

## FLUORIDE INFLUENCE ON THE GROWTH, MORPHOLOGICAL AND BIOCHEMICAL PARAMETERS, AND ELEMENTAL COMPOSITION OF *TRITICUM AESTIVUM* L. IN A MODEL EXPERIMENT

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**ABSTRACT:** Anthropogenic pollution of the environment by fluoride (F) can lead to abnormality symptoms in some species of plants even at relatively low impact levels. Currently, an active accumulation of data on the effect of F on plant functions take place. The purpose of the present work was complex investigation of influence of different F concentrations in soil on growth, morphological and biochemical parameters and elemental composition of the wheat plants (*Triticum aestivum* L.). Seed germination, survivorship rate, external appearance, length and thickness of leaf blade, chlorophyll, electrolyte leakage, catalase and acid phosphatase activity and elemental composition (As, Ba, Ca, Cd, Co, Cr, F, Fe, K, Mg, Na, Ni, Pb, Se, Sr, Ti, V, Y, Zn and rare-earth elements) were studied at different levels of F in soil in range of 100 – 7600 mg/kg. Methods of spectrophotometry, conductometry, inductively coupled plasma mass-spectrometry and atomic absorption spectrometry were used. The most sensitive biochemical markers for estimation of the plant F stress were found to be electrolyte leakage and acid phosphatase activity. Noticeable change of these parameters started at F soil concentration of 600 and 1100 mg/kg respectively. Concentration of Mg, Ca and Sr in the plants was established to decrease at increasing F in soil. Distribution of concentration Al, As, Ba, Co, Cr, Fe, K, Ni, Pb, Sb, Th, Ti, V, Y, and light rare-earth elements was described by curve with maxima at 1100 mg/kg; maximum concentration of Fe, K, and Ni was observed at 2100 mg/kg of F in soil. Possible 3-stage's scheme for the development F stress in wheat plant was suggested. Both the decrease of the F bioaccumulation factor, electrolyte leakage, acid phosphatase activity and the increase of the content of molybdenum that is a component of plant enzymes catalyzing key stages of nitrogen, carbon, and sulfur metabolism at high F soil levels could be parts of adaptation mechanism of the wheat plants (*Triticum aestivum* L.) to F stress.

Keywords: Acid phosphatase; Catalase; Chlorophyll; Electrolyte leakage; Fluoride stress; *Triticum aestivum* L.

### INTRODUCTION

Hydrogen fluoride and fluoride (F) entering the environment, mainly with emissions from the production of aluminum, mineral fertilizers, silicate materials and the combustion of various fuels have a high toxicity to plants.<sup>1</sup> Sensitivity of different plant species is variable to F exposure. Yarrow (*Achillea*), field mustard (*Sinapis arvensis*), common chicory (*Cichorium intybus*), aiten (*Juniperus communis*), tea are F tolerable species and pine, larch, narrow-leaved catoptric (*Typha angustifolia*) are F sensitive species.<sup>2</sup> The resistant species could accumulate up to 1500 mg/kg of F on dry basis without any visible alterations while sensitive ones had some morphological abnormality symptoms already at the level 40 mg/kg.<sup>3</sup>

There are not quite clear the mechanisms responsible for the accumulation of F by plants and their response and adaptation to F stress till now. Currently very limited

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number of studies deals with this aspect using methods of mass spectrometric identification of metabolic products<sup>3</sup>, determination of the activity of enzymatic systems<sup>4</sup>, assessment of physiological<sup>5</sup> and histological changes.<sup>6</sup> Ionomics studies the total elemental composition of an organism to solve biological problems. It could be efficient tool for understanding behavior of plants in condition of F exposure together with traditional methods.

In the present work influence of different levels of F exposure on morphology, changes in biochemical parameters and elemental composition of wheat plants (*Triticum aestivum* L.) were studied in a model experiment. Activity of acid phosphatase, concentration of protein, chlorophyll, micro- and macroelements, electrolyte leakage were determined in the plant samples. Correlations between these parameters and F concentration in soil were estimated.

## MATERIAL AND METHODS

### 1.1. Chemicals and standard solutions

Sodium fluoride (high purity grade), sodium acetate, acetic acid,  $\beta$  – mercaptoethanol, polyvinylpyrrolidone (PVP), potassium dichromate, dimethylsulfoxide (DMSO), calcium carbonate (all chemically pure grade), p-nitrophenylphosphate and Folin-Ciocalteu's reagent (both BioChemica grade, Panreac, Spain) were used for model experiment and plant sample analysis. Nitric acid (69% m/m, Suprapur, Merck, Germany) and hydrogen peroxide (30%, high purity grade) were used for plants digestion. All reagents except from those marked were acquired from Prime Chemicals Group, Russia, and used with no extra purification.

An indium standard solution (1000 mg/mL, High-Purity Standards, USA) was used for preparing working solution of internal standard for microelements' determination by inductively coupled plasma mass-spectrometry (ICP-MS). Cesium chloride and lanthanum chloride (Fluka, cat j 20982) both were used as ionized buffer at Ca determination by flame atomic absorption spectrometry (AAS). Multi-element standard solutions (10 mg/L) (ICP-MS-68A Solution B; ICP-MS-E; ICP-MS-B; High-Purity Standards, Charleston, USA) were used to prepare a series of calibration solutions using 3% m/m HNO<sub>3</sub>.

### 1.2. Conditions of model experiment

The seeds of *Triticum aestivum* L. were preliminary examined and unhealthy seeds were removed. Sowing was carried out into soil that originally contained of N - 1.5%, P<sub>2</sub>O<sub>5</sub> – 0.74%, K<sub>2</sub>O – 0.56%, CaO – 9.86%, MgO – 0.26%, F – 100 mg/kg, pH<sub>KCl</sub>=5.5. Plastic pots were filled by 600 g of the soil. Then different portions of sodium fluoride were added into the pots so that final F concentrations were 100, 600, 1100, 2100, 5100 and 7600 mg/kg. Each soil sample was vigorously mixed. There were sown 100 healthy seeds into each pot. The pots were placed on the laboratory desk next to the window. Accelerated seed germination and enhanced plant growth were achieved by irradiation with LED-U150-16W lamps (Uniel, China) with maximum of radiation at wavelengths of 440 and 660 nm. Wetting was performed daily using 50 cm<sup>3</sup> of distilled water per a container. Plants grew under

controlled conditions ( $t_{\text{air}} = 25 \pm 2^\circ\text{C}$  and humidity =  $40 \pm 10\%$ ) between February and April, 2020. Each experiment was carried out in three replicates. Plants were harvested at 60th day after planting.

### *1.3. Sample preparation for determination of enzymes activity and protein concentration*

Samples of fresh leaves (500 mg) were homogenized thoroughly with 500  $\mu\text{L}$  of sodium acetate extraction buffer (0.1 M, pH 5.5, 2.5% PVP) and 5  $\mu\text{L}$  of  $\beta$ -mercaptoethanol under  $0^\circ\text{C}$ . The homogenates were centrifuged at 10 000 rpm for 45 min at  $4^\circ\text{C}$ .<sup>7</sup> The supernatants were collected and used for the determination of enzymes (acid phosphatase, catalase) activity and protein concentration.

### *1.4. Sample preparation for determination of elements' concentration*

The aerial part of the plants was cut, weighed and dried at room temperature for 24 hours and then at  $70^\circ\text{C}$  for 72 hours. Thereafter the samples were ground in a laboratory mill Stegler LM-250 (Stegler, China).

### *1.5. Determination of protein concentration*

Concentration of protein was determined for calculation of enzymes activity. Protein concentration was determined using capability of  $\text{Cu}^{2+}$  to form complex compound with peptides in alkaline medium.<sup>8</sup> The reduction of  $\text{Cu}^{2+}$  to  $\text{Cu}^+$  is carried out during this process. The reaction of the  $\text{Cu}^+$  with Folin-Ciocalteu's reagent leads to formation of the complex of  $\text{Cu}^+$  that has absorbance maximum at 750 nm. The amount of the complex is proportional to protein content in the sample to be analyzed.

### *1.6. Determination of acid phosphatase activity*

Acid phosphatase (EC 3.1.3.2) activity was determined via measuring the rate of hydrolysis of a model substrate of para-nitrophenylphosphate.<sup>9</sup> The amount of the acid phosphatase that catalyzes the formation of 1  $\mu\text{mol}$  of p-nitrophenol for 1 min at  $37^\circ\text{C}$  per 1 mg of total protein was taken as a unit of the enzyme activity.

### *1.7. Determination of catalase activity*

Catalase (EC 1.11.1.6) activity was determined via measuring the rate of degradation of hydrogen peroxide.<sup>10</sup> The value of the enzyme activity was expressed as a constant of the first order velocity per milligram of total protein in the supernatants. Residual concentration of hydrogen peroxide that didn't decompose under effect of catalase after a certain interval from the beginning the reaction was determined via reduction of the dichromate ion in the acetic acid medium to the  $\text{Cr}^{3+}$ -ion by the hydrogen peroxide. It was done by measurement of the change in the absorbance of the solution at 570nm.

### *1.8. Determination of chlorophyll concentration*

Chlorophyll concentration was determined in accordance with Barnes et al.<sup>11</sup> 200 mg of fresh plant sample was pounded with a pestle together with 100 mg of calcium carbonate and 1 mL of extract solution (2,5g/L PVP in DMSO). Obtained mixture was quantitatively transferred into the tube and 5 mL of extract solution was added to the mixture. Then the tube was hold in the oven at  $60^\circ\text{C}$  for 6 hours. Absorbance of

the obtained solution was measured at  $\lambda = 665$  nm and  $\lambda = 648$  nm. Chlorophyll concentration was calculated in accordance with Barnes et al.<sup>11</sup>

Spectrophotometer 'Scan Drop<sup>a</sup> (Analytik Jena) was applied for determination of acid phosphatase and catalase activity and chlorophyll concentration. All measurements were carried out using quartz cell with absorbance layer of 1 cm.

### 1.9. Determination of electrolyte leakage

The membrane damage of plant parts was measured by following the method<sup>12</sup>. Ten discs (0.5 cm diameter) were cut from the fresh leaves (five plants per variant) and washed with deionized water to remove surface-adhered electrolytes three times. The discs of the leaves were placed in tube containing 5 mL of deionized water, the tube was closed and incubated at 10°C for 24 hr. The initial electrical conductivity of the solution ( $EC_1$ ) was determined using a conductometer Expert-002, (Econix-Expert Ltd, Russia). Then the samples were incubated in a water bath at 95°C for 20 min to release all electrolytes, cooled down to 25°C and the final electrical conductivity ( $EC_2$ ) was measured. The electrolyte leakage (EL) was calculated as:

$$EL = \frac{EC_1}{EC_2} \times 100 (\%)$$

Where:

- EL = Electrolyte leakage (%)
- $EC_1$  = Initial electrical conductivity of the solution
- $EC_2$  = Final electrical conductivity of the solution

### 1.10. Bioaccumulation factor calculation

The bioaccumulation factor (BF) value of F in shoots was calculated in accordance with Saini et al.<sup>13</sup> and Li et al.<sup>14</sup>:

$$BF = \frac{F \text{ concentration in shoot}}{F \text{ concentration in soil}}$$

Where:

- BF = Bioaccumulation factor
- F = Fluoride

### 1.11. Determination of element concentrations in plants

Element concentrations were measured by (i) Inductively coupled plasma mass spectrometry (ICP-MS): (aluminum (Al), antimony (Sb), arsenic (As), barium (Ba), cadmium (Cd), cobalt (Co), copper (Cu), chromium (Cr), manganese (Mn) molybdenum (Mo), nickel (Ni), lead (Pb), selenium (Se), strontium (Sr), tin (Sn), thallium (Tl), titanium (Ti), thorium (Th), vanadium (V), yttrium (Y), zinc (Zn) and rare-earth elements); (ii) Atomic absorption spectroscopy (AAS): iron (Fe), calcium

(Ca), magnesium (Mg); and (iii) Flame photometry: (sodium (Na), potassium (K), after plants' digestion by a mixture of nitric acid and hydrogen peroxide at 190°C for 30 minutes using high pressure vessels XP-1500 Plus and microwave oven MARS-5 (CEM Corp., USA). The measurements were carried out using the high-resolution mass spectrometer Element 2 (Thermo Fisher Scientific of GmbH, Germany), atomic absorption spectrometer KVANT-2A (CORTEC, Russia) and photometer FPA2-01 (ZOMF, Russia). The concentration of internal standard (In) in the solutions to be analyzed by ICP-MS was 1 mg/L and the concentration of LaCl<sub>3</sub> and CsCl as ionized buffer in the solutions at Ca determination by AAS was 0.1% and 0.01%, respectively.

Total F concentration was measured by ionometry after sample dissolution using the alkali fusion.<sup>15</sup> Fluoride-selective electrode ELIT-221, reference silver chloride electrode EVL-1MZ and ion analyzer Anion4100 (InfraSpac-Analyte, Russia) were applied for the measurements with instrument measurement accuracy  $\pm 0,1$  mV.

Nitrogen (N) was determined by Dumas' method using analyzer Dumatherm (Gerhardt, Germany) after burning the plant sample in oxygen at high temperature in the presence of catalyst.

Reference materials of elodea EK-1, birch leaves LB-1, grass mixture TR-1 (Institute of Geochemistry, Russia) and legumes grass meal ЪСЃЃ 10-209-2015 (Institute of Agrochemistry, Russia) were analysed for accuracy control at elements' determination in plants samples.

### *1.12. Statistical analysis*

Relationship between analyzed parameters and components was studied via plotting the regression lines and calculating the correlation coefficients (r) and the significance levels of obtained correlations (p-value). Statistical significance of differences between values corresponding to different F soil concentrations was carried out using Two-Sample T-Test (P-Value). Plotting the regression lines and calculation of correlation coefficients was done using Microsoft Excel. P-Values and p-values were calculated using software Minitab16 (Minitab Inc., USA).

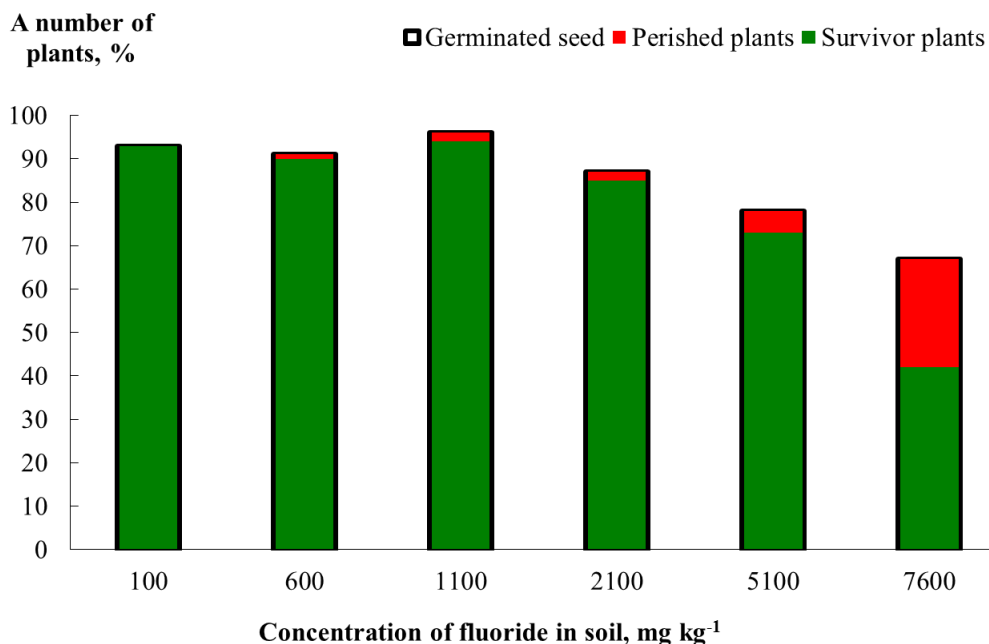
## **RESULTS**

### *3.1. Influence of F on seed germination and morphological characteristics of wheat plants*

The number of germinated seeds and plants that were perished after germination during three weeks into starting the experiment as total biomass, length and thickness of leaf blade at end of the experiment were estimated. Gradual degradation of germination and studied morphological markers was observed at increasing the F stress (Table 1). This is especially evident when F concentration in soil achieved 2100 mg/kg and more. The germination was only 67% at 7600 mg/kg and a number of survivor plants were 51% less comparing to ones at 100 mg/kg considering plants perished after germination (Figure 1). A decrease in the plant biomass at 7600 mg/kg was revealed to be about 60% in comparison with 100 mg/kg (Table 1).

**Table 1.** Germinating capacity and morphological features of wheat plants at different levels of fluoride stress

Concentration of F in soil (mg/kg)	Number of germinated seeds	Number of plants that perished after germination	Total biomass (g)	Length of leaf blade (mm)		Thickness of leaf blade (mm)	
				Mean±SD, n = 5		Mean±SD, n = 5	
100	93	0	44.3	221±35		0.31±0.07	
600	91	1	32.7	228±19		0.29±0.05	
1100	96	2	33.3	218±34		0.30±0.06	
2100	87	2	31.5	194±49		0.20±0.04	
5100	78	5	31.2	181±47		0.20±0.05	
7600	67	25	17.8	138±56		0.19±0.03	



**Figure 1.** Responses of the wheat plants to different levels of fluoride (F) content in the soil showing the relative levels of germination and the proportions of perished and surviving plants.

Also 20 days after starting the experiment the chlorosis developed in the wheat plants that was grown in soil with a F concentration of 2100 mg/kg and more. Initially light spots appeared at the lamina tissue which then became darker and larger (Figures 2A and 2B). The greatest changes were observed in wheat plants grown with soil F concentrations of 5100 and 7600 mg/kg. In these cases the necrotic

areas were reddish-brown and were clearly bounded from the healthy lamina tissue (Figures 3A and 3B).

2A



2B



**Figures 2A and 2B.** 2A and 2B: Appearance of chlorosis.

**3A**



**3B**



**Figures 3A and 3B.** Necrotic areas on the lamina tissue of wheat plants at F soil concentrations of 3A:5100 mg/kg and 3B:7600 mg/kg.

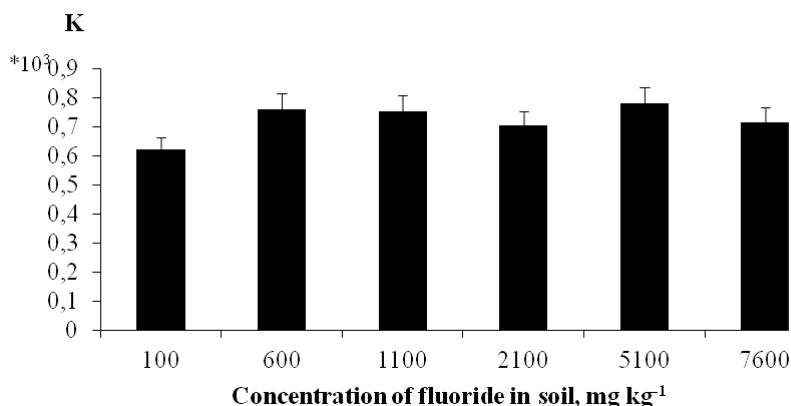


Length of leaf blade corresponding to F concentration of 7600 mg/kg was found to be significantly lower in comparison with ones at 100, 600 and 1100 mg/kg (P-Values 0.031, 0.027 and 0.035 respectively). For the samples at 5100 mg/kg this difference was found to be less compared to ones at 100 and 600 mg/kg (P-Values 0.175 and 0.096 respectively). The thickness of leaf blade is likely to be a bit more sensitive to the fluoride stress and reliably decreased at 2100 mg/kg and with a higher F concentration in soil (Table 1). Significances of differences (P-Values) between data corresponding 100, 600 and 1100 mg/kg and 2100, 5100 and 7600 mg/kg were in range 0.001 – 0.003.

Thus, the data obtained indicate that the change in the studied morphological parameters of wheat plants becomes noticeable only at the levels of F concentration in the soil significantly exceeding both its average content of 321 mg/kg as indicated in<sup>1</sup> and critical range of 500–1000 mg/kg in accordance with Tandelov<sup>16</sup>, Pomazkina et al.<sup>17</sup> and Vazhenin et al.<sup>18</sup>. The results are consistent with the data of the study, which showed only a slight decrease in the germination of wheat seeds up to 20 times the maximum permissible concentration of water-soluble F in the soil<sup>19</sup>. This indicates the limited suitability of morphological features and the necessity of researching the more sensitive biochemical criteria for assessing the F effect on plants.

### 3.2. Influence of F on the biochemical parameters of plants

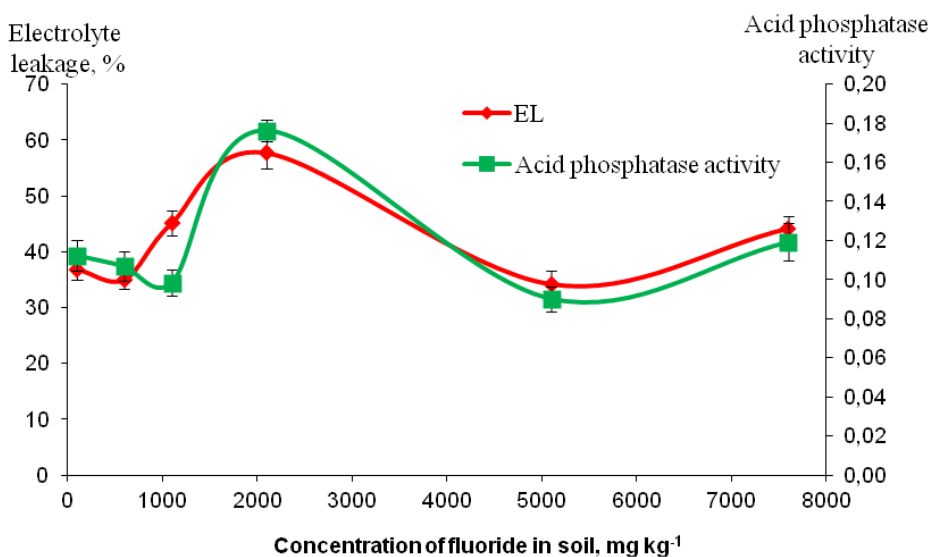
In the present work there was studied activity of acid phosphatase and catalase that are the most sensitive to F exposure for a number of plants.<sup>20-21</sup> There are contradictory data about F influence on enzyme activity.<sup>22-24</sup> It is possible that this may be due to specificity of species that were studied by different authors as well as differences in the experimental conditions. It was found that there was both inhibition and an increase in the activity of enzymes involved in glycolysis, respiration and photosynthesis under F exposure<sup>25</sup>. The catalase enzyme accelerates the decomposition of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to form water and oxygen, thereby protecting plants from the accumulation of reactive oxygen species.<sup>26</sup> According to our data, catalase activity raised slightly with increasing the F concentration in soil (Figure 4).



**Figure 4.** Dependence of catalase activity, K, on F concentration in soil. K is rate constant for the hydrogen peroxide decomposition reaction, min<sup>-1</sup> mg<sup>-1</sup>(total protein).

Fluoride does not have a redox or oxidant agent property and is possible that it acts indirectly upon plant enzyme plant systems resulting in some enhancement of the catalase activity. Our results are in compliance with increasing the catalase activity for *Prosopis juliflora* and *Vigna radiata* (L.) under F stress.<sup>27, 28</sup>

Acid phosphatase is involved in mineral nutrition and the transport of metabolites of plants.<sup>29</sup> This enzyme is located mainly in plasma membranes and vacuoles of plants.<sup>30</sup> So, it was interesting to study the relationship between acid phosphatase and electrolyte leakage that describes the state of the plasma membranes and vacuoles.<sup>31, 32</sup> An influence of F soil concentration on acid phosphatase activity and electrolyte leakage is found to be the same (Figure 5).

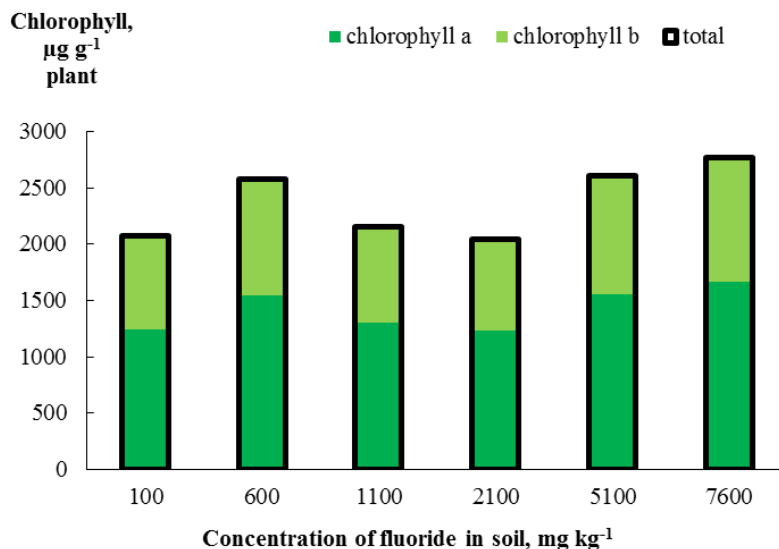


**Figure 5.** Dependence of electrolyte leakage and acid phosphatase activity ( $\text{mol L}^{-1} \text{s}^{-1} \text{mg}^{-1}$  of total protein) on F concentration in soil.

Increasing the F at least up to 600 mg/kg did not result in change of these parameters. The minimum and maximum responses on F exposure were found to be at 1100 and 2100 mg/kg of F, respectively, for electrolyte leakage and acid phosphatase activity. An increase in the electrolyte leakage indicates an injury of the permeability of the cell membranes. This abnormality could be caused by deterioration of both their structure and the lipid complex state.<sup>33</sup> So, electrolyte leakage can be used for an integral characterization of degree of F stress which plants are exposed to<sup>34,35</sup> and is likely to be a more sensitive indicator of the complex effects of F exposure in wheat plants compared to the other biochemical parameters.

Distribution of chlorophyll in wheat plant at different levels of F exposure was found to be variable (Figure 6). According to Weerasooriyagedar et al.<sup>36</sup> the effect of F on plant metabolism is characterized by a diminution in the assimilation of nutrients, suppression of starch synthesis and a decrease in the content of chlorophyll.

The inhibitory F influence on the accumulation of chlorophyll was described for cereals and *Oryza sativa*.<sup>37, 38</sup> But complex dependence of chlorophyll content on F concentration in substrate was found for wheat plant.<sup>37</sup> So, literature data and our results indicate complicated mechanism of wheat plant adaptation to F exposure.



**Figure 6.** Distribution of chlorophyll in wheat plant at different F exposures.

### 3.3. Influence of F on the elemental composition of plants

Results for Eu, Tb, Ho, Tm, Yb, Lu and U in the analysed samples of wheat plants at different F levels in soil were found to be less than limit of determination of (0.002 – 0.004) µg/g. Concentrations of most other studied elements in the plants at F soil content of 100 mg/kg were found to be close with ones at 600 mg/kg and to be raised at increasing the soil F up to 1100 mg/kg (Tables 2A and 2B). Further increasing the soil F resulted in decrease of the elements in the plant. This distribution at different F soil levels is especially evident for Al, As, Ba, Co, Cr, Pb, Sb, Th, Ti, V, Y and rare-earth elements (Table 2, Figure 7A). Similar behavior with maximum at 2100 mg/kg was found for Fe, K and Ni (Tables 2A and 2B, Figure 7B). But increase of Ni and K was found to start at 600mg/kg.

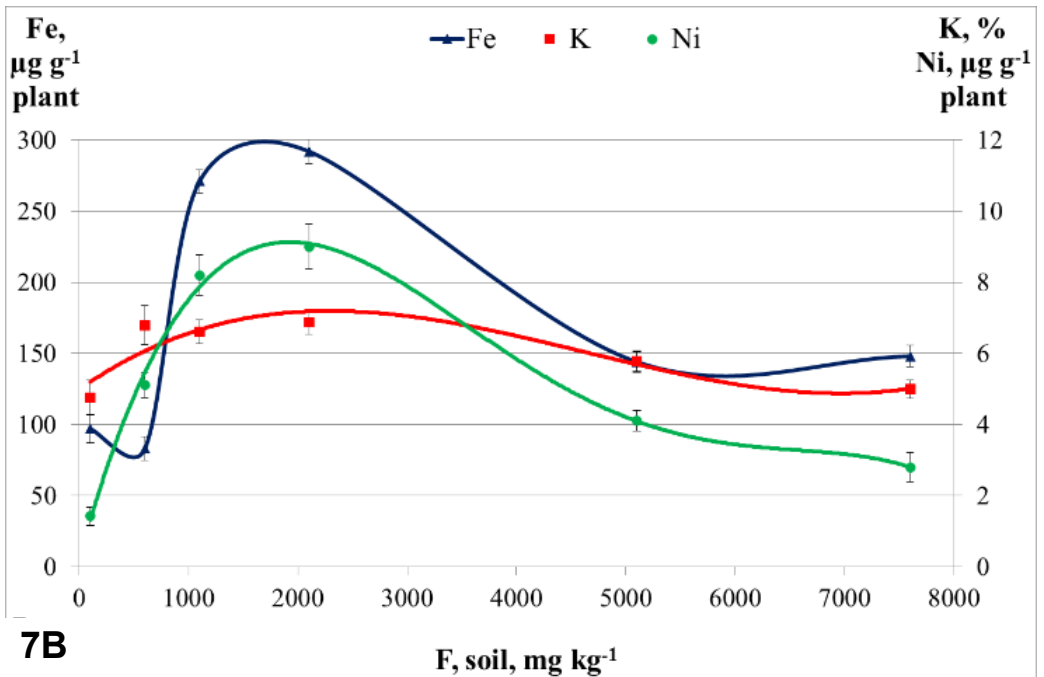
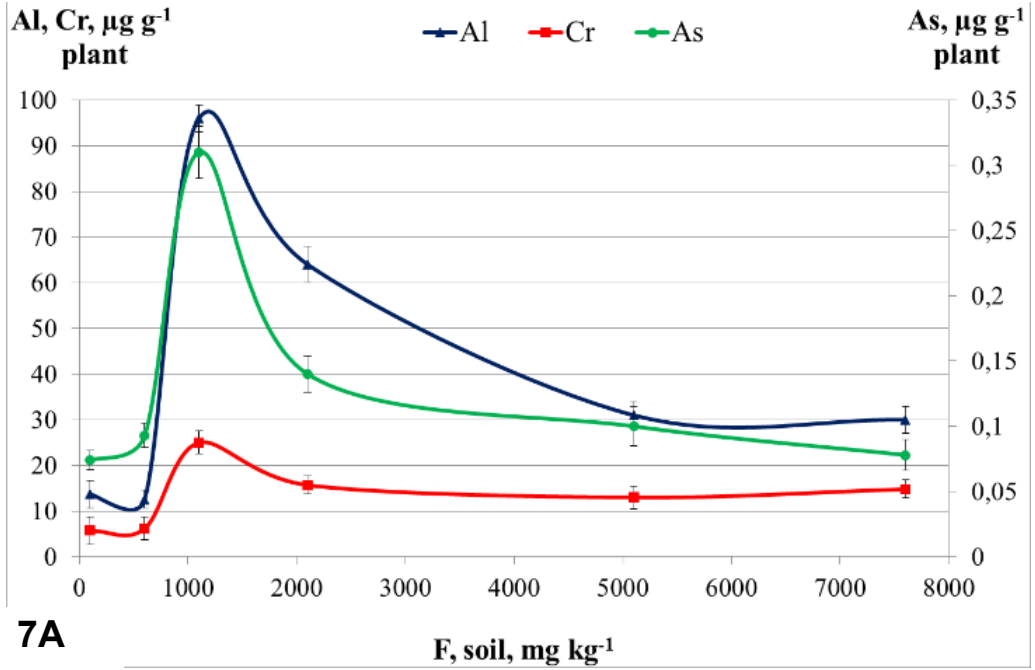
Increasing the F soil content was revealed to result in decrease of Mg concentration in plants (Figure 7C,  $r = -0.973$ , p-value 0.001) possibly because of formation of low-solubility and low-availability for plants of  $MgF_2$  and  $MgF^+$ . Similar dependences were obtained for Ca and Sr that exhibit close to Mg chemical properties (Figure 7C - insert,  $r = -0.937$ , p-value 0.006 and  $r = -0.954$ , p-value 0.003, respectively). This finding is in good agreement with significant decrease in magnesium uptake by yellow mombin (*Spondias mombin*)<sup>39</sup> and common almond (*Amygdalis communis*)<sup>40</sup> under F stress. Also there are literature data about similar behavior of Mg and Sr in soil-plant system<sup>41, 42</sup>.

**Table 2A.** Element concentrations in wheat plants at different F levels in soil (mg/kg)

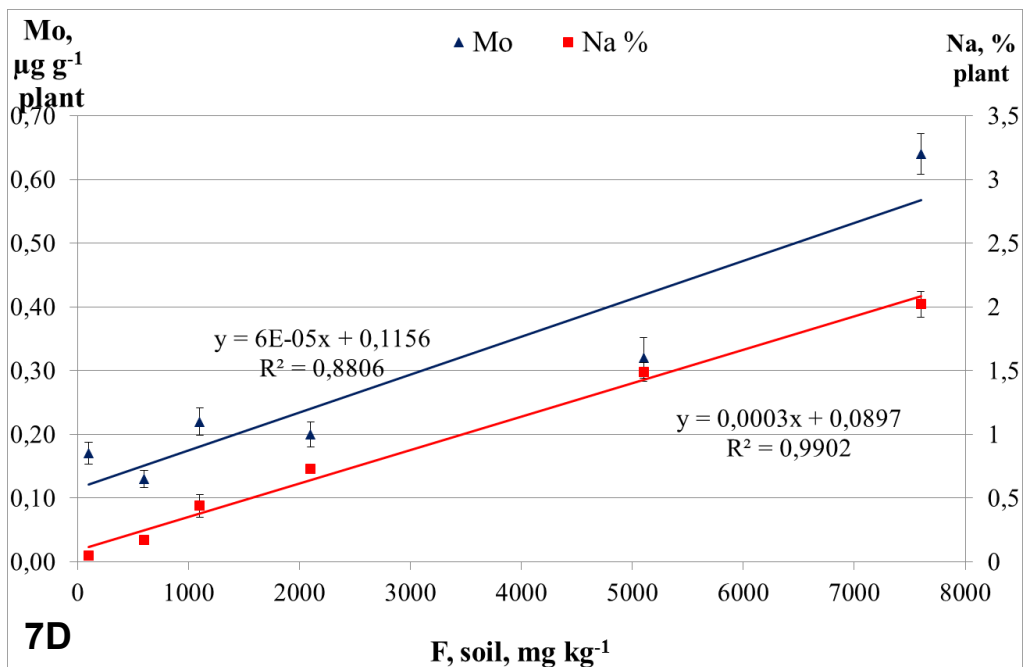
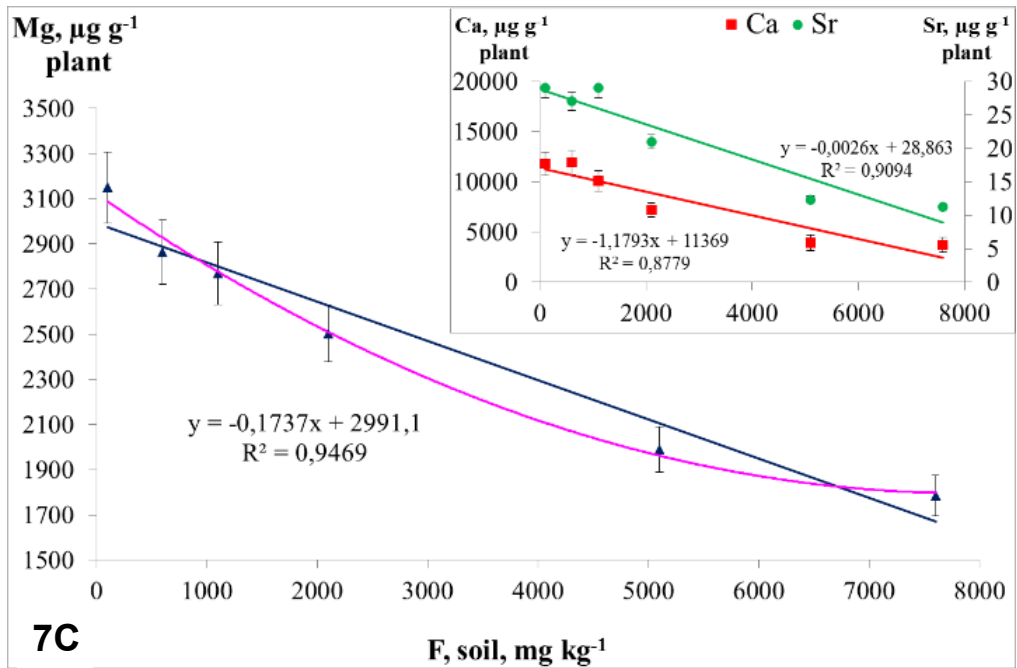
Element	Concentration of F in soil (mg/kg)					
	100	600	1100	2100	5100	7600
Al	13.7	12.6	96	64	30	31
As	0.074	0.093	0.31	0.14	0.10	0.078
Ba	5.6	5.5	9.7	7.7	4.6	3.6
Ca	11798	11930	10051	7200	3945	3717
Cd	0.081	0.10	0.10	0.078	0.059	0.053
Co	0.019	0.022	0.11	0.067	0.037	0.047
Cr	5.7	6.2	25	15.7	13.0	14.8
Cu	3.8	3.3	4.5	4.4	3.5	3.8
F	5	28	15	30	90	100
Fe	97	83	271	292	144	148
K	47633	67941	66114	68811	57845	49871
Mg	3151	2863	2769	2505	1990	1786
Mn	38	48	69	93	95	86
Mo	0.17	0.13	0.22	0.20	0.32	0.64
N	47000	42000	45000	48000	43000	41000
Na	534	1717	4382	7312	14935	20203
Ni	1.42	5.1	8.2	9.0	4.1	2.8
Pb	0.29	0.32	3.3	1.16	0.81	0.61

**Table 2B.** Element concentrations in wheat plants at different F levels in soil (mg/kg)

Element	Concentration of F in soil (mg/kg)					
	100	600	1100	2100	5100	7600
Sb	<0.02	0.04	0.16	0.07	0.03	0.03
Se	0.039	0.070	0.046	0.065	0.049	0.037
Sn	<0.07	<0.07	0.15	0.12	<0.07	0.10
Sr	29	27	29	21	12.3	11.2
Th	<0.002	<0.002	0.013	0.005	0.003	0.004
Ti	1.21	1.21	7.6	4.0	2.5	2.5
Tl	0.006	0.006	0.006	0.004	0.004	0.003
V	0.049	0.057	0.34	0.18	0.10	0.13
Y	0.007	0.007	0.069	0.030	0.017	0.020
Zn	44	40	42	41	39	41
La	0.037	0.031	0.33	0.11	0.085	0.1
Ce	0.055	0.046	0.51	0.19	0.12	0.13
Pr	0.006	0.005	0.050	0.020	0.014	0.021
Nd	0.021	0.019	0.17	0.068	0.047	0.078
Sm	0.003	0.003	0.026	0.010	0.007	0.009
Gd	0.003	<0.004	0.020	0.008	0.005	0.008
Dy	<0.002	<0.002	0.013	0.006	0.004	0.004
Er	<0.002	<0.002	0.005	0.003	0.002	0.002



**Figures 7A and 7B.** Relationship between element concentrations in wheat plants and F soil content.



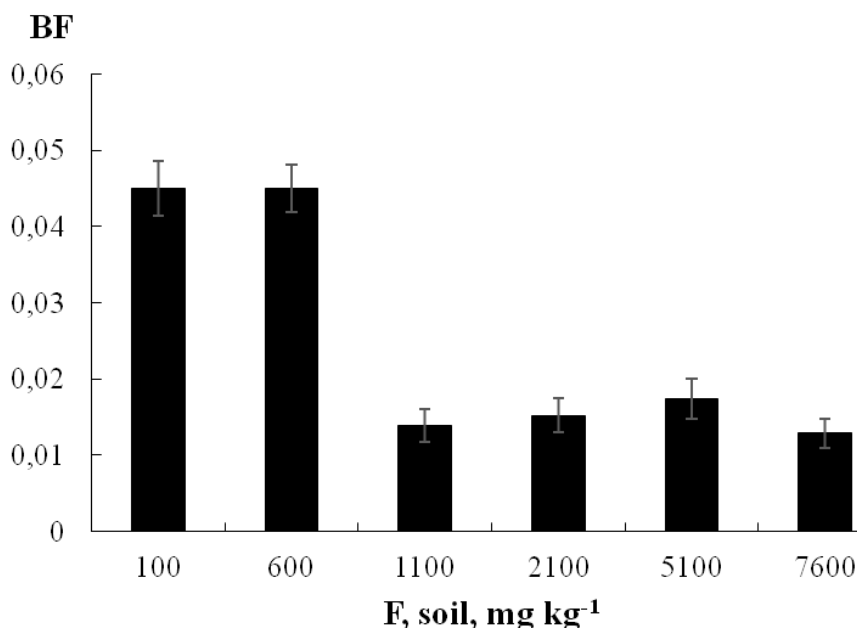
**Figures 7C and 7D.** Relationship between element concentrations in wheat plants and F soil content.

Decreasing the concentration of mobile ions of alkali earth elements in the presence of F significantly reduces the adaptive capacity of plants. This is especially so with magnesium that is well known to play an important biological role. Also calcium that is a secondary messenger could determine the concentrations of other elements via affecting the membrane functions through the interaction with calmodulin.<sup>43-44</sup>

It is interesting that confidence level of nonlinear approximation of dependency of Mg from F ( $r = -0.997$ , p-value 0.019) is less than linear ones but still significant. The nonlinear approximation leads to some constant value – 1800 mg/kg that could be interpreted as least necessary concentration of Mg for the plant vitality.

Concentration of Mo was raised in wheat plants with increasing the F soil content (Figure 7D,  $r = 0.938$ , p-value 0.006). It is consistent with the literature data about Mo accumulation in tea leaves (*Camellia sinensis*) in experiments simulating F stress by hydroponics method<sup>45</sup>. An increase of the Na concentration in wheat plants may be associated with the rise of the Na content in the soil together with F due to the addition of ions as NaF (Figure 7D,  $r = 0.995$ , p-value <0.001).

A drastic fall of the F bioaccumulation factor was obtained with an increase in the F concentration in soils (Figure 8). It was likely to be due to the plants having adaptive mechanisms to form barrier functions for protection against increased concentrations of F. We have previously observed the effect of reducing the accumulation factor with increasing soil contamination in the Moscow region<sup>46</sup>. Thus, the revealed antisymbatic dependence of bioaccumulation factor on F soil concentration contradicts the concept of passive diffusion of F from soil solutions.<sup>4</sup>



**Figure 8.** Dependence of bioaccumulation factor (BF) in the wheat plant on F concentration in soil.



### 3.4. Discussion of F stress development on wheat plant (*Triticum aestivum* L.)

The data of morphological characteristics, biochemical parameters and elemental composition obtained in this work were used to suggest a possible scheme for the development F stress in wheat plant (*Triticum aestivum* L.).

**Stage 1.** High value of accumulation coefficient indicates about effective uptake of F by wheat plant (*Triticum aestivum* L.) at F soil content up to 600 mg/kg. This intake of F is likely to be promoted by  $\text{Ca}^{2+}$ -ATPase and  $\text{H}^{+}$ -ATPase as described by Peng et al.<sup>48</sup>. However, the molecular mechanisms are still poorly documented. When the concentration of F in the soil reached the levels of 600 mg/kg, the plant showed the first symptoms of F stress. They were decreasing the nitrogen content (P-value 0.001) and opposite, increasing the activity of catalase (P-value 0.048), the content of chlorophyll (P-value 0.001) and potassium (P-value <0.001) at the F concentration in soil of 600 mg/kg compared to 100 mg/kg, possibly due to some compensatory mechanism.

**Stage 2.** Concentration of various elements reliably increased at increasing F soil content in range of 600 to 1100 mg/kg. Statistical significance (P-value) of differences between concentrations of elements at F soil concentrations of 600 and 1100 mg/kg was found of  $p \leq 0.001$  for Al, As, Cr, Fe, Y, V, Ti; 0.002 for Ba and Pb, and 0.004 for Co. It could be explained by enhancing the mobility of elements ions due to disintegration of soil colloids structure because of specific sorption of F.<sup>49,50</sup> As a result, more ions are released from the soil and become available for the plants. Decreasing the concentration of  $\text{Ca}^{2+}$  is followed by the disruption of the abiotic stress-responsive signaling process.<sup>51</sup> Value of electrolyte leakage at F concentration in soil of 1100 mg/kg was more than that at 600 mg/kg (P-value 0.001).

Both the accumulation of nitrogen in the plant tissues and the increased electrolyte leakage could be considered as non-specific reactions to F stress.<sup>52</sup> A strong reduction in the accumulation coefficient (Figure 8) may indicate the changing mechanism of F entry into the plant.

**Stage 3.** Further increasing the concentration of F in the soil is likely to result in implementation of adaptive biochemical processes. Electrolyte leakage and acid phosphatase activity reach their maximum values at a F soil content of 2100 mg/kg and decrease with F soil levels of 5100 and 7100 mg/kg to the values that were found for low F soil levels. P-values of differences between electrolyte leakage at F soil concentrations of 2100 and 5100 mg/kg were calculated to be <0.001 and 0.008 for F soil concentrations of 2100 and 7100 mg/kg. P-values for acid phosphatase activity were calculated to be <0.001 for both cases. Concentration of Mo was less at F soil of 2100 mg/kg compared to 5100 mg/kg (P-value 0.029) and compared to 7100 mg/kg (P-value 0.001). Mo is accumulated in wheat plants as a structural component of molybdopterin that is a cofactor of a number of plant enzymes. These enzymes catalyze the key stages of nitrogen, carbon, and sulfur metabolism<sup>53</sup>, and this may possibly be important in plant adaptation to F stress.

## CONCLUSIONS

Electrolyte leakage and acid phosphatase activity could be used as the most sensitive biochemical markers for estimation of F stress. A possible 3-stage scheme for the development of F stress in wheat plant was suggested. The decrease of both

the F bioaccumulation factor and electrolyte leakage and acid phosphatase activity at high F soil levels could be part of the adaptation mechanism of the wheat plant (*Triticum aestivum* L.) to F stress.

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