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Partial Loss of Cytochrome C by Mitochondria of Pea Seedlings Under Conditions of Water Scarcity

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ABSTRACT

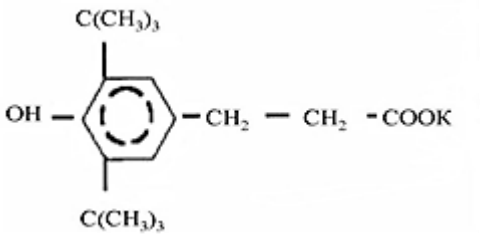
We investigated the effect of water deficiency and treatment of pea seedlings with 10^{-11}M solution of a synthetic phenolic antioxidant - potassium phenosan on the functional state of mitochondria of pea seedlings. Water deficiency, triggering lipid peroxidation, leads to a decrease in the content of unsaturated fatty acids containing 18 and 20 carbon atoms in the mitochondrial membranes, decreases the maximum oxidation rates of NAD-dependent substrates and succinate also the electron transport rates at the terminal stage (the cytochrome oxidase stage) of the mitochondrial respiratory chain. In addition, water deficiency causes mitochondrial swelling. Introduction into the incubation medium of mitochondria 5×10^{-6}M of cytochrome C or 10^{-11}M of potassium phenosan restores the electron transfer rates at this stage of the respiratory chain. The antioxidant prevents mitochondrial dysfunction. It is assumed that a breach of the bioenergetics characteristics of mitochondria in conditions of water deficiency apparently connected with the oxidation of unsaturated fatty acids mainly the linoleic acid, which are included in the composition of the cardiolipin .Potassium phenosan, preventing lipid peroxidation, restores the functional activity of mitochondria.

Keywords: Water Deficiency, Mitochondria, Cardiolipin, Cytochrome C

I. INTRODUCTION

Water is part of cells of all plant tissues, it transports nutrients from roots to leaves and on the contrary. In the plant tissues are includes approximately 70-80% water. Therefore, its quantity in various habitats is a limiting factor for plants and animals and determines the nature of the flora and fauna of this area. The lack of water violates normal vital activity of plants. The shortage of water primarily leads to a reduction in the cells of the free water, which alters the hydration shell of proteins of the cytoplasm and affects the functioning of proteins-enzymes. While inhibited cell division and especially the stretch that leads to the formation of small cells. The result of delayed growth of the plant, especially the leaves and stems. Plants, which experienced strong short-term drought, and couldn't return to normal metabolism [1]. Implementation of anti-stress programs require large energy expenditures [2]. Thus, the energy exchange plays an important role in adaptive reactions of the organism. In our work, we paid attention mainly on mitochondria, as these organelles like in plants so in animals play a major role in the response of the organism to the action of stress factors. Changes in the environment lead to changes in the lipid composition of mitochondrial membranes. Thus there is a transformation of degree of saturation and the amount of free fatty acids [3], that may be a signal of action of the stressful factor. It is known that temperature-dependent changes of fatty acid composition supports the fluidity of the lipid fraction of the membranes at a level, which allowing to maintain associated with membrane enzymes in functionally-active state at a quite wide temperature ranges [4,5]. In addition, the mitochondrial membrane of frost-resistant plants, containing a large amount of unsaturated fatty acids that allow these organelles change own volume in a wide temperature range that provides the higher cell energy potential. Similar data are known also for plants that are resistant to other influences: high temperatures, drought, infection, and ethanol [6]. Note that in conditions of water scarcity the most significant changes occur in the content of unsaturated fatty acids with 18 carbon atoms [7, 8] and, that particularly important in the content of linoleic acid. This acid is one of the main fatty acids included in the composition of cardiolipin – phospholipid, providing efficient functioning of the mitochondrial respiratory chain due to the formation a

sufficiently close contacts between the respiratory complexes with the formation of supercomplexes of respiratory carriers [9]. Excessive generation of ROS by mitochondria under stress leads to the interaction of ROS with polyunsaturated fatty acids that included in the lipid composition of membranes, such as linoleic, linolenic and arachidonic acid, and activation of lipid peroxidation (LPO). Peroxidation of linoleic acid has the consequence of decreasing the amount of this phospholipid in the inner membranes of mitochondria that underlies the damaging effects of reactive oxygen species on the functional state of mitochondria [9]. In addition, peroxidation of membrane lipid, primarily cardiolipin, leads to oxidation of thiol groups of proteins, mitochondria swelling, release of cytochrome C and possibly apoptosis induction [10, 11]. Based from this, it could be assumed that the drugs with adaptogenic properties must influence the generation of ROS by mitochondria. Primarily for this role are applicable antioxidants. The object of investigation was been selected antioxidant from the class of spatial hindered phenol - potassium phenosan (potassium 3, 5-di-tert- butyl-4-hydroxyphenyl propionate) :



Potassium Phenosan

Since water deficiency reduces functional activity, as chloroplasts as mitochondria [12], it was interesting to find out how this antioxidant affects the functional state of mitochondria of pea seedlings subjected to 2-day water deficiency. The test of protective properties of the drug was performed using a 10^{-11} M potassium phenosan (in the concentration in which the antioxidant decreases the LPO intensity to control values in model experiments [13]).

II. METHODS AND MATERIAL

Plant material

The study was carried out on mitochondria isolated from pea seedlings (P. *sativum*), variety Flora-2 obtained in standard conditions and in the conditions of water deficiency.

Pea seeds germination

Pea (*Pisum sativum* L., cv. Flora 2) seeds were washed with soapy water and 0.01% $KMnO_4$. Control seeds were then soaked in water, experimental seeds – in 10^{-11} M potassium phenosan for 1 h. Then the control group seeds were placed on wet filter paper, where they were in the dark at a temperature of 24^o C. The experimental group were placed on filter paper, wetted 10^{-11} M solution of potassium phenosan. After 2 days, half of control (WD) and of potassium phenosan treated seeds (WD+PHEN) were transferred on dry filter paper. Another half of control plants were retained on wet filter paper, where they were kept for 6 days. After 2 days of water deficit the sprouts group WD were transferred to moist filter paper, where they remained for the next two days at 24^0 C. Seedling of WD+PHEN group transferred to filte r pape r soaked in 10^{-11} M solution of potassium pfenosan. On the sixth day, mitochondria were isolated from seedling epicotyls.

Isolation of mitochondria

Isolation of mitochondria from 6-day-old epicotyl of pea seedlings (P. sativum) performed by the method [12] in our modification. The epicotyls having a length of 1.5 to 5 cm (20-25 g) were placed into a homogenizer cup, poured with an isolation medium in a ratio of 1:2, and then were rapidly disintegrated with scissors and homogenized with the aid of a press. The isolation medium comprised 0.4 M sucrose, 5 mM EDTA, and 20 mM KH_2PO_4 (pH 8.0), 10 mM KCl, 2 mM 1, 4-Dithio-di-theiritol, and 0.1% fatty acids-free (FA-free) BSA .The homogenate was centrifuged at 25000g for 5 min. The precipitate was re-suspended in 8 ml of a rinsing medium comprised: 0.4 M sucrose, 20 mM KH_2PO_4, 0.1% FA-free BSA (pH 7.4) and centrifuged at 3000g for 3 min. The supernatant was centrifuged for 10 min at 11000 g for mitochondria sedimentation. The sediment was re-suspended in 2-3 ml of solution contained: 0.4 M sucrose, 20 mM KH_2PO_4 (pH 7.4), 0.1 % FA-free BSA and mitochondria were precipitated by centrifugation at 11000 g for 10 min.

Rate of mitochondria respiration

Respiration in mitochondria was recorded polarographically (an LP-7 polarograph, Czech Republic) using a Clark oxygen electrode. The incubation medium contained 0.4 M sucrose, 20 mM

Hepes–Tris (pH 7.2), 5 mM KH_2PO_4, 4 mM $MgCl_2$, and 0.1% BSA (28°C).The rate of respiration was expressed in ng atom O/(mg protein min).

Fatty acid methyl esters (FAMEs)

FAMEs were produced by acidic methanolysis of mitochondrial membrane lipids [14, 15]. Mitochondrial suspension (200 μL) was placed in a special hermetically closed tube, 5 mL of methanol was added, and the sample was placed in the freezer for 1 h. Thereafter, 600 μL of acetyl chloride was added, and the sample was boiled for 1 h with stirring. FAMEs were additionally purified by TLC on glass plates with silica gel KSK (Russia). FAMEs were extracted with hexane, and solutions obtained were analysed.

FAME identification

FAME identification was performed by chromato mass spectrometry (GCMS) using a Hewlett-Packard-6890 spectrophotometer with a HP-5972 mass-selective detector and after the retention times [16]. FAME were separated in the HP-5MS capillary column (30 m ×0.25 mm, phase film thickness of 0.25 μm) at programmed temperature increase from 60 to 285°C at the rate of 5°C/min. Evaporator temperature is 250°C, detector temperature is 280°C. Mass spectra were obtained in the regime of electron impact ionization at 70 eV and the scan rate of 1 s/10 mass in the scan mass range of 40–450 a.u.m.

FAME Quantification.

FAME quantification was performed using a Kristall 2000M chromatograph (Russia) with flame-ionization detector and quarts capillary column SPB-1 (50 m×0.32 mm, phase film thickness of 0.25 μm). FAME analysis was performed at programmed temperature increase from 120 to 270°C at the rate of 4°C/min. Temperature of injector and detector – 270°C; the helium carrier gas rate was 1.5 mL/min. Each sample contained 2 μL of the hexane extract. The FAME content in samples was calculated as the ratio of peak area of a corresponding acid to the sum of peak areas of all found FAMEs.

Lipid peroxidation (LPO) activity

LPO activity was assessed by fluorescent method [17]. Lipids were extracted by the mixture of chloroform and methanol (2:1). Lipids of mitochondrial membranes (3–5 mg of protein) were extracted in the glass homogenizer for 1 min at 10°C. Thereafter, equal volume of distilled water was added to the homogenate, and after rapid mixing the homogenate was transferred into 12mL centrifuge tubes. Samples were centrifuged at 600 g for 5 min. The aliquot (3 mL) of the chloroform (lower) layer was taken, 0.3 mL of methanol was added, and fluorescence was recorded in 10mm quartz cuvette with a spectrofluorometer (FluoroMaxHoribaYvon, Germany). Background fluorescence was recorded using a mixture of 3mL chloroform and 0.3 mL methanol. The excitation wavelength was 360 nm, the emission wave length was 420–470 nm. The results were expressed in arbitrary units per mg protein.

The index of double bond (DB)

The index of double bond (IDS), which characterizes the degree of unsaturation of lipids, was calculated according to the formula: $IDS=\Sigma Pjnj/100$, where Pj is the contents of the fatty acids (FA) (in %), nj is the number of double bonds in each acid. Also we used the unsaturation coefficient (K) as the ratio of the sum of unsaturated FA to the sum of saturated FA.

Atomic force microscopy (AFM)

Samples of mitochondria fixed with 2% glutaraldehyde for 1 hour, followed by washing with water by centrifugation and precipitation**.** Mitochondria were applied to the surface of the silicon substrate and were dried on air. The study was performed on a SOLVER P47 SMENA at a frequency of 150 kHz in tapping mode. NSG11 used cantilever with a radius of curvature of 10 nm. Some geometrical parameters of mitochondrial image was determined using the "Image Analysis". The section was made at a height of 30 nm. The volume of a mitochondrial image is equal to the product of the sectional area of the mitochondrial image and the average height of the image in the region of section and is calculated by an Image Analysis program to the coordinate data and scanning pitch.

Statistics

Tables and figures present means values and their standard deviations. The number of experiments was 10.

Reagents

Sucrose, rotenone, antimycine A, N,N,N',N'-tetramethyl- p-phenylenediamine (TMPD), ascorbate, glutaraldehyde (Sigma-Aldrich, USA), BSA (Bovine serum albumin) (Fraction V, free fatty acids) (Sigma, USA), FCCP (carbonyl cyanide-p-trifluorometoxyphenylhydrazone, , KCl (Potassium chloride purees),1,4–Dithio-dl-theritol (Fluka, Germany), HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) (Biochemica Ultra, for molecular biology) (Fluka, Germany), Figure2: The unsaturation coefficient of C18 fatty acids in conditions of water `deficienty and seed treatment of pea with potassium phenosan.

Tris (hydroxymethyl)aminjmethan) (MP Biomedicalis, LLC, Germany), (Fluka, Germany), chloroform, methanol (Merk, Germany).

III. RESULTS AND DISCUSSION

The water deficiency led to LPO activation in the mitochondrial membranes of pea seedlings: the fluorescence intensity of LPO products increased 3 times (Figure 1).

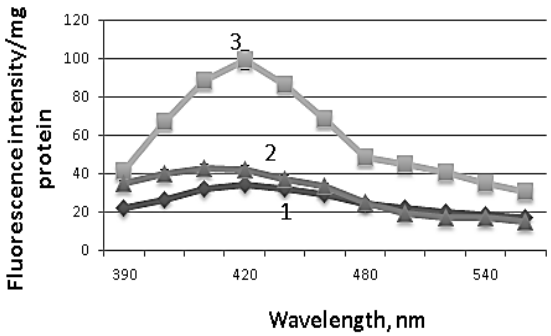


Figure 1: The fluorescence spectra of LPO products in the mitochondrial membranes of pea seedlings exposed to water deficiency and in the mitochondrial membranes from pea seedlings treated by potassium phenosan and exposed to water deficit Y-axis: fluorescence intensity, arbitrary units/mg protein; X-axis: wavelength, nm 1-Control; 2- Water deficiency + Potassium Phenosan (PHEN); 3- WD -. Water deficiency.

These data are consistent with the published data on the effect of a water stress on the activation of free radical oxidation in membranes of wheat seedlings [18, 19]. The treatment of pea sprouts by investigated antioxidant resulted in the decrease in the intensity of fluorescence of LPO products almost to control values. Activation of LPO caused substantial changes in the content of C_{18} and C20 fatty acids (FA) in membranes of mitochondria.

The content of unsaturated fatty acids (F A) has decreased, and the content of saturated fatty acids - increased. The index of double bonds reduced to 1.45± 0.02 to 1.30 ± 0.01, and the coefficient of unsaturation of fatty acids containing 18 carbon atoms decreases from 23.54 ± 0.07 to 15.15 ± 0 22.(Figure 2). While the contribution of linoleic acid, which is part of cardiolipin-one of the major phospholipids of the inner membrane of mitochondria in the total percentage of C18 unsaturated fatty acids decreased upon 5% (Figure.3), and the contribution of linolenic acid decreased by 26%. It must be emphasized that the lipids, including α- linolenic acid, are vital to maintain normal membrane structure and further accelerate the development of seedlings [20]. Changing the content of fatty acids with

18 carbon atoms due to dehydration are also observed in membrane lipids of potato, suspension of cells, and membrane lipids of the leaves of Arabidopsis thaliana and apricot [7,8,21]. In all these cases, insufficient watering was accompanied by a decrease in the contents of linoleic and linolenic acids and an increase in the content of stearic acid in membrane.

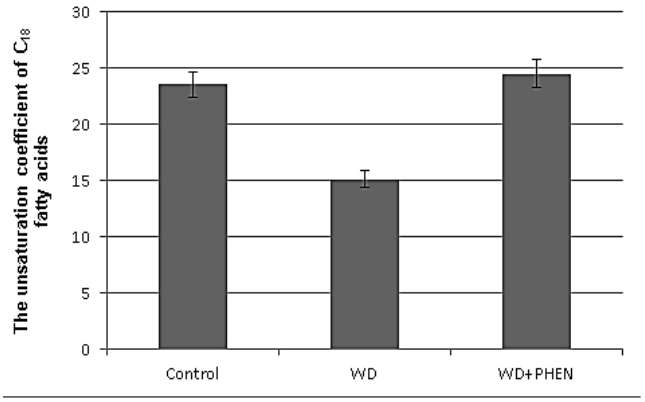


Figure 2 : The unsaturation coefficient of C18 fatty acids in conditions of water `deficienty and seed treatment of pea with potassium phenosan.

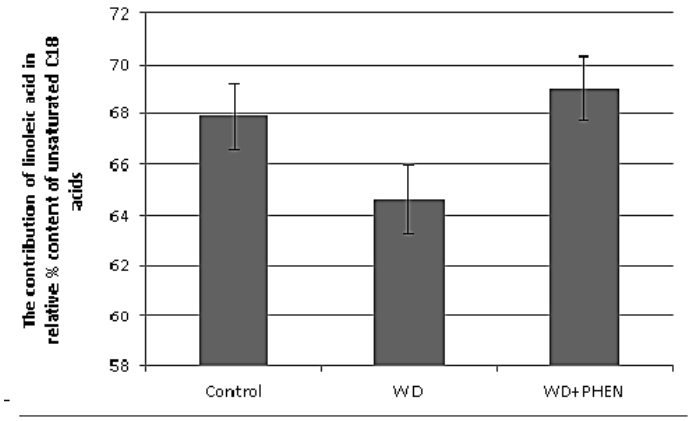


Figure 3 : The contribution of linoleic acid in relative content of unsaturated C_{18} fatty acids in membranes of pea seedling mitochondria.

Substantial change s occurred also in the relative content of fat ty acids with 20 carbon atoms. As in t he case of C_{18} FA, content of unsatura ted C_{20} FA decreased, and the content of saturated C_{20} fatty acids was increased: double bonds index decreases from 0.0531 to 0.0317 ±0.001 ± 0.001, and the coeffic ient of unsaturation of FA, containing 20 carbon atoms, decreases 3.65 ± 0,03 on 1.20 ± 0.16 (Figure 4).

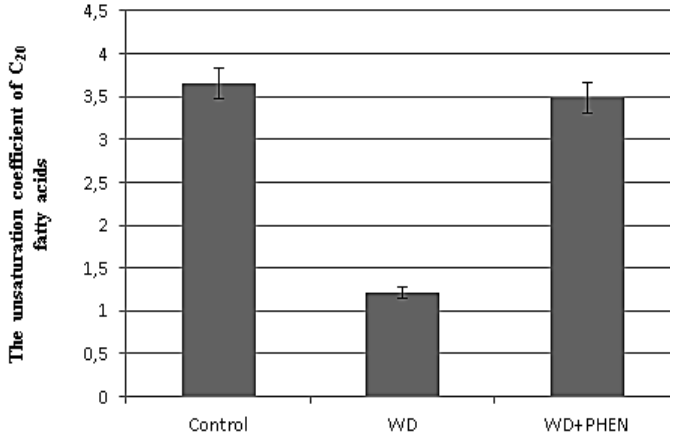


Figure 4. The unsaturation coefficient of C20 fatty acids in conditions of water `deficienty and seed treatment of pea with potassium phenosan

Treatment of pea sprouts with potassium phenosan prevents the change in the fatty acid composition of mitochondrial membranes caused by activation of lipid peroxidation. Changes in physicochemical properties of mitochondrial membranes probably may entail changing and lipid-protein interactions and, consequently, the activity of membrane-bound enzymes, in particular, enzymes of the mitochondrial respiratory chain. Indeed, water deficiency resulted in decreasing the maximum oxidation rates of NAD-dependent substrates. Rates of oxidation of NAD-dependent substrates in the presence of FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) decreased about 40% and 30% reduction in RCR in the oxidation of these substrates by pea seedlings mitochondria (table 1).

Table 1. Effects of insufficient watering (WD) and potassium phenosan (WD+PHEN) on the rate of NAD-dependent substrate oxidation by mitochondria isolated from pea seedlings, ng-mol / (mg protein min

Group	State 2	State 3	State 4	RCR	FCCP	KCN
Control	19.5 ±2.5	70.0 ±4.1	29.20 ±1.4	2.40± 0.01	74.0 ±5.8	4.4. 5 5±1 5 .5 5
WD	11.0 ±1.8	45.4 ±2.8	27.5± 1.0	1.65± 0.02	44.3 ±2.2	6.0 0 ±2. 0
WD+PHEN (10^{-11} M)	20.1 ±2.8	69.5 ±2.9	28.7± 1.3	2.42± 0.02	70.3 ±3.2	5.1 6 ±1. 6

The incubation medium contained 0.4M sucrose, 20 mM HEPES-Tris buffer, 5 mM KH_2PO_4, 4 mM $MgCl_2$ and 0.1% BSA, 10 mM malate, 10 mM glutamate. Other additives: 125µM ADP, 10^{-6}M FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone), 1mM KCN The results of 10 experiments are presented.

However, he is less impact on the rate of oxidation of succinate. So the maximum oxidation rates of this substrate were reduced by 15% and RCR - by 3.6% (table 2), indicating greater stability to oxidative stress the complex II of the respiratory chain, particularly to water shortages, and agrees with data obtained on the mitochondria of the cell culture of *Arabidopsis* [22]. Note that potassium phenosan has prevented changes in the efficiency of oxidative phosphorylation caused by water deficiency. Also, this antioxidant has prevented decreasing the oxidation rates of NAD-dependent substrates and succinate in the presence FCCP.

Table 2. Effects of insufficient watering (WD) and potassium phenosan (WD+PHEN) on the rate of succinate oxidation by mitochondria isolated from pea seedlings, ng-mol / (mg protein min)

Group	State 2	State 3	State 4	RCR	FCCP	KCN
Control	42.3 ± 3.8	113.2± 2.5	45.7 ±3.1	2.48 ± 0.03	129.0± 7.2	3.2 ±1.4
WD	31.7.0±2.6	96.0 ±3.5	40.2 ±4.1	2.38 ±0.02	109.6±5.2	4.0 ±1.7
WD+PHEN (10^{-11} M)	38.7 ± 4.1	115.0±1.4	46.0 ±1.7	2.50 ± 0.03	130.0±2.9	3.8 ±2.0

The incubation medium contained 0.4M sucrose, 20 mM HEPES-Tris buffer, 5 mM KH2PO4, 4 mM MgCl2 and 0.1% BSA, 8 mM succinate + 5 mM glutamate. Other additives: 125μM ADP, 10-6M FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone), 1mM KCN The results of 10 experiments are presented.

The dysfunction of the electron transport chain of mitochondria under conditions of water scarcity, probably associated with the oxidation of unsaturated fatty acids included in the composition of cardiolipin, mainly linoleic acid, and thus with a possible decrease of the content of this phospholipid in the inner membrane of mitochondria [23].

Confirmation of this hypothesis is a 2-fold reduction in the electron transport rates in the final cytochrome oxidase section of mitochondrial respiratory chain pea seedlings in situations of water scarcity (Figure 5).

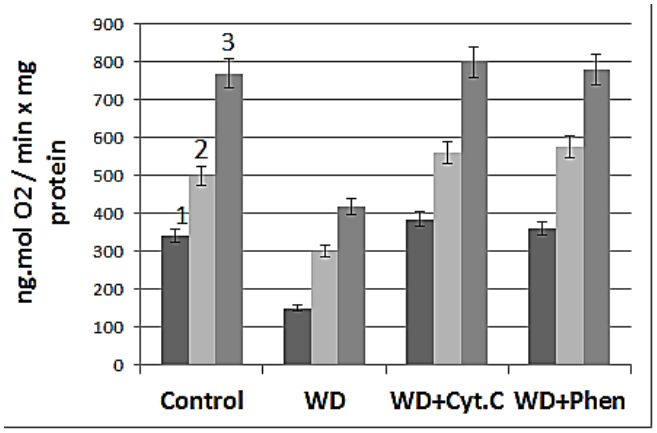
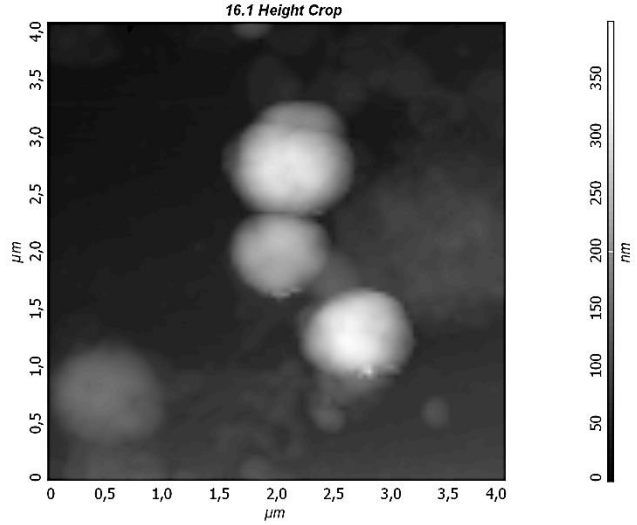


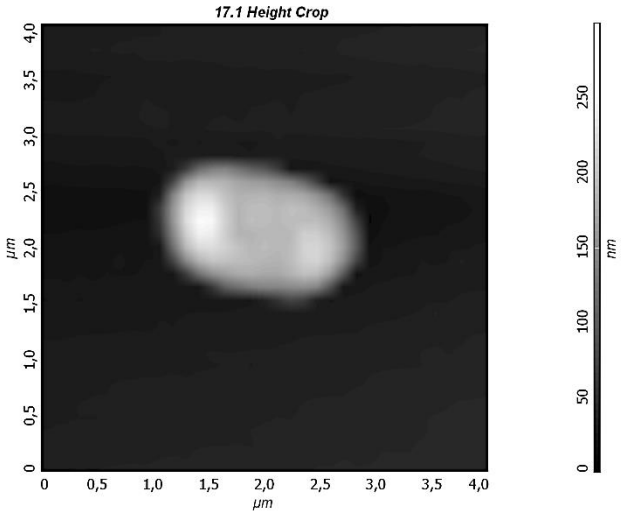
Figure 5 : The oxidation rate of ascorbate in the presence of TMPD (with mitochondria pea seedlings. X-axis: the different groups of plants; Y-axis: in the oxidation rate ng. mol O_2 / min × mg protein. The incubation medium contained: 0.4M sucrose, 20 mM HEPES-Tris buffer, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 10mM ascorbate, 60μM rotenone, 5 μM antimycine A, 0.5μM FCCP (carbonyl cyanide-*p*-trifluorometoxyphenylhydrazone). 1-200 μM TMPD N,N,N',N'- tetramethyl- p-phenylenediamine); 2- 400 μM TMPD; 3- 600 μM TMPD. Cytochrome C was added to the incubation medium in a concentration of 5 × 10^{-6}M.

Introduction to the incubation medium of these mitochondria, 5× 10^{-6}M of cytochrome C led to recovery rates of oxidation of pair ascorbate+ TMPD to control values, indicating that the output from the mitochondria into the cytosol part of cytochrome C , which is caused by oxidation of cardiolipin in the inner membrane of these organelles. This suggestion conform data obtained by atomic force microscopy (AFM),that indicate a possibility peroxidative swelling of mitochondria [24, 25].

AFM images of pea seedlings mitochondria subjected to a two-day water deficiency, were significantly changed and differed from the control samples. Statistical analysis of the amount of pre-fixed with glutaraldehyde mitochondria of this group of seedlings shows the appearance of a solitary mitochondria larger volume (Vav. =110,23(μm)^2 × nm) (Figure 6 b) compared with the control group (Vav. = 80,05(μm)^2 × nm) (Fig. 6 a), indicating mitochondrial swelling. . The number of divisible mitochondria in group WD decreased significantly.



(a)



(b)

Figure 6. The AFM image of the mitochondria isolated from the pea seedlings of the "Control" group (a) and water deficiency group (b). The two-dimensional AFM image of mitochondria (10x10 $\mu м^2$) isolated from 6-day pea seedlings.

Thereby potassium phenozan, protecting against peroxidation the unsaturated fatty acids contained in the lipid composition of membranes, had prevented changes in the fatty-acid composition of membranes of seedlings grown under water deficit (WD+PHEN). The content of C_{18} unsaturated FA, which play a critical role in plant resistance to unfavourable environmental factors [28], remained at control values. Did not differ from the control values also and the relative content of C_{20} fatty acids. Changes in the fatty-acid composition of mitochondrial membranes was accompanied by changes in the maximum rates of oxidation of NAD-dependent substrates and succinate. Remained high rate of oxidation of substrates and had prevented the decline in efficiency of oxidative phosphorylation caused by water deficit (table 1, 2). Herewith it were recovered the electron transport rates in the terminal phase of the mitochondrial respiratory chain (Figure.4).

Thus potassium phenozan, preventing the peroxidation of phospholipids, primarily cardiolipin, apparently, prevents the dissociation of supercomplexes respiratory chain of mitochondria [26], thereby providing the effective work of electron-transport chains of mitochondria. We can also assume that in water deficit conditions, cytochrome C plays a key role in defining the functional state of mitochondria of pea sprouts: on the one hand, reside in the mitochondrial intermembrane space of mitochondria, he can play the role of an antioxidant,, oxidizing superoxide radical into molecular oxygen. On the other hand, being in contact with cardiolipin, it can accelerate the oxidation of this phospholipid in the presence of H2O2,that facilitates the release of cytochrome C from mitochondria (Vladimirov et al., 2006).

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