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Effect of high CO_2 and ocean acidification on photosynthesis and response to oxidative stress in seagrasses

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Declaração de autoria do trabalho.

Declaro ser a autora deste trabalho que é original e inédito.

Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Abstract

Climate change scenarios comprise significant modifications of the marine realm, notably ocean acidification and temperature increase, both direct consequences of the rising atmospheric CO_2 concentration. These changes are likely to impact marine organisms and ecosystems, namely the valuable seagrass-dominated coastal habitats. The main objective of this thesis was to evaluate the photosynthetic and antioxidant responses of seagrasses to climate change, considering CO_2, temperature and light as key drivers of these processes. The methodologies used to determine global antioxidant capacity and antioxidant enzymatic activity in seagrasses were optimized for the species *Cymodocea nodosa* and *Posidonia oceanica*, revealing identical defence mechanisms to those found in terrestrial plants. The detailed analysis and identification of photosynthetic pigments in *Halophila ovalis*, *H.stipulacea*, *Zostera noltii*, *Z marina*, *Z. capricorni*, *Cymodocea nodosa* and *Posidonia oceanica*, sampled across different climatic zones and depths, also revealed a similarity with terrestrial plants, both in carotenoid composition and in the pigment-based photoprotection mechanisms. *Cymodocea nodosa* plants from Ria Formosa were submitted to the combined effect of potentially stressful light and temperature ranges and showed considerable physiological tolerance, due to the combination of changes in the antioxidant system, activation of the VAZ cycle and accumulation of leaf soluble sugars, thus preventing the onset of oxidative stress. *Cymodocea nodosa* plants living in a naturally acidified environment near submarine volcanic vents in Vulcano Island (Italy) showed to be under oxidative stress despite the enhancement of the antioxidant capacity, phenolics concentration and carotenoids. *Posidonia oceanica* leaves loaded with epiphytes showed a significant increase in oxidative stress, despite the increase of antioxidant responses and the allocation of energetic resources to these protection mechanisms. Globally, the results show that seagrasses are physiologically able to deal with potentially stressful conditions from different origins, being plastic enough to avoid stress in many situations and to actively promote ulterior defence and repair mechanisms when under effective oxidative stress.

Key Words: Seagrasses; antioxidant activity; oxidative stress; photosynthetic pigments; carotenoids composition; global change

Resumo

Os cenários de alterações climáticas prevêem mudanças significativas no ambiente marinho, tais como a acidificação dos oceanos e o aumento da temperatura, consequências diretas do aumento da concentração atmosférica de CO_2. Prevê-se que estas alterações tenham impacto nos ecossistemas e organismos marinhos, nomeadamente nos valiosos habitats costeiros dominados por ervas marinhas.

O principal objectivo desta tese foi o de avaliar as respostas fotossintéticas e antioxidantes às alterações climáticas, considerando o CO_2, a temperatura e a luz como fatores-chave destes processos. As metodologias usadas para a determinação da capacidade antioxidante e atividade enzimática nas ervas marinhas foram otimizadas para as espécies *Cymodocea nodosa e Posidonia oceanica*, revelando que os mecanismos de defesa antioxidante assemelham-se aos encontrados em plantas terrestres. A análise e identificação detalhada dos pigmentos fotossintéticos em *Halophila ovalis*, *H. stipulacea*, *Zostera noltii*, *Z. marina*, *Z. capricorni*, *C. nodosa e P. oceanica*, colhidas em diferentes zonas climáticas e a diferentes profundidades, também revelaram uma semelhança com as plantas terrestres, tanto em termos de composição em carotenóides como relativamente aos mecanismos de fotoproteção baseados nos pigmentos fotossintéticos.

Plantas de *C. nodosa* da Ria Formosa foram submetidas ao efeito combinado de luz e temperatura em intensidades potencialmente stressantes, mostrando uma considerável tolerância fisiológica devida a uma combinação de alterações no sistema antioxidante, ativação do ciclo VAZ e acumulação de açúcares solúveis nas folhas, prevenindo assim o stress oxidativo. As plantas de *C. nodosa* que vivem em ambiente naturalmente acidificados, próximo de chaminés submarinas de origem vulcânica na Ilha de Vulcano (Itália), mostraram suscetibilidade perante as condições ambientais. Apesar do aumento da capacidade antioxidante, concentração em fenóis e carotenóides, as plantas expostas a emissões de elevado CO_2 revelaram estar sob stress oxidativo. Folhas de *P. oceanica* apresentaram um aumento significativo do stress oxidativo em resposta à presença de epífitos, apesar do aumento na resposta antioxidante e da alocação das reservas energéticas para os mecanismos de proteção. Globalmente, os resultados demonstram que as ervas marinhas encontram-se fisiologicamente habilitadas para lidar com situações potencialmente exigentes de diferentes origens, sendo plásticas o suficiente para evitar muitas situações de stress e promover ativamente a defesa e a reparação celular quando em situações de stress oxidativo efetivo.

Palavras-chave: Ervas marinhas; atividade antioxidante; stress oxidativo; pigmentos fotossintéticos; composição em carotenóides; alterações climáticas.

Palavras-chave: Ervas marinhas; atividade antioxidante; stress oxidativo; pigmentos fotossintéticos; composição em carotenóides; alterações climáticas.

Contents

Abbreviations list

(A+Z)/(V+A+Z) – Xanthophyll de-epoxidation index
$^{1}O_2$ - Singlet oxygen
^{3}Chl - Chlorophyll triplet state
A - Antheraxanthin
AAO - Ascorbate oxidase
AAPH - 2,2'-Azobis(2-amidinopropane) dihydrochloride
ABAP - 2,2′-Azobis(2-methylpropionamidine) dihydrochloride
ABTS$^{\bullet+}$ - Stable oxidant agent
ANOVA – Analysis of variance
AOS - Active oxygen species
APX - Ascorbate peroxidase
AsA - Ascorbate
AsA-GSH - Ascorbate-glutathione cycle
CarotT - Total carotenoids
CAT - Catalase
Chla - Chlorophyll a
Chlb – Chlorophyll b
ChlT - Total chlorophyll
CO_2 - Carbon dioxide
DHA - Dehydroascorbate,
DHAR - Dehydroascorbate reductase
ET - Antioxidants that acts by electron transfer
Fv/Fm - Potential quantum yield of PSII
GPX - Guaiacol peroxidase
GR - Glutathione reductase
GSH - Reduced form of glutathione
GSSH - Oxidized form of glutathione
H_2O_2 - Hydrogen peroxide
H_2S - Hydrogen sulphide
HAT - Hydrogen atoms transfer
HO$^{\bullet}$ - Hydroxyl radical
L - Lutein

L/(Lx+L) - Lutein de-epoxidation index
LHC - Light harvesting antennae complexes
Lx – Lutein-epoxide
MDA - Malondialdehyde
MDHA - Monodehydroascorbate,
MDHAR - Monodehydroascorbate reductase
NAD(P)H - reduced form of nicotinamide adenine dinucleotide phosphate
NADPH - Nicotinamide adenine dinucleotide phosphate
$\mathbf{O_2^{\bullet -}}$ - Superoxide anion radical
OA – Ocean acidification
ORAC - Oxygen reactive absorbance capacity
PAR – Photosynthetically active radiance
PD – Pre-dawn
PODs - Peroxidases
PSI - Photosystem I
PSII – Photosystem II
PSs - Photosystems
PVPP - Polyvinyl polyvinylpolypyrrolidone
ROI - Reactive oxygen intermediates
$\mathbf{ROO^{\bullet}}$ - Peroxide radicals
ROS - Reactive oxygen species
SOD - Superoxide dismutase
TEAC - Trolox equivalent antioxidant capacity
Trolox® - 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
V - Violaxanthin
VAZ - violaxanthin (V) + anteraxanthin (A) + zeaxanthin (Z)
VDE - Violaxanthin de-epoxidase
Z - zeaxanthin
ZE - Zeaxanthin-epoxidase

Chapter 1

General introduction

General introduction

Seagrasses are marine flowering plants, widely distributed along temperate and tropical coastlines of the world (Short *et al.*, 2007). Seagrass meadows provide valuable ecosystem services in shallow marine habitats, constituting a very important carbon sink, besides serving as feeding and nursery grounds for many species, stabilizing sediments with its below-ground complex rhizosphere and reducing suspended sediments and dissolved nutrients in water, thus improving overall water quality (Short and Neckles, 1999). Although seagrasses have the same basic requirements for growth as terrestrial angiosperms, their underwater habitat imposes some additional constraints to resource availability (Hemminga and Duarte, 2000). Factors such as light, temperature and nutrients have a direct effect on many biochemical processes at the plant level, thus influencing the overall productivity of seagrass meadows (Hemminga and Duarte, 2000; Lee *et al.*, 2007). Light requirements are a limiting factor for seagrass development and can control its distribution (Ralph *et al.*, 2007). Although light requirements vary spatially, temporally and with species (Procaccini *et al.*, 2012), the average lower limit for survival has been estimated to be around 11% of the surface irradiance (Duarte, 1991).

Photosynthetic pigments

All photosynthetic organisms synthesize carotenoids, natural and multifunctional pigments located on plant photosystems (DellaPenna, 1999a). These are responsible not only for the colours and aroma of leaves, fruits and flowers (Cazzonelli, 2011) but they play a very important role on the adaptation of plants to the light environment, being accessory pigments for light harvesting and essential components of photoprotection mechanisms (DellaPenna, 1999a). In terrestrial plants, the carotenoids pool is composed by lutein, β-carotene, violaxanthin, neoxanthin, antheraxanthin and zeaxanthin (DellaPenna, 1999a). Lutein-epoxide (DellaPenna, 1999a) and α-carotene can be also present in some species, but many lack these two pigments (Demmig-Adam *et al.*, 1996). Among marine macroalgae, the carotenoid composition can be diverse and is often specific to a certain taxonomic group (Demmig-Adams *et al.*, 1996). According to Casazza and Mazzella (2002), who firstly addressed this subject, seagrass pigment composition, appears to be similar to that of land plants. The ability of plants to survive in places were light is a limited resource, is connected with the carotenoids light-harvesting function, since they absorb light in spectral regions minimally absorbed by chlorophylls (Yamamoto and Bassi, 1996). Neoxanthin, lutein (Lichtenthaler, 1987; Lichtenthaler and Babani, 2004) and violaxanthin are the main carotenoids responsible by light harvesting (Cazzonelli, 2011). In some algae, light harvesting is also performed by specific carotenoids, such as fucoxanthin, siphonaxanthin and peridinin (Demmig-Adams *et al.*, 1996).

In opposition to light limitation, certain environments or times of the day are associated with very high light intensities, which can easily overcharge plants with more energy than the photosynthetic system is able to process (Ralph *et al.*, 2002). In such conditions, plants need either to intercept the excess of energy or, if it is absorbed, dissipate it in order to avoid damages to the photosynthetic apparatus (Yamamoto and Bassi, 1996). Interception of excess energy can be conducted by β-carotene, which thus protects chlorophyll a (Lichtenthaler 1987; Strzalka *et al.*, 2003). Energy dissipation as heat occurs mainly through the operation of the xanthophyll (VAZ) cycle pigments at the antenna level (Gilmore *et al.*, 1995). In this cycle, energy is dissipated through anteraxanthin (A) and zeaxanthin (Z), after the de-epoxidation of violaxanthin (V) in these pigments, induced by the lumen acidification and the presence of ascorbate (Yamamoto and Bassi, 1996). The VAZ cycle exists in all land plants and green algae (Adams III *et al.*, 2006) and it is also described in seagrasses (Ralph *et al.*, 2002). Besides the VAZ-cycle, two additional xanthophyll-based cycles can perform identical functions, the lutein-epoxide cycle, so far only found in some land plant species (Bungard *et al.*, 1999; Matsubara *et al.*, 2011) and the diadinoxanthin-cycle, only known to exist in some algae (Demmig-Adams *et al.*, 1996; Jahns *et al.*, 2009). All xanthophyll cycles are induced by high light to dissipate energy and are reversible with its decrease or after the dissipation of energy excess (Jahns *et al.*, 2009).

Carotenoids also have antioxidant functions. β-carotene, neoxanthin (Strzalka *et al.*, 2003), violaxanthin (Peterman *et al.*, 1997; Strzalka *et al.*, 2003) and lutein (DellaPenna, 1999b; Namitha and Negi, 2010) protect the photosynthetic apparatus from photodamage by quenching the triplet states of chlorophyll (Strzalka *et al.*, 2003). Zeaxanthin can act preventively by avoiding the formation of the chlorophyll singlet (^{1}Chl*) (Demmig-Adams *et* *al.*, 1996; Osto *et al.*, 2012) and can also remove epoxy groups from the oxidized double bands of thylakoid fatty acids of (Lichtenthaler and Babani, 2004). The antioxidant functions of carotenoids are also linked with the scavenging of superoxide anion radicals (Cunningham and Gantt, 1998), ROO* (Strzalka *et al.*, 2003), hydroxide radicals (Huang *et al.*, 2010) and the reaction with nitrogen oxides (Namitha and Negi, 2010), and in this way protecting pigments and unsaturated fatty acids from oxidative damage (Strzalka *et al.*, 2003). Alternatively, β-carotene also acts as a chain-breaking antioxidant to terminate lipid oxidation and β-carotene and lutein act together to decrease the cellular release of lactate dehydrogenase and protect cells from lipid peroxidation and membrane damage (Namitha and Negi, 2010). Carotenoids also serve as precursors for plant growth regulators and phytohormones, such as abscisic acid (ABA) (Cazzonelli, 2011), vitamin A (Cunningham and Gantt, 1998) and other signalling molecules that influence plant development and stress responses (Farré *et al.*, 2010).

Oxidative stress and antioxidant response

Despite all the carotenoid operation, performing as a first line of defence against oxidative stress, situations occur where the excess of light can lead to the development of oxygen reduced forms, also known as oxygen reactive species (ROS) (Scandalios, 2005). Even under optimal conditions these are routinely generated in low levels by the regular plant metabolic processes (Wojtaszek, 1997) and act as key regulators in growth and reproduction, besides signalling for antioxidant defence (Dabrowska *et al.*, 2007; Pessarakli, 2011). However, ROS levels should be kept below certain limits (Desikan *et al.*, 2005), as its excess can be highly toxic and cause oxidative stress (Pessarakli, 2011). Stressful conditions such as high light, drought, high salinity, extreme cold, heat shocks, excess of heavy metals (Pessarakli, 2011), high UV levels and pathogen infections are prone to increase ROS concentrations (Blokhina *et al.*, 2003), leading to oxidative stress (Scandalios, 2005). ROS include superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and oxygen singlet ($^1O_2$) (Desikan *et al.*, 2005; Pessarakli, 2011).

Plants have mechanisms to regulate the intracellular concentrations of ROS, known as antioxidants (Mittler, 2002). Antioxidants can be biologically defined as molecules that when present in small concentrations can protect, prevent or reduce the oxidative destruction of biomolecules (Halliwell, 1990). The antioxidant system comprises several mechanisms, both enzymatic and non-enzymatic, that scavenge ROS (superoxide dismutase, catalase, peroxidases and glutathione peroxidase, low molecular mass non enzymatic antioxidants), detoxify lipid peroxidation products (glutathione S-transferases, phospholipid-hydroperoxide, glutathione peroxidase and ascorbate peroxidase), and regenerate active forms of antioxidants (monodehydroascosbate reductase, dehydroascorbate reductase and glutathione reductase) (Blokhina *et al.*, 2003). The antioxidant system operates in different subcellular compartments and responds in a structured form when cells are exposed to demanding situations or under effective oxidative stress (Foyer and Shigeoka, 2011; Sharma *et al.*, 2011).

Due to a growing interest in the use of natural compounds for medical and pharmaceutical proposes, marine algae antioxidants have been the target of many studies, aiming to identify and isolate the compounds with commercial interest (Ruberto *et al.*, 2001; Zubia *et al.*, 2007; Vadlapudi *et al.*, 2012; Kelman *et al.*, 2012; Farasat *et al.*, 2013. These studies have in fact revealed important antioxidant activities, especially in brown (Demirel *et al.*, 2009; Indu and Seenivasan, 2013) and blue green algae (Pan *et al.*, 2011), as reviewed by Tierney *et al.* (2010). In seagrasses, antioxidant extracts of *Posidonia oceanica* (Gokce and Haznedanoglu, 2008) and *Zostera marina* (Khasina *et al.*, 2003), have proved effective against some human diseases. *Thalassia hemprichii*, *Syringodium isoetifolium*, *Enhalus acoroides* (Kannan *et al.*, 2010; Santoso *et al.*, 2012), *Cymodocea rotundata* (Santoso *et al.*, 2012) and *Halodule pirifolia* (Kannan *et al.*, 2010) extracts have also been probed for their antioxidant activity as reducing agents (DPPH assay).

Apart from medical purposes, antioxidant analysis can provide information about the plant physiological responses to environmental stressors. In land plants this topic has been widely explored with a large number of published studies (Gill and Tuteja, 2010). In marine macroalgae, Collén and Pedersén (1994) firstly reported algae antioxidant activity as a response to mechanical stress caused by grazers and pathogens, with an oxidative burst further down-regulated by the activity of catalase. Algae antioxidant response triggered by high light (Collen and Davison, 1999; Choo *et al.*, 2004) freezing, desiccation (Collen and Davison, 1999) and different combined stresses have also been reported (Choo *et al.*, 2005).

In sharp contrast with terrestrial plants and marine macroalgae, there are only a few published studies on the occurrence of oxidative stress or antioxidant responses in seagrasses. Despite the widely recognised ecological importance of seagrasses and their known worldwide decline (Short and Neckles, 1999), little effort has been done towards the direct assessment of seagrass oxidative stress and the corresponding physiological defence mechanisms. Most of the studies that evaluated seagrass responses to potential stressors were based on indirect observations, namely photosynthetic parameters derived from chlorophyll a fluorescence (e.g. Ralph *et al.*, 1998 ; Campbell *et al.*, 2006) or models that relate seagrass photosynthesis with light, temperature and geochemistry (Eldridge *et al.*, 2004). Also simple ecological parameters such as decreases on growth and survival have been considered as stress indicators in seagrasses submitted to high temperatures (McMilan, 1984), light limitation (Fitzpatrick and Kirkman, 1995) or that co-occur with algae (Holmer *et al.*, 2011). However, stress is not a generalizable term but rather a condition to which plants may or not be submitted if and when the first lines of antioxidant defence have failed and ulterior mechanisms such as ROS scavenging are operating in deficit. Furthermore, the presence of potentially stressful factors does not necessarily entail the occurrence of stress, but eventually the mere operation of avoidance mechanisms, such as the VAZ cycle, e.g.. As well, when the antioxidant system has the capacity to down-regulate the ROS formed, this means that the plants may not be in effective stress, as showed by Li *et al.* (2012) and Sales *et al.* (2013).

Important efforts have been made in order to understand the environmental conditions that are potentially demanding for seagrasses, and although some studies use ecological parameters to infer stress, the actual biochemical and physiological stress mechanisms are hardly known at all in seagrasses. A major knowledge gap concerns the ecophysiological aspects of stress in seagrasses, i.e, how the antioxidant responses relate to the environmental conditions. The first published study describing the enzymatic antioxidant response triggered by ROS (Sureda *et al.,* 2008), revealed that superoxide dismutase, catalase and glutathione peroxidase presented higher activity in *Posidonia oceanica* leaves as a response to the increase of ROS induced by the presence of epiphytes. Li *et al.* (2012) found higher superoxide dismutase and guaiacol peroxidase activity in *Thalassia hemprichii* when exposed to Zn, Cd and Pb. The increase of ROS coupled to the acclimation of the photosynthetic apparatus to light limitation was reported by Silva *et al.* (2013) for *Z. marina* and *C. nodosa*. Despite these initial contributions, all the response mechanisms to potential stressors such as high light, temperature and CO_2 are yet unknown, which is particular relevant in a climate change scenario (Soares-Cordeiro *et al.*, 2010).

Impact of global changes in seagrasses

As the oceans temperature increases, the distribution patterns of seagrasses are predicted to change (Short and Neckles, 1999). Foreseeable responses to high temperature also include increased respiration rates (Short and Neckles, 1999) and changes in growth rates (Lee *et al.*, 2007; Koch *et al.*, 2013). Alongside with coastal development and eutrophication events (Burkholder *et al.*, 2007), increased storms frequency and intensity (Connolly, 2009) will increase turbidity, hence decreasing the light reaching seagrass meadows with expected consequences in photosynthesis, productivity (Short and Neckles, 1999) and colonization depth (Duarte, 1991). High temperature is likely to potentiate the high light stress (Huner *et al.*, 1998), but this combination is to be expected in the future. Indeed, in natural conditions, the responses of plant to light already have a seasonal variation, mostly in temperate latitudes (Fitzpatrick and Kirkman, 1995). In land plants, under low temperatures and high light intensities, the de-epoxidation of violaxanthin to zeaxanthin can be greatly retarded (Demmig-Adams *et al.*, 1989; Zhang *et al.*, 2011). As well, when other processes are thermodynamically restricted due to temperature, the importance of the reaction centre and LHCII in energy dissipation has been proposed as a complement to the operation of the xanthophyll-cycle (Bravo *et al.*, 2007). This kind of information is not available for seagrasses. Regarding the effect of light in seagrasses, it is known that high light triggers the activation of the xanthophyll cycle in *Z. marina* to dissipate the excess of energy and avoid photodamage (Ralph *et al.*, 2002). By opposition, under light limitation, *Z. marina* and *C. nodosa* respond distinctly in terms of LHCII carotenoids (Silva *et al.*, 2013). Moreover, *Z. marina* is more sensitive to light limitation, responding with the increase in ROS (Silva *et al.*, 2013). The combined effects of temperature and light in the carotenoids composition and in the operation of the xanthophyll cycle remains unknown, as well as the antioxidant defence mechanisms under these conditions. The relationship between seagrass mortality and global warming, especially in Mediterranean species (Marbà and Duarte, 2010) and the light impact on meadow limits (Hemminga and Duarte, 2000) underline the fact that these changes are and will continue to create demanding situations to seagrasses, whose physiological understanding is essential.

Ocean acidification (OA) is a key aspect of the climate change scenarios. It can be defined as the decline in seawater pH due to the sustained absorption of CO_2 (Raven, 2005) that leads to changes in seawater chemistry, namely the carbonate balance (Barry *et al.*, 2013). Predictions indicate that until 2100 the partial pressure of dissolved CO_2 (pCO_2) will reach values 2 to 3 times higher than the current ones, which means more than 700ppm (Stocker *et al.*, 2013). OA will lead to changes in environment naturally with impacts in marine organisms and communities, which will either adapt or succumb to the new conditions (Cooley *et al.,* 2009). According to some authors, OA can benefit seagrass photosynthesis (Raven, 2005; Hendriks *et al.,* 2014). This is supported mostly by short-term studies (45 days) in *Zostera marina*, but long-term experiments (one year) showed an acclimation effect of photosynthesis and a decrease in growth (Palacios and Zimmerman, 2007). In contrast, an increase in photosynthetic activity was reported for *Cymodocea nodosa* in naturally acidified environments (Apostolaki *et al.,* 2014). Several studies also relate high CO_2 levels with reduced RuBisCO content in plants (Stitt, 1991; Cheng *et al.,* 1998; Stitt and Krapp, 1999; Gordillo *et al.*, 2011). The uncertainty about the effects and consequences of OA in seagrasses needs to be addressed carefully, bearing in mind the complexity of metabolic processes.

Land plants growing under high CO_2 present lower antioxidant activity (Vurro *et al.,* 2009; Levine and Paré, 2009) but also an increase in enzymatic substrate regeneration (Vurro *et al.,* 2009) and an enhancement of antioxidant response when acclimated plants are submitted to acute stress (Gillespie *et al.,* 2011). Plant energetic resources are limited, hence the acclimation and/or defence strategies are driven by necessity (Herms and Mattson, 1992), which may explain an eventual low-cost strategy to growth under CO_2 environment, not requiring the activation of more expensive metabolic processes (Vurro *et al.,* 2009). However, the response of the antioxidant system in seagrasses to high CO_2 levels is still completely unknown.

In recent years, submarine volcanic CO_2 vents have been proposed as natural laboratories to investigate the effects of ocean acidification in marine ecosystems (Hall-Spencer *et al.,* 2008). One of the most studied european vents is located in Vulcano Island, in Italy, where *Cymodocea nodosa* can be found growing along a natural gradient between high and normal CO_2 concentrations. Previous studies on those vents reported, higher photosynthetic activity, maximum electron transport rate and compensation irradiance on *C. nodosa* plants exposed to high CO_2 (Apostolaki *et al.,* 2014). However, the authors also found lower shoot density and lower biomass in the same plants, despite the higher photosynthetic rates (Apostolaki *et al.*, 2014).

Main objectives

Taking into account the predicted changes for the marine environment, the importance of the antioxidant system in the adaptation of plants to these changes, the big knowledge gap concerning the antioxidant system of seagrasses and the global importance of these plants in the marine realm, the objectives of this thesis are:

i) to adapt and optimize for seagrasses the methodologies to determine the activity of the antioxidant system enzymes and evaluate the global antioxidant capacity.

ii) to characterize the composition in photosynthetic pigments of seagrasses naturally adapted to different climatic zones and depths. With this characterization we expect to obtain a broad outline regarding seagrass chlorophylls and carotenoids.

iii) to evaluate the photosynthetic and antioxidant responses of *Cymodocea nodosa* to the combined effect of light and temperature, using potentially stressful ranges of both parameters, providing a comprehensive assessment of the mechanisms involved.

iv) to determine the effect of epiphytes in *Posidonia oceanica*, namely on (i) the photosynthetic rates, (ii) the energetic balances (iii), the composition and balance of photosynthetic pigments and (iv) in the antioxidant responses and oxidative stress.

v) to assess the response of *Cymodocea nodosa* to high CO_2, in plants growing near submarine volcanic vents, in terms of photosynthetic operation, pigment composition, antioxidant response, potential oxidative stress and energetic balance.

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Chapter 2

Optimization of methods for the determination of antioxidant enzymes activity and total antioxidant capacity in seagrasses

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(To be submitted)

Optimization of methods for the determination of antioxidant enzymes activity and total antioxidant capacity in seagrasses

Abstract

Oxygen is essential for aerobic organisms; however, in its energetically more reactive forms is amongst the most toxic substances for life. Oxygen reactive species (ROS) occurs on plant metabolic processes as a natural and unavoidable consequence of aerobic metabolism even under optimal conditions since they are regularly generated at low levels in non-stressed plant cells. However, ROS must be kept under controlled levels because its enhanced generation provokes oxidative stress, the imbalance between the production and the antioxidant response to keep them under low levels. Stressful conditions caused by a diverse amount of biotic or abiotic threats are prone to provoke oxidative stress leading to lipid peroxidation, and other cellular damages. The aim of this work was to give an overview on plant antioxidant system against oxidative stress and to adapt and optimize the methodologies described to terrestrial plants, in order to determine the activity of the antioxidant system enzymes and evaluate global antioxidant capacity in seagrasses. With that, we pretend to develop the tools that will allow us to have a better understanding on seagrass defensive systems under demanding natural or imposed conditions. Antioxidant enzymes ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, catalase, glutathione reductase and superoxide dismutase were optimized were optimized for *Zostera noltii*, *Zostera marina* and *Cymodocea nodosa* and of *Posidonia oceanica.* Methods to assess global antioxidant capacity, ORAC and TEAC of two *C. nodosa* and two *P. oceanica* ecotypes, were optimized. Global antioxidant assays reflect which group of mechanisms are being activated by the plant in a certain moment and as a response to environmental conditions.

Introduction

Oxygen is essential for aerobic organisms; however, in its energetically more reactive forms is amongst the most toxic substances for life (Desikan *et al.*, 2005; Scandalios, 2005). These energetically more reactive forms of oxygen are commonly known as reactive oxygen species (ROS), reactive oxygen intermediates (ROI) or active oxygen species (AOS) (Desikan *et al.*, 2005). On plant metabolic processes ROS occur as a natural and unavoidable consequence of aerobic metabolism (Sharma *et al.*, 2011), even under optimal conditions (Foyer and Shigeoka, 2011), as they are regularly generated at low levels in non-stressed plant cells (Wojtaszek, 1997). In plants, ROS are continuously formed due to the leakage of electrons from the mitochondrial, chloroplastic and plasma membrane electron transport chains to O_2, as a by-product of different metabolic pathways, such as respiration, photosynthesis and photorespiration, or as a consequence of the exposure of dioxygen to high energy (Sharma *et al.*, 2012). While in green plant parts at light chloroplasts and peroxisomes are the main ROS producers (Foyer and Noctor, 2003), this role is played by mitochondria in non-green plant parts at darkness (Møller, 2001). In low or moderate concentrations, ROS regulate gene expression and several biochemical processes, acting as key regulators in plant growth and development, and playing important roles on the sensing and reaction to environmental conditions (Wojtaszek, 1997; Demidchik *et al.*, 2002; Eghbal *et al.*, 2004; Desikan *et al.* 2005; Dabrowska *et al.*, 2007; Sharma *et al., 2011*). However, ROS must be kept under controlled levels (Desikan *et al.*, 2005; Sharma *et al., 2011*) because its enhanced generation disrupts cellular homeostasis and provokes oxidative stress. Oxidative stress is defined as the disturbance of the balance between the production of ROS and the antioxidant defences that avoid ROS over-accumulation (Mittler, 2002). Stressful conditions as high light, drought, salt stress, chilling, heat shock, excess of heavy metals (Sharma *et al.*, 2011), UV stress and pathogen infection, are prone to provoke oxidative stress (Blokhina *et al.*, 2003) leading to lipid peroxidation, oxidation of proteins, damage to nucleic acids, enzyme inhibition, and activation of programmed cell dead (Mittler, 2002; Scandalios, 2005; Sharma *et al.*, 2011).

The level of ROS is controlled by different kinds of enzymatic and non-enzymatic antioxidants. Ascorbate, glutathione, tocopherol, carotenoids and phenolic compounds are well-known non-enzymatic antioxidants (Scandalios, 2005; Sharma *et al.*, 2011), while catalase (CAT) superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) are the most studied antioxidant enzymes in higher plants (Mittler, 2002).

Plants respond distinctly to different environmental stresses (Lu *et al.*, 2008) and the level of antioxidant expression can increase in a coordinated way for an effective protection of plants avoiding cellular damage under unfavourable conditions (Lu *et al.*, 2008). A disturbance on coordination or the inefficiency of one enzyme, can lead to tissue damage. The activity of different antioxidant enzymes is connected with the susceptibility of the plant species to a specific environmental stress (Lu *et al.*, 2008) and the balance between all antioxidant systems should be strongly controlled (Mittler, 2002). Organisms that can adjust to changes in the environment are likely to exhibit a greater degree of adaptation than those who cannot (Scandalios, 2005; Sharma *et al.*, 2011) and for that reason, the increase in SOD, CAT, APX as a response to stressful conditions has been associated with enhanced plant resistance (Lee *et al.*, 2007; Yong *et al.*, 2008). Nevertheless, not always all enzymes increase simultaneously as a response to stress (Edwards *et al.*, 1990).

The study of the oxidative stress and antioxidants has long received great attention in terrestrial plant physiology being the object of hundreds of scientific studies as Asada *et al.*, (1974), Foyer and Halliwell (1976) Mittler and Zilinskas (1991) and several reviews as Bowler *et al.* (1992), Foyer and Noctor (2000), Arora *et al.* (2002) and Sharma *et al.* (2012), to cite just a few. In seagrasses, however, this issue has long been forgotten, no matter its obvious importance. Seagrass tissues are known to possess high antioxidant activity, but this feature has been used to study seagrasses as a source of antioxidants with pharmacological purposes (Regalado *et al.*, 2011; Hamdy *et al.*, 2012; Rengasamy *et al.*, 2012) and rarely to study its ecophysiological importance. Generally, the studies concerning the use of seagrasses as a source of antioxidants use generic methodologies to evaluate its total antioxidant capacity. In seagrass (eco)physiology, the total antioxidant capacity gives important information but there is also the need to identify and quantify the diverse antioxidants, enzymatic or not. To the best of our knowledge, the studies published about oxidative stress in seagrasses are scarce and isolated in time. In 2002, Ferrat *et al.* determined catalase and glutathione S-transferase enzymatic activities and the level of lipid peroxidation in *Posidonia oceanica* contaminated with mercury. Athiperumalsami *et al.* (2008) scanned several seagrasses species for bioactive compounds like phenolics and ascorbic acid and Sureda *et al.* (2008) studied the antioxidant response of epiphyted *P. oceanica*. In this work a commercial kit was used to analyse lipid peroxidation, and superoxide dismutase, catalase and glutathione peroxidase activities were determined as well as H_2O_2 production. These authors also observed a global increase on the antioxidant indicators and antioxidant enzyme activities and hypothesized that the antioxidant status of *P. oceanica* may play a role in the control of epiphytism. Athiperumalsami *et al.* (2010) did some experiments aiming to evaluate the antioxidant capacity of seagrasses and seaweeds from the Gulf of Mannar, India. Hydrogen peroxyde and nitric oxide scavenging capacity were assayed, and vitamins A, B3, C and E as well as β-carotene, tannin and phenol were quantified. In 2012 two different papers (Gavin and Durako, 2012; Griffin and Durako, 2012) used the Trolox equivalent antioxidant capacity (TEAC) assay in *Halophila johnsonii* to assess the effecs of low salinity stress. Also in 2012, Li *et al.* determined superoxide dismutase and guaiacol peroxidase activities to determine the effect of excessive metals in *Thalassia hemprichii*, with the final objective of using this species in phytoremediation. Finally, Jiang *et al.* (2013) determined the activities of the same enzymes to study the effects of nitrate enrichment and salinity reduction on seagrasses previously grown in low light.

From the plant physiological point of view, the choice depends on the target. Hence, to evaluate an overall antioxidant system, it is important to choose the methodologies, which, alone or in combination, will provide the broader perspective (Huang *et al.*, 2005).

The methodologies to evaluate antioxidant capacity are vast and used mostly in food science due to the importance to human nutrition and health (Pellergrini *et al.*, 2003; Wu *et al.*, 2004; Miguel *et al.*, 2010). These methodologies are normally used in land plants analysis and need to be properly adjusted to meet the particularities of seagrasses.

The aim of this work was to provide an overview of plant antioxidant system against oxidative stress and to adapt and optimize for seagrasses the methodologies already described for terrestrial plants, in order to determine the activity of the antioxidant system enzymes and evaluate global antioxidant capacity in seagrasses. From this starting point, it will be possible to develop the tools for a better understanding of the seagrass defensive systems under demanding and potentially stressful conditions.

Reactive oxygen species

Activation of oxygen may occur by two different mechanisms: i) stepwise monovalent reduction and ii) absorption of enough energy to reverse the spin on one of the oxygen unpaired electrons. Stepwise monovalent reduction leads to the formation of superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$), while energy transfer leads to the formation of singlet oxygen ($^1O_2$) (Sharma *et al.*, 2012).

$O_2^{\bullet-}$ is produced when one electron is added to ground state oxygen (Sharma *et al.*, 2011) and its half-life in biological systems is 1 µs (Møller *et al.*, 2007). Superoxide travels 30 nm in one half-life if the diffusion coefficient is assumed to be 10^{-9} m^2s^{-1}, and is moderately reactive, with both oxidizing and reducing proprieties (Sharma *et al.,* 2011; Møller *et al.*, 2007). It oxidises sulphur compounds, O-diphenols, ascorbate, cytochrome C, metal ions and metal complexes (Sharma *et al.*, 2011). Under abiotic stress conditions, plasma membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH)-oxidases can use NADPH to produce superoxide (Mittler, 2002; Sharma *et al.*, 2011). In plants, chloroplasts and mitochondria are major sites of $O_2^{\bullet-}$ production. In mitochondria, the unbalance between substrate availability and ATP requirement results in the generation of $O_2^{\bullet-}$ in the reactions associated with the complexes I and III of the respiratory electron transport chain (Møller, 2001). In chloroplasts, the depletion of $NADP^+$ accelerates the transport of electrons from photosystem I to molecular oxygen generating superoxide (Asada 1999), which can also be produced at photosystem II (Møller *et al.*, 2007). The addition of one more electron to $O_2^{\bullet-}$ and further protonation give rise to hydrogen peroxide (Halliwell and Gutteridge, 1984; Asada 2006). In chloroplasts, the production of H_2O_2 from O_2 is composed by these two reaction steps, known as Mehler reaction, and is enzymatically catalysed by superoxide dismutase (SOD) (Asada, 2006):

$$2O_2 + 2(e^-) \rightarrow 2\ O_2^{\bullet-}$$

$$2\ O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

In plant cells, hydrogen peroxide is mainly produced during photosynthesis, in the chloroplast, and during photorespiration, in the peroxisome (Slesak *et al.*, 2007). H_2O_2 is also produced by cellular respiration in the extracellular matrix. It is the most important and stable ROS (Desikan *et al.* 2005) with a half-life in biological systems of 1ms and high mobility (1 μm in one half-life if the diffusion coefficient is assumed to be 10^{-9} m^2s^{-1}) (Møller *et al.*, 2007). As a signalling molecule, regulates basic acclimatization and developmental processes in plants (Sharma *et al.*, 2011), mediating signal transduction in several metabolic pathways, and activates transcription factors, regulating this way gene expression as well as several cell-cycle processes (Morita *et al.*, 1999; Slesak *et al.*, 2007); it also regulates the defence against pathogen attacks (Mittler, 2002) and is a potent inhibitor of photosynthesis even at low concentrations, bellow 10 μM (Foyer and Shigeoka, 2011). In high concentrations, H_2O_2 causes oxidative stress, marked by the increase in lipid peroxidation and membrane permeability (Sharma *et al.*, 2011) and oxidation of cysteines (Møller *et al.*, 2007). However, as a reactive oxygen species it is well-known not by its reactivity *per se*, but as a precursor of the hydroxyl radical through the Fenton reaction (Sharma *et al.*, 2011).

Hydroxyl radical is the most reactive ROS (Sharma *et al.*, 2011) and is generated by the Haber-Weiss reaction in which hydrogen peroxide reacts with superoxide to form $HO^\bullet$. This reaction is catalysed by free transition metallic ions such as Fe^{2+}, Cu^+ or Mn^{2+} (Halliwell and Gutteridge, 1984):

$$H_2O_2 + O_2^{\bullet -} \rightarrow OH^- + O_2 + HO^\bullet$$

$HO^\bullet$ is the most instable ROS, with a half-life in biological systems of 1 ns, and travels 1 nm in one half-life if the diffusion coefficient is assumed to be 10^{-9} m^2s^{-1} (Møller *et al.*, 2007). The hydroxyl radical is a strong oxidizing agent that reacts with proteins, lipids, DNA, carbohydrates, and can initiate and self-perpetuate lipid peroxidation and even lead to cell dead, since it is not eliminated by the enzymatic system (Møller *et al.*, 2007; Sharma *et al.*, 2011).

Dioxygen may also be excited to the singlet state when pigments such as chlorophylls are excited by light. After excitation, chlorophyll is elevated to its singlet. If this excitation energy is not efficiently used by chlorophylls to photosynthesis, the spins of the electrons in the excited state can rephase and give rise to the chlorophyll triplet state ($^3Chl^\bullet$). $^3Chl^\bullet$ can react with ground state oxygen and produce $^1O_2$ (Halliwell and Gutteridge, 1984; Krieger-Lindsay, 2005). Singlet oxygen has a half-life of 1 μs and travels 30 nm in one half-life (assuming that the diffusion coefficient is 10^{-9} m^2s^{-1}), being highly destructive (Scandalios, 2005; Møller *et al.*, 2007). It oxidises polyunsaturated fatty acids, DNA, and proteins (Møller *et al.*, 2007). In plants, the main site of production of $^1O_2$ is the photosystem II in the chloroplast. High light exposure, drought and low CO_2 concentrations favour the oxygen singlet formation (Krieger-Lindsay, 2005)

Antioxidant system in plants

Antioxidant is a substance that in small concentrations can prevent or significantly delay the oxidation of easily oxidable materials, like lipids, membranes, DNA, and proteins (Miguel, 2009). It can also be a substance that is able to sequester metal ions or even repair damaged systems (Miguel, 2009). A good antioxidant should be non-toxic in its oxidized form, and capable of being recycled or regenerated in some way, successfully recovering electrons from another source (Gest *et al.*, 2012).

Due to ROS duality as both signalling and toxic agents, plants require mechanisms that are able to regulate ROS intracellular concentrations, by the fine modulation of low levels or by scavenging the excess of ROS during stress (Mittler, 2002). To control ROS levels and protect the cells under stressful conditions, plants comprise several enzymatic and non-enzymatic antioxidant mechanisms that scavenge ROS (superoxide dismutase, catalase, peroxidases and low molecular mass non enzymatic antioxidants), detoxify lipid peroxidation products (glutathione S-transferases, phospholipid-hydroperoxide, glutathione peroxidase and ascorbate peroxidase), and regenerate the active forms of antioxidants (monodehydroascosbate reductase, dehydroascorbate reductase and glutathione reductase) (Blokhina *et al.*, 2003).

Antioxidant classification

Antioxidants are a research target on many scientific fields, as biology, physiology, nutrition and health. According to that antioxidants can be divided based on mechanistic terms (Miguel, 2009), catalytic activity (Mitller, 2002; Pradedova *et al.*, 2011), molecular weights, levels of defence and struggle against ROS action, hydrophobicity, and hydrophily (Pradedova *et al.*, 2011). Until now, and albeit the multiple attempts to classify antioxidants, there is no stated and universal classification to follow (Pradedova *et al.*, 2011).

In the present work, antioxidants were divided based on their catalytic activity in enzymatic and non-enzymatic. Nevertheless we should always keep in mind that antioxidant expression is triggered based on specific needs and not necessarily by a specific order (Sharma *et al.*, 2011).

Non-enzymatic antioxidants

Non-enzymatic antioxidants include ascorbate, glutathione, tocopherols, carotenoids and phenolic compounds (Scandalios, 2005; Sharma *et al.*, 2011). They interact with several cellular components, and play pivotal roles in defence as co-factors of enzymes, and influence plant growth, development, senescence and cell death (Sharma *et al.*, 2011).

Ascorbic acid, also named ascorbate or vitamin C, is the most abundant low molecular-weight antioxidant in plants and is 90% located in cytoplasm but is exported to apoplast in a substantial amount (Larson, 1988; Sharma *et al.*, 2011).

Apoplastic ascorbate represents the first line of defence against potentially damaging external oxidants because of its ability to directly react with superoxide and hydrogen peroxide. Ascorbate is particularly abundant in photosynthetic tissues and occurs in its reduced form (ascorbate, AsA), and oxidized forms (monodehydroascorbate,MDHA and dehydroascorbate, DHA) (Sharma *et al.*, 2011). Together with glutathione, ascorbate plays an important role in hydrogen peroxide removal working as a substrate for ascorbate peroxidase (APX) and being regenerated through the ascorbate-glutathione cycle (Foyer and Shigeoka, 2011). Additionally, ascorbate functions as a substrate for violaxanthin de-epoxidase (VDE) in the violaxanthin-antheraxanthin-zeaxanthin (VAZ)-cycle in chloroplasts (Asada, 1999; Niyogi, 1999; Jahns *et al.*, 2009).

The reduced form of the tripeptide glutathione (GSH), is the major low-molecular-weight non-protein thiol present in most plants, synthetized in cytosol, chloroplasts (Sharma *et al.*, 2011), vacuoles and mitochondria (Blokhina *et al.*, 2003). GSH is the main storage form of sulphur, acts as potent detoxifier of xenobiotics and serves as precursor of phytochelatins (Noctor *et al.*, 1998).

In collaboration with its oxidized form (GSSH), glutathione maintains the redox balance in cellular compartments, which is important as a fine-tuning of cellular redox environment under normal conditions, and works as a defence and signalling molecule under stressful conditions (Bhokhina *et al.*, 2003). It reacts non-enzymatically with hydrogen peroxide, singlet oxygen, superoxide radical and hydroxyl radical (Larson, 1988). Nevertheless, the most important role of glutathione is to regenerate ascorbate, via the ascorbate-glutathione cycle (Noctor and Foyer, 1998). Glutathione is also important on controlling the cellular heavy metal concentrations by chelating and sequestering them in vacuoles (Sharma *et al.*, 2011).

Tocopherols and carotenoids are important antioxidants in plant cells (Sharma *et al.*, 2011) and are essential components of biological membranes with both antioxidant and non-antioxidant functions (Bhokhina *et al.*, 2003). Tocopherols represent a group of lipophilic antioxidants that are synthesized only by photosynthetic organisms (Sharma *et al.*, 2011). From this group, vitamin E (α-tocopherol) is a chain breaking antioxidant, that is able to repair oxidizing radicals and prevent chain propagation during lipid oxidation (Bhokhina, *et al.*, 2003). Tocopherols function as membrane stabilizers and act as oxygen radicals, lipid peroxy radicals and singlet oxygen scavengers, this way protecting pigments, proteins, and polyunsaturated fatty acids from oxidative damage (Sharma *et al.*, 2011).

As antioxidants, carotenoids have the ability to scavenge, prevent or minimize the production of triplet chlorophyll, oxygen singlet and to detoxify several forms of ROS (Demmig-Adams *et al.*, 1996; Niyogi *et al.*, 1997; Strzalka *et al.* 2003). They also are precursors of signalling molecules who influence plant development and stress responses (Sharma *et al.*, 2011). The role of carotenoids as antioxidants will be developed in more detail in Chapter 3.

Plants have developed the ability to produce an enormous number of phenolic secondary metabolites, which are not required in the primary processes of growth and development but are of vital importance for their interaction with the environment, reproductive strategy and defence mechanisms (Cheynier *et al.*, 2013). The accumulation of phenolic compounds in plants submitted to stress allows their survival and adaptation to environmental disturbances, not all directly related to antioxidant function (Cheynier *et al.*, 2013). In fact, antioxidant activity of an extract cannot be predicted based on total phenolic content since generally there is no correlation between them (Kahkonen *et al.*, 1999).

Phenolic antioxidant activity resides in their chemical structure. They contain an aromatic ring with –OH or OCH_3 substituents that together contribute to their biological activity

(Sharma *et al.*, 2011). The most effective antioxidants within phenolic compounds are flavonoids, phenylopropanoids (Sharma *et al.*, 2011), tannins and lignin precursors (Blokhina *et al.*, 2003). Flavonoids are known to outperform antioxidants as ascorbate and α-tocopherol due to their strong capacity to donate electron and hydrogen atoms (Rice-Evans *et al.*, 1997; Hernandez *et al.* 2009), capacity of stabilize and delocalize the unpaired electron (chain-breaking function), and finally their ability to chelate transition metal ions (Rice-Evans *et al.*, 1997; Bhokhina *et al.*, 2003).

Defensive phenolic compounds can directly scavenge ROS, quench singlet oxygen, decompose peroxides and act in the hydrogen peroxide-scavenging in a phenolic-ascorbate-peroxidase system, and chelate metals on plants under heavy metal stress (Sharma *et al.*, 2011; Cheynier *et al.*, 2013).

Enzymatic defence system

Plant enzymatic antioxidants include: superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), the enzymes of ascorbate-glutathione cycle (AsA-GSH), such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Noctor and Foyer, 1998; Sharma *et al.*, 2011). These enzymes operate in different subcellular compartments and respond in concert when cells are exposed to oxidative stress (Foyer and Shigeoka, 2011; Sharma *et al.*, 2011).

Superoxide dismutase (SOD) belongs to the group of metalloenzymes and catalyses the disproportionation of superoxide into oxygen and hydrogen peroxide in different compartments of the cell (Sharma *et al.*, 2011). Within the cell, SOD constitutes the first line of defence against superoxide (Alscher *et al.*, 2002). SOD converts superoxide in hydrogen peroxide which can then be removed by APX or CAT (Dabrowska *et al.*, 2007). The overproduction of SOD can result in the enhancement of stress tolerance in many plant species (Sharma *et al.*, 2011).

Peroxidases (PODs) belong to a large family of enzymes that convert H_2O_2 to water and oxygen (Sharma *et al.*, 2011). They are effective quenchers of reactive intermediary forms of oxygen and peroxy radicals under stress conditions, namely in defence against pathogens (Sharma *et al.*, 2011). Besides APX, guaiacol peroxidase (GPX) is another POD but less subtract-specific than APX. GPX decomposes H_2O_2 by oxidation in co-substrates such as phenolic compounds and/or ascorbate (Edwards *et al.*, 1990). GPX is not present in chloroplasts (Asada, 1992) and is more associated with H_2O_2 scavenging in roots than in leaves (Neto *et al.*, 2006).

The removal of H_2O_2 by APX involves the ascorbate-glutathione cycle (Asa-GSH cycle or Halliwell-Asada pathway); this cycle comprehends successive reactions of oxidation and reduction of AsA, GSH and NADPH catalysed by the enzymes APX, MDHAR, DHAR and GR. The ascorbate-glutathione cycle occurs in at least four subcellular locations: cytosol, chloroplast, mitochondria and peroxisomes (Sharma *et al.*, 2011) and plays a critical role in chloroplast redox protection (Dabrowska *et al.*, 2007). APX uses ascorbate as an electron donor to reduce H_2O_2 to water. The resulting monodehydroascorbate is reduced back to ascorbate either spontaneously, using electrons from ferredoxin, or enzimatically by MDHAR and using electrons from NAD(P)H or another donor as, for instance, a b-type cytochrome (Foyer and Shigeoka, 2011; Sharma *et al.*, 2011). Monodehydroascorbate can also be spontaneously disproportionated to dehydroascorbate and, in this case, ascorbate is regenerated by the enzyme DHAR. To reduce dehydroascorbate to ascorbate, DHAR uses electrons from reduced glutatione (GSH) leaving oxidised glutathione (GSSG). In order to continue regenerating the ascorbate, it is necessary to reduce GSSG back to GSH. This reaction is catalysed by glutathione reductase (GR) which gets reducing power from NAD(P)H. APX uses two molecules of ascorbate as electron donors to H_2O_2 elimination (Dabrowska *et al.*, 2007). APX activity declines if H_2O_2 is in nanomolar range (Miyake and Asada, 1996).Under low concentrations of ascorbate of less than 20 μM, the chloroplastic APX is lost even in the presence of H_2O_2 (Foyer and Shigeoka, 2011). Though, the depletion of ascorbate in the chloroplast has been considered as a limitation for photosynthetic efficiency in stress conditions (Foyer and Shigeoka, 2011) making the regeneration of ascorbate a key point in the scavenging of ROS and avoidance of $HO^\bullet$ generation.

Cross-compartment protection might be found in peroxisomes and mitochondria, regarding H_2O_2 elimination, because this ROS can be transported across biological membranes (Dalvetova *et al.*, 2005). APX is an important member of peroxidases (Dabrowska *et al.*, 2007; Sharma *et al.*, 2011) containing the heme prosthetic group in which iron plays and important role in the catalytic site (Dabrowska *et al.*, 2007) and can be divided in at least five isoforms identified in plants: cytosolic, mitochondrial, peroxisomal and chloroplastic (Asada, 1992; Caverzan *et al.*, 2012)..

Ascorbate peroxidase is important in H_2O_2 scavenging (Dabrowska *et al.*, 2007; Maruta *et al.*, 2010; Caverzan *et al.*, 2012), but also reacts with singlet oxygen and superoxide, as well as with glutathione and tocopherol radicals (Noctor and Foyer, 1998). Enzyme isoforms expression can be induced by pathogen attack, mechanical pressure, tissue injury, UVB radiation, water deficiency, low or high salt stress, excess of excitation energy at the photosynthetic apparatus, high or low temperatures, excess of oxygen after a period of anoxia, atmospheric pollution, excess of metal ions, nutrient deficiency and herbicides (Yoshimura *et al.*, 2000; Dabrowska *et al.*, 2007; Wang *et al.*, 2011). The expression of the different isoforms can be differentially changed by the specific demanding conditions and the same stress can induce different concentrations of each isoform (Dabrowska *et al.*, 2007).

MDHAR is one of the key enzymes in ascorbate-glutathione cycle, playing an essential role in plant stress protection (Lunde *et al.*, 2006). The first product of ascorbate oxidation is MDHA, a very stable radical, which therefore limits the chances of an oxidation chain reaction (Gest *et al.*, 2012). Within the cell, MDHAR can rapidly reduce MDHA to AsA using as electron donor a b-type cytochrome, reduced ferredoxin or NAD(P)H (Sharma *et al.*, 2011). MDHAR activity can be detected in several cellular compartments as chloroplasts, mitochondria, peroxisome, cytosol and also associated with plasma membrane and cell wall (Lunde *et al.*, 2006).

DHAR is a monomeric thiol enzyme and another enzymatic key component of the ascorbate-glutathione cycle system (Sharma *et al.*, 2011). Despite the possibility of enzymatic and non-enzymatic regeneration of ascorbate from monodehydroascorbate, some dehydroascorbate is always produced when ascorbate is oxidized in plant tissues (Sharma *et al.*, 2011). DHA is then reduced to AsA by DHAR, using GSH as reducing substrate (Foyer and Shigeoka, 2011). Substrate regeneration may play a secondary but not less important role in antioxidant defence, since it seriously affects the level of ROS in plants (Chen and Gallie 2006). In fact, it was shown that DHAR activity increases when plants are exposed to stress (Sharma *et al.*, 2011), and its suppression affects not only ROS level, but also photosynthetic activity, leaf growth and development (Chen and Gallie 2006).

GR catalyses the last Halliwell-Asada enzymatic pathway, by reducing one molecule of GSSH (oxidised glutathione), generated by DHAR in two molecules of GSH and using NADPH as co-factor (Foyer and Shigeoka, 2011; Sharma *et al.*, 2011). There are at least eight isoforms of GR (Edwards *et al.*, 1990).This enzyme is always present in mesophyll cells of the leaf, in the cytosol, chloroplasts and mitochondria, although 90% of its activity occurs in chloroplasts (Edwards *et al.*, 1990; Sharma *et al.*, 2011).

The interactions of APX, CAT, SOD and their involvement in ROS scavenging is very complex (Noctor and Foyer 1998) and the balance between these enzymes is critical for the determination of the steady-state level of superoxide radicals and hydrogen peroxide (Mittler, 2002) and to the generation of the hydroxyl free radical (Lu *et al.*, 2008).

Catalase (CAT) is a tetrameric heme-containing enzyme found in all aerobic organisms that catalyse the dismutation of hydrogen peroxide (H_2O_2) in water and oxygen without consuming reducing equivalents (Sharma *et al.*, 2011). CAT scavenges the H_2O_2 generated during mitochondrial electron transport, β-oxidation of fatty acids and, most important, during photorespiratory oxidation, but its properties suggest that the enzyme is inefficient in removing low concentration of H_2O_2 (Sharma *et al.*, 2011). In higher plants, CAT is predominantly localized in peroxisomes and glyoxissomes but there are a few reports about its presence associated with mitochondria (Feierabend, 2005). No matter its restricted localization, CAT is indispensable for ROS detoxification during stress, when the level of hydrogen peroxide gets too high (Mittler, 2002).

Although CAT and APX are both H_2O_2 scavengers, they are not completely redundant since they do not compensate for the lack of each other as shown by the susceptibility of plants with reduced levels of CAT or APX to environmental stresses and pathogen attack (Mittler, 2002). APX is considered to be the primary H_2O_2 scavenging enzyme in cytosol and chloroplasts while CAT activity is important in peroxisomes for scavenging H_2O_2 coming from photorespiration (Asada, 1992). Also, APX and CAT have different affinities for H_2O_2 (μM and mM ranges, respectively), suggesting that they belong to two distinct classes of peroxide-scavenging enzymes: APX might be responsible by fine modulation for signalling while CAT can control ROS removal during stress (Mittler, 2002). However, the presence of AsA-GSH cycle in all cellular compartments and the affinity of APX to H_2O_2 suggest that this enzyme is crucial for the control of this ROS level too (Mittler, 2002).

Usually an organelle has more than one enzyme able to scavenge a single ROS (Caverzan *et al.*, 2012) and the suppression of one enzyme may be reflected on the induction of other enzymes; for instance, in plants where APX was suppressed, SOD, CAT and GR were induced, whereas the suppression of CAT induced the production of APX and GPX (Mittler, 2002).

Methods optimization

Antioxidant enzyme activity

Plant material

Antioxidant enzymes were extracted from leaves of *Zostera noltii*, *Zostera marina* and *Cymodocea nodosa* from Ria Formosa (Portugal) and of *Posidonia oceanica* from Cabo de Gata (Spain) were collected, cleaned from epiphytes rinsed with distilled water, immediately frozen in liquid nitrogen and stored in -80ºC prior to analysis.

To evaluate antioxidant enzymatic activity, methods to assess ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) were optimized. Enzyme extractions and assays for enzyme activity determination were always done in the same day. Each sample was read at least three times, enzyme activity was determined using the slope of absorbance in function of time and calculated in units (U) as follows, except for SOD (Barrote, 2005):

$$U\ (\mu mol\ min^{-1}) = \Delta_{Abs}/\varepsilon \times V/V_a \times F_c \times V_c \times 1/b \times 10^3$$

Where:

Δ_{Abs} – slope of the absorbance in function of the time (min^{-1})

ε - Extinction coefficient ($mM^{-1}\ cm^{-1}$)

V – Total volume in the cuvette (μl)

V_a – Volume of extract (µl)

F_c - Dilution factor due to sample purification in the sephadex columns

V_c – Extraction volume (l)

b - Thickness of the cuvette (cm)

Slopes were only considered if R^2 was equal or superior to 0.90.

Activity of APX, DHAR and MDHAR

Assays to determine enzymatic antioxidant activity of APX, DHAR and MDHAR were based on Polle and Morawe (1995), with some modifications. Different extract concentrations were tested, as well as distinct extract volumes in reaction mixture.

400, 600 and 800 g of frozen leaf samples were powdered in liquid nitrogen, polyvinyl polyvinylpolypyrrolidone (PVPP) and sodium ascorbate, and then extracted with 5 mL of 100 mM potassium phosphate buffer, pH 7.8, with 2% Triton-X and 10 mM ascorbate. Ascorbate was added to avoid inactivation of enzymes (Nakano and Asada, 1987). Extracts were centrifuged at 4°C and 3500xg for 30 min. Supernatant was purified by filtration with sephadex PD-10 G-25 column (GE Healtcare), previously equilibrated with 20 mL of 100 mM potassium phosphate buffer pH 7.0 with 1 mM ascorbate (Polle and Morawe, 1995). The purified enzymatic extracts were used to determine enzymatic activity.

APX and DHAR protocols were already tested in *Zostera noltii* so they were applied for *Cymodocea nodosa*, *Zostera marina* and *Posidonia oceanica* analysis.

APX activity was measured at 25°C, following during 3 min the decrease in absorbance at 290 nm (Beckman Coulter DU-650 spectrophotometer, Brea CA, USA) in 1 ml of a reaction mixture containing 50 mM potassium phosphate buffer pH 7.0, 8 mM ascorbate, 20 mM oxygen peroxide and extract (Nakano and Asada, 1987). The volume of extract varied from 50-125 µL, according to the species. APX activity was calculated after subtraction of the control rates, where enzyme extract was replaced by the potassium phosphate buffer, using $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$ as extinction coefficient. One unit (U) of APX is equivalent to the enzyme necessary to oxidize 1 µmol of ascorbate per min.

DHAR activity was measured at 25ºC following for 3 min the increase in absorbance at 265 nm (Beckman Coulter DU-650 spectrophotometer, Brea CA, USA) in 1 ml of a reaction mixture containing 60 mM potassium phosphate buffer pH 6.1, 5 mM reduced glutathione, 800 µM dehydroascorbic acid and 50-100 µl of extract according to the species (Polle and Morawe, 1995). DHAR activity was calculated after subtraction of the control rates, where enzyme extract was replaced by the potassium phosphate buffer, using $\varepsilon = 14\ mM^{-1}cm^{-1}$ as extinction coefficient. One unit (U) of DHAR is equivalent to the enzyme necessary to reduce 1 µmol of dehydroascorbate per min.

To analyse MDHAR activity it was necessary to produce monodehydroascorbate (MDHA) through ascorbate oxidase (AAO) in the presence of ascorbate (Barrote, 2005). AAO (Sigma) was diluted following the instructions of the manufacturer: 1 mg of AAO in 20 ml of sodium acetate 200 mM, pH 6.2. AAO activity was then evaluated in 1 ml of a reaction mixture containig 100 mM Tricine/NaOH, pH 8.0, with 200 µM NADH and 800 µM ascorbate, following the decrease in absorbance at 290 nm, due to ascorbate oxidation to MDHA. AAO solution was diluted in the reaction mixture in order to obtain an AAO activity of 7 nkat. After several trials, it was not possible to measure the activity of ascorbate oxidase (AAO) and obtain MDHA at a constant rate and this invalidated the measurement of MDHAR (Polle and Morawe, 1995; Barrote, 2005).

After finding the right amounts of leaf tissue to use and the best dilutions of the extract in the reaction mixture, APX and DHAR enzymatic activity was successfully measured in *Zostera marina, Cymodocea nodosa, and Posidonia oceanica.* The best results, that had to correspond to a linear relationship between the evolution of absorbance and time, were achieved extracting 800 mg of leaves in 5 ml of extraction buffer; enzymes activity was read using 100-150 µL of extract in the reaction mixture, according to the species and ecotypes. The results obtained are indicated in table 2.1.

Activity of CAT, GR and SOD

Catalase activity was assayed as in Aebi (1984) and glutathione reductase activity was assayed as in Polle and Morawe (1995), both with some modifications. Superoxide dismutase was assayed as in Polle *et al.* (1989) and Misra (1985) with modifications. Different extract concentrations were tested, as well as distinct extract volumes in reaction mixture and additional chemicals.

600 and 800 and 1000 mg of frozen leaf samples were powdered in liquid nitrogen, polyvinyl polypyrrolidone (PVPP) and then extracted with 5 mL of 100 mM potassium phosphate buffer, pH 7.8, with 2% Triton-x, 1 mM Phenylmethanesulfonyl fluoride (PMSF) and 1 mM DL-Dithiothreitol (DTT). Extracts were centrifuged at 4°C and 3500xg for 30 min. Supernatant was purified by filtration with sephadex PD-10 G-25 column (GE Healtcare), previously equilibrated with 20 mL of 20 mM potassium phosphate buffer pH 7.0. The purified enzymatic extracts were used to determine CAT and GR activity.

CAT activity was measured at 25°C, following for 3 min the decrease in absorbance at 240 nm (Beckman Coulter DU-650 spectrophotometer, Brea CA, USA) in 1 ml of a reaction mixture containing 500 mM potassium phosphate buffer pH 7.0, 40 mM hydrogen peroxide and extract (Barrote, 2005). Extract volume varied from 30-70 µL, according to the species. CAT activity was calculated after subtraction of the control rates, where enzymatic extract was replaced by the potassium phosphate buffer, using $\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$ as extinction coefficient. One unit (U) of CAT is equivalent to the protein necessary to reduce 1 µmol of hydrogen peroxide per min.

GR activity was measured at 25ºC following the decrease in absorbance at 340 nm (Beckman Coulter DU-650 spectrophotometer, Brea CA, USA) during 3 min, in 1 ml of a reaction mixture containing 500 mM heppes buffer pH 8.0 with 5 mM EDTA and 2.5 mM NADPH, 5 mM oxidised glutathione and 100-150 µl of extract according to the species (Barrote, 2005). GR activity was calculated after subtraction of the control rates, where enzyme extract was replaced by potassium phosphate buffer, using the extinction coefficient $\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$.One unit (U) of GR is equivalent to the protein necessary to oxidase 1 µmol of NADPH per min.

CAT and GR activity were measured in *Z. marina* and *C. nodosa* samples with 600 mg and 800 mg FW, respectively (Table 1). Nevertheless, in *P. oceanica* it was not possible to measure the activity of these enzymes in any extract concentration. Although GR activity in *Z. noltii* was measured with 5 mM of NADPH, better slopes were obtained with 2.5 mM in *Z. marina* and *C. nodosa*, thus in the optimized protocol we fixed NADPH in the later concentration.

To determine SOD activity, epinephrine is autoxidized at pH 10.2 and 30ºC. In this reaction epinephrine is linearly converted in adenochrome and another intermediate component. This reaction is inhibited by SOD. Thus, the activity of the enzyme can be measured by comparing the evolution of the adenochrome formation with and without SOD in the reaction mixture following the evolution of absorbance at 480nm (Barrote, 2005). Before SOD activity measurements, distinct volumes of a 20 mM solution of epinephrine in HCl 0.05 N were added to a reaction mixture of sodium carbonate buffer 125 mM at pH 10.4 with EDTA 0.250 mM and potassium phosphate buffer 20 mM pH 7.8, in order to obtain an increase in absorbance rate of 0.025 min^{-1} at 480 nm. Each epinephrine assay was read at least three times. The selected volume of epinephrine in HCl was then added to a reaction mixture, with the same composition as previously, where a fraction of the phosphate buffer was replaced by the volume of extract previously determined. All the spectrophotometric readings were performed in a total volume of 1ml.

SOD activity was measured by following during 3 min the changes in absorbance at 480 nm (Beckman Coulter DU-650 spectrophotometer, Brea CA, USA), in comparison with epinephrine rates at 30ºC. Each sample was read at least three times. One unit (U) is defined as the quantity of SOD required to produce 50% inhibition of the accumulation rate of autoxidation of epinephrine products under the already described conditions (Misra, 1985) and is calculated as follows (Barrote, 2005):

$PI = 1 - A/E$

$VE_{50\%}$ (µl) = $0.5 \times V_a/PI$

U_{SOD} (U ml^{-1}) = $1000/VE_{50\%}$

Where:

PI – Fraction of inhibition by SOD

E – Absorbance evolution in the reaction mixture only with epinephrine (min^{-1})

A – Absorbance evolution in the reaction mixture containing both epinephrine and foliar extract

V_a – volume of extract used in the reaction mixture (µl)

Measurements were done with crude extracts and with extracts purified in the sephadex column. Different extraction concentrations were tested: 600 mg, 800 mg and 1000 mg of plant leaves in 5 ml and 4 ml of the extraction solution. Based on Baptista *et al.* (2007), Zhen *et al.* (2009) and Zhang *et al.* (2010) 0.04% (v/v) mercaptoethanol was added to the extraction solution. Based on Stepien and Klobus (2005) and Rivas *et al.* (2008), 2 mM EDTA were added to extraction solution. Some combinations of extract concentrations and tested conditions allowed the measurement of SOD activity, but the results were not consistent in any species and were not considered reliable. This method needs further investigation before it can be used in seagrasses.

The activity of APX was always higher than that of the other enzymes in all species, between 17 and 85-fold higher than CAT activity (Tab. 1). Direct inter-specific comparisons are not possible, since activities were determined in different conditions. Nevertheless, differences in the relative proportions of APX:DHAR suggest the existence of distinct and species-specific strategies. For example, APX and CAT activities in *Z. noltii* were higher than in the other species, but DHAR activity (ascorbate regeneration) was substantially lower (APX:DHAR ratio was 20 in *Z. noltii* and 1.5-3 in other species). Among the seagrasses tested, *Z. noltii* is the only intertidal species and, as consequence, the one submitted to higher light intensities together with low water availability, being more prone to ROS increase during low tide. On the other hand, *C. nodosa* and *P. oceanica*, with higher DHAR activity, should be able to maintain a higher ascorbate pool but presented almost half of the APX activity. Distinct plant conditions lead to different strategies to overcome potentially stressful conditions, keeping in perspective that antioxidant defence entails a significant energetic cost (Herms and Mattson, 1992; Andersen, 2003; Lattanzio *et al.*, 2009).

Sureda *et al.* (2008) analysed the activity of SOD, CAT and GPX in *Posidonia oceanica* leaves. These authors measured SOD activity by using the xanthine/xanthine oxidase system to generate a superoxide anion, and Li *et al.* (2012) measured SOD activity using the nitro-blue tetrazolium (NBT) method, instead of using the epinephrine system.

Table 1. Enzymatic activity of antioxidant enzymes ascorbate peroxidase (APX), dehydroascorbate redutase (DHAR), glutathione redutase (GR) and catalase (CAT) from leaf extract of *Zostera noltii* (Zn), *Zostera marina* (Zm), *Cymodocea nodosa* (Cn) and *Posidonia oceanica* (Po) . Values represents mean ± SE (n=3) in U.mg DW^{-1}.

	APX (U.mg DW^{-1})	DHAR (U.mg DW^{-1})	GR (U.mg DW^{-1})	CAT (U.mg DW^{-1})
Zn	96.06 ± 1.16	4.81 ± 0.38	2.38 ± 0.30	2.09 ± 0.09
Zm	23.32 ± 0.87	6.09 ± 1.50	4.11 ± 0.13	1.32 ± 0.05
Cn	52.92 ± 5.45	19.59 ± 3.37	1.13 ± 0.13	0.62 ± 0.06
Po	15.91 ± 0.90	13.13±1.26		

Total antioxidant capacity

A number of methods to assess total antioxidant capacity of an extract can be found in the literature (Prior *et al.*, 2003; Thaipong *et al.*, 2006; Nassar *et al.*, 2007). Nevertheless, different antioxidants have distinct mechanisms of action, hence to evaluate the antioxidant capacity of a specific species in a certain condition it is important to use more than one methodology (Huang *et al.*, 2005).

The mechanistic classification divides the antioxidants scavenging capacity according to its way of action in two groups: antioxidants that act by hydrogen atoms transfer (HAT) and antioxidants that act by electron transfer (ET) (Miguel, 2009; Dunnoné *et al.*, 2009). Hydrogen atoms transfer-based assays apply a competitive reaction scheme in which antioxidant and substrate compete for thermally generated peroxyl radicals, while electron transfer-based essays measure the capacity of an antioxidant to reduce an oxidant (Huang *et al.*, 2005).

To assess the global antioxidant capacity of seagrasses, we choose the oxygen reactive absorbance capacity (ORAC) as an HAT method and the trolox equivalent antioxidant capacity (TEAC) as an ET method.

Antioxidants were extracted from leaves of *Posidonia oceanica* collected in Panarea (Italy) and in Cabo de Gata (Spain), and *Cymodocea nodosa* collected in Vulcano (Italy) and in Ria Formosa (Portugal). Leaves were collected, cleaned from epiphytes, rinsed with distilled water, immediately frozen in liquid nitrogen and stored in -80ºC prior to analysis. Extracts were obtained as in Booker and Miller (1998) and Migliore *et al.* (2007) and three different dilutions were tested: 200 mg, 300 mg and 500 mg of frozen leaf samples, were powdered in liquid nitrogen, suspended in 3 mL of 0.1N hydrochloric acid (HCl), and kept under constant agitation at 4°C, overnight, followed by centrifugation at 4700xg for 30 min. After centrifugation supernatant was collected and the pellet was suspended again in 3 mL of 0.1 N HCl. This procedure was repeated twice. Supernatant collected (10 mL) was used for quantification procedure. Extracts were used simultaneously to test both methods.

ORAC method was performed according to Huang *et al.* (2002): 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was replaced by 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (ABAP). 8.16 x10^{-5} mM fluorescein (Sigma) was dissolved in 75 mM potassium phosphate buffer and added directly to the leaf extract in microplate wells. This mixture was heated at 37ºC and read in a Synergy TM 4 multi-detection microplate reader with a 485 nm 20 nm bandpass, excitation filter and a 528 nm, 20 nm bandpass, emission filter. Reaction was initiated by the addition of 153 mM ABAP (2,2′-Azobis (2-methylpropionamidine) dihydrochloride) (Sigma). Results were expressed as Trolox® equivalents (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid). To prepare the calibration curve, trolox was dissolved in 75 mM potassium phosphate buffer in a range of concentrations from 6.25 to 50 µM and read following the same procedure as with samples.

In TEAC essay, $ABTS^{\bullet+}$ was produced by the reaction of 7 mM ABTS with potassium persulphate in a final concentration of 2.45 mM according with Re *et al.* (1999). $ABTS^{\bullet+}$ diluted in absolut ethanol (A734 nm = 0.800 ± 0.020) was added to the extract and, after an incubation period of 6 minutes, absorbance was read at 734 nm in a Beckman Coulter DU-650 spectrophotometer (Brea CA, USA), against a blank sample. Results were expressed as Trolox® equivalents (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid). To prepare the calibration curve trolox was diluted in ethanol, in a range of concentrations from 500 to 1500 µM and read following the same procedure with as samples.

For the species tested, the quantity of extract to use was fixed in 300 mg of fresh weight, since on this concentration extracts fitted on trolox calibration curve and concentrations were higher than with 200 mg and 500 mg. For ORAC method, samples were previously diluted 10-fold prior to analysis.

ORAC and TEAC methods can reveal both non-enzymatic and enzymatic antioxidants acting as scavengers in the groups described by Pradedova *et al.* (2011), which give us a global perspective of plant antioxidant defence. The antioxidant activity of two *C. nodosa* and two *P. oceanica* ecotypes, determined through the ORAC and TEAC methods, is depicted in Table 2. Samples were collected from the plants in their natural conditions. Global antioxidant assays reflect the plant defence mechanisms in a certain moment or conditions.

Leaves from the *C. nodosa* Italian ecotype presented a higher antioxidant capacity with ORAC assay than the Portuguese one (Table 2) which, in contrast had higher TEAC concentrations. In the *P. oceanica* Italian ecotype, ORAC was twice higher than in the Spanish ecotype, which presented four-fold more TEAC. The mechanism up-regulated by the plant is induced by the specific ROS to scavenge, according to environmental impacts. However, these results also suggest that different ecotypes might activate preferentially a certain mechanism related with the composition of plant antioxidants. The HAT assay includes antioxidants such as carotenoids, phenolic compounds, ascorbate and tocopherols, while the ET assay includes carotenoids, phenolic compounds, superoxide dismutase, ascorbate peroxidase and the reducing agents ascorbate, glutathione and NADPH. Differences in plant antioxidant composition and activities are related with the conditions where plants live, that are able to create reactive oxygen species either as signalling molecules or as consequence of demanding environments. The information relative to the mechanism activated by the plant, can give us a broad perspective of the group of the preferential ways on plant defence, however, carotenoids can work with both mechanisms according to the ROS, and under the phenolics each compound will act distinctively.

Table 2 – Oxygen radical absorbance capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC) in 2 *Cymodocea nodosa* (Cn) ecotypes (From Portugal and Italy) and 2 ecotypes of *Posidonia oceanica* (Po) (Spain and Italy). Values represent mean ± SE (µmol g DW^{-1}) (n=3).

	ORAC (µmol g DW^{-1})	TEAC (µmol g DW^{-1})
Cn Portugal	278.7 ± 39	221.6 ± 14.3
Cn Italy	480 ± 21	101.02 ± 3.6
PO Spain	112.8 ± 3.8	217.7 ± 16.6
PO Italy	284.8 ± 17	58.3 ± 6.2

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Chapter 3

Photosynthetic pigment content in seagrasses

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(To be submitted)

Photosynthetic pigment content in seagrasses

Abstract

There are as few as 60 different species of seagrasses all around the world, living under different climates and light environments. Their vertical distribution and survival is determined by the light availability and spectral composition. Seagrass adaptations to the aquatic environment include, among other, structural changes in leaves, such as a chloroplast-rich epidermis and an un-pigmented mesophyll. The few published data on seagrass photosynthetic pigments suggest a much higher similarity with land plants than with marine algae. Nevertheless, the available information regarding seagrass pigments is indeed extremely scarce, notably when compared to their terrestrial counterparts or to algae. Therefore, the aim of the present work was to amplify the knowledge regarding the photosynthetic pigment composition of seagrasses. For that purpose, we sampled and analysed several seagrass species in different geographical locations, namely *Halophila ovalis* and *Zostera capricorni* (Sidney Bay, Australia), *H. stipulacea* (Gulf of Acqba, Israel) *Z. noltii* and *Z. marina* (Ria Formosa, Portugal), *Cymodocea nodos*a (Ria Formosa, Portugal and Vulcano, Italy) and *Posidonia oceanica* (Cabo de Gata, Spain). Our results showed that the photosynthetic pigment composition of the seagrasses analysed is similar to land plants, and that they lack any of the carotenoids specifically found in algae. Lutein was the major carotenoid in all seagrasses except in *C. nodosa*, where β-carotene was the main carotenoid followed by lutein. All major pigments were present in the same order of magnitude as usually found in land plants. However, the ratio of chlorophylls to carotenoids was globally lower. Our results also indicate that all seagrasses use the VAZ cycle as a response to light changes but we found no unequivocal evidence of the lutein-epoxide cycle, which is present on land plants but not in algae.

Introduction

Seagrasses evolved from terrestrial angiosperms that colonized the sea about 100 million years ago. They rank amongst the most productive ecosystems in the biosphere and are believed to enhance the biodiversity of coastal waters, being ecologically very important. There are as few as 60 different species of seagrasses all around the world, living under different climates and light environments (Short *et al.*, 2007). These plants are able to thrive in a large variety of ambient conditions, from intertidal meadows (e.g. *Zostera noltii* in Ria Formosa, Portugal) where plants experience alternate periods of very high light during emersion with lower light intensities when submerged, to pronounced vertical distribution gradients with drastic differences in light quantity and quality (e.g. up to depths of 40 m as *Posidonia oceanica*, in the Mediterranean Sea (Via *et al.*, 1998; Proccacini *et al.*, 2010; Dattolo *et al.*, 2013) and *Halophila stipulacea*, in the Red Sea (Sharon *et al.*, 2009) . Despite this apparent versatility, seagrasses are experiencing a world-wide decline in many cases due to increased turbidity (Orth *et al.*, 2006; Waycott *et al.*, 2009). Depth and water turbidity are some of the factors that influence light availability and quality (Short and Neckles, 1999) and although it is well established that light availability is one of the limiting factors to the vertical distribution and survival of seagrasses (Hemminga and Duarte, 2000; Ralph *et al.*, 2007), light quality may also play an important role.

Underwater, both the intensity and the quality, *i.e.* the spectral composition of light, change with depth. While the light intensity decreases exponentially, spectral distribution varies in accordance with the absorption properties of the water. In general, the wavelengths corresponding to the red light are extinguished above 10 m, orange is extinguished above 20 m and only yellow, violet, green and blue reach deeper (Kirk, 2011). While algae evidence a chromatic adaptation reflected by a variable pigment composition (Kirk 2011), where carotenoids can be diverse and many times specific to a certain taxonomic group (Demmig-Adams *et al.*, 1996), seagrasses seem to have the same and conservative pigment composition as land plants (Casazza and Mazzella, 2002). According to Cummings and Zimmerman (2003), although seagrasses possess adaptations for life underwater, they lack specialized accessory pigments for the efficient harvesting of green light that leads to a package effect and the loss of linearity between light harvesting efficiency and pigment loading. Consequently, an increase in pigment concentrations does not always result in better photosynthetic efficiency.

Chlorophylls a and b display two major light absorption peaks, one in the red region (680 nm), and another one in the blue region (450 nm) and carotenoids absorb essentially in the blue region (Taiz and Zeiger, 2003). Therefore, changes in the proportion and localization of the different pigments in the photosynthetic apparatus affect (i) the wavelengths and the amount of light that is captured, (ii) the proportion of excitation energy that is in fact used in photosynthesis, (iii) the amount of excitation energy that is dissipated as heat and (iv) the quenching of the excited states of chlorophyll (Ruban *et al.*, 2007; Kirk, 2011; Ruban *et al.*, 2011; Ballotari *et al.*, 2013) These changes may contribute either to improve the light capture efficiency underwater or to photo-protect the photosynthetic apparatus when light intensity is too high.

In land plants, the content of leaf photosynthetic pigments changes with age, differs from tissue to tissue (Pilarki *et al.*, 2007), species to species and with the ambient conditions (Ramalho *et al.*, 2002; Keskitalo *et al.*, 2005; Matsubara *et al.*, 2009; Abney *et al.*, 2013). For example, shaded chloroplast have more chlorophyll, the chlorophyll a/b ratio is lower and the total chlorophyll/total carotenoid ratio is higher (Lichtenthaler and Babani, 2004). At high light intensities the xanthophyll cycle is activated (Gilmore *et al.*, 1995; Demmig-Adams *et al.*, 1996; Jahns *et al.*, 2009). Some plants display the lutein-epoxide ↔ lutein cycle with the accumulation of lutein-epoxide under low light (Ladygin 2008, Osmond *et al.*, 2008).

Much is known about the pigment content of land plants (Demmig-Adams *et al.*, 1996; Yamamoto and Bassi, 1996; Farré *et al.*, 2010; Namitha and Negi, 2010) and algae (Kirk, 2011) under different environmental conditions (Strzalka *et al.,* 2003; Ashraf and Harris, 2013) but there are only a few studies on seagrasses. Casazza and Mazzela (2002) screened but did not quantify chlorophylls a and b and the different carotenoids of *C. nodosa*, *H. stipulacea*, *P. oceanica* and *Z. noltii*. In other reports the photosynthetic pigments of seagrasses were quantified but refer mainly to chlorophyll a and b, whereas carotenoids were analysed as a bulk or not considered at all (Rotini *et al* 2013; Liu *et al.* 2011; Sharon *et al.* 2009; Kahn and Durako 2009; Cayabyab and Enriquez 2007; Durako *et al.*, 2003). Other authors (Ralph *et al.*, 2007 and references therein) addressed the effects of light reduction on the chlorophyll content in *Z. capricorni*, *H. wrightii*, *H. ovalis* and *Thalassia testudinum*. In general, these studies concluded that, as well as in land plants, light reduction caused the increase in total chlorophyll content and the decrease in the chlorophyll a/b ratio. To the best of our knowledge, Flanigan and Critchley (1996), Ralph *et al.* (2002) and Silva *et al.* (2013)

are the only published works that quantify seagrass violaxanthin-anteraxanthin-zeaxanthin (VAZ) cycle pigments (in *Z. capricorni*, Flanigan and Critchley, 1996) and β-carotene, VAZ cycle pigments and other xanthophylls like lutein, lutein-epoxide, and neoxanthin (in *Z. marina*, Ralph *et al.*, 2002; Silva *et al.*, 2013, and in *C. nodosa*, Silva *et al.*, 2013).

This work aims to contribute to the knowledge on the photosynthetic pigment content in seagrasses through the characterization and quantification of pigment composition in distinct seagrass species, namely: *Halophila ovalis* and *Zostera capricorni* (Sidney Bay, Australia), *H. stipulacea* (Gulf of Acqba, Israel) *Z. noltii* and *Z. marina* (Ria Formosa, Portugal), *Cymodocea nodos*a (Ria Formosa, Portugal and Vulcano, Italy) and *Posidonia oceanica* (Cabo de Gata, Spain).

The photosynthetic apparatus of higher plants

In higher plants, the photosynthetic apparatus is enclosed in the chloroplast and comprises the cytochrome b_6f (cyt b_6f) and two large pigment protein complexes, known as photosystem I (PSI) and photosystem II (PSII), both embedded in the chloroplast membranes or thylakoids. Photosynthetic pigments include the chlorophylls, that are the specialized light-absorbing pigments, and carotenoids, which are integral constituents of the thylakoid membranes and, besides absorbing light and transferring it to chlorophylls, play important roles in the photoprotection of the photosynthetic apparatus. In higher plants, only chlorophylls a and b can be found, while there are several different carotenoids, as the xanthophyll group (violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, lutein-epoxide and lutein) and α- and β-carotene (Lichtenthaler and Babani, 2004).

Photosystems I and II capture light and convert its energy into electrons circulating in the photosynthetic electron transport chain, which final products are ATP and NADPH. These molecules are then used in the Calvin cycle to reduce CO_2 to triose-phosphates, which are the carbon skeletons of several biomolecules. PSI and PSII structure comprises a reaction centre surrounded by the light harvesting antennae complexes (LHCs); LHCs capture light and transfer its excitation energy to the reaction centres (Demmig-Adams and Adam III, 1996; DellaPenna, 1999b). Reaction centres contain chlorophyll a and β-carotene, and the adjacent core complex proteins along with lutein in small amounts (Namitha and Negi, 2010). LHCs are the primary light harvesters (DellaPenna, 1999b) and are constituted by a large family of related proteins that bind xanthophylls, usually lutein, violaxanthin and neoxanthin, and chlorophyll a and b. The light harvesting complex II (LHCII) is associated to PSII and is more abundant than the light harvesting complex I (LHCI) which is associated to PSI (DellaPenna, 1999b). While PSI is more enriched in β-carotene, PSII have more lutein. Nevertheless, β-carotene can also be found in PSII, surrounding the reaction centre (Demmig-Adams *et al.*, 1996).

Chlorophylls organization and biosynthesis

Chlorophylls are the major photosynthetic pigments (Lichtenthaler, 1987). Chemically, chlorophylls are mixed prenyllipids and their chemical structure consists in a non-isoprenoid porphyrin ring attached to a long isoprenoid phytyl tail (Lichtenthaler, 2009). The ring structure contains some loosely bound electrons and is the part of the molecules responsible by electron transitions and redox reactions (Taiz and Zeiger, 2003) and the phytyl tail gives chlorophylls the lipidic properties (Lichtenthaler, 1987). The biosynthesis of chlorophyll starts from a glutamate molecule in its activated form to produce, through a sequence of reductions, condensations and oxidations, one molecule of protoporphyrin. Mg^{2+} is then inserted into the protoporphyrin molecule to form protochlorophylide which is afterwards reduced to chlorophyllide (Scoefs and Bertrand, 2005). This is the only reaction of chlorophyll biosynthesis that requires light (Willows, 2007) because is catalysed by a light dependent NADPH:oxidoreductase (Willows, 2007). Chlorophyllide molecule can either form chlorophyll a by esterification or chlorophyll b by oxidation prior to esterification (Scoefs and Bertrand, 2005). Chlorophyll a has an important role in chlorophyll b synthesis because it is likely that the synthesis of chlorophyll b is mediated by one radical from the tyrosine enzyme, which is quenched by chlorophyll a (Eggink *et al.*, 2004).

Chlorophyll a can be converted into chlorophyll b by chlorophyll a oxygenase (CAO) and the opposite reaction may also occur. The interconversion of chlorophyll a to b works like a cycle, similar to the xanthophyll cycle (Rüdiger, 2002). Thus, the cycle occurs from chlorophyllide a to chlorophyllide b, followed by esterification to chlorophyll b, reduction to chlorophyll b and again deesterification to chlorophyllide a (Rüdiger, 2002). Chlorophyll a and b are usually found in the proportion of 3:1, but this ratio may be changed by environmental factors (Lichtenthaler, 1987) through chlorophyll cycling (Rüdiger, 2002).

Chlorophyll biosynthesis is regulated in three main points: i) at the beginning of the synthesis, ii) on the magnesium incorporation, and iii) on the reduction of protochlorophylide to chlorophyllide (Willows, 2007).

Chlorophylls are localized in thylakoid membranes of chloroplasts, more specifically in the reaction centers and in the antenna of LHCI and LHCII in both plant photosystems (Lichtenthaler and Babani, 2004). During the last steps of the biosynthesis, chlorophyll and chlorophyllide can interact with proteins and form chlorophyll-protein complexes, which in turn are the building-blocks of the photosynthetic apparatus (Willows, 2007). The chlorophyll-proteins that only contain chlorophyll a, are hydrophobic and are more closely associated with the reaction centre of the photosystems (Green, 1988). The complex containing both chlorophyll a and b is associated with the LHCs. The major of these light-harvesting complexes is LHCII (Green, 1988).

Chlorophylls functions

Chlorophyll gives the green colour to plants and is essential for the first steps of the photochemical reactions of photosynthesis (Lichtenthaler and Babani, 2004). Chlorophyll a and chlorophyll b in LHCs are responsible for the light absorption and transference of excitation energy to the chlorophyll a in the reaction centres. Chlorophyll b also plays an important role in LHCII membrane stability (Enggink *et al*., 2001) and it is required at the beginning of chloroplasts formation for the assembly of LHCs (Eggink *et al*., 2004). It is also important in the photoprotection of chlorophyll a (Lichtenthaler, 1987). In the reaction centres, chlorophyll a converts light quantum into electrons that enter in the photosynthetic electron chain (Lichtenthaler and Babani, 2004).

In seagrasses living in distinct environments, the concentration in chlorophyll can vary about five-fold in order to maintain light harvesting efficiency (Cummings and Zimmerman, 2003). This plasticity in chlorophyll content plays an important role in seagrass adaptations to different light conditions because the cost of chlorophyll synthesis is somewhat compensated by the increase in light harvesting (Cummings and Zimmerman, 2003). However, the light spectrum reaching the leaves may affect the production of chlorophyll precursors. In fact, while red and blue light lead to an enhancement of photosynthesis, green light might have a negative effect (Fan *et al*., 2013). This is important in the adaptation of seagrasses to the environment, since the changes in water spectral quality with depth may affect the biosynthesis of chlorophyll and influence seagrasses development and growth. The effect of depth in the biosynthesis of chlorophylls was detected by Mohamed and Geneid (2007) in several seagrass species. Oxidative stress can also inhibit some steps of chlorophyll biosynthesis (Aarti *et al.*, 2006).

Carotenoid organization and biosynthesis

Carotenoids are natural pigments, located in plant photosystems (DellaPenna, 1999a) and synthetized in the plastids of all photosynthetic organisms (Cunningham and Gantt, 1998). They are responsible for the colours and aroma of leaves, fruits and flowers (Cazzonelli, 2011) and play important functions in light capture and photoprotection of the photosynthetic apparatus. There is evidence that some carotenoids have the plasticity to replace each other's functions. This is the case of the replacement of lutein by violaxanthin (DellaPenna, 1999b) and vice-versa (Zhirong *et al.*, 2009) and, because the location of neoxanthin and lutein in LHCII is similar, these pigments may play identical roles, as it was reported in some studies in which lutein was successfully replaced by neoxanthin for the reconstitution of LHCII (Gruszecki *et al.*, 1999). All carotenoids are bound to thylakoids, with exception of violaxanthin which, to some extent, is bound to the chloroplast envelope (Lichtenthaler, 1987).

Carotenoid biosynthesis starts with the unsaturation of phytoene, a C40 compound, to form lycopene. Lycopene can then be cyclized to β-carotene or to α-carotene, both oxygen free (DellaPenna, 1999a). Carotenes are the precursors of the oxygenated xanthophylls (Lichtenthlaler, 1987). α-carotene is the precursor of lutein-epoxide (taraxanthin) and lutein by hydroxylation (Lichtenthaler 1987), while β-carotene can be submitted to a series of hydroxylations and epoxidations to synthetize zeaxanthin, antheraxanthin, violaxanthin and neoxanthin (DellaPenna, 1999a).

In higher plants, carotenoids composition is remarkably conserved throughout evolution. By order of abundance, major carotenoids are lutein, β-carotene, violaxanthin, and neoxanthin (DellaPenna, 1999a). Lutein concentrations can fluctuate between 40% of all carotenoids to more than 65%, while violaxanthin and neoxanthin concentrations in LHCs can vary considerably (DellaPenna, 1999b). Most carotenoids are ubiquitous in higher plants, but α-carotene and lutein-epoxide are not found in a wide range of species (Demmig-Adam *et al.*, 1996; Matsubara *et al.*, 2009).

Carotenoid functions

Carotenoids are multifunctional compounds. Besides being structural components for LHCs assembly and stability, and precursors for vitamins and abscisic acid synthesis (Cazzonelli, 2011), carotenoids are accessory pigments for light harvesting, and play important roles in photoprotection acting both in dissipating excess excitation energy and as antioxidants (DellaPenna, 1999a, Cazzonelli, 2011).

Light harvesting

Carotenoids are extremely important in light harvesting since they can absorb in spectral regions where chlorophylls absorb minimally (Yamamoto and Bassi, 1996). The absorption of light energy by carotenoids elevates its ground electron state to an excited singlet state. Subsequently the energy transfer takes place through singlet-singlet photophysical processes until it reaches a chlorophyll a molecule (Yamamoto and Bassi, 1996) who is responsible by photosynthetic quantum conversion (Lichtenthaler and Babani, 2004). Lutein, neoxanthin, (Lichtenthaler 1987; Lichtenthaler and Babani, 2004; Jahns and Holzwarth 2012) and violaxanthin (Cazzonelli, 2011) are the main carotenoids in charge of light collection. Lutein also plays a crucial role for the stability of antenna proteins and contributes to light harvesting by transferring excitation energy to chlorophyll. Lutein-epoxide accumulation occurs particularly in deeply shaded plants or leaves and has been proposed as an efficient light-harvesting xanthophyll (Jahns and Holzwarth, 2012). Although β-carotene can act as a light absorbing pigment (Namitha and Negi, 2010), its main function is to protect chlorophyll a in the reaction centres against oxidative stress (Lichtenthaler, 1987). α-carotene displays the same functions as β-carotene in the species where it is found (Lichtenthaler and Babani, 2004). In algae, light collection may also be performed by other carotenoids, specific to some taxonomic algal groups, as fucoxanthin, siphonaxanthin and peridinin (Demmig-Adams *et al.*, 1996).

Photoprotection – excess energy dissipation as heat

Plants are frequently exposed to light intensities higher than those they have the capacity to use in photosynthesis. Photoprotective processes prevent or minimize the damages caused to the photosynthetic apparatus as a consequence of excess light (Yamamoto and Bassi, 1996). Carotenoids act in photoprotection dissipating excess energy as heat and as antioxidants, as they quench the triplet of chlorophyll (^{3}Chl) and the singlet of oxygen ($^1O_2$) formed in such light conditions (Niyogi 1999, Jahns *et al.*, 2012).

Currently there are three xanthophyll-cycles described: i) the Violaxanthin-Anteraxanthin-Zeaxanthin cycle (VAZ cycle), that occurs in seagrasses (Ralph *et al.*, 2002) and all land plants and green algae (Jahns *et al.*, 2009), ii) the lutein-epoxide cycle, which until now was only found in some land plant species (Bungard *et al.*, 1999; Matsubara *et al.*, 2011) and iii) the diadinoxanthin-cycle, which have been described only in some algae (Demmig-Adams *et al.*, 1996; Jahns *et al.*, 2009). In all these xanthophyll cycles the de-epoxidation of the xanthophylls is induced by high light and, as a consequence, the light-harvesting efficiency of the LHCs is reduced (Jahns *et al.*, 2009). Conversely, low light induces the epoxidation of the de-epoxidised xanthophylls (Jahns *et al.*, 2009).

In higher plants, the process of energy dissipation as heat occurs mainly through VAZ cycle pigments at the antenna level (Gilmore *et al.*, 1995), in three sequential steps: first the induction of the acidification of the thylakoid lumen, then, in the presence of ascorbate, the induction of the de-epoxidation of violaxanthin (V) to antheraxanthin (A) and then to zeaxanthin (Z) and finally, the dissipation of energy as heat because of the conformational changes of the LHCII due both to the presence of zeaxanthin and to the protonation of some the LHCII components (Yamamoto and Bassi, 1996; Ruban and Horton, 1999). This process depends on lumen acidification to pH=5 and is modulated by ascorbate (Gilmore *et al.*, 1995; Yamamoto and Bassi, 1996) that acts as a reductant and co-substrate to the enzyme violaxanthin-de-epoxidase (VDE). This enzyme catalyses the de-epoxidation reactions of the VAZ cycle (Jahns *et al.*, 2009). VAZ cycle is a reversible process and the epoxidation of Z back to A and V occurs as soon as light intensity decreases. Epoxidation activity is optimal near pH 7.0 and requires NADPH and oxygen (Yamamoto and Bassi, 1996). While the de-epoxidation process can take minutes, the reverse process may take hours or even days if light is combined with other kind of stresses (Demmig-Adams and Adam III, 1996). VDE is firmly bound to the thylakoid membrane at pH 5.0 and it is probably free in the lumen at pH 7.0 (Yamamoto and Bassi, 1996).

Cyanobacteria, Cryptophyta and most of Rhodophyta, do not possess any xanthophyll cycle (Jahns *et al.*, 2009). However, red and green-brown algae can still form zeaxanthin for photoprotection, directly from β-carotene (Demmig-Adams *et al.*, 1996). Zeaxanthin *de novo* biosynthesis from β-carotene is also described in terrestrial plants under high light (Lichtenthaler and Babani, 2004).

The VAZ cycle is not only activated by high light but also by several abiotic stresses, occurring even without light, like desiccation, anoxia and high-temperatures (Fernández-Martin *et al.*, 2011).

Other xanthophyll cycle found in some higher plants is the lutein↔lutein-epoxide cycle (lutein-epoxide cycle or Lx-cycle), described as an accessory mechanism to VAZ-cycle for energy dissipation as heat (Esteban *et al.*, 2009). By its operation, lutein-epoxide is de-epoxidased into lutein by violaxanthin-de-epoxidase (VDE). When light decreases, zeaxanthin-epoxidase (ZE), catalyses the reverse reaction, to regenerate lutein-epoxide again (García-Plazaola *et al.*, 2007). Therefore, both Lx and VAZ cycles are catalysed by the same enzymes (García-Plazaola *et al.*, 2007). However, the activation of Lx-cycle requires a higher acidification of the lumen than VAZ cycle, and is less effective (Niyogi *et al.*, 1997). Lutein epoxide is rapidly de-epoxidased to lutein when plants are exposed to light, while lutein epoxidation back to lutein-epoxide is much slower than the epoxidation steps in the VAZ cycle (Matsubara *et al.*, 2011). Therefore the identification of the lutein (L) ↔lutein-epoxide (Lx) as a xanthophyll-cycle may not be easy to detect. In fact, some studies indicate that the operation of this cycle is species-specific (Esteban *et al.*, 2007), and epoxidation does not always occur after the decrease of light intensity; in some cases, lutein-epoxide must be *de novo* synthesised from α-carotene (García-Plazaola *et al.*, 2007). Lx-cycle may play an important role in shade adapted plants, promoting quick energy dissipation when shaded leaves are suddenly exposed to strong light, as the conversion of Lx to L may convert efficient light harvesting centres to photoprotective centres (Esteban *et al.*, 2007; Matsubara *et al.*, 2007).

Photoprotection - antioxidant activity

When there is too much light, the excitation energy reaches the chlorophyll in the reaction centres at a higher rate than the rate at which it is used in photosynthesis (Demmig-Adams *et al.*, 1996). Consequently, the first quinone acceptor (QA) stays reduced and the excited states of chlorophyll (^{1}Chl) accumulate at PSII, meaning that the formation of the chlorophyll triplet (^{3}Chl*) is favoured. ^{3}Chl can easily and immediately interact with molecular oxygen to generate the short-living but highly reactive oxygen singlet ($^1O_2$) (van Mighem *et al.*, 1995; Demmig-Adams *et al.*, 1996), capable of attacking pigments and oxidising proteins, lipids and nucleic acids (Demmig-Adams and Adams III, 2000). Carotenoids participate in the inactivation of ^{1}Chl, ^{3}Chl and $^1O_2$, avoiding oxidative damage (Niyogi *et al.*, 1997; Choudhury and Behera, 2001; Strzalka *et al.* 2003; Ruban *et al.*, 2007; Zubik *et al.*, 2011).

When the antenna system becomes overexcited, β-carotene from the reaction centers (Choudhury and Behera, 2001) and neoxanthin (Strzalka *et al.* 2003; Zubik *et al.*, 2011) and lutein (DellaPenna, 1999b; Ruban *et al.*, 2007; Namitha and Negi, 2010, Jahns and Holzwarth, 2012) from light harvesting complexes protect the photosynthetic system from photodamage quenching ^{3}Chl and avoiding oxidative stress due to $^1O_2$ generation. Furthermore, zeaxanthin also have the ability to de-excite ^{1}Chl (Demmig-Adams *et al.*, 1996; Osto *et al.*, 2012), quench ^{3}Chl (Namitha and Negi, 2010) and act in a preventive way by removing epoxy groups from the oxidized double bonds of fatty acids of thylakoids (Lichtenthaler and Babani, 2004).

In the literature, there are several references to other carotenoid antioxidant functions as for instance the scavenging of superoxide anion radicals ($O_2^{\bullet-}$) (Cunningham and Gantt, 1998), peroxide radicals ($ROO^\bullet$) (Strzalka *et al.*, 2003), hydroxyl radicals ($HO^\bullet$) (Huang *et al.*, 2010) and nitrogen oxides (Namitha and Negi, 2010) protecting biomolecules from oxidative damage (Strzalka *et al.*, 2003).

Structural function

The physical properties of the photosynthetic membranes constrain their molecular dynamics. Thylakoid membrane fluidity affects their permeability and the mobility of electron and proton carriers. Membrane physical properties also determine oxygen mobility and the scavenging of ROS by lipid soluble antioxidants, as plastoquinone and α-tocopherol (Strzalka *et al.*, 2003).

Carotenoids condition the physical proprieties of membranes regarding both stability fluidity and permeability (Gruszecki *et al.*, 1999; Gruszecki *et al.*, 2005; Namitha and Negi, 2010). Lutein and zeaxanthin act preventing lipid peroxidation (Namitha and Negi, 2010) and changes in their concentration may increase or decrease the fluidity of the thylakoid membrane depending on the phase of the membrane (Gruszecki *et al.*, 2005). β-carotene and zeaxanthin affect the membrane permeability to glucose while lutein and neoxanthin are required for the correct assembly of LHCII and for its structural stability (Gruszecki *et al.*, 1999). Upon overexcitation, LHCII may suffer a configuration change from the trimeric to a monomeric form, to make the dissipation of energy more efficient. Violaxanthin stabilizes the trimeric configuration, while zeaxanthin promotes the monomeric state (Gruszecki, 2010).

The increase in proton concentration that occurs in the lumen of the thylakoids under light conditions is prone to induce structural membrane changes. It prompts the protonation of antenna complexes proteins and induces structural changes that bring chlorophyll closer to zeaxanthin, increasing excess energy dissipation as heat (Gilmore *et al.*, 1995; Demmig-Adams and Adam III, 1996). Structural changes can also be induced by zeaxanthin and antheraxanthin leading to energy dissipation by direct protonation of chlorophyll (Demmig-Adams and Adam III, 1996).

Carotenoids as precursors of other biomolecules

Carotenoids are precursors of phytohormones, as abscisic acid (ABA) (Cazzonelli, 2011), vitamins, as vitamin A (Cunningham and Gantt, 1998), and other signalling molecules that influence plant development and stress responses (Farré *et al.*, 2010). β-Carotene, also known as pro-vitamin A is the precursor of vitamin A (Asensi-Fabado and Munné-Bosch, 2010; Farré *et al.*, 2010) and terpenoids responsible for the control of branching and symbiotic associations (Cazzonelli, 2011). Whereas violaxanthin and neoxanthin are the precursors of ABA, β-carotene can be rate-limiting for this phytohormone biosynthesis, influencing on plant resistance to drought and to oxidative stress (Cazzonelli, 2011).

Material and methods

Plant material

Photosynthetic pigments were extracted from leaves of *Halophila stipulacea* (Gulf of Aqaba, Israel, collected in May, 2010), growing at 17 m and 40 m, *Posidonia oceanica*, (Cabo de Gata, Spain, collected in February, 2012), growing at 4-5 m, *Cymodocea nodosa*, (Vulcano, Italy, collected in May, 2013), *Halophila stipulacea and Zostera capricorni* (Sidney Bay, Australia, collected in May, 2011) growing at 4 m, all sampled at pre-dawn and solar noon. *Zostera noltii, Zostera marina, Cymodocea nodosa* (Ria Formosa, Portugal, collected in July, 2010) were sampled in one single period of the day, during morning (10h30).

After collection, leaves were cleaned from epiphytes (when necessary), rinsed with distilled water, blotted dry, frozen in liquid nitrogen and stored at -80°C until analysis. Each sample was composed by the middle part of green leaves from several shoots, discarding all brown, old or damaged parts.

Results were expressed in µmol m^{-2}. Area (m^{-2}) was calculated with ImageJ 1.46r software (Wayne Rasband National Institute of Health, USA), in leaf segments photographed with a scale.

Photosynthetic pigments extraction and quantification

Photosynthetic pigments were extracted as it is described in Abadía and Abadía (1993). Chlorophyll a and b were quantified spectrophotometrically (Beckman Coulter DU-650 spectrophotometer, Brea CA, USA) using the equations of Lichtenthaler and Buschmann (2001). The carotenoids neoxanthin, violaxanthin, lutein-epoxide (taraxanthin), antheraxanthin, lutein, zeaxanthin, β-carotene and α-carotene were analysed by an isocratic High Performance Liquid Chromatography (HPLC) according to Larbi *et al.* (2004) after de las Rivas *et al.* (1989).

Photosynthetic pigment extraction was done in 200 mg frozen leaf tissue ground in liquid nitrogen and sodium ascorbate. Pigments were extracted in 5 ml of acetone 100% buffered with $CaCO_3$. The extracts were sequentially filtered with 5.0 µm LS membranes and hydrophobic PTFE 0.2µm filters and stored in the dark at -20ºC until analysis. The entire extraction procedure was performed under low light environment to avoid pigment degradation.

HPLC calibration (de las Rivas *et al.*, 1989) was performed with commercial pigments from CaroteNature, Lupsingen, Switzerland. 1 mg of pigment standards was regenerated in 20 ml absolute ethanol. Four dilutions were made from this stock solution to obtain the standards used to build the calibration curve. Prior to injections, all standard solutions went through the same filtration procedures as extracts. Standards concentrations were determined spectrophotometrically (Beckman coulter DU 650 spectrophotometer), at the correspondent absorption peak wavelengths in ethanol 100%. Neoxanthin, violaxanthin, lutein-epoxide, antheraxanthin, lutein, zeaxanthin, β-carotene and α-carotene were read at 439, 443, 442, 444, 445, 450, 453 and 445 nm, respectively, and concentrations were determined using the following coefficients of extinction ($E^{1\%}_{1cm}$): 2243, 2550, 2800, 2500, 2550, 2540, 2620 and 2710 (Val *et al.*, 1986; Barua and Olson, 2001; Liu *et al.*, 2011). Calibration curves were done injecting each standard eight-fold.

HPLC analysis were performed in an Alliance Waters 2695 separation module (Milford MA, USA), with a Waters 2996 photodiode array detector and a Waters Novapak C18 radial 8x100mm compression column (4µm particle size). 20 µL of standard solutions or leaf extract were injected with an auto sampler. During the injection period, both standard solutions and extracts were maintained at 5ºC. Mobile phase was pumped at a 1.7 ml min^{-1} flow. Mobile phase A, acetonitrile: methanol:TEA (7.5:1:0.7), was fluxed trough the column, in a 3.5 min isocratic step, followed by mobile phase B, acetonitrile:methanol:mili-q water:ethyl acetate (7:0.96:0.04:8) in a 6.5 min isocratic step. Between injections, the column was equilibrated with mobile phase A for 5 min. All eluents were prepared with HPLC grade solvents (VWR Hipersolv Chromanorm) and were filtered and sonicated prior to use. During all chromatographic analysis, the column was kept in a steady temperature of 24ºC.

Peak areas were monitored at 450 nm, and concentrations were calculated based on peak areas obtained from standard solutions.

Statistical analysis

All results are presented as mean values ± standard error. T-student tests or one-way analysis of variance (ANOVA) were used to detect significant effects of time and species (at $p < 0.05$). T-student tests were used to compare differences amount time, for each species and pigment. ANOVA was used to detect differences between species. The Student-Newman-Keuls post-hoc test was used to test for significant differences between treatments. All data treatment and statistical analysis was performed using the SigmaStat/SigmaPlot (SPSS Inc., v.11) software package.

Results and Discussion

All seagrass extracts were separated in chlorophyll a and b and eight to nine carotenoids depending on the seagrass species (Fig. 3.1). Photosynthetic pigments were eluted from the HPLC column in the following order: neoxanthin (1 and 2), violaxanthin, lutein-epoxide (taraxanthin), antheraxanthin, lutein, zeaxanthin, chlorophyll b, chlorophyll a, α-carotene and β-carotene. According to our identification, and as could be expected because seagrasses evolved from land angiosperms, all the analysed seagrasses showed the same pigment composition as land plants (DellaPenna, 1999a). No other chromatographic peaks, that could indicate the presence of other lipossoluble pigments like the xanthophylls specific to some taxonomic algae groups (Demmig-Adams *et al.*, 1996; Jahn *et al.*, 2009), were found (Fig. 3.1). α-Carotene and lutein-epoxide are not present in all in land plants (Matsubara *et al.*, 2009), and the same happens in seagrasses where lutein-epoxide was not identified in *Zostera capricorni* and α-carotene was only identified in *Posidonia oceanica* (Tab. 3.1).

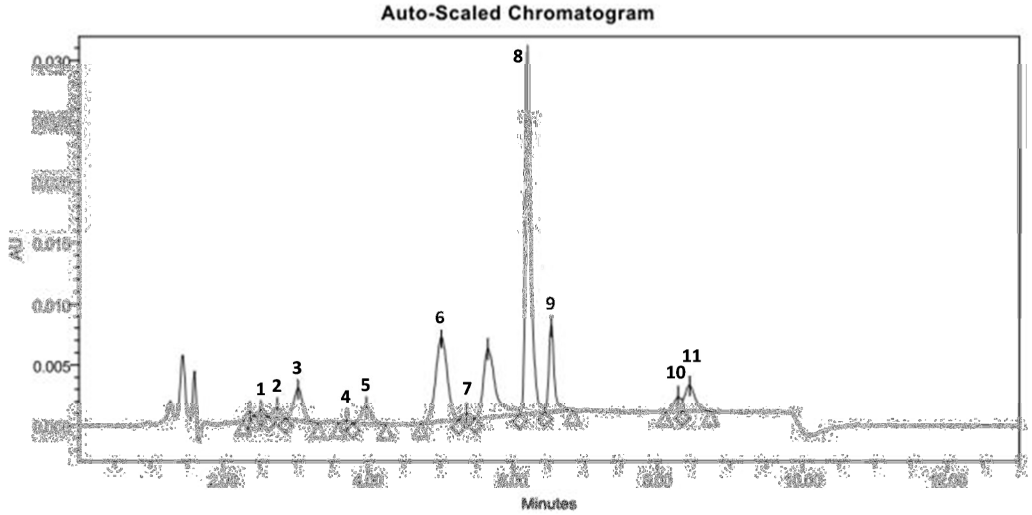

Figure 3.1. Example of HPLC chromatographic profile of seagrass photosynthetic pigments of *Posidonia oceanica*. Peak identification: 1-neoxanthin1; 2-neoxanthin 2; 3-violaxanthin; 4-lutein-epoxide; 5-anteraxanthin; 6-lutein; 7-zeaxanthin; 8-chlorophyll b; 9-chlorophyll a; 10-β-carotene; 11-α-carotene.

Neoxanthin, violaxanthin, lutein and β-carotene have been previously detected, but not quantified, in *P. oceanica*, *H. stipulacea*, and *Z. noltii* by Casazza and Mazzella (2002), as well as the presence of α-carotene in *P. oceanica*,. Chlorophyll a and b and separated carotenoids have been already detected and quantified in *Z. marina* and *C. nodosa* by Silva *et al.* (2013), in *Z. marina* by Ralph *et al.* (2002), but not lutein-epoxide or α-carotene. Flanigan and Chritchley (1996) quantified the xanthophylls of the VAZ cycle in *Z. capricorni*. To the best of our knowledge, this is the first time that the photosynthetic carotenoids were quantified in *H. ovalis*, *H. stipulacea*, *Z. noltii* and *P. oceanica*.

The total chlorophyll concentrations of the species sampled here varied between 46.50 and 336.8 μmol.m^{-2} (Tab. 3.1), according to the species and time of the day (Tab. 3.1). Five-fold variations of chlorophyll concentration has also been reported for seagrasses elsewhere in order to optimize light harvesting efficiencies of plants growing in much different light environments (Cummings and Zimmerman, 2003). Factors such as light reduction (Ralph *et al.*, 2007) or low temperatures (Niu *et al.*, 2012) can have a positive impact on chlorophyll content in seagrasses. As observed here for *P. oceanica*, lower values of chlorophyll a compared with terrestrial plants were also found in Enríquez *et al.* (1992). The seagrasses sampled here showed a wider variation of chlorophyll concentration (32.6 to 464.55 µmol.m^{-2}) than land plants (250 to 900 µmol.m^{-}2, Matsubara *et al.*, 2009).

Total carotenoids represent 9 - 32% of the total photosynthetic pigments in the seagrasses analysed (Table 1). In *H. ovalis*, *H. stipulacea* (at 17 and 40m depth), *Z. noltii*, *Z. marina* and *Z. capricorni* and *P. oceanica*, the most abundant identified carotenoid was lutein (85-170 mmol molChlT^{-1}), while in *C. nodosa* ecotypes (Ria Formosa, Portugal and Vulcano, Italy), the most abundant carotenoid was β-carotene (58-113 mmol molChlT^{-1}). Both carotenoids were in the same order of magnitude as they can be found in terrestrial plants (Matsubara *et al.*, 2009). Neoxanthin, and violaxanthin were also found in high concentrations (18-76 and 23-55 mmol molChlT^{-1}). α-carotene was only identified in *P. oceanica* where it represented twice the concentration of β-carotene. Albeit α-carotene is found in a great number of terrestrial angiosperms, the ratio α-/β-carotene is commonly below one. Ratios of two as those displayed by *P. oceanica* were found in only a few number of shade adapted species, as for instance, *Costus villosissimus*, *Strychnos panamensis* and *Cassipourea elliptica* (Matsubara *et al.*, 2009). As it was told before, α-carotene is the precursor of lutein and lutein-epoxide. The higher amount of this pigment is probably connect with an enhancement of light capture (in the case of lutein-epoxide) or of photoprotection (in the case of lutein).

Table 3.1. Seagrass photosynthetic pigments and relationships under pre-dawn and noon. Values represent mean ± SE; different letters indicate significant differences between pre-dawn (PD) and noon (n=3 or 5, $p<0.05$).

Species	***Halophila ovalis***		***Halophila stipulacea***		***Halophila stipulacea***		***Zostera noltii***	***Zostera marina***	**Zostera capricorni**		***Cymodocea nodosa***	***Cymodocea nodosa***		***Posidonia oceanica***	
Location	Sidney Bay, Australia, May 2011		Gulf of Aqaba, Israel, May 2010		Gulf of Aqaba, Israel, May 2010		Ria Formosa, Portugal, Julho, 2010	Ria Formosa Portugal Julho 2010	Sidney Bay Australia, May 2011		Ria Formosa, Portugal, Julho 2010	Vulcano, Aeolian Islands, Italy, May 2013		Cabo de Gata, Spain, September 2012	
Depth (m)	4	4	17	17	40	40	Intertidal	3	4	4	3	3	3	4-5	4-5
Sampling	PD	Noon	PD	Noon	PD	Noon	Morning	Morning	PD	Noon	Morning	PD	Noon	PD	Noon
Chlorophyll a (Chla) (µmol.m^{-2})	336.79a ± 198.11	98.61a ± 44.84	38.97a ± 6.30	30.13a ± 2.58	22.53a ± 1.49	27.93a ± 4.12	271.86 ± 21.27	242.10 ± 10.58	243.29a ± 75.92	122.96a ± 37.74	44.98 ± 8.06	123.71a ± 18.86	76.50a ± 20.31	46.36a ± 7.20	52.25a ± 7.45
Chlorophyll b (Chlb) (µmol/m^2)	127.76a ± 62.01	41.57a ± 19.61	16.63a ± 2.77	12.57a ± 1.43	9.62a ± 0.48	12.81a ± 1.91	92.72 ± 7.25	98.97 ± 4.33	81.55a ± 26.59	41.9a ± 13.65	15.30 ± 2.90	34.19a ± 4.74	19.01b ± 4.80	20.69a ± 3.34	22.80a ± 3.34
Total chlorophyll (ChlT) (µmol.m^{-2})	464.55a ± 260.03	140.18a ± 64.44	55.60a ± 9.06	42.70a ± 3.950	32.16a ± 1.91	40.74a ± 5.98	364.59 ± 28.52	341.06 ± 14.91	324.85a ± 102.45	164.88a ± 51.39	56.29 ± 7.07	157.90a ± 23.60	95.51a ± 25.11	67.05a ± 10.52	75.06a ± 10.77
Chla/b	2.37a ± 0.28	2.40a ± 0.05	2.36a ± 0.03	2.43a ± 0.10	2.34a ± 0.08	2.19a ± 0.09	2.93 ± 0.00	2.45 ± 0.00	3.00a ± 0.07	2.97a ± 0.05	2.72 ± 0.25	3.60b ± 0.09	3.97a ± 0.08	2.25a ± 0.04	2.30a ± 0.04
α-Carotene (mmol.molChlT^{-1})	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	74.89a ± 4.62	74.65a ± 4.60
β-carotene ((mmol.molChlT^{-1})	61.84a ± 22.49	100.63a ± 0.23	4.90a ± 0.55	4.99a ± 0.72	3.97a ± 0.29	3.68a ± 0.26	82.34 ± 0.00	81.10 ± 0.00	82.89a ± 41.33	114.78a ± 23.16	113.19 ± 7.29	57.94a ± 13.24	111.33a ± 27.86	33.55a ± 5.13	33.87a ± 2.91
Neoxanthin (N) ((mmol.molChlT^{-1})	18.30a ± 6.69	27.04a ± 1.78	25.03a ± 5.95	20.14a ± 4.51	20.06a ± 1.74	20.18a ± 5.02	31.46 ± 2.51	37.01 ± 0.6	22.43a ± 10.75	29.55a ± 7.56	25.61 ± 2.11	15.21a ± 2.94	26.9a ± 7.26	76.62a ± 9.73	74.93a ± 5.19

Violaxanthin (V) (mmol.molChlT^{-1})	28.84a ± 8.22	23.77a ± 1.03	61.11a ± 18.75	35.03a± 5.93	41.33a ± 8.49	39.49a ± 1.63	69.25 ± 9.2	38.36 ± 2.9	59.53a ± 34.56	45.32a ± 12.30	47.04 ± 2.97	43.14a ± 9.35	55.69a ± 12.34	44.55a ± 3.70	41.55a ± 3.26
Anteraxanthin (A) (mmol.molChlT^{-1})	2.44b ± 0.92	14.98a ± 0.20	16.95b ± 3.81	29.55a± 6.06	9.59a ± 1.85	13.41a ± 1.54	5.30 ± 0.71	22.00 ± 1.17	5.07b ± 1.76	14.83a ± 4.89	42.55 ± 4.62	2.72b ± 0.52	23.41a ± 6.83	3.06b ± 0.25	4.55b ± 0.49
Zeaxanthin (Z) (mmol.molChlT^{-1})	nd	37.88 ± 16.60	9.27b ± 3.97	20.59a± 5.16	2.46a ± 1.39	4.32a ± 0	nd	23.97 ± 1.45	1.98b ± 0.0	6.56a ± 0.0	43.95 ± 7.97	2.22b ± 0.29	19.38a ± 6.06	0.07b ± 0.00	0.20a ± 0.00
V+A+Z (mmol.molChlT^{-1})	31.28b ± 9.14	76.62a ± 15.77	87.32a ± 20.70	85.17a ± 17.06	55.36a ± 4.00	52.00a ± 9.25	74.55 ± 8.21	84.34 ± 8.20	31.28a ± 9.13	76.62a ± 15.77	128.22 ± 10.20	48.08a ± 10.09	98.48a ± 24.43	47.60a ± 3.88	46.10a ± 3.20
(A+Z)/(V+A+Z)	0.07b ± 0.01	0.67a ± 0.08	0.34b ± 0.03	0.58a ± 0.02	0.17b ± 0.02	0.28a ± 0.03	0.07 ± 0.0	0.55 ± 0.0	0.15b ± 0.09	0.28a ± 0.04	0.62 ± 0.02	0.11b ± 0.01	0.42a ± 0.03	0.052b ± 0.00	0.15a ± 0.01
Lutein (L) (mmol.molChlT^{-1})	84.82a ± 32.50	140.79a ± 14.44	139.5a ± 38.50	121.58a ± 24.38	122.32a ± 8.65	129.25a ± 30.42	96.50 ± 8.20	96.67 ± 8.20	94.92a ± 50.50	123.87a ± 23.02	101.27 ± 8.99	34.90a ± 6.70	69.01a ± 17.66	159.53a ± 16.9	168.88a ± 14.13
Lutein-epoxide (Lx) (mmol.molChlT^{-1})	1.13a ± 0.12	1.43a ± 0.01	5.74a ± 2.07	2.46a ± 0.15	2.26a ± 0.09	3.36a ± 0.50	9.35 ± 3.71	4.19 ± 0.36	nd	nd	0.80 ± 0.00	2.13a ± 0.44	1.95a ± 0.41	4.14a ± 0.23	3.52a ± 0.14
L/(Lx+L) (mmol mmol^{-1})	0.99a ± 0.00	0.99a ± 0.00	0.97b ± 0.01	0.99a ± 0.00	0.97b ± 0.00	0.99a ± 0.00	0.91 ± 0.00	0.95 ± 0.00	-	-	0.99 ± 0.01	0.94b ± 0.00	0.97a ± 0.00	0.98a ± 0.00	0.98a ± 0.00
Chlt/carotT (µmol/ µmol)	4.00a ± 0.40	3.94a ± 0.30	3.35a ± 0.04	3.35a ± 0.13	3.48a ± 0.11	3.83a ± 0.18	4.31 ± 0.00	4.16 ± 0.00	5.04a ± 0.79	3.46a ± 0.11	3.23 ± 0.25	2.70a ± 0.51	2.56a ± 0.59	3.84a ± 0.11	4.08a ± 0.14
% (Total Carotenoids/ total pigments)	15.85a ± 5.24	25.66a ± 1.52	24.01a± 4.79	29.26a± 3.35	24.10a± 1.48	22.31 a ± 2.99	22.73 ± 0.00	23.27 ± 0.00	19.21a ± 8.13	24.45a ± 3.76	32.73 ± 5.97	9.60a ± 1.90	16.85a ± 3.58	28.55a ± 1.90	28.55a ± 1.21

The foliar concentration of neoxanthin, lutein and β-carotene, did not change from pre-dawn to noon. The unaltered concentration of these pigments, no matter the light intensity, was also reported in *Z. marina* (Ralph *et al.*, 2002) and on several terrestrial species (Bungard *et al.*, 1999; Demmig-Adams *et al.*, 1996; Lichtenthaler and Babani, 2004; Matsubara *et al.*, 2009). Lutein-epoxide is not an ubiquous pigment in the leaves of higher land plants (Matsubara *et al.*, 2009) and the same might be true for seagrasses as it was not detected in *Z. capricorni*. Lx concentrations in the remaining seagrasses analysed were always below 10 mmol mol^{-1} ChlT and similar to those reported for the majority of land plants (García-Plazaola *et al.*, 2007).

The VAZ cycle was activated by light in all species sampled (Figure 3.2). A significant increase in antheraxanthin and zeaxanthin at expense of violaxanthin decrease was observed from pre-dawn to noon (Table 3.1). It is noteworthy that at 17 m depth the VAZ deepoxidation index ((A+Z)/(V+A+Z)) of *H. stipulacea* was twice the values displayed at 40 m, both at pre-dawn and noon. Moreover, at 17 m the deepoxidation index at pre-dawn was higher than at 40 m at noon (Table 3.1). Both observations reflect the influence of the attenuation of light intensity with depth on the deepoxidation index, and are an example of pigment modulation influenced by the light environment to meet the energetic demand/photoprotection needs of the plant. VAZ cycle activation as a response to light was already reported in *Z. capricorni* (Flanigan and Critchley, 1996) and in *Z. marina* (Ralph *et al.* 2002).

Only two xanthophyll cycles have been so far identified in higher plants: the VAZ cycle and the lutein-epoxide cycle (Bungard *et al.*, 1999; García-Plazaola *et al.*, 2007; Matsubara *et al.*, 2011). From the nine seagrass species/ecotypes analysed, the L/(Lx+L) ratio increased significantly in *H. stipulacea* (17m and 40m) and *C. nodosa* (Italy) from pre-dawn to noon (Table 1). However, the activation of the lutein-epoxide cycle is not evident in these species as the changes on the L/(Lx+L) ratio where not due to the decrease of Lx and concomitant increase in L as they were supposed to if the referred cycle was involved (García-Plazaola *et al.*, 2007). More research is needed to clarify this issue.

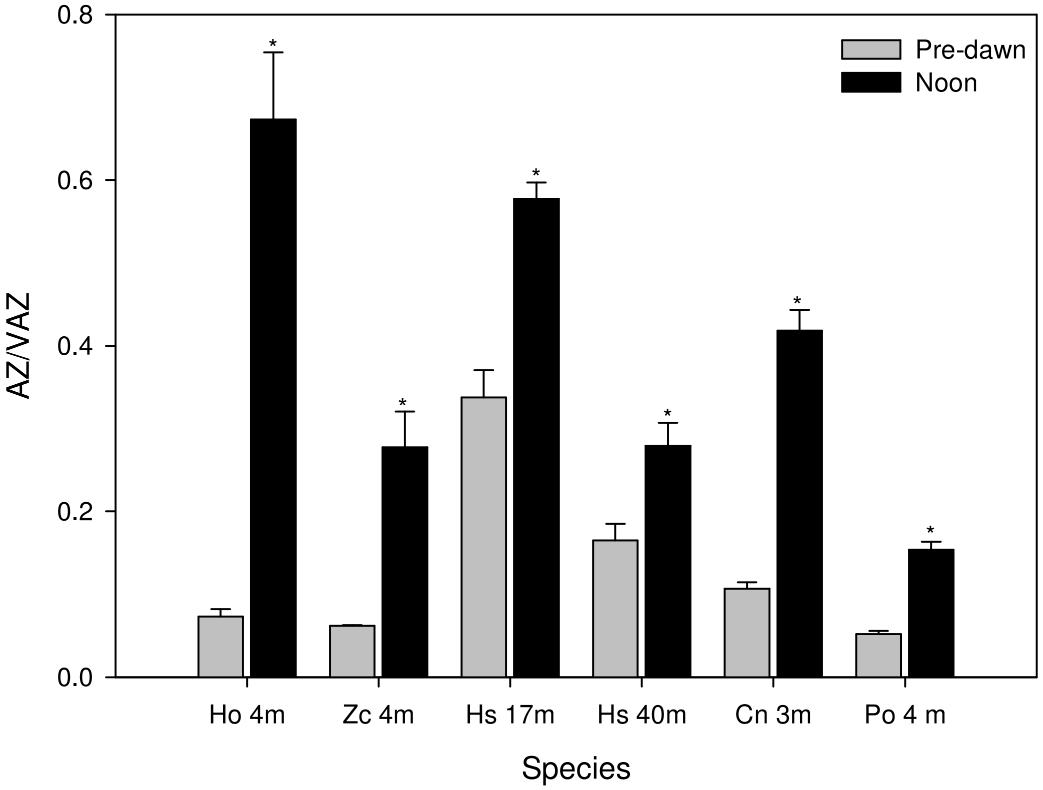

Figure 3.2. de-epoxidation index [(A+Z)/(V+A+Z)] in seagrasses *Halophyla ovalis* (Ho) at 4m, *Zostera capricorni* (Zc) at 4m, *Halophyla stipulacea* at 17m and 40m (Hs 17m and Hs 40m, respectively), *C. nodosa* (Cn) at 3m and *Posidonia oceanica* (Po) at 4m sampled at pre-dawn (PD) and noon. Values represent means ± SE; * indicates significant differences between pre-dawn and noon (n=3 or 5, $p<0.05$).

There are other xanthophyll cycles identified in algae. One of them is the diadinoxanthin-cycle present in diatoms, phaeophytes, dinophytes and haptophytes (Demmig-Adams *et al.*, 1996; García-Plazaola *et al.*, 2007; Jahns *et al.*, 2009), and the other is a cycle involving L-siphonaxanthin interconversions that was identified in the green alga *Caulerpa racemosa* (García-Plazaola *et al.*, 2007). The pigments involved in these xanthophyll cycles were not identified in the seagrasses analysed in the present work.

The chlorophyll a/b ratio is commonly used as an indicator of the adaptation of the photosynthetic apparatus to changes in light intensity (Lichtenthaler *et al.*, 1981; Lichtenthaler and Babani 2004). The chlorophyll a/b ratio of *H. ovalis* and *P. oceanica* was below 2.7, which is typical of low light adaptation in land plants (Lichtenthaler and Babani, 2004). Albeit seagrasses are generally considered as shade plants (Larkum *et al.*, 2006), this results are somehow puzzling as these seagrasses were collected from sites with transparent shallow water where light intensities are typically high. *H. stipulacea* also exhibited chlorophyll a/b ratios below 2.7 and there were no differences between the samples collected at 17m and 40m deep. This is contrary to the expectation of a decreasing ratio with depth in response to the attenuation of light intensity. On the other hand, the two *C.nodosa* ecotypes exhibited different chlorophyll a/b ratios, following the land plants patterns: 2.72 in Ria Formosa (Portugal), where the turbidity of the water is high and light intensity is often low, and 3.60 in the transparent shallow water of Vulcano (Italy). Accordingly, *Z. marina* from Ria Formosa (Portugal) exhibited a low chlorophyll a/b ratio of 2.45. Together with *C. nodosa* from Vulcano (Italy), *Z.noltii* (intertidal, Ria Formosa, Portugal) and *Z. capricorni* (Sidney Bay, Australia), exhibited chlorophyll a/b ratios higher than 2.9 which in land plants is considered typical of high light adapted plants (Lichtenthaler and Babani, 2004).

In land plants, total chlorophyll to total carotenoids ratio (chlT/carotT) is used as an indicator of greenness of plants, lying between 4.3-5.5 in sun exposed plants and between 5.5-7.0 in the shade exposed plants (Lichtenthaler and Buschmann, 2001; Lichtenthaler and Babani, 2004). All the leaves analysed were healthy and green but, besides *Z. capricorni* which presented a ratio of 5.04 at pre-dawn, all the other plants presented ratios between 3.5 and 2.6. This could indicate chlorophyll degradation (Lichtenthaler and Buschmann, 2001) but could also simply indicate a response to an environment distinct from land plants. Ralph *et al.* (2005) also found ratios chlT/ caroT bellow 4 in *P. australis.*

The restriction of chloroplasts to the epidermal cell layer of seagrasses leaves (Hemminga and Duarte, 2000) makes easier the access to carbon and light, being advantageous in low light environments. However, in tropical, shallow or transparent waters, chloroplasts will be exposed to an excess of light and that can lead to photoinhibition (Cummings and Zimmerman, 2003). Seagrasses leaves are efficient light capturing organs, with a leaf absorbance capacity similar to land plants (Cummings and Zimmerman, 2003). Based on our results, it is likely that the low chla/chlb and ChlT/carotT ratios reflect an adaptive enhancement in light harvesting pigments, where the interchangeable forms of carotenoids may function either in light harvesting or in photoprotection (Adams III *et al.*, 2006; Kirk, 2011).

In this work, we showed that the photosynthetic pigment composition of the seagrasses analysed is similar to land plants. Likewise, all major pigments were present in the same order of magnitude as usually found in land plants. Lutein was the major carotenoid in all seagrasses with the exception of both *C. nodosa* ecotypes, where β-carotene was the major carotenoid followed by lutein. The ratio of chlorophylls to carotenoids in seagrasses is globally lower than in land plants. Nevertheless, the proportions of photosynthetic pigments in marine plants can change with environmental conditions and species to achieve the best light efficiencies or photoprotection. None of photosynthetic carotenoids specifically identified in some marine macroalgae were present in seagrasses. Based on our results we can say that all the seagrasses analysed activate the VAZ cycle as a response to light increase and this response was more expressive in shallow water, as expected. Although there was a significant change of the ratio L/(L+Lx) in *C. nodosa* and *H. stipulacea*, there is no clear evidence of the occurrence of the lutein-epoxide cycle in seagrasses.

4. References

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Chapter 4

Short-term effects of light and temperature on the photosynthetic performance and antioxidant responses of the seagrass *Cymodocea nodosa*

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(To be submitted)

Short-term effects of light and temperature on the photosynthetic performance and antioxidant responses of the seagrass *Cymodocea nodosa*

Abstract

Understanding the mechanisms of a species' response to stress it's vital to anticipate its behavior on a climate change perspective. Increased temperature combined with high light levels can be potentially stressful to species by interfering with its biochemical mechanisms and enhance the production of oxygen reactive species (ROS). Antioxidant system ability to control ROS levels and energy dissipation through xanthophyll-cycle, are some of biochemical mechanisms that can prevent oxidative stress and allow plant adjustment to new conditions. The aim of this work was to evaluate the photosynthetic and antioxidant responses of *Cymodocea nodosa* from Ria Formosa (Portugal), to the combined effect of light and temperature, using potentially stressful ranges of both parameters. Leaves were analyzed for pigment responses, namely VAZ-cycle activation, antioxidant defenses measured both by trolox equivalents antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC), ascorbate peroxidase activity (APX), malondialdehyde (MDA) concentrations, potential quantum yield of PSII and soluble sugars variations. *C. nodosa* presented an integrated physiological response to the imbalance created by the stressful conditions, with changes in pigments, antioxidant system and sugar content. Due to high acclimation capacity, the experimental conditions did not cause serious damage in the plants. However, low temperature, particularly in combination with high light appears to be specially demanding for *C. nodosa*. This reveals that *C. nodosa* photoprotection mechanisms are well prepared to deal with severe conditions, preventing damages of the photosynthetic system that could compromise the plants performance.

Introduction

Understanding the mechanisms of a species' response to stress it’s vital to anticipate its behavior on a climate change perspective (Soares-Cordeiro *et al.*, 2010). Organisms that can adjust their physiological mechanisms to changes in the environment, particularly those that increase the stress levels, will be in advantage in relation to those with less adjustment capacity (Scandalios, 2005).

During their normal growth and development processes, plants produce reactive oxygen species (ROS) in different ways (Wang *et al.*, 2011). Even under optimal growth conditions, metabolic processes such as electron transport systems produce reactive oxygen species (Yoshiura et al, 2000). However, when the environment becomes abnormally stressful, ROS can reach high concentrations (Wang *et al.*, 2011), able to create oxidative stress (Blokhina *et al.*, 2003). Strong oxidative stress conditions can be responsible by oxidative degradation of some macromolecules and peroxidation of lipids leading to malondialdehyde (MDA) formation (Valenzuela, 1991).

The increase in ROS triggers the antioxidant system in order to prevent the imbalance between the reactive oxygen species created and the ability of the system to scavenge them (Huner *et al.*, 1998; Sandalios, 2005). Antioxidants have the ability to detoxify ROS (Aghadam *et al.,* 2011) through complex physiological pathways that comprise both enzymatic and non-enzymatic antioxidants (Johnston *et al.,* 2006). Non-enzymatic antioxidants include tocopherols, phenolic compounds, ascorbic acid and carotenoids while enzymatic antioxidants are mainly composed by superoxide dismutases, peroxidases, catalases and enzymes that oxidize or reduce ascorbate (Dabrowska *et al.*, 2007). Both antioxidant systems works together to control ROS in the organism (Dabrowska *et al.*, 2007).

Since different antioxidants have distinct mechanisms of action, to properly evaluate the antioxidant capacity of a specific species in any particular situation it is important to use a comprehensive array of methods that cover the possible antioxidant pathways. According to Huang *et al.* (2005), the methods used to evaluate the antioxidant scavenging capacity can be divided in two groups: assays based in hydrogen atoms transfer, like oxygen reactive absorbance capacity (ORAC) and assays based on electron transfer, like trolox equivalent antioxidant capacity (TEAC), phenolic compounds and ascorbate. The TEAC assay has been widely used to determine antioxidant capacity of terrestrial plant extracts (Dunnoné *et al.*, 2009; Sun *et al.*, 2012). The oxygen radical absorbance (ORAC) assay has been extensively used by pharmaceutical, nutritional and food industries to determine total antioxidant capacity (Huang *et al.*, 2002). Regarding enzymatic antioxidant activity, studies in terrestrial plants evidence the increase in peroxidase activity (APX) under oxidative stress conditions (Yoshiura *et al.*, 2000; Fryer *et al.*, 2003). APX has the function of scavenging oxygen peroxide (H_2O_2), which is continuously formed in the cells (Miyake and Asada, 1996). This enzyme uses ascorbate as electron donor for the reduction of H_2O_2 to water (H_2O), detoxifying the system from this well-known ROS (Lu *et al.*, 2008).

Concerning non-enzymatic antioxidant protection, plants can dissipate the excess of energy through carotenoids from the xanthophyll cycle. These oxygenated carotenoids have photoprotection functions (Niyogi *et al.*, 1997). The xanthophyll cycle is composed by violaxanthin (V), anteraxanthin (A) and zeaxanthin (Z). Antheraxanthin and zeaxanthin are obtained by the reversible de-epoxidation of violaxanthin, under high irradiance, and are responsible for photosynthetic down-regulation, due to heat dissipation (Ralph *et al.*, 2002). A range of factors can influence the degree of violaxanthin de-epoxidation, like temperature, light quality and water stress (Eskling and Akerlund, 1998).

The recently observed effect of global warming on seagrass mortality, especially in Mediterranean species (Marbà and Duarte, 2010), highlight the importance of investigating the physiological responses of seagrasses to heat stress, particularly under high light levels that contribute to the production of ROS. *Cymodocea nodosa* is a Mediterranean subtidal species that spreads into the Atlantic to the Canary and Madeira Islands (Cancemi *et al.*, 2002). This species has its Northern limit in the coast of Portugal. In the Ria Formosa coastal lagoon in the south coast of Portugal, the species develops in shallow areas being exposed at high spring tides. Particularly in spring and summer, high surface irradiances are coincident with higher temperatures at low tide, creating physiologically demanding conditions for seagrasses. The combined effect of light and temperature is known to have the potential to magnify stress reactions, when compared with isolated effects. Consequently, experimental approaches have to consider the synergistic effect of both variables.

In this study we evaluated the photosynthetic and antioxidant responses of *Cymodocea nodosa* to the combined effect of light and temperature, using potentially stressful ranges of both parameters. By covering pigment responses, antioxidant activity, antioxidant capacity (trough different pathways), potential quantum yield of PSII and soluble sugars variations, our goal was to provide a comprehensive assessment of how this species reacts to the imposed stress.

Materials and methods

Sampling site and biological material

Cymodocea nodosa plants were collected in May and June 2011 in Ria Formosa coastal lagoon (South Portugal: 37°N, 008°W), where this species forms shallow subtidal meadows, with about 3m depth. Water temperature varies seasonally between 14 and 22ºC (unpublished data). Photosynthetically active radiation (PAR), in Ria Formosa, varies seasonally between 115 and 578$\mu mol_{quanta} m^{-2} s^{-1}$ (Cabaço *et al.*, 2009). Plants were harvested and immediately re-planted in glass aquariums, using sterilized fine sand as substrate. Aquariums were filled with natural salt water and aerated continuously.

Experimental design

Plants were submitted to the combined effects of light (10, 150 and 450 $\mu mol_{quanta} m^{-2} s\text{-}^{1}$) and temperature (10ºC, 20ºC and 40ºC) (n=3 per treatment) in a factorial four-day experiment. Different light levels were accomplished by shading aquariums with mesh-lined PVC structures. Temperature was set and maintained via the plant growth-chamber controller (Aralab 1000 Thin), and each temperature was tested separately. Plant samples for biochemical analysis were collected at the end of the experiment. Field plants at the moment of the harvest were also collected for biochemical analysis.

After sampling, plant leaves were separated, cleaned and immediately frozen in liquid nitrogen and stored in -80ºC prior to biochemical analysis. Simultaneously to plant sampling and in the middle of the experiment (2nd day), chlorophyll a fluorescence measurements were carried out for each treatment.

Photosynthetic potential and pigments

Measurements of maximum potential quantum yield of PSII (Fv/Fm) were performed using a pulse amplitude modulated fluorometer (Diving-PAM, Heinz Walz, Effeltrich, Germany). Plants were dark-adapted for 30 min., previously to fluorescence measurements.

Frozen leaf tissue (200 mg) was powdered with liquid nitrogen and sodium ascorbate and then extracted with 5 mL of acetone 100% and $NaHCO_3$ (Abadía and Abadía, 1993). Extracts were filtered with 5.0 µm LS membrane filters and then by PTFE 0.2 µm hydrophobic ones. Extractions were performed under a low light environment. Chlorophyll a and b were quantified spectrophotometrically, using the equations of Lichtenthaler and Buschmann (2001) while carotenoids were analysed by isocratic high performance liquid chromatography (HPLC).

HPLC analysis of extracts and standards (20 µL) were carried out in an Alliance Waters 2695 separation module, with a Waters 2996 photodiode array detector and a Waters Novapak C18 radial 8x100 mm compression column (4 µm particle size). During injection period, extracts were maintained at 5°C. The mobile phase was pumped at a 1.7 mL flow. The mobile phase A, acetonitrile: methanol:Triethylamine (TEA) (7.5:1:0.7), was fluxed trough the column, in a isocratic 3.5min step, followed by mobile phase B, acetonitrile:methanol:mili-q water:ethyl acetate (7:0.96:0.04:8) in a 6.5min isocratic step. Between injections, column was equilibrated with mobile phase A for 5 min. All eluents were prepared with HPLC grade solvents (VWR Hipersolv Chromanorm), filtered and sonicated prior to use. During all chromatographic analysis, column was kept in a steady temperature (24°C). Calibration was done with commercially available pigments (CaroteNature, Lupsingen, Switzerland).

Peak areas were monitored at 450nm. Concentrations were calculated based on peak areas from standards in known concentrations and the absorbance measured in a spectrophotometer (Beckman coulter DU 650 spectrophotometer). For calibration curves, all standard dilutions were injected eight times for each pigment.

Malondialdehyde (MDA)

For MDA extraction, about 300 mg of frozen leaf tissue were powdered in liquid nitrogen and suspended in 80% aqueous ethanol. Extracts were centrifuged at 3000xg for 10 min.

Quantification was performed according to Hodges *et al*. (1999). The supernatant was added to a solution of 20% trichloroacetic acid (TCA) with 0.65% thiobarbituric acid (TBA) and 0.015% butylated hydroxytoluene (BHT). Two blanks were made either without TBA or with 80% ethanol instead of sample extract. All samples and blanks reaction mixtures were incubated at 90°C for 25 min., then cooled for 15 min. and again centrifuged. Absorbances of the supernatants were read at 440 nm, 532 nm, 600 nm using a Beckman Coulter DU-650 spectrophotometer and MDA equivalents were calculated as in Hodges *et al*. (1999).

Antioxidant capacity

Extraction for oxygen reactive absorbance capacity (ORAC), and trolox equivalents antioxidant capacity (TEAC) was performed simultaneously, frozen leaf samples (ca. 300mg), were powdered in liquid nitrogen and suspended in 0.1 N hydrochloric acid (HCl), and kept under constant agitation at 4°C, overnight, following centrifugation at 4700xg for 30min. Quantifications were performed in the supernatant.

ORAC was quantified according to Huang *et al.* (2002). 75mM phosphate buffer and 8.16 x10^{-5}mM fluorescein (Sigma) were added to the extract. This mixture was heated at 37ºC and read in a Synergy ™ 4 multi-detection microplate reader with a 485 nm, 20 nm bandpass, excitation filter and a 528 nm, 20 nm bandpass, emission filter. Reaction was initiated by the addition of 153 mM ABAP (2,2′-azobis (2-methylpropionamidine) dihydrochloride) (Sigma). Results were expressed as Trolox® equivalent (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid).

For TEAC quantification, ABTS$^{\bullet+}$ was produced by the reaction of 7 mM ABTS with potassium persulphate in a final concentration of 2.45 mM according with Re *et al.* (1999). Diluted ABTS$^{\bullet+}$ (A_{734nm}= 0.800± 0.020) was added to the extract and, after an incubation period of 6 min., absorbance was read at 734 nm in a Beckman Coulter DU-650 spectrophotometer, against a blank sample. Results were expressed as Trolox® equivalents (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid).

Ascorbate peroxidase (APX)

800 mg of frozen leaf tissue was powdered with liquid nitrogen with polyvinyl polypyrrolidone (PVPP) and sodium ascorbate and then extracted in 5 mL of 100 mM

potassium phosphate buffer (pH 7.8) with 2% triton-x and 10 mM ascorbate. Extracts were centrifuged at 4°C, 3500xg for 30 min. Supernatant was purified by filtration with sephadex PD-10 G-25 columns (GE Healtcare) (Polle *et al.*, 1993), previously equilibrated with 20 mL of 100 mM potassium phosphate buffer (pH 7.0) with 1 mM ascorbate. APX activity was measured at 25°C, by following for 3 min. the decrease in absorbance at 290 nm of a mixture containing 50 mM potassium phosphate buffer (pH 7.0), 8 mM ascorbate and 20 mM oxygen peroxide (adapted from Polle and Morawe, (1995). APX activity was calculated after subtraction of the control rates, where the enzyme extract was replaced by the potassium phosphate buffer, using $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$. One unit (U) of APX is equivalent to the protein necessary to oxidize 1 µmol of ascorbate per min. Enzyme activity was expressed in U mg-1 soluble protein.

Soluble protein

The purified APX extract was also used to determine the soluble protein concentration, by a dye binding assay (Coomassie Brilliant Blue G-250 dye) (Bradford, 1976), against a Bovine Serum Albumin standard (BioRad).

Soluble sugars

Dried leaf tissue (10 mg) were powdered on a ball mill (RETSCH MM 300, Grainner), extracted in 80% ethanol at 80°C for 10 min. and centrifuged at 2000 g for 5min. (adapted from Burke *et al.*, 1996). The supernatant was collected and the pellet was ressuspended in ethanol for additional extraction. This procedure was repeated three consecutive times. The supernatants from the three-step extraction were mixed together and the amount of soluble sugars was determined by a phenol-sulphuric assay (adapted from Dubois *et al.*, 1956) using glucose standards.

Statistical analysis

Statistical analysis were performed with the SIGMA-PLOT V.11 statistical software package (Systat software, Inc, 2008). Two-way ANOVAs were performed to test significant differences ($p<0.05$). Significant differences between individual means were tested with the Student- Newman-Keuls Post-Hoc test.

Results

Photosynthetic potential and pigments

Light and temperature had a significant effect on the potential quantum yield of PSII (Fv/Fm) (Fig. 4.1., Tab. 4.1.). Fv/Fm decreased with high light at all temperatures, particularly at the lowest one (10°C). The combination of the lowest temperature and the highest light level represented a highly stressful condition for *C. nodosa* photosynthesis, as revealed by the level of Fv/Fm that decreased to values of 0.26 after 4 days. The continuous decrease of the values of maximum potential yield of photosynthesis under these conditions, indicate that if conditions do not improve the plants will be on the verge of survival.

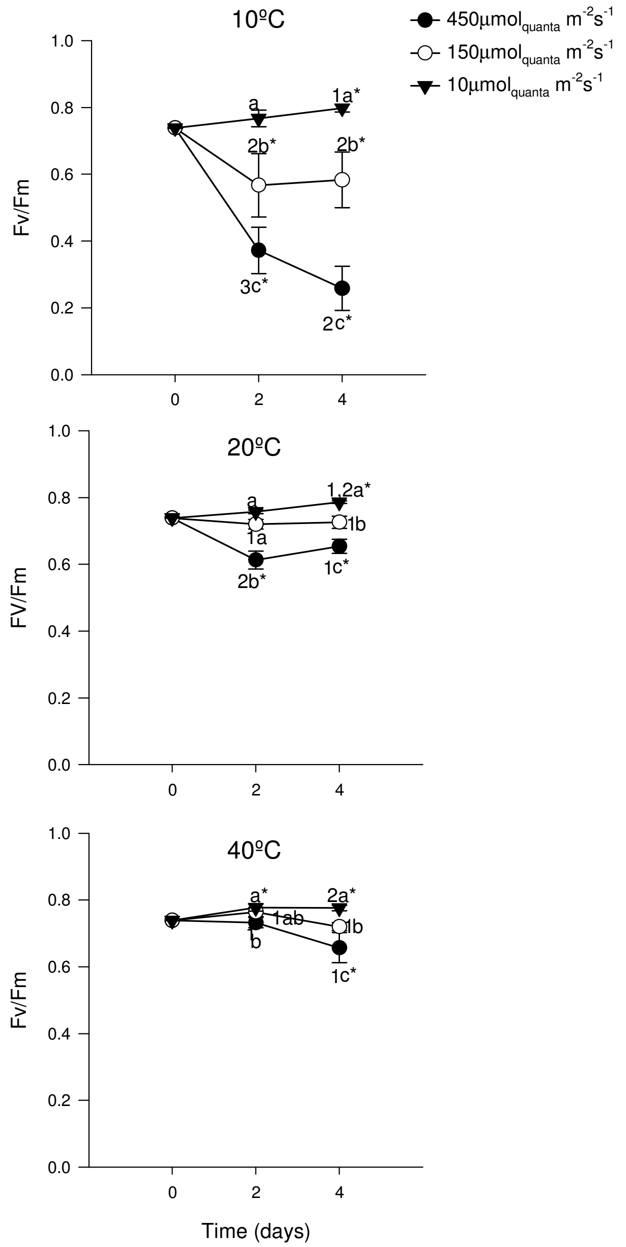

Figure 4.1. – *Cymodocea nodosa* potential quantum yield of PSII (Fv/Fm) in plant leaves under field conditions (field) and submitted to combined temperatures (10ºC, 20 ºC and 40ºC) and light levels (10, 150 and 450µmol$_{quanta}$ m^{-2}s^{-1}). Different letters indicate significant differences between light levels under the same temperature, different numbers indicate significant differences between temperatures under the same light level (respectively at day two and at day four); * indicates significant differences between the days (n=3, $p<0.05$).

Table 4.1.- Significance values of the effects of temperature and light on fluorescence (Fv/Fm), de-epoxidased pigments xanthophyll cycle pigments ratio (AZ/VAZ), chlorophyll a :chlorophyll b ratio (chla/chlb), malondialdehyde concentrations (MDA), oxygen absorbance capacity (ORAC), trolox equivalents antioxidant capacity (TEAC), ascorbate peroxidase (APX), and soluble sugars in *Cymodocea nodosa* leaves, tested with two-way ANOVAs at the end of the experiment (day four). Significant effects are in bold.

Variable	**Temperature (T)**	**Light (L)**	**Interactions (T x L)**
Fv/Fm	**$P < 0.001$**	**$P < 0.001$**	**$P < 0.001$**
AZ/VAZ	**$P < 0.001$**	**$P < 0.001$**	**$P < 0.001$**
Chla/Chlb	**$P < 0.001$**	**$P < 0.05$**	**$P = 0.101$**
MDA	$P = 0.245$	$P = 0.925$	$P = 0.217$
ORAC	**$P = 0.05$**	$P = 0.385$	$P = 0.393$
TEAC	**$P < 0.001$**	$P = 0.147$	$P = 0.146$
APX	$P = 0.240$	**$P < 0.05$**	$P = 0.241$
Soluble sugars	**$P < 0.001$**	**$P = 0.05$**	**$P < 0.05$**

The violaxanthin de-epoxidation ratio of *C. nodosa* increased with light under all temperatures as expected, except at 10º C under the highest light levels of 450 µmol$_{quanta}$ m^{-2}s^{-1} where it decreased sharply. Interestingly, the synergistic effects of low temperature and high light also decreased the maximum potential quantum yield of photosynthesis to lowest values (Fig. 4.2.).

Temperature had a stronger impact than light intensity in the chlorophyll a/b ratio (chla/chlb) of *C. nodosa* leaves (Fig. 4.2. B, Tab. 4.1.). In fact, the chla/chlb ratio at 40°C was about 30% lower than at 10°C in under all light levels. Chla/chlb ratio was only influenced by the light level in leaves submitted to 20°C. In this situation, plants under 150 µmol$_{quanta}$ m^{-2}s^{-1} presented significantly higher chla/chlb than plants under 10 µmol$_{quanta}$ m^{-2}s^{-1}.

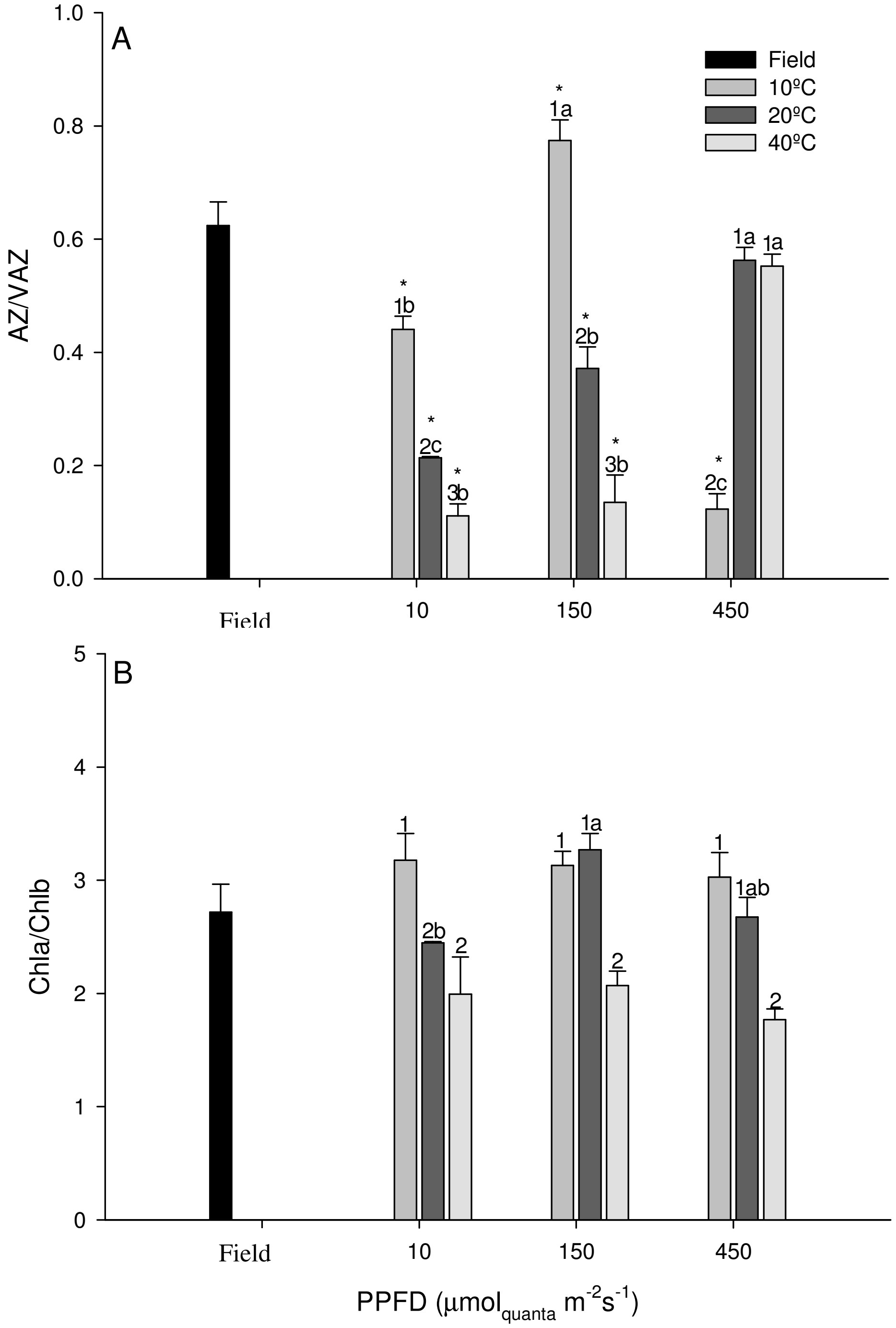

Figure 4.2. – *Cymodocea nodosa* de-epoxidased pigments xanthophyll cycle pigments ratio (AZ/VAZ) (A) and chlorophyll a :chlorophyll b ratio (B) in plant leaves under field conditions (field) and submitted to combined temperatures (10ºC, 20 ºC and 40ºC) and light levels (10, 150 and 450µmol$_{quanta}$ m^{-2}s^{-1}). Different letters indicate significant differences between light levels under the same temperature at day four, different numbers indicate significant differences between temperatures under the same light level at day for; * indicates significant differences between field and treatments (n=3, p<0.05).

Malondialdehyde (MDA) and antioxidant response

Light and temperature did not significantly affect the lipid peroxidation (MDA), and the radical absorbance capacity (ORAC) of *C. nodosa* (Fig. 4.3 B, A, Tab. 4.1). As well, there were no significant differences between plants under field and experimental conditions.

The antioxidant capacity (TEAC) of *C. nodosa* did not vary with light but generally decrease with temperature (Fig. 4.3.C, Tab. 4.1.). At 20ºC, the antioxidant capacity was similar to field samples, regardless of the light level.

Temperature had a significant effect in ascorbate peroxidase activity (APX), which was higher at 40ºC at all light levels and also relatively to the field samples (Fig 4.3. D, Tab. 4.1.). Although there were some differences among light levels on plants at 10°C and 20°C, there was no clear pattern of these effects on APX activity.

Soluble sugars

Leaf soluble sugars generally increased significantly with light (Fig. 4.4., Tab. 4.1.), except at 20°C where the opposite trend was observed. At 20°C and 450 μmol_{quanta} $m^{-2}s^{-1}$, as well as under all light intensities at 40°C, leaf soluble sugars decreased significantly when compared to field plants.

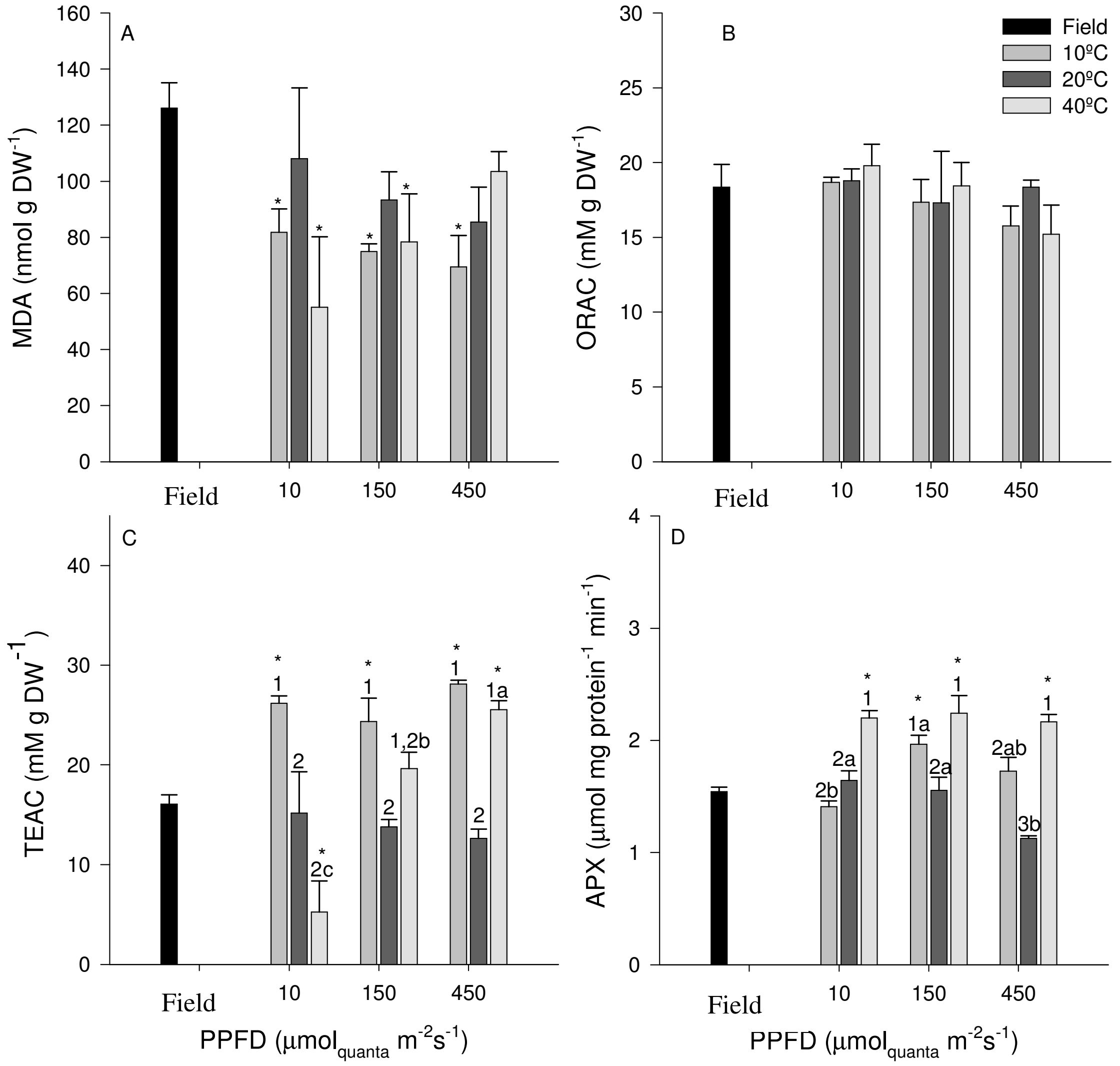

Figure 4.3. – *Cymodocea nodosa* Malondialdehyde (MDA) concentration (A), Oxygen Reactive Absorbance Capacity (ORAC) concentrations (B), Antioxidant capacity (TEAC assay) (C) and Ascorbate peroxidase (APX) (D) activity in plant leaves under field conditions (field) submitted to combined temperatures (10ºC, 20 ºC and 40ºC) and light levels (10, 150 and 450µmol$_{quanta}$ m^{-2}s^{-1}). Different letters indicate significant differences between light levels under the same temperature at day four, different numbers indicate significant differences between temperatures under the same light level at day for; * indicates significant differences between field and treatments (n=3, $p<0.05$).

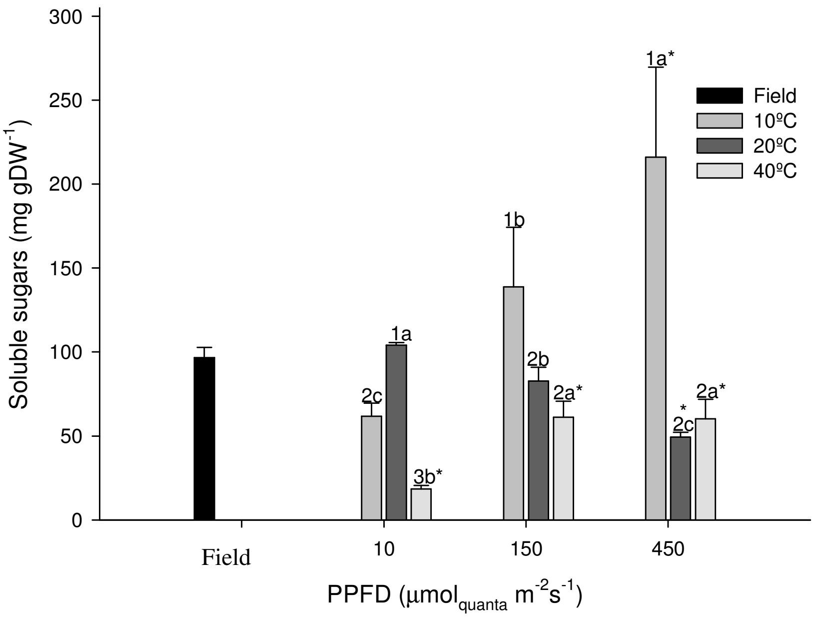

Figure 4.4. – *Cymodocea nodosa* soluble sugar concentrations in plant leaves under field conditions (field) and submitted to combined temperatures (10ºC, 20 ºC and 40ºC) and light levels (10, 150 and 450µmol$_{quanta}$ m^{-2}s^{-1}). Different letters indicate significant differences between light levels under the same temperature at day four, different numbers indicate significant differences between temperatures under the same light level at day for; * indicates significant differences between field and treatments (n=3, p<0.05).

Discussion

Cymodocea nodosa reacted to the potentially stressful conditions with changes in pigments, antioxidant system and soluble sugar content. Due to this response capacity, the experimental conditions did not cause oxidative stress to the plants, but low temperature, particularly in combination with high light appears to be specially demanding for *C. nodosa*.

The AZ/VAZ ratio of *C. nodosa* increased with light and temperature indicated the need to dissipate energy generated by the combination of high light and the increase in temperature (Fig. 4.2.A). This is in agreement with Chartrand *et al.* (2012), which also showed the increase of violaxanthin-de-epoxidised pigments in plants exposed to high light levels. At 10ºC at the highest light level of 450 µmol$_{quanta}$ m^{-2}s^{-1} the AZ/VAZ ratio decreased significantly revealing a synergistic effect of low temperatures and high light intensities. This was also discribed by Huner *et al.* (1998) in both terrestrial plants and algae. In natural conditions, the plant responses to light have a seasonal variation (Fitzpatrick and Kirkman,

1995) that depends also on the interaction with other variables such as temperature. Under lower temperatures and high light intensities, the de-epoxidation of violaxanthin to zeaxanthin can be greatly retarded (Demmig-Adams *et al.*, 1989; Zhang *et al.*, 2011), probably caused by the decrease in the activity of the zeaxanthin-de-epoxidase enzyme under low temperatures (Zhang *et al.*, 2011). Under these conditions (10ºC and 450 μmol_{quanta} $m^{-2}s^{-1}$), we could not quantify the content zeaxanthin in *C. nodosa* as it was below the detection levels. This xanthophyll is known to have a photoprotective effect against the excess of energy in PSII (Xu *et al.*, 2000) and its absence can be connected with phoinhibition, photodamage and the consequent decrease in photochemical efficiency of PSII (Fv/Fm) (Demmig-Adams *et al.*, 1989). This is corroborated by the extremely low Fv/Fm values under the same conditions (10ºC and 450 μmol_{quanta} $m^{-2}s^{-1}$), a clear indication of photoinhibition (Silva and Santos, 2003).

The chla/chlb ratio in *C. nodosa* plants under field conditions was 2.7 (Fig. 4.2.B). In land plants this ratio is usually around 3.0, but shade-adapted plants can show values varying between 2.4 and 2.7 (Lichtenthaler, 1987; Lichtenthaler and Burkart, 1999). In *C. nodosa*, Zavodnik *et al.* (1998) indicated a strong seasonal variation of this ratio from about 2.5 between spring and autumn to 1.5 during winter, which suggests a decrease under both low light and temperature. In *Z. marina*, a chla/chlb ratio of 2.0 was not affected by temperatures between 10ºC and 30ºC (Niu *et al.*, 2012). Our results showed no alteration of the chla/chlb ratio with light, but rather a marked tendency for the ratio to be higher at 10ºC (about 3.1). At 40ºC, the chla/chlb ratio decreased (about 1.9) and the plants invested proportionally more in chlorophyll b production than in chlorophyll a (data not shown), which contributed to the lower chla/chlb ratio, suggesting that the biosynthesis of chlorophyll b was stimulated by the temperature increase. Chlorophylls are the predominant pigments in LHCII (Aarti *et al.*, 2006) and there are some evidences of the role of chlorophyll b in the size and stability of LHCII (Eggink *et al.*, 2004). Aienl *et al.* (2011) proposed for a terrestrial species the use of chla/chlb ratio as an indicator of species tolerance, indicating that a decrease in the chla/chlb ratio under high temperatures could be closed associated with species susceptibility to those conditions. On the other hand, the increase of chla/chlb ratio under low temperatures can be a possible strategic way to dissipate the energy that can damage the photosynthetic system. In fact, the importance of the reaction center and LHCII in energy dissipation has been proposed as complement, when other processes are thermodynamically restricted due to cold temperatures (Bravo *et al.*, 2007).

C. nodosa antioxidant capacity was evaluated through ORAC and TEAC assays (Fig. 3.B, C) and through APX activity (Fig. 3.D). Concerning oxygen radical absorbance capacity (ORAC), there were no differences between light levels, temperatures or even between experimental and field conditions. Nonetheless, antioxidant capacity results (TEAC assay) indicate an increment on the ability to scavenge radicals in leaves under low temperatures and at high temperature, when combined with high irradiance. An increase in total antioxidant capacity (TEAC assay) during cold acclimation was reported for turfgrass, a land species (Sarkar *et al.*, 2009).

Ascorbate peroxidase (APX) activity was significantly higher in *C. nodosa* leaves submitted to 40ºC, independently of the light level, and at 10ºC under moderate or high light intensity. This might indicate the ability of the system to detoxify ROS potentially formed under those experimental conditions (Cui, 2006; Reusch *et al.*, 2008). Published studies showed that under stressful conditions an increase in APX concentration is expected (Shigeoka *et al.*, 2002). APX has the role to convert hydrogen peroxide (H_2O_2) in water (Cavernan *et al.*, 2012). H_2O_2 is a well-known ROS, produced under conditions of stress, such as extreme temperatures (Mittler *et al.*, 2004; Scandalios, 2005) or high light (Mittler, 2002). The increase of that specific ROS works as a stress signal that triggers the increase of APX concentration (Morita *et al.*, 1999). Kornyeyev *et al.* (2003) also verified the increased of APX concentration in cotton leaves under the combined effect of low temperature and high light, connecting the increase in enzymatic overexpression with the increase of H_2O_2 under those conditions. Spinach also showed an increase in APX concentration as response to high light stress (Yoshiura *et al.,* 2000).

Malodialdehyde (MDA) is a product of lipid peroxidation of the membranes, and so, it is used as a stress indicator (Hodges *et al.,* 1999). The fact that there were no differences in *C. nodosa* MDA between leaves at distinct light levels, indicates that the protective mechanisms of the species acted efficiently in order to prevent stress damage (Fig. 3.A). MDA concentrations in leaves under extreme temperatures and 10 and 150$\mu mol_{quanta} m^{-2}s^{-1}$ and at 40ºC and 450μmol_{quanta} $m^{-2}s^{-1}$ were even lower than in leaves under field conditions, which can be a reflex of all the protective mechanisms that were activated under the experimental conditions.

Soluble sugar concentration increased with the combination of the lower temperature and the higher light level (10ºC and 450 μmol_{quanta} $m^{-2}s^{-1}$). This might reflect the well-known increment in soluble sugars under low temperatures (Guy *et al.*, 1992) that is believed to protect plant tissues from damage, by acting as osmo-protectants during cold acclimation (Yuanyuan *et al.*, 2009)

The results from this work suggest that *Cymodocea nodosa* is highly tolerant to thermal and light stresses, due to effective physiological responses under adverse conditions. The most stressful condition for the species was low temperature, especially when combined with the high light intensity. Under these conditions, the photoproctective mechanism of violoxanthin de-epoxidation was not triggered and Fv/Fm values clearly indicated photoinhibition. This is a potentially damaging situation that was probably attenuated by an increase of the antioxidant activity (APX activity), of the antioxidant capacity (TEAC assay), of soluble sugars and of chlorophyll a/b ratio in order to scavenge oxidative species thus protecting the system and avoiding damage. This reveals that *C.nodosa* photoprotection mechanisms are well prepared to deal with severe conditions, preventing damages of the photosynthetic system that could compromise the plants performance. Therefore under climate change predictions indicating a decline in light reaching the meadows and the increase in temperature, *C. nodosa* will be able to not only survive but to well adapt to new conditions.

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Chapter 5

Photosynthetic pigment composition and antioxidant responses of *Posidonia oceanica* plants under contrasting epiphyte loads

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(To be submitted)

Photosynthetic pigment composition and antioxidant responses of *Posidonia oceanica* plants under contrasting epiphyte loads

Abstract

Epiphytes pose physical barriers to light penetration into seagrass leaves affecting photosynthesis but also constitute aggressions that may increase the production of oxygen reactive species (ROS) levels potentially leading to oxidative stress. Here we compare the photosynthetic rates, the energetic balances, the composition and balance of photosynthetic pigments and the antioxidant responses and oxidative stress of leaves of the seagrass *Posidonia oceanica* with contrasting epiphyte loads developing at identical depths and light exposures in Cabo de Gata Natural Park, southern Spain. In general, high-epiphyted leaves showed lower net photosynthetic rates and chlorophyll a/b ratios than low-epiphyted leaves, indicating a negative effect of epiphytes on photosynthesis and light harvesting. Under high solar noon irradiance the xanthophyll cycle was activated in both high- and low-epiphyted leaves but the de-epoxidation-ratio (AZ/VAZ) was lower in high-epiphyted leaves. Even though these plants were under a lower light stress, they showed higher oxidative stress as revealed by the increase of the antioxidant capacity (TEAC and ORAC assays) and by the higher activity of the antioxidant enzymes ascorbate peroxidase and dehydroascorbate. These results suggest that despite their protection role against excessive light, leaf colonization by epiphytes reduces plant productivity probably because of the induction of oxidative stress, which will demand the allocation of energetic resources to anti-oxidant mechanisms.

Introduction

Seagrass epiphytes are a complex community composed by plants, bacteria, micro and macroalgae, heterotrophic organisms and organic and inorganic detritus (Brush and Nixon, 2002) which use leaves as a physical support structure.

Associations between epiphytes and hosts can involve organism's excretion of polymeric substances, hydrogen bonding, hydrophobic and polar group interactions, coordination with metals and cations, steric interferences, specific reactions between substrate and surface functional groups and surface appendages (Michael *et al.*, 2008). Algae epiphytes can either attach to the outer layers of the host (holo-epiphytes) or can anchor deeply in the host tissue (amphi-epiphytes) (Ducker and Knox, 1984) in a parasitic relationship responsible by host cellular reactions to infections (Goff and Coleman, 1984). Non-parasitic settlement of epiphytes may require the formation of a bacterial biofilm in the initial state of biofouling to facilitate attachment (Michael and Smith, 1995; Shikuma and Hadfield, 2006; Prabhakaran *et al.*, 2012) as a part of specific reactions (Zardus *et al.*, 2008).

The most commonly documented effect of epiphytes on seagrasses is the creation of a physical barrier causing shading and light attenuation (Van Montfrans *et al.*, 1984; Brush and Nixon, 2002; Aho and Beck, 2011). The cover of epiphytes can have a positive effect on intertidal seagrasses by reducing desiccation under low tide and protecting plants against UV radiation (Aho and Beck, 2011). However it can also be detrimental, mostly to subtidal seagrasses, by having a negative impact on photosynthesis (Aho and Beck, 2011). In fact, Oh *et al.* (2009) reported the light interception and the consequent decrease in photosynthesis from epiphytes in *Zostera marina* leaves.

Apart from the shading effect, epiphyted marine angiosperms can also experience reduction on nutrients availability (Frankovich and Fourqurean 1997; Sureda *et al.*, 2008), decrease in the CO_2 diffusion rate (Michael *et al.*, 2008; Sureda *et al.*, 2008), decline in phosphorous assimilation and diurnal changes in pH and oxygen content, due to the barrier caused by epiphytes (van Montfrans *et al.*, 1984). The presence of epiphytes can also be responsible for leaf chlorosis and necrosis (Ballesteros *et al.*, 2007), wounds formed when receptacles shed (Longtin and Scrosati, 2009) and sloughing of the surface layer (Duarte, 1989). It is also known to be one of the consequences of wounding in land plants, the production of considerable amounts of oxygen reactive species (ROS) (Wojtaszek, 1997). This is commonly known as oxidative burst and the amount of ROS produced can easily surpass the plants antioxidant capacity and induce oxidative stress (Wojtaszek, 1997). On the other hand, the ROS upsurge itself can act as a protection mechanism either by being directly toxic to epiphytes (Wojtaszek, 1997) or by signalling and/or triggering other defence reactions (Wojtaszek, 1997; Sureda *et al.*, 2008).

Marine plants developed defence strategies that influence leaf colonization by epiphytes and their distribution on the leaf surface (Michael *et al.,* 2008). Among the pre- and post-settlement defence strategies are the production of anti-fouling compounds (plant secondary metabolites), some of them toxic and therefore deterrent to epiphytes, others leading to changes in wettability (Walters *et al.*, 2003) or even by having antibacterial activity against biofilm production (Marhaeni *et al.*, 2010). Plant flexibility also plays an important role in trying to maintain leaves clean, by the dislodgement of epiphytes as the water agitates the leaves (Walters *et al.*, 2003), and the increase of the leaf turnover and renewal can also reduce the permanency of epiphytes on plants (Orbita and Mukai, 2009). In general, the presence of epiphytes is demanding and stressful to plants inducing antioxidant defence (Sureda *et al.*, 2008).

Under a moderate stress level, ROS increase triggers acclimation responses such as the modulation of gene expression, the upregulation of the antioxidant system and the downregulation of the photosystem II turnover rate in order to decrease the production of reactive species and protect the cell apparatus (Karuppanapandian *et al.*, 2011), especially the photosynthetic system (Foyer and Shigeoka, 2011). Under high stress conditions ROS concentration increases to levels higher than those that the plant antioxidant system is able to compensate, leading to the unbalance between ROS production and antioxidant capacity (Wojtaszek, 1997), a situation known as oxidative stress (Wojtaszek, 1997; Blokhina *et al.*, 2003; Sureda *et al.*, 2008).

Malondialdehyde is formed during oxidative degradation of some macromolecules and peroxidation of lipids. Its formation is associated with strong oxidative stress conditions and can be responsible by cell damage (Valenzuela, 1991).

The antioxidant system comprises several different ways of combat, namely enzymatic and non-enzymatic antioxidants, and anatomic changes (Mitller, 2002). It is vital for plant survival under stressful conditions that all antioxidant forms operate in an inter-balanced form

(Blokhina *et al.,* 2003). Enzymatic antioxidants are mainly composed by superoxide dismutases, peroxidases, catalases and enzymes that oxidise or reduce ascorbate (Dabrowska *et al.*, 2007). Non-enzymatic antioxidants include tocopherols, phenolic compounds, ascorbic acid and carotenoids. The antioxidant activity of carotenoids includes their ability to quench the oxygen singlet (Telfer, 2005), modifications in photosynthetic pigments (Yamamoto and Bassi, 1996) or induced by them (Namitha and Negi, 2010) and also their ability to trap peroxyl radicals (Stahl and Sies, 1996). The carotenoids quenching activity can also prevent lipid peroxidation (Stahl and Sies, 1996). Besides the antioxidant capacity, carotenoids can also dissipate the excess of energy as heat, through activation xanthophyll (VAZ) cycle (Gilmore *et al.*, 1995). Light induces the de-epoxidation of violaxanthin (V) into antheraxanthin (A) and zeaxanthin (Z) and energy is dissipated as heat by the two de-epoxidased pigments (Gilmore *et al.*, 1995; Yamamoto and Bassi, 1996).

Since different antioxidants have distinct mechanisms of action, it is important to use methods that cover the possible antioxidant pathways to properly evaluate the antioxidant capacity. According to Huang *et al.* (2005), the methods used to evaluate the antioxidant scavenging capacity can be divided in two groups: assays based in hydrogen atoms transfer, like oxygen reactive absorbance capacity (ORAC) and assays based on electron transfer, like trolox equivalents antioxidant capacity (TEAC), phenolic compounds, superoxide dismutase, and ascorbate peroxidase.

Posidonia oceanica (L.) Delile, is a seagrass growing in coastal Mediterranean waters (Duarte 1991), characterized by its slow growth and long-living (Arnaud-Haond *et al.*, 2012). It is a species declared by the European Habitats Directive (92/43/CEE) as a habitat of priority interest (Boudouresque, 2004). The leaves from this species are frequently colonized by a distinct epiphytic fauna and flora (Gobert, 2002). Epiphytes found on *P. oceanica* leaves include many protists, foraminifera, diatoms, cyanobacteria and bacteria (Gobert, 2002). Specifically in terms of flora, encrusting coralline algae can be found as early colonizers (Gobert, 2002). *P. oceanica* sensitiveness to light limitation can produce a stress reaction leading to reduced growth rates, smaller shoot sizes or even meadow declines (Ruiz and Romero, 2003).

As described above, it is well established that epiphytes cause light attenuation in seagrass leaves and induce an enzymatic antioxidant response. However, the physiological response mechanisms operating remain unclear. Here we compared the effect of epiphytes in *Posidonia* *oceanica*, namely on (i) the photosynthetic rates, (ii) the energetic balances through soluble sugar and starch content (iii), the composition and ratios of photosynthetic pigments and (iv) in the antioxidant responses through ORAC and TEAC assays and enzymatic activities and oxidative stress through MDA concentrations.

Materials and methods

Sampling design

The measurements were carried out in Cabo de Gata Natural Park (southern Spain, 36° 48' 47.337" N 2° 3' 0.875" W) where epiphyted and non-epiphyted *Posidonia oceanica* plants can be found at identical depths and light exposure. The two meadows have a different orientation, being exposed to different winds. Therefore, the plants are submitted to distinct hydrodynamics, which is the most probable cause for differences in epiphytic state. Under higher water motion in one of the sites, epiphytes colonization might be more difficult.

Epiphyted and non-epiphyted plants were sampled at pre-dawn and solar noon, in shallow meadows (4-5m depth). Leaf samples (n=5) for biochemical analysis were cleaned of epiphytes (when necessary), rinsed with distilled water, blotted dry and frozen in liquid nitrogen. Plants for photosynthetic measurements (whole shoots) were kept immersed in seawater prior to incubations.

Photosynthetic active radiation (PAR) was measured continuously along the incubations with an Odissey light sensor (Dataflow systems, New Zealand).

Net photosynthesis

Net photosynthesis was evaluated as oxygen evolution rates in closed incubation chambers. Incubations were performed at different times of the day and also at night, for dark respiration measurements. Sets of 5 chambers of epiphyted and non-epiphyted plants (one whole-shoot per chamber) were always incubated in parallel. Incubation chambers built from gas impermeable plastic were filled with ambient seawater, sealed tight and left incubating for 2-hour periods. Wave action promoted external movement of the chambers and consequent medium agitation. Oxygen evolution rate was quantified as the difference in dissolved oxygen concentration between initial and final water samples, normalised by the chamber volume and the incubated biomass. Dissolved oxygen was quantified by the Winkler method, as modified by Labasque *et al.* (2004). Sample absorbance was read at 466 nm in a Healthcare Novaspect plus visible spectrophotometer (Brea CA, USA). Oxygen concentrations were determined against potassium iodate standard solutions considering a 3:2 O_2:KIO_3 ratio.

Photosynthetic pigments

Frozen leaf tissue (200 mg) was powdered in liquid nitrogen and sodium ascorbate, and extracted under low light in 5 mL of acetone 100% neutralized with $CaCO_3$ (Abadía and Abadía, 1993). Extracts were filtrated with membrane filters 5.0 µm LS followed by hydrophobic 0.2 µm PTFE. Chlorophyll *a* and *b* were quantified spectrophotometrically (Beckman Coulter DU-650 spectrophotometer, Brea CA, USA) using the equations of Lichtenthaler and Buschmann (2001). Carotenoids were analysed by isocratic high performance liquid chromatography (HPLC) (de las Rivas *et al.*, 1989; Larbi *et al.*, 2004). HPLC calibration (de las Rivas *et al.*, 1989) was made with commercial pigments (CaroteNature, Lupsingen, Switzerland). Liquid chromatography analysis was performed in an Alliance Waters 2695 separation module (Milford MA, USA), with a Waters 2996 photodiode array detector and a Waters Novapak C18 radial 8x100 mm compression column (4 µm particle size). 20 µL of extract were injected via an auto-sampler. Samples were stored at 5ºC prior to injection and the column was kept at 24°C during chromatographic analysis. All the eluents were prepared with HPLC grade solvents (VWR Hipersolv Chromanorm), filtered and sonicated prior to use. Peak areas were monitored at 450 nm and concentrations were calculated based on peak areas obtained for standards at known concentrations.

Soluble carbohydrates and starch

Leaf soluble carbohydrates were determined in 60 mg of frozen leaf tissue, powdered in liquid nitrogen and extracted in 10 mL of 80% ethanol at 80°C for 30 min. After extraction, 5 mL of the supernatant were cleaned with activated charcoal, centrifuged at 4700xg for 8 min. (Stitt *et al.*, 1978; Stitt *et al.*, 1989) and reserved for analysis. The insoluble residue was washed in deionized water, heated at 100ºC for 10 min. and hydrolysed to glucose using amyloglucosidase and α-amylase enzymatic solutions, as described in Smith and Zeeman (2006).

Soluble carbohydrates from the first extraction and glucose resulting from starch hydrolysis were quantified by a phenol-sulphuric assay (adapted from Dubois *et al.*, 1956) using glucose standards. Results were expressed as glucose equivalents.

Malondialdehyde (MDA) quantification

MDA extraction and quantification were performed according to Hodges *et al.* (1999). 300 mg of frozen leaf tissue were powdered in liquid nitrogen and suspended in 5 mL of 80% ethanol. Each sample was divided in two reading-replicates. Each of these was obtained after homogenization and centrifugation of the extract (4°C, 3000xg, 10 min). 1.0 mL of the supernatant was added to 1.0 mL of 20% trichloroacetic acid (TCA) with 0.65% thiobarbituric acid (TBA) and 0.01% butylated hydroxytoluene (BHT) solution. Two blanks were prepared in which the sample was replaced by 80% ethanol. All the samples and blanks were first heated (90°C, 25 min.), then cooled (ice bath, 15 min.), and again centrifuged (4°C, 3000xg, 10 min.). Absorbance of the supernatants was read at 440 nm, 532 nm and 600 nm (Beckman Coulter DU-650 spectrophotometer, Brea CA, USA) and MDA equivalents were calculated as in Hodges *et al.* (1999).

Phenolic compounds, oxygen radical absorbance capacity (ORAC), and Trolox equivalent antioxidant activity (TEAC)

Frozen leaf tissue samples (ca. 300 mg), were powdered in liquid nitrogen, suspended in 0.1 N hydrochloric acid (HCl), kept overnight under constant agitation at 4°C, and then centrifuged at 4700xg for 30 min. The same supernatant was used for phenolic compounds quantification and ORAC and TEAC assays.

Phenolic compounds were quantified by the Folin-Ciocalteu method (Booker and Miller, 1998; Migliore *et al.*, 2007). 0.25 N Folin-Ciocalteu reagent and 7.5% Na_2CO_3 were added to the supernatant. Absorbance was read at 724 nm in a Beckman Coulter DU-650 spectrophotometer, against a blank. Chlorogenic acid was used as standard and the assay results were expressed as chlorogenic acid equivalents.

ORAC was quantified according to Huang *et al.* (2002). 75 mM phosphate buffer and 8.16 $x10^{-5}$ mM fluorescein were added to the extract. This mixture was heated at 37ºC and read in a Synergy TM 4 multi-detection microplate reader with a 485 nm excitation filter (20 nm bandpass) and a 528 nm emission filter (20 nm bandpass). The reaction was initiated by the addition of 153 mM ABAP (2,2′-azobis (2-methylpropionamidine) dihydrochloride). Results were expressed as Trolox® equivalents (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid).

For TEAC assay, $ABTS^{\bullet+}$ was produced by the reaction of 7 mM ABTS with potassium persulfate in a final concentration of 2.45 mM according with Re *et al.* (1999). Diluted $ABTS^{\bullet+}$ (A734 nm= 0.800 ± 0.020) was added to the extract and, after an incubation period of 6 min., absorbance was read at 734 nm in a Beckman Coulter DU-650 spectrophotometer, against a blank sample. Results were expressed as Trolox® equivalents.

Activity of antioxidant enzymes

Frozen leaf tissue samples (1 g) were powdered in liquid nitrogen, polyvinyl polypyrrolidone (PVPP) and sodium ascorbate and then extracted with 5 mL of 100 mM potassium phosphate buffer (pH 7.8) with 2% Triton-x and 10 mM ascorbate. Extracts were centrifuged at 4°C and 3500xg for 30 min. The supernatant was purified by filtration in a sephadex PD-10 G-25 column (GE Healtcare), previously equilibrated with 20 mL of 100 mM potassium phosphate buffer (pH 7.0) with 1 mM ascorbate (Polle and Morawe, 1995). The purified enzymatic extracts were used to determine the activities of the enzymes ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR), and also to determine the soluble protein content.

APX activity was measured as the decrease of absorbance at 290 nm (Beckman Coulter DU-650 spectrophotometer) of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 8 mM ascorbate, 20 mM hydrogen peroxide and 125 µl of extract. The reaction was followed during a 3 min. period at 25°C (Nakano and Asada, 1987). APX activity was calculated after subtraction of the control rates, where the enzyme extract was replaced by the potassium phosphate buffer, using $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$. One unit (U) of APX is equivalent to the protein necessary to oxidize 1 µmol of ascorbate per min. Enzyme activity was expressed in U mg^{-1} soluble protein.

DHAR activity was measured as the increase in absorbance at 265 nm of a reaction mixture containing 60 mM potassium phosphate buffer (pH 6.1), 5 mM reduced glutathione, 800 µM dehydroascorbic acid and 50 µl of extract. The reaction was followed during a 3 min period at 25ºC (Polle and Morawe, 1995). DHAR activity was calculated after subtraction of the control rates, where the enzyme extract was replaced by the potassium phosphate buffer, using $\varepsilon = 14\ mM^{-1}\ cm^{-1}$. One unit (U) of DHAR is equivalent to the protein necessary to reduce 1 µmol of dehydroascorbate per min. Enzyme activity was expressed in U mg^{-1} soluble protein.

Soluble protein was quantified by a dye-binding assay (Bradford, 1976), using a commercial reagent (BioRad Protein Assay, Hercules, California, USA). Bovine serum albumin was used as standard.

Statistical analysis

All results are presented as mean values ± standard error. Data was submitted to one- or two-way analysis of variance (ANOVA). The Student-Newman-Keuls post-hoc test was used to test for significant differences between treatments. All data treatment and statistical analysis was performed using the SigmaStat/SigmaPlot (SPSS Inc., v.11) software package.

Results

Net photosynthesis

Net photosynthetic rates followed the diel cycle of light intensity (Fig. 5.1.) and showed the same pattern on both sites by significantly increased from 13h-15h to 16h-18h, and decreased from 16h-18h to 18h-20h, still being significantly higher in 13h-15h than in 18h-20h incubations. However, except for 16-18h, when the light intensity was 768 $\mu mol_{quanta}\ m^{-2}s^{-1}$ (maximum photosynthetic rate), there were no significant differences in photosynthetic rates between epiphyted plants and non-epiphyted ones. As well, there were no significant differences in dark respiration (Fig. 5.1., 22h-24h incubation). Higher light intensities of 1086 $\mu mol_{quanta}\ m^{-2}s^{-1}$ did not corresponded to maximum photosynthetic rates.

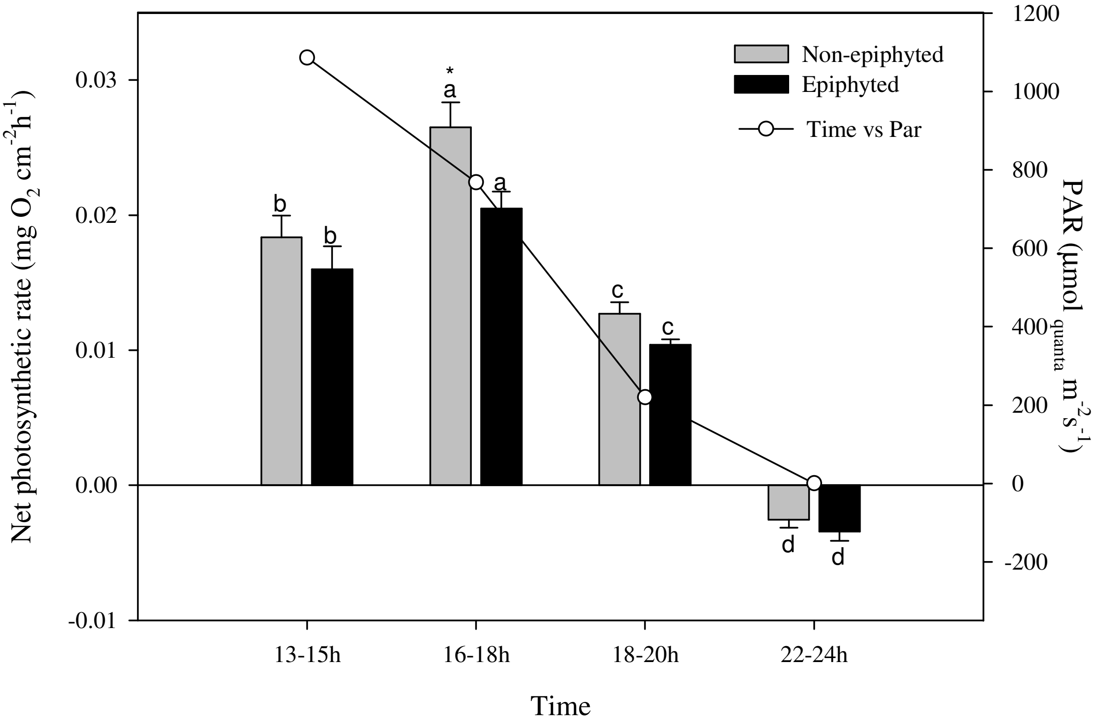

Figure 5.1. – Net photosynthetic rate of *Posidonia oceanica* along the day (local time, UTC+1). Different letters indicate significant time differences ($p<0.05$); * indicates significant differences between epiphyted and non-epiphyted shoots.

<u>Photosynthetic pigments</u>

Chlorophyll *a/b* ratio was significantly higher in non-epiphyted plants (2.3), due proportionally less chlorophyll b content, than in epiphyted ones (2.0) (Fig. 5.2. A) and was not influenced by the time of the day. The foliar concentration of the VAZ pool pigments (violaxanthin (V) + anteraxanthin (A) + zeaxanthin (Z)) was significantly higher in non-epiphyted than in epiphyted plants at pre-dawn, but no differences were detected at noon. Foliar VAZ pool didn't significantly change from pre-dawn to noon in neither epiphyted nor non-epiphyted plants (Fig. 5.2. B). Nevertheless, (A+Z)/(V+A+Z) ratio significantly increased between pre-dawn and noon in both epiphyted (1.6 fold) and non-epiphyted plants (2.6 fold) (Fig. 5.2. C). The de-epoxidation state of the VAZ-cycle pigments in epiphyted-plants at noon was almost twice than in non-epiphyted ones.

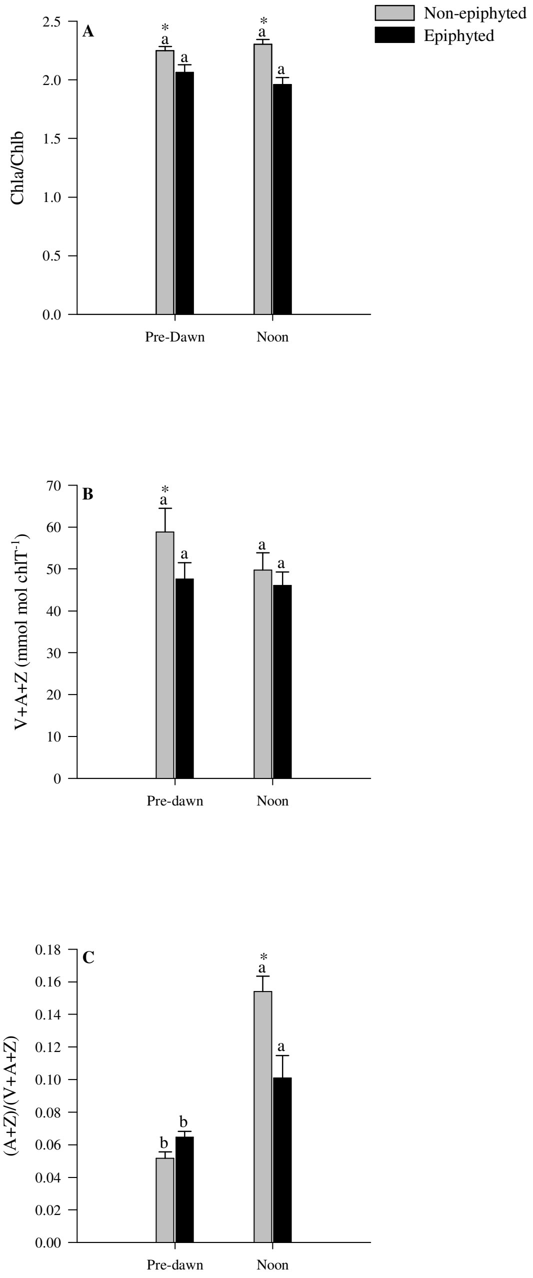

Figure 5.2. – Chlorophyll *a/b* ratio (A), xanthophyll cycle pool (violaxanthin (V) + anteraxanthin (A) + zeaxanthin (Z)) (B) and de-epoxidation index (A+Z/V+A+Z) (C) in non-epiphyted and epiphyted *Posidonia oceanica*, leaves at pre-dawn and noon. Different letters

indicate significant differences between pre-dawn and noon; * indicates significant differences between epiphyted and non-epiphyted shoots (n=5, p<0.05).

Soluble carbohydrates and starch

The soluble carbohydrates content at pre-dawn was significantly higher in epiphyted leaves while the starch content was significantly lower (Fig. 5.3.). At noon there were no significant differences between epiphyted and non-epiphyted leaves. Regarding daily variations, only non-epiphyted leaves showed significantly higher starch content at pre-dawn than at noon.

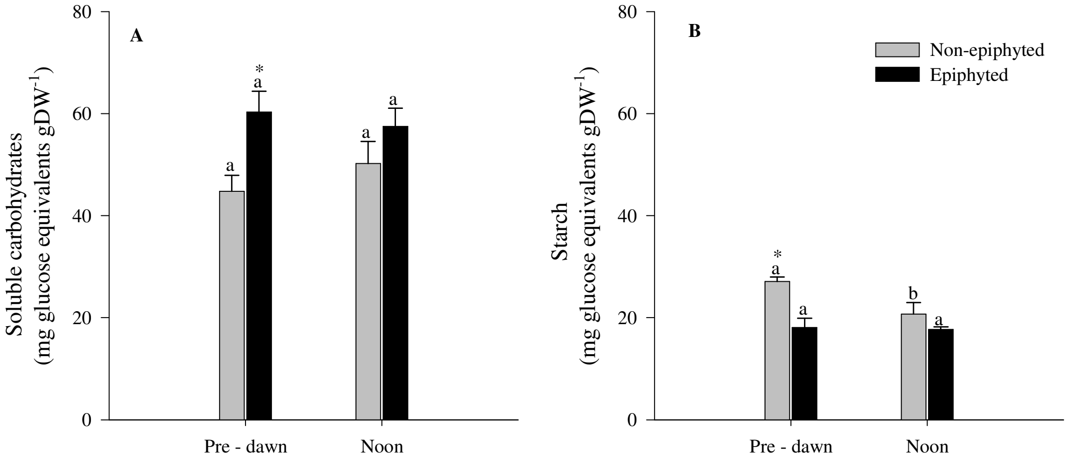

Figure 5.3. – Leaf soluble sugars (A) and starch (B) in non-epiphyted and epiphyted *Posidonia oceanica* shoots at pre-dawn and noon. Different letters indicate significant differences between pre-dawn and noon; * indicates significant differences between epiphyted and non-epiphyted shoots (n=5, p<0.05).

Malondialdehyde (MDA)

The level of peroxidation of membrane lipids (MDA), increased significantly (1.5 fold) between pre-dawn and noon in non-epiphyted leaves but remained unaltered in epiphyted plants (Fig. 5.4.). Differences between epiphyted and non-epiphyted plants were only found at pre-dawn where MDA was significantly higher in epiphyted plants (1.3 fold).

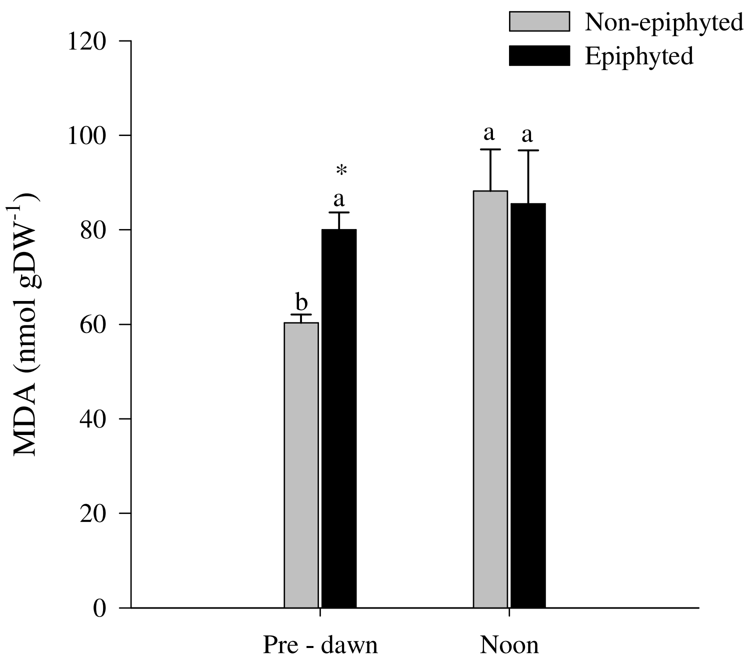

Figure 5.4. – Malondialdehyde (MDA) concentration in non-epiphyted and epiphyted *Posidonia oceanica* leaves, at pre-dawn and noon. Different letters indicate significant differences pre-dawn and noon; * indicates significant differences between epiphyted and non-epiphyted shoots (n=5, $p<0.05$).

Phenolic compounds, oxygen radical absorbance capacity (ORAC) and trolox equivalent antioxidant activity (TEAC)

The concentration of leaf phenolic compounds was the same in both epiphyted and non epiphyted plants, and was influenced by the period of the day (Fig. 5.5.). In fact, the amount of these compounds was significantly higher at pre-dawn than at noon in both plants' types.

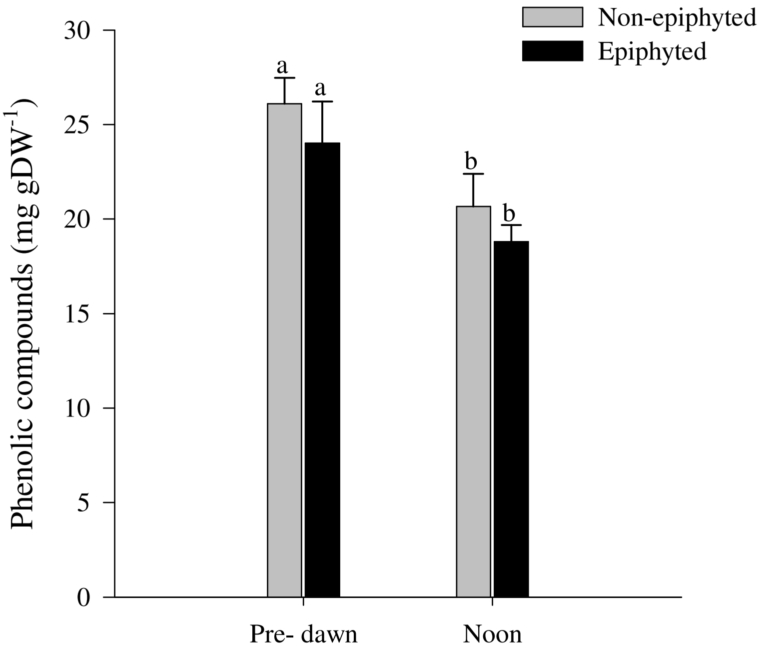

Figure 5.5. – Phenolic compounds concentration in non-epiphyted and epiphyted *Posidonia oceanica* leaves, at pre-dawn and noon. Different letters indicate significant differences pre-dawn and noon; * indicates significant differences between epiphyted and non-epiphyted shoots (n=5, $p<0.05$).

Antioxidant response was globally higher in *P. oceanica* epiphyted leaves, than in non-epiphyted ones (Fig. 5.6.). Both the antioxidant capacity (TEAC assay) and the oxygen radical absorbance capacity (ORAC) were significantly higher (1.4 to 2-fold) in epiphyted leaves, both at pre-dawn and noon (Fig. 5.6. A and 5.6. B). At pre-dawn the antioxidant capacity was significantly higher than at noon in both epiphyted and non-epiphyted plants. Nonetheless, the oxygen radical absorbance capacity was only significantly higher in epiphyted-plants at pre-dawn.

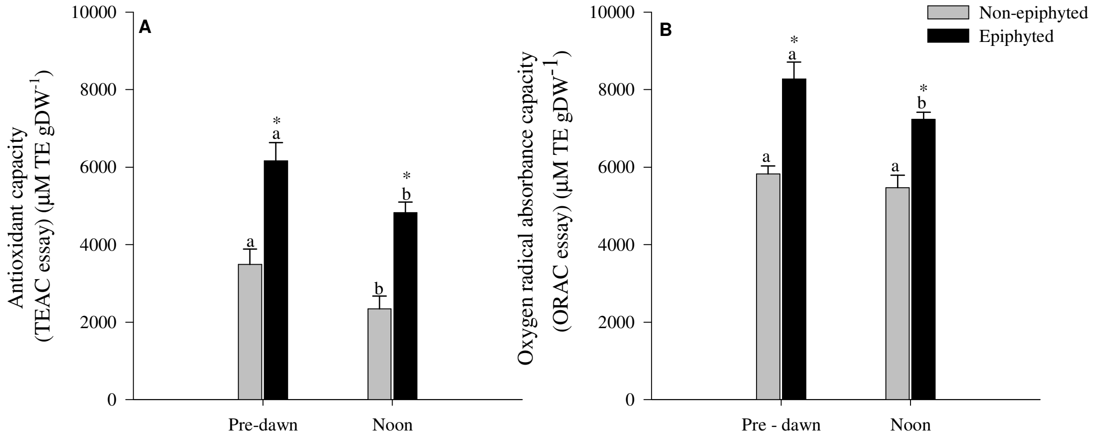

Figure 5.6. – Antioxidant capacity (TEAC) (A) and oxygen radical absorbance capacity (ORAC) (B) in non-epiphyted and epiphyted *Posidonia oceanica* plant leaves, at pre-dawn and noon. Different letters indicate significant differences between pre-dawn and noon; * indicates significant differences between epiphyted and non-epiphyted shoots (n=5, $p<0.05$).

Activity of antioxidant enzymes

Ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) activities were significantly higher in epiphyted than in non-epiphyted plants collected at noon. However, on plants collected at pre-dawn, the enzymatic activity was the same. At noon, APX activity was 1.3-fold higher in epiphyted than in non-epiphyted plants (Fig. 5.7. A) as a result of the significant increase on APX activity from pre-dawn to noon in epiphyted plants. Also at noon, DHAR activity (Fig. 5.7. B) was significantly higher in epiphyted leaves but in this case the difference resulted from a significant decrease on DHAR activity in non-epiphyted plants, as there were no changes on DHAR activity in epiphyted plants. There were no significant differences in the activity of both enzymes between epiphyted and non-epiphyted plants in the pre-dawn period.

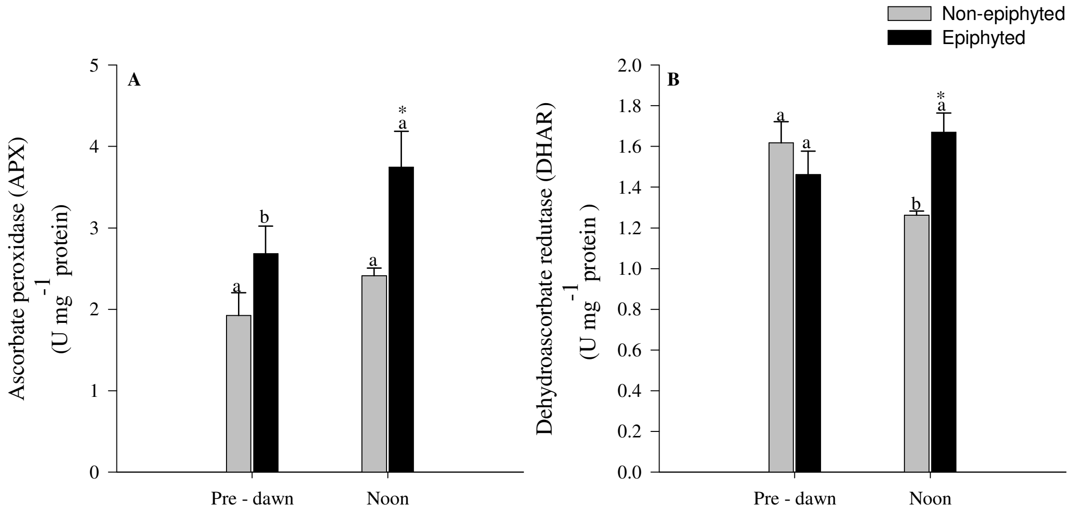

Figure 5.7. – Ascorbate peroxidase (APX) activity (C) and dehydroascorbate (DHAR) activity (D) in non-epiphyted and epiphyted *Posidonia oceanica* plant leaves, at pre-dawn and noon. Different letters indicate significant differences between pre-dawn and noon; * indicates significant differences between epiphyted and non-epiphyted shoots (n=5, $p<0.05$).

Correlations between phenolic compounds and antioxidant capacity

The concentrations of phenolic compounds were significantly correlated ($R^2=0.90$) with the oxygen radical absorbance capacity (ORAC) in epiphyted leaves at pre-dawn (Table 5.1) suggesting that specifically in these leaves and period of the day, the composition of the phenolic extract is more connected with antioxidant activity. Apart from this situation, the concentration of phenolic compounds showed no correlation with antioxidant capacity, neither through ORAC or TEAC assays.

Table 5.1 – Coefficient of determination (R^2) between phenolic compounds (mg.g DW^{-1}) and ORAC (µmol.gDW^{-1}) and between phenolic compounds (mg.g DW^{-1}) and TEAC (µmol.gDW^{-1}) in non-epiphyted and epiphyted *Posidonia oceanica* plant leaves, at pre-dawn and noon.

Time	**Epiphytic state**	**Phenolics/TEAC (mg.µmol^{-1})**	**Phenolics/ORAC (mg.µmol^{-1})**
Pre-dawn	Non-epiphyted	0.78 ($p = 0.05$)	0.59 ($p > 0.05$)
Noon	Non-epiphyted	0.77 ($p = 0.05$)	0.52 ($p > 0.05$)
Pre-dawn	Epiphyted	0.60 ($p > 0.05$)	**0.90 ($p < 0.05$)**
Noon	Epiphyted	0.30 ($p > 0.05$)	0.27 ($p > 0.05$)

Discussion

Maximum net photosynthetic rates were lower in *Posidonia oceanica* leaves with epiphytes. However results also showed that these leaves needed less photoprotection. Simultaneously, epiphyted plants were under oxidative stress, despite the increase of the antioxidant responses. The higher soluble sugar content on epiphyted leaves suggests the allocation of energetic resources to these protection mechanisms.

Both epiphyted and non-epiphyted plants attained their maximum photosynthetic rates at 16-18h (Fig. 5.1.), which did not coincide with maximum light intensity. At noon period (13-15h), light intensity was probably higher enough to surpass the limit that plants were able to use for photosynthetic processes. When this happens, oxygen reactive species (ROS) are produced and dynamic photoinhibition can be induced (Adams III *et al.*, 2006). Simultaneously, plants induce changes in dynamic components from photosystems, in order to dissipate the excess of energy as heat (Ragni *et al.*, 2008; Turpan, 2012) thus diverting energetic resources from photosynthesis to photoprotective mechanisms (Andersen, 2003).

Maximum photosynthetic rates were lower in epiphyted plants. Lower photosynthetic rates have long been related with the physical barrier created by epiphytes on the leaf surface (Sand-Jensen, 1977; Brush and Nixon 2002), which hampers light penetration, turning it into a limiting factor (Alcoverro, 1995). Recently, Oh *et al.* (2009) showed that epiphytes induce a permanent loss of chlorophyll a fluorescence signal in the place where they are located on the leaf, based on that, they proposed that such disruption on photosynthesis, can lead to permanent damages on the long-term.

Dark respiration was similar in both types of plants, showing no response to the presence of epiphytes. Photosynthesis and respiration are usually inter-balanced processes, meaning that an increase or decrease in either one is likely to entail a similar trend in the other (Flexas *et al.*, 2006).

The lowest chlorophyll a/b ratio was found in epiphyted leaves (Fig. 5.2. A), which is in agreement to what was expected since plants adapted to lower light intensities usually have lower chlorophyll a/b ratios (Lichtenthaler and Burkart, 1999). The main driver of the proportionally, the difference in chlorophyll b was higher than in chlorophyll a between non-epiphyted and epiphyted plants. The lower chlorophyll a/b ratio in epiphyted plants corresponds to an additional enlargement of the PSII antennae system (Lichtenthaler and

Buschman, 2001) due to a proportionally higher content in chlorophyll b. In non-epiphyted plants there was proportionally more chlorophyll a, which apart from the antennae, also exists in the reaction centres (Lichtenthaler and Buschman, 2001). This emphasizes the idea that epiphyted plants were more light-limited and invested more in harvesting light in a chlorophyll basis than non-epiphyted plants.

If light limitation can be a problem to plants, the excess of light can be stressful, passible to create oxidative stress, photoinhibition and also permanent photo-damages (Demmig-Adams and Adams III, 1992). The xanthophyll VAZ (violaxanthin (V) + anteraxanthin (A) + zeaxanthin (Z)) cycle is an important mechanism to protect plants from the excess of light. The de-epoxidation of violaxanthin into anteraxanthin and zeaxanthin is a dynamic and quick response to light changes (Jahns, 1995; Demmig-Adams and Adams III, 1992; Han *et al.*, 2004). In land herbs, the VAZ-pool size depends upon light intensity and varies according to species (Walters and Horton, 1994; Niinemets *et al.*, 2003). Usually plants adapted to high irradiance have higher concentrations of VAZ-cycle pigments than the ones adapted to low irradiances (Han *et al.*, 2004). In this work, the VAZ-pool size at pre-dawn was significantly higher in non-epiphyted plants, but at noon the VAZ-pool size was similar in epiphyted and non-epiphyted plants (Fig. 5.2. B) due to a slight decrease in non-epiphyted plants VAZ content. These results show that the epiphytes didn't seem to influence significantly the foliar VAZ pool content. However, with the light increase towards noon, both types of plants showed significant increases of the de-epoxidation-state (A+Z)/(V+A+Z) (Fig. 5.2. C). This emphasizes the hypothesis of photosynthetic dynamic, thus reversible, photoinhibition, under solar noon conditions. In fact, it is known that under high light intensities, the compensatory increase in the (A+Z)/(V+A+Z) ratio leads to a greater midday depression of PSII efficiency and lower photosynthetic rates, also called dynamic photoinhibition (Adams III *et al.*, 2006).

It is also noteworthy that epiphyted plants presented a significantly lower (A+Z)/(V+A+Z) ratio at noon than non-epiphyted-ones, which is probably related with the fact that epiphyted leaves have a lower light exposed surface and therefore a lower need for photoprotection.

Epiphyted plants presented lower starch and higher soluble sugar concentrations compared with non-epiphyted plants (Fig. 5.3. A, B) at pre-dawn, probably because the presence of epiphytes led to energetic requirements for the upregulation of some physiological processes, which at night must be obtained at the expense of reserves. In fact, during this period, in epiphyted leaves, the higher amount of soluble sugars is proportional to the lower starch amount when compared to non-epiphyted ones. The distribution of leaf carbohydrates can be adjusted as a response to energetic requirements according to environmental signals, from growth to stress response (Andersen, 2003). Processes that increase the demand for carbohydrates, such as antioxidant defence, lead to the depletion of storage in the form of starch (Andersen, 2003). The allocation of energetic resources is done according to the major physiological needs, and there is an inverse relationship between allocation to growth and to non-growth processes (Lattanzio *et al.*, 2009), being plant defence the priority energetic sinks (Close and McArtur, 2002).

MDA formation is associated with strong oxidative stress conditions and can be responsible by cell damage (Valenzuela, 1991). At pre-dawn, MDA concentrations were significantly higher in epiphyted leaves (Fig. 5.4.), corroborating the hypothesis that epiphyted plants were under oxidative stress. Sureda *et al.* (2008) also identified an increase in MDA concentration and in antioxidant response to epiphytes in *P.oceanica*. With higher light levels (noon) MDA concentration in non-epiphyted leaves increased to the same level as in epiphyted plants. This means that under high light conditions, non-epiphyted plants were also confronted with a stressful situation that the increase in the de-epoxidation-state helped to attenuate through energy dissipation, but still not enough to prevent oxidative stress. By leaving less leaf area exposed, the cover of epiphytes somewhat protected plants from solar noon light, prevented further increases in oxidative stress.

In order to control ROS bursts and avoid oxidative stress, plant defences comprise a complex set of enzymatic and non-enzymatic antioxidants and secondary metabolites, among which are some phenolic compounds (Larcher, 2003). Phenolic compounds can have multiple biological functions related with the reproductive strategy (Ning *et al.*, 2010), adaptation and survival to environmental disturbances (Ning *et al.*, 2010; Cheynier *et al.*, 2013), antimicrobial and anti-fouling proprieties (Jesen *et al.*, 1998), effect on oxidative bursts, photosynthetic and respiration rates (Cheynier *et al.*, 2013), and also antioxidant activity (Kahkonen *et al.*, 1999; Ahn *et al.*, 2007). In this last role, phenolics have the capacity to reduce free radical formation and scavenge ROS (Rosch *et al.*, 2003). Nevertheless, phenolic compounds can be polymerized by peroxidases during pathogen attacks to create morphological barriers against possible infections (Close and McArtur, 2002). Its deposition as lignin in cell walls increases their mechanical strength and improves plants response against pathogens and wounding (Booker and Miller, 1998). Some, but not all, phenolic species can have a direct effect in herbivores (Close and McArtur, 2002), however, they are mostly linked to photodamage prevention (Close and McArtur, 2002; Lattanzio *et al.*, 2009).

Our results show identical total phenolic concentrations in epiphyted and non-epiphyted leaves, yet higher at pre-dawn than at noon (Fig. 5.5.). Being a complex group of compounds with diverse functions, total phenolic concentrations do not necessarily reflect antioxidant capacity (Kahkonen *et al.*, 1999; Miguel *et al.*, 2010). Plants submitted to different environmental conditions and with different physiological requirements will most likely have a qualitatively distinct phenolic composition. Consequently, although the two types of plants presented the same concentration in total phenolic compounds, this does not mean that the compounds were the same. In fact, epiphyted plants could have expressed phenolic compounds for defence purposes, while non-epiphyted plants could have applied their resources in phenolic compounds with other functions. Actually there was only a strong and significant correlation between the concentration of phenolic compounds and oxygen radicals absorbance capacity (ORAC) on epiphyted plants at pre-dawn (Table 5.1.), which suggests that in these conditions phenolic compounds could be performing antioxidant functions, which makes sense in face of the high oxidative stress showed by these plants. Under the remaining conditions the weak or non-significant correlation between phenolic compounds and antioxidant capacity (ORAC and TEAC assays) suggests allocation of phenolics to functions other than antioxidant defence.

Significantly higher antioxidant scavenging capacity was found in epiphyted leaves (Fig. 5.6. A, B) as indicated by the antioxidant radical scavenging capacity (TEAC assay) and the absorbance of oxygen species capacity (ORAC assay). These assays reflect the antioxidant reducing capacity and the hydrogen atom donation capacity, respectively (Huang *et al.*, 2005).

Our results reveal that the existence of epiphytes in *P. oceanica* leaves led to higher TEAC and ORAC concentrations and from this increase in the antioxidant response we can infer that epiphytes were responsible for a burst of reactive oxygen species (ROS). Moreover, the antioxidant capacity of epiphyted plants (ORAC and TEAC assays) was significantly higher at pre-dawn than at noon, meaning that this response was more related with the presence of epiphytes than with the effect of light, which in turn agrees with the higher MDA concentrations on the same plants.

It has been suggested that hydrogen peroxide (H_2O_2) can be released by plants as a direct chemical defence against pathogens and epiphytes (Choo *et al.*, 2004). In marine plants, H_2O_2 is released by diffusion from cells to seawater, being toxic to those organisms (Choo *et al.*, 2005). H_2O_2 is also a compound which, similarly to other ROS, acts as a signalling mechanisms for the antioxidant system to act in response to bursts of oxidative species (Forman and Torres, 2002; Sureda *et al.*, 2008). The antioxidant defence enzymes, ascorbate peroxidase (APX) and dehydroascorbate redutase (DHAR), are main scavengers of H_2O_2 (Sureda *et al.*, 2008). In fact, APX is an H_2O_2-peroxidase that uses ascorbate as an electron-donor in plants and algae (Miyake and Asada, 1996), to convert H_2O_2 into water (Rijstenbil, 2002) and DHAR is part of the ascorbate recycling system. APX activity was higher in epiphyted plants (Fig. 5.7. A, B). The ability of the antioxidant system to fight a stressful condition can be a good indicator of its resistance to it. The enhancement of the global antioxidant capacity (TEAC and ORAC assays) and the enzymatic activity (APX and DHAR) may potentially turn plants more resistant to epiphytes if these mechanisms can efficiently scavenge ROS before they cause severe damages. Resistance induced by antioxidants could be expected to lower lipid peroxidation levels (Sales *et al.*, 2013). However, MDA concentrations demonstrate that epiphyted plants were struggling against stress.

Most of the epiphytes found in *P.oceanica* leaves were calcareous. As carbon dioxide (CO_2) concentration in the ocean is increasing, the inherent changes in the carbonate balance are leading to a concomitant decrease in seawater pH (Barry *et al.*, 2013). Calcifying marine organisms are among the most susceptible to this process, since the pH decrease will affect their ability to form and maintain calcium carbonate skeletons (Budenbender *et al.*, 2011; Barry *et al.*, 2013) and ultimately compromise the survival of many species. The existence of boundary layers allows plants to alter pH at their surfaces (Hurd *et al.*, 2011) and seagrasses photosynthesis is known to be able to increase pH at leaf surface (Hendriks *et al.*, 2014), somehow mitigating ocean acidification impacts in calcareous algae and other calcareous epiphytes normally associated to seagrasses (Hendriks *et al.*, 2014). Nevertheless, a significant reduction in epiphytic coralline algae cover was found on seagrasses growing in natural acidified seawater, in CO_2 vents with pH bellow 7.9 (Martin *et al.*, 2008). Since coralline algae are major colonizers of seagrass leaves, the decrease in the area covered by these epiphytes can largely affect the total epiphytic load in seagrasses (Martin *et al.*, 2008). In this particular aspect, while negative for calcifying epiphytes, ocean acidification can be beneficial for *P.oceanica* meadows, since with less or no epiphytes the plants will probably become more productive, with lower energy requirements for antioxidant responses and hence the possibility to allocate more resources to other processes such as growth and reproduction.

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Chapter 6

Cymodocea nodosa antioxidant responses and photosynthetic pigment composition at a natural CO_2 vent used to investigate the effects of ocean acidification

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(To be submitted)

Cymodocea nodosa antioxidant responses and photosynthetic pigment composition at a natural CO_2 vent used to investigate the effects of ocean acidification

Abstract

Ocean acidification (OA) is the decline in seawater pH due to the sustained absorption of atmospheric CO_2. Predictions indicate that until 2100, ocean concentrations in CO_2 will be to 2 to 3 times higher than the current average values, reaching values above 700 ppm. This will lead to changes in environment, with impacts in marine organisms and communities, which will either adapt or succumb. According to some authors, OA can benefit seagrass photosynthesis, however, differences between short and long term experiments indicate that this subject still needs to be carefully investigated, especially regarding the effects on the metabolic processes. In recent years, volcanic CO_2 vents have been considered as potential natural laboratories to investigate the effects of ocean acidification in marine ecosystems and some studies involving seagrasses have been conducted in the proximity of these vents. One of these vents is located in Vulcano Island (Italy). In this work we investigated the antioxidant response and pigment composition and interconversion on *Cymodocea nodosa* plants from Vulcano Island, growing under normal and high-CO_2 levels. Our aim is to provide a better understanding of the plants photo-physiological processes under those environmental conditions, and also to evaluate the suitability of Vulcano CO_2 vents as natural laboratories for the research of high-CO_2 effects on seagrasses. Results show that plants from the high-CO_2 site were submitted to significant levels of oxidative stress, as indicated by the high concentrations of malondialdehyde (MDA) at pre-dawn and at noon, despite the high global antioxidant capacity (TEAC and ORAC assays), the high concentrations of phenolic compounds and the high levels of activity of the enzymes APX and DHAR. Identical concentrations of photosynthetic pigments in both sites at noon showed similar light harvesting capacity, but the lower soluble protein concentration in high-CO_2 plants suggests a downregulation of photosynthesis. Coupled to a permanent and energetically expensive process of antioxidant defence, low photosynthetic rates of the high-CO_2 plants can help to explain the reduced expansion capacity of these meadows found on previous works. Besides the high-CO_2 levels, the interaction of other factors such as low nitrogen, H_2S, trace substances and toxic metals

needs to be considered when assessing the responses of autotrophs near natural CO_2 vents, as they are the most likely cause for oxidative stress that may confound the responses. Hence, the use of volcanic CO_2 vents as natural laboratories for investigating the effects of high-CO_2 on seagrass performance needs to be dealt cautiously, given the presence of factors other than CO_2, which can easily origin confounding responses, particularly when these factors are site-specific and have no foreseeable association with high-CO_2 in the predicted climate change scenarios.

Introduction

Ocean acidification (OA) is the decline in seawater pH due to the sustained absorption of atmospheric CO_2 (Raven, 2005), which leads to changes in the oceans carbonate balance, i.e. the proportions of carbon dioxide, bicarbonate and carbonate, the dissolved forms of inorganic carbon (Barry *et al.*, 2013). As the partial pressure of CO_2 (pCO_2) in the ocean increases, predictions indicate that until 2100 it will be to 2 to 3 times higher than the current average values, reaching values above 700 ppm (Stocken *et al.*, 2013). OA will lead to changes in environment, with impacts in marine organisms and communities, which will either adapt or succumb to the future conditions (Cooley *et al.*, 2009).

According to some authors, OA can benefit seagrass photosynthesis (Raven, 2005; Hendriks *et al.*, 2014). In fact, a short term study (45 days) in mesocosm-cultivated *Zostera marina* showed that high-CO_2 stimulated an increase in photosynthesis, which authors hypothesized to correspond to an enhancement of light harvesting, electron-transport and carbon fixation capacities, and consequently a reduction in light requirements (Zimmerman *et al.*, 1997). Nonetheless, a long-term experiment (one year) showed no effect of high-CO_2 on growth, leaf length or sugar content in the same species (Palacios and Zimmerman, 2007). Therefore the effects of OA in seagrasses still need to be carefully investigated, especially regarding the effects on the metabolic processes.

In recent years, volcanic CO_2 vents have been considered as potential natural laboratories to investigate the effects of ocean acidification in marine ecosystems (Hall-Spencer *et al.*, 2008) and some studies involving seagrasses have been conducted in the proximity of these vents (Martin *et al.*, 2008; Arnold *et al.*, 2012; Vizzini *et al.*, 2013; Apostolaki *et al.*, 2014), taking advantage of the long-term adaptation that plants necessarily have to high-CO_2 conditions.

One of these vents is located in the Mediterranean island of Vulcano, in Italy, where *Cymodocea nodosa* can be found growing under high and normal CO_2 conditions. Previous studies on those vents reported higher photosynthetic activity, maximum electron transport rate and compensation irradiance on *C. nodosa* (Apostolaki *et al.,* 2014). However, authors also found lower shoot density and lower biomass, despite the higher productivity (Apostolaki *et al.*, 2014). Another work indicated that plants from high-CO_2 had lower concentrations of phenolic compounds (Arnold *et al.*, 2012). Phenolics are responsible by the interaction with environment for reproductive strategy (Ning *et al.*, 2010), adaptation and survival to environmental disturbances (Ning *et al.*, 2010; Cheynier *et al.*, 2013), can affect oxidative bursts, photosynthetic and respiration rates (Cheynier *et al.*, 2013), have antioxidant activity (Kahkonen *et al.*, 1999; Ahn *et al.*, 2007) and have relevant antimicrobial activity and anti-fouling proprieties (Jesen *et al.*, 1998).

Vizzini *et al.* (2013) revealed the contamination of Vulcano sediments and *C. nodosa* leaves with low concentrations of trace elements that must be considered as a potential stress factor. Boata *et al.* (2013) showed that besides CO_2, also methane, hydrogen sulphide (H_2S), metals and toxic trace elements are present near the vents. Hydrogen sulphide (H_2S) is a low temperature product of hydrothermal activity. In the Vulcano venting sites its concentration is variable, but can be considerably high (Sedwick and Stüben, 1996; Armend *et al.,* 2003). Other factors such as nitrogen limitation, which according to Apostolaki *et al.,* (2014) is below the minimum nitrogen requirement, may influence *C. nodosa* growth near the vents. Nevertheless, these factors do not necessarily make this site less suitable for low pH studies, as long as data interpretation is carefully made (Boatta *et al.,* 2013; Apostolaki *et al.,* 2014) and if it is considered that plants are submitted not only to low pH conditions, but also to non-negligible and potentially adverse stress conditions.

In land plants there are some studies that relate high-CO_2 environments with antioxidant and oxidative stress responses. Antioxidants are substances able to prevent or significantly delay the oxidation of lipids, membranes, DNA, and proteins (Miguel, 2009). They have the ability to detoxify oxygen reactive species (ROS) (Aghadam *et al.,* 2011), which are usually developed under optimal metabolic processes but that under demanding conditions may increase (Wang *et al.*, 2011), creating an imbalance between antioxidant capacity and ROS concentration, known as oxidative stress (Huner *et al.,* 1998; Scandalios, 2005), which leads to lipid peroxidation of membranes (Hodges *et al.*, 1999). Malondialdehyde (MDA) is a product of lipid peroxidation, which can be used to quantify the negative effects of oxidative stress (Hodges *et al.*, 1999).

The complex antioxidant system comprises both enzymatic, like ascorbate peroxidase, dehydroascorbate reductase, glutathione reductase and catalase and non-enzymatic, like thocoferols, phenolic compounds and carotenoids, protection mechanisms (Pradedova *et al.*, 2011). Since different antioxidants have distinct mechanisms of action, it is important to use methods that cover the possible antioxidant pathways to properly evaluate plant response to demanding situations. Methods to evaluate the antioxidant scavenging capacity can be divided in two groups: assays based in hydrogen atoms transfer, like oxygen reactive absorbance capacity (ORAC) and assays based on electron transfer, like trolox equivalents antioxidant capacity (TEAC) and phenolic compounds (Huang *et al.*, 2005).

Land plants growing under high-CO_2 environments usually present lower antioxidant activity (Vurro *et al.,* 2009; Levine and Paré, 2009) but also increases in enzymatic substrate regeneration (Vurro *et al.,* 2009) and enhancement of antioxidant responses when submitted to an acute stress (Gillespie *et al.,* 2011). This might configure a less costly strategy to growth under CO_2 environment, without the need to activate expensive metabolic processes (Vurro *et al.,* 2009). How the antioxidant system of seagrasses respond to high-CO_2 levels is still unknown.

Although marine plants seem to respond to high-CO_2 concentrations with higher photosynthetic rates, carbon fixation can be compromised, since several studies associate high-CO_2 with lower RuBisCO content (Stitt *et al.*, 1991; Cheng *et al.,* 1998; Stitt and Krapp, 1999; Gordillo *et al.*, 2011). To the best of our knowledge, there are no published reports of the effects of high-CO_2 on the composition of seagrass photosynthetic pigments, which is a major knowledge gap, given their potential effect on photosynthetic rates and the subsequent implications for the plant energy balance.

Carotenoids are photosynthetic pigments that can either work in light collection or in photoprotection, according to plants needs and light conditions. As a result of high light, the carotenoid violaxanthin, (V), can be rapidly de-epoxidased into antheraxanthin (A) and zeaxanthin (Z), which in turn, dissipate the excess of energy, as heat, in a so called xanthophyll cycle (Demmig-Adams and Adams III, 1996).

In this work we investigated the antioxidant response and pigment composition and interconversion on *C. nodosa* plants from Vulcano Island, growing under normal and high-CO_2 levels. Biochemical analysis of the plants is aimed to provide a better understanding of the plants photo-physiological processes under those environmental conditions. We also wanted to evaluate the suitability of Vulcano CO_2 vents as natural laboratories for the research of high-CO_2 effects on seagrasses.

Materials and Methods

Site characterization

Levante Bay in Vulcano Island, (Italy) is characterized by the existence of underwater vents, which ejects mainly CO_2 (97-99%) but also sulphite (H_2S) in minor concentrations (2.2%) (Boatta *et al.*, 2013). The place presents a linear pH gradient along the coast. The water is acidic near the emission points, with a pH 6.37, showing a pH gradient until it reaches normal values of pH 8.16, at 400 m from the vents (Boatta *et al.*, 2013). These levels can change according to wind direction and consequent water agitation (Boatta *et al.*, 2013). Minor concentrations on trace elements, toxic metals and methane were also detected in the bay water, especially at 150 to 350 m from the main vents and should not be undervalued regarding to its potential toxicity for living organisms (Vizzini *et al.*, 2013; Boatta *et al.*, 2013). Meadows of *Cymodocea nodosa* can be found along the pH gradient (Vizzini *et al.*, 2013).

Two sampling sites were selected, one site at high-CO_2, with a pCO_2 of 839 µatm corresponding to low pH (7.89) and another one at reference-CO_2, with a pCO_2 of 430 µatm corresponding to pH (8.17), located at 200 m and 420 m from the main vent respectively, and both at 3 m depth. Seawater on both sites presented a salinity of 37 psu and a temperature of 19.3ºC.

Experimental design and plant sampling

Cymodocea nodosa plants were sampled in the two selected sites, reference and high-CO_2 on May 2013. In both sites, plants (leaves and rhizomes) were collected at pre-dawn and at solar noon. Leaves and rhizomes were separated and samples (n=5) for biochemical analysis were cleaned from epiphytes, rinsed with distilled water, blotted dry and immediately frozen in liquid nitrogen. Samples were stored at -80ºC prior to biochemical analysis.

Malondialdehyde (MDA)

For MDA extraction, 300 mg of frozen leaf tissue was powdered in liquid nitrogen and suspended in 80% aqueous ethanol. Extracts were centrifuged at 3000xg for 10 min. Quantification was performed according to Hodges *et al.* (1999). The supernatant was added to a solution of 20% trichloroacetic acid (TCA) with 0.65% thiobarbituric acid (TBA) and 0.015% butylated hydroxytoluene (BHT). Two blanks were performed either without TBA or with 80% ethanol instead of the sample extract. All samples and blanks reaction mixtures were incubated at 90°C for 25 min., cooled for 15 min. in an ice bath and centrifuged at 3000xg. Absorbances of the supernatants were read at 440 nm, 532 nm and 600 nm using a Beckman Coulter DU-650 spectrophotometer and MDA equivalents were calculated as in Hodges *et al.* (1999).

Phenolic compounds

Extraction for phenolic compounds, oxygen reactive absorbance capacity (ORAC) and radical trolox equivalent antioxidant capacity (TEAC) was performed simultaneously. 300 mg of frozen leaf tissue was powdered in liquid nitrogen, suspended in 0.1 N hydrochloric acid (HCL), kept overnight at 4°C under constant agitation and then centrifuged at 4700xg for 30 min. The supernatant was used for the different quantification procedures.

Phenolic compounds were quantified by the Folin-Ciocalteu method, as described in Booker and Miller (1998) and Migliore *et al.* (2007). 0.25 N Folin-Ciocalteu reagent and 7.5% Na_2CO_3 were added to the supernatant. Absorbance was read at 724 nm in a Beckman Coulter DU-650 spectrophotometer (Brea CA, USA), against a blank sample. The assay results were expressed as chlorogenic acid equivalents.

Antioxidant global capacity

ORAC and TEAC were extracted simultaneously with phenolic compounds, as described above. ORAC was quantified according to Huang *et al.* (2002). Results were expressed as Trolox® equivalents (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid).

For TEAC assay, $ABTS^{\bullet+}$ was produced by the reaction of 7 mM ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium) with potassium persulfate in a final concentration of 2.45 mM according with Re *et al.* (1999). Diluted $ABTS^{\bullet+}$ (A_{734nm}= 0.800 ± 0.020) was added to the extract and after an incubation period of 6 min., absorbance was read at 734 nm in a Beckman Coulter DU-650 spectrophotometer, against a blank sample. Results were expressed Trolox® equivalent (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid).

Activity of enzymatic antioxidants

Frozen leaf tissue (800 mg) was powdered in liquid nitrogen, polyvinyl polypyrrolidone (PVPP) and sodium ascorbate, and then extracted with 5 mL of 100 mM potassium phosphate buffer (pH 7.8) with 2% Triton-x and 10 mM ascorbate. Extracts were centrifuged at 4°C and 3500xg for 30 min. The supernatant was purified by filtration with sephadex PD-10 G-25 column (GE Healtcare), previously equilibrated with 20 mL of 100 mM potassium phosphate buffer pH 7.0 with 1 mM ascorbate (Polle and Morawe, 1995). The purified enzymatic extracts were used to determine de activity of ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR).

APX activity was measured as the decrease of the absorbance at 290 nm (Beckman Coulter DU-650 spectrophotometer, Brea CA, USA) of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 8 mM ascorbate, 20 mM hydrogen peroxide and 125 µl of extract. The reaction was followed for 3 min. at 25ºC (Nakano and Asada, 1987). APX activity was calculated after subtraction of the control rates, where the enzyme extract was replaced by the potassium phosphate buffer, using $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$. One unit (U) of APX is equivalent to the protein necessary to oxidize 1 µmol of ascorbate per min. Enzymatic activity was expressed in U mg^{-1} soluble protein.

DHAR activity was measured as the increase of the absorbance at 265 nm (Beckman Coulter DU-650 spectrophotometer, Brea CA, USA) of a reaction mixture containing 60 mM potassium phosphate buffer (pH 6.1), 5 mM reduced glutathione, 800 µM dehydroascorbic acid and 50 µL of extract. The reaction was followed for 3 min at 25ºC (Polle and Morawe, 1995). DHAR activity was calculated after the subtraction of the control rates, where the enzyme extract was replaced by the potassium phosphate buffer, using $\varepsilon = 14\ mM^{-1}\ cm^{-1}$.One unit (U) of DHAR is equivalent to the protein necessary to reduce 1 µmol of dehydroascorbate per min. Enzymatic activity was expressed in U mg^{-1} soluble protein.

To determine glutathione reductase (GR) activity, 800 mg of frozen leaf tissue was powdered in liquid nitrogen, polyvinyl polypyrrolidone (PVPP) and then extracted with 5 mL of 100 mM potassium phosphate buffer (pH 7.8) with 2% Triton-x, 1 mM Phenylmethanesulfonyl fluoride (PMSF) and 1 mM DL-Dithiothreitol (DTT). Extracts were then centrifuged at 4°C and 3500xg for 30 min. Supernatant was purified by filtration with sephadex PD-10 G-25 column (GE Healtcare), previously equilibrated with 20 mL of 20 mM potassium phosphate buffer (pH 7.0).

GR activity was measured as the decrease in absorbance at 340 nm (Beckman Coulter DU-650 spectrophotometer, Brea CA, USA) of a reaction mixture containing 500 mM heppes buffer pH 8.0 with 5 mM EDTA and 2.5 mM NADPH, 5 mM oxidised glutathione and 100-150 µL of extract. The reaction was followed by 3 min. at 25ºC (Polle and Morawe, 1995; Barrote, 2005). GR activity was calculated after subtraction of the control rates, where the enzyme extract was replaced by deionized water, using the extraction coefficient $\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$.One unit (U) of GR is equivalent to the protein necessary to oxidase 1 µmol of NADPH per min. Enzymatic activity was expressed in U mg^{-1} soluble protein.

Hydrogen peroxide (H_2O_2)

To extract hydrogen peroxide, frozen leaf tissue (30 mg) was ground in liquid nitrogen and extracted with 5 mL of trichloroacetic acid 0.1% (adapted from Velicova *et al.*, 2000). Quantification was performed as described in Valicova *et al.* (2000) through a calibration curve performed with hydrogen peroxide 30%, from 0.1-1 mM (according to Brennan and Frankel, 1977).

Photosynthetic pigments

Frozen leaf tissue (200 mg) was powdered in liquid nitrogen and sodium ascorbate, and extracted under low light in 5 mL of acetone 100% neutralized with $CaCO_3$ (Abadía and Abadía, 1993). Extracts were filtrated with membrane filters 5.0 µm LS followed by hydrophobic 0.2 µm PTFE.

HPLC calibration (de las Rivas *et al.,* 1989) was performed with commercially available pigments (CaroteNature, Lupsingen, Switzerland). Liquid chromatography analysis was performed in an Alliance Waters 2695 separation module (Milford MA, USA), with a Waters 2996 photodiode array detector and a Waters Novapak C18 radial 8x100 mm compression column (4 µm particle size), maintained at 24ºC during analysis. Samples were stored at 5ºC prior to injection. For sample analysis, 20 µL of extract were injected via an auto-sampler. All eluents were prepared with HPLC grade solvents (VWR Hipersolv Chromanorm), filtered and submitted to sonication prior to use. Peak areas were monitored at 450 nm and concentrations were calculated based on peak areas obtained for standards at known concentrations.

Soluble protein

Soluble protein was quantified in the enzymatic extract, through a dye-binding assay (Bradford, 1976), using a commercial reagent (BioRad Protein Assay, Hercules, California, USA). Bovine serum albumin was used as standard.

Non-structural carbohydrates

For soluble sugars and starch determinations, 60 mg of frozen leaf or rhizome tissue were powdered in liquid nitrogen and extracted with 10 mL in 80% of ethanol at 80°C, for 30 min. After extraction, 5 mL of the supernatant were cleaned with activated charcoal, centrifuged at 4700xg for 8 min. (Stitt *et al.*, 1978; Stitt *et al.,* 1989) and reserved for analysis. For starch quantification the insoluble residue was washed with deionized water and then heated at 100ºC for 10 min, as described in Smith and Zeeman (2006). An enzymatic solution of amyloglucosidase and α-amylase was used to hydrolyse starch to glucose (Smith and Zeeman, 2006). Soluble sugars and glucose obtained from starch hydrolysis were quantified by a phenol-sulphuric assay (adapted from Dubois *et al.*, 1956) using glucose standards. Results were expressed as glucose equivalents.

Statistical analysis

All results are presented as mean values ± standard error (N=5). One- or two-way analysis of variance (ANOVA) and Student-Newman-Keuls post-hoc tests were used to assess significant differences (at $p < 0.05$). All data treatment and statistical analysis was performed using the SigmaStat/SigmaPlot (SPSS Inc., v.11) software package.

Results

Oxidative stress indicated by membrane lipid peroxidation (MDA) was significantly higher in leaves exposed to high-CO_2 than in those of reference conditions, both at pre-dawn and noon (1.5- and 1.3-fold, respectively) (Fig. 6.1A). On both sites, MDA concentrations decreased significantly from pre-dawn to noon, (1.3- and 1.4-fold, in high-CO_2 and reference conditions, respectively).

The concentration of foliar phenolic compounds was always higher in leaves from the high-CO_2 site (1.2- and 1.6-fold, respectively at pre-dawn and noon) (Fig. 6.1B). However, while in leaves from the reference site the phenolics concentration decreased between pre-dawn and noon (1.1-fold), in leaves from high-CO_2 its concentration increased significantly (1.2-fold).

Antioxidant capacity determined through TEAC assay was higher in leaves from high-CO_2 (1.2- and 1.5-fold, respectively at pre-dawn and noon) (Fig. 6.1C) and did not varied from pre-dawn to noon. ORAC was not significantly different between reference and high-CO_2 sites (Fig. 6.1D). Nevertheless, while in leaves from the reference site ORAC was similar between pre-dawn and noon, under high-CO_2 the leaves presented lower concentrations at pre-dawn than at noon.

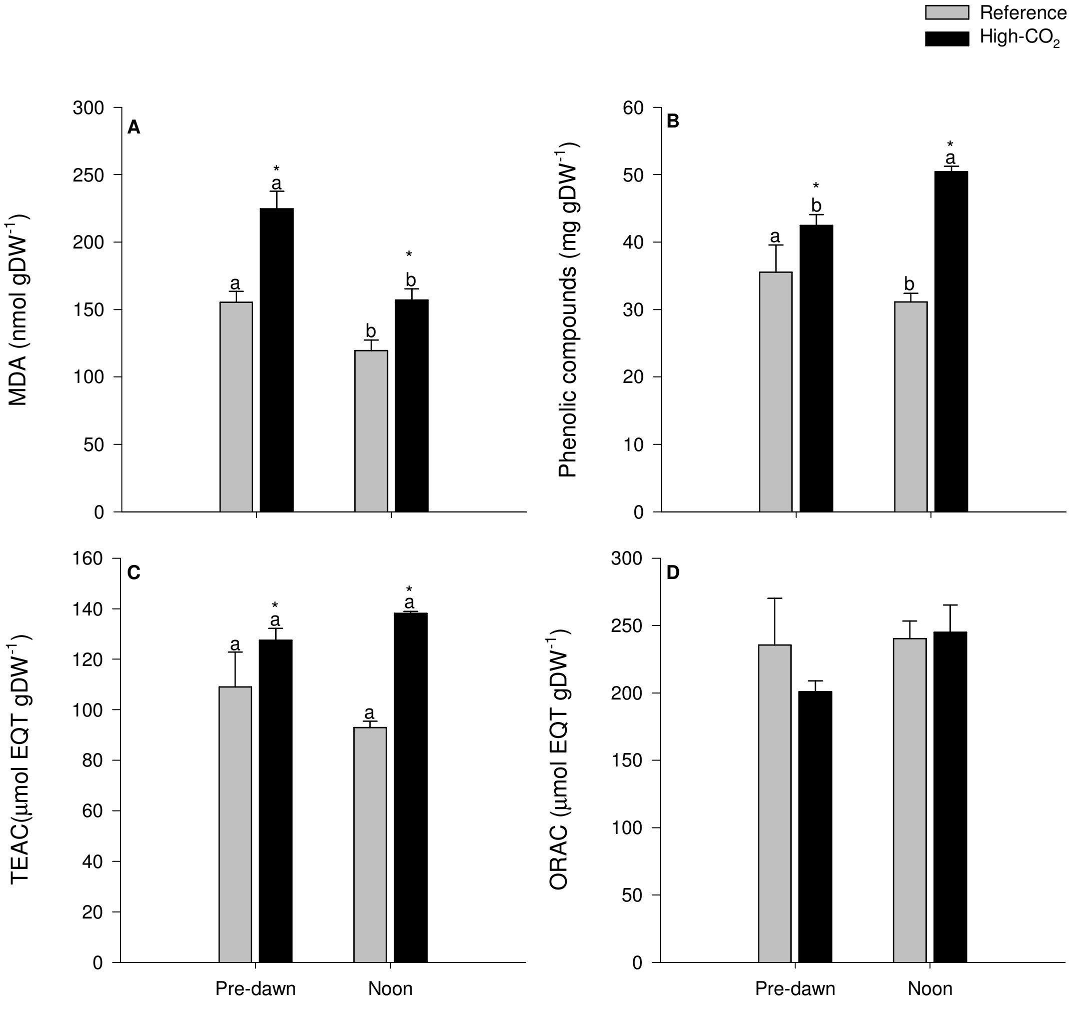

Figure 1 - Malondialdehyde (MDA) (A), Phenolic compounds (B) and Antioxidant capacity (TEAC assay) concentrations (C) Oxygen radical absorbance capacity (ORAC) concentrations (D) in *Cymodocea nodosa* leaves, in reference and high-CO_2 conditions, at pre-dawn and noon. Different letters indicate significant differences between pre-dawn and noon; * indicates significant differences between leaves under reference and high-CO_2 conditions (n=5, $p<0.05$).

The activity of ascorbate peroxidase (APX) was not different between sites at pre-dawn (Fig. 6.2.A). At noon, the APX activity was different between sites because it decreased about 1.5 fold from pre-dawn at the reference site. The activity of dehydroascorbate reductase (DHAR) was always significantly higher in high- CO_2 leaves, (Fig. 6.2B) both at pre-dawn and noon (about 1.5-fold), but with no significant time differences within sites. No significant effects of site or time were found both in the activity of glutathione reductase (GR) (Fig. 6.2C) and in the concentration of hydrogen peroxide (H_2O_2) (Fig. 6.3).

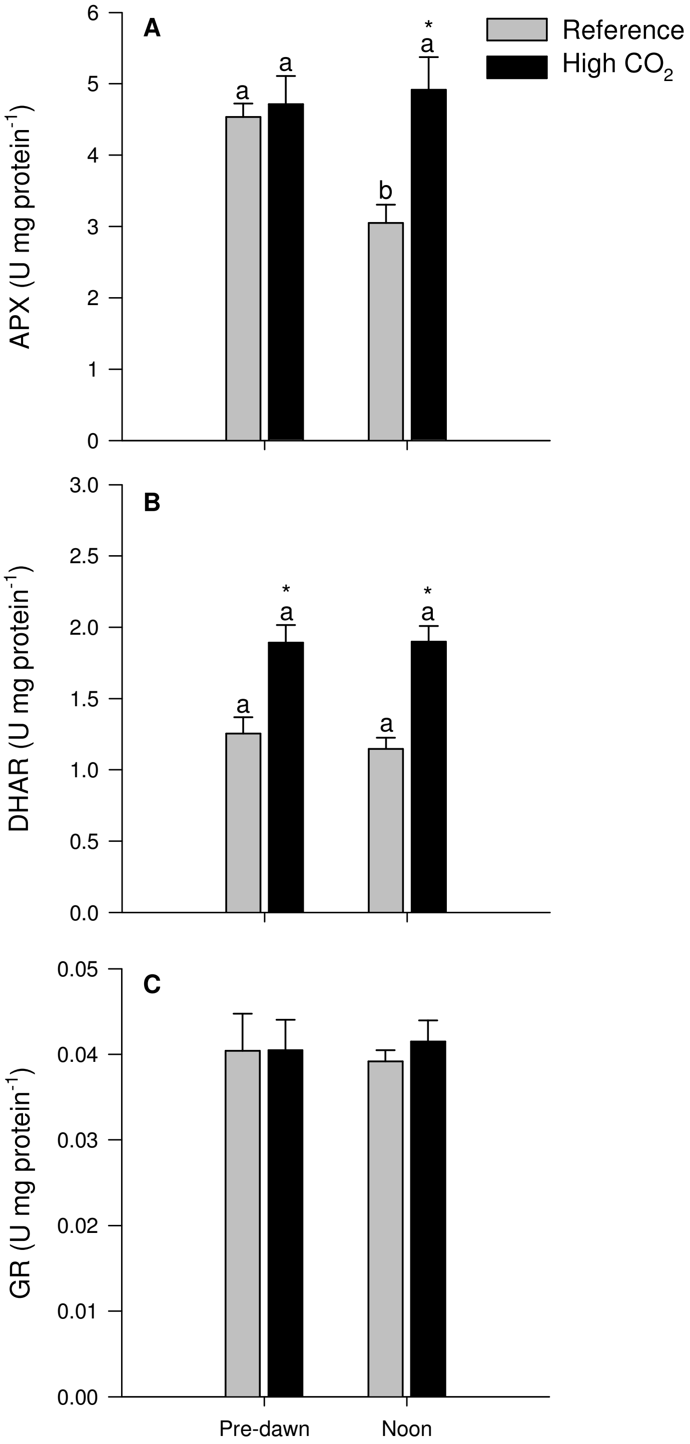

Figure 6.2 – Ascorbate peroxidase (APX) (A), Dehydroascorbate reductase (DHAR) and Glutathione reductase (GR) activity (C) in *Cymodocea nodosa* leaves, in reference and high- CO_2 conditions, at pre-dawn and noon. Different letters indicate significant differences between pre-dawn and noon; * indicates significant differences between leaves under reference and high- CO_2 conditions (n=5, $p<0.05$).

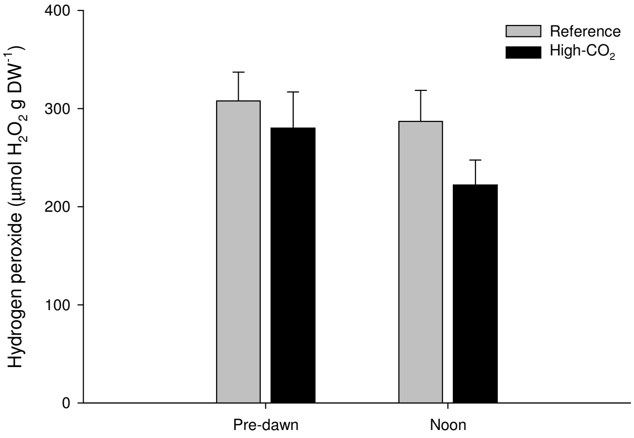

Figure 6.3 – Hydrogen peroxide (H_2O_2) concentrations in *Cymodocea oceanica* leaves, in reference and high-CO_2 conditions, at pre-dawn and noon. Different letters indicate significant differences between pre-dawn and noon; * indicates significant differences between leaves under reference and high-CO_2 conditions (n=5, $p<0.05$).

The same seven carotenoids were identified in leaves from reference and high-CO_2 sites (Table 6.1). Following the same order on both sites, major carotenoids were β-carotene, violaxanthin and lutein at pre-dawn, representing in reference and CO_2 site, respectively 32, 24, 20% and 32, 26, 26% of the total carotenoids. At noon, on both sites, lutein became the second most abundant carotenoid, replacing violaxanthin.

Both neoxanthin and lutein showed significantly higher concentrations in leaves from high-CO_2 at pre-dawn (respectively 1.7- and 1.5-fold higher than at noon).

Leaves from reference and high-CO_2 also showed a distinct pattern regarding β-carotene concentration. Actually, whereas there were no differences between its concentration in reference leaves between pre-dawn and noon, in leaves from high-CO_2 site the β-carotene concentration decreased significantly from pre-dawn to noon (1.5-fold).

Table 6.1 – Photosynthetic pigments in *Cymodocea oceanica* leaves, in reference and high-CO$_2$ conditions, at pre-dawn and noon. Different letters indicate significant differences between pre-dawn and noon; * indicates significant differences between leaves under reference and high-CO$_2$ conditions (n=5, p<0.05).

Pigments	**Reference**		**High-CO$_2$**	
(µmol m^{-2})	Pre-dawn	Noon	Pre-dawn	Noon
Neoxanthin	2.18 ± 0.23 a	1.92 ± 0.17 a	2.59 ± 0.23 a	1.56 ± 0.32 b
Violaxanthin	6.07 ± 0.65 a	4.19 ± 0.41 b	6.96 ± 0.62 a	3.97 ± 0.52 b
Lutein-epoxide	0.30 ± 0.03 a	0.15 ± 0.02 b	0.38 ± 0.04 a	0.21 ± 0.04 b
Antheraxanthin	3.39 ± 0.05 b	1.65 ± 0.11 a*	0.39 ± 0.03 b	1.15 ± 0.06 a
Lutein	4.99 ± 0.50 a *	4.98 ± 0.50 a	7.00 ± 0.86 a	4.74 ± 0.56 b
Zeaxanthin	0.32 ± 0.02 b	1.33 ± 0.17 a	0.55 ± 0.09 b*	1.09 ± 0.14 a
β-Carotene	8.12 ± 1.02 a	8.07 ± 0.54 a*	8.61 ± 0.83 a	5.94 ± 0.60 b

As well, there were no effects of site and time on the de-epoxidation of violaxanthin (V) in antheraxanthin (A) and in zeaxanthin (Z) (AZ/VAZ) (Figure 6.4). AZ/VAZ ratio increased about 3-fold from both sites from pre-dawn to noon. As expected, there was a decrease in the violaxanthin concentrations from pre-dawn to noon, (Table 6.1) (1.5-and 1.7-fold in reference and high-CO$_2$, respectively), and an increase in antheraxanthin and zeaxanthin between the same periods. While the concentrations of antheraxanthin at noon were 0.50 µmol m^{-2} higher in leaves from the reference site, at the same time the concentrations of zeaxanthin were similar on leaves from both sites. Nevertheless, the concentration of this pigment at pre-dawn was significantly higher in leaves from the high-CO$_2$ site. Concentrations of lutein-epoxide decrease likewise on the leaves from both sites from pre-dawn to noon (Table 6.1).

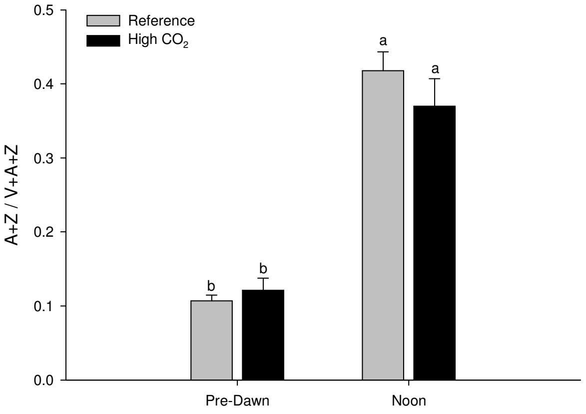

Figure 6.4 – Xanthophyll cycle de-epoxidation index (A+Z/V+A+Z) (violaxanthin (V) + anteraxanthin (A) + zeaxanthin (Z)) in *Cymodocea nodosa* leaves, in reference and

high-CO_2 conditions, at pre-dawn and noon. Different letters indicate significant differences between pre-dawn and noon; * indicates significant differences between leaves under reference and high-CO_2 conditions (n=5, p<0.05).

Soluble protein concentration was always significantly lower (about 1.3-fold) in the high CO_2 site (Fig. 6.5). No differences from pre-dawn to noon were found.

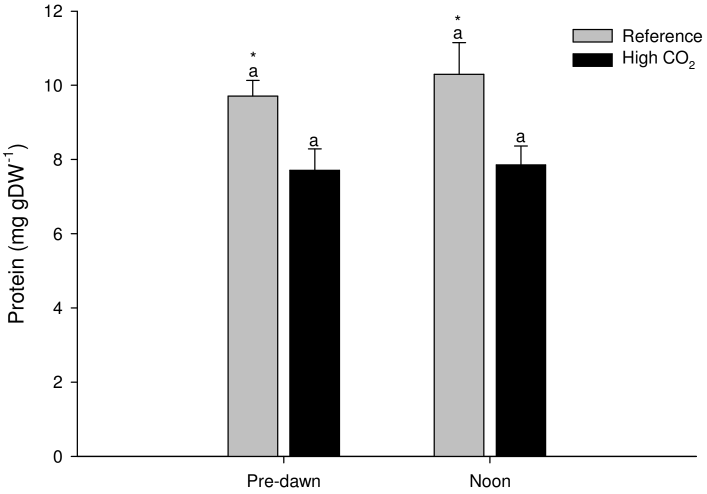

Figure 6.5 – Soluble protein concentrations in *Cymodocea nodosa* leaves, in reference and high CO_2 conditions, at pre-dawn and noon. Different letters indicate significant differences between pre-dawn and noon; * indicates significant differences between leaves under reference and-high CO_2 conditions (n=5, p<0.05).

Soluble sugars in leaves from the reference site did not vary from pre-dawn to noon (Fig. 6.6 A), but in the high-CO_2 site they increased significantly. The opposite pattern was observed in rhizomes (Fig. 6.6C), where soluble sugars did not change from pre-dawn to noon in high-CO_2, but increased significantly in the reference site. No effects of time of day, site and plant part (leaves / rhizomes) were observed on starch concentration (Fig. 6.6B, D).

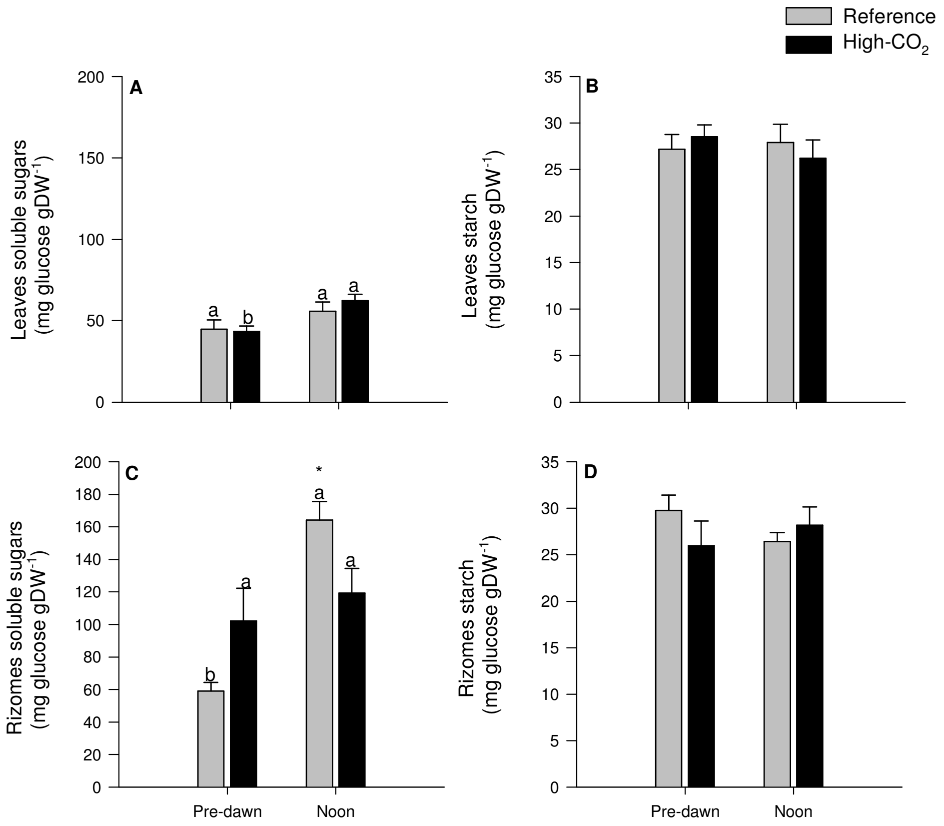

Figure 6.6 – Soluble sugars and starch concentrations in *Cymodocea oceanica* leaves (A, C) and rhizomes (B, D), in reference and high-CO_2 conditions, at pre-dawn and noon. Different letters indicate significant differences between pre-dawn and noon; * indicates significant differences between leaves under reference and high-CO_2 conditions (n=5, p<0.05).

4. Discussion

Our results revealed that *C. nodosa* plants from the high-CO_2 site were submitted to significant levels of oxidative stress. This is supported by the high concentrations of malondialdehyde (MDA) at pre-dawn and at noon, despite the high global antioxidant capacity (TEAC and ORAC assays), the high concentrations of phenolic compounds and the high levels of activity of the enzymes APX and DHAR. The higher neoxanthin, lutein, and zeaxanthin concentrations in plants from high-CO_2 at pre-dawn can also be related to the increased antioxidant protection required by these plants.

MDA is a product of the peroxidation of membrane lipids and is usually associated with oxidative stress (Hodges *et al.,* 1999), often under high light conditions. However, the fact that MDA concentrations did not increase towards noon, and actually decreased on both sites (Fig. 6.1A), suggests that the oxidative stress was induced by factors other than light. High concentrations of methane, hydrogen sulphide (H_2S), metals (Boatta *et al.,* 2013) and toxic trace elements (Apostolaki *et al.,* 2014) have been reported for this venting site and were also found in *C. nodosa* leaves (Vizzini *et al.,* 2013). All these substances are potential causes for the high levels of oxidative stress observed.

The higher concentration of *C. nodosa* phenolic compounds observed in the high-CO_2 site can be related with antioxidant defence (Fig. 6.1B). Some phenolic compounds such as flavonoids, phenylopropanoids, hydroxycinnamic acids (Sharma *et al.,* 2011), tannins and lignin precursors are effective antioxidants (Blokhina *et al.,* 2003). They have the ability to scavenge ROS during stress events and act as metal chelators in plants under heavy metal stress (Sharma *et al.,* 2011). In contrast to our results, Arnold *et al.* (2012) found lower concentrations of phenolic compounds in *C. nodosa* growing close to the vents in Vulcano than on plants from the reference site. A number of reasons can justify these opposite results. Under high-CO_2 levels, the allocation of the different defensive compounds (among which are several phenolics) can be significantly influenced by other circumstantial external factors (Coviella *et al.,* 2002), As it has been documented (Boatta *et al.,* 2013) the area affected by the vents' emission and the CO_2 gradient changes widely with the water circulation in the bay, as driven by the wind direction and water agitation. Differences in temperature, light and the time of sampling are also examples of factors that can concur to the opposite pattern observed in the phenolics concentration between the present work and Arnold *et al.* (2012). Although in the present work the phenolics concentration was always higher in leaves from high-CO_2, the difference between sites was more noticeable at noon. This may indicate that if a plant is already submitted to a certain stress, light increase can turn the situation even more demanding. The increase of phenolics towards noon could even be used to alleviate the stress, explaining the MDA decrease at that time.

The antioxidant capacity (TEAC assay) of *C. nodosa* was always higher in high-CO_2 site regardless of light (Fig. 6.1C). The oxygen radical absorbance capacity (ORAC assay) increased towards noon in the high-CO_2 site, but there were no differences between the two sites (Fig. 6.1D). Antioxidants are triggered by an increase in reactive oxygen species (ROS) in order to protect the plant against oxidative stress (Gill and Tuteja, 2010). TEAC and ORAC assays together provide a broad perspective on the global ROS scavenging capacity of the plant. Short- and medium-term experiments in land plants showed that the antioxidant capacity decrease under high-CO_2 (Levine and Paré, 2009; Vurro *et al.,* 2009; Gillespie *et al.*, 2011). Nonetheless, these plants showed an increase in antioxidant response when submitted to an acute stress due to high-ozone concentrations, suggesting the existence of a distinct regulation of the antioxidant system (Gillespie *et al.*, 2011). This supports our hypothesis that plants from the high-CO_2 site triggered the antioxidant defence as a response to factors other than CO_2, namely the high concentrations of H_2S and/or trace elements. The activity of ascorbate peroxidase (APX) decreased towards noon in the reference site while it remained constant in the high-CO_2 site (Fig. 6.2A), indicating a synergistic effect of high light and high-CO_2 on APX activity. The concentrations of hydrogen peroxide (H_2O_2) remained constant along the day on both sites (Fig. 6.3), which indicates that the differences in APX activity between sites at noon were able to maintain H_2O_2 concentrations under control (Asada, 1999; Pang and Wang, 2010).

The activity of dehydroascorbate reductase (DHAR) was higher on *C. nodosa* leaves from the high-CO_2 site (Fig. 6.2B), both at pre-dawn and at noon suggesting that the higher ascorbate regeneration by DHAR activity in high-CO_2 leaves resulted from a demand for more antioxidant activity on the leaves, supporting the hypothesis that plants near the CO_2 vents were under higher oxidative stress. . DHAR recycles dehydroascorbate (DHA) to ascorbate (AsA) in the AsA-GSH cycle, regulating the redox status of the cells (Chen *et al.,* 2003; Sharma *et al.,* 2011). Once formed, and if not rapidly recycled, DHA will be hydrolysed and lost (Chen *et al.,* 2003). AsA is the major antioxidant on plants. It contributes to the redox status of the cells (Chen *et al.,* 2003) and constitutes as substrate for APX and for violaxanthin de-epoxidase in the xanthophyll-cycle (Asada, 1999; Niyogi, 1999; Jahns *et al.,* 2009). Being a non-enzymatic antioxidant, ascorbate represents the first line of defence against potentially damaging external oxidants, with the ability to react directly with superoxide and hydrogen peroxide (Sharma *et al.,* 2011). AsA synthesis is regulated by the AsA pool, which is maintained according to external and internal stimuli (Pallanca and Smirnoff, 2000).

The activity of glutathione reductase (GR) was identical on both sites and periods of the day (Fig. 6.2C). GR regenerates reduced glutathione (GSH) from oxidised glutathione (GSSG) (Neto *et al.,* 2006), using NADPH as subtract (Sharma *et al.*, 2011). GSH level is influenced by sulphur nutrition (Han *et al.,* 2012) and although regeneration is usually rapid, under situations were sulphur dioxide (SO_2) or hydrogen sulphide concentrations (H_2S) are high, the system responds with an increase in cysteine concentrations (Wonisch and Schaur, 2001) and GSH burst as a mean to detoxify cells by assimilation of sulphur compounds into cysteine (Anderson and McMahon, 2001). Exogenous H_2S can then up-regulate the glutathione metabolism, and through this way protect or alleviate plants from stress (Shan *et al.,* 2012; Christou *et al.,* 2013; Shan *et al.,* 2014). The assimilation of sulphur is much higher in marine algae than in terrestrial plants (Leustek and Saito, 1999). In our high-CO_2 sampling site the H_2S concentration is also high (Sedwick and Stüben, 1996; Armend *et al.,* 2003; Boatta *et al.,* 2013) and the GSH biosynthesis is a strong explanation for the observed GR results. Glutathione also serves as the first line of defence against ROS, toxins, xenobiotics and heavy metals (Leustek and Saito, 1999), hence GSH synthesis can also alleviate high-CO_2 leaves from the stressful effects of trace elements and toxic metals present in the volcanic emissions.

The concentrations of lutein and neoxanthin were higher on *C. nodosa* leaves from high-CO_2 site, at pre-dawn, following the same pattern as MDA, suggesting that the enhancement on those pigment concentrations is connected with the prevention of lipid peroxidation (Tab. 6.1). Lutein and neoxanthin are responsible by structural stability (Gruszecki *et al.*, 1999) and by quenching ROS (Namitha and Negi, 2010) of LHCII, which can have a protecting effect by preventing lipid peroxidation during over-excitation periods (Namitha and Negi, 2010).

The concentration of β-carotene decrease in in high-CO_2 site from pre-dawn to noon, to values bellow from the reference site, suggesting a downregulation of pigments from this biosynthetic way on high-CO_2 leaves. β-carotene is the precursor of zeaxanthin, antheraxanthin, violaxanthin and neoxanthin (DellaPenna, 1999). The decrease in neoxanthin and violaxanthin, along with energy allocation to other processes might be the explanation for β-carotene downpregulation.

When exposed to high light, at noon, plants in both sites reacted as expected showing a decrease in lutein-epoxide and violaxanthin concentrations and an increase in AZ/VAZ ratio (Fig. 6.4) similar on leaves from both sites indicating the need to dissipate an identical amount of energy, since the de-epoxidation of antheraxanthin and zeaxanthin was equivalent (Gilmore *et al.,* 1995; Yamamoto and Bassi, 1996). Nevertheless, at pre-dawn, the concentration of zeaxanthin was higher in high-CO_2 leaves, which indicates that even in the absence of light these leaves had a higher need to dissipate energy. High zeaxanthin concentration in the absence of high light have been associated with other types of stress and is associated with this pigment antioxidant function (Fernández-Marín *et al.,* 2011). Zeaxanthin can remove epoxy groups from the oxidized double bands of fatty acids of thylakoids (Lichtenthaler and Babani, 2004), prevent the formation of (Ruban and Johnson, 2010) and scavenging ROS (Betterle *et al.,* 2010), and together with violaxanthin it also plays an important role in membrane stability (Havaux, 1998).

Soluble protein was always higher in leaves from the reference site and constant throughout the day (Fig. 6.5). More than 40% of the total leaf soluble protein is RuBisCO (Taiz and Zeiger, 2003). Photosynthetic acclimation to high-CO_2 can be characterized by a reduction in RuBisCO content, usually corresponding to adjustments on the photosynthetic capacity (Wittenbach, 1983; Simova-Dtoilova *et al.,* 2001). The decrease in RuBisCO under high-CO_2 can be explained by a decrease in the carboxylation process (Stitt *et al.*, 1991), signaled by the accumulation of sugars in the leaf, and eventually by a nitrogen-mediated signaling when nitrogen limitation occurs (Stitt and Krapp, 1999). In fact the combination of high-CO_2 with nitrogen limitation can potentiate both the decrease in the carboxylation capacity and in RuBisCO concentration (Stitt and Krapp, 1999). Since high-CO_2 promotes leaf growth (Jiang *et al.,* 2010), these plants require a higher nutrient availability and therefore will experience more acutely the effects of nutrient limitation (Stitt and Krapp, 1999).

According to Apostolaki *et al.* (2014), the water around the Vulcano CO_2 vents has lower levels of nitrogen, compared with the reference sites. It is therefore possible that together with high CO_2, nitrogen limitation could have potentiated the observed lower concentration of soluble leaf protein.

The concentration of leaf soluble sugars was the same on both high-CO2 and reference sites (Fig. 6.6A). In contrast, sugar concentration on rhizomes from the reference site increased significantly between pre-dawn and noon (Fig. 6.6C) and that was not observed in the CO_2 site. On the other hand, starch maintained constant concentrations on plants from both sites along the day (Fig. 6.6B,D). This means that plants from reference and CO_2 sites have different energy use and allocation strategies, which is also evidenced by the fact that, at noon, while plants from high-CO_2 accumulate more sugars in leaves, reference plants accumulate more in the rhizomes. Probably, the accumulation of sugars in leaves in the high-CO_2 site is mainly due to higher energetic demands, whereas reference plants, with lower immediate energy demands, can translocate part of the noon-produced sugars to the rhizomes. Leaves growing at high-CO_2 tend to accumulate higher levels of non-structural carbohydrates, both sugars and starch (Moore *et al.,* 1998; Ehleringer *et al.,* 2002). This pattern was verified not only in land plants (Stitt *et al.*, 1991), but also in seagrasses like *Talassia hemprichii* (Jiang *et al.,* 2010), *Zostera marina* (Zimmerman *et al.,* 1997) and *Talassia testudinum* (Campbell and Fourqurean, 2013). Nevertheless, *Cymodocea nodosa* from the CO_2 site at Vulcano revealed lower carbon content in leaves (Apostolaki *et al.,* 2014). The carbohydrate accumulated under high-CO_2 environment is thought to be species specific in the sense that while some species store more starch others prefer to accumulate sugars (Makino and Mae, 1999). The high carbohydrate accumulation and the lower photosynthetic rates found in many species under high-CO_2 can be related with photosynthetic rates that exceed the sink capacity for growth (Makino e Mae, 1999; Mohile and Wakte 2011), but may also be related with the sucrose cycling in leaves through the enzymes acid invertase and hexokinase (Moore *et al.,* 1998). Hexokinase acts as a sucrose sensor and directly affects the expression of photosynthetic genes (Urbonaviciute *et al.,* 2006). Exposure to high-CO_2 may lead to an increased carbon flux to sucrose. Under sink-limited conditions, this may result in an increased accumulation of hexose from the vacuolar or apoplastic hydrolysis of sucrose, mediated by acid invertase. That accumulation will signaling to photosynthesis downregulation (Cheng *et al.,* 1998 and references therein).

In conclusion, despite the observed increases in TEAC, DHAR, phenolic compounds, carotenoids and probably GSH synthesis, leaves from the high-CO_2 site were undoubtedly under oxidative stress. Identical concentrations of photosynthetic pigments in both sites at noon showed similar light harvesting capacity, but the lower soluble protein concentration in high-CO_2 plants suggests a downregulation of photosynthesis. Coupled to a permanent and energetically expensive process of antioxidant defence, low photosynthetic rates of the high-CO_2 plants can help to explain the reduced expansion capacity of these meadows found by Apostolaki *et al.* (2014). In fact, antioxidant defence consumes significant resources, suggesting that under stressful conditions these will be at least partially diverted to antioxidant defence, in detriment of other metabolic functions (Herms and Mattson, 1992). Besides the high-CO_2 levels, the interaction of other factors such as low nitrogen (Apostolaki *et al.,* 2014), often associated with high-CO_2 environments (Stitt and Krapp, 1999), also H_2S, trace substances and toxic metals (Boatta *et al.*, 2013; Apostolaki *et al.*, 2014) needs be taken into account and considered as the most likely cause for oxidative stress on these plants. Hence, the use of volcanic CO_2 vents as natural laboratories for investigating the effects of high-CO_2 on seagrass performance needs to be dealt with cautiously, given the presence of factors other than CO_2, which can easily give origin to confounding responses, particularly when these factors are site-specific and have no foreseeable association with high-CO_2 in the predicted climate change scenarios.

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Main conclusions

1 - Antioxidant defence mechanisms identical to those described for land plants were found in the seagrasses *Posidonia oceanica, Cymodocea nodosa, Zostera marina* and *Zostera noltii* and were activated in response to light, temperature and CO_2-rich volcanic emissions.

2 - The antioxidant capacity of *Posidonia oceanica, Cymodocea nodosa, Zostera marina* and *Zostera noltii* was achieved through the operation of reducing agents associated to the presence of carotenoids, phenolic compounds, superoxide dismutase, ascorbate peroxidase, ascorbate, glutathione and NADPH, and through the operation of hydrogen donors such as carotenoids, phenolic compounds, ascorbate and tocopherols. The antioxidant enzymes ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutatione reductase (GR) and catalase (CAT) were also found to be active in these species**.**

3 - The higher activity of APX and CAT found on *Z. noltii* leaves when compared with *Zostera marina*, *Cymodocea nodosa* and *Posidonia oceanica*, suggests higher antioxidant response from intertidal species to demanding situations of high light and temperature.

4 - The subtidal species *Zostera marina*, *Cymodocea nodosa* and *Posidonia oceanica* invested more in the regeneration of reducing agents than intertidal species, as indicated by DHAR and GR activities. These results suggest the preferential use of low energetic cost mechanisms, when the conditions are not severe.

5 - The photosynthetic pigments of all the seagrass species analysed, *Halophila ovalis*, *Halophila stipulacea*, *Zostera noltii*, *Zostera marina*, *Zostera capricorni*, *Cymodocea nodosa* and *Posidonia oceanica*, were similar to land plants. They showed the same carotenoids, similar major compounds and no presence of carotenoids specifically found in algae species. This indicates that marine angiosperms maintained their ancestral land photosynthetic characteristics in the adaptation to seawater.

6 - The photosynthetic pigments found in the analysed seagrass species were chlorophylls a and b, α-carotene, β-carotene neoxanthin, violaxanthin antheraxanthin,

zeaxanthin, lutein and lutein-epoxide (taraxanthin). α-Carotene was only identified in *Posidonia oceanica* and lutein-epoxide was not identified in *Zostera capricorni*. These carotenoids are also not ubiquitous in terrestrial species. As in land plants, the concentrations of neoxanthin, lutein and β-carotene were not affected by light intensity. Nevertheless, seagrass light adaptation strategies might be slightly different from land species, since lutein was not always the major compound and the proportion of chlorophylls to carotenoids was lower.

7 - The VAZ-cycle was activated in all seagrasses as a photoprotection mechanism under high light (solar noon). The lutein-epoxide cycle was only detected in two out of nine species and ecotypes (*C. nodosa* and *H. stipulacea*), which suggests that it only exists in some seagrasses and under particular conditions. The diadinoxanthin-cycle, found in some algae, was not detected in the tested seagrass species.

8 - *Cymodocea nodosa* is very tolerant to thermal and light extremes, combining an increase in the antioxidant response, VAZ cycle and soluble sugar accumulation. The most challenging condition for this species was lower temperature, especially when combined with the higher light intensity. Under these conditions, Fv/Fm values indicated photoinhibition that coupled with the absence of zeaxanthin implied a potential for photo-damage. Nevertheless, ascorbate peroxidase activity, antioxidant capacity (TEAC assay), soluble sugars and chlorophyll a/b ratio increased to scavenge oxidant species (ROS), thus avoiding oxidative stress.

9 - The presence of epiphytes on *Posidonia oceanica* leaves resulted in a reduction of net photosynthetic rates probably due to lower light availability, but also in an increase in oxidative stress despite the enhancement of the antioxidant responses and the allocation of energetic resources to these protection mechanisms. Oxidative stress may thus result from the physical or chemical aggression of epiphytes, which were mostly calcifyers. Ocean acidification may release seagrasses from this stress.

10 - *Cymodocea nodosa* plants living near the submerged volcanic vents of Vulcano Island (Italy) showed to be under oxidative stress despite the enhancement of the antioxidant capacity (TEAC), DHAR, phenolic compounds, carotenoids and probably GSH synthesis. The low concentration of soluble proteins indicates a down-regulation of photosynthesis. This shows that the use of volcanic vents to test the effects of CO_2 on

seagrasses has to be taken very carefully as other stressful factors such as the presence of H_2S, trace substances and toxic metals may confound the results. These other components are the most probable cause for the oxidative stress observed in *C. nodosa*, forcing plants to permanently increase their basal level of antioxidants, at the cost of lower growth and expansion rates.