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Antioxidant response of some Georgian wheat species to simulated acid rain

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Abstract

Influence of artificial acid precipitations on the content of antioxidants, namely ascorbic acid, carotenoids, anthocyanins, total phenols and proline, and the activity of antioxidative enzymes, peroxidase and catalase, was investigated in endemic species of Georgian wheat: *Triticum timopheevi* Zhuk. (Georgian–Zanduri), and three cultivars of *T. persicum* V.–var. fuliginosum Zhuk. (Georgian–black Dika), var. rubiginosum Zhuk. (Georgian–red Dika) and var. stramineum Zhuk. (Georgian–white Dika). The experimental plants were sprayed with water solution of a mixture of sulfuric and nitric acids 2:1, with pH1.5, since the stem rising phase, at intervals of 5 days, till the end of vegetation. Control variants of the same species were sprayed with distilled water. Analyses were made in the flowering phase, a week after the last spraying. Spraying wheat plants with high acidity (pH1.5) solution caused an increase of ascorbic acid content in white Dika (by 29%, compared to control), synthesis of carotenoids and anthocyanins was activated in white Dika and red Dika (by 31% and 73% - carotenoids, and by 5% and 17% anthocyanins, respectively). Content of proline enhanced in leaves of all acid sprayed plants (by 7%, 30% and 72% compared to corresponding controls). Content of soluble phenols increased in all varieties of *T. persicum* compared with their controls (by 28%, 15%, and 37%). Activity of catalase raised in white Dika and black Dika, while activity of peroxidase was higher in Zanduri and white Dika, compared with their controls. We conclude that high acidity (pH1.5) stress caused changes in the metabolism of the antioxidative system of the Georgian wheat species, but these changes were not unidirectional. Alterations in antioxidative system, which is responsible for plant resistance to stress, indicate that Georgian endemic wheats are resistant to high acidity (pH1.5), and may be recommended as reliable material for cultivation under progressively polluting environmental conditions.

Key words: acid precipitations; ascorbic acid; catalase; Georgian wheat; peroxidase; proline; soluble phenols.

Abbreviations: AR – acid rain, SOD – superoxide dismutase, APX – ascorbate peroxidase, GPX – guaiacol peroxidase, GSH – glutathione, ASA – ascorbic acid, POD –peroxidase, CAT – catalase.

Introduction

Among the environmental pollutants, acidification of precipitations ("acid rain"), caused by increased amount of gases like SO_2, NO_X etc. (Rogozin et al., 2000; Dukhovsky et al., 2003; Chupakhina and Maslennikov, 2004) is one of the serious problems today. Acid rain changes physiological and biochemical processes in plant, comprising visible damages (chlorosis and necrosis of tissues) and invisible effects, such as decline in photosynthesis, disorder in water balance, changes in enzyme activity etc. (Ferenbaugh, 1976; Evans, 1982; Khan, Devupra, 2004; Ni et al., 2006; Rapava et al., 2007a, 2007b). Revealing acid rain-resistant cultivars and clearing the physiological mechanisms of their stability is one of the urgent needs in agriculture. It is well known that under stress conditions the defensive antioxidant system is activated, which protects the plant against stress and enables it to realize its own vital potential (Dmitriev, 2003). According to literary data the higher the antioxidant activity of plant, the more resistant it is to stress (Smirnoff, 1998). There exists enough number of investigations on the influence of acid precipitations on agricultural plants, such as vegetables, legumes, or cereals (Dukhovskiy et al., 2003; Singh, Agrawal, 2004;; Zeng et al., 2005; Ni et al., 2006; Rapava et al., 2007a, 2007b; Mai et al., 2008). Measuring the responses of different anti-oxidants in 2-year-old birch (*Betula pendula* Roth) seedlings subjected to simulated acid rain (pH 4.0) showed that acid rain increased superoxide dismutase (SOD), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) activities in leaves (Koricheva et al., 1997). The effect of simulated acid rain (pH 1.8) on activities of peroxidase and catalase in bean plants had shown that AR significantly increased peroxidase and decreased catalase activities for the first hours after treatment. Later, both enzyme activities were enhanced that could contribute to the scavenging and detoxification of active oxygen species (Velikova et al., 2000). The effect of acid rains implementation, including pH 2-5 on the matured strawberry fruits' levels of A, E, and C vitamins demonstrated that vitamin levels of sprayed plants decreased in respect of pH (Munzuroglu et al., 2005). Study of the resistance of 4 species in Magnoliaceae family and 2 varieties of *Myrica rubra* to acid rain showed that acid rain treatment at pH 5.0 is unlikely to cause a significant damage, and even have an obvious promotion to *M. platypetala*. When treated by acid rain with pH<3.5, visible foliar injuries were induced, membrane lipid preoxidation and permeability increased, chlorophyll content and the pH values of cell sap were significantly declined. With the decrease of pH value of simulated acid rain, the content of glutathione（GSH）and ascorbic acid（ASA）and the activity of superoxide dismutase（SOD）and glutathione reductase（GR）were significantly declined in two species leaves, but the activity of peroxidase（POD）increased in *Michelia platypetala* leaves (China papers, 2010 http://mt.china-papers.com/).

Leaves of 2-year *H. mitabilis* seedlings treated by pH 2.0 and 3.0 acid solutions showed visible injury spots to various extents. The membrane permeability (MP) increased significantly after acid rain stress with an extremely significant negative correlation to pH value (r= - 0.961). The maximum catalase (CAT) activity and peroxidase (POD) activity were observed at pH 2.0 (48.75mg·min^{-1}·g^{-1}·FW and 77.69U·min^{-1}·g^{-1}·FW, respectively), while that of superoxide dismutase (SOD) activity were observed at the pH 3.0(216.80U·g^{-1}·FW)(Wang et al., 2011). The unique agricultural properties of Georgian wheat species — early ripening, easy threshability, resistance to lodging and diseases, modest demands on the environment, a high content of protein and the amino acid lysine in the grains — are shared among the different species and varieties. They possess features of both wild and cultivated species; therefore they occupy a special place in the world's collection of wheat, not least as valuable material for genetic investigation (Menabde, 1950; Gorgidze, 1977). No information exists concerning the resistance of Georgian wheat species to acid stress. In our early experiments with endemic species of Georgian wheat species we have studied the influence of artificial acid rain of pH2.5 on the antioxidative system and have concluded that acid stress (pH2.5) caused changes in the metabolism of the antioxidative system of the studied species. Changes in the activity of antioxidative enzymes, alteration in carotenogenesis, the content of anthocyanins, tocopherol and ascorbic acid were not unidirectional. Each species possesses distinctive characteristic mechanisms of adaptation to acid stress (Chkhubianishvili et al., 2008). In the presented work the purpose of the study was to investigate the effect of severe acid stress (pH1.5) on the activity of antioxidative defense system of Georgian wheat species and to reveal the threshold of resistance of studied plants to acid stress.

Results and discussion

Ascorbic acid

Experimental results have shown that differences between the studied variants were significant for the content of ascorbic acid (p=0.0002) (table 1). The lowest content of ascorbic acid among the control plants was detected in Zanduri, the highest – in black Dika (Table 1). High acidity caused increase of ascorbate in white Dika (by 29%), while in black variety on the contrary – its decrease by 28%. Acid spraying diminished ASA content also in Zanduri leaves (by 46%), while in red Dika it remained at the control level. These results give a clearer picture of the difference between species and varieties by particular indices, compared with early data obtained with pH2.5 acid stress (Chkhubianishvili et al., 2008). Ascorbic acid is the principle component of redox reactions of the living organism. It takes an active part in cell immunity, and is responsible for plant resistance against stress factors. Thereby, its level may be used as a characteristic of plant's physiological state (Chupakhina, 1997; Munsuroglu et al., 2005). Presumably high content of ascorbate in sprayed variants of white Dika demonstrates its higher resistance against the severe acid stress (pH1.5) by this index, compared with other studied variants. The lowest index of Zanduri may indicate that the resistance of this species to severe acid stress is at the threshold.

Carotenoids

It is established that carotenoids together with light-antenna function protect the photosynthetic apparatus against stress by scavenging the active oxygen (Strzhalka et al., 2003). According to the obtained results, the control variant of Zanduri significantly prevailed Dika varieties by the content of carotenoids (Table 1). Spraying with acid solution reduced the amount of carotenoids in black Dika and Zanduri (by 29% and 27% respectively), while in white and red varieties of Dika in contrary – the process of carotinogenesis was activated (by 26% and 36% respectively). Data demonstrate that black Dika and Zanduri are less resistant to increased acid stress (pH1.5) by this index, compared with red and white varieties of Dika. Tukey's all-pairwise comparison test did not reveal any significant differences among the means (p=0.007). The protective mechanism of antioxidants is diverse: one group blocks free radicals, another splits the reaction chain between free radicals, while yet another reduces the oxidized substances. A promising approach to solve the problem is synergism, defined as the coincident and mutually reinforcing effect of two or more antioxidants as an adaptive strategy against acid stress (Corning, 1998; Riabushkina, 2005). The positive correlation between the stimulation of carotinogenesis and ascorbic acid synthesis under the acid stress in our experiments may be considered as cooperation between two antioxidants against free radicals, rising stress resistance of plants.

Proline

Accumulation of the amino acid proline is one of the indications to plant adaptation to unfavorable conditions. It regulates the osmotic equilibrium between the cytoplasm and vacuole. The experiments show a positive correlation between proline accumulation and stress intensity (Greenway, Munns, 1980). In our studies the highest content of proline was revealed in white Dika, the least – in black Dika (Table 1). Acid stress activated proline biosynthesis in leaves of experimental plants. In white and red forms of Dika stimulation was stronger (30-38%). Obtained results demonstrate that the protective role of proline against acid stress was significant in all varieties of Dika. White and red forms of Dike were distinguished among studied variants by this index too. Tukey's all-pairwise comparison test did not reveal any significant differences among the means (p=0.05).

Antioxidative enzymes

It is known that abiotic stress can induce an imbalance of the redox homeostasis of cell. Activities of antioxidative enzymes (CAT, POD, etc.) change with the intensity of the stress factor (Vuleta et al., 2009). According to some authors various reactions of species to acid stress depends on difference in the ability of CAT and POD to remove free radicals. In particular, POD is regarded as more sensitive to acid stress than CAT. (Lihong et al., 2008). Spraying with high acidity solution (pH1.5) influenced the activity of POD differently (Fig.1). The enzyme's activity increased by more than 41% in leaves of white Dika, while opposite results were obtained in cases of black and red varieties of Dika (Fig. 1).

Table 1. Influence of leaves spraying with pH1.5 acid solution on the content of antioxidants in leaves of Georgian endemic wheats ($p<0.05$).

Wheat species	Ascorbic acid, (mg% fresh weight)		Carotenoids, ($mg \cdot g^{-1}$ fresh weight)		Proline, ($mg \cdot g^{-1}$ dry weight)		Proline in seeds, ($mg \cdot g^{-1}$ dry weight)	
	Control	Acid sprayed	Control	Acid sprayed	Control	Acid sprayed	Control	Acid sprayed
T. timopheevi,	a 59.7±2.0	a 32.3±2.9	a 2.89±0.3	a 2.11±0.1	a 1.44±0.1	a 1.5±0.1	a 2.8±0.1	a 2.0±0.2
T. persicum, var. stramineum	c 120.0±2.0	c 155.0±5.6	a 1.08±0.1	a 1.42±0.1	a 1.8±0.3	a 2.34±0.2	ab 2.80±0.2	ab 3.20±0.2
T. persicum, var. fuliginosum.	bc 136.0±6.1	bc 98.0±7.9	a 1.09±0.1	a 0.78±0.1	a 1.34±0.1	a 1.44±0.2	b 2.89±0.3	b 4.00±0.3
T. persicum, var. rubiginosum	b 96.0±0.1	b 96.0±0.1	a 0.96±0.3	a 1.31±0.1	a 1.44±0.1	a 2.00±0.4	ab 2.800.33	ab 3.4±0.5

Table 2. Influence of leaves spraying with pH1.5 acid solution on the content of anthocyanins and soluble phenols in leaves of Georgian endemic wheats ($p<0.05$)

Wheat species	Anthocyanins, ($mg \cdot g^{-1}$ fresh weight)		Soluble phenols, ($mg \cdot g^{-1}$ fresh weight)	
	Control	Acid sprayed	Control	Acid sprayed
T. timopheevi,	a 0.271±0.17	a 0.200±0.03	a 7.00±0.46	a 4.59±0.17
T. persicum, var. stramineum	a 0.192±0.01	a 0.202±0.03	a 6.66±0.63	a 8.58±0.34
T. persicum, var. fuliginosum	a 0.369±0.03	a 0.329±0.05	a 6.91±0.34	a 8.00±0.58
T. persicum, var. rubiginosum	a 0.279±0.03	a 0.329±0.02	b 10.08±1.33	b 13.83±1.0

In Zanduri leaves enhancment of the enzyme's activity by 5.5% was noticed (Fig.1). Tukey's all-pairwise comparison test did not reveal any significant differences among the means ($p=0.05$). In our early experiments, pH2.5 acidity caused abating of POD activity in all studied forms of wheat (Chkhubianishvili et al., 2008). So strengthening of the intensity of acid stress (from pH2.5 to pH1.5) has emphasized differences in adaptive mechanisms between species. Aactivation of POD in leaves of white Dika and Zanduri under the severe acid stress (pH1.5) may be regarded as demonstration of stimulation of protective mechanisms associated with the activity of antioxidative enzymes. POD is known as poly functional enzyme, taking active part in cell metabolism (Andreeva, 1988; Cevahir et al., 2004; Bakalovic et al., 2006; Gazarian et al., 2006). Its main function is protection against the harmful effect of hydrogen peroxide. Moreover, its activation may be connected with the realization of protective abilities of plant and retention of cell homeostasis. Decrease in POD activity under stress indicates to inhibition of metabolic processes and enzyme synthesis (considered as a protective reaction against stressor) (Sergeichik, 1988; Vuleta et al., 2009). We assume that attenuation of POD activity under the acid stress plays special role in adaptation. It makes possible to regulate growth and metabolic processes in plant. (Chkhubianishvili et al., 1994; Gabara, 2003). In Fig. 2 the results of CAT activity under the acid stress are demonstrated. High acidity (pH 1.5) caused activation of the enzyme in two varieties of Dika. Especially white Dika must be mentioned, demonstrating also the significant activation of POD. CAT protects the plants against the high concentrations of hydrogen peroxide (Seregin, Ivaniov, 2001). Comparison of activities of CAT and POD in our experiments makes possible to suppose that under the high acidity stress, distribution of protective functions between these two enzymes takes place.

Total proteins

Content of total proteins in seeds of experimental plants was studied for investigation of acid stress influence on productivity. Our early experiments on endemic species of wheat have demonstrated that spraying plants with pH2.5 acid solution stimulated protein accumulation in seeds (Chkhubianishvili et al., 2008). This tendency was seen in recent experiments too (Fig.3). From literary data it is known that under the stress conditions, synthesis of most proteins is inhibited in plants (Vogelman, 1993; Boston et al., 1996), while synthesis of shock proteins is activated. It may be supposed that increase in total protein content in seeds of experimental plants is connected with their protective role.

Total phenols

Phenolic substances according to their antioxidant activity play a significant role in cell protection against stress (Dixon, Paiva, 1995; Robles et al., 2003). Among them are anthocyanins – effective protectors of cell against stress-induced free oxygen species (Merzliak, 1999; Neill, Could, 2003). It is established that phenolic substances *in vitro* are more effective antioxidants, than tocopherol and ascorbate (Grace, Logan, 2000). Among the control variant of experimental plants in white variety of Dika, content of anthocyanins was especially low (Table 2). Spraying with pH1.5 acid solution caused increase of this index in all varieties of Dika, while in Zanduri it diminished by 26.2%. Total amount of phenols was highest in red Dika (Table 2). Negative effect of acid spraying on the content of total

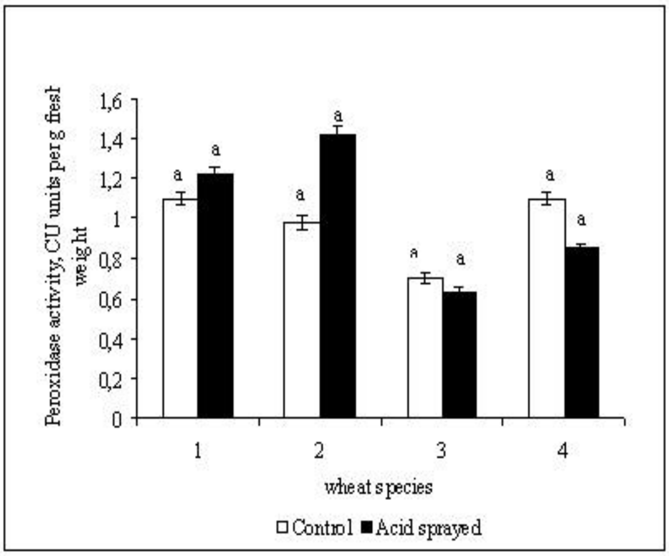

Fig. 1
Influence of acid precipitations on peroxidase activity in leaves of wheat species
1. Triticum timofeevi,
2. Tritikum persicum v. stramineum,
3. Triticum persicum v. fuliginosum ,
4 Triticum persicum v. rubiginosum (p=0.05)

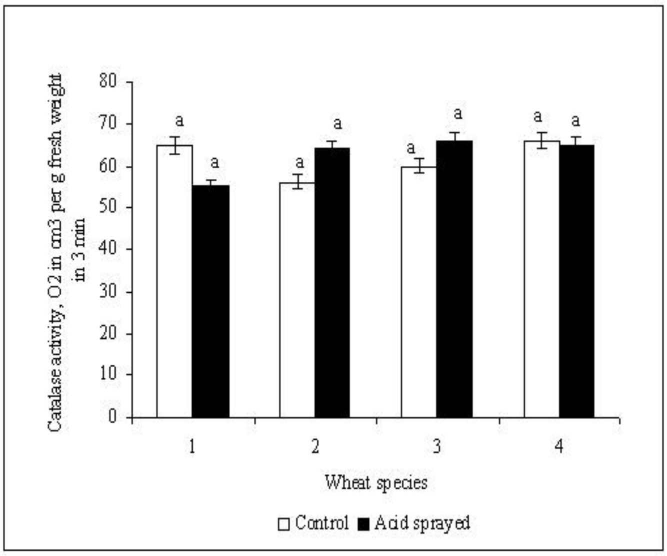

Fig. 2
Influence of acid precipitation on catalase activity in leaves of wheat species.
1. *Triticum timofeevi,*
2. *Triticum persicum* v. stramineum,
3. *Triticum persicum* v. fuliginosum,
4. *Triricum persicum* v. rubiginosum (p=0.05)

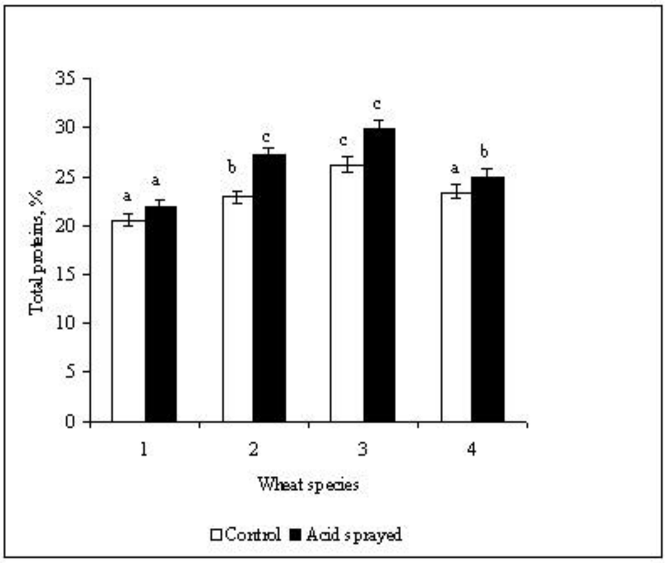

Fig.3
Influence of acid precipitations on the content of total protein in leaves of wheat species
1. *Triticum timofeevi,*
2. *Tritikum persicum* v. stramineum,
3. *Triticum persicum* v. fuliginosum,
4 *Triticum persicum* v. rubiginosum (p=0.0015)

phenols was revealed in Zanduri, while in all varieties of Dika stimulation by 16-37% was evident. Some authors mention activation of soluble phenols synthesis in leaves of winter wheat *T. aestivum* L. during cold-hardening of plant (Zagoskina et al, 2005). Stimulating effect of hardening was explained by synergetic activity of flavonic and phenylpropanoid ways (Zaprometov, Zagoskina, 1987). Presumably flavonoid and phenylpropanoid ways of accumulation of soluble phenols occupy one of the leading places in adaptation of Dika varieties to high acidity. Accordingly, this mechanism of plant protection against the oxidative stress has a special role in the studied plants. Because of poly antioxidant constitution of Dika varieties we can speak about antioxidative cooperation, when several substances belonging to soluble phenols are more effective against the oxidative stress (Zaprometov, 1974). Generally stress tolerance is ability of plants to endure unfavorable conditions and give generation (Larcher, 2003). From this point of view we can say that all experimental plants appeared to be resistant to severe acid stress (pH1.5), but at different level. The level of their tolerance may be evaluated according the changes in antioxidative system on the one side, and by the productivity, on the other. Spraying with pH1.5 acidity solution caused increase of productivity (the weight of 500 grains) in all varieties of Dika, and its decrease – in Zanduri (Rapava et al., 2010).

Materials and methods

Plant material, growth and stress conditions

Experimental studies were performed at the Digomi experimental base of the Institute of Botany of Ilia State University, Georgia, under field conditions. The following endemic species of wheat were used as test objects: spring wheat *T. timopheevi* Zhuk. (Georgian Chelta-Zanduri), and three varieties of *T. persicum* V. – var. stramineum Zhuk. (Georgian white Dika), var. rubiginosum Zhuk. (Georgian red Dika) and var. fuliginosum Zhuk. (Georgian black Dika).
Experimental plants were sowed in rows. Three row for each variant. Control and acid sprayed variants were situated in separate blocks. The experimental plants were sprayed with a water solution of a mixture of sulfuric and nitric acids 2:1, pH1.5, since the stem rising phase (Z30 by Zadok scale), at intervals of 5 days, till the end of vegetation (Z77 by Zadok scale) (totally 9 sprayings). Plants sprayed with distilled water served as controls for each studied species or cultivar. Analyses were made in the flowering phase (Z60-65 by Zadok scale), one week after the last spraying. Material for analysis was picked randomly both for control and acid sprayed variants. Analyses were made in five biological replicates.

Ascorbic acid

A titration method was used to measure the content of ascorbic acid. 2 g of fresh leaf material was mashed in 15 ml of 2% hydrochloric acid and 10 ml of 2% metaphosphoric acid, and filtered. One ml of the filtrate was added to 25 ml of distilled water and titrated with a 0.001 M solution of dichlorphenolindophenole (Ermakov et al., 1987).

Carotenoids

200 mg of fresh leaf material was mashed with 96% ethanol. The optical extinction of the extract was measured on a spectrophotometer SPEKOL 11 (Carl Zeiss, Germany). Pigment concentration was calculated by Wettstein formula (Wettstein, 1987).

Proline

0.5 g of dry leaves were mashed in 10ml of 3% sulphosalicylic acid and filtered. 2 ml of the filtrate was added to 2 ml of acid ninhydrin and 2 ml of ice acetic acid. After 1 h exposition on a water bath the extract was cooled and added with 4 ml of toluene and divided in a separating funnel. Optical density of upper layer was measured on a spectrophotometer at 520 nm (Bates et al., 1973).

Antioxidant enzyme assay

Activity of peroxidase was determined using guaiacole. Optical density of guaiacole oxidized products was measured at wavelength of 470 nm over a period of 2 min (Ermakov et al., 1987). Catalase activity was measured gasometrically: the volume of the oxygen released after adding H_2O_2 (30%) to water extract of experimental leaves was determined (Pleshkov, 1985). *Anthocyanins* One g of fresh leaves was placed in a mix of 20 ml of 96% ethanol and 2 ml of 1% HCL for 24 h. The extinction of the obtained extract was measured at 529 nm (Ermakov et al., 1987).

Total phenols

A 0.5 g of fresh leaves was boiled in 80% ethanol for 15 min. After centrifugation the supernatant was saved, and residues of leaves were mashed in 60% ethanol and boiled for 10 min. Obtained extract was added to the first supernatant and evaporated. The sediment was dissolved in distilled water. One ml of the received solution was added with the Folin-Ciocalteu reagent and optical density was measured at 765 nm. The Chlorogenic acid served as control (Ferraris et al., 1987).

Total protein assay

Content of proteins was determined after Lowry (1951) in seed ripening phase (Z94 by Zadok scale).

Statistical analysis

Mean values, standard deviations and statistical significance (p) of experimental results (in five biological replications) are given in figures and tables. One way ANOVA and Tukey's multiple comparison tests were used to test differences between the means. All calculations were performed using statistical software Satistix8 (Analytical Software, Tallahassee, FL).

Conclusions

The positive correlations between accumulation of anthocyanins and soluble phenols, proline and carotenoids, also activation of antioxidant enzymes revealed under the acid stress may be the reason for high efficiency of antioxidants in the studied plants. This was especially expressed by the acid-tolerance of white and red forms of Dika. Zanduri and black variety of Dika appeared to be more sensitive to stress. Sensitivity of Zanduri to acid stress was also revealed through growth parameters and decrease in grain weight. According to obtained results it may be concluded that the time interval between sprayings was enough for the recovery of vital potential in Dika to resist the acid stress. In Zanduri the process of reactivation seemed to be restricted. Decrease in content of phenols, proline, and catalase activity, and increase in peroxidase activity had negative effect on yield. From the data it is clear that the compensatory mechanisms under stress conditions are not always realized by all the antioxidants to an equal extent, but depend on the plant species. We consider that high acidity stress stimulates the activity of defensive antioxidant system in Georgian wheat species. Obtained results may be taken into account in the selection of acid resistant species of wheat. Tested species of Georgian wheats may be recommended as reliable material for cultivation under progressively polluting environmental conditions.

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