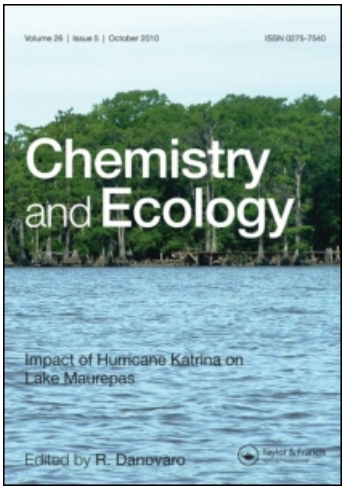

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Cytophysiological features of *Posidonia oceanica* as putative markers of environmental conditions

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CYTOPHYSIOLOGICAL FEATURES OF *POSIDONIA OCEANICA* AS PUTATIVE MARKERS OF ENVIRONMENTAL CONDITIONS

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(In final form 19 February 2004)

Posidonia oceanica beds form one of the most important coastal ecosystems in the Mediterranean basin and represent a good indicator of the seawater quality, being strongly sensitive to human perturbations. The aim of the present study is to verify whether changes in cytophysiological features of this seagrass could mark early altered environmental conditions. In this context, we investigated in juvenile and intermediate leaves: (1) the presence and structure of phenolic compounds, due to their role in the defence response against biotic and abiotic stress; (2) the presence and distribution of cytokinins, because of their key role in leaf growth and senescence as well as in relation to their interaction in light signalling. Significant and measurable increases in the amount of phenols as well as changes in cytokinins distribution were detected in *Posidonia* leaves collected from meadows subjected to disturbed environmental conditions as compared to those of preserved ones. These results clearly indicated that these variables could be used as early biomarkers of environmental quality.

Keywords: *Posidonia oceanica*; Phenolics; Cytokinins; Environmental stress

1 INTRODUCTION

Posidonia oceanica (L.) Delile is an endemic phanerogam to the Mediterranean basin which forms widespread meadows along the coastal infralittoral and plays a pivotal role in the coastal system. In fact, this marine phanerogam is responsible for high primary production and water oxygenation, and constitutes refuges and nurseries for economically relevant animals (Ott, 1980; Harmelin-Vivien, 1982; Mazzella *et al.*, 1995). In addition, this seagrass forms a natural belt, which protects adjacent coasts against erosive wave action (Jeudy de Grissac and Bouderesque, 1985; Colantoni, 1995).

P. oceanica is particularly sensitive to marine pollution (e.g., discharge of sewage, industrial wastes) development activities (e.g., dams, ports, breakwaters), and trawling, all these impacts having the potential to determine the regression of *P. oceanica* meadows (Boudouresque and Meinesz, 1983; Ardizzone and Pelusi, 1984; Astier, 1984; Meinesz *et al.*, 1985). As a consequence, changes in distribution patterns, density, biomass, and dynamic features

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of seagrass communities have been widely used to obtain an integrated response to disturbances (Pergent *et al.*, 1995; Marbà *et al.*, 1996; Guidetti and Fabiano, 2000). Recently, leaf production and rhizome elongation rates have also been employed to evaluate the anthropogenic impact of the decreased water clarity (Guidetti, 2001).

In animals, changes in physiological and biochemical variables, which allow early detection of pollution before the appearance of any visual sign of damage, have been largely developed (Contour-Ansel and Louget, 1986). On the contrary, in plants, biomarkers have been applied more recently; these include measurable responses such as photosynthetic activity, enzymatic processes of nutrition, secondary metabolite synthesis, oxidative stress, and/or detoxication mechanisms (Ferrat *et al.*, 2003).

Similarly to land plants, which, in heavily polluted areas, exhibit changes in physiological and biochemical traits (Malhotra and Khan, 1984; Darral, 1989; Dixon and Paiva, 1995), aquatic plants could also act as biomonitors of environmental quality, through the use of biomarkers (Ferrat *et al.*, 2003). In this context, phenolic compounds, tannins, and related phenolic substances, which are common in marine plants, could provide insights into sea quality. In marine vascular plants and brown algae, these reducing substances are components of cell walls and, in seagrasses, can act as putative osmoregulators (Schoenwaelder and Clayton, 1999). In addition, phenolics have several secondary roles: many of them are antimicrobial agents, presumed to protect macrophytes against pathogen attacks (Vergeer and Develi, 1997); some have been identified as herbivore deterrents (Pavia and Toth, 2000), and others have been verified to protect macrophytes against harmful ultraviolet radiation (Pavia *et al.*, 1997).

A second variable that we considered as a possible environmental marker are cytokinins, due to the wide spectrum of their actions (Brault and Maldiney, 1999; Mok and Mok, 1994, 2001), which include the enhancement of secondary metabolites synthesis (Biddington and Thomas, 1973; Mérillon *et al.*, 1991; Brault and Maldiney, 1999) and the control of cell proliferation (Laureys *et al.*, 1998; Riou-Khamlichi *et al.*, 1999; Francis and Sorrell, 2001). Moreover these hormones are clearly involved in chloroplast differentiation (Parthier, 1979; Chin-Atkins *et al.*, 1996), leaf senescence (Brault and Maldiney, 1999; Ori *et al.*, 1999; McCabe *et al.*, 2001; Pyung *et al.*, 2003), as well as in plant photomorphogenesis (Chaudhury *et al.*, 1993).

Noteworthy, in the marine environment, light could be a limiting factor in the presence of pollution and water turbidity. Plants are particularly sensitive to the light environment, which they scrutinize with photoreceptors; thus, in natural environments, the phytochromes control the shade-avoidance response (Smith, 1995; Maloof *et al.*, 2000). A recently published study by Fankhauser (2002) dealing with light/hormones interactions showed that cytokinins modulate phytochromes B-mediated light signalling. This result strongly suggests a role for cytokinins in the response of plant to diminished light intensity.

On these bases, we have compared here the amount and localization of phenolic compounds (BFs) and cytokinins in *P. oceanica* leaves, sampled from meadows in disturbed and preserved sites of the Calabria coast (Italy). These meadows were differing also in macroscopic features, such as shoot density (according to Giraud, 1977). This study is aimed at verifying whether the above-mentioned variables undergo changes in the marine phanerogam *P. oceanica* in response to disturbed environmental conditions, allowing, in this way, their use as biomarkers.

2 MATERIALS AND METHODS

2.1 Sampling

P. oceanica shoots were sampled by scuba divers from two sites of the Tyrrhenian Sea in March 2002, at about 12 m depth. The two sites present different environmental conditions:

the presence of a wet basin and anthropogenic waste in the first site (Site A), with a clear regression of the meadow (class III 'sparse meadow', according to Giraud (1977)); and a well-preserved environment in the other site (Site B, *i.e.* the control) (Class II 'dense meadow', according to Giraud, 1977). Five sampling areas were chosen at distance of 25 m both in the disturbed site (site A) and in the control site (site B).

The leaves of *P. oceanica* were separated into: adult leaves (with blade and sheath), intermediate (with blade >50 mm and without/or sheath <2 mm) and juvenile leaves (young leaf with blade <50 mm and without sheath) (Giraud, 1977). On young and intermediate leaves, the localization of both phenolics compounds and cytokinins was performed.

2.2 Histolocalization of Phenolic Compounds (BPs)

The presence of BPs was investigated on cross-sections of both young and intermediate leaves by using differential staining methods, according to Gutmann (1995). Analyses were performed on three leaf portions: the basal region (undifferentiated and light green), the medial region, and the apical region (differentiated and green). The total surface covered by the BPs compound in the mesophyll cells was estimated by scoring all the serial sections of three different leaves (for each sampling area ($n = 15$), both at site A and site B, by using a Leica Q500/W image analyser equipped with a CCD camera. Statistical differences were evaluated by Student's *t*-test. The area covered by BPs, with respect to the whole mesophyll area, was calculated and expressed as a percentage.

The BPs were classified into different morphological classes in relation to their organization and localization in the leaf mesophyll cells.

2.3 Localization of Cytokinins

Immunolocalization of zeatin was performed, on cross-sections, in the basal and apical parts of juvenile and intermediate leaves, according to the procedure described by Dewitte *et al.* (1999). Samples were fixed in a 0.5% (v/v) gluteraldehyde and 3% (w/v) paraformaldehyde mixture in PBS (135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM K_2HPO_4, pH7.2) at 4 °C for 3 h. Infiltration was achieved by vacuum during the first 30 min. Thick sections (20–25 μm) were cut using the vibratome (Leica VT1000E, Germany) and collected in PBS on ice in the wells of a tissue culture plate. Subsequently, floating vibratome sections were incubated in blocking buffer [0.5% (w/v) BSA, 0.1% (v/v) fish gelatin, 1% (v/v) normal goat serum, 20 mM glycine in PBS] three times for 10 min and in a 0.07% saponin solution in PBS for 20 min. Afterwards, the sections were incubated with a primary antibody against zeatin in a dilution of 1:100 in blocking buffer overnight at 4 °C, followed by 1 h at room temperature.

After three washings (three times for 10 min) with PBS, sections were incubated for 3 h at room temperature with the secondary antibody goat anti-rabbit IgG (1:100, in blocking buffer) conjugated with alkaline phosphatase (Boehringer, Germany). After three washings (three times for 10 min) with PBS, sections treated with alkaline phosphatase conjugates were rinsed with Tris–HCl buffer (0.1 M Tris, 2 mM $MgCl_2$, pH 9.5) and left to react in the presence of nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Boehringer, Germany) for 5 min. The enzymatic reaction was stopped by 2 mM EDTA in PBS. Samples were mounted in a PBS/glycerine mixture (1:1 v/v) and immediately observed under a Leica DMRB photomicroscope. The number of labelled cells with respect to the total mesophyll cells was estimated and expressed as a percentage, by scoring all the serial sections of three different leaves for each sampling area ($n = 15$), at both site A and site B.

3 RESULTS

3.1 Phenolics

The presence of BPs was investigated in cross-sections of juvenile and intermediate *P. oceanica* leaves. Our observations on leaf mesophyll allowed us to distinguish four different typologies of phenolic inclusions, in relation to their organization and structure in the vacuole and/or in the cell (Fig. 1). Essentially, we have distinguished chain-like, ring-like, reticulate-like, and drop-like inclusions, depending on the grade of BP aggregation in the cell (Fig. 1a–d).

Noticeable differences were observed among BP typologies in mesophyll cells of juvenile leaves collected in the disturbed and the preserved site, whereas no difference was recorded in intermediate leaves (Fig. 2). As far as juvenile leaves are concerned, the most significant differences dealt with a higher number of drop-like inclusions (*i.e.* cell totally impregnated by BP inclusions) at the disturbed site (Fig. 2a) than at the control site (Fig. 2b). Significant differences ($P < 0.001$) in the amount of BPs, estimated as the covered cell area, were also detected between the two sites: a greater occurrence of BPs was evident in the plants from site A compared to site B (Fig. 2e). Moreover, the percentage area covered by all BPs inclusions with respect to the whole leaf mesophyll, was notably higher at site A than at site B, with the higher value in the apical part in both juvenile and intermediate leaves (Fig. 2f).

3.2 Cytokinins

Similarly to BP analysis, immunolocalization of zeatin (Z) was performed on cross-sections in the basal and apical part of juvenile and intermediate leaves. The results presented here show clear differences between leaves collected from the disturbed site (Site A) and those from the control site (Site B).

As a general rule, the number of reactive cells was higher in juvenile leaves from the control site (80%) than in those from the disturbed site (50%) (Fig. 3). On the contrary, in intermediate leaves, the labelled cells show a similar percentage (60%) for both the control and the disturbed site. Furthermore, a preferential distribution of these cells in the basal zone (growing part) of both juvenile and intermediate leaves was observed (Fig. 3c, d, g and h). However, the most relevant differences deal with cytokinin distribution. Namely, in the basal zone of juvenile control leaves, the reactive cells were detected in the whole mesophyll

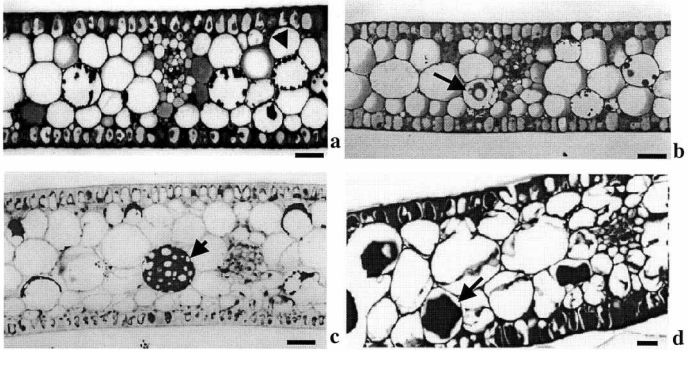

FIGURE 1 Histolocalization of phenolic compounds on a cross-section of *P. oceanica* leaves. The different typologies of phenolic inclusions are visible: (a) chain-like; (b) ring-like; (c) reticulate-like; (d) drop-like. Arrows indicate the phenolic inclusions. Bars: 23 μm.

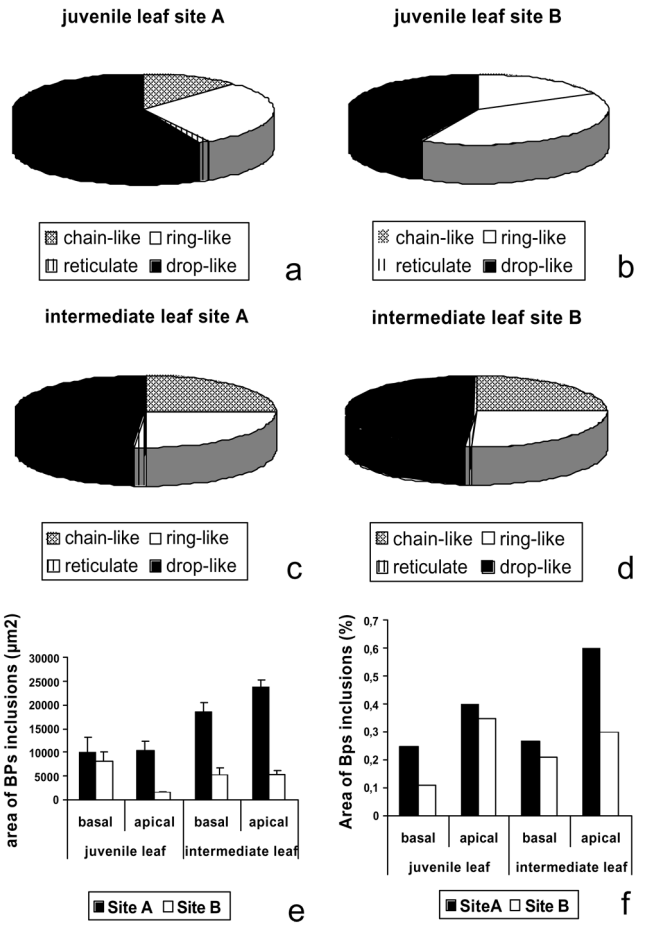

FIGURE 2 Typologies of phenolic inclusions (%) in (a, b) juvenile and (c, d) intermediate *P. oceanica* leaves sampled in the site A (disturbed site) and site B (control site). (e) Total area of BPs and (f) percentage of cell-covered area estimated in mesophyll cells of the basal and apical portion of the juvenile and intermediate *P. oceanica* leaves sampled at site A (disturbed site) and B (control site).

(Fig. 3d). By contrast, in the leaves of the disturbed site, the signal was detected only in the sub-epidermal cells and in vascular bundles (Fig. 3c). No difference was detected between leaves collected in the control and disturbed sites by comparing the apical portion (Fig. 3a and b).

Concerning the intermediate leaves, some clusters of cells remain positive for zeatin antibody in the basal part of control leaf (Fig. 3h) whereas, in the leaf from the disturbed site, the reaction was confined to sub-epidermal layers and vascular bundles (Fig. 3e and g).

4 DISCUSSION

Plants integrate with many environmental conditions and are useful in providing evidence of environmental problems reflecting dysfunctions of ecosystems (Godefroid, 2001). In terrestrial habitats, many examples are reported where plant bioindicators have been used to assess environmental pollution based on their presence/absence and their abundance.

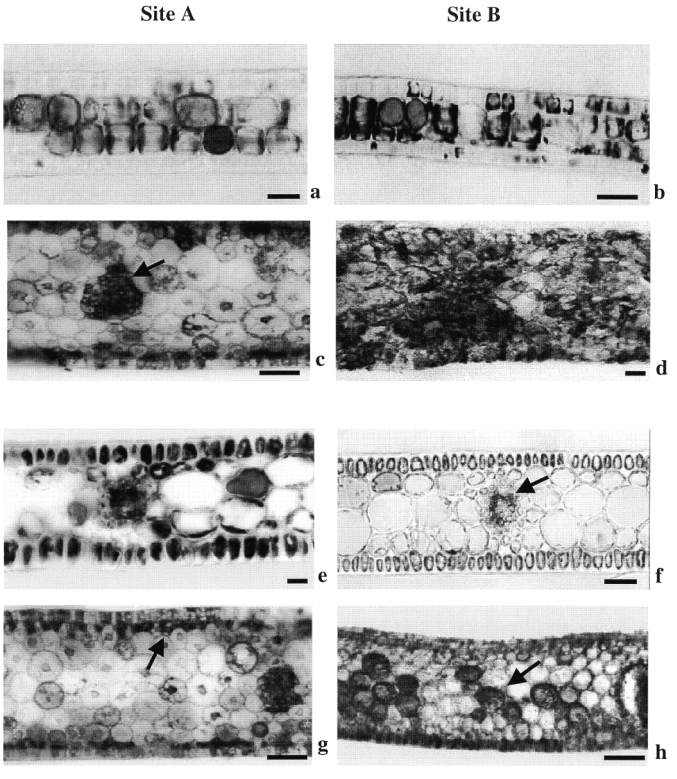

FIGURE 3 Immunolocalization of zeatin on a cross-section of (a–d) juvenile and (e–h) intermediate *P. oceanica* leaves collected at site A (disturbed) and site B (control). (a, b, e, f) Apical portion of the leaf; (c, d, g, h) basal portion of the leaf. Arrows indicate reactive cells. Bars: 15 μm.

However, few data are available on marine habitats; these are mainly based on the use of macrophytes (Magnoliophyta) in the biomonitoring of trace-metal contamination (Pergent, 1991; Pergent-Martini and Pergent, 2000).

In this context, we may point out that in spite of their crucial role in the ecosystems, plants have been (until now) largely underused in evaluating the quality of a medium, probably because of the difficulties in correlating visual symptoms with cytophysiological biomarkers. Only recently, have plant biomarkers been developed and more extensively used overall for terrestrial plants.

It is worth noting that a clear relation between chemical compounds (e.g., secondary metabolites) and environment state has been detected for terrestrial plants (Darral, 1989; Christie *et al.*, 1994; Dixon and Paiva, 1995; Bussotti *et al.*, 1997; Loponen *et al.*, 2001; Pasqualini *et al.*, 2003), whereas few studies have been conducted on aquatic plants. Production of a phenolic compound, assessed using HPLC, was reported in *P. oceanica* leaves collected within an area colonised by the chlorophyte *Caulerpa taxifolia* (Cuny *et al.*, 1995). An increase of four phenolic acids (Gallic, caffeic, *p*-coumarinic, and ferulic acid) was also detected in the seagrass *Zostera marina*, following infection with *Labyrinthula zosterae* (Vergeer and Develi, 1997). In addition, in leaves of *Zostera marina*, grown *in vitro*, the biosynthesis of the phenolic compound was either positively or negatively affected by light intensity and temperature, respectively (Vergeer *et al.*, 1995).

Our results provide evidence for an increased production of phenolic compounds in the marine seagrass, *P. oceanica*, following perturbed environmental conditions. The cytological

approach that we used allowed us to detect not only an enhancement of BP synthesis, but also clear variations in their aggregation state, probably related to qualitative changes. In fact, in leaves from the disturbed area, a remarkable increase in the number, as well as in the typology of phenolic inclusions, was detected. These results strongly support the involvement of phenolic compounds in plant strategy against environmental injuries. In line with this assumption, in a previous study, qualitative and quantitative changes of phenolic compounds, assessed by HPLC, have been reported in *P. oceanica* foliar tissue collected in sites exhibiting overgrazing by herbivores, presence of anthropogenic waste (chemical and inorganic), interspecific competition with the alga *C. taxifolia* and intraspecific competition (dense meadows) (Agostini *et al.*, 1998).

Remarkable differences were also detected studying cytokinin presence and distribution in leaves from preserved *vs.* disturbed sites. In the basal portion of both young and intermediate leaves, collected at the control site, numerous clusters of hormone-reactive cells were observed, whereas they were reduced and/or completely absent in juvenile and intermediate leaves from disturbed sites. Since in *P. oceanica* leaves the basal zone contains meristematic cells, which allow continuous leaf growth, our observations fit well with the major role played by these hormones in promoting cell proliferation (Laureys *et al.*, 1998; Riou-Khamlichi *et al.*, 1999; Francis and Sorrell, 2001), a feature of the meristematic state. In accordance with this observation is the relationship between cytokinins and some developmental genes related to the maintenance of meristematic feature. For example, in leaves of the KNAT1 transgenic plant of lettuce, meristematic cell clusters, which appear reactive to cytokinin antibody, ectopically developed leaf-like structures (Frugis *et al.*, 1999). Thus, the presence of a high number of reactive cells in the basal growing zone of *Posidonia* leaf from the control site is clearly consistent with its regular development. Conversely, in the disturbed site, the leaf growth appears limited and/or delayed, strongly suggesting that the absence of hormone is related to environmental disturbance.

The involvement of cytokinins in photo-morphogenic induction, leaf development, chloroplast differentiation and light-regulated gene expression is widely documented (Chory *et al.*, 1994; Chin-Atkins *et al.*, 1996). Recently, a response regulator working downstream of a cytokinin receptor (ARR4), which modulates phytochrome B-mediated light signalling, has been isolated (Fankhauser, 2002). Since in the disturbed site light could be a limiting factor due to water turbidity, changes in the cytological pattern of cytokinins distribution could be explained in view of the aforementioned link with phytochrome B-mediated light signalling. Hence, the preferential dislocation of the hormone into the external cell layers could relate specifically to altered light conditions. Although it is not within the scope of this paper to define which specific environmental factors determine the behaviour patterns described, it may be that the presence of a wet basin and anthropogenic disturbances could be the cause of the recorded differences, also in relation to water turbidity.

Finally, it should be noted that studies carried out under natural conditions are rare. To our knowledge, our *in situ* work provides the first evidence for the potential use of both phenolic typology and cytokinin distribution as cytophysiological biomarkers to monitoring environmental conditions in *P. oceanica* meadows.

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