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# ROS as Signaling Molecules and Enzymes of Plant Response to Unfavorable Environmental Conditions

Dominika Boguszevska<sup>1</sup> and Barbara Zagdańska<sup>2</sup>

<sup>1</sup>*Plant Breeding and Acclimatization Institute-  
National Research Institute Division Jadwisin,*

<sup>2</sup>*Warsaw University of Life Sciences  
Poland*

## 1. Introduction

Research concern plant response to unfavourable environmental conditions is becoming increasingly important and most climate-change scenarios suggest an increase in aridity in many areas of the globe (Petit et al. 1999, Blum 2011). On a global principle, drought (taking into account soil and/or atmospheric water deficit) in combination with coincident high temperature and radiation, determines the most important environmental constraints to plant survival and to crop productivity (Boyer 1982). Agriculture is a major user of water resources and abiotic stress is the principal cause of decreasing the average yield of major crops by more than 50%, which causes losses worth hundreds of millions of dollars each year. Understanding mechanism of abiotic stress tolerance and defense is important for crop improvement. Many works concerning this problem were developed over last decades, discussing subjects from plant strategies to control water status under drought (Schulze 1986) to the physiological and biochemical processes underlying plant response to water shortage (Chaves 1991, Cornin and Massacci 1996) and oxidative stress (Smirnoff 1998). In this chapter various aspects of reactive oxygen species and enzymes in plant response to drought (abiotic stress) are discussed.

## 2. Environmental stresses

Stress in physical terms is defined as mechanical force per unit area applied to an object. In biological systems stress can be defined as an adverse force, effect, or influence that trends to inhabit normal systems from functioning.

A wide range of unfavourable environmental conditions may induce stresses in plants that alter plant growth, development and metabolism, and even may lead to plant death. These stresses include mechanical damage, herbicides, UV radiation, salt, low/high temperature, soil drought, flooding, high speed wind, nutrient loss and anaerobic conditions are very important stress factors limiting crop productivity (Lawlor, 2002). Among them drought is a major abiotic factor that limits agricultural crop production.

To sense these environmental signals plants have evolved a complex signalling network, which may also cross-talk. Stress signal transduction pathway start with signal perception by receptors like phytochromes, histidine kinases, receptor like kinases, G-protein-coupled receptors (GPCR), hormone receptors etc., which generate secondary signalling molecules like inositol phosphatase, reactive oxygen species (ROS), abscisic acid (ABA), etc. Mentioned secondary molecules can modulate the intracellular  $\text{Ca}^{2+}$  level and initiate protein phosphorylation cascades i.e. mitogen-activated protein kinase MAPK, calcium dependent protein kinase CDPK, protein phosphatase, SOS3/protein kinase S etc. Drought and salinity occur their influence on cell mainly by disrupting the ionic and osmotic equilibrium leading to a cascade of events, which can be grouped under ionic and osmotic signalling pathway. These stresses are marked by symptoms of stress injury. Stress injury may occur through denaturation of cellular proteins/enzymes or through production of ROS. In response to injury stress plants start detoxification process which include changes in expression of LEA/dehydrin-type gene synthesis of proteinases, enzymes for scavenging ROS and other detoxification proteins.

### 3. Plant defence against reactive oxygen species

Reactive oxygen species are continuously produced during aerobic metabolism as byproducts of different metabolic pathways which are localized in some cellular compartments such as chloroplast, mitochondria and peroxisomes – organelles with a highly oxidizing metabolic activity or with intense rate of electron flow. Therefore, plants are well equipped with antioxidants and enzymes scavenging ROS to keep their levels low under favourable conditions of growth. However, under unfavourable environmental conditions production of reactive oxygen species may increase and lead to oxidative stress in many plant species (Smirnoff 1993, Mullineaux & Karpinski 2002, Miller et al., 2010).

Reactive oxygen species inactivate enzymes and damage important cellular components. ROS are responsible for protein, lipid and nucleic acid modification and are thought to play a major role in ageing and cell death (Jacobson 1996). To avoid the accumulation of these compounds to toxic level, animals and plants possess several detoxifying enzymatic systems that comprise a variety of antioxidant molecules and enzymes. Two main classes of **plant defenses** have been described and can be classified as non-enzymatic and enzymatic systems. The first class - non-enzymatic constituents, including lipid-soluble and membrane-associated tocopherols, water soluble reductants, ascorbic acid and glutathione, and enzymatic constituents, including superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6), peroxidase (EC 1.11.1.7), ascorbate peroxidase (EC 1.11.1.11) and associated antioxidant enzyme, glutathione reductase (EC 1.1.4.2).

Under steady state conditions, the ROS molecules are scavenged by antioxidant mechanisms (AOS) mentioned earlier (Fig. 1). The equilibrium between the production and the scavenging of ROS may be disturbed by stress factors. However, it is widely discussed that the relationship between metabolism and redox state is complex and subtle (Hare et al., 1998; Bohnert & Jensen 1996). Oxidation involves different signals that plants use to make appropriate adjustments of gene expression or cell structure in response to environmental factors. Increased level of intercellular ROS due to increased production and/or insufficient antioxidant protection can cause significant modification of cell structure or even lead to cell death. In many cases, the situation of enhancing oxidation, which itself is negative may be a signal for adjusting plant metabolism to the new conditions of growth.

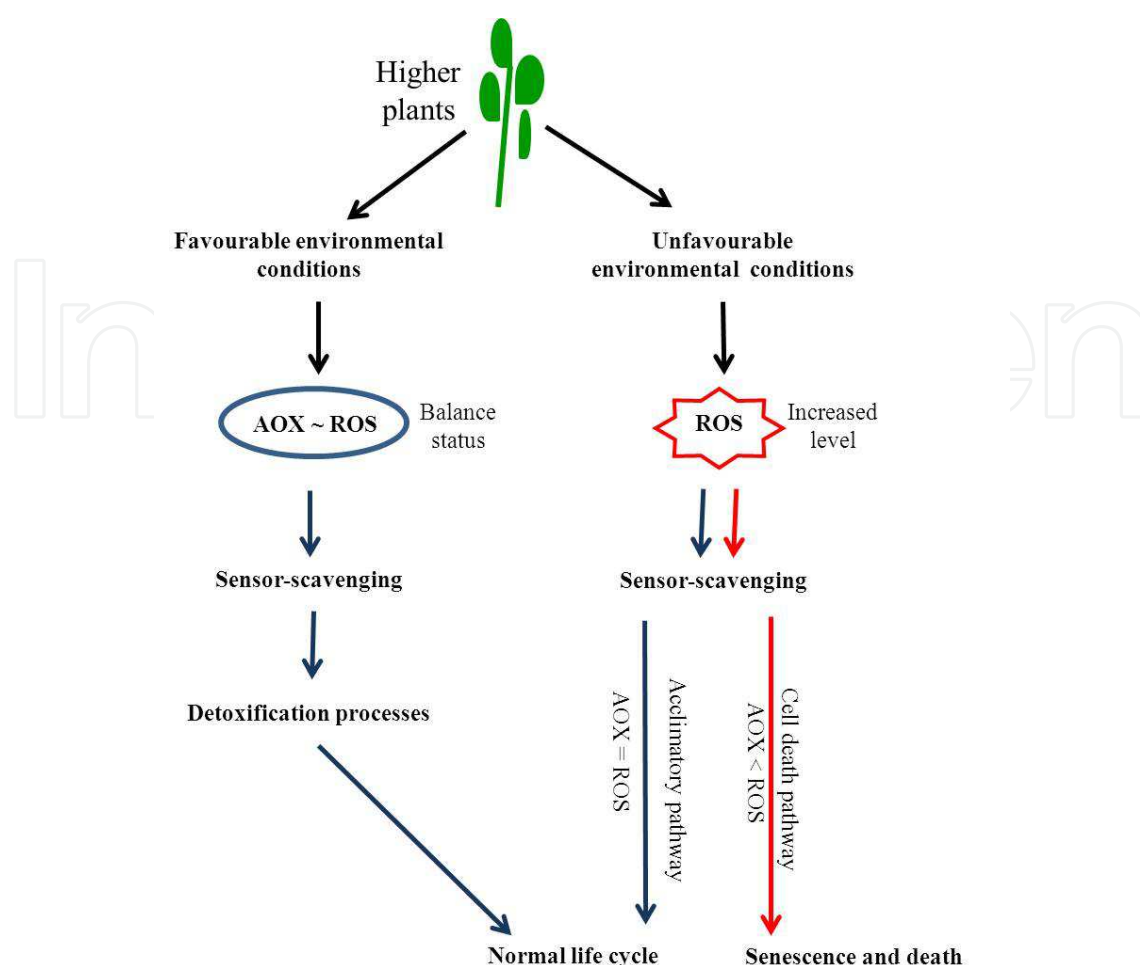


Fig. 1. Antioxidants and redox signaling in plants

#### 4. Reactive oxygen species involving in plant defence

Stress of any kind, biotic or abiotic leads to an increased level of ROS production and/or to inactivation of antioxidants, particularly enzymes. The oxidative stress that accompanies unfavorable environmental conditions should not be viewed as symptom of cellular dysfunction but it can represent a perquisite signal for a plant to induce proper acclimation mechanisms (Jaspers & Kandasjarvi, 2010; Miller et al. 2010). Reactive oxygen species can also play a role of “oxidation signalling molecules” (Foyer & Noctor 2009). Signals of ROS originating at different organelles have been shown to induce large transcriptional changes and cellular reprogramming that can either protect the plant cell or induce programmed cell death (Foyer & Noctor 2005).

It is important to remember that whether ROS will act as damaging, protective or signalling factor depends on sensible equilibrium between ROS production and scavenging at the proper site and time. It is known that ROS can damage cells and initiate different responses like expression of new genes. Evoking of cell response highly depends on several factors. One of them is subcellular localization for formation of ROS. Stress-induced ROS accumulation is counteracted by enzymatic and nonenzymatic antioxidant. Thus, plant stress tolerance may be improved by increasing of *in vivo* levels of antioxidants enzymes.





The most common ROS are hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), superoxide ( $\text{O}_2^{\cdot-}$ ), the hydroxyl radical ( $\text{HO}\cdot$ ) and singlet oxygen ( $^1\text{O}_2$ ).

The major site of **superoxide radical** ( $\text{O}_2^{\cdot-}$ ) production is the reaction centers of photosystem I (PSI) and a photosystem II (PSII) in chloroplast thylakoids. In mitochondria, complex I, II and complex III in the electron transport chain contribute to superoxide radical production. The terminal oxidases-cytochrome c oxidase and the alternative oxidase react with  $\text{O}_2$ , four electrons are transferred and  $\text{H}_2\text{O}$  is released. There is situation when  $\text{O}_2$  can react with other ETC components and there in only one electron transferred with the result of  $\text{O}_2^{\cdot-}$  release. It has been shown that in plants 1-2% of  $\text{O}_2$  consumption leads to  $\text{O}_2^{\cdot-}$  production (Puntarulo, et al. 1988).

**Singlet oxygen** is the first excited electronic state of  $\text{O}_2$ . Insufficient energy dissipation during photosynthesis can lead to formation of chlorophyll (Chl) triplet state. And the Chl triplet state can react with  $3\text{O}_2$  to give up very reactive singlet oxygen. It has been proved that singlet oxygen formation during photosynthesis can have damaging effect on PSI and PSII and on whole machinery of photosynthesis.

**Hydroxyl radicals** ( $\text{HO}\cdot$ ) are the highest reactive ROS. It can be produced from  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$  at neutral pH and ambient temperature by iron-catalyzed.

**Hydrogen peroxide** ( $\text{H}_2\text{O}_2$ ) is produced by univalent reduction of  $\text{O}_2^{\cdot-}$ .  $\text{H}_2\text{O}_2$  is moderately reactive. It has been proved that excess of hydrogen peroxide leads to oxidative stress. This molecule may also inactivate enzymes by oxidizing their thiol groups. Moreover,  $\text{H}_2\text{O}_2$  play dual role in plants. At low concentration it can act as a signal molecule involved in acclimatory signaling triggering tolerance to different biotic and abiotic stresses. At high concentration it leads to programmed cell death (Quan et al., 2008). It has been proved that  $\text{H}_2\text{O}_2$  act as a key regulator of in a wide range of physiological processes like photorespiration and photosynthesis (Noctor & Foyer 1998), stomatal movement (Bright et al., 2006), cell cycle (Mittler et al., 2006) and growth and development (Foreman 2003).  $\text{H}_2\text{O}_2$  is taking as a second messenger for signals generated by means of ROS due to its relatively long life and high permeability across membranes. Many of the general stress genes are regulated by a signaling pathways using  $\text{H}_2\text{O}_2$  as the messenger (Möller & Sweetlove 2010).

## 5. Major cellular sources of reactive oxygen species

ROS are produced continuously as byproducts of various metabolic pathways that are localized in different cellular compartments such as chloroplasts, mitochondria and peroxisomes.

Plant **mitochondria** called “energy factories” are known, apart from chloroplasts, as a main place of ROS production like  $\text{H}_2\text{O}_2$  and also the ROS target (Rasmusson et al., 2004). Plant mitochondria have specific electron transfer chain (ETC) components and functions in processes like photorespiration. The mitochondrial ETC harbors electrons with sufficient free energy to directly reduced  $\text{O}_2$  which is the unavoidable primary source of mitochondrial ROS generation in aerobic respiration (Rhoads et al., 2006). Nevertheless, ROS production in mitochondria takes place also under normal respiratory condition but can be enhanced due to various biotic and abiotic stress factors. Complex I and II is well

known as a place of  $O_2^{\cdot-}$  production. In aqueous solution  $O_2^{\cdot-}$  is moderately reactive but this  $O_2^{\cdot-}$  can be reduced by SOD dismutation to  $H_2O_2$  (Quan, 2008; Möller 2001; Grene 2002). Next  $H_2O_2$  can react with  $Fe^{2+}$  and  $Cu^+$  to produce highly toxic  $HO\cdot$ , and these uncharged  $HO\cdot$  can penetrate membranes and leave the mitochondrion (Rhoads et al., 2006). Abstraction of hydrogen atom by ROS, especially by  $HO\cdot$ , starts peroxidation of mitochondrial membrane PUFA (polyunsaturated fatty acid). The consequence of this is formation of cytotoxic lipid aldehydes, alkenals and hydroxyalkenals etc. However, plant mitochondria may control ROS generation by means of energy-dissipating systems. Therefore mitochondria may play a central role in plant adaptation to abiotic stress.

ROS formation is possible also in **chloroplasts**, where photosynthesis takes place, which contain a highly organized thylakoid membrane system that harbours all components of light-capturing photosynthetic apparatus. Oxygen generated in chloroplast during photosynthesis is able to accept electrons passing through the photosystem resulting on  $O_2^{\cdot-}$  formation. The presence of ROS producing centers like triplet Chl, electron transfer chains in PSI and PSII make chloroplast a site of ROS ( $O_2^{\cdot-}$ ,  $^1O_2$ ,  $H_2O_2$ ) production.

Small, usually spherical microbodies bounded by a single lipid bilayer membrane called **peroxisomes** – organelles with an essentially oxidative type of metabolism are sites of intracellular ROS production. Similar to mitochondria and chloroplasts, peroxisomes produce  $O_2^{\cdot-}$  radicals in their normal metabolism. There are two sites of  $O_2^{\cdot-}$  production in peroxisomes (Rio et al., 2002). One of them is in the organelle matrix, where xanthine oxidase (XOD) catalyzes the oxidation of xanthine and hypoxanthine to uric acid. The second site takes place in peroxisome membranes.

Other important sources of ROS production in plants take places in cytoplasm, endoplasmic reticulum and in apoplast at plasma membrane level or extracellular (Gill & Tuteja 2010).

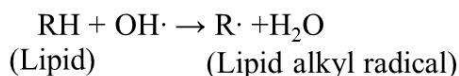
## 6. Reactive oxygen species-mediated damage to macromolecules

It has long been known that the stress-induced formation of reactive oxygen species have been associated with non-specific damage to DNA, proteins and lipids which potentially can result in death of the cell and even the organism.

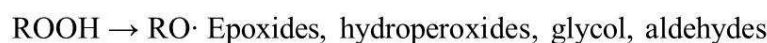
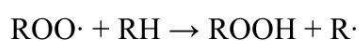
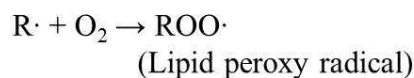
**Oxidative damage to lipids or lipid peroxidation (LPO).** The peroxidation of lipids is one of the most damaging processes occurs every living organism. During LPO, products are formed from polyunsaturated precursors that include small hydrocarbon fragments like ketons, MDA etc. Lipid peroxidation takes place when in both cellular and organelle membranes above-threshold levels of ROS are reached. The process of LPO involved three stages: initiation, progression and termination. (Fig.3). The overall effects of LPO are to decrease membrane fluidity, increase the leakiness of the membrane to substances that normally cross it other than through specific channels and damage membrane proteins, inactivating receptors, enzymes and ion channels (Gill & Tuteja 2010).

**Oxidative damage to protein.** Proteins are the most abundant cellular component oxidized by ROS constituting up to 68% of the oxidized molecules in the cell (Rinalducci et al., 2008). Protein oxidation is a covalent modification induced by ROS or by products of oxidative stress. Protein oxidation mostly is irreversible, however, a few involving sulfur-containing amino acid are reversible (Ghezzi & Bonetto 2003). The most susceptible residues to oxidation

## Initiation step



## Propagation step



## Termination step

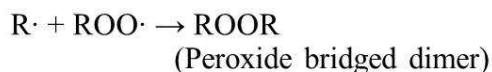


Fig. 3. The main steps in lipid peroxidation according to Gill & Tuteja (2010).

are the sulphur containing cysteine and methionine. The thiol of cysteine may be oxidized by hydroxyl radicals, superoxide and hydrogen peroxide to a disulfide that can be readily reversible. Oxidation of methionine in many proteins has little effect on protein structure and function. An example of the reversible oxidation of methionine is the inactivation of the small heat shock protein in chloroplasts that is reactivated by thioredoxin in reaction catalysed by methionine sulfoxide reductase (Gustavsson et al., 2002). Tyrosine oxidation can alter residue hydrophobicity with consequent effect on protein structure. Tryptophan oxidation is an irreversible protein modification (Rinalducci et al., 2008). The most commonly occurring oxidative modification of proteins, after oxidation of sulphur-containing amino acids, is protein carbonylation. The oxidation of number of protein amino acid side-chains particularly Arg, His, Lys, Pro, Thr and Trp give free carbonyl groups which may inactivated, cross-linking or breakdown of proteins (Möller et al., 2007; Foyer & Noctor 2009). In leaves of wheat (*Triticum aestivum* L.) mitochondria contained more oxidatively modified proteins than chloroplasts and peroxisomes (Bartoli et al., 2004).

**Oxidative damage to DNA.** Due to biotic and abiotic stresses DNA is exposed to damage. Endogenously generated damage to DNA is known as “spontaneous DNA damage”, which is produced by reactive metabolites ( $\text{HO}\cdot$ ,  $\text{O}_2^{\cdot-}$  and  $\cdot\text{NO}$ ). High level of ROS can influence on damage to cell structures, nucleic acids, lipids and proteins. It has been considered that one of the most reactive is  $\text{HO}\cdot$  causing damage to all components of DNA molecules. This molecule damages purine, pyrimidine and deoxyribose backbone.  $^1\text{O}_2$  damages guanine, and  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot-}$  do not react at all. Result of DNA damage can be various physiological effects like reduced protein synthesis, cell membrane destruction, damage to photosynthetic proteins what consequently leads to growth and development disorders (Britt 1999).



7. Enzymatic antioxidants

A wide range of unfavorable environmental conditions like mentioned drought, extreme temperatures, salt stress etc. can induce stresses that alter seriously plant metabolism and may increase production of ROS ( $\text{H}_2\text{O}_2$ ,  $\text{O}_2^{\cdot-}$ ,  $^1\text{O}_2$ ,  $\text{HO}\cdot$ ) inducing an oxidative stress in organelles. Plants are unable to escape exposure to these environmental constraints and evolved mechanisms in order to survive. To prevent appearance of these toxic compounds and their consequences plants have a variety of constitutively expressed antioxidant defense mechanisms to scavenge the ROS generated. A lot of researches have been done to emphasize the importance of the cellular antioxidant machinery in protection against various stresses (Dalton, et. al., 1999, Tuteja 2007, Tuteja 2009). ROS-scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and associated antioxidant enzymes, glutathione reductase (GR) and antioxidants such as “big three” antioxidants (ascorbic acid, glutathione and the pyridine nucleotides) and many redox-active phenolics, carotenoids and tocopherols are essential for ROS detoxification. The components of cellular “antioxidant machinery” and their role in plant protection against various abiotic stresses have been summarized in Fig.4.

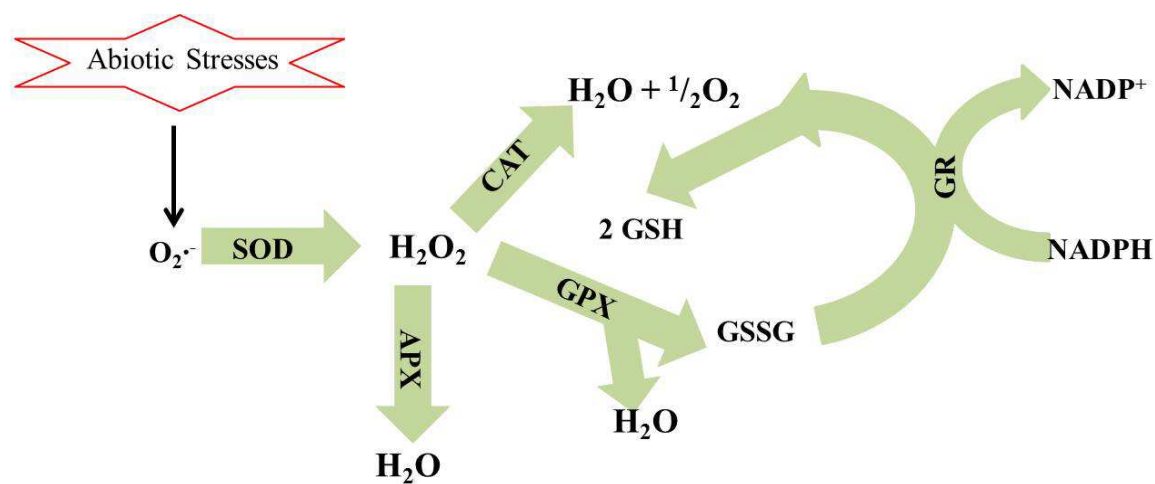


Fig. 4. ROS and antioxidant defense mechanism.

Enzymatic antioxidants include SOD, CAT, APX, GPX (guaiacol peroxidase), MDHAR (monodehydroascorbate reductase), DHAR (dehydroascorbate reductase) and GR. Reactions catalyzed by enzymatic antioxidants are presented in Table 1.

Enzymatic antioxidant	Enzyme code	Reaction catalyzed
Superoxide dismutase (SOD)	EC 1.15.1.1	$\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-} + 2\text{H}^+ \rightarrow 2\text{H}_2\text{O}_2 + \text{O}_2$
Catalase (CAT)	EC 1.11.1.6	$\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \frac{1}{2} \text{O}_2$
Ascorbate peroxidase (APX)	EC 1.11.1.11	$\text{H}_2\text{O}_2 + \text{AA} \rightarrow 2 \text{H}_2\text{O} + \text{DHA}$
Guaiacol peroxidase (GPX)	EC 1.11.1.7	$\text{H}_2\text{O}_2 + \text{GSH} \rightarrow \text{H}_2\text{O} + \text{GSSG}$
Monodehydroascorbate reductase (MDHAR)	EC 1.6.5.4	$\text{MDHA} + \text{NAD(P)H} \rightarrow \text{AA} + \text{NAD(P)}^+$
Dehydroascorbate reductase (DHAR)	EC 1.8.5.1	$\text{DHA} + 2\text{GSH} \rightarrow \text{AA} + \text{GSSG}$

Table 1. Reaction catalyzed by major ROS-scavenging antioxidant enzymes

**Superoxide dismutase** (EC 1.15.1.1) is the primary scavenger in the detoxification of active oxygen species in plants discovered by Irwin Fridovich and Joe McCord (1969). SOD constitutes the first line of defense against ROS. Specialization of function among SODs may be due to combination of the influence of subcellular localization of the enzyme and upstream sequences in genomic sequence. SODs remove  $O_2^{\cdot-}$  by catalyzing its dismutation, one  $O_2^{\cdot-}$  is reduced to  $H_2O_2$  and another to  $O_2$  (Table 1). SODs are metalloproteins and based on their metal cofactor they are classified into three known types: the copper/zinc (Cu/Zn-SOD), the manganese (Mn-SOD) and the iron (Fe-SOD) that are localized in different cellular compartment (Mittler 2002). The activity of SOD isoenzymes can be detected by negative staining and identified on the base of their sensitivity to KCN and  $H_2O_2$ . Cu/Zn-SOD is sensitive to both inhibitors; the Mn-SOD is resistant on both inhibitors, whereas Fe-SOD is resistant to KCN and sensitive to  $H_2O_2$ . The distribution of SOD isoenzymes is also distinctive. The Cu/Zn-SOD is found in the cytosolic fraction and also in chloroplasts in higher plants. Mn-SOD is found in the mitochondria of eukaryotic cells and in peroxisomes. And the Fe-SOD is usually present in chloroplasts, but they are not often found in plants. The up regulation of SODs has been observed in plants subjected to both abiotic (Boguszevska et al. 2010) and biotic stresses (Torres 2010, Świątek et al., submitted for publication). Overexpression of SODs in transgenic plants resulted in higher salt or drought tolerance (Badawi et al., 2004). Thus, SODs have a critical role in the survival of plants under environmental stresses.

**Catalase** (EC 1.11.1.6) was the first antioxidant enzyme to be discovered and characterized (Mhamdi et al. 2010). Catalase is a heme-containing enzyme that catalyzes the dismutation of two molecules of hydrogen peroxide to water and oxygen (Table 1). All forms of the enzyme are tetramers with each monomer of 50-70 kDa. CAT has one of the highest turnover rates for all enzymes: one molecule of CAT can convert about 6 million molecules of  $H_2O_2$  to  $H_2O$  and  $O_2$  per minute. Multiple forms of catalase have been described in many plants. Monocots and dicots contain three catalase genes. The CAT1 gene is mainly expressed in pollen and seeds, CAT2 in photosynthetic tissues but also in roots and seeds and CAT3 in vascular tissues and in leaves. CAT isozymes, CAT1 and CAT2 are localized in peroxisomes and the cytosol, whereas CAT3 is mitochondrial isozyme. Catalase is a light-sensitive protein that has a high rate of turnover and environmental stresses which reduce the rate of protein turnover, such as salinity, heat shock or cold, cause the depletion of catalase activity. This may have significance in the plant's ability to tolerate the oxidative components of these environmental stresses (Boguszevska et al., 2010, Mhamdi et al., 2010). However, the response of CAT2 to soil drought differed from that of mite-infestation. Mite feeding decreased the CAT2 activity band whereas dehydration of leaves increased it only slightly and induced the CAT3 activity band (Świątek et al., submitted for publication). Catalase activity increased in response to low temperatures in germinating embryos of maize lines and the increase in total catalase activity was due to accumulation of the CAT1 and CAT2 isozymes whereas the CAT3 activity decreased (Auh & Scandalios 1997). Kukreja et al. (2005) reported increase of CAT activity in *C. arietinum* roots under salinity stress whereas, in the other study, Sharma & Dubey (2005) reported a decrease in CAT activity in rice seedling under drought stress. It remains unclear whether variability in catalase response to different unfavourable conditions may be of importance in plant stress tolerance level.

**Ascorbate peroxidase** (EC 1.11.1.11) exists as isoenzymes and plays an important role in the metabolism of  $H_2O_2$  in higher plants. It is clear that a high level of endogenous ascorbate is essential to maintain effectively the antioxidant system that protects plants from oxidative damage due to biotic and abiotic stresses. APX is involved in scavenging of  $H_2O_2$  into water-water and ascorbate-glutathione cycles and utilizes ascorbate as an electron donor (Table 1). There are five different isoforms of APX base on the localization: thylakoid tAPX, glyoxysome membrane APX (gmAPX), chloroplast stromal soluble form (sAPX) and cytosolic form of APX (cAPX). It has been shown enhanced expression of APX in plants growing under unfavourable environmental conditions.

**Guaiacol peroxidase** (EC 1.11.1.7). Guaiacol peroxidase represent an important peroxidase group which oxidise a large number of organic compounds such as phenols, aromatic amines, hydroquinones etc. but the commonly used reducing substrates are guaiacol or pyrogallol. In most plants, about 90% of the peroxidase activity is referred to as guaiacol ('anionic') peroxidase (Foyer et al., 1994). This haeme-containig protein decomposes indole-3-acetic acid (IAA) and has a role in the biosynthesis of lignin and defense against biotic stresses consuming  $H_2O_2$ . It is found in cytoplasm and apoplast. The activity of GPOX depends on plant species and stress condition.

**Glutathione reductase** (EC 1.6.4.2) is a flavo-protein oxidoreductase. It is an enzyme that is thought to play an essential role in defence system against ROS (Gill & Tuteja 2010, Noctor et al. 2010). Reducing glutathione disulfide (GSSG) to the sulfhydryl form (GSH), which is an important cellular antioxidant in defense against ROS, it sustains the reduced status of GSH. Glutathione disulfide contains of two GSH linked by a disulphide bridge which can be converted back to GSH by GR (Reddy 2006). Glutathione reductase is localized mainly in chloroplasts and small amount of this enzyme has been found in mitochondria and cytosol. By catalyzing the reduction of GSH, GR is an enzyme involved in regulation of cell energy metabolism. Glutathione reductase catalyzes the NADPH-dependent reduction of disulfide bond of GSSG what is important in the maintaining of GSH pool. Increased level of GR has been observed in plants subjected to metal, drought and salt stresses.

**Glutathione peroxidase** (EC 1.11.1.9) is the name of an enzyme family with peroxidase activity. The biological function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. There are several isozymes encoded by different genes, which vary in cellular location and substrate specificity. Millar at al., (2003) identified a family of seven related proteins in the cytosol, chloroplasts, mitochondria and endoplasmatic reticulum in *Arabidopsis*. Activity of GPX decreased in roots and did not change significantly in the leaves of *Pisum sativum* exposed on Cd-stress (Dixit et al., 2001), whereas activity of this enzyme increased in cultivars of *Capsicum annuum* under Cd-stress (Leon et al., 2002).

**Monodehydroascorbate reductase** (EC 1.6.5.4). In plants, the monodehydroascorbate reductase (MDHAR) is an enzymatic component of the glutathione-ascorbate cycle that is one of the major antioxidant systems of plant cells for the protection against the damages by reactive oxygen species (ROS). The MDHAR activity has been described in several cell compartments, such as chloroplasts, cytosol, mitochondria, glyoxysomes, and leaf peroxisomes.

**Dehydroascorbate reductase** (EC 1.8.5.1) contributes to the regulation of the symplastic and apoplastic ascorbate pool size and redox state of the cell. Dehydroascorbate is reduced to ascorbate by DHAR in a reaction requiring glutathione. Thus, dehydroascorbate reductase catalyzes the regeneration of ascorbate from its oxidized state and serves as an important regulator of ascorbate recycling. It has been shown that guard cells in DHAR-overexpressing plants exhibited a reduced level of  $\text{H}_2\text{O}_2$ , decreased responsiveness to  $\text{H}_2\text{O}_2$  or abscisic acid signaling, and greater stomatal opening (Chen & Gallie 2005). On the contrary, suppression of DHAR expression resulted in higher levels of  $\text{H}_2\text{O}_2$  in guard cells and enhanced stomatal closure under normal growth conditions or following water deficit. Increasing DHAR expression also provided enhanced tolerance to ozone. Plants suppressed in DHAR expression exhibited a reduced rate of  $\text{CO}_2$  assimilation with slower growth and reduced biomass accumulation (Chen & Gallie 2005).

## 8. Characterization of non-enzymatic antioxidants

The non-enzymatic antioxidants refer to the biological activity of numerous vitamins, secondary metabolites and other phytochemicals aimed to protect plants against ROS activity. Among the most important non-enzymatic antioxidants are ascorbic acid (AA), glutathione (GSH), proline,  $\alpha$ -tocopherols, carotenoids and flavonoids.

**Ascorbic acid (vitamin C)** is the most abundant, powerful and water soluble antioxidant which minimizes or prevents damage caused by ROS in plants. Ascorbic acid (AA) is one of the most studied one and has been detected in majority of plant cell types, organelles and appoplast (Smirnoff & Wheeler 2000). Ascorbic acid reacts not only with  $\text{H}_2\text{O}_2$ , but also with  $\text{O}_2$ , OH and lipid hydroperoxidases (Wu 2007). In turf grass AA concentration significantly increases during water deficiency (Shao et al., 2006). Ascorbic acid can also directly scavenge  $^1\text{O}_2$ ,  $\text{O}_2^{\cdot-}$  and HO $\cdot$  and regenerate tocopherol from tocopheroxyl radicals providing membrane protection. Moreover, antioxidants like AA and glutathione are involved in neutralization of secondary products of ROS reaction. Fundamental role of AA in the plant defense system is to protect metabolic processes against  $\text{H}_2\text{O}_2$ . Summing up AA reacts non-enzymatically with superoxide, hydrogen peroxide and singlet oxygen (Smirnoff & Wheeler 2000).

**Glutathione (GSH)** is a tripeptide ( $\alpha$ -glutamyl-cysteinyl-glycine), which is considered as the most important intracellular defense against ROS-induced oxidative damage. Glutathione has been detected in all cell compartments such as cytosol, chloroplasts and endoplasmatic reticulum (Foyer & Noctor 2003). Glutathione is the major source of non-protein thiol groups. The nucleophilic nature of the thiol group is important in the formation of mercaptide bonds with metals for reacting with selected electrophiles. Glutathione is involved in control of  $\text{H}_2\text{O}_2$  levels. The change in the ratio of its reduced (GSH) to oxidized (GSSG) form during the degradation of  $\text{H}_2\text{O}_2$  is very important in certain signaling pathway. It has been considered that GSH/GSSG ratio, indicative of the cellular redox balance, may be involved in ROS perception (Li & Jin 2007). Glutathione is important in plant chloroplasts because it helps to protect the photosynthetic apparatus from oxidative damage.

**Tocopherols**, lipid soluble antioxidants are known as potential scavengers of ROS and lipid radicals. Tocopherols are major antioxidant in biomembranes, where they play both antioxidant and non-antioxidant functions. The main role of tocopherols is protection of membrane stability, including quenching or scavenging ROS like  $^1\text{O}_2$ . Tocopherols are



localized in plants in the thylakoid membrane of chloroplasts. Among four tocopherols isomers  $\alpha$ -tocopherol (vitamin E) has the highest antioxidative activity because of presence of three methyl groups in its molecular structure (Kamal-Eldin & Appelqvist 1996).

**Carotenoids** are pigments that are found in plants and microorganisms. There are over 600 carotenoids in nature. Carotenoids are lipid soluble antioxidants that plays multitude of function in plant metabolism including oxidative stress tolerance. Carotenoids take part in three different functions in plants. First one, they absorb the light at wavelength between 400 and 550 nm and transfer it to the Chl. Secondly, they protect photosynthetic apparatus by quenching a triplet sensitizer (Chl3),  $^1\text{O}_2$  and other harmful free radicals which are naturally formed during photosynthesis (an antioxidant function). Thirdly, they are important for the PSI assembly and the stability of light of light harvesting complex protein as well as thylakoid membrane stabilization (structural function) (Sieferman-Harms 1987)

**Flavonoids** are widely distributed in plants leaves, floral part and pollens. They often accumulate in the plant vacuole as glycosides or as exudates on the leaves surface and other aerial part of the plant. There are four flavonoid classes depending on their structure: flavonols, flavones, isoflavones and anthocyanines. Flavonoides belong to one of the most reactive secondary metabolites of plants (Olsen et al., 2010). Flavonoids play important role as ROS scavenger by locating and neutralizing radicals before they damage cell structure. Flavonoides have function as flowers, fruits and seed pigmentation, they play protective role before UV light, drought and cold and defense against pathogens. Flavonoids play an important role in plant fertility and germination of pollen. They are involved in plant signaling with interaction with plant microbes (Olsen et al., 2010, Gill & Tuteja 2010). It has been proved that they are involved in plant responses to both, biotic or abiotic stresses such as wounding, drought and metal toxicity (Cle et al., 2008).

**Proline**,  $\alpha$ -amino acid is an antioxidant and potential inhibitor of programmed cell death. It has been suggested that free proline act as osmoprotectant, a protein stabilizer, a metal chelator, an inhibitor of lipid peroxidation and  $\text{OH}\cdot$  and  $^1\text{O}_2$  scavenger. Increased proline accumulation appears especially during salt, drought and metal stresses (Trovato et al., 2008). Therefore proline is not only an important signaling molecule, but also an effective ROS quencher. It has been found that the important role of proline is in potentiating pentose-phosphatase pathway activity as important component of antioxidative defense mechanism (Hare & Cress 1997).

## 9. Drought-responsive antioxidant enzymes in potato

The involvement of antioxidant enzymes in limitation of ROS production has been examined in potato cultivars differing in the drought tolerance (Boguszevska et al., 2010). Additionally, it has been interested whether the ROS-itscavenging antioxidant enzymes may be critical for protecting plants against water deficiency in the soil and/or whether they may be responsible for the timely activation of the accliamatory response. In our experiment three weeks after tuberization, half of potato plants grown in pots were subjected to soil drought by cessation of watering during two weeks. The other parts of plants were still watering (control plants). After this dry treatment the plants were rewatered and grown under the same conditions as control plants until maturity. To assess relative water content (RWC) in leaves of control and dehydrated plants at the end of dry period, mature leaves



from the third level from the top of the plant, comparable in size, were sampled. Leaves were weighted immediately (fresh weight, FW), floated in dark for 24 h to achieve turgidity (saturated weight, SW), then oven dried (105°C) for 24 h and weighted again (dry weight, DW). Relative water content of leaves was calculated according to Weatherly (1951):  $RWC (\%) = [(fresh\ weight - dry\ weight) / (saturated\ weight - dry\ weight)] \times 100\%$

Electrophoretic separation was performed using 4% stacking gel and 10% polyacrylamide resolving gel as described by Laemmli (1970). Samples (30 µg) were diluted in loading buffer in relation 1: 1 (50mM Tris-HCl pH 6.8; 0.1 % bromophenol blue, 10% glycine). Gel electrophoresis was run at 4°C for about 1.5h with constant current of 30 mA.

Peroxidases were visualised by incubating the gel in 50 mM potassium phosphate buffer, pH 5.0 containing 2 mM benzidine and 3 mM H<sub>2</sub>O<sub>2</sub> until appearance of orange bands.

Superoxide dismutase activity was detected following the method of Beauchamp & Fridovich (1997). Gels were soaked in 50 mM sodium phosphate buffer, pH 7.8 containing 0.098 mM nitroblue tetrazolium and 0.03 mM riboflavin. After 20 min in the dark, gels were immersing in 50 mM sodium phosphate buffer pH 7.8 containing 28 mM TEMED and exposed to a light source at room temperature. In a separate experiments 3 mM KCN and 5 mM H<sub>2</sub>O<sub>2</sub> were included during activity staining steps to distinguish amongst Cu/ Zn-SOD (inhibited by KCN), Fe-SOD (inactivated by H<sub>2</sub>O<sub>2</sub>) and Mn-SOD (resistant to both inhibitors).

Catalase (CAT, E.C. 1.11.1.6) was stained according to the method of Racchi & Terragona (1993). After native PAGE gels were washed in deionized water, incubated in 0.003% (w/v) H<sub>2</sub>O<sub>2</sub> for 10 min, and stained in 1% ferric chloride and 1% potassium ferricyanide solution.

The obtained results clearly indicated that the investigated cultivars differed in tolerance to applied two-week soil drought (Table 2). According to physiological criterion of dehydration tolerance (RWC expressed in %) the more tolerant cultivar was Owacja, whereas susceptible cultivar was Jutrzenka. However, the more intensive wilting and lower regeneration ability of cv Owacja resulted in 56% yield decrease of potato tubers of cv Owacja and significantly lower yield decrease (34%) of cv. Jutrzenka. Therefore, from agricultural point of view, cv. Jutrzenka was more tolerant than that of cv. Owacja.

Specification	cv. Owacja	cv. Jutrzenka
Yield decrease (%)	56	34
Wilting during drought (0,5 – 9 scale)	0,5	2
Regeneration after drought	1	3
$RWC_{Control} - RWC_{Drought} (\%)$	Δ 26	Δ 31

Table 2. Characterization of drought resistance of potato cultivars.

To keep ROS at constant level and maintain redox homeostasis within the plant the activities of diverse antioxidant enzymes are up-regulated in response to abiotic stresses such as drought, salinity (Miller et al., 2010) and many others (Gill & Tuteja, 2010; Jaspers & Kangasjärvi 2010) as well as in response to biotic stresses (Maserti et al., 2011, Torres 2010). Among them, plant peroxidases have also been shown to be upregulated under various abiotic treatments (Foyer & Noctor 2009). Peroxidase activity bands in leaves of droughted

plants increased in both cultivars (Fig. 5). However, the increase being higher in drought susceptible cultivar (Owacja with 59% yield decrease) than in the drought resistant (Jutrzenka with 34% yield decrease) cultivar. Soil drought did not induce the new isoforms of peroxidase.

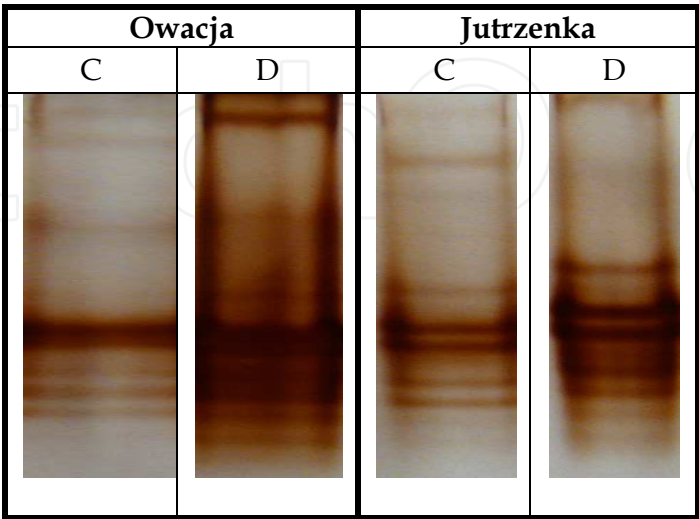


Fig. 5. Peroxidase activity in control, fully turgid leaves (C) and in dehydrated leaves (D) of two potato cultivars

In contrast to the dehydrated leaves, the response of peroxidase from potato tubers to soil drought was negative or stable (Fig. 6). The activity of peroxidase bands remained at the same level in Jutrzenka tubers and decreased in Owacja, but with one band being more active.

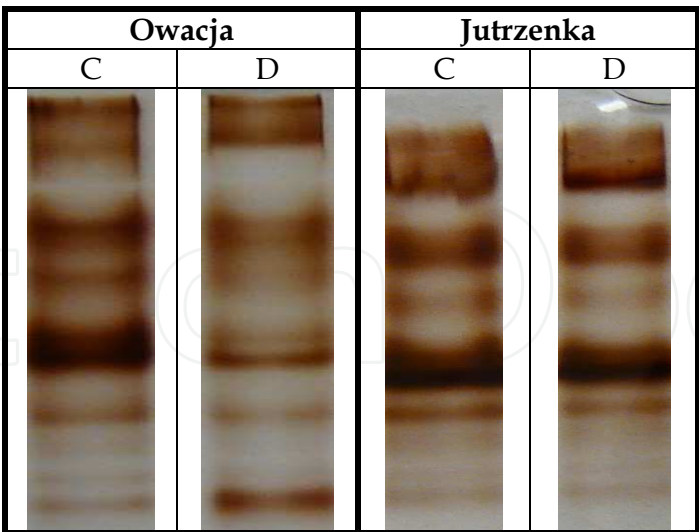


Fig. 6. Peroxidase activity in potato tubers of control, fully turgid plants (C) and in tubers of plants subjected to dehydration (D) of two potato cultivars.

Increased activity of peroxidase band activity more in leaves of drought susceptible cultivar than in resistant cultivar may suggest their involvement, at least in part, in defence against dehydration-induced injuries of plant tissues. One can speculate that this enzyme may

locally detoxify over-abundance of  $\text{H}_2\text{O}_2$  by cell wall lignification and chlorotic lesion formation. Higher responsiveness of leaf and tuber peroxidase of sensitive potato plants exposed to soil drought seems to suggest the involvement of this enzyme in limitation of  $\text{H}_2\text{O}_2$  amount.

The only enzyme able to dismutate  $\text{O}_2^-$  to  $\text{H}_2\text{O}_2$  and  $\text{O}_2$  is SOD. An increase in SOD activity under diverse abiotic stresses has been shown in several plants (Gill & Tuteja 2010). In extracts obtained from potato cultivars, three activity bands of superoxide dismutase (SOD) were observed (Boguszewska et al., 2010). In the case of two other potato cultivars, the similar pattern of SOD activity bands was observed (Fig. 7). In response to drought, a stable amount of activity bands was observed in tubers of cv. Jutrzenka, whereas in tubers of cv. Owacja the increased amount of activity bands of SOD was clearly visible. Thus, the response of potato to soil water shortage referring to SOD activity bands was only visible in sensitive cultivar. Substantial increase in the activity of SOD at tuberisation stage of potato plants under soil drought may suggest that they play an important role in  $\text{O}_2^-$  scavenging and  $\text{H}_2\text{O}_2$  formation within cells and are involved in an adjustment of plants to this environmental stress as it was suggested earlier (Matters & Scandalios 1986). In contrast to the sensitive plants, practically unchanged activity of SOD bands in plants of drought tolerant cultivars seems to suggest that that under water deficiency cell compartments most probably differ in  $\text{H}_2\text{O}_2$  concentration.

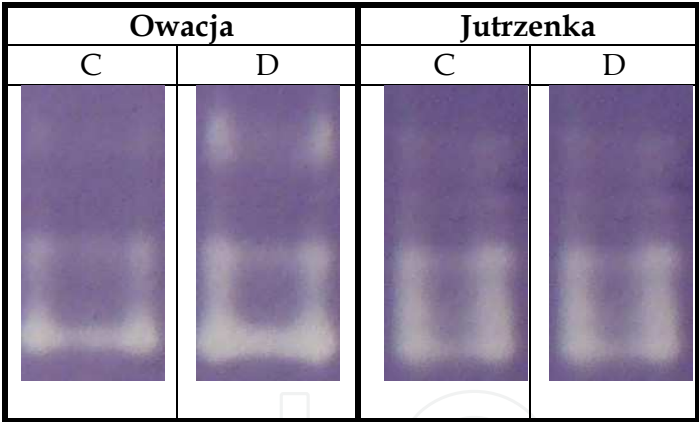


Fig. 7. SOD activity in potato tubers of control, fully turgid plants (C) and in plants subjected to dehydration (D) of two potato cultivars

The last enzyme investigated was catalase. Catalase is a key enzyme catalysing  $\text{H}_2\text{O}_2$  decomposition (Apel & Hirt 2004, Mittler et al., 2004). In tubers of both potato cultivars two forms of catalase, namely CAT-2 and CAT-3, fairly the same in activity were detectable (Fig. 8). In tubers of drought treated plants the activity of two catalase activity band decreased, more in cv Jutrzenka than in cv. Owacja. Such observation may indicate that the concentration of  $\text{H}_2\text{O}_2$  has been limited to the level that was putatively not able to activate catalase efficiently. Although, the reason of CAT decrease is not clear yet, our results are consistent with the previous suggestion that in plants subjected to abiotic stress the diminished activity of catalase and peroxidase in tubers of drought sensitive cultivar resulted in localized increases of ROS and mediates in drought injuries of cells.

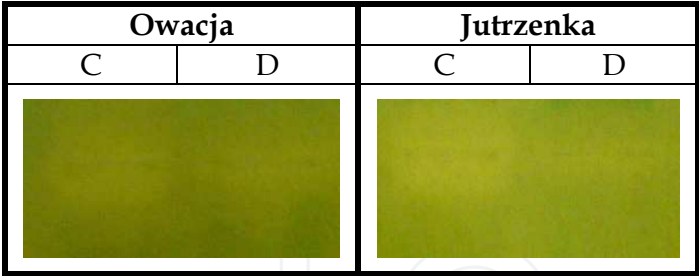


Fig. 8. CAT activity in potato tubers of ccontrol, fully turgid plants (C) and in tubers of plants subjected into dehydration (D) of two potato cultivars

In conclusion, ROS-scavenging enzymes investigated in potato tubers of two cultivars differing in drought tolerance respond differently to a two-week soil drought. A strong increase in the activity of peroxidase in leaves of potato of sensitive cultivar exposed to drought suggests that this cultivar suffers from oxidative stress and high amounts of ROS may be limited insufficiently as indicated higher wilting intensity and weak regeneration ability of this cultivar. On the contrary, a strong increase of SOD together with a decrease of peroxidase and catalase activities in tubers of sensitive cultivar may indicate on changed conversion of  $O_2^-$  to  $H_2O_2$ . This findings show that SOD rather than peroxidase and catalase are a target antioxidant protein protecting tuber tissues against oxidative damage.

The present experiments and those made earlier (Boguszewska et al., 2010) showed that the yield of potato tubers (agricultural yield) dependend more on the regeneration ability of rewatered plants after soil drought treatments than on the water loss from the leaves. Although leaf relative water content (RWC) is considered a reliable and widely-used indicator of the plant sensitivity to dehydration (Rampino et al., 2006, Sanchez-Rodriguez et al., 2010), the correlation between RWC in leaves of ten potato cultivars investigated and yield decrease was poor. Moreover, the observed differences in RWC in leaves of investigated potato cultivars indicated that neither the time course of dehydration nor the attained leaf RWC values related to tuber yield. The observed differences in leaf RWC attained at the same drought period indicated only that the investigated cultivars differed in characteristics responsible for dehydration avoidance.

However, the RWC reflected guaiacol peroxidase activity in droughted potato leaves i.e. a high correlation was observed between the relative increase in guaiacol peroxidase activity (i.e. POX activity in droughted leaves minus POX activity in turgid leaves) and RWC in potato leaves. These results are in full agreement with a substantial increase in guaiacol POX activity in maize seedlings acclimated to suboptimal growth temperature (Prasad 1996). It may indicate that a high activity of guaiacol POX induced by such diverse stresses as soil drought and suboptimal growth temperature is a common response of plants to unfavourable environmental conditions. The activity bands of guaiacol peroxidase confirmed the spectrophotometric measurement of POX activity: the bands with the highest activity and an increase in number of activity bands were observed in in dehydrated leaves of cultivar with a yield decrease of 49% whereas in leaf extracts from cultivar with a yield decrease of 26%, only two new activity bands appeared. In contrast, the response of POX in potato tubers was cultivar dependent i.e. it was either negative (activity of peroxidase bands decreased) or remained at the level of control, turgid plants. Increased activity of POX and

SOD in leaves and tubers of dehydrated cultivars tolerant to soil drought seemed to counteract the accumulation of ROS and in effect protected plants against loss of yield.

## 10. Perspectives

As discussed above, significant progress has been made in understanding the biological role of reactive oxygen species in plant growth and development. Moreover, a growing number of data that have been identified indicate on participation of ROS in plant responses to both, abiotic and biotic stresses. It is becoming increasingly evident that many different stresses activate endogenous production of ROS and that these are not only a deleterious effect of oxidative metabolism but are necessary for plant intracellular communication system. ROS signaling and integration into many others signaling networks together with antioxidants has been shown to be involved in acclimation ability of plants to unfavourable environmental conditions and their responses to pathogen attack. It seems to be of importance to decipher ROS signaling mechanisms because it may lead to the development of agricultural important plants more tolerant to suboptimal conditions of growth. Thus, it should have a significant impact on enhanced and stable yield of plants under less favourable conditions of growth to provide plants for fibbers, improving human health, human food and feed for animals.

It is hoped that the next decade will address many of the unanswered questions pertinent to plant functioning under adverse environmental conditions. We think that one of the most important questions still unanswered is mechanism(s) of ROS sensing and signal transduction. Up to date, sensors of ROS are mostly unknown. We think that the next important question concerns the way in which antioxidants provide the most important information on plant redox state and how they affect gene expression associated with plant responses to both, biotic and abiotic stresses to maximize plant defence systems. It is hoped also that the coming years will shade more light on involvement of photosynthetic and respiratory electron transport chains in ROS signaling pathways, post-transcriptional regulation of gene expression and modification of proteins important for plant survival.

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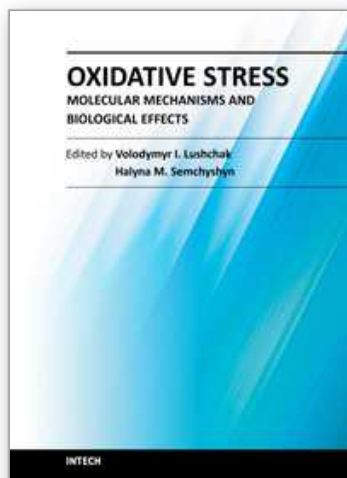
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## **Oxidative Stress - Molecular Mechanisms and Biological Effects**

Edited by Dr. Volodymyr Lushchak

ISBN 978-953-51-0554-1

Hard cover, 362 pages

**Publisher** InTech

**Published online** 25, April, 2012

**Published in print edition** April, 2012

Since the discovery of free radicals in biological systems researchers have been highly interested in their interaction with biological molecules. Denoted in 1980, and due to fruitful results and ideas, oxidative stress is now appreciated by both basic and applied scientists as an enhanced steady state level of reactive oxygen species with wide range of biological effects. This book covers a wide range of aspects and issues related to the field of oxidative stress. The association between generation and elimination of reactive species and effects of oxidative stress are also addressed, as well as summaries of recent works on the signaling role of reactive species in eukaryotic organisms. The readers will gain an overview of our current understanding of homeostasis of reactive species and cellular processes they are involved in, as well as useful resources for further reading.

### **How to reference**

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Dominika Boguszezewska and Barbara Zagdańska (2012). ROS as Signaling Molecules and Enzymes of Plant Response to Unfavorable Environmental Conditions, *Oxidative Stress - Molecular Mechanisms and Biological Effects*, Dr. Volodymyr Lushchak (Ed.), ISBN: 978-953-51-0554-1, InTech, Available from:  
<http://www.intechopen.com/books/oxidative-stress-molecular-mechanisms-and-biological-effects/ros-as-a-signaling-molecules-and-enzymes-of-plant-response-to-unfavorable-environmental-conditions>

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Phone: +86-21-62489820  
Fax: +86-21-62489821

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