COMPARISON OF THE EFFECTS OF SOIL TREATMENT WITH NaF AND KF ON ANTIOXIDANT ENZYMES IN WINTER WHEAT (*TRITICUM AESTIVUM* L.) SEEDLINGS

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ABSTRACT: This paper assesses the impact of the treatment of soil with NaF and KF on activity of antioxidant enzymes involved in the Halliwell-Asada cycle: glutathione reductase (GR), dehydroascorbate reductase (DHAR), ascorbate peroxidase (APX), and antioxidant enzymes that decompose hydrogen peroxide: catalase (CAT), and guaiacol peroxidase (POD) in winter wheat cv. Skagen seedlings. The fluoride was added to loamy sand at concentrations of 10 mmol/kg DM soil and 30 mmol/kg DM soil. This study demonstrated that soil treatment with fluorine salts caused mainly increase in the activity of the studied enzymes. The highest changes occurred in DHAR and POD activity. The influence of NaF was typically higher than that of KF, while based on η^2 it was determined that the activity of GR and POD was mostly shaped by the salt concentration added to soil, and those of DHAR, APX, and CAT by the time of measurement after sowing. The conducted principal component analysis demonstrated that the activity of all determined enzymes was negatively correlated with the first principal component.

Keywords: Catalase; Fluoride; Guaiacol peroxidase; Halliwell-Asada cycle; Potassium; Sodium; Wheat.

INTRODUCTION

In the Earth crust, fluoride ranks thirteenth in terms of abundance, which amounts to 0.077% of the earth's crust.[1] Fluoride is introduced into the atmosphere during volcanic exhalations; however, anthropogenic sources constitute a significant source of these compounds in the environment.[2] From the atmosphere, fluoride returns to Earth with dust, snow, rain, or fog. Fluoride, penetrating the ground and surface waters, is widely distributed in soils.[3] An additional source of fluorine compounds in the soil and plants are phosphorus fertilizers.[4] In addition, fluoride is introduced into the soil from sewage. Considering the solubility of certain fluorine compounds in the environment, they are absorbed by plants and transported to their aboveground parts.[5]

Fluoride is known as a metabolic inhibitor, interfering with the overall responses of plants including seed germination, growth and productivity, biomass accumulation, photosynthesis, enzyme activities, protein synthesis, gene expression patterns, and reactive oxygen species (ROS) production.[6] However, the stimulating effects of fluoride are known (although much less widespread), for example in relation to one of the key enzymes of metabolism, which is adenylate cyclase.[7]

Plants have developed different protective mechanisms, enabling removal of ROS, including prevention or minimization of ongoing cell damage. The antioxidant system consists of enzymes (e.g., superoxide dismutase, catalase, guaiacol peroxidase, and enzymes involved in the Halliwell-Asada cycle: glutathione reductase, dehydroascorbate reductase, ascorbate peroxidase), as well as low-

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molecular weight compounds (ascorbate, cysteine, glutathione, polyphenols).[8,9] Majority of the studies on the impact of fluoride on induction of oxidative stress in plant cells have used NaF.[1,3,8,10,11] However, Saini et al.[5] state that other salts containing fluoride, including KF can be equally toxic.

Potassium is the key ion determining the level of osmotic potential in plant cells, and excess of sodium ions may lead to depolarization of the cellular membrane.[12] Moreover, potassium is one of important nutrient elements in crop growth, which modifies dozens of enzyme activations and controls stomatal movement of photosynthesis.[13] A presumption exists that sodium ions replace potassium during photophosphorylation process.[14] Thus, disturbance of the balance between the potassium and sodium content may also have a significant impact on the excessive production of ROS.[15] Moreover, Saxena et al.[16] demonstrated that leaching of fluoride in soils is significantly influenced by the type of cations found.

Thus, it is of interest to compare the effect of soil treatment with NaF and KF on the activities of antioxidant enzymes involved in the Halliwell-Asada cycle: glutathione reductase (GR), dehydroascorbate reductase (DHAR), ascorbate peroxidase (APX), and antioxidant enzymes that decompose hydrogen peroxide: catalase (CAT), and guaiacol peroxidase (POD) – in green parts of winter wheat, cv. Skagen seedlings.

MATERIAL AND METHODS

Plant growth conditions: The experiment was conducted on soil samples obtained from the arable and humus level of typical red soils from the area of the Agricultural Experimental Station in Lipnik. This soil is characterized by granulometric composition of loamy sand, with the content of C_{org} 8.71 g/kg and N_{total} 0.72 g/kg. The soil obtained was sieved through a sieve of 2 mm mesh size and aqueous solutions of NaF and KF were added into the soil. Concentrations of NaF and KF were 10 and 30 mmol/kg dry matter (DM) soil. The amounts of sodium, potassium, and fluoride introduced into soil are given in Table 1. The soil thus prepared was used to fill flower pots, with each pot subsequently sown with 10 seeds of winter wheat, cv. Skagen. Control consisted of plants growing in soil without fluorine salt addition. The soil humidity was maintained at about 60% of the maximum water holding capacity. During the experiment, the plants were illuminated with Son-T Agro 400 W sodium lamp from Philips, with the radiation intensity at the soil level in the vases of 170 µmol/m^2/sec PAR (photosynthetically active radiation). The photoperiod was established at 12 hours of day and night. On days 14, 21, and 28 after sowing, the activity of GR, DHAR, APX, CAT, and POX was determined in the green seedling parts with the use of spectrophotometry.

Assays of antioxidant enzymes: One gram of fresh plant mass was homogenized in frozen mortars with the buffer at the appropriate pH. The extracts were centrifuged at 14,800 × *g* at 4ºC. The obtained supernatants were used to determine the activity of antioxidant enzymes. Activity of glutathione reductase (GR, EC 1.8.1.7) was determined using the method of Smith et al.[17] Activity of the dehydroascorbate reductase (DHAR, EC 1.8.5.1) and ascorbate peroxidase (APX, EC 1.11.1.11) were determined with the method of Nakano and Asada.[18] Activity of catalase (CAT, EC 1.11.1.6) was determined using the Lück[19] method. Activity of the guaiacol peroxidase (POD, EC 1.11.1.7) was determined using the Chance and Maehly[20]

method, The measurements were performed using a UV-1800 spectrophotometer from Shimadzu (Kyoto, Japan).

Table 1. The amounts of sodium, potassium, and fluoride introduced into the soil

Salt	10 mmol/kg DM soil			30 mmol/kg DM soil		
	Na^+ (mg/kg DM soil)	K^+ (mg/kg DM soil)	F^- (mg/kg DM soil)	Na^+ (mg/kg DM soil)	K^+ (mg/kg DM soil)	F^- (mg/kg DM soil)
NaF	230.00	-	190.00	690.00	-	570.00
KF	-	390.00		-	1,170.00	

Statistical analysis: The results were processed statistically in the Statistica 13.1 program (StatSoft, Kraków, Poland). Significance of the observed differences was verified using a two-way analysis of variance followed by the post-hoc Tukey's HSD test ($P<0.05$). The contribution of independent variables to dependent variables was determined by calculating coefficient η^2 in ANOVA. The results were also subjected to principal component analysis (PCA) with the use of multidimensional methods. The influence of NaF and KF on antioxidant enzyme activity levels was determined using the following formula:

$$I_F = (P_o - C_o)/C_o$$

where I_F – influence of fluorine salts, P_o – antioxidant enzyme activities in seedlings grown in soil treated with fluorine salts, C_o – antioxidant enzyme activities in seedlings grown in untreated soil.

RESULTS AND DISCUSSION

Application of NaF and KF to soil at two concentrations (10 and 30 mmol/kg DM soil) resulted in a statistically significant increase in the activity of GR and POD on day 14. The observed effect increased with the increase in salt concentration in the soil, and NaF and KF at concentration of 30 mmol/kg DM soil resulted in GR activity increase of 84.72% and 46.50%, respectively, and in POD activity of 80.05% and 141.44%, respectively, as compared with the control (Table 2). The stimulatory effect of both the fluorine salts on the activity of GR and POD in the subsequent measurement time points remained constant only at the concentration of 30 mmol/kg DM soil. However, in the case of GR, it occurred only on day 14 and for NaF and KF, it was 24.50% and 18.70%, respectively. On the other hand, for POD, it remained constant on day 21 (64.81% and 66.23% for NaF and KF, respectively, compared to control), as well as on day 28 day (56.82% and 58.90% for NaF and KF, respectively, compared to control). A statistically significant increase of activity following application of both fluorine salts at a concentration of 30 mmol/kg DM soil during the entire experiment was demonstrated for APX. For NaF and KF, it was 20.85–35.16% and 18.79–31.21%, respectively, compared to control.

Table 2. Activity of antioxidant enzymes in winter wheat seedlings grown in soil treated with NaF and KF (GR=glutathione reductase; DHAR=dehydroascorbate reductase; APX=ascorbate peroxidase; CAT=catalase; POD=guaiacol peroxidase; NaF=sodium fluoride; KF=potassium fluoride; 10NaF= 10 mmol NaF/kg dry matter [DM] soil; 30NaF=30 mmol NaF/kg dm soil; 10KF= 10 mmol KF/kg dry matter [DM] soil; 30KF=30 mmol KF/kg dm soil; Values are mean±SD)

Treatment	Antioxidant enzyme				
	GR	DHAR	APX	CAT	POD
			Day 14		
Control	1.774 ± 0.022f	0.146 ± 0.007h	2.357 ± 0.131j	1.325 ± 0.087de	34.909 ± 1.151e
10NaF	2.454 ± 0.116bc	0.563 ± 0.012g	2.714 ± 0.068ij	1.680 ± 0.017b	50.526 ± 2.552d
30NaF	3.277 ± 0.141a	0.647 ± 0.050g	3.366 ± 0.014h	1.946 ± 0.016a	62.854 ± 0.317b
10KF	2.383 ± 0.215bcd	0.206 ± 0.013h	2.450 ± 0.092ij	1.413 ± 0.077de	51.805 ± 0.446cd
30KF	2.599 ± 0.068b	0.163 ± 0.030h	2.800 ± 0.102i	2.083 ± 0.112a	84.285 ± 3.773a
			Day 21		
Control	2.147 ± 0.121cde	2.052 ± 0.061cd	3.797 ± 0.158fg	1.208 ± 0.064e	51.630 ± 1.704cd
10NaF	2.055 ± 0.046ef	2.668 ± 0.106b	4.016 ± 0.154ef	1.289 ± 0.024de	52.358 ± 1.300cd
30NaF	2.673 ± 0.185b	3.136 ± 0.119a	5.132 ± 0.138b	1.258 ± 0.039de	85.091 ± 4.613a
10KF	2.101 ± 0.053de	2.237 ± 0.045c	3.568 ± 0.162gh	1.339 ± 0.052cd	55.962 ± 3.731bcd
30KF	2.535 ± 0,044b	1.810 ± 0.143e	4.982 ± 0.176bc	1.280 ± 0.050de	85.850 ± 3.226a
			Day 28		
Control	2.003 ± 0.102ef	1.924 ± 0.101de	5.040 ± 0.163bc	1.366 ± 0.018de	53.551 ± 3.102cd
10NaF	2.087 ± 0.027def	0.655 ± 0.031g	4.694 ± 0.104cd	1.419 ± 0.066cd	50.840 ± 2.850d
30NaF	2.186 ± 0.016cde	0.512 ± 0.007g	6.091 ± 0.293a	1.578 ± 0.107bc	83.978 ± 3.414a
10KF	2.046 ± 0.076ef	0.876 ± 0.012f	4.345 ± 0.038de	1.340 ± 0.036de	59.868 ± 2.879bc
30KF	2.094 ± 0.080de	0.652 ± 0.048g	6.233 ± 0.114a	1.416 ± 0.040cd	85.091 ± 2.996a

Data are expressed as a mean ± SD of three replicates, values marked with the same letters within columns (for each enzyme) form homogeneous groups at the level of $p<0.05$ (post-hoc Tukey's HSD test): a – homogeneous group with the highest activity of each enzyme, consecutive letters within the columns (b, c, d, e, f for GR; b, c, d, e, f, g, h for DHAR; b, c, d, e, f, g, h, i, j for APX; b, c, d, e, for CAT; b, c, d, e, for POD) indicate homogeneous groups with decreasing activity differing from each other at the level of $p<0.05$ (LSD for GR 0.327; for DHAR 0.219; for APX 0.139; for CAT 0.191; for POD 9.024), GR – glutathione reductase (µmol TNB/g FW/min), DHAR – dehydroascorbate reductase (µmol AA/g FW/min), APX – ascorbate peroxidase (µmol AA/g FW/min), CAT – catalase (µmol H2O2/g FW/min), POD – guaiacol peroxidase (µmol purpurogaline/g FW/min), TNB – 2-nitro-5-thiobenzoic acid, AA – ascorbic acid, FW – fresh weight

In activity of DHAR, a statistically significant stimulating effect was demonstrated on days 14 and 21 after the application of NaF at both concentrations and the highest level was observed on the first measurement time point (for concentrations of 10 and 30 mmol/kg DM soil it was 285.62% and 331.51%, respectively, compared to control). On the other hand, the activity of CAT, compared to control, showed statistically significant increase on day 14 after the application of NaF at the concentration of 10 mmol/kg DM soil (26.79%) and 30 mmol/kg DM soil (46.87%), and KF at the concentration of 30 mmol/kg DM soil (57.21%); on day 21 after the application of KF at the concentration of 10 mmol/kg DM soil (10.84%); and on day 28 after the addition of NaF at the concentration of 30 mmol/kg DM soil (15.52%)

Increase in the activity of antioxidant enzymes caused by the presence of fluorine salts in the soil may indicate increased ROS production and induction of oxidative stress in the seedlings of winter wheat. The obtained results are compliant with those reported by Tak and Asthir.[21] The researchers have also demonstrated that the effect of fluorine compounds on the parameters of oxidative stress in winter wheat seedlings depends on its cultivar: HD 3086 cultivar has a higher up-regulation of the antioxidant defense system induced by NaF than the WH 1105 cultivar. Early seedling growth stages are physiologically complex processes and are severely affected by fluoride.[6] Wang et al.[22] demonstrated a positive correlation between the content of fluoride in soil and the concentration of these ions in wheat tissues. Increase in activity of antioxidant enzymes under the influence of fluoride present in the soil was further demonstrated for numerous plant species, for example, *Morus alba*[8], *Prosopis juliflora*[5], *Oryza sativa*[23], *Camellia sinensis*[1], and *Olea europaea*.[11] However, reports on inhibition of activity of antioxidant enzymes under the influence of fluorine salts have been published.[3,24] In this study, inhibition of DHAR activity on day 21 following the treatment with KF at a concentration of 30 mmol/kg DM soil and on day 28 after the application of the salt in both concentrations, as well as inhibition of APX activity on day 28 after introduction of KF at a concentration of 10 mmol/kg DM soil occurred. As proposed by Yadu et al.,[6] reduction in activity of antioxidant enzymes may indicate excessive concentration of ROS, resulting in disturbances of functioning of the oxidative stress defense system.

Comparing the median and mean values of I_F, the influence of NaF on the activity of GR, DHAR, APX, and CAT was found to be higher than that of KF. A reversed relationship was found for the activity of POD (Figure 1). In addition, except from DHAR, following the soil treatment with KF, increase in effect of fluorine salts on the activity of antioxidant enzymes along with the increase in their soil concentration was found. Biczak et al.[25,26] demonstrated that the cation type in ionic liquids containing anions of fluoride had a considerable impact on the accumulation of fluoride and activity of antioxidant enzymes in *Hordeum vulgare* and *Raphanus sativus*.

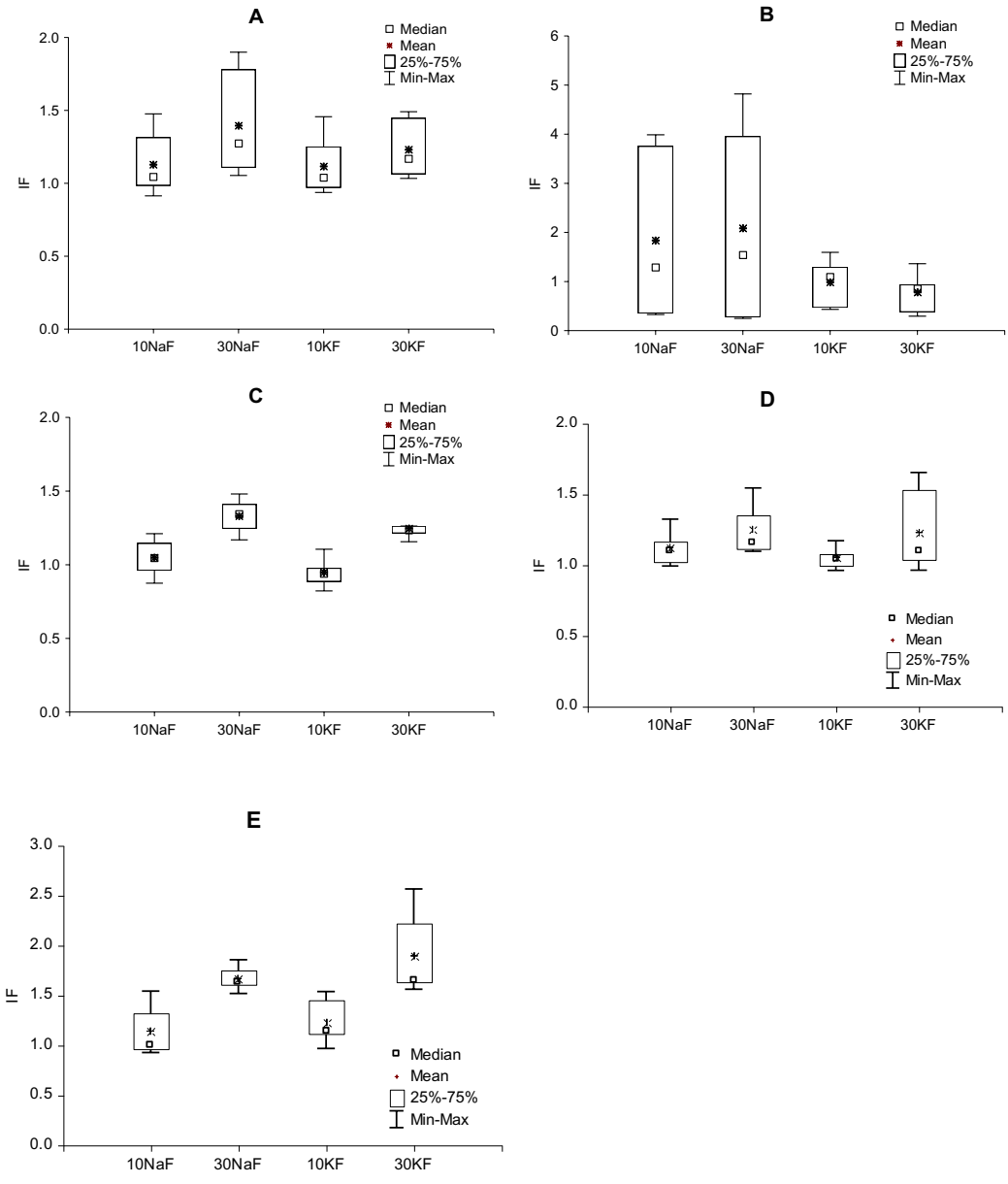

Figure 1. Median and mean influence of NaF and KF on activity of antioxidant enzymes in winter wheat seedling: A – glutathione reductase, B – dehydroascorbate reductase, C – ascorbate peroxidase, D – catalase, E – guaiacol peroxidase.

The two alkali cations Na^+ and K^+ have similar relative abundances in the earth crust but display very different distributions in the biosphere. In all living organisms, K^+ is the major inorganic cation in the cytoplasm, where its concentration is usually several times higher than that of Na^+. Accumulation of Na^+ at high concentrations in the cytoplasm results in deleterious effects on cell metabolism, e.g., on photosynthetic activity in plants.[27] However, it can be a beneficial element, by replacing K^+ as vacuolar osmoticum for instance.[28] In contrast, K^+ is an essential

element and plant growth requires large quantities of K^+ ions that are taken up by roots from the soil solution, and then distributed throughout the plant. The availability of K^+ ions in the soil solution, slowly released by soil particles and clays, is often limiting for optimal growth in most natural ecosystems.[29] In contrast, due to natural salinity or irrigation with poor quality water, detrimental Na^+ concentrations, toxic for all crop species, are present in many soils, representing 6% to 10% of the earth's land area.[27] Moreover, an increased sodium content in the soil may cause its sodicity.[30,31] Soil sodicity resulted in osmotic stress in plants due to the presence of high soluble salt concentrations. It also generates adverse soil physical conditions such as high bulk density, low porosity, low oxygen concentrations, and prolonged waterlogging associated with the reduced hydraulic conductivity.[32,33] Our previous studies showed that soil sodicity induced oxidative stress in plants.[34] Differences in impact of NaF and KF on antioxidant enzyme activities in winter wheat seedlings may therefore result from the above reasons. This is important because many authors have shown that fertilizers used in agriculture, including compound fertilizers with potassium ions, may contain significant amounts of fluorine. [35-38]

However, coefficient η^2 in ANOVA showed that activities of GR and POD were affected to the greatest extent by the concentration of fluorine salts, while the activities of DHAR, APX, and CAT were most affected by time of measurement (Figure 2). Comparison of the influence of the type of salt on the studied antioxidant enzymes allowed to determine that it is highest for GR, yet it amounted to only 5.40% change in activity compared to the control.

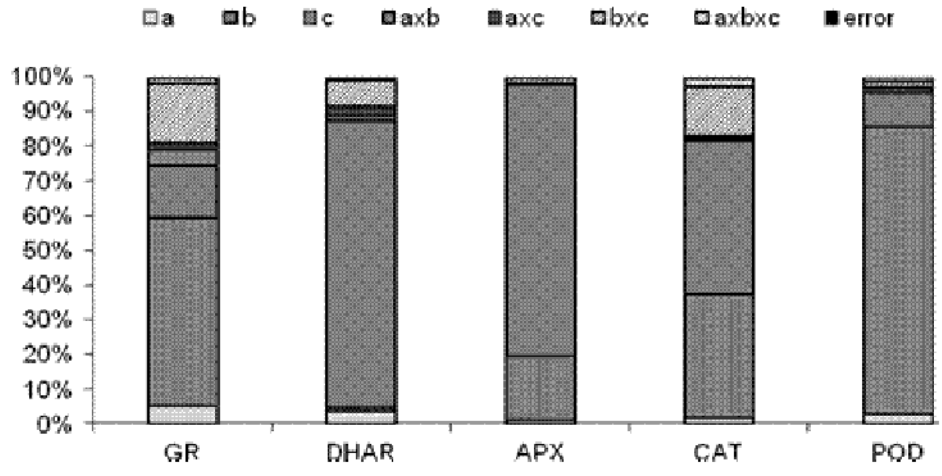

Figure 2. The share of independent variables in the evolution of the activity of antioxidant enzymes in winter wheat seedlings (Á2): a – type of fluorine salt, b – concentration of fluorine salt, c – time of measurement.

The effect of the degree of soil treatment with fluorine salts on the activity of antioxidant enzymes in winter wheat seedlings was interpreted by using principal component analysis (PCA) (Figure 3).

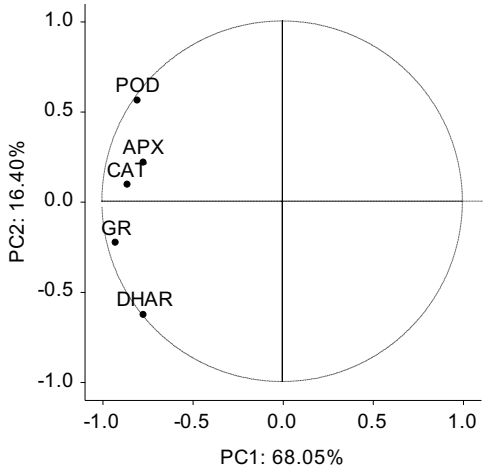

Figure 3. Activity of antioxidant enzymes in winter wheat seedlings grown in soil treated with NaF and KF as presented by the principal component analysis (PCA) method, GR – glutathione reductase, DHAR – dehydroascorbate reductase, APX – ascorbate peroxidase, CAT – catalase, POD – guaiacol peroxidase.

PCA allowed precise determination of the scale of antioxidant enzyme changes which occurred in winter wheat seedlings because of the presence of the fluorine salts in the soil. The distribution of vectors around the axis which represents the first factor is described by 68.05%. The activity of antioxidant enzymes correlated negatively with the PC1. The position of the vectors proves the occurrence of a response of APX, GR, and CAT activity to soil treatment with fluorine salts. Correlation of DHAR and POD activities with those of the aforementioned enzymes was slightly less. Moreover, DHAR activity was negatively correlated with PC2 (Table 3).

Table 3. Pearson product-moment correlation coefficients between activities of antioxidant enzymes in winter wheat seedlings grown in soil treated with NaF and KF

Enzyme	PC1	PC2
GR	–0.922	–0.234
DHAR	–0.763	–0.628
APX	–0.769	0.215
CAT	–0.856	0.092
POD	–0.802	0.562

GR – glutathione reductase, DHAR – dehydroascorbate reductase, APX – ascorbate peroxidase, CAT – catalase, POD – guaiacol peroxidase.

CONCLUSIONS

Soil treatment with NaF and KF caused significant changes in the activity of antioxidant enzymes in winter wheat cv. Skagen seedlings. The most commonly used fluorine salts resulted in stimulation of the studied enzymes, which may indicate the presence of oxidative stress. Activity of DHAR and POD exhibited the greatest changes. It can be stated that NaF, to a minor degree, had greater impact on the

activity of antioxidant enzymes. However the conducted η^2 analysis demonstrated that type of salt had the smallest impact on determined antioxidant enzymes.

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