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Fatty acid composition and activity of the mitochondrial respiratory chain complex I of pea seedlings under water deficit

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In this work the effect of insufficient watering and plant growth regulator melaphen (melamine salt of bis(oxymethyl)-phosphonic acid) on the fatty acid composition and the energy of 5-day etiolated pea seedling mitochondria (*Pisum sativum*) were studied.

Isolation of mitochondria from 5-day sprouts epicotyls was performed by differential centrifugation. The rate of mitochondria respiration was measured with the aid of Clarke oxygen electrodes and LP-7 polarograph (Czech Republic). Fatty acid methyl esters (FAMEs) were produced by acidic methanolysis of mitochondrial membrane lipids and FAMEs quantification was performed using a Kristall 2000M chromatograph (Russia) with flame-ionization detector and quarts capillary column SPB-1 (50 m × 0.32 mm, a nonpolar phase layer – 0.25 μm).

It has been shown that insufficient watering results in alteration of fatty acid composition in mitochondrial membranes of seedlings. The ratio of the content of C_{18} – unsaturated fatty acids to the stearic acid content decreases by 1.5 times. Significant changes are observed in the content of fatty acids with 20 carbon atoms: the ratio of unsaturated fatty acids to saturated fatty acids decreases by 3.3 times. The changes in fatty acid composition of mitochondrial membranes are in correlation with changes in maximum rates of NAD-dependent substrates oxidation (the Pearson's coefficient of correlation for C_{18} fatty acids is 0.7651, for C_{20} fatty acids – 0.963).

Key words: *Pisum sativum* L. germs, fatty acid composition, mitochondria, respiratory chain, insufficient watering

Abbreviations: LPO – lipid peroxidation; FCCP – carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone; V_3 – the rate of substrate oxidation in the presence of ADP (state 3 according to Chance); V_4 – the rate of substrate oxidation at the ADP exhausting (state 4 according to Chance); IW – insufficient watering; MF – melaphen

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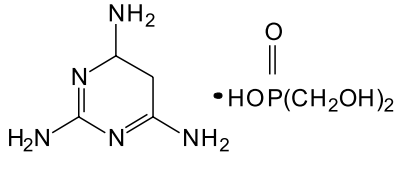
INTRODUCTION

The development and, probably, survival of plant in any case are more dependent on availability of water than on any other environmental factor. At present, numerous data has been accumulated demonstrating that even weak water deficit affects plant metabolism and thus their growth and development (Ribas-Carbo et al., 2005). Metabolism of plants survived even short-term strong drought could not be recovered (Boyer, 1982). Water deficit modified cell membranes which affected their functions and disturbed cell metabolism (Kizis et al., 2001). The alterations occur at the level of glycolipids, monogalactosyl-diacyl-glycerol and digalactosyl-diacyl-glycerol (Junior et al., 2008; Sahsah et al., 1988). The content of unsaturated fatty acids decreases in these lipids which result in the decreasing the membrane "fluidity", alteration in the lipid-protein ratio, and eventually in the activity changes of the enzymes associated with membrane, first of all enzymes which enter into complex of electron-transport chain of mitochondria and chloroplasts (Shugaeva et al., 2007). The energy metabolism plays a significant role in adaptive response of the organism. Mitochondria play a key role in the energy, oxidation-reduction and metabolic processes in cell (Atkin, Macherel, 2009). As known from the literature, regulators of plant growth and development improve their tolerance to biotic and abiotic stresses, to water deficit in particular (Zhirmunskaya, Shapovalov, 1987). One of such growth regulators is melaphen – a melamine salt of bis(oxymetyl)phosphonic acid (Fattakhov, Reznik, Konovalov, 2002):



The aim of this work is to study the effect of insufficient watering and the plant growth regulator – melaphen on the fatty acids composition of lipid fraction of mitochondrial membranes and bioenergetical function of 5-day pea seedling mitochondria.

MATERIALS AND METHODS

Plant material

The study was carried out on mitochondria isolated from pea seedlings (*Pisum sativum*) obtained in standard conditions and in conditions of insufficient watering.

Pea seeds germination

The seeds from the control group were washed with soap solution and 0.01% $KMnO_4$ (potassium permanganate) solution and left in water for 60 min. The seeds from the experimental group were placed in 2×10^{-12} M melaphen solution for 60 min. After 1-day exposure, half of the seeds from the control group and half of the seeds treated with melaphen were placed onto a dry filter paper in open cuvettes. Two days later the seeds were placed into closed cuvettes with periodically watered filter paper and left for 2 days. On the 5th day the amount of germinated seeds was calculated and mitochondria isolated.

Isolation of mitochondria

Isolation of mitochondria from 5-day sprouts epicotyls was performed by a method of (Popov, Ruuge, Starkov, 2003) 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 rapidly disintegrated with scissors and homogenized with the aid of a press. The isolation medium comprised: 0.4 M sucrose, 5 mM EDTA, 20 mM KH_2PO_4 (pH 8.0), 10 mM KCl, 2 mM dithioerythritol, and 0.1% BSA (free of fatty acids). The homogenate was centrifuged at 25 000 g for 5 min. The precipitate was re-suspended in 8 ml of a rinsing medium and centrifuged at 3 000 g for 3 min. The suspension medium comprised: 0.4 M sucrose, 20 mM KH_2PO_4, 0.1%. BSA (free of fatty acids), pH 7.4. The supernatant was centrifuged for 10 min at 11 000 g for mitochondria sedimentation. The sediment was re-suspended

in 2–3 ml of solution and contained: 0.4 M sucrose, 20 mM KH_2PO_4 (pH 7.4), 0.1% BSA (without fatty acids) and mitochondria were precipitated by centrifugation at 11 000 g for 10 min. The suspension of mitochondria (about 6 mg of protein/ml) was stored in ice.

The rate of mitochondria respiration

The rate of mitochondria respiration was measured with the aid of Clarke oxygen electrodes and LP-7 polarograph (Czech Republic). Mitochondria were incubated in a medium containing 0.4 M sucrose, 20 mM HEPES-Tris buffer (pH 7.2), 5 mM KH_2PO_4, 4 mM $MgCl_2$, 5 мМ EDTA and 0.1% BSA, 10 mM mlate + glutamate, pH 7.4. The rate of respiration was expressed in ng-atom O/(mg protein min).

Fatty acid methyl esters (FAMEs)

Fatty acid methyl esters (FAMEs) were produced by acidic methanolysis of mitochondrial membrane lipids (Carreau, Dubacq, 1979) or using one-step methylation of fatty acids, excluding the extraction of lipids (Wang et al., 2000). MEFA were purified by the method of thin layer chromatography on the silica plates and hexanol elution. For a quantitative control of the methanolysis process an internal standard, pentadecane, was used.

FAME identification

FAME identification was performed by gas chromatography-mass-spectrometry (GC-MS) using a Hewlett-Packard-6890 spectrophotometer with a HP-5972 mass-selective detector and the use of retention times (Golovina, Kuzmenko, 1977). FAME were separated in the HP-5MS capillary column (30 m × 0.25 mm, phase layer – 0.25 μm) at programmed temperature increase from 60 to 285 °C at the rate of 5 °C/min. Evaporator temperature was 250 °C, detector temperature – 289 °C. Mass spectra were obtained in the regime of electron impact ionization at 70 eV and the scan rate of 1 sec per mass decade 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, a nonpolar phase layer – 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.

Unsaturation index

Unsaturation index was calculated as a total percentage of unsaturated fatty acids with a certain number atoms multiplied by the number of double bonds, and divided by 100%. For example, for fatty acids with 18 carbon atoms unsaturation index is equal to (18:2ω6) × 2 + (18:3ω3) × 3 + 18:1ω9 + 18:1ω7/100.

Lipid peroxidation (LPO) activity

Lipid peroxidation (LPO) activity was assessed by fluorescent method (Fletcher, Dillard, Tappel, 1973). 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 12-mL 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 10-mm quartz cuvettes with a spectrofluorometer

(FluoroMaxHoribaYvon, Germany). The excitation wavelength was 360 nm, the emission wavelength was 420–470 nm. The results were expressed in arbitrary units per mg protein. The use of this method permits recording both fluorescence of 4-hydroxynonenals and the fluorescence of MDA. The emission wavelength depends on the nature of the Schiff's bases: the Schiff's bases formed by 4-hydroxynonenals have fluorescence wavelength 430–435 nm; those formed by MDA – 460–470.

Statistics

Tables and figures present the means and their standard deviations (M + m). In Figs. 2 and 3, correlations between unsaturation coefficients of C 18 and C 20 fatty acids and the rates of NAD-dependent substrate oxidation are presented; they were calculated using Statistica v. 6 software for Windows.

Reagents

The following reagents were used: potassium carbonate, methanol, chloroform (Merck, Germany), hexane (Panreac, Spain), acetyl chloride (Acros, Belgium), sucrose, Tris, EDTA, FCCP, malate, glutamate, FA-free BSA (Sigma, United States), Hepes (MB Biomedicals, Germany).

RESULTS

Insufficient watering resulted in 3-fold increase in content of LPO products in pea seedling mitochondrial membranes (Fig. 1).

The treatment of seeds with a 2×10^{-12} M melaphen solution decreased the content of LPO products to the control values. Insufficient watering promoted LPO accompanied by modification of the fatty acid composition of pea seedling mitochondrial membranes. Water deficit led to the increase in the relative content of saturated and a decrease in the content of unsaturated fatty acids in mitochondrial membranes of pea seedlings (Table 1).

The relative content of linoleic acid was reduced by 11%, that of linolenic acid – by 19%. The content of stearic acid increased by 41%, which resulted in the decrease in the total content of C_{18} unsaturated fatty acids relative to the content of stearic acid from 16.61 ± 0.30 to 10.59 ± 0.20. Similar effect of water deficit on the fatty acid composition of the mitochondrial membranes from maize, potato, and leaves of *Arabidopsis thaliana* and apricot was observed earlier (Junior et al.,

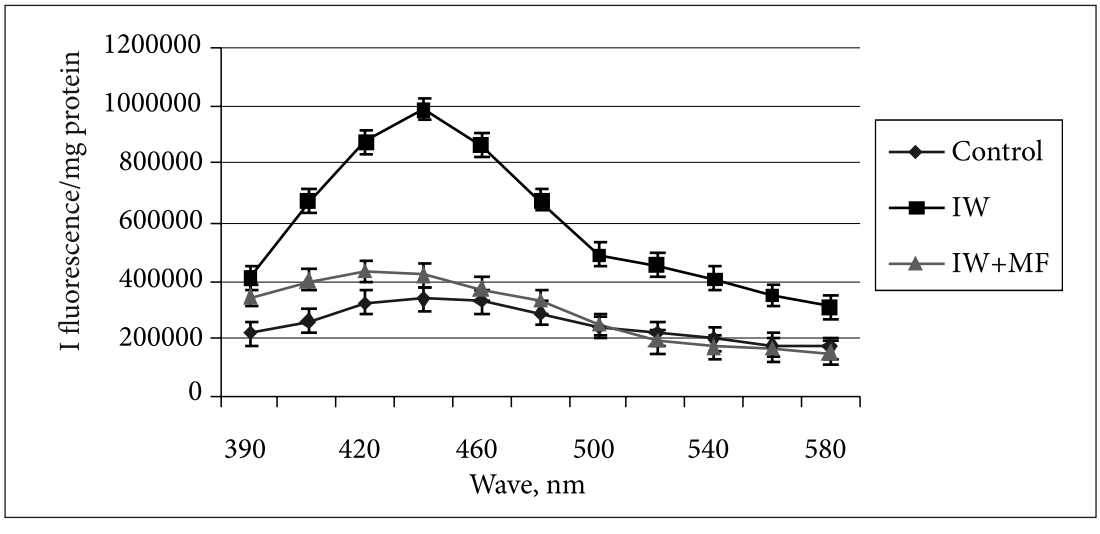


Fig. 1. Fluorescence spectra of LPO products in mitochondrial membranes of 5-day pea seedlings under condition of insufficient watering (IW) and the treatment of seeds with melaphen (IW + MF)

Table 1. Effects of insufficient watering (IW) and melaphen (MF) on the relative fatty acids content in mitochondrial membrane lipids of pea seedlings, % (the results of 6 experiments are presented; 3 repeats of each analysis were used)

Fatty acid	Control	Control + MF	IW	IW + MF
12:0	0.34 ± 0.03	0.34 ± 0.01	0.94 ± 0.30	0.34 ± 0.20
14:0	0.68 ± 0.03	0.64 ± 0.02	0.67 ± 0.20	0.69 ± 0.20
16:1ω7	0.36 ±0,03	0.36 ± 0.004	0.47 ± 0.13	0.42 ± 0.005
16:0	18.64 ± 0.75	18.63 ± 0.05	20.74 ± 0.11	18.96 ± 0.50
17:0	0.45 ± 0.05	0.78 ± 0.12	0.66 ± 0.10	0.45 ± 0.16
18:2ω6	50.72 ± 0.80	50.74 ± 0.40	45.22 ± 0.10	50.65 ± 0.01
18:3ω3	11.3 ± 0.02	10.67 ± 0.01	9.18 ± 0.30	10.81 ± 0.09
18:1ω9	5.27 ± 0.40	5.25 ± 0.37	6.77 ± 0.20	5.22 ± 0.01
18:1ω7	0.81 ± 0.10	0.79 ± 0.24	0.61 ± 0.03	0.73 ± 0.05
18:0	4.10 ± 0.18	4.14 ± 0.32	5.83 ± 0.38	4.10 ± 0.15
20:2ω6	0.82 ± 0.01	0.80 ± 0.02	0.30 ± 0.05	0.82 ± 0.01
20:1ω9	2.22 ± 0.01	2.79 ± 0.01	1.57 ± 0.01	2.6 ± 0.03
20:1ω7	1.45 ± 0.01	1.14 ± 0.01	1.00 ± 0.01	1.52 ± 0.01
20:0	1.23 ± 0.03	1.20 ± 0.03	2.52 ± 0.20	1.30 ± 0.05
22:0	1.23 ± 0.11	1.20 ± 0.03	2.52 ± 0.20	1.04 ± 0.05
24:0	0.37 ± 0.02	0.55 ± 0.005	0.98 ± 0	0.35 ± 0.10

2008; Makarenko et al., 2003; Gigon et al., 2004; Leone et al., 1996; Guo Yun-ping, Li Jia-rui, 2002). The authors detected a considerable decrease of the levels of linoleic and linolenic acids and an increase of the level of stearic acid in the membranes.

Substantial changes also occurred in the relative content of fatty acids with 20 carbon atoms. The pool of 20:2ω6 reduced by 2.7 times, 20:1ω 9–1.3 times. At the same time, the content of eicosanoic acid (20:0) increased more than twofold. As a result, the ratio of pool unsaturated fatty acids containing 20 carbon atoms (20:1ω7 + 20:1ω9) + (20:2ω 6) × 2 to eicosanoic acid in mitochondrial membrane lipids decreased from 3.65 ± 0.03 to 1.20 ± 0.16.

The observed alterations possibly influence lipid–protein relation and thus alter the activity of the enzymes associated with the membrane. Indeed, insufficient watering results in a decrease of the maximal rates of NAD-dependent substrates oxidation. The rate of the pair glutamate + malate oxidation in the presence of uncoupling agent (FCCP) drops from 70.0 ± 4.6 down to 48.9 ± 3.2 ng oxygen atom/mg of protein min and the respiratory control rate (RCR) decreases from 2.27 ± 0.1 to 1.7 ± 0.2 (Table 2).

The treatment of seeds with a 2×10^{-12} M melaphen solution before germination prevents the alteration of the oxidative phosphorylation efficiency caused by insufficient watering. Moreover, the preliminary treatment with melaphen reduces the rates of NAD-dependent substrates oxidation in the presence of ATP or FCCP to the control values (Table 2). Apparently, the described alterations are related with the physicochemical state of mitochondrial membranes.

Indeed the treatment with melaphen protects the unsaturated fatty acid from LPO and prevents thereby from changes in the fatty acid composition of seedling membranes in condition of insufficient watering (Table 1). In the group of seedlings subjected to insufficient watering combined with melaphen treatment (group IW + MF) concentrations of such saturated fatty acids as lauric, palmitic, and stearic acids are lower by 65%, 7.5% and 30%, respectively, than in seedlings subjected to insufficient watering only (group IW). The level of C_{18} – unsaturated fatty acids playing an important role in plant resistance to the effect of adverse factors of

Table 2. Effects of insufficient watering (IW) and melaphen (MF) on the rate of NAD-dependent substrate oxidation by mitochondria isolated from pea seedlings, ng-atom/(mg protein min)

Treatment	V_0	V_3	V_4	V_3/V_4	FCCP
Control	20.0 ± 1.5	68.0 ± 4.1	30.0 ± 2.0	2.27 ± 0.1	60.0 ± 4.6
IW	14.0 ± 2.0	48.6 ± 3.0	40.2 ± 1.0	1.7 ± 0.2	41.9 ± 3.2
IW + MF	19.8 ± 3.0	66.0 ± 2.4	27.5 ± 1.3	2.4 ± 0.2	60.3 ± 5.2

Notes: Incubation medium contained 0.4 M sucrose, 20 mM Hepes-Tris, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 5 mM EDTA, 10 mM malate + glutamate, pH 7.4. ADP (200 μM) and FCCP (0.5 μM) were added. V_0 – the rate of oxygen decay in the presence of 10 mM malate + glutamate as substrate; V_3 – the rate of substrate oxidation in the presence of ADP (state 3, 200 μM ADP); V_4 – the rate of substrate oxidation after added ADP consumption (state 4); FCCP – the rate of substrate oxidation in the presence of FCCP (carbonylcyanide –*p*-trifluoromethoxyphenylhydrazone). The results of 10 experiments are presented, M ± m

environment (Demin et al., 2000) increased. Thus, the level of linoleic acid increases by 12% and linolenic acid – by 15% (Table 1). The ratio of the sum of unsaturated C_{18} acids to saturated $C_{18:0}$ acids increases 1.5-fold in comparison with the group of insufficient watering. The level of C_{20} fatty acids also changed. The level of 20:2ω6 acid increases 2.73 times and that of 20:1ω9 acid – 2.28 times. The level of eicosanoic acid decreases 1.92 times. As a result, the ratio of the sum of unsaturated C_{20} acids to saturated $C_{20:0}$ acids returns to the control values. The changes in the fatty acid composition of mitochondrial membranes were accompanied by changes in maximum rates of NAD-dependent substrates oxidation. A decrease in unsaturation coefficient of fatty acids in mitochondrial membranes led to decreasing the rates of NAD-dependent substrates oxidation and efficiency of oxidative phosphorylation.

On the basis of the presented data it may be supposed that a prevention of unsaturated fatty acids peroxidation, in particular C_{18} and C_{20} acids in membranes of plant tissues leads to

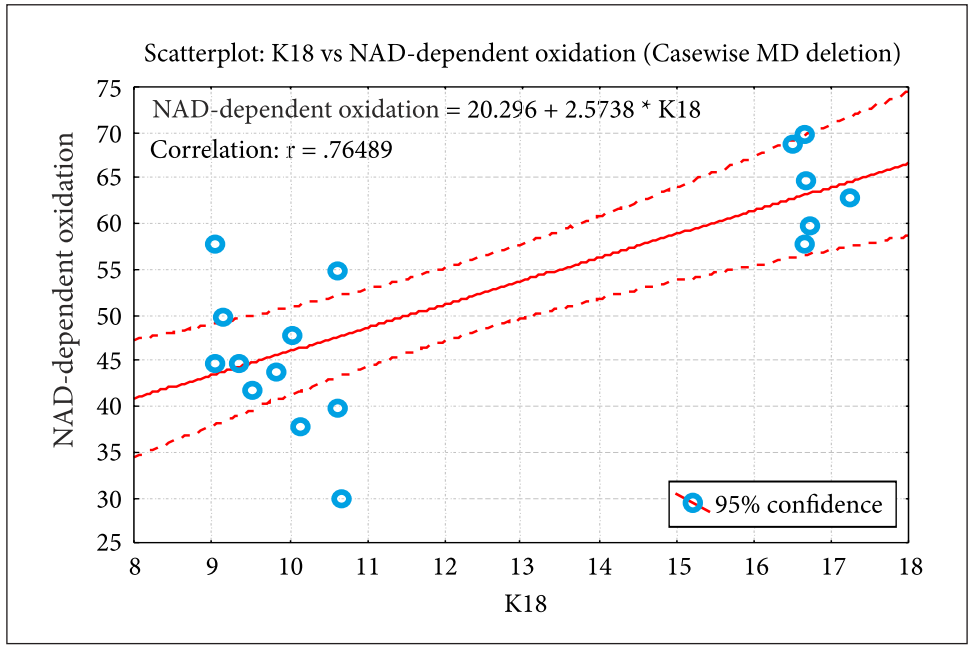


Fig. 2. Correlation between the unsaturation coefficient of C_{20} fatty acids and maximum rates of NAD-dependent substrate oxidation. Y-axis shows the maximum rates of NAD-dependent substrate oxidation; X-axis – unsaturation coefficient of C_{18} fatty acids

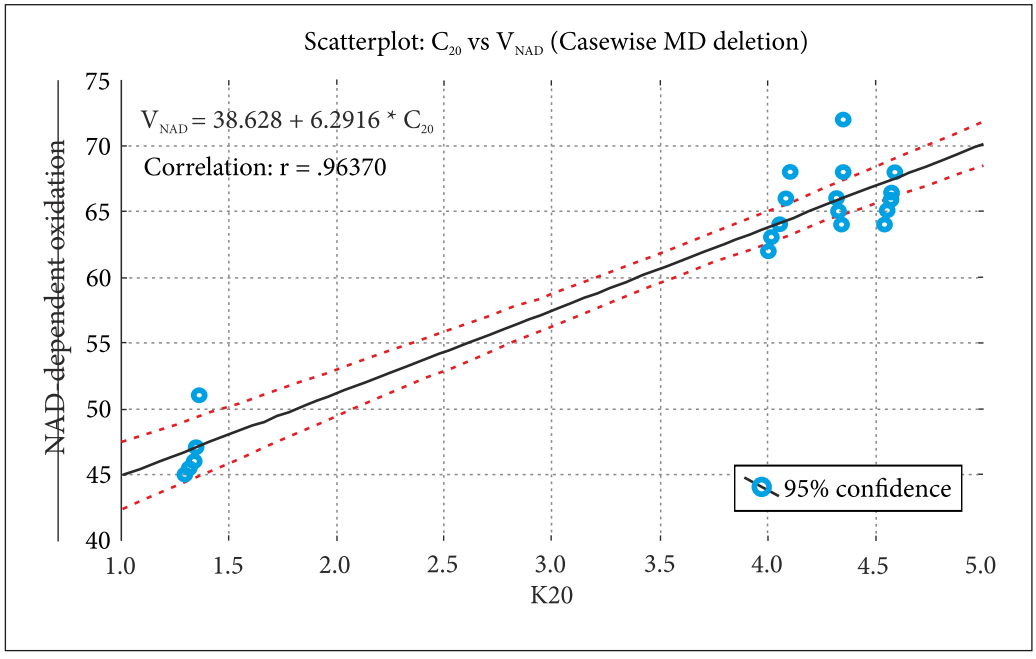


Fig. 3. Correlation between the unsaturation coefficient of C_{20} fatty acids and maximum rates of NAD-dependent substrate oxidation. Y-axis shows the maximum rates of NAD-dependent substrate oxidation; X-axis – the ratio (20:2ω6) × 2 + 20:1ω9 + 20:1ω7/20:0)

enhancement of plant resistance to insufficient watering. In fact, a close correlation was observed between the unsaturation coefficient of C_{18} fatty acids in mitochondrial membranes (Σunsaturated C_{18} fatty acids/$C_{18:0}$) and maximum rates of NAD-dependent substrate oxidation (correlation coefficient r = 0.765) (Fig. 2). An even greater correlation is observed between the unsaturation coefficient of C_{20} fatty acids (20:2ω6) × 2 + 20:1ω9 + 20:1ω7/20:0) and maximum rates of NAD-dependent substrate oxidation (r = 0.964) (Fig. 3).

Changes in physical and chemical properties of mitochondrial membranes resulting in changes in the energy metabolism also affected the physiological indices, e. g. seedling growth (Fig. 4).

As it is evident from Fig. 4, pea seed treatment with melaphen stimulated root growth by 5 times and sprouts growth by 3.5

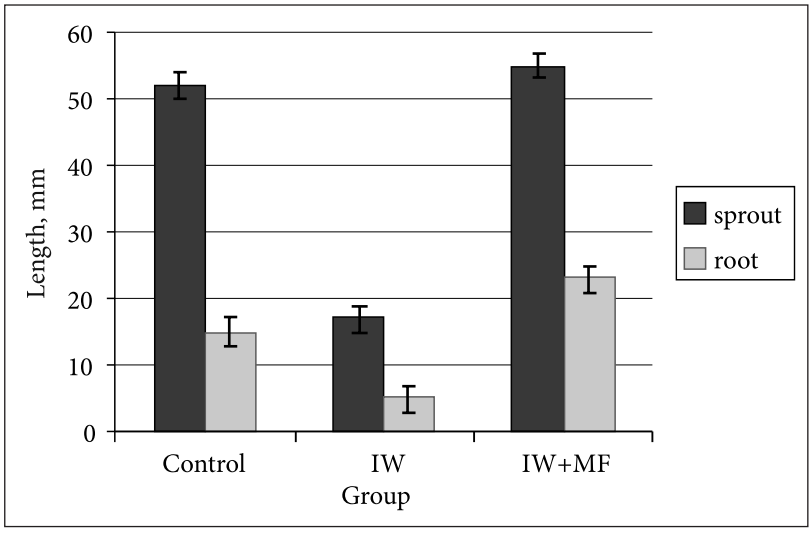


Fig. 4. The lengths of sprouts and roots of 5-day pea seedling under condition of insufficient watering (IW) and treatment of pea seeds in this condition with melaphen (IW + MF)

times under conditions of water deficit. The observed stimulation of seedling root growth under insufficient watering has a great adaptive significance.

DISCUSSION

Thus, under condition of insufficient watering melaphen decreased the intensity of lipid peroxidation in mitochondrial membranes. As a result, the pool of unsaturated fatty acids containing 18 and 20 carbon atoms in the lipid phase of mitochondrial membranes remained unchanged. The prevention of changes in fatty acid composition of mitochondrial membranes affected the bioenergetic indices: a high activity of the NADH-dehydrogenase complex of the respiratory chain of mitochondria was maintained.

On the basis of the obtained data it is possible to suggest that tolerance to water stress is determined by the cell antioxidant system protecting unsaturated C_{18} fatty acids and unsaturated very-long-chain fatty acids against modifications induced by the oxidative stress, e. g. activation of free radical processes (Torres-Franklin et al., 2009). Changes in the C_{20} fatty acids in the mitochondrial membrane lipids are noted for the first time. Just the unsaturation coefficient of C_{20} fatty acids was correlated with the highest rates of NAD-dependent substrate oxidation. At the same time, mitochondria of storage organs and seeds are characterized by relatively low rates of oxidation of NAD-dependent substrates. The result of maintenance high activity of NAD-dependent dehydrogenases is the support the energy processes in cell that promotes the resistance of plant to varying environmental conditions. Under conditions of insufficient watering, protective effect of melaphen is apparently determined by maintenance in the content of unsaturated fatty acids with 18 and 20 carbon atoms in lipid phase of mitochondrial membranes.

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RIEBALŲ RŪGŠČIŲ SUDĖTIES IR VEIKLOS POKYČIAI MITOCHONDRIJŲ KVĖPAVIMO GRANDINIŲ KOMPLEKSUI I DĖL NEPAKANKAMO ŽIRNIŲ DAIGŲ LAISTYMO

Santrauka

Šiame darbe buvo studijuoti nepakankamo laistymo padariniai žirnio (*Pisum sativum*) daigų augimui ir poveikis mitochondrijų kvėpavimo grandinių kompleksui I.

Praėjus 5 dienoms nuo tyrimo pradžios buvo vykdomas diferencinis daigų mitochondrinių izoliatų centrifugavimas. Mitochondrijų kvėpavimas buvo išmatuotas panaudojus Clarke deguonies elektrodus ir LP-7 polarografą (Čekijos Respublika). Sočiųjų riebalų rūgščių metilo esteriai (FAMEs) ir mitochondrijos membranų lipidai buvo atskirti Kristall 2000M chromatografu (Rusija) su liepsnos jonizacijos detektoriumi ir kapiliarine kolonėle SPB-1 (50 m × 0,32 mm, neporėtos fazės sluoksnis – 0,25 μm).

Rezultatai rodo, kad nepakankamas laistymas yra viena iš sočiųjų riebalų rūgščių sudėties pokyčių žirnių daigų mitochondrijų membranose priežasčių. Nesočiųjų riebalų rūgščių (C_{18}) santykis, palyginti su stearino rūgštimi, sumažėjo 1,5 karto. Reikšmingi pakitimai pastebėti sočiosiose riebalų rūgštyse, kurios sudarytos iš 20 anglies atomų: nesočiųjų riebalų rūgščių santykis, palyginti su sočiosiomis riebalų rūgštimis, sumažėjo iki 3,3 karto. Sočiųjų riebalų rūgščių mitochondrijų membranų pakitimai koreliuoja su maksimaliais NAD rodikliais substrato oksidacijos metu (riebalų rūgščių Pearsono koreliacijos koeficientas C_{18} yra 0,676, o C_{20} – 0,963).

Raktažodžiai: *Pisum sativum* L. gemalas, sočiųjų riebalų rūgščių sudėtis, mitochondrija, kvėpavimo grandinė, nepakankamas laistymas