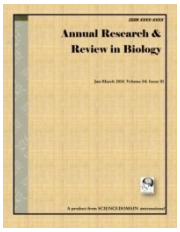

Annual Research & Review in Biology
4(19): 3007-3025, 2014

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Combined Effect of Insufficient Watering, Moderate Cooling, and Organophosphorous Plant Growth Regulator on the Morphology and Functional Properties of Pea Seedling Mitochondria

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Mini-Review Article

Received 21st March 2014
Accepted 15th May 2014
Published 26th May 2014

ABSTRACT

Aims: In nature, plants are exposed to not one but several environmental factors. In this connection, the problem of studying the rearrangements of plant cell metabolism under exposure to several abiotic factors is of special importance. Various stresses lead to dysfunction of mitochondria that underlie the development of cell death and the whole organism. It is known that regulators of plant growth and development increase the resistance of plants to both biotic and abiotic stressors. One of such growth regulators is melaphen, a melamine salt of bis (hydroxymethyl) phosphinic acid. The aim of this work was investigation the effects of combined action of insufficient watering, moderate cooling to 10-14ºC and plant growth by regulator melaphen (melamine salt of bis(oxymethyl)-phosphinic acid) on the atomic force microscopy images of isolated mitochondria 5 day-old seedlings of pea (*Pisum sativum* L).
Place and Duration of Investigation: Emanuel Institute of Biochemical Physics Russian

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Academy of Sciences, Moscow, Russia and Timiryazev Institute of Plant Physiology Russian Academy of Sciences, Moscow, Russia, between June 2009 and October 2012.
Methodology: Functional state of mitochondria were researched by rate of mitochondria respiration, by the level of lipid peroxidation, the structural characteristics of mitochondrial membranes and by mitochondrial morphology, which was studied by method of atomic force microscopy.
Results: An atomic force microscopy (AFM) technique has revealed a statistically significant change in the shape of mitochondria (swelling mitochondria) exposed to insufficient watering and moderate cooling, which was associated with activation of lipid peroxidation in mitochondrial membranes and with disturbance of bioenergetics functions of these organelles. Treatment of pea seeds with $2x10^{-12}$ M solution melaphen prevents mitochondrial swelling and associated with that dysfunction of organelles
Conclusion: It was suggested that the effect of the drug on the morphology of mitochondria was associated with its antiradical and antioxidant properties.

Keywords: Mitochondria; plant growth regulators (PGR); insufficient watering; cooling; the atomic force microscopy; lipid peroxidation.

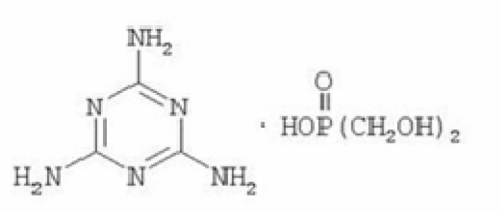
1. INTRODUCTION

The adverse environmental factors (drought, flood, high temperature, heavy metals, mechanical damages, herbicides, etc.) lead to disturbance of the antioxidant-prooxidant equilibrium in plant cells in the direction of excessive production of reactive oxygen species (ROS) by mitochondria and chloroplasts and may result in severe functional disorders as there are injured various components of plant cells [1,2]. Since under stress conditions mitochondria in animals [3] and mitochondria and chloroplasts in plants [4] are the main source of ROS, we paid attention to the role of mitochondria in the resistance of plants to stresses. ROS can inhibit or reduce the activity of enzymes of mitochondria containing Fe-S clusters, such as NADH-dehydrogenase (complex I), ATP-synthetase (complex V), and aconitase [5,6]. The accumulation of H_2O_2 in these organelles may induce the interaction of hydrogen peroxide with Fe^{2+} of mitochondria, which will evidently promote the formation of OH• via the Fenton's reaction [7]. The interaction of $OH^{\bullet}$ with polyunsaturated fatty acids of membrane lipids, e.g., linoleic acid, linolenic acid, and arachidonic acid, leads to activation of lipid peroxidation (LPO). The formed LPO products such as 4-hydroxy-2, 3-nonenals (HNE) have been shown to inhibit mitochondrial pyruvate dehydrogenase (PDC) and 2-oxoglutarate dehydrogenase (OGDC) by modification of lipoic acid residues [8], which leads to mitochondrial dysfunction and ultimately cell death. In animal cells and yeast, these organelles are combined into an extensive network, referred to as «mitochondrial reticulum" [9], in higher plants, the mitochondria singly and have either a spherical or cylindrical shape [10,11]. Under stress conditions, mitochondria form branched networks (anoxia) [12] or aggregate and form dense clusters around chloroplasts or in other regions of cytosol (heat shock, UV irradiation, effect of strong oxidants) [13]. Creation of a "giant mitochondria» is accompanied by an increase in the generation of ROS [14,15]. Antioxidants prevent the formation of a "giant mitochondria" and increase the generation of ROS by these organelles [13,16 and 17]. The standard procedure for selection of the mitochondria in a sucrose solution leads to the complete destruction intermitochondrial contacts. For this reason, the mitochondria are presented in separate vesicles before 1.2μm in diameter. The morphology of isolated mitochondria possibly reflects their functional state [18], consequently, the degree of the plants adaptation to changing environmental conditions.

Plants grow in constantly changing environmental conditions and undergo combined effects of various abiotic and biotic natural factors. But a response to a combined effect of these factors may be very different from that to the effect exerted by each of these factors. Some of the mechanisms are specific for a certain environmental factor, whereas the others are common (nonspecific) for a variety of factors; This determines the ability of plants under the action of one stress factor to increase the resistance to the other stress factor (the phenomenon of cross-adaptation) [19,20 and 21]. We can assume that on minor changes in the strength of the effect, a combined effect of several abiotic factors (e.g., insufficient watering and low temperature) will allow plants more successfully adapt to extreme conditions. Plant growth regulators (PGR) are preparations that promote the resistance of plants to stress factors. The use of plant growth and plant development regulators is one of the most efficient ways of increasing the crop production and strengthening their resistance to stress factors and pathogens. The natural plant growth stimulants are phytohormones and their synthetic analogues.

The use of phytohormones in the farming industry is associated with many problems. In fact, the manufacture of phytohormones and cleaning them from impurities are costly processes, which make the use their in practice disadvantageous. Moreover, phytohormones are unstable compounds and decompose easily under the action of ambient environment. It should also be noted that some phytohormones correspond with animal and human hormones in their physiological functions; this is confirmed by data of their biosynthesis [22]. Consequently, the safety of using them shall be verified. In this regard, the synthesis and selection analogs of natural phytohormones having desired properties is carried out for the prevention of lodging of crops, for the acceleration of maturation, and the improvement of fruit setting. These preparations facilitate the mechanical harvesting, increased crop capacity and quality of agricultural products [23,24]. Hence, the use of biologically active substances (BASs), which are also plant growth regulators, for promoting protective mechanisms of plants is not only of fundamental but also of great practical importance.

We supposed that the main property of these preparations is their ability to reduce the excessive production of ROS and, consequently, reduction the intensity of lipid peroxidation processes in biological membranes and, mainly, in membranes of mitochondria and chloroplasts. We verified this the supposition on a plant growth and development regulator – melaphen, i.e., melamine salt of (bis (oxymethyl)-phosphinic acid synthesized in the Institute of Organic and Physical Chemistry of the Kazan Scientific Center.



In our study, we investigated the combined effect of moisture deficiency with a moderate cooling to 10-14ºC, and the processing of peas with plant growth regulator melaphen on lipid peroxidation (LPO) and AFM images of isolated mitochondria 5 day-old pea (*Pisum sativum L)*. The AFM method provides a quantitative assessment of parameters of images of mitochondria and then process them using statistical methods.

2. MATERIALS AND METHODS

2.1 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 insufficient watering.

2.2 Germination of Pea Seeds

The seeds from the control group were washed with soap solution and 0.01% $KMnO_4$ solution and left in water for 60min. The seeds from the experimental group were placed in the 2 $x10^{-12}$ M melaphen solution for 60min. Thereafter, seeds were transferred into covered trays on moistened filter paper in darkness for a day. After 1-day exposure, half of the seeds from the control group (Drought Cooling) and half of the seeds treated with melaphen (Mph Drought Cooling) were placed onto a dry filter paper in open cuvettes, where they were kept at 14ºC for two day. Two days later the seeds were placed into closed cuvettes with periodically watered filter paper and left for 2 days. The control group of seedlings was kept at 22ºC throughout the experiment (Control). On the 5-th day the amount of germinated seeds was calculated and mitochondria isolated.

2.3 Isolation of Mitochondria

Isolation of mitochondria from 5-day-old epicotyl of pea seedlings (*P. sativum*) or from sugar beet root storage (*Beta vulgaris* L., variety Verhnyachenskaya 31) performed by the method [25] in our modification.

The epicotyls having a length of 1.5 to 5cm (20-25g) or 30g of sugar beet root storage 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: 0.4M sucrose, 5mM EDTA, and 20mM KH_2PO_4 (pH 8.0), 10 mM KCl, 2mM 1, 4-Dithio-di-theiritol, and 0.1% fatty acids-free (FA-free) BSA .The homogenate was centrifuged at 25000g for 5min. The precipitate was re-suspended in 8 ml of a rinsing medium comprised: 0.4M sucrose, 20mM KH_2PO_4, 0.1% FA-free BSA (pH 7.4) and centrifuged at 3000g for 3 min. The supernatant was centrifuged for 10min at 11000g for mitochondria sedimentation. The sediment was re-suspended in 2-3ml of solution contained: 0.4M sucrose, 20mM KH_2PO_4 (pH 7.4), 0.1 % FA-free BSA and mitochondria were precipitated by centrifugation at 11000g for 10min. The isolation medium for rat liver mitochondria comprised 0.25M sucrose, 5mM MOPS, pH 7.4 [26] .The primary centrifugation lasted 10min at 600g; the secondary, for 10 min at 10000 g. The sediment was re-suspended in 5-7ml of solution contained: 0.25M sucrose, 5mM MOPS, pH 7.4. The precipitate was suspended in 0.5ml of the isolation medium.

2.4 Rate of Mitochondria Respiration

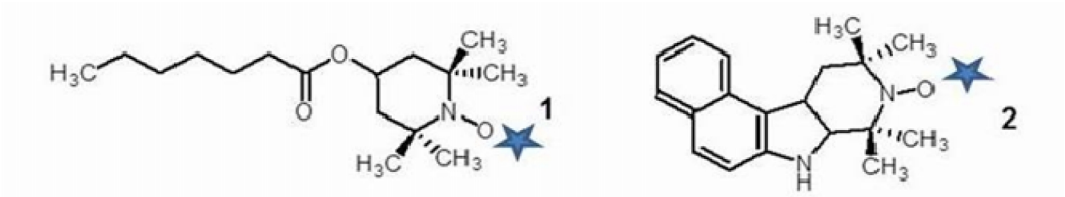
Respiration in mitochondria we recorded polarographically (An LP-7 polarograph, Czech Republic) using Clarke oxygen electrode. Sugar beet root and pea sprout mitochondria were incubated in a medium containing: 0.4M sucrose, 20mM HEPES-Tris buffer (pH 7.2), 5mM KH_2PO_4, 4mM $MgCl_2$ and 0.1% BSA. The rat liver mitochondria were incubated in a medium contained 0.25M sucrose, 10mM Tris-HCl, 2mM $MgSO_4$, 2mM KH_2PO_4 and 10 mM KCl, pH 7.4 (28° C). The rate of respiration was expressed in ng-moll O_2/mg protein min.

2.5 The Level of Lipid Peroxidation

The level of lipid peroxidation (LPO) was evaluated by the fluorescence method [27]. 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 10ºC. Thereafter, equal volume of distilled water was added to the homogenate, and after rapid mixing the homogenate was transferred into 12-mL centrifuge tubes. Samples were centrifuged at 600g for 5min. The aliquot (3mL) of the chloroform (lower) layer was taken, 0.3mL of methanol was added, and fluorescence was recorded in 10-mm quartz cuvettes with a spectrofluorometer (Fluoro Max Horiba Yvon, Germany). The excitation wavelength was 360nm, the emission wavelength was 420--470nm. The results were expressed in arbitrary units per mg protein. The using of this method permits recording both fluorescence of 4-hydroxy-2, 3-nonenals (HNE) and the fluorescence of MDA. The emission wavelength depends on the nature of the Schiff's bases: The Schiff's bases formed by 4-hydroxy-2, 3-nonenals have fluorescence wavelength 430-435nm; those formed by MDA, 460-470.

2.6 The Structural Characteristics of Membranes

The structural characteristics of membranes were studied by EPR-spectroscopy with spin probes. As probes, we used stable nitroxyl radicals synthesized in the Institute of Chemical Physics of the Russian Academy of Sciences: 2,2,6,6-tetramethyl-4-capryloyloxypiperidine-1-oxyl (probe I) and 5,6-benzo-2,2,6,6-tetramethyl-1,2,3,4-tetrahydro-γ-carboline-3-oxyl (probe II), [28,29]. The microviscosity in different domains of the membrane were assessed by the instrumentality of rotational correlation time of spin probes : 2,2,6,6-tetramethyl-4-capryloyloxypiperidine-1-oxyl (probe I) and 5,6-benzo-2,2,6,6-tetramethyl-1,2,3,4-tetrahydro -γ-carboline-3-oxyl (probe II), which differ in their localization in protein-lipid membranes:



Probe I 2,2,6,6 teteramethyl-4-capryloyloxy piperidin-1-oxyl
Probe II 5,6-bonze-2,6,6,6-teteramethyl-1,2,3,4-tetrahy-y-corboline -3-oxyl

According to [28-30], probe I is predominantly localized in the free lipid bilayer of membranes within 2-4 Å from the surface; probe II is localized in the zone of lipids bound to protein domains (annular lipids).

Melaphen in the concentration of $2x10^{-7}$ to $2x10^{-22}$ M was added to a membrane suspension (3-5mg protein/ml) and incubated for 30 min at 4ºC. The membrane samples without the preparation were used as control. Then a probe, the final concentration of which was 10^{-5} M was added to the membrane suspension. The mixture was incubated for another 30 min at 4ºC. The EPR spectra were recorded with an ER-200D SRC EPR spectrometer ("Bruker") at room temperature. From the obtained spectra, according to the formulas for fast-rotating probes there was calculated the time of rotational correlation time probes ($\tau_c \times 10^{-10}$ s), which has the meaning of the period of re-orientation of radical to the angle π/2 [30]. The results were expressed in arbitrary units.

2.7 Atomic Force Microscopy (AFM)

Samples of mitochondria for AFM were prepared on a polished silicon wafer. Before air drying the mitochondria on the silicon substrate washed with buffer without BSA, fixed with 2 % glutaraldehyde for 2 minutes followed by washing with water.

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 10nm.

An analysis of mitochondria AFM images under study permits us to determine the volume of individual mitochondria. 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. The section was made at a height of 30nm. It is also possible to determine the other parameters – the average height and area of the AFM image in the region of section of mitochondria. In the analysis and processing the data file, there was used Statistica 6. In the analysis there were used individual mitochondria.

2.7.1 Statistics

Tables and figures present means values and their standard deviations. The number of experiments was 10 in Figs. 6, 7 and 9. In Fig. 8 is presented correlation microviscosity in the zone of lipids bound with protein domains and fluorescence intensity of lipid peroxidation products; they were calculated using Statistic *v.6* software for Windows.

2.8 Reagents

Sucrose, rotenone, antimycine A, N,N,N',N'- tetramethyl- *p*-phenylenediamine (TMPD), ascorbate, glutaraldehyde , salicylhydroxamic acid (SHAM), adenosine 5′-triphosphate disodium salt (ATP)(Sigma-Aldrich, USA), BSA (Bovine serum albumin) (Fraction V, free fatty acids) (Sigma, USA), FCCP (carbonyl cyanide-p-trifluorometoxyphenylhydrazone, MOPS (3- (n-Morpholino) propanesulfonic acid), 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), Tris (hydroxymethyl) aminjmethan) (MP Biomedicalis, LLC, Germany), (Fluka, Germany), chloroform, methanol (Merk, Germany).

3. RESULTS AND DISCUSSION

A combined effect of insufficient watering and moderate cooling (14ºC) (Drought Cooling) resulted in changes in the mitochondria morphology of pea (*P. sativum*), variety Flora-2, isolated from 5-day etiolated seedlings as compared with the control (22ºC) and with the seedlings whose seeds were pretreated with melaphen (2×10^{-12}M) (Mph Drought Cooling). In the Drought Cooling group there was observed an increase in the volume of AFM images (Fig. 1, Table 1) of some mitochondria and the number of divisible mitochondria decreased significantly (Fig. 2). Several areas of two-dimensional and three-dimensional AFM images of mitochondria (in different scan spaces) are shown in Fig. 1 (2x2μм^2) and Fig. 2 (10x10 μм^2).

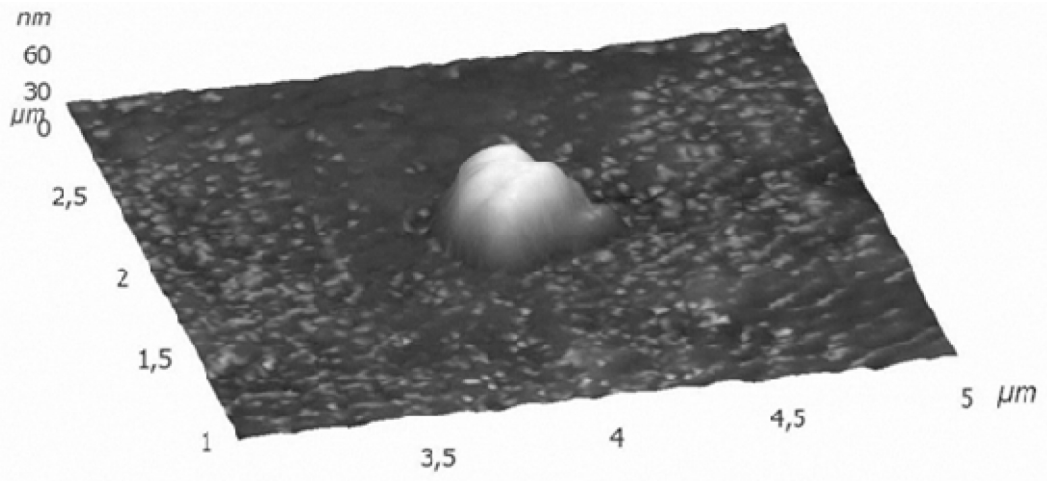


Fig.1(a). The AFM image (3D) of the individual mitochondria isolated from the pea seedlings of the "Control" group

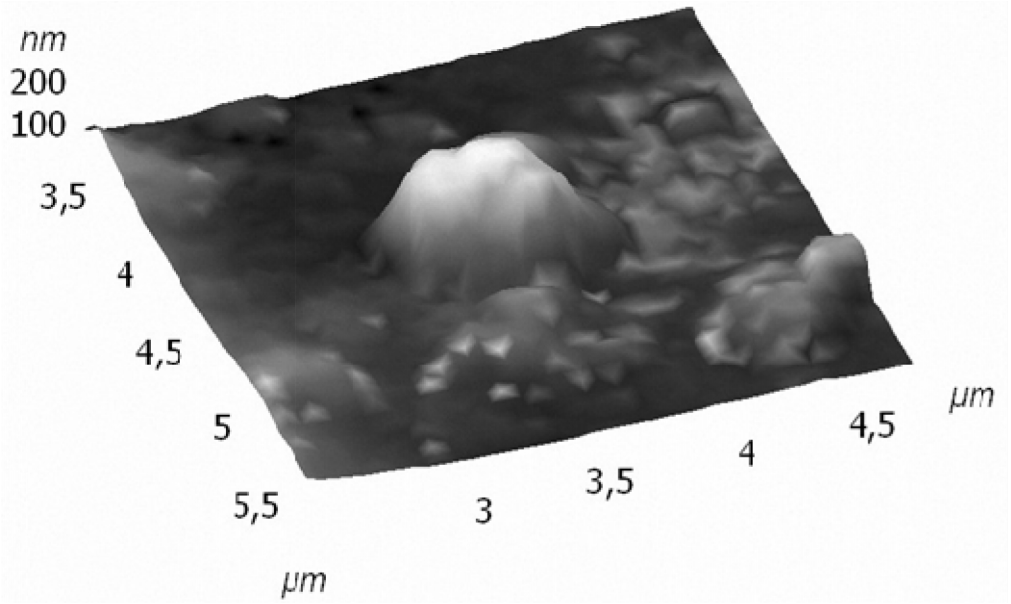


Fig. 1(b). The AFM image (3D) of the individual mitochondria isolated from the pea seedlings of the" Drought Cooling" group

The divisible mitochondria were observed mainly in the AFM images of control mitochondria samples and in mitochondria samples from the seedlings whose seeds were pretreated with melaphen (Mph Drought Cooling) (Fig. 1a, d). The swelling mitochondria were observed in the Drought Cooling group (Fig. 1c), as in the case with the treatment of *Arabidopsis thaliana* leaves by paraquat or hydrogen peroxide [31].

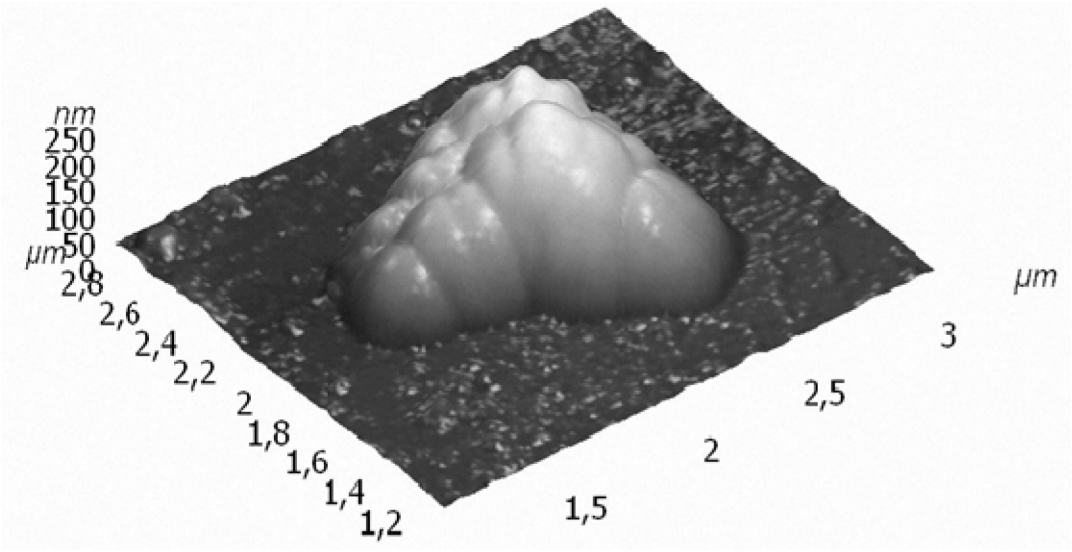


Fig.1(c). The AFM image of the swelling mitochondria isolated from the pea seedlings of the"Drought Cooling" group

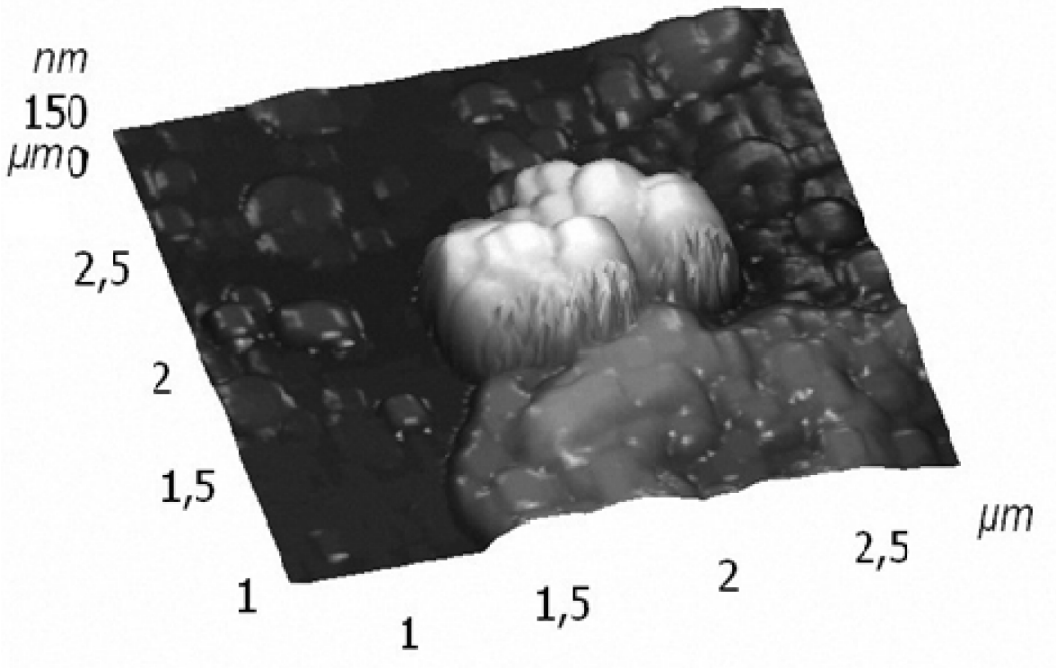


Fig.1 (d). The AFM image of the division of mitochondria isolated from the pea seedlings of the "Mph Drought Cooling" (2 x10^{-12} M melaphen) group

In the two-dimentional AFM images of pea seedling mitochondria (10×10μм^2) there is evidently observed a site of divisible mitochondria (Fig. 2a).

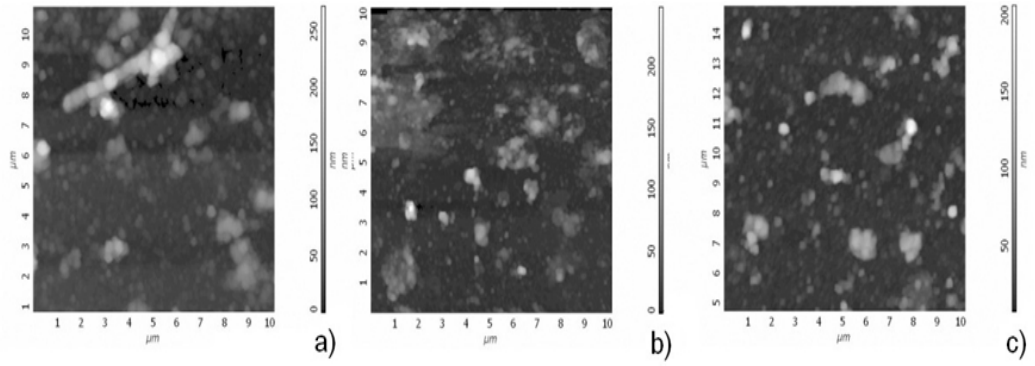


Fig. 2. The two-dimensional AFM image of mitochondria (10x10 μм^2) isolated from 5-day pea seedlings (a) in the Control group, (b) in the Mph Drought Cooling group (2 x10^{-12} M melaphen), and (c) in the Drought Cooling group

An analysis of the AFM images of individual mitochondria performed with the aid of Image Analysis and the statistical processing them has shown that a combined effect of insufficient watering and moderate cooling resulted in increasing the average volume of AFM images of pea seedling mitochondria. However, the pretreatment of pea seeds with melaphen (2×10^{-12} M) prevented altering the geometric parameters of the AFM images of mitochondria exposed to the insufficient watering and moderate cooling conditions and these parameters remained similar to those of the control group (Table 1).

Table 1. The combined effect of insufficient watering, moderate cooling and melaphen (2×10^{-12}M) on the volume (V) of AFM image of pea seedling mitochondria

Samples	Average V, (μм)^2 ×nm	95%	-95%
Control	81.05	92.11	69.99
Mph Drought Cooling	86.43	94.51	78.38
Drought Cooling	115.13	127.23	103.03

These results are in agreement with data of Logan D.C. and Scott I. obtained by the treatment of *A. thaliana* leaves with methyl-viologen [16].

A comparison of the published data with the obtained results can be assumed that a combined effect of moderate cooling and insufficient watering of pea seedling mitochondria promotes the increase of generation of ROS and subsequent swelling of mitochondria [32]. Indeed, simultaneous cooling and deficit of moisture led to LPO activation in the mitochondrial membranes of pea seedlings. In this case, the fluorescence intensity of LPO products increased 3 and 2.5 times, respectively (Fig. 3).

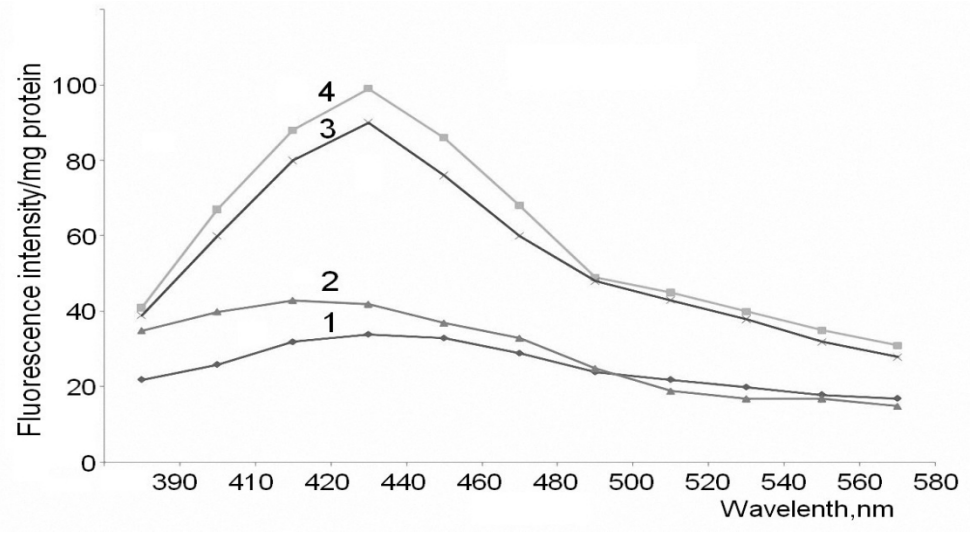


Fig. 3. The fluorescence spectra of LPO products in the mitochondrial membranes of pea seedlings exposed to combined action of insufficient moisture and moderate cooling and in the mitochondrial membranes from pea seedlings treated by melaphen or melamine and exposed to combined action of insufficient moisture and moderate cooling

Y-axis: fluorescence intensity, arbitrary units/mg protein; X-axis: wavelength, nm 1-Control; 2- Mph Drought Cooling (2 x10^{-12} M melaphen); 3- Melamine (2 x10^{-12} M) Drought Cooling; 4- Drought Cooling

Evidently, a combined effect of insufficient watering and moderate cooling did not heighten the resistance of pea seedlings to the action of each factor separately as is seen both from the changes in the mitochondria morphology and from the generation of ROS by these organelles. Similar data were obtained in the study of a combined effect of drought and heat shock on the ultrastructure of mitochondria and chloroplasts of leaves of *Triticum aestivum* [33]. Soak the seeds in a 2×10^{-12}M melaphen solution resulted in a decrease of LPO products in the membranes of the mitochondria: the fluorescence intensity of lipid peroxidation products decreased almost to the control level. Such treatment prevents changes to the morphology of mitochondria, approaching the size of mitochondria towards control. The effect was accompanied by an increase in the number of divisible mitochondria similar to that in the control. Note that the heterocycle (melamine), which is part of the melaphen, produces almost no effect on the fluorescence intensity of lipid peroxidation products. On this basis, it can be assumed that the melamine has no protective action in conditions of a combined effect of insufficient moisture with moderate. However, when it forms a salt with a phosphinic acid (melaphen), the preparation shows protective properties. Melaphen heightens the resistance of plants to the stress factors, showing either the antioxidant properties [34], or by activating the alternative oxidase [35] or PmitoK$_{ATP}$ [36,37]. We verified the this supposition using a model system "aging" of mitochondria from sugar-beet storage roots which consist in prolonged incubation (35min) of mitochondria in hypotonic medium containing 1mM KH_2PO_4 at room temperature. As a control, we investigated the fluorescence intensity of the samples containing only chloroform, methanol and solutions of melaphen or melamine into a wide range of concentrations. Note, that fluorescence intensity of the samples containing the different concentrations of melaphen or melamine and samples containing only chloroform and methanol were identical. On this basis, it can be assumed that the main contribution to the intensity of the fluorescence does

chloroform. Obtaining data indicate a slight contribution of chloroform in the fluorescence intensity (curve 1). In this the incubation of mitochondria in the hypotonic medium promoted the generation of ROS, which manifested itself in 3- to 4-fold increase in the intensity of fluorescence of LPO products. Moreover, melamine, which is part of melaphen in all tested concentrations no effect on the fluorescence intensity of lipid peroxidation products (curve 3). While the introduction of melaphen into the incubation medium affected the intensity of fluorescence of LPO products depending on the functional state of mitochondria and the concentration of the preparation in the incubation medium. The preparation in concentrations $2\times10^{-7;}$ 2×10^{-12} and 2×10^{-18} to 2×10^{-22} M reduced the intensity of fluorescence of LPO products to the control values (Fig. 4). Thus, these data suggest the possibility of reducing the intensity of lipid peroxidation with help low and ultra-low concentrations melaphen. Furthermore, we can conclude that the protective properties of melaphen, apparently, caused by POL inhibition under stress conditions. Since melamine, which is part of this drug has little effect on intensity of lipid peroxidation, what can evidence about that it has no protective properties.

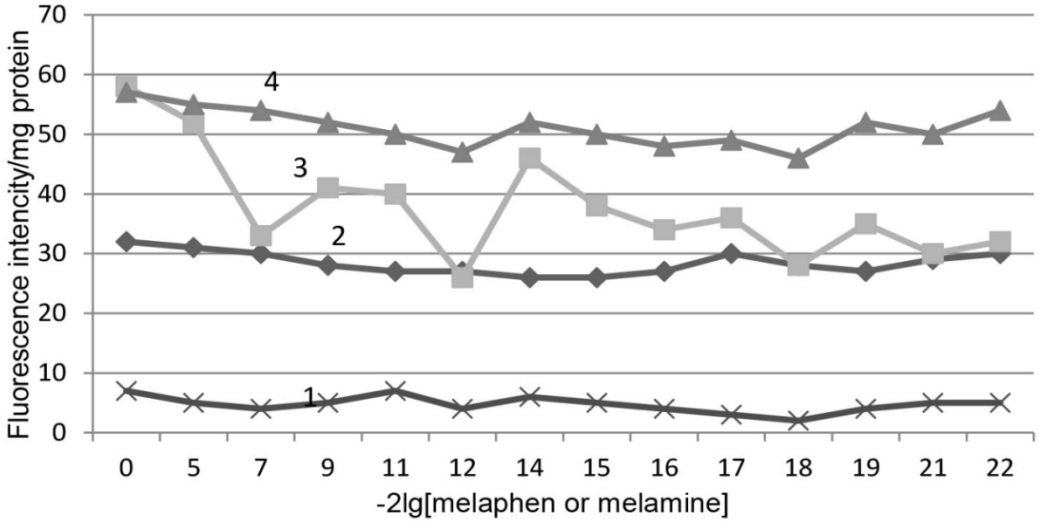


Fig. 4. The fluorescence intensity of LPO products after introduction of different concentrations of melaphen or melamine into the incubation medium of the mitochondria isolated from the sugar-beet storage roots

Y-axis: fluorescence intensity, arbitrary units/mg protein; X-axis: the concentration of melaphen or melamine. 1-fluorescence intensity of the samples containing the different concentrations of melaphen or melamine ; 2-control; 3- introduction into the incubation medium of different concentrations of melaphen; 4- introduction into the incubation medium of different concentrations of melamine

It should be noted that introduction of an alternative oxidase inhibitor – salicylhydroxamic acid (SHAM) into the incubation medium of mitochondria did not affect the melaphen activity towards fluorescence intensity of LPO. The introduction of a $PmitoK_{ATP}$ inhibitor – ATP into the incubation medium of mitochondria did not affect on effects of melaphen on the intensity of LPO (Fig. 5).

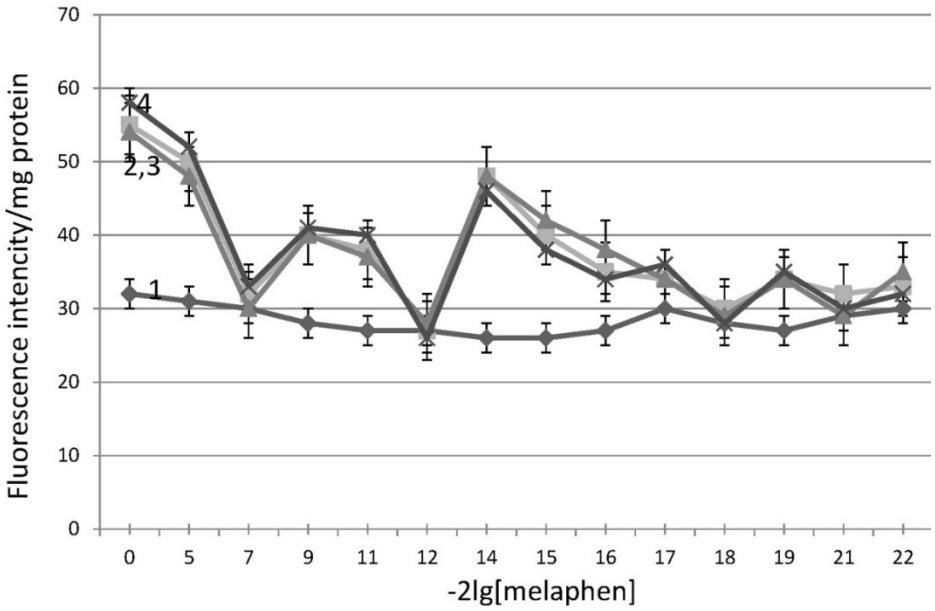


Fig. 5. The fluorescence intensity of LPO products after introduction of different concentrations of melaphen into the incubation medium of the mitochondria isolated from the sugar-beet storage roots

Y-axis: fluorescence intensity, arbitrary units/mg protein; X-axis: the concentration of melaphen, M. Additions: SHAM – 1 mM; ATP – 250 μM. 1-control; 2-SHAM; 3-ATP; 4-melaphen

The obtained results show evidently that the decrease in the intensity of fluorescence of LPO products in model system is associated with the antiradical and antioxidant activity of the preparations. Indeed, the rate constant of the interaction of melaphen with the superoxide radical was $1.67\times10^{4}(Ms)^{-1}$ and was comparable with that of nitro tetrazolium blue [38]. The effective rate constant of the interaction of melaphen with the peroxy radicals (k_7) per the oxidation of ethylbenzene (60ºC) using as the initiator - Azobisisobutyronitrile (AIBN) was 1.64×10^{6} $(Ms)^{-1}$ [39]. While for melamine, (a component of the molecule of melaphen), constant k_7 was $0.24x10^{4}(Ms)^{-1}$ [40]. It was safe to suppose that the preparation in low concentrations was embedded into the biological membranes and reacted with the ambient lipids [41]. Indeed, the incubation of biological membranes with melaphen resulted in changes in the membranes microviscosity; the changes exhibited the concentration dependence. There was observed a decrease in the microviscosity of the free lipid bilayer measured with a probe localized within 2 to 4 Å from the surface (probe I); whereas the microviscosity of the annular lipids (bound to the protein clusters) measured with probe II increased (Table 2, Figs. 6, 7).

The low concentrations of the preparation that caused maximum changes in the micro-viscosity in the region of annular lipids of the plant membranes (2×10^{-7} M) and animal (2×10^{-12} M) origin differed; whereas the ultra-low concentrations of the preparation produced identical alteration of microviscosity of annular lipids on plant and animal membranes.

Table 2. The relative changes in the microviscosity of a lipid bilayer of membranes under the action of low concentrations of melaphen. (The data are represented as a ratio of Experiment to Control)

Object of research	Incubation time (min)	Probe	Concentration "Melaphen", M			
			2×10^{-7}	2×10^{-9}	2×10^{-11}	2×10^{-12}
Liver SMP	30	1	0.94	1.07	1.07	0.99
(Sub mitochondrial particles)	30	2	1.13	1.15	1.30	1.38
	90	1	1.07	1.02	0.78	0.76
	90	2	0.93	1.09	1.15	1.47
Microsomes	30	1	1.02	1.05	1.08	1.01
	30	2	1.11	1.09	1.20	1.21
	90	1	0.84	0.78	0.86	0.92
	90	2	1.36	1.15	1.29	1.40

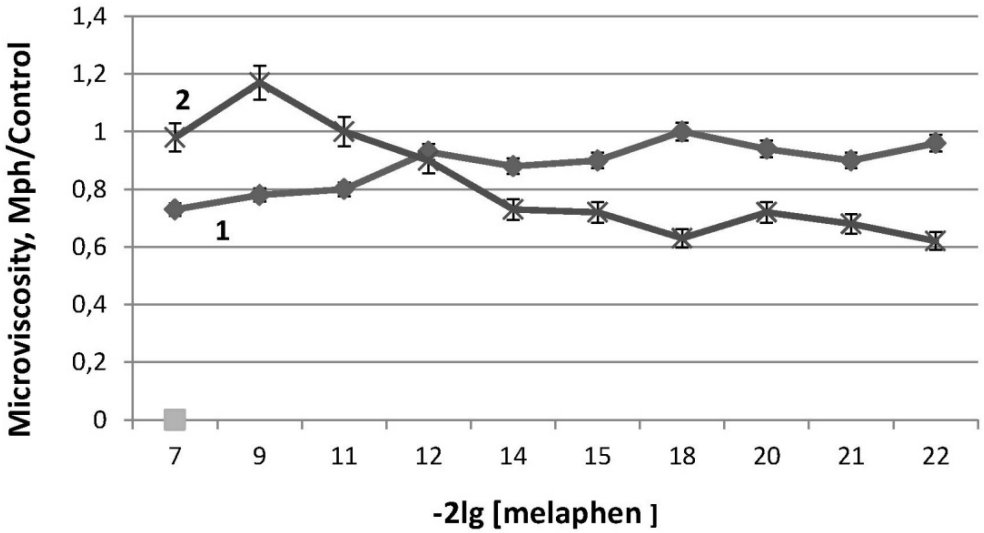


Fig. 6. The microviscosity of the free lipid bilayer: (1) erythrocytes; (2) mitochondria from sugar-beet storage roots

Y-axis: microviscosity in Mph/Control (Mph-melaphen); X-axis: the concentration of melaphen, M

In particular, in the range of ultra-low concentrations of melaphen, a pronounced increase in the microviscosity of lipids bound to the protein domains was observed in membranes of the plant and animal origin in the ranges of 2×10^{-14} to 2×10^{-22} M, what may be attributed to the peculiar mechanisms of the action of the ultra-low concentrations of the preparation. These mechanisms may be associated by the formation of supramolecular Nano associates initiated by the dissolved substance, because changes in the concentration dissolved substance may cause rearrangement of the Nano associates and to render the influence on their biological effects [42].

The changes in the microviscosity in the region of annular lipids of membranes correlated with the LPO inhibition (r=0.8763; the confidence level 95%) (Fig. 8).

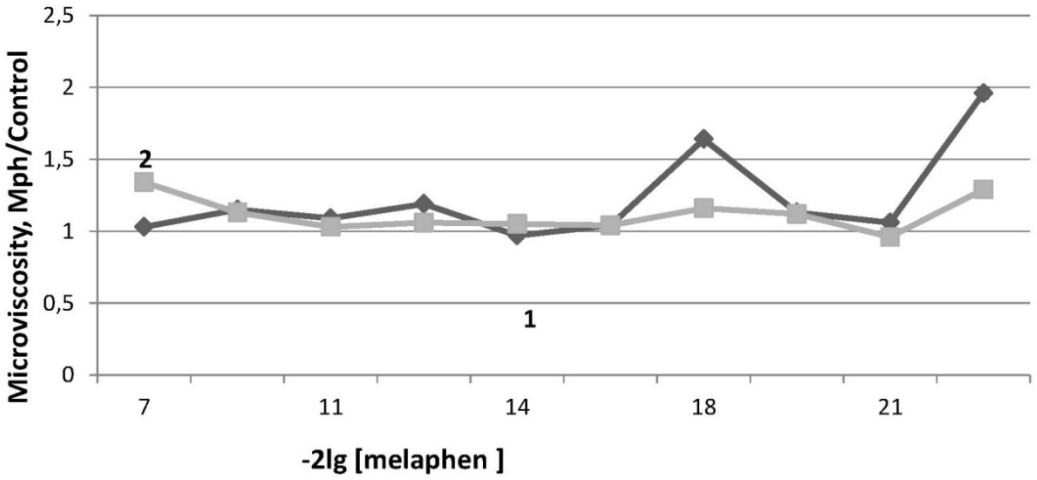


Fig. 7. The microviscosity in the zone of lipids bound with protein domains. Conventional signs as in Fig. 6

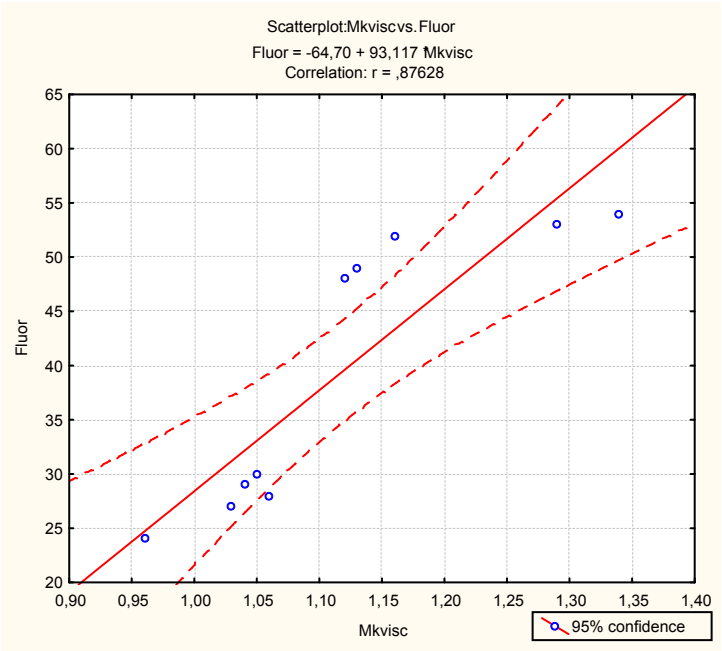


Fig. 8. Correlation between microviscosity of lipids bound to protein clusters and fluorescence intensity of lipid peroxidation products

Y-axis: - fluorescence intensity of lipid peroxidation products in arbitrary units/mg protein; X-axis: - ratio (Mph/control) in the area of annular lipids of mitochondrial membranes

The decrease in the LPO intensity may be associated with an increase in the electron transport rate in the respiratory chain of mitochondria. Indeed, melaphen affects the electron transport rate at the terminal (cytochrome oxidase) stage of the respiratory chain animal and plant origin mitochondria. The introduction of melaphen into incubation medium of the

mitochondria stimulated the electron transport at this stage of the electron-transport chain, both in the mitochondria from sugar-beet storage roots and rat liver mitochondria. The rates of oxidation of ascorbate in the presence of TMPD (tetramethyl-p-phenylenediamine) and $2x10^{-18}$M melaphen by liver mitochondria increased from 194.25±10.20 to 267.00±12.00 nmol O_2/mg. protein. min. At the same concentrations, melaphen promoted the electron transport at the cytochrome oxidase site of the respiratory chain of sugar beet root mitochondria. The rates of oxidation increased from 395.41±10.6 to 650.0±22.3nmol O_2/mg.protein.min. (Fig. 9). The rates of oxidation of these substrates in the presence of $2x10^{-12}$M melaphen increased by 23% and in the presence of $2x10^{-1}$8M melaphen- by 60% (Fig. 9).

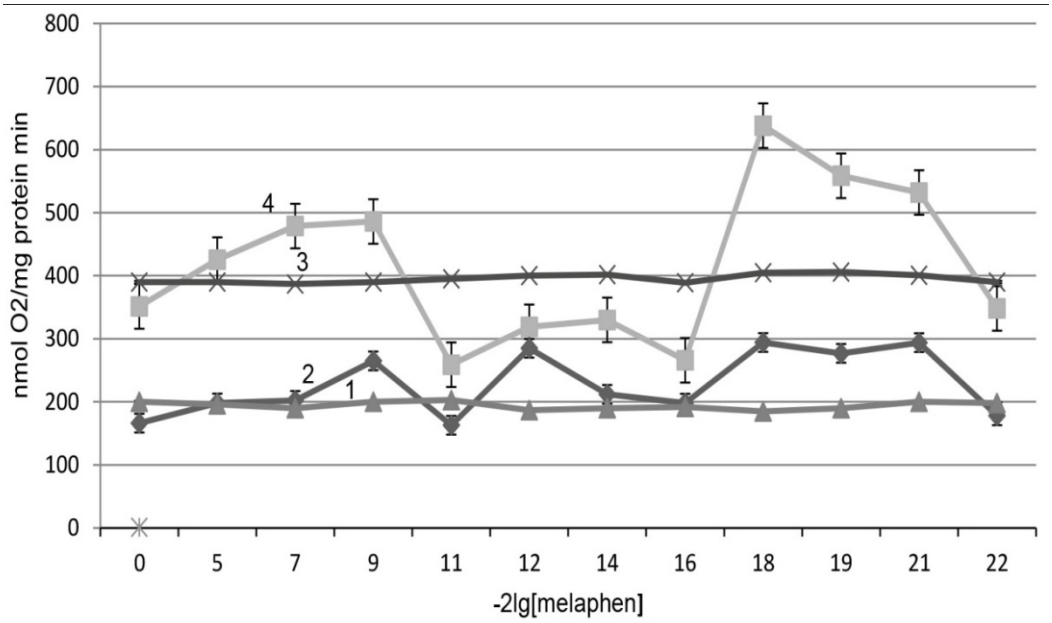


Fig. 9. The rates of electron transport on the terminal stage of the respiratory chain of mitochondria after introduction of different concentrations of melaphen into the incubation medium

Notation: 1-rat liver mitochondria without adding the preparation; 2-rat liver mitochondria; 3-mitochondria from sugar-beet storage roots without adding the preparation; 4-mitochondria from sugar-beet storage roots. The incubation medium for rat liver mitochondria contained: 0.25M sucrose, 10 mM Tris-HCl, 2 mM $MgSO_4$, 2 mM KH_2PO_4, 10 mM KCl, 2mM ascorbate, 5μM rotenone, 0.9μM antimycine A, 0.5μM FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) and 800 μM TMPD (pH 7.4). The incubation medium for mitochondria from sugar-beet storage roots 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 and 800 μM TMPD (pH 7.2)

These data are in agreement with the data obtained by Platonova T. A. [43], who showed that melaphen treatment of plants affects the differentiation of mitochondria. There is observed an increase in the number of condensed mitochondria enriched with cristas, which is evidence about the enhancement of the activity of the mitochondrial apparatus.

The activation of the energy metabolism must not only decrease the intensity of the free radical processes but also to increase the resistance of plants to stress factors. It should be

noted that treatment of potato tubers (*Solanum tuberosum*) with 10^{-5}M melaphen affected the activity of enzymes of the antioxidant system: The activity of peroxidase was increased two times the activity of catalase, 1.3 times [44,45]. It can be assumed, that the drug in low concentrations (10^{-5}-10^{-7}M) was embedded into biological membranes and it reacted with the ambient lipids. In these concentrations, drug affects the activity of antioxidant enzymes. However, we no found the influence of ultra-low concentrations of melaphen (10^{-9}-10^{-22}M) on the activity of antioxidant enzymes (unpublished data). Nevertheless, melaphen in this concentration range affects on the bioenergy characteristics of mitochondria.

4. CONCLUSION

To summarize, we have arrived at a conclusion that melaphen, can to change the physicochemical state (the structural organization) of mitochondrial membranes and/or can accelerate the electron transport rate at the terminal (cytochrome oxidase) stage of the respiratory chain, thereby to reduce the generation of ROS by these organelles and prevented the induction of LPO and damages of mitochondrial membranes both in experiments in “ageing” mitochondria and in experiments in whole plants. It is safe to suppose that the drug reduced the LPO intensity and preserved thereby a high functional activity of mitochondria, which underlies of the resistance of plants to stress factors, in particular to a combined effect of insufficient watering and moderate cooling, what manifests itself on the morphology of mitochondria.

It should be noted that the discrete character of the concentration dependences of the effect of ultra-low doses of melaphen obtained in our experiments (Figs. 4-7, 9) is qualitatively consistent with published data on the effect of ultra-low doses of biologically active substances (BAS) on living systems of various degrees of complexity [46-48]. There exist a great number of hypotheses as to the mechanisms of the effect of ultra-low concentrations of BAS in the literature. In our opinion, the results obtained for melaphen may be interpreted in terms of the physicochemical behavior of highly diluted solutions of the preparation. A. I. Konovalov [42], that melaphen in a concentration of 10^{-10}-10^{-4}mol/l forms Nano associates of the size of about 200nm with participation of water. The concentration dependences obtained by the authors for a size and electro kinetic potential (ζ-potential) of Nano associates formed in aqueous solutions of melaphen at low and ultra-low concentrations [42], are comparable with the biological effects of the preparation as described in this work and published previously [38-39], which is the evidence for a role of melaphen Nano associates in the biological effects thereof. The most plausible evidence for the fact that ultra-low concentrations of melaphen that affect the energy of mitochondria are discrete in character is the formation of associates of a different polarity, and, probably, of different level of complexity depending on the melaphen concentration in a solution [42].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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