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Chapter VI

Plant Responses to Stresses: Role of Ascorbate Peroxidase in the Antioxidant Protection

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Abstract

The change in activities and numerous molecular forms of antioxidant enzymes is one of the parameters of plant biological tolerance against constantly changing environmental conditions. A group of genes involved in the synthesis of ascorbate is used in genetic engineering for the improvement of plant tolerance to biotic and abiotic stresses. The level of H_2O_2 in plant cells is controlled by two enzymes: catalase and ascorbate peroxidase (APX), though the key role in decomposition of H_2O_2 belongs to APX. Ascorbate peroxidase converts H_2O_2 into H_2O and O_2. The APX activity and electrophoresis spectra of its isoenzyme content have been investigated in plants subjected to abiotic (soil drought) and biotic (virus infections) stresses. The analysis of electrophoretic spectra of four isoforms in wheat leaves at the beginning of drought (flowering phase) revealed four isoforms. The amounts of APX isoforms increased to 7 during the wax ripeness phase both in irrigated plants and plants subjected to drought. The long-term drought led to the increase in the intensity of high-molecular isoforms and emergence of three additional isoforms of the enzyme. In this instance, the APX activity level remained higher in drought tolerant wheat genotypes compared with the sensitive ones. At the same time, in both tolerant and sensitive varieties, enhanced APX activity compared with the control variants was observed at the end of drought, which is apparently related to the synthesis of new molecules of the enzyme (i.e. it is stipulated by

the changes of genome expression under stress). The immune enzymatic method revealed the presence of CMV virus in *C. sativus* L. plants and TMV in *L. esculentum* Mill. plants. The obtained results show significant differences in APX activities in all virus infected leaves compared with controls. The effect of the viral infection caused approximately a 1.6-fold increase of the APX activity in *L. esculentum* Mill. and a 2.1-fold increase in *C. sativus* L. compared with corresponding controls. Pathogenesis was accompanied by an increase in the quantity of APX isoforms, which reached 5 in leaves of *L. esculentum* Mill. and by the formation of 2 new isoforms and intensification of the enzyme electrophoresis spectra in infected leaves of *C. sativus* L. compared with the control variants. Thus, an increase in ascorbate peroxidase activity and *de novo* synthesis of its numerous molecular forms under stress conditions testify its involvement in the antioxidant defense system.

Keywords: Ascorbate-glutathione cycle, ascorbate peroxidase, soil drought, pathogens, Cucumber mosaic virus, Tomato mosaic virus, wheat genotypes, vegetable crops

Introduction

A significant part of agricultural plants perishes annually in consequence of the abiotic and biotic stress effects. One of the central problems of the modern plant biology is the investigation of genetically controllable mechanisms of plant tolerance to different stresses. Plants possess common mechanisms in their physiological tolerance to drought, low temperature and salinization. The cell degradation caused by different stresses triggers a range of biological, physiological and structural changes in plant cells. These changes especially occur in the expression of numerous genes. The primary change of the total plant metabolism subjected to any stress is emergence of free radicals in cytoplasm and other cell components, i.e. emergence of the so called oxidative stress. Development of photooxidative processes in a plant organism is accompanied by an excessive accumulation of active oxygen forms (AOF) in cells. The content of AOF is under multilevel control of antioxidant system enzymes (Mitteler, 2002; Apel and Hirt 2004). One of the parameters of plant biological tolerance to constantly changing environmental conditions is the change of activities and numerous molecular forms of antioxidant enzymes. Superoxide dismutase, which declines superoxide concentrations, plays a principal role in elimination of AOF (Merzlyak, 1989), as well as catalase, peroxidase and enzymes, involved in the ascorbate-glutathione cycle that remove an excess of peroxides (Scandalios, 1993).

Two strategic approaches using genetic engineering techniques currently exist for improvement of the plant tolerance to abiotic and biotic stresses. The first approach is based on the introduction into genome and overexpression of a gene or groups of genes, determining plant tolerance to stress. Groups of genes involved in the ascorbate synthesis are used in the second approach. Ascorbate peroxidase deactivates H_2O_2 by converting it into H_2O and O_2. The H_2O_2 level in plant cells is controlled by two enzymes: catalase and ascorbate peroxidase (APX), although a key role in the destruction of H_2O_2 belongs to APX (Asada, 1992).

Ascorbate peroxidase (EC 1.11.1.11) is related to a peroxidase family that includes the microbial peroxidase, yeast cytochrome c peroxidase and prokaryotic catalase-peroxidase (Takeda et al., 2000; Shigeoka et al., 1980; Wilkinson et al., 2002).

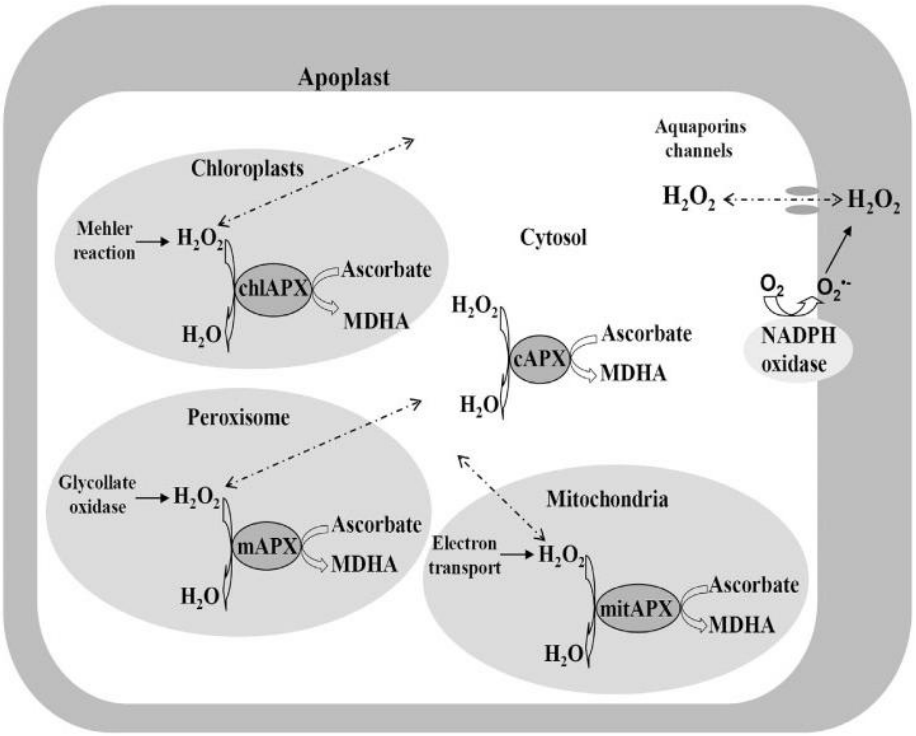


Figure 1. The ascorbate-glutathione cycle (*Halliwell- Asada cycle*). Enzymes of the ascorbate-glutathione cycle. The Mehler reaction-formation and accumulation of H_2O_2 in chloroplasts under stress conditions (Caverzan et al., 2012).

APX differs from guaiacol peroxidase not only in the specific donor, but also in the amino acid sequence and physiological functions. Ascorbate peroxidase (Mr 28-58 kDa) contains heme iron. It was detected in all intracellular compartments such as cytosol, chloroplasts, mitochondria and plant cell thylakoids (Mittler and Zilinskas, 1992; Bunkelman and Trelease, 1996; Ishikawa et al., 1996, 2008; Jimenes et al., 1998). Stromal and thylakoid-bound ascorbate peroxidases were isolated from chloroplasts. The thylakoid form is located predominantly adjacent to the acceptor part of PS I, providing the interception of H_2O_2 molecules close to their formation place.

The ascorbate peroxidase reaction is a central process of the whole cycle of reactions directed to the elimination of the main active oxygen forms in chloroplasts. The ascorbate-glutathione cycle is an example for such a cycle (Figure 1).

It functions in different compartments of cells: cytosol, peroxisomes, mitochondria, but mainly in chloroplasts of the plants lacking catalase, being the only means of H_2O_2 elimination. The cycle includes the following enzymes: ascorbate peroxidase, monodehydroascorbate peroxidase, dehydroascorbate peroxidase and glutathione reductase. During the functioning of the cycle, H_2O_2 is reduced in the presence of ascorbic acid. The oxidized glutathione is reduced in the presence of NADPH. The main enzyme of the cycle is ascorbate peroxidase. The enzyme is highly specific to ascorbate and rapidly loses activity in the absence of it. The ascorbate peroxidase reaction results in the formation of monodehydroascorbate-radical (MDA), which is reduced to ascorbate (Asc) by the stromal monodehydroascorbate reductase (EC 1.6.5.4):

$$H_2O_2 + 2Asc \rightarrow 2MDA + 2H_2O \quad (1)$$

$$2MDA + NAD(P)H+H^+ \rightarrow 2Asc + NAD(P)^+ \quad (2)$$

The thylakoid monodehydroascorbate reductase reacts to ferrodoxin instead of NAD(P)H. Disproportionation of monodehydroascorbate reductase leads to the formation of a relatively stable product dehydroascorbate (DHA), which is converted into ascorbic acid in the presence of dehydroascorbate reductase. At the same time oxidization of the reduced glutathione (GSH) occurs together with the formation of the appropriate dithiol (GSSG) (Foyer and Noctor, 2005):

$$2GSH + DHA \rightarrow Asc + GSSG \quad (3)$$

Photoreduction of O_2 in the electron-transport chain (ETC) of chloroplasts, or the Mehler reaction, is a well-known physiological alternative pathway of the electron transport in photosynthesis, including photosynthesis under abiotic stress conditions. However, there is still no consensus on the importance of the Mehler reaction. The question remains: does the activation of the photoreduction of O_2 promote the enhancement of the plant tolerance or lead to the initiation of the oxidative stress. In recent years extensive data have been obtained in a range of laboratories. Analysis of these data confirms the necessity of the formation and even accumulation of H_2O_2 in chloroplasts under stress conditions (Mubarashkina et al., 2006). The accumulation of H_2O_2 in chloroplasts has been shown to be the key factor of the systemic acquired acclimation of plants, in particular under conditions of high light intensity. It was observed that high H_2O_2 concentrations in chloroplasts could defend photosynthetic apparatus from photoinhibition. There are numerous reports on the photosystem I (PS I) as the main place of the formation of $O_2^{\bullet-}$ and H_2O_2 as a result of the electron transfer to O_2. At the same time according to the redox potentials of ETC carriers, the Mehler reaction is practically possible in any site of the ETC. Considering the necessity of the protonation of $O_2^{\bullet-}$, photoreduction of O_2 to H_2O_2 most probably occurs at the PS II level or the pool of plastoquinones.

Some modern investigations have confirmed this hypothesis proposed by scientists earlier. The study of the O_2 photoreduction in chloroplasts of plants, grown under different stress conditions and differing in their tolerance to unfavorable factors revealed the tendency to redistribution of electron flows in the ETC, namely, to decline in the O_2 photoreduction intensity at the PS I level and activation of the H_2O_2 photoformation in tolerant plants. Thus, the investigation of the photosynthetic electron transport in two different wheat varieties with contrasting drought tolerance showed the activation of O_2 photoreduction with the formation of H_2O_2 in the Mehler reaction in leaves of a tolerant variety and its decline in a sensitive variety under drought conditions. In contrast, the electron transport to O_2 with the formation of $O_2^{\bullet-}$ at the acceptor side of PS I decreased 7 times in chloroplasts of a tolerant variety and changed slightly in a sensitive variety as water deficiency of leaves increased (Allen and Hall, 1974).

Drought belongs to one of the most prevalent and crucial unfavorable environmental factors, which causes a sharp decline in the productivity of most agricultural crops (Abedi and Pakniyat, 2010; Passioura and Angus, 2010; Aliyev, 2012). Scientists of the Stanford University established that a 2°C increase in temperature above the average reduced the maturation period of wheat by nine days. This led to a 20% yield decline. Severe water deficiency in soil detains

biosynthesis of organic compounds and intensifies hydrolysis leading to the disturbance of growth processes (Sayar et al., 2008; Tas and Tas, 2007; Khan and Naqvi, 2010). The process of oxidative phosphorylation and ATP synthesis is also disturbed. The activity of proteases increase leads to a decline in protein content and an increase in low-molecular products of their decomposition; the carbohydrate exchange is disturbed (Al-Khatib and Paulsen, 1999). Soil drought can also facilitate the generation of active oxygen forms (AOF), especially when it is accompanied by high solar insolation (Fu and Huang, 2001). Over the past decades, drought tolerance of plants has been successfully increased in many countries (Fischer et al., 2006, Burke et al., 2006). Plant tolerance to water deficiency can certainly be provided by different mechanisms, the efficiencies of which depend on climatic conditions (Lafitte et al., 2007; Takase et al., 2011). A clear relationship between drought tolerance and contents of osmotics has been revealed, and introduction of genes encoding enzymes, catalyzing the formation of osmotic active products into the plant genome, is being used over the last few years. For example, tolerance to increased temperatures improved when the peroxisomal APX gene is expressed in transgenic Arabidopsis plants (Shi et al., 2001). Transgenic tobacco plants with the expression of the *tAPX* gene in chloroplasts were characterized by an enhanced tolerance to freezing and high light intensities (Yabuta et al., 2002). Tobacco transformants expressing SOD cDNA of alfalfa appeared to be resistant to paraquat, heavy metals, hydrogen peroxide and sodium chloride and can grow back after 35-day periods of drought (Oberschall et al., 2000).

Anthropogenic impacts on biota have currently become one of the most important environmental factors. Therefore an assessment of the adaptive capacity of living organisms that inhabit in radiation biocenoses is particularly relevant. The effect of pollutants on the production of AOF has been extensively studied lately. Ions Fe^{2+}, Fe^{3+} and/or Cu^{2+} have been shown to catalyze the formation of free radicals in the Fenton and Haber-Weiss reactions (Dat et al., 2000; Mitteler, 2002). In the Fenton (equations 4, 5) and Haber-Weiss (equation 6), reactions with the involvement of the mentioned ions superoxide radical and hydrogen peroxide form the hydroxyl radical, which is the most powerful oxidizer (Dat et al., 2000; Mitteler, 2002):

$$O_2^{\bullet-} + Fe^{+3}\,(Cu^{+2}) = O_2 + Fe^{+2}\,(Cu^{+}) \quad (4)$$

$$Fe^{+2}\,(Cu^{+}) + H_2O_2 = Fe^{+3}\,(Cu^{+2}) + HO^{\cdot} + HO^{-} \quad (5)$$

$$O_2^{\bullet-} + H_2O_2 = HO^{\cdot} + HO^{-} + O_2 \quad (6)$$

In addition to the mentioned metals, zinc, nickel, aluminum, cadmium, lead, etc. also cause an active generation of AOF (Devi, Prasad, 2005; Fang, Kao, 2000). Cell compartmentation of this process is still unclear. Polesskaya (Polesskaya, 2007) suggested that the reason for the oxidative stress under these conditions could be the effect of the simultaneous toxic diffuse of metal ions on the most enzymatic systems and membranes, as well as declines in the efficiency of antioxidant systems as a consequence of the diversion of reduced glutathione to the synthesis of phytochelatins, which detoxify them as a result of conjugation with pollutants. Transgenic plants of *Pinus virginiana* Mill. with increased antioxidant enzyme (ascorbate peroxidase, glutathione reductase) activities were described (Tang et al., 2005). These plants were shown to possess an enhanced tolerance to different stresses, which increase their survivability, particularly in metal-polluted soils. To maintain

homeostasis in the presence of heavy metal toxic concentrations plants synthesize phytochelatins from glutathione. They bind heavy metals and form complexes with Cd, Ag, Pb, Zn, Cu and Hg, providing their detoxification (Clemens et al., 1999). However, activities of the antioxidant enzymes of plants from nature ecosystems, living for a long time under conditions with increased but not lethal contents of such metals as nickel, zinc, iron and manganese have not been studied sufficiently. Radionuclides penetrating into plant tissues out of soil cause significant changes in cell metabolism, operating as stress factors, which is the reason for increased APX activity when soil is polluted with different natural radionuclides. Then plants proceed to stress state and their signal warning systems are activated. APX is one of the components of such systems. Therefore the increase in its activity in response to stressors, in this case - radionuclides - is quite clear. This enzyme could probably be considered as a marker of the plant stress state when soil is contaminated with different radionuclides.

In addition to the data on the intensive generation of active oxygen forms under the influence of abiotic factors, a large amount of experimental data on the induction of AOF under biotic stress have been compiled. The intensification of the generation of AOF causes damage to plants by bacteria, fungi and microorganisms (Kolupayev, 2007; Maksimov, Cherepanov, 2006; Mohamed, Raldugina et al., 2006; Tarchevskiy, 2002). In vegetables more than 100 specific viruses of different taxonomic groups that differ in their biological, economic, serological properties and modes of transmission were registered. Seven exciters, spread by contact, insects and through soil were identified only in cucumber. 36 viruses and their strains from 12 genera were registered in tomato (Keldish, 2006). Viral infections of crops cause significant yield losses, impairment of the quality of agricultural products and are considered as a serious threat for food security. This problem concerns all food, feed and industrial crops cultivated in any region of the Earth and is especially actual for vegetatively propagated plants, since progressive accumulation of viruses in a range of generation leads to the complete infection and degradation of a variety. Annual losses of one crop in a certain region caused by viruses are often expressed in hundreds of millions (and billions) of dollars (Kegler et al., 1986; Hull and Davies, 1992; Waterworth and Hadidi, 1998; Strange and Scott, 2005).

The important problem of modern biology is the study of mechanisms of the induction of plant resistance to the effects of pathogens. In response to the pathogen infection, defense reactions, such as enhancement of the barrier properties (lignification), expression of genes and synthesis of proteins, including enzymes and especially peroxidases are switched on (Rogozhin, 2010). The role of peroxidases in plant defense mechanisms during pathogenesis was extensively studied (Maksimov, Cherepanova, 2006; Minibayeva, Gordon, 2003; Tarchevskiy, 2002; Baker et al., 1997; Bolwell et al., 2002; Zhao, Sakai, 2003; Kuznaik, Sklodowska, 2005). It was established that some peroxidases were located in the apoplastic space of plant cells (Betwick et al., 1998). The peroxidase levels were shown to be higher in cells of plant varieties resistant to pathogens compared with the sensitive ones. Similar data was obtained particularly for potato (Graskova et al., 2005) and pea (Kozyavina, 2012).

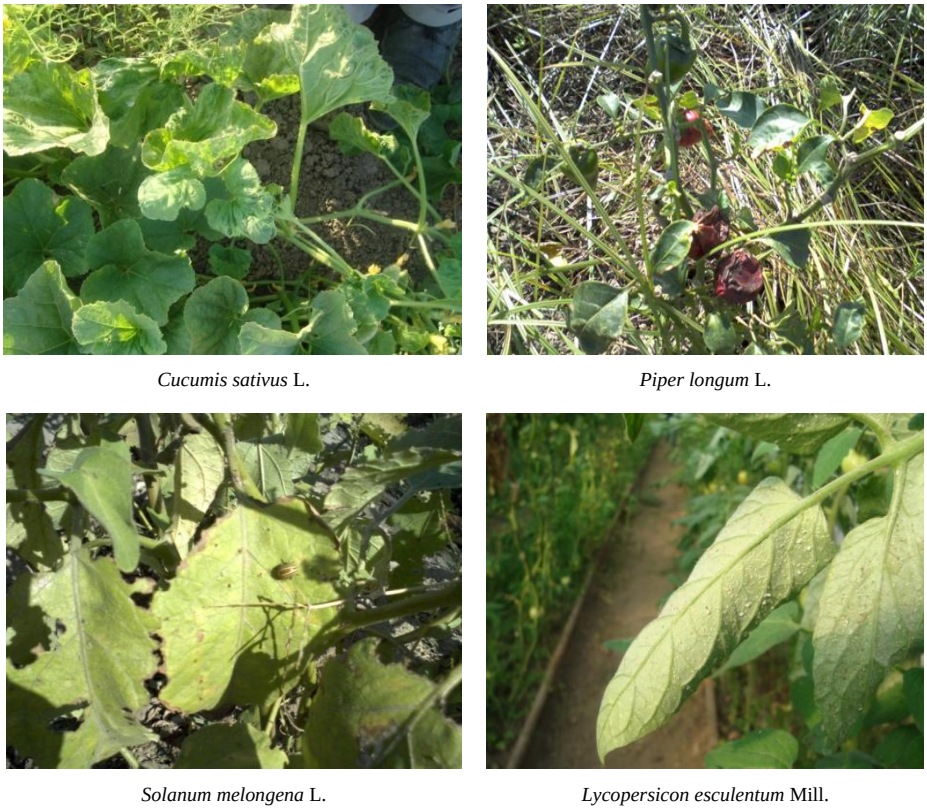
Therefore the investigation of the protective role of ascorbate peroxidase in plant adaptation to the changing environmental conditions, including drought and pathogen microbiota, is highly relevant.

Materials and Methods

Six durum (*Triticum durum* Desf.) and bread (*Triticum aestivum* L.) wheat genotypes contrasting for drought tolerance, such as Barakatli-95, Gyrmyzy bugda, Azamatli-95 (drought tolerant), Garagylchyg-2, Gyrmyzy gul-1, Giymatli-2/17 (drought sensitive) were chosen as investigation objects. Plants were grown in the field of the Absheron Experimental Station of the Research Institute of Crop Husbandry (Baku, Azerbaijan) under conditions with a normal water supply and water deficiency (Figure 2). Control plants were grown under normal irrigation conditions. The use of just such a model system allowed us to study dynamics of the activities of antioxidant enzymes at the earliest stages of drought and till the end of the vegetation.

Figure 2. Wheat genotypes grown under conditions of severe water deficiency at the experimental station of the Research Institute of Crop Husbandry.

The objects of study were also samples of various vegetable crops grown in field conditions at the Experimental Station of the Research Institute of Vegetable Growing (Baku, Azerbaijan), including mainly pepper, tomato and eggplant with symptoms of viral diseases. At first phenological and phytopathological investigations were carried out. The first symptoms of the infection include deformation of young leaves, yellow engraving and enlightenment of veins. As the infection progressed symptoms of chlorosis, mosaic, mottling and distortion of leaves were observed (Figure 3). An *immunoenzyme analysis*-based *test system* revealed the presence of viruses CMV (Cucumber mosaic virus) in *Cucumis sativus* L. and TMV (Tomato mosaic virus) in *Lycopersicon esculentum* Mill. (Clark, Adams, 1977).



Cucumis sativus L. *Piper longum* L.

Solanum melongena L. *Lycopersicon esculentum* Mill.

Figure 3. Distortion, chlorosis, mosaic, yellow gravity and enlightenment of veins in young leaves of viral infected vegetable crops.

Activities and molecular forms of APX were studied in wheat genotypes subjected to drought and vegetable crops infected with viruses. A sample of plant tissue was ground in 50 mM K-phosphate buffer (pH 7.8) containing 1 mM PMSF, 1 mM ascorbic acid and 1% polyvinylpyrrolidone. The homogenate was filtered through 4 layers of cheesecloth and then centrifuged at 15,000xg at 4°C for 15 min. The obtained supernatant was used for the analysis. The activity of ascorbate peroxidase (EC 1.11.1.11) was determined according to (Verma and Dubey, 2003). The reaction mixture contained 50 mM K-phosphate buffer (pH 7.0), 0.2 mM ascorbic acid, 0.2 mM EDTA, and the enzyme extraction. The reaction was initiated by adding 20 mM hydrogen peroxide. The optical density was measured at 290 nm for 120 s. with a spectrophotometer ULTROSPEC 3300 PRO ("AMERSHAM", USA). The

enzyme quantity changing the optical density of the reaction mixture by 0.001 units per unit time was assumed as the enzyme activity unit.

Qualitative changes of the enzyme activity were investigated with native polyacrylamide gel (PAGE) using the Laemmli method (Laemmli, 1970) of gel electrophoresis with some modifications. An equal amount of the enzyme extract was mixed with bromophenol blue and glycerol to a final concentration of 12.5% (v/v). Electrophoresis in 10% PAAG with a thickness of 0.75 mm and length of 16 cm was performed at 4°C for 3 hours at a steady current of 30 mA. 2 mM sodium ascorbate was added to the electrode buffer to detect APX activity. For visualization of ascorbate peroxidase, the gel was incubated in a solution containing 50 mM potassium phosphate buffer (pH 7.0) and 2 mM Na-ascorbate for 30 min. Then the gel was incubated in a solution containing 50 mM potassium - phosphate buffer (pH 7.0), 4 mM sodium ascorbate and 2 mM H_2O_2, for 20 min and stained in a solution containing 50 mM potassium phosphate buffer (pH 7.8), 28 mM TEMED and 2.45 mM nitroblue tetrazolium, for 15 min by mixing with a shaker (Mittler and Zilinskas, 1993). The protein amount was determined by the method of Sedmak (Sedmak and Grossberg, 1977). Bovine serum albumin was used for the construction of standard curve.

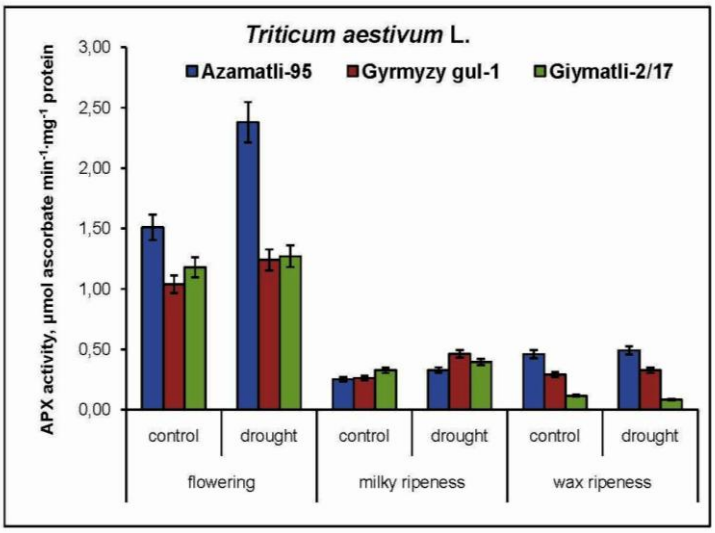
Statistical analysis. Data of the determination of the ascorbate peroxidase activity are presented as arithmetic means and standard deviations of two separate experiments carried out with three biological replicates.

Results and Discussion

Ascorbate peroxidase is one of the key components of the cell protection system against harmful effects of H_2O_2. The role of ascorbate peroxidase is very important in the oxidative process. Its activity and izoenzyme content strongly depend on the effects of both biotic and abiotic external factors. The enzyme is sensitive to changes in the composition of soils to pollution and salinization, the impact of viral and bacterial pathogens. The APX activity also increased as metabolism intensified - during the spring active growth and flowering period (when generative organs and fruit formed) (Rogozhin, 2004). The study showed that the magnitude of the constitutive activity of ascorbate peroxidase was high in wheat leaves at the beginning of the experiment (i.e. flowering stage) (Figure 4). The enzyme activity decreased in the phase of milk ripeness; afterwards it increased in the phase of wax ripeness in drought tolerant and decreased in drought sensitive genotypes. The enzyme activity decrease can be explained by the rapid inactivation of its pool during the catalytic reaction. Resynthesis of new enzyme molecules (or isozymes), apparently provided the activity increase in the wax ripeness phase. The synthesis of new molecules could be initiated by the formed peroxide considering its signaling role (Garifzyanov et al., 2011).

Dynamics of the APX functioning differed in irrigated plants of both tetraploid and hexaploid varieties of wheat. The maximum APX activity in tetraploids was observed at the end of the flowering phase, while in hexaploids it was detected at the end of the earring phase. The most maximum APX activity among all studied genotypes was found in drought tolerant genotypes Azamatli-95 and Gyrmyzy bugda at the end of the flowering stage (1.51 and 1.49 μmol/mg min, respectively).

(A)



(B)

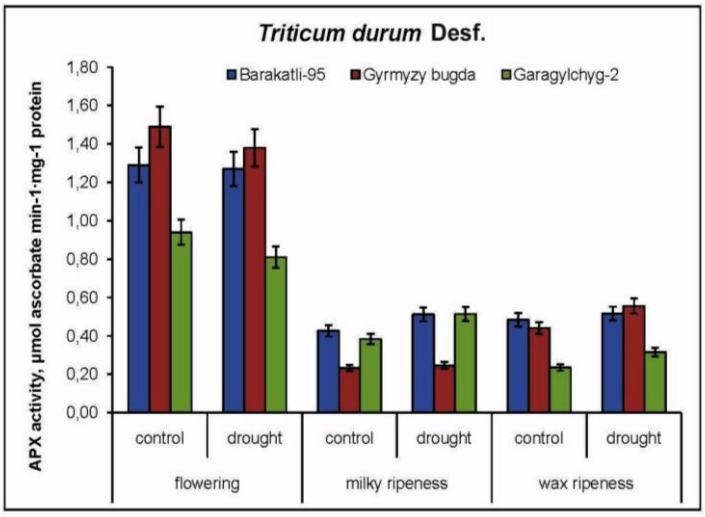


Figure 4. Dynamics of the ascorbate peroxidase activity in bread (A) and durum (B) wheat genotypes subjected to long-term soil drought.

The enzyme activities in control and drought stressed variants of tetraploid varieties did not differ significantly at the end of the flowering phase. In contrast, in hexaploids subjected to stress, the enzyme activity increased significantly at the end of the flowering phase compared with the control. However, in drought sensitive Giymatli-2/17 the enzyme activity was higher compared with the control variants during the whole ontogenesis (except for the wax ripeness phase). Thus, the reaction of the systems providing defense against harmful effects of water stress in investigated varieties of wheat were different. The level of the enzyme activity appeared to depend on the varieties of wheat, duration of drought and stages of leaf development.

The electrophoretic separation of numerous molecular forms of ascorbate peroxidase revealed significant qualitative (presence of additional bands in electrophoregram) and quantitative (changes of the color intensity of the individual bands) changes in expressivity of individual isoforms of APX during the soil drought (Figure 5A, B).

Gene expression is a rapid response to the influence of stress factors. The electrophoretic separation showed that the amount of APX isoforms varied in leaves depending on the growth stages of the plants. The least amount was observed at the beginning of the flowering phase (4 isoforms), the most - at the end of the wax ripeness phase (7 isoforms). During the drought, the amounts of APX isoforms reached seven in both variants (irrigated and drought-stressed). The increase in the intensity of the high-molecular isoform and appearance of 3 additional APX isoforms were observed in the wax ripeness phase (Figure 5B).

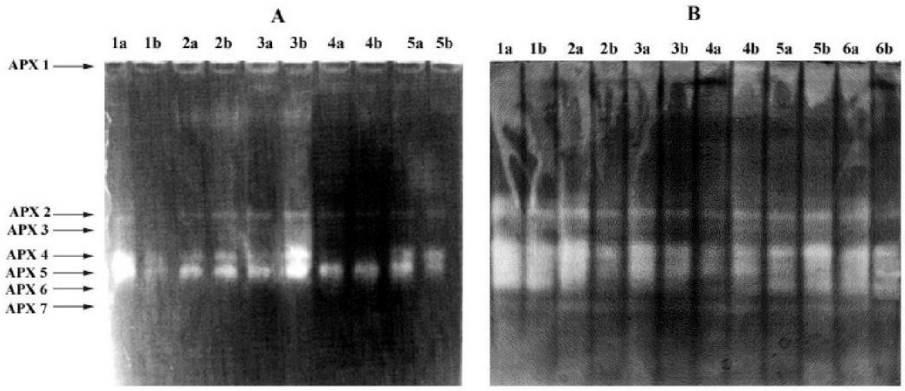


Figure 5. Electrophoretic spectra of ascorbate peroxidase in leaves of wheat grown under drought conditions. A - flowering phase, B - wax ripening phase, 1 - Garagylchyg-2, 2 - Gyrmyzy gul-1, 3 - Azamatli-95, 4 - Giymatli-2/17, 5 - Barakatli-95, 6 - Gyrmyzy bugda: a - irrigated, b - subjected to drought; Electrophoresis was carried out in 10% PAAG using tris-glycine buffer, pH 8.3 (with adding 2mM sodium ascorbate) at 4°C for 3 hours at 30mA stable current. 35μg of protein samples were applied to a gel lane.

It should be noted that the constitutive level of the APX activity was higher in tolerant genotypes, Azamatli-95 and Barakatli-95, than in sensitive ones. Moreover, at the end of the drought, increases in the APX activity compared with the controls were observed both in tolerant and sensitive varieties (except for Giymatli-2/17), which is apparently related to the synthesis of new enzyme molecules (i.e. due to the change in genome expression under stress). The enzyme activity increase under stress could be due to the activation of its latent forms or/and synthesis of new enzyme molecules.

Thus, the simultaneous increase in the activity of APX and the corresponding proteins were observed under salt stress in chloroplasts of pea (Gomez et al., 2004), and wheat (Navari-Izzo et al., 1998), in leaves of a tolerant variety of tomato *Lycopersicon penneli* (Mittova et al., 2003), confirming an intensification of the enzyme synthesis under the influence of stress factors.

The increase in the APX activity, which we observed in wheat leaves, could be related to an enhancement of H_2O_2 concentrations. Adapted wheat plants were shown to be characterized by enhanced activities of antioxidant enzymes (Selate and Khanna-Chopra, 2006). In contrast, activities of ascorbate peroxidase, superoxide dismutase and enzymes of

the ascorbate-peroxidase cycle decreased, causing accumulation of H_2O_2 excessive levels in cells of unadapted plants under drought. This caused a dysfunction of chloroplasts and mitochondria due to damage to their membranes, and, therefore, all metabolic processes in cells and the growth of plants in general.

It was recently found that the activation in expression of an ascorbate peroxidase gene *APX2* in leaves of Arabidopsis happened in the presence of the extracellular H_2O_2 pool (Bechtold et al., 2008). A treatment of cultivable soybean cells with exogenous H_2O_2 also led to changes in transcription levels of *cAPX* (Kausar et al., 2012).

These results confirm that the expression of *cAPX* genes are regulated at the transcriptional level and activated in response to H_2O_2 in cells. The accumulation of H_2O_2 through redox-signaling can play an important role in the regulation of expression of *cAPX* genes under stress conditions. The increased activity of ascorbate peroxidase confirms its apparent participation in the antioxidant system under drought conditions. Therefore, an intensification of the gene expression of antioxidant enzymes catalase (Choi et al., 2008) and ascorbate peroxidase (Park et al., 2008) by means of genetic engineering methods increased tolerance of rice plants to drought. The activities of ascorbate peroxidase in all investigated samples of infected leaves differed significantly from that in control variants (Figure 6). Effects of the viral infection caused approximately a 1.6-fold increase in the enzyme activity in studied samples of *Lycopersicon esculentum* Mill. reaching 0.36 µmol/mg min. As shown in figure 6, slight changes occurred in the APX activity in the drought stressed *Solanum melongena* L. and *Piper longum* L. compared with the control variants. Thus, in infected samples of *Solanum melongena* L. and *Piper longum* L. the enzyme activity increased only by 24% and 26% compared with the controls and was estimated to be 0.34 and 0.44 µmol/mg min, respectively.

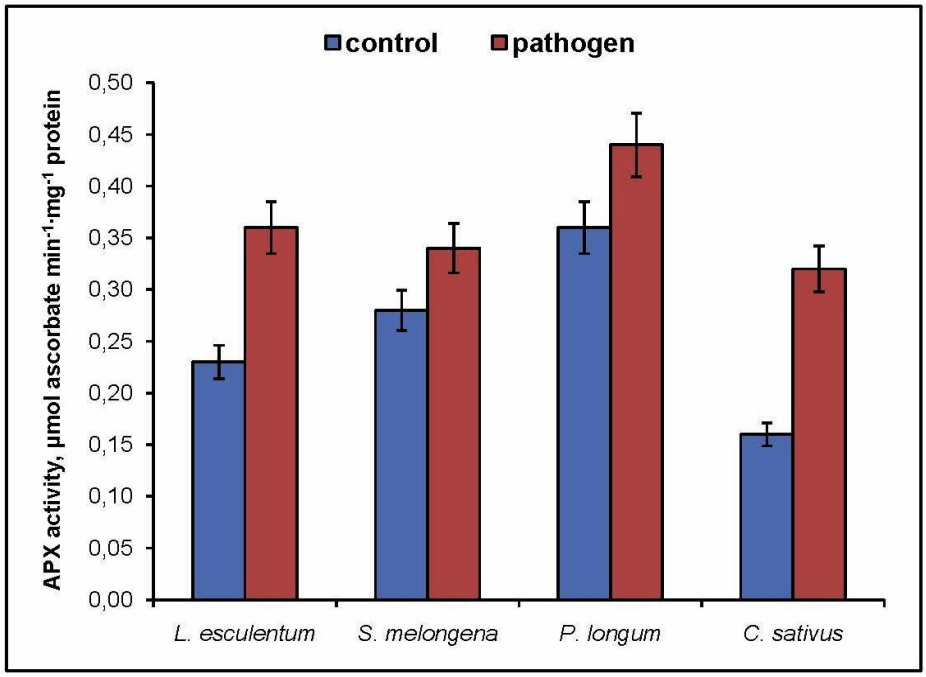


Figure 6. Activity of ascorbate peroxidase in leaves of vegetable crops infected with the viruses.

Table 1. Changes in isoform amounts in leaves of the vegetable crops infected with viruses

Plants	Isoform amounts in control plants	Isoform amounts in infected plants	Changes in isoform amounts after the infection
Lycopersicon esculentum Mill.	3	5	+2
	3	4	+1
Solanum melongena L.	3	4	+1
Piper longum L.	3	3	-
Cucumis sativus L.			

"+" - increase in the number of isoforms; "–" - the number does not change.

According to figure 6, the APX activity in infected *Cucumis sativus* L samples was enhanced significantly. In these samples the activity increased 2.1 times, reaching 0.32 μmol/mg min. Varieties of crops, as shown in the majority of reports, are characterized by a higher initial level of the antioxidative activity or are able to increase this activity more rapidly and they are more tolerant to the oxidative damage under stress conditions (Zhang, Kirkham 1996; Kang, Salveit 2002).

Pathogens infecting plants cause a rapid activation of peroxidase, often accompanying the emergence of numerous new forms and vanishing of the other forms of this enzyme (Nagy et al., 2004; Mayer et al., 2001). As shown in table 1, new APX forms did not appear in infected *Cucumis sativus* L. plants. In this case, 3 isoforms of APX were detected. The observed intensification of lane colors in the gels confirms the increase in the enzyme activity in this plant after the infection with pathogens. In the other investigated plants the infection led to the increase in the amounts of isoforms.

The intensity and composition of the electrophoretic spectrum of APX in leaves of the infected *Lycopersicon esculentum* Mill. changed compared with that of the normally developed plants. Thus, 3 isoforms were observed in the electrophoresis of the control variant. Pathogenesis under the influence of the viral infection was accompanied by the increase in amounts of APX isoforms to five and the formation of two new high-molecular forms. According to the results of the analysis during the pathogenesis the constitutive level of the enzyme activity in *Solanum melongena* L. leaves was higher compared with the control variant. Three low-molecular forms of APX occur in healthy plants, while four isoforms and an additional intensive high-molecular isoform were detected in plants with viral infections. The constitutive level of the APX activity in *Piper longum* L. also changed in infected plants, since three isoforms and an additional high-molecular form were observed in the stressed variant. The obtained data suggest that in plant cells exposed to the pathogenesis of the viral infection, the accumulation of peroxides at the early stages of the infection was inhibited by the increase in the activity of peroxidases.

Conclusion

The enhanced activity of ascorbate peroxidase and *de novo* synthesis of its various molecular forms at different stresses, testify to its undoubted involvement in the antioxidant

defense system. Using APX as a marker of the stress state allows better characterization of the protective mechanisms of plants and finding approaches for the diagnostics of the tolerance to stress factors in different agricultural crops. The obtained data can be used to develop the strategy and tactics of breeding programs to create varieties tolerant to abiotic and biotic stressors.

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