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Deleterious effect of TRIS buffer on growth rates and pigment content of *Gracilaria birdiae* Plastino & E.C. Oliveira (Gracilariales, Rhodophyta)

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RESUMO – (Efeito deletério do tampão TRIS nas taxas de crescimento e no conteúdo pigmentar de *Gracilaria birdiae* Plastino & E.C. Oliveira (Gracilariales, Rhodophyta)). O presente trabalho avaliou os efeitos do tampão Tris (hydroxymethyl)-aminomethane (TRIS) e a interação com a concentração de nutrientes no desenvolvimento em *Gracilaria birdiae*, espécie presente no litoral brasileiro utilizada comercialmente na produção de ágar. As respostas às diferentes condições de cultivo foram avaliadas por meio das taxas de crescimento e conteúdo pigmentar (clorofila *a*, ficoeritrina, ficocianina e aloficocianina). A solução de nutrientes de Provasoli com e sem adição de TRIS foi testada nas concentrações de 12,5, 25 e 50%. O pH foi também monitorado. *G. birdiae* cresceu melhor em ausência de TRIS e em baixas concentrações de nutrientes, 12,5 e 25% (taxas de crescimento de 10,8-11,3%.dia^{-1}). As maiores concentrações de ficoeritrina e clorofila *a* foram observadas na ausência de TRIS em 12,5 e 25% (Ficoeritrina, 649,6-698.0 μg g^{-1} de biomassa fresca; Clorofila *a*, 156,0-168,6 μg g^{-1} de biomassa fresca), evidenciando o efeito deletério do TRIS no crescimento e nos conteúdos de clorofila *a* e ficoeritrina. Os dados demonstram ainda a importância da utilização de concentrações de nutrientes adequadas em cultivos em laboratório, dependendo das características intrínsecas de cada espécie.

Palavras-chave: meio de cultura, TRIS (hydroximetil)-aminometano, *Gracilaria birdiae*, crescimento, pigmentos

ABSTRACT – (Deleterious effect of TRIS buffer on growth rates and pigment content of *Gracilaria birdiae* Plastino & E.C. Oliveira (Gracilariales, Rhodophyta)). This work evaluated the effects of Tris (hydroxymethyl)-aminomethane (TRIS) buffer and its interaction with nutrient concentration on the development of *Gracilaria birdie,* a common species on the Brazilian coast that has been exploited for agar production. Responses to different conditions were assessed through growth rates and pigment content (chlorophyll *a*, phycoerythrin, phycocyanin and allophycocyanin). Provasoli's nutrient solution with and without TRIS addition was tested at concentrations of 12.5, 25 and 50%. The pH was also monitored. *G. birdiae* grew better in the absence of TRIS and at low nutrient concentrations, 12.5 and 25% (growth rates of 10.8-11.3%.day^{-1}). Higher contents of phycoerythrin and chlorophyll *a* were observed without TRIS at 12.5 and 25% (Phycoerythrin, 649.6-698.0 μg g^{-1} fresh biomass; Chlorophyll *a*, 156.0-168.6 μg g^{-1} fresh biomass). These findings highlight the deleterious effect of TRIS on growth and phycoerythrin and chlorophyll *a* content. They also demonstrate the importance of appropriate nutrient concentration for laboratory cultures, depending on the intrinsic characteristics of each species.

Key words: culture medium, TRIS (hydroxymethyl)-aminomethane, *Gracilaria birdiae*, growth, pigments

Introduction

In vitro cultures have significantly contributed to phycology development in the last decades. Life histories of many species of seaweeds have been accessed through these techniques, as well as physiological and genetic processes. *In vitro* cultures have also been important tools for selection and domestication of species (van der Meer 1982). Environmental factors, such as light, nutrients, salinity and temperature, can be manipulated in the laboratory, which allows researchers to evaluate their effects on vegetative and reproductive development of different species or strains (Oliveira *et al.* 1995; Plastino 2003). Although *in vitro* cultures have been commonly utilized, some basic points still need further investigation, such as the adequacy of culture media for a selected species.

Many seaweed culture media still have a number of potentially undesirable characteristics, when their chemical composition is examined. Firstly, concentrations of some ions, notably phosphate and some micronutrients are substantially greater in the media than in natural seawater (Woelkerling *et al.* 1983). This is the case in seawater enriched with Provasoli's solution (PES - McLachlan 1973) and with von Stosch's solution (VS - Edwards 1970), commonly applied in rhodophytes *in vitro* cultivation. Secondly, concentrations of some other ions, especially calcium, sulphate and borate, while generally similar to those in seawater, appear to be totally unnecessary for ensuring stability of the medium

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(Woelkerling *et al.* 1983). Thirdly, some media employ organic buffers which are metabolizable, such as glycylglycine (McLachlan 1973) and Tris (hydroxymethyl)-aminomethane, TRIS (Hanisak 1979), which seem to have negative effects on life material. Of these, TRIS is the most controversial, because its effectiveness of stabilized pH has not been clearly demonstrated (Woelkerling *et al.* 1983). In addition, the deleterious effect of TRIS has been observed for some phytoplankton species (Harrison *et al.* 1980) and freshwater algae (Smith & Foy 1974). Studies on seaweeds and seagrasses have proposed that TRIS has a deleterious effect on photosynthesis by inhibition of mechanisms such as the transport of HCO_3^- across the plasma membrane (Axelsson *et al.* 2000; Hellblom *et al.* 2001) or O_2 evolution in Photosystem II (Sofrová *et al.* 1978; Rickert *et al.* 1991; Jegerschold & Styring 1996). This buffer also stimulates bacterial growth, leading to a negative effect on cultures (Fábregas *et al.* 1993).

Although most reports indicate a negative effect of TRIS, some authors have raised some of its beneficial effects, by enhancing the availability of carbon, vitamins and hormones when promoting the increase of bacteria in the growth media (Ogata 1966; Provasoli & Pintner 1980; Woelkerling *et al.* 1983). The question is if the beneficial effects of TRIS would compensate the damage caused by its addition, especially in the photosynthetic apparatus.

TRIS is a component of the PES medium, which is probably the most utilized for laboratory seaweed culture (Oliveira *et al.* 1995). Similarly to the majority of culture media, it was originally developed for cold area species from nutrient-rich waters. However, it has been widely used, in spite of the geographic origin of the alga. If it were taken into account, it would be expected that species from oligotrophic tropical waters do not need such high nutrient concentrations for their laboratory cultures. Nevertheless, PES has been commonly utilized for species from the tropical Brazilian coast (e.g. Berchez & Oliveira 1990; Yokoya & Oliveira 1992).

This work was planned to analyze the effects of TRIS buffer and its interaction with nutrient concentration on growth and pigment content of a tropical species. The target organism was *Gracilaria birdiae*, one of the main species exploited for agar production in Brazil (Plastino & Oliveira 2002), which has been the subject of color strain investigations in our laboratory (e.g. Ursi *et al.* 2003; Plastino *et al.* 2004). *G. birdiae* occurs from the northeast to the southeast coast of Brazil (Plastino & Oliveira 2002), which is predominantly characterized by oligotrophic warm waters (Boltovskoy *et al.* 1999).

Material and methods

Apical segments of three infertile tetrasporophytes of *Gracilaria birdiae* Plastino & E.C. Oliveira obtained in the laboratory were utilized in the experiments. These tetrasporophytes were originated from cystocarpic specimens derived from a tetrasporophyte collected in a natural population from Anchieta Beach (20°80'S and 40°65'W), Espírito Santo state (Brazil) (Plastino *et al.* 2004). Unialgal cultures were established as described by Plastino & Oliveira (1990).

Standard culture conditions were 25 ± 1 ºC, 95 ± 10 µmol photon m^{-2} s^{-1} (Osram 40 W daylight fluorescent tubes), 14-10 light-dark cycle, with 30 min aeration every hour, and sterile seawater (32 psu, 7.90 pH) enriched with culture medium renewed weekly.

Three different concentrations of PES (McLachlan 1973) were employed: 12.5, 25, and 50%. These concentrations were also tested utilizing PES without TRIS addition (PES-TRIS). Three replicates each with three apical segments from three different individuals were cultivated separately in flasks with 900 ml of enriched seawater.

Growth rates were evaluated by weekly weighing for 28 days. Fresh biomass was utilized to calculate the growth rates (Lignell & Pedersén 1989). Initial biomass of each replicate was 10 mg.

The pH values were measured after PES dilution in seawater using a pH meter (Denver mod. 10234). After one week of cultivation, the apical segments were removed from the flasks and the pH of the medium was measured again.

Pigments were quantified by spectrophotometry using an HP 8452A spectrophotometer. Pigment extractions were carried out at the end of the growth experiment at 4 °C, according to Kursar *et al.* (1983) with modifications (Plastino & Guimarães, 2001). Briefly, the samples were disrupted by grinding with liquid nitrogen and 50 mmol/L phosphate buffer, pH 5.5. Crude extracts were centrifuged at 36,000 g for 25 min to get the phycobiliproteins. Chlorophyll *a* was extracted after dissolving the pellet in 90% acetone, and centrifuging at 12,000 g for 15 min. Pigment concentrations were calculated according to Kursar *et al.* (1983) for phycobiliproteins (allophycocyanin, phycocyanin and phycoerythrin), and Jeffrey and Humphrey (1975) for chlorophyll *a.*

Growth rates and pigment concentrations were analyzed by two-factor ANOVA (independent variables: PES concentrations and presence or absence of TRIS). Newman-Keuls *post hoc* tests were applied when necessary.

Results

Growth rates – The growth rates of *Gracilaria birdiae* were affected by both PES concentration and presence or absence of TRIS (interaction between factors F = 4.352, P < 0.05). Branches showed higher growth rates when cultivated in PES without TRIS and low nutrient concentration, 12.5 and 25% (Fig. 1, Tab. 1). Similar growth rates were observed in PES, regardless of concentration (Fig. 1, Tab. 1), whereas in the absence of TRIS, *G. birdiae* grew better in 12.5 and 25% than in 50%.

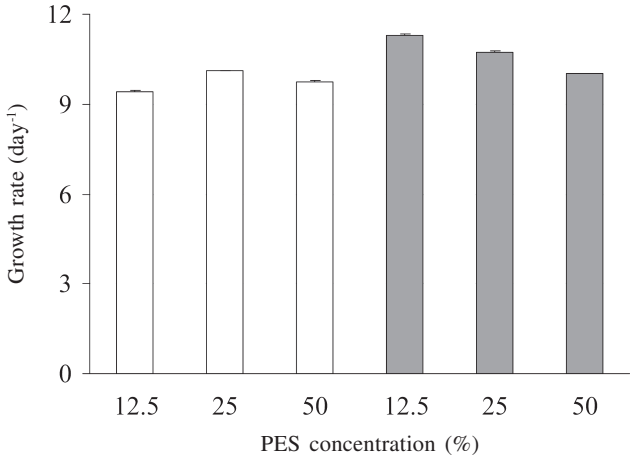

Figure 1. Growth rates of *Gracilaria birdiae* Plastino & E.C. Oliveira after 28 days of cultivation in seawater enriched with different concentrations (50, 25, and 12.5%) of PES with (PES) and without TRIS (PES-TRIS). Mean (n = 3) ± Standard deviation. (□ = PES; ■ = PES-TRIS).

Table 1. Newman-Keuls post hoc test of the two-factor ANOVA of growth rates of *Gracilaria birdiae* Plastino & E.C. Oliveira after 28 days of cultivation in seawater enriched with different concentrations (50, 25, and 12.5%) of PES with (PES) and without TRIS (PES-TRIS). Independent variables: PES concentration and presence or absence of TRIS.*, significant differences (P<0.05).

	PES 50%	PES 25%	PES 12.5%	PES-TRIS 50%	PES-TRIS 25%
PES 25%	0.632				
PES 12.5%	0.423	0.337			
PES-TRIS 50%	0.544	0.764	0.346		
PES-TRIS 25%	0.087	0.112	0.031*	0.150	
PES-TRIS 12.5%	0.017*	0.034*	0.006*	0.034*	0.261

pH measurement – The buffer effect of TRIS was evident when culture medium was added to seawater. The initial pH values in seawater enriched with PES were similar to the original seawater pH (7.9), while the initial values in seawater enriched with PES-TRIS were slightly higher (Tab. 2). Despite the differences in initial pH, the values observed after one week of cultivation were similar between seawater enriched with PES and PES-TRIS, showing that the buffer effect of TRIS was not maintained through cultivation (Tab. 2). During one week, pH values of seawater with PES-TRIS varied less than the pH values of seawater enriched with PES. The pH variation was sensitive to PES concentration as well as to the presence or absence of TRIS (interaction between factor F = 18.881, P > 0.05). No difference in pH variation was observed among the nutrient solution concentrations when branches were cultivated in PES-TRIS. However, this difference was verified in PES, in which variation increased with rising concentrations of nutrient solution (Tab. 2).

Table 2. pH variation (Initial pH - Final pH) of seawater utilized in *Gracilaria birdiae* Plastino & E.C. Oliveira cultivation for one week. Seawater was enriched with different concentrations (50, 25, and 12.5%) of PES with (PES) and without TRIS (PES-TRIS). Mean (n = 3) ± Standard deviation.

	Initial pH	Final pH	Ph variation
PES 50%	7.89 ± 0.00	8.05 ± 0.01	0.16 ± 0.01
PES 25%	7.96 ± 0.00	8.08 ± 0.01	0.12 ± 0.01
PES 12.5%	8.02 ± 0.00	8.09 ± 0.01	0.07 ± 0.01
PES-TRIS 50%	8.12 ± 0.00	8.11 ± 0.01	0.01 ± 0.01
PES-TRIS 25%	8.14 ± 0.00	8.12 ± 0.02	0.02 ± 0.02
PES-TRIS 12.5%	8.13 ± 0.00	8.10 ± 0.00	0.03 ± 0.00

Pigment content – Concentrations of allophycocyanin and phycocyanin did not vary in any PES concentration tested (allophycocyanin, F = 1.475, P > 0.05; phycocyanin F = 3.896, P > 0.05), nor in the presence or absence of TRIS (allophycocyanin, F = 2.142, P > 0.05; phycocyanin, F = 2.301, P > 0.05) (Fig. 2).

Both chlorophyll *a* and phycoerythrin concentrations were affected by PES concentration and presence or absence of TRIS, as demonstrated by the significant interaction between these factors (chlorophyll *a*, F = 5.540, P < 0.05; phycoerythrin F = 6.973, P < 0.05). The highest concentrations of chlorophyll *a* were observed in branches cultivated in seawater enriched with 12.5 and 25% of PES-TRIS (Fig. 2). The chlorophyll *a* content of branches cultivated in all concentrations of complete PES was similar to those of branches cultivated in PES-TRIS at 25% and 50%. Higher concentrations of phycoerythrin were observed among branches cultivated in seawater enriched with 12.5 and 25% of PES-TRIS, and in branches cultivated in complete PES at 50% (Fig. 2). Although pigment content varied according to culture condition, there was no color variation among branches.

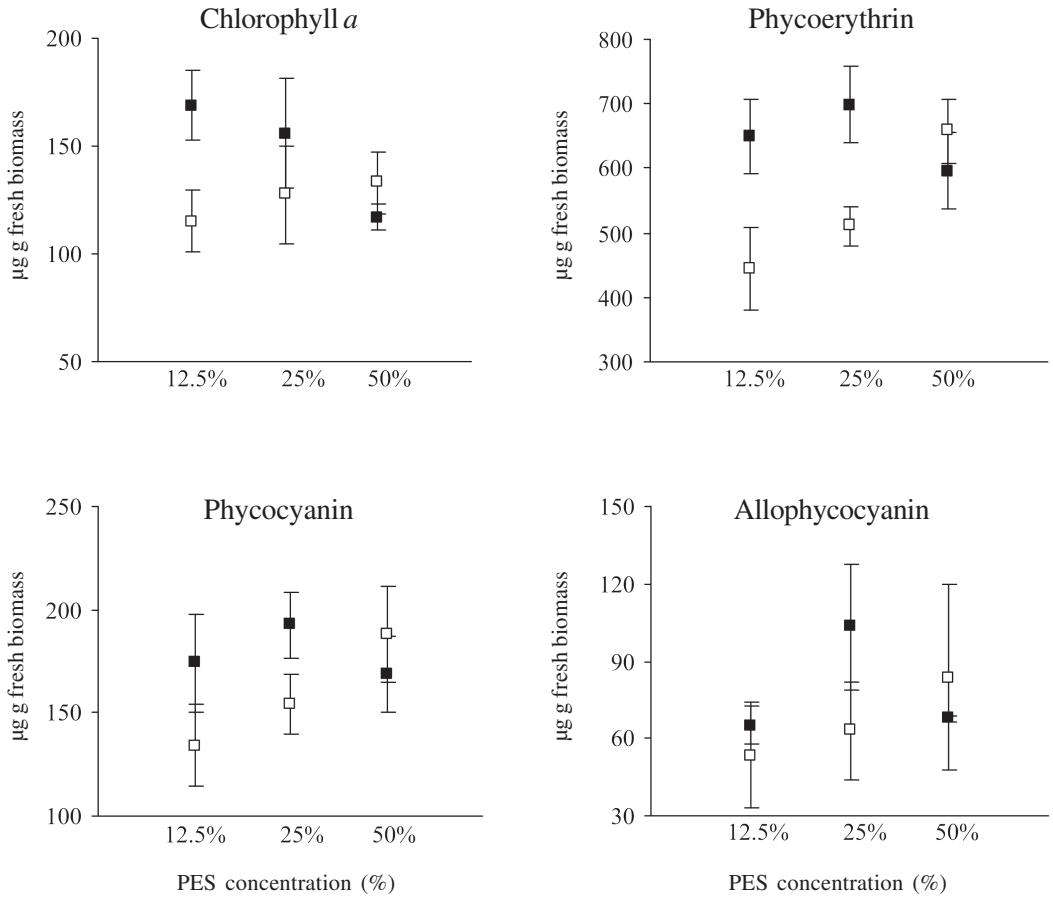

Figure 2. Chlorophyll *a*, phycoerythrin, phycocyanin and allophycocyanin concentrations of *Gracilaria birdiae* Plastino & E.C. Oliveira cultivated in seawater enriched with different concentrations (50, 25, and 12.5%) of PES with (PES) and without TRIS (PES-TRIS). Means (n = 3) ± Standard deviation.

Discussion

The higher growth rates of *Gracilaria birdiae* observed in PES without TRIS when compared to PES with TRIS corroborates the idea that the eventual benefits of this buffer in minimizing pH fluctuation may be smaller than its deleterious effects. However, it is clear that a consensus about TRIS effects does not exist because they vary among different organisms. From seven species of seaweeds cultivated in artificial marine culture medium (MCM - Woelkerling *et al.* 1983) with and without TRIS addition, five grew better in medium without the buffer, one showed worse growth in this condition and the other one was not sensitive to TRIS. The negative effect of this buffer was also reported for *Kappaphycus alvarezii*, which has shown lower growth rates in PES than in PES-TRIS (Paula *et al.* 2001), as observed for *G. birdiae*. The growth of some phytoplancton species was also lowered in cultures containing TRIS (Harrison *et al.* 1980).

Color variation among branches of *G. birdiae* cultivated in different conditions has not been observed. By contrast, *Kappaphycus alvarezii* (Paula *et al.* 2001) and *G. domingensis* (personal observation) showed changes in color when cultivated in media with TRIS. Although quantitative analysis of pigment was not performed on these species, it is reasonable to suppose that TRIS interfered in their pigment content, as observed here for *G. birdiae*.

Pigment analysis of *G. birdiae* showed that neither allophycocyanin nor phycocyanin was affected by TRIS or PES concentration, in contrast with phycoerythrin and chlorophyll *a*. The inner position of allophycocyanin and phycocyanin in the phycobilisome structure (Talarico 1996) may protect them from TRIS action, while phycoerythrin and chlorophyll *a* are probably more assessable to this buffer.

The type of interaction between TRIS and pigments is unknown. Our assumption is that this buffer may be negatively acting on nitrogen acquisition/assimilation, since phycoerythrin and chlorophyll *a* are highly nitrogenated molecules. Previous studies showed that low nitrogen availability diminishes the contents of phycoerythrin and chlorophyll *a* (Lapointe 1981; Lapointe *et al.* 1984).

A positive correlation was observed between chlorophyll *a* concentration and growth rates of *G. birdiae*, which is probably explained by an increase in the photosynthetic efficiency as the concentration of this pigment increased. In contrast with chlorophyll *a*, the content of phycoerythrin did not seem to be strongly related to the growth of *G. birdiae*. These results are expected since chlorophyll a plays a fundamental function in photosynthesis, while phycoerythrin is not essential for light-harvesting proposes if the light level is sufficient for algal metabolism. The metabolic pathway that involves phycoerythrin synthesis is complex. Apart from a light-harvesting function in photosynthesis, this pigment has a function in nitrogen storage (Lapointe 1981). This secondary function can explain the high concentration of phycoerythrin in 50% of PES, despite TRIS presence. The high nitrogen content in the medium might have compensated the negative effect of TRIS, maintaining the stability of phycoerythrin concentration.

Besides the deleterious effects of TRIS on growth and phycoerythrin and chlorophyll *a* content of *G. birdiae*, our results suggested that TRIS is not essential for buffering purposes. Its buffer effect was evident when culture medium was added to seawater. However, final pH values were similar between seawater enriched with PES and PES-TRIS, which showed that this effect was not maintained during the cultivation period. Studies on *Callithamnion byssoides* (Woelkerling *et al.* 1983) and on a variety of phytoplankton species (Harrison *et al.* 1980) also concluded that TRIS was not efficient in stabilizing pH during cultivation.

An increase of seawater pH during one week of cultivation was observed when PES was utilized, but it was not verified in PES-TRIS. These results suggest that pH increase might be attributed to bacterial growth and not to ordinary algal metabolism. The stimulatory effect of TRIS on bacterial growth has already been verified in microalgal cultures (Fábregas *et al.* 1993).

Our results suggested that *G. birdiae* might be adapted to a low nutrient content, since the higher growth rates were verified in higher PES dilutions. It demonstrates the need for adequate nutrient concentration, depending on intrinsic characteristics of each species. Dilutions of the original culture media can be more appropriate for laboratory culture of several species, especially those from oligotrophic warm waters. Therefore, it is not recommended the use of TRIS in future cultures of *G. birdiae*. Furthermore, this buffer can potentially impair the development of other species, being necessary further studies to evaluate how general this negative effect can be.

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