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DOKUZ EYLÜL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

THE RELATIONSHIP BETWEEN CAULERPENYNE SECRETION AND ANTIOXIDANT STATUS IN *CAULERPA SP.*

by

Levent ÇAVAŞ

March, 2005

İZMİR

THE RELATIONSHIP BETWEEN CAULERPENYNE SECRETION AND ANTIOXIDANT STATUS IN *CAULERPA* SP.

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by

Levent ÇAVAŞ

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İZMİR

Ph.D. THESIS EXAMINATION RESULT FORM

We have read the thesis, entitled **"THE RELATIONSHIP BETWEEN CAULERPENYNE SECRETION AND ANTIOXIDANT STATUS IN *CAULERPA* SP."** completed by **Levent ÇAVAŞ** under supervision of **Prof. Dr. Kadir YURDAKOÇ** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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THE RELATIONSHIP BETWEEN CAULERPENYNE SECRETION AND ANTIOXIDANT STATUS IN *CAULERPA SP.*

ABSTRACT

Antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), *in vivo* play important roles for removal of damaging effects caused by reactive oxygen species in living systems. The activities of these antioxidant enzymes, lipid peroxidation (LPO) levels as a membrane damage marker and caulerpenyne (CYN) levels were investigated in *Caulerpa* species (*C.taxifolia, C. racemosa, C. prolifera*) were kept in aquaria with artificial seawater, and *C.racemosa, Padina pavonica, Enteromorpha sp., Cystoseria barbata, Oxynoe olivaceae, Lobiger serradifalci* and *Haminoe hydatis* which were collected from Seferihisar-Izmir. According to results, the highest SOD enzyme activity was found in *C.prolifera* compared to other *Caulerpa* species. *Caulerpa racemosa* which had invasive property for Seferihisar coastline between 2003 and 2004 had maximum antioxidant enzyme activities and minimum lipid peroxidation levels compared to other Mediterranean macrophytes such as *Padina pavonica, Enteromorpha sp.* and *Cystoseria barbata.* Maximum CYN level was observed in the *C. prolifera* compared to other species. On the other hand, *C.racemosa* which was collected in May-2003 had maximum CYN levels compared to other seasons. In this Ph.D. thesis, antioxidant status of the important *Caulerpa* consumers, *Oxynoe olivaceae* and *Lobiger serradifalci* and a sea slug which was very common near *Caulerpa* meadows called *Haminoe hydatis* were also investigated. Inasmuch as *Caulerpa sp* has higher antioxidant enzyme activities compared to other macrophytes, invasion of properties of *Caulerpa sp* may be considered to correspond with antioxidant status.

Key words: antioxidant enzymes, biological invasion, *Caulerpa* sp., sea slugs

CAULERPA TÜRLERİNDE CAULERPENİN SALINIMI VE ANTİOKSİDAN DURUM ARASINDAKİ İLİŞKİ

ÖZ

Antioksidan enzimlerden; süperoksit dismutaz (SOD), katalaz (CAT), glutatyon peroksidaz (GSH-Px) canlı sistemlerde reaktif oksijen türlerinden kaynaklanan hasar etkilerinin giderilmesinde önemli roller oynamaktadırlar. Bu antioksidan enzimlerin aktiviteleri ve hücre membran hasarının göstergesi olan lipid peroksidasyon (LPO) ve caulerpenin (CYN) düzeyleri akvaryum içerisinde suni deniz suyunda yetiştirilen *Caulerpa* türlerinde (*C.taxifolia*, *C.racemosa*, *C.prolifera*) ve Seferihisar kıyılarından mevsimsel olarak toplanan *C.racemosa, Padina pavonica, Cystoseria barbata, Enteromorpha sp., O.olivacea, L.serradifalci* ve *H.hydatis*'te ölçülmüştür. Sonuçlara göre en yüksek SOD enzim aktivitesi diğer *Caulerpa* türlerine kıyasla *Caulerpa prolifera*'da tespit edilmiştir. Seferihisar kıyılarında 2003-2004 yılları arasında toplanan ve yayılımcı özellik gösteren *C.racemosa*'da *Padina pavonica, Cystoseria barbata, Enteromorpha sp* gibi diğer Akdeniz makrofitlerine kıyasla daha yüksek antioksidan enzim aktiviteleri ve düşük lipid peroksidasyon düzeyleri olduğu saptanmıştır. Maksimum CYN düzeyleri *C.prolifera*'da ölçülmüştür. Diğer yandan, Mayıs-2003 te toplanan *C.racemosa* da diğer sezonlara göre daha yüksek CYN düzeyi tespit edilmiştir. Bu doktora tezinde ayrıca *Caulerpa* tüketicileri olan *L.serradifalci, O.olivacea* ve *Caulerpa* biyotopunda yaygın olarak rastlanan *H.hydatis* türlerinin de antioksidan düzeyleri incelenmiştir. *Caulerpa* türleri genel olarak diğer Akdeniz makrofitlerine kıyasla daha yüksek antioksidan enzim aktivitelerine sahip olduklarından ötürü bu türlerin yayılımında antioksidan durumun ilişkisi olduğu söylenebilir.

Anahtar Kelimeler: antioksidan enzimler, biyolojik yayılım, *Caulerpa* türleri, deniztavşanları

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CHAPTER ONE
INTRODUCTION

1. Introduction

1.1 Invasive *Caulerpa* species in Mediterranean

For the last two decades, the Mediterranean Sea has been under invasion of two exotic algae species, *Caulerpa taxifolia* and *Caulerpa racemosa* (Verlaque, Boudouresque & Meinesz, 2000). *C. taxifolia* was introduced to the Mediterranean due to the accidental release of it from a public aquarium in Monaco. When recorded for the first time in 1984, its distribution had been in the order of $1m^2$ around Monaco shores, however in 1990 the expansion had gone up to 3 hectares and by the year of 2000, it had invaded more than hundred independent localities in six countries; Monaco, France, Spain, Italy, Croatia and Tunisia (Meinesz et al., 1993, Meinesz et al., 2001). Yet, the same invasive form was also reported from California (Jousson et al., 2000). Molecular studies showed that this invasive strain is world-wide common in public aquaria and is closely related to a native population found in Manly Harbour, Moreton Bay, Australia (Schaffelke, Murphy & Uthicke, 2002). The only way of reproduction observed for *C. taxifolia* in the Mediterranean is budding. In addition, the fragmentation of the thallus is the most important factor that enhances its spread (Žuljevic & Anatolic, 2000). The *C. taxifolia* fronds are usually fragmented by waves or human activities such as anchoring, trawling, fishing nets, etc. (Sant, Delgado, Rodriguez, Prieto& Ballesteros, 1996). The drifting fragments can grow and form a new colony or strengthen the existing canopy (Smith &Walters, 1999).

1.2 Chronological Information on *Caulerpa* Phenomena in Mediterranean (from Madl & Yip, 2003)

1.2.1 *Caulerpa taxifolia*

"The chronology of the Mediterranean invasion of *C.taxifolia* reads like a tale of missed chances and spoiled opportunities:

1984: a few meters deep, A. Meinesz, Professor at the University of Nice-Sophia Antipolis, localizes a patch of C.taxifolia about a $1m^2$ in size situated just the base of the Oceanographic Museum in Monaco (Meinesz & Hesse, 1991);

1989: repeated surveys reveal that the original site of infestation extends to cover an area about 1ha in size;

1990: in summer of that year, the alga has spread along the southeastern coast of France. First outcrops are also spotted some 100km west of Monaco at Cap Martin and Toulon;

1991: by now the radius of infestation stretches as far as 400km west of Monaco at St.Cyprien close to the Spanish border;

1992: early that year and for the first time, the alga is spotted at sites opposing the westward drifting Ligurian current: e.g. Imperia (Italy); on the western front it continues its spread westward to hit the coast of the Balearic Island of Majorca (Spain);

1993: the south-eastward extension of the algae continues; sites of infestations are registered at the island of Elba (Italy) and as far south at Messina in the strait of Sicily (Italy);

1995: eventually the algae reach the Adriatic Sea; first sightings are reported at Malinksa and Stari Grad (Croatia);

1997: by the end of that year, the tumor-like growth of individual patches of *C.taxifolia* carpets along the south-eastern coast of France have drastically increased in size and eventually merge to form huge meadows;

2000: in ever more rapid events, *C.taxifolia* reaches the southern shores of the Mediterranean and is sighted at Sousse (Tunisia). In the very same year a large patches of this invasive species is identified in the lagoon off San Diego (California, USA) (Williams & Grosholz, 2002). At the same time patches of this invasive strain are sighted in Port Hacking, Lake Conjola and Careel Bay (in New-South Wales, AUS);

2001: eventually other sites if infestation are identified at sites along the south-eastern coast of Australia: e.g. Narrawallee Inlet, Burrill Lake, Lake Conjola and Berringer Lake, Careel Bay, Pittwater, Lake Macquarie all in New South Wales and West Lakes & Port River in South Australia".

1.2.2 *Caulerpa racemosa*

1926: First observation of *C.racemosa* in Mediterranean (Sousse Harbour, Tunisia) (Hamel, 1926).

1931 - 1976: It was not regarded as a potential invader (Hamel, 1931; Mayhoub, 1976).

1991: Observation of a distichously branched form of *C. racemosa* unknown to the Mediterranean (Nizamuddin, 1991).

2002: This unknown taxon occurred in at least 11 Mediterranean countries (Albania, Croatia, Cyprus, France, Greece, Italy, Libya, Malta, Spain, Tunisia and Turkey) and all the large islands (Balearic Islands, Corsica, Crete, Cyprus, Sardinia and Sicily) (Verlaque, 1994; Verlaque et al, 2003).

2003: DNA ITS analysis showed identity of invasive *C. racemosa*: the new combination *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman *et* Boudouresque.

Although its way of introduction is not yet clear, it is thought that *C. racemosa* is an immigrant from the Red Sea (Verlaque & Fritayre, 1994). It is a widely observed species in tropical and subtropical seas and is invading the Mediterranean basin at a faster rate than *C. taxifolia*. Its presence in the Mediterranean was first reported in 1926 from Tunisia (Hamel, 1926). Three *C. racemosa* variations have been reported from the Mediterranean; *C. racemosa var. turbinata-uvifera* in the North African coast and eastern Mediterranean, var. *lamourouxii f. requienii* in the eastern Mediterranean and "invasive" variation known to be spreading all throughout the Mediterranean since 1990 (Verlaque et al., 2000). This invasive form shows a fast invasion rate since it reproduces not only by fragmentation but also sexually (Panayotidis & Zulcevic, 2001). Moreover, its ability to adapt to temperature changes, depth and sediment structure enhances its distribution. This invasive form was previously regarded as close to *C. racemosa var. occidentalis*, but renamed as *C. racemosa var. cylindracae* in the light of recent molecular studies (Verlaque et al., 2003). *Caulerpa scalpelliformis* is another *Caulerpa* species and its existence was reported in Antalya Harbour in the year 1995. However, after 1995, this species seemed to disappear in this coastline (Ertan, Turna & Cormaci, 1998).

1.3 Biological Information on *Caulerpa* species (from Madl & Yip, 2003)

1.3.1 Taxonomy

KINGDOM Protista (Gk. protos, first) by Raven et al., 1992 or Plantae (L. plant);
DIVISION / PHYLUM Chlorophyta (Gk. chloros, green + phyto, plant)

CLASS Ulvophycea (L. ulva, sedge + phyto, plant)

ORDER *Caulerpa*les (L. caulis, stalk) referring to the stalk of the algae

FAMILY *Caulerpa*ceae (L. caulis, stalk)

GENUS *Caulerpa* (L. caulis, stalk)

SPECIES taxifolia (L. taxi, yew-like + folia, leaves) referring to the dark-green and flattened foliage

Caulerpa species that are member of order *Caulerpa*les, consists of marine (sub-) tropical macrophytes. They have multinucleate siphonous forms, without cross-walls or segregative division, filled with numerous plastids (e.g. amyloplasts which is unpigmented plastids for starch storage). Therefore, forming one huge cell is open to loss substantial plasma. However, *Caulerpa* species are equipped with efficient wound healing properties (rapid wound damage, recovery occurs in second involving acting-mediated contraction and a plug of cell wall materials). Cell wall consists of mostly xylan rather than cellulose and is reinforced with a multi axial construction and sometimes even stabilized with calcium carbonate deposits as in Halimeda sp (Strassburger, 1998). *Caulerpa* belongs to siphonoxanthin and siphonein as photosynthetically active pigments.

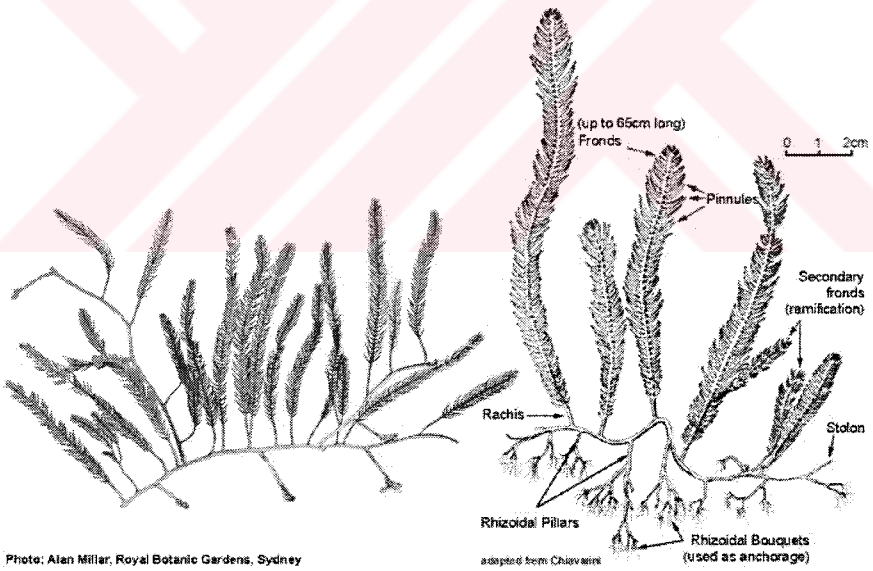


Figure 1.1. *Caulerpa taxifolia*

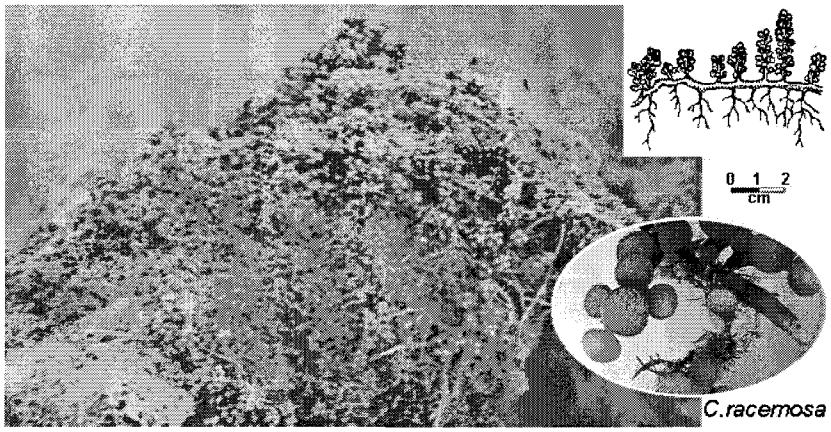


Figure 1.2. ***Caulerpa racemosa***

Caulerpa racemosa: algae with long stolons; its racemous-rhizoids point downward. Photosynthesis occurs in the clustered aggregations that, opposing the rhizoids, merely reach a few cm in height. Growth pattern of phtotosynthetic thalli can vary from bifurcated with tiny branches to entirely rounded bubbles. This alga is likewise extremely invasive in Mediterranean subtidal habitats and as with C.taxifolia causes major modifications to benthic communities (Verlaque & Fritayre, 1994; Villele & Verlaque, 1995; Bellan-Santini et al., 1996; Ceccherelli & Cinelli, 1997; Piazzi & Cinelli, 2001). It is currently in the process of spreading into the western Mediterranean (Piazzi et al., 1994, 1997; Gambi & Terlizzi, 1998; Modena et al., 2000; Verlaque et al., 2000) and it seems to be capable of even out-competing *C.taxifolia* (Ceccherelli, Piazzi & Balata, 2002).

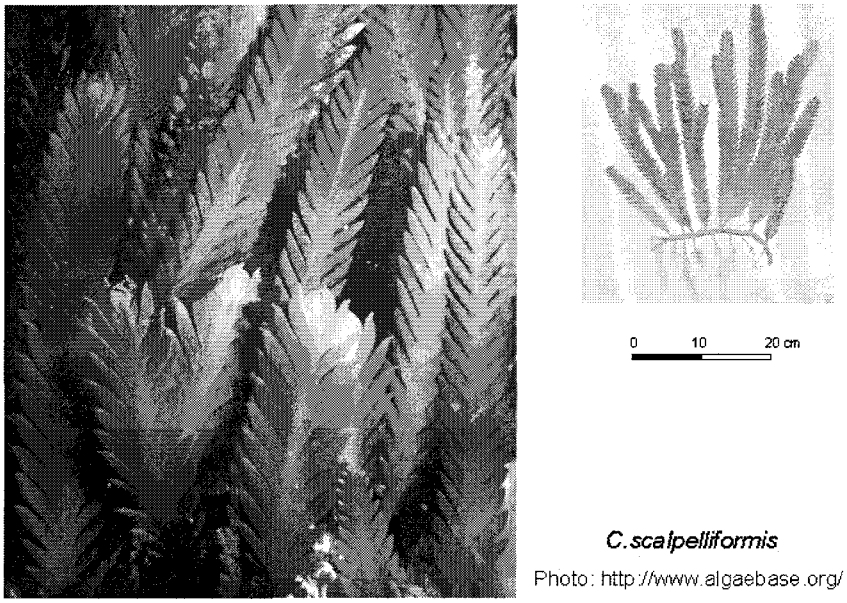


Figure 1.3. ***Caulerpa scalpelliformis***

Caulerpa scalpelliformis: Straight fronds that are about 10-20 cm long. It closely resembles *C.taxifolia* but can be distinguished by the form of its branches which are curved towards the interior in the latter and straight in the former. The rachis of the fronds is quite dominant and thicker than the pinnules. Another feature that distinguishes it from *C.taxifolia* is its pointed pinnules.



Figure 1.4. ***Caulerpa prolifera***

Caulerpa prolifera: This native alga has few but robust stolons with the branching blades protruding perpendicularly into the water in intervals of 1-2 cm. The superior part of each blades house the assimilatory pigments. The blades can reach a length of up to 15 cm and can in some cases be even 13 cm wide. Usually they have an oval or

linearly elongated appearance with a smooth border following a helical pattern towards the apex (Madl & Yip, 2003).

1.3.2 Reproduction

In the aquarium strain of *C.taxifolia* only male gametes have been observed and this strain is apparently only vegetative spreading (Zuljevic & Antolic, 2000). Sexual reproduction of this aquarium strain appears to be a stochastic event (Clifton & Clifton, 1999) as it has been only observed in temperatures above 25°C (Zuljevic & Anatolic, 2000).

Caulerpa's life cycle is therefore poorly understood. But when it does occur it involves "holocarpy", in which the entire protoplasm gives rise to biflagellate gametes at once; i.e. almost all of the protoplast of a thallus is converted into gametangia containing the biflagellate dissimilar (anisogamy) motile gametes, which are discharged through papillae. Upon merger of the gametes, the zygote develops into a protonema, which then forms a typical diploid (2n) thallus (Silva, Azevedo & Manso, 1996). Most siphonous green algae are primarily diploid; the gametes are the only haploid cells in the life cycle (Raven, Evert & Eichhorn, 1992). Since *C. taxifolia*'s life cycle is poorly understood, it could well be that the mature sporophyte releasing quadri-flagellated zoospores that in turn would give rise to the gametophyte (n), is inexistent.

According to Meinesz, under lab-conditions *C.taxifolia* is able to disperse a shower of male and female gametes that pair up and fuse to form a zygote (new plant). But over more than 10 years that he has observed this species in the Mediterranean, he has never seen evidence of sexual reproduction. In the wild, though, the only reproductive cells released are male, confirming existing evidence that all *C.taxifolia* in the Mediterranean are clones of that single aquarium plant release in 1984 (Meinesz & Hesse, 1991).

Genetically, this invasive species shows relatively little variation, thus vegetative reproduction by fragmentation is the most common mode of proliferation (asexual or

clonal propagation). The break-up of thalli (mediated via anchor damage, fishing gear or storm activity as small as 1 cm^2) gives rise to new colonies that usually appear between 2 and 10 m deep during summer and fall when growth rates are highest (Meinesz et al., 1995; Madl & Yip, 2003).

1.3.3 Control Strategies and Secondary Metabolites Secreted from *Caulerpa*

With their toxic properties and potential to overgrow natural biocoenoses such as Posidonia or Zostera meadows, exotic *Caulerpa* species are thought to be a threat for the ecology and biodiversity of the Mediterranean Sea (Boudouresque, Lemee, Mari & Meinesz, 1996; Romero, 1997). Many attempts have been made to control the spread of *C.taxifolia* and several different methods have been applied, including manual eradication, coverage with photo resist foil, application of hot water and osmotic stress (Jaffrennou & Odonne, 1994; Escoubet, Dupeux, & Escoubet, 1998; Gavach et al., 1999, Robert & Gravez, 1998). However, these elaborate efforts succeed only in small scale and yet, the invasion of *C. taxifolia* could not be still stopped.

The potential use of four Sacoglossans (Mollusca: Opisthobranchia); *Oxynoe olivacea*, *Oxynoe azuropunctata*, *Lobiger serradifalci* and *Elysia subornata* as biological control agents against *C.taxifolia* (and *C.racemosa*) has been studied (Meinesz et al., 1996; Thibaut et al., 1998; Thibaut & Meinesz, 2000; Thibaut et al., 2001). *Oxynoe azuropunctata* and *Elysia subornata* are host-specific feeders on *Caulerpa spp.* and inhabit tropical northwest Atlantic. In laboratory experiments, it was observed that they consume *C.taxifolia* fronds 3-4.5 cm and 5-6 cm per day, respectively, but could not survive in the Mediterranean winter conditions. On the other hand, the Mediterranean species, *Oxynoe olivacea* and *Lobiger serradifalci* which were known to feed on native *C. prolifera*, have been found to switch their diet to exotic *Caulerpa sp.* But their low feeding rates and planktothrophic larval development make them inefficient biocontrol agents. Besides, *L. serradifalci* fragments the *C. taxifolia* fronds while feeding, thus enhances its dispersion (Žuljevic et al, 2001; Madl & Yip, 2003).

The consumption of oxygen by aerobic organisms results in potentially deleterious reactive oxygen species (ROS) which may react in an aggressive manner, destroying cellular compartments, tissues and finally organisms and populations (Halliwell & Gutteridge, 1999). Altered oxygen metabolism generally leads to a disease or an environmental adaptation (Hippeli, Heiser & Elstner, 1999). The balance between oxidants and antioxidants is vital in all aerobic organisms. Although they seem to be inefficient, the native *O. olivacea* and *L. serradifalci* are the only two sacoglossans in our hands, which may have an effect on the expansion of invasive *Caulerpa spp.* in the Mediterranean. Therefore not only the feeding rate, but also their metabolic activities are of great importance. The antioxidant status of an organism gives vital information about the capability of it to resist the environmental stress. Any data concerning to antioxidant status of sea slugs could not be found in our literature survey. SOD converts the $O_2^{\cdot-}$ (superoxide) radical produced by aerobic respiration and some other various metabolic activities, into oxygen and H_2O_2 (hydrogen peroxide). CAT and GSH-Px are the major antioxidant enzymes that catalyze the decomposition of H_2O_2 to H_2O. These three enzymes constitute the major elements of the cellular defense system against oxidative stress. Membranes, lipoproteins and fatty acids contain double bonds which can be peroxidised in the presence of ROS. The concentration of the resulting lipid oxidation products is the most commonly used criteria in the evaluation of oxidative stress (Cavas, Yurdakoc & Yokes, 2005).

"The accidental introduction of *Caulerpa* taxifolia (Vahl) C. Agardh into the Mediterranean in 1984 (Meinesz & Hesse, 1991) and its success in conquering the new habitat (Meinesz et al., 2001) has only recently put the focus back on these seaweeds. In addition to its light and temperature tolerance (Garcia, Rodríguez-Prieto, Delgado, Ballesteros,1996; Komatsu, Meinesz & Buckles, 1997) and fast growth (Komatsu, 1994), its chemical defense also has facilitated the invasion (Guerriero et al., 1994; Bellan-Santini et al., 1996). Recent monitoring revealed that *C. taxifolia* is not the only invasive *Caulerpa* species spreading in the Mediterranean. One introduced variety of *C. racemosa*, *C. racemosa var. occidentalis* (J. Agardh) Borgesen, with high temperature tolerance, was identified and has been spreading

continuously since the early 1990s (Verlaque et al., 2000). Besides those introduced and invasive species, there exist the indigenous non-invasive *C. prolifera* (Forsskal) Lamouroux that has been proven to be useful in comparison studies for investigations of the factors determining the invasive potential (Sant et al., 1996). The role of the sesquiterpene caulerpenyne (1) (Figure 1.5), in the chemical defense of species of *Caulerpa* has been investigated in numerous studies. Caulerpenyne acts as a feeding deterrent to tropical sea urchins (McConnell et al., 1982) and has been related to the poor food quality that *C. taxifolia* has for Mediterranean sea urchins (Boudouresque et al., 1996).

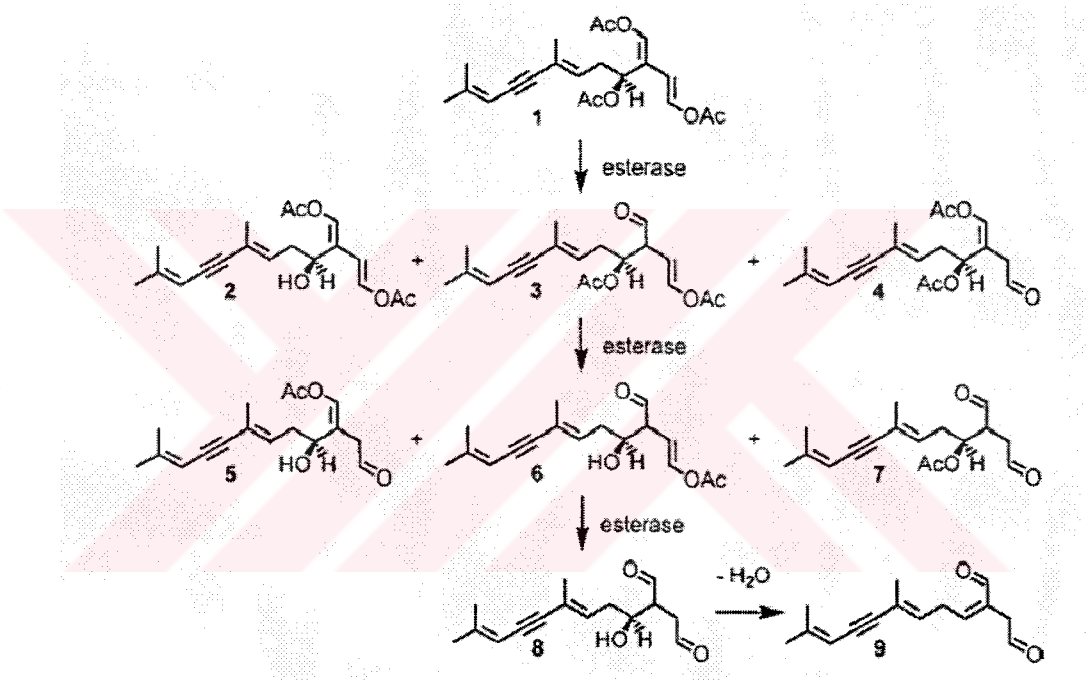


Figure 1.5 Stepwise deacetylation of caulerpenyne 1 by esterases from *C.taxifolia* (4 = oxytoxin 1, 9 = oxytoxin 2) (Jung et al, 2002).

It inhibits the growth of microorganisms, interferes with the development of fertilized sea urchin eggs, and is toxic to larvae and adults of potential herbivores (Paul & Fenical, 1986; Pesando, Lemee, Ferrua, Amade & Girard, 1996; Lemee et al., 1997; Ricci, Capovani & Dini, 1999). On the other hand, studies with herbivorous tropical fishes have shown that caulerpenyne does not defend the plants against these macro herbivores (Meyer & Paul, 1992). Several studies have focused also on the caulerpenyne content in various *Caulerpa* species (Paul & Fenical, 1986;

Meyer & Paul, 1992; Cimino et al, 1999; Amade & Lemee, 1998). Throughout this order, caulerpenyne was found to be the major or one of the major secondary metabolites, the overall content varied depending on species, location, and season. The aquarium derived strain of *C. taxifolia* (Jousson et al., 2000; Wiedenmann, Baumstark, Pillen, Meinesz & Vogel., 2001), spreading in the Mediterranean, can contain up to 1.9% of the algal dry weight (DW) of caulerpenyne, but this value depends on season and depth (Amade & Lemee, 1998). In contrast, Pacific *C. racemosa* species collected in Guam contained only 0.2–0.5% DW. Not only does the caulerpenyne content vary among different species but also within different parts—fronds and stolons—of one individual alga (Meyer & Paul, 1992). While these investigations have mainly focused on caulerpenyne, recent evidence suggests that the presence of caulerpenyne-derived sesquiterpenes in wounded algal tissue also have to be taken into account. It has been found that upon mechanical wounding of *C. taxifolia*, caulerpenyne is rapidly transformed into reactive aldehydes (Jung & Pohnert, 2001). This reaction is comparable to the wound-activated conversion of the diterpene halimedatetraacetate into the potent feeding deterrent halimedatrial in algae of the related genus Halimeda (Paul & van Alystre, 1992). The identified products from *C. taxifolia* and the use of model substrates suggest an enzymatic transformation as shown in Figure 1.5 (Jung & Pohnert, 2001). The resulting aldehydes 4 and 9 from *C. taxifolia* are of the oxytoxin family and have been previously found in defensive secretions and parapods of specialized mollusks feeding on *C. prolifera* (Cimino et al, 1999). Under attack, these mollusks secrete toxic mucus or, if molested further, detach their tail or parapods to distract the enemy and escape. The mucus and the parapods contain the aldehydes 4 and 9 as well as caulerpenyne and are capable of deterring various predators (Cimino et al, 1999; Thibaut et al., 2001). Although directly involved in the defense of slugs against predatory fishes (Cimino et al, 1999), the role of these metabolites in a wound-activated algal defense has yet to be evaluated. Facing the spread of *C. taxifolia* in the Mediterranean, the question arose as to whether the wound-activated transformation of caulerpenyne acting immediately after tissue damage is an important key to success for this species alone or whether this mechanism is universally found among *Caulerpa*les (Jung et al, 2002)". According to our literature

search, no published material existed on the relationship between antioxidant status and caulerpenyne content in the invasive *Caulerpa* sp. existed in the literature.

Antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), *in vivo* play important roles for removal of damaging effects caused by reactive oxygen species in living systems. The activities of these antioxidant enzymes, lipid peroxidation (LPO) levels as a membrane damage marker and caulerpenyne (CYN) levels were investigated in *Caulerpa* species (*C.taxifolia, C. racemosa, C. prolifera*) were kept in aquaria with artificial seawater, and *C.racemosa, P.pavonica, Enteromorpha sp., Cystoseria sp., Oxynoe olivaceae, Lobiger serradifalci* and *Haminoe hydatis* which were collected from Seferihisar-Izmir.

CHAPTER TWO
OXIDANTS AND ANTIOXIDANTS

2. Oxidants and Antioxidants (Bozkaya, 2001)

2.1 Oxidants and Antioxidants in Living Organism

Oxygen is vital for life, yet it is also potentially toxic. To all aerobic organisms it is vital for the production of energy by its controlled reduction to water, yet to most forms of life it caused numerous manifestations of toxicity at greater than normal partial pressure. And also, there is a very strong thermodynamic driving force for the reactions between oxygen and biochemical compounds in the body, like proteins, carbohydrates, lipids and nucleic acids. If such reactions go to completion, water, carbon dioxide and a number of waste products are formed as end products concomitantly with the release of large amounts of energy. Fortunately such reactions occur spontaneously very slowly due to reaction barriers (Halliwel & Gutteridge, 1984). Oxidation of biological compounds is, however, the source of energy of living organisms. However, the direct four-step reduction of oxygen to water is almost unique, and when oxygen reacts spontaneously or catalysed by enzymes it is for mechanistic reasons forced to react one step at a time (Halliwel & Gutteridge, 1984).

Free radicals are molecules or fragments of molecules containing unpaired electrons in their outer orbitals, which represent a free valence. Unpaired electrons tend to acquire a pair; therefore, free radicals are chemically highly reactive and have a short half-life. Free radical reactions are nonenzymatic chain reactions. Free radicals of oxygen play an important role in biology and medicine. They are generated from oxygen by excitation (singlet oxygen $^1O_2$) or reduction (superoxide anion radical $O^{\cdot}_2$, hydrogen peroxide H_2O_2, hydroxyl radical $^{\cdot}OH$). Not all reactive products of the reduction or excitation of oxygen are free radicals (e.g. singlet oxygen, hydrogen peroxide), but their reactivity and reactions resemble those of free radicals. These products, together with the true free radicals are called reactive oxygen intermediates (ROIs) (Feher, Cosmos & Vereckei, 1987).

The importance of oxygen-derived radicals, reactive oxygen species, and antioxidants in health and disease is now recognised by every branch of medicine and biological science. Overwhelming evidence indicates that free radicals play a role in most major health problems of the industrialised world, including cardiovascular diseases, cancer, neurologic disease, and ageing and that antioxidants play a critical role in wellness, health maintenance, and the prevention of chronic and degenerative disease. Oxidants also play a role in some aspects of health, as in the oxidative burst of neutrophyls and macrophage which allows them kill foreign organisms (Halliwel & Gutteridge, 1984).

Potentially damaging oxygen free radicals can be generated within the body, by a variety of processes. The superoxide free radical, particular, is thought to be formed enzymically during oxygen reperfusion following ischemia, during phagocytosis or through the action of toxic chemicals (Willson, 1993).

It is clear that free radical reactions are amongst the panoply of mechanisms leading human cancer and this presents opportunities for intervention to prevent the processes of initiation, or progression. Certainly there is a growing body of preventive role in at least certain types of cancer.

Broadly speaking, there are three potential roles for free radicals and active states of oxygen in environmental carcinonesis: the agent itself may be a free radical or active state of oxygen; these active species may serve as the intermediary by which an environmental chemical produces genetic damage; or the metabolism of the chemical through a free radical dependent step may lead to the formation of a carcinogen (Farber, 1981; Bozkaya, 2000).

2.2 The Production of Superoxide Radicals in Biological Systems

An electroparamagnetic resonance signal, characteristic for superoxide radical, was observed by the rapid-freezing technique in the oxidation at pH 10 of xanthine

by dioxygen catalyzed by xanthine oxidase (EC 1.2.23.2) (Knowles et al, 1969). The enzymatic reduction of dioxygen by aldehyde oxidase (EC 1.2.3.1) produces also the superoxide radical.

The plasma-membrane bound superoxide-generating flavoprotein (superoxide synhetase) of human polymorphonuclear neutrophyls (PMN) has been solubilized by Triton X-100 (Gabig & Bahior, 1979) and deoxycholate (Tauber & Goetzl, 1979) and purified. The superoxide synthetase catalyses the reaction:

$$2O_2 + NAD(P)H + H_2O \longrightarrow NAD(P) + 2O_2^{\cdot-} + H_3O^+$$

Nicotine adenine dinucleotide phosphate (NAD(P)H) seems to react on the cytosolic side of the membrane, while the superoxide anion in generated at the outer side (Green, Schaefer & Makler, 1980).

Following stimulation by the yeast cell wall extract zymosan, phagocytosing human leukocytes show enhanced oxygen consumption, the cyanide-insensitive "respiratory burst", after a lag period of 30-40s. The production of superoxide anions, measured by the reduction of Fe(III)-cytochrome c, followed the same course (Markett et al, 1980). The formation of superoxide radicals was directly demonstrated by spin trapping on stimulated human neutrophils (Green et al, 1980).

A similar enzymatic activity was found in stimulated pulmonary alveolar macrophages (Joseph et al, 1980) and underwater rugby players from humans (Cavas, 2005), tadpoles (Cavas & Tarhan, 2003), and rats (Balasubramanian, Somasundaram & Felix, 2004), and in retinal pigment epithelial cells from chick embryos (Ueno et al, 1979)

A cyanide-sensitive NAD(P)H dependent superoxide generating system was described, however, for the nuclear membrane of hepatoma 22a ascites cells grown in mice (Peskin, Zbarsky & Konstantinov, 1980).

Superoxide radicals can also be produced in the reduction of dioxygen by strong reducing agents, like iron-sulfur proteins, flavins, phenylhydrazine, semiquinones, and thiols (Halliwell & Gutteridge, 1999). Some of the reactions of dioxygen, like the herbicide paraquat in Drosophila (Chen, Rio, Haddad & Ma, 2004).

The production of superoxide radicals was also observed in the "autoxidation" of oxyhemoglobin the methemoglobin: with shark hemoglobin by induction of the oxidatin of adrenaline to adrenochrome (Gotoh & Shikama, 1976). Similarly with human hemoglobin a proton assisted displacement of superoxide by nucleophilic anions has been described (Afanas'Ev, 2004).

Finally superoxide radicals can also be generated photochemically in chloroplasts in the presence of ascorbate or of paraquat. The formation was demonstrated by spin trapping on illumination of spinach chloroplasts in the presence of oxygen (Henmi, Miyao & Yamamoto, 2004). Superoxide radicals are formed, moreover, in the near-ultraviolet photooxidation of tryptophan, as indicated by the increase of the H_2O_2 in the presence of SOD (McCormick, Fischer, Pachlatko & Eisenstark, 1976), and of melanin with light of 320-600 nm as shown by spin trapping (Halliwell & Gutteridge, 1999).

In aprotic solvents the superoxide anion is a very weak oxidant: the uptake of a proton from the substrate is followed by the dismutation of $HO_2^{\cdot}$ (Sawyer & Gibian, 1979). Thus stable solutions of $O_2^{\cdot-}$ can be prepared by controlled-potential coulometry (Sawyer & Gibian, 1979; Bozkaya, 2000).

2.3 Control of Free Radical Reactions in Biological Systems

Since living organisms contain several free radicals under physiological conditions, there must initiate mechanisms keeping their reactions under control in situations like electron transport, melanin production or photosynthesis. Molecular structures of vital importance like the cell membrane.

DNA and proteins have constituents which are very sensitive to radical reaction, such as the a-methylene carbon atom of Polyunsaturated fatty acids (PUFAs) as well as allylic hydrogen, and the thymidine base (Halliwell & Gutteridge, 1999).

Free radicals formed during electron transport are kept under control by the enzymes and carriers of the transport chain, which are bound to the membrane and are closely packed together. The PUFAs of the membrane are localized in its hydrophobic region, far from the site of electron transport, which takes place in the hydrophilic region. Thus the protection of PUFAs is inherent in membrane structure. This defense may be damaged if the cell membrane or mitochondria are injured, giving raise to pathological free radical reactions. PUFAs of the membrane phospholipids are "packaged" by cholesterol. Hydrogen bonds in membranes also provide protection against free radicals. However, hydrophobic chemical pathogens (e.g. CCl_4, DDT, halothane and some carcinogenic substances) can penetrate the hydrophobic region and initiate free radical reactions (Craig et al, 2003).

Some normally occurring free radicals are under control because they are intermediates formed during reactions of enzyme-substrate complexes. Owing to their fixed localization and short half-life, they have little chance to interact with other substances (Halliwell & Guttregidge, 1999).

The rate of radical reactions is influenced by the amount of membrane unsaturated fatty acids and the degree of their instauration. These, in turn, depend to a lesser extent on the fat content of the diet, and predominantly on genetic factors (Demopoulos, 1982)

Catalase dismutases hydrogen peroxide. The enzyme is found in variable amounts in tissues in the body (Marklund, Westman, Roos & Carlsson, 1984), in general in peroxisomes together with hydrogen peroxide-producing oxidases. Erythrocytes contain much catalase. Glutathione peroxidases are intracellular enzymes catalyzing the reaction between glutathione and hydrogen peroxide or organic hydroperoxides (Halliwell & Gutteridge, 1999).

In the cytochrome oxidase complex, oxygen is directly reduced in four steps:

$$O_2 + 4\,e^- + 4H^+ \longrightarrow 2H_2O$$

Nonenzymatic reduction occurs in one step at a time, "Univalent Pathway":

$$O_2 \xrightarrow{e} O_2^{\cdot-} \xrightarrow{e+2H^+} H_2O_2 \xrightarrow{e+H^+} {}^{\cdot}OH \xrightarrow{e+H^+} 2\,H_2O$$

Superoxide anion radical — hydrogen peroxide — hydroxyl radical

The Fenton reaction

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{+3} + {}^{-}OH + {}^{\cdot}OH$$

The Haber-Weiss reaction:

$$H_2O_2 + O_2^{\cdot-} \xrightarrow{Cu,\ Fe} {}^{-}OH + {}^{\cdot}OH + O_2$$

Reaction catalyzed by catalase:

$$H_2O_2 + H_2O_2 \longrightarrow O_2 + 2H_2O$$

Reaction catalyzed by glutathione peroxidase

$$H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O$$

(H_2O_2 (or organic peroxide) + 2GSH reduced glutathione → GSSG oxidized glutathione + $2H_2O$)

A new enzyme reported in 1969 whose function is the disproportionation of superoxide radicals to oxygen and hydrogen peroxide and was named superoxide dismutase (SOD) (McCord & Fridovich, 1969):

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

SOD was subsequently found to be a universal enzyme which exists in three different metalloforms where each form incorporates a transition metal ion at the active site. SOD is not known to react catalytically with any substrate other than the superoxide radical. Prior to discovery of SOD many biochemists regarded the possible existence of oxygen radicals in the cellular environment with extreme scepticism. The proof of the existence of an enzyme which catalysed the removal of such a radical forced a change of view, and subsequently had profound and far reaching effects on the study of reactive oxygen species in living biological systems (Asada et al, 1980; Barkley & Gregory, 1990).

2.4 The Structure of Superoxide Dismutase (from Bozkaya 2000; from Michalski, 1996)

2.4.1 Isoenzymes of Superoxide Dismutase

Although the nature of the enzymatic activity of Cu,Zn-SOD was discovered over two decades ago, the protein had been known much earlier. It was first isolated as a copper protein from bovine erythrocyte (erythrocuprein) and liver (hepatocuprein) in 1938. This was followed by the discovery of a whole family of copper-containing proteins that were present in various organs (Carrico & Deutsch, 1969). Much later, as a the result of various observations carried out by McCord and Fridovich (McCord & Fridovich, 1969) on the reduction of cytochrome C by xanthine oxidase reaction, erytrocuprein was identified as copper zinc superoxide dismutase. Cu,Zn-SOD is a soluble enzyme found mainly in the cytosol of eukaryotic cells. It has a molecular mass of 32000 and it contains one Cu^{2+} and one Zn^{2+} in the active site. Cu,Zn-SODs are generally very stable enzymes, tolerating exposure to organic solvents and

retaining activity in 8.0 M urea (Forman & Fridovich, 1973). Cu,Zn-SODs from some sources are not as stable in the presence of denaturants (Barra et al, 1979; Nakano et al, 1995). In general, the properties and structure of Cu,Zn-SODs have been remarkably resistant to evolutionary modifications are very similar (Halliwell &Gutteridge, 1999).

Cu,Zn-SODs have also been investigated from several bacterial species, including *Escherichia coli* (Imlay & Linn, 1986) and its presence has been indicated in some protozoan parasites (Michalski & Prowse, 1996). Bacterial Cu,Zn-SODs have properties similar to those of eukaryotic Cu,Zn-SODs. In *E.coli*, Cu,Zn-SODs seems to be important for aerobic growth in mutants that cannot produce Mn-SOD and Fe-SOD (Michalski & Prowse, 1996). It remains to be elucidated whether there are sources of O_2 within the periplasm or whether the periplasmic SOD protects against extracellular source of $O_2^{\cdot-}$ (Michalski & Prowse, 1996).

Manganese containing SOD was first isolated form *E.coli*, (McCord & Fridovich 1969) and has since been isolated from a wide range of bacteria (Imlay & Fridovich, 1991). The bacterial enzyme contains manganese in its active site (one Mn per subunit, in the resting state), usually has a molecular mass of 40000 to 46000, and is a dimer made up of identical subunits. High molecular mass tetrameric MnSODs have been identified in several species of bacteria with molecular masses of 110000 to 140000 (Barkley & Gregory, 1990). Mn-SOD has also been obtained from mitochondria. The mitochondrial enzyme is strikingly similar to the MnSOD from prokaryotes, with the notable difference that it is always tetrameric (Halliwell & Gutteridge, 1999). Mn-SODs are much more susceptible to denaturizing by heat or chemicals (including solvents and detergents) than are CuZnSODs (Asada et al, 1990).

Fe-containing SOD was first isolated from *E. coli* and subsequently from many other bacteria (Halliwell & Gutteridge, 1999). No animal tissues have been found to contain Fe-SOD of 43 plant families examined, Fe-SOD was found in only three (Bridges & Salin, 1981). Like mitochondrial Mn-SOD, some bacterial Fe-SODs were

found to be tetrameric. "From the bacterium *E.coli,* four SOD enzymes can be purified including Cu,Zn-SOD and intracellular MnSOD. A third SOD was found to be an iron-containing enzyme (Fe-SOD). The fourth *E.coli* SOD enzyme is a hybrid enzyme containing subunits of the manganese enzyme and of the iron enzyme in the same dimeric molecule" (Halliwell & Gutteridge, 1999). Yet these enzymes from *E.coli* exhibit substantial differences in their solution properties and absolute metal cofactor specificity (Carlioz et al, 1988).

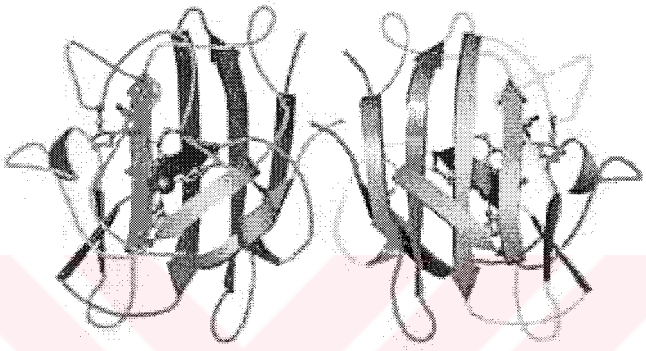


Figure 2.1 Three dimensional structure of superoxide dismutase

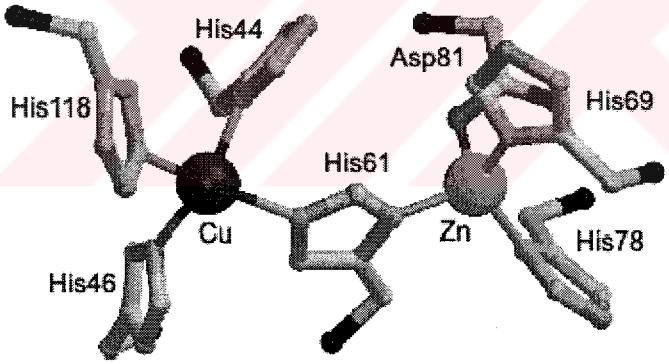


Figure 2.2 Active site of Cu,Zn-SOD.

$Fe(III)\text{-enzyme} + O_2^{\cdot-} \rightarrow Fe(II)\text{-enzyme} + O_2$

$Fe(II)\text{-enzyme} + O_2^{\cdot-} + 2H^+ \rightarrow Fe(III)\text{-enzyme} + H_2O_2$

Net Reaction: $O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$

2.4.2 The Inhibitors of SOD

Extracts of tissues or cells will ordinarily contain more than one type of SOD, and means for individually assaying SODs in such mixtures have been devised. These usually depend upon reagents that inhibit or inactivate one of the SODs, while having no effect on the others. Thus cyanide inhibits Cu,Zn-SODs, but not Fe-SOD or Mn-SOD. One may use 5 mM CN to specifically suppress the activity of Cu,Zn-SODs in extracts. Hydrogen peroxide on the other hand, inactivates Cu,Zn-SODs and Fe-SOD but has no effect on Mn-SOD. Alternately, Mn-SOD activity can be entirely eliminated by 2% sodium dodecyl sulfate, which has no effect on Cu,Zn-SODs. And also, all of SODs are inhibited by sodium azide.

The two active sites on the Cu,Zn-SODs enzyme function independently although separated subunits have not been found to be active. It is thought that the reaction of one copper-site with the substrate prevents the site on the other subunit from catalytic function, i.e. antico-operative interaction between the two sites. Cyanide completely inhibits Cu,Zn-SODs by binding to the inner co-ordination sphere of the copper.

SOD is powerful enough to increase the rate of dismutation of $O_2^{\cdot-}$ by several orders of magnitude at physiological pHs.

The catalytic activity of Cu,Zn-SODs has been shown by direct assay to be constant over the pH range 5-9.5 and to decrease rapidly above pH 10 where as the activity of Mn-SOD is substantially suppressed at the higher pH (This section retrieved from Bozkaya, 2001, therefore, original literatures related to this section can be found in thesis of Bozkaya, 2001) .

2.5 The Structure of Catalase

Catalases are metallo-enzymes that degrade H_2O_2 via the following reaction:

Fe(III)-enzyme + H_2O_2 → compound I + H_2O

compound I + H_2O_2 → Fe(III)-enzyme + H_2O + O_2

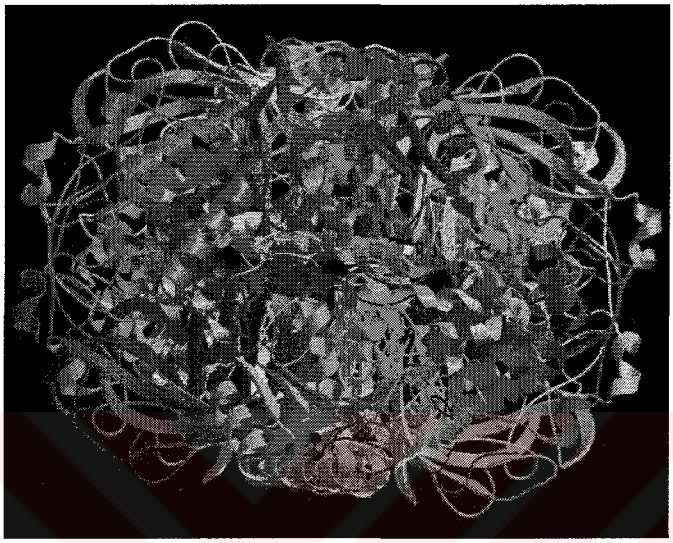


Figure 2.3 Three dimensional structure of catalase.

The decomposition of two molecules of H_2O_2 takes place in two steps. The first involves contact between the ferric state of the enzyme (Fe-E) and H_2O_2 to produce an intermediate from of the enzyme termed Compound I. Compound I than interacts with a second molecule of H_2O_2 (Halliwell & Gutteridge, 1999).

"Enzymes are very large and complex organic molecules that are synthesized by the cell to perform very specific functions. These biological catalysts are important because they speed up the rate of the reaction they catalyze that would otherwise be too slow to support life. Catalase is an enzyme present in the cells of plants, animals and aerobic (oxygen requiring) bacteria. It promotes the conversion of hydrogen peroxide, a powerful and potentially harmful oxidizing agent, to water and molecular oxygen.

Catalase is located in a cell organelle called the peroxisome. Peroxisomes in animal cells are involved in the oxidation of fatty acids, and the synthesis of cholesterol and bile acids. Hydrogen peroxide is a byproduct of fatty acid oxidation. White blood cells produce hydrogen peroxide to kill bacteria. In both cases catalase prevents the hydrogen peroxide from harming the cell itself. Peroxisomes in plant cells are involved in photorespiration (the use of oxygen and production of carbon dioxide) and symbiotic nitrogen fixation (the breaking apart of the nitrogen molecule N_2 to reactive nitrogen atoms). Hydrogen peroxide is produced as an intermediate during these chemical processes and must be removed to prevent damage to cellular machinery. Aerobic (oxygen requiring) bacteria produce hydrogen peroxide as a byproduct of metabolism. This fact is used when identifying bacteria. If hydrogen peroxide is added to a bacterial colony and bubbles are produced, this is evidence of oxygen production and confirms that the colony is aerobic "(Catalase, 2004).

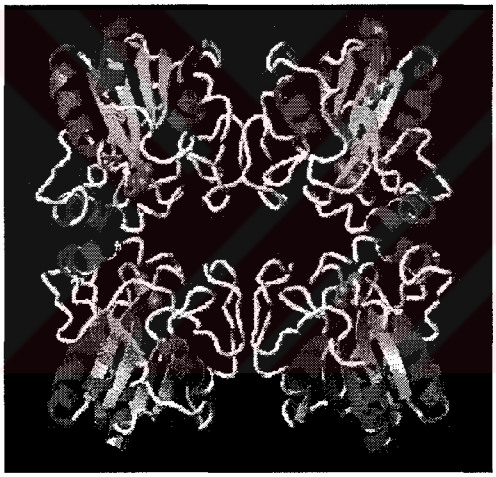
2.5.1 Inhibitors of CAT

Catalase can be inhibited by azide, cyanide and HOCl, but these are non-specific: they inhibit many other enzymes. A more useful inhibitor is aminotriazole (Halliwell & Gutteridge, 1999). Freezing and lyophilization cause inactivation. The reports indicate catalases to be inactivated by sunlight under aerobic conditions. Catalase inactivation by peroxide has been reported (Hippeli et al, 1999; Halliwell & Gutteridge, 1999; Brioukhanov & Netrusov, 2004).

2.6 Glutathione Peroxidase

"Glutathione peroxidase (EC 1.11.1.9) belongs to the family of selenoproteins and plays an important role in the defense mechanisms of mammals, birds and fish against oxidative damage by catalyzing the reduction of a variety of hydroperoxides, using glutathione as the reducing substrate. Four distinct species of glutathione peroxidase have been identified in mammals to date, the classical cellular enzyme, the phospholipid hydroperoxide metabolizing enzyme, the gastroinestinal tract enzyme and the extracellular plasma enzyme.

Their primary structures are poorly related. It has been shown that they are encoded by different genes and have different enzymatic properties. The physiological role of the human plasma enzyme remains still unclear due to the low levels of reduced glutathione in human plasma and the low reactivity of this enzyme. A number of structural differences were observed between the human plasma enzyme and the bovine cellular enzyme whose structure had been solved previously. Structural comparison indicated that all the residues which were previously suggested to be involved in glutathione binding have been mutated or deleted in the plasma enzyme. The observed structural differences between the two enzymes suggest differences in substrate binding and specificity." (Ren, Tibbelin & Akesson, 2005).



2.4 Three dimensional structure of glutathione peroxidase

During the catalytic mechanism of GSH-Px selenol (protein-Se) reacts with peroxide to give a selenenic acid (protein-SeOH)

$Protein\text{-}Se^- + ROOH + H^+ \rightarrow ROH + protein\text{-}SeOH$ (selenenic acid)

GSH then binds, followed by

$protein\text{-}SeOH\text{-}GSH \rightarrow H_2O + protein\text{-}Se\text{-}SG$

The second GSH binds, followed by,

$protein\text{-}Se\text{-}SG\text{-}GSH \rightarrow protein\text{-}SeH\text{-}GSSG \rightarrow protein\text{-}Se^- + H^+ + GSSG$ (Halliwell & Gutteridge, 1999).

2.6.1 Isoenzymes and other properties of Glutathione Peroxidase

As it well defined by Halliwell and Gutteridge (1999), Glutathione peroxidases consists of four protein subunits, each of which contains one atom of the element selenium (Se) at its active site. Selenium is in group VI of the periodic table, and somewhat resembles in its chemistry.

It is present at the active sites as selenocysteine, the amino acid cysteine in which sulphur atom has been replaced by selenium (R-SeH instead of R-SH).

Selenocysteine is encoded in the gene as TGA (UGA in mRNA), also a stop codon. To prevent misreading as "stop" special conformations of the transcribed mRNA are recognised by a translation factor that ensures insertation of a selenocysteinyl-tRNA by the ribosome. This tRNA is first loaded with serine, which is then converted by an enzyme into selenocysteine.

Traces of selenium are essential in the diet of animals: an important role of dietary selenium is to provide the selenium-containing cofactor for the glutathione peroxidase enzyme family.

Selenium plays other roles. For example in thyroid hormone biosynthesis it acts as an essential component of a type I iodothyronine-5-deiodinase enzyme, which converts thyroxine (T_4) to the more biologically-active thyroid hormone T_3.

Selenium-containing peroxidases comprise a family of enzymes (the glutathione peroxidase superfamily), of which at least four types exist. One is the classical glutathione peroxidase (Halliwell & Gutteridge, 1999). Glutathione peroxidase activities are very low in plants family and marine plants as presented in this thesis.

2.7 Glutathione and Glutathine Disulfide (Kidd, 2004)

The tripeptide thiol glutathione (γ-glutamylcysteinylglycine, GSH) has facile electron-donating capacity, linked to its sulfhydryl (-SH) group (Meister, 1994 a,b). Glutathione is an important water-phase antioxidant and essential cofactor for antioxidant enzymes; it provides protection also for the mitochondria against endogenous oxygen radicals. Its high electron-donating capacity combined with its high intracellular concentration endows GSH with great reducing power, which is used to regulate a complex thiol-exchange system (-SH / -S-S-). This functions at all levels of cell activity, from the relatively simple (circulating cysteine/-SH thiols, ascorbate, other small molecules) to the most complex (cellular -SH proteins).

Glutathione exists in two forms: The antioxidant "reduced glutathione" tripeptide is conventionally called glutathione and abbreviated GSH; the oxidized form is a sulfur-sulfur linked compound, known as glutathione disulfide or GSSG. The GSSG/GSH ratio may be a sensitive indicator of oxidative stress.

The reduced glutathione molecule consists of three amino acids - glutamic acid, cysteine, and glycine - covalently joined end-to-end. The sulfhydryl (-SH) group, which gives the molecule its electron-donating character, comes from the cysteine residue. Glutathione is present inside cells mainly in its reduced (electron-rich, antioxidant) GSH form. In the healthy cell GSSG, the oxidized (electron-poor) form, rarely exceeds 10 percent of total cell glutathione. Intracellular GSH status appears to be a sensitive indicator of the cell's overall health, and of its ability to resist toxic challenge

Glutathione is homeostatically controlled, both inside the cell and outside. Enzyme systems synthesize it, utilize it, and regenerate it as per the gamma-glutamyl cycle. Glutathione is most concentrated in the liver (10 mM), where the "P450 Phase II" enzymes require it to convert fat-soluble substances into water-soluble GSH conjugates, in order to facilitate their excretion. While providing GSH for their specific needs, the liver parenchymal cells export GSH to the outside, where it serves

as systemic source of -SH/reducing power. GSH depletion leads to cell death, and has been documented in many degenerative conditions.

2.8 Lipid Peroxidation

Lipid peroxidation has been defined by A.L. Tapple as "the oxidative deterioration of polyunsaturated lipids". Polyunsaturated fatty acids (PUFAs) are those that contain two or more carbon-carbon double bonds. =C=C=.

Oxygen-dependent deterioration, leading to rancidity, has been recognised since antiquity as a problem in the storage of fats and oils and was often dealt with since using spices. Rancidity is even more relevant today with the popularity of "polyunsaturated" margarines and cooking oils, and the importance of paints, plastics, lacquers, waxes and rubber, all of which can undergo oxidative damage.

The fist attempts to study tis problem began in 1820 when de Sauusre, using a simple mercury manometer, observed that a layer of walnut oil on water exposed to air absorbed three times its own volume of air in the course of 8 months. This initial lengthy period was followed by a second phase of rapid air-absroption, the oil taking up 60 times its own volume of air in 10 days.

During the following 3 months, the rate of air uptake gradually diminished, so that the oil eventually taken up 145 times its own volume. Parallel with these changes, the oil became viscous and evil-smelling. Commenting on these experiments a few years later, the famous chemist Berzelius suggested that O_2 uptake might account for not only the oxidation of oil exposed to air but also a host of similar phenomena. In particular, it was thought that autooxidation might be involved in the spontenous ignition of wool after its lubrication with linseed oil, a common cause of fires in textile mills at that time. Berzelius also discovered the elements selenium, which helps to protect against lipid peroxidation *in vivo*.

The sequence of reactions which is now recognised as the core of lipid peroxidation was worked out in detail by scientist at the British Rubber Producers Association research laboratories in the 1940s. The relevance of these reactions to biological systems was not appreciated until much later, however (Halliwell & Gutteridge, 1999).

Dietary fats have to be digested, adsorbed and transported around the body. Initiation of lipid peroxidation is caused by attack upon a lipid of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene ($-CH_2-$) group. Fatty acids with one or no double bonds are more resistant to such attack than are the PUFAs, yet PUFA side chain are present in many membranes and lipi proteins (Wagner et al, 1994).

An adjacent double bond weakens the energy of attachment of the hydrogen atoms present on the next carbon atom (the allylic hydrogen), especially if there is a double bond on either side of the $-CH_2-$ giving bis-allylic hydrogens.... Since a hydrogen atom has only one electron, abstraction of $H^{\cdot}$ From a $-CH_2-$ group leaves behind an unpaired electron on the carbon ($-C^{\cdot}H-$).

$H^{\cdot}$ abstraction is easiest from bis-allylic methylene groups. For example, the oxidizability of docosahexaenoic acid (22:6) in vitro is five times greater that that of linoleic acid. One consequence of this is that fish-oil supplements, widely advocated by health food stores, undergo oxidation easily because they contain substaintial amounts of eicosapentaenoic and docosahexaenoic acid.

The carbon radical is usualy stabilized by a molecular rearrangement to form a conjugated dienes. Carbon radicals can undergo various reactions, e.g. if two of them collided within a membrane they might cross-link the fatty acid side-chains (Frank et al., 1989).

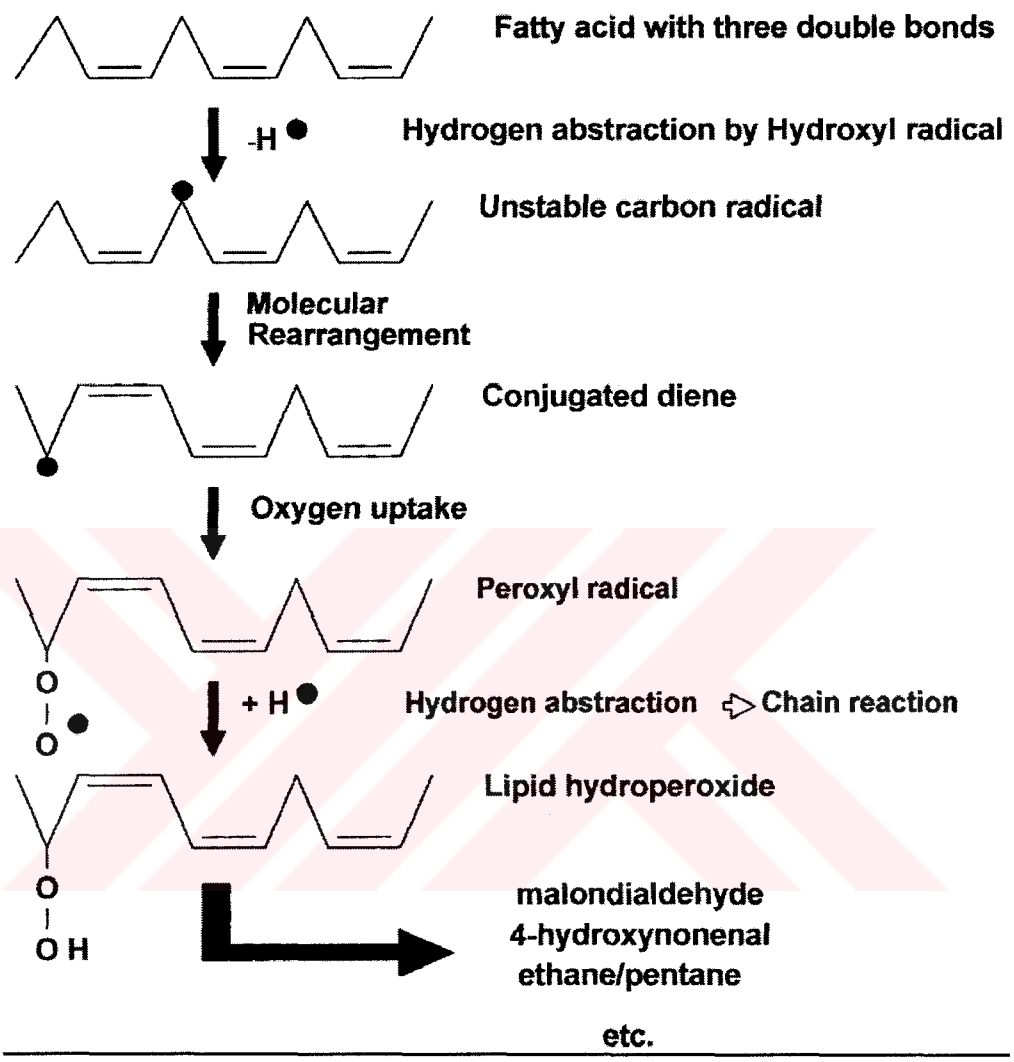


Figure 2.5 Schematic view of Lipid peroxidation (Young & McEneny, 2001)

2.9 Oxidative stress in plants (from Mittler, 2002)

Ever since the introduction of molecular oxygen (O_2) into our atmosphere, by O_2-evolving photosynthetic organisms, about two billion years ago, reactive oxygen species have been the unwelcome companions of aerobic metabolism. In contrast to O_2, these partially reduced or activated derivatives of oxygen are highly reactive and toxic, and can lead to the oxidative destruction of cells. Consequently, the evolution of all aerobic organisms has been dependent upon the development of efficient ROS-scavenging mechanisms. In recent years a new role for ROS was identified: the control and regulation of biological processes such as programmed cell death, hormonal signaling, stress responses, and development. These studies extend our understanding of ROS and suggest a dual role for ROS in plant biology: (a). Toxic byproducts of aerobic metabolism, and (b). Key regulators of metabolic and defense pathways.

2.10 Production and scavenging of ROS in cells: "the ROS cycle" (from Mittler, 2002)

The steady state level of ROS in different cellular compartments is determined by interplay between multiple ROS-producing pathways, and ROS-scavenging mechanisms. These are controlled by the ROS-signal transduction pathway and constitute the "basic cycle". During normal growth and development this pathway monitors the level of ROS, produced by aerobic metabolism, and controls the expression and activity of ROS-scavenging pathways (Fig. 2.3). The basic ROS cycle may also perform fine metabolic tuning, e.g., suppression of photosynthesis, to reduce the production rate of ROS. There are many potential sources of ROS in plants (Table 2.1). Some are reactions of normal aerobic metabolism, such as photorespiration, while others belong to pathways enhanced during abiotic stresses, such as photorespiration. In recent years new sources of ROS were identified in plants, including NADPH oxidases, amine oxidases, and cell wall-bound peroxidases. These are tightly regulated and participate in the control of processes such as programmed cell death, stress response, and pathogen defense.

Table 2.1 Mechanisms for production and scavenging of ROS in cells. AOX, alternative oxidase; APX, ascorbate peroxidase; CAT, catalase; GPX, glutathione peroxidase; SOD, superoxide dismutase.

Production and scavenging of ROS in plants		
Production of ROS	**Localization**	**Primary ROS**
Photosynthesis ET, PSI or PSII	Chloroplast	$O_2^{\cdot-}$
Respiration ET	Mitochondria	$O_2^{\cdot-}$
Glycolate oxidase	Peroxisome	H_2O_2
Excited chlorophyll	Chloroplast	$O_2^{\cdot-}$
NADPH oxidase	Plasma membrane	$O_2^{\cdot-}$
Fatty acid oxidation	Peroxisome	H_2O_2
Oxalate oxidase	Apoplast	H_2O_2
Xantine oxidase	Peroxisome	$O_2^{\cdot-}$
Peroxidase, Mn	Cell wall	H_2O_2 $O_2^{\cdot-}$
Amine oxidase	Apolast	H_2O_2
Scavenging of ROS		
Superoxide dismutase	Chl, Cyt, Mit, Per, Apo	$O_2^{\cdot-}$
Ascorbate peroxidase	Chl, Cyt, Mit, Per, Apo	H_2O_2
Catalase	Peroxisome	H_2O_2
Glutathione peroxidase	Cytosol	H_2O_2, ROOH
Peroxidases	Cell wall, cytosole, vacuol	H_2O_2
Thioredoxin peroxidase	Chl, Cyt, Mit	H_2O_2
Ascorbic acid	Chl, Cyt, Mit, Per, Apo	H_2O_2 $O_2^{\cdot-}$
Glutathione	Chl, Cyt, Mit, Per, Apo	H_2O_2
α-Tocopherol	Membranes	ROOH
Carotenoids	Chl	$O_2^{\cdot-}$
Alternative oxidase	Chl, Mit	$O_2^{\cdot-}$

Under optimal growth conditions the production of ROS in cell is estimated at a constant rate of 240 μM s^{-1} $O_2^{\cdot-}$, and a steady state level of 0,5 μM H_2O_2. However, stresses that disturb the cellular homeostasis of cells result in the enhanced production of ROS (up to 720 μM s^{-1} $O_2^{\cdot-}$, and a steady state level of 5-15 μM H_2O_2). These include drought, salt, chilling, heat shock, heavy metals, UV radiation, air pollutants such as ozone and SO_2, mechanical stress can pose a threat to cells, and many stress conditions enhance the expression of ROS-scavenging enzymes. However, it is also thought that during stress ROS are actively produced by cells (e.g., by NADPH oxidases), and act as signals for the induction of defense pathways. Thus, ROS may be viewed as cellular by-products of stress metabolism, as well as secondary messengers involved in the stress-response signal transduction pathway. This view, of the "extended ROS cycle" is presented in Fig.2.2.

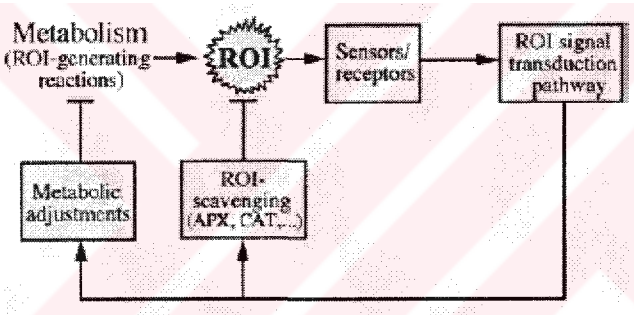


Figure 2.1 The basic ROS cycle. This cycle modulates the cellular levels of ROS during normal metabolism. Some of the key ROS scavenging enzymes of plants, ascorbate peroxidase (APX), and catalase (CAT) are indicated.

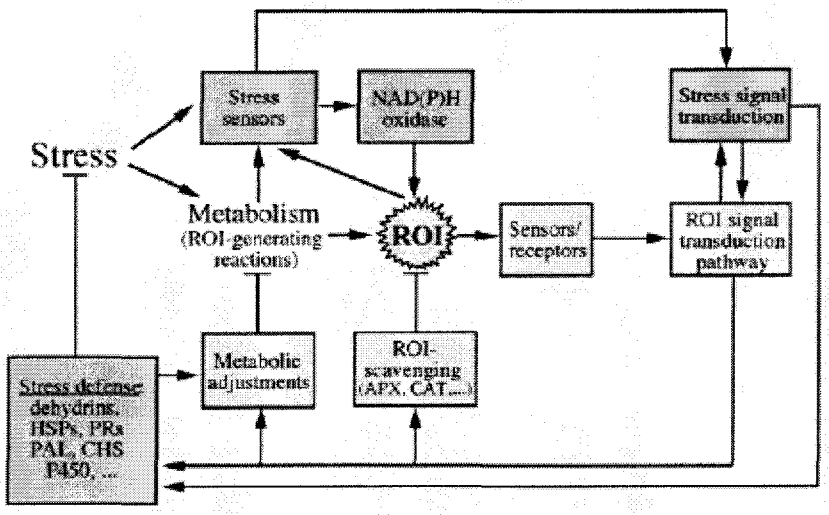


Figure 2.2 The extended ROS cycle. This cycle operates in plants during biotic or abiotic stresses. HSPs, heat shock proteins; PR, pathogenesis related proteins, phenylalanine ammonia-lyaze; CHS, chalcone synthase; P450, cytochrome P450.

Inasmuch as ROS are toxic but also participate in key signaling events, plant cells require different mechanisms to regulate their intracellular ROS concentrations by scavenging of ROS. Major ROS scavenging mechanisms of plants are summarized in Table 2.1. These include superoxide dismutase, ascorbate peroxidase, and catalase. The balance between SOD and APX (and / or CAT) activity in cells is considered to be crucial for determining the steady-state level of O_2^- and H_2O_2. This balance, together with sequestering of metal ions such as Fe and Cu by ferritin and copper-binding proteins, is thought to be important to prevent the formation of the highly toxic $HO^{\cdot}$ Via metal-dependent Haber-Weiss or the Fenton reactions.

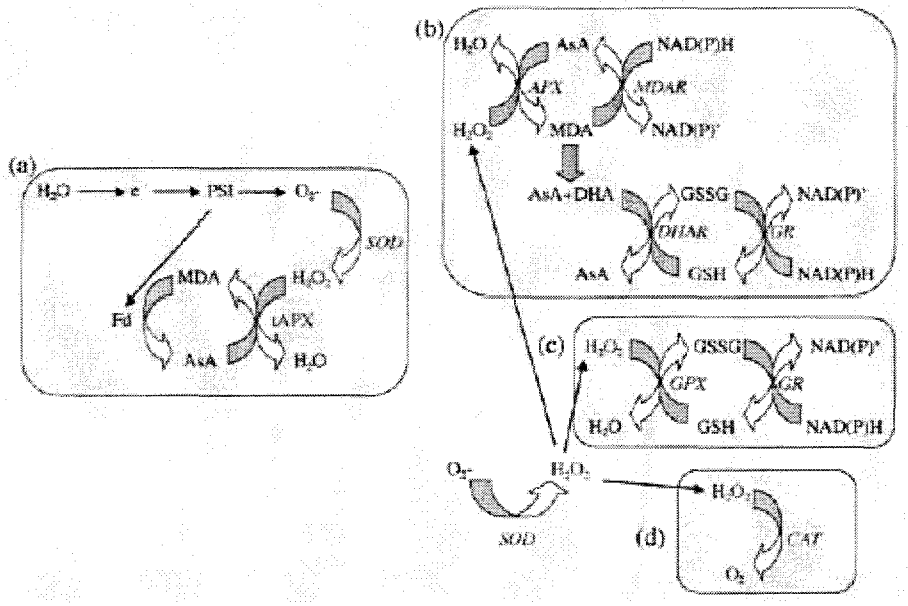


Figure 2.3 The main cellular pathways for ROS removal in plants. (a). SOD and thylakoid-bound APX (tyl-APX) in the chloroplast as part of the water-water cycle. (b). SOD and APX in the stroma, cytosol, mitochondria, and apoplast of plants, as part of the ascorbate-glutathione pathway. (c). Glutathione peroxidase (GPX) and its glutathione regenerating cycle. (d). Catalase in peroxisomes. AsA, ascorbate; GSH and GSSG, reduced and oxidized glutathione; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; MDA, monodehydroascorbate; MDAR, monodehydroascorbate reductase; GR, glutathione reductase; PSI, photosystem I; Fd, ferredoxin; e-, electron.

Antioxidants such as ascorbic acid and glutathione, found at very high concentrations in chloroplasts and other cellular compartments (5-20 mM ascorbic acid and 1-5 mM glutathione), are also important for defense of plants against oxidative stress. Consequently, mutants with suppressed ascorbic acid levels, and transgenic plants with suppressed ROS-scavenging enzymes, are hypersensitive to pathogen attack and abiotic stress conditions. In addition, over-expression of ROS-scavenging enzymes increases the tolerance of plants to abiotic stresses. ROS production can also be decreased in cells by the alternative channeling of electrons in the electron transport chains of the chloroplasts and mitochondria by a group of enzymes called alternative oxidases.

2.11 ROS at the interface between biotic and abiotic stresses (from Mittler, 2002)

ROS play a central role in the defense of plants against pathogens. During this response, ROS are produced by plant cells via enhanced enzymatic activity of plasma membrane-bound NADPH oxidases, cell wall-bound peroxidases and amine oxidases in the apoplast. H_2O_2 produced during this response is thought to diffuse into cells through aquaporins and together with salicylic acid (SA) and nitric oxide (NO) activate many of the plant defenses, including the induction of programmed cell death. The activity of APX and CAT is suppressed during this response by salicylic acid and nitric oxide, the expression of APX is post-transcriptionally suppressed, and the expression of CAT is down regulated at the steady state m-RNA level. Thus, the plant simultaneously produces more H_2O_2 and diminishes its own capability to scavenge H_2O_2, resulting in the over-accumulation of H_2O_2 and the activation of PCD. This response serves as an excellent example to how the steady state level of ROS can dramatically increase in cells when the basic ROS cycle is severed.

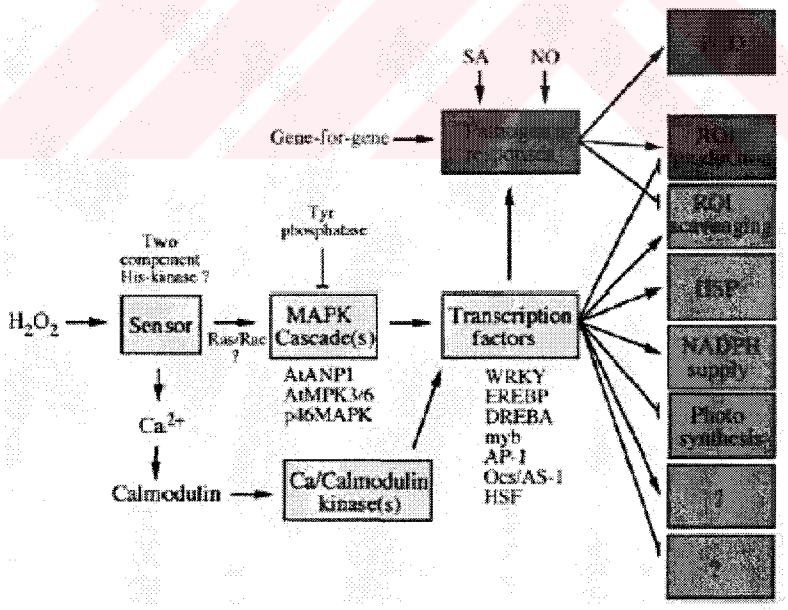


Figure 2.4 A hypothetical model of the signaling pathway activated in plants in response to external application of oxidants. SA, salicylic acid; NO, nitric oxide; PCD, programmed cell death; HSP, heat

shock protein; MAPK, mitogen-activated protein kinase; Tyr, tyrosine; HSF, heat shock transcription factor.

2.12 ROS signal transduction pathway (from Mittler, 2002)

Recent studies identified a number of components that may be involved in the ROS signal transduction of plants. These include the MAPKKK, AtANP1 (also NPK1), the MAPKs, AtMPK3/6, and Ntp46MAPK and calmodulin. A hypothetical model depicting some of the players involved in this pathway is shown in Fig 2.4. A sensor that might be a two component Histidine-kinase, or a receptor-like protein kinase, is thought to sense H_2O_2. Calmodulin and a MAPK cascade are then activated resulting in the induction/activation/suppression of a number of transcription factors. These regulate the response of plants to oxidative stress. Cross-talk with the pathogen-response signal transduction pathway (gene-for-gene) also occurs and may involve interactions between different MAPK pathways, feedback loops, and the action of salicylic acid and nitric oxide.

CHAPTER THREE
MATERIAL AND METHODS

3. Material and Methods

3.1 Algae Materials

The *Caulerpa* species (*C.taxifolia, C. racemosa, C.racemosa lamourixii, C. prolifera*) were kept in aquaria (in Ulm University) with artificial seawater, and *C.racemosa, P.pavonica, Enteromorpha sp., Cystoseria sp., Oxynoe olivaceae, Lobiger serridifalci* and *Haminoe hydatis* which were collected from Seferihisar-Izmir and were grown in Dokuz Eylul University, Biochemistry Laboratory. *Caulerpa* samples were grown in 40-80W (low-high light) and at 21°C and 26°C (warm-cold), respectively and then samples were named below:

C.racemosa LLWS (Low light-Warm)
C.racemosa HLWS (High light-Warm)
C.racemosa HLWB (High light-Warm- lamourixii sbp)
C.racemosa Cold
C.taxifolia LLW (Low light-Warm)
C.taxifolia HLW (High light-Warm)
C.taxifolia Cold
C.prolifera LLW (Low light-Warm)
C.prolifera HLW (High light-Warm)
C.prolifera Cold

3.2 Biochemical Assay Methods

3.2.1 Superoxide dismutase

All solutions used in this study were made up with bidistilled water, according to the following scheme:

Phosphate Buffer: 0.1M, pH = 5,5 – 8,0

2,774 g K_2HPO_4 and 1,7025 g KH_2P0_4 were dissolved in 250 mL of water and pH was adjusted with 0,1M NaOH solution.

Potassium Pyrogallate Solution

50 g KOH was dissolved in 100 mL of water and sparged with N_2. Then 5 g pyrogallol was added into this solution. The pyrogallate solution was used as oxygen sorbent in the N_2 flow system. 1,028 mg 6-OHDA was dissolved in 1 mL of KCl solution (1 mM, pH 2,00) which was prepared before used and sparged with nitrogen. During the experiments stock solution was reserved in N_2 environment which was passed through the pyrogallate solution at $+4^0$C.

Definition of Unit of Superoxide Dismutase Activity in the Standard Activity Assay Condition

Simple assay system for SOD based on the inhibitory effects of SOD on the spontaneous auto-oxidation of 6-hydroxydopamine (Crosti, Servidei, Bajer & Serra, 1987).

Auto-oxidation rate of 6-OHDA ($4x\ 10^{-4}$ M) in 0.1 M phosphate buffer pH = 7,4 which was saturated by air-0_2 (8,2 mg/L) was determined by observing of the absorbance changes at 490 nm in 15 sec time intervals at 25^0C. SOD activity assays were carried out under the same conditions by adding solution required to drop the half of the initial absorbance value of 6-OHDA at the 90 sec. 1 IU is the amount of SOD required to inhibit the initial rate of 6-OHDA auto-oxidation by 50%.

Reaction mechanism;

$$6\text{-OHDA} + O_2 \rightarrow SQ^* + O_2^* + H^+$$

$$H^+ + 6\text{-OHDA} + O_2^* \rightarrow SQ^* + H_2O_2$$

$$SQ + O_2 \rightarrow Q + O_2^* + H^+ \rightarrow SQ^* + O_2^* \rightarrow Q + H_2O_2$$

3.2.2 Catalase

Spectrophotometric determination

CAT activity is determined by measuring the decrease of absorbance at 240 nm in the reaction mixture (1mL) containing 50 mM phosphate buffer pH = 7,0 and 10 mM H_2O_2 (Aebi, 1985). Catalase activity was measured by the rate of reduction of hydrogen peroxide as measured spectrophotometrically at 240 nm. One unit of enzyme activity was defined as the amount of enzyme that decomposes 1 μmol of hydrogen peroxide per min.

Reagents:

Phospate buffer: 50 mmol/L, pH 7.0

Hydrogen peroxide: 30 mmol/L

Activity Measurement :

Pipette succesively into the cuvvette	Blank	Sample	Concentration in assay mixture
Phosphate buffer (1) Sample Hydrogen peroxide (2)	1,0 mL 2,0 mL -----	----- 2,0 mL 1,0 mL	Phos. 50 mM Volume fract 0,67 H_2O_2
Start the reaction by addtion of H_2O_2. Initial absorbances should be approx. A=0.500. Mix well with a plastic paddle; follow the decrease in absorbance with a recorder for ca. 30 s.			

Total Peroxidase (Chemiluminescence Determination) by Kits

$$\text{Uric acid} + O_2 \xrightarrow{\text{Uricase}} \text{allantoin} + H_2O_2 + CO_2$$

$$H_2O_2 + \text{Luminol} + \text{ferricyanide} \rightarrow \text{aminophtalate anion} + \text{Photon} + N_2 + 2H_2O$$

Total peroxidase activity was measured in Lumat LB 9507 luminometer by using peroxidase chemiluminescence (ECL) kit (Amersham Corp., Piscataway,NJ). Its working prensible was mentioned above reactions. Activity unit was measured dependent on light emission.

3.2.3 Glutathione peroxidase

GSH-PX activity is measured in a coupled system by measuring the decrease of NADPH-absorbance at 340 nm. The reaction mixture (1,060 mL) contains in order of addition: 1,0 mL reagent consisting of glutathione 4 mmol/L, glutathione reductase > 0,5 U/L, NADPH 0,28 mmol/L and 50 mM phosphate buffer pH 7,2. After adding 20 mL of diluted sample, 40 mL cumene hydroperoxide is added and vigorously mixed. The decrease of the absorbance at 340 nm is measured in minute intervals and corrected against blank samples (Paglia & Valentine, 1967).

Reaction mechanism:

$$2GSH + ROOH \xrightarrow{GSH\text{-}Px} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP + 2GSH$$

Activity Measurement

Pipette into cuvette	**Diluted sample**	**Reagent blank**
Diluted sample	0,02 mL	---
Distilled water	---	0,02 mL
Reagent*	1,00 mL	1,00 mL
Cumene	0,04 mL	0,04 mL
Mix, read initial absorbance of sample and reagent blank after one minute and start timer simultaneously. Read again after 1 and 2 minutes. Substract reagent blank value from that of the sample.		

*content of reagent: GSH (4mmol/L), GR (> 0,5U/L), NADPH (0,28 mmol/L)

GSH-Px activity was defined according to the decrease of absorbance of NADPH at 340 nm. 1 unit of GSH-Px is the formation of 1 μmol $NADP^+$ from NADPH per min at pH 7.2.

3.2.4 Lipid peroxidation

The degree of LPO is measured using the Yagi (1994) method using thiobarbituric acid reactive substances. Samples are weighed and homogenized in ice-cold 1,15% KCl, and 10% (w/v). Homogenate (0,1 mL) is added in a glass tube. The following substances are added in order: 0,2 mL of 8,1% sodium dodecyl sulphate, 1,5 ml of 20% acetic acid solution (pH = 3,5), 1,5 mL of 0,8% aqueous solution of thiobarbituric acid solution and 0.7 mL of distilled water. The mixture is heated at 95 ^{0}C for 60 min in an oil bath. After cooling with tap water, 1,0 mL of distilled water and 5,0 mL of a mixture of n-butanol and pyridine (15:1, v/v) are added and mixed vigorously. After centrifugation of 4000 rpm for 15 min, the n-butanol layer is taken for absorbance measurement at 532 nm (Yagi, 1994).

3.2.5 Protein Assay Method

Protein concentrations were estimated with the coomassie blue dye-binding assay according to the method of Bradford (1976).

Coomassie Brillant Blue Solution

Coomassie Brillant Blue G-250 (100 mg) was dissolved in 50 mL 95% ethanol. To this solution, 100 mL 85% (w/v) phosphoric acid was added. The resulting solution was diluted to final volume of one liter. Final concentrations in the reagent were 0,01% (w/v) Coomassie Brillant Blue 0-250, 4,7% (w/v) ethanol and 8,5% (w/v) phosphoric acid.

Stock Standard Protein Solution

10 mg BSA (crystalline) was diluted in to 100 mL with 0,02 M phosphate buffer, pH=7.4. Protein solutions containing 1,2 to 6 μg protein in a volume up to 0,1 mL were pipetted in to test tubes.

One milliliter of protein reagent was added to the test tube and the contents were mixed. Absorbance at 595 nm was measured after two minutes.

In 1 mL cuvettes against a reagent blank prepared from 0,1 mL of buffer solution in 1 mL assay volume. The amounts of protein were plotted against the corresponding

3.2.6 Caulerpenyne Determination (from Jung et al, 2002)

In the experiments, MS-grade solvents were purchased from Riedel de Haen (Seelze, Germany). HPLC-APCI-MS (atmospheric pressure chemical ionization): A Hewlett-Packard HP1100 was equipped with a Macherey-Nagel normal phase Nucleosil 50–7 column (250 x 4 mm) for LC separation; a Finnigan LCQ with APCI interface was used for detection. High-resolution gas chromatography–mass spectrometry was performed on a Micromass MasSpec double focusing magnetic sector mass spectrometer (geometry EBE) connected to a Hewlett Packard HP6890 II gas chromatograph, equipped with a DB-5 (J&W Scientific) capillary column (30 m x 0.25 mm, 0.25 μm) (Jung et al, 2002).

3.2.7 Extraction and Quantification of Caulerpenyne (from Jung et al, 2002)

For the determination of the caulerpenyne content in intact tissue, enzymatic degradation was prevented by freezing 3 g of the algae (fronds and stolons) with excess liquid nitrogen. The cold material was ground to a powder and treated with 3 mL ethyl acetate containing 1 mg/mL 4-methoxyacetophenone (Amade & Lemee, 1998) as an internal standard. During warm-up to 0 °C, grinding was continued and the cold mixture was centrifuged 5 min at 15,000g for phase separation. The organic phase (20 μL) was used directly for quantification with normal-phase (NP)-HPLC-MS [diode array detector (DAD), detection wavelength 252 nm]. HPLC gradient was: hexane–ethyl acetate; 1 mL/min: 0%, 12 min 25%, 18 min 35%, 18 min 0%, 25 min 0 % ethyl acetate. Reextraction of the algal residues with 2 x 3 mL of ethyl acetate showed an extraction efficacy of > 95% caulerpenyne in the first step. For

comparison, other extraction methods were tested on the frozen algal powder. One method used 3 mL of methanol containing 1 mg/mL 4-methoxyacetophenone instead of ethyl acetate as solvent. 20 µL of the alcoholic solution were analyzed directly with NP-HPLC-MS. Repeated extractions (3x) showed poor and unreliable extraction efficacy for the first extraction step (78–91%). The other method tested using a ternary mixture of water, chloroform, and methanol as solvent. To 3 g ground algal material obtained after freezing with liquid nitrogen, 1,25 mL chloroform and 2,5 mL methanol containing 3 mg 4-methoxyacetophenone were added and shaken for 2 min. For phase separation, 1,25 mL water and 1,25 mL chloroform was added. After centrifugation, 20 µL of the organic phase were applied to NP-HPLC-MS. While the extraction efficacy was around 95 %, the caulerpenyne purification was complicated by elevated amounts of extracted lipids. Purity of the caulerpenyne signal was monitored by using DAD and MS detection (APCI). For quantification, a linear calibration curve obtained with purified (column chromatography with SiO_2 and Florisil) caulerpenyne was used. The response was linear from 0.1 to 10 g caulerpenyne.

Transformation Experiments: For measurements at t=0, the algal material was shock-frozen with liquid nitrogen before extraction as described above. Injury was simulated by manually grinding 2–3 g of the algae for 30 sec to homogeneity. The homogenate was shock-frozen after defined intervals to prevent further enzymatic degradation and was then extracted with ethyl acetate (1 mL/g algal fresh weight) containing the internal standard. LC quantification was performed as described above. For the relative quantification of oxytoxin 2, the APCI MS ion at m/z = 231 (Jung and Pohnert, 2001) was monitored and compared to the same ion of caulerpenyne. The identity of oxytoxin 2 in the fresh extracts of wounded algae was verified by GC-HR-MS: 230,1305; calculated: 230,1307 [$C_{15}H_{18}O_2$]; EI-MS (70eV; m/z (%): 230(100), 215(42), 201(15), 187(59), 159(45), 143(15), 131(17), 115(14) (Jung et al, 2002).

3.2.8 Derivatization of Crude Algal Extracts (from Jung et al, 2002)

Trapping experiments using 4 g algal material and 1 mL of a saturated ethanolic 2,4-dinitrophenylhydrazine (DNPH) solution were performed as described previously (Jung et al, 2002).

3.2.9 Homogenisation Procedure:

2 g alga sample was homogenised with 2 mL buffer solution related to antioxidant enzymes.

SOD = pH 7.4 , 100 mM Kpi

CAT = pH 7.0, 50 mM Kpi

LPO = pH 7.0, 1.15 % KCl

GSH-Px = pH 7.4 , 100 mM Kpi

For homogenisation, preliminarly, samples at –86°C were homogenised with mortar and pestle then IKA-Jankel homogenisator was also used for extra-homogenisation. For enzyme activities, homogenates were centrifuged at 10.000 rpm for 10 min at + 4°C.

3.3. Statistical Analysis

SPSS 9.0 and Minitab 13.20 statistics programs were used to evaluate the data. One-way ANOVA test was used to compare the values. Meanwhile, Tukey's pairwise comparison test was utilized to compare the samples' differences between each other. The values are the means of three separate experiments of three individuals. The statistical difference set up at $p < 0.05$. The Pearson correlation test was used to check possible correlation among antioxidant enzymes and LPO levels.

CHAPTER FOUR

RESULTS

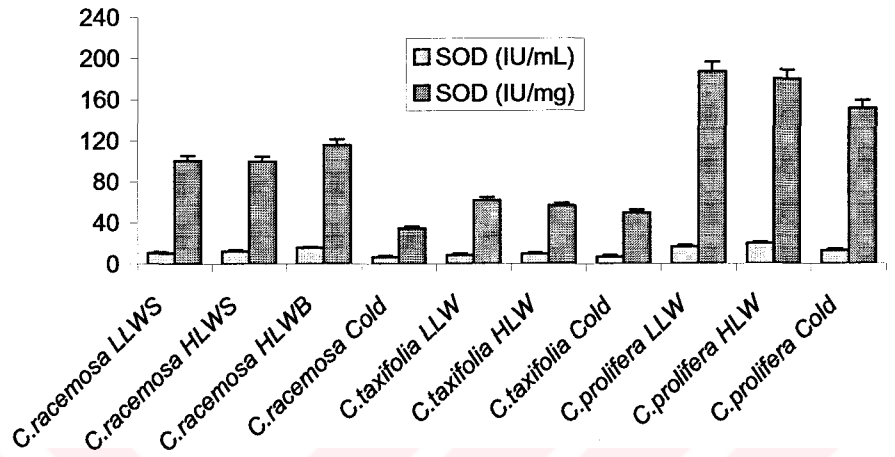


Figure 4.1 Volumetric and specific SOD activities in *C.racemosa, C.taxifolia* and *C.prolifera* dependent on various light and temperature conditions. The values are the means of three separate experiments. Statistical analyses among enzyme activities were depicted in Table 4.1.

Table 4.1 Insignificant ($p>0.05$) differences in SOD activities among *Caulerpa* species.

Groups	**P value**
C.racemosa LLWS – *C.racemosa* HLWS	1,000
C.taxifolia LLW – *C.taxifolia* HLW	0,414
C.taxifolia HLW – *C.taxifolia* Cold	0,311
C.prolifera LLW – *C.prolifera* HLW	0,096

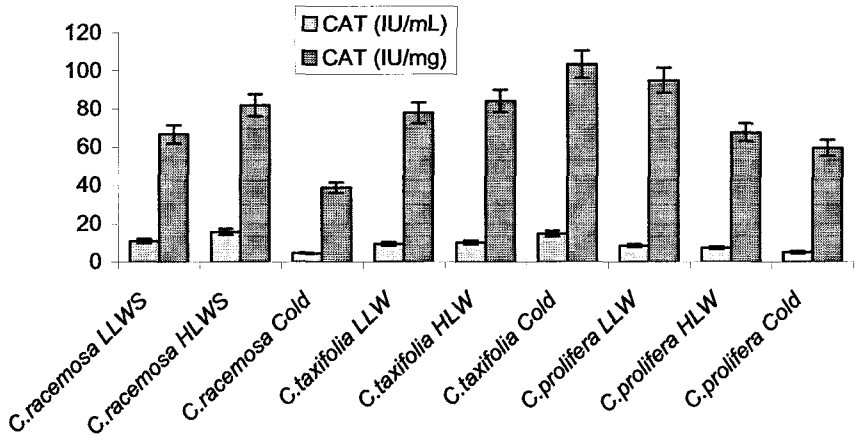


Figure 4.2 Volumetric and specific CAT activities in *C.racemosa, C.taxifolia* and *C.prolifera* dependent on various light and temperature conditions. The values are the means of three separate experiments. Statistical analyses among enzyme activities were depicted in Table 4.2.

Table 4.2. Insignificant (p>0,05) differences in CAT activities among *Caulerpa* species.

Groups	**P value**
C.racemosa LLWS – *C.prolifera* HLW	1,000
C.racemosa LLWS – *C.prolifera* Cold	0,232
C.racemosa HLWS – *C.taxifolia* LLW	0,914
C.racemosa HLWS – *C.taxifolia* HLW	0,999
C.racemosa HLWB – C.prolifra Cold	0,975
C.taxifolia LLW – *C.taxifolia* HLW	0,527
C.taxifolia Cold – *C.prolifera* LLW	0,190
C.prolifera Cold – *C.prolifera* HLW	0,202

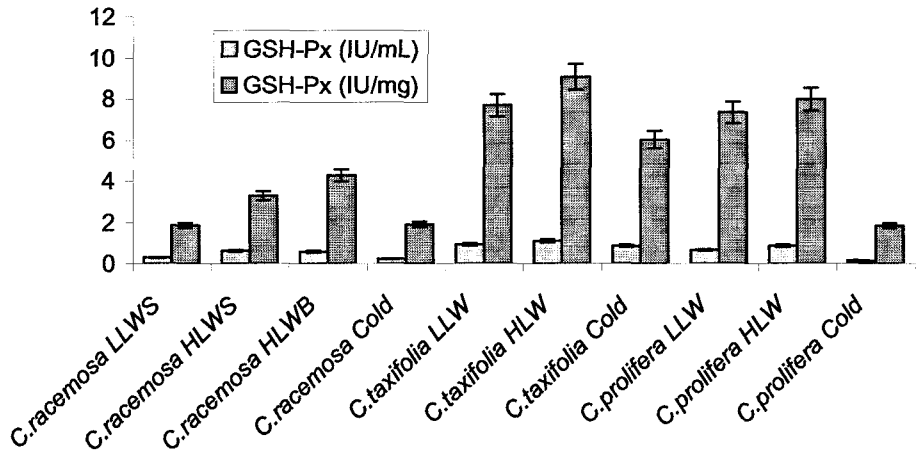


Figure 4.3 Volumetric and specific GSH-Px activities in *C.racemosa, C.taxifolia* and *C.prolifera* dependent on various light and temperature conditions. The values are the means of three separate experiments. Statistical analyses among enzyme activities were depicted in Table 4.3.

Table 4.3 Insignificant ($p>0.05$) differences in GSH-Px activities among *Caulerpa* species.

Groups	**P value**
C.racemosa LLWS – *C.racemosa* Cold	1,000
C.racemosa LLWS – *C.prolifera* Cold	1,000
C.taxifolia LLW – *C.prolifera* LLW	0,899
C.taxifolia LLW – *C.prolifera* HLW	0,917
C.prolifera LLW - *C.prolifera* HLW	0,191

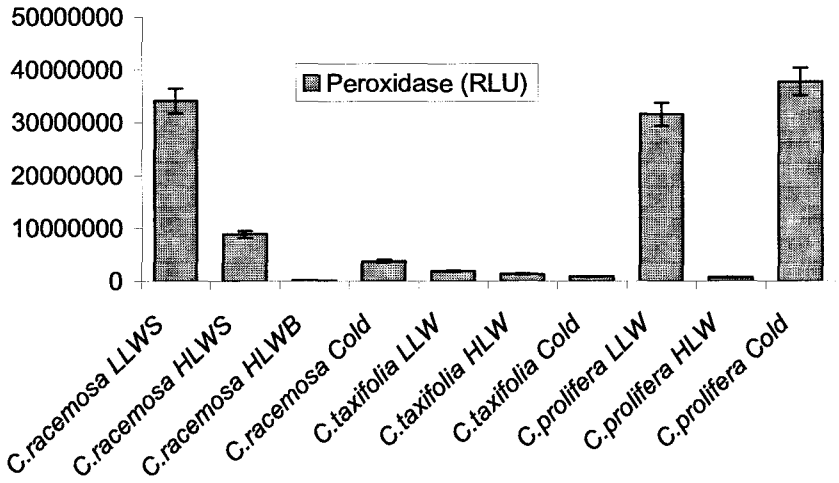


Figure 4.4 Total Peroxidase activities in *C.racemosa, C.taxifolia* and *C.prolifera* dependent on various light and temperature conditions. The values are the means of three separate experiments. Statistical analyses among enzyme activities were depicted in Table 4.4.

Table 4.4 Insignificant (p>0,05) differences in Peroxidase activities among *Caulerpa* species.

Groups	**P value**
C.racemosa LLWS – *C.prolifera* Cold	0,070

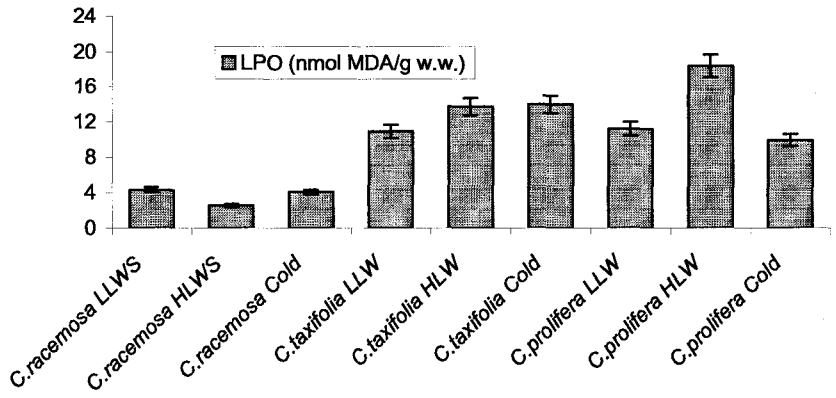


Figure 4.5 Lipid peroxidation levels in *C.racemosa, C.taxifolia* and *C.prolifera* dependent on various light and temperature conditions. The values are the means of three separate experiments. Statistical analyses among LPO levels were depicted in Table 4.5. The unit is defined as nmol MDA/ g wet weight.

Table 4.5 Insignificant (p>0,05) differences in LPO levels among *Caulerpa* species.

Groups	**P value**
C.racemosa LLWS – *C.racemosa* HLWS	0,483
C.racemosa LLWS – *C.racemosa* HLWB	0,355
C.racemosa LLWS – *C.racemosa* Cold	1,000
C.racemosa HLWS – *C.racemosa* Cold	0,697
C.racemosa HLWB – *C.racemosa* Cold	0,204
C.taxifolia LLW - *C.taxifolia* HLW	0,067
C.taxifolia LLW – *C.prolifera* LLW	1,000
C.taxifolia LLW – *C.prolifera* Cold	0,587
C.taxifolia HLW – *C.taxifolia* LLW	0,067
C.taxifolia HLW – *C.taxifolia* Cold	1,000
C.taxifolia HLW – *C.prolifera* LLW	0,088
C.prolifera LLW – *C.prolifera* Cold	0,500

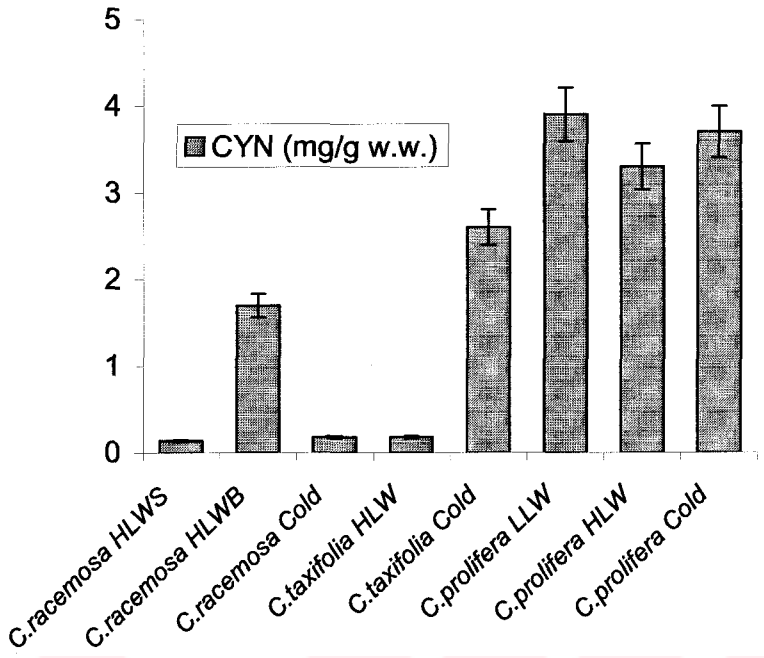


Figure 4.6 Caulerpenyne (CYN) contents in *C.racemosa, C.taxifolia* and *C.prolifera* dependent on various light and temperature conditions. The values are the means of three separate experiments. Statistical analyses among CYN levels were depicted in Table 4.6. The unit is defined as mg CYN / g wet weight.

Table 4.6 Insignificant ($p>0.05$) differences in CYN levels among *Caulerpa* species.

Groups	**P value**
C.racemosa LLWS – *C.racemosa* HLWS	0,993
C.racemosa LLWS – *C.taxifolia* HLW	0,355
C.racemosa LLWS – *C.taxifolia* Cold	1,000
C.racemosa Cold – *C.taxifolia* LLW	0,990
C.prolifera LLW – *C.prolifera* Cold	0,815
C.prolifera HLW – *C.prolifera* Cold	0,085

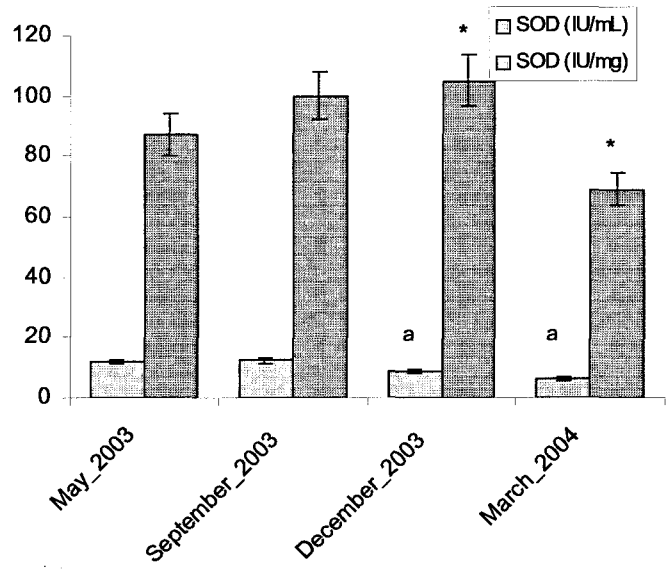


Figure 4.7 Seasonal changes of SOD enzyme activities in *C.racemosa*. The values are the means of three separate experiments. * and a represent significant ($p<0,05$) difference from specific and volumetric activities in May_2003, respectively.

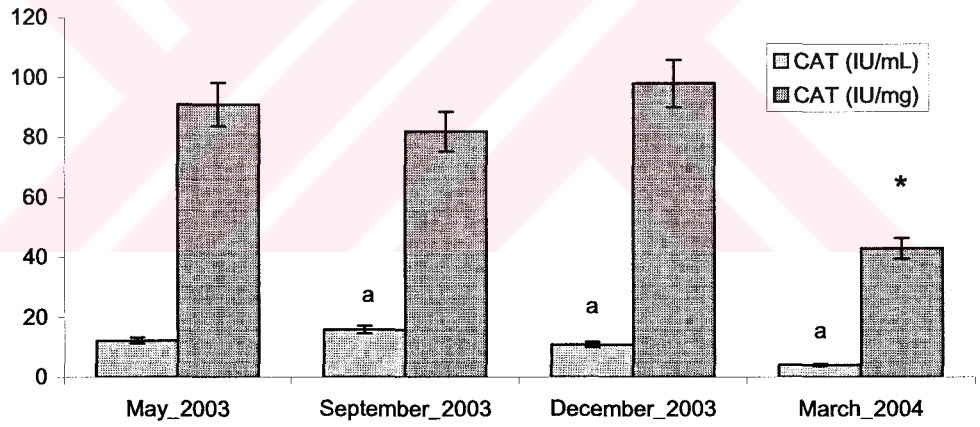


Figure 4.8 Seasonal changes of CAT enzyme activities in *C.racemosa*. The values are the means of three separate experiments. * and a represent significant ($p<0,05$) difference from specific and volumetric activities in May_2003, respectively.

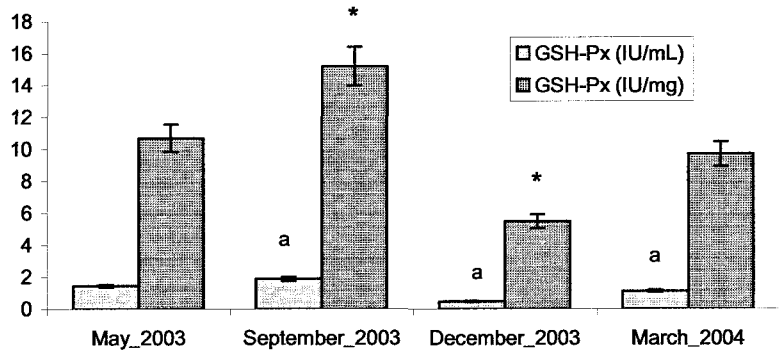


Figure 4.9 Seasonal changes of GSH-Px enzyme activities in *C.racemosa*. The values are the means of three separate experiments. * and a represent significant ($p<0,05$) difference from specific and volumetric activities in May_2003, respectively.

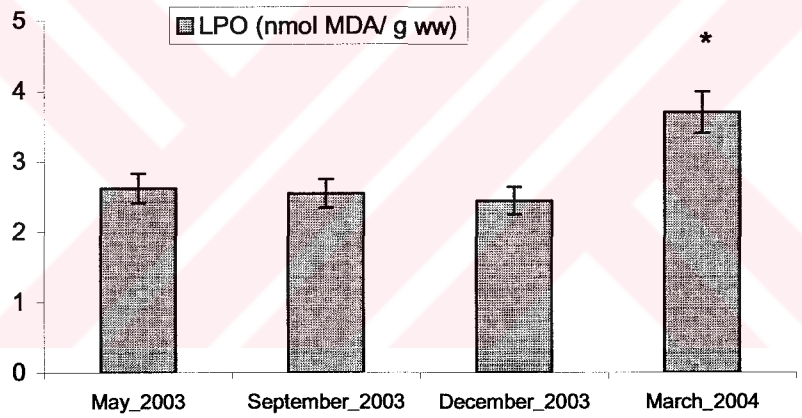


Figure 4.10 Seasonal changes of Lipid peroxidation levels in *C.racemosa*. The values are the means of three separate experiments. * represents significant ($p<0,05$) difference from LPO level in May_2003.

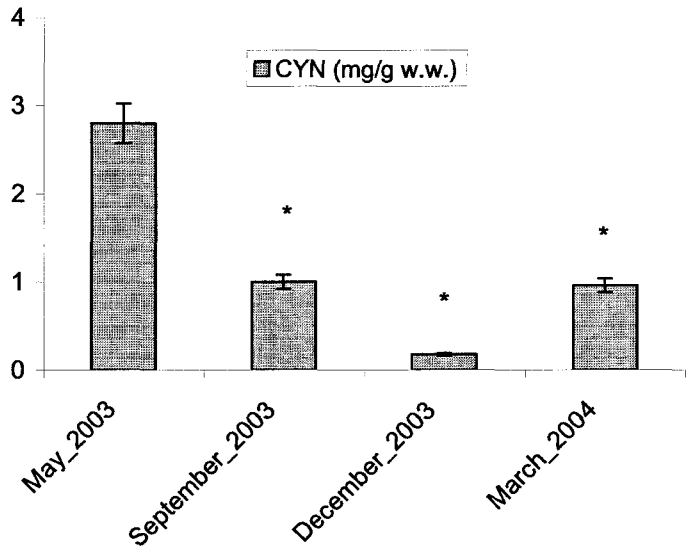


Figure 4.11 Seasonal changes of caulerpenyne levels in *C.racemosa*. The values are the means of three separate experiments. * represents significant ($p<0,05$) difference from CYN in May_2003.

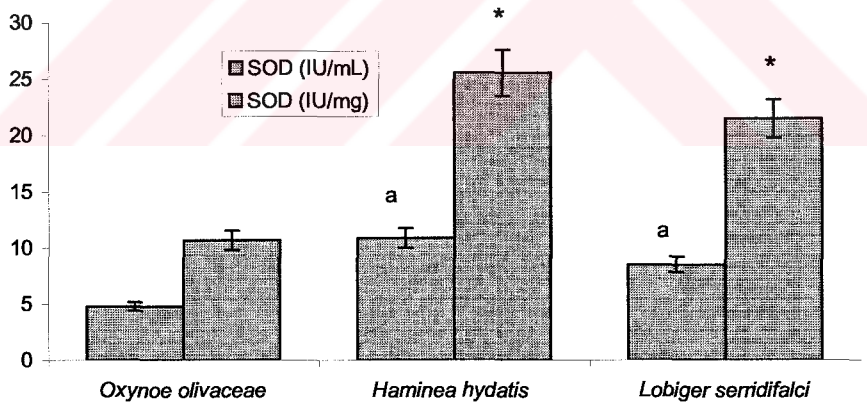


Figure 4.12 SOD enzyme activities in the Sea slugs. The values are the means of three separate experiments. * and a represent significant ($p<0,05$) difference from specific and volumetric activities in *Oxynoe olivacea*.

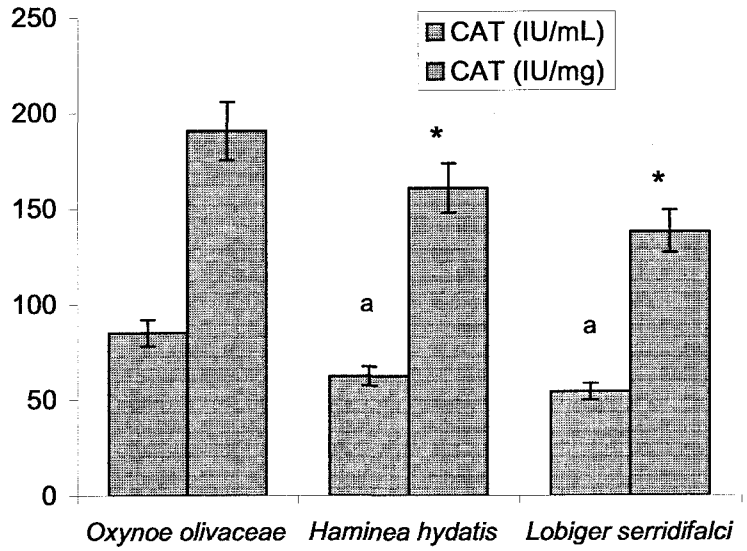


Figure 4.13 CAT enzyme activities in the Sea slugs. The values are the means of three separate experiments. * and a represent significant ($p<0,05$) difference from specific and volumetric activities in *Oxynoe olivacea.*

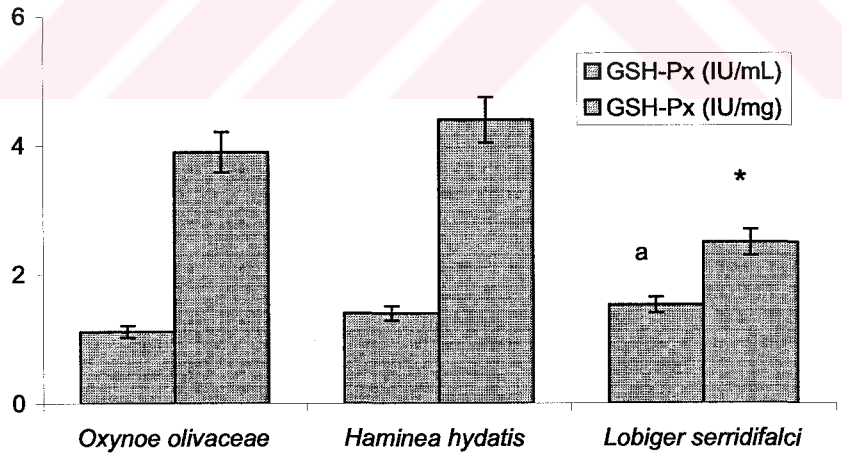


Figure 4.14 GSH-Px enzyme activities in the Sea slugs. The values are the means of three separate experiments. * and a represent significant ($p<0,05$) difference from specific and volumetric activities in *Oxynoe olivacea.*

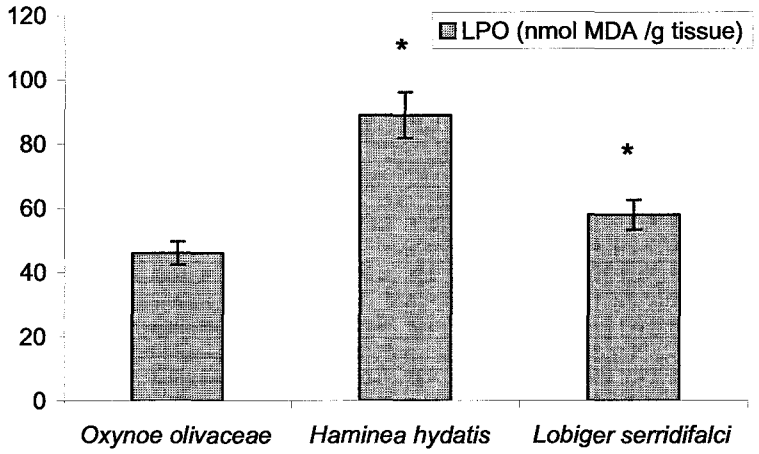


Figure 4.15 LPO levels in the Sea slugs. The values are the means of three separate experiments. * represents significant ($p<0,05$) difference from LPO level in *Oxynoe olivacea*.

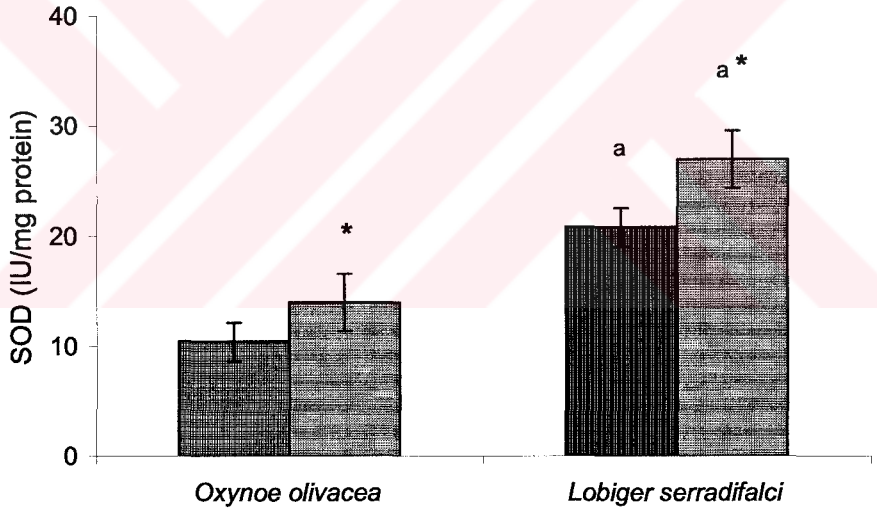


Figure 4.16 Specific superoxide dismutase (SOD) enzyme activities in Oxynoe olivacea and Lobiger serradifalci. Horizontal and vertical lines represent SOD enzyme activities at 20 ^{0}C and 27 ^{0}C, respectively. The given values are the means of three separate experiments.

* significantly ($p<0,05$) different from cold condition

a, significantly ($p<0,05$) different from *Oxynoe olivacea*

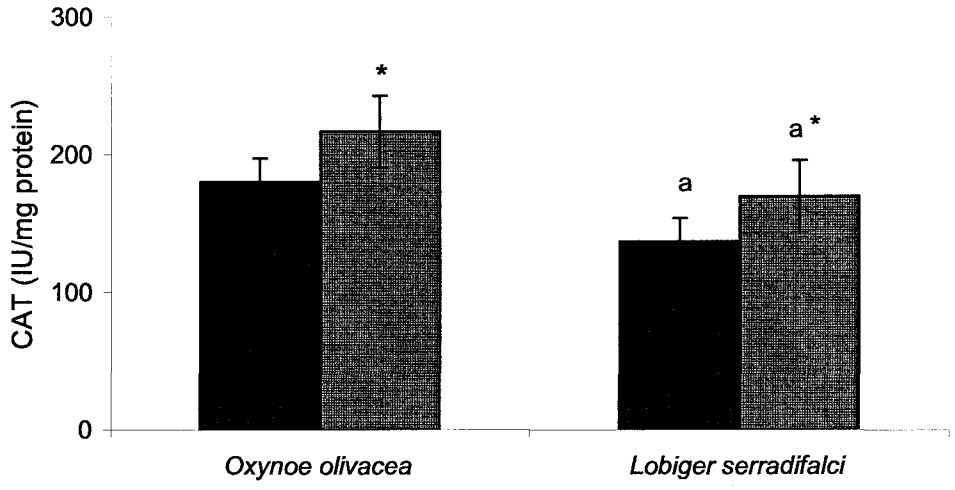


Figure 4.17 Specific catalase (CAT) enzyme activities in *Oxynoe olivacea* and *Lobiger serradifalci*. Horizontal and vertical lines represent CAT enzyme activities at 20^{0}C and 27^{0}C, respectively. The given values are the means of three separate experiments.

* significantly (p<0,05) different from cold condition

a, significantly (p<0,05) different from *Oxynoe olivacea*

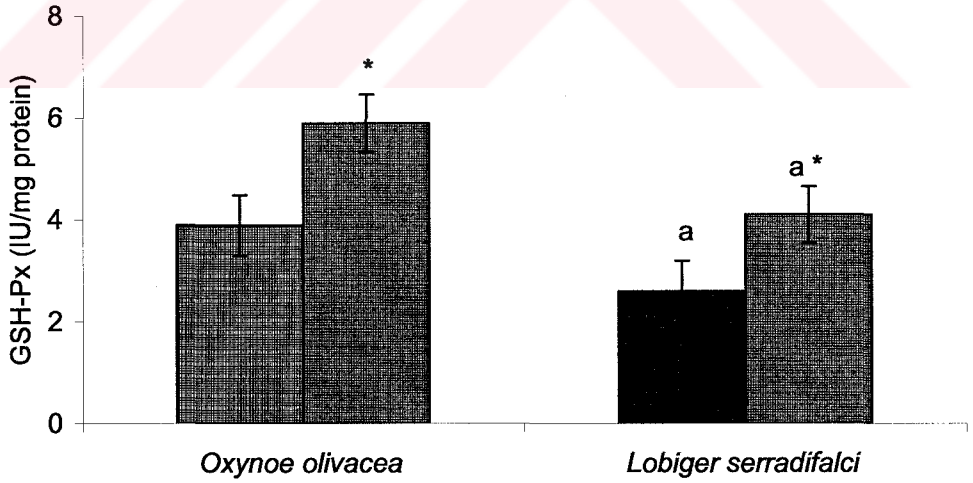


Figure 4.18 Glutathione peroxidase (GSH-Px) activities in Oxynoe olivacea and Lobiger serradifalci. Horizontal and vertical lines represent GSH-Px activities at 20 ^{0}C and 27 ^{0}C, respectively. The given values are the means of three separate experiments.

* significantly (p<0,05) different from cold condition

a, significantly (p<0,05) different from Oxynoe olivacea

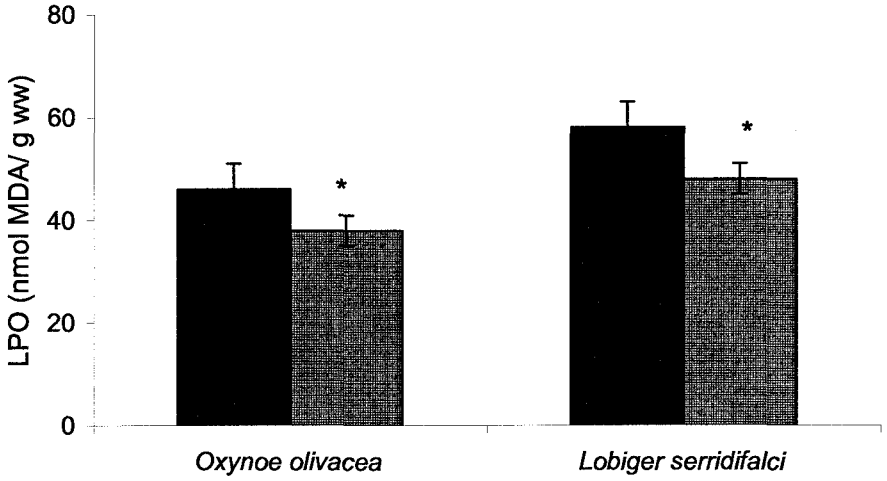


Figure 4.19 Lipid peroxidation (LPO) levels in Oxynoe olivacea and Lobiger serradifalci. Horizontal and vertical lines represent LPO levels at 20 ^{0}C and 27 ^{0}C, respectively. The given values are the means of three separate experiments. * significantly (p<0,05) different from cold condition.

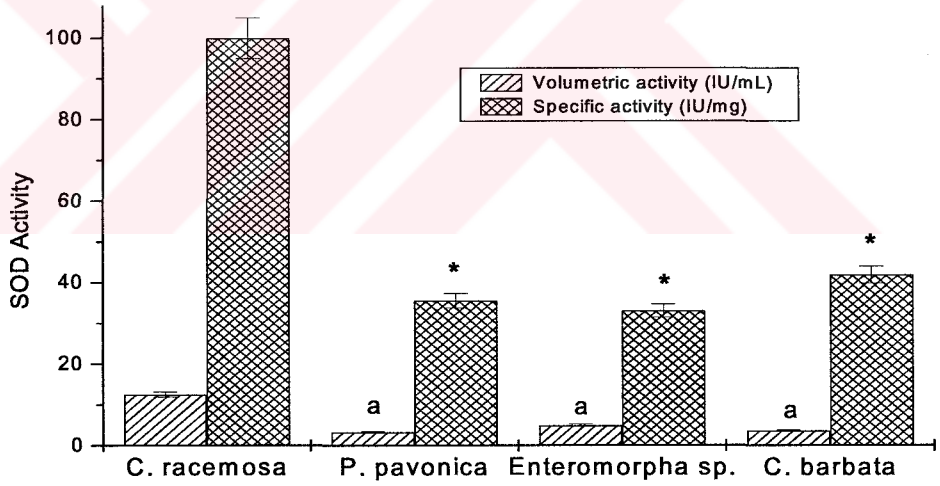


Figure 4.20 Superoxide dismutase (SOD) enzyme activities in *C.racemosa, P.pavonica, Enteromorpha* sp and *C.barbata*. The values are the means of three separate experiments. a and * represent significant differences (p<0,05) compared to volume and specific activities of *C.racemosa*, respectively.

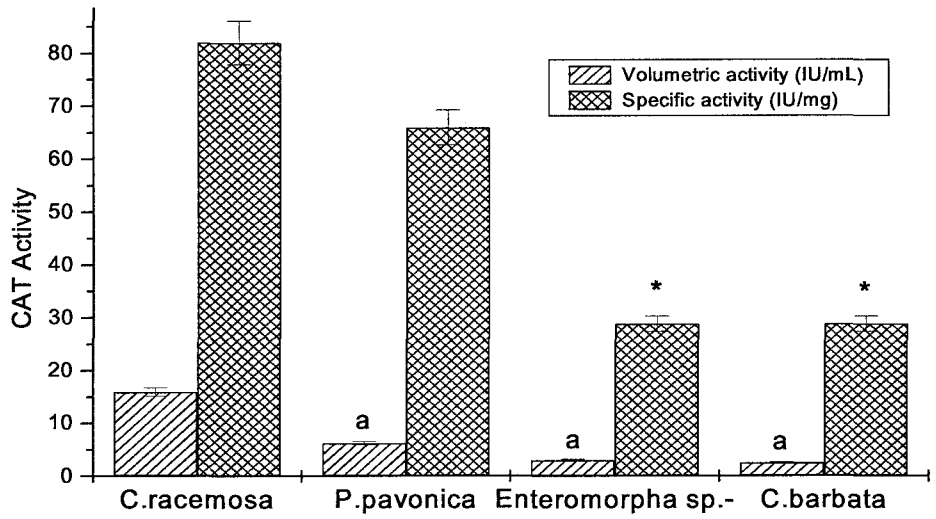


Figure 4.21 Catalase (CAT) enzyme activities in *C.racemosa, P.pavonica, Enteromorpha* sp and *C.barbata.* The values are the means of three separate experiments. a and * represent significant differences ($p<0,05$) compared to volume and specific activities of *C.racemosa*, respectively.

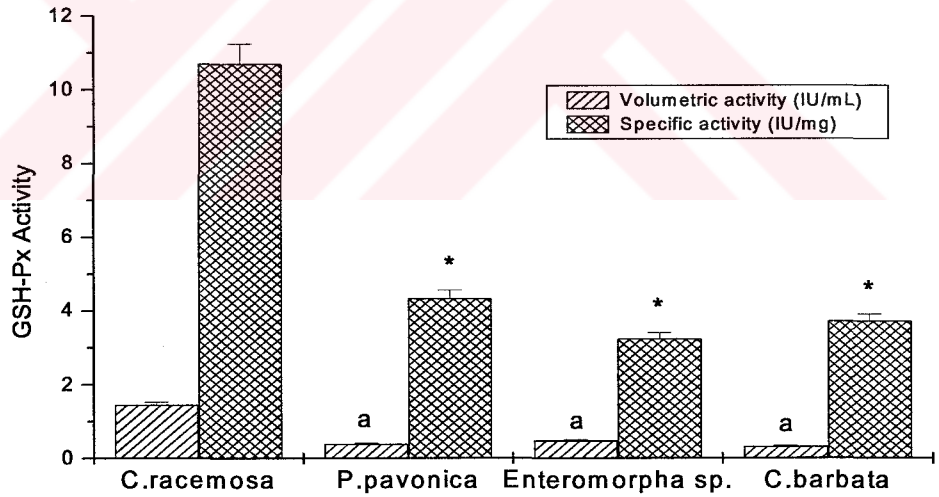


Figure 4.22 Glutathione peroxidase (GSH-Px) enzyme activities in *C.racemosa, P.pavonica, Enteromorpha* sp and *C.barbata.* The values are the means of three separate experiments. a and * represent significant differences ($p<0,05$) compared to volume and specific activities of *C.racemosa*, respectively.

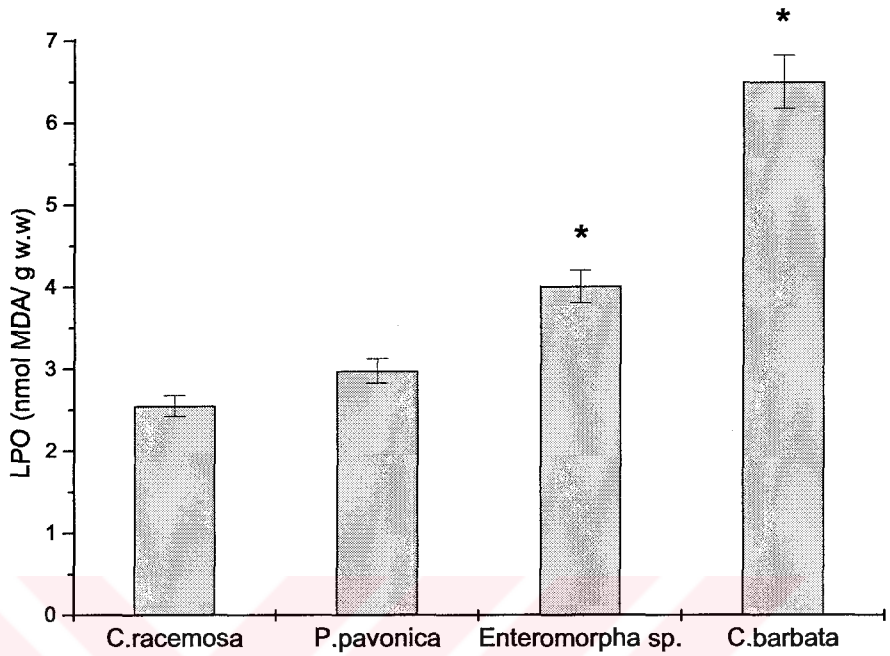


Figure 4.23 Lipid peroxidation levels in *C.racemosa, P.pavonica, Enteromorpha* sp and *C.barbata.* The values are the means of three separate experiments. * represents significant differences ($p<0,05$) compared to LPO level of *C.racemosa*, respectively.

CHAPTER FIVE
CONCLUSION

Antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase and lipid peroxidation and caulerpenyne contents (in only *Caulerpa* species) in killer alga *Caulerpa taxifolia*, invasive form of *Caulerpa racemosa, Caulerpa prolifera*, *Padina pavonica*, *Enteromorpha sp, Cystoseria sp,* and some sea slugs such as *Oxynoe olivacea*, *Lobiger serradifalci* and *Haminoe hydatis* which are live on *Caulerpa* species were investigated in the present Ph.D. thesis.

As can be seen from Figure 4.1 and Table 4.1, maximum SOD enzyme activities were found in *C.prolifera* ($p<0,05$) compared to *C.racemosa* and *C.taxifolia.* SOD activity of *C.prolifera* in low light and warm conditions was also significantly ($p<0,05$) higher than those of other conditions compared to *C.prolifera.* In the similar conditions, *C.taxifolia* showed also higher SOD activity whereas SOD activity of *C.racemosa* in low light and warm condition was lower than those of other conditions. Higher SOD activity observed in *C.prolifera* might be explained with over superoxide radical production compared to other *Caulerpa* species. It is interesting to note that lower SOD activities were observed in *C.taxifolia* which has highest invasive capacity compared to other *Caulerpa sp.* This result can be explained by higher CYN secretions in this species. On the other hand other, non-enzyme based antioxidant molecules such glutathione, ascorbate, vitamin E and A may underlie this result in *Caulerpa taxifolia.* In cold conditions, lower SOD enzyme activities were observed in C.racemosa which may have a mean that possible eradication efforts might be applied against this species during winter season. Lower SOD enzyme activities which can not dismutate the over production of superoxide radical production and may cause the formation of other oxygen-based free radicals.

One of the vital enzymes of antioxidant system is catalase. In contrast to SOD enzyme activities, CAT activities were partially higher than those of other *Caulerpa* species. Specifically, the results showed that catalase activity in *C.taxifolia* in cold condition was higher than those of other *Caulerpa* species. This result supported the origin of *C.taxifolia* is aquarium. As defined in several papers (Meinesz et al, 2001,

Wiedenmann et al, 2001) aquarium originated *C.taxifolia* is resistant to cold water. On the other hand, lowest CAT activity was observed in *C.racemosa* which was grown in cold condition. Results may show that hydrogen peroxide production in *Caulerpa taxifolia* was higher than those of other *Caulerpa* species (Figure 4.2 and Table 4.2).

Another primary antioxidant enzyme is glutathione peroxidase (GSH-Px). GSH-Px activities showed similarities with CAT enzyme activities. Higher GSH-Px enzyme activities were found in Killer alga (*C.taxifolia*) compared to other *Caulerpa* species in this study. Although GSH-Px activities in *C.racemosa* and *C.prolifera* in cold conditions were significantly ($p>0.05$) lower than in other conditions, *C.taxifolia* growth in cold condition was not significantly different compared to other conditions. Higher GSH-Px activities in *C.taxifolia* in cold condition may also show its cold resistance. Insignificant differences in GSH-Px enzyme activities among *Caulerpa* species were depicted in Table 4.3. There were no significant differences ($p>0.05$) between *C.racemosa* LLWS and *C.racemosa* Cold; *C.racemosa* LLWS and *C.prolifera* Cold; *C.taxifolia* LLW and *C.prolifera* LLW; *C.taxifolia* LLW and *C.prolifera* HLW; *C.prolifera* LLW and *C.prolifera* HLW.

As can be seen in from Figure 4.4, maximum total peroxidase activities were determined in C.*racemosa* LLWS and *C.prolifera* Cold. It is interesting to note that total peroxidase activities in killer alga were significantly ($p<0,05$) higher than those of other species. These results may show that importance of catalase and glutathione peroxidase activities in *C.taxifolia*. On the other hand, ascorbate peroxidase might be a reason of the observation higher total peroxidase in both *C.racemosa* and *C.prolifera*. Higher total peroxidase activities in both *C.racemosa* and *C.prolifera* may correspond to the higher production of hydrogen peroxide in these species. It was reported that the hydrogen peroxide secrete by frond surface of these species to prevent growth of epiphytes (Choo et al, 2004).

Lipid peroxidation level as a marker of membrane damage caused by oxygen free radicals was highest in *C.prolifera* in high light and warm conditions. On the other hand, lowest LPO level was observed in *Caulerpa racemosa* in higher light and

warm conditions. Similar amounts of LPO were found among *C.taxifolia*, however these values significantly ($p<0,05$) higher than those of *C.racemosa*. Except for *C.prolifera*, there was no significantly ($p>0,05$) differences in LPO levels among *C.prolifera* and *C.taxifolia*. Lower LPO levels observed in *C.racemosa* might have been caused by possibly higher antioxidant molecules such as ascorbate, glutathione and some antioxidant vitamins such as Vitamin E and A in this species (Figure 4.5 and Table 4.5).

According to Figure 4.6, The higher caulerpenyne levels compared to *C.racemosa* were found in *C.taxifolia* and *C.prolifera*. The CYN levels in *C.taxifolia* and *C.prolifera* were two-fold higher than that of *C.racemosa*. Our results strongly supported the previously published report by Jung et al, (2002). The CYN levels among *Caulerpa* species show differentiation dependent on light–dark and warm-cold conditions. *Caulerpa* species tended to be increase CYN secretion in the high-light conditions. This result may correspond with photosynthetic activities in *Caulerpa* species. The highest CYN contents observed in *C.racemosa* HLWB might have been caused by the biotope difference of this species. Pacific originated *C.racemosa* must secrete higher CYN in order to prevent its predators compared to other local *C.racemosa* varieties. It is very interesting to note that CYN contents in C.prolifera have competitive amounts compared to *C.taxifolia*. In case that C.prolifera would have invasive trait, it can be said that this species will be more harmful and effective invasive capacity in Mediterranean.

In order to understand the variations in antioxidant status in invasive *Caulerpa racemosa* whether it is dependent on season or not, the antioxidant enzyme activities such as superoxide dismutase, catalase, glutathione peroxidase and lipid peroxidation levels and caulerpenyne concentrations were measured in *Caulerpa racemosa* samples which were collected in May-2003, September-2003, December-2003 and March-2004 in Seferihisar coastline.

As can be seen from Figure 4.7, SOD enzyme activities were gradually increased from May-2003 to December-2003 than a sharp decrease was observed in March-2004. The highest and lowest SOD enzyme activities in *Caulerpa racemosa* were

found in December-2003 and March-2004 samples as 105,1 ± 5,6 IU/mg and 69 ± 3,7 IU/mg, respectively. There was a significant difference ($p<0,05$) between these two months.

According to Figure 4.8, similar to SOD, the highest and lowest CAT activities were observed in December-2003 and March-2004 samples. CAT enzyme activities in May-2003, September-2003, December-2003 and March-2004 were 91 ± 4,9 IU/mg, 82 ± 4,7 IU/mg, 98 ± 7,1 IU/mg and 43 ± 3,3 IU/mg, respectively. Although there were significant ($p<0,05$) differences between December-2003/September-2003 and December-2003/March-2004, no statistical ($p>0,05$) difference existed between December-2003 and May-2003 samples.

Glutathione peroxidase activities in *Caulerpa racemosa* dependent on different months were depicted in Figure 4.9. As can be seen from Figure 4.9, GSH-Px enzyme activities showed a fluctuation between May-2003 and March-2004. On contrary to SOD and CAT enzyme activities, the lowest GSH-Px enzyme activities was found in *Caulerpa racemosa* samples that was collected in December-2003. On the other hand, the highest GSH-Px enzyme activity was found in September-2003. The GSH-Px enzyme activities in May-2003, September-2003, December-2003 and March-2004 were 10,7 ± 1,13 IU/mg, 15,2 ± 2,27 IU/mg, 5,5 ± 0,89 IU/mg and 9,7 ± 2,4 IU/mg, respectively.

Figure 4.10 shows lipid peroxidation levels in *Caulerpa racemosa* samples. As can be seen in Figure 4.10, LPO levels showed a gradually decreasing trend from May-2003 to December-2003 than a sharp ($p<0,05$) increase was observed in *Caulerpa racemosa* samples that was collected in March-2004. The LPO levels in March-2004 was significantly ($p<0,05$) higher than those of other months. LPO levels in May-2003, September-2003, December-2003 and March-2004 were 2,92 ± 0,13 nmol MDA/g w.w., 2,55 ± 0,17 nmol MDA/g w.w., 2,14 ± 0,20 nmol MDA/g w.w., and 3,70 ± 0,25 nmol MDA/g w.w., respectively.

Figure 4.11 shows caulerpenyne concentrations in *Caulerpa racemosa* samples that were collected in May-2003, September-2003, December-2003 and March-2004. According to Figure 5, there was a gradual decrease from May-2003 to December-2003 and than a sharp increase was observed. CYN concentrations in *Caulerpa racemosa* in May-2003, September-2003, December-2003 and March-2004 were 2,8 ± 0,4 mg/g w.w., 1,00 ± 0,2 mg/g w.w., 0,18 ± 0,01 mg/g w.w., and 0,96 ± 0,01 mg/g w.w., respectively.

Reactive oxygen species in photosynthetic plants are of great importance for some important metabolic events such as key signaling and regulation of metabolic activity. In the light of this information, ROS based-oxidative stress might give a vital clue about invasion of *Caulerpa racemosa* in Mediterranean Sea. In the present study, SOD enzyme activities showed a gradual increase from May-2003 to December-2003 than significant decrease was found in *Caulerpa racemosa* in March-2004. Similarly, solar energy is increased from May to September. High irradiance might have been caused superoxide radical production in the chlophylls of *Caulerpa racemosa*. In order to scavenge the produced superoxide radical anion, *Caulerpa racemosa* might has been tended to increase SOD enzyme activities up to September-2003. Increasing superoxide radicals, however, increasing water temperature might have been caused the highest SOD enzyme activity in December-2003. Lower solar radiation and therefore lower superoxide radical production might have been caused the lowest SOD enzyme activities in March-2004. Except for September-2003 samples, CAT activities showed a similar trend like SOD activities. A gradual increase between May-2003 and December-2003 was observed and than sharp decrease was also found in CAT activities. These results can be evidence for hydrogen peroxide over-production in May and December. On the other hand, other peroxidase, GSH-Px enzyme activities in *Caulerpa racemosa* showed different trend compared to SOD and CAT enzyme activities. A fluctuation was found in GSH-Px enzyme activities in *Caulerpa racemosa* samples. Contrast to CAT activity in September-2003, GSH-Px activity reached maximum in September-2003 in *Caulerpa racemosa*. The observed highest activities in SOD and CAT in December-2003, the lowest GSH-Px activities were determined in this month. Since over-

expressed CAT activities might have been decomposed the produced hydrogen peroxide, GSH-Px activities might have been remained lower value in this month. The observation of different trends in both CAT and GSH-Px enzyme activities can be evidence of production of different types of peroxides such as lipid hydroperoxides. Lipid peroxidation levels decreased from May-2003 to December-2003 with increased SOD and CAT enzyme activities.

According to results, GSH-Px enzyme activities could not strictly affect the LPO levels in *Caulerpa racemosa*. On the other hand, there was a negative correlation between LPO levels and both SOD and CAT activities. Decreased SOD and CAT activities caused the increased LPO levels in March-2004. On the other hand, increased GSH-Px activities in this month could not prevent the induced LPO levels in *Caulerpa racemosa*.

It is very interesting to note that CYN concentration showed the similar trend with LPO levels. Decreasing of solar radiation from May to December may have been caused the decreased CYN levels. Results may show that the biosynthesis of caulerpenyne might have been increased with decreasing antioxidant power of *Caulerpa racemosa* to protect itself from its predators. Since lower antioxidant activities, lower caulerpenyne concentrations and higher lipid peroxidation levels in March, possible eradication methods might also be applied in this month against this potential invader in Mediterranean Sea.

With the greatly increased number of marine biological invasions occurring globally it is inevitable that attention would turn to marine bio-control. For this purpose, four ascoglossans as biological control agents against *C.taxifolia* has been examined. According to Thibaut et al, *Lobiger serridifalci* and *Oxynoe olivaceae* may be used for eradication of *C.taxifolia*. These species have very important characteristics, one of them is metabolizing of caulerpenyne secreted by *C.taxifolia*. These species use Caulerpenyne to produce their own chemical weapons. Cimino et al (1999) reported that these species modify caulerpenyne to transform their toxins called oxytoxin-1 and oxytoxin-2.

In order to understand the relationship between antioxidant statuses among sea slugs that were in our hand, we determined similar parameters in these sea slugs. According to our results, SOD enzyme activity in *Haminoe hydatis* was higher than those of other two sea slugs. However, CAT activity in *Oxynoe olivaceae* was higher than those of other two sea slugs. GSH-Px enzyme activity showed an arrangement which followed the order : *Haminea hydatis* > *Oxynoe olivaceae* > *Lobiger serridifalci.* On the other hand, in contrast to higher antioxidant enzyme activities that were observed in *H.hydatis* except CAT activity, maximum LPO levels were also observed in *H.hydatis*. This result might be explained by stimulation of LPO in *H.hydatis* might be depend on other systems (Figure 4.12 – 4.15).

In order to understand the effects of temperature on *Oxynoe olivacea* and *Lobiger serradifalci,* we carried out experiments on two different climatical conditions. In both species, the SOD activity at 27 ^{0}C was found to be higher than the activity observed at 20 ^{0}C ($p<0,05$). At both temperature, *L. serradifalci* showed a higher SOD activity compared to *O. olivacea*, and this difference was statistically significant at both temperatures ($p<0,05$). SOD activities in *O.olivacea* and *L.serradifalci* at 20 ^{0}C and 27 ^{0}C were 10,5 ± 0,6 IU/mg and 14 ± 1,5 IU/mg; 20,9 ± 0,9 IU/mg and 27,0 ± 2,0 IU/mg, respectively (Figure 4.16).

CAT activities were also found to decrease significantly with decreasing temperature ($p<0,05$), but CAT activity in O. olivacea was significantly higher than that of *L. serradifalci* at both temperatures ($p<0,01$ at 20 ^{0}C; $p<0,05$ at 27 ^{0}C). CAT activities in O. olivacea and *L. serradifalci* at 20 ^{0}C and 27 ^{0}C were 170,0 ± 10.0 and 217 ± 3,5 IU/mg; 137,0 ± 3,6 and 180,2 ± 5,0 IU/mg, respectively (Figure 4.17).

GSH-Px activities are depicted in Figure 4.18. O. olivacea showed significantly higher GSH-Px activities in both warm and cold conditions, when compared to *L. serradifalci* ($p<0,05$). As it was observed in SOD and CAT activities, GSH-Px activity was found positively correlated wit temperature ($p<0.05$). GSH-Px activities in O. olivacea and *L. serradifalci* at 20^{0}C and 27 ^{0}C were 4,1 ± 0,2 and 5,9 ± 0,8 IU/mg; 2,6 ± 0,3 and 3,9 ± 0,3 IU/mg, respectively.

LPO levels at 20 ^{0}C were significantly higher than LPO levels observed at 27 ^{0}C ($p<0,05$ for both species). But when the LPO levels at a given temperature were compared between species, no significant differences were observed ($p>0,05$). LPO levels in O. olivacea and *L. serradifalci* at 20 ^{0}C and 27 ^{0}C were 48,0 ± 2,7 and 38,0 ± 3,6 nmol MDA/ g tissue; 58,3 ± 3,5 and 46,0 ± 4,0 nmol MDA/ g tissue, respectively (Figure 4.19).

In the framework of this study, antioxidant status and lipid peroxidation levels of *O. olivacea* and *L. serradifalci* were investigated at different temperature conditions. In both species, SOD enzyme activity was found in positive correlation with temperature, and SOD activity in *L. serradifalci* was significantly higher than the activity observed in *O. olivacea*. CAT and GSH-Px activities were also found to increase by temperature. But on the contrary to SOD activity, CAT and GSH-Px activities in *L.serradifalci* were statistically lower than those recorded in *O.olivacea* at both temperatures. An increase peroxidase activity (GSH-Px and CAT activities) indicate an increase in the formation of hydrogen peroxide and lipid hydroperoxides. In order to evaluate the membrane damage caused by ROS, LPO levels at two different temperature conditions were also determined. Our data reveal that in both of the species LPO levels decrease significantly by increasing temperature. In addition, the LPO levels in *L. serradifalci* seem to be greater than the LPO levels in *O. olivacea* at both temperature conditions. Almost two-fold increased SOD activity in *L. serradifalci*, compared to *O. olivacea*, suggests a higher conversion of superoxide to H_2O_2 in *L. serradifalci*. A higher level of lipid peroxidation is expected as a consequence of high H_2O_2 concentration and low peroxidase activity. However, the difference in LPO levels between the two species is not as apparent as it is in SOD, CAT and GSH-Px activities, thus, statistically not significant. This indicates that other antioxidant systems compensate the low peroxidase activity in defense mechanisms of *L. serradifalci*.

An increase in SOD activity is a sign of enhanced superoxide production. The electron transport chains of mitochondria and endoplasmic reticulum are considered as the major intracellular sources of superoxide. Although the components of these chains pass the bulk of electrons onto the next component of the chain some appear

to "leak" electrons to oxygen (Halliwell and Gutteridge, 1999). Thus the rate of superoxide production is increased at elevated ambient oxygen concentrations. There is enough of evidence that the increase in metabolic rate and higher calorie intake enhance the oxidative stress in tissues, which supports the idea that the more electrons are passed on to the electron transport chain, the more superoxide radicals are produced (Sohal and Weindruch, 1996). On the other hand, electron transport chains are not the only sources of superoxide production. Various superoxide-generating systems have been well defined (Silva et al., 1996; Sorescu and Griendling, 2002).

The solubility of oxygen in sea water decreases as the temperature increases. Therefore, the changes in SOD activity cannot be explained by the enhanced superoxide production due to elevated oxygen concentration in our experimental system. However, the increase in ambient temperature may lead to a higher feeding rate and a higher metabolic activity. Concentrations of the oxidant molecules found in the food may also result in a change in the antioxidant status of these slugs, since the SOD activity observed in *C. racemosa* and *C. prolifera* is also positively correlated with temperature. Caulerpenyne, a cytotoxic sesquiterpenoid, is the major metabolite secreted by the members of genus *Caulerpa* for defensive purposes. The toxic effects of CYN have been demonstrated on various species. This effect can vary among species during developmental stages (Lemée et al., 1993; Lemée et al., 1997). Ascoglossans feeding on *Caulerpa* spp. were found to modify CYN into a couple of more toxic compounds, oxytoxin-1 and -2 for their own defense systems (Gavagnin et al., 1994). It was reported that CYN production is positively correlated temperature (Amade and Lemée, 1998; Dumay et al., 2002), suggesting that increasing CYN concentration alters the antioxidant status of these sacoglossans, that should be verified by further studies.

In conclusion, antioxidant status of the sacoglossan species, *Oxynoe olivacea* and *Lobiger serradifalci* are modified with ambient temperature. In both of the species antioxidant enzyme concentrations (SOD, CAT and GSH-Px) are positively correlated with temperature, but different enzyme systems are affected to variable

extent when the species are compared. LPO levels are decreased at higher temperatures, but there is no significant distinction between the species investigated in the framework of this study. These results demonstrate that the oxidative consequences of a given environmental condition may vary among different species. Furthermore, it is not reliable to predict which antioxidant enzyme system is affected, if only the LPO levels are taken into consideration. Our findings support the idea proposed by Dotan et al, 2004, that is, "lipid peroxidation cannot be used as a universal criterion of oxidative stress".

In order to compare antioxidant status of *Caulerpa racemosa* which is highly propagated in Turkish coastline with other sea plants which were collected from same habitat of *Caulerpa racemosa*, *Padina pavonica*, *Cystoseria sp.*, and *Enteromorpha sp* were also collected in September, the antioxidant enzyme activities such as SOD, CAT, GSH-Px and lipid peroxidation levels were determined in invasive *C.racemosa*, *P.pavonica*, *C.barbata* and *Enteromorpha* sp that were collected from Seferihisar-Turkey.

As can be seen in Figure 4.20, the highest both volumetric and specific SOD activities were found in invasive *C.racemosa* samples. SOD activities in *C.racemosa* were significantly ($p<0,05$) higher than those of other sea plants. No statistical differences ($p>0,05$) in SOD activities were found among *P.pavonica, Enteromorpha sp* and *Cystoseria barbata.*

The CAT activities related to invasive *C.racemosa, P.pavonica, C.barbata* and *Enteromorpha* sp were depicted in Figure 4.21. CAT activities showed a gradual decrease from *C.racemosa* to *C.barbata*. Similar to SOD activities, the highest both volumetric and specific CAT activities were found in *C.racemosa* samples. The CAT activity in *P.pavonica* were significantly ($p<0,05$) higher than those of both *Enteromorpha sp.* and *C.barbata*. On the other hand, no statistical ($p>0,05$) was found between *P.pavonica* and *C.racemosa*. There were no statistical difference ($p>0,05$) in CAT activities between *Enteromorpha sp* and *C.barbata*.

As can be seen from Figure 4.22, the highest both GSH-Px activities as well as in SOD and CAT were found in *C.racemosa* samples. Volumetric and specific GSH-Px enzyme activities in *C.racemosa* were about 3- and 2-fold higher than those other sea plants, respectively. Although no statistical difference ($p>0,05$) was found between *P.pavonica* and *C.barbata*, volumetric GSH-Px enzyme activity in Enteromorpha sp was significantly ($p<0,05$) higher than those of *P.pavonica* and *C.barbata*. Specific GSH-Px activity in P.pavonica was significantly ($p<0,05$) higher than that of *Enteromorpha sp.*

Lipid peroxidation levels were shown in Figure 4.23. While the lowest LPO level was observed in *C.racemosa*, the highest LPO level was found in *C.barbata*. The LPO levels in *C.racemosa*, *P.pavonica*, *Enteromorpha sp* and *C.barbata* were 2,55 ± 0,13 nmol MDA/g w.w.; 2,98 ± 0,15 nmol MDA/g w.w.; 4,01 ± 0,20 nmol MDA/g w.w.; and 6,5 ± 0,33 nmol MDA/g w.w., respectively.

Antioxidant status of an aerobic organism is of great importance and might be used to evaluate its metabolic power and resistance to environmental adaptation. Over produced reactive oxygen species might play important roles in the modification of genetic trait or in the possibly mutation by affecting DNA. In the light of this information, the antioxidant status of invasive species might give some vital clues for understanding of its invasion. In the present study, the highest SOD activities were found in *C.racemosa* as compared to other sea plants. This result may show that superoxide radical production in *C.racemosa* might be higher than those of other sea plants. Over-produced superoxide radical in *C.racemosa* that can not be dismutated by SOD might damage especially on DNA as well as other important cell components such as proteins and polyunsaturated fatty acids (PUFA) of cell membrane (Imlay and Linn, 1986; Gardner and Fridovich; 1991, Bradley and Min, 1992) and may cause both genotypic or phenotypic differentiation.

As can be seen from Figure 4.21, CAT activities in *C.racemosa* were also significantly higher than those of other sea plants. Higher CAT activities may correspond with higher hydrogen peroxide production in *C.racemosa* compared to

other sea plants. Hydrogen peroxide production in an aerobic metabolism is of great importance because it might be transformed to another harmful radical such as hydroxyl radical in the existence of some metals such as iron (Hippeli et al, 1999). Higher CAT activities observed in *C.racemosa* compared to other sea plants may show that this species scavenges hydrogen peroxide more efficiently than other species. On the one hand, possible over-production of hydrogen peroxide related to higher CAT and GSH-Px activities in *C.racemosa* might have been used to remove epiphytes and other predators. Accordingly, we observed lots of sea slugs species such as Lobiger serradifalci and Oxynoe olivacea on *C.racemosa* during our diving in Seferihisar coastline. On the other hand, higher production of hydrogen peroxide may be used by *C.racemosa* to form some volatile halogenated compounds. Inhibition of epiphytes' growth on the surface of some algae species by these halogenated compounds has been shown in some reports (Collén and Pedersen, 1994; Choo *et al*, 2004; Abrahamsson *et al*, 2003). Our results may support these previously findings.

As can be seen from Figure 4.22, maximum GSH-Px activities were found in *C.racemosa* as well as SOD and CAT. The higher GSH-Px activities might be a reason of over production of lipid hydroperoxides. On the other hand, *C.racemosa* might have been tended to increase this enzyme expression to protect PUFA of cell membrane effectively in the adaptation to new habitat. Higher CAT activities might support higher GSH-Px activities in *C.racemosa* compared to other sea plants. Higher antioxidant enzyme activities in *C.racemosa* resulted in lower LPO levels compared to other sea plants. LPO levels showed that the cell damage by ROS in *C.racemosa* was minimum compared to other sea plants. In this case, there might be some explanations, first, the over-produced ROS in *C.racemosa* might have been immediately scavenged by antioxidant system, second, *C.racemosa* might have been used these unscavenged ROS to adapt to new environment for invasion by effecting its DNA structure. This might be resulted phenotypic and genotypic differentiation in *C.racemosa*. If *C.racemosa* is taken into consideration chronologically, this species shown different characteristics in different habitats, therefore, its Latin name (variety and forms) was modified several times (Verlaque *et al*, 2003). According to our

results, ROS may underlie in the both phenotypic and genotypic differentiations in *C.racemosa*. When other sea plants are taken into consideration, since they can contain other antioxidant molecules such as vitamin E and A, ascorbate, GSH, carotenoids, their metabolism might overcome ROS by having possible higher these non-enzyme based antioxidant molecules. Therefore, further characterization of antioxidant system in these sea plants is strongly warranted.

In conclusion, since higher antioxidant enzyme activities and lower lipid peroxidation levels observed in invasive *C.racemosa* compared to other sea plants in the same habitat, it is obvious that this species is more tolerant and powerful to war against ROS compared to other sea plants in the present study. The results clearly show that invasion of this species might continue unless its strong antioxidant system would not effected by any inhibitors or environmental changes e.g.

REFERENCES

Abrahamsson, K., Choo, K. S., Pedersen, M., Johansson G. & Snoeijs P., (2003). Effects of temperature on the production of hydrogen peroxide and volatile halocarbons by brackish-water algae. *Phytochemistry,* 64, 725-734.

Aebi, H. (1985). Catalase, In Methods in Enzymology (Packer, L., Ed.), 121-126, San Diego, Academic Press.

Afanas'Ev, IB.(2004) Mechanism of superoxide-mediated damage relevance to mitochondrial aging. Annuals New York Academy of Sciences. 1019:343-5. Review.

Amade, P., & Lemée, R., (1998). Chemical defense of the Mediterranean alga *Caulerpa taxifolia*: variations in caulerpenyne production. *Aquatic Toxicology*, 43, 287-300.

Asada, K., Kanematsu, S., Okaka, S., & Hayakawa, T., (1980). In: Chemical and Biochemical Aspects of Superoxide and Superoxide Dismutase (Bannister J.V. and Hill A.O. Eds.), North-Holland, New York, Elsevier.

Asada, K., (1999). The water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annuals Review of Plant Physiology and Plant Molecular Biology.* 50, 601-639.

Balasubramanian, T, Somasundaram, M, & Felix AJ.(2004). Taurine prevents Ibuprofen-induced gastric mucosal lesions and influences endogenous antioxidant status of stomach in rats. *Scientific World Journal.* 4, 1046-54.

Barkley, K.B., & Gregory, E.M. (1990). Tetrameric manganese superoxide dismutases from anaerobic Actinomyces. *Archives Biochemistry Biophysics*, 280 (1),192-200.

Bellan-Santini, D., Arnaud, P. M., Bellan, G.,& Verlaque, M., (1996). The influence of the introduced tropical alga *Caulerpa taxifolia*, on the biodiversity of the

Mediterranean marine biota. *Journal of Marine Biological Association U.K.* 76, 235-237.

Bors, W., Saran, M., Lengfelder, E., Spottl, R., & Michel, C., (1974). The relevance of the superoxide anion radical in biological systems. *Topics Radiation Research. Quarterly*. 9(3),247-309.

Bozkaya, L.A., (2000). Purification, immobilization and characterization of superoxide dismutase from chicken heart. Master of Science Thesis, Dokuz Eylul University, Institute of Educational Sciences.

Bradford, M.M., (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*. 72, 248-252.

Bradley, D. E.,& Min, D. B., (1992). Singlet Oxygen Oxidation of Foods. *Critical Reviews in Food Science and Nutrition*.31, 211-236.

Bridges, S.M., & Salin, M.L., (1981). Distribution of iron-containing superoxide dismutase in vascular plants. *Plant Physiology*. 68, 275 278.

Brioukhanov, A.L., & Netrusov, A.I., (2004). Catalase and superoxide dismutase: distribution, properties, and physiological role in cells of strict anaerobes. *Biochemistry (Mosc)*. 69(9), 949-62.

Boudouresque, C. F., Lemee, R., Mari X, & Meinesz A., (1996). The invasive alga *Caulerpa taxifolia* is not a suitable diet for the sea urchin *Paracentrotus lividus*. *Aquatic Botany*. 53, 245–250.

Carlioz, A., Ludwig, M.L., Stallings, W.C., Fee, J.A., Steinmann, H.M., and Touati, D., (1988). *Journal of Biological Chemistry*, 263, 1555

Carrico, R.J., & Deutsch, H.F. (1969). Isolation of human hepatocuprein and cerebrocuprein. *Journal of Biological Chemistry*. 244, 6087–6093

Catalase three dimensional structure (n.d.). Retrieved August 21, 2004, from http://crystal.uah.edu/%7Ecarter/enzyme/catalase.htm.

Catalase (n.d.), Catalase FAQ, Retrieved August 24, 2004, from http://www.seps.org/cvoracle/faq/catalase.html

Cavas, L., & Tarhan, L., (2003). The relationship between total sialic acid levels and antioxidant status in the tadpoles of *Bufo viridis* and *Rana ridibunda ridibunda*. *Cell Biochemistry Function.* 21(3), 257-61.

Cavas, L., (2005). Does underwater rugby stimulate the over-production of reactive oxygen species? *Cell Biochemistry Function.* 23(1), 59-63.

Cavas, L., Yurdakoc, K., & Yokes B., (2005). Antioxidant status *Lobiger serradifalci* and *Oxynoe olivacea* (Opisthobranchia, Mollusca). *Journal of Experimental Marine Biology Ecology.* 314, (2), 227-235.

Ceccherelli, G., & Cinelli, F., (1997). Short-term effects of nutrient enrichment of the sediment and interactions between the seagrass *Cymodocea nodosa* and the introduced green alga *Caulerpa taxifolia* in a Mediterranean bay. *Journal of Experimental Marine Biology and Ecology.* 217, 165-177.

Ceccherelli, G., Piazzi, L., & Balata, D., (2002). Spread of introduced *Caulerpa* species in macroalgal habitats. *Journal of Experimental Marine Biology and Ecology.* 280, 1-11.

Chen, L., Rio, D.C., Haddad, G.G., & Ma, E., (2004). Regulatory role of dADAR in ROS metabolism in *Drosophila* CNS. Brain Research Reviews. 24, 131(1-2), 93-100.

Choo, K., Snoeijs, P., & Pedersen, M., (2004). Oxidative stress tolerance in the filamentous green algae *Chladophora glomerata* and *Enteromorpha ahlneriana*. *Journal of Experimental Marine Biology and Ecology.* 298, 111-123.

Cimino, G., Fontana, A., Gavagnin, M., (1999). Marine opisthobranch molluscs: chemistry and ecology in sacoglossans and dorids. *Current Research in Organic Chemistry* 3, 327–372

Cimino, G., Crispino, A., Di Marzo, V., Gavagnin, M., & Ros, J. D., (1990). Oxytoxins, bioactive molecules produced by the marine opisthobranch mollusc *Oxynoe olivacea* from a diet-derived precursor. *Experientia*. 46, 767-770.

Clifton, K. E., & Clifton, L. M., (1999). The phenology of sexual reproduction by green algae (Bryopsidales) on Caribbean coral reefs. Journal of Phycology. 35, 24-34.

Collén, J., Ekdahl, A., Abrahamsson, K., & Pedersén M., (1994). The involvement of hydrogen peroxide in the production of volatile halogenated compounds by Meristiella gelidium. *Phytochemistry*. 36, 1197-1202.

Craig, L., Taylor, R.K., Pique, M.E., Adair, B.D., Arvai, A.S., Singh, M., et al, (2003). Type IV pilin structure and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. *Molecular Cell*. 11(5), 1139-50.

Crosti, N., Servidei, T., Bajer, J., & Serra, A., (1987). Modification of the 6-hydroxydopamine technique for the correct determination of superoxide dismutase. *Journal of Clinical Chemistry Clinical Biochemistry*. 25, 265-6

Demopoulos, H.B., (1982). Oxygen free radicals in central nervous system ischemia and trauma. In: Autor AP ed. Pathology of Oxygen. New York: Academic Press; 127-155.

Dotan, Y., Lichtenberg, D., & Pinchuk I., (2004). Lipid peroxidation cannot be used as a universal criterion of oxidative stress. *Progress in Lipid Research*. 43, 200-227.

Dumay, O., Pergent, G., Pergent-Martini, C., & Amade, P., (2002). Variations in Caulerpenyne contents in *Caulerpa taxifolia* and *Caulerpa racemosa*. *Journal of Chemical Ecology*, 28, 343-352.

Ertan, O., Turna, I., Cormaci, M., (1998). A new record for the Marine Algal Flora of Turkey: *Caulerpa scalpelliformis* (Brown ex Turner) C.Agardh (Caulerpaceae, Chlorophyceae). *Turkish Journal of Botany*. 22, 285-287.

Escoubet, S., Dupeux, D., & Escoubet, P., (1998). Utilisation du chlorure de sodium comme moyen d'eradication de *Caulerpa taxifolia* (Vahl) C.Agardh. In Proceedings of the Third international workshop on Caulerpa taxifolia, Marseille, France, 19-20 September 1997 (ed C.F., Boudouresque et al.), pp. 117-124. France : GIS Posidonic Publications.

Farber, E., (1981) Chemical carcinogenesis. *N.Engl. J.Medicine*. 305, 23, 1379.

Frank H., (1989). Mass spectrometric detection of cross linked fatty acids formed during radical-induced lesion of lipid membranes. *Biochemistry Journal*, 260, 873

Forman, H.J., & Fridovich, I., (1973). Superoxide dismutase. *Archives Biochemistry and Biophysics*, 158: 396-400

Feher, J., Csomos G., & Vereckei, A., (1987). Free Radical Reactions, Springer Verlag Berlin, 9.

Gabig, T.G., & Babior, B.M, (1979). The O2(-) -forming oxidase responsible for the respiratory burst in human neutrophils. Properties of the solubilized enzyme. *Journal of Biological Chemistry*, 254: 9070 - 9074

Gambi, M.C., & Terlizzi, A., (1998). Record of large population of *Caulerpa racemosa* (Forsskal) J.Agardh (Chlorophyceae) in the Gulf of Salerno (Southern Tyrrhenian Sea, Italy). *Biology of Marine Mediterranean*. 5(1), 553-556.

Gardner, P. R., & Fridovich, I., (1991). Superoxide sensitivity of the Escherichia coli aconitase. *Journal of Biological Chemistry*. 266, 1478-1483.

Garcia, E., Rodríguez-Prieto, C., Delgado, O., & Ballesteros, E., (1996). Seasonal light and temperature responses of *Caulerpa taxifolia* from the north western Mediterranean. *Aquatic Botany*. 53, 215-225.

Gavach, C., Bonnal, L., Uchimura, M., Sandeaux, R., Sandeaux, J., Souard, R., et al, (1999). Destruction de *Caulerpa taxifolia* par la technique de la couver-ture a`ions cuivriques. Developpement preindustriel et premier essais. In: GIS Posidonie (Eds), Third International Workshop on *Caulerpa taxifolia*, Marseille, pp.101-104.

Gavagnin, M., Marin, A., Castelluccio, F., Villani, G. & Cimino, G. (1994). Defensive relationships between *Caulerpa prolifera* and its shelled sacoglossan predators. *Journal of Experimental Marine Biology and Ecology*. 175, 197-210.

Gotoh, T., & Shikama, K., (1976). Generation of the superoxide radical during autoxidation of oxymyoglobin. Journal of Biochemistry (Tokyo). 80(2), 397-9.

Green, T.R., Schaefer, R.E., & Makler, M,T., (1980). Orientation of the NADPH dependent superoxide generating oxidoreductase on the outer membrane of human PMN's. *Biochem Biophys Res Commun*. 14;94(1):262-9.

Guerriero, A., D'Ambrosio, M., Guella, G., Dini, F., & Pietra, F., (1994). Secondary metabolites of the green seaweed *Caulerpa taxifolia* introduced into the Mediterranean sea, and a comparison with ciliates of the genus Euplotes. In: Boudouresque C.F., Meinesz A., Gravez V., (Eds.); "First International Workshop on *Caulerpa taxifolia*", GIS Posidonie Publication.

Halliwell, B., & Gutteridge, J.M.C., (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemistry Journal* 219, 1–14

Halliwell, B., & Gutteridge, J.M.C., (1999). Free Radicals in Biology and Medicine, 3rd ed. Oxford Univ. Press. New York.

Hamel, G., (1926). Quelques alguesrares ou nouvelles pour la flore méditerranéenne. Bulletin Muséum National d'Histoire Naturelle. 32, 420.

Hamel, G., (1931). Sur le Cladostephus dubius Bory. In Travaux crytogamiques dédie à Louis Mangin, 309-312. Laboratoire de Cryptogamie, Muséum National d'Histoire Naturelle, Paris.

Henmi, T., Miyao, M., & Yamamoto, Y. (2004). Release and reactive-oxygen-mediated damage of the oxygen-evolving complex subunits of PSII during photoinhibition. *Plant Cell Physiology*. 45(2), 243-50.

Hippeli, S., Heiser, I., & Elstner, E.F., (1999). Activated oxygen and free oxygen radicals in pathology: New insights and analogies between animals and plants. *Plant Physiology and Biochemistry*. 37, 167-178.

Imlay, J. A., & Linn, S., (1986). Bimodal pattern of killing of DNA-repair-defective or anoxically grown *Escherichia coli* by hydrogen peroxide. *Journal of Bacteriology*. 166(2), 519-27.

Imlay, J.A., & Fridovich, I., (1991). Assay of metabolic superoxide production in *Escherichia coli*. *Journal of Biological Chemistry* 266, 6957-6965

Jaffrennou, B., & Odonne, L., (1994). Procede de maitrise et de reduction du developpement de *Caulerpa taxifolia* en Mediterranee. In First international workshop on *Caulerpa taxifolia*, Nice, France, 17-18 January 1992 (ed. C. F., Boudouresque et al.), pp. 339-340. France: GIS Posidonic Publications.

Joseph, M., (1980) from Pagrus major cDNA and enzyme stability. *Clinical Experimental Immunology*. 40, 416

Jousson, O., Pawlowski, J., Zaninetti, L., Zechman, F.W., Meinesz, A., Boudouresque, C.F., (2000). Invasive alga reaches California. *Nature*. 408, 157-158.

Jung, V., & Pohnert, G., (2001). Rapid wound-activated transformation of the green algal defensive metabolite caulerpenyne. *Tetrahedron*. 57, 7169-7172.

Jung, V., Thibaut, T., Meinesz, A, & Pohnert, G., (2002). Comparison of the wound-activated transformation of caulerpenyne by invasive and noninvasive *Caulerpa* species of the Mediterranean. *Journal of Chemical Ecology*. 28, 2091-2105.

Kidd P. (2005) Glutathione: Systemic Protectant Against Oxidative and Free Radical Damage, Retrieved August 24, 2004, from http://www.thorne.com/altmedrev/fulltext/glut.html

Knowles, P.F., et al, (1969). Characterization of copper/zinc-superoxide dismutase from *Biochemistry Journal*, 111,53

Komatsu, T., Meinesz, A., & Buckles, D., (1997). Temperature and light responses of alga Caulerpa taxifolia introduced into the Mediterranean Sea. *Marine Ecological. Progress Series*. 146,145-153.

Komatsu, T., Molenaar, H., Blachier, J., Buckles, D., Lemèe, R., & Meinesz, A., (1994). Premières donnèes sur la croissance saisonnière des stolons de *Caulerpa taxifolia* en Mèditerranèe, pp. 179-283. In: First International Workshop on *Caulerpa taxifolia*, Nice, January, 17-18, 1992. GIS Posidonia publication.

Lemée, R, Pesando, D., Durand-Clement, M., Dubreuil, A., Berardi, R., Meinesz, A., et al, (1993). Premiers éléments d'étude sur la toxicité de Caulerpa taxifolia introduite en Méditerranée occidentale. Algologie. 14, 88.

Lemée, R, Pesando, D., Issanchou, C., & Amade, P., (1997). Microalgae: A model to investigate the ecotoxicity of the green alga *Caulerpa taxifolia* from the Mediterranean Sea. *Marine Environmental Research*. 44, 13-25.

Madl, P., & Yip, M., (2003). Literature Review of *Caulerpa taxifolia*. Contribution for the 31th BUFUS Newsletter. http://www.sbg.ac.at/ipk/avstudio/pierofun/ct/ct-1.htm

Marklund, S.L., Westman, N.G., Roos, G., & Carlsson, J., (1984). Radiation resistance and the CuZn superoxide dismutase, Mn superoxide dismutase, catalase,

and glutathione peroxidase activities of seven human cell lines. *Radiation Research.* 100(1), 115-23.

Markett, M., Allaz, M.J., Frei, J., (1980) Journal of Federation of Biochemical Societies Letters. 113, 225.

Mayhoub, H., (1976). Resherches sur la végétation marine de la cote syrienne. Etude expérimentale sur la morphogenèse et le développement de quelques espèces peu connues. Thesis Natural Sciences, Caen University, France.

McConnell, O. J., Hughes, P. A., Targett, N. M., & Daley, J., (1982). Effects of secondary metabolites from marine algae on feeding by the sea urchin, *Lytechinus variegatus. Journal of Chemical Ecology.* 8, 1437–1453.

McCord, J.M., & Fridovich, I., (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry.* 25, 244(22) 6049-55.

McCormick, J.P., Fischer, J.R., Pachlatko, J.P., & Eisenstark, A., (1976). Characterization of a cell-lethal product from the photooxidation of tryptophan: hydrogen peroxide. *Science.* 6, 191(4226), 468-469.

Meinesz, A., & Hesse, B., (1991). Introduction et invasion de l'algue tropicale C.taxifolia en Mediterranee Nord-Occidentale. *Oceanologica Acta.* 14, 415-426.

Meinesz, A., Vaugelas, De J., Benichou, L., Caye, G., Cottalorda, J. M., Delahaye, L., Febvre, M., Garcin, S., Komatsu, T., Lemée, R., Mari, X., Molenaar, H., Perney, L., Venturini, A., (1993). Suivi de l'invasion de l'algue tropicale Caulerpa taxifolia en Méditerranée. Situation au 31 Décembre (1992). Rapport Laboratoire Environnement marin littoral, Université de Nice-Sophia Antipolis. GIS Posidonie, Marseilles, France.

Meinesz, A., Benichou, L., Blachier, J., Komatsu, T., Lemee, R., Molenaar, H., & Mari, X, (1995). Variations in the structure, morphology and biomass of *Caulerpa taxifolia* in the Mediterranean sea. *Botanica Marina*, 38, 499-508.

Meinesz, A., Melnick, J., Blachier, J., & Charrier, S., (1996). Etude preliminaire en aquarium de deux ascoglosses tropcaux consommant *Caulerpa taxifolia*: une voi de recherche pour la lutte biologique. In Proceedings of the second international workshop on *Caulerpa taxifolia*, Barcelona, Spain, 19-20 December 1994 (ed. M.A. Ribera et al.), pp. 157-161. Spain : Universitat Barcelona Publications.

Meinesz, M., Belsher, T., Thibaut, T., Antolic, B., Ben, M.K., Boudouresque, C-F., et al., (2001). The introduced alga *Caulerpa taxifolia* continues to spread in the Mediterranean. *Biological Invasions*. 3, 201-210.

Meister, A., (1994a). Glutathione, ascorbate, and cellular protection. *Cancer Research*. 54, 1969S-1975S.

Meister, A., (1994b). Minireview: Glutathione-ascorbic acid antioxidant system in animals. *Journal of Biological Chemistry*. 269(13), 9397-9400.

Meyer, K.D., & Paul, V.J., (1986). Intraplant variation in secondary metabolite concentration in 3 species of *Caulerpa* and its effects on herbivorous fishes. *Marine Ecological Prog. Series,* 82: 249-57.

Michalski W.P. (1996) Chromatographic and electrophoretic methods for analysis of superoxide dismutases. *Journal of Chromatography* B, 684, 59-75.

Millar, A. & Talbot, B., (2002). Proc. International Caulerpa taxifolia Conference, San Diego, CA in January 2002.

Mittler R. (2002) Oxidative stress in plants. Retrieved February 2, 2002, from http://www.public.iastate.edu/~botany/rmittler/oxistress.pdf

Modena, M., Matricardi, G., Vacchi, M., & Guidetti, P., (2000). Spreading of Caulerpa racemosa (Forsskal) J.Agardh (Bryopsidaceae, Chlorophyta) along the coasts of the Ligurian Sea. *Cryptogamie Algologie*. 21, 301-304.

Nizamuddin, M., (1991). The green algae of Libya. Elga Publication. Bern.

Paglia, D.E, & Valentine, W.N., (1967) studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory Clinical Medicine.* 70, 158-165.

Panayotidis, P., & Zuljevic, A., (2001). Sexual reproduction of the invasive green alga *Caulerpa racemosa* var. *occidentalis* in the Mediterranean Sea. *Ocaenologica Acta.* 24, 199-203.

Paul, V.J., & Hay M.E., (1986). Seaweed susceptibility to herbivory: chemical and morphological correlates. *Marine Ecology Press Series.* 33, 255-264.

Paul, V.J, & Fenical, W. (1986). Chemical defense in tropical green-algae, order Caulerpales. Marine Ecology Press Series. 34, 157–169.

Pesando, D., Lemee, R., Ferrua, C., Amade, P., & Girard, J.P., (1996). Effects of caulerpenyne, the major toxin from *Caulerpa taxifolia* on mechanisms related to sea urchin egg cleavage. *Aquatic Toxicology.* 35, 139–155.

Peskin, A.V., Zbarsky, I.B., Konstantinov, A.A., (1980). A novel type of superoxide generating system in nuclear membranes from hepatoma 22a ascites cells. *FEBS Letters*, 117(1):44-8.

Piazzi, L., & Cinelli, F., (2001). Distribution and dominance of two introduced turf-forming macroalgae on the coast of Tuscany, Italy, north-western Mediterranean Sea in relation to different habitats and sedimentation. *Botanica Marina.* 44(5), 509-520.

Piazzi, L., Balestri, E., & Cinelli, F., (1994). Presence of *Caulerpa racemosa* in the north-western Mediterranean. *Cryptogamie Algologie.* 15, 183-189.

Piazzi, L., Balestri, E., Magri, M., & Cinelli, F., (1997). Expansion de l'algue tropicale *Caulerpa racemosa* (Forsskal) J.Agardh (Bryopsidophyceae, Chlorophyta) le long de la côte toscane (Italie). *Cryptogamie Algologie.* 18, 343-350.

Piazzi, L., & Gravez, V., (1998). Controle de l'algue *Caulerpa taxifolia* dans le Parc National de Port-Cros (Var, France). In Proceedings of the Third international workshop on Caulerpa taxifolia, Marseille, France, 19-20 September 1997 (ed C.F., Boudouresque et al.), pp. 79-87. France : GIS Posidonic Publications.

Por, F.D., (1978). Lessepsian Migrations. The influx of Red Sea biota into the Mediterranean by way of the Suez Canal. Springer Publ., Berlin.

Pryor, W.A., & Castle, L., (1984). Chemical methods for the detection of lipid hydroperoxides. *Methods of Enzymology*. 105, 293-9.

Raven, P.H., Evert, R.F., & Eichhorn, S.E. (1992). Biology of Plants. (New York: Worth Publishers).

Ren B., Tibbelin G., & Akesson B., (2005) Glutathione peroxidase, Retrieved August 24, 2004, from http://www.csb.ki.se/xray/gpox.html

Ricci, N., Capovani, C., & Dini, F., (1999). Behavioural modifications imposed to the ciliate protist Euplotes crassus by caulerpenyne: the major toxic terpenoid of the green seaweed, *Caulerpa taxifolia*. *European Journal of Protistology*. 35, 290–303.

Robert, P. & Gravez, V., (1998). Contrôle de l'algue *Caulerpa taxifolia* dans le Parc National de Port-Cros (Var, France). Third international workshop on *Caulerpa taxifolia*, Boudouresque C.F., Gravez V., Meinesz A., Palluy F. édit., GIS Posidonie publ., France.

Romero, J., (1997). Consequences de l'expansion de *Caulerpa taxifolia* en Méditerranée. In: Académie des Sciences Paris (ed.) Dynamique d'espèces marines invasives: application à l'expansion de *Caulerpa taxifolia* en Méditerranée. Académie des Sciences Paris. 45, 241-254.

Sant, N., Delgado, O., Rodriguez, Prieto, C., & Ballesteros, E., (1996). The spreading of the introduced saeweed *Caulerpa taxifolia* (Vahl) C. Agardh in the

Mediterranean Sea: testing the boat transportaion hypothesis. *Botanica Marina*. 39, 427-430.

Sawyer, D.T., & Gibian, M.J., (1979). Electrochemical Properties of Superoxide Ion in Aprotic Media. *Tetrahedron*, 35, 1471.

Schaffelke, B., Murphy, N., & Uthicke, S., (2002). Using genetic technigues to investigate the sources of the invasive alga *Caulerpa taxifolia* in three new locations in Australia. *Marine Pollution Bulletin*. 44, 204-210.

Silva, I.J., Azevedo, M.S., & Manso, C.F., (1996). Superoxide Anion Radical Generation During Oxidation of Various Amines by Diamine Oxidase. *Free Radical Research*. 33, 167-175.

Silva, P.C., (2002). Overview of the Genus *Caulerpa*; Herbarium of the Univ. of California, Berkeley (CA) – USA. (retrieved February 02, 2004, from http://sgnis.org/publicat/silv2002.htm)

Slater, A.F.G., Stefan, C., & Nobel, I., (1995). Signalling mechanisms and oxidative stress in apoptosis. *Toxicology Letters*. 82/83, 149-153.

Smith, C.M., Walters, L.J., (1999). Fragmentation as a strategy for *Caulerpa* species: fates of fragments and implications for management of an invasive weed. *Marine Ecology*. 20, 307-319.

Sohal, R.S., & Weindruch, R., (1996). Oxidative Stress, Caloric Restriction, and Aging. *Science*. 273, 59-63.

Sorescu, D., & Griendling, K.K., (2002). Reactive oxygen species, mitochondria, and NAD(P)H oxidases in the development and progression of heart failure. *Congestive Heart Failure*. 8(3), 132-40.

Strassburger, E., (1998). Lehrbuch der Botanik 33rd ed.; Fischer Verlag; Stuttgart – FRG

Superoxide dismutase three dimensional structure (n.d.). Retrieved August 21, 2004, from http://srs.dl.ac.uk/Annual_Reports/AnRep02_03/mnd-fig2.jpg

Superoxide dismutase three dimensional structure (n.d.). Retrieved August 21, 2004, from http://www.altonweb.com/cs/downsyndrome/sodfig2.gif

Tauber, A.I. & Goetzl, E.J. (1979) Structural and catalytic properties of the solubilized superoxide-generating activity of human polymorphonuclear leukocytes. Solubilization, stabilization in solution, and partial characterization. *Biochemistry,* 18:5576-5584.

Thibaut, T., Meinesz, A., Burtaire, L., Charrier, S., Mangialajo, L., Ierardi, S. & Vidal V., (1998). Biological control of *Caulerpa taxifolia* in the Mediterranean Sea: use of tropical and Mediterranean ascolossans. In Proceedings of the third international workshop on *Caulerpa taxifolia*, Marseille, France, 19-20 September 1997 (ed. C.-F. Boudouresque et al.), pp.105-111. France, GIS Posidonia Publications.

Thibaut, T., & Meinesz, A., (2000). Are the Mediterranean ascoglossan molluscs Oxynoe olivacea and Lobiger serradifalci suitable agents for a biological control against the invading alga *Caulerpa taxifolia*? *Comptes Rendus de l'Académie des Sciences*, Paris, 323, 477-488.

Thibaut, T., Meinesz, A., Amade, P., Charrier, S., De Angelis, K., Ierardi, S., Mangialajo, L., Melnick, J., & Vidal, V., (2001). *Elysia subornata* (Mollusca) a potential control agent of the alga *Caulerpa taxifolia* (Chlorophyta) in the Mediterranean Sea. *Journal of Marine Biological Association of United Kingdom.* 81, 497-504.

Verlaque, M., (1994). Inventaire des plantes intoduites en Méditerranée: origines et répercussions sur l'environnement et les activités humaines. *Oceanologica Acta.* 171, 1-23.

Verlaque, M., & Fritayre, P., (1994). Modifications des communautés algales méditerranéennes en présence de l'algue envahissante *Caulerpa taxifolia* (Vahl) C.Agardh. *Oceanologica Acta.* 17, 659-672.

Verlaque, M., Boudouresque, C. F., & Meinesz, A., (2000). The *Caulerpa racemosa* Complex (Caulerpales, Ulvophyceae) in the Mediterranean Sea. *Botanica Marina*, 43, 49-68.

Verlaque, M., Belsher, T., & Deslous-Paoli, J. M., (2003). On the identity and origin of the Mediterranean invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta). *European Journal of Phycology*. 38, 325-339.

Verlaque, M., Durand, C., Huisman, J. M., Boudouresque, C. F., & Parco, Y., (2003). On the invasive *Caulerpa racemosa* complex. *European Journal of Phycology*. 38, 325-339.

Villele, X. & Verlaque, M. (1995). Changes and degradation in a Posidonia oceanica bedinvaded by the introduced tropical alga *Caulerpa taxifolia* in the North Western Mediterranean. *Botanica Marina,* 38, 79-87.

Wagner, B.A. et al (1994). Free radical mediated lipid peroxidation in cells: oxidizability is a function of cell lipid bis-allilic hydrogen content. *Biochemistry*, 33, 4449

Wiedenmann, J., Baumstark, A., Pillen, T.L., Meinesz, A., & Vogel, W., (2001). DNA fingerprints of *Caulerpa taxifolia* provide evidence for the introduction of an aquarium strain into the Mediterranean Sea and its close relationship to an Australian population. *Marine Biology*. 138, 229-234.

Williams, S. L., & Grosholz, E. D., (2002). Preliminary reports from the *Caulerpa taxifolia* invasion in southern California. *Marine Ecology Progress Series*. 233, 307-310.

Willson, R.L. (1993). Trevor Slater, Free radical redox chemistry and Antioxidants. In: Free radicals from science to Medicine, Birkhauser Verlag, Basel, Switzerland.

Yagi, K., (1994). In Free Radicals in Diagnostic Medicine, Armstrong, D., (ed.), Plenum Press, New York.

Young I.S., & McEneny J. (2001) Lipoprotein oxidation and atheriosclerosis. *Biochemical Society Transactions*, 29, 358-62

Žuljevic, A., & Anatolic, B., (2000). Synchronous release of male gametes of *Caulerpa taxifolia* (Caulerpales: Chlorophyta) in the Mediterranean Sea. *Phycologica*. 39, 157-159.

Žuljevic, A., Thibaut, T., Elloukal, H., & Meinesz, A., (2001). Sea slug disperses the invasive *Caulerpa taxifolia*. *Journal of Marine Biological Association* U.K. 81, 343-344.