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THE EFFECT OF OXIDATIVE STRESS ON STRUCTURAL AND FUNCTIONAL DEPENDENCE OF PHOTOSYSTEM II

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Abstract

The action of methyl viologen (MV) on pumpkin leaves (*Cucurbita pepo* L.) increased the content of reactive oxygen species (ROS) that caused disruption of the electron transport chain PSII. After the treatment by MV the seedlings were treated by protein synthesis inhibitor – chloramphenicol (CAP). The millisecond delay fluorescence of Chl*a* induction curves analysis (msec-DF Chl*a*) is shown to change of intensity of phase of msec- DF Chl*a*, possible in result of suppression D1 protein *de novo* formation.
The changes of proteins profiles especially in PSII polypeptides content of chloroplasts in dependence on action of methyl viologen and chloramphenicol were observed. The high correlation between changes of polypeptides in range of PSII polypeptides and changing of stationary phase of msec-DF Chl*a* were shown.

Keywords*: Cucurbita pepo* L. *oxidative damage; millisecond delay fluorescence of Chla; reactive oxygen species; methylviologen*
Abbreviations. *Chl – chlorophyll; LHC – light harvesting complex; OEC – oxygen evolving complex; PS – photosystem; ROS – reactive oxygen species.*

1. Introduction

The reaction centers of PSI and PSII in chloroplast thylakoids are known to be a main site of ROS generation under oxidative stress [1] that induced a significant damage to cell biological structures and physiological-biochemical processes run in them [2]. ROS, accumulating in chloroplasts [3] affects first to PS II and its main manganese complex-Mn_4O_5Ca-cluster [4, 5]. The fall of PS II activity is regards to result of balance disturbance between its restoration and damage since in intact cells these processes are take place simultaneously [6, 7]. The oxygen of main triplet state ($^3O_2$) is excited to singlet state ($^1O_2$) by chlorophyll of reaction centers of triplet excited state ($^3P680^*$) [8]. The target of ROS is known to be D_1 protein that is one of subunit of PS II RC, that catalyzed of water splitting and realized of primary functions of charge separation and chlorophyll electron of reaction centers separation [9]. The D_1 protein degradation at definite conditions may be triggered by long lived oxidative forms such as $P680^+$ (first donor and $Tyr\text{-}Z^+$)

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and take place at oxygen absence [10]. Degradation and re-synthesis of D_1 protein is a process significantly faster compare to other thylakoid proteins and it is a reason of impossibility to detect of new synthesized proteins to restoration of photosynthetic activity [6, 11, 12, 13]. The inhibitor of protein synthesis is widely used in this connection. Chloramphenicol is one from there which connecting to great subunit of 70S ribosome has blocked its peptidyl-transferase activity [12]. In present work the damage mechanism of electron transport chain of PS II under increased content of ROS and its connection with D_1 protein degradation were investigated.

2. Materials and methods

The investigation object was pumpkin leaves (*Cucurbita pepo* L.) two week, growth at 22°C and 80% humidity from second tier and its isolated from their chloroplasts were used. To create an oxidative stress in leaves and chlo- roplasts 50 and 100 μM methylviologen (MV) (1,1′-Dimethyl-4,4′-bipyridinium) that is known create a reactive oxygen species and 10 mg/ml inhibitor of protein synthesis chloramphenicol (CAP) were used during comparative analysis.

Chloroplast preparation. The plant leaves in the beginning were homogenized in buffer contained 40mM HEPES (pH 7.4) 0.3M sorbitol, 10mM $MgCl_2$, 1mM EDTA, 1M glicine -betain.

The suspension was centrifuged during 5 min at 1100g. The sediment was treated by osmotic buffer 10mM HEPES (pH 7.4), 5mM sorbitol and 10 mM $MgCl_2$ to obtain suspension. The suspension was precipitated at 2000g. Obtained final sediment was suspended in buffer containing 10mM HEPES (pH 7.4), 0,5M sorbitol, 10mM $MgCl_2$ and 5mM NaCl. Isolation of chloroplasts at all steps was carried out at 4°C under weak green illumination [9].

Method of millisecond delay fluorescence of chlorophyll a. The functional state of photosynthetic apparatus was evaluated by millisecond delay fluorescence (msec-DF Chl*a*) method. Msec-DF of Chl*a* was measured with phosphoroscope in such way that 0,3 ms excitation light pulse was following by 1,25 ms of dark and 0,3 ms of registration of the delayed light emission. Method of msec-DF Chl*a* makes it possible fast evaluation of plant stability and weak sites in photosynthetic chain under stress conditions [14].

The method of polyacrylamide gel electrophoresis. Electrophoresis was fulfilled in "Mini Protean" (Bio-Rad) and while using in finished gel "mini- protean TGX stain-free precast" which does not contain SDS. Then running buffer (pH 8.3) was containing 25 mM Tris-HCl, 192 mM glycineand 0.1% SDS. Sample buffer was consisted62.5 mM Tris-HCl (pH 6.8), 25% glicerol, 2% SDS, 0.01% bromophenol blue. Before the use this was mixed with 50 μl β- merkaptoetanol 950 μl of buffer. The prepared chloroplast samples were mixed with buffer samples in 1:1 ratio and placed to the gel plate. The separation of proteins was fulfilled during 35-45 min at 200 V maximum. 0.2 g of Coomassie (Brilliant) Blue G-250, 80ml of methanol and 20 ml of acetic acid were used for staining of polypeptides.

3. Results and discussion

The activity of electron transport chain of PS II was evaluated by changes of induction transients of msec-DF of Chl*a* that in intact system are in tight connection with metabolic processes and regulated by cell at accordance with its energetic requirements.

The activity of PS II ETC in incubated with methyl viologen leaves is obtained to fall. The first phase (f.ph) intensity after 4 h incubation was equal to 53% activity. Slow phase (sl.ph) after 2 h adaptation loss to 62% and to 4 h adaptation is restored to 53%. Chloramphenicol action under leaves infiltration also suppressed an activity of ETC PS II. After 4 h adaptation an activity of f.ph was equal to 53% and sl.ph-73%.

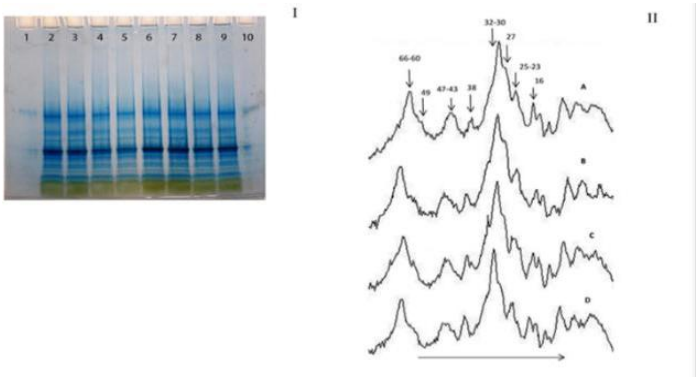

Fig. 1. I The effect of inhibitors in polypeptides of pumpkin leaves. 1;10-BSA marker, 2;3-control (unaffected green leaves), 4;5- leaves measured after contain in 5min 50μM MV, 6;7- leaves restored within 16 hours after being kept in 5 min 50μM methylviologen (MV+16 h), 8;9- leaves contained 5 min 50μM MV and then contained 5min 10 mg/ml chloramphenicol and restored 16 hours (MV+CAP+16 h). II Chloroplast polypeptide profiles and densitogram from pumpkin: A) control; B) 5 min 50μM MV; C) 5 min 50μM MV+16 h restoration; D) 5 min 50 μM MV+5min 10 mg/ml CAP+16h restoration

Table 2 shows the change in the average density of the 43-47 kDa protein zone corresponding to dimer LHPC of PSII and the 30-32 kDa protein zone associated with the PSII core. Quantitative changes in the polypeptide profiles and the ratio of protein zones observed under the action of MV and CAP are compatible with the action of other oxidants. So the effect of NaCl was shown in a change in the ratio of chlorophyll-protein complexes and polypeptides in the zone of 38-49 kDa and in the zone of 25-30 kDa, which quantitatively increased under the action of the thylakoid membrane stabilizer Polistimulin K. Changes in the polypeptide profiles suggest their role in the protection of the thylakoid membrane, which is confirmed by studies of fluorescence characteristics [12, 13].

Table 2. Quantitative changes in protein zones in the range of 43-47 kDa and 30-32 kDa. 1;2-control (unaffected green leaves), 3;4- leaves measured after contain in 5min 50μM MV, 5;6- leaves restored within 16 hours after being kept in 5 min 50μM methylviologen (MV+16 h), 7;8- leaves contained 5 min 50μM MV and then contained 5min 10 mg/ml chloramphenicol and restored 16 hours (MV+CAP+16 h)

Number Protein Zones	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
43-47 kDa	135.8	135.79	110.5	108.06	127.47	138.71	132.04	137.33
30-32 kDa	185.30	148.98	138.28	124.67	143.45	155.75	144.34	161.20

Under action of MV to leaves during 5 min on induction curve of msec-DF Chl*a* the sharp change in slow phase of fluorescence probably connected with disturbance by ROS oxidation-restoration equilibrium between donor and acceptor sides (Fig. 1.II.B). Toxic action of MV may be cause by its action on photochemical reactions in chlorophyll-protein complexes due its damage. This is confirmed by analysis that point out of significant changes in polypeptide profiles (Fig. 1II.B, 2.II.B).

Action of MV during 5 min in protein spectrum leads significant changes. The polypeptides at 30-32 kDa, charactering of D_1 protein position was quan- titatively decreased (Fig. 1.II.B).The decreasing of polypeptides at 60-66 zone was negligable. After 16 h adaptation after action of MV (5 min) a broadening of zone at 60-66 kDa is observed, a peak at 49 kDa was concentrated and well-defined peak at 32 kDa protein marked. The zone at 23-25 kDa protein was enlarged and divided on 2 peaks (Fig. 1.II.C).

Action of Chl in case of increased content in chloroplast of ROS produced by MV after 16 h of restoration the decreasing of polypeptides in range of 60- 66 kDa, decreasing and widening of zone 43-47 kDa where 3 peaks is well- defined. The increasing of zones at range of 30-32 kDa (Fig. 3.II.D).

For elucidation of dependence an activity of PS II from changes of polypeptides, especially at region of main protein of PSII (D_1 protein) under oxidative stress a Fig. 4 is presented by us. The restoration

processes, delayed by action CAP also will assist to establishment of this dependence.

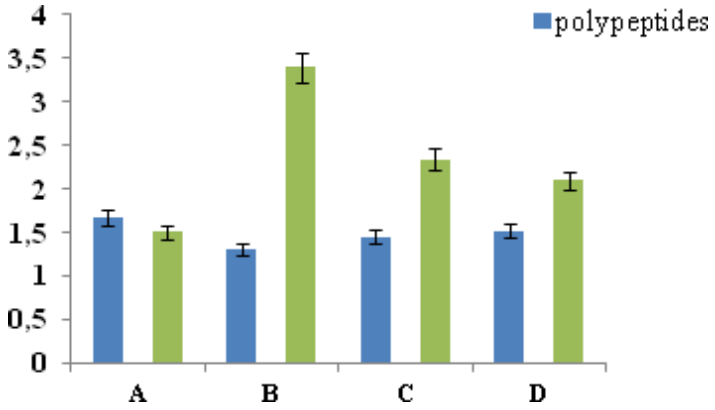

Fig. 2. ■ - Dependence of change of condition of the stationary phase of 30-32 kDa polypeptides (relative amount of polypeptides related to reaction center
of PSII (D1 protein) (see Fig.3.1)) and ■ - msec-DF Chl*a* (P680*+ QA→QB) during the oxidative stress caused treatment by 50μM MV at 5 min: A) control (unaffected green leaves), B) leaves measured after treatment by 50μM MV at 5 min, C) leaves restored within 16 hours after being kept in 5 min 50μM methylviologen (MV+16 h), D) leaves treated by 50μM MV during 5 min and then by 5min with 10 mg/ml chloramphenicol and restored 16 hours (MV+CAP+16 h)

On the Fig. 2A dependence of PSII activity state from polypeptides changes especially at range of main protein of PSII – D_1 under oxidative stress generated by MV. The sharp increase of stationary fluorescence (Fig. 2B) and definite fall of value proportional to level of D_1 protein indicate to formation of reactive oxygen species.

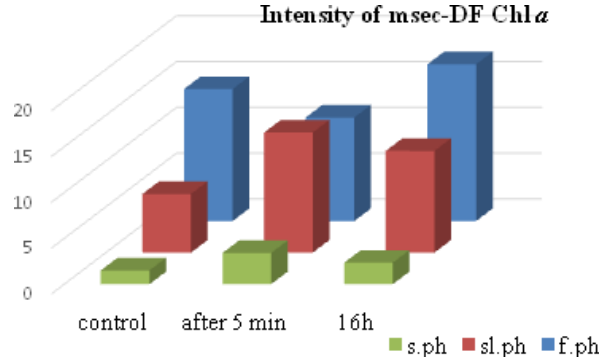

Fig. 3. Changes of delayed fluorescence of steady state (s.ph.), slow (sl.ph.) and fast (f.ph) phases in control, after treatment of 5 min by MV and after washing off MV 16 h of restoration

The restoration processes (16 h after action of MV (Fig. 5) are cha- racterized by some increase of polypeptide at zone 30-32 kDa and fall of sta- tionary fluorescence (Fig. 2C), that is evident about increasing of electron transfer on acceptor side of PSII chain. The introduction to system of CAP pro- tein synthesis inhibitor significantly affect on occurred changes, characterized PSII activity (Fig. 2D).
The Fig. 4 represent correlation plot of relative amount of polypep- tides related to RC (reaction center) PSII (probably proportional to D1 protein) upon the steady state level (s.ph) of induction curve of msec-DF of Chl *a*, during oxidative stress in leaves of pumpkin induced by action of MV and restoration process in different conditions.

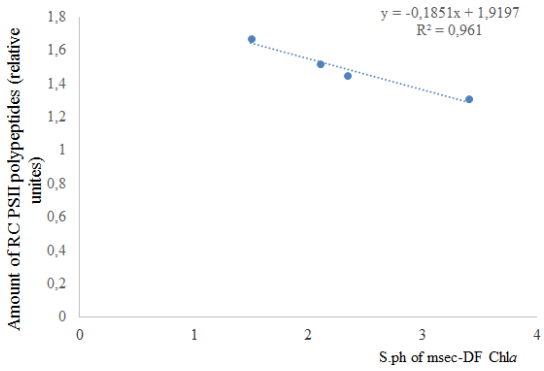

Fig. 4. Correlation between the average values of the PSII reaction center obtained from the average density of polypeptides at 32-33 kDa and the intensity of the stationary phase (s.ph) Chl *a* ms-GF. R^2 – Pearson coefficient. Here, X-axe is Chl*a* ms - GF is the relative unit of the induction curve of the stationary phase (s.ph), Y-axe is average value of PSII reaction center 32-33 kDa polypeptides

We could fit it with line as function and find strong enough reverse cor- relation between these two parameters with Pearson coefficient R^2=0.961. With increasing the relative amount of polypeptides associated to RC PSII during 16hours restoration (Fig. 1, 2), level of stationary phase intensity sharp decreasing. We suppose that this is due to the turnover at D1 protein during restoration of pumpkin leaves and recovery equilibrium between PSII activity and synthesis of new protein of reaction centers of PSII.

Chloramphenicol has inhibited of protein synthesis in chloroplasts and enhanced an activity of photosynthetic ferment ribuloza-1,5-di carboxylase, preventing light-dependent synthesis of photosynthetic activity in leaves [8]. ROS production by methyl viologen damaged ETC of PSII *in vivo* and formed strong reactive O_2 and OH has inhibited of restoration of damaged PS II.

The CAP and MV inactivated of processes connected with capture and disintegration of charge separation with formation of initial radical pair P680* Phe (f.ph) and electron transfer on primary quinine acceptor Q_A (sl.ph). The chloramphenicol action to these processes was less aggressive relatively to action of MV. The sharp increase of stationary fluorescence and sharp fall of value polypeptides, probably proportional to D_1 protein level indicate on significant influence of MV to structural changes and activity of ETC PS II. The MV-produced ROS damaged PS II *in vivo* formatted strong reactive forms O_2 and OH has inhibited whole restoration of damaged PS II [15]. The restoration processes (16 h after action of MV (Fig. 5) are characterized by some increase of polypeptide at zone 30-32 kDa and fall of stationary fluorescence (Fig. 4C), that is evident about increasing of electron transfer on acceptor side of PSII chain. The introduction to system of CAP protein synthesis inhibitor significantly affect to occurred changes, characterized PSII activity (Fig. 4). Introduction to system of protein synthesis inhibitor CAP insignificantly influenced on character of occurred changes in site characterized of PS II activity (Fig. 4D). The effect of CAP is similar with effect of MV but less aggressively suppressed of msec-DF Chl*a* parameters. At the case action of CAP the steady state level of DF say about preservation of oxidative-stationary equilibrium between photosystems. Probably CAP leads to suppression of D_1 protein formation *de novo*, breaked by oxidative stress disturbance of equilibrium between RC PSII inactivation and synthesis of new centers.

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