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RESEARCH PAPERS

Photoprotective Mechanisms in Photosystem II of *Ephedra monosperma* during Development of Frost Tolerance

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Abstract—Dissipation of light energy absorbed by photosystem II (PSII) in assimilating shoots of an evergreen shrub *Ephedra monosperma* was investigated during its transition from the vegetative to frost-tolerant state under natural conditions of Central Yakutia. The dynamics of modulated chlorophyll fluorescence and carotenoid content was analyzed during seasonal decrease in ambient temperature. The seasonal cooling was accompanied by a stepwise decrease in photochemical activity of PSII ($F_v/F_m = (F_m - F_0)/F_m$). The decrease in F_v/F_m occurred from the beginning of September to the end of October, when the temperature was lowered from 10 to –8°C. During winter period the residual activity of PSII was retained at about 30% of the summer values. The seasonal decrease in temperature was accompanied by a significant stimulation of pH-independent dissipative processes in reaction centers and antenna of PSII. The increase in energy losses was paralleled by a proportional increase in zeaxanthin content on the background of decreasing content of violaxanthin and β-carotene as possible zeaxanthin precursors. At the same time, inhibition of light-induced non-photochemical quenching in the PSII antenna was observed. The results suggest that principal photoprotective mechanisms during seasonal lowering of temperature are: (1) inactivation of PSII and dissipation of excitation energy in PSII reaction centers and (2) zeaxanthin-mediated energy dissipation in the antenna complexes. The first mechanism seems to prevail at early stages of seasonal cooling, whereas both mechanisms are recruited from the onset of sustained freezing temperatures.

Keywords: Ephedra monosperma, low temperature adaptation, pigments, violaxanthin cycle, chlorophyll fluorescence quenching

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INTRODUCTION

During cold seasons when the light energy absorbed by plant pigments cannot be fully utilized in photochemical reactions, excessive irradiance promotes formation of chlorophyll (Chl) triplet states and reactive oxygen species (ROS), thus increasing the probability of destruction of assimilating cells [1–3]. One of the main means for maintaining safe balance between absorption of light energy by photosynthetic apparatus (PSA) and energy consumption by metabolic sinks is the adaptive decrease in photochemical efficiency of photosystem II (PSII) and, accordingly, the increase in energy dissipation [3–6]. The best characterized mechanism is light-induced non-photochemical quenching, including its energy-dependent component (*qE*) that performs the main protective function during summer season [2, 4].

Seasonal long-term photoprotective mechanisms in evergreen plants are less examined. Earlier studies showed that low temperatures promote the increase in photoinhibitory component of quenching (*qI*) of excessive energy [5, 6]. Activation of *qI* is associated with the cessation of plant growth and the beginning of cold hardening. In overwintering coniferous plants and evergreen shrubs, direct correlation was found between dissipative processes in PSII and the level of photoprotective proteins PsbS and Elips/Hlips [7, 8]. The energy dissipation mechanisms operating in winter period comprise a significant increase in the pool of deepoxidized pigments of the violaxanthin cycle (VXC), photoinhibition and partial disassembling of reaction centers (RC) of PSII, as well as the transfor-

Abbreviations: Anth—antheraxanthin; Car—carotenoids; β-Car—β-carotene; Chl—chlorophyll; LHC—light-harvesting complex; Lut—lutein; Neo—neoxanthin; NPQ—non-photochemical quenching of chlorophyll fluorescence; PAR—photosynthetically active radiation; PFD—photon flux density; PSII—photosystem II; PSA—photosynthetic apparatus; Qa—primary quinone acceptor of electrons; RC—reaction centers; Via—violaxanthin; VXC—violaxanthin cycle; Xanth—xanthophylls; Zea—zeaxanthin.

mation of light-harvesting antenna of PSA into energy-dissipating sites (centers) due to formation of aggregated pigment–protein complexes [5–8].

In our previous study [9] we described rearrangements of pigment apparatus in the shoots of an evergreen shrub *Ephedra monosperma* that were caused by development of low-temperature tolerance during autumn–winter–spring season under severe climatic conditions of Yakutia. The autumnal hardening was accompanied by the increase in the content of VXC carotenoids and in the level of their de-epoxidation. In the peripheral cells of assimilating shoot parenchyma, accumulation of a photoprotective secondary carotenoid (Car), rhodoxanthin was noted. This carotenoid effectively screens a substantial part of photosynthetically active radiation (PAR) in the blue–green spectral region, thus shading the chloroplasts. We considered these rearrangements of pigment apparatus as adaptive processes aimed at activation of energy-dissipating, antioxidant, and photoscreening pigment systems. However, data on pigment composition are insufficient to clarify the mechanisms by which excessive excitation energy is dissipated in PSA. Fluorescence methods, including pulse-amplitude modulation (PAM) fluorometry, are widely used for analysis of energy deactivation in PSII; the latter technique allows characterization of photochemical and non-photochemical quenching in PSII.

The aim of this work was to examine dynamic changes of energy dissipation in PSII to heat in assimilating shoots of an evergreen shrub *Ephedra monosperma* during formation of frost tolerance. To this end, we measured parameters of chlorophyll fluorescence under natural and laboratory conditions and analyzed the composition and content of photosynthetic pigments.

MATERIALS AND METHODS

Plant material and conditions of plant growth. *Ephedra monosperma* C.A. Meyer (family Ephedraceae Dumort.) is an evergreen shrub with a long rhizome, up to 20–25 cm in height. It inhabits stepped areas, stony slopes of river valleys, and pine forests of southern, eastern, and central Yakutia. *E. monosperma* is a light-loving and drought-resistant plant with numerous highly branched shoots and reduced scaly leaves. Experiments were performed with current-year shoots of plants growing in the Botanical Garden of the Institute for Biological Problems of Cryolithozone, Siberian Branch, Russian Academy of Sciences (Yakutsk, 62°15′ N, 129°37′ E). Experiments were carried out in 2011–2012. Air temperature was recorded with a thermograph DS 1922L iButton (Dallas Semiconductor, United States) at 1-h intervals. Irradiance was measured with a LI-190 sensor (LI-COR, United States) at 30-min intervals. The daily incidence of solar radiation was determined by averaging data recorded at half-hour intervals from the dawn to sunset and was expressed in micromol photons per meter square per second. The average air temperature over the growth period (May–September) was slightly above 14°C, the total precipitation was 163 mm. The minimal air temperature in the winter period did not drop below −48°C (Fig. 1). The depth of the snow cover in December–January was 44–48 cm; the temperature in the snow at a depth of 15 cm from the surface varied from −20 to −24°C.

Chlorophyll fluorescence measurements. Parameters of chlorophyll *a* (Chl) fluorescence were determined in experiments with current-year shoots using a PAM 2500 field fluorometer (Walz, Germany) permitting multibeam actinic excitation. The minimal fluorescence (F_0) was excited in dark-adapted samples using weak (probing) light pulses with photon flux density (PFD) of 0.1 μmol/(m^2 s) ($\lambda = 630$ nm, pulse duration 1 μs, modulation frequency 200 Hz). The maximal fluorescence (F_m) was induced in dark-adapted samples by superimposing the probing light pulses at a frequency of 100 kHz with a powerful saturating flash. Saturating flashes (PFD 8000 μmol/(m^2 s), peak emission at 630 nm, pulse duration 400 ms) served to convert the RC of PSII into the closed state (the state with a fully reduced primary quinone acceptor Q*a*), at which the entire excitation energy dissipates to heat and fluorescence emission. In light-adapted samples, we monitored the steady-state fluorescence F_s (measurements of F_s are technically similar to recording F_0) and the maximum fluorescence F_m' (similarly to recording F_m). From these basic parameters we calculated the derived characteristics of photochemical and non-photochemical events in PSII.

The maximal quantum yield of photochemical energy transduction in PSII was determined with dark-adapted samples from the formula: $F_v/F_m = (F_m - F_0)/F_m$. In summer months and September the measurements were conducted under field conditions 1 h before the sunrise. In the period from October to January, Chl fluorescence parameters were assessed in the laboratory. To this end, the shoots were excised 1 h prior to sunrise and transported to the laboratory within 30 min in a light-proof thermostatic bag at natural air temperature. Next, fluorescence measurements were performed immediately, without adaptation of plant material to room temperature.

In order to examine recovery of PSII activity, the shoots were excised before sunrise at −38°C (on January 17, 2012) and were immediately transported in the light-proof thermostatic bag to the laboratory. The selected plant specimens were placed into water and allowed to stay at room temperature under illumination at PFD of 25–30 μmol/(m^2 s). After incubating samples for 6, 24, 48, and 72 h, parameters of Chl fluorescence were determined. At the same time, a part of plant material was fixed with liquid nitrogen for the assay of pigment composition.

Deactivation of excitation energy in PSII to heat was quantified by the quantum yield of non-photo-

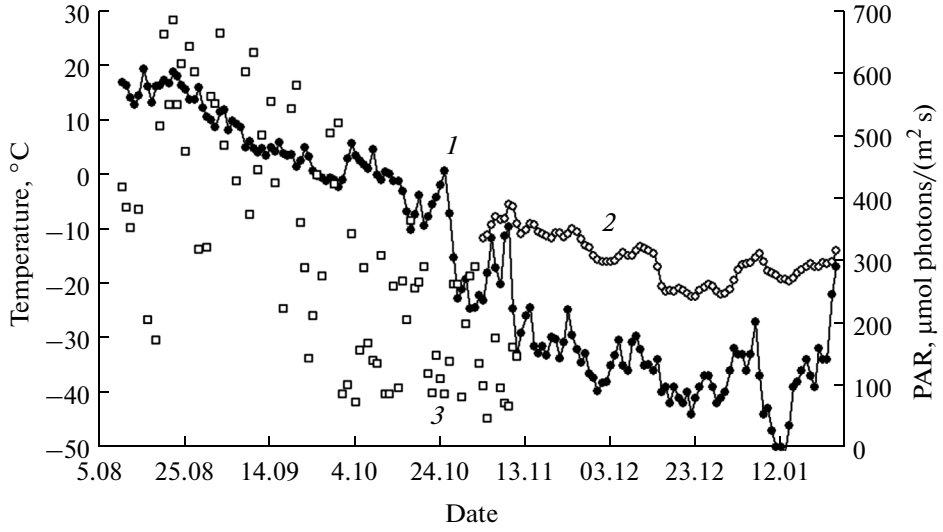

Fig. 1. Seasonal changes in air temperature and photosynthetically active radiation (PAR) in Central Yakutia (62°15′ N, 129°37′ E) in 2011–2012.

Temperature and PAR data were averaged over the day/night cycle and the daylight period, respectively. Temperature was measured at 1-h intervals; PAR, at 30-min intervals. *1, 2* —air temperatures in the experimental plot at a height of 2 m above the ground and at a depth of 15 cm beneath the snow surface, respectively; *3* —PAR.

chemical quenching $\phi_{NPQ} = F_s/F'_m - F_s/F_m$ and by the quantum yield of constitutive (non-regulated) energy losses $\phi_{f,D} = F_s/F_m$ [10]. In the period from July to September these characteristics were assayed under field conditions in vivo around the noon (11:00 to 13:00 solar time). To accomplish these measurements, the current-year shoots were adapted to darkness for 30 min, and F_m and F_0 were first determined. Next, the samples were sequentially illuminated for 5 min by actinic light at PFD of 5, 28, 65, 112, 190, and 305 μmol/(m^2 s). At the end of each 5-min cycle sufficient to attain steady-state photosynthesis, F_s and F'_m parameters were recorded. The quantum yields ϕ_{NPQ} and $\phi_{f,D}$ were calculated for each illumination cycle. In the period from October to January, measurements were performed in the laboratory using the shoots excised before the noon and dark-adapted at room temperature for 50 min.

The light-response curves of *qE* (energy-dependent component of NPQ) were measured under field conditions by exposing dark-adapted intact shoots to various irradiances. The induction of *qE* was achieved by illumination of a sample for 10 min at PFD of 112, 250, 305, 460, 637, and 903 μmol/(m^2 s). Values of *qE* at various light intensities were calculated from the equation: $qE = F_m/F'_m - F_m/F''_m$, where F'_m is the maximal fluorescence yield at the end of the illumination period and F''_m is the maximal fluorescence yield after 10-min dark relaxation [4].

Analysis of pigments. The content and composition of pigments was determined in assimilating partial shoots of the current year. The samples were fixed with liquid nitrogen immediately after sampling in natural plant habitats; they were placed in a Dewar vessel and transported to the laboratory. Chlorophylls and carotenoids (Car) were extracted from plant material with acetone at 8–10°C. The homogenate was centrifuged for 20 min at 8000 *g*. The content of Chl and Car in the supernatant was determined spectrophotometrically using an Agilent 8453E spectrophotometer (Agilent Technologies, Germany) by assessing absorbance at 662, 644, and 470 nm.

For analysis of Car content, the current-year shoots were cut into 4- to 5-cm segments and immediately transported to the laboratory in a thermostatic bag at natural air temperature. The shoots were placed on a wet paper sheet and adapted to darkness for 30 min at room temperature; then, they were fixed in liquid nitrogen and desiccated in a VirTis lyophilizer (United States). The lyophilisates were stored at –80°C and used for pigment analysis by HPLC. Individual carotenoids were separated by the reverse-phase HPLC (Knauer, Germany) using the modified method [11] after extracting the liophylisates with 100% acetone. The calibration plots were obtained with standard samples of pure substances (pigments) (Sigma and Fluka, United States).

Data were statistically processed by single-factor ANOVA (analysis of variance) at the significance level of 0.05 using Microsoft Excell 2003. Data in tables and figures are mean values and standard deviations. Biochemical characteristics were determined in 3–5 replicates with two assays per replicate.

RESULTS

Experiments were conducted in the period from the mid-July 2011 to the middle of January 2012. During this period the daily mean air temperature decreased from 21–23°C to –39 or –45°C (Fig. 1). The mean night temperatures were lower by 3–5°C. The beginning of frosts was noted around September 25. Persistent lowering of night temperature below 0°C level occurred in the beginning of October. The snow cover was established in the beginning of November. The thickness of snow cover was 20–25 cm in the middle of November; 40–45 cm, in December–January. The shoots of *E. monosperma* were largely covered with snow in the beginning of November. Our measurements showed that temperature in December–January under the snow at the shoot level was 18–22°C higher than air temperature.

Measurements of Chl fluorescence, performed under field conditions before the sunrise, revealed stepwise seasonal changes in F_0 and F_m related to lowering of environmental temperature. When the daily mean temperature decreased from values around 7.1 ± 2.5°C in the beginning of September to a lower chilling level (3.1 ± 1.2°C in the end of September), a decrease in F_m and an insignificant increase in F_0 were observed (Fig. 2a). When the daily mean temperature decreased further in the fourth week of October, sharp drops in F_m and F_0 were noted. The Chl content remained largely unchanged during this period. In the period of November–January, when the snow cover was established, the values of F_0 and F_m showed no significant changes. The changes in F_0 and F_m reflected the seasonal dynamics of maximal PSII photochemical activity (F_v/F_m) (Fig. 2b). For example, F_v/F_m was 0.82 during summer period. This parameter started decreasing in September and stopped lowering at the level of 0.35 in the end of October. In November–January, when the plants were covered with snow and exposed to temperatures from –11 to –22°C, F_v/F_m values decreased to 0.32–0.33 and remained at this level, which indicates the retention of residual PSII activity. The largest seasonal changes in F_v/F_m occurred upon temperature lowering from 8.0 to –10°C (Fig. 3). The fast drop in maximal photochemical activity was observed at temperatures near and slightly below 0°C, when F_0 and F_m decreased to the lowest levels. Changes in these parameters were almost independent on natural oscillations of solar radiation.

In order to clarify the ability of *E. monosperma* plants to develop photoprotective processes related to heat dissipation of the light energy absorbed, we monitored the dynamics of the quantum yield of constitutive energy losses ($\phi_{f,D}$), the quantum yield of non-photochemical quenching (ϕ_{NPQ}), and the content of violaxanthin cycle (VXC) pigments (Fig. 4). The values of $\phi_{f,D}$ and ϕ_{NPQ} were determined at PFD of 305 μmol/(m^2 s) (see Materials and Methods). The autumnal cooling induced the sustained increase in $\phi_{f,D}$ from 0.3 in summer months to 0.6 in the end of October, when the temperature dropped to the range between –7 and –10°C. In the period of November–January the $\phi_{f,D}$ values remained almost unchanged. The value of ϕ_{NPQ} was 0.10 in summer months and increased to the highest value of 0.24 in the end of September during cooling of air temperature to 3–4°C. After a slight decrease in October, $\phi_{f,D}$ remained at 0.17 until the end of the observation period (January). It should be noted that determinations of $\phi_{f,D}$ and ϕ_{NPQ} in October–January were performed after 50-min incubation of shoots at room temperature. During this period the partial repair of PSII and resynthesis of carotenoids were not excluded.

During the period of cold hardening (from the end of August to the middle of October) the Chl ($a + b$) content decreased markedly, from 2.82 ± 0.23 to 2.07 ± 0.24 mg/g dry wt. The total pool of carotenoids comprising VXC pigments, lutein (Lut) and neoxanthin (Neo) remained quite stable, reaching 0.65–0.70 mg/g dry wt. The fractional content of zeaxanthin (Zea) increased dramatically from 1–2% (in summer period) to 16.6% (in the end of September) (Fig. 4b). The relative content of Zea went on rising up to 22.7% in the middle of October, when the mean daytime and nighttime temperatures were 1.6 ± 2.5 and –0.4 ± 2.1°C, respectively, and variations of natural irradiance were 240 ± 150 μmol photons/(m^2 s). The increase in the content of this photoprotective carotenoid was accompanied by lowering in the content of violaxanthin (Vio) and β-carotene (β-Car), the total drop in the content of Vio and β-Car corresponded to the increment in Zea content (Fig. 4b). This observation indicates that Vio and β-Car were partly transformed into Zea. During subsequent lowering of air temperature in November–January, the composition of Car remained almost unchanged (Fig. 4b).

The seasonal dynamics of energy-dependent component of non-photochemical quenching *qE* was monitored by measuring the induction curves of Chl fluorescence in vivo under actinic illumination with PFD levels of 112, 250, 305, 460, 637, and 903 μmol photons/(m^2 s); the subsequent dark relaxation of fluorescence parameters was also recorded (see Materials and Methods). As seen in Fig. 5, the extent of *qE* decreased significantly during seasonal lowering of temperature. Remarkably, at daily mean temperatures equal or higher than 7°C, the light response plot of *qE* was linear in the range up to 903 μmol photons/(m^2 s). When the temperature decreased to 4°C the light response plot become nonlinear showing the saturation at about 400 μmol/(m^2 s). In this case the slope of *qE* against PFD in the range 112–305 μmol/(m^2 s) was higher than that in light response curves recorded at warmer environmental temperatures.

In January we monitored the restoration of maximal photochemical activity and other characteristics of PSII over a 72-h period after the transfer of plant

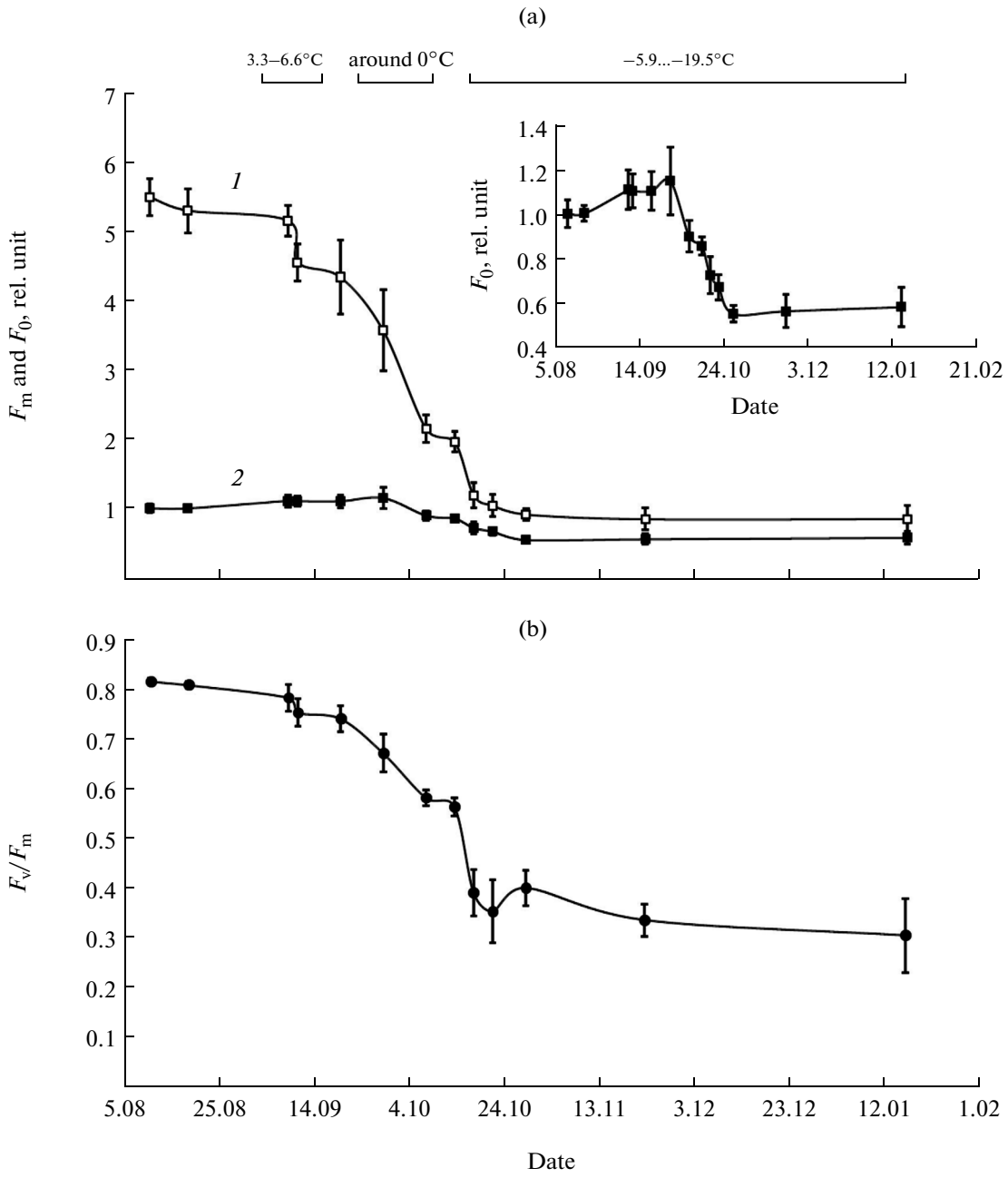

Fig. 2. Seasonal changes in minimal (F_0) and maximal (F_m) chlorophyll fluorescence and the maximal PSII efficiency (F_v/F_m) in the shoots of *E. monosperma*.

Absolute values of F_0 and F_m were normalized by taking into account the chlorophyll content to F_0 level recorded on August 10, 2011. Data are mean values ±SD standard errors ($n = 7–9$). Figures above the horizontal bars designate ranges for fluctuations of daily mean temperature in the respective calendar dates. *1*—F_m, *2*—F_0.

material from an outdoor environment to laboratory conditions (Fig. 6). The Chl ($a + b$) content and the total Car content in shoots remained constant throughout the experiment. At the time point 0 and after 6, 24, and 48 h of incubation at room temperature, F_v/F_m values were 0.31, 0.52, 0.72, and 0.79, respectively. Within the 2-day period the photochemical activity of PSII restored to levels typical of the beginning of September (Fig. 6a). During recovery of F_v/F_m, we observed the increase in F_0, indicative of relaxation of quenching in the antenna complexes, and the decrease in $\phi_{f,D}$, the quantum yield of primary constitutive losses of excessive Chl excitation energy (Fig. 6b). At the same time, the fractional content of Zea and antheraxanthin (Anth) decreased by 11 and 5%, respectively. These changes were accompanied by

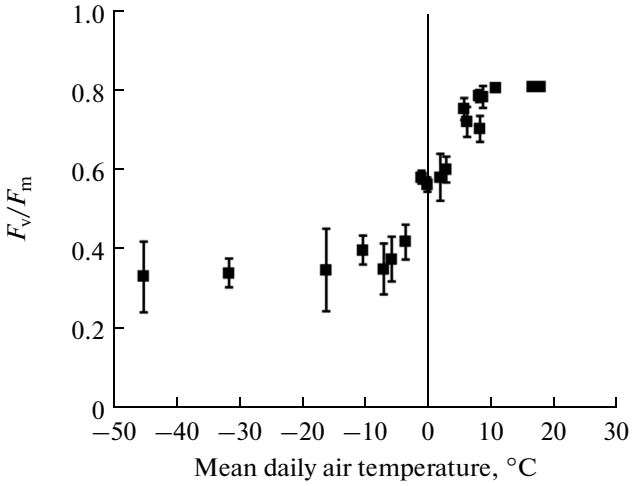

Fig. 3. Effect of air temperature on seasonal decrease in maximal PSII efficiency (F_v/F_m) in *E. monosperma* plants. Data are mean values ±SD standard errors (n = 7–9). The mean daily temperature was calculated by averaging data over 3 days before the onset of measurements.

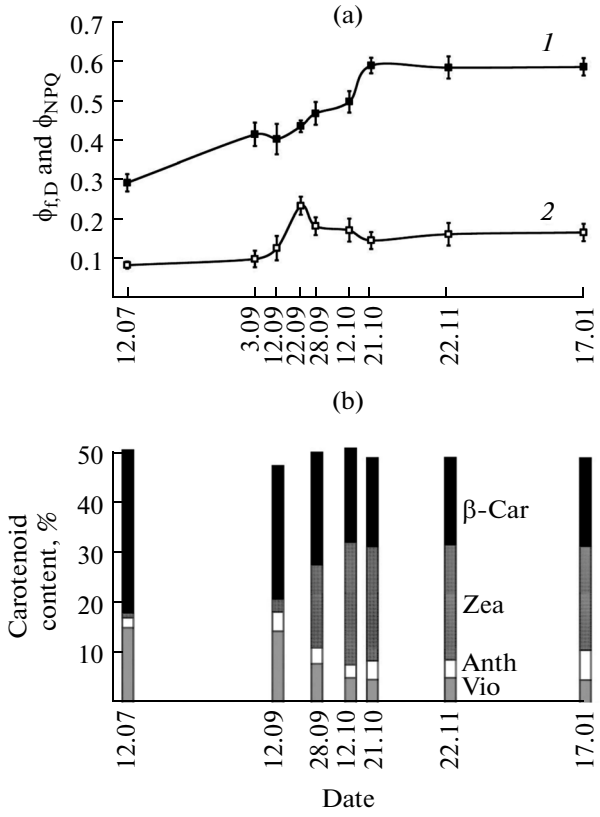

Fig. 4. Seasonal changes in parameters of (a) non-photochemical quenching and (b) pigment composition in the current-year shoots of *E. monosperma*. The induction of $\phi_{f,D}$ (*1*) and ϕ_{NPQ} (*2*) parameters was started by actinic illumination with PFD of 305 μmol/(m^2 s). In July–September fluorescence parameters were measured with undetached shoots under field conditions; in the period from October to January measurements were conducted on excised shoots after allowing them to stay at room temperature for 50 min. In order to assay the composition of carotenoids, the excised shoots were incubated for 30 min in darkness at room temperature prior to fixation by liquid nitrogen. Data are mean values ±SD standard deviations (n = 3–5).

the increase in Vio content by 13% and β-Car content by 2% (Fig. 6c). The relative content of Zea in shoots (about 8.6% of the total Car content) remained constant, even after 72 h of incubation. Apparently, this fraction was uninvolved in energy-dependent quenching of Chl fluorescence.

DISCUSSION

The excess absorbed energy in PSII is quenched by at least three types of processes. Energy-dependent quenching *qE* developing in the time frame of seconds and minutes, results from the pH decrease in the thylakoid lumen and from associated protonation of PsbS protein and de-epoxidation of Vio with the production of Zea [2, 4]. The *qE* quenching dissipates rapidly in darkness. The state transition quenching, *qT* is caused by the decrease in absorption cross section of PSII owing to migration of a part of a phosphorylated population of LHCII from PSII to PSI. This component has no significant role during cold hardening of plants [12]. Under stress conditions, when fast photoprotective processes are insufficient for dissipating energy in PSII, photoinhibition takes place, which is accompanied by the increase in a slow photoinhibitory component of energy quenching (*qI*). This type of quenching can develop in evergreen plants during cold seasons under natural conditions [5–8]. A certain part of *qI* may represent quenching in the RC of PSII; however, in evergreen plants this quenching component is presumably related to pH-independent sustained quenching of the antenna [5].

We found that the total Chl content decreased from the end of August to the middle of October by 25–30%, which promoted the decrease in light absorption by PSA per unit surface area. These changes were accompanied by a 2.5-fold drop of maximal photochemical activity of PSII (F_v/F_m) in the period from beginning of September to the end of October (Figs. 1, 2b). The largest decrease in F_v/F_m occurred in the temperature range from 8.0 to −10°C (Fig. 3). Judging from our observations, natural variations of solar radiation had no influence on the rate of autumnal F_v/F_m decrease, which is consistent with data published for evergreen coniferous plants inhabiting northern boreal ecosystems of Siberia, Scandinavia, and Eastern Europe [3, 13, 14]. According to our results, the seasonal dynamics of F_v/F_m changes comprised three consecutive phases (Fig. 2): (1) a slow decline in the period from beginning of September to the end of fourth week of September, (2) a rapid drop from the end of September to the beginning of fourth week in October, and

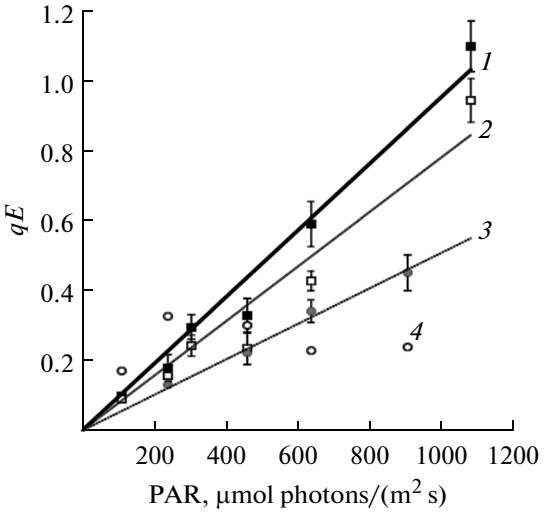

Fig. 5. Plots of qE as a function of photon flux density during seasonal lowering of temperature.

Measurements were performed with undetached assimilating shoots of *E. monosperma* under field conditions ($n = 3$). The mean daily air temperature at measurement dates were: *1*—23.9, *2*—11.3, *3*—7.3, and *4*—4.0°C.

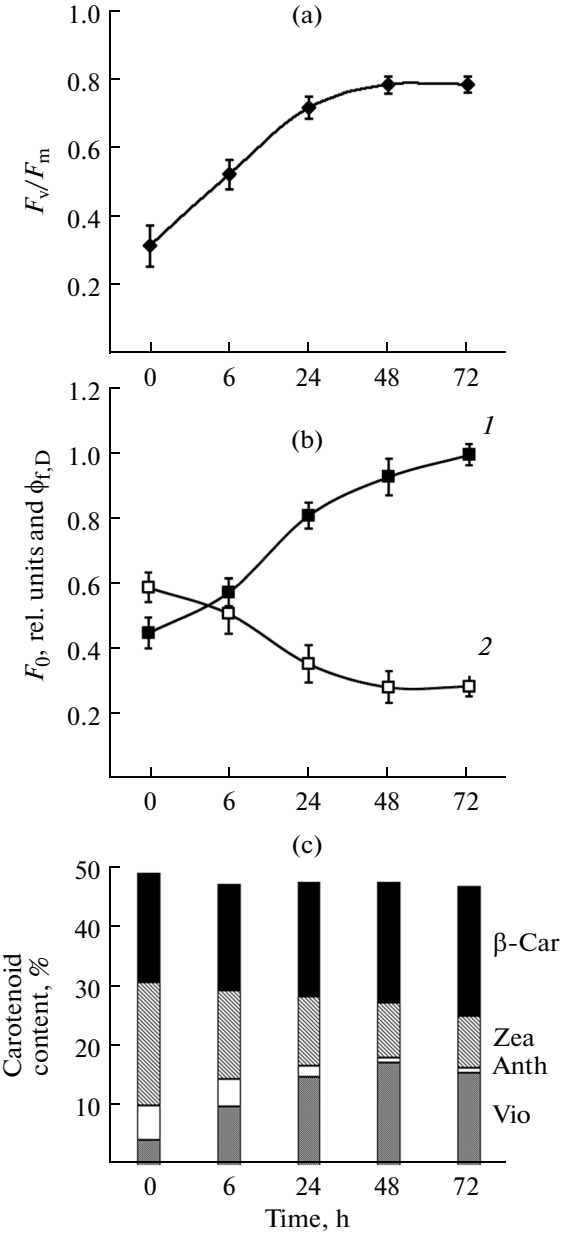

Fig. 6. Recovery of (a) PSII maximal photochemical activity, F_v/F_m, (b) parameters F_0 (*1*) and $\phi_{f,D}$ (*2*), and (c) composition of Car in winter-collected shoots of *E. monosperma* at room temperature under illumination with white light at PFD of 30–40 μmol/(m^2 s).

The abscissa axis designates time after transferring the plant material to laboratory conditions (21–22°C). Data are mean values ±SD standard deviations ($n = 3$–5).

(3) persistently low levels in the period from the end of October to January. In our view, such a dynamics reflects three sequential stages at which the PSII complexes undergo seasonal low-temperature adaptation through dominance of different photoprotective mechanisms.

The first stage was observed in September upon lowering the mean daily temperature to 2–8°C (Figs. 1, 2). The F_v/F_m level decreased by 13% with respect to its summer values (Fig. 2). The retention of comparatively high F_v/F_m values during this period is apparently related to the increased heat dissipation of energy in RC of PSII. The increase in the yield of the nonradiative route of backward electron transfer from Q_A^- to $P680^+$ in RC due to the modified redox potentials of electron carriers at the acceptor side of PSII was found previously for young plants of *Pinus sylvestris* during long term adaptation to chilling temperatures [15]. This type of recombination is safe because it is not accompanied by the production of Chl triplet forms, singlet oxygen, and RC degradation [16]. Nevertheless, our observations imply that this mechanism does not provide entirely stable F_v/F_m values at 2–8°C. In September the decrease in F_v/F_m was mainly caused by the F_m decrease and by a slight, though reliable increase in F_0 (Fig. 2). The decrease in F_m is usually ascribed to energy quenching in PSII centers resulting from RC photoinhibition, i.e., degradation of D1 protein or oxygen-evolving complex and formation of the fluorescence quencher $P680^+$ [7, 8, 17]. The transient increase in F_0 caused by the autumnal thermal stress was previously reported for *Pinus sylvestris* [3]. The increase in F_0 emission is commonly ascribed to the increased number of inactivated RC of PSII following Q_A reduction, as well as to the detachment of LHC II from PSII [3, 18]. Slight elevation of ϕ_{NPQ} in September is insufficient to prevent photoinhibition and the associated increase in $\phi_{f,D}$ (Fig. 4). The quantum yield of NPQ (ϕ_{NPQ}) reflects the portion of energy dissi-

pated by means of ΔpH- and xanthophyll-dependent mechanism of quenching [10]. In our experiments this parameter is equivalent to the quantum yield of energy-dependent quenching *qE* (ϕ_{qE}). The quantum yield of constitutive losses ($\phi_{f,D}$) represents non-regulated energy dissipation in RC and LHC of PSII, this mechanism being insensitive to light and pH control [10]. In evergreen overwintering plants this characteristic reflects primarily the quantum yield of photoinhibitory component (ϕ_{qI}). Prolonged photoinhibition may affect the structure of RC and PSII antenna, thus increasing $\phi_{f,D}$ [5–8, 10]. The decrease in F_v/F_m observed in September was accompanied by the increase in $\phi_{f,D}$, which confirms the assumed photoinhibition of PSII centers. The quenching in PSII antenna complexes seem to be insignificant at early stages of F_v/F_m decrease, because the F_0 level remained undiminished and Zea relative content at mean daily temperature of 7–8°C was rather low, about 3% (Figs. 2, 4). The PSA capacity of energy-dependent quenching (*qE*) was found to decrease in September (Fig. 5). This decrease might be caused by insufficient ability of alternative electron transport pathways to generate the trans-thylakoid ΔpH on the background of depressed carbon fixation, because the intersystem electron transport operates slowly when the plastoquinone diffusion is retarded at low temperatures.

The second stage, i.e., the rapid decrease in F_v/F_m was observed in the period from the end of September to the third week of October, when the daily mean temperature was lowered gradually from about 0°C to −8 or −10°C. During this period we observed the concurrent decrease in F_0 and F_m (Fig. 2). This stage was preceded by the increase in Zea and Anth content to 24 and 4%, respectively, when sustained temperatures near 0°C were attained in the mid-October (Figs. 1, 4). Accumulation of Zea results from inhibition of the backward reaction of VXC de-epoxidation. During recovery of PSII photochemical activity in winter-collected shoots of *E. monosperma* under laboratory conditions, we observed the decrease in Zea content and $\phi_{f,D}$ occurring synchronously with the increase in F_0 (Fig. 6). Apparently, the role of Zea in energy quenching in PSII antenna complexes becomes substantial on lowering centigrade temperatures to near-zero and freezing levels. Indeed, accumulation of Zea, PsbS, Elip proteins, and the enhanced phosphorylation of LHC II are characteristic traits of adaptation to low temperature in many frost-tolerant plant species [5, 7, 8]. These proteins and Zea are thought to participate in the formation of specialized sites responsible for energy dissipation in the antenna complexes and for stabilization of aggregated trimers of LHC II. Such quenching mechanisms in the antenna may also comprise conformational changes in proteins of minor antenna CP24, CP26, and CP29 upon Zea binding [19, 20]. We show for the first time that, unlike VXC pigments, the $\phi_{f,D}$ value went on rising until temperature was lowered to the range from −8 to −11°C (Fig. 4). Under severe temperature conditions, the core complex of PSII RC is likely to undergo partial disassembling. The decrease in the content of D1 protein, oxygen-evolving complex, and pheophytin was observed in *Arctostaphylos uva-ursi*, overwintering under the snow cover, as well as in *Abies lasiocarpa, Picea engelmannii, Pinus contorta, P. ponderosa* and *Pseudotsuga menziesii* upon lowering air temperature to the range from −7.9 to −13.8°C [6–8].

The third stage of cold adaptation of PSII complexes occurs at further lowering of daily mean temperature to −15 and −23°C; it is characterized by nearly constant F_v/F_m close to 0.32–0.33 in the period from the end of October to the middle of January (Fig. 2). When the plants become covered with snow in the beginning of November, direct incidence of solar radiation is excluded, which alleviates the impact of low temperatures. Nevertheless, we found that F_m and F_0 values for shoots residing beneath the snow cover in November–December were about 19 and 15% lower, respectively, than at the temperature range from −7 to −10°C in the beginning of the fourth week of October. The constitutive energy quenching in antenna complexes and effective energy transfer from LHC to RC of PSII are due to conformational changes of protein matrices, to which the pigment molecules are linked [5, 19, 20]. Apparently, after temperature is lowered to the range from −15 to −23°C, the RC undergo further decomposition, while pigment–protein macromolecular structures residing in the lipid phase experience hindrance to conformational changes involved in appearance of favorable configurations for photoinhibitory quenching.

As seen in Fig. 6, during formation of frost tolerance, assimilating shoots of *E. monosperma* accumulate a certain pool of Zea that apparently escapes deepoxidation and is not involved in energy-dependent (*qE*) quenching of Chl *a* fluorescence. Under stress conditions the excess of accumulated Zea is mainly located in a weakly bound peripheral V1 site of LHC II oligomers (peripheral antenna) [21]. This Zea pool performs a double function: it quenches the excess energy and acts as an antioxidant agent. Remarkably, the ability of Zea to protect thylakoid membranes against lipid peroxidation by means of scavenging ROS and free radicals might be higher than that of other xanthophylls and approaches to that of α-tocopherol. It is this Zea pool that is most susceptible to epoxidation [21]. The site of location and the role of Zea pool poorly susceptible to epoxidation are not yet clarified.

The results of our experiments provide evidence on sequential activation of different photoprotective mechanisms that are mobilized upon the increase in heat dissipation of excitation energy in PSII during transition of *E. monosperma* plants from active growth to the state of winter dormancy. We found that quenching in the RC of PSII is the main dissipative

mechanism during autumnal adaptation of plants to chilling temperatures; this mechanism develops on the background of weakening of pH-dependent mechanisms of non-photochemical quenching of Chl fluorescence, such as VXC and protonation of PsbS protein. At temperatures near 0°C and lower, light-insensitive non-regulated energy dissipation to heat in the antenna complexes of PSII via Zea-mediated pathways becomes the dominant mechanism, together with appearance of inactive PSII forms. In the shoots of *E. monosperma* accumulation of Zea fraction uninvolved in energy-dependent quenching of Chl fluorescence was documented. Involvement of photoprotective energy-dissipation mechanisms is an indispensable component of the intricate process of frost tolerance formation in evergreen plants; it is related to structural–functional rearrangement of photosynthetic apparatus in assimilating shoots.

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