and minimum sulfate content was produced by young branches of *G. tikvahiae* at low temperature (Craigie & Wen, 1984). Agar yield was reported to increase after a dark treatment of one-month (Rincones & Pedersen, 1993).

Sulfate is not a limiting factor for seaweeds under normal conditions. It is present in seaweed proteins, polysaccharides, sulfolipids and low molecular sulfated compounds (DeBoer, 1981). Cell division has a special requirement for sulfate in the growth medium (O'Kelly, 1974). The effect of sulfate starvation on agar biosynthesis has not been studied. It might however increase the understanding of the agar biosynthetic process and location.

The purpose of this study was to define the pre-harvest optimal starvation period of ammonium, phosphate and sulfate for *G. conferta* pond cultivation, and to determine if there is a controllable two-phase process of growth followed by the accumulation of agar.

Materials and methods

Gracilaria conferta (Schousboe) J. & G. Feldman was cultured for three weeks in aerated outdoor 40 L tanks (200 g FW per tank) with continuously filtered (100 m) ambient seawater (1 exchange hr^{-1}) from the adjacent Mediterranean coast for the nitrogen and phosphate starvation experiments (n = 2; Friedlander & Ben-Amotz, 1991). *Gracilaria* was pulse fed weekly with 1 mM ammonium and 0.1 mM phosphate. The seaweeds were pre-incubated for one week with normal feeding. Afterwards they were harvested and processed every 3 days in the nitrogen and phosphate starvation experiment. This simultaneous effect of ammonium and phosphate starvation on the daily yield of agar was tested in four different seasons. In a dark starvation experiment, the treated tanks were covered with a dark screen. The same species was cultured for three weeks in aerated indoor 4-L flasks (10 g FW per flask) with one weekly water exchange under 25 °C and 100 μmol photon cm^{-2} s^{-1} for the sulfate starvation experiments (n = 3). This experiment was conducted using artificial seawater with a PEM enrichment medium (Provasoli, 1968) without sulfate. The seaweeds were harvested once a week or only in the end of the experiment. After termination of the sulfate starvation experiment, tips (upper 3 mm) were separated from the rest of the thallus for agar extraction.

Table 1. Effect of nitrogen and phosphate starvation on different variables of *G. conferta*: 2 way ANOVA (F values)

Variable	Temp	Day	Interaction	n
Agar yield	36.36***	0.03 ns	2.71***	110
Gel strength	74.26***	4.79***	5.29***	117
Molar ratio	709.8***	4.16**	4.05***	72
Sulfate %	39.45***	14.15***	9.76***	74

Agar was extracted by alkali treatment and its yield was calculated as the product of average daily FW yield, % DW and % agar in DW per tank (n = 3; Craigie & Leigh, 1978). Agar gel strength was determined with a standardized gelometer. Galactose, anhydrogalactose and sulfate were determined according to Friedlander & Zelikovitch (1984). The C/N ratio was determined with a C, H, N analyzer (Perkin-Elmer). Statistical analysis included 2- and 3-way ANOVA and calculation of correlation between variables (Sokal & Rohlf, 1981). Some calculations included a high number of data (about n = 120), which consisted of measured effects of starvation days, seasons, number of tanks and repetitions. Differences between treatments were measured by Duncan's multiple range test (Cary, 1985).

Results

Nitrogen and phosphate starvation

The simultaneous effect of ammonium and phosphate starvation days on the daily yield of agar was tested in four different seasons (Figure 1). In spring and summer, significantly higher daily agar yield occurred after 3–6 days of starvation, whereas in autumn (not significant) and winter (significant) it occurred after 9–15 days of starvation (best fit by quadratic function). The highest level of agar was not stable in summer, whereas in the other seasons the highest level was stable for 3 days and more. Maximal daily agar yield

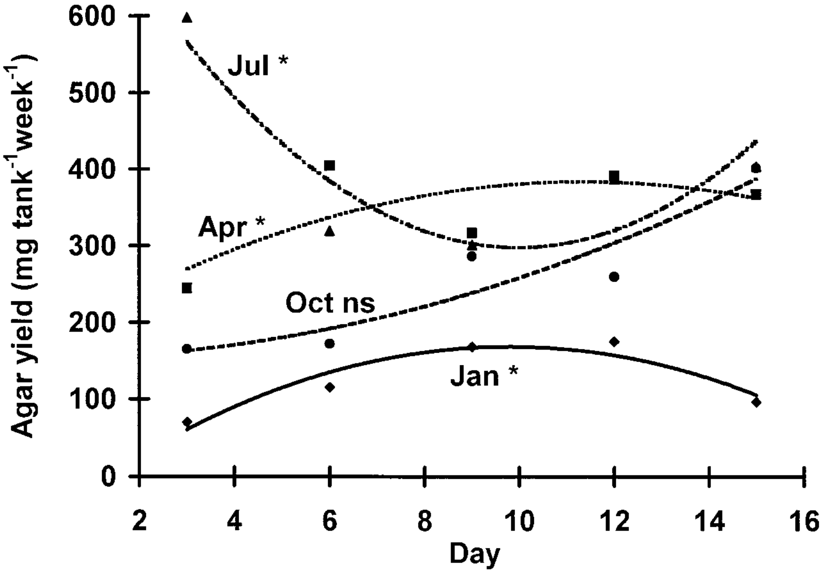

Figure 1. Effect of nitrogen and phosphate starvation days on the daily agar yield (mg tank^{-1} week^{-1}) in *G. conferta* through the year (* significant at p <0.05; n.s., non-significant). Quadratic correlation: Jan R^2 = 0.896; Apr R^2 = 0.495; Jul R^2 = 0.804; Oct R^2 = 0.870.

reached 175, 400, 600 and 400 mg tank^{-1} in January, April, July and October, respectively. The overall analysis of variance showed a significant main effect of season (temperature) and of interaction between season and starvation days, but not of days alone (Table 1).

Gel strength was less affected by nitrogen and phosphate starvation (Figure 2). Maximum gel strength was only obtained in January on day 6, whereas in the other seasons the level was significantly stable during 15 days of nitrogen and phosphate starvation (best fit by quadratic function). Maximal gel strength reached 975, 870, 700 and 1040 g cm^{-2} in January, April, July and October, respectively. Thus gel strength decreased significantly during spring and summer temperatures, and increased by winter and autumn conditions. However, the overall analysis of variance showed significant main effects of season (temperature), days of starvation and of their interaction (Table 1). Gel strength (Y) was negatively correlated with daily agar yield (X), (Y = –0.714X+1016.9; R^2 = 0.359).

The molar ratio of anhydrogalactose to galactose showed high values in autumn and winter and low values in spring and summer (Figure 3). The effect of starvation days was significant in all seasons except of spring. ANOVA showed significant main effects of season (temperature), duration of starvation (days) and of their interaction on the molar ratio (Table 1). This molar ratio was positively correlated with gel strength and negatively correlated with daily agar yield (Table 2).

Table 2. Correlation between variables of nitrogen and phosphate starved *G. conferta*

Variable		Gel strength	Molar ratio	Sulfate
Yields	r	–0.377	–0.609	0.014
	p	0.0001	0.0001	0.907
	n	109	68	69
Gel strength	r		0.51	0.491
	p		0.0001	0.0001
	n		67	68
Molar ratio	r			0.304
	p			0.009
	n			73

Sulfate content did significantly increase after the beginning of nitrogen and phosphate starvation, except of summer when there was no starvation effect (Figure 4). ANOVA showed significant main effects of season (temperature), duration of starvation and their interaction (Table 2). Although correlations with other variables were relatively low, sulfate content was pos-

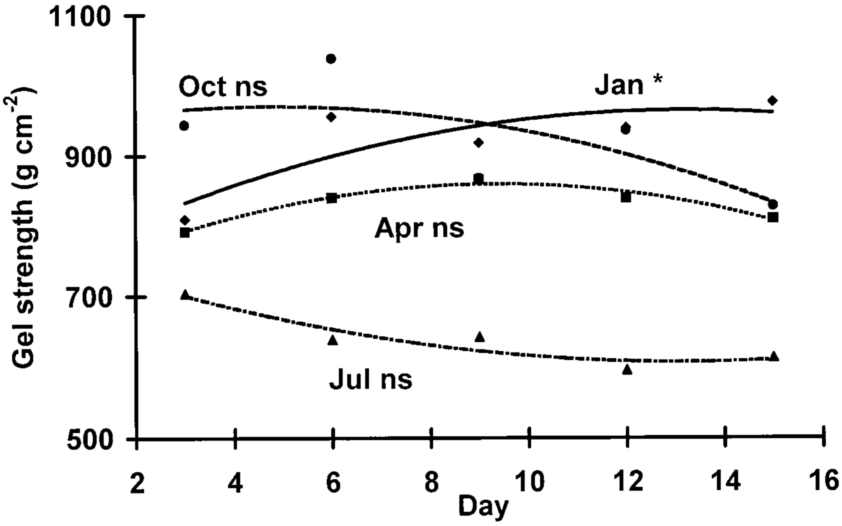

Figure 2. Effect of nitrogen and phosphate starvation days on gel strength (g cm^{-2}) in *G. conferta* agar through the year (* significant at $p < 0.05$; n.s., non-significant). Quadratic correlation: Jan $R^2 = 0.699$; Apr $R^2 = 0.959$; Jul $R^2 = 0.886$; Oct $R^2 = 0.507$.

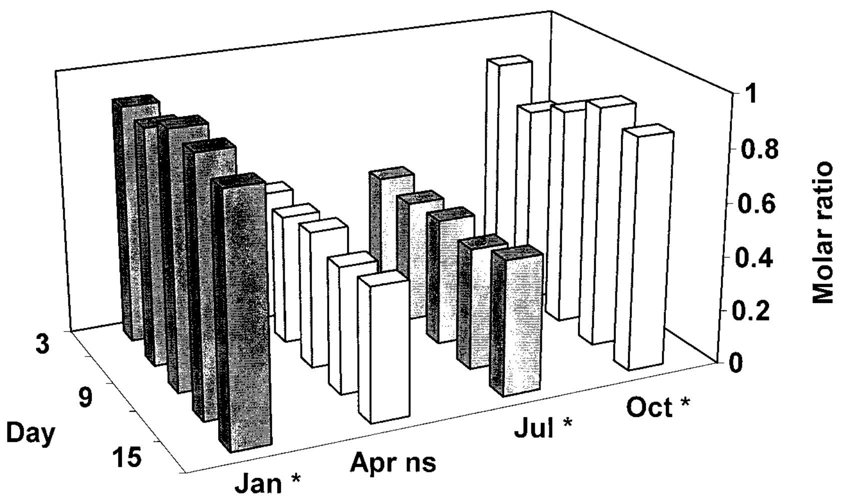

Figure 3. Effect of nitrogen and phosphate starvation days on the molar ratio (AHG/GAL) in *G. conferta* agar through the year (* significant at $p < 0.05$; n.s., non-significant).

itively related to agar gel strength and to molar ratio (Table 2).

The effect of nitrogen starvation was also tested under light and dark conditions (Figure 5). During 16 days of the starvation experiment the daily agar yield under dark starvation was significantly lower as compared to light starvation. Light and dark starvation did not consistently effect gel strength. However, C/N ratio was always lower in the dark as compared to light starvation, with significant differences after 10–13 days of starvation.

Separated nitrogen and phosphate starvation

Separated starvation effects of ammonium and phosphate showed a significant decrease in daily agar yield and gel strength under nitrogen starvation and a significant decrease in gel strength also under phosphate

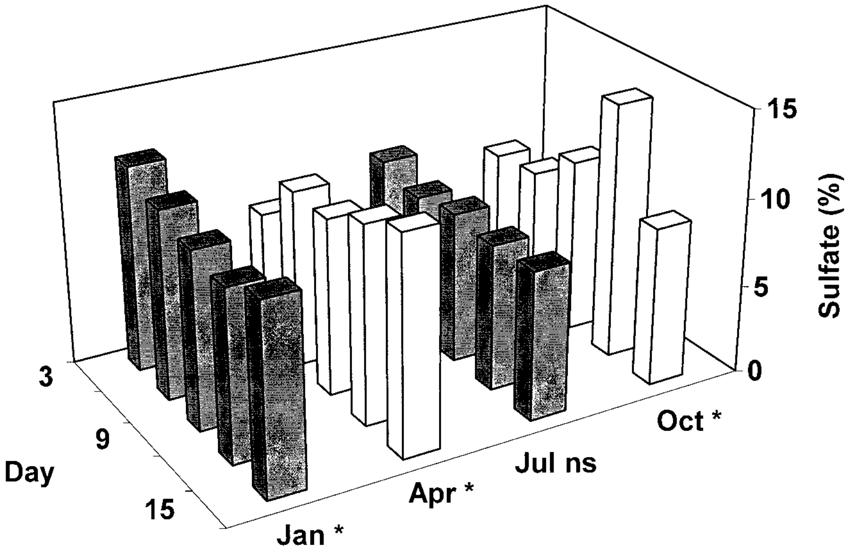

Figure 4. Effect of nitrogen and phosphate starvation days on the sulfate concentration (%) in *G. conferta* agar through the year (* significant at p <0.05; n.s., non-significant).

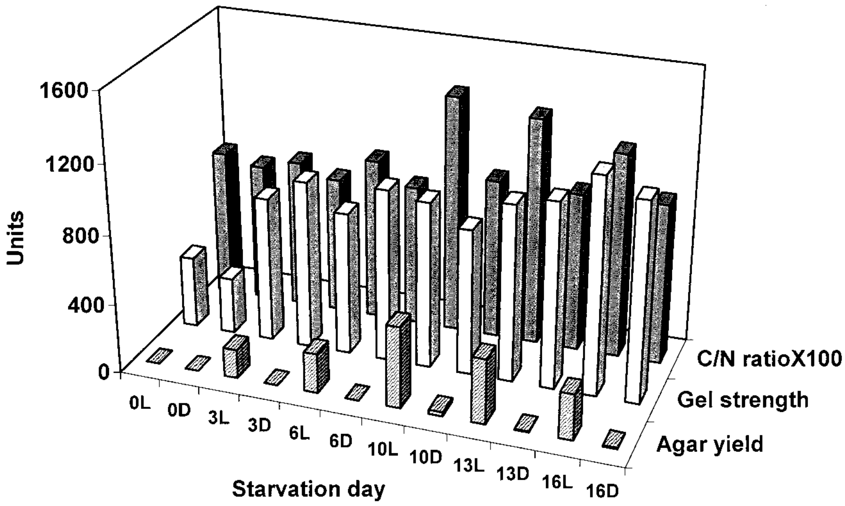

Figure 5. Effect of light and dark starvation on agar yield, gel strength and C/N ratio in *G. conferta* (agar yield in mg tank^{-1} week^{-1}; gel strength in g cm^{-2}).

starvation as compared to the integrated feeding of both factors (Figure 6).

Sulfate starvation

After termination of the sulfate starvation experiment the seaweeds were separated into tips and the rest of the thallus. The daily agar yield significantly decreased in the starved tips in both seasons as compared to fed tips and to starved thallus, whereas the starved thallus did not respond consistently as compared to the fed thallus (Figure 7). The overall agar yield was higher in summer than in winter. ANOVA showed that the major effects, season (temperature), seaweed parts and starvation were significant, and also most of their interactions (Table 3).

The molar ratio between anhydrogalactose and galactose (data not presented) was significantly lower

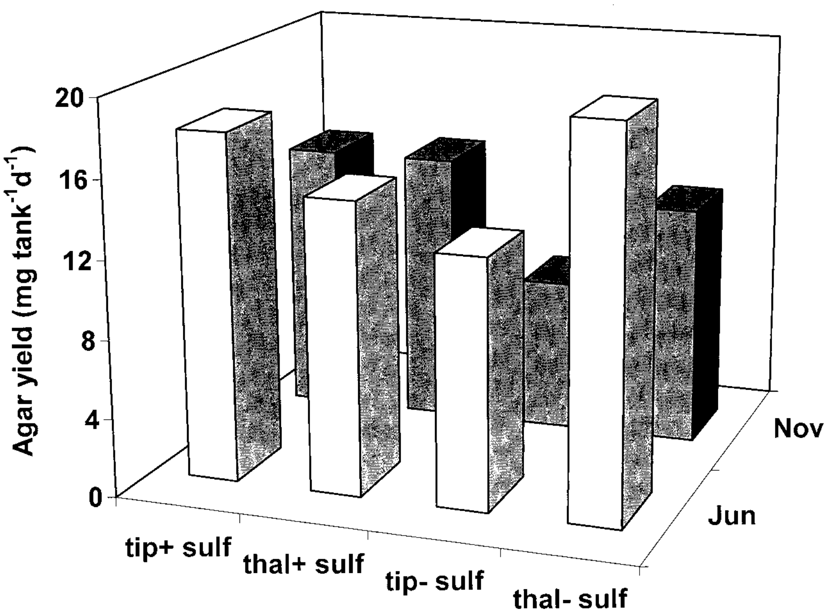

Figure 7. Effect of sulfate starvation on agar yield in *G. conferta*. Yield was separately determined in tips and thalli with or without (+/-) sulfate (sulf).

Table 3. A) Effect of sulfate starvation on different variables on *G. conferta*: 3-way ANOVA (F values). B) Correlation between variables of sulfate-starved *G. conferta*

A

Variable	Temp	Sulfate	Part	T/S	T/P	S/P	T/S/P
Agar yield	52.2***	40.7***	21.6***	13.3***	1.5 ns	12.7**	6.9**
Molar ratio	1177***	2.98 ns	1.09 ns	7.72**	15.0***	10.4**	1.22 ns
Sulfate	225.6***	2.4 ns	0.44 ns	5.85*	0.65 ns	13.5***	1.79 ns

B

Variable		Molar ratio	Sulfate
Yield	r	–0.405	0.601
	p	0.0292	0.0065
	n	29	19
Molar ratio	r		–0.895
	p		0.001
	n		34

in summer than in winter. ANOVA showed that only the season main effect and interactions were significant, whereas the main effects of starvation and seaweed parts were not significant (Table 3). The molar ratio negatively correlated also here with daily agar yield.

The overall sulfate content was higher in summer than in winter (data not presented). ANOVA showed also here that only the seasonal main effect and interactions were significant, whereas the other main effects of starvation and seaweed parts were not significant (Table 3). Sulfate content positively correlated with daily agar yield and negatively with molar ratio (Table 3). The effect of continuous sulfate starvation on a full *Gracilaria* thallus was studied during 3 weeks, including parameter determinations every week (Figure 8). Daily agar yield and percentage of dry weight showed a significant decrease, whereas

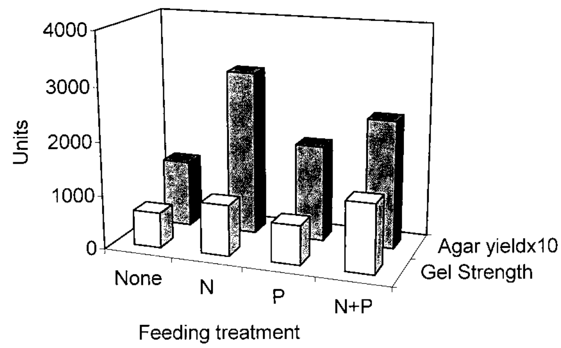

Figure 6. Effect of separated nitrogen and phosphate feeding on agar yield (mg tank^{-1} week^{-1}) and gel strength (g cm^{-2}) in *G. conferta*.

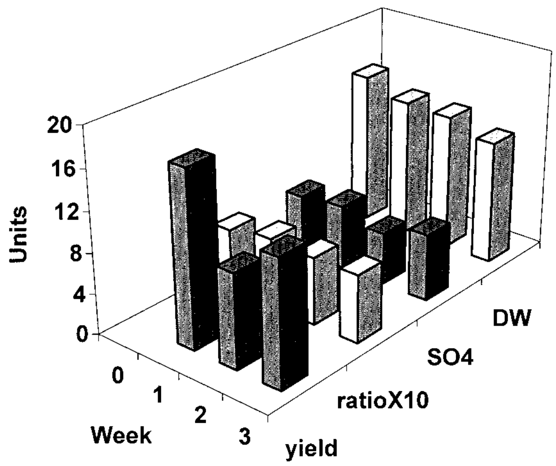

Figure 8. Effect of three weeks sulfate starvation on agar yield, AHG/GAL ratio (ratio × 10), sulfate percentage (SO_4) and dry weight (DW) in *G. conferta*.

molar ratio and sulfate content were practically stable.

Discussion

The effect of temperature and nutrient starvation of nitrogen, phosphate and sulfate on agar parameters of *G. conferta* was tested in this study. The parameters were agar yield, gel strength, molar ratio and sulfate content. The feeding experiment in which nitrogen was separated from phosphate and sulfate suggests that under local conditions of *G. conferta* cultivation, nitrogen is the dominant limiting nutrient while phosphate and sulfate are secondary ones.

The analysis of agar yields and gel strength showed that the starvation effect of all three nutrients was temperature dependent, suggesting that temperature was the major limiting growth factor. Increase in temperature had a positive effect on agar yield and a negative effect on gel strength, and optimal starvation time differed between summer and winter. *G. tikvahiae* and *Gracilaria* G-16S showed similar responses (Craigie & Wen, 1984; Lewis & Hanisak, 1996). As agar yield of sulfate starved tips was lower than fed tips and starved and fed thallus, it may be suggested that tips initially biosynthesized sulfated agarose, whereas the mature thallus transformed it into the final neutral agarose. Analysis of all starvation experiments confirmed that high agar biosynthesis in summer produced mainly a non-mature sulfated agar. Therefore, it may be suggested that nitrogen and phosphate starvation had a positive effect on daily agar yield, while sulfate starvation had no such advantage. However, a potential positive effect of additional sulfate treatment on the non-mature agar accumulation should still be studied.

Temperature and nutrient starvation had a limited but significant effect on the agar molar ratio. This supports the effect of temperature in culture experiments with *G. tikvahiae* (Craigie & Wen, 1984). The correlation results show that during high agar production the biosynthesis did not produce mature agar with a high molar ratio and high gel strength, but rather a low molar ratio agar in which desulfation and production of anhydrogalactose had still not occurred. The high molar ratio, which appeared in the winter, reflected mature agar, whereas the summer agar, which showed lower molar ratio, presented higher biosynthetic activity. The sulfate content showed that during nitrogen and phosphate starvation the synthesized agar accumulated more the sulfated agarose form, rather than its neutral agarose one. The higher sulfate content and lower molar ratio in summer reflects the high biosynthetic activity of non-mature highly sulfated agar, although summer conditions affected this seaweed experiment only in the pre-experiment outdoor culture. One of the correlation results contradicted the previous expectations, and suggests that the sulfate content in the last biosynthetic phase included also sulfate residues like C2 and C4 derivatives, which were not precursors of anhydrogalactose, like the C6 sulfate derivatives.

The overall starvation response in this study suggests a biphasic process of agar yield production in *G. conferta*. A growth phase mainly in summer with a high proportion of agar biosynthesizing tips, and an agar maturation phase mainly in winter with less active tips. The decision of harvest timing will have to consider the antagonistic effect of temperature and starvation days on gel strength and agar yield, according to the expected priorities of the producer. Regarding the effect of dark treatment on agar yield it can be definitely concluded that dark conditions contribute to the decrease of agar yield under conditions of starvation, contradicting previous experiments (Rincones & Pedersen, 1993).

In summary, this study showed that the effect of nitrogen and phosphate starvation period on daily agar yield of *G. conferta* was strongly modified by season. Furthermore, it was found that biosynthesis of mature, high gel strength agar with a high molar ratio was limited by summer temperatures and not by nitrogen starvation. It was also found that the nitrogen and phosphate starvation probably increased the sulfation step in the biosynthesis of agar, which was revealed in *G. conferta* tips, whereas the mature thallus derived from these tips, synthesized the final non-sulfated agarose. Considering this new evidence, it is finally suggested that *G. conferta* has a biphasic growth. A first phase of overall growth with high sulfated agarose biosynthesis under conditions of summer and nitrogen starvation, and a second phase of agar maturation under winter and non starvation conditions.

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