Enzymatic regulation of photosynthetic and light-independent carbon fixation in *Laminaria setchellii* (Phaeophyta), *Ulva lactuca* (Chlorophyta) and *Iridaea cordata* (Rhodophyta)

Regulación enzimática de la fotosíntesis y la fijación de carbono en obscuridad por *Laminaria setchellii* (Phaeophyta), *Ulva lactuca* (Chlorophyta) e *Iridaea cordata* (Rhodophyta)

ALEJANDRO CABELO-PASINI & RANDALL S. ALBERTE

1Instituto de Investigaciones Oceanológicas, Universidad Autónoma de Baja California, Apartado Postal 453, Ensenada, Baja California 22800, México, e-mail: acabello@faro.ens.uabc.mx
2Phycogen, Inc., 4 Moulton street, Suite 440, Portland, Maine 04101, U.S.A.

ABSTRACT

Carbon is acquired through photosynthetic and non-photosynthetic processes in marine algae. However, little is known about the biochemical regulation of these metabolic pathways along the thallus of seaweeds. Consequently, the objective of this study was to assess the distribution of in vivo carboxylation pathways and to relate them to the in vitro activity of ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBISCO), phosphoenolpyruvate carboxykinase (PEPCK), and phosphoenolpyruvate carboxylase (PEPC) in the Phaeophyte *Laminaria setchellii*, the Chlorophyte *Ulva lactuca*, and the Rhodophyte *Iridaea cordata*. Chlorophyll-a levels did not vary in *U. lactuca* and *I. cordata*. However, pigment levels were significantly lower in the meristematic region of *L. setchellii* probably as a result of a lack of differentiation of the chloroplasts in this region. Similarly, net photosynthesis did not vary in the thallus of *U. lactuca* and *I. cordata*, while it increased from the stipe and meristem towards the lamina of *L. setchellii*. In contrast to photosynthesis, light-independent carbon fixation rates were significantly greater in the meristematic region of *L. setchellii* suggesting a compensating mechanism for carbon incorporation in photosynthetically limited tissue. The activity of RUBISCO and PEPCK followed a pattern similar to that of in vivo carboxylation processes indicating that in vivo carbon assimilation is regulated by the activity of the carboxylating enzymes throughout the thallus of *L. setchellii*.

Key words: enzymatic activity, chlorophyll, light-independent carbon fixation, photosynthesis.

RESUMEN

La incorporación de carbono en algas marinas se lleva a cabo mediante procesos fotosintéticos y no-fotosintéticos. Sin embargo, poco se sabe sobre la regulación bioquímica de estas rutas metabólicas en el tejido de algas marinas. En consecuencia, el objetivo de este estudio fue el de evaluar la distribución de la carboxilación in vivo y relacionarlas a la actividad in vitro de ribulosa 1,5-bisfosfato carboxilasa/oxigenasa (RUBISCO), fosfoenolpiruvato carboxilasa (PEPCK) y fosfoenolpiruvato carboxilasa (PEPC) en la Phaeophyta *Laminaria setchellii*, la Chlorophyta *Ulva lactuca* y la Rhodophyta *Iridaea cordata*. Los niveles de clorofila-a no variaron en *U. lactuca* e *I. cordata*. Sin embargo, los niveles de pigmentos fueron significativamente menores en la región meristemática de *L. setchellii* probablemente debido a una falta de diferenciación de los cloroplastos de esta región. De una manera similar, la fotosíntesis neta no varió en el talo de *U. lactuca* e *I. cordata*, mientras que se incrementó desde el estipe y el meristema hacia la lamina de *L. setchellii*. En contraste con la fotosíntesis, la fijación de carbono en oscuridad fue significativamente mayor en la zona meristemática de *L. setchellii* sugiriendo un mecanismo para la compensación de la incorporación de carbono en tejido fotosintéticamente limitado. La actividad enzimática de RUBISCO y PEPCK se comportó de una manera similar a los procesos carboxilantes in vivo, indicando que la fotosíntesis y la fijación de carbono en oscuridad son reguladas por la actividad de las enzimas carboxilantes a lo largo del talo de *L. setchellii*.

Palabras clave: actividad enzimática, clorofila, fijación de carbono en oscuridad, fotosíntesis.
INTRODUCTION

The bulk of carbon is assimilated through photosynthetic processes in all autotrophic organisms, including marine algae (Kremer & Kuppers 1977). Besides photosynthetic carbon assimilation, there are other carboxylation processes that incorporate inorganic carbon through metabolic pathways that are independent of light reactions (Cabello-Pasini & Alberte 1997). Photosynthetic and light-independent carbon fixation (LICF) processes in marine algae have been shown to vary as a function of seasonal changes in irradiance and temperature, and carbohydrate levels in the tissue (Cabello-Pasini & Alberte 1997). Fluctuations in photosynthetic and non-photosynthetic carboxylation processes also vary as a function of tissue type in some Phaeophyta, however, essentially nothing is known about the regulation of LICF processes in different tissue of Chlorophytes and Rhodophytes.

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBISCO, EC 4.1.1.39) is the enzyme responsible for incorporating the carbon assimilated through the Calvin-Benson cycle in all autotrophic organisms. In contrast to photosynthesis, phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.49), and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) are the enzymes responsible for carbon assimilation via LICF reactions in most marine macroalgae (Kremer & Kuppers 1977, Cabello-Pasini 1996). Although the biochemistry of carboxylation enzymes has been studied extensively in crop species, little is known about the activity of these enzymes in the thallus of marine algae (Kremer & Kuppers 1977, Cabello-Pasini 1996). Vascular plants generally have well differentiated tissues, which show distinct physiological and biochemical functions (Ting 1982). In contrast, seaweeds are less differentiated, usually having photosynthetic holdfasts, stipes and blades as well as pigmented and non-pigmented cells and tissues (Pritchard & Bradt 1984). Among marine macrophytes, the Laminariales generally display the greatest tissue complexity with a well defined meristematic cells which divide rapidly and drive older tissue distally (Lobban et al. 1985). In this algal group, immature plastids are found in the meristem, and chloroplast differentiation follows the age gradient of the tissue (Grevby et al. 1989). This suggests that the photosynthetic potential in some algae might be limited by the lack of plastid development or reduced pigment levels.

In the Laminariales, photosynthetic and LICF processes have been shown to vary in response to the pattern of development and the type of tissue. Photosynthesis generally increases distal to the meristem in some Laminaria species, whereas LICF and respiration are greatest in the meristematic region (Kuppers & Kremer 1978, Kremer 1980, Kuppers & Weidner 1980, Arnold & Manley 1985, Cabello-Pasini 1996). Although the activity of RUBISCO and PEPC have also been found to vary throughout the thallus in the Laminariales, the spatial patterns of expression and localization of these enzymes are unknown (Kuppers & Kremer 1978).

The causative factors dictating differences in carboxylation processes are unknown and might play an important role in the ecophysiology of some marine macrophytes. Such fluctuations in carboxylation patterns might be the result of variations in enzymatic activity along the tissue of seaweeds. Consequently, the objective herein was to assess the in vivo carboxylation capacities along the thallus of Laminaria setchellii, Ulva lactuca and Irisidea cordata, and relate these to fluctuations in the activity of RUBISCO, PEPC and PEPC.

MATERIAL AND METHODS

Algal material

The Phaeophyte Laminaria setchellii, the Chlorophyte Ulva lactuca and the Rhodophyte Irisidea cornea were collected at Pacific Grove, California (36° 37' N, 121° 54' W) during the summer of 1993. The thalli were maintained in outdoor tanks with running water, constant aeration and under natural solar illumination. Experiments were conducted within 3 days of plant collection.

Pigment analysis

The concentration of chlorophyll-a in the thallus of L. setchellii was determined after dimethyl sulfoxide (DMSO) and acetone extractions of the tissues. Triplicate samples (1 cm²) were taken in the stipe, meristem and blade, and pigments were extracted at room temperature with 2 ml of DMSO for 15 min. Tissues were rinsed with 0.5 ml of distilled water and the rinse added to the DMSO extract. Samples were re-extracted with a 3:1:1 dilution of acetone:methanol:water in darkness for 2 h in a shaker table. Chlorophyll-a from the base, mid-blade and edge of the blade (approximately 1 cm², n = 3) of U. lactuca and L. cornea was extracted with 90 % (v/v) acetone and after
disrupting the tissue with a glass tissue homogenizer. The homogenate was centrifuged at 12,000 x G for 15 min and the pellet discarded. Blanks were generated using the same solvent volumes but omitting the tissues. The concentration of chlorophyll-a was determined in the three species by spectrophotometry using the equations of Seely et al. (1972).

In vivo carbon fixation

Rates of maximum oxygenic photosynthesis (P_{max}) and dark respiration were determined polarographically at 15 °C in triplicate samples from the three species. The stipe, meristem and mid-blade of _L. setchellii_, and the base, mid-lamina and edge of the blades of _U. lactuca_ and _I. cornea_ were sampled with a cork borer (1 cm³). Tissue samples were weighed and preincubated in FSW (0.2 μm) for 15 min in darkness. Maximum rates of photosynthesis (P_{max}) were then determined at 250 μmol quanta m⁻² s⁻¹ photosynthetically active radiation (PAR) for approximately 10 min. Dark respiration rates were determined in all tissue samples before and after the photosynthetic incubations. Respiration was determined in the oxygen electrodes by incubating the tissue in darkness for approximately 10 min.

Maximum in vivo photosynthesis (P_{max}) and in vivo LICF were determined by quantifying ¹⁴C assimilation as described by Cabello-Pasini & Alberte (1997). Tissue samples (1 cm³) were taken from the three species as described above and incubated in darkness with running seawater for at least 4 h to eliminate exudates. In vivo P_{max} was determined by incubating the tissues in filtered seawater (FSW, 0.2 μm) with 1 μCi mL⁻¹ of NaH¹⁴CO₃ (specific activity = 55 mCi mmol⁻¹) at 250 μmol quanta m⁻² s⁻¹ PAR for 15 min at 15 °C. In vivo LICF was determined similarly but tissue was incubated in darkness for 30 min. Tissues were rinsed with FSW after the incubation period and solubilized with 2 volumes of 0.5 N NaOH, 5 % (v/v) Triton-X-100 in darkness for 24 h. Pigments were oxidized with a 1:1 (v:v) dilution of 35 % H₂O₂ for 24 h and the non-fixed ¹⁴C volatilized overnight with 0.75 N HCl (final concentration). Ecolume (ICN) was added to the samples, vortexed until all the material was suspended and carbon assimilation determined by scintillation counting. Sample channel ratio corrections for quenching were applied to all the data. Counts were converted to nmol C gFW⁻¹ min⁻¹ and LICF/ P_{max} ratios calculated for each section of the thalli.

Enzymatic activity

In vitro PEPCK, PEPC and RUBISCO activities were determined by ¹⁴C-carboxylation of phosphoenolpyruvate (PEP) and ribulose bisphosphate (RUBP), respectively, modifying the method of Kremer & Kuppers (1977). Samples (n = 3, 1 cm²) were taken along the thalli of the three species as described above and the tissues frozen in liquid N₂. The tissues were grounded individually to a fine powder with mortar and pestle containing acid-washed quartz sand. Grounded tissue was homogenized with 20 volumes (w/v) of extraction buffer (EB, 50 mM MOPS [pH 7.2], 10 mM MgCl₂, 2 mM DTT, 5 mM EDTA, 5 mM MnCl₂, 0.5 mM 4-[1-aminoethyl]benzenesulfonyl fluoride [AEBSF], 0.5 % [v/v] Triton-X-100), and incubated in ice for 30 min. Samples were then centrifuged at 10,000 x G for 15 min at 4 °C and the supernatant saved for enzymatic analysis. Protein concentration was determined in the cell free extracts as described by Bradford (1976) and standardized against bovine serum albumin.

The in vitro PEPCK, PEPC and RUBISCO enzymatic assay system consisted of a 1:10 (v/v) dilution of cell free extract and assay buffer (AB, 50 mM MOPS [pH 7.2], 10 mM MgCl₂, 2 mM DTT, 5 mM EDTA, 5 mM MnCl₂, 10 mM NaHCO₃, 5 mM ADP, 5 mM NaCl and 2 μCi NaH¹⁴CO₃ [58 mCi/ mmol]). In the PEPC assay, ADP and MnCl₂, were omitted. PEPC and PEPC reactions were started with 5 mM PEP (final concentration) and incubated at 24 °C for 15 min. Blanks were obtained by omitting PEP from the reaction assay. In vitro RUBISCO activity was assayed following the same protocol but omitting MnCl₂ and ADP from the assay buffer. The assay for RUBISCO activity was started with 5 mM RUBP (final concentration) and incubated at room temperature for 15 min. Blanks were generated by omitting RUBP from the reaction assay. Assay reactions were stopped with 0.25 N HCl (final concentration) and incubated overnight on a shaker table. Ecolume was added to samples and carbon incorporation determined through scintillation counting as described above.

Statistical analysis

Significance of variations in chlorophyll levels, net photosynthesis, respiration, LICF levels, and enzymatic activity was determined by one-way analysis of variance (ANOVA) after testing for homoscedasticity and normality of the data (Sokal & Rohlf 1981). The Tukey’s test was conducted to evaluate statistical differences between specific tissue samples.
RESULTS

Chlorophyll-a levels

Chlorophyll-a levels in the thallus of *L. setchellii* fluctuated significantly relative to variations observed in *U. lactuca* and *I. cornea* (Fig. 1). The concentration of chlorophyll-a in the stipe and the meristem of *L. setchellii* were significantly lower (P < 0.05) than those observed in the mid-blade. In contrast to *L. setchellii*, levels of chlorophyll-a did not vary throughout the thallus of *U. lactuca* and *I. cornea*. In general, the concentration of chlorophyll-a in *U. lactuca* were significantly greater (P < 0.05) than those observed in the thallus of *L. setchellii* and *I. cornea*.

![Chlorophyll-a Levels](image)

**Fig. 1:** Chlorophyll-a levels as a function of the type of tissue in *Laminaria setchellii*, *Ulva lactuca* and *Iridaea cordata*. Bars indicate the mean of three replicates and error bars indicate one standard deviation.

Niveles de clorofila-a en función del tipo de tejido en *Laminaria setchellii*, *Ulva lactuca* e *Iridaea cordata*. Las barras indican el promedio de tres réplicas y las barras de error indican una desviación estándar.

**Fig. 2:** Net oxygenic photosynthesis (white bars) and dark respiration (filled bars) as a function of the type of tissue in *Laminaria setchellii*, *Ulva lactuca* and *Iridaea cordata*. Bars indicate the mean of three replicates and error bars indicate one standard deviation.

Fotosíntesis oxigenica neta (barras blancas) y respiración en oscuridad (barras negras) en función del tipo de tejido en *Laminaria setchellii*, *Ulva lactuca* e *Iridaea cordata*. Las barras indican el promedio de tres réplicas y las barras de error indican una desviación estándar.

**In vivo carboxylation**

Oxygenic photosynthesis and respiration fluctuated throughout the thallus of *L. setchellii* while no variations were observed in the thalli of *U. lactuca* and *I. cordata* (Fig. 2). In *L. setchellii*, oxygenic photosynthesis was low (P < 0.05) in the stipe and high in the blade, while no fluctuations were observed in the thalli of the other species. Maximum respiratory rates in the thallus of *L. setchellii* were observed in the meristem and minimum rates were recorded in the stipe (P < 0.05). In the other two species, there were no fluctuations in the respiratory rates throughout their thalli. In general, photosynthetic and respiratory rates were greater in *U. lactuca* than in *L. setchellii* and *I. cordata*.

The pattern of photosynthetic 14C-carbon incorporation was similar to that of oxygenic photo-
synthesis in all three species (Fig. 3). In *L. setchellii*, ¹⁴C incorporation was 3-fold greater (P < 0.05) in the blade than in the stipe and meristematic tissue, while in *U. lactuca* and *I. cordata*, ¹⁴C incorporation did not vary throughout their thalli. In vivo LICF rates in the thallus of *L. setchellii* were 3-fold greater in the meristem than in the stipe and blade. In contrast, LICF rates did not vary throughout the thallus of *U. lactuca* and *I. cordata*.

The incorporation of ¹⁴C through non-photosynthetic processes relative to photosynthetic carbon incorporation varied significantly (P < 0.05) in *L. setchellii*, while no fluctuations were observed in *U. lactuca* and *I. cordata* (Fig. 4). In the stipe and blade of *L. setchellii*, carbon incorporation through LICF processes was approximately 8% of photosynthetic incorporation while it reached 30% in the meristematic tissue. In *U. lactuca* and *I. cordata*, LICF incorporated less than 1% of the carbon assimilated photosynthetically throughout their thalli.

**Fig. 4:** Incorporation of carbon through non-photosynthetic methods relative to photosynthetic carboxylation as a function of tissue type in *Laminaria setchellii, Ulva lactuca* and *Iridaea cordata*.Bars indicate the mean of three replicates and error bars indicate one standard deviation.

Incorporación de carbono mediante procesos no fotosintéticos en relación a la carboxilación fotosintética en función del tipo de tejido en *Laminaria setchellii, Ulva lactuca* e *Iridaea cordata*. Las barras indican el promedio de tres réplicas y las barras de error indican una desviación estándar.

**Fig. 3:** Net photosynthetic ¹⁴C incorporation (white bars) and light-independent carbon fixation (filled bars) as a function of the type of tissue in *Laminaria setchellii, Ulva lactuca* and *Iridaea cordata*. Bars indicate the mean of three replicates and error bars indicate one standard deviation.

Incorporación de ¹⁴C fotosintético (barras blancas) y fijación de carbono en ausencia de luz (barras negras) como función del tipo de tejido en *Laminaria setchellii, Ulva lactuca* e *Iridaea cordata*. Las barras indican el promedio de tres réplicas y las barras de error indican una desviación estándar.

**Enzymatic activity**

*Rubisco* and *PEPCK* activity fluctuated throughout the thallus of *L. setchellii* following a pattern similar to that of in vivo photosynthetic and non-photosynthetic carbon incorporation (Fig.
5). RUBISCO activity rates were 5-fold greater (P < 0.05) in the blade than in the stipe and meristematic region, while PEPCK activity rates were 7-fold greater in the meristem than in the stipe and blade. Similar to in vivo photosynthetic and I.JICF rates, RUBISCO and PEPC activity did not vary (P > 0.05) in the thallus of *U. lactuca* and *I. cordata*.

There was a wide fluctuation in the PEPCK/RUBISCO activity ratio along the thallus of *L. setchellii*, while the same activity ratio did not fluctuate in the thallus of *U. lactuca* and *I. cordata* (Fig. 6). The activity of PEPCK was approximately 4-fold greater than that of RUBISCO in the meristematic region of *L. setchellii*, while similar activities were observed in the rest of the thallus. In contrast to that observed in *L. setchellii*, PEPC activity represented only 2 to 3 % of RUBISCO activity in the thallus of *U. lactuca* and *I. cordata*.

**Fig. 6**: Enzymatic activity of PEPCK relative to RUBISCO activity as a function of tissue type in *Laminaria setchellii* and enzymatic activity of PEPC relative to RUBISCO activity as a function of tissue type in *Ulva lactuca* and *Irishaea cordata*. Bars indicate the mean of three replicates and error bars indicate one standard deviation.

Actividad enzimática relativa de PEPC en función de la actividad enzimática de RUBISCO en diferentes tejidos de *Laminaria setchellii*, y actividad enzimática relativa de PEPC en función de la actividad enzimática de RUBISCO en diferentes tejidos de *Ulva lactuca* e *Irishaea cordata*. Las barras indican el promedio de tres réplicas y las barras de error indican una desviación estándar.

**DISCUSSION**

Variation in the photosynthetic and non-photosynthetic carbon acquisition has been
observed in the thallus of marine algae, especially in the Laminariales (Kuppers & Kremer 1978, Arnold & Manley 1985). Similarly, pigment levels have been shown to vary as a function of tissue age within a shoot, and within the same leaf in marine seagrasses (Mazzella & Alberte 1986). Such fluctuations in pigment levels might regulate, at least partially, the incorporation of carbon through photosynthetic processes. Indeed, the fluctuation of pigment constituents as a function of light treatments was demonstrated to regulate photosynthetic acquisition in Gracilaria tikvahiae (Lapointe & Duke 1984). In contrast to photosynthesis, the fixation of carbon throughout non-photosynthetic pathways in marine algae does not depend on pigment levels, and, as a consequence, carboxylation patterns in the thalli are probably regulated through other processes. This study demonstrates that photosynthetic and non-photosynthetic carbon fixation are regulated directly by enzyme activity, especially in the Phaeophyte Laminaria setchellii.

While there were no fluctuation in chlorophyll-a levels and photosynthetic carbon assimilation throughout the thallus of U. lactuca and I. cordata, the increase in Pn in the lamina of L. setchellii was consistent with the observed increase in chlorophyll-a levels. This suggests that photosynthetic carbon acquisition might be regulated, at least partially, by the concentration of pigments in the tissue. These results are consistent with pigment and photosynthetic fluctuations found in the tissue of other seaweeds and seagrasses (Lapointe & Duke 1984, Lewey & Gorham 1984, Mazzella & Alberte 1986, Gómez et al. 1995). The relatively low pigment levels in the meristematic region of L. setchellii are probably the result of lack of chloroplast differentiation often observed in this genus (Greby et al. 1989).

The patterns of in vivo Pn and LICF recorded here demonstrate that most of the 14C fixed in the three species studied occurs through photosynthesis. However, there appears to be a differential carbon acquisition in the thallus of L. setchellii. This is consistent with the high degree of morphological and histological complexity observed in kelps (Pritchard & Bradt 1984). Since the meristem is a region of rapid cell division and tissue growth, it might be limited by the low carbon acquisition through photosynthetic processes. It is likely that the rapid LICF rates partially compensate for the lack of photosynthetic carbon assimilation in this metabolically active region. In U. lactuca and I. cordata, LICF processes represent less than 1% of the carboxylation metabolism, and, as a consequence, they probably do not play a key role in the overall carbon assimilation pathway in these seaweeds. Finally, these results clearly indicate that estimates of photosynthesis and LICF in whole thalli of kelps can not be generated from measurements on single discs taken from blades, as conducted in other studies (Arnold & Manley 1985).

The different Pn and LICF activity patterns indicate that carboxylation patterns might respond to different regulatory processes within the same plant. In vitro RUBISCO, PEPC and PEPC activity closely followed the in vivo photosynthetic and non-photosynthetic carboxylation patterns in all species studied. These results are consistent with those obtained from other marine seaweeds (Kuppers & Kremer 1978, Cabello-Pasini 1996). Photosynthetic rates have been shown to vary as a function of RUBISCO activity (Mott et al. 1984), and as a consequence, fluctuations of in vivo photosynthetic activity are probably the result of regulatory processes linked to both pigment levels and RUBISCO activity in the tissue. While photosynthetic carbon acquisition is clearly influenced by pigment levels in the tissue, LICF must be regulated by other mechanism. These results demonstrate that non-photosynthetic carbon incorporation is likely regulated by the activity of PEPC in L. setchellii and PEPC in U. lactuca and I. cordata, and are consistent with results observed in other seaweeds (Kremer & Kuppers 1977, Cabello-Pasini 1996).

Respiratory rates followed a pattern similar to that observed for photosynthesis in U. lactuca and I. cordata. However, maximum respiratory rates in L. setchellii were observed in the meristematic and laminar region, which is consistent with results obtained by Cabello-Pasini (1996). This suggests that although chloroplasts in the meristem are not well differentiated, mitochondria and respiratory pathways are fully developed in young tissue of the Laminariales. This is consistent with the relatively high LICF rates found in the meristematic region, which probably compensate for limited photosynthetic carbon incorporation.

Carbon assimilation in marine algae is largely accomplished by light-dependent photosynthesis. However, there are active and significant light-independent carboxylation pathways operating as well (Cabello-Pasini 1996). Our results indicate that photosynthetic and non-photosynthetic carboxylation pathways are regulated, at least partially, by the activity of RUBISCO and PEPC. Furthermore, differences between the in vivo and in vitro carboxylation in the thallus of Laminaria setchellii suggest structural, biochemical and functional differences that impact the dynamics of production of kelp species.
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