Ph.D. Thesis

EFFECTS OF ENVIRONMENTAL STRESS FACTORS ON THE ANTIOXIDANT DEFENCE SYSTEMS AND MOLIBDOENZYMES IN PLANTS

by

Nina Katalin Barabás

Promoters: Professor László Erdei
Professor Herman Lips*

BRC–JATE Joint Laboratory of Stress Physiology and Transport in Plants,
Institute of Biophysics, Biological Research Center,
Hungarian Academy of Sciences, Szeged
Department of Plant Physiology, Faculty of Natural Sciences,
József Attila University, Szeged
*Biostress Research Laboratory, Jacob Blaustein Institute for Desert Research,
Ben-Gurion University of the Negev, Sede Boqer Campus, Israel

SZEGED, 1999

ACKNOWLEDGMENT

I wish to express my sincere gratitude to my scientific supervisor, Prof. László Erdei for his great support and help during the time until the completion of this thesis. I am very grateful to Prof. Herman Lips for giving me the opportunity to work in his laboratory and for his guidance during the time spent in Israel. I am thankful to Prof. Ferenc Zsoldos for one year supervision and for the useful advises.

I wish to thank to all my colleagues from the BRC -JATE Joint Laboratory of Stress Physiology and Transport in Plants (Szeged, Hungary) and the Biostress Research Laboratory (Sede Boqer Campus, Israel) especially to Zsolt Szegletes, Anikó Pestenácz, Krisztina Fülöp, Dr. Rustem Omarov, Dr. Moshe Sagi, Genlin Jiao and Ruth Shaked for their cooperation and patience.

My thanks also to Mónika Ördög, Erika Pál, Genia Shichman and Xiao Ping Fu for their technical assistance and to Etelka Nagy for her professional help.

The warmest thanks to my parents and Virgis for their moral support, and to all those friends and acquaintances who helped me during all this time.

Special thanks to András Dér for the Fourier transformation and to Tomokazu Koshiba (Tokyo Metropolitan University, Japan) for providing with maize AO antibodies.

I am indebted to the József Attila University, Szeged, for granting me the Ph.D. fellowship, and to the Jacob Blaustein International Center, Israel, for the graduate fellowship.

My gratitude to AID/CDR program for project CA 15-125 and to the Fohs Foundation (U.S.A.) for their support of part of this research and to the Hungarian – Israeli Intergovernmental S&T Cooperation Program (ISR-5/96). This project was supported by the Hungarian Scientific Research Foundation (OTKA T 017254, T 021192 and T 022070) and by the Ministry of Education (MKM 360/96).

A KÖRNYEZETI STRESSZFAKTOROK A NÖVÉNYEK ANTIOXIDATÍV VÉDEKEZŐRENDSZERÉRE ÉS A MOLYBDOENZIMEKRE GYAKOROLT HATÁSA

Az ózonréteg folyamatos elvékonyodása miatt egyre növekvő mértékű UV-B sugárzás éri a Föld felszínét. Emiatt vizsgáltuk e sugárzás hatását a növények antioxidatív védekezőrendszerére. Igen jelentős problémaként jelentkezik a mezőgazdaságban az öntözésre használt vízből a só felhalmozódása. A Földön az öntözött területeknek körülbelül egy harmada sószennyezett. Ezért vizsgáltuk a só (NaCl) hatását a növény "bioritmusára" és a superoxid dizmutáz (SOD) enzim aktivitására. A nitrát és az ammónium ion fontos ásványi tápanyagok, emellett néhány kulcsfontosságú enzim aktivitását szabályozó szignálként is szolgálnak. Ez szolgált kiindulásként, hogy tanulmányoztuk ezen ionok es a só hatását a Mo-enzimek -úgymint aldehid oxidáz (AO), xanthine dehidrogenáz (XDH), nitrát reduktáz (NR) - aktivitására és eloszlására a növényekben.

A kísérletek során folyadékkultúrában nevelt búza (*Triticum aestivum* L., cv.Tiszatáj) és kukorica (*Zea mays* L., cv. Jubily) növényeket használtunk. Az antioxidáns enzimek (kataláz, guaiacol peroxidáz, SOD, glutation reduktáz-GR) valamint a NR aktivitását spektrofotometriásan mértük. Az oxidált glutation (GSSG) mennyiségét HPLC-s technikával határoztuk meg. A periódikusság vizsgálatára Fourier analízist használtunk. A Mo-enzimek (AO és XDH) aktivitását natív polyakrilamid gélelektroforézis után határoztuk meg. Mennyiségi analízishez komputerizáltuk (scanneltük) a géleket, és az értékeléshez a következõ programokat használtuk fel: Image Quant version 3.19.4 és NIH Image 1.6. Az AO enzimfehérje mennyiségét immunoblot analízissel határoztuk meg, AO antitest segítségével.

Azt tapasztaltuk, hogy a kataláz, guaiacol peroxidáz és SOD aktivitása csökkent, míg a GSSG mennyisége, valamint a GR aktivitása növekedett erõs UV-B sugárzás hatására. A növényekben kialakuló válaszreakciók közül a vészreakciót (alarm fázis) és az adaptációs fázist figyeltük meg. A vészreakció során a szár és levélnövekedés gátlása, flavonoid pigmentek felhalmozódása, az antioxidáns enzimek aktivitásának csökkenése, a GSSG szintjének növekedése és a GR

aktivitásának ugrásszerű növekedése figyelhetõ meg. Míg az UV-B sugárzáshoz való adaptációt az antioxidáns enzimek aktivitásának kiegyensúlyozódása, magas GR aktivitás és csökkkent GSSG szint jellemzi. A flavonoid pigmentek szintézise az alarm fázisban egy *syntoxikus* elem, amely hozzájárul a tűrõképesség növeléséhez, míg az antioxidánsok mennyiségének és/vagy aktivitásának növekedése *catatoxikus* elemnek tekinthetõ, mely hatékonyan eltávolítja a stresszor oxidatív komponenseit (Leshem és Kuiper, 1996). Általában véve az az enyhe stressz, amit a fokozott UV-B sugárzásnak való kitétel okozott a jelen tanulmányban, *eu-stressznek* (Lichtenthaler, 1996) tekinthetõ, amely stimuláló stresszhatásként fogható fel, aktiválva a sejt anyagcseréjét és növelve az ellenálló- vagy alkalmazkodó képességét az élõlénynek. Az az enyhe mértékű UV-B sugárzás növekedés, amit az ózon mennyiségének csökkenése okoz a sztratoszférában, nincs végzetes hatással a növények (legalábbis a búza esetében) repair mechanizmusára, anyagcseréjére és növekedésére.

A só hatással volt a SOD aktivitásváltozásának ritmusára a búzanövénykék levelében. A vizsgálatok során a 150 mM NaCl-al kezelt minták esetében a napiritmusban amplitúdónövekedés mutatkozott. Ezenfelül egy körülbelül 13 órás ultradián ritmus is jelentkezett, amely kimutatható a kontroll növényeknél, a sókezelt minták estében azonban nem. Ezekután a következõket állapíthatjuk meg: i) a SOD aktivitásváltozásának mind cirkadián mind pedig ultradián ritmusa van, ii) a sókezelés hatással volt az oszcilláció amplitúdójára, míg a cirkadián ritmust változatlanul hagyta, iii) az ultradián ritmus érzékenynek mutatkozott a stresszhatásra. Feltételezték, hogy a stresszválasz növényekben kiváltható a biokémiai és élettani folyamatok ritmusának módosításával és deszinkronizálásával, mivel ez az ellenállóképesség csökkenését idézi elõ.

A Mo-enzimek aktivitása határozottan magasabb volt a sós körülmények között nevelt növények estében. A Mo-hidroxilázok (AO és XDH) aktivitása magasabb volt azokban az esetekben, amikor ammónium szolgált nitrogénforrásként. Immunoblot analízis után nagyobb mennyiségű AO enzimfehérjét találtunk az ammóniumon tartott növények gyökércsúcsában. A sókezelésnek a nitráton nevelt növényeknél nem volt hatása a fehérjetartalomra, annak ellenére, hogy az aktivitásban növekedést tapasztaltunk sós körülmények között. A Mo-enzimek specifikus aktivitása (aktivitás protein mg^{-1} min^{-1}) magasabb volt a gyökércsúcsban

(0-1 cm szakasz), míg a transzverzális tengely mentén a sztélében találtunk magasabb aktivitást. Az AO aktivitás elhelyezkedése a gyökércsúcsban és a sztélében a gyökérnövekedés két tényezőjével hozható kapcsolatba: (a) Feltételezhető, hogy a gyökércsúcsnak a hosszanti növekedés fenntartásához ABA-ra van szüksége (Saab et al., 1992) és (b) Az ABA szintézise a kifejlett gyökérzónák sztéléjében kapcsolatban állhat a xylémparenchima sejtekkel. Ezekből a sejtekből a hormon gyorsan elszállítódhat a hajtásba a xylémnyalábokon keresztül. Gélelektroforézissel történő elválasztás után AO fehérjék 4 sávját találtuk (AO1-4) melyek különböző alifás és aromás aldehideket tudtak oxidálni. Az ammónián és sós körülmények között nevelt kukorica gyökerében az össz AO aktivitás főleg az AO3 és AO4 formákból adódott. A gyökércsúcs és a sztéle főleg AO3-at, míg nyomokban AO1-et és AO2-t tartalmazott. SDS PAGE után végzett immunoblot analízis kimutatta, hogy a fő 150 kD-os AO alegység mellett két polypeptid egyenként 72 és 85 kD-os molekulasúllyal található specifikusan a cortexben. Az AO polymorfizmusa a növények gyökerében részben kapcsolatban állhat az izoformák meghatározott gyökérrégiókban való elhelyezkedésével. Habár ebben a stádiumban még nem állapíthatjuk meg az egyes sávok pontos anyagcserében betöltött szerepét.

A sterssz (só) és az ammónium ion hatással volt a Mo-enzimekre a különböző gyökérzónákban. Ez a változatosság az AO, XDH és NR megoszlásában kapcsolatban állhat a különböző gyökérzónák és szövetek specifikus funkciójával valamint a Mo-enzimek a növények változó környezeti körülményekhez való alkalmazkodásában betöltött szerepével.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABA	abscisic acid
AO	aldehyde oxidase
BCIP	5-bromo-4chloro-3indolyl phosphate
CDPK	Ca^{2+} -dependent protein kinase
DTT	dithiothreitol
DW	dry weight
EDTA	disodium ethylenediaminetetraacetate
EU	enzyme unit
FAD	flavine adenine dinucleotide (oxidized form)
FW	fresh weight
GAS	general adaptation syndrome
GR	glutathione reductase
GSH	glutathione (reduced form)
GSSG	glutathione disulfide (oxidized form)
HPLC	high performance liquid chromatography
kD	kilodalton
MoCo	molybdenum cofactor
MTT	3[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium-bromide
$NADH_2$	nicotinamide adenine dinucleotide
NBT	nitroblue tetrazolium
NR	nitrate reductase
PAGE	polyacrilamide gel electrophoresis
PMS	phenazine methosulfate
PMSF	phenylmethyl sulfonylfluoride
PS I and II	photosystem I and II
PVPP	polyvinylpolypyrrolidone
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
XDH	xanthine dehydrogenase

1. INTRODUCTION

We are living in a time when the word "stress" occurs frequently in our daily life. Human, animal and plant kingdoms are all touched by this "syndrome". The present work wants to be a little contribution to the numerous, but yet not sufficient studies dealing lately with plant stress and plant stress detection.

Nowadays, a large part of plant physiology research is covered by investigations on plant stress concepts or particular aspects of plant stress. This interest is explained by the alterations happened in biosphere during the last decades: depletion of stratospheric ozone which has as a primer effect the increase of UV-B radiation reaching the Earth's surface; modifications in the composition of atmosphere leading to changes in climate, materialized in the "green house effect"; presence of saline soils and continuous desertification processes, etc. And all these in conditions of increasing population. Every year, environmental stress causes considerable losses in crop quality and productivity. One of the important mechanisms by which plants are damaged during adverse environmental conditions is the excess production of active oxygen species, such as superoxide anion, hydrogen peroxide and hydroxyl radicals, giving rise to the so called "oxidative stress". Plants have evolved non-enzymatic protection mechanisms that efficiently scavenge these active oxygen species, such as ascorbic acid, glutathione, α-tocopherols, carotenoids, as well as enzymatic defense strategies including superoxide dismutase, catalase, peroxidases and glutathione reductase.

Aldehyde oxidase (AO) and xanthine dehydrogenase (XDH), two molybdenum cofactor containing enzymes, could also be involved in stress adaptation processes: through the production of abscisic acid (ABA) by AO, a known stress phytohormon implicated in the control of physiological and molecular processes involved in the response of plants to environmental stresses such as freezing, drought and salinity; XDH by producing uric acid, which is an effective scavenger of reactive oxygen species produced under stress conditions.

Due to the significant role of plants in our daily life (food for human and animals, material for certain industries etc.) it was felt that a better understanding of plant adaptation to different environmental stress conditions and of the mechanisms

involved in these processes is of great importance. This knowledge constitutes the basis for a modern plant breeding system and commercial application for engineering new, stress tolerant plants.

Wheat and corn were used as experimental material, due to their value as crop species. The aims of the present work were:

1. To investigate the effect of UV-B radiation corresponding to about 10% ozone depletion, as oxidative stress for wheat, at the physiological and biochemical levels, by answering the questions:

   i) What are the stress responses to enhanced UV-B treatment in wheat?

   ii) Which mechanisms are involved in the protective responses?

   iii) Do wheat plants adapt to these circumstances?

2. To examine the effect of salinity, as environmental stress factor, on the biological clock in plants, specifically, the changes of rhythmic behavior of the antioxidant enzyme superoxide dismutase in wheat under salinity treatment.

3. To characterize the effect of salinity and nitrogen source on aldehyde oxidase, xanthine dehydrogenase and nitrate reductase activities and their distribution in adventitious or nodal roots of maize, and within the different zones of root with defined physiological functions and of progressive maturation.

2. LITERATURE REVIEW

2.1 General concepts of stress

2.1.1 Stress theory

Stress can be defined as an external factor that exerts a disadvantageous influence on plant (Taiz and Zeiger, 1998). Because *stress* is defined solely in terms of plant responses, thus, it is sometimes called "strain" in conformance with engineering terminology. The concept *stress* is intimately associated with that of "stress tolerance", which is the plant's fitness to cope with an unfavorable environment.

H. Selye (1936, 1956) developed the original general stress concept for living organisms. He defined the *stress* as "A group of characteristic symptoms produced by non-specific stimuli, which result in increased cell function and hormonal production. Although it serves as protection for the organism, it can be harmful by the increasing consumption of the *adaptation energy*". Selye's stress theory can be summarized in two sentences: "All agents can act as stressors, producing both stress and specific action" and "There exist stressor specific responses and non-specific general responses". J. Levitt (1980) defined stress as "Any environmental factor potentially unfavorable to living organisms". Larcher (1987) summarized the stress concept of plants and he stated that "Every organism experiences stress, although the way in which it is expressed differs according to its level of organization". From the botanist's point of view he described stress as a "State in which increasing demands made upon a plant lead to an initial destabilization of functions, followed by normalization and improved resistance" and also "If the limits of tolerance are exceeded and the adaptive capacity is overworked, the result may be permanent damage or even death". He also stated that "Stress contains both destructive and constructive elements and that stress is a selection factor as well as a driving force for improved resistance and adaptive evolution".

Lichtenthaler (1988) extended the stress concept of plants by differentiating between *eu-stress* and *dis-stress*, in which case *eu-stress* is an activating, stimulating

stress and a positive element for plant development, whereas *dis-stress* is a severe stress that causes damage, and thus has a negative effect on plant and its development. He stated that "A mild stress may activate cell metabolism, increase the physiological activity of a plant, and does not cause any damaging effects even at a long duration. Such mild stimulating stress is favorable for the plants".

Leshem and Kuiper (1996) introduced the concept of *syntoxic* and *catatoxic* elements. *Syntoxic stimuli* create a state of passive tolerance permitting a peaceful co-existence with the stressors, while *catatoxic stimuli* or agents are acting mainly through the production of "detoxifying" enzymes, free radical scavengers, antioxidants, nitric oxide, etc., which actively attack the stressor. However, the borderline between the two categories is not always clear-cut and a certain degree of overlapping – and in certain situations – even of interchange, may exist.

Erdei et al. (1998) advanced a new direction in the theory of stress, in which it was hypothesized that stress responses in plants, as described by the stress concept of Selye and adopted in plant physiology, can be evoked by the modification and desynchronization of oscillations of biochemical and physiological processes resulting in a decreased state of resistance.

2.1.2 Phases of he stress response. General Adaptation Syndrome

According to the classical mammalian "general adaptation syndrome" (GAS) concept, different type of stress evoke similar adaptation responses (Selye, 1936). Leshem and Kuiper (1996) hypothesized the existence of a "GAS" in plants, as a response to various environmental stress conditions. This implies that different types of stress as heat, cold, drought, salinity, anoxia etc. would evoke similar or even identical stress coping mechanisms, and that tolerance to one type of stress can be induced by sublethal exposure of the organism to a different type of stress. The logic behind the hypothesis of a putative existence of a "GAS" in plants is that any plant (seasonal, annual, or perennial) during its life cycle is exposed to wide seasonal or diurnal vicissitudes of environmental conditions. It thus appears that an inherent multiple stress resistance mechanism is developmentally advantageous and may be pleiotropically encoded by evolutionary selection (Leshem et al., 1998).

The stages of the general adaptation syndrome in plants (Lichtenthaler, 1996, modified from Selye, 1956 to include plant systems) are presented in Figure 1:

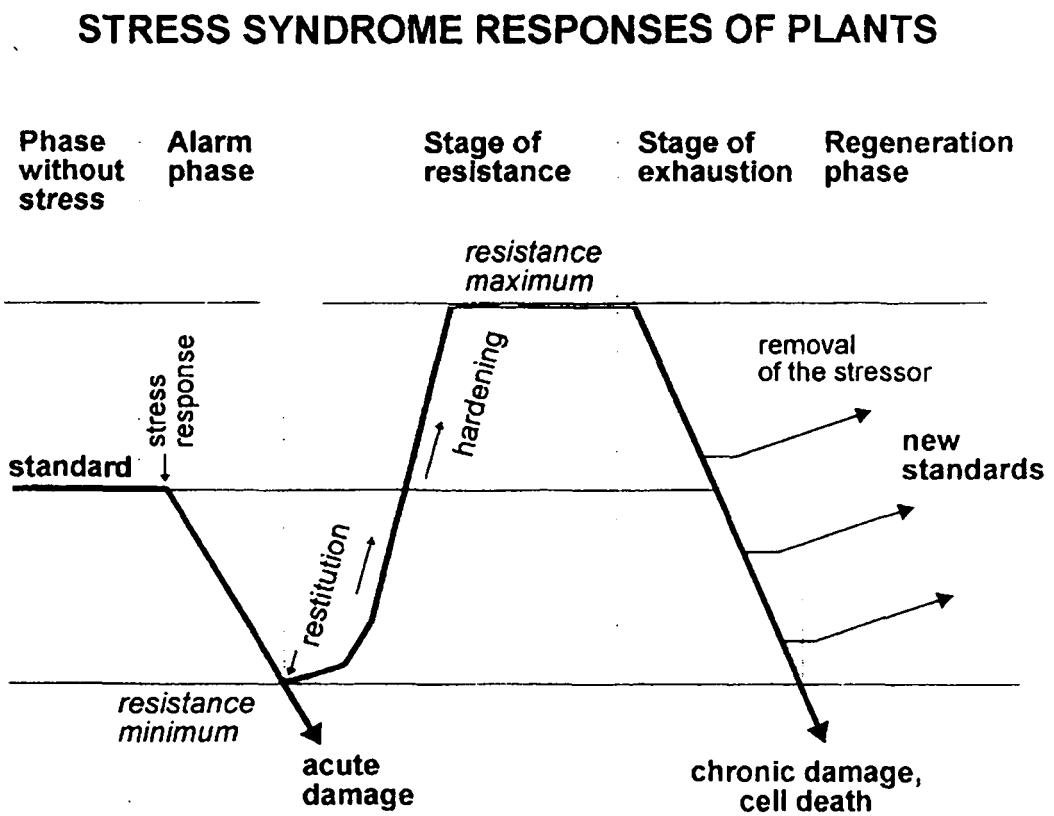

Figure 1: Stages of the general adaptation syndrome (GAS), (Lichtenthaler, 1996). Plants growing at a physiological standard condition will respond to and cope with stress. After removal of the stressor(s), new standards of physiology can be reached depending on the time of stressor removal as well as the duration and intensity of the stress.

Response Phase (beginning of stress)

Alarm reaction: the somatic response to the first exposure to a localized or general stressor and initially lowers basal state of resistance. Characteristics: a decline of vitality, deviation of the functional norm, catabolic processes

exceed anabolism. If the stressor is sufficiently strong (e.g. extremes of temperatures, salinity), death may result.

Restitution Phase (continuing stress)

Stage of resistance or acclimation: if continued exposure to the stressor is compatible with adaptation, resistance mechanisms of either "syntoxic" (coexistence with stress) or a "catatoxic" (stressor removal) nature ensues (Leshem and Kuiper 1996). Characteristics: adaptation processes, repair processes, hardening or reactivation. The cellular manifestations characteristic of the alarm reaction have virtually disappeared or are strictly held in abeyance, and resistance rises above normal.

End Phase (long term stress)

State of collapse or exhaustion: following long-term exposure to the stressor, adaptation capability is eventually exhausted. Signs of the alarm reaction reappear, and the organ or the whole organism senesces and/or dies. Characteristics: stress intensity too high, overcharge of adaptation capacity, chronic disease or death.

Regeneration phase

Partial or full regeneration: of the physiological function when the stressor is removed and the damage was not too high. New physiological standards are established when the intensity of the stress is not too high and plant life cycle is completed under those environmental conditions (e.g. shorter life span, smaller size etc.).

2.1.3 Stress constrains and stressors

There are many stress events and a multitude of stress factors that occur in the life cycle of a plant. These stress factors or stressors are listed in Table 1 under the grouping of natural (I) and anthropogenic (II) stress factors (Lichtenthaler, 1996). Another classification of the environmental stress factors is by grouping them in biotic (infection or competition by other organisms) and abiotic stressors (physicochemical factors as temperature, water, radiation, chemicals, etc). These

stress factors rarely act individually and separately on the plant. Usually, several stressors act simultaneously, inducing the "GAS" and "Co-stress" response in plants (Leshem et al., 1998).

Table 1: Natural and anthropogenic stress factors acting on terrestrial vegetation (Lichethaler, 1996)

I. Natural stress factors	*II. Anthropogenic stress factors*
High irradiance (photoinhibition, photooxidation)	Herbicides, pesticides, fungicides
Heat (increased temperature)	Air pollutants(e.g.SO_2, NO, NO_2, NO_x)
Low temperature (chilling)	Ozone (O_3) and photochemical smog
Sudden and late frost	Formation of highly reactive oxygen species ($^1O_2$ radicals $O_2^{\cdot-}$ and $OH^{\cdot}$, H_2O_2)
Water shortage (desiccation)	Photooxidants (e.g. peroxyacylnitrates)
Natural mineral deficiency (e.g. nitrogen shortage)	Acid rain, acid fog, acid morning dew
Long rainy periods	Acid pH of soil and water
Insects	Mineral deficiency of the soil, often induced by acid rain (shortage of the basic cations K, Mg, Ca, often Mn and sometimes Zn)
Viral, fungal and bacterial pathogens	Over-supply of nitrogen (dry and wet NO_3 deposits)
	Heavy metal load (lead, cadmium, etc.)
	Overproduction of NH_4^+ in breeding stations (uncoupling of e^- transport)
	Increased UV-radiation (UV-B & UV-A)
	Increased CO_2 level and global climate change

2.2 Oxidative stress and antioxidant defense mechanism

2.2.1 Basic considerations

In recent years "oxidative stress" came to denote a disturbance in the prooxidant – antioxidant balance in the favor of the former (Sies, 1985). There are numerous sites of oxygen activation in the plant cell, which are highly controlled and tightly coupled to prevent release of intermediate products. Under stress situation, it is likely that this control or coupling breaks down leading to occurrence of activated oxygen species like superoxide anion, hydroxyl radical, hydrogen peroxide, etc. (Table 2). Such oxidative stress has been shown to occur in plants exposed to high and low temperatures, particularly in combination with high light intensities, drought, exposure to air pollutants (e.g. ozone, sulfur dioxide), ultraviolet light, and herbicides such as paraquat (Foyer and Mullineaux, 1994).

Oxidative stress is probably a common occurrence in plants especially when we consider that plants have minimal mobility and control of their environment. These uncoupling events are not detrimental provided that they are short in duration and that oxygen scavenging systems are able to detoxify various activated oxygen forms (Elstner, 1990). If production of oxygen free radicals exceeds the plant's scavenging capacity, deleterious degenerative reactions occur, the typical symptoms being loss of osmotic responsiveness, wilting and necrosis. At the subcellular level, membrane disintegration and protein aggregation are typical symptoms. Therefore, the equilibrium between the production and the scavenging of activated oxygen species is critical to the maintenance of active growth and metabolism and overall environmental adaptation (Leshem and Kuiper, 1996).

Detoxification of reactive oxygen species is one of the prerequisites of aerobic life, and the multiple lines of defense, which have evolved form veritable antioxidant defense systems. These include scavenging enzymes as the SOD family, peroxidases and catalase, and non-enzymatic components such as tocopherols, ascorbic acid, glutathione, carotenoids, polyamines and flavonoids (Table 3).

Table 2. Reactive oxygen species of interest in oxidative stress (Sies, 1985)

COMPOUND	*REMARKS*
$O_2^{\cdot-}$, superoxide anion	One-electron reduction state, formed in many autoxidation reactions (e.g. flavoproteins; redox cycling)
$HO_2^{\cdot}$, perhydroxy radical	Protonated form of $O_2^{\cdot-}$, more lipid-soluble
H_2O_2, hydrogen peroxide	Two-electron reduction state, formed from $O_2^{\cdot-}$ ($HO_2^{\cdot}$) by dismutation, or directly from O_2
$HO^{\cdot}$ ($OH^{\cdot}$), hydroxyl radical	Three-electron reduction state, formed by Fenton reaction, metal(iron)-catalysed Haber-Weiss reaction; highly reactive
$RO^{\cdot}$, alkoxy radical	Oxygen-centered organic (e.g. lipid) radical
$ROO^{\cdot}$, peroxy radical	Formally formed from organic (e.g. lipid) hydroperoxide, ROOH, by hydrogen abstraction
ROOH	Organic hydroperoxide (e.g. lipid-, thymine-OOH)
$^1\Delta_g O_2$ (also O_2^*)	Singlet molecular oxygen, first excited state, 22 kcal/mol above ground state (triplet) $^3O_2$; red (dimol) or infrared (monomol) photoemission
^3RO (also RO^*)	Excited carbonyl, blue-green photoemission (e.g., formed via dioxetane as intermediate)

Table 3. Antioxidant defense in biological systems (Sies, 1985)

COMPOUND	*REMARKS*
Non-enzymatic	
α-Tocopherol (vitamin E)	Membrane-bound
Ascorbate (vitamin C)	Water soluble
Flavonoids	Plant antioxidants (rutin, quercetin, etc.)
Chemicals	Food additives, e.g. BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene)
β-Carotene (vitamin A)	Singlet oxygen quencher
Urate	Singlet oxygen quencher, radical scavenger?
Plasma proteins	e.g., Coeruloplasmin
Enzymatic	
Superoxide dismutases	CuZn enzyme, Fe enzyme, Mn enzyme
GSH peroxidases	Selenoenzyme: non-Se enzyme: some GSH S-transferases, e.g., isoenzymes B and AA Cytosol and mitochondrial matrix
Catalase	Heme enzyme. Predominantly in peroxisomal matrix
Ancillary enzymes	
NADPH-quinone oxidoreductase (DT-diaphorase)	Two electron reduction, dicoumarol-sensitive
Epoxide hydrolase	
Conjugation enzymes	UDP-glucuronyltransferase; Sulfotransferase; GSH S-transferases
GSSG reductase	NADPH dependent enzyme
NADPH supply	Glucose-6-phosphate dehydrogenase 6-Phosphogluconate dehydrogenase Isocitrate dehydrogenase Malic enzyme Energy-linked transhydrogenase
Transport systems	GSSG export Conjugate export

Superoxide dismutases

Superoxide dismutases (SOD, EC 1.15.1.1) have been identified as an essential component in an organism's defense mechanism (reviewed by Bowler et al., 1992). SODs are a family of metalloenzymes that react with superoxide radicals at almost diffusion-limited rates to produce hydrogen peroxide:

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

The three known types of SOD are classified by their metal cofactor: the copper/zinc (Cu/Zn SOD), manganese (MnSOD), and iron (FeSOD) forms. Experimentally, these isoenzymes can be identified by their differential sensitivities to KCN and H_2O_2. Cu/ZnSOD is characterized as being sensitive to both H_2O_2 and KCN, FeSOD is sensitive only to H_2O_2, and MnSOD is resistant to both inhibitors. Subcellular fractionation studies have been performed in many plant species and in general plants contain a mitochondrial matrix-localized MnSOD and a cytosolic Cu/ZnSOD, with FeSOD and/or Cu/ZnSOD present in the chloroplast stroma. All of the enzymes appear to be nucleus encoded and, where necessary, are transported to their organellar locations by mean of NH_2-terminal targetting sequences. SOD genes are differentially regulated and respond to a variety of stress conditions, such as paraquat application, drought (Perl-Treves and Galun, 1991), chilling (Karpinski et al., 1994), pathogens (Zacheo and Bleve-Zacheo, 1988), etc. The large increases in SOD transcript levels upon stress treatment, in general, correlate with much more moderate increases in SOD activity. Possibly, stress causes a more rapid turnover of SOD proteins, thereby necessitating activation of gene expression to maintain SOD levels (Inze and Montagu, 1995).

Catalase

Catalase (EC 1.11.1.6) is a tetrameric, heme-containing enzyme, and one of the most active catalysts produced by nature. It decomposes hydrogen peroxide at an extremely rapid rate, corresponding to a catalytic center of about 10^7 min^{-1} (Scandalios, 1994) according to the following reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

In plants, catalase is found predominantly in peroxisomes (and also in glyoxysomes) where it functions chiefly to remove the H_2O_2 formed during photorespiraton (or during β-oxidation of fatty acids in glyoxysomes) (Lazarow and Fujiki, 1985). Catalase plays a central role in stress tolerance. Extensive work of Scandalios and co-workers (in Foyer and Mullineaux, 1994) have revealed the presence of three differentially regulated catalase in maize. The *Cat1* gene product seems to play a role in removing photorespiratory H_2O_2, whereas the expression pattern of *Cat3* suggests that the encoded protein has a role in scavenging H_2O_2 generated by β-oxidation of fatty acids in glyoxysomes. The *Cat2* gene product most likely has a specific role in protecting the cell from oxidative stress. Exposure of plants to ambient ozone, sulfur dioxide, and UV-B radiation, all lead to a rapid decline in *Cat1* steady-state transcript level and a concomitant rapid increase in *Cat2* transcript levels (Willekens et al., 1994).

Peroxidases

Peroxidases reduce hydrogen peroxide to water by a peroxidatic reaction using a photoreductant (AH_2):

$$H_2O_2 + 2AH_2 \rightarrow 2\ H_2O + 2A$$

In angiosperms, AH_2 has been identified as ascorbate. In plant cells ascorbate peroxidase is present mainly in chloroplasts, but also in the cytosol (Asada, 1992). Guaiacol peroxidase (EC 1.11.1.7) is a typical plant peroxidase, as represented by horseradish peroxidase and it is localized in cell walls and vacuoles, but not in chloroplasts. The physiological functions of this peroxidase include the biosynthesis of lignin and ethylene, and the degradation of indoleacetic acid, rather than scavenging of hydrogen peroxide.

Glutathione and Glutathione reductase

Glutathione, γ-glutamyl-cysteinyl glycine (GSH), is the major low molecular weight thiol compound in most plants. It can be found in the chloroplasts as well as in the cytosol (Foyer and Halliwell, 1976; Klapchek et al., 1987). GSH enhances tolerance to numerous xenobiotics and oxidative agents. In addition to its major role in sulfur transport, glutathione is involved in a number of cellular processes in plants, including acting as a protein disulfide reductant, detoxifying xenobiotics (e.g. herbicides) by conjugation either spontaneously or by the activity of glutathione-*S*-transferase, and regulating gene expression in response to environmental stress and pathogen attack (reviewed by Creissen et al., in Foyer and Mullineaux, 1994). Many of the important metabolic, regulatory, and antioxidative roles of glutathione result in its oxidation to glutathione disulfide (GSSG). The reduction of GSSG to GSH is carried out by the enzyme glutathione reductase.

Glutathione reductase (GR, EC 1.6.4.2), a flavoprotein oxidoreductase, is a NADPH dependent enzyme, which catalyses the reduction of oxidized glutathione (GSSG) to glutathione (GSH):

$$NADPH + H^+ + GSSG \rightarrow NADP^+ + 2\ GSH$$

GR is localized mainly in the chloroplasts, but has been found also in mitochondria and cytosolic cellular compartments (Foyer et al., 1991). GRs are encoded by nuclear genes and play important function in the ascorbate-glutathione cycle (Figure 2). Several isoforms have been detected with isoelectric points ranging from 5.6 to 6.5 in pea leaves: five isoforms in the chloroplast fraction while three isoforms were identified in the mitochondrial fraction. In addition, on native PAGE, up to six bands of GR activity were detected. GR activity is influenced by various environmental factors known to increase oxyradical formation. These include exposure to gaseous pollutants or xenobiotics, extremes of temperature, pathogen attack, and senescence (reviewed by Creissen et al., 1994).

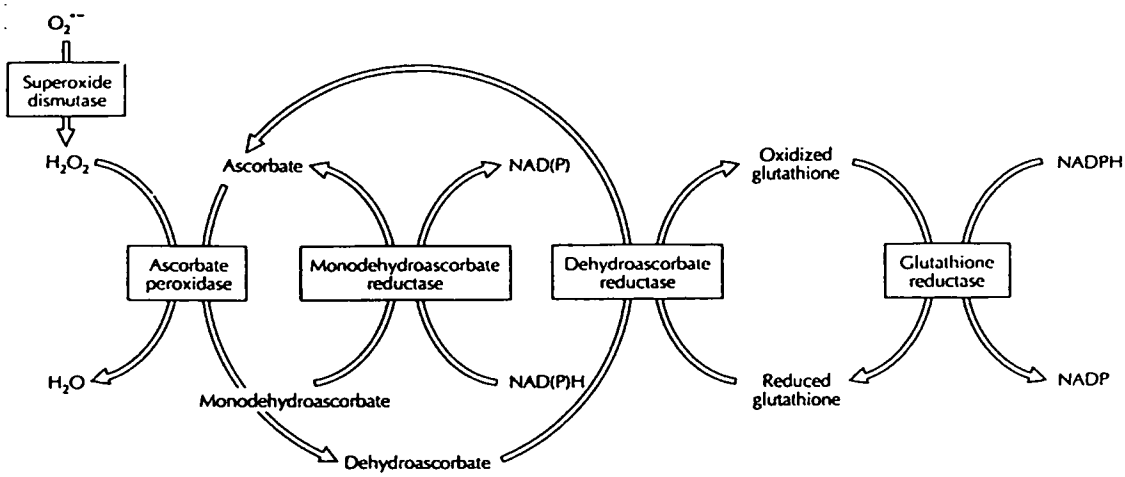

Figure 2: The ascorbate-glutathione cycle. Hydrogen peroxide is removed by ascorbate peroxidase and ascorbate is regenerated by the ascorbate-glutathione cycle, involving monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase. Ascorbate is first oxidized to monodehydroascorbate. If monodehydroascorbate is not rapidly reduced again to ascorbate by monodehydroascorbate reductase, it will spontaneously disproportionate into ascorbate and dehydroascorbate. Dehydroascorbate recycles ascorbate using reduced glutathione that is regenerated through the action of glutathione reductase in an NADPH-dependent reaction (Inze and Montagu, 1995).

2.2.2 UV-B as oxidative stress in plants

Ultraviolet (UV, λ = 10 – 400 nm) radiation emanating from the sun travels unaltered until it enters the Earth's atmosphere. Here, absorption and scattering by various gases and particles modify the radiation profoundly, so that by the time it reaches the terrestrial and oceanic biosphere, the wavelengths which are most harmful to organisms are largely filtered out. Human activities are now changing the composition of the atmosphere, raising serious concerns about how this will affect the wavelength distribution and quantity of ground-level UV radiation. The sensitivity of organisms to UV radiation is in general a function of wavelength. Wavelengths shorter than 280 nm are absorbed almost completely by the Earth's atmosphere and therefore are not important for biological processes at the surface. The UV radiation relevant to environmental biology is restricted to UV-B (280 – 315 nm) and UV-A (315 – 400 nm) ranges. Ozone changes affects mostly short-

wavelength UV-B radiation, while cloud cover changes affect both UV-B and UV-A; therefore the impact of these changes on the rates of biological and chemical processes may be quite different (Madronich, 1993).

Depletion of stratospheric ozone (O_3) layer, mainly due to the catalytic reactions by chlorinated fluorocarbons (Rowland, 1989) and oxides of nitrogen, chlorine and bromine (Molina et al., 1996), has as a primer effect the increase of UV-B irradiation reaching the Earth's surface. Using the Total Ozone Mapping Spectrometer (TOMS) data furnished by the Nimbus Satellite over 11 years, a significant decrease in total ozone was reported in all seasons in both the Northern and Southern hemispheres, at middle and high latitudes. The declines in the lower stratosphere, below 25 km, are about 10 % per decade (Stolarski et al., 1992).

Numerous studies have shown that UV-B radiation can affect fundamental physiological processes in plants, such as growth rate, morphology, biomass allocation, photosynthesis, transpiration, DNA integrity (reviewed by: Bornman and Teramura, 1993; Tevini, 1993; Caldwell et al., 1995), flavonoid biosynthesis (Rao and Ormrod, 1995) and even attractiveness to herbivore insects (Ballaré et al., 1996). These are, in part, due to the direct effects of UV-B radiation on photosystem II, nucleic acids, enzymes, pigments and growth regulators (Caldwell and Flint, 1994); or, to its indirect effects administered through the formation of reactive oxygen species (Peak et al., 1983) like the superoxide ($^{\cdot}O_2^-$) and hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) (Table 2). The level of damage caused by these factors depends on the effectiveness of the cellular defence mechanism which includes scavenging enzymes such as superoxide dismutase, peroxidases and catalase, and nonenzymatic components such as ascorbic acid and glutathione (Table 3) (Asada, 1994; Foyer et al., 1991). Superoxide radicals are produced by the reduction of molecular oxygen at PSI via Mehler reaction, when electrons are transported to molecular oxygen. This radical is rapidly dismuted by SOD associated with PSI and the resulting H_2O_2 is scavenged by thylakoid-bound ascorbate peroxidase. Reactive oxygen species that escape destruction at the thylakoid, are scavenged by stromal SOD and ascorbate peroxidase. H_2O_2, produced by glycolate oxidase, is degraded by catalase mainly in peroxisomes.

The primary products in ascorbate peroxidase reaction, monodehydroascorbate radicals, are converted to ascorbic acid through reactions with ferredoxin or monodehydroascorbate reductase. Reduction of dehydroascorbate to ascorbic acid is catalysed by dehydroascorbate reductase via ascorbate:glutathione pathway (Figure 2), (Asada, 1994). In this reaction GSH is oxidised to GSSG, and the level of GSH is maintained by the NADPH dependent enzyme, GR. Glutathione thiyl radicals ($GS^{\cdot}$) have been shown to be produced by interaction of GSH with various radicals (Winterbourn, 1993), while the accumulation of GSSG indicates oxidative circumstances. There is much evidence to suggest that almost all active oxygen-scavenging enzymes are also present in non-photosynthetic tissues (Bielawski and Joy, 1986; Tanaka et al., 1988, 1994; Foyer et al., 1991).

2.2.3 Salinity as oxidative stress: the implication of circadian rhythms

In plants, salinity can induce: alterations in the metabolism of proteins and nucleic acids, photosynthesis and respiration (Cheeseman, 1988; Greenway and Munns, 1980; Jolivet et al., 1990; Lambers, 1985); accumulation of polyamines (Erdei et al., 1995; Erdei et al., 1996a) and oxidative stress in plants (Bartosz, 1997). Kayupova and Klyshev (1987) reported that in pea roots NaCl induced the generation of the powerful oxidant hydroxyl radical, $^{\cdot}OH$, suggesting that activated oxygen species could play an important role in the mechanism of salt injury. Studies were done on the effect of salt on the activity of superoxide dismutase in sunflower (Barabás et al., 1994), pea (Corpas et al., 1993) and rice (Fadzilla et al., 1997), however, little is known about the influence of NaCl on the rhythmic behavior of this enzyme. Preliminary results suggested that the environmental stress factors (e.g. salinity) may influence the circadian rhythm of the of antioxidant enzymes (catalase, guaiacol peroxidase, SOD, GR) activities in wheat (Erdei et al., 1996b).

Rhythmicity is a general characteristic of biological phenomena (Lumsden, 1991; Piechulla, 1993; Hall, 1995; Roenneberg, 1996; Golden et al., 1997). In plants, most of the physiological and biochemical processes are synchronized by repeating fluctuation patterns characterized by periods (time between comparable points of the cycle) ranging from seconds to annual seasons (Figure 3).

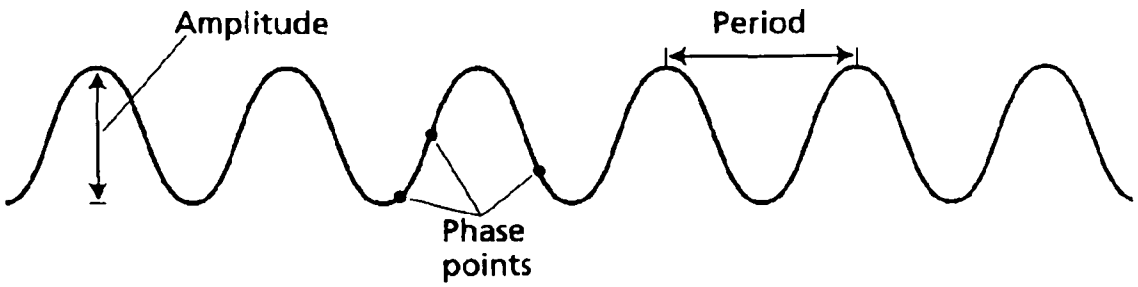

Figure 3: A typical circadian rhythm. The period is the time between comparable points in the repeating cycle; the phase is any point in the repeating cycle recognizable by its relationship with the rest of the cycle; the amplitude is the distance between the peak and through (Taiz and Zeiger, 1998).

The most significant fluctuations of these are the circadian (periods from 20 to 28 hours) and ultradian (periods shorter than circadian) oscillations. Although the strongest factor for the entrainment of the central pacemaker (defined as an autoregulative gene expression, Roenneberg, 1996) is the light in a temperature-compensated manner, other environmental factors are also known as modifiers of the parameters of rhythmicity. Thus, low temperature (Kocsy et al., 1997), a slight physical stress (Koukkari et al., 1997), nitrogen starvation and UV-B irradiation (Peterson-Mahrt et al., 1994, 1995), the form of N-source (Leport et al., 1996), salt stress (Herppich et al., 1995) and iron deficiency (Spiller et al., 1987) may influence rhythm parameters.

Environmental information finds its way towards the central clock by entrainment (input), resulting in an altered rhythm of the messenger RNA accumulation (transcriptional control, Piechulla, 1993) and consequently, the corresponding biochemical or physiological phenomena coupled to it (output). Stressors may also affect, however, the output rhythms by the modification of the abundance or the activity of the oscillating proteins (enzymes) by acting on their translation, or, even more directly, by influencing their activity. Finally, both period and phase of the central oscillator can be modified by these variables via feedback loops (Roenneberg, 1996).

Mechanisms, independent of the central clock, can also cause fluctuations. About two decades ago, membrane-transport based models were suggested to be in the background of oscillating processes (Njus et al., 1974). Although it cannot be generalized, it is quite plausible to suppose that ultradian fluctuations, e.g. in tropistic (Shabala and Newman, 1997) or stomatal movements (Erdei et al., 1998), depend on underlying ion transport mechanisms.

2.3 Molibdoenzymes in plants as "stress enzymes"

Molibdenum is an essential element for plants. Six different genes are known as responsible for the enzymes participating in the synthesis of the molybdenum cofactor (MoCo) (Mendel, 1997) which complexes molybdenum and pterin. MoCo constitutes the active site of at least four enzymes: nitrate reductase (NADH:NR, EC 1.6.6.1.), aldehyde oxidase (AO, EC 1.2.3.1), xanthine dehydrogenase (XDH, EC 1.2.37) and sulfite oxidase (SO; EC, 1.8.3.1) (Figure 4).

MoCo is widely distributed in living organisms including higher plants (Mendel, 1997). AO may play important roles in plant development and adaptation to environmental stresses by catalyzing the final steps in the biosynthesis of two phytohormones through the oxidation of abscisic aldehyde to abscisic acid (ABA) (Walker-Simmons et al., 1989; Leydecker et al., 1995) and indole-3-acetaldehyde to indole-3-acetic acid (IAA) (Bandurski et al., 1995; Koshiba et al., 1996). Four bands of AO proteins were reported in barley plants (Omarov et al., 1999), three AO isoforms were detectable in *Arabidopsis* (Schwartz et al., 1997) and ryegrass (Sagi et al., 1998b), and two major bands in *N. plumbaginifolia* (Akaba et al., 1997). At least two maize cDNAs were cloned in maize where they are differentially expressed in a tissue specific manner, with zmAO-1 expressed at a higher level in roots (Sekimoto et al., 1997). In Arabidopsis seedlings, AO1 was suggested to be responsible for IAA biosynthesis (Seo et al., 1998).

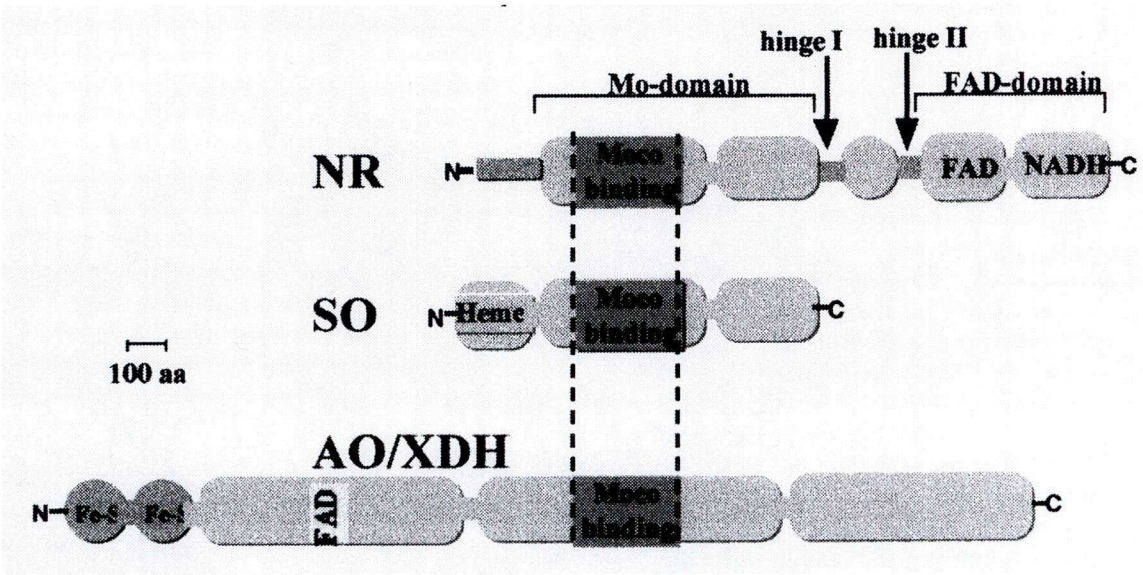

Figure 4: Structural comparison of the four Mo-enzymes: NR – nitrate reductase, AO – aldehyde oxidase, XDH – xanthine dehydrogenase, and SO – sulfite oxidase. 1.Nitrate reductase: reduces nitrate to nitrite as the first step to ammonium production, which will be assimilated into aminoacids by GS. NR is the rate-controlling step of the assimilation of nitrate into organic nitrogen compounds. 2.Aldehyde oxidase: responsible for the oxidation of a number of aldehydes but principally at the final steps of the biosynthesis of two plant hormones: abscisic- aldehyde to ABA and indole-acetaldehyde to IAA. 3.Xanthine dehydrogenase: has been characterized as a dehydrogenase, which catalyzes the first oxidative step in purine catabolism and the oxidation of purine derivatives originating from nucleic acid degradation. It is important in the antioxidative defense reactions of the plant. 4. Sulfite oxidase: is an important Mo-enzymes in animals where it is involved in the degradation of sulfur containing amino acids (Lips et al., 1999, as modified from Mendel and Schwarz, 1998).

ABA is implicated in the control of physiological and molecular processes involved in the response of plants to environmental stresses such as freezing, drought and salinity (Zeevaart and Creelman, 1988). Exposure of barley and cotton plants to NaCl reduced transpiration and increased ABA levels in leaves and xylem sap (Kefu et al., 1991). Maize root system subjected to drought resulted in elevated ABA synthesis (Zhang and Tardieu, 1996). Ammonium nutrition and salinity increased ABA levels in plant tissues, while nitrate had a reverse effect (Peuke et al., 1994). The main site of ABA synthesis under environmental stress conditions is the root from where it is transported to the shoot, especially under stress conditions (Wolf et

al., 1990; Bano et al., 1993). Since ABA affects water relation parameters (Erdei and Taleisnik, 1993) and K^+ transport processes (Dhakal and Erdei, 1986), these physiological and transport parameters exert their influence on the K^+-shuttle responsible for the translocation of nitrate to the shoot (Lips et al., 1970). Inorganic nitrogen ions such as ammonium and nitrate, may be not only mineral nutrients but also signals affecting the level of activity of NR, AO and XDH (Omarov et al., 1999; Sagi et al., 1998b). XDH is an oxidoreductase of importance in purine metabolism. In plants, XDH is a NAD^+ dependent enzyme, catalyzing the first oxidative step in the catabolism of purine and purine derivatives resulting from nucleic acid degradation. In higher plants, XDH takes part in ureide biosynthesis (Nguyen, 1986) through the *de novo* synthesis of purines from glutamine (Triplett et al., 1980). In pea leaves, XDH activity increased sharply in parallel to superoxide dismutase and other oxygen related enzymes during senescence (Pastori and del Rio, 1997). Enhancement of XDH in maize nodal roots of plants grown with ammonium correlated with the increment of some antioxidant enzymes (SOD, GR) (Barabás et al., 1999a). XDH may be involved in the defense of plant tissues against oxidative stress induced by salinity, most probably through the production of uric acid, as observed in ryegrass (Sagi et al., 1998b). Uric acid is an effective scavenger of reactive oxygen species produced under stress conditions in many organisms (Becker et al., 1989; Radi et al., 1990). NR is the first enzyme in the nitrate assimilation pathway, reducing nitrate to nitrite with NADH or NADPH as electron donors (Campbell, 1988). Mo in NR is a dioxi ion. AO and XDH require the replacement of one of the oxygen atoms connected to Mo by sulfur atom by the enzyme Mo-hydroxylase sulfurylase (MHS) (Figure 5).

Recent studies have pointed out the influence of salinity and nitrogen source on Mo-enzymes activities: salinity increased the activity of AO in barley (Omarov et al., 1998), ryegrass (Sagi et al., 1998b) and maize (Barabás et al., 1999b), XDH and NR in ryegrass (Sagi et al., 1998a,b) and maize (Barabás et al., 1999b) and NR in tomato plants (Gao et al., 1996). The increased allocation of MoCo to the AO-apoprotein followed by the Mo-sulfurylation activating the enzyme were considered key regulatory events allowing the rise of ABA level and the plant adaptation to changing environmental conditions (Lips et al., 1999).

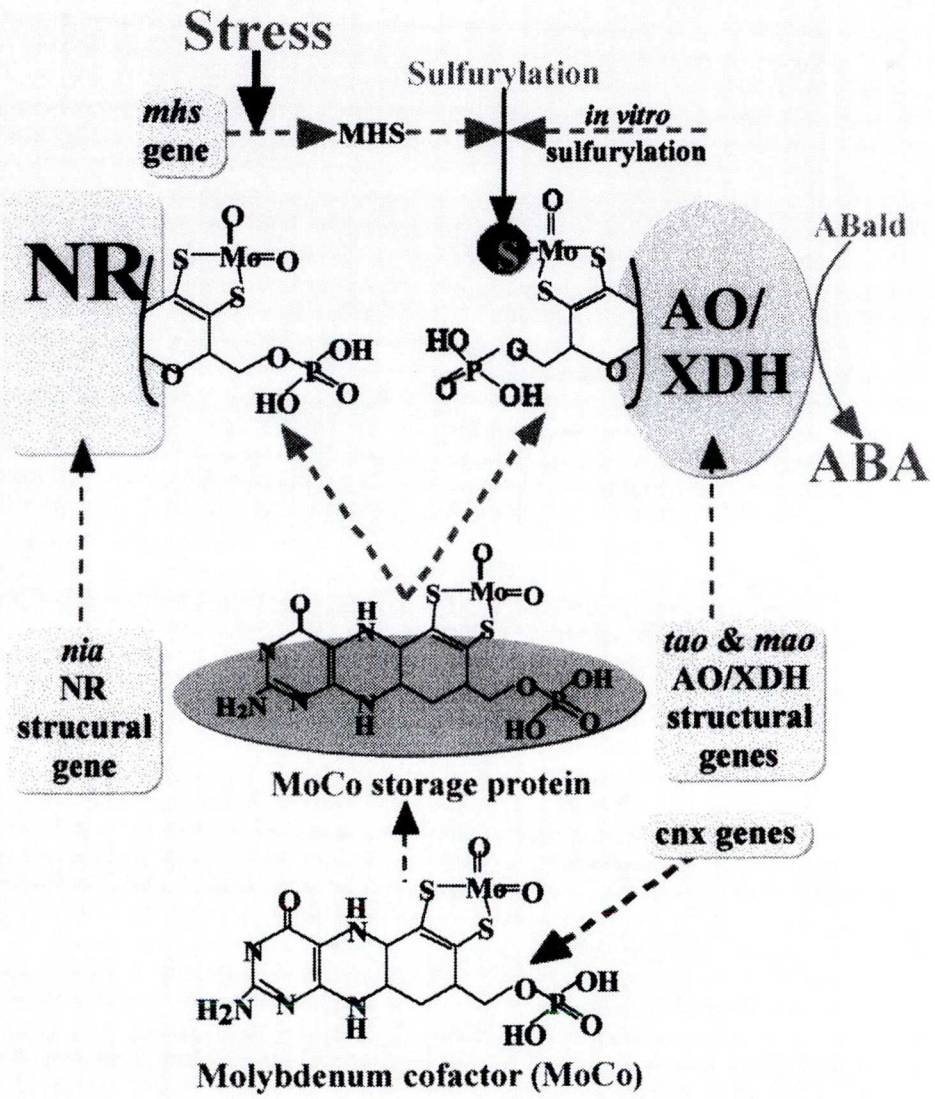

Figure 5: Synthesis of the three major Mo-enzymes in plants (Lips et al., 1999, as modified from Mendel and Schwarz, 1998)

The plant root is a well-organized organ in which cells divide, elongate and differentiate along its longitudinal and transversal axes. Cells along these axes differ in both developmental stage and function, showing prominent differences in their capacity for nutrient absorption, translocation and assimilation (Lazof et al. 1992; Cruz et al., 1995). This heterogeneity must be considered when interpreting biochemical and molecular studies on root tissue. Zone and tissue specific gene expression has been reported (Rost and Bryant, 1996). In-situ hybridization localized *nia*mRNA mainly in root tips and the stele of chicory roots at high nitrate concentration (Palms et al., 1996). Post-embedding immunogold labeling showed location of NR protein in the cytoplasm of root epidermal and cortical cells, as well as in the parenchyma and pericycle cells of the stele (Federova et al., 1994). Studies have been reported on the comparison of AO levels in the apical and mature regions of maize coleoptiles (Koshiba et al., 1996) as well as on the organ specific distribution of AO in *Arabidopsis thaliana* (Seo et al., 1998) and on AO gene expression in tomato organs (Ori et al., 1997). However, very little is known about the distribution of AO and XDH along and across the root and its main anatomical areas: cortex and vascular cylinder, root apex and mature zones.

3. MATERIAL AND METHODS

3.1 Plant material and growth conditions

To determine the effect of UV-B treatment

Wheat caryopses (*Triticum aestivum* L. cv. Tiszatáj, Cereal Research Institute, Szeged, Hungary) were germinated on moist filter paper in the dark for 48 h. Seedlings were then hydroponically cultivated in modified Hoagland complete nutrient solution (Erdei et al., 1984) in two phytotrons (Conviron PGW 36) with 23/18 °C and 13/11 hours day/night photoperiod for 11 days. One phytotron served as control while the other one was supplemented with UV-B light emitting tubes. In both phytotrons, irradiance was 60 Wm^{-2} at shoot level (Sylvania type power tubes F48/T12/CW/VHO and Gro-Lux WS F48/T12/GRO/VHO/WS). In one of the phytotrons, the set of tubes was supplemented with Philips sun lamps (TL 100W/01) providing 2.5 Wm^{-2} radiation of major spectral emittance between 310-315 nm (λ_{max}= 311/312 nm) (Santos et al., 1993), corresponding to the increase in UV-B irradiation due to about 10% stratospheric ozone depletion.

To simulate realistic growth conditions from the field, seedlings were treated with UV-B immediately after germination. For studying early responses to UV-B treatment, sampling was made daily between day 4 and 8 and at the 11^{th} day of growth in both phytotrons. Three independent series of wheat seedlings were grown; from each of them, independent samples were taken for enzyme activity measurements, oxidised glutathione level and fresh weight, dry weight determinations.

To determine the effect of salinity treatment on the rhythmic behavior of SOD

Wheat caryopses (*Triticum aestivum* L. cv. Tiszatáj, Cereal Research Institute, Szeged, Hungary) were grown hydroponically in complete nutrient solution in a phytotron (Conviron PGW 36) with 23/18°C and 12 h photoperiod for 14 days. Irradiance was 60 W m^{-2} at shoot level (Sylvania type power tubes F48/T12/CW/VHO and Gro-Lux WS F48/T12/GRO/VHO/WS). Treatments with NaCl at 0, 50, 100 and 150 mM concentrations started on the 12th day and were

followed by hourly sampling for 60 hours. After the measurement of fresh weight of roots and shoots, samples were placed into liquid N_2 and stored at -70 ^{0}C until further processing.

To determine the effect of salinity on the distribution and activity of MoCo-enzymes

Seeds of maize *(Zea mays* L., cv. Jubily) were surface sterilized with ethanol for 3 min and germinated in 0.2 mM $CaSO_4$ in the dark at room temperature. The resulting young seedlings were transferred to moisture filter paper for two weeks. Germinated seedlings were transferred into aerated hydroponic culture, wrapping a small part of the stem in foam rubber and inserting the entire plant through plastic lids into the tanks containing nutrient solution. The plants were grown in 20 l containers for a further 3 weeks before use with nutrient solutions as previously described (Savidov et al., 1997). Nutrient solutions were replaced once a week and the pH of the medium (6.5) was monitored daily. Uniform maize plants, 8 pot^{-1}, were grown in 4 replications for each treatment in a completely randomized block design. The experiments were conducted in a greenhouse, with an average day temperatures of 20 - 25°C, and of 8 - 12°C during the night. Midday light intensity was 900-1000 $\mu mol\ m^{-2}\ s^{-1}$. The nitrogen sources consisted of 4 mM $NaNO_3$ or 2 mM $(NH_4)_2SO_4$ in a half-strength modified Hoagland nutrient solution (Hoagland and Arnon, 1938). Salinity treatments consisted of 50 mM NaCl.

Excision of root segments: Maize nodal roots, used in all determinations, enabled the separation of cortex and stele. Roots were selected from the first or second node, without lateral root formation and up to a length of about 12 cm. Similar nodal roots were excised and placed on a glass plate wetted with growth medium. The roots were cut with a razor blade into 3 segments: 0-1, 1-2, and 2-10, where the numbers represent the distance from the apex in cm. For cortex and stele separations, the first two cm of the root were removed leaving the mature zone of the root (2-10 cm) from which the cortex was stripped manually. This could be readily performed because of the partial lignification of the stele. Similar root segments of 10 nodal roots were collected together and constituted one of 3 replicates.

3.2 Tissue extraction

For detection of oxidative enzymes

Wheat leaves and roots (1 g FW) were placed in liquid nitrogen then homogenised in a prechilled mortar and pestle under ice cold conditions in 4 ml of 50 mM TRIS buffer, pH=7.0, containing 1 mM EDTA, 3 mM $MgCl_2$ (for determination of catalase and guaiacol peroxidase activities), and in 4 ml of 50 mM potassium phosphate buffer, pH=7.0 with 1 mM EDTA (for determination of SOD and GR activities). In both cases, 1 % (w/v) insoluble polyvinilpolypyrrolidone (PVPP) was added. The homogenate was centrifuged at 27,000 g for 15 min at 4 °C. The supernatant was used for enzyme assays and protein determination (Dindsha et al., 1981).

For detection of glutathione

Samples of plant tissue (2 g FW) were placed in liquid nitrogen, pulverised to fine powder with a mortar and pestle, and quickly transferred to 8 ml of ice cold aqueous solution of 1 M perchloric acid and 1 mM of bathophenanthroline disulfonic acid (BPDS) (Reed et al., 1980). The extract was centrifuged at 12,100 g for 20 min at 4 °C, and supernatant was collected (Siller-Cepeda et al., 1991). For determination of GSH and GSSG, perchloric acid extracts were carboxy-methylated and derivatized using 2,4-dinitro-1-fluorobenzene (Fariss and Reed, 1987).

For detection of MoCo enzymes

Maize nodal root samples were extracted immediately after harvesting. Crude extracts used for the assays of AO and XDH in native gel electrophoresis were prepared by maceration with acid washed sand in a mortar with ice-cold extraction medium. The extraction medium consisted of 250 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol (DTT), 10 mM reduced glutathione (GSH), 5 µM FAD and 3% (w/v) PVPP. Samples of 1 g root tissue were extracted in 3 ml buffer (1:3 w/v). For NR determination, the extraction buffer contained 25 mM Tris-HCl (pH 8.4), 3 mM EDTA, 10 µM FAD, 5 µM sodium molybdate, 1 mM DTT, 5 mM

cystein, 10μM antipain, 0.1 mM phenylmethyl sulfonylfluoride (PMSF), and 3% (w/v) washed PVPP (Sigma chemicals) (Sagi et al., 1998c). Extraction ratio was 1:8 (w/v). The homogenized plant material was centrifuged in a Centrikon T-124 refrigerated centrifuge at 27,000 g and 4^{o}C for 15 min. The resulting supernatant was used in subsequent assays.

3.3 Analytical determinations

3.3.1 Enzyme assays

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of Nitro Blue Tetrasolium (NBT) in the presence of riboflavin in the light (1 EU=50 % inhibition). The colour change was monitored at 560 nm. Isoenzymes were identified by using native gel electrophoresis and on the basis of their differential inhibition by H_2O_2 and KCN (H_2O_2 inhibits Cu/Zn-SOD and Fe-SOD but not Mn-SOD; KCN inhibits Cu/Zn-SOD, but not Fe-SOD or Mn-SOD) (Beauchamp and Fridovich, 1971; Dindsha et al., 1981).

Catalase (EC 1.11.1.6) activity was measured spectrophotometrically by following the decrease of H_2O_2 quantity in time at 240 nm (1 EU=mmol H_2O_2 decomposed in one minute) (Upadhyaya et al., 1985).

Guaiacol peroxidase (EC 1.11.1.7) activity was determined by monitoring the increase in absorbance at 470 nm as guaiacol was oxidised (1 EU=μmol guaiacol oxidised in one minute) (Upadhyaya et al., 1985).

Glutathione reductase (GR, EC 1.6.4.2) activity was determined by monitoring absorbance increment at 412 nm when DTNB (5,5'-dithio-*bis*(2-nitrobenzoic acid)) was reduced by GSH, generated from GSSG. Standard GR was Type III from Baker's Yeast. The rate was calculated from the linear portion of the curve and expressed as a rate/5 min (Smith et al., 1988).

Aldehyde oxidase (AO, EC 1.2.3.1) activity was detected in polyacrylamide gels by staining after native electrophoresis carried out with 7.5% acrylamide gels (Laemmli,

1970) in the absence of SDS at 4°C. The gel was immersed after electrophoresis in 0.2 M phosphate buffer, pH 7.5, for 10 min followed by gentle shaking at room temperature in a reaction mixture containing 0.1 M Tris-HCl (pH 7.5), 0.1 mM phenazine methosulfate (PMS), 1 mM 3[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT) and 1 mM substrate (acetaldehyde, heptaldehyde, benzaldehyde or indole-3-aldehyde). Native PAGE was carried out with a Protean II xi Cell (Bio-Rad, USA).

Xanthine dehydrogenase (XDH, EC 1.2.37) XDH activity was detected after native gel-electrophoresis using hypoxanthine as a substrate (Mendel and Muller, 1976).

NADH-nitrate reductase (NADH:NR, EC 1.6.6.1) was assayed in a reaction mixture containing 30 mM K-phosphate buffer (pH 7.5), 25 mM KNO_3 and 0.25 mM NADH. The reaction started with the addition of 50 µl of the enzyme extract. The total volume of the assay solution consisted of 300 µl and the enzyme reaction took place at 28°C for 15 min. The assay was terminated by addition of 50 µl of a 1:1 (v/v) mixture of 0.3 mM phenazine methosulphate and 1 M Zn acetate followed by vigorous mixing to remove residual NADH. Nitrite was determined with 1 ml of a 1:1 (v/v) mixture of 1% (w/v) sulfanilamide in 3.0 M HCl and 0.02% (w/v) N-naphthyl-(1)-dihydrochloride. Absorbance at 540 nm was measured after 20 min. NR activity was expressed as µmol NO_2^- g^{-1} fresh weight h^{-1} or nmol NO_2^- mg^{-1} protein min^{-1} (Sagi and Lips, 1998c).

3.3.2. Quantitation of glutathione

Chromatographic conditions: dinitro-phenyl derivates were separated and measured using a gradient HPLC (BioRad) system equipped with a BioRad Amino-5S column and UV detector (Spectra Physics) at 365 nm. Following a 100 µl injection of the centrifuged solution containing derivatized sample, the mobile phase was 75 % solution A and 25 % solution B for 10 min followed by a 30 min linear gradient to 5 % A and 95 % B. Flow rate was 1.0 ml/min. Solution A contained 80 % methanol, solution B was composed of 0.55 M sodium acetate, 12.6 % acetic acid and 64 % methanol (Fariss and Reed, 1987).

3.3.3 Protein determination and statistics

Total soluble protein content was estimated by the Bio-Rad micro assay modification of the Bradford procedure (1976) and according to Lowry et al. (1951) using crystalline bovine serum albumin as a reference. Each treatment was analyzed with at least three replicate tissue samples bulked from at least five plants. All treatments were repeated at least three times.

3.4 Western blot analysis for aldehyde oxidase

The AO proteins extracted from the plant material were subjected to Western blotting. Ground tissue (1 g FW) was extracted with 1 ml Tris-HCl buffer, pH 7.5, 3 mM DTT, 1 mM EDTA, 5 mM GSH and 50 µM leupeptin. After centrifugation, the resulting supernatant was added to SDS-buffer at a ratio of 1:4 (v/v). SDS-PAGE was performed in a 10% polyacrylamide gel (Laemmli, 1970). The resulting gel with the separated proteins was then electrophoretically transferred onto a nitrocellulose membrane (0.2 µm pore size; Schleicher and Schüll, Dassel, Germany). Blotting time was 1 h at 2 mA cm^{-2}. Blots were blocked for 90 min in 5% (w/v) bovine serum albumin in TBS. Immunodetection of AO was carried out with polyclonal mouse antibodies raised against the purified maize AO (Koshiba et al., 1996) after a 500-fold dilution in TBS and secondary antibodies (anti-mouse IgG, Sigma) diluted 1000-fold in TBS. The antigen/primary antibody complex was detected by binding of alkaline-phosphatase-linked goat anti-mouse IgG (Sigma, USA). Phosphatase activity was developed by staining with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT, Sigma Fast™ tablets). Molecular weight of proteins was estimated with a mixture of protein standards: myosin (202 kD), galactosidase (109 kD), bovine serum albumin (78 kD) and ovalbumin (46.7 kD). Binding of the antibodies to maize root AO proteins was detected by immunoprecipitation using protein-A-Sepharose CL-4B (Pharmacia). All of the activity (detected after native-PAGE) in the samples was removed when the

antibodies and Sepharose were added, while all of the activity remained in the supernatant when control mouse serum was used.

3.5 Estimation of AO and XDH activities

Enzyme activities of the Mo-hydroxylases were estimated on the basis of MTT reduction, which resulted in the development of specific formazane bands. The quantity of formazane was directly proportional to enzyme activity during a given incubation time, with substrate and tetrazolium salt supplied in excess (Rothe, 1974). Quantitative analyses were made by scanning the formazane bands in the gel, with a computing laser densitometer (Molecular Dynamic) using Image Quant version 3.19.4 and NIH Image 1.6.

3.6 Fit of periodicity by Fourier transformation

For the calculation of different periodicities, Fourier analysis was carried out by a radix-2 fast Fourier transform algorithm implemented in the MATLAB 4.0 programming environment (The Mathworks Inc., Cochituate Place, 24 Prime Park Way Natick, Mass. 01760). According to the nature of the N-point Fourier transform, the amplitudes ("weights") can be expanded in terms of full-scale fractions, N/k, where "k" is a natural number not bigger than N/2. In other words, the number of data points (N) determines the resolution causing problems only at the low frequency limit, however, unresolved components can be assembled by the linear combination of N/k terms. Frequency filtering was performed by an inverse Fourier transform of the truncated frequency spectra, employing the same algorithm.

4. RESULTS AND DISCUSSION

4.1 Oxidative stress

4.1.1. Effects of excess UV-B radiation on the antioxidant defense mechanisms in wheat (Triticum aestivim. L.) seedlings

We presumed that enhanced UV-B irradiation (280-320 nm) reaching the Earth's surface, corresponding to about 10 % ozone depletion, gives rise to oxidative stress for plants at the physiological and biochemical levels. From this general hypothesis, the following questions were to be answered: i) what are the stress responses to enhanced UV-B treatment in wheat; ii) which mechanisms are involved in the protective responses; and iii) do plants adapt to these circumstances.

To answer these questions we investigated the changes in the antioxidant defence systems in wheat as affected by UV-B radiation, by screening the activities of superoxide dismutase, catalase, guaiacol peroxidase and glutathione reductase enzymes, as well as the level of glutathione disulfide (GSSG) since the elevation of this compound is a good indicator of oxidative stress. Modifications of growth parameters and the appearance of ultraviolet light absorbing pigments were also studied.

Growth

As an early response to excess UV-B light, the inhibition of stem and leaf elongation occurred at the beginning of the treatment. Reduction in the length and fresh weight, accompanied by almost unchanged dry weight (Figures 6 and 7) suggests that UV-B irradiation inhibited the cell elongation process rather then the mass growth. The reduction of elongation by UV-B irradiation has also been observed in spring barley and wheat, however, dicotyledonous species showed a range of sensitivities (Dumpert and Knacker, 1985). Similar plant responses were found under solar UV-B irradiation in *Datura ferox* (Ballaré et al., 1996). However, according to our observations the inhibitory effect of UV-B irradiation on the elongation of wheat seedlings decreased by the end of experimental time (Figure 6).

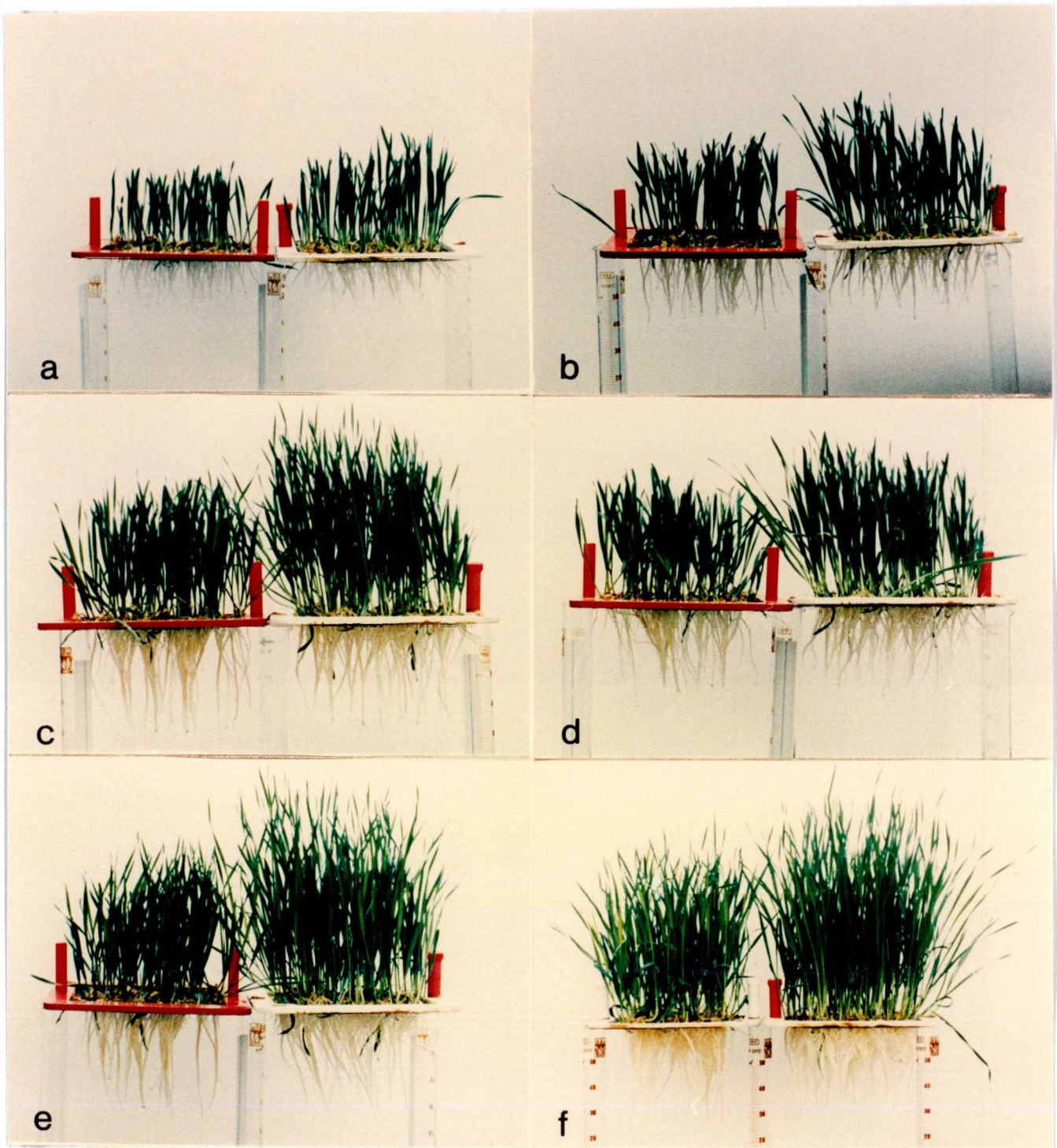

Figure 6: Growth of wheat seedling populations as affected by excess UV-B irradiation. Day of UV-B treatment: (a) 4th, (b) 5th, (c) 6th, (d) 7th, (e) 8th, (f) 11th. Right side, control plants; left side UV-B treated plants.

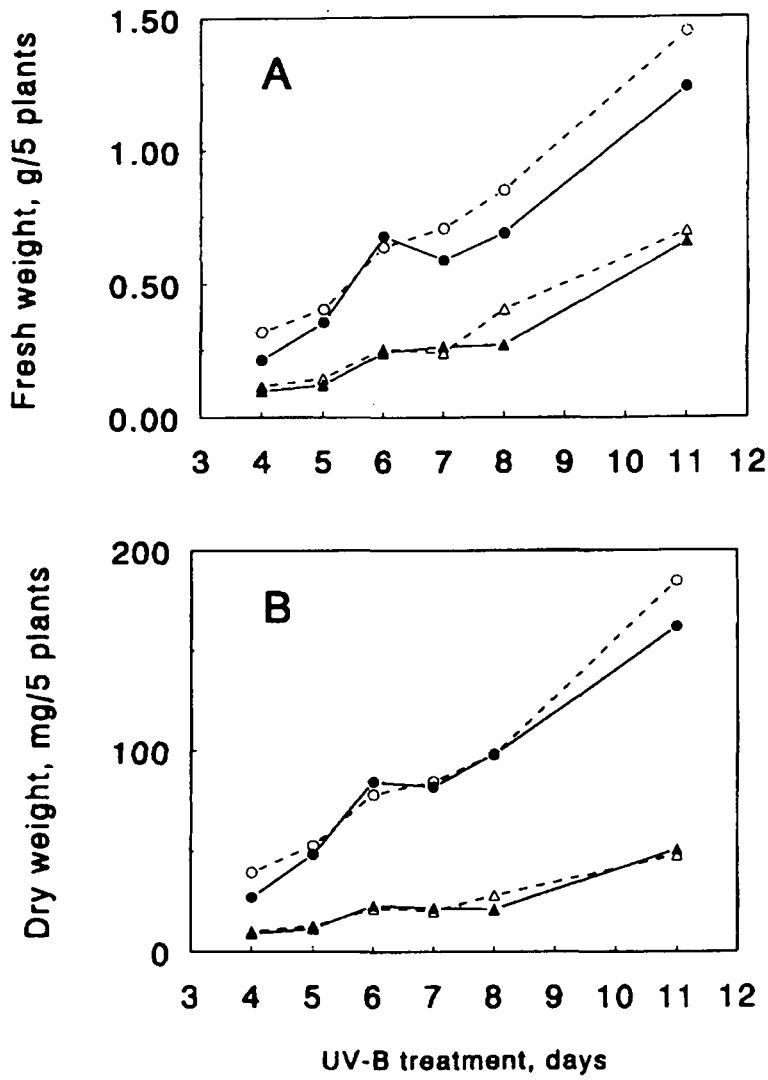

Figure 7: Growth on fresh (A) and dry (B) weight basis of wheat seedlings as affected by UV-B irradiation. Representative growth series, batches of 5 plants. Open symbols, control; filled symbols, treated samples; circles, leaves; triangles, roots.

Pigment accumulation

UV-B exposure induced the accumulation of ultraviolet light absorbing pigments in the epidermal cells of the wheat stem (Figure 8).

UV-B Control

Figure 8: Pigment accumulation in the epidermal cells of wheat stem; 4th day of UV-B treatment.

These pigments were reported to be of flavonoid type (McClure, 1975) and they play an important role by reducing the damage of photosynthetic function (Tevini et al., 1991). Such compounds, which serve as optical screens, absorbing UV while transmitting the photosynthetically active radiation (400-700 nm), were also

reported to accumulate in greater amount in a more resistant cucumber variety than in the less resistant one (Adamse and Britz, 1996). Rao and Ormrod (1995) showed out that an *Arabidopsis* genotype of blocked flavonoid biosynthesis was more sensitive to UV-B irradiation than the wild genotype, although the former one maintained efficient scavenging system. In parsley (*Petroselinum crispum*) cell suspension cultures, the induction by UV light of expression of genes for flavonoid biosynthesis enzymes has been also demonstrated (Chappell and Hahlbrock, 1984). In our present experiments, however, the accumulation of the flavonoid pigments was temporary only, since stem pigmentation disappeared by the 11^{th} day of treatment. This can be attributed to the induction or normalisation of other adaptive mechanisms, like antioxidant enzyme activities leading to a balanced metabolism under the new conditions. Normalisation of growth and pigmentation under the applied excess UV-B irradiation indicates that the stress in question was an *eu-stress* rather than *dis-stress* according to the extended stress concept introduced recently by Lichtenthaler (1996).

Enzyme activities and antioxidant levels

Since increased formation of oxygen radicals could be expected in photosynthetic systems under high light conditions (Hideg and Vass, 1996) and UV-B irradiation (Foyer et al., 1994), the activity of SOD was followed during the treatment (Figure 9).

It is seen that although this enzyme was damaged in seedlings in their 1^{st} leaf developmental stage, by the 7^{th} day SOD activity was restored and reached control level. By using native gel electrophoresis and different inhibitors (see Material and Methods) the studied SOD was identified as Cu/Zn SOD isoenzyme.

At the stage of panicle initiation, reduced SOD activity was observed in both the UV-B tolerant and susceptible cultivars of rice, grown under excess UV-B irradiation (Olszyk et al., 1996). UV-B irradiation affected transcript level as well: Strid (1993) reported that the expression of SOD was drastically decreased in pea (*Pisum sativum*) after exposure to supplementary UV-B radiation. The primary target of UV-B irradiation thus may be located well before the level of translation or even transcription.

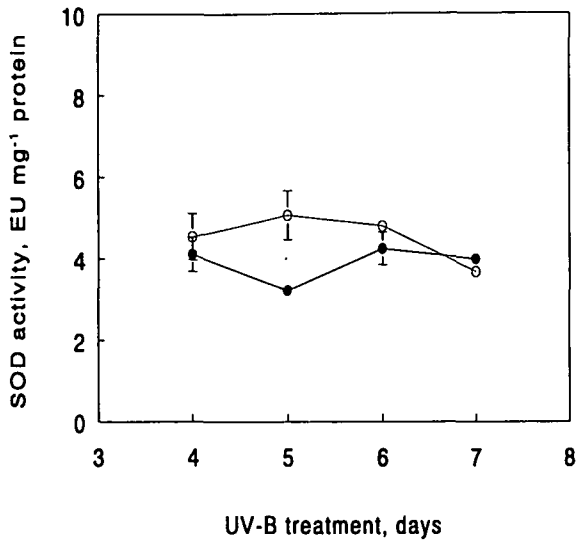

Figure 9: Effects of UV-B irradiation on the activity of superoxide dismutase in leaves of wheat seedlings. Mean ±S.D. of 3 independent determinations. Symbols without bars mean that S.D. do not exceed the size of symbol. Open symbols, control; filled symbols, treated samples.

Additionally, however, it has to be taken into account that there are also nonenzymatic mechanisms that convert superoxide anion into H_2O_2 (Asada and Takahashi, 1987). For example, $^{\bullet}O_2^-$ can react with two NAD(P)H molecules to produce H_2O_2 (Nadezhdin and Dunford, 1979). Several electron donors can act in this manner, including ascorbate (Cabelli and Bielski, 1983) and glutathione (Anderson et al., 1983). Also, electrons directly from the electron transport chains of the photosystems can reduce $^{\bullet}O_2^-$ to H_2O_2 (Allen, 1975).

The product of SOD reaction is H_2O_2, which is eliminated by peroxidases and catalase. However, H_2O_2 is formed in numerous metabolic reactions and its formation is not restricted to dismutation of superoxide radicals only. Figure 10A shows that catalase activity increased with age in the early growth period in both control and UV-B treated seedlings, whereas it culminated in a plateau in control plants on days 6 and 7 followed by a fast drop. UV-B treatment decreased catalase

activity starting from the 7th day of growth, which remained at a level somewhat lower than that of the control. Catalase activity was also decreased by UV-B in cucumber seedlings (Krizek et al., 1993) and rice (Olszyk et al., 1996)

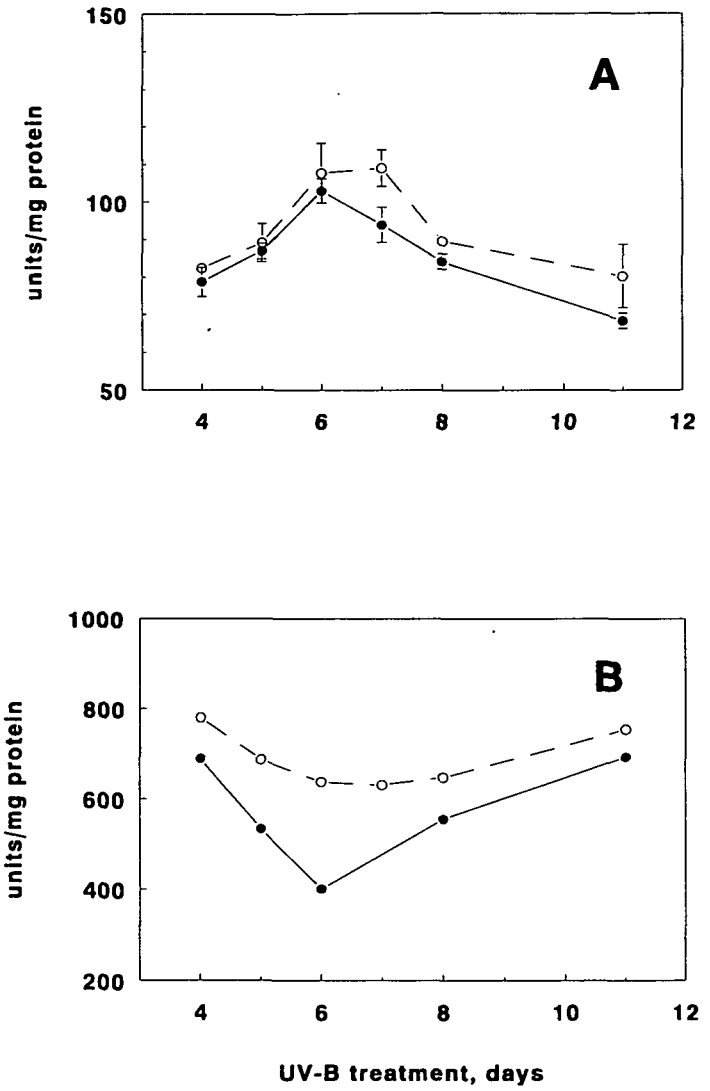

Figure 10: Effects of UV-B irradiation on catalase (A) and guaiacol peroxidase (B) activity in leaves of wheat seedlings. Mean ±S.D. of 3 independent determinations. Symbols without bars mean that S.D. does not exceed the size of symbol. Open symbols, control; filled symbols, treated samples.

Guaiacol peroxidase is a typical plant peroxidase, localised in cell walls and vacuoles, but not in chloroplast. Its physiological functions include the biosynthesis of lignin and ethylene, and the degradation of indoleacetic acid, rather than scavenging of H_2O_2 (Asada, 1994). In the present study, exposure to UV-B dropped the activity of guaiacol-peroxidase in the leaves of wheat seedlings (Figure 10B), similarly to that reported for barley leaves (Liu and McClure, 1995).

In the hydrated tissues, free radical production is normally regulated by compounds with scavenging function such as SOD, catalases and peroxidases. It is possible that water loss by cells alters the equilibrium between free radical production and enzymatic defence reactions in favour of the first one (Quartacci and Navari-Izzo, 1992). In our experiments, reduction in the levels of antioxidant enzymes (SOD, catalase, peroxidase) under excess of UV-B irradiation, could be brought about by the decreased cell water content, which is indicated by the reduction in leaf length and fresh weight and the almost unchanged dry mass (Figures 6 and 7).

In plants, one of the most powerful antioxidant system is the glutathione redox mechanism with its coupling enzymes towards NADP/NADPH and the ascorbate cycle. The excess UV-B irradiation applied, brought about oxidative circumstances in both leaves and roots as shown by the intensively increasing GSSG levels in both organs (Figures 11A and 12A). However, GSSG levels decreased by the 7^{th} day of age in a close relationship with GR activity which increased with substrate abundance and was maintained at a higher level in UV-B irradiated plants, both in leaves and roots, than in the untreated control (Figures 11B and 12B). By the 11^{th} day of growth, GR activities were at the same level both in treated and control plants.

In pea leaves, GR, which together with SOD and ascorbate peroxidase forms part of an enzymatic chain for removal of $^{\bullet}O_2^-$ and H_2O_2, was induced by UV-B irradiation (Strid, 1993). GSSG also accumulated during UV-B exposure to levels 9-fold higher than in nontreated plants. One plausible explanation was that decreased amounts of mRNA transcripts for SOD (Cu/Zn-SOD of the chloroplast) upon exposure of the pea plants to supplementary UV-B, determined the increase in GR, which could in this case be required to prevent deleterious effects due to lowered SOD activity (Strid, 1993). GR activity was also reported to increase in maize

seedlings grown under UV-B radiation (Masi et al., 1996).

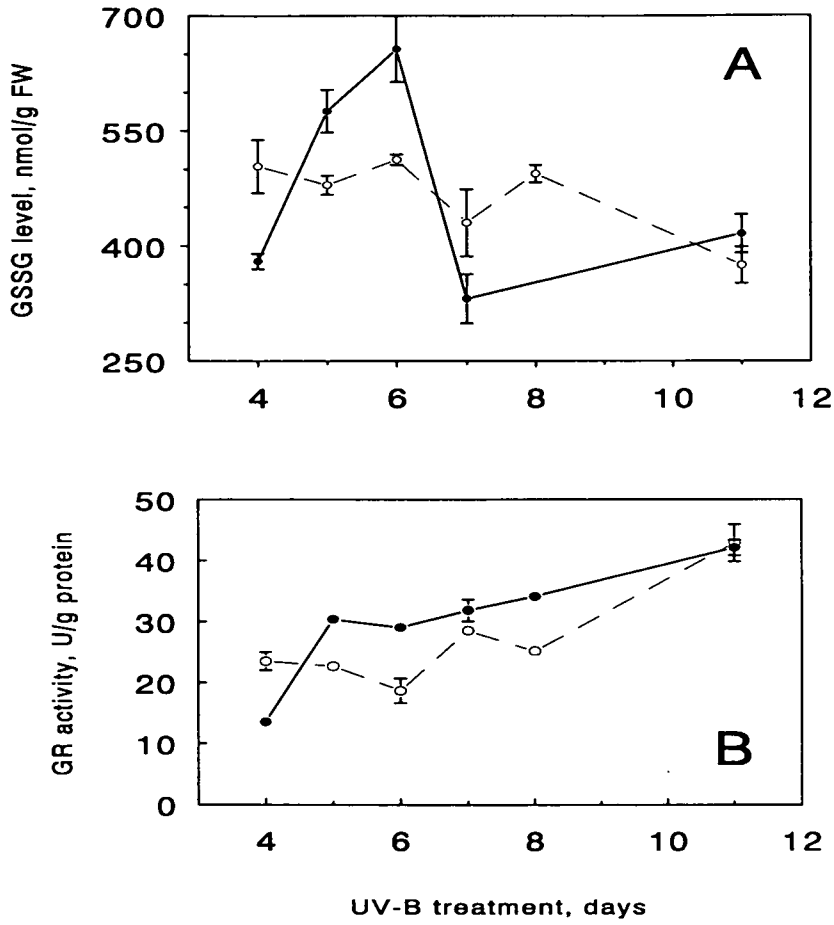

Figure 11: Effects of UV-B irradiation on the glutathione disulfide level (A) and on glutathione reductase activity (B) in leaves of wheat seedlings. Mean ±S.D. of 3 independent determinations. Symbols without bars mean that S.D. does not exceed the size of symbol. Open symbols, control; filled symbols, treated samples.

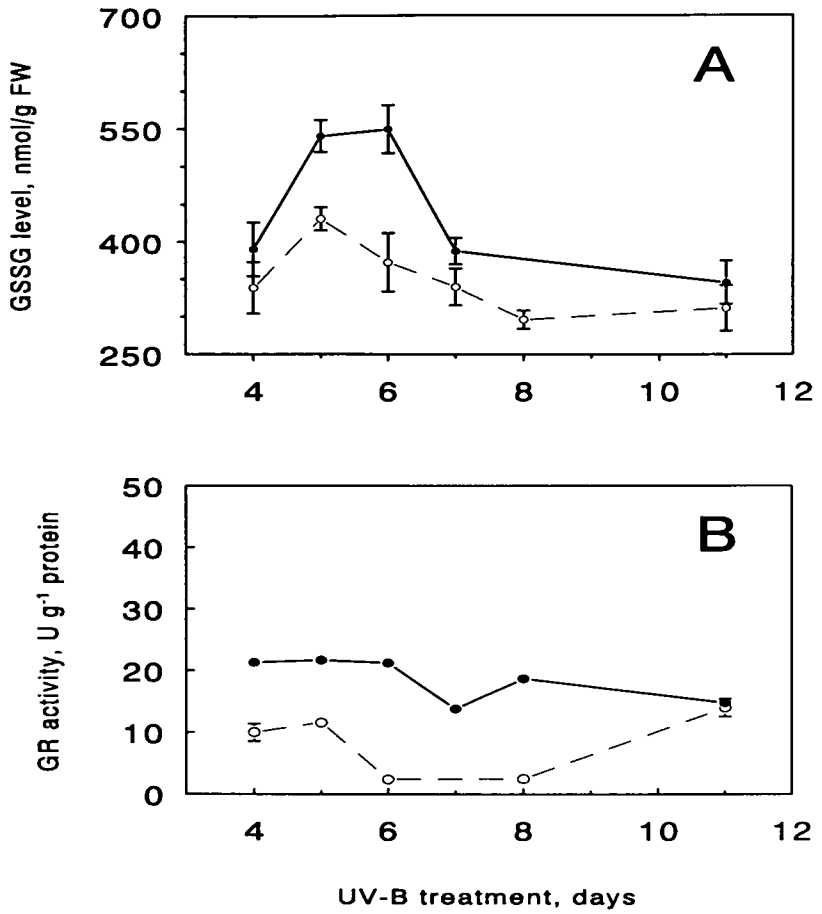

Figure 12: Effects of UV-B irradiation on the glutathione disulfide level (A) and on glutathione reductase activity (B) in roots of wheat seedlings. Mean ±S.D. of 3 independent determinations. Symbols without bars mean that S.D. does not exceed the size of symbol. Open symbols, control; filled symbols, treated samples.

Concerning the overall antioxidant enzyme activities, it can be summarised that during UV-B irradiation, the activities of the enzymes investigated, after reaching a transitional minimum value, caught up the control level, *i. e.* acclimatisation has occurred. The predominant component among them was the glutathione system, which, being one of the most powerful antioxidant in plants, brought about an intensive "detoxification" and remediation of cellular environment.

Literature data suggest that excess UV-B irradiation acts at the level of gene regulation (Strid, 1993), and the induction of antioxidant enzymes is differential in response to UV-B and O_3 exposition (Rao and Ormrod, 1995; Rao et al. 1996).
The activity of a Ca^{2+}-dependent 52 kDa protein kinase responding with enhanced activity to osmotic treatment in sorghum (Pestenácz and Erdei, 1996) was also found to increase in wheat seedlings at day 7 after exposure to UV-B; in addition, more pronounced activity stimulation was observed in a 65 kDa band, which was maintained during the subsequent experimental time (Barabás et al., 1998). The protein kinases are known to be members of the signal transduction cascade.

It is to be stressed in this context that in our experiments, substantial changes took place in the activities of enzymatic and non-enzymatic defence systems around day 7. The activation of CDPK by this time (after 3-day treatment with excess UV-B irradiation) suggests its participation in the signalling pathway (Barabás et al., 1998).

The time course of events in daily timescale indicates two main phases in the adaptation process to UV-B irradiation in young wheat plants. In terms of the Selyean stress concept (Selye, 1956) these phases can be considered as parts of the general adaptation syndrome (GAS): The alarm reaction is represented by the inhibition of stem and leaf elongation, accumulation of flavonoid pigments, decrease in antioxidant enzyme activities, increased GSSG level and triggering of GR activity, while normalisation of growth, disappearance of stem pigmentation, levelling of antioxidant enzyme activities, high GR activity and decreased level of GSSG were found as parts of adaptation to UV-B irradiation. In other words, synthesis of flavonoid pigments during the alarm phase is a *syntoxic* element which contributes to the increase in tolerance while the increase in the levels and/or activities of antioxidants can be regarded as *catatoxic* elements which actively eliminate the oxidative components of the stressor (Leshem and Kuiper, 1996). In general,

moderate stress caused by the excess UV-B irradiation in the present experiments can be categorised as *eu-stress* (Lichtenthaler, 1996) which is a stimulating stress that activates cell metabolism, increases resistance or acclimatisation The final message of the present work is that the moderate increase in UV-B irradiation due to the present depletion of ozone in the stratosphere, at the surface do not bring about fatal damage in plant (at least in wheat) repair mechanisms, metabolism and growth.

4.1.2 Effects of salinity treatment on the changes of rhythmic behavior of superoxide dismutase in wheat seedlings

In order to assess the influence of environmental stress as effector on rhythmic phenomena in plants, we researched the effect of salinity (0, 50, 100 and 150 mM NaCl) on the activity of superoxide dismutase as function of time in winter wheat seedlings.

When investigating the activity of SOD (identified as Cu/Zn isoenzyme) in the leaves of wheat, very scattering series of data was obtained and without further analysis, besides an overall period, no difference between treated and control samples could be seen (Figure 13A). The analysis, however, disclosed an increase in the amplitude of the circadian period fraction in the 150 mM NaCl-treated samples (Figure 13B). Additionally, an ultradian rhythm of about 13 hours, visible in control plants, disappeared in the salt treated series. The amplitude of the circadian period has changed intensively but without showing a consequent trend: at intermediate salt concentrations the amplitude decreased, while it increased at the highest treatment of 150 mM NaCl (Figure 13B).

The Fourier transform of SOD activity (Figures 13A and B) suggests the presence of more than one period governing the oscillating phenomenon in question. In order to make it more obvious, frequency filtering was performed by an inverse Fourier transform of the truncated frequency spectra, and the resulting "smooth" curve was compared to the original experimental curve. Figure 14 shows the existence of composed fluctuation in SOD activity in the roots of NaCl treated wheat plants.

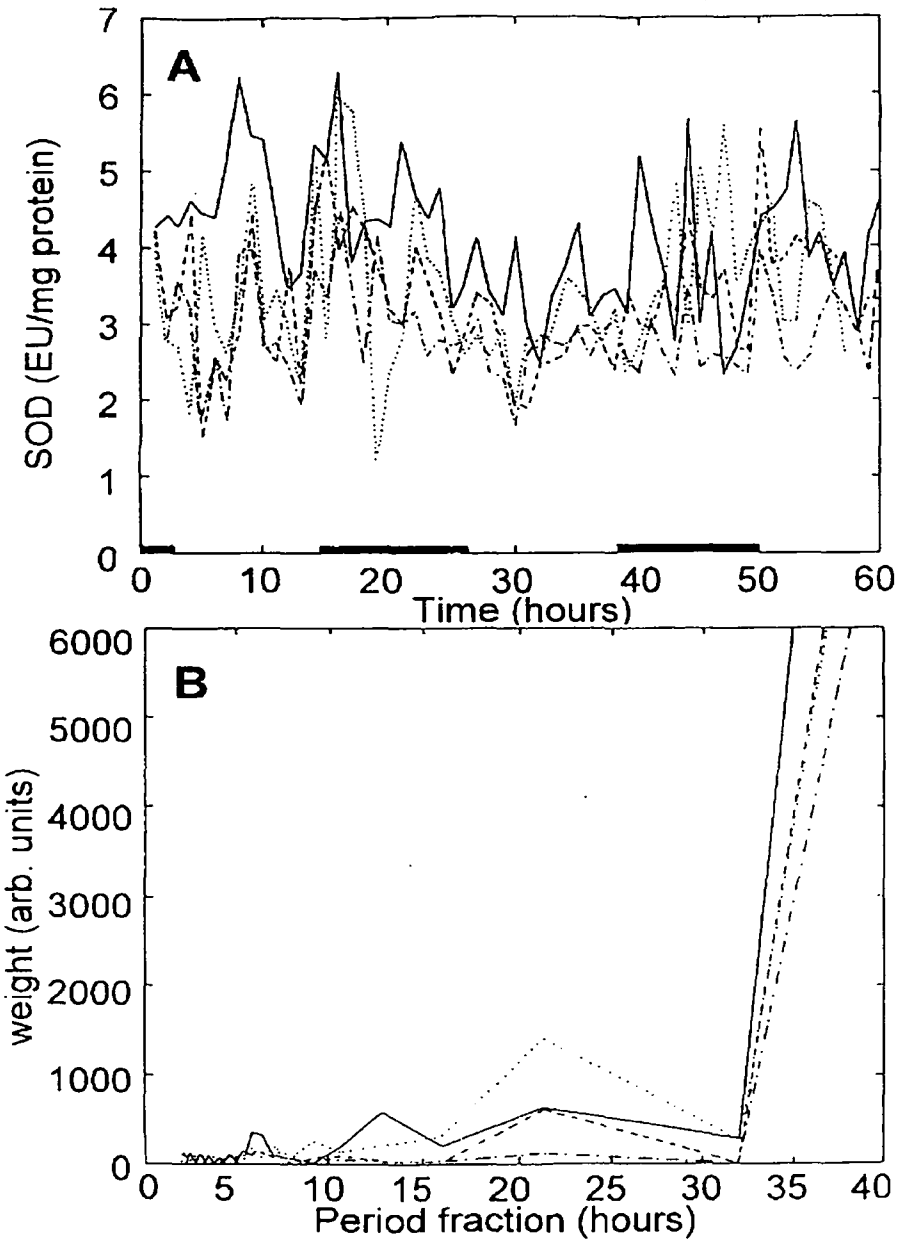

Figure 13: (A) Changes in the levels of superoxide dismutase activity in the leaves of control and NaCl-treated wheat plants. Samples were taken hourly for 60 hours. Dark periods (12 h) are represented the thick sections of the abscissa. Symbols: full line, control (0 mM NaCl); dashed line, 50 mM NaCl; dash-and-dot line, 100 mM NaCl; dotted line, 150 mM NaCl. (B) The Fourier components of the curves in (A). Symbols are as in (A).

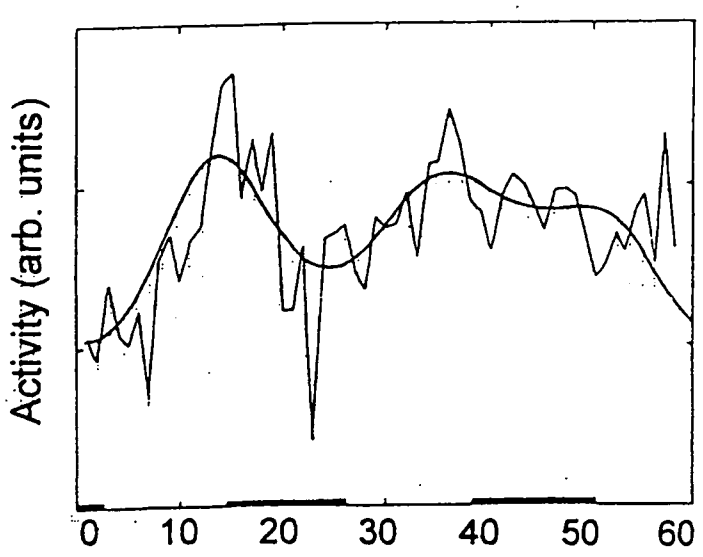

Figure 14: A composed oscillation as shown by the "smooth" curve, obtained by frequency filtering, compared to the original experimental curve. SOD activity, roots, 150 mM NaCl.

Investigating the effects of stressors on oscillations of biochemical and physiological processes, the following targets have to be considered: i) the molecular mechanism of the central pacemaker itself; ii) the elements of the signal transduction pathway from the environment to it; iii) the mechanism of control of the underlying variables; and iv) fluctuations which are not necessarily coupled to the oscillator.

Molecular mechanisms of the oscillator, input and output

Characterization of the molecular identity and mechanism of the central oscillator cannot be separated from the investigation of the closely connected steps of input and output pathways. In higher plants, there are a large number of genes under the control of a circadian clock. Most significantly, the family of *Lhc* genes, which code the components of the light harvesting complexes of photosystems I and II, showed concerted fluctuation at transcript level in tomato (Piechulla, 1993). However, because of the great variation in periodicity in other species like *Petunia*

and *Arabidopsis*, no uniform model can be constructed for transcriptional control.

Analyses of the 5'-deletion mutants of *Lhc* genes were a breakthrough in understanding circadian clock controlled gene expression (see references in Piechulla, 1993). It was found that the 5'-upstream region of the *Lhc* genes could direct clock controlled gene expression indicating the interaction between trans-factors and cis-elements in transcriptional control.

Since trans-acting factors are located in the cytoplasm, their role as signal transducers to the nucleus can be initiated by their phosphorylation by specific kinases and/or phosphatases (Hunter and Karin, 1992; Proud, 1992). Plant Ca^{2+} - dependent protein kinases (CDPKs) are known to be associated with the activation of proteins involved in the signal transduction pathway (Harper et al., 1991). A water-stress inducible 52 kDa CDPK was found in *Sorghum*, which shared the cascade mechanism with abscisic acid (Pestenácz and Erdei, 1996). More recently, it was observed that the activities of a CDPK, similar to that found in *Sorghum*, and an additional one having 65 kDa molecular mass, were stimulated by excess UV-B irradiation in wheat seedlings (Barabás et al., 1998). In this way, CDPKs are reasonable candidates in the information transfer from stressors towards the nucleus, possibly *via* phosphorylation of trans-acting protein factors.

Translational control. Biochemical and physiological oscillations: remote steps of output or independent pacemakers?

In many cases, the controlling mechanism of rhythmicity is apparently not coupled to transcription. In the unicellular alga *Gonyaulax,* for example, two distinct oscillations of bioluminescence were observed with different phases and amplitudes, suggesting the operation of two "clocks". However, the level of mRNA of the luciferin binding protein (LBP) remained constant, while the LBP itself fluctuated. The protein synthesis inhibitor cycloheximide blocked the oscillation of LBP and acted as 'Zeitgeber' causing phase shift. Translational control of periodicity was also observed in other species, e.g. in *Acetabularia* (Schweiger and Schweiger, 1977). The fact that translation is controlled also by protein *trans*-acting elements that bind to the mRNA as activator or repressor is being turned out by now (Mittag and Hastings, 1996).

Although certain enzymes of the antioxidant defense system, e.g. catalase isoforms (Redinbaugh et al. 1990) or SOD in *Gonyaulax* (Colepicolo et al., 1991), are under the control of clock controlled genes, the activities of others can be influenced by stressors like UV-B or ozone, *via* modifying substrate affinity through the abundance of different isoforms of the enzyme (Rao et al., 1996). In the present experiments, the period of the circadian rhythm was not influenced, however, the shorter ultradian fluctuations seemed to disappear (SOD, Figure 13B). Amplitudes, on the other side, were either increasing (SOD, Figure 13) or decreasing, like in the accumulation of total soluble carbohydrates (Erdei et al., 1998). In the first case, the reason can be the change in substrate abundance and/or in enzyme affinity.

It could be concluded that: i) SOD activity fluctuated with circadian and ultradian periodicities; ii) salinity treatment influenced the amplitude of oscillations and left the circadian period unchanged; iii) ultradian fluctuations proved to be sensitive to stress treatment. It was hypothesized that stress responses in plants, as described by the stress concept of Selye and adopted in plant physiology, can be evoked by the modification and desynchronization of oscillations of biochemical and physiological processes resulting in a decreased state of resistance.

4.2 Molibdoenzymes: Distribution of the Mo-enzymes aldehyde oxidase, xanthine dehydrogenase and nitrate reductase in maize (*Zea mays* L.) nodal roots as affected by nitrogen and salinity

Roots are frequently the first part of the plant to sense environmental stress conditions, such as salinity, and the first line of adaptation reactions.

We characterized the effect of salinity and nitrogen source on aldehyde oxidase, xanthine dehydrogenase and nitrate reductase activities and the distribution of these enzymes in adventitious or nodal roots of maize, and within the different zones of root with defined physiological functions and of progressive maturation. In order to get more information and a clear picture about AO enzyme as the enzyme involved in the last step of ABA synthesis, imunoblot analysis of AO proteins and studies correlation of substrate and tissue specificity for AO activity were completed.

4.2.1 The effect of salinity and nitrogen source on AO, XDH, NR

Four bands with AO activity (AO1-4) were detected in native PAGE (Figure 15A). The band with the highest mobility and activity, as estimated by density measurements, was AO-4. Salinity treatment (50 mM NaCl) and the presence of ammonium resulted in a significant increase of the total AO activity in maize nodal roots (Figure 15A). AO3 and AO4 were the main bands enhanced in the roots by ammonium as the nitrogen source as well as in nitrate fed plants under salinity treatment (Figure 15A), and were the principal contributors to total AO activity in the nodal roots of maize. XDH activity, similar to AO, was enhanced by salinity and by ammonium in the nutrient solution as the sole nitrogen source (Figure 15B).

NADH-NR activity in nodal roots of nitrate fed plants increased with salinity, when calculated either on a fresh weight or on a protein basis (Figure 16A,B). The increase was more pronounced in tips than in mature parts of the root. On the transversal root axis, NR activity increased in the stele while it decreased slightly in the cortex (Figure 16).

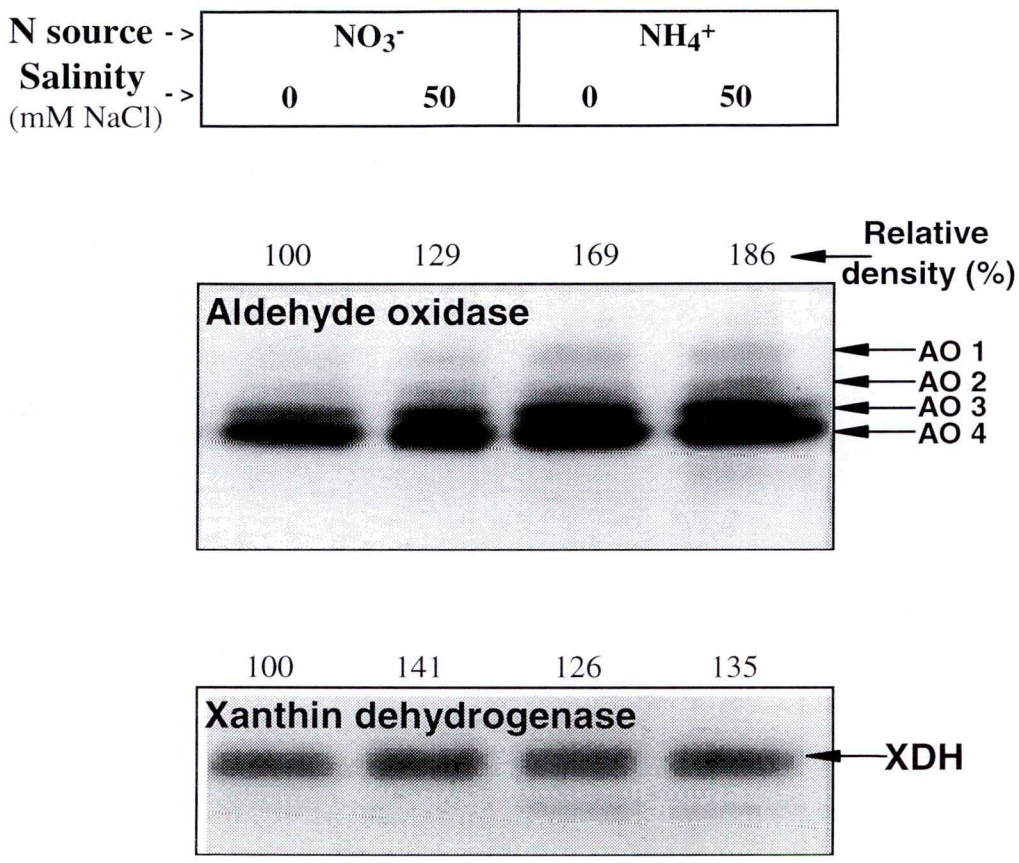

Figure 15: AO and XDH activities in crude extract of maize nodal roots as affected by N source and salinity (50 mM NaCl). Substrate staining of AO and XDH bands was done with indole-3-aldehyde and hypoxanthine, respectively. Controls (100%) were the enzyme activities of extracts obtained from plants grown with nitrate in the absence of salinity. Numbers above the lanes indicate percentage values obtained by density scanning and analysis by computer software (NIH Image 1.6). The zymogram represents one of at least three different experiments with similar results.

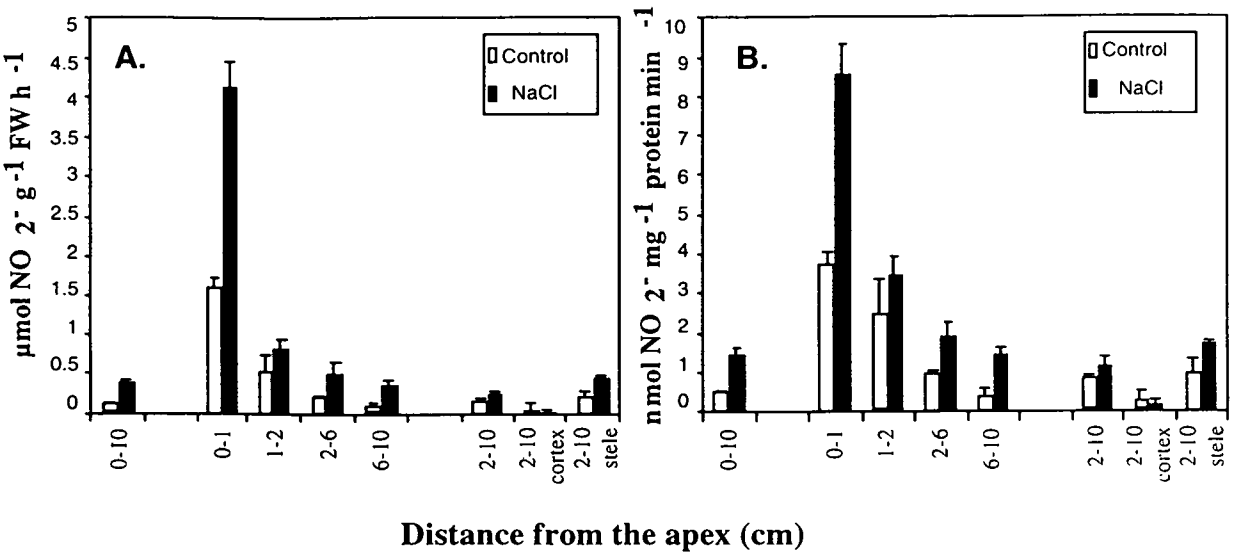

Figure 16: Nitrate reductase activity in different zones of maize nodal roots as affected by salinity. Roots were grown in medium supplied with 4 mM $NaNO_3$ (control) and salinity treatment consisted of 50 mM NaCl. Enzyme activity was calculated on fresh weight (A) and protein (B) basis. Bars indicate standard error of the mean (n=3).

4.2.2 Distribution of Mo-enzymes through different parts of the root

Zone and tissue specific distribution of AO, XDH and NR activities were studied in nodal roots of maize plants grown in nutrient medium containing 4 mM NH_4^+ and 50 mM NaCl, since these conditions were previously found to enhance AO and XDH activities (Figure 15).

AO activity was unequally distributed through the different tissues of maize nodal roots with the highest levels of enzyme activity in tips and the stele (Figure 17). Activity staining of AO after native PAGE revealed four bands designated AO1 to AO4 (Figure 17). Comparative analysis of AO activity in the different root zones showed that the overall AO activity in maize nodal root was given mainly by the

AO3 and AO4 protein bands or isoenzymes. The tips and the vascular cylinder contained mainly AO3 and only traces of AO1 and AO2. AO activity was expressed on a fresh weight basis with acetaldehyde as substrate (Figure 17). Similar results were obtained when AO activity was determined on a total soluble protein basis with other aldehydes as substrates (Figure 20).

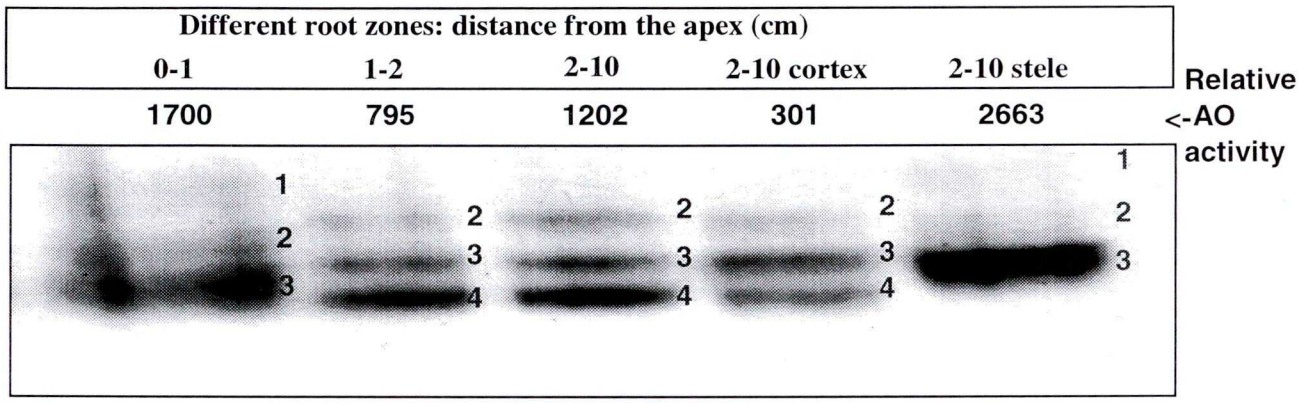

Figure 17: AO activity in different zones of maize nodal roots grown in 4 mM NH_4^+ + 50 mM NaCl. Supernatant samples (100µl/lane) were subjected to native PAGE, followed by AO activity staining with acetaldehyde as substrate. After staining, the gel was scanned and the relative intensities of the bands estimated by (NIH Image 1.6) software.

NR and XDH, showed a similar distribution pattern, with their principal concentration in the first cm of the maize nodal root (tips), and on the transverse axis in the stele (Figures 16 and 18).

4.2.3 Immunoblot analysis of AO proteins

Immunoblot analysis of AO proteins after SDS-PAGE of different parts of maize nodal roots, with polyclonal antibodies raised against maize AO protein

(Koshiba et al.1996), revealed a major band (a) of about 150 kD in extracts of root tips and stele, and two minor polypeptides (band b and c) with molecular masses of about 72 and 85 kD in the cortex (Figure 19).

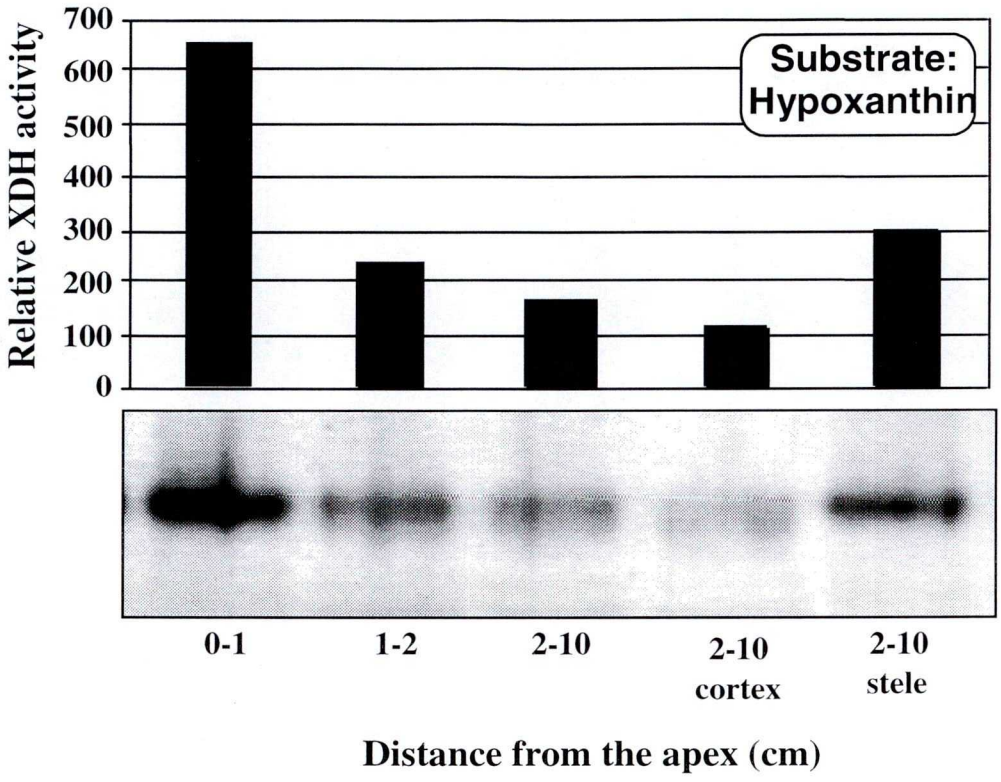

Figure 18: Zymogram of XDH activity in different zones of maize nodal roots grown in 4 mM NH_4^+ and 50 mM NaCl. Crude protein supernatants (80 µg/lane) were subjected to native-PAGE, followed by staining for XDH activity with hypoxanthine as substrate. Band intensities were expressed in relative units, after scanning with a personal laser densitometer and evaluated by Image Quant. Program. The zymogram represents one of at least three experiments with similar results.

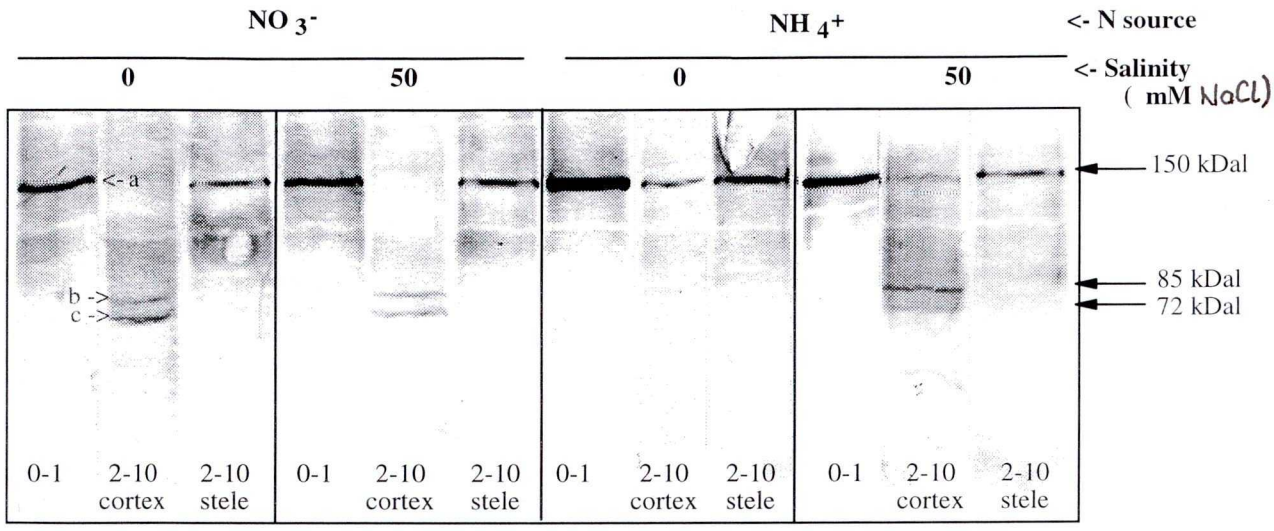

Figure 19: Immunoblot analysis of AO proteins after SDS-PAGE. Crude extract samples of different parts of maize nodal roots (tips: 0-1 cm; cortex and stele from mature zone: 2-10 cm from the apex) grown with NO_3^- or NH_4^+ as nitrogen source, with or without salinity (50 mM NaCl) treatment. About 40 µg total soluble protein was loaded in each lane. The results represent one of at least three different experiments with similar results.

Western blots revealed enhanced levels of 150 kD polypeptide in extracts of root tips of ammonium-grown plants. In the cortex, the 150 kD polypeptide was observed only in plants receiving ammonium as their nitrogen source (Figure 19). The amount of AO protein varied among different tissues and under different growth conditions. The increment of AO activity in ammonium fed plants (Figure 15A) is in agreement with the accumulation of AO protein in the root tips of ammonium fed plants (Figure 19). The enhancement of AO activity under salinity treatment of nitrate fed plants (Figure 15A), was not accompanied by increased levels of AO protein in the tips (Figure 19) and seems to be related to activation of existing enzyme molecules.

4.2.4 Correlation of substrate and tissue specificity for AO activity

The four bands (AO1-4) of AO activity observed, could be detected with different substrates (Figure 20). AO3 band was observed preferentially in stele of roots while AO1 and AO2 exhibited mere traces. When acetaldehyde was used as substrate, the root tip was the richest in total AO activity, while the second cm and the mature part of the root did not show significant differences. On the transversal axis, AO was located mainly in the stele (Figure 20A). Benzaldehyde uncovered the highest AO activity in the tip (0 to 1 cm) and mature part as compared to the second cm of the root, and in the mature section it was significantly higher in stele than in cortex (Figure 20B).

AO supplied with heptaldehyde, did not show significant differences along longitudinal axis of the root, although it was slightly higher in the mature zone, where most of the enzyme activity resulted from AO4. The central cylinder had significantly higher total AO activity than the cortex, resulting mainly from the AO3 band (Figure 20C). No significant differences were found in the total AO activity on the longitudinal axis, when indole-3-aldehyde was supplied as the substrate. On the transversal axis, even if AO3 was slightly higher in the stele, the total AO activity did not show differences between cortex and stele (Figure 20D).

4.2.5 Possible involvement of Mo-enzymes in plant adaptation to changing environmental conditions

AO activity in maize nodal roots increased under salinity treatment and when ammonium was the sole nitrogen source (Figure 15A). Similar responses were reported recently for barley (Omarov et al., 1998) and ryegrass (Sagi et al., 1998b). After native PAGE and staining with indole-3-aldehyde for AO activity, four bands or isoforms of AO where detected in maize nodal roots (Figure 15A) with the strongest activities produced by the AO3 and AO4 bands. Salinity and ammonium enhanced activity of AO (Figure 15A) although in different ways: ammonium

increased the level of AO protein while salinity seemed to activate pre-existing enzyme proteins.

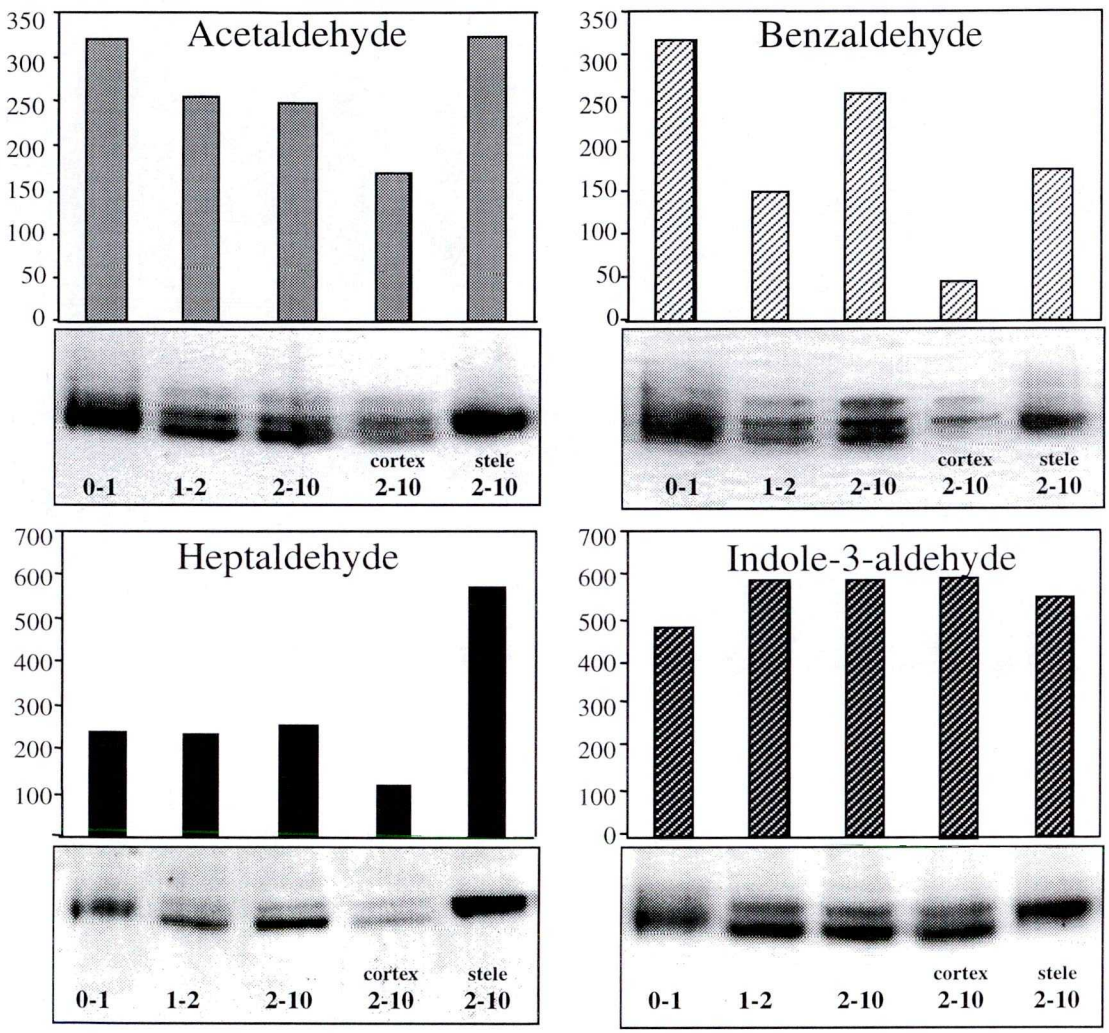

Figure 20: AO activity in different zones of maize nodal roots grown in 4 mM NH_4^+ and 50 mM NaCl. Crude protein supernatants (80 µg) were subjected to native-PAGE, followed by staining for AO activity with acetaldehyde, benzaldehyde, heptaldehyde and indole-3-aldehyde as substrates. Band intensities were expressed in relative units, after scanning with a personal laser densitometer and evaluated by Image Quant. Program.

ABA level increased in plant tissues (Peuke et al., 1994; Omarov et al., 1999) under the same conditions. This correlation supports the possible role of AO3 and AO4 in ABA biosynthesis in maize nodal roots. The centers of AO activity, located in tips and the stele of roots, fits the two known requirements of ABA in developing roots: (a) The root tip seems to require ABA for sustain elongation growth (Saab et al., 1992) and (b) Synthesis of ABA in the stele of mature root zones may be related to the xylem parenchyma cells responsible for xylem loading of compounds transported with the xylem sap.

To which extent different zones of the maize nodal root contribute to the overall pattern of Mo-enzymes activities? Is there any tissue specificity of the four AO isoforms?

Tips and central cylinder of maize roots had a significantly higher level of total AO activity, especially observed when using acetaldehyde and benzaldehyde as substrates (Figures 17 and 20A,B). Maize root tips were found to accumulate more ABA in response to dehydration than mature root sections (Zhang and Tardieu, 1996), which is in agreement with our results showing high AO activity in the root tips. A significant contribution of the stele to the total AO activity in mature zones of the root has been observed in the present study. AO activity is underestimated in stele, since the xylem vessels account for a large fraction of the tissue volume (6-9%) (data not shown) and fresh weight. It can be assumed that AO activity in stele may be located mainly in the xylem parenchyma cells. These cells control the composition of the xylem sap by loading specific solutes onto the xylem vessels. It has been observed that under ammonium nutrition and salinity, xylem loading preferences change from nitrate to organic nitrogen compounds (Cramer et al, 1995).

Activity staining of root AO after native PAGE obtained from NH_4^+ and NaCl grown plants, revealed that the isoforms AO3 and AO4 were the main contributors to total AO activity, and that these bands were localized mainly in the tips and the stele (Figure 17). The difference in mobility during native PAGE of AO3 in tips and stele of roots, may be the result of partial proteolitic degradation of the active enzyme molecule, either during extraction, or due to some specific regulatory

mechanisms as observed in barley roots (Omarov et al, 1999). Tips and stele tissues contain a large number of small, protein rich, unvacuolated cells, while the cortex is made up mainly of large, vacuolated parenchyma cells. Immunoblot analysis of AO proteins after SDS-PAGE revealed in cortex two small polypeptides (band b and c) with molecular masses of about 72 and 85 kD, (Figure 19). These polypeptides, which appear in NO_3^- grown plants or plants exposed to salinity, may be residues of a break up of the main AO 150 kD monomer, two of which are required for the active AO enzyme. Similar minor AO bands were detected in maize coleoptiles and roots (Koshiba et al., 1996), tomato (Ori et al., 1997) and following AO digestion with trypsin (Omarov and Lips, personal communication).

The increased activity of AO in roots may be the result of enhanced gene expression of the AO apoprotein resulting in increased levels of AO observed in ammonium-grown plants but not in nitrate-grown plants. Salinity may induce posttranslational activation of the existing enzyme molecules as observed in tomato genotypes (Sagi et al., unpublished). Enhanced AO activity in ammonium fed plants (Figure 15A) corresponded to higher levels of AO protein in the root tips (Figure 19) suggesting a regulatory role of NH_4^+ ions as a signal for *de novo* synthesis of AO proteins in root tips and xylem parenchyma cells. On the other hand, the enhancement of AO activity by salinity of nitrate fed plants (Figure 15A), which was not supported by an increase of AO protein in neither tips nor stele (Figure 19), points to a possible post translational regulation effect of the type observed for NR (Kaiser and Huber, 1996; Bachmann et al., 1996; Moorhead at al., 1996). Protein phosphorylation has been shown to be the mechanism mediating the posttranscriptional regulation of NR in response to light and CO_2 in spinach (Labrie and Crawford, 1994). The increase of AO activity in maize nodal roots under salinity (Figure 19) could be attributed to increased activity of the Mo-hydroxylase sulfurylase required for the addition of sulfur which activates AO and XDH (Rajagopalan and Johnson 1992; Sagi and Lips, unpublished). In ryegrass roots, immunoblot analysis of AO proteins made after native-PAGE, showed higher AO band densities in root extracts of plants grown in the presence of salinity and ammonium than in nitrate fed plants (Sagi et al., 1998b).

XDH activity was enhanced by salinity and ammonium (Figure 15B).

Increase of XDH under similar conditions was previously reported in ryegrass where the enhancement correlated with a higher content of ureides. It was suggested that the role of XDH during stress may be related to the need for a more efficient use of available carbon skeletons to assimilate ammonium into organic nitrogen compounds with a much lower C/N ratio for their transport through the xylem to the shoot (Sagi et al., 1998b). In pea leaves, XDH activity increased sharply in parallel to superoxide dismutase and other oxygen related enzymes during senescence (Pastori and del Rio, 1997). Enhancement of XDH in maize nodal roots of plants grown with ammonium correlated with the increment of some antioxidant enzymes (SOD, GR) (Barabás et al., 1999a). XDH may be involved in the defense of plant tissues against oxidative stress induced by salinity, most probably through its product uric acid, which is an effective scavenger of active oxygen species in many organisms (Becker et al., 1989; Radi et al., 1990).

NR activity in nodal roots of nitrate fed plants increased with salinity, when calculated either on a fresh weight or protein basis, and this increment was more evident in the root tip and in stele (Figure 16A,B). NR activity increased in tomato (Gao et al., 1996) and ryegrass (Sagi et al., 1998a) roots, while it decreased in barley (Omarov et al., 1998) roots exposed to salinity. Along the longitudinal axis of maize nodal root, NR was found to be located mainly in the tips (0-1 cm). Higher NR activity was reported in root tips than in the older segments of carob (Cruz et al., 1995), and maize seedlings (Wallace, 1973).

NR activity was higher in the stele (Figure 16) than in cortex in the present study, perhaps suggesting that most of the nitrate reaching the stele did not dot transverse the cortex symplast, inducing NR along the way, but moved through the cortex apoplast bypassing the cortex parenchyma cells.

The distribution pattern of Mo-enzymes seems to be related to their physiological functions on the various zones of the root. The tip is the site of root apical meristem with actively dividing cells, essential for root growth. The root tip seems to cater for its own organic nitrogen needs, independently from the main plant body, by inducing high levels of nitrogen assimilation enzymes whose products (amino acids) are used on the spot and not exported to the shoot. The apical portion of the primary maize root has been considered a sink for concurrently absorbed N

(Lazof et al., 1992). The second center of AO activity was the vascular cylinder and most probably the xylem parenchyma cells, which control the xylem loading and composition.

Mo-enzymes were not homogeneously distributed along the longitudinal and transversal axes of the maize nodal roots, but were preferentially located in the tips and in the stele mature root zones. Stress (salinity) and ammonium ions, affected Mo-enzymes in the different root zones. This diversity of AO, XDH and NR distribution may be tightly related with specific metabolic functions of root zones and tissues and to the involvement of Mo-enzymes in the adaptation of plants under changing environmental conditions.

5. CONCLUSIONS

5.1. Effects of excess UV-B radiation on the antioxidant defense mechanisms in wheat seedlings

During UV-B irradiation, the activities of the investigated antioxidant enzymes, after reaching a transitional minimum value, caught up with the control level, i.e. acclimatisation had occured. The predominant component among them was the glutathione system, which, being one of the most powerful antioxidant in plants, brought about an intensive "detoxification" and remediation of the cellular environment.

Catalase, guaiacol proxidase and superoxide dismutase activities decreased, while GSSG level and glutathione reductase activity increased under UV-B irradiation.

The responses indicated two main phases in the adaptation process to UV-B irradiation in young wheat plants. In terms of the Selyean stress concept (Selye, 1956) these phases can be considered as parts of the general adaptation syndrome (GAS):

1 - the alarm reaction is represented by the inhibition of stem and leaf elongation, accumulation of flavonoid pigments, decrease in antioxidant enzyme activities, increased GSSG level and triggering of GR activity,

2 - normalisation of growth, disappearance of stem pigmentation, levelling of antioxidant enzyme activities, high GR activity and decreased level of GSSG were found as parts of adaptation to UV-B irradiation.

Synthesis of flavonoid pigments during the alarm phase is a *syntoxic* element that contributes to the increase in tolerance, while the increase in the levels and/or activities of antioxidants can be regarded as *catatoxic* elements, which actively eliminate the oxidative component of the stressor (Leshem and Kuiper, 1996).

In general, moderate stress caused by the excess UV-B irradiation in the present study can be categorised as *eu-stress* (Lichtenthaler, 1996), which is a stimulating stress that activates the cell metabolism and increase resistance or

acclimatisation.

Moderate increase in UV-B irradiation due to the present depletion of ozone in the stratosphere, at the surface do not bring about fatal damage in plant (at least in wheat) repair mechanisms, metabolism and growth.

5.2 Effects of salinity treatment on the changes of rhythmic behavior of superoxide dismutase in wheat seedlings

The activity of SOD decreased under NaCl salinity (0, 50, 100 and 150 mM) treatment in young winter wheat.

SOD activity showed a circadian rhythm when followed as the function of time, in both control and treated seedlings. Fourier analysis disclosed an increase in the amplitude of the circadian period fraction of SOD activity in the 150 mM NaCl treated samples.

The oscillation of SOD activity in the roots of wheat seedlings were modulated by the stressor (NaCl) suggesting a connection between environmental stress and biological rythmicity:

i) antioxidant enzyme activity (SOD) fluctuate with circadian and ultradian periodicities;

ii) salinity treatment influenced the amplitude of oscillations and left the circadian period unchanged; and

iii) ultradian fluctuations proved to be sensitive to stress treatment.

On the basis of these observations and literature data it can be hypothesized that the stressor may modify and desynchronize the biochemical and physiological oscillations by acting either on the input (from receptor till oscillator) or on the output (from the oscillator till the oscillating component) pathways. In these terms, *adaptation* means re-setting of the new circadian and/or ultradian rhythms of biochemical/physiological processes and the restoration of their concerted action results in maximal resistance for the organism under the new environmental conditions.

5.3 Molibdoenzymes: effects of salinity and nitrogen ions on the level and distribution of aldehyde oxidase, xanthine dehydrogenase and nitrate reductase

Mo-enzymes activities in maize nodal roots were considerably higher under mild saline conditions and activities of the Mo-hydroxylases (AO and XDH) were enhanced by ammonium as nitrogen source.

Four bands or isoforms of AO (AO1-4) were detected after native PAGE with indole-3-aldehyde in maize roots showing the strongest activities in the AO3 and AO4 bands. Salinity and ammonium enhanced activity of AO3 and AO4 in different ways: ammonium increased the level of AO protein while salinity seemed to activate preexisting enzyme molecules. Since ABA level increased in plant tissue under the same conditions, it can be concluded the possible role of AO3 and AO4 in ABA biosynthesis in maize nodal roots.

Mo-enzymes were not homogeneously distributed along the longitudinal and transversal axes of the maize nodal roots, but were mainly located in the tips and in the stele of mature root zones. The location of the centers of AO activity in the tips and the stele fits two needs of the developing root: (a) The root tip seems to require ABA for sustain elongation growth (Saab et al., 1992) and (b) Synthesis of ABA in the stele of mature root zones may be related to the xylem parenchyma cells from which the hormone can be rapidly transported to the shoot via the xylem vessels.

Stress (salinity) and ammonium ions, affected Mo-enzymes in the different root zones. Immunoblot analysis with antibodies raised against AO protein revealed increased level of AO protein in root tips of ammonium fed plants while salinity treatment of nitrate fed plants did not affect the enzyme protein level, in spite of the salinity-enhanced activity of these enzymes. Therefore, salinity seems to activate latent or down-regulated enzymes.

SDS-PAGE followed by immunoblotting, revealed besides the major 150 kD subunit of AO, two polypeptides with molecular masses of 72 and 85 kD located specifically in the cortex.

This diversity of AO, XDH and NR distribution may be tightly related with specific metabolic functions of root zones and tissues and to the involvement of Mo-enzymes in the adaptation of plants under changing environmental conditions.

6. SUMMARY

Due to the continuous depletion of the stratospheric ozone (O_3) layer there is an increase of UV-B radiation reaching the Earth's surface. Therefore, changes in the antioxidant defense systems as affected by excess UV-B irradiation were investigated. An extensive problem in agriculture is the accumulation of salts from irrigation water, about one-third of the irrigated land on Earth is affected by salt. In this view, the influence of salinity (NaCl) on rhythmic phenomena, particularly on the activity of superoxide dismutase (SOD) in plants was examined. Nitrate and ammonium ions are important mineral nutrients, which serve also as signals, which modulate the level and the activity of several key enzymes. Hence, the level and distribution of Mo-enzymes aldehyde oxidase (AO), xanthine dehydrogenase (XDH) and nitrate reductase (NR) as affected by nitrogen ions and salinity were studied.

Hydroponically grown wheat (*Triticum aestivum* L., cv. Tiszatáj) and maize (*Zea mays* L., cv. Jubily) were used as experimental material. Changes in the antioxidative enzymes (catalase, guaiacol peroxidase, SOD, glutathione reductase-GR) and NR activities were followed spectrophotometrically. The level of glutathione disulfide (GSSG) was determined by HPLC technique. The periodicity was calculated by Fourier analysis. Mo-enzymes (AO and XDH) were detected after native polyacrylamide gel-electrophoresis. Quantitative analyses were made by scanning the formazane bands in the gel, with a computing laser densitometer (Molecular Dynamic) using Image Quant version 3.19.4 and NIH Image 1.6. The level of AO protein was investigated by immunoblot analysis with antibodies raised against AO protein.

It was found that catalase, guaiacol peroxidase and SOD activities decreased, while GSSG level and GR activity increased under excess of UV-B irradiation. In plant responses, the alarm and the adaptation phases were recognised. During the alarm reaction, inhibition of stem and leaf elongation, accumulation of flavonoid pigments, decrease in antioxidant enzyme activities, increased GSSG level and triggering of GR activity were observed, while normalisation of growth, disappearance of stem pigmentation, levelling of antioxidant enzyme activities, high GR activity and decreased level of GSSG were found as parts of adaptation to UV-B

irradiation. Synthesis of flavonoid pigments during the alarm phase is a *syntoxic* element that contributes to the increase in tolerance, while the increase in the levels and/or activities of antioxidants can be regarded as *catatoxic* elements, which actively eliminate the oxidative component of the stressor (Leshem and Kuiper, 1996). In general, moderate stress caused by the excess UV-B irradiation in the present study can be categorised as *eu-stress* (Lichtenthaler, 1996), which is a stimulating stress that activates the cell metabolism and increase resistance or acclimatisation. Moderate increase in UV-B irradiation due to the present depletion of ozone in the stratosphere, at the surface do not bring about fatal damage in plant (at least in wheat) repair mechanisms, metabolism and growth.

Salinity influenced the rhythmic behavior of SOD activity in the leaves of wheat seedlings. The analysis disclosed an increase in the amplitude of the circadian period fraction in the 150 mM NaCl-treated sample. Additionally, an ultradian rhythm of about 13 hours, visible in control plants, disappeared in salt treated series. It could be concluded that: i) SOD activity fluctuated with circadian and ultradian periodicities; ii) salinity treatment influenced the amplitude of oscillations and left the circadian period unchanged; iii) ultradian fluctuations proved to be sensitive to stress treatment. It was hypothesized that stress responses in plants, as described by the stress concept of Selye and adopted in plant physiology, can be evoked by the modification and desynchronization of oscillations of biochemical and physiological processes resulting in a decreased state of resistance.

Mo-enzymes activities were considerably higher under mild saline conditions. Activities of the Mo-hydroxylases (AO and XDH) were enhanced by ammonium in the nutrient solution. Immunoblot analysis with antibodies raised against AO protein revealed increased level of AO protein in root tips of ammonium fed plants while salinity treatment of nitrate fed plants did not affect the enzyme protein level, in spite of the salinity-enhanced activity of these enzymes. Therefore, salinity seems to activate latent or down-regulated enzymes. Mo-enzymes specific activity (activity mg^{-1} protein min^{-1}) was highest in root tips (0-1 cm segments) while on the transversal axis maximal activity was observed in the stele or vascular cylinder. The location of the centers of AO activity in the tips and the stele fits two

needs of the developing root: (a) The root tip seems to require ABA for sustain elongation growth (Saab et al., 1992) and (b) Synthesis of ABA in the stele of mature root zones may be related to the xylem parenchyma cells from which the hormone can be rapidly transported to the shoot via the xylem vessels. Activity staining of AO after native PAGE separations of root extracts revealed four bands of AO reacting proteins (AO1-4) capable of oxidizing a number of aliphatic and aromatic aldehydes. Total AO activity in maize nodal roots grown with ammonium and salinity was given mainly by AO3 and AO4 bands. Tips and stele contained mainly AO3 and only traces of AO1 and AO2. SDS-PAGE followed by immunoblotting, revealed besides the major 150 kD subunit of AO, two polypeptides with molecular masses of 72 and 85 kD located specifically in the cortex. Part of the polymorphism of AO in plant roots may be related to the allocation of isoforms to defined regions of the root, although defined metabolic roles of the different bands could not be established at this stage.

Stress (salinity) and ammonium ions, affected Mo-enzymes in the different root zones. This diversity of AO, XDH and NR distribution may be tightly related with specific functions of root zones and tissues and to the involvement of Mo-enzymes in the adaptation of plants under changing environmental conditions.

7. REFERENCES

ADAMSE, P. and S.J. BRITZ: Rapid fluence-dependent responses to ultraviolet-B radiation in cucumber leaves: The role of UV-absorbing pigments in damage protection. J. Plant Physiol. *148*, 57-62, (1996).

AKABA, S., N. KAWAKAMI, M.T. LEYDECKER, T. MOUREAUX, and T. KOSHIBA: Purification and properties of aldehyde oxidase from Nicotiana plumbaginifolia. Plant Physiol. *114S*, 153, (1997).

ALLEN, J.F.: A two step mechanism for the photosynthetic reduction of oxygen by ferredoxin. Biochem. Biophys. Res. Commun. *66*, 36-43, (1975).

ANDERSON, J.W., C.H. FOYER, and D.A. WALKER: Light dependent reduction of hydrogen peroxid by intact spinach chloroplast. Biochim. Biophys. Acta, *724*, 69-74, (1983).

ASADA, K. and M. TAKAHASHI: Production and scavenging of active oxygen in photosynthesis. In: D.J. KYLE, C.B. OSMOND, and C.J. ARNTZEN, (eds.), Photoinhibition: Topics in Photosynthesis. Vol. 9., pp. 227-287. Elsevier, Amsterdam. ISBN 0-444-80890-6, (1987).

ASADA, K.: Ascorbate proxidase – hydrogen peroxide scavenging enzyme in plants. Physiol. Plant. *85*, 235, (1992).

ASADA, K.: Production and action of active oxygen species in photosynthetic tissue. In: C.H. FOYER, P.M. MULLINEAUX, (eds.), Causes of photooxidative stress and amelioration of defence systems in plants, pp. 77-104. CRC Press, Boca Raton, FL, (1994).

BACHMANN, M., N. SHIRAISHI, W.H. CAMPBELL, B.B. YOO, A.C. HARMON, and S.C. HUBER: Identification of Ser-453 as the major regulatory phosphorylation site in spinach leaf nitrate reductase. The Plant Cell *8*, 505-17, (1996).

BALLARÉ, C.L., A.L. SCOPEL, A.E. STAPLETON, and M.J. YANOVSKY: Solar ultraviolet-B radiation affects seedling emergence, DNA integrity, plant morphology, growth rate and attractiveness to herbivore insects in *Datura ferox*. Plant Physiol. *112*, 161-170, (1996).

BANDURSKI, R.S., J.D. COHEN, J. SLOVIN, and D.M. REINECKE: Auxin biosynthesis and metabolism. In: P.J. Davies (ed.) Plant Hormones. Physiology, Biochemistry and Molecular Biology, Kluwer Academic Dordrecht, Netherland, pp. 39-65, (1995).

BANO, A., K. DÖRFFLING, D. BETTIN, and H. HAHN: Abscisic acid and cytokinins as possible root-to-shoot signals in xylem sap of rice plants in drying soil. Austr. J., Plant Physiol. *20*, 109-15, (1993).

BARABÁS, N.K. and L. ERDEI: Enzyme assays: catalase, peroxidase and superoxide dismutase in sunflower (*Helianthus annus* L.) under salt, osmotic and heavy metal stresses. Biol. Plant. *36S*, 259, (1994).

BARABÁS, N.K., ZS. SZEGLETES, A. PESTENÁCZ, K. FÜLÖP, and L. ERDEI: Effects of excess UV-B irradiation on the antioxidant defence mechanisms in wheat (*Triticum aestivum* L.) seedlings. J. Plant Physiol. *153*, 146-153, (1998).

BARABÁS, N.K., K. FÜLÖP, R.T. OMAROV, L. ERDEI, and S.H. LIPS: The effect of nitrogen source and salinity on the spatial distribution of glutathione reductase and Mo-enzymes in maize (*Zea mays* L.) nodal roots. In: Nitrogen in a sustainable ecosystem from the cell to the plant. Kluwer Acad. Publ., Amsterdam (in press) (1999a).

BARABÁS, N.K, R.T. OMAROV, L. ERDEI and S. H. LIPS: Distribution of the Mo-enzymes aldehyde oxidase, xanthin dehydrogenase and nitrate reductase in maize (*Zea mays* L.) nodal roots as affected by nitrogen and salinity. J.Exp.Bot. (submitted) (1999b).

BARTOSZ, G.: Oxidative sress in plants. Acta Physiol. Plant. 19(1), 47-64, (1997).

BEAUCHAMP, C. and I. FRIDOVICH: Superoxide dismutase: improved assays and an assay applicable to acrylamide gel. Anal. Biochem. *44*, 276-287, (1971).

BECKER, B.F., N. REINHOLZ, T. OZCELIK, B. LEIPERT, and E. GERLACH: Uric acid as radical scavenger and antioxidant in the heart. Pflug. Arch. Eur. J. Physiol. *415*,127-135, (1989).

BIELAWSKI, W. and K.W. JOY: Properties of glutathione from chloroplast and root of pea: Phytochem. *25*, 2261-2265, (1986).

BORNMAN, J.F. and A.H. TERAMURA: Effects of ultraviolet-B radiation on terrestrial plants. In: A.R.YOUNG, L.O. BJÖRN, J. MOAN, and W. NULTSCH (eds.), Environmental UV Photobiology, pp. 427-471. Plenum Press, New York (1993).

BOWLER, C., M. VAN MONTAGU, and D. INZÉ: Superoxide dismutase and stress tolerance. Ann. Rev. Plant Physiol. Plant Mol. Biol., *43*, 83-116, (1992).

BRADFORD, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248-54, (1976).

CABELLI, D.E. and B.H.J. BIELSKI: Kinetics and mechanism for the oxidation of ascorbic acid/ascorbate by $^{\bullet}O_2^-$ radicals, A pulse radiolysis and stopped-flow photolysis study. J. Phys. Chem. *87*, 1809-1812, (1983).

CALDWELL, M.M. and S.D. FLINT: Solar ultraviolet radiation and ozone layer change: implication for crop plants. In: K.J. BOOTE, J.M. BENNETT, T.R. SINCLAIR and G.M. PAULSON (eds), Physiology and Determination of Crop Yield, pp. 487-507. American Society of Agronomy, Madison, WI (1994).

CALDWELL, M.M., A.H. TERAMURA, M. TEVINI, J.F. BORNMAN, L.O. BJÖRN, and G. KULANDAIVELU: Effects of increased solar ultraviolet radiation on terrestrial plants. Ambio *24*, 166-173, (1995).

CAMPBELL, W.H.: Nitrate reductase and its role in nitrate assimilation in plants. Physiol. Plant. *74*, 214-219, (1988).

CHAPPELL, J. and K. HAHLBROCK: Transcription of plant defence genes in response to UV light or fungal elicitor. Nature *311*, 76-78, (1984).

CHEESEMAN, J.M.: Mechanisms of salinity tolerance in plants. Plant Physiol. *87*, 547-550, (1988).

COLEPICOLO, P., V.C.P.C. CAMARERO, and J.W. HASTINGS: A circadian rhythm in the activity of superoxide dismutase in the photosynthetic alga *Gonyaulax polyedra*. Chronobiol. Int., *9*, 266-268, (1991).

CORPAS F.J., M. GOMEZ, J.A. HERNÁNDEZ, and L.A. DEL RIO: Metabolism of activated oxygen in peroxisomes from two *Pisum sativum* L. cultivars with different sensitivity to sodium chloride. J. Plant Physiol. *141*, 160-165, (1993).

CRAMER, M.D., A. SCHIERHOLT, Y.Z. WANG and S.H. LIPS: The influence of salinity on the utilization of root anaplerotic carbon and nitrogen metabolism in tomato seedlings. J. Exp. Bot. *46*, 1569-1577, (1995).

CREISSEN, G.P., A. EDWARDS, M. VAN MONTAGU, and D. INZÉ: Glutathione reductase and ascorbate peroxidase. In C.H. Foyer, P.M. Mullineaux, eds, Causes of Photooxidative Stress and Amelioration of Defence Msystems in Plants. CRC Press, Boca Raton, FL, pp. 343-364, (1994).

CRUZ, C., S.H. LIPS, and M.A. MARTINS-LOUÇÃO: Uptake regions of inorganic nitrogen in roots of carob seedlings. Physiol. Plant. *95*, 167-175, (1995).

DHAKAL, M.R and L. ERDEI: Long-term effects of abscisic acid on K^+ transport in young wheat plants of different K^+ status. Physiol. Plant. *68*, 637-640, (1986).

DHINDSA, R.S., P. PLUMB-DHINDSA, and T.A. THORPE: Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. *32*, 93-101, (1981).

DUMPERT, K. and T. KNACKER: A comparison of the effects of enhanced UV-B radiation on some crop plants exposed to greenhouse and field conditions. Biochem. Physiol. Pflanzen *180*, 599-612, (1985).

ELSTNER E.F.: Der Sauerstoff: Biochemie, Biologie, Medizin. – BI Wissenschaftverlag, Mannheim (1990).

ERDEI, L., Z. OLÁH, and A. BÉRCZI: Phases in potassium transport and their regulation under near-equilibrium conditions in wheat seedlings. Physiol. Plant. *60*, 81-85, (1984).

ERDEI, L. and E. TALEISNIK: Changes in water relation parameters under osmotic and salt stresses in maize and sorghum. Physiol. Plant. *89*, 381-387, (1993).

ERDEI, L., A. PESTENÁCZ, N.K. BARABÁS, and ZS. SZEGLETES: Adaptive responses of plants under stress conditions. Acta Phytopath. Entomol. Hung. *30*, 27-37, (1995).

ERDEI, L., ZS. SZEGLETES, N.K. BARABÁS, and A. PESTENÁCZ: Responses in polyamine titer under osmotic and salt stress in sorghum and maize seedlings. J. Plant Physiol. *147*, 599-603, (1996a).

ERDEI, L., ZS. SZEGLETES, N.K. BARABÁS, A. PESTENÁCZ, A. KOVÁCS, and B. TÓTH: Effects of environmental stress factors on the circadian rhythm in wheat. Plant Physiol. Biochem. (special issue) p. 267, (1996b).

ERDEI, L., ZS. SZEGLETES, N.K. BARABÁS, A. PESTENÁCZ, K. FÜLÖP, L. KALMÁR, A. KOVÁCS, B. TÓTH, and A. DÉR: Environmental stress and the biological clock in plants: changes of rhythmic behavior of carbohydrates, antioxidant enzymes and stomatal resistance by salinity. J. Plant Physiol. *152*, 265-271, (1998).

FARRIS, M.V. and D.J. REED: High-performance liquid chromatography of thiols and disulfides: Dinitrophenol derivatives. Meth. Enzymol. *143*, 101-109, (1987).

FEDEROVA, E, J.S. GREENWOOD, and A. OAKS: In-situ localization of nitrate reductase in maize roots. Planta *194*, 279-286, (1994).

FOYER, C.H. and B. HALLIWELL: The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta *133*, 21-25, (1976).

FOYER, C.H. and P.M. MULLINEAUX (Eds): Causes of photooxidative stress and amelioration of defense systems in plants. Boca Raton: CRC Press (1994).

FOYER, C., M. LELANDAIS, C. GALAP, and K.J. KUNERT: Effects of elevated cytosolic glutathione reductase activity on the cellular glutathione pool and photosynthesis in leaves under normal and stress conditions. Plant Physiol. *97*, 863-872, (1991).

FOYER, C.H., P. DESCOURVIERES, and K.J. KUNERT: Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. Plant Cell Environ. *17*, 507-523, (1994).

GAO, Z., M. SAGI, and S.H. LIPS: Assimilate allocation priority as affected by nitrogen compounds in the xylem sap of tomato. Plant Physiol. Biochem. *34(6)*, 807-815, (1996).

GOLDEN, S.S., M. ISHIURA, C.H. JOHNSON, and T. KONDO: Cyanobacterial circadian rhythms. Ann. Rev. Plant Physiol. Plant Mol. Biol. *48*, 327-354, (1997).

GREENWAY, H., and R. MUNNS: Mechanisms of salt tolerance in nonhalophytes. Ann. Rev. Plant Physiol. *31*, 149-190, (1980).

HALL, J.C.: Tripping along the trail to the molecular mechanisms of biological clocks. Trends Neurosci. *18,* 230-240, (1995).

HARPER, J.F., M.R. SUSSMAN, G.E. SCHALLER, C.L. PUTNAM-EVANS, H. CHARBONNEAU, and A. HARMON: A calcium-dependent protein kinase with a regulatory domain similar to calmodulin. Science *252,* 951-954, (1991).

HERPPICH, M., D.J. VON WILLERT, and W.B. HERPPICH: Diurnal rhythm in citric acid content preceded the onset of nighttime malic acid accumulation during metabolic changes from C_3 to CAM in salt-stressed plants of *Mesembryanthemum crystallinum.* J. Plant Physiol. *147,* 38-42, (1995).

HIDEG, É. and I. VASS: UV-B induced free radical production in plant leaves and isolated thylakoid membranes. Plant Sci. *115,* 251-260, (1996).

HOAGLAND, D.R. and D.I. Arnon: The water culture method for growing plants without soil. California Agricultural Experimental Station Circular *347,* 1-32, (1938).

HUNTER, T. and M. KARIN: The regulation of transcription by phosphorylation. Cell *70,* 375-387, (1992).

INZÉ, D. and M. VAN MONTAGU: Oxidative stress in plants. Current Opinion Biotech. *6,* 153-158, (1995).

JOLIVET, Y., J.C. PIREAUX, and P. DIZENGREMEL: Changes in properties of barley leaf mitochondria isolated from NaCl-treated plants. Plant Physiol. *94,* 641-646, (1990).

KAISER, W.M. and S.C. HUBER: Correlation between apparent activation state of nitrate reductase (NR), NR hysteresis and degradation of NR protein. J. Exp. Bot. *48,* 1367-1374, (1996).

KARPINSKI, S., B. KARPINSKA, G. WINGSLE, and J.E. HALLGREN: Molecular responses to photooxidative stress in *Pinus sylvestris.* I. Differential expression of nuclear and plastid genes in relation to recovery from winter stress. Physiol. Plant. *90,* 358-366, (1994).

KAYUPOVA, G.A., and L.K. KLYSHEV: Effect of chloride salinization on some intracellular processes in pea roots. Vestn. Akad. Nauk. Kaz. SSR *4,* 67-71 (1987).

KEFU, Z.H., R. MUNNS and R.W. KING: Abscisic acid levels in NaCl-treated barley, cotton and saltbush. Austr. J. Plant Physiol. *18*, 17-24, (1991).

KLAPCHEK, S., C. LATUS, and L. BERGMANN: Localization of glutathione syntetase and distribution of glutathione in leaf cells of *Pisum sativm* L. J.Plant Physiol. *131*, 123-131, (1987).

KOCSY, G., G. OWTTRIM, K. BRANDER, and CH. BRUNOLD: Effects of chilling on the diurnal rhythm of enzymes involved in protection against oxidative stress in a chilling-tolerant and a chilling-sensitive maize genotype. Physiol. Plant. *99*, 249-254, (1997).

KOSHIBA, T., E. SAITO, N. ONO, N. YAMAMOTO, and M. SATO: Purification and properties of flavin - and molybdenum-containing aldehyde oxidase from coleoptiles of maize. Plant Physiol. *110*, 781-89, (1996).

KOUKKARI, W.L., C. BINGHAM, J.D. HOBBS, and S.H. DUKE: In search of a biological hour. J. Plant Physiol. *151*, 352-357, (1997).

KRIZEK, D.T., G.F. KRAMER, A. UPADHYAYA, and R.M. MIRECKI: UV-B response of cucumber seedlings grown under metal halide and high pressure sodium/deluxe lamps. Physiol. Plant. *88*, 350-358, (1993).

LABRIE, S.T. and N.M. Crawford: A glycine to aspartic acid change in the MoCo dómain of nitrate reductase reduces both activity and phosphorilation levels in Arabidopsis. J. Biol. Chem. *269(20)*, 14497-14501, (1994).

LAEMMLI, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-85, (1970).

LAMBERS, H.: Respiration in intact plant tissue. Its regulation and dependence on environmental factors, metabolism and invaded organisms. In: DOUCE R. and D.A. DAYS (eds.): Encyclopedia of Plant Physiology, vol. 18, pp. 418-473, Springer-Verlag, Berlin (1985).

LARCHER, W.: Streß bei Pflanzen. Naturwissenschaften *74*, 158-167, (1987).

LAZAROW P.B. and Y. FUJIKI: Biogenesis of peroxisomes. Ann. Rev. Cll Biol. *1*, 489-530, (1985)

LAZOF, D.B., T.W. RUFTY, and M.G. REDINBAUGH: Localization of nitrate absorbtion and translocation within morphological regions of the corn root. Plant. Physiol. *100*, 1251-1258, (1992).

LEPORT, L., A. KANDLBINDER, B.BAUR, and W.M. KAISER: Diurnal modulation of phosphoenolpyruvate carboxylation in pea leaves and roots related to tissue malate concentrations and to the nitrogen source. Planta *198*, 495-501, (1996).

LESHEM, Y.Y., P.J.C. KUIPER, L. ERDEI, S. LURIE, and R. PERL-TREVES: Do Selye's mammalian "GAS" concept and "Co-stress" response exist in plants? Stress of Life: From Molecules to Man. Annals of the New York Academy of Sciences, Vol. 851, pp. 199-208, New York (1998).

LESHEM, Y.Y. and P.J.C. KUIPER: Is there a GAS (general adaptation syndrome) response to various types of environmental stress? Biol. Plant. *38*, 1-18, (1996).

LEVITT, J.: Responses of Plants to Environmental Stresses. Vol. 1, Academic Press, New York, (1980).

LEYDECKER, M.T., T. MOUREAUX, Y. KRAEPIEL, K. SCHNORR, and M. CABOCHE: Molybdenum cofactor mutants, specifically impaired in xanthine dehydrogenase activity and abscisic acid biosynthesis, simultaneously overexpress nitrate reductase. Plant Physiol. *107*, 1427-31, (1995).

LICHTENTHALER, H.K. and C. BUSCHMANN: The role of chlorophyll fluorescence in the detection of stress conditions in plants. CRC Critical Reviews in Analytical Chemistry *19*, Suppl. I, 29-85, (1988).

LICHTENTHALER, H.K.: Vegetation stress: An introduction to the stress concept in plants. J. Plant Physiol. *148*, 4-14, (1996).

LIPS, S.H., A. BEN ZIONI, and Y. VAADIA: K^+ recirculation in plants and its importance for adequate nitrate nutrition. Recent Advances in Plant Nutrition. (Ed.) R.M. Samish. Gordon and Breach Science Publishers. New York-London-Paris. ISBN 0 677 12360 4. Vol.1, pp. 207-215, (1970).

LIPS, S.H., R.T. OMAROV, and M. SAGI: Mo-enzymes at the crossroads of signal transmission from root to shoot. In: Nitrogen in a sustainable ecosystem from the cell to the plant. Kluwer Acad. Publ., Amsterdam (in press) (1999).

LIU, L. and J.W. McCLURE: Effects of UV-B on activities of enzymes of secondary phenolic metabolism in barley primary leaves. Physiol. Plant. *93*, 734-739, (1995).

LOWRY, O.H., N.J. ROSEBROUGH, A.L. FARR, and R.J. RANDALL: Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265-275, (1951).

LUMSDEN, P.J.: Circadian rhythms and phytochrome. Ann. Rev. Plant Physiol. Plant. Mol. Biol. *42*, 351-371, (1991).

MADRONICH, S.: The Atmosphere and UV-B Radiation at Ground Level. In: In: A.R.YOUNG, L.O. BJÖRN, J. MOAN, and W. NULTSCH (eds.), Environmental UV Photobiology, pp. 1-39. Plenum Press, New York (1993).

MASI, A., M. FERRETTI, C. PASSERA, and R. GHISI: Antioxidative responses in *Zea mays* seedlings grown under UV-B radiation. Plant Physiol. Biochem. (special issue), S18-114, pp. 264-265 (1996).

McCLURE, J.W.: Physiology and function of flavonoids. In: HARBORNE, B., T.J. MARBY and H. MARBY (eds.): The Flavonoids, Part 2, pp. 970-1055, Academic Press, New York, NY (1975).

MENDEL, R R.: Molybdenum cofactor of higher plants: Biosynthesis and molecular biology. Planta *203*, 399-405, (1997).

MENDEL, R.R. and A. Muller: A common genetic determination of xanthine dehydrogenase and NR in *N. tabacum*. Biochem. Physiol. Pflanzen *170*, 538-541, (1976).

MENDEL, R.R. and G. SCHWARZ: Molybdoenzymes and molybdenum cofactor in plants. Critical Rev. Plant Sci. (in press), (1998).

MITTAG, M. and J.W. HASTINGS: Exploring the signaling pathway of circadian bioluminescence. Physiol. Plant. *96*, 727-732, (1996).

MOLINA, M.J., C.E. KOLB, and L.T. MOLINA: Gas-phase and heterogeneous chemical kinetics of the troposphere and stratosphere. Ann. Rev. Phys. Chem. *47*, 327-367, (1996).

MOORHEAD, G., P. DOUGLAS, N. MORRICE, M. SCARABEL, A. AITKEN, and C. MACKINTOSH: Phosphorylated nitrate reductase from spinach leaves is inhibited by 14-3-3 proteins and activated by fusiococcin. Current Biology *6*, 1104-13, (1996).

NADEZHDIN, D. and H.B. DUNFORD: Oxidation of nicotinamide adenine dinucleotide by hydroxyperoxyl radical, a flash photolysis study. J. Phys. Chem. *83*, 1957-1961, (1979).

NGUYEN J.: Plant xanthine dehydrogenase: its distribution, properties and function. Physiol.Veg. *24*, 163-281, (1986).

NJUS, D., F.M. SULZMAN, and J.W. HASTINGS: Membrane model for the circadian clock. Nature *248*,116-120, (1974).

OLSZYK, D., Q. DAI, P. TENG, H. LEUNG, Y. LUO, and S. PENG: UV-B effects on crops: Response of the irrigated rice ecosystem. J. Plant Physiol. *148*, 26-34, (1996).

OMAROV, R.T., M. SAGI, and S.H. LIPS: Regulation of aldehyde oxidase and nitrate reductase in roots of barley (*Hordeum vulgare* L.) by nitrogen source and salinity. J. Exp. Bot. *49*, 897-902, (1998).

OMAROV, R.T., S. AKABA, T. KOSHIBA, and S.H. LIPS: Aldehyde oxidase in roots, leaves and seeds of barley (Hordeum vulgare L.). J. Exp. Bot. (in press) (1999).

ORI, N., Y. ESHED, P. PINTO, I. PARAN, D. ZAMIR, and R. FLUHR: TAO1, a representative of the molybdenum cofactor containing hydroxylases from tomato. J. Biol. Chem. *272*, 1019-1025, (1997).

PALMS, B., P. GOUPIL, J. DE ALMEIDA ENGLER, D. VAN DER STRAETEN, M. VAN MONTAGU, AND S. RAMBOUR: Evidence for the nitrate-dependent spatial regulation of the nitrate reductase gene in chicory roots. Planta *200*: 20-27, (1996).

PASTORI, G.M. and L.A. DEL RIO: Natural senescence of pea leaves: an activated oxygen-mediated function for peroxisomes. Plant Physiol. *113*, 411-418, (1997).

PEAK, J.G., M.G. PEAK, and R.W. TUVESON: Ultraviolet action spectra for aerobic and anaerobic inactivation of *Escherichia coli* strains specifically sensitive and resistant to near ultraviolet radiation. Photochem. Photobiol. *38*, 541-543, (1983).

PERL-TREVES, R. and E. GALUN: The tomatoCu/Zn superoxide dismutase genes are developmentally reglated and respond to light and stress. Plant Mol. Biol. *17*, 745-760, (1991).

PESTENÁCZ, A. and L. ERDEI: Calcium-dependent protein kinase in maize and sorghum induced by polyethylene glycol. Physiol. Plant. *97*, 360-364, (1996).

PETERSON-MAHRT, S.K., N.G.A. EKELUND, and S. WIDELL: Effects of UV-B radiation and nitrogen starvation on enzyme activities in isolated plasma membranes of *Euglena gracilis*. Physiol. Plant. *95*, 515-522, (1995).

PETERSON-MAHRT, S.K., N.G.A. EKELUND, and S. WIDELL: Influence of UV-B radiation and nitrogen starvation on daily rhythms in phototaxis and cell shape of *Euglena gracilis*. Physiol. Plant. *92*, 501-505 (1994).

PEUKE, A.D., W.D. JESCHKE, and W. HARTUNG: The uptake and flow of C, N and ions between roots and shoots in Ricinuscommunis L. III. Long-distance transport of abscisic acid depending on nitrogen nutrition and salt stress. J. Exp. Bot. *45*, 741-747, (1994).

PIECHULLA, B.: 'Circadian clock' directs the expression of plant genes. Plant Molec. Biol. *22*, 533-542, (1993).

PROUD, C.G.: Protein phosphorylation in translational control. In: E.R. STADTMAN, B.P. CHOCK, and A. LEVITZKI (eds.), Current Topics in Cellular Regulation, pp. 243-369. Academic Press Inc., San Diego, CA (1992).

QUARTACCI, M.F. and F. NAVARI-IZZO: Water stress and free radical mediated changes in sunflower seedlings. J. Plant Physiol. *139*, 621-625, (1992).

RADI, R., H. RUBBO, L. THOMSON, and E. PRODANOV: Luminol chemiluminescence using xanthine and hypoxanthine as xanthine oxidase substrates. Free Radic. Biol. Med. *8*, 121-126, (1990).

RAJAGOPALAN, K.V. and J.L. JOHNSON: The pterin molybdenum cofactor. J. Biol. Chem. *267*, 10199-10202, (1992).

RAO, M.V. and D.P. ORMROD: Impact of UV-B and O_3 on the oxygen free radical scavenging system in *Arabidopsis thaliana* genotypes differing in flavonoid biosynthesis. Photochem Photobiol. *62*, 719-726, (1995).

RAO, M.V., G. PALIYATH, and D.P. ORMROD: Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol. *110*, 125-136, (1996).

REDINBAUGH, M., M. SABRE, and J.G. SCANDALIOS: Expression of the maize Cat3 catalase gene is under the influence of a circadian rhythm. Proc. Natl. Acad. Sci. USA *87*, 6853-6857, (1990).

REED, D.J., J.R. BABSON, P.W. BEATTY, A.E. BRODIE, W.W. ELLIS, and D.W. POTTER: High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. Anal. Biochem. *106*, 55-62, (1980).

ROENNEBERG, T.: The complex circadian system of *Gonyaulax polyedra*. Physiol. Plant. *96*, 733-737, (1996).

ROST, T.L. and J.A. BRYANT: Root organization and gene expression patterns. J. Exp. Botany *47(304)*, 1613-1628, (1996).

ROTHE, G.M.: Aldehyde oxidase isoenzymes (E.C. 1.2.3.1) in potato tubers (Solanum tuberosum). Plant Cell Physiol. *15*, 493-499, (1974).

ROWLAND, F.S.: Chlorofluorocarbons and the depletion of stratospheric ozone. Am. Sci. *77*, 36-45, (1989).

SAAB, I.N., R.E. SHARP, and J. PRITCHARD: Effect of inhibition of abscisic acid accumulation on the spatial distribution of elongation in the primary root and mesocotyl of maize at low water potentials. Plant Physiol. *99*, 26-33, (1992).

SAGI, M., A. DVORAT, T. KIPNIS, AND S.H. LIPS: Nitrate reductase phosphoenolpyruvate carboxylase and glutamine synthetase in annual ryegrass as affected by salinity and nitrogen. J. Plant Nutrition *21(4)*, 707-723, (1998a).

SAGI, M, R.T. OMAROV, AND S.H. LIPS: The Mo-hydroxylases xanthin dehydrogenase and aldehyde oxidase in ryegrass as affected by nitrogen and salinity. Plant Sci. *135*, 125-135, (1998b).

SAGI, M. and S.H. LIPS: The level of nitrate reductase and MoCo in annual ryegrass as affected by nitrate and ammonium. Plant Sci. *135*, 17-24, (1998c).

SANTOS, I., J.M. ALMEIDA, and R.SALEMA: Plants of *Zea mays* L. developed under enhanced UV-B radiation. I. Some ultrastructural and biochemical aspects. J. Plant Physiol. *141*, 450-456, (1993).

SAVIDOV, N.A., N.P. L'VOV, M. SAGI, and S.H. LIPS: Molybdenum cofactor biosynthesis in two barley (*Hordeum vulgare* L.) genotypes as affected by nitrate in the tissue and in the growth medium. Plant Sci. *122*, 51-59, (1997).

SCANDALIOS, J.G.: Regulation and Properties of Plant Catalases. In C.H. FOYER, P.M. MULLINEAUX, eds, Causes of Photooxidative Stress and Amelioration of Defence Msystems in Plants. CRC Press, Boca Raton, FL, pp. 275-315, (1994).

SCHWARTZ, S.H., K.M. LÉON-KLOOSTERZIEL, M. KOORNEEF, J.A.D. ZEEVAART: Biochemical characterization of the *aba2* and *aba3* mutants in *Arabidopsis thaliana*. Plant Physiol. *114*, 161-166, (1997).

SCHWEIGER, H.G. and M. SCHWEIGER: Circadian rhythms in unicellular organisms. Int. Rev. Cytol. *51*, 315-342, (1977).

SEKIMOTO, H., M. SEO, N. DOHMAE, K. TAKIO, Y. KAMIYA, and T. KOSHIBA: Cloning and molecular characterization of plant aldehyde oxidase. J. Biol. Chem. *272*, 15280-15285, (1997).

SELYE, H.: A syndrome produced by various nocuous agents. Nature 138, 32-34, (1936)

SELYE, H.: The Stress of Life. McGraw-Hill Book. Co. Inc., New York, 1956.

SEO, M., S.H. AKABA, T. ORITANI, M. DELARUE, C. BELLINI, M. CABOCHE, and T. KOSHIBA: Higher activity of an aldehyde oxidase in the auxin-overproducing superroot1 mutant of Arabidopsis thaliana. Plant Physiol. *116*, 687-693, (1998).

SHABALA, S.N. and I.A. NEWMAN: Proton and calcium flux oscillations in the elongation region correlate with root nutation. Physiol. Plant. *100*, 917-926, (1997).

SIES, H.: Oxidative stress: Introductory Remarks. In: Oxidative Stress. Ed.: Sies, H., Academic Press Inc. (London) Ltd., London, ISBN 0-12-642760-7, pp. 1-8, (1985).

SILLER-CEPEDA, J.H., T.H.H. CHEN, and L.H. FUCHIGAMI: High performance liquid chromatography analysis of reduced and oxidised glutathione in woody plant tissues. Plant Cell Physiol. *32*, 1179-1185, (1991).

SMITH, I.K., T.L. VIERHELLER, and C.A. THORNE: Assay of glutathione reductase in crude tissue homogenates using 5,5'-Dithio*bis*(2-nitrobenzoic acid). Anal. Biochem. *175*, 408-413, (1988).

SPILLER, S.C., L.S. KAUFMAN, W.F. THOMPSON, and W.R. BRIGGS: Specific mRNA and rRNA levels in greening pea leaves during recovery from iron stress. Plant Physiol. *84*, 409-414, (1987).

STOLARSKI, R., R. BOJKOV, L. BISHOP, C. ZEREFOS, J. STAEHELIN, and J. ZAWODNY: Measured trends in stratospheric ozone. Science *256*, 342-349, (1992).

STRID, A.: Alteration in expression of defence genes in *Pisum sativum* after exposure to supplementary ultraviolet-B radiation. Plant Cell Physiol. *34*, 949-953, (1993).

TAIZ, L. and E. ZEIGER: Plant Physiology II edition. Ed. Sinauer Associates, Inc. Publ., Sunderland Mass. ISBN 0-87893-831-1, (1998).

TANAKA, K., H. SAJI, and N. KONDO: Immunological properties of spinach glutathione reductase and inductive biosynthesis of the enzyme with ozone. Plant Cell Phys. *29*, 637-642, (1988).

TANAKA, K., T. SANO, K. ISHIZUKA, K. KITTA, and Y. KAWAMURA: Comparison of properties of leaf and root glutathione reductases from spinach. Physiol. Plant. *91*, 353-358, (1994).

TEVINI, M., J. BRAUN, and G. FIESER: The protective function of the epidermal layer of rye seedlings against UV-B radiation. Photochem. Photobiol. *53*, 329-333, (1991).

TEVINI, M.: Effects of enhanced UV-B radiation on terrestrial plants. In: M. TEVINI (ed.), UV-B radiation and ozone depletion: Effects on humans, animals, plants, micro-organisms and materials. Chapter 5, pp.125-153. Lewis Publishers, Boca Raton, FL, (1993).

TRIPLETT, E.W., D.G. BLEVINS, and D.D. RANDALL: Allantoic acid synthesis in soybean root nodule cytosol via xanthine dehydrogenase. Plant Physiol. *65*, 1203-1206, (1980).

UPADHYAYA, A., D. SANKHLA, T.D. DAVIS, N. SANKHLA, and B.N. SMITH: Effect of paclobutrazol on the activities of some enzymes of activated oxygen metabolism and lipid peroxidation in senescing soybean leaves. J. Plant Physiol. *121*, 453-461, (1985).

WALKER-SIMMONS, M., D.A. KUDRA, and R.L. WARNER: Reduced accumulation of ABA during water stress in a molybdenum cofactor mutant of barley. Plant Physiol. *90*, 728-733, (1989).

WALLACE W.: The distribution and characteristics of nitrate reductase and glutamate dehydrogenase in the maize seedlings. Plant Physiol. *52*, 191-196, (1973).

WILLEKENS, H., W. VAN CAMP, M. VAN MONTAGU, D. INZÉ, H. JR. SANDERMANN, and C. LANGEBARTELS: Ozone, sulfur dioxide, and ultraviolet B have similar effects on mRNA accumulation of antioxidant genes in *Nicotiana plumbaginifolia* (L.). Plant Physiol. *106*, 1007-1014, (1994).

WINTERBOURN, C.C.: Superoxide as an intracellular radical sink. Free Radical Biol. Med. *14*, 85-94, (1993).

WOLF, O, W.D. JESCHKE, and W. HARTUNG: Long distance transport of abscisic acid in NaCl-treated intact plants of *Lupinus albus*. J. Exp. Bot. *41*, 593-600, (1990).

ZACHEO, G and T. BLEVE-ZACHEO: Involvment of superoxide dismutases and superoxide radicals in the susceptibility and resistance of tomato plants to *Meloidogyne incognita* attack. Physiol.Mol.Plant Pathol. *32*, 313-322, (1988).

ZEEVAART, J.A.D. and R.A. Creelman: Metabolism and Physiology of abscisic acid Ann.Rev.Plant Physiol.Plant Mol.Biol. *39*, 439-473, (1988).

ZHANG, J. and F. TARDIEU: Relative contribution of apices and mature tissue to ABA synthesis in droughted maize root systems. Plant Cell Physiol. *37(5)*, 598-605, (1996).