Activity and isoenzyme content of ascorbate peroxidase in wheat genotypes (*Triticum aestivum* L.) under heat stress conditions

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Wheat is a major cereal crop and considered a source of basic calories and protein for more than 80% of the world population. Heat is the major abiotic stress limiting wheat production. Antioxidant enzymes play a key role in the elimination of reactive oxygen species at elevated temperatures and play an important role in the tolerance of wheat to heat stress. İn this purpose the impact of rising temperature on wheat production is gaining concern worldwide. The activity and isoenzyme content of ascorbate peroxidase (APX), in the leaves of four bread wheat genotypes (tolerant genotypes Zirva 85 and Murov 2, stress-sensitive genotypes Gyzyl bugda and Aran), have been studied under conditions of short-term heat stress. Elevated temperatures lead to visible changes in enzyme activity. Heat stress caused a significant increase in APX activity in tolerant genotype Zirva 85. Polyacrylamide gel electrophoresis (PAGE) showed 7 isoforms of APX. Short-term heat stress caused an increase in the intensity of isoform staining in tolerant genotypes Zirva 85 and Murov 2.

Keywords: *Bread wheat, APX, isoenzyme content, heat stress*

INTRODUCTION

The most visible environmental problem in agriculture is the rise in global temperatures. By the end of 21st century, the average ambient temperature is predicted to rise by 1–6°C (Poudel et al., 2020). An increase in global temperature could have a significant impact on agricultural productivity in line with the severity of stresses associated with high temperature, drought, salinization, waterlogging. The situation of heat stress is aggravated by an increase in soil temperature as a result of an increase in air temperature associated with a decrease in soil moisture. Thus, heat stress has become a major threat to successful global crop production (Chaudhary et al., 2020; Lobell and Gourdji, 2012). Wheat is a staple food crop and usually grows over 200 million hectares worldwide. In the first half of the vegetation period, wheat plants often suffer from unfavorable environmental conditions such as drought, heat, frost, etc., which is reflected in deviations in physiological processes leading to a plant productivity decline. Crop plants in field conditions are dependent on different biotic and abiotic factors. Abiotic stresses cause high leakage of electrons towards oxygen during photosynthetic and respiratory processes leading to enhancement in reactive oxygen species (ROS) generation (Farooq et al., 2019). ROS are ubiquitous molecules produced as a consequence of normal cellular metabolism (Patil et al., 2021). In plants exposed to heat stress, destructive ROS are often formed, including singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), which are responsible for the occurrence of oxidative stress (Blum et al., 2001). Oxidative stress markedly increases membrane peroxidation and decreases membrane thermal stability in many plants, including wheat (Chakraborty and Pradhan, 2011). The most stable of the reactive oxygen species is hydrogen peroxide (H_2O_2), which plays a major role in the coordination of tolerance reactions, including the hypersensitivity reaction (Huseynova et al., 2016). To avoid potential damage to cellular components caused by ROS, as well as to maintain growth, metabolism, development, and total productivity, the balance between ROS production and removal must be strictly regulated at the intracellular level. The balance between ROS production and detoxification is maintained by enzymatic and non-enzymatic antioxidants (Caverzan et al., 2016). Changes in activity and content of antioxidant enzymes were reported as a key part of the anti-stress response to manage oxidative stress (Geng et al., 2018). Enhanced antioxidative protection leads to stress resistance and adaptation to stress

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conditions (Sarker and Oba, 2018). Reactive oxygen species produced during heat stress cause damage to membranes, lipids, proteins, DNA, etc. According to Badawi et al., 2007; Caverzan et al., 2012; Hasanuzzaman et al., 2020; Mohi-ud-Din et al., 2021, in wheat plant genotypes exposed to heat stress an increase in the activity of antioxidant enzymes was observed. However, other reports show that an increase in ambient temperature environment (from 25 to 40 ° C) during the period of grain filling leads to a decrease in the leaves the activities of these enzymes (Wu et al., 2020). A decrease in their activity with exposure to high temperatures may indicate that these plants are sensitive to high temperature exposure (Almeselmani et al., 2009). It should be noted that, in general, the response of enzymes to unfavorable exposure depends on the initial level of enzyme activity, which, in turn, is determined by the physiological state of the plant (Savicka et al., 2018). Thus, the variability in enzymatic and non-enzymatic antioxidants could be useful for breeding genotypes tolerant to a number of factors that can cause stress to plant cultivation. So, changes in enzymatic and non-enzymatic antioxidants can be beneficial for cultivation genotypes tolerant to a number of factors that can cause stress when growing plants.

MATERIALS AND METHODS

Plant material. Four bread wheat genotypes (*Triticum aestivum L.*) provided by the Gobustan RES of the Research Institute of Crop Husbandry tolerant genotypes Zirva 85 and Murov 2, stress-sensitive genotypes Gyzyl bugda and Aran were used in the present study. The seeds were treated with a 3% peroxide solution for 20 min and soaked in the dark for 24 h. Then the germinated seeds were planted in vessels with soil (1 soaked seed was sown in each vessel). Plants were grown for 12 days in an automated mini-phytotron with a controlled temperature of 19°C-23°C with a relative air humidity of about 50%, at a photoperiod of 8/16 (dark/light). 12-day-old plants were subjected to heat stress. For preadaptation, the plants were exposed to heat shock for 30 min at 38°C, the temperature gradually increased to 40°C, and the stress lasted 30 min, then the plants were subjected to severe heat shock by raising the temperature to 42°C for 2h. Such a scheme of experiments envisaged the possibility of developing acquired thermal tolerance and the effect of "soft" and "hard" thermal regimes on plants. The leaves were wrapped in foil and immediately frozen in liquid nitrogen. The obtained samples were stored in a refrigerator at -80°C.

Isolation of the enzyme extract. 0.5 g of leaf sample was crushed in liquid nitrogen and homogenized in 100 mM Na-phosphate (pH 7.8) buffer solution containing 1 mM EDTA, 2 mM PMSF, 1% PVP and 0.1% Triton X-100. Samples are centrifuged at 4 C at a rate of 15,000 g for 20 minutes. The obtained supernatant was used to determine the activity of antioxidant enzymes and isoenzyme content.

Determination of the activity of antioxidant enzymes. The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was determined spectrophotometrically based on the decomposition of H_2O by the enzyme ascorbate peroxidase at a wavelength of 290 nm for 1 minute (Nakano and Asada,1981). The reaction medium consists of 0.1 mM EDTA (pH 8.0), 0.05 mM ascorbate, 0.1 mM hydrogen peroxide, 50 mM Na phosphate buffer (pH 7.6) and 100 µl enzyme extract. The activity was calculated based on the decrease in the optical density during the first 30 seconds of the reaction and was expressed in µmol / (mg protein min) with a molar extinction coefficient ε=2.8 $mM^{-1}cm^{-1}$.

Determination of the isoenzyme spectrum of ascorbate peroxidase. The study of isoenzyme composition of antioxidant enzymes was studied at a constant electric current (30 mA) at a temperature of 4ºC for 3 hours using the PAAG electrophoresis method. For APX, 10% gel was used. During APX electrophoresis, 2 mM Na-ascorbate solution was added to the buffer. At the end of the process, the gels were stained with color buffers suitable for each enzyme. Qualitative changes in the enzyme activity were determined using native polyacrylamide gel electrophoresis (PAGE) according to the method of Davis (1964). The enzyme extract in 50% glycerol with 1% bromophenol blue was applied to the 8% polyacrylamide gel. Electrophoresis was carried out for 3h at 4°C with a steady current of 30 mA, using the device SE 250 (Amersham Biosciences, USA). Following electrophoretic separation, the gels were stained for different isoenzymes. Dyeing of APX takes place in three stages: in the first stage, 150 ml of 50 mM Na-phosphate (pH 7.0) and 2 mM Na-ascorbate solution are prepared (Mittler et al., 1993). The gel is stirred in this solution for 30 minutes in the dark, shaker, changing every 10 minutes. The volume of the second gel is 50 ml and consists of 50 mM Na-phosphate (pH 7.0), 4 mM Na-ascorbate, 2 mM H_2O_2. The gel stays in the second color under the same conditions for 20 minutes. In the third stage, it is washed under the same conditions with 50 mM Na-phosphate (pH 7.0) buffer for 1 minute. The volume of the fourth dye consists of 50 ml, 50 mM Na-phosphate (pH 7.0) buffer, 28 mM TEMED, 2.45 mM NBT. After

5 minutes of irradiating the gel with this solution, it is possible to observe the formation of light spots on a dark blue background.

RESULTS AND DISCUSSION

Heat stress tolerance in cereals is associated with an increase in the activity of antioxidant enzymes (Almesalmani et al., 2009; Mohi ud Din et al., 2020). The activity of various antioxidant enzymes (ascorbate peroxidase APX, catalase CAT, guaiacol peroxidase GPO, benzidine peroxidase BPO) is sensitive to temperature and activation occurs in different temperature ranges, but with an increase in temperature, the activity of these enzymes increases. The activity of these enzymes also depends on the tolerance or sensitivity of various varieties of grain crops, the stage of their growth, and the vegetation period (Chakraborty and Pradhan, 2011). Esfandiari et al. (2007) reported that high activity of APX in wheat cultivars was related to stress tolerance but there was no data on the correlation between the activity of APX in wheat plant leaves and abiotic stress tolerance. Kumar et al. (2019) reported that the high activity of antioxidant enzymes might be related to stress tolerance. According to Devi (2008), high activity of APX in wheat plant leaves could be associated with stress tolerance. Plant ability to endure stress conditions and recover after stress is based on the severity of stress and genotypic differences. The increased activity of antioxidant enzymes and the content of non-enzymatic antioxidants cause enhancement of the elimination processes of ROS in plants. It was found that under heat stress the activity of the enzyme changed differently in tolerant and sensitive genotypes (Fig.1).

The study shows that after heat stress the activity of the APX enzyme in stress-sensitive genotypes Aran and Gyzylbugda decreased by 1.3 and 1.5 times, respectively. The enzyme activity increased relatively 1.8 times in tolerant genotype Zirva 85 and 1.4 times in genotype Murov 2. Heat stress caused a significant increase in enzyme activity in tolerant genotype Zirva 85. Elevated enzyme activity and content of APX caused ROS scavenging processes in plants. Plant ability to get over stress is based on the severity of stress and genotypic differences. Peroxidation of cell membrane lipids by ROS causes changes in metabolism and generation of ~~the~~ secondary metabolites such as phenolic compounds, which contribute control of abiotic stress. It has been suggested that peroxidase activity determines plant tolerance under stress conditions in the early stages of plant development (Aliyeva, et al., 2020). Almeselmani et al. (2009), Orabi et al. (2019) proved an increase in the activity of antioxidant enzymes at elevated temperatures, as well as showed that the mechanism of antioxidant protection plays an important role in the tolerance of wheat genotypes to heat stress. It was also detected that the activity of APX, CAT, BPO, GPO significantly increased during all stages of growth in heat-tolerant varieties in response to heat stress, while sensitive varieties showed a significant decrease in the activity of the same enzymes under heat stress.

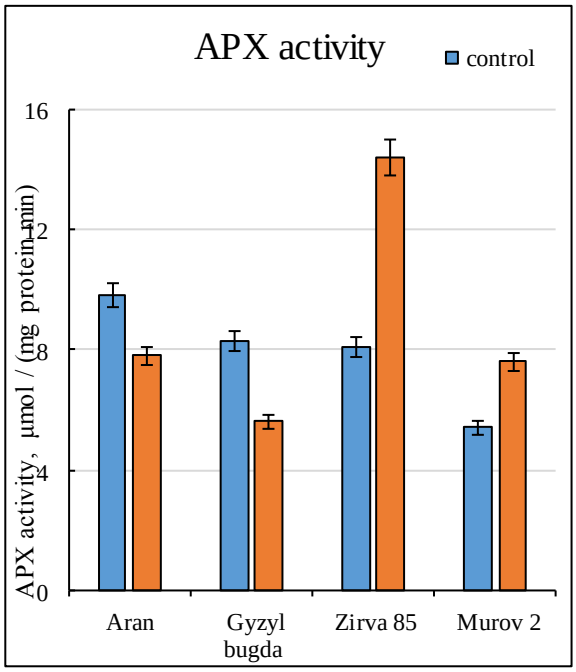

Fig. 1. Changes in APX enzyme activity depending on heat shock in bread wheat genotypes (Zirva 85, Aran, Gyzyl bugda, Murov2).

Isoenzyme content of APX was demonstrated in Fig.2 in bread wheat genotypes under heat stress. It was found that high temperature in bread wheat genotypes Zirva 85 and Murov 2 caused an increase in the intensity of isoform staining in the stress variants, while in other varieties (Gyzyl bugda and Aran) sharp visual changes were not observed. Electrophoretic analysis of polyacrylamide gel showed 7 isoforms of APX. It was found that heat shock induced the activity of this enzyme in tolerant genotypes. The study of the isoenzyme content of the APX enzyme showed that the expression of this enzyme increased in the genotypes Zirva 85 and Murov 2, and the intensity of the isoforms APX1, APX3 and APX4 was enhanced by the effect of heat shock (Fig. 2). Increased activity of enzymes in stress-tolerant genotypes plays an important role in the protection of structural elements of plant cell components, especially the photosynthetic apparatus (Laus et al., 2022).

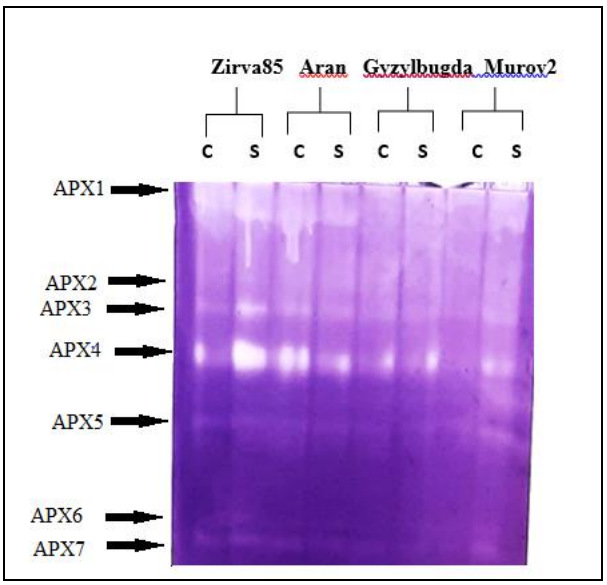

Fig. 2. Isoenzyme content of ascorbate peroxidase (APX) in bread wheat genotypes under heat stress. A separating gel of 7% acrylamide was used for APX analysis: **C**-control, **S**-stress.

The expression of genes encoding APX is stimulated by processes such as drought, salt stress, strong light, high and low temperatures, pathogenic attacks, synthesis of abscisic acid, and so on. (Caverzan et al., 2012). According to the results, it was suggested that an increase in APX enzyme expression to combat oxidative stress in heat shock tolerant genotypes.

CONCLUSION

Summarizing all the above, we can conclude that high-temperature lead to significant changes in physiological and biochemical processes, which impacts the synthesis of APX in plants. The highest level of enzyme activity was detected in the stress-tolerant genotype; however lowest rate was in the sensitive one. These changes may be part of a defense reaction or a result of plant damage.

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İstilik stresi şəraitində buğda genotiplərində (*Triticum aestivum* L.) askorbat peroksidaza fermentinin fəallığı və izoferment tərkibi

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Buğda əsas dənli bitki olub dünya əhalisinin 80%-dən çoxu üçün kalori və zülal mənbəyi hesab olunur. İstilik buğdanın məhsuldarlığını məhdudlaşdıran əsas abiotik stres faktorlardan sayılır. Antioksidant fermentlər yüksək temperatur şəraitində oksigenin fəal formalarının aradan qaldırılmasında və buğdanın distilik stresinə davamlılığında mühüm rol oynayırlar. Bu məqsədlə Dörd yumşaq buğda genotipinin (stresə davamlı - Zirvə 85 və Murov 2, stresə həssas - Qızıl buğda və Aran) yarpaqlarında qısamüddətli istilik stressi şəraitində askorbat peroksidazanın (APO) fəallığı və izoferment tərkibi tədqiq edilmişdir. Yüksək temperatur fermentlərin fəallığında əhəmiyyətli dəyişikliklərə səbəb olur. Yüksək temperatur tolerant Zirvə-85 genotipində APO-nun fəallığının ciddi dərəcədə artmasına gətirib şəxarmışdır. Poliakrilamid gel elektroforez analizi nəticəsində elektroforeqramda APO-nun 7 izoforması aşkar olunmuşdur. Qısamüddətli istilik stresi tolerant Zirvə-85 və Murov-2 genotiplərində izoformaların intensivliyinin artmasına təsir etmişdir.

Açar sözlər: *Yumşaq buğda, APO, izoenzim tərkibi, istilik stresi*