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Patterns of *Posidonia oceanica* (L.) Delile tannin cells and leaf characteristics according to environmental conditions

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Abstract

There is little research on the distribution and evolution of tannin cells, specialized in the sequestration of phenolic compounds, in the leaves of *P. oceanica*, depending on the developmental stage and environmental conditions. This work aims to evaluate the density of tannin cells along the vertical axis of leaves (basal, middle and apical regions) at four sites corresponding to an anthropogenic gradient estimated from the ecological status of *P. oceanica* meadows: *Moderate* (El Djamila), *Good* (Bou Ismaïl) and *very good* (Kouali and Aïn Tagouraït). Leaf thickness and width were measured in each region to express the density of tannin cells per mm². Data analysis shows that the density of tannin cells decreases with increasing leaf age and that the highest densities are recorded in the apical regions, especially when the leaf apex is entire. The density of leaf tannin cells is significantly correlated ($R = -0.977$, $p = 0.022$) with the Ecological Quality Ratio (EQR) corresponding to the ecological status of *P. oceanica* meadows and reflecting the impact of environmental pressures. This sensitivity to environmental conditions opens interesting prospects for using tannin cell density as a descriptor (environmental biomarker) in coastal monitoring programs based on *P. oceanica* meadows.

Keywords: Seagrass; Biomarker; Monitoring; Southern Mediterranean.

Introduction

The marine Magnoliophyta *P. oceanica* is regularly used to report on the Mediterranean coastal environment's quality using descriptors that can respond to various biotic and abiotic stresses. This ability of *P. oceanica* to integrate the environmental quality can be measured from the sub-individual scale (e.g., biomarker approach) to the scale of the ecosystem as a whole (e.g., Biointegrator approach; Micheli *et al.*, 2005; Pergent-Martini *et al.*, 2005; Personnic *et al.*, 2014; Procaccini *et al.*, 2017).

Several studies have monitored the state of health of *P. oceanica* meadows and proposed multi-parameter approaches. These approaches consider morphological, cytological, physiological, biochemical and genetic characteristics and constitute relevant diagnostic and decision support tools (Pergent-Martini *et al.*, 2005; Martínez-Crego *et al.*, 2008; Rotini *et al.*, 2013; Cozza *et al.*, 2019; Gnisci *et al.*, 2020). *P. oceanica* contains tannin cells specialized in the sequestration of phenolic compounds (Pellegrini & Pellegrini, 1993; Arnold & Targett, 2002; Dumay *et al.*, 2004; Pergent *et al.*, 2008; Castellano *et al.*, 2012). These compounds are considered biomarkers of environmental quality (Mannino & Micheli, 2020; Astudillo-Pascual *et al.*, 2021).

However, the role of these phenolic compounds in the defense mechanisms of *P. oceanica* against abiotic and biotic stresses is still poorly documented. The few studies conducted to date allowed us to clarify their implications for biotic interactions during the (i) interspecific competition with the invasive species *Caulerpa taxifolia* (M. Vahl) C. Agardh and *Caulerpa cylindracea* Sonder (Cuny *et al.*, 1995; Ferrat *et al.*, 2003; Dumay *et al.*, 2004; Pergent *et al.*, 2008), (ii) intraspecific competition (Rotini *et al.*, 2013) and (iii) grazing (Vergés *et al.*, 2007a, b, 2008; Boumaza *et al.*, 2014). Abiotic stresses, such as nitrogen enrichment associated with aquaculture and mercury contamination, also appear to increase total phenol (Ferrat *et al.*, 2003; Leoni *et al.*, 2007; Ferrat *et al.*, 2012) and flavonoids (Cannac *et al.*, 2006) in *P. oceanica* leaves. The total phenol content of *P. oceanica* rhizomes also seems to respond to effluent discharges (Migliore *et al.*, 2007; Rotini *et al.*, 2011, 2013). The total phenol content in the leaves of *P. oceanica* appears to be related to the number of tannin cells present in the cells (Cuny *et al.*, 1995; de Villèle & Verlaque, 1995; Dumay *et al.*, 2004; Pergent *et al.*, 2008; Boumaza *et al.*, 2012).

In terms of organization and aggregation of the phenolic compounds, a variation in the typology of phenolic inclusions in tannin cells has also been reported in response to effluent discharge. This finding suggests the involvement of specific phenolic compounds in the defense mechanism of *P. oceanica* against this type of stress (Cozza *et al.*, 2004). Moreover, the number of tannin cells seems to increase in the presence of effluent discharges (Pergent, 1988; Agostini *et al.*, 1998; Cozza *et al.*, 2004; Boumaza *et al.*, 2012) and during interspecific competition with *C. taxifolia* and *C. cylindracea* (Cuny *et al.*, 1995; de Villèle &Verlaque 1995; Dumay *et al.*, 2004).

In this context, this study aims to clarify the distribution and abundance of tannin cells and certain characteristics of *P. oceanica* leaves in response to the quality of the water body in which they develop and to consider using these descriptors in environmental monitoring programs.

Material and Methods

Four *P. oceanica* meadows with different ecological statuses, reflecting the coastal water quality, were considered in Bou Ismaïl Bay (Algeria, Southern Mediterranean) (Fig.1; Table 1). The Ecological Quality Ratio (EQR) of these meadows was established from the PREI index (Gobert *et al.*, 2009) and calculated based on the measurement of five descriptors (density of the meadow, leaf area, ratio between epiphyte biomass and leaf biomass, depth and type of lower limit) (Sengouga *et al.*, 2019). The El Djamila site has a *Moderate* ecological status and the Bou Ismaïl site shows a *Good* ecological status. The two sites of Aïn Tagouraït and Kouali show a *Very Good* ecological status with environmental conditions close to the reference conditions. At each site, five orthotropic shoots of *P. oceanica* were randomly sampled at least 5 m apart to reduce the likelihood of collecting shoots connected to the same plagiotropic axis. Samples were collected by scuba diving during the summer of 2018 at a depth of 10 ± 2 m.

After dissection of the shoots, the leaves were classified according to their rank of insertion within the shoot and numbered from rank 1 (the oldest, outermost leaf) to the last rank (the youngest, innermost leaf). The state of the leaf apex was also examined (entire leaves, broken/grazed leaves). For each leaf (Fig. 2A), transverse sections were made manually, using a razor blade at (i) the basal region of the leaves, approximately 3 cm above the ligule, where the tissue is white to light green, (ii) the middle region of the leaves, at the level of the chlorophyll tissue with few epiphytes, especially in young leaves, and (iii) the apical region of the leaves, about 2 to 3cm from the upper edge, where the tissue is generally covered with epiphytes, particularly at the level of the oldest leaves.

A total of 260 cross-sections were made. The counting of tannin cells was carried out under a microscope, previously calibrated, at X20 magnification over the entire surface of the cross-section. The cross-section area

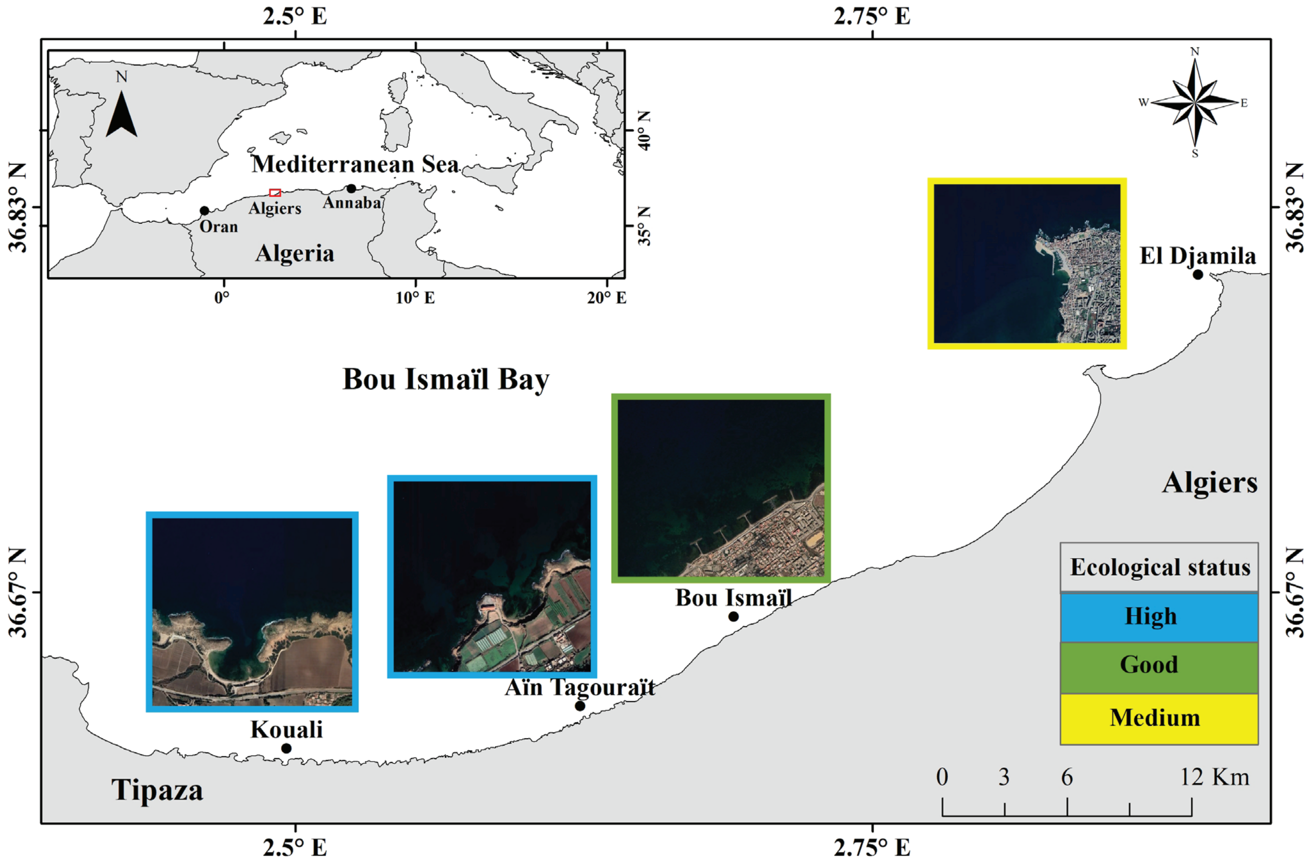

Fig. 1: Geographic location of the four sampling sites on the coast of Bou Ismaïl Bay (Algeria, southern Mediterranean).

Table 1. Geolocation, anthropogenic pressures, Ecological Quality Ratio (EQR) and ecological status of the four study sites (Sengouga *et al.*, 2019).

Sites	**Geographical coordinates**	**Anthropogenic pressures**	**EQR (Ecological Quality Ratio)**	**Ecological status**
Kouali	36.59308°N 2.49498°E	Reference zone	0.86	High
Aïn Tagouraït	36.61315°N 2.62393°E	Mussel farming stopped since 2016	0.80	High
Bou Ismaïl	36.65445°N 2.69235°E	Urban and industrial effluents Coastal protection structures	0.69	Good
El Djamila	36.80238°N 2.89297°E	Fishing and pleasure port Urban and industrial effluents Presence of *C. cylindracea*	0.36	Medium

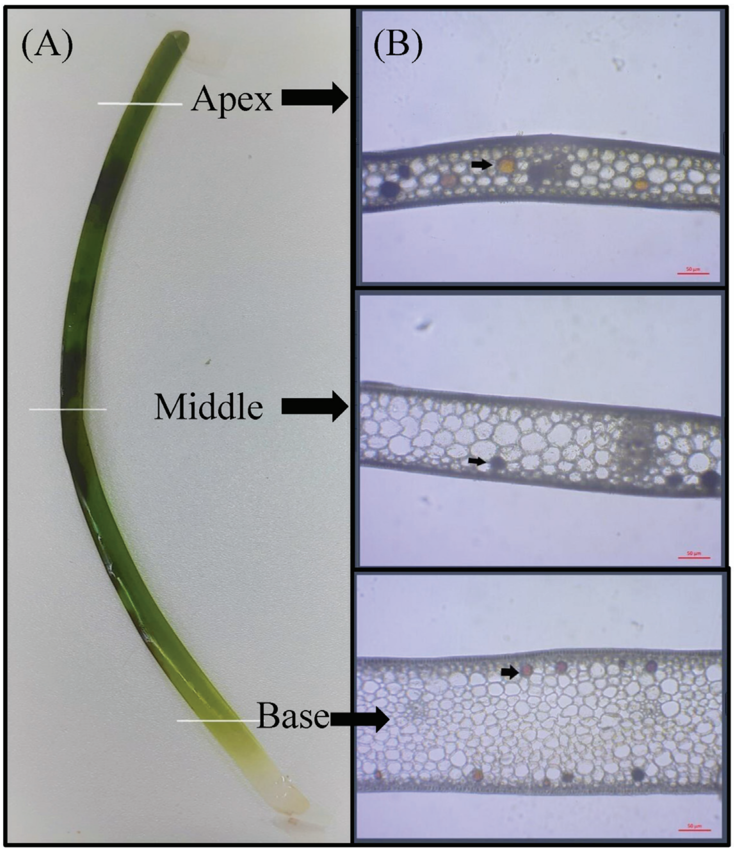

Fig. 2: (A) Leaf region of *P. oceanica* used to count tannin cells (black arrows): Base, Middle and Apex. (B) Light microscopy of typical cross-section regions (scale bar = 50 µm). Magnification: 20X.

was estimated by multiplying the width of the leaf by its thickness, measured at the center of the section, assuming that the cross-sections are rectangular. The density of tannin cells in the leaves of *P. oceanica* is then expressed per mm^2. The youngest leaves, occupying ranks 6 and 7, which are not very present within the shoots, are not considered.

Statistical analyses were performed in the R4.1.2 environment, open source (https://www.r-project.org/). The differences between the sites and the variables measured were analyzed using a one-way ANOVA insofar as the data complied with the hypotheses of the parametric tests (independent samples, normality of the distributions of the quantitative variables, homogeneity of the variances). Tukey's post-hoc test was applied to identify differences between means. The t-test was used to evaluate differences in tannin cell density in entire and grazed/broken leaves. Pearson's correlation was used to assess relationships between EQR and tannin cell density. Statistical tests were performed at the 95% significance level.

Results

Leaf characteristics

Leaf thickness and cross-section area show significant differences along the vertical axis of the leaves (ANOVA; F = 81.41, df = 2, $p < 0.001$ and ANOVA; F = 65.55, df = 2, $p < 0.001$; respectively). Tukey's post-hoc test results show that leaf thickness is significantly greater in basal regions, followed by middle and apical regions (Fig. 2B, 3A). However, there is no significant difference between apical and middle regions for the cross-section area (Fig. 3B). The thickness and cross-section area increase significantly as leaf age increases (rank decreases) and stabilize for the oldest leaves of ranks 1 and 2 (Table 2, Fig. 3C, 3D). Conversely, the average leaf width shows no significant differences either along the vertical axis (ANOVA; F = 0.068, df = 2, $p = 0.934$), nor according to the rank of the leaves (ANOVA; F = 2.119, df = 4, $p = 0.0794$).

Inter-site comparisons of mean leaf thickness show significant differences (ANOVA; F = 3.38, df = 3, $p = 0.0189$). Tukey's post-hoc test distinguishes three groups of sites according to the leaf thickness [Kouali and El Djamila], [Bou Ismaïl] and [Aïn Tagouraït] (Fig. 3E). Leaf width and cross-section area also show significant differences (ANOVA; F = 43.49, df = 3, $p < 0.001$ and ANOVA; F = 5.79, df = 3, $p < 0.001$, respectively). Tukey's post-hoc test allowed us to identify two groups of sites according to these two descriptors [Kouali and Aïn Tagouraït] and [Bou Ismaïl and El Djamila] (Fig. 3F, 3G).

Tannin cell density patterns

The average tannin cell density (expressed in number of cells per mm²) along the vertical axis of the leaves shows significant differences (ANOVA; F = 16.45, df = 2, $p < 0.001$). Tukey's post-hoc test shows that the maximum values are recorded in the apical regions, while the basal and middle regions show no significant differences (Fig. 4A). The mean density of tannin cells shows significant differences according to leaf rank (ANOVA; F = 5.86, df = 4, $p < 0.001$). It increases significantly when leaf age decreases (rank increases) (Tukey post-hoc test; Fig. 4B; Table 2).

Inter-site comparison of mean tannin cell density shows significant differences (Fig. 4C; ANOVA; F = 12.11, df = 3, $p < 0.001$). The El Djamila site has the highest tannin cell density with 34.0 ± 23.1 cells.mm^{-2}, followed by Bou Ismaïl (20.9 ± 16.7 cells.mm^{-2}), Kouali (17.3 ± 17.7 cells.mm^{-2}) and Aïn Tagouraït (15.3 ± 17.6 cells.mm^{-2}). Tukey's post-hoc test individualizes two groups of sites [Kouali, Aïn Tagouraït and Bou Ismaïl] and [El Djamila] (Fig. 5). The same trend is recorded considering the number of tannin cells (ANOVA; F = 9.9, df = 3, $p < 0.001$; data not shown).

Table 2. Summary of mean values (± standard deviation) of *P. oceanica* leaf characteristics (thickness and cross-section area) and Tannin cell density by rank (1 to 5) in different leaf regions (Apex, Middle and Base).

Thickness (mm)					
	Rank 1	Rank 2	Rank 3	Rank 4	Rank5
Apex	0.16 ± 0.06	0.14 ± 0.03	0.12 ± 0.02	0.11 ± 0.02	0.10 ± 0.01
Middle	0.19 ± 0.06	0.20 ± 0.07	0.19 ± 0.06	0.15 ± 0.04	0.13 ± 0.02
Base	0.52 ± 0.17	0.49 ± 0.21	0.39 ± 0.21	0.22 ± 0.08	0.13 ± 0.05
Cross-section area (mm²)					
	Rank 1	Rank 2	Rank 3	Rank 4	Rank 5
Apex	1.63 ± 0.68	1.43 ± 0.43	1.20 ± 0.30	1.06 ± 0.26	1.08 ± 0.15
Middle	1.95 ± 0.70	2.04 ± 0.83	1.88 ± 0.71	1.45 ± 0.52	1.34 ± 0.27
Base	5.35 ± 2.16	5.13 ± 2.62	3.98 ± 2.49	2.15 ± 1.00	1.33 ± 0.55
Tannin cell density (per mm²)					
	Rank 1	Rank 2	Rank 3	Rank 4	Rank 5
Apex	22.39 ± 20.92	24.98 ± 22.40	31.58 ± 23.56	38.33 ± 14.38	40.10 ± 22.37
Middle	12.43 ± 12.35	15.48 ± 15.31	14.52 ± 14.49	16.38 ± 13.30	25.34 ± 15.48
Base	9.85 ± 9.03	14.00 ± 15.36	20.02 ± 24.50	23.26 ± 22.50	27.66 ± 23.56

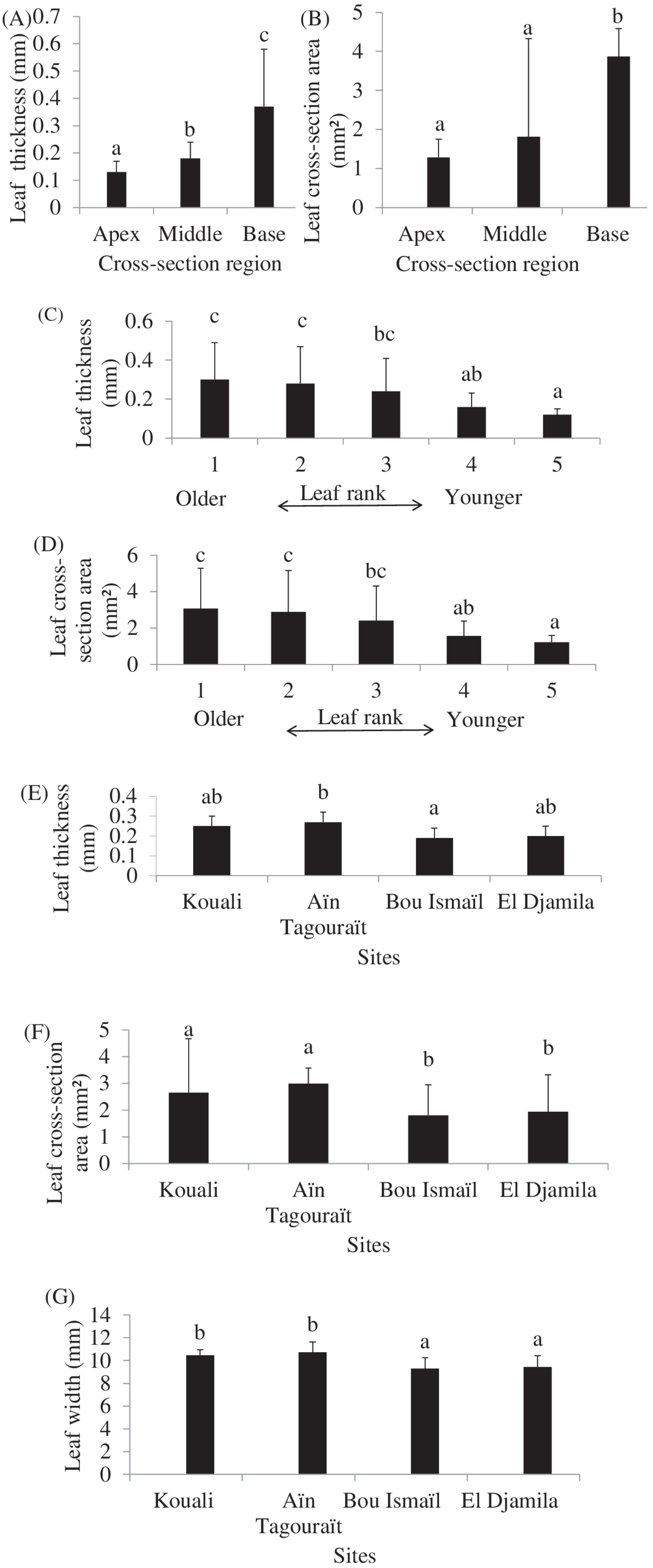

Fig. 3: *P. oceanica* leaf characteristics by cross-section region (A, B), leaf rank (C, D) and sites (E, F, G) (mean ± standard deviation). Lowercase letters indicate different homogenous groups (Tukey's post-hoc test).

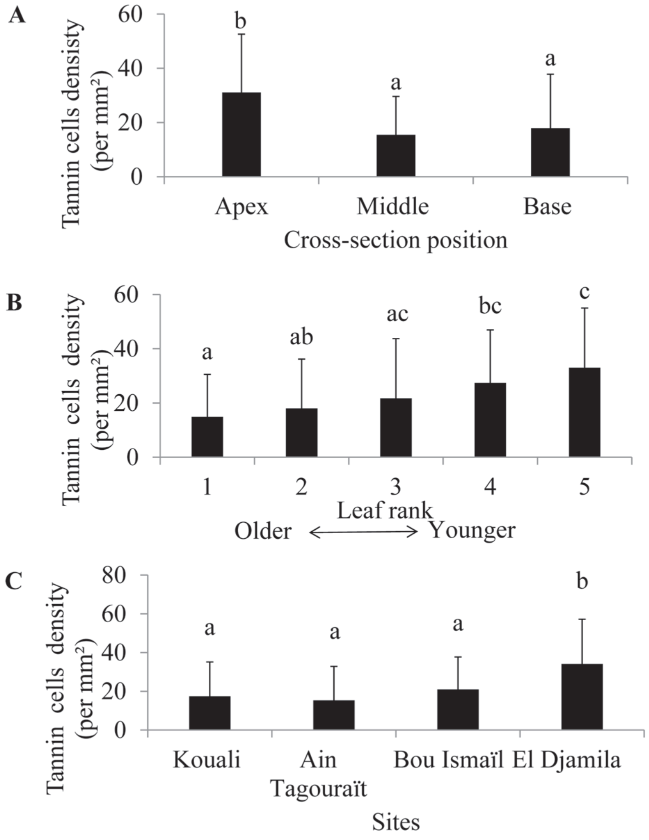

Fig. 4: Tannin cell density in *P. oceanica* leaves by cross-section position (A), leaf rank (B) and sites (C) (mean ±standard deviation). Lowercase letters indicate different homogenous groups (Tukey's post-hoc test).

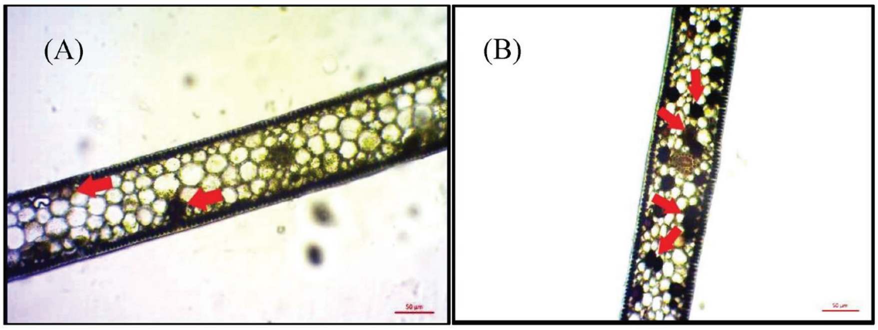

Fig. 5: Tannin cells (red arrows) in *P. oceanica* leaves at the sites with the highest (Kouali) (A) and lowest (El Djamila) (B) ecological status (scale bar = 50 µm). Magnification: 10X.

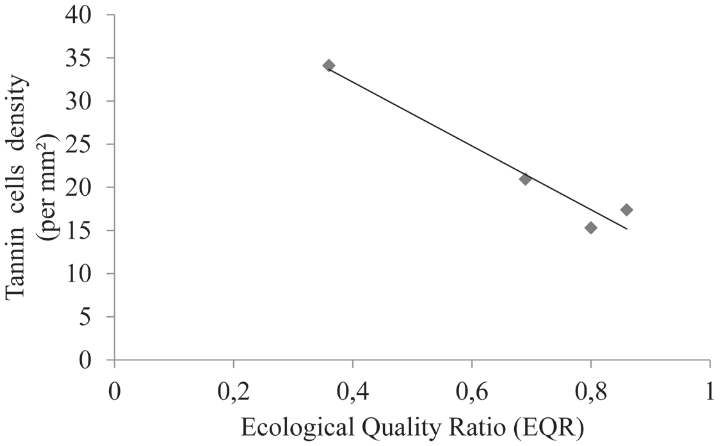

Fig. 6: Negative correlation between the Ecological Quality Ratio (EQR) and Tannin cell density in *P. oceanica* leaves at four sites in Bou Ismaïl Bay (Pearson R test = 0.977, $p = 0.022$).

A significant inverse correlation is highlighted between the EQR of the different sites and the tannin cell density (Fig. 6; R Pearson = -0.977, $p = 0.022$). This trend is also recorded considering the number of tannin cells (R Pearson = -0.901, $p = 0.091$; data not shown).

The mean tannin cell density showed significant differences between entire and grazed/broken leaves (t-test; t = 3.83, df = 91.9, $p < 0.001$). The maximum value was recorded in entire leaves (30.8 ± 22.8 cells.mm^{-2}; n = 64 leaves) and the minimum value was recorded in broken/grazed leaves (18.7 ± 18.7 cells.mm^{-2}; n = 186 leaves). This trend is recorded at each site (Fig. 7).

Discussion

The thickness of *P. oceanica* leaves shows a vertical gradient that decreases from the basal to the apical region. This observation confirms studies carried out in other areas of the Mediterranean (Colombo *et al.*, 1983; Dalla Via *et al.*, 1998), including for other marine Magnoliophyta species such as *Cymodocea nodosa* (Ucria) Asch (Schubert *et al.*, 2018) and *Thalassia testudinum* Banks and Solander ex König (Enríquez, 2005). The trend observed in this study may be related to the growth of the *P. oceanica* leaves from a basal meristem that allows the plant to continue growing when the apical older regions become senescent and less chlorophyllous (Kuo & den Hartog, 2006; Ruocco *et al.*, 2019). In addition, the reduction in thickness at the apical region of the leaves could be linked to the significant covering by epiphytes, as observed in *Zostera marina* L. (Vettori & Marjoribanks, 2021).

The thickness increases with age, especially in basal regions, then stabilizes for the oldest leaves (ranks 1 and 2). This trend differs slightly from that reported by de los Santos *et al.* (2016), who first noted an increase in thickness with the age of the leaves, then a continuous decrease in the oldest leaves due to a decrease in nitrogen, carbon and C:N ratios in the tissues of the old leaves of *P. oceanica*. Our results are similar to those reported by Vettori & Marjoribanks (2021), who observed the same trend when studying the biomechanical properties of *Z. marina* leaves.

Cross-section areas follow the same trend as thickness since leaf width does not show significant variation with leaf age, as also pointed out by de los Santos *et al.* (2016). The highest values of width and cross-section area were recorded at sites with a *Very good* ecological status (Kouali and Aïn Tagouraït), while the lowest values were recorded at sites with a *Good* or *Moderate* status (Bou Ismaïl and El Djamila, respectively). Moreover, the average leaf thickness does not seem to be linked to the ecological status of the meadows. Fernández-Torquemada *et al.* (2020) reported that certain changes in the morphological characteristics of *P. oceanica* could not be linked to the state of health of the meadows. Other factors such as light availability or hydrodynamics could explain at least part of the variability in leaf thickness as reported for *Z. marina* by Paul & de los Santos (2019).

The tannin cell density in *P. oceanica* leaves varies according to their stage of development (age) and along the leaf's vertical axis. The density of tannin cells in the leaves of *P. oceanica* shows a significant decrease when their age increases (rank decreases). This tendency is linked to the fact that the metabolism is more active in young leaves, and therefore the process of biosynthesis of phenolic compounds is more intense (Cariello & Zanetti, 1979; de Villèle & Verlaque, 1995; Boumaza *et al.*, 2014). Phenolic compounds do not only function as defensive secondary metabolites; plants also produce them for their growth and development (Pratyusha, 2022). They have an important structural role in cell wall expansion during plant growth, as observed in algae belonging to class Phaeophyceae (Schoenwaelder & Clayton, 1999; Schoenwaelder, 2001). The decrease in tannin cell density in aged leaves does not appear to be related solely to a cessation of biosynthesis of these compounds when the leaf ages. According to Mannino & Micheli (2020), phenolic compounds regularly undergo turnover or degradation and are released directly by exudation when *P. oceanica* leaves become old. Little is known about the impact of environmental stresses on the content and composition of defense compounds, in particular tannin cells,

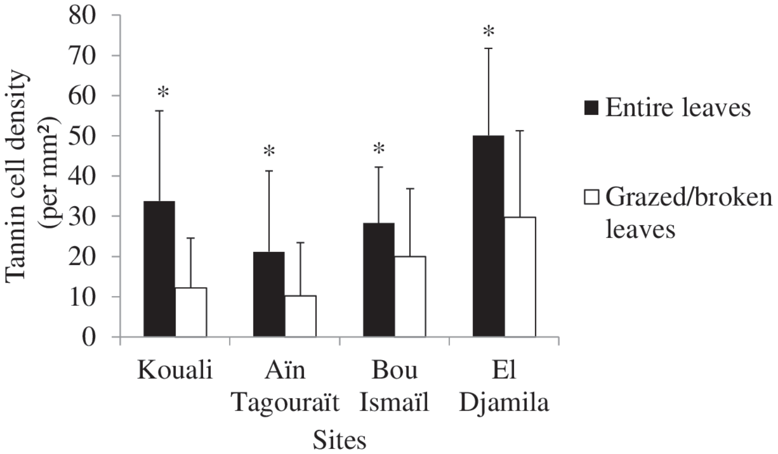

Fig. 7: Tannin cell density (mean ± standard deviation) in entire and grazed/broken *P. oceanica* leaves at each site (*= $p < 0.05$).

in young and senescent plant tissues. They could affect the production of secondary metabolites and their resorption during the leaf senescence process (Top *et al.*, 2017).

The tannin cell density is higher in the apical regions compared to the middle and basal regions of the leaves. The same trend was reported by Cariello & Zanetti (1979) in *P. oceanica*; this distribution can be explained by the Optimal Defence Hypothesis (ODH) (Rhoades, 1979). The ODH predicts that defense substances, such as phenolic compounds, are concentrated in the tissues most exposed to grazing, namely the apical regions of the oldest leaves (outermost leaves) and/or the tissues with the highest fitness value: the youngest growing leaves (innermost leaves). Furthermore, it has been shown that the apical regions of *P. oceanica* leaves, the oldest ones, accumulate more trace metal elements (TMEs) compared to the basal regions (Conti & Iacobucci, 2008; Cozza *et al.*, 2013); the increase in the number of tannin cells in these apical regions could be related to the chelating power of phenols towards metal elements (Waterman & Mole, 1994).

Grazing does not seem to impact the biosynthesis of tannin cells, contrary to the hypothesis that the density of tannin cells would increase during the herbivore / plant interaction (Pergent *et al.*, 2008). The tannin cell density is significantly higher in leaves with the apex still entire (not grazed/broken) and confirms the observations of Agostini *et al.* (1998), Leoni *et al.* (2006) and Vergés *et al.* (2008), who do not show a direct relationship between the synthesis of phenolic compounds and the grazing pressure. The decrease in tannin cell density in leaves that have lost their apex could be explained by the fact that phenolic compounds are released into the environment when the leaves are damaged either by grazing or by hydrodynamics (Mannino & Micheli, 2020). Phenolic compounds' role in controlling marine plants against herbivores is highly controversial. While some authors demonstrate that these phenolic compounds are directly involved in defense against grazing (Van Alstyne, 1988; Yates & Peckol, 1993; Pavia *et al.*, 1997; Stiger *et al.*, 2004), others refute this hypothesis (Steinberg, 1984, 1986; Steinberg *et al.*, 1991; Arnold *et al.*, 2008).

The site's tannin cell density increase is characterized by the lowest ecological status (El Djamila). More generally, the inverse correlation between tannin cell density and EQR value reflects a direct relationship between tannin cell synthesis and reduced quality of the water body in which *P. oceanica* grows. These results confirm the studies conducted (i) in a highly anthropized site in the Bay of Algiers (Tamentfoust; Boumaza *et al.*, 2012) and (ii) in areas subject to discharges from the urban sewage of the city of Marseille (Pergent, 1988) and Calabria coast (Cozza *et al.*, 2004). The El Djamila site is characterized by the presence of the green algae *C. cylindracea,* which is likely to cause additional stress linked to interspecific competition with *P. oceanica* and contribute to an increase in the synthesis of tannin cells (Dumay *et al.*, 2004; Pergent *et al.*, 2008). Biosynthesis of phenolics would allow plants to better adapt to hostile environments (Lattanzio, 2013; Chowdhary *et al.*, 2021) as phenolics are involved in the plants' strategy to overcome abiotic and biotic environmental stressors and avoid cell and tissue damage (Yeshi *et al.*, 2022). Under stress, the diversity of phenolic compounds increases in *P. oceanica* to protect the plant from reactive oxygen species (ROS) that can be generated by anthropogenic pressures and/or interspecific competition (Castellanno *et al.*, 2012). Thus, Agostini *et al.* (1998) demonstrated qualitative and quantitative variations of these phenolic compounds in leaf tissue of *P. oceanica* at sites with herbivore overgrazing, chemical contamination and inter-specific and intra-specific competition.

Conclusion

The increase in the tannin cell density in *P. oceanica* leaves at the most impacted site (ecological status *Moderate*) seems to constitute the plant's response to these environmental conditions. The consideration of tannin cell density could be a new descriptor reflecting a response of the *P. oceanica* meadow to the quality of the waterbody in which it develops. Its advantage for monitoring *P. oceanica* meadows is enhanced because it is easy, quick to measure, and reproducible. However, it will still be necessary to confirm this potential by studying a larger number of sites for each ecological status in different areas with various environmental conditions, particularly those reputed to have *Poor* and *Bad* ecological status, but also to look for other factors: grazing, epiphytes and seasonal variation. For inter-individual variability and measured variability within individuals (on leaf age and relevant part of the leaf), a sampling plan based on an adequate number of subsamples is recommended, and a standardized protocol should be proposed. In addition, the development of a five-level interpretation grid (*Very Good* to *Bad*) is essential to integrate this descriptor with existing multiparameter indices as a basis for assessing the vitality of *P. oceanica* meadows and the water body in which they develop (Romero *et al.*, 2007; Martínez-Crego *et al.*, 2008; Marbà *et al.*, 2012).

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