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Alkaline phosphatase activity as a tool for assessing nutritional conditions in the seagrass *Posidonia oceanica* (L.) Delile*

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SUMMARY: The effects of experimental phosphorus enrichments on alkaline phosphatase activity (APA) of the seagrass *Posidonia oceanica* (L.) Delile were tested. Short-term additions (as phosphate, 12 hours, in the laboratory) decreased APA by 18-28%, depending on the plant part considered (roots, young leaves or old leaves). The values of APA after this treatment were well correlated with internal phosphorus pools (as P concentration in plant tissues). Long-term additions (as phosphate, added to the sediment, 1 month in situ) decreased APA by 40-75%, also depending on the plant part. We conclude that alkaline phosphatase activity is a good indicator of P deficiency in this seagrass. We used this indicator to assess the P-nutritional status in a series of meadows in the NW Mediterranean, finding a high geographical variability, but correlations between APA and basic features of the meadows (carbonate content of the sediment, organic content of the sediment, shoot density, etc.) were not significant. Consequently, phosphorus deficiency does not seem to be directly related to these descriptors.

Key words: Seagrasses, *Posidonia oceanica*, nutrient limitation, alkaline phosphatase, phosphorus.

RESUMEN: LA ACTIVIDAD FOSFATASA ALCALINA COMO INSTRUMENTO PARA LA EVALUACIÓN DE LAS CONDICIONES NUTRICIONALES EN LA FANERÓGAMA MARINA *POSIDONIA OCEANICA* (L.) DELILE. – Se analizaron los efectos de adiciones experimentales de fósforo sobre la actividad fosfatasa alcalina (APA) de la fanerógama marina *Posidonia oceanica* (L.) Delile. Las adiciones a corto plazo (como fosfato, 12 horas de duración y en el laboratorio) disminuyeron la APA en un 18-28%, según la parte de la planta considerada (hojas jóvenes, hojas viejas y raíces). Los valores de APA obtenidos después de la aplicación de dicho tratamiento se correlacionaron bien con el fósforo interno de las plantas (como concentración de fósforo en los tejidos vegetales). Las adiciones a largo plazo (como fosfato añadido al sedimento, durante 1 mes y en el campo) hicieron disminuir la APA en un 40-75 %, dependiendo también de la parte de la planta considerada. Se concluye que la actividad fosfatasa alcalina es un buen indicador de la deficiencia de fósforo en esta especie. Asimismo, se usó este indicador para evaluar el estado nutricional respecto al fósforo de una serie de praderas de *P. oceanica* del Mediterráneo Nor-occidental; la variabilidad geográfica hallada fue elevada, pero las correlaciones entre la APA y algunos descriptores básicos de las praderas (carbonato y materia orgánica del sedimento, densidad de haces, etc.) no resultaron significativas; en consecuencia, la deficiencia de fósforo no parece estar directamente ligada a los factores mencionados.

Palabras clave: Fanerógamas marinas, *Posidonia oceanica*, limitación por nutrientes, fosfatasa alcalina, fósforo.

INTRODUCTION

The assessment of the extent of nutrient limitation of seagrass growth is a basic question in coastal ecology, which is being addressed by an increasing number of authors (Orth and Moore, 1986; Harlin and Thorne-Miller, 1981; Dennison, 1987; Short 1987; Short *et al.*, 1990; Pérez *et al.*, 1991; Pérez *et al.*, 1994; Powell *et al.*,1989; Fourqurean *et al.*, 1992; Fourqurean, 1992). However, in general,

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intensive work (such as nutrient additions or nutrient budgets: e.g. Fourqurean *et al.* (1992), Pedersen and Borum (1993), among others) is needed to achieve this assessment, and thus extensive data on nutrient limitation of seagrass growth are very scarce. The indirect evaluation of nutritional status seems an alternative to the classic nutrient addition experiments; and the activity of key enzymes may be appropriate for this indirect approach.

Aquatic plants generally contain external alkaline phosphatases (Kuenzler and Perras, 1965). These enzymes are able to hydrolyze organic phosphate monoesters, releasing inorganic phosphate and increasing the availability of this nutrient for plant growth. Alkaline phosphatase activity increases when plant growth is phosphorus limited, both in phytoplankton (Kuenzler and Perras, 1965; Berman *et al.*, 1990; Gage and Gorham, 1985; Hino, 1988; Feuillade *et al.*, 1990) and in macroalgae (Atkinson, 1987; Weich and Granéli, 1989; Lapointe, 1989; Hernández, 1992) and this feature has been used in indirect evaluations of phosphorus deficiency. Recent findings also indicate significant alkaline phosphatase activity in seagrasses (Hernández, 1992; Pérez and Romero, 1993; Hernández *et al.* 1994), and, again, a link between this activity and phosphorus availability (Pérez and Romero, 1993).

In the Mediterranean sea, the seagrass *Posidonia oceanica* occupies large areas at depths between 0 and 40 m (Ros *et al.*, 1985), with a substantial contribution to the primary production of coastal waters. In recent studies, a phosphorus limitation of seagrass growth in some Mediterranean meadows has been reported (Pérez *et al.*, 1991; Alcoverro *et al.*, 1995).

Here, we examine the validity of measurements of alkaline phosphatase activity (APA) as an indicator of phosphorus nutritional status by:

(i) analyzing some basic features of the kinetics of this enzyme relevant to the sensitivity of the assay (i.e. influence of pH and temperature);

(ii) assessing the response of the alkaline phosphatase activity to short-term (i.e. 12 hours) and long.term (i.e. 1 month) inorganic phosphate addition;

(iii) measuring the alkaline phosphatase activity in a series of *Posidonia oceanica* meadows encompassing both geographical and environmental variability; and

(iv) examining correlations between enzyme activity and some descriptors of the nutritional status of the environment (both from the plant and from the sediment).

METHODS

Optimization of phosphatase activity measurement

The effects of assay pH and temperature were tested using plants from a single site (Medes Islands, 5 m depth, see below), collected by scuba-diving and transported in refrigerated seawater to the laboratory.

Phosphatase activity was first measured within a pH range from 3.7 to 9.7, using p-nitrophenyl phosphate (p-NPP) as substrate. The pH of the incubation media was adjusted by the addition of different quantities of two buffers: citrate-hydrochloric for pH 3.7-7 and Tris buffer for pH 8-10. Following incubation, pH was adjusted at 9.5 and absorbance was measured at 410 nm in a Perkin-Elmer Lambda2 UV/VIS spectrophotometer.

To assess the effect of the assay temperature on the phosphatase activity, we chose the pH giving the highest activity (pH=9.7, see results), and performed a set of incubations at 9, 15, 20, 24 and 30 ^{o}C. Q_{10} was computed for the range 9-24^{o}C following Valiela (1984).

On the basis of these results, further assays were performed at pH=9.7 and 24^{o}C. Thus, data presented in this paper correspond to alkaline phosphatase activity (APA).

In all cases, the volume of the incubation medium was 200 ml (seawater from the sampling site plus reagents), to which approximately 0.2-0.4 g dry weight of different plant parts (young leaves, i.e. inner leaves without macroscopic epiphytes; old leaves, i.e. outer leaves including epiphytes; roots) were added. Incubation lasted for two hours, under natural saturating irradiance (i.e. above 400 $\mu E\ m^{-2}\ s^{-1}$, Alcoverro, unpublished results); assay vessels were manually stirred at 10' intervals during the 2 h of incubation.

Four replicates were assayed for each experimental condition; after incubation, plants were dried at 70^{o}C until constant weight, and results are expressed as $\mu mol\ PO_4$ released g dry $wt^{-1}\ h^{-1}$.

More details on the analytical procedure can be found in Kuenzler and Perras (1965), and Lapointe (1989); specific details concerning its application to seagrasses are explained in Pérez and Romero (1993).

Alkaline phosphatase activity spatial variability

To study APA variability, seven areas with *Posidonia oceanica* meadows, covering a wide geo-

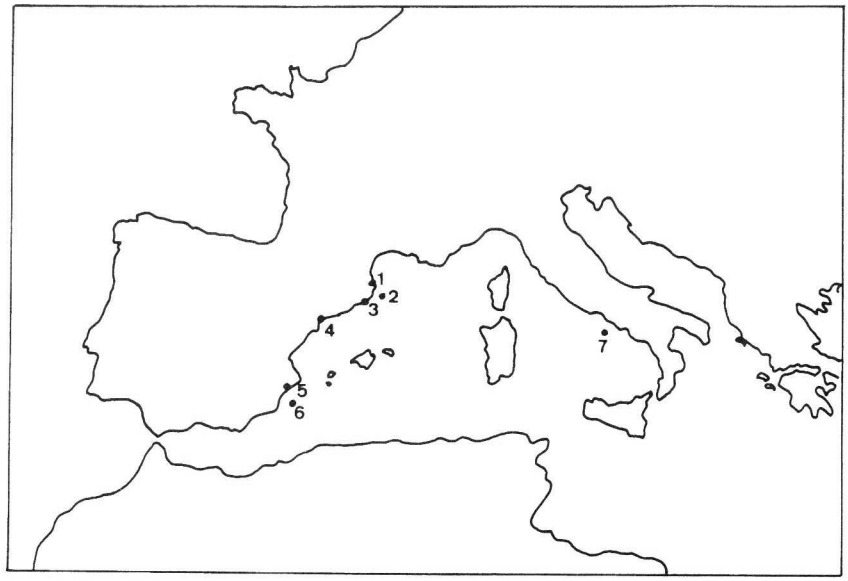

FIG. 1. – Geographical situation of the meadows studied. (1) Port-Lligat, (2) Medes Is., (3) Giverola, (4) Ametlla, (5) Campello, (6) Tabarca Is., (7) Ischia Is.

graphical and environmental range (Fig. 1), were selected. Whenever possible, samples were collected at different depths within the same area, resulting in a total of 17 sampling sites (Table 1). Additional details on these sites have been previously reported (Romero, 1989; Buia *et al.*, 1992; Sanchez-Lizaso, 1993; Alcoverro *et al.*, 1995).

Sampling was performed in summer, the period of maximum nutrient limitation (Alcoverro *et al.*, 1995). At each site, entire shoots of *Posidonia* were collected by hand and immediately transported in refrigerated seawater to the laboratory. At the same sites, surface (0-3 cm) sediment samples were collected by hand.

TABLE 1. – General features of the *Posidonia oceanica* meadows sampled. Phosphorus and nitrogen concentrations (relative to dry weight) of young leaves are given, as well as the organic and carbonate content of the sediment (OM and $CaCO_3$ respectively).

SITE	DEPTH	DENSITY	% N	% P	% OM.	% $CaCO_3$
	m	shoots m-2	(young leaves)			
PORT LLIGAT	1	949	1.697	0.113	2.39	26.32
	4	324	1.308	0.109	1.71	13.11
MEDAS	5	628	2.738	0.142	1.03	82.98
	13	340	2.628	0.075	1.19	81.43
GIVEROLA	5	-	2.375	0.19	-	-
	9	-	1.879	0.135	-	-
L'AMETLLA	4	-	1.815	0.105	1.16	30.67
	7	-	1.708	0.098	1.08	38.28
CAMPELLO	3	263	1.309	0.082	1.73	89.11
	8	153	1.441	0.111	2.89	75.65
TABARCA	5	1074	1.682	0.055	1.76	40.58
	10	458	1.518	0.09	2.17	61.45
	20	378	1.643	0.099	1.54	36.65
ISCHIA	5	400	2.263	0.137	2.37	17.49
	10	310	1.887	0.126	4.47	18.86
	20	250	2.192	0.114	3.38	12.97
	30	100	1.629	0.091	2.87	27.98

In the laboratory, a set of shoots was kept in an aquarium with P-enriched seawater (10 µM), and a second set was kept in another aquarium with natural seawater as control. After 12 hours, shoots from both sets were sorted into young leaves, old leaves and roots (plant parts hereafter) as described in Pérez and Romero (1993), and alkaline phosphatase assays were performed as described.

The influence of long-term inorganic phosphorus availability on alkaline phosphatase activity was assessed by experimental field additions of phosphorus. At each of two sites (Port Lligat, 4 m deep and Medes Islands, 5 m deep: see Table 1), two plots were marked: one was fertilized by introducing slow-release fertilizers (2 g P m^{-2}) in the sediment and the other was kept as control. The APA was measured in shoots collected from the two plots one month after fertilization.

Subsamples of plant parts from all sampling sites and fertilized plots were dried (70ºC until constant weight) and analyzed for N and P concentration. Nitrogen concentration in plant tissues was determined by a Carlo-Erba autoanalyzer; phosphorus was determined by ICP, following wet acid digestion in a microwave oven (Sommers and Nelson, 1972; Mateo and Sabate, 1993).

The organic matter in the sediment was measured as the weight decrease after ashing (450 ºC, 5 hours). The carbonate content of the sediment was obtained as the difference in carbon content (as measured by a Carlo-Erba autoanalyzer) in sediment before and after treatment with hydrochloric acid (Navarro *et al.*, 1993).

Statistical procedures

The effects of pH and temperature were tested using a two-way (plant part and pH or temperature) ANOVA. The effects of 12 hour P-treatment, spatial variability and plant part were tested using a three-way ANOVA. The effects of in situ fertilization were tested using three-way (site, fertilization and plant part) ANOVA. When necessary, Tukey post-hoc test was used to test differences between means.

RESULTS

Influence of pH and temperature on APA

Phosphatase activity was influenced by the pH of the assay medium ($p< 0.001$, Fig. 2). The response differed following the different plant parts (as shown by the significant interaction: $p<0.001$); apparently, young leaves and roots presented two peaks of activity, at pH=4.6 and pH=9.7, but while low pH maximum was significant for young leaves (Tukey post-hoc test, $p=0.02$), it was not the case for roots ($p=0.9$). Old leaves only presented a maximum at pH=9.7.

FIG. 2. – Effect of pH on phosphatase activity of the different parts (old leaves, young leaves and roots) measured. Vertical bars are standard error of the mean, with n=4.

Temperature had a significant effect on APA ($p<0.001$). Increasing temperatures increased APA until a certain threshold (24 ºC), after which an inhibitory effect was found (Tukey post-hoc test, $p=0.02$; Fig. 3). A Q_{10} of 1.4 was computed for the temperature range of 9-24 ºC.

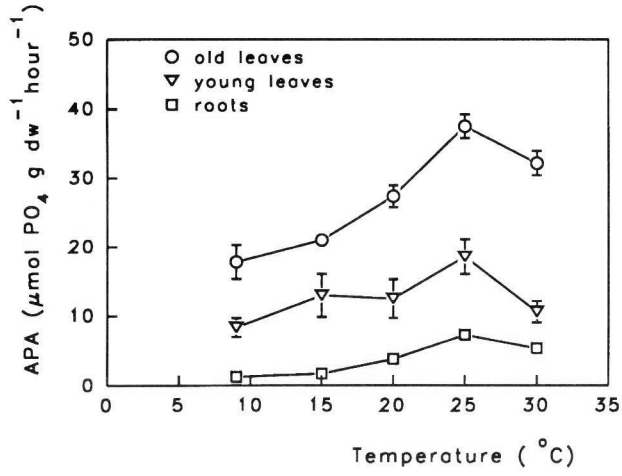

FIG. 3. – Influence of temperature on alkaline phosphatase activity of the different parts (old leaves, young leaves and roots) measured. Vertical bars are standard error of the mean, with n=4.

TABLE 2. – Results of the ANOVA performed on data of APA spatial variability.

SOURCE OF VARIABILITY	df	MEAN SQUARES	% VARIABILITY	F	p
SITE	14	113.7	33.4	54.97	<0.001
P - TREATMENT	1	136.6	2.9	66.04	<0.001
PLANT PART	2	815.9	34.2	394.53	<0.001
SITE X TREAT.	14	15.5	4.6	7.48	<0.001
SITE X PART	28	18.3	10.8	8.84	<0.001
TREAT. X PART	2	16.8	0.7	8.11	<0.001
S x T x P	28	6.3	3.7	3.02	<0.001
ERROR	225	2.07	9.8		

Variability in APA

The effects on APA variability of plant part, 12 hour P-treatment and site of collection are summarized in Table 2. To perform this analysis, the data from Giverola sites were omitted due to the lack of data for the treated plants.

Most of the variability is explained by the site of collection and the plant part. Alkaline phosphatase activity is, in general, highest in old leaves and lowest in roots, but differences among plant parts depend on site (as shown by the significant interaction part x site, Table 2). 12 hour P-treatment resulted in a significant decrease in APA; the extent of the reduction depended on the plant part (as shown by the significant interaction, Table 2), and averaged 21% for old leaves, 28% for young leaves and 18% for roots (Fig. 4).

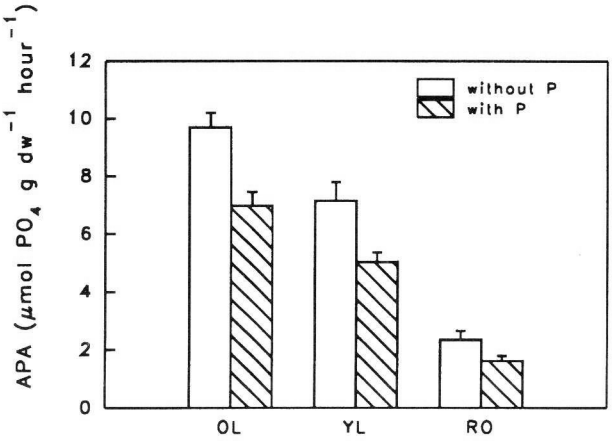

FIG. 4. – Effect of short-term P inorganic addition on alkaline phosphatase activity of the different parts (old leaves, young leaves and roots) measured. Data from the different meadows have been pooled, and verticals bars are standard errors.

The long-term (1 month) addition of inorganic phosphorus to the sediment also showed a significant effect (ANOVA, $p < 0.001$), decreasing APA by 40-75% relative to controls (Fig. 5). This decrease was only significant for young and old leaves (interaction: $p<0.001$, and Tukey post hoc test, $p < 0.001$ in all cases). However, the increase in phosphorus concentration in plant tissues after fertilization was only weakly significant (ANOVA, $p=0.056$).

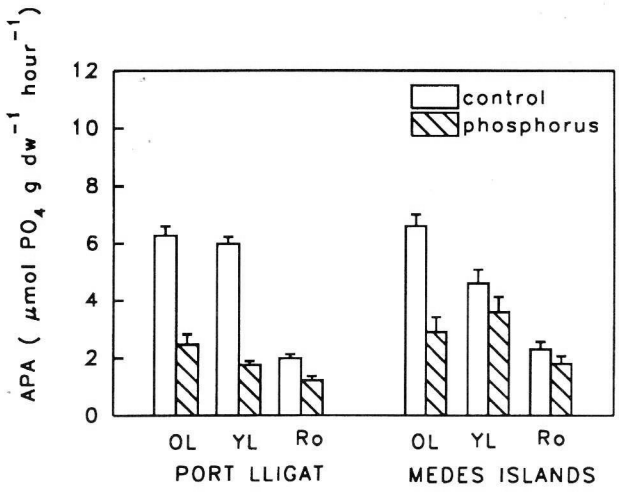

FIG. 5. – Effect of long-term P inorganic additions on alkaline phosphatase activity in the two meadows studied. Vertical bars represent standard errors.

DISCUSSION

Phosphatase activity was maximum at the highest pH assayed (9.7), similar to that found for other seagrasses (Hernández, 1992). The response to temperature showed a maximum at 24 ºC and then a decrease. This temperature is close to the maximum

values that these plants are exposed to in their natural environment. Other seagrasses living under higher temperatures did not show this decrease (Hernández *et al.*, 1994). The Q_{10} value found in the range 10-24 ºC is similar to that found in other macrophytes and lower than 2, indicating low sensitivity to temperature increments (Price and Stevens, 1982; Hernández, 1992).

Differences in APA between the different plant parts follow the same pattern previously reported (Pérez and Romero, 1993). The high values found in old leaves are due, at least in part, to the associated epiphytes: it is known that APA is generally higher in algae than in seagrasses e.g. (Lapointe, 1989).

Our results also confirm the decrease in alkaline phosphatase activity in *Posidonia oceanica* after phosphate addition. This decrease occurs following not only long-term (i.e. 1 month) treatment (Pérez and Romero, 1993) but also short-term treatment (i.e. 12 hours), being higher in the former (40-75 %) than in the later (22% on average). The dependence of APA on P-nutritional status is thus complex. To confirm this, we examined the correlation between APA and phosphorus concentration in plant tissues (which has been used as an indicator of nutritional status: Duarte, 1990; Pérez *et al.*, 1991; Fourqurean *et al.*, 1992). Althought no correlation was found between APA and phosphorus concentration in leaves of untreated plants ($r = -0.14$, $p = 0.52$), a significant negative correlation was found between APA measured after short-term phosphorus additions and phosphorus concentration in leaves (Fig. 6, $r = -0.56$, $p < 0.01$). This suggests a double control on alkaline phosphatase activity: a short-term control (i.e. decrease in measured activity after a 12 h P-treatment), which is probably linked to an inhibition of the activity of the enzyme, and a long-term control (i.e. decrease in measured activity after 1 month P-treatment), probably linked to an inhibition of enzyme synthesis. However it should be noted that the precise nature of the control of APA has not yet been established (Cembella *et al.*, 1983), and it seems clear that control is exercised at different interacting levels (enzyme activity, protein synthesis, gene expression) involving different time scales. In any case, we have evidence that the APA remaining after a 12 hour P-treatment (constitutive APA) depends on internal phosphorus pools (Fig. 6), and can be considered as an indicator of P nutritional status averaged over, at least, weeks. This is in agreement with the results of Hernández *et al.*, (1994), who found a good correlation between APA and phosphorus concentration in tissues from plants not previously treated but growing in a phosphorus-rich estuarine environment.

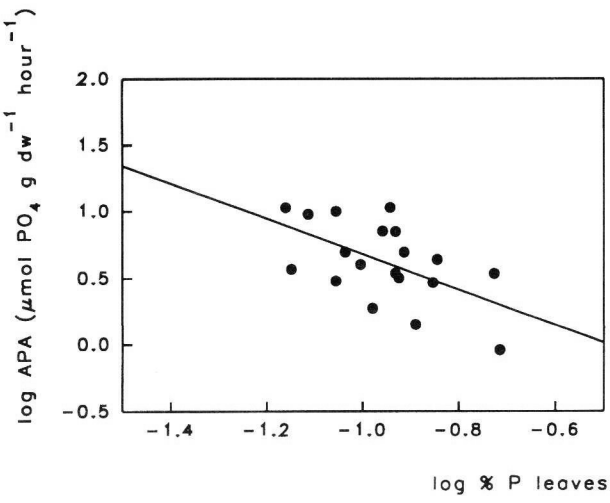

FIG. 6. – Relationship between P concentration in leaves and alkaline phosphatase activity after the P- treatment.

The spatial variability observed in APA (33% of total, Table 2) cannot be explained by the meadow features considered (shoot density, depth, carbonate content...Table 1). Correlation between such features and constitutive APA (as defined above) were non-significant in all cases. Thus, accepting that constitutive APA is a good indicator of basal nutritional conditions, the lack of correlation between APA and carbonate content of the sediment seems to be in contradiction with results of Short *et al.* (1985). Probably, there are other factors obscuring the relation between carbonate and phosphorus: as has been mentioned by other authors (Howarth et al., in press), the effectiveness of trapping of P by sediments may depend also on rates of sulfate and iron reduction, which in turn depend on organic matter inputs and temperature.

In summary, alkaline phosphatase activity depends on the nutritional status of the plant. The APA remaining after 12 hour of P treatment is correlated with the internal phosphorus pools, while the APA of untreated plants depends on both internal and external (i.e. phosphate concentration in the water column) phosphorus supplies. Geographical variability in APA and, consequently, in nutritional status of the plants, is not directly linked to the basic features of the sediment, such as organic and carbonate content, and thus these descriptors, in the geographical range considered (i.e. NW Mediterranean) cannot be used as predictors of possible phosphorus limitation of growth.

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