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Adaptative response of *Posidonia oceanica* (L.) Delile leaves to salt water and to depth: *Po*PIP1;1 aquaporin involvement and anatomical strategies evolution

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Summary

Part I

Introduction

Chapter 1: General characteristics

1.1 Macrostructural aspects

Posidonia oceanica is an endemic seagrass of the Mediterranean Sea named, after the ruler of the sea, Poseidon, and commonly called Neptune grass, it is a seagrass which plays an important role in the Mediterranean ecosystem. It is a monocot with stem modified in rhizome, adventitious roots for the attachment to substratum, ribbon-like leaves.

Figure 1 Picture of a *Posidonia oceanica* cutting

Leaves are arranged in bundles consisting of 5 to 10 leaves attached to vertical rhizomes. The leaves are broad (5 to 12 mm) and the length usually varies from 20 to 40 cm, but may be up to 1 m.

The rhizomes show either a plagiotropic (horizontal) or an orthotropic (vertical) growth. Generally, when the meadows reach a high level of leaf density, the vertical growth starts to increase light capture whereas, during the colonization phase, the horizontal growth prevails to reach a fast meadow spreading.

Long lasting these two kinds of growth form a net, made by rhizomes and roots, where the sediments suspended in the seawater are captured leading two consequences:

1. Stabilization of substratum on which the sea grass grows and reduction the suspension of sediment by currents and waves
2. Retention of suspended living and dead particles, becoming a sort of filter for coastal waters.

The particles trapping capacity of seagrasses is enhanced by the organisms living on the leaves either through filter feeding and active capture, or through the direct attachment of the suspended particles to the mucus-covered seagrass surfaces which result from their activity.

Thus the seagrasses can, at a certain extent, control the transparency of the water column. Increased light availability at the bottom facilitates the life of seagrasses themselves and that of other benthic plants which will further affect the water transparency.

So, we can easily understand the importance of *Posidonia oceanica* meadows in the Mediterranean shoreline: sediments vegetated by seagrasses are less likely to be mobilized by waves and currents, so that seagrasses can reduce the erosion of the coastline.

Detached seagrass leaves, which are lost either at the end of their life or earlier due to waves and storms, and their accumulation in the beaches represent another way by which seagrasses play a role in the shoreline protection. In fact the leaves accumulations dissipate the wave energy and directly protect beach sediments from the impact of waves.

Furthermore, *Posidonia oceanica* meadows lead to the increasing of both habitat diversity and biodiversity of coastal zone. In fact, it provides habitat for numerous organisms which cannot live in un vegetated sites.

The leaf canopy and the network of rhizomes and roots provide substratum for attachment (which is scarce in unconsolidated bottoms), stabilize the sediment, and reduce irradiance producing an array of microhabitats not present in unvegetated environments. In addition, the three-dimensional structure of seagrasses creates hiding places to avoid predation. As a result, the abundance and diversity of the fauna and flora living in seagrass meadows are consistently higher than those of adjacent unvegetated areas.

Seagrass meadows are key habitats in the life cycle of many organisms. The populations of crustaceans (e.g. shrimps) and fishes living in seagrass meadows are typically composed by a high proportion of larvae and juvenile individuals suggesting that seagrass meadows are preferred nursery habitats. Increased food availability and/or refuge from predation explains the importance of seagrass meadows as nursery and feeding habitats for these organisms, some of them target of highly important commercial fisheries. In addition, migrating birds use shallow and intertidal seagrass meadows as resting and feeding areas during their travels. Brent geese, widgeons and pintails feed preferentially on seagrasses, other birds feed on associated fauna.

Seagrasses are characterized by high rates of primary production. As any other photosynthetic organism, they fix carbon dioxide using the light energy and transform it into organic carbon either to sustain seagrass growth or to product biomass. High rates of biomass production imply high rates of oxygen production, a byproduct of photosynthesis, which is released toward the surrounding waters. The biomass of some seagrasses decomposes slowly and long lasting certain species (*i.e. Posidonia oceanica*) store a significant amount of carbon in the

sediment. Seagrass primary production is only 1% of total primary production in the oceans but they are responsible for 12% of the total amount of carbon stored in ocean sediments. This uncoupling between carbon dioxide fixation by photosynthesis and release by respiration indicates that seagrasses play a significant role in the regulation of the global carbon cycle (Duarte and Cebriàn, 1996).

The primary production of epiphytic algae growing on seagrasses and of benthic algae living in seagrass meadows is comparable to that of the seagrasses themselves. Together with the secondary production of associated fauna, the seagrass ecosystems become as productive as many agricultural crops and land forests. The coastal zone is a dynamic environment and currents and waves detach part of seagrass biomass which is transported to adjacent marine and terrestrial ecosystems. These increases of organic materials can locally be quite high, thus significantly contributing to the growth of fauna communities of adjacent coast habitats.

1.2 Reproduction

Posidonia oceanica is constituted by a tracing rhizome plagiotrope from which others branch can be originated either plagiotrope or orthotrope, carrying several leaf bundles.

Thereafter, plant rhizomes can intersected themselves producing a net structure where it is difficult to distinguish each single units. This process of development is defined "stolonization". Portions of rhizomes, detached from sea hydrodynamism, can originate cuttings which contribute to the seagrass propagation by vegetative reproduction. This is the main way of reproduction, since *Posidonia oceanica* produces flowers only induced by high temperature in summer and by a temperature of 20°C in October (Caye and Meinesz, 1984; Thélin et Boudouresque, 1985; Pergent *et al.*, 1989a; Stoppelli and Peirano, 1996). However, normally less than one flower is produced per 10 square meters per year.

The hermaphrodite flowers are grouped in a particular inflorescence green in color, which is carried by an axis inserted in the center of the leaf bundles and wrapped by two flower bracts. (Fig. 2).

Figure 2: Flowers and fruits on a plant of *Posidonia oceanica*

In the shallow meadows, until 15 m of depth, flowers arise in September-October period. In last autumn it is possible to observe the beginning of fruits development, which ripened in the March-April. In deeper meadows, beyond 15 m of depth, this cycle is delayed of approximately two months (Mazzella *et al.*, 1984).

The fruits of *Posidonia oceanica* have been called “olive of sea” as they look both in shape and size like the fruit of olive tree. The ripened fruit detaches itself from the mother plant and floats on the water surface, because the external pericarp is porous and rich of fat substances. Thus, the fruits can be transported from either wind or water currents far from the meadow of origin. At pericarp broken, the seed falls down and, in favorable environmental conditions, it begins the germination process. This kind of seed dispersion allowed the plant to colonize wide environments.

However, the fruits often did not reach maturation and therefore they degenerate, like others vegetative portions of the plant, assuming one tawny-blackish staining and it can persist various months within the bundle.

In 2009 (Nicastro, personal observations), meadows of *Posidonia oceanica* in Calabria undergo abundant flowering, which produced numerous fruits observable on the beach after drift events.

Figure 3: On the left, *Posidonia oceanica* fruits on the beach; on the right seedling showing juvenile leaves, a primary root and a little adventitious root.

1.3 Annual cycle of growth

Vegetative growth in *Posidonia oceanica* begins in winter and reach the maximum level in June, when the irradiance did not reached the highest peaks of season (Fig. 5) (c) (Alcoverro *et al.*, 2001).

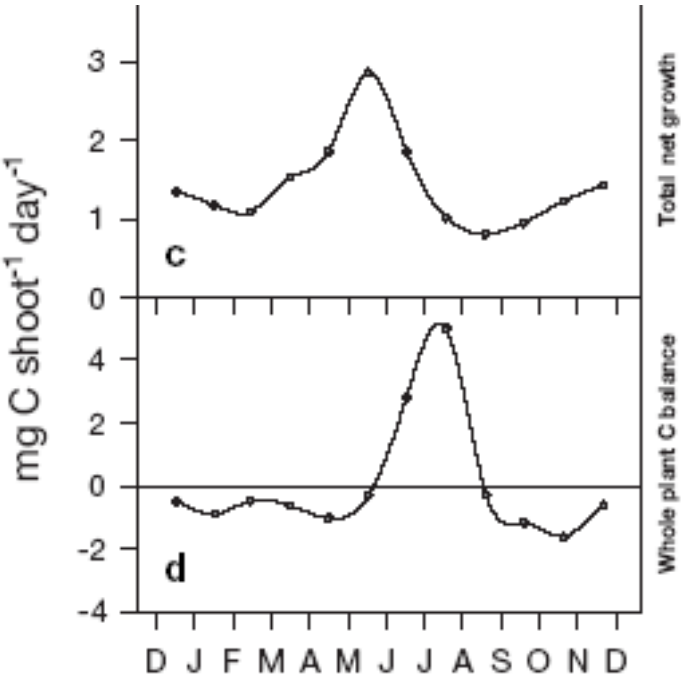
The maximum leaf length is reached during the period June-July, and decreases in the period between August-September when, in the distal part of leaves, necrosis becomes visible.

Until December-January, when it is recorded an high amount of leaf abscission (Tab. 2).

June-July	August-September	December-Junary
Maximum leaf length	Start of tissue necrosis in the leaf distal portion	Leaf abscissione

Table 1. Annual cycle of growth of *Posidonia oceanica leaves*

The leaf growth in *Posidonia oceanica* is of fundamental importance, because, in summer, the major leaf length can retain a greater amount of nutrients, when the surrounding environment is poor of nutrient.



The ability of leaves to withhold the nutrients is such that water between the leaves has chemical characteristics (concentration of nutrients) and physical (temperature factor) different from the remaining column of water. During winter a higher hydrodynamism due to the smaller leaf length mixes all sediment in the waters diminishing this phenomenon (Gobert *et al.*, 2002).

The process of leaf abscission is maximum from July to November and a new production of leaves begins to replace the old ones in October (Frankignoulle and (Bouquegneau, 1987).

The emergence of new roots starts at the end of spring (June), during the summer they branch, but it is during the autumn that they reach the maximum degree of development.

During winter the root growth stops and old woody roots assume a mechanical anchor function (Caye and Rossignol, 1983) (Tab. 3).

	Spring	**Summer**	**Autumn**	**Winter**
Cycle of root growth	New roots emergence	Roots branching	Maximum root development	Growth arrest and roots becoming anchor organs

Table 2 Growth cycle of roots in *Posidonia oceanica*

During Autumn when the radical development is maximum, the degree of branching roots is high, and the nitrogen absorbed in excess is stored particularly in the rhizome (Lepoint *et* ., 2002 ; Pellegrini, 1971 a,b).

The hortotrope rhizomes, which lakes functional roots and show long leaves, displays a lower growth than plagiotrope rhizomes.

It can therefore be assumed that adventitious roots in *Posidonia oceanica* are able to participate in the mineral nutrition at least in the autumn, when their development is highest and the woodiness process has not yet happened (Caye and Rossignol, 1983).

The amount of nitrogen absorbed by roots is 35 % of the annual requirement of nitrogen in *Posidonia oceanica* (Lepoint *et al.*, 2004).

1.4 Carbon metabolism in Posidonia oceanica

The total budget of carbon (Fig. 4 d) is the result combined among the net profit of carbon (photosynthesis), its loss (respiration) and the vegetative growth. The request of carbon by the portions not photosynthetic of plants (roots, rhizome and scales) reaches values higher in May, as a result of the increase of root breathing.

The growth, as previously mentioned, is greater during May-June. Overall, net budget of C alternate periods with negative values (Autumn-Winter) and periods with positive values (Summer); these positive values correspond to an accumulation of carbohydrates excess as sucrose and starch products.

At the beginning of winter, this storage of carbohydrates support the request respiratory and the growth after the massive loss of leaves in autumn, when the irradiance values and the budget carbon is not, therefore, still positive.

The photosynthesis fixation of carbon is highest during the period between June and September, when the light regime are optimal, leading the carbon amount to positive values. This carbohydrates accumulation will support the *Posidonia oceanica* growth in the next season.

Therefore, there is a curious asynchrony between the availability of nutrients and the best light conditions. In fact, in July-August period, when the amount of carbon reaches positive values, the availability of nutrient is low; whereas in winter, , when the light regimes are not the best to gain carbon, nutrients availability is high.

The Carbon storage allows the plant:

a) to reach a substantial biomass photosynthetic in the July-August (months at higher irradiance),
b) to sustain an important part of the annual cycle growth during the period of maximum concentration of nutrients (winter).

The seasonal changes in the storage amounts (sucrose and starch) differently affect the various part of the plant and in different classes of leaf age. Higher values were found in the rhizome, while the lower in old leaves. In any case the values are more high during the period between July and September, and lower in the period between February and May (Alcoverro *et al.*, 2001).

May-June	June-September	September-January	February-May
Growth peak	Carbohydrate accumulation	Greater availability of nutrients	Use of stored carbohydrate
High request for Carbon	Greater photosynthetic capacity	Minor photosynthetic capacity	Vegetative growth

Table 3: Seasonal growth and carbon metabolism in *Posidonia oceanica.*

In conclusion, the period of the greatest absorption and accumulation of nutrients corresponds to Winter-Spring, whereas in the period of summer-autumn *Posidonia oceanica* absorbs less nutrients from the environment and it uses, its internal storage of nutrients.

This trend can be associated with the natural variation in the concentration of nutrients in the surrounding environment (the largest concentration of nutrients being available in Winter-Spring).

However, in *Posidonia oceanica* the organ appointed to absorption of nutrients is mainly the leaf whereas in terrestrial plants is the root.

Thus, in autumn, due to the leaf abscission, a fewer absorption of nutrients occurs to which correspond the new leaves production and the increased radical development.

1.5. Ecology

Like land plants, in *Posidonia oceanica* the processes of germination and the successive vegetative growth is conditioned, by the physico-chemical and granulometric characteristics of the substrate.

In fact, the seeds of *Posidonia oceanica*, can occasionally germinate on deep rocky, but generally they develop on sands substrates with irregular granulations preventively fertilized from the colonization of pioneers organisms enriching the sediment of organic substances. During this evolutionary process, the substratum must be first colonized by the brown algae *Cystoseira*; then it follows an intermediate colonization by *Cymodocea nodosa* meadows.

On this stabilized layer organically enriched by *Cymodocee* products, the seeds of *Posidonia oceanica* can germinate and develop tiny root, while the plagiotrope rhizomes begin to grow horizontally to a rhythm of approximately 5-10 cm for year.

This driven a gradual formation of a net between roots and rhizomes in which the sediments are captured, in fact, sands, sandstone, organic detritus and calcareous algae, such as *Melobesia* fill up the spaces between rhizomes and fertilize the substrate.

At the same time, orthotropes rhizomes grow up and contrast the sedimentation that risks to cover the young seedlings, thus building the base of the meadows.

The biological characteristics and the evolutionary dynamics of *Posidonia* biocenoses are conditioned either by soil factors connected to the substrate nature and also by abiotic parameters as light and hydrodynamism.

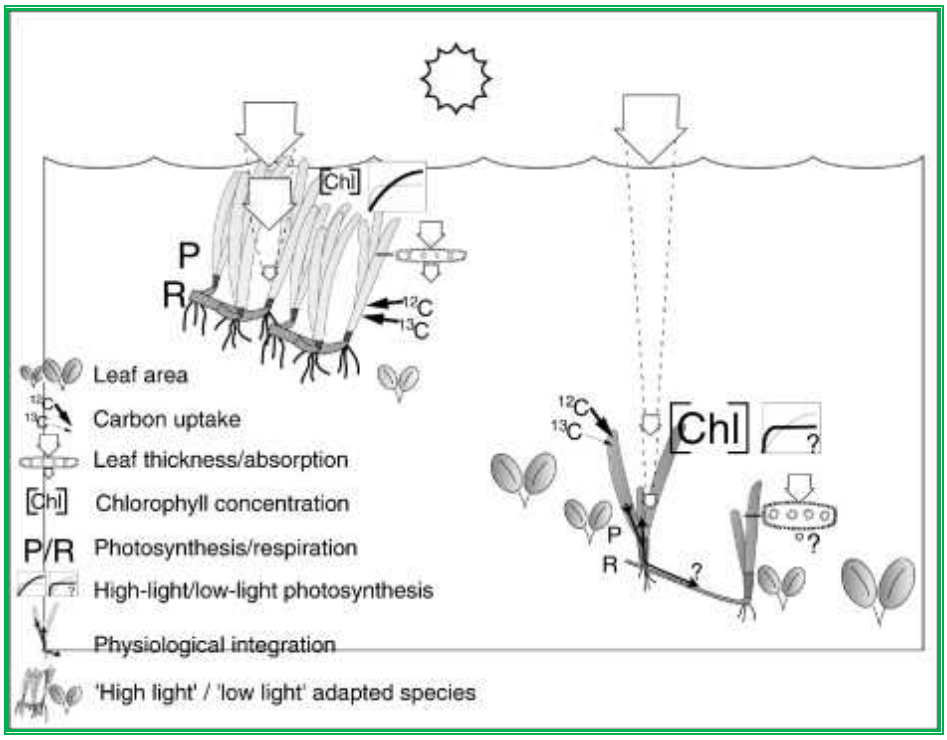


Fig. 9 Conceptual model showing the physiological and morphological adaptations to various light regimes in superficial and deep meadow. The superficial meadows show elevated density of leaf bundles, well developed hypogeal portion and high photosynthesis rates. The deep meadows show wider leaves and contain a greater amount of chlorophyll pigments.

The rate of quantitative and qualitative penetration of light induces physiological modifications in *Posidonia oceanica* leaves such as the concentration of chlorophyll in chloroplasts. In fact, it has been demonstrated that in leaves growing to 30m of deep, where the light intensity is low, the concentration of the chlorophyll is three time higher than in leaves growing in 5m deep water, to be able to utilize at best the reduced light regime.

Also the waters hydrodynamism induce morphologic variations in *Posidonia oceanica* leaves which appear small, where hydrodynamism is greater, and long in deep and calm waters (Fresi *et al.*, 1979; Ralph *et al.*, 2007).

1.5.1 The Matte

One of the particular characteristics of the rhizomes of *Posidonia oceanica* is its vertical growth that contrasts the progressive silting caused by the continuous sedimentation. This kind of growth allows the sea grass to use the maximum of available space and light. The two rhizome growths (horizontal and vertical) allow the plant to colonize contiguous areas and to determine an elevation from the bottom originating a typical formation called with the French term of "matte". (Fig.10)

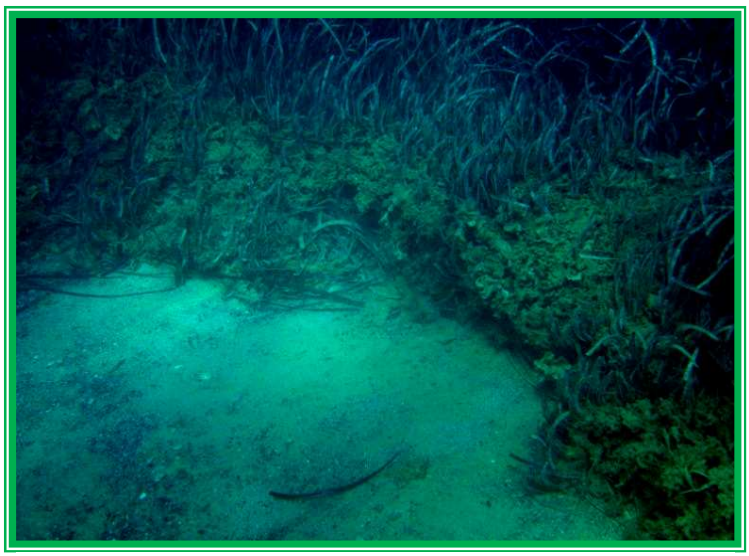


Figure 10 *Matte* of *Posidonia oceanica*

The *matte* is constituted from a complicated interlace formed by more layers of old plants rhizomes and from sediment caught between these, its top is covered by the bundles of living leaves. The rhythm of elevation of the *matte* depends on the growth rhythm of *Posidonia oceanica*, which is strictly related to the exposure of the meadow to the hydrodynamism and to the regimen of the currents. In some zones where the sediment deposition is greater, the meadow, in order to oppose the silting, can grow up until reaching the emersion of the leaves from sediment and forming a kind of natural barrier. However, in particularly exposed zones, the *matte* and sediments can be eroded with consequent regression and dead of the meadows.

The *matte* elevation has been estimated about 1 meter to one century. If it is considered that there are "mattes" high 4-5 m, we can make one idea of the meadows longevity.

Where a worsening of the environmental conditions provoke the dead of the plants, the *matte* persists with the interlace of dead rhizomes and roots. Therefore, its surface can be colonized from both algae or other small phanerogames like *Cymodocea nodosa e Zostera noltii*.

1.5.2 The Meadow

The extension and the morphology of the *Posidonia oceanica* meadows, both in depth and along the coast, is due to the interaction of different factors like sedimentation, leaf density,

the exposure of the meadows to hydrodynamism, the currents and their intensity, the water transparency and the climate.

Moreover this marine phanerogam shows the ability to modify, often in a remarkable way, both sandy or rocky substrate characteristics.

This phenomenon is mostly due to the refraining action of leaf layer on the water movements, reducing their intensity.

Thus the particles suspended in the water column settle with more facility and therefore *Posidonia oceanica* can be considered a real "trap" for fine sediments. It was observed, in fact, that the substrate where the meadow are present is more and more muddy than one not colonized from the plant.

The refraining action of the leaves reduces also the impact of the waves on the shoreline, therefore the meadows of *Posidonia oceanica* constitute one important natural belt of control and protection of the coasts from the erosive action of the waves.

Namely, it was estimated that the regression of one meter of meadow can provoke the loss of 15-18 m of sandy shoreline.

An effective parameter, to estimate the health state of a *Posidonia oceanica* meadow is the evaluation of leaf bundles density, defined as the number of bundles over each square meter of substrate.

The different health stages of meadows have been distinguished, according to this parameter, by *Giraud* (1977):

Generally the density is elevated in the superficial meadows (until 15 m approximately) and goes progressively decreasing in depth meadows.

In relation to the density, it can also be distinguished the uniformity and continuity of meadows, with a more or less regular distribution and without visible interruptions at long the interval of depth where they extend.

Some meadows look irregular, with numerous interruptions and with a not uniform distribution of the density. Other, can be constituted by single groups of plants confering to the meadow a typical aspect of "spots".

Regarding the meadows description it can be distinguish a *superior limit* and an *inferior limit*.

The superior limit is the point in which the meadow begins leaving from the coast line, while the inferior limit is the point in which the meadow stop to grow.

The superior limit of one meadow is always definite, the density is rather elevated and often it forms a consisting *matte*.

The inferior limit instead can have various conformations, between which it can be distinguished:

a) *Progressive limit*: Where the plant covering on the bottom is inferior to 50%; the density of the leaf bundles diminishes progressively and these tend to place parallel with the slope direction; the *matte* generally are absent and the rhizomes show a plagiotrope growth.
This type of limit indicates that the light is the factor that mainly regulate the growth and the colonization to greater depth.

b) *Definite limit*: the covering of the plants on the bottom is minor of 50%; the meadow shows margin interruption very definite, the *matte* are generally absent. This type of limit indicates that the growth is prevented from the type of sediment or the nature and morphology of the bottom.

c) *erosion limit*: the covering of the plants on the bottom can be also much elevated (100%); the meadow finishes abruptly, often it evidences scales formed from the *matte* that appears deeply recorded. This type of limit indicates that the presence of currents prevents to the meadow to extend itself and indeed, in some cases, they cause erosion. (Fig.10)
The *matte* erosion, due to the movement of waters can provoke the formation of “channels” and “glades”. Therefore, the channels called “*inter matte*” are channels inside the meadow, in which the original substrate of system is make bare, whereas the glades, have a quite circular shape, due probably to local turbulences of the current in proximity of solid bodies (like rocks formations) or of “matte” reliefs.

The extension of these structures depends on the intensity and persistence of the marine currents.

In these glades and channels “inter-matte”, in autumn, large amounts of brown leaf detritus are

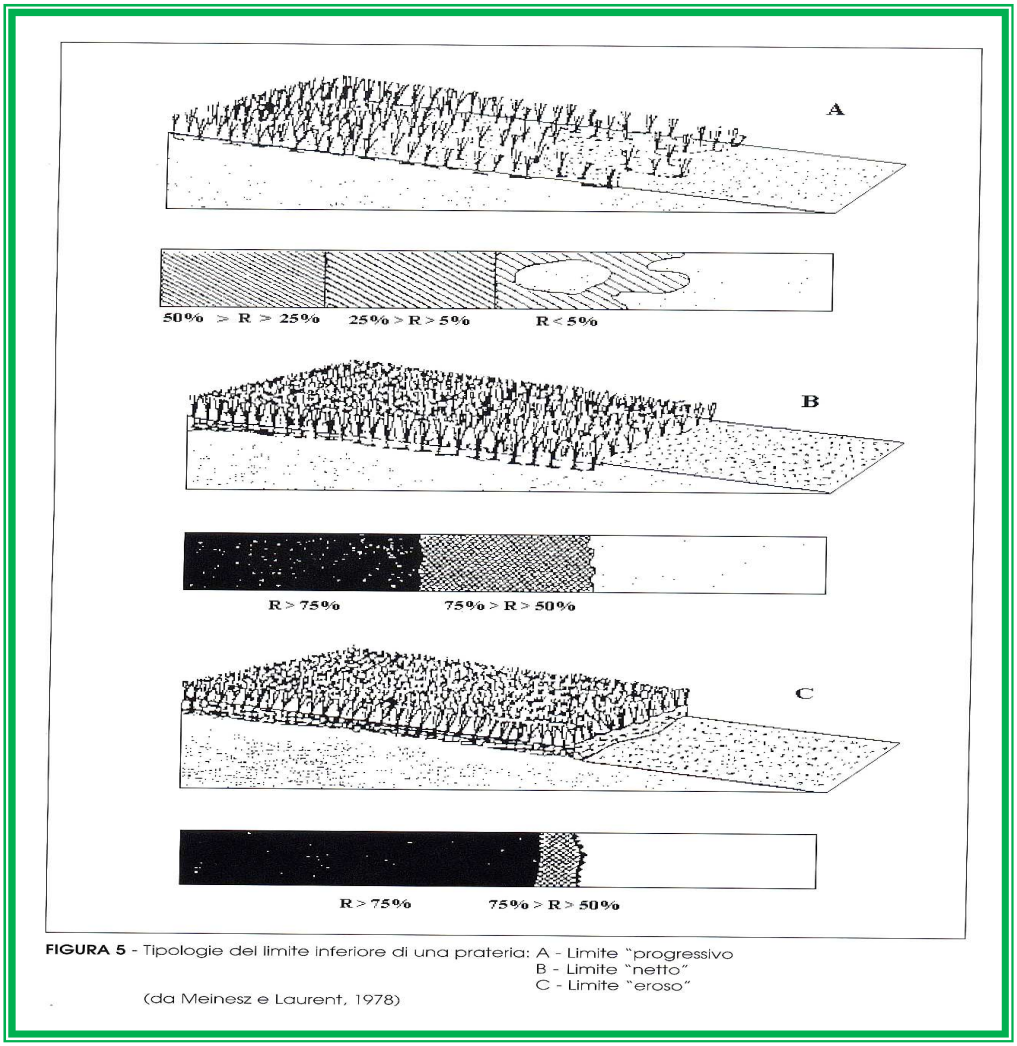


FIGURA 5 - Tipologie del limite inferiore di una prateria: A - Limite “progressivo
B - Limite “netto”
C - Limite “eroso”

(da Meinesz e Laurent, 1978)

Figure 11 Type of inferior limits of a meadow

accumulated and, with the first seas storm, it is poured on the beaches where form characteristic formations said with the French term “*banquettes*”.

Figure 12 Characteristic *banquettes*

Another type of particular formation is the "*atolls"*, these formations are found only in some meadows of the tunisian and sicilian coasts. These atolls remember the coralline formations of the Pacific and Indianan oceans, in fact *Posidonia oceanica* is arranged to ring around a glade in the centre, probably in relation, once again, to the movements of the water (Calvo and Fradà Orestano, 1984; Calvo *et al.*,, 1996).

1.6 *Law protection of Posidonia oceanica*

The international Convention on the Biodiversity of Rio de Janeiro in 1992, constitutes the main reference concerning the safeguard of the Biodiversity.

Biodiversity, or biological diversity, means the variability between the organisms living of all the species in an ecosystem and also the variability of the present ecosystems in an area, both those earthlings and those of aquatic, and obviously the complexity of which they make part.

Inside the *biotic* diversity of our planet, it is possible to distinguish three main levels: the genetic diversity (*intra specific*), the specific diversity (*inter specific*) and the ecosystem diversity.

The genetic diversity is between organisms of the same species;

the specific diversity regards organisms of different species;

the ecosystem diversity is the variety between ecosystems constituted from one biotic part and abiotic member: this can be considered the level of diversity that comprises the two previous levels, genetic and specific (*Primack and Carotenuto 2003*).

Numerous initiatives and legislative instruments have been adopted, at national and international level, for the safeguard of species and natural habitats, with positive results.

In Italy, the D.P.R. n.178 of 27 March 2001, prescribed the organization of the Minister of the Environment and the Protection of the Territory, assigns to the Direction for the Defense of the sea, Department for the water resources, the competences in matter of Marine Biodiversity protection and of the marine species and also of the marine environment in its complex.

The D.P.R. is focused to realize actions for the protection and the management of species marked by international agreements, like priority for the Mediterranean, whose conservation is particularly threatened.

In particular, among the species to hold under control it can be remembered, all the cetaceans presents in Italian seas, the marine turtles, the meadows of *Posidonia oceanica*.

In line with the directives of the Convention on the Biodiversity, in the world-wide encounter in 1992 at Rio de Janeiro, it has been recognized the necessity to the marine biological diversity.

Italy has ratified the Convention on the Biodiversity with the L. n.124 of 14 February 1994.

The Directive Habitat 92/43/CEE, regarding the conservation of the natural and seminatural habitats, and also the wild flora and fauna, prescribed that the member states of the Union characterize on their own territory areas that accommodate animals and vegetables species, and habitat whose conservation is considered a priority of European relief, like *Posidonia oceanica* considered, a species of priority conservation.. In Italy this directive was approved with D.P.R. n. 357 of the 8 September 1997 and according also to directive 79/409/CEE, with the collaboration of the Regions, it has marked to the European Commission, a directory of the S.I.C.,Sites of Communitarian Importance and Z.P.S. Special Protection Zones, among which there are numerous marine SIC.

Convention of Barcelona relative to the protection of the Mediterranean Sea from pollution (1978) ratified with the law of 21 January 1979 n. 30, as a result of the amendment from the Conference of the Plenipotentiary ones of the Contracting Parts, happened to Barcelona in 1995, changes title becoming the *"Convention for the protection of the marine environment and the coastal region of the Mediterranean "* and widens its geographic application comprising inner marine waters of the Mediterranean and the coastal areas.

The Convention maintains its programmatic nature, whose performance must be realized by specific protocols concerning several forms of pollution.

The protocol regarding the Especially Protected Areas in the Mediterranean (Protocol ASP), takes into account the protected species and those commercially exploited. It prescribe the institution of Areas Specially Protected of Mediterranean Importance (ASPIM), with criteria taking into account the degree of biodiversity, the peculiarity of the habitat and the presence of rare, threatened or endemic species

Unfortunately, in the last decades *Posidonia oceanica* meadows were subjected to a worrying regression due to its particular sensitivity to local environmental degradation, caused by human activities (Duarte, 2002; Green & Short, 2003; Orth *et al.*, 2006; Short & Wyllie-Escheverria, 1996). By the mid-1990s, loss of 900 km^2 of seagrass meadows were documented (Short & Wyllie-Escheverria, 1996), mainly through coastal development, eutrophication and boating activities. Exacerbating these traditional anthropogenic' disturbances, global climate change will further lead to decreases in water clarity, changes of salinity and increases of either the means or the extremes sea temperature.

It is worrying, also, the diffusion in the Mediterranean Sea of exotic species, like algae of genus *Caulerpa* (*Caulerpa taxifolia* and *Caulerpa racemosa*) that are in competition with *P. oceanica* for the same ecological niches, but they grow much faster than the seagrass.

This sensitivity to disturbance environmental factors, with the genetic weakness, the long persistence and the slow vegetative growth make *Posidonia oceanica* a good bio indicator.

The use of organisms as bio indicators for monitoring water quality has been officially suggested from the EU Water Framework Directive (WFD, Directive 2000/60/EC) from 2006 (ref.). Marine invertebrates, phytoplankton organisms, seaweeds and seagrasses have been identified as 'Biological Quality Elements (BQEs)'. *Posidonia oceanica*, in particular, has been included in the WFD as model system (Casazza *et al.*, 2006).

Chapter 2: Aim of the work

In the last years, several strategies to protect this system have been undertaken, but in spite of the numerous studies on this fascinating plant, at present its complex physiology is little known. However, behind all possible strategies of safeguard there is the need of continuous physiological studies, because all efforts are useless without a solid knowledge of the plant system. *Posidonia oceanica*, which was originated as land plant, to become compatible with submerged marine habitat, set up a series of anatomic and citophysiological changes allowing their life in salt water such as:

- Presence of air lacuna, mainly in the leaves, to allow their floating in seawater and the gas diffusion in the inner tissues.
- Lack of stomata in leaf epidermis whose cells contain numerous large chloroplasts.
- Nutrients absorption through the leaf surface, whereas adult roots became woody to anchor the plant to substratum.
- Inorganic carbon uptake by mechanism based either on direct utilization of HCO_3^- via a plasma-membrane ATPase (Beer and Rehnberg, 1997) or by external carbonic anhydrase (Invers *et al.*1999).

The aim of the present work was to be a little card in the puzzle's physiology knowledge of the system *Posidonia oceanica*, focusing our attention on two main questions:

1) How can a Phanerogame, originated as land plant, survive in salt water?
2) How can a sea grass, which lives from 5m to 40m depth, to adapt at different bathymetries?

First point. At present the mechanisms, which prevent the salt entry or allow its extrusion from the leaf surface, are quite unknown. Recently Maestrini *et al* (2004) suggested that aquaporin could be involved in saltwater adaptation. In fact they isolated two aquaporin genes constitutively expressed in *Posidonia* leaves and founded in hyper saline regime a higher level of *Po*PIP1;1 transcript. In order to gain insight on the site of aquaporin action our objective is to localize in cross leaf sections, a PIP1 antibody (against *Arabidopsis* PIP1;1 peptide, which shares 84% of identities with *Po*PIP1;1).

Second point. In this case the broad focus is the identification of anatomic variations in *Posidonia* leaves growing at different depths. The objective is to perform a comparative evaluation of morphologic parameters, through analysis of Advanced Geometric

Morphometry, in order to determine what changes can be associated to different light, temperature and/or CO_2 conditions. The goal will be to relate structural leaf blade aspects with the depth adaptation.

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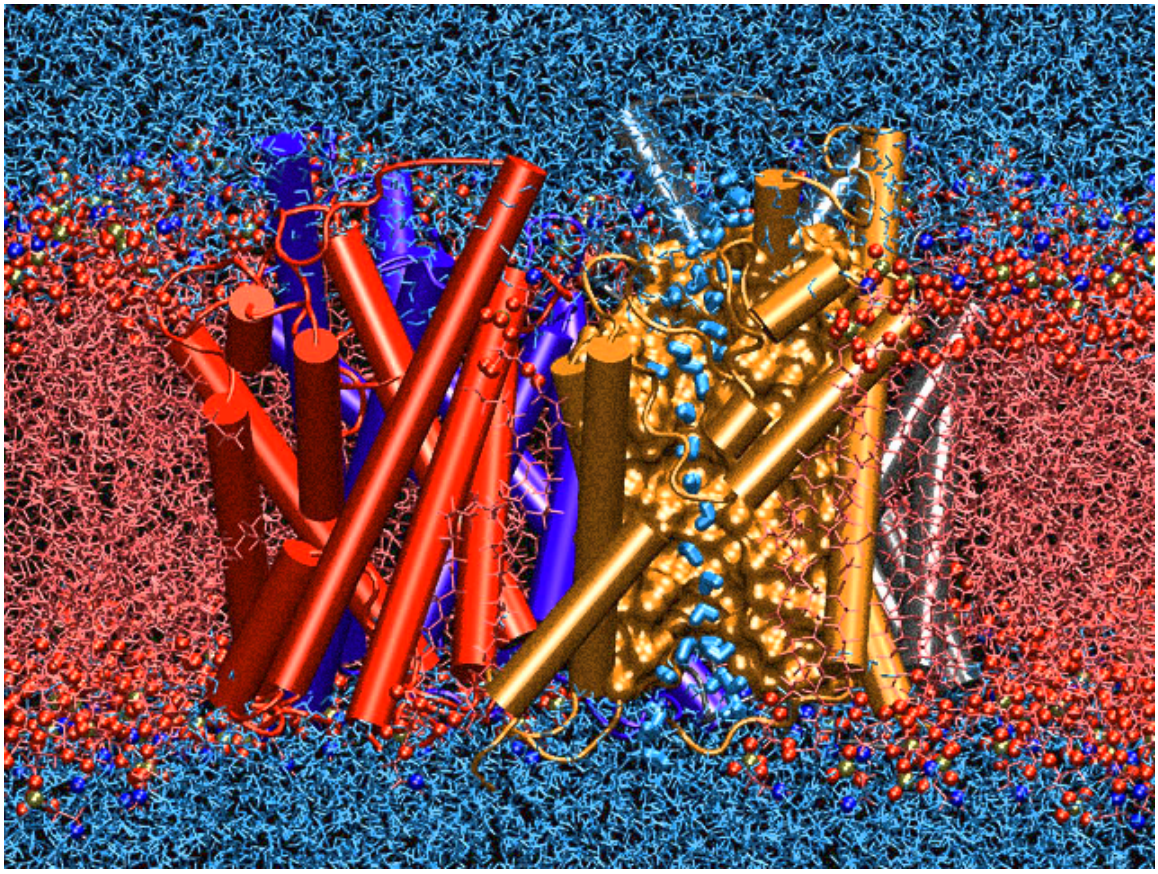
Part II

Adaptative response of Posidonia oceanica to salt water:PoPIP1;1 aquaporin involvement



Chapter 1: Aquaporins

1.1 General features

In the past, it was commonly accepted that water simply diffuses through lipid bi layers although it is a polar molecule, more recently protein water channels were identified not only in animals and plants, but also in insects, fungi, and bacteria.

These ubiquitous proteins, so-called aquaporins, are membrane-intrinsic pores facilitating the movement of water along an osmotic gradient.

The reason for water's free diffusion assumption is not evident, as artificial lipid bi layers had been found to be much less permeable to trans-bi layer water movement when compared with biological membranes, such as the plasma membranes of erythrocytes and renal epithelial cells (Sidel and Salomon, 1957). However, since no protein water channels or transporters have been identified, it was believed that water crossed biological membranes without the aid of specific channels or transporters. The net transport of water across biological membranes is dependent on hydrostatic and osmotic gradients (Steudle and Henzler. 1995). The hydraulic conductivity of a membrane is proportional to the osmotic water permeability (Pf), which is a property of each specific membrane, measured by imposing an osmotic or hydrostatic gradient across the membrane. The diffusion permeability (Pd), which is a specific property of membrane, is the measure of the free diffusion of water across a membrane, as measured by the exchange of water without an imposed gradient. For a synthetic lipid bi layer, Pf is roughly equal to Pd. However, for biological membranes, Pf has been found to be greater than Pd (Ray, 1969; Henzler, T. and Steudle, E. (1995). For example, regarding the movement of water across the erythrocyte membrane, a high Pf/Pd ratio was detected. The Arrhenius activation energy (i.e. the temperature dependence) of the osmotic water permeability in the erythrocytes was similar to that in the free water diffusion and much lower than the activation energy for diffusion water permeability of an artificial lipid bi layer. Taken together, these data suggests that trans membrane aqueous pores are responsible for most of the water transport across the erythrocyte membrane. Unlike the diffusion by water permeability, which cannot be inhibited by chemical means, the water permeability osmotic driven can be inhibited by chemical reagents, which covalently modify cysteine residues, suggesting that proteins are components of the trans membrane pores.

The first protein with water transport activity to be identified was CHIP28 (channel forming integral protein of 28 kDa), a major erythrocyte plasma membrane protein (Preston, 1992).

CHIP28 had been serendipitously isolated and cloned were found to have a high sequence homology to a previously isolated and cloned plasma membrane protein, the major intrinsic protein (MIP) of bovine lens fiber cell membrane (Gorin, 1984). Oocytes were injected with cRNA corresponding to the CHIP28 mRNA, and thereafter incubated for few days to allow the protein synthesis machinery synthesize and target CHIP28 on oocyte plasma membrane. When placed in a hypotonic solution the transgenic oocytes swelled much faster than control oocytes, demonstrating that the abundance of the CHIP28 protein enhanced the permeability to water (Preston and Agre 1991). The CHIP28 protein, identified in erythrocytes and epithelial cells (renamed aquaporin 1; AQP1) was the first example of a water channel protein (Preston *et al.*, 1992).

When the oocytes, transiently expressing a water channel protein, were transferred to a hypotonic solution (five times more dilute) they swelled within a few minutes, whereas 50% of control oocytes swell after an hour. Genes encoding proteins homologous to the MIP of the eye lens-fiber cell membrane belong to the MIP family of proteins were found in organisms ranging from bacteria to fungi, plants, insects and mammals. Using the oocyte expression system, most of the MIP homologues tested were found as aquaporin water channels. However, two MIP family members, GlpF in *E. coli* and Fps1 in yeast were found to transport glycerol but no water (Maurel, C. *et al.*,1994; Luyten, K. *et al.* 1995). Furthermore, a few MIPs have mixed specificity, for example the human AQP3 allows the passage of glycerol as well as water (Ishibashi, K. *et al.* 1994), and NOD26 of soybean allows the passage of glycerol and formamide in addition to water (Rivers *et al.*, 1997). Based on the biochemical and structural analysis of two-dimensional crystals of AQP1, a 6A three-dimensional structure has been resolved (Walz *et al.*, 1997). AQP1 contains six trans membrane domains with both the C- and the N-terminal at the cytoplasm side of the membrane, a topology that is conserved in all MIP homologues (Fig. 1).

AQP1 appears to exist as tetramers in the native membrane, although each monomer has water transport activity (Engel *et al.*, 1994).

Amino acid sequence comparisons of the corresponding plant and animal proteins reveal that they are evolutionarily related and belong to the MIP family (Reizer *et al.*, 1993).

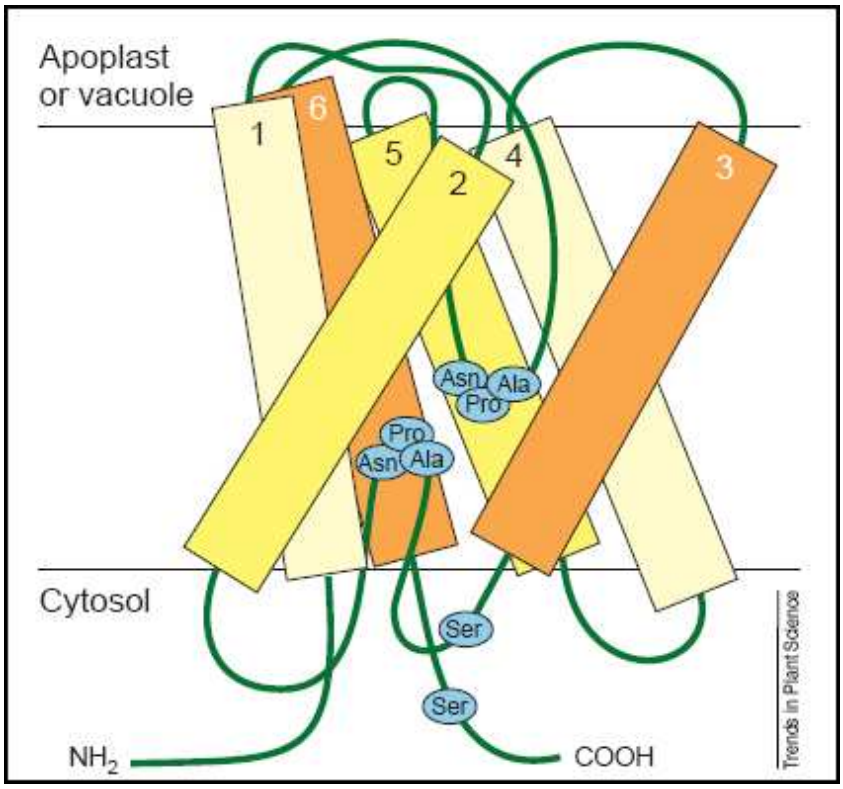


Figure 13 Basic structure of aquaporins. The model is valid for both plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs). The side with the N- and C-termini always faces the cytosol whereas the other side either faces the apoplast, in the case of PIPs, or the vacuole, in the case of TIPs. There are six transmembrane helices. The first cytosolic loop and the third extracytosolic loop, each of which contain a conserved Asn-Pro-Ala (NPA) box, are relatively hydrophobic and probably dip into the membrane from opposite sides creating a seventh transmembrane structure. The colouring of the helices reflects the internal homology, where the N-terminal half of the protein is homologous to the C-terminal half, although the two halves are inserted inversely in the membrane. Thus, helix 1 corresponds to helix 4, helix 2 to helix 5, and helix 3 to helix 6. Conserved serine residues that are known to be phosphorylated and dephosphorylated *in vitro*, *in planta* and in the oocyte system, thereby regulating the water transport activity of aquaporins, are also depicted. The serine residue (Ser) in the first cytosolic loop is present in all PIP1 and PIP2 subfamily members. In place of this serine, the majority of TIP subfamily members have a threonine residue. The serine in the C-terminal domain is present in all PIP2 subfamily members.This schematic model is based on the structure for AQP1 (Heymann *et al.*, 1998).

In mammalians aquaporins are called AQP1-AQP9 and the water permeability of cell membrane is tissue specific (Johansson *et al.*, 2000).

The majority of members of this highly conserved group of membrane proteins have a molecular mass between 26 and 29 kDa and contain the two characteristic, highly conserved amino acid motifs asparagine–proline–alanine *(*NPA) (Park and Saier, 1996).

The complete protein consists of six membrane-spanning a helice, linked by loops. Since the first three membrane helices and loops resemble the other half of the MIP, an intra gene duplication has been suggested. This leads to a tandem conformation of three alfa helices, each half including one NPA motif (Pao *et al.*, 1991; Reizer *et al.*, 1993) in a loop that is slightly more hydrophobic. The in vivo conformation of peptide results in an overlapping of the NPA regions inside the membrane. This structure has been described as ‘the hourglass model’ (Jung *et al.*, 1994) and is suggested to form a pore complex of about 3 A° diameter for the passage of water molecules. Although there are reports demonstrating that a single aquaporin can be functional, like the case of the glycerol facilitator GlpF in the native membrane (Lagrée *et al.*, 1998), they were found to

form tetramers (Verbavatz *et al.*, 1993; Walz *et al.*, 1994) and dimers (Mariaux *et al.*, 1998) (Fig. 13).

1.2 Aquaporins in plants

In plant genome, there are more genes than in other organisms (Kaldenhoff *et al.*, 2007), in fact the genome of *Arabidopsis thaliana* encodes 35 full-length aquaporins homologues (Johanson *et al.*, 2001; Quigley *et al.*, 2001). Thirteen of them belong to the plasma membrane intrinsic protein (PIP) subfamily and predominantly are part of the plasma membrane (PM).

This underlines their importance for plant water movements such as the transport of assimilates through sieve elements and the closure and opening of stomata in leaves.

Based on sequence homology, plant aquaporins can be subdivided in four sub groups which to some extent correspond to distinct sub-cellular localizations: PIPs, TIPs, NIPs, SIPs and XIP (Johanson *et al.*, 2001; Quigley *et al.*, 2001; Chaumont *et al.*, 2001; Sakurai *et al.*, 2005; Danielson and Johanson, 2008).

The tonoplast intrinsic proteins (TIP) and plasma membrane intrinsic proteins (PIP) correspond to aquaporins that are abundantly expressed in the vacuolar and plasma membranes, respectively.

PIPs are further subdivided into two phylogeny subgroups, PIP1 and PIP2. Because of their abundance, PIPs and TIPs represent central pathways for trans cellular and intracellular water transport (Maurel *et al.*, 2002; Wallace *et al.*, 2006).

PIP1 isoforms of *Arabidopsis thaliana* expressed heterologously in *Xenopus laevis* oocytes or other expression systems show no or very low aquaporin activity (Chaumont et., 2000; Fetter *et al.*, 2004; Moshelion *et al.*, 2002;Biela *et al.*, 1999;Temmei *et al.*, 2005),whereas, they seem to increase the permeability for small solutes like glycerol or gases such as CO_2 (Uehlein *et al.*, 2003; Biela *et al.*, 1999).

Experimental studies provide evidence that *Xenopus laevis* oocytes, expressing tobacco NtAQP1, show a CO_2 uptake 45% higher of control oocytes injected only with water (Uehlein *et al.*, 2003). The CO_2 transport was initially shown for the human AQP1 associated to gas channel function (Nakhoul *et al.*, 1998).

However effects on the membrane lipid composition or expression pattern of intrinsic genes that could modify oocyte CO_2 permeability were excluded (Cooper and W.F. Boron, 1998).

Nevertheless, the authors did not relate the effects to the increase in CO_2 transport rate, but to the facilitated water transport. Consequently PIP1 aquaporins could be transporters for small solutes and/or gases, or they need to be activated in the plant in order to function as water channels.

In the TIPs family three sub-families: αTIP, δTIP, γTIP have been distinguished (Schäffner, 1998).

A third subgroup comprises Nodulin26- like intrinsic membrane proteins (NIP), i.e. aquaporins that are close homologues of GmNod26, an abundant aquaporin in the peri bacteroid membrane of symbiotic nitrogen-fixing nodules of soybean roots (Wallace *et al.*, 2006). NIPs are present in non-leguminous plants, where they have been localized in plasma and intracellular membranes (Ma *et al.*, 2006; Mizutani *et al.*, 2006; Takano *et al.*, 2006). The small basic intrinsic proteins (SIP) define the fourth plant aquaporin subgroup and were first uncovered from genome sequence analysis (Johanson *et al.*, 2002). SIPs form a small class of 2–3 divergent aquaporin homologues and are mostly localized in the endoplasmic reticulum (ER) (Ishikawa *et al.*, 2005; Chaumont *et al.*, 2001; Johanson and Gustavsson, 2002; Johanson *et al.*, 2001).

Subgroup	*Physcomitrella*[a]	*Arabidopsis*[b]	*Zea*[c]
PIP1	2	5	6
PIP2	3	8	7
TIP	4	10	11
NIP	1	9	4
SIP	2	3	3

Figure 14 **Number of representatives of aquaporin subgroups identified in *Physcomitrella patens, Arabidopsis thaliana* and *Zea mays***

However, this classification is somewhat misleading, as PIP aquaporins have been identified in organelle membranes and some TIP aquaporins in the plasma membrane (Kaldenhoff *et al.*, 2007; Maurel, 2007).

Some aquaporins also facilitate the membrane transport of other small, uncharged molecules, such as ammonia, boric acid, hydrogen peroxide, glycerol or urea, across membranes (Baiges *et al.*, 2002; Beitz *et al.*, 2006; Biela *et al.*, 1999; Holm *et al.*, 2004; Jahn *et al.*, 2004; Liu *et al.*, 2003; Meinild *et al.*, 1998; Tyerman *et al.*, 2002).

An EST library of the moss *Physcomitrella patens* revealed that its aquaporins fall into the same subfamilies of higher plants, indicating that the main radiation of plant aquaporins was already established when land plant evolution began (Fig. 14).

The partial identification of *Physcomitrella patens* aquaporins demonstrates that the diversification into PIP, TIP, NIP and SIP subfamilies, as well as the differentiation into PIP1 and PIP2 classes, pre-dates the divergence of bryophytes and tracheophytes (Fig. 15) (Borstlap, 2002).

The X intrinsic proteins (XIP) subfamily was recently identified in the moss *Physcomitrella patens,* but functions and cellular localization is yet unknown (Danielson and Johanson, 2008).

The evolution of plants is marked by two major changes in plant water relations (Walter and Stadelmann, 1968; Raven, 1997). The first occurred when Charophyceae algae left their aqueous environment, giving rise to small land plants that, like the extant bryophytes, were poikilohydric (dependent by atmospheric moisture), and evolved desiccation tolerance to survive dry periods. The second change was linked to the emergence of homoiohydric plants – the Tracheophyte (vascular plants) – which acquired a root system to exploit soil water resources and xylem vessels and tracheids to rapidly conduct water to leaves and other aerial part of plants.

In the specific case of marine phanerogams, there is a third evolution step which regards their return into the marine environment.

1.3 Aquaporins in Posidonia oceanica

Posidonia oceanica is a stenohaline specie, that is not present when the salinity is below 33 ppt (Ben Alaya, 1972). This species tolerates more the high than the low salinity (Ben Alaya, 1972). In fact in the lagoon of Tunisie (Mediterranean Eastern basin), this plant can endure salinities over 40 ppt and the hyper saline environment provides optimum growth conditions (Pergent and Zaouali, 1992).

However, recently (Meinesz *et al.*, 2009) discovered an isolated meadow of *Posidonia oceanica* in the Marmara Sea, where salinity ranges is comprised between 21.5 and 28 ppt. Very probably, these *Posidonia oceanica* beds can be considered a relic population composed of genotypes adapted to brackish waters and growing clonally since a long time (Meinesz *et al.*, 2009).

Posidonia oceanica roots absorb water only in the juvenile phase, thereafter they lose this function to become anchorage organs to the substratum. Thus, the water necessary for the photosynthesis reaches the leaf mesophyll through the epidermis and is transported by scarcely differentiated xylem elements (Tomlinson, 1979). In this context it is of fundamental importance for cells life and function that the absorbed water mast be salt deprived.

However at present no information explaining the mechanism which prevents the salt entry in epidermis cells and/or the extrusion of salt from the cell surface is available.

In *Posidonia oceanica* two genes of aquaporins have been recently isolated by Maestrini *et al.* (2004): *Po*PIP1;1 and *Po*TIP1;1 (of 1548 bp and 1212 bp) showing high similarity to plasma

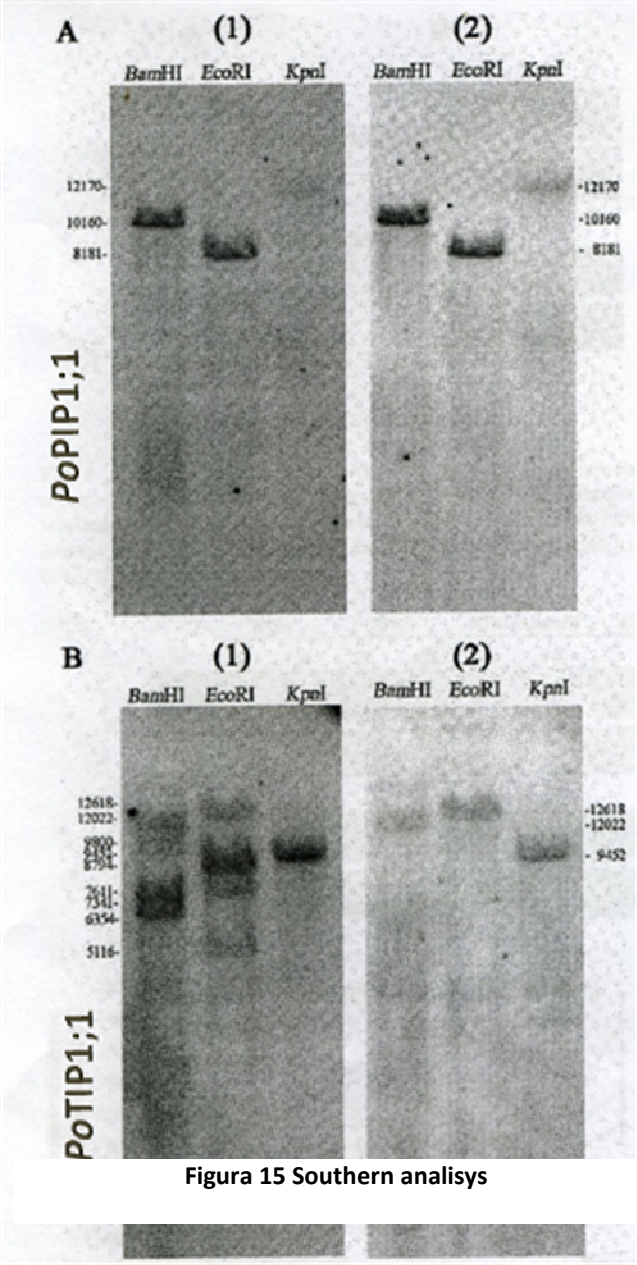


Figura 15 Southern analisys

membrane- and tonoplast-intrinsic protein-encoding genes, respectively. *Po*PIP1;1 is unique in the genome of *P. oceanica*, while *Po*TIP1;1 belongs to an aquaporin subfamily of at least four members (Fig 15) Both genes are constitutively expressed in the leaves, with higher levels of transcripts in young than in differentiated leaf tissues. Variations of salt concentration in aquarium determined different *Po*PIP1;1 and *Po*TIP1;1 transcript accumulation, indicating the existence of adaptation mechanisms related to gene expression also in marine plants adapted to very high salt concentrations. Hyposalinity induced lower levels of PIP1 transcripts, while hypersalinity determined more PIP1 transcripts than normal salinity. TIP1 transcripts increased in response to both hypo- and hypersalinity after 2 days of treatment and went back to control levels after 5 days (Fig. 16) (Maestrini *et al.*, 2004).

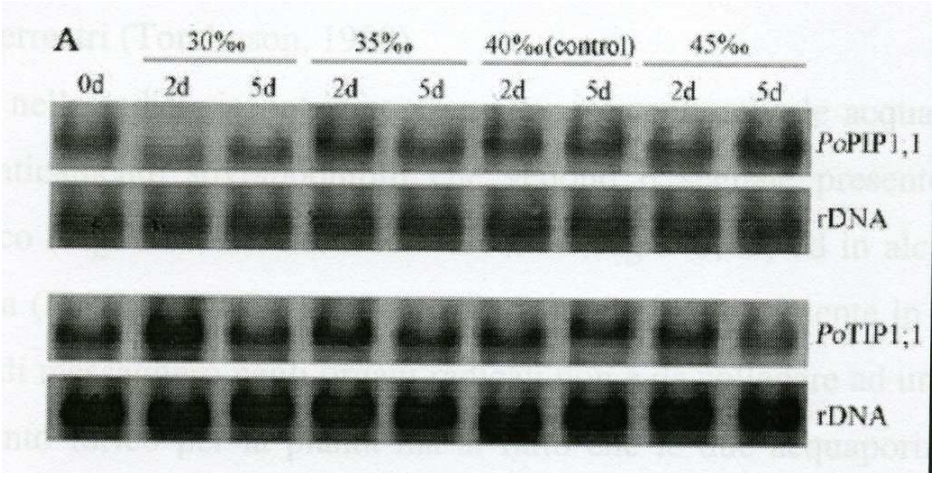


Figure 26 Northern blot on *Posidonia oceanica* leaves subjected to different saline stress (Maestrini et al., 2004)

These results were confirmed by in situ hybridization with *Po*PIP1;1 mRNA carried on *Posidonia oceanica* leaves subjected to saline stress for two days. Hypersalinity (45‰) induced an increase of *Po*PIP1;1 mRNA in leaf epidermis and in vascular bundles (Fig. 17) (Cozza and Pangaro, 2009).

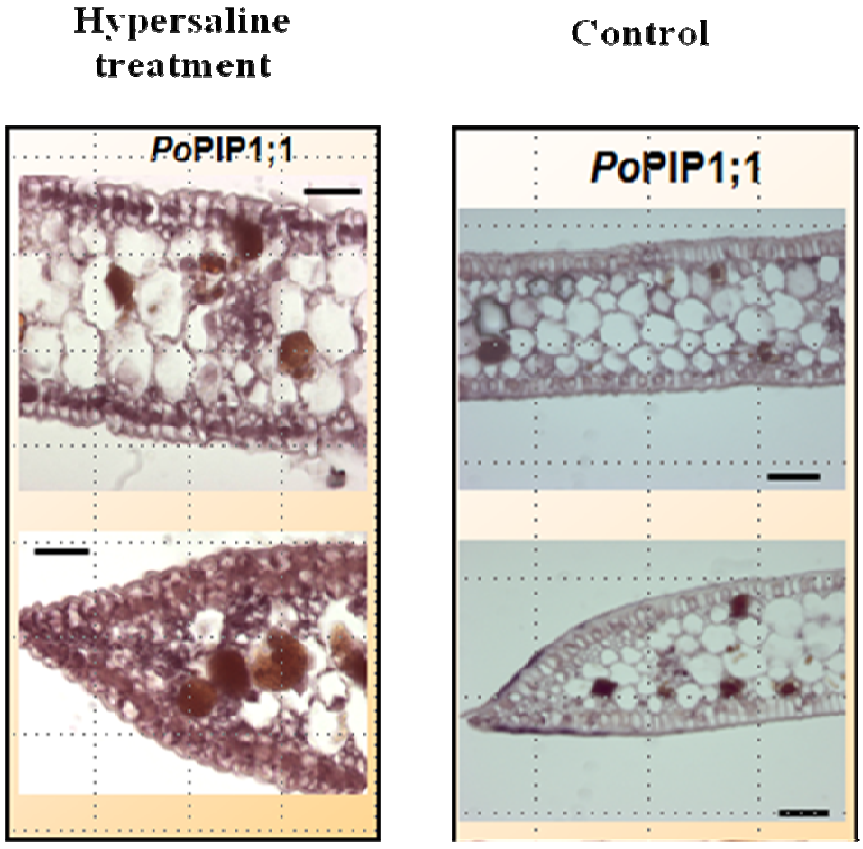


Figure 17 In situ hybridation of *Po*PIP1;1 mRNA on cross section of *Posidonia oceanica* leaves.

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Chapter 2: Materials and methods

Experimental procedure

In order to gain insight on the role of aquaporins, in seagrasses adaptive mechanisms to salt water, we apply the immunolocalization of PIP1;1 aquaporin in *Posidonia oceanica* leaves through western blot, immunolocalization with TRITC for light microscope and immunogold for T.E.M..

2.1 Material

Posidonia oceanica cuttings were collected, by SCUBA diving, in a pristine meadow sited in Cirella at depth of -5 m. A first group of cuttings was subjected to salt treatment (45‰) for two days, whereas a second control group was kept in the salt water concentration of collection site for the same time. After salt treatment the leaf samples were fixed in paraformaldehyde 4% in PBS 1x and subjected to vacuum treatments with a vacuum pump.

In the year 2009 the *Posidonia* meadows of Calabria showed a notable flowering and a lot of fruits were found on the beach after the occurrence of drift events.

This event allows us to perform salt treatment also on seedlings with the purpose to investigate the variations of mRNA transcription through rtPCR and the precise localization trough immunogold in joung growing leaves.

Before salt treatment the seeds were extracted carefully from dissected fruits, washed in sterilized natural sea water, and then placed in a folded filter paper inside semitransparent covered plastic containers, saturated with sterilized natural seawater (40 ‰) according to Belzunce *et al.*, 2008. Seeds were, then, placed in a germinating chamber

The seeds were checked weekly, to be sure that the seawater saturation was maintained.

After two week they were placed in aquarium with natural sea water (40‰) for an additional week. Then they were submitted to salt treatment as follow

Three cutting and seven juvenile seedlings were placed in sea water with ipersaline concentration (45‰) and the same number was kept in normal seawater (40‰, control) for two days. The salt concentration was obtained using synthetic salt for aquaria and de ionized water (45,5 gr/l for iper saline water and 40,5 gr/l for normal seawater).

2.2 Immunolocalization procedure

The antibody, used for immunolocalization analysis, have been furnished by Dr.s Maurel C. and Santoni V. from the Institute of Biochimie et Physiologie Moléculaire des Plantes, Agro-M/CNRS/INRA/UM2, Montpellier, France. It was polyclonal and was raised in rabbit against an *Arabidopsis thaliana* PIP1;1 peptide in the portion N-terminal (MEGKEEDVRVGANKFPERQ) that shares a sequence similarity of 83% with *Po*PIP1;1.

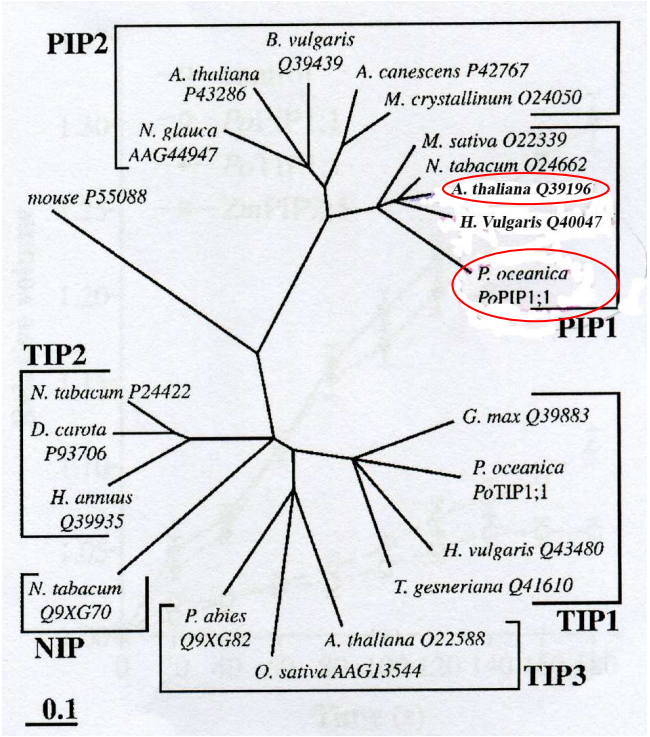


Fig. 18 Phylogenetic tree, based on deduced protein sequences of aquaporin-encoding genes and obtained by neighbor-joining analysis. The scale bar represents genetic distance(Maestrini et al., 2004)

2.3 Protein extraction and Western blot

The specifity of the antibody was tested by western blot analysis.

1 g of leaf sample were ground in liquid N_2 to obtain a powder that was further ground to a fine powder by the aid of quartz sand (silicon dioxide) and then transferred in 2-ml microtubes. In the tissue powder, 2 ml of 20% aqueous TCA containing 1% PMSF (phenylmethylsulfonylfluoride), as a protease inhibitor, was added. After vortexing, the resultant pellet was washed once more with 20% TCA+1% PMSF. The pellet was rinsed with cold 80% acetone, vortexed and centrifuged until the supernatant became colorless. The final pellet was dried at room temperature. Proteins were extracted using the phenol extraction method described by Meyer *et al.* (1988) and optimized for recalcitrant plant tissue by Wang *et al.* (2003).

About 0.1 g of the dry leaf tissue powder was resuspended in 0.8 ml phenol (Trisbuffered, pH 8.0; Sigma St Louis, MO, USA) and 0.8 ml dense SDS buffer (30% sucrose, 2% SDS, 0.1M Tris–HCl buffer, pH 8.0, 5% 2-mercaptoethanol) in a 2 ml microtube. Samples were vortexed for 30 s and centrifuged at 13,000 rpm for 5 min. The upper phenol phase was removed, and pipetted to fresh microtubes (0.3 ml for each 2-ml tube), 5 volumes of cold methanol plus 0.1M ammonium acetate was added and the mixture was stored at –20°C for 30 min. The precipitated proteins were recovered by centrifugation at 13,000 rpm for 5 min, and then washed with cold methanolic ammonium acetate twice and with cold 80% acetone twice. The

final pellet was dried and dissolved in Laemmli (1970) sample buffer, used to cast 4.75 stacking and 12.5 resolving gel.

Proteins were quantified spectrophotometrically by the Bradford method (1976). The pellet was incubated, from 2 h to overnight, in loading buffer. After denaturation at 95°C for 3 min, proteins were resolved under constant 120 V in a Bio-Rad mini- Protean II apparatus until bromophenol blue reached the bottom of the gel. Gels were processed using the Bio-Rad software.

Western blot was performed using a policolonal antibody against an *Arabidopsis thaliana* PIP1;1 peptide in the portion N-terminal (MEGKEEDVRVGANKFPERQ) that shares a sequence similarity of 83% with *Po*PIP1;1 (1:500), and as the secondary antibody anti-rabbit alkaline phosphatase, AP conjugated (1:1500). The detection was performed with the NBT/BCIP (4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl- phosphate) reagent kit.

2.4 Immunofluorescence

Samples from the basal portion of intermediate leaves were fixed in formaldehyde and thereafter dehydrated in ethanol, cleared in xylene and embedded in paraffin wax.

Cross sections , 8 μm tick, were cut, mounted on gelatin coated slides and dewaxed.

Immunofluorescence staining was carried out as described by Coons *et al.* (1955). After washing with PBS, the sections were incubated for 10 min in a moist chamber at 20°C with 20% normal goat serum to block aspecific sites. Unwashed sections were incubated overnight at 4°C with anti-PIP1,1 antibody developed in rabbit (1:2500), except two slides incubated with only goat serum to verify the labeling specificity.

The day after the slides were submitted to several washes in PBS and incubated with Tetramethyl Rhodamine Isothiocyanate-conjugated g-globulins goat anti-rabbit (TRITC 1:200, Sigma- Aldrich) for 30 min at room temperature.

Finally the sections were washed with PBS and mounted in Vectashield (Vector Lab. Burlingame, CA).

The observations were carried out with a Leica TCS SP2 Confocal Laser Scanning Microscope.

2.5 Immunogold

The immunogold reaction was tested both on the younger basal portion of leaves and on the leaves of *Posidonia oceanica* seedlings since the expression of *Po*PIP1;1 gene was found to be higher in the young tissues by Maestrini *et al*. (2004) (Fig.3).

All specimens were fixed with formaldehyde (4%) in phosphate buffer (PBS 1x pH 7.3-7.4) and submitted to vacuum treatments with a vacuum pump and then transferred in formaldehyde 1% in PBS 1x pH 7.3-7.4, 3x 10'.

Thereafter sample were washed with cold buffer (PBS pH 7.3) and dehydrated with a graded alcohol series up to 70% alcohol at 4°C in continuous agitation, then the specimens were embedded in LR White resin with catalyst.

Sections were cut with a diamond knife on a ultra mictotome and collected on grids.

Immuno-staining was performed treating the grid-mounted sections with the following solutions:

a) 3x5' in 0,15 M glycine in PBS 0.1 M pH 7,6 for inactivation of aldehyde sites.
b) 10' in 1% BSA in PBS 0.1 M pH 7,6 for inactivation of aspecific sites
c) Antibody anti-PIP1 in BSA 1% (1:400) overnight at 4°C in a moist chamber, except control grids which were maintained in BSA 1% overnight at 4°C
d) 3x2' Washing in BSA 1%
e) 30' Antibody IgG anti rabbit developed in goat gold-conjugated in BSA 1% (1:100)
f) 5x1' Washing in BSA 1%
g) 2x5' washing in double distilled water

All sections were counterstained with uranyl acetate and lead citrate, then they were metallized with metal graphite and, finally, were examined at Transmission Electron Microscope (T.E.M.).

Chapter 3: Results

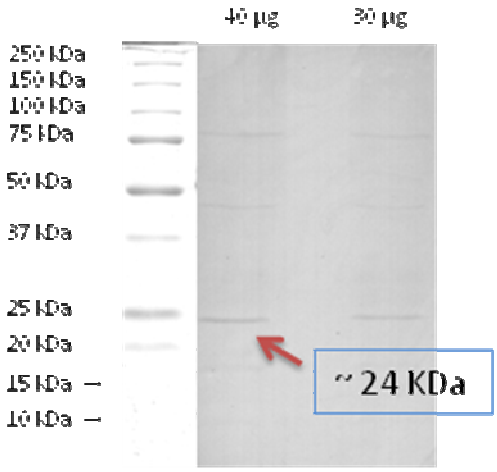


Figure 19 Western blot on *Posidonia oceanica* proteins extract.

Western blot analysis showed that the antibody used was specific. The more evident band is about 24 KDa (Fig. 19), the molecular weight of a aquaporin monomer. The other band were about 50 KDa and 75 KDa, that are multiple of 25 KDa, so they represent, very probably, dimer and trimer associations.

The immuno fluorescent staining, performed on cross leaf sections of *Posidonia oceanica*, revealed that, in spite of the presence of auto fluorescence (green), (Figure 22), the antibody signal appeared clear. In fact doing a merge it can be easily distinguished the yellow auto fluorescent signal from the real red-orange PIP1;1, signal.

The antibody signal appeared more intense in the leaf sections of cuttings subjected to hypersaline treatment, than in control ones.

The parenchyma cells of mesophyll showed the higher label amount, but the signal was also present in vascular tissue and in epidermis cells (Fig. 20, 21 and 22).

The TEM observations clearly revealed the PIP1;1 antibody localization either on plasma membrane and on chloroplasts (Fig. 23, 24 and 25).

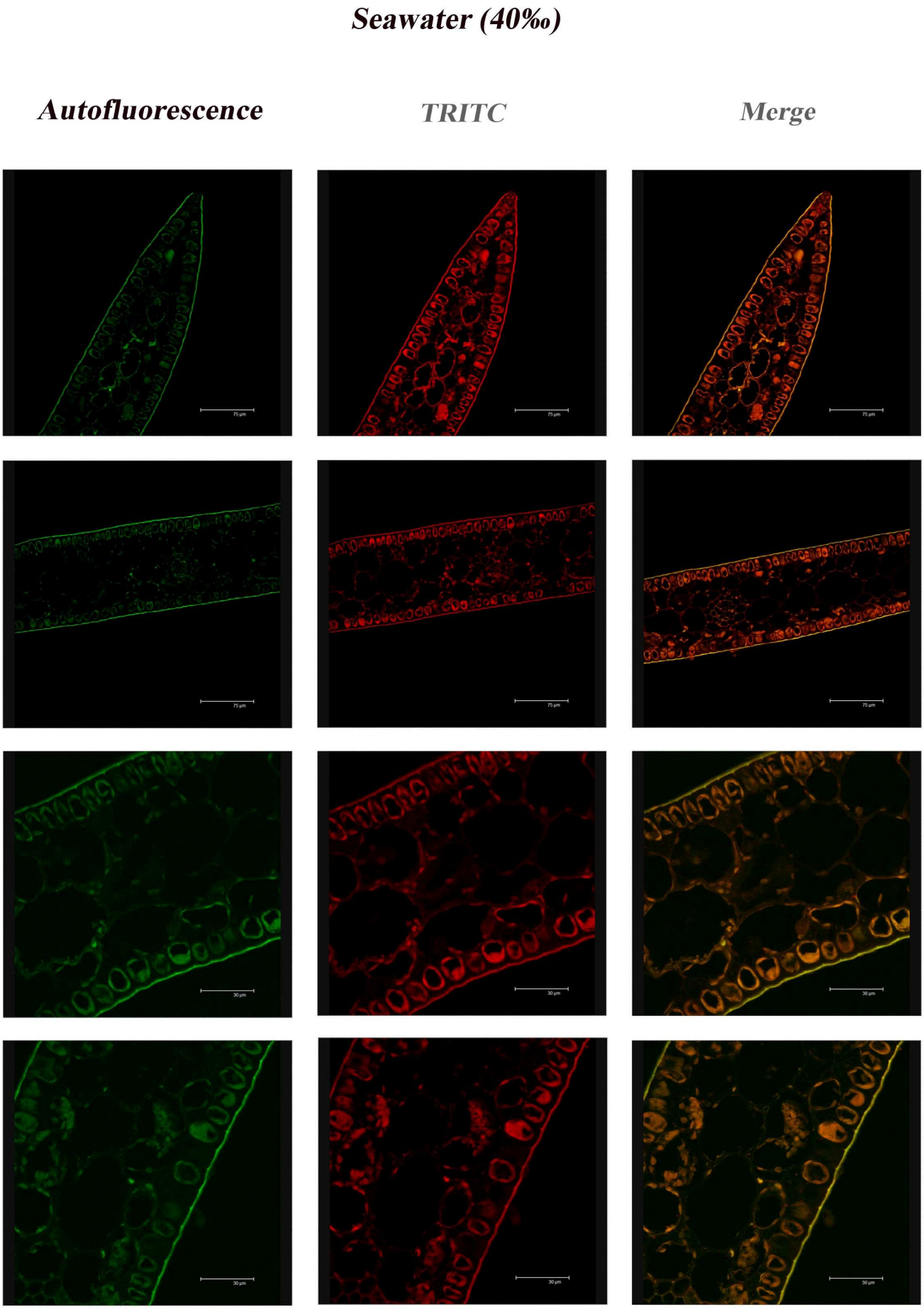


Figure 30 Immuno localization of PIP1;1 on cross sections of *Posidonia oceanica* leaves maintained in natural seawater salinity. On the left, sections showing only auto fluorescence are reported; In the middle sections with TRITC signal; on the right sections with the merging between auto fluorescence and real antibody signal.

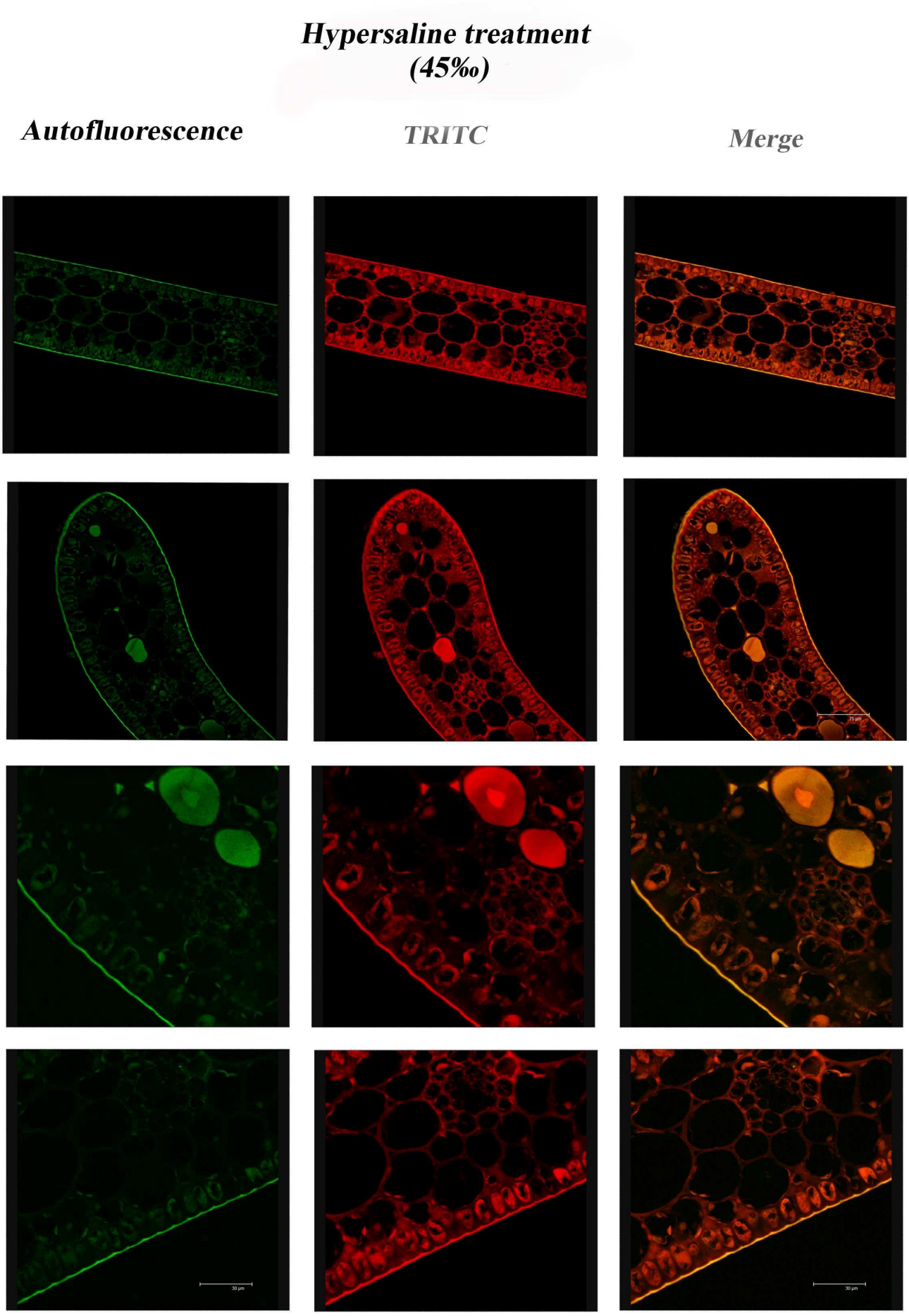


Figure 21 Immunolocalitation of PIP1;1 on cross sections of *Posidonia oceanica* leaves subjected to hypersaline stress. On the left side, sections showing autofluorescence are reported, on the middle sections with TRITC signal, on the right side sections with the merging between autofluorescence and real antibody signal.

Control

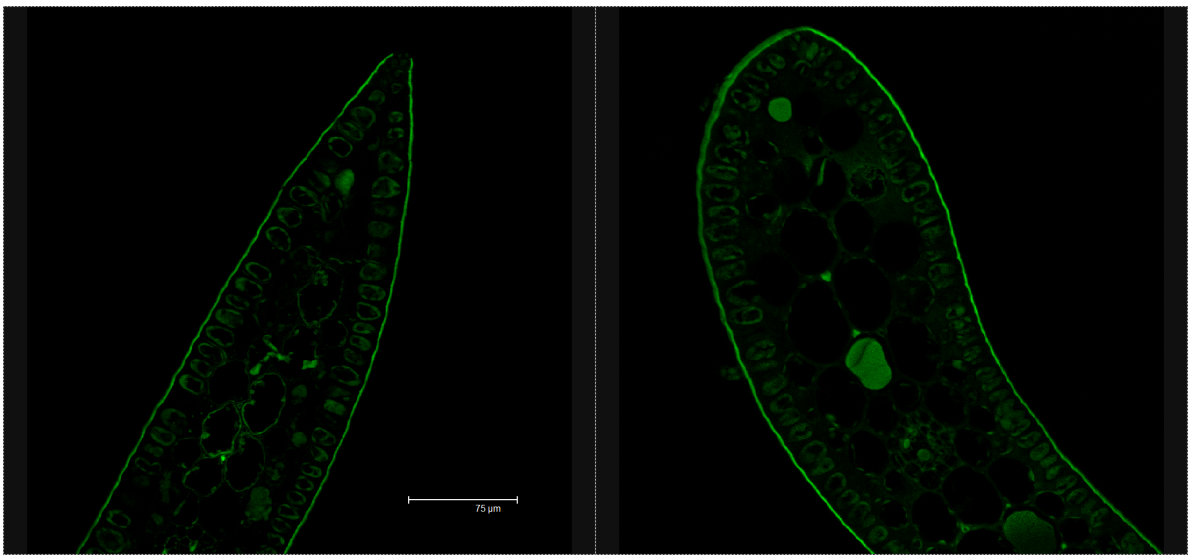


Figure 22 Sections of control without the secondary antibody showing the specificity of signal reaction

PIP1;1 on juvenile leaves chloroplasts

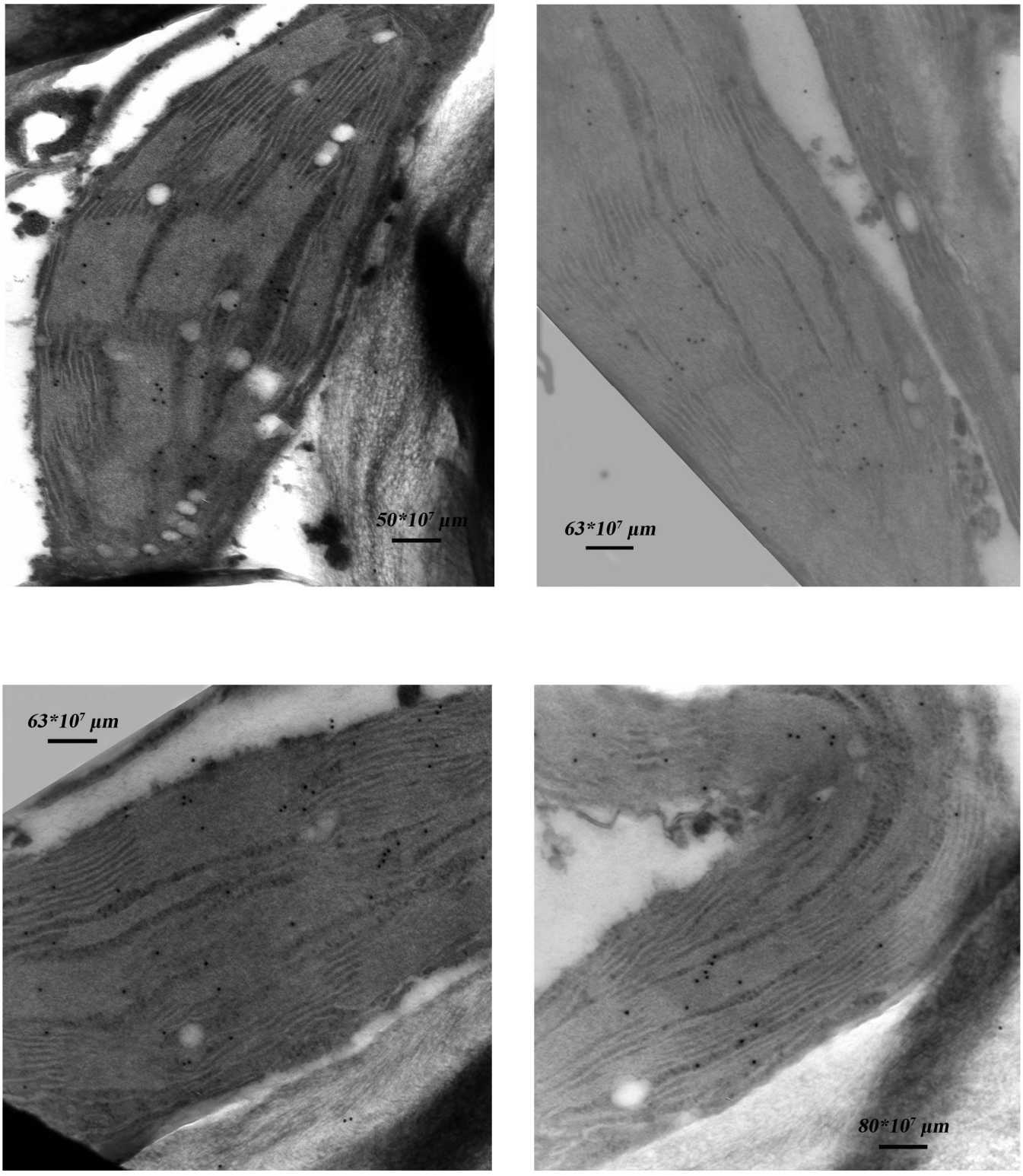


Figure 23 PIP1;1 Immunogold reaction on seedling *Posidonia oceanica* leaf chloroplasts

PIP1;1 Immunogold on adult leaves

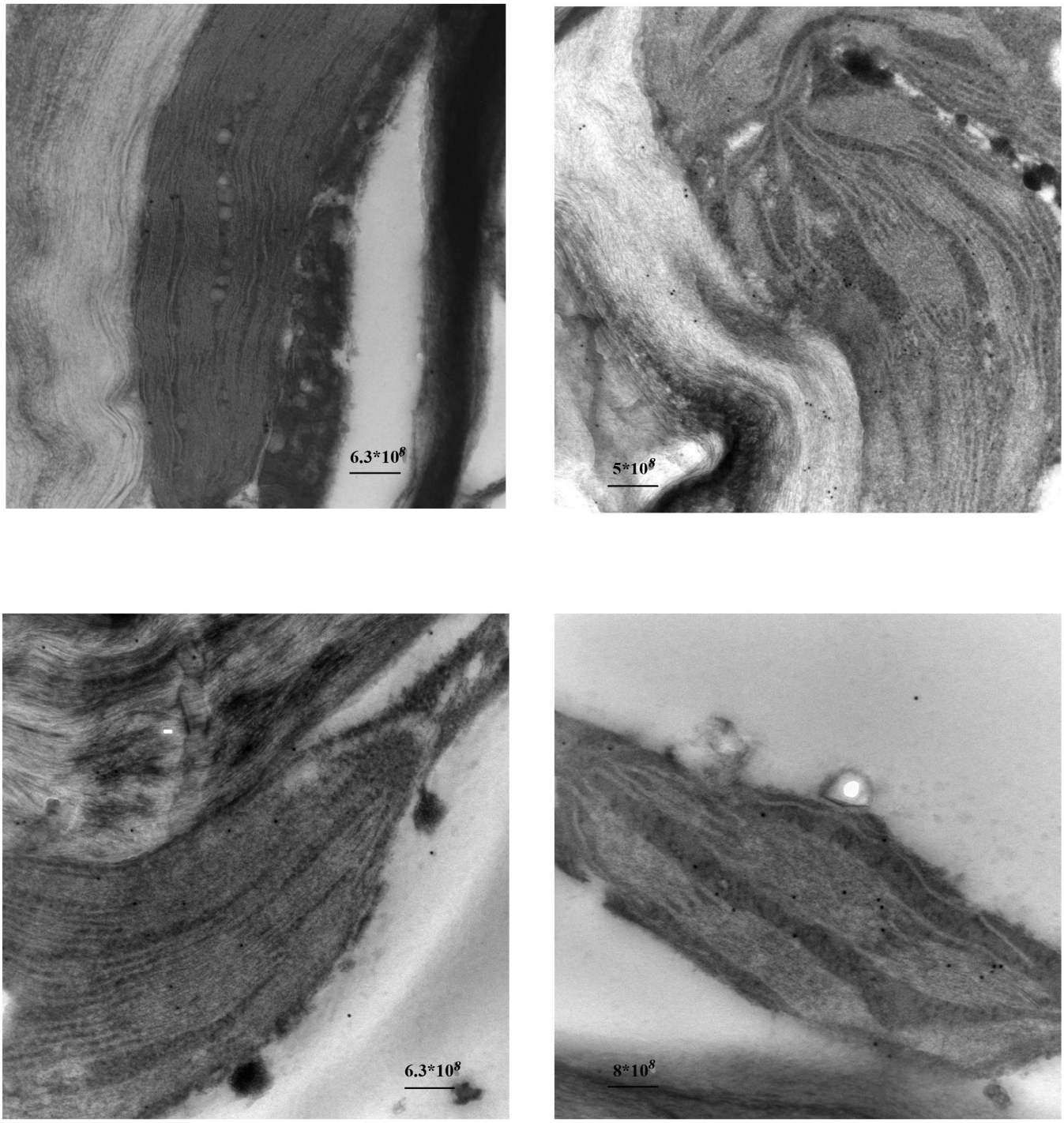


Figure 24 PIP1;1 Immunogold reaction on adult *Posidonia oceanica* leaf chloroplasts

Control

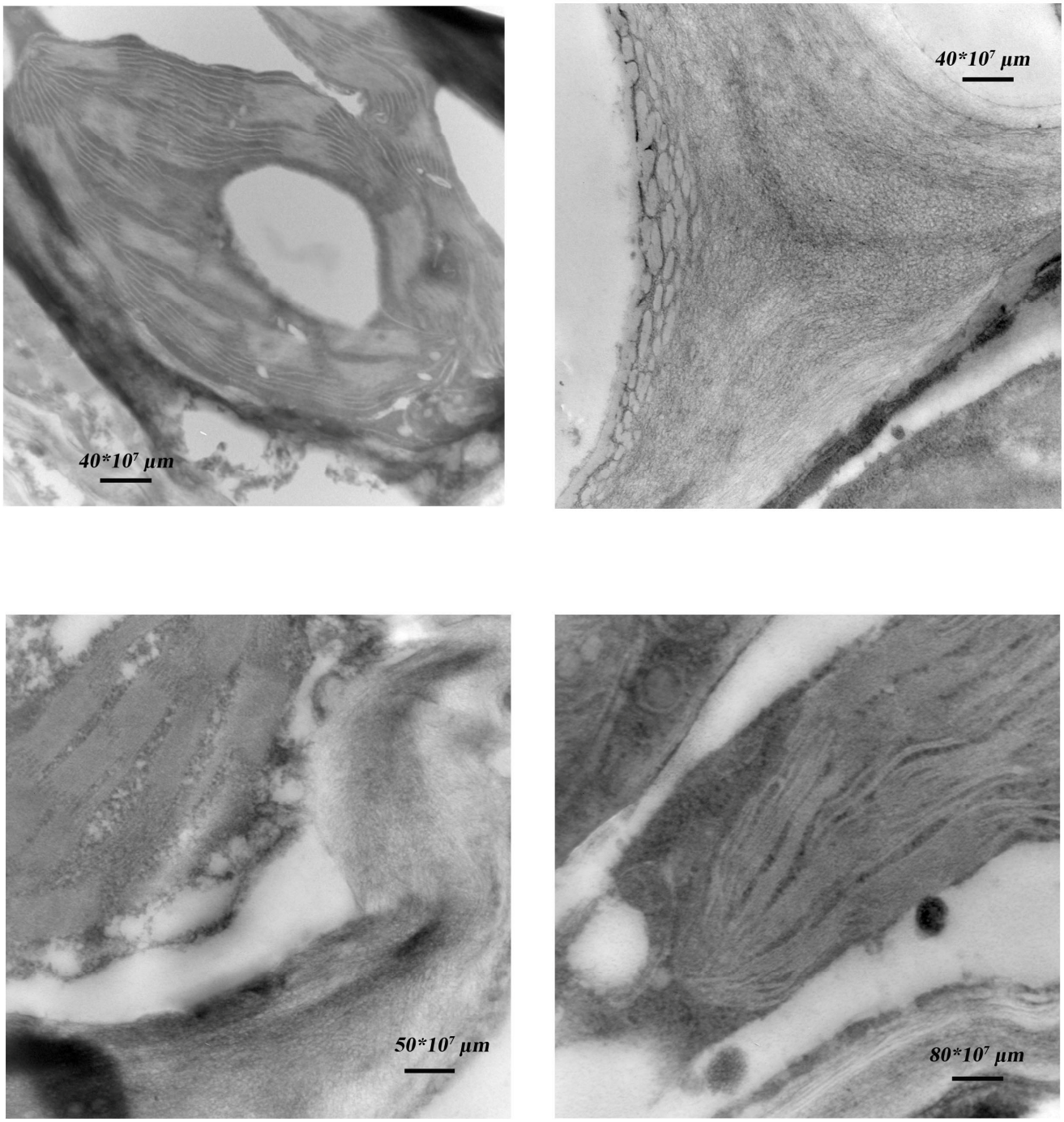


Figure 25 Control sections without the secondary antibody, where no signal is present

Chapter 4: Discussion and conclusion

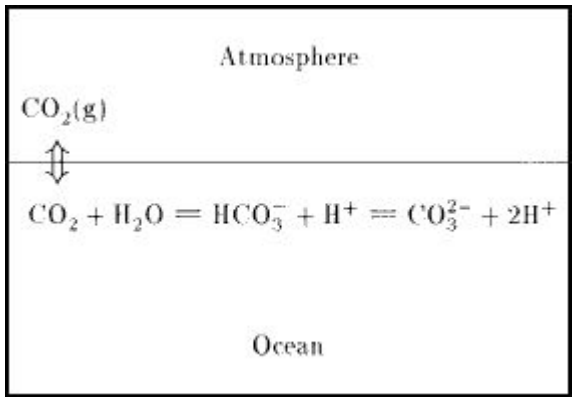
The obtained results confirmed the previous literature data indicating that under hyper saline stress conditions an over-expression of *Po*PIP1;1 aquaporin occurs (Maestrini *et al.* 2004), in fact we found that the anti-PIP1;1 antibody against *Arabidopsis thaliana* labeling was greater after ipersaline treatment. In addition, the antibody signal was localized in the same leaf tissues showing *Po*PIP1;1 mRNA immunolocalization(Cozza and Pangaro 2009). Thus indicating that very probably the aquaporin action occurs in the same site of its protein synthesis.

Given that the PIP1;1 antibody was present in epidermis, mesophyll parenchyma and vascular leaf bundles this tissues localization suggested its relevant role in the preservation of osmotic balance in the seagrass. Thus, *Po*PIP1;1 could allow the water entry in the leaf cells as well as prevention the salt entry. However in *Xenopus laevis* oocytes the heterologous expression of *Po*PIP1;1 did not show a volume increasing like the heterologous expression of *Po*TIP1;1 (Maestrini *et al.*, 2004), suggesting that *Po*PIP1;1 role could be more complex. Another possibility could be that this protein needs to be chemically modified by components not present in *Xenopus laevis* oocytes or that the strong activation of these acquaporine occurs only after subunit-subunit interactions in plant cells (Chaumont *et al.*, 2000; Fetter *et al.*, 2004; Suga and Maeshina 2004).

In addition the chloroplast localization of PIP1;1 antibody signal that we found by TEM immuno gold analysis strongly suggest that this acquaporine could be also a CO_2 channel like *Nt*AQP1 in *Nicotiana tabacum* (Uehlein *et al.*, 2008).

*Nt*AQP1 belongs to subfamily of PIP1 and it is present both in plasma membrane, and in the inner membrane of chloroplast, although a classical chloroplast transit peptide are not present. Moreover, *Nt*AQP1 seems to switch function from a water transport facilitating channel to a CO_2 transport facilitating channel, depending on its cellular location or on the intrinsic CO_2 permeability of the membrane where it resides (Uehlein *et al.*, 2008).

Thus also *Po*PIP1;1 could play a different role depending by its cellular location, acting either as water channel in the plasma membrane, as demonstrated by the enhanced immuno localitation after salt treatment or as CO_2 channel in the chloroplast membranes.



$$DIC \equiv \Sigma CO_2 = [CO_2] + [HCO_3^-] + [CO_3^{2-}]$$

$$K_1^* = \frac{[HCO_3^-][H^+]}{[CO_2]}$$

$$K_2^* = \frac{[CO_3^{2-}][H^+]}{[HCO_3^-]}.$$

Figure 26 Carbon balance in natural seawater

In marine environment CO_2 uptake and distribution is a factor of primary importance for a seagrass.

In fact, in land plants during photosynthesis, CO_2 moves from the atmosphere (Ca) surrounding the leaf to the sub-stomatal internal cavities (Ci) through stomata, and thereafter to the carboxylation site inside the chloroplast stroma (Cc) in mesophyll cells.

In marine sea grasses carbon dioxide diffuses trough water ~ 10000- fold more slowly than trough air (Stumm and Morgan, 1996) and unlike many other gases (oxygen for instance), it reacts with water and forms a balance of several ionic and non-ionic species (collectively known as dissolved inorganic carbon, or DIC). These are dissolved free carbon dioxide ($CO_{2\,(aq)}$), carbonic acid (H_2CO_3), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), and they interact with water as shown in Fig. 26:

Stoichiometric equilibrium constants depend on temperature, pressure, salinity and pH (Zeebe and Wolf-Gladrow, 2001).

In seawater the pH is regulated by the charge balance of a number of positive (e.g. Na^+, K^+, Mg^{2+}, Ca^{2+}) and negative (e.g. CO_3^{2-} itself, Cl-, SO_4^{2-}, Br^-) ions. Normally, the balance of these species leaves a net positive charge. To compensate this carbonate system, the excess of positive charge shifts the balance of carbonate species towards negative ions. The result of which is a reduced concentration of the free carbon dioxide and carbonic acid species, which in turn leads to an oceanic uptake of carbon dioxide from the atmosphere to restore the balance.

The millimolar concentrations of dissolved inorganic carbon (DIC) in seawater are about

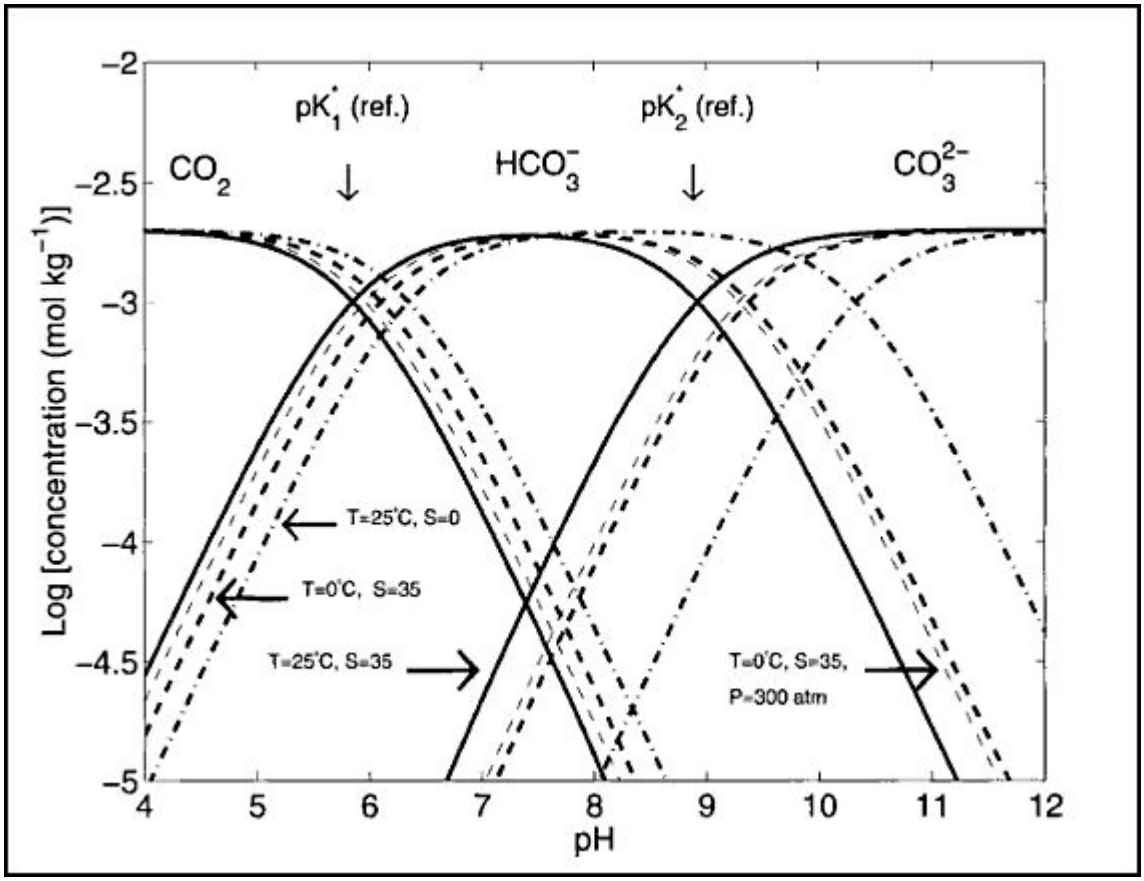


Figure 27 Carbon balance depending on pH values

three orders of magnitude greater than the concentration of other dissolved inorganic resources, generally considered to limit plant growth, such as nitrate or phosphate, which are typically in the micromolar range.

However, the primary carbon source for RUBISCO (aqueous dissolved carbon dioxide, CO_{2aq}) represents only 0.5–1% of the total DIC pool, while HCO_3^- represents ca. 90% of the DIC pool for seawater at pH 8.1–8.3 (Fig. 27) (Stumm and Morgan, 1981). Seagrasses generally live in seawater at a pH of ca. 8.2 and a bicarbonate concentration of ca 2 mol m^{-3} (Larkum *et al.*, 1989), while the CO2 concentration is very little (ca 10mmol m^{-3} at 25°C). Thus, the demand for inorganic carbon in marine photosynthesis is often met by the use of HCO_3^- (Burns and Beardall, 1987; Raven, 1991; Berman-Frank *et al.*, 1994; Maberly, 1990; Madsen and Sand-Jen-sen, 1991; Beer, 1994; Raven *et al.*, 1995).

Several studies indicated that there are at least two systems of HCO_3^- uptake:

(i) an indirect mechanism mediated by the activity of an external carbonic anhydrase and

(ii) a direct mechanism of HCO_3^{2-} uptake. External carbonic anhydrase (CA) catalyzes the dehydration of HCO_3^- to $CO_{2(}$aq) , which diffuses readily into the cell (Badger and Price, 1994).

Using different approaches (e.g. CA inhibition, inhibition of HCO_3^- uptake, etc..) it has been demonstrated that photosynthetic use of HCO_3^- is a common, but variable feature of marine angiosperms (Millhouse and Strother, 1986b; Durako, 1993; James and Larkum, 1996; Beer, 1996b; Beer and Rehnberg, 1997; Bjo¨rk *et al.*, 1997).

In *Posidonia oceanica*, as well as in *Cimodocea nodosa*, the indirect use of bicarbonate mediated by external carbonic anhydrase plays an important role in the photosynthesis (Invers *et al.*, 1999). In fact, the lack of an efficient mechanism of bicarbonate utilization in these seagrasses could explain the decrease in photosynthesis at the increasing of pH values, and the described limitation of production of seagrasses by inorganic carbon in conditions of light (Invers *et al.*, 1997) and nutrient availability (Beer and Koch, 1996).

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Part III

Adaptative response to depth

Chapter 1 Anatomical strategies evolution

Posidonia oceanica meadows extend from near the surface down to 40m depth (Bay, 1984), thus, the plants located at different depths are adapted to very dissimilar environmental conditions. When the light meets the sea surface, a certain amount is reflected depending on the incidence angle.

When the incidence angle is close to the 90° (i.e. perpendicular to the surface of the water) the penetration is greater and the reflected fraction minor. The incidence angle changes during the day, among the seasons and with the latitude.

The light that penetrates in water changes its luminous intensity (measured in Lux) and its spectral composition (wavelength) as a result of different absorption levels (fig.1).

The first obstacle which light meet in water is relates to the its reflection by suspended inorganic and organic particulate. Namely, water rich of suspended material show a decreasing of transparency that determines, a rapid light intensity extinction. In August, in temperate areas in August, a light intensity equal 150,000 LUX reaches the surface of water. This intensity is halved to half a metre of depth.

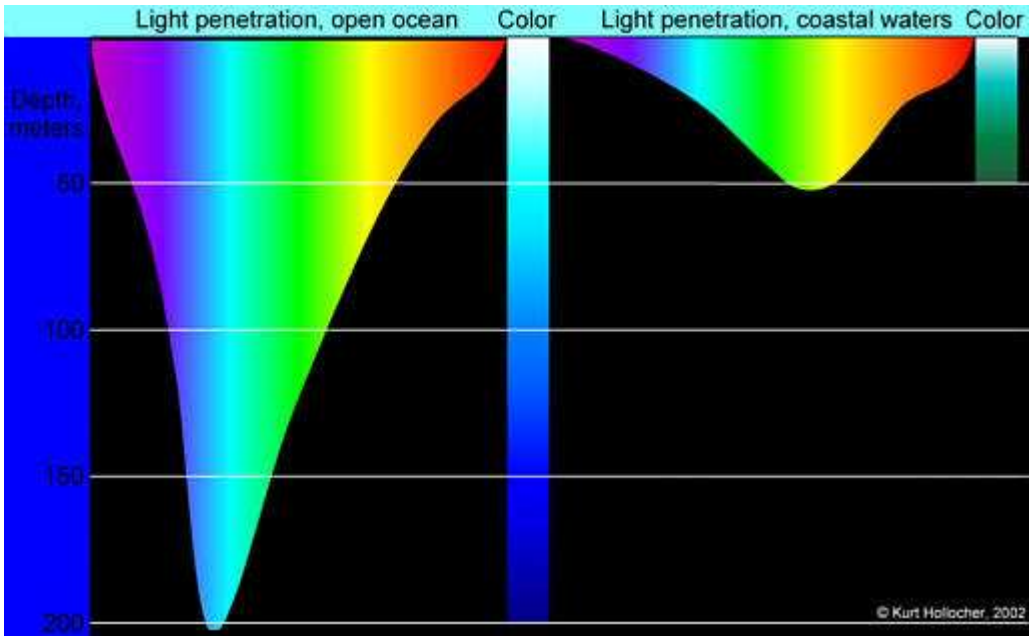


Figura 1 Schematic water penetration representation the different visible wavelengths as a result of the depth. To 20 m of depth, there is a drastic light reduction

The different waves of the visible spectrum penetrate differentially in sea water and reach different depth. The ultraviolet and infrared stop in the first centimetres of depth, thereafter progressively the red, the orange, the yellow, the green, and the violet extinguished whereas the blue reaches more depth.

This is the reason of open sea intense blue color.

In order to allow the photosynthesis capacity of a seagrasses meadow, the light must penetrate the column of water reaching photosynthetic cells of the leaves and through leaf epiphytes stratum (Dalla Via *et al.*, 1998).

In each of these steps the light is attenuated, but also because of additional factors such as the amount of material in suspension in water increased either by anthropogenic pollution or atmospheric phenomena (fig.2) (Ralph *et al.*, 2007).

So, the plants adapted to high depth are subjected to reduced light regimes that affects the photosynthetic efficiency.

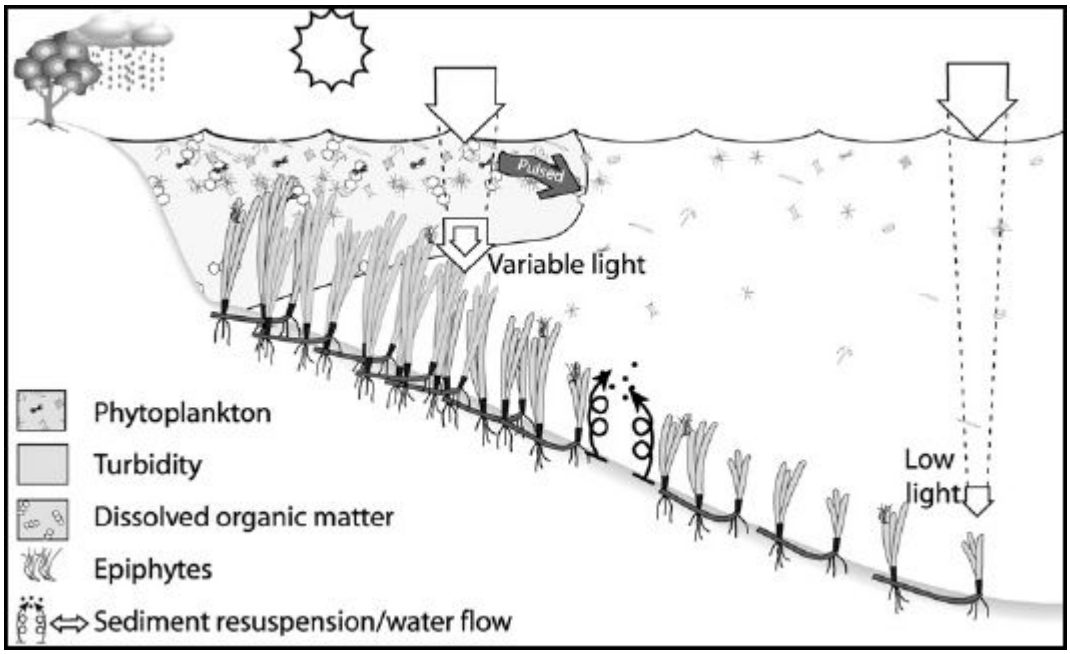


Figure 2 Causes of light reduction affecting *Posidonia oceanica* photosynthesis

The leaf density decreases approximately 72 % already to 10 m depth, also leaf portion in senescence decreases, slices while leaf number in bundles and width of leaf increase in depth in order to increase the active photosynthetic surface (Dalla Via *et al.*, 1998).

Generally in the small phanerogams, species showing scarce total biomass colonize deeper waters (e.g., *Halophila* in Australia and Florida; Coles *et al.*, 2000), whereas the greater species, like *Posidonia oceanica*, which can accumulate storage substances, can survive to low luminous intensity periods (Czerny and Dunton, 1995; Longstaff *et al.*, 1999).

In fact, in this species there is an asynchrony between growth and light absorption, because the maximum photosynthetic capacity is reached in summer when *Posidonia oceanica* produce carbohydrates for storage, while the growth begin at the end of winter using the substances produced in summer (Hall *et al.*, 1991; Dixon and Leverone, 1995). This is

particularly evident in the plants living at greater depth. Probably for this reason the cuttings collected in depth and replanted in shallow water show a good survival potential.

In fact, the cuttings collected from a meadows at 3 m of depth and transplanted in deep-sea (36 m approximately) showed low survival (14 %), whereas the cuttings collected from 30 m and transplanted to minor depth (3 m, 14m, 20 m) show a survival rates of about 96-100%.

In addition, no cutting collected from 3 m depth undergoes branching, whereas those collected from 30 m and transplanted in lower waters show branching and develop adventitious roots. (Molenaar et Meinesz, 1992).

1.1 Anatomy of Posidonia oceanica

1.1.1 Anatomy of leaf

Leaves are arranged in bundles, consisting of five to ten leaves, with older leaves outside, which differ in form and size from the younger inner leaves.

They are bright green, but become brown with age. They show from 13 to 17 parallel veins. The terminus of the leaf is rounded or sometimes absent because of damage.

According to Giraud, there are three kind of leaves in a bundle: adult, intermediate, and young. All leaves whose length is less of 5 cm are named young, adult leaves can be distinguish from intermediate because adult leaves have a ligule (Giraud, 1977)

In a leaf we can distinguish a base whitish and the photosynthesizing green remaining portion, respectively sheath and blade. The leaves are broad (5 to 12 mm) and the length usually varies from 20 to 40 cm but may be up to 1 m.

The form is typically elongated and flattened, so as they have a high surface-volume ratio, which, assumes a vital importance and evolutionary significance in a marine environment where spread both of O_2 and CO_2 are ten thousand times lower than those of the air medium (Stumm and Morgan, 1996).

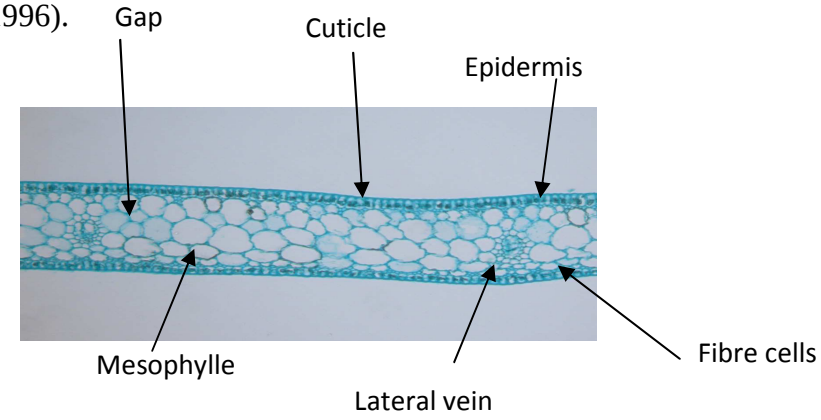


Figure 3: Cross leaf section of *Posidonia oceanica*

The leaves lack stomata and they have a cuticle with a complicated ultrastructure which regulates the ionic diffusion. The leaves are very flexible and have within the parenchyma a continuous "aerenchyma" system, which is also present in the rhizome and in roots ensuring a good movement of gas within the different organs (Fig.3) (Hemminga and Duarte, 2000).

The leaves show a basal growth due to the presence of a meristem zone.

Thus the younger part of the leaf is located close to the basal portion, while the oldest is located in the leaf apex, which is subject to degenerative phenomena, leading to a brown color and to the breakage of the leaf terminal portions.

The anatomy of leaf blade consists of a single layer of epidermisl cells and a parenchyma of 3-5 layers of cells, which contain a series of parallel vascular bundles.

In the leaf mechanical tissue are also present including a single marginal strand made up from a group of up to 30 fibers cells and other strands of 3-8 fibers cells just beneath the epidermis (Kuo, 1978).

In adult leaf, each epidermal cell shows a large central vacuole containing polyphenolic substances (Cozza *et al.*, 2004).

The cytoplasm is rich in ribosomes, mitochondria, endoplasmic reticulum (Kuo, 1978) and contain numerous chloroplasts, like Pteridophyta and other aquatic plants or plants that live in shady areas. This aspect is very probably, due to the little diffusion of CO_2 in the aquatic environment rather than related to light regime (Albergoni *et al.*, 1978).

Chloroplasts in epidermis cells are large with well-developed grana and many osmiophilic droplets, but they are normally devoid of starch grains. Cell wall consist of cellulose and pectin. Electron micrographs show that there are two distinct layers in the wall. The outer layer is thick and has a packed micro fibrillar structure, occasionally exhibiting some cross-hatching. The inner layer does not show fibrillar structures.

The cells of mesophyll are large with a thin wall, they contain a single large vacuole which occupies most of cell lumen and an exceedingly thin peripheral cytoplasm. Chloroplasts are large with well-developed grana and sometimes contain starch grains.

The fiber cells show a thick wall (ca. 1-2μm) that is strongly bi refracting, but not auto fluorescent indicating that cellulose and hemicellulose are present but without lignin. Lumen of fibre cells is small, ca. 1-2 μm in diameter (Kuo, 1978).

Each leaf is characterized by parallel veins (from 11 to 15), being the central one the most important. In cross section the veins show a sheath bordering the phloem whereas the xylem is very limited (Albergoni *et al.*, 1978).

Sheat's cells contain vacuoles with poli phenolic substances, and the citoplasme is rich in ribosomes and mitochondria. Each vascular bundles has 5-8 sieve tubes. Sieve tubes and sieve aereaa are similar in structure to those of land plants and show callose.

In the sieve tubes endoplasmic reticulum, mitochondria and plastids are present.

The xylem is represented by a lacuna surrounded by a group of xylem parenchyma cells. The xylem wall is highly hydrolyzed and only a small portion of lignified secondary thickening is present. All veins contain at least one xylem element. Both companion and xylem parenchyma cells have endoplasmatic reticulum, mitochondria, plastids and Golgi bodies.

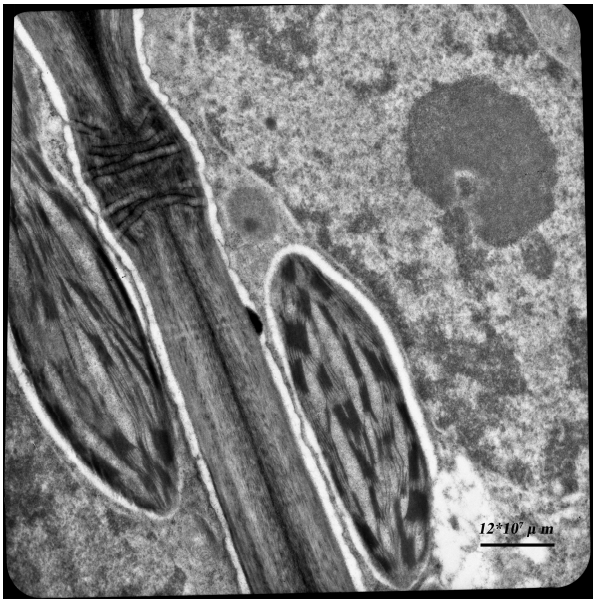


Figure 4 Plasmodesmata between two mesophyll cells.

The main veins are linked by transverse veins consisting of at least one line of tracheids and sieve tubes and a group of vascular parenchyma cells. Bundles sheats cells are also present. The size of each cell type is much smaller than those in longitudinal veins.

Numerous plasmodesmata can be observed in the wall of leaf cells: between adjacent epidermal cells (Fig. 4), between epidermal and mesophyll cells, between mesophyll cells, between mesophyll and vascular bundle sheath cells and between the cells of sheath and vascular parenchyma. They appear single or grouped, but they don't form pit fields like that present in the thick walls of land plants.

The sheath differs from the blade, concerning epidermis and mechanical tissues. The air lacunae are larger in the sheath than in the blade and epidermal cell wall is covered by a thin cuticle (27-50 nm thick) not porous. The epidermal and parenchyma cells lack chloroplasts entirely, they are highly vacuolated and sometimes contain polyphenolic materials. As in the leaf blade, a group of distinct lignified sheath cells surrounds each vascular bundle.

In transition region between sheath and blade, the epidermal cells become more elongated with thinner walls, so they look like to of sheath cells. The vascular bundles are continue from the blade to the sheath whereas the fibres are not continuous (Kuo, 1978).

1.1.2 Anatomy of rhizome

The rhizomes of *Posidonia oceanica* can easily be distinguished from those of the other Mediterranean seagrass species, since it is covered by dense, hairy remains of old, leaf sheaths after abscission. The rhizome internodes are short (0.5 to 2 mm) due to the slow horizontal growth of the plant, and the thickness of rhizomes varies between 5 and 10 mm. The roots are 3-4 mm thick, up to 40 cm long and richly branched ashore on the beaches.

The rhizome of *Posidonia oceanica* represents an unusual case of "poly stele", in fact in cross section it shows two different type of stele: a central atacto stele with peri xylematic bundles, at both side of which, there are three or four lateral steles consisting of one single perixylematic bundle.

All the bundles lie in a plane perpendicular to that of leaves tracks, which alternatively emerge from both sides of the central stele, to form the central rib of each leaf (Albergoni *et al.*, 1978) (Fig.5).

In *Posidonia oceanica*, the xylem is very reduced with elements represented only by spiral tracheids. The rhizome endoderm shows the Caspary band without further modifications of the wall, even in the older parts.

The structure of lateral steles is not well known. They are perixylematic bundles without medulla surrounded by either pericycle and endodermis as the central one. So they look like the haplostele of some Pteridophyta, but they are perixylematic bundles whereas in Pteridophyta the bundles are periphloematic.

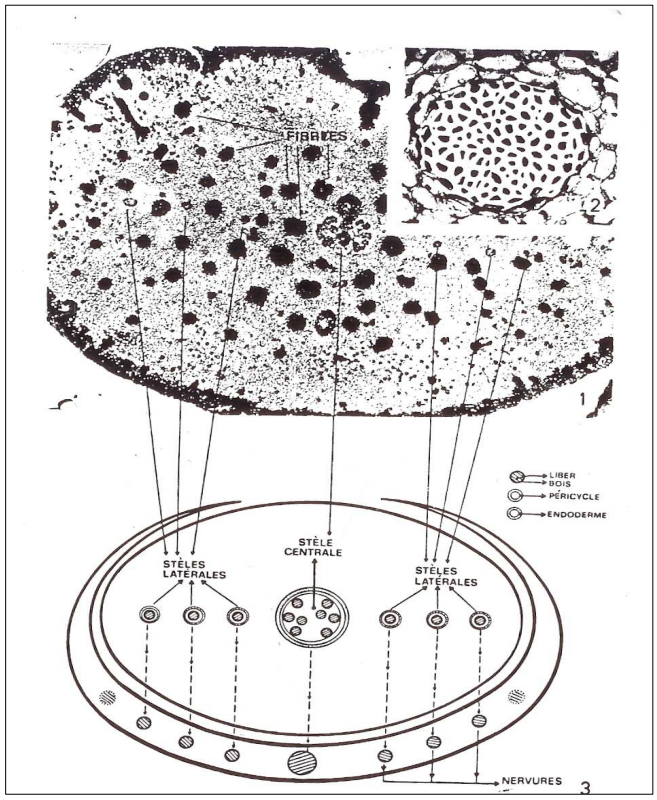


Figure 5 Section of a *Posidonia oceanica* rhizome

Each lateral stele has a net area of differentiation.

The shoot consisting of a flattened dome which represents the convergence point of lateral steles and of corresponding differentiation areas that remain distinctly separated.

The differentiation process is not synchronous, in fact it starts early in the more central, stems. Along the rhizome, the lateral bundles fork in an irregularly way branching angle and any

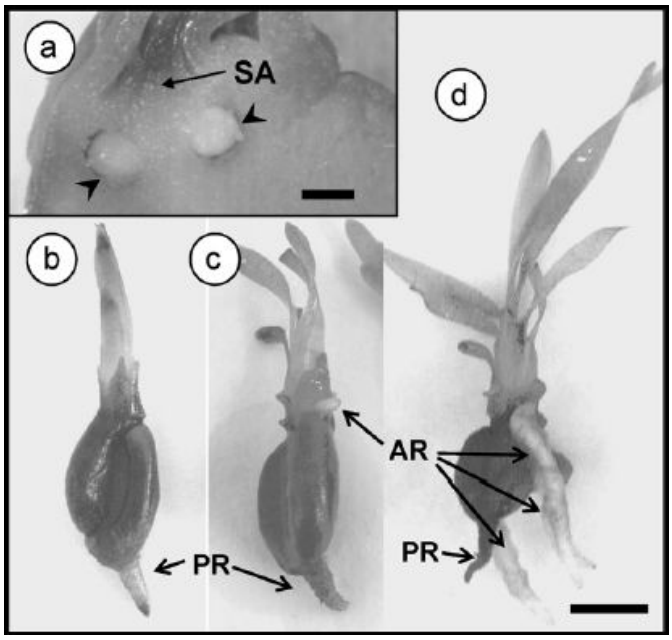


Figura 6 *Posidonia oceanica* seeds showing initiation, emergence and initial growth of root system. (a) Longitudinal fresh section of mature P. oceanica seed showing the two spherically shaped adventitious root primordia (arrows). The seedling shoot apex (SA) is visible above the hypocotyls node where the adventitious root primordia are formed. Scale bar = 1 mm.
(b) Two-week seedling; only primary root (PR) has emerged. (c) Three-week seedling. Primary root has emerged, and first adventitious root (AR) has begun emergence. (d) Seven-week seedling. Primary root remains relatively unchanged and various adventitious roots have emerged and grown. For (b– d) scale bar = 10 mm.

subsequently merges with that from contiguous bundles, forming a grid independent of the central stele.

There is not relationship between the frequency of this phenomenon and the presence of nodes which, on the contrary are very regularly distribuited.

All the bundles are involved in the formation of leaf veins, the external lateral branches of the stele determine the outermost vein of the leaf (however along the rhizome these branches do not present endodermis and they can be considered as leaf traces).

Each bundle determines the formation of one single leaf vein, therefore all the veins can be directly traced to steles. However generally the steles are seven or nine, whereas the leaf veins are eleven, thirteen or fifteen. Thus, in adult leaf the more external veins, originate from leaf parenchyma (Fig. 5).

The mechanical tissue consist of groups of fibers lignified and apparently arranged without any order that recalls the bundles distribution of an actinostele structure (Albergoni *et al.*, 1978).

1.1.3 Anatomy of seedlings Root

As in other *Posidonia* species (Kuo and Kirkman, 1996), *Posidonia oceanica* seeds have high potential for germination and survival due to their nutrient reserves and embryo structure, which includes both a primary root and two prominent lateral adventitious root primordia (Belzunce *et al.*, 2005).

The seedling root system of the seagrass *Posidonia oceanica* consists of a primary root and up to four adventitious roots. Under culture, germination and early growth starts with the emergence of the primary root in the first week. Therefore two adventitious root primordia emerged after 3-5 weeks whereas the further adventitious roots grow later. Primary roots

reached 17 mm after 4 weeks, but their growth decreased rapidly. In contrast the adventitious roots showed a continuous elongation pattern (Fig. 6).

The root apex of both primary and adventitious roots shows a well-defined root cap which appears different in the two root types. Namely in adventitious roots the cap, consisted of a prominent cone-shaped mass of cells that extended approximately 200–400 mm from the apical meristem. Whereas in the primary roots the cap extension was 110–170 mm from the meristem region. In general, root cap cells showed large nuclei and thick walls but they did not contain amiloplasts as described in mature root cap cells of adult *Posidonia australis* (Kuo and Cambridge, 1978). In adventitious roots the meristem region, was larger than in primary roots and consisted of a broader zone with many layers of apical initials with more densely stained nuclei.

In both primary and adventitious roots transverse sections a wide cortex is observable, which consists of a single layer epidermis with large cells thin-walled and tangentially elongated. Root hairs, with maximum lengths of approximately 300 mm were present at scattered, separated positions along the root epidermis.

Both of types root showed a predominance of cortex zone, which represent 99% of total cross-section area.

However the total cross-sectional area of adventitious roots was slightly higher (1.1 _ 0.4 mm^2) than that of primary roots (0.75 _ 0.38 mm^2). For both root types the cortical thin-walled parenchyma cells appeared roughly isodiametric (Fig. 3c and d) without air spaces and lacunae and they did not contain starch grains.

The outermost cell layers of cortex constitute a hypodermis compact layers of hexagonal shaped cells smaller than the inner cortical cells.

Generally, in primary roots, the hypodermis, consists of more cell layers than in the adventitious roots, whose cell walls were notably thick as demonstrated by their intense UV autofluorescence.

The stele, which represented 1% of the total root cross-section area, displays, anatomical differences between primary and adventitious roots, particularly in the degree of vascular tissue differentiation.

In fact, in the adventitious roots the xylem elements, in polyarch arrangement, show differentiated secondary walls, whereas in primary roots the vascular tissue is not completely differentiated.

During seed germination first the primary root emerges and grews rapidly, followed by the successive emergence of up to four adventitious roots which grew slowly.

This pattern would suggest that the early primary root emerges to facilitate the initial anchorage, whereas successively adventitious roots assure the final settlement.

Several studies, performed on different seagrasses, indicates that young roots are effective in water and nutrient absorption (Kuo and Cambridge, 1978; Hemminga *et al.*, 1994; Duarte *et al.*, 1998). Seagrass root structure appears to be species specific, with some degree of intraspecific variability (Barnabas, 1994b), and show multiple functional adaptations to the environment (Barnabas, 1991; Pérez *et al.*, 1994).

The thick multilayers hypodermis prevent salt entering in the cortex tissues, representing an apoplastic barrier essential for osmotic adjustment in the marine environment. The thick walls of hypodermis cells observed in older primary roots of young *Posidonia oceanica* seedlings can also provide strength, like the mechanical layer showed by seagrasses in surf-exposed habitats (Barnabas, 1994a).

The primary roots of the young *Posidonia oceanica* seedling had a notable endodermis, in contrast to the adventitious roots. The presence of an endodermis has been confirmed in the roots of adult seagrass species (Tomlinson, 1969; Kuo and Cambridge, 1978; Cambridge and Kuo, 1982; Barnabas and Arnott, 1987; Barnabas, 1991, 1994a,b).

In *Posidonia oceanica* the large cortex observed in seedlings, and the aerenchyma presents in adult plant, aids in ensuring a supply of oxygen by increasing porosity (Jackson and Amstrong, 1999; Striker *et al.*, 2007).

Additionally, a substantial cortex provides a large, metabolically inexpensive structure for support. In young *Posidonia oceanica* seedling roots we observed the narrow stele area as described in adult seagrass roots (Barnabas and Arnott, 1987; Barnabas, 1991, 1994b; Connell *et al.*, 1999). In contrast, Wahl and Ryser (2000) reported that the stele occupied 11.7–21% of the root cross section area of nineteen land grass species, ten to twenty times greater than the 0.75–1% of the stele area that were observed in the seagrasses. Very probably, the small stele area of *Posidonia oceanica* is due to the reduced vascular development, which is typical of aquatic plants (Peterson, 1992). The anatomy of *Posidonia oceanica* seedling root is similar to that land plants roots, but differs for the amounts of cells and tissues present and for or their degree of differentiation. These differences represent structural adaptations to the submerged marine environment which, are in line with those previously described in either adult seagrasses or other aquatic plants, with the exception of the absence of aerenchima (Belzunce *et al.*, 2008).

Adult root has a typical actinostelic structure with a variable number of archs (from 8 to 17) and also medulla’s cells lignifie.

Endodermis is characterized by thick lignified bands along the lateral wall arranged without a regular pattern. The inner parenchyma also lignified and the rhizodermis is transformed in mechanical tissue for the lignification of its cell walls (Albergoni *et al.*, 1978).

Thus, in *Posidonia oceanica* the roots lose their typical function of nutrients uptake and reserves to become organ whose main function is to anchor the plant to the substratum.

Since leaf aerenchyma is continuous up to the roots through the rhizome the oxygen, produced in the leaves during photosynthesis, reaches the roots. A little part of oxygen is released into the sediments, creating a microenvironment rich in oxygen, which promotes nitrification (NH_4^+ NO_2^- NO_3^-) and the absorption of some metals and minerals (Iizumi *et al.*, 1980).

Chapter 2: Material and methods

Experimental procedure

In order to gain insight on the adaptement of *Posidonia oceanica* to different depths we apply a morphoanatomical approach on leaves of this marine phanerogam through two different types of study: traditional quantitative analysis and the Geometric morphometry which is a more modern approach.

2.1 Material examined

Leaves of *Posidonia oceanica* were collected by SCUBA diving from a pristine meadow in Cirella (CS) that extends from 3m to about 30m and transported in laboratory in cans full of natural seawater.

This work was performed in two different time. The first time (July 2008) ten intermediate leaves from two different depths (-5m and -25m) were analyzed. The second time, twenty intermediate leaves from three different depth (-5m, -15m and -25m) in May 2009, were collected.

2.2 Data collection

In the first time it was taken a piece of 1 cm of each leaf, both in the basal portion of leaf (1cm above the interface sheath-blade) and in the middle portion (5cm above the interface sheath-blade).

In the second time, the intermediate leaves, from the three different depth, were separated in five length classes: from 20cm to 30cm, 31cm to 40cm, 41cm to 50cm, 51cm to 60cm, 61cm to 70cm.

Using a multiparametric probe (ver.2.00, IP151D – IDROMAR), it was been possible to record ecological parameters, such as: salinity, pH, conductivity, temperature and oxygen saturation.

For each leaf, it was taken a sample of 1cm at 5cm from the interface sheath-blade.

All the leaf samples were fixed in Carnoy (glacial acetic acid: absolute ethanol 1:3), thereafter submitted to vacuum treatments with a vacuum pump, dehydrated in ethanol, cleared in xylene and embedded in paraffin wax.

Cross sections, 20 μm tick, were cut by Leica RM 2155 microtome, mounted on gelatin coated slides and dewaxed. The sections were stained with fast green, dehydrated in ethanol, cleared in xylene and mounted in Canada balsam.

For each sections were taken photos using a Leica camera (0,63x magnification) connected to a Leica DMRD microscope (100x magnification)and with the software Leica application suite.

Photos were taken in a sequential way with overlapping margins, using the ribs as optical reference. Thus, it was possible rebuild the section with the function photomerge of the software Photoshop. Then, in order to made the geometric morphometric analysis, it was necessary to align the reconstructed sections placing ribs on a plain by Photoshop.

Figura 9: Reconstructed section

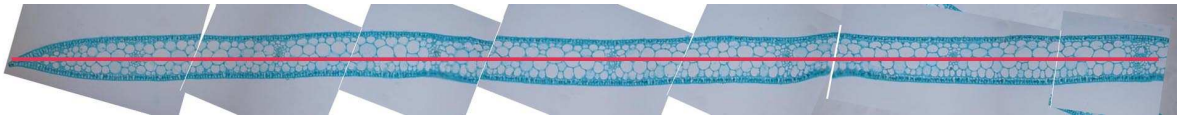


Figura 10: Aligned section

2.3 Quantitative morphometry

In order to gain a general overview on the leaf section a preliminarly quantitative analyses was undertaken on reconstructed sections consisting on the determinations of quantitative parameters regarding leaf epidermis and mesophyll, both at central rib level and at fifth rib level:

- Number of epidermal cells including in a segment of 200 µm
- Number of mesophill cells layers from epidermis to the central or fifth rib
- Number of mesophill cells within a square of 4 10^4 μm^2 area
- Number of mesophill cells from upper to lower epidermis including in a rectangle with shorter side of 200 µm
- Number of mesophill cells layers from upper to lower epidermis

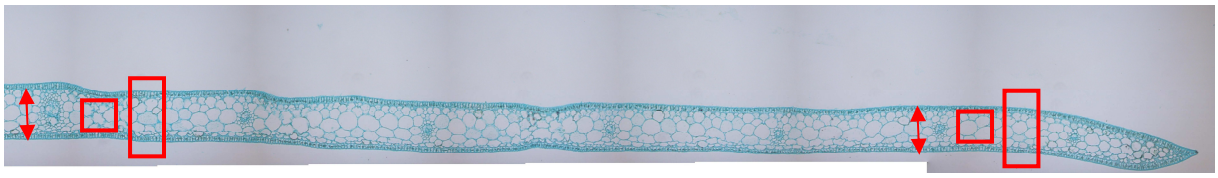


Figure 4 Simplified representation of histological parameters measured

Further, it was measured also the leaf width and thickness (at either central or peripherical level), the distance between leaf external edge and sixth, fifth and central rib and the distance between fifth and sixth ribs, thanks to the landmark method explained in the following paragraph. Distances to landmarks were measured on half-leaves (to avoid redundancy introduced by symmetry) of all leaves collected.

Some leaves sampling from - 5m and - 25m were "peeled, in order to study the photosynthetic surface at an epidermis level. The number of chloroplasts for each epidermis cell and their area was measured.

2.4 Geometric Morphometric Analysis.

Morphometry is the study of shape variation and its covariation with other variables (Bookstein, 1991; Dryden & Mardia, 1998).

In the late 1980's and early 1990's however, a shift occurred in the approach to quantify morphological structures as well as to analize the data. This shift emphasized methods that captured the geometry of the morphological structures of interest, and preserved this information throughout the analyses. In 1993 a review of the field of morphometrics called this new approach 'geometric morphometrics' a "revolution in morphometrics" (Rohlf & Marcus, 1993) defined as the fusion of geometry and biology (Bookstein, 1982b).

One of the best method to captured the geometry of the morphological structure is that using *landmark data.*

Landmark-based geometric morphometric methods begin with the collection of two- or three-dimensional coordinates of biologically definable landmarks defined by Cartesian coordinates (x and y). The identification of landmarks is simple, since biological objects are composed of many structural components whose location can be precisely defined.

In leaves of *Posidonia oceanica*, collected for geometric morphometry analisys, eight landmarks have been identified on the basis of homologies and parahomologies:

Anatomical landmark	Description	Type

1	Central rib	Homologous
2	External leaf edge	Para-homologous
3	Fifth rib	Homologous
4	Sixth rib	Homologous
5	Point in the epidermis above the central rib	Para-homologous
6	Point in the epidermis under the central rib	Para- homologous
7	Point in the epidermis above the sixth rib	Para- homologous
8	Point in the epidermis under the sixth rib	Para- homologous

Table 1 Description and classification of selected landmarks

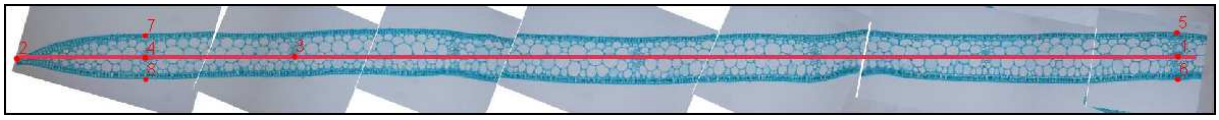


Figura 11 Landmarks on section

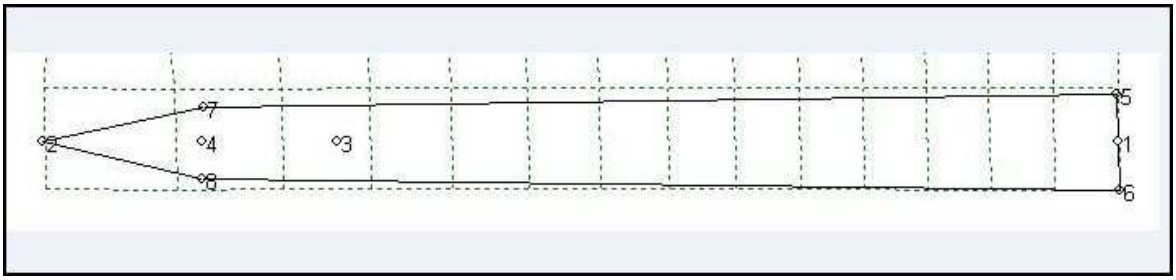


Figura 12 Configuration of a leaf section

The central, fifth and sixth ribs are surely homologous characters identifiable in each leaf sections. Several sections, but not all sections, have a seventh supernumerary rib that was not considered in this analysis.

The others landmark have to be considered parahomologous characters, because of they are points in the epidermis that are not indentifiable by a kind of visible referements, but only in relation with other point: landmark 5 and 6 are identifiable in relation to central rib, 7 and 8 by sixth rib, 2 (external leaf edge) is arbitrary fixed as the point most extern of leaf edge.

Before to analyze these coordinates as shape variables, the non-shape variation of the specimens in position, orientation, and scale, must be mathematically removed.

Superimposition methods eliminate non-shape variation in configurations of landmarks by overlaying them according to some optimization criterion. In this work, Generalized Procrustes Analysis method (GPA: called also Generalized Least Squares, GLS) was used.

This method superimposes landmark configurations using least-squares estimates for translation and rotation parameters. First, the centroid of each configuration is translated to the origin, and configurations are scaled to a common, unit size (by dividing by centroid size: Bookstein, 1986).

Finally, the configurations are optimally rotated to minimize the squared differences between corresponding landmarks (Gower, 1975; Rohlf & Slice, 1990). The process is iterated to compute the mean shape, which is inestimable prior to superimposition.

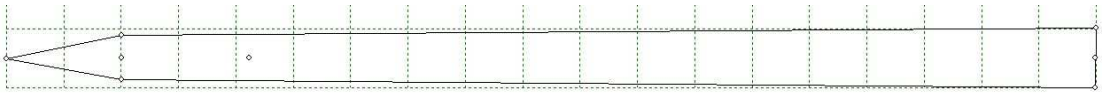


Figure 13 Mean shape (consensus configuration)

After superimposition, shape differences can be described by the differences in coordinates of corresponding landmarks between objects, by the TPS (thin-plate spline) method can be used to map the deformation in shape from one object to another (Bookstein, 1991), in this particular case was used to quantify the variation in the shape of leaves collected from the three different depth (Rohlf 1997).

Differences in shape represented in this fashion are a mathematically rigorous realization of D'Arcy Thompson's (1917) idea of transformation grids, where one object is deformed or "warped" into another. Differences in shape among objects can then be described in terms of differences in the deformation grids depicting the objects. The parameters describing these deformations (partial warp scores) can be used as shape variables for statistical comparisons of variation in shape within and between populations.

The configuration of references of the specimens by host were combined to analyze only the differences between the means. The warps, represented by the thin-plate splines function, were determined and decomposed in shape descriptors which may have uniform components that describe stretching, compression or scission (global variation) and localized components corresponding to changes that occur at specific regions. The principal warps are the eigenvectors of the matrix of the deformation energy and each one describes a possible change in the shape applicable to the main configuration. The superimposed configurations were then projected on the principal warps describing the differences in shape as deviations of the main configuration (Bookstein 1989, Rohlf 1993). The projections or scores generated

indicated how much of each principal warp was needed to accomplish the deformations. The scores, also called partial warps, described each leaf of the specimens as a linear combination between principal warps and the standardized coordinates x and y for each anatomic landmark. These scores can be used in multivariate analysis (Rohlf 1993, Rohlf *et al*. 1996). The relative warps, which are the main components of the matrix that combine scores of partial warps and uniform components, were obtained using the matrix of the scores of the partial warps.

2.5 Data analysis

The analyses of superimposing and relative warps were performed using the Tps Relw program (Rohlf 1998b). For the regression of the relative warps with depth TPS Regr was used, calculating the multivariate regression of Wilks on independent variables (Wilks' λ) with test that hypothesis zero was nil (p) and permutation tests with 1000 random permutations (λ_0).

The size analysis, *i.e*. the distance between landmark, and correlations between size and histological parameters (Sperman correlation) were calculated through Past. Correlation between shape (a multivariate parameters) and either size or histological parameters were performed using TPS PLS. This software was utilized also to calculate regressions of size and histological parameters by depth, appraising R^2, R^2 adjusted (R^2 adj.) and p (probability of hypothesis is nil).

All programs of the "TPS" series and PAST used in this research work are *freeware* (http://life.bio.sunysb.edu/morph).

Chapter 3 Results

3.1 *Geometric morphometry analisys*

The analisys were conducted in two different time.

In the first time the geometric morphometry analisys were conducted both at basal and at middle level of leaves collected from two different depths: -5m and -25m. However, the distribution of relative warps obtained by TPS Relw analysis show that there is not a substantial difference in the leaf shape at these two different levels, confirmed also by significant high regression between leaf shape data, at two leaf level, performed with TPS PLS software

For this reason, analysis were repeated in a second time on leaves, collected from the same meadow, but at three different depths (-5m, -15m and -25m), focusing the attention only on the middle zone of leaves. In this way, it was possible to increase the number of leaves analyzed with an important improvement of statistical analisys. The relative warp analysis, performed using the Tps Relw software, have done the PCA that follows:

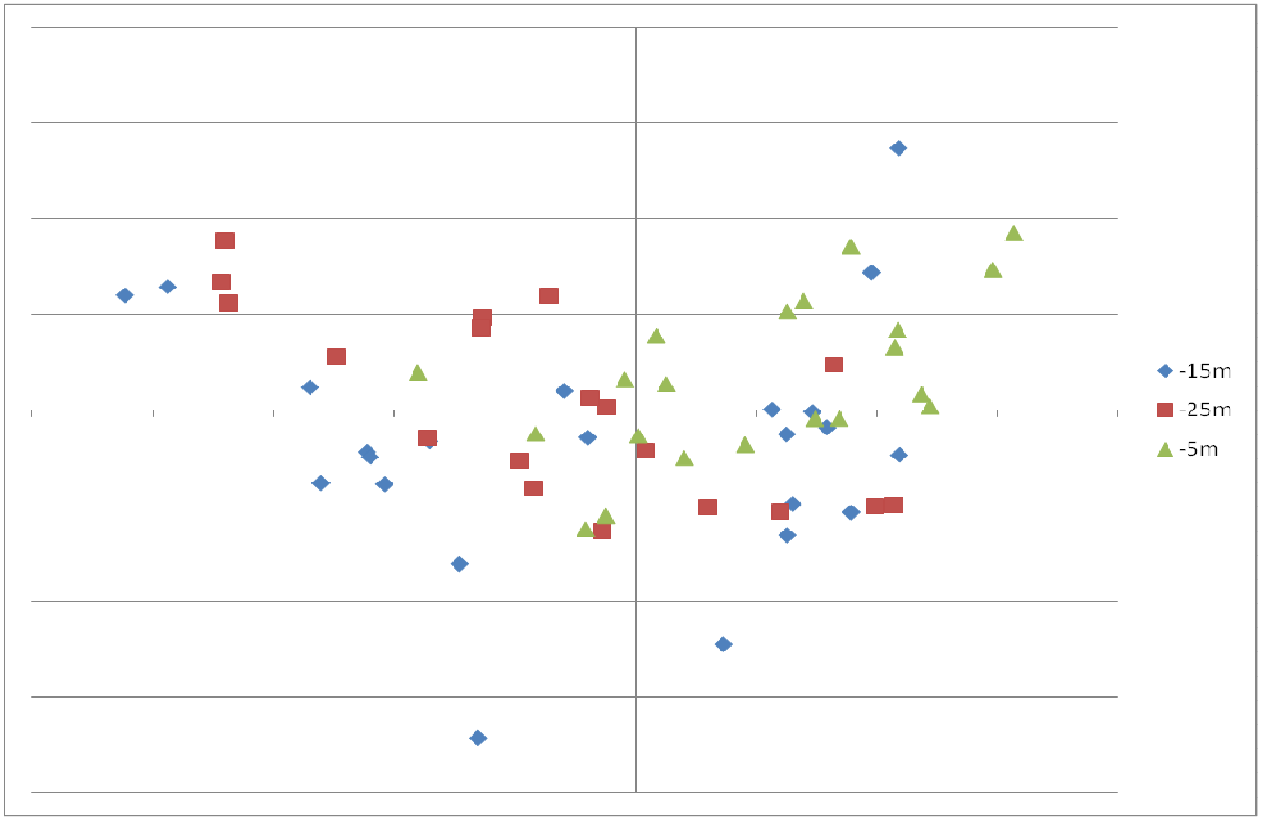


Figure 14 PCA of individuals according to the report form-depth distribution

Most of the variability (88,42 %) is explained by the first main component of shape variability, represented by the x axis, *i.e.* the position of ribs and, in particular, the mutual arrangement of 5th and 6th rib respect central rib and the leaf edge.

From left to right of the x axis the configuration change from a shape tapered with 5th and 6th rib more far between them, but closer to the central rib, to a shape less tapered with peripheral ribs closer together and with the leaf edge, but more far from the central rib.

The second main component of shape variability (represented by y axis) explains 7.40 % of the variability.

In PCA reported in figure 14, it is possible to distinguish individuals coming from the three different depth dispersed according to how much their leaf shape configuration differs from the consensus configuration which is in the cross between x and y axes.

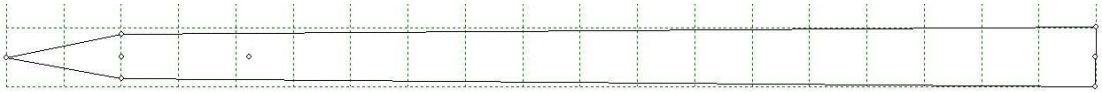


Figure 15 Consensus configuration

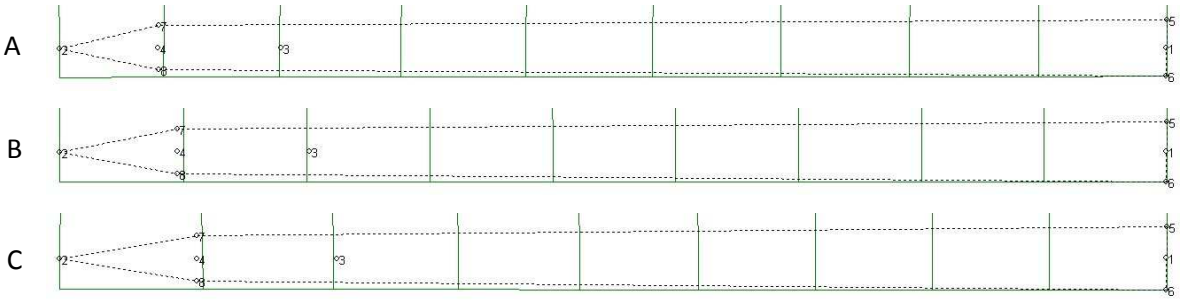


Figure 16 Configurations of three leaf coming from the three different depth: A: -5m; B: -15m; C: -25m.

Figure 16 shows that the most variability regard the relative disposition of ribs respect themselves and the leaf external edge. Here, it is talking about "shape" of leaf reconstructed through the identification of the eight Landmarks chosen on the basis of the homologous and parahomologous characters and not about leaf size.

The linear model of dependency depth-shape, explains only a small percentage of the leaf shape variability, since 87 % of variability remains unexplained being influenced by other factors clearly not measured.

However, the percentage of dependency depth-form is statistically significant: $\lambda = 0, 56$; $p = 0, 0016$ (permutation test: $\lambda > \lambda_0 = 0{,}6\%$) indicating that the model is reliable and that the depth certainly affects the leaf morphology, although is not the only factor to influence.

3.2 *Size parameters analysis*

When the leaf size is considered, depending on the depth, there are some statistically significant factors in regression with depth.

	R^2	R^2 adj.	p
Lenght	**20,30%**	**19,00%**	**0,0002**
Width (1-2)	0%	-1,60%	0,9085
Thickness (5-6)	5,30%	3,70%	0,0706
Thickness (7-8)	1,2%	-0,4%	0,3912
1-3	5,00%	3,40%	0,079
2-3	**13,60%**	**12,20%**	**0,0029**
2-4	**12,10%**	**10,70%**	**0,0052**
3-4	10,00%	8,5%	0,0118

Table 2 Regression of dimensional parameters by depth. The most significant data were in bold blue

Surely the leaf length is significantly dependent on the depth in a positive manner, *i.e.* it increases with the bathymetry increasing. It was found also a significant correlation between length and leaf shape (r=0,528; P= 1%).

This data are not in agreement with literature data (Dalla Via *et al.*, 1998), which report that the leaf length decreases with the depth.

Probably, this discrepancy could be due to the characteristics of the collection site (Calvì Bay) or to the seasonal period of leaf sampling. In fact, in Dalla Via *et al.*, (1998) the collection period was September when leaf abscission begin and older leaves are replaced, whereas in the case of the present work the collection period was June, the period of maximum leaf length.

On the contrary, the leaf thickness, both at central and at peripheral level (Landmark 5-6, and 7-8), and the width of the leaf (Landmark 1-2) are not significant depth-dependent.

However, it is interesting to notice that thickness is correlated with leaf shape, in particular, more the central zone of the leaf than the peripheral (r=0,64; P=0,1% and r=0,343; P=1.1%, respectively).

Very relevant appear to be the distance between leaf edge (landamark 2) and peripheral ribs (5^{th} and 6^{th} rib, respectively landmarks 2 and 3). In fact, both the distance (represented in the

table as distance 2-3 and 2-4) are significantly dependent from the depth as these distance increased with the sea depth explaining the tapered shape prevailing in the leaves coming from higher depth. Furthermore, several sections of leaves from highest depth showed a seventh supernumerary rib.

3.3 *Quantitative parameters analysis*

Traditional quantitative analysis, were also carried out on sections of leaves coming from the three different bathymetry.

The quantitative parameters were subjected to regression with depth and also correlated with themselves and with the dimensional parameters.

3.3.1 *Regression*

The more meaningful tests of regression was obtained when the parameters regarding both epidermis and mesophyll at level of the leaf edge were considered. In fact, the number of epidermis cells and of mesophyll cells layers between the upper and lower epidermis, as well as the number of mesophyll cells included in a rectangle with smaller side of 1000 µm (obviously related with the number of mesophyll cells for unit of measure), were positively dependent by depth.

Also the number of mesophyll cells for measure unit and between upper and lower epidermis at level of the central rib increased with depth.

Thus indicating that, a greater number of photosynthetic cells were present in leaves of plants living at higher depth as, in *Posidonia oceanica*, not only the mesophyll cells, like in land plants, show photosynthetic activity but also the epidermis cells whose cells contain large and numerous chloroplasts, in part explaining the good photosynthesis rate in spite of not optimal light l regimes.

Also the number of mesophyll cells for chosen unit of area ($4\ 10^{4}\ \mu m^2$) and from upper and lower epidermis, at both peripheral and central level, increased with depth. However, the leaf thickness, as indicated by the distance between Landmark 5-6 and 7-8, did not dependent by depth, thus the leaves adapted to higher depth show more numerous mesophyll cells, but smaller than the leaves coming from shallow water.

The number of epidermis cell at peripheral level of leaf is also significatively correlated with leaf shape (r=0,451; P=0,002%), like also layers of mesophyll cells at peripheral level (r=0,468;P=0,17%).

	R^2	R^2 adj.	p
N°epi cells 5 th rib	**25,7%**	**24,5%**	**0,0001**
N°meso cell from epi u. to epi l. P.L.	**18,3%**	**17,0%**	**0,0005**
Layers of meso cells from epi u. to epi l. P.L.	**20,6%**	**19,3%**	**0,0002**
N°meso cells/ u.m. P.L.	**19,6%**	**18,3%**	**0,0003**
Layers of meso cells from epi to 5th rib	4,1%	2,5%	0,1127
N°epi cells 1 st rib	10,1%	8,60%	0,0111
N°meso cell from epi u. to epi l. C.L.	13,7%	12,3%	0,0028
Layers of meso cells from epi u. to epi l. (C.L.)	10,9%	9,4%	0,0083
N°meso cells/ u.m. C.L.	14,3%	12,8%	0,0023
Layers of meso cells from epi to 1st rib	10,0%	8,5%	0,0117

Table 3 Regression of quantitative parameters by depth. The most significant data were in bold blue (C.L., central level; P.L. peripheral level)

3.3.2 *Correlation between quantitative parameters*

Data of correlations of quantitative parameters with themselves and with dimensional parameters are reported in the appendix.

Most of meaningful values are easily recognizable, *i.e.* the thickness and the mesophyll parameters, mesophyll parameters (at both level analyzed) between themselves, or thickness at central level and at peripheral level of leaf.

Some data appeared interesting: the number of epidermis cells for unit of measure, for example, is correlated with leaf length, but unrelated with any other histological parameters. This correlation seems to have no logical reason, but both parameters are positively correlated with depth.

The leaf width was correlated with leaf thickness (at both level), but not with leaf length.

The distances between external leaf edge and sixth and fifth ribs were strongly correlated between them (r=0,95322; p=0,0073), like also the leaf width and the distance between

central rib and fifth rib. These data suggested that the relative disposition of ribs in leaf is strongly determined.

The quantitative analysis showed a strong dependency of leaf length and thickness, indicated by the correlation of these two parameters either between themselves or between the mesophyll parameters at the middle level of the leaf section, but mainly with those concerning the peripheral level.

The mesophyll parameters at the central level were strongly related to whose at peripheral level. Thus indicating that the leaf length and thickness are strongly related inducing an increase of the mesophyll cells number both at the level of the central and of the fifth rib. Particularly it seems that the thickness of the central portion of the leaf section determine the thickness at the peripheral level (5^{th} rib).

However, whereas the leaf length, the epidermis and mesophyll parameters, either at central or peripheral level, depend in a positive way by the depth the leaf thickness is not dependent by the depth.

So, the plants adapted to high depth show longer leaves with a greater number of epidermic and mesophyll cells for unit of measure, which of course displays a lower dimension.

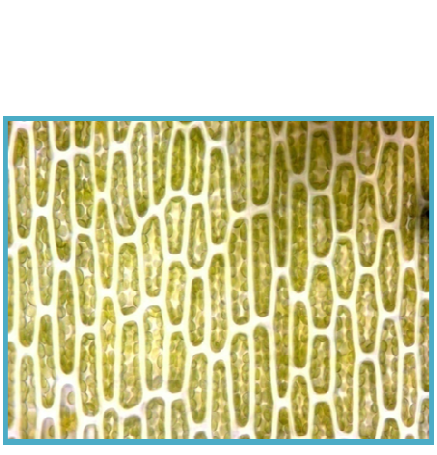
At peripheral leaf edge a greater variability was detected, in fact the quantitative parameters related to the fifth rib, were correlated with several factors and the 4 Landmark, that corresponding to the sixth rib, contributes more than others to the variability on the first main component of variability represented by the x axis in PCA of figure 13.

3.4 *Photosynthetic Surface*

In order to study the photosynthetic surface at epidermis level, a group of leaves, sampling at - 5m and - 25m, were “peeled”, the number of cells counted and the area of chloroplasts for each epidermal cell and the cell aerea recorded (Fig. 17).

By multiplying the mean area of the chloroplast and the number of chloroplasts for cell, an estimation of the photosynthetic surface for cell can be obtained.

Results show that the epidermal cells of leaves sampled at higher bathymetry look smaller



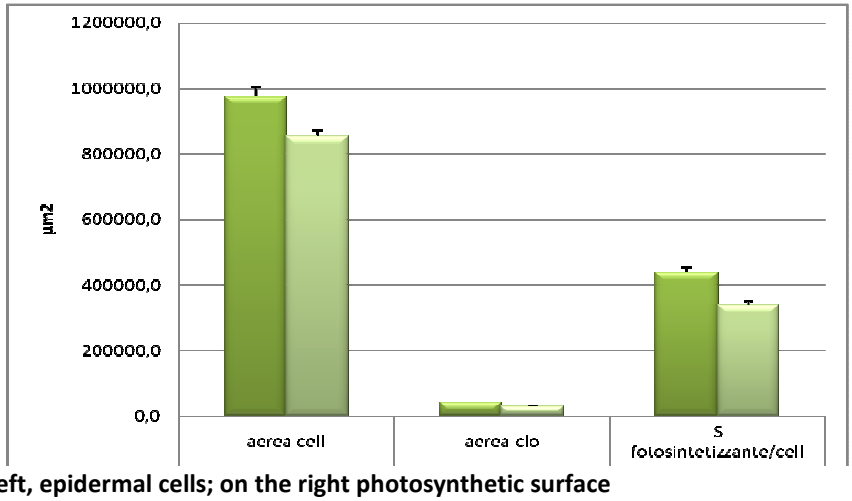


Figure 17 On the left, epidermal cells; on the right photosynthetic surface

than the leaves sampled from -5m. The number of chloroplasts for cell does not changes, they are smaller in leaf epidermis of higher depth.
However the leaves sampled at higher depth show smaller but more have more numerous epidermal cells able to optimize the photosynthetic surface.

Chapter 4: Discussion and conclusions

The study of leaf morphology in *Posidonia oceanica* by geometric morphometry, as well as the quantitative and dimensional parameters measured are innovative. Namely, only the leaf length (Dalla Via *et al*.,1998), the leaf thickness and the number of mesophyll cells layers (Colombo *et al*., 1982) were reported in literature.

According to these previous data, leaf length and thickness were negatively dependent by depth, as they decreased in leaves of plants adapted to higher depth, whereas the number of mesophyll cells layers did not change with the depth, but showed a reduction in the average size of the mesophyll cells (Colombo *et al*., 1982).

The data, that we obtained, are not completely in line with these literature data. In fact, the leaf length is dependent by depth in a positive way as it increases with sea depth, whereas leaf thickness, appeared not dependent by depth, but by leaf length. Thus shorter leaf is potentially less thick and *vice versa*, a short leaf sampled both from high and low depth is less thick than a long leaf sampled at the same depth.

Our data also showed that a reduction in the average size of the mesophyll cells occurs, but not as consequence of thickness. In fact, the number of mesophyll cells for unit of measure increase with depth.

We have, also, detected significant differences in the leaf morphology in relation to depth, although this did not fully explain the variability of the form observed.

The more tapering shape of leaves sampled from higher depth and the major number of either epidermal or mesophyll cells for unit of measure, could represent a symptom of a modified leaf morphogenetic pattern to achieve photosynthesis in not optimal light condition, which occur at -25m.

The evaluation of parameter, such as, epidermis cells number for unit of measure, was strongly dependent by depth, and correlated either with leaf shape, and with leaf length. However, it is not easy suggest the biological significance of the last correlation, but we can suppose that they are in correlation because share a dependence by the same factor: depth.

In marine phanerogams, a main photosynthetic adaptation to marine submergence is the conversion of the leaf epidermis in the primary site of photosynthesis, accompanied by the loss of stomata and extreme reduction of the cuticle (Larkum *et al*., 1989).

Either mesophyll or epidermis parameter measured changed with depth, indicating a smaller area of these cells, principally at the peripheral leaf edge. This probably could be a

consequence of the selfshading of leaves in a same bundle. Selshading is a phenomenon studied at different level: meadow, canopies, but also bundles (Zimmerman, 2003).

The disposition of leaf in a bundle causes a selfshading of leaves mainly in the basal portion, whereas the leaf margin is more exposed to light. This fact could influence the morphogenesis of leaf tissue.

The distances between the fifth and the sixth ribs and between the leaf edge increased with depth, whereas the leaf width did not increase. Furthermore, in leaves collected at higher depth it was found a seventh supernumerary rib.

Leaf longer, more tapered, showing smaller epidermis and mesophyll cells, with more ribs seem to be typical of leaves growing in deeper waters.

In the evolutive process of adaptation to the depth, leaf morphology and anatomical differences can play a decisive role. Indeed, the leaf morphology can contribute in the regulation of the light absorption efficiency through changes in the optical path of light (Enriquez, 2005).

In addition, the leaf morphology could be also correlated to other important leaf functions like the exchange of gases and solutes. The photosynthesis in an aquatic environment is strongly limited by the availability of CO_{2aq} and HCO^-_3 whose proportions of diffusion in water is 4 - 5 times smaller in that not in air (Raven 1984).

The amount of water that surrounds the leaves depends not only by the external hydrodynamism, but also from the leaf morphology and especially the leaf size and shape. (Nowell and Jumars, 1984). Although many studies have been conducted to examine the effect of the water movement on the photosynthesis (Koch, 1994; Henriquez and Rodríguez-Román, 2006), the link between leaf morphology and photosynthesis is little known, as a result to the stabilization effect of the water layer surrounding leaves, and consequently on the limitation of the carbon availability. Therefore, it is significant to approach the leaf morphology in response to photo-acclimation (Ralph *et al.*, 2007).

A link between morphology and light absorption was shown in phytoplankton (Morel and Bricaud, 1981; Agustí, 1991a, b), in some species of macroalgae (Ramus,1978, 1990) and in the marine Macrophytes (Henriquez *et al.*, 1994). However, even if a link between morphologic parameters is not well defined in the seagrass *Posidonia oceanica*, it is undeniable that the observed differences in clearly indicated the phenological plasticity of this plant to survive in different environmental conditions.

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Appendix I

	Lenght	Width	Thickness C.L.	Thickness P.L.	Central rib-Fifth rib	Ext. leaf edge -5th rib	Ext. Leaf edge -6th rib
Lenght	0	0,13766	2,90E-10	3,11E-06	0,36073	0,35292	0,615
Width	0,18913	0	4,25E-06	3,65E-06	5,55E-15	0,0044667	0,011434
Thickness C.L.	**0,69381**	**0,54308**	0	2,78E-19	0,00022746	0,18863	0,50335
Thickness P.L.	**0,54942**	**0,54622**	**0,85775**	0	2,17E-05	0,52724	0,86299
Central rib-Fifth rib	0,1171	0,797	**0,44849**	0,50767	0	0,024555	0,019006
Ext Leaf edge -5th rib	0,119	**0,35363**	0,16781	0,081146	-0,28311	0	2,87E-35
Ext Leaf edge -6th rib	0,064594	**0,31674**	0,085879	-0,022184	-0,2948	**0,95952**	0
N° epidermic cells C.L.	0,13948	-0,03806	0,063873	0,10165	-0,26857	**0,35548**	**0,29206**
N° epidermic cells P.L.	**0,40162**	-0,12601	0,16546	0,11541	-0,38347	**0,39379**	**0,38115**
N° Mesophyll cells in rectangle C.L.	**0,54955**	0,17148	**0,54141**	**0,42523**	0,068656	0,16596	0,14671
N° Mesophyll cells for unit of measure C.L.	**0,46851**	0,03198	0,3147	**0,22019**	-0,064918	0,15132	0,13449
N° of mesophyll cells layers C.L.	**0,62047**	**0,36354**	**0,73536**	**0,5746**	**0,26928**	0,16026	0,13166
N° of mesophyll cells until central rib	**0,44516**	**0,3083**	**0,50356**	**0,52696**	**0,30378**	0,019117	-0,012269
N° Mesophyll cells in rectangle P.L.	**0,53811**	0,091051	**0,50469**	**0,54182**	0,011301	0,12709	0,091384
N° Mesophyll cells for unit of measure P.L.	**0,54204**	0,0866	**0,50534**	**0,53855**	-0,0010938	0,13922	0,10008
N° of mesophyll cells layers P.L.	**0,50795**	**0,25088**	**0,56429**	**0,55016**	0,13104	0,19545	0,16049
N° of mesophyll cells until fifth rib	**0,44095**	**0,22802**	**0,58759**	**0,53474**	0,16125	0,11236	0,042872

	N° epidermic cells C.L.	N° epidermic cells P.L.	N° Mesophyll cells in rectangle C.L.	N° Mesophyll cells for unit of measure C.L.	N° of mesophyll cells layers C.L.	N° of mesophyll cells until central rib	N° Mesophyll cells in rectangle P.L.	N° Mesophyll cells for unit of measure P.L.	N° of mesophyll cells layers P.L.	N° of mesophyll cells until fifth rib
Lenght	0,27561	0,001105	3,09E-06	0,0001077	5,83E-08	0,000256	5,40E-06	4,47E-06	2,14E-05	0,0002979
Width	0,76712	0,32508	0,17902	0,80351	0,003404	0,013962	0,47789	0,49976	0,047338	0,072272
Thickness C.L.	0,61896	0,19501	4,61E-06	0,012006	6,73E-12	2,59E-05	2,47E-05	2,40E-05	1,46E-06	4,13E-07
Thickness P.L.	0,42794	0,36776	0,000513	0,082913	8,45E-07	9,13E-06	4,52E-06	5,29E-06	3,00E-06	6,34E-06
Central rib-Fifth rib	0,033315	0,00192	0,59288	0,61322	0,032833	0,015505	0,92995	0,99321	0,30599	0,20676
Leaf external edge -5th rib	0,004248	0,001408	0,19362	0,23648	0,20959	0,88178	0,32092	0,27651	0,12475	0,3806
Leaf external edge -6th rib	0,020199	0,002057	0,25122	0,2933	0,30367	0,92397	0,47628	0,43514	0,20894	0,73866
N° epidermic cells C.L.	0	5,62E-06	0,35287	0,19372	0,48724	0,058358	0,050983	0,036505	0,13227	0,057832
N° epidermic cells P.L.	**0,53728**	0	0,006112	0,0013199	0,023359	0,042913	0,000464	0,0003457	0,0053294	0,0053303
N° Mesophyll cells in rectangle C.L.	0,11901	**0,3418**	0	1,09E-20	6,14E-22	6,62E-07	7,36E-10	3,27E-10	1,16E-13	1,11E-10
N° Mesophyll cells for unit of measure C.L.	0,16593	**0,39588**	**0,87307**	0	1,01E-09	0,000116	3,78E-08	1,46E-08	6,42E-08	2,82E-07
N° of mesophyll cells layers C.L.	0,089134	**0,28542**	**0,88517**	**0,67835**	0	5,82E-08	1,61E-07	1,05E-07	5,61E-13	1,65E-11
N° of mesophyll cells until central rib	0,23981	**0,25593**	**0,57909**	**0,46669**	**0,6205**	0	4,55E-07	5,22E-07	1,94E-08	2,71E-09
N° Mesophyll cells in rectangle P.L.	0,247	**0,42821**	**0,68232**	**0,62724**	**0,60388**	**0,58587**	0	3,41E-81	1,09E-12	7,24E-06
N° Mesophyll cells for unit of measure P.L.	0,26407	**0,43672**	**0,69234**	**0,64162**	**0,61094**	**0,58343**	**0,99876**	0	2,89E-13	5,35E-06
N° of mesophyll cells layers P.L.	0,19171	**0,34702**	**0,77291**	**0,61893**	**0,75912**	**0,63741**	**0,75298**	**0,76502**	0	5,57E-09
N° of mesophyll cells until fifth rib	0,24029	**0,34702**	**0,70511**	**0,59427**	**0,72613**	**0,66534**	**0,53193**	**0,5383**	**0,65544**	0

Table 3: Correlation between quantitative and dimensional parameters. The most significant data were in bold blue (C.L., central level; P.L. peripheral level)

Appendix II: Publication and conference contributions

- Nicastro S., Perrotta I., Filadoro D., Mazzuca S., Brunelli E., Innocenti A.M. Adaptive response to salt stress in seagrass: Pip1;1 aquaporin antibody localization in *Posidonia oceanica* leaves. Oral presentation, *Mediterranean Seagrass Workshop 2009 6-10 September Hvar, Croazia.*
- Mazzuca S., Spadafora A., Filadoro D., Nicastro S., Pignataro V., Innocenti A.M.. *Posidonia oceanica*, una potenziale pianta modello per lo studio dell'ecosistema marino costiero del Mediterraneo. Un approccio proteomico funzionale. *I° Italian Plant Proteomics Workshop*, Vitorchiano (Vt), 20-21 October 2008.
- M. Cardilio, S. Nicastro, F. Rende, A.M. Innocenti. Seasonal effect of nitric oxide on *Posidonia oceanica* cuttings. *102° Congresso della società botanica italiana*. Palermo orto botanico 26 – 29 September 2007.
- Innocenti A. M., Cardilio M., Nicastro S. and Rende S. F.:Metodo per ottimizzare la sopravvivenza e per stimolare la crescita delle talee di *Posidonia oceanica*, patent n. CS2007A000018 , 19/04/2007.
- Cardilio M., Nicastro S., Rende F., Innocenti A.M.. Nitric oxide effect in transplanted cuttings of *Posidonia oceanica* (L.) Delile. Proceedings of the 3rd Mediterranean Symposium on Marine Vegetation; 27-29 March 2007 – Marseilles- *Actes du 3ème Symposium Mediterraneen sur la Vegetation Marine* 27-29 Mars 2007 – Marseilles.
- Rende S.F.,. Cardilio M,. Nicastro S, Innocenti A.M., "Stimolazione della crescita in talee di *Posidonia oceanica* (L.) Delile coltivate in acquario". Cambiamenti globali, diversità ecologica e sostenibilità. Viterbo – Civitavecchia *XVI Congresso Nazionale Società Italiana di Ecologia* 19 – 22 settembre 2006, Poster.
- Rende S.F.,. Cardilio M,. Nicastro S, Innocenti A.M., "Stimolazione della crescita in talee di *Posidonia oceanica* (L.) Delile coltivate in acquario." *Atti del XVI Congresso della Società Italiana di Ecologia* Viterbo/Civitavecchia 2006.