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RESEARCH PAPERS

Photosynthesis, Respiration, and Thermal Energy Dissipation in Leaves of Two Phenotypes of *Plantago media* L. under Environmental Conditions

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Abstract—The ability to maintain the balance between the absorbed light energy and energy used in photosynthesis is a key factor of plant adaptation to variable environmental conditions. In this work, diurnal variations in photosynthesis, respiration, thermal energy dissipation, and the activity of the antioxidant system were studied in hoary plantain (*Plantago media* L.) growing on an open slope (sun plants) and under natural shading in the herbage (shade plants). The highest leaves net photosynthetic rate (Pn) was observed early in the morning and amounted to 2.6 and 9.2 μmol CO_2/m^2 s in shade and sun plants, respectively. In the daytime, the Pn values of sun plants decreased significantly (threefold) along with the decrease in stomatal conductance; changes of both parameters developed concurrently with the increase in insolation and air temperature. The Pn changes in leaves of shade plants were less pronounced and weakly dependent on stomatal conductance. The leaves of shade plants contained comparatively high amounts of soluble carbohydrates, whereas the sun plant leaves accumulated larger amounts of starch. In the daytime, nonphotochemical quenching (NPQ) of chlorophyll *a* fluorescence in photosystem II of sun plant leaves could be as large as 2.6 rel. units, which was four- to fivefold higher than NPQ in shade plants. In leaves of sun plants in the morning and evening hours, the ratio of cytochrome pathway (CP) and the alternative (AP) respiratory pathways was approximately 1.0, whereas this ratio decreased to 0.4 during the day, synchronously with an increase in NPQ. The CP/AP ratio in shade plant leaves remained constant throughout the diurnal cycle and equaled 1.4, indicating a comparatively high energy efficiency of respiration in shaded plants growing under the grass canopy. The leaves of sun plants featured an increased content of superoxide anion radical and hydrogen peroxide as well as the elevated activity of antioxidant enzymes (superoxide dismutase, guaiacol peroxidase) that control the accumulation of reactive oxygen species. The results emphasize the importance of coordinated changes in energy-dissipating processes and the activity of the antioxidant system for maintaining the energy and redox balance in phototrophic tissues during long-term adaptation of plants to environmental conditions, excessive insolation in particular.

Keywords: *Plantago media*, antioxidant enzymes, respiration, respiratory pathways, energy dissipation, nonphotochemical quenching, chlorophyll fluorescence, photosynthesis

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INTRODUCTION

The plasticity of a genotype, i.e., the plants' ability to acquire different phenotypes, is of key importance for plant adaptation to diverse environmental conditions [1]. Many plant species form sun and shade phenotypes that differ in anatomical and morphological features and functional properties. The phenotypic polymorphism allows plants to efficiently use light energy of different intensities and prevent the photooxidative stress development upon excessive insolation. Photooxidative stress results from the imbalance between the absorption and the use of light energy, which is accompanied by the increased generation of reactive oxygen species (ROS) and inhibition of photosynthesis [2]. The excessive ROS destroy the photosynthetic apparatus (PSA) components and inhibit the repair of proteins in photosystems [3]. In other words, photoinhibition is result of the imbalance between the

Abbreviations: AP—alternative respiratory pathway; CP—cytochrome respiratory pathway; ETC—electron transport chain; ETR—electron transport rate; GPX—guaiacol peroxidases; NPQ—nonphotochemical quenching of chlorophyll *a* fluorescence; PAR—photosynthetically active radiation; Pn—net photosynthesis; PPFD—photosynthetic photon flux density; PSII—photosystem II; PSA—photosynthetic apparatus; ROS—reactive oxygen species; SLW—specific leaf weight; SOD—superoxide dismutase; TBARS—thiobarbituric acid reactive substances; V_{cyt}—rate of cytochrome respiration; V_{alt}—rate of alternative respiration; WUE—water use efficiency.

rates of PSA photodestruction and PSA repair, primarily, the repair of photosystem II (PSII).

Plants have developed various ways to protect PSA from photoinhibition [4–7], such as the partial thermal dissipation of the absorbed light energy, the activation of alternative electron flows (water-water cycle and cyclic electron flow around PSI), and the enhancement of photorespiration. Recently, considerable evidence has appeared on the involvement of mitochondrial respiration in maintaining the energy and redox balance of phototrophic cells [8, 9]. The alternative oxidase (AOX) prevents excessive ROS generation in the mitochondrial electron-transport chain (ETC) and enhances plant resistance [10] by shunting electron outflow from NAD(P)H for direct reduction of O_2 to H_2O. The risk of oxidative stress under certain conditions determines the increasing role of the antioxidant system that controls pro-/antioxidant homeostasis and the cellular ROS content [11]. However, the operation of cellular mechanisms as a unified complex of complementary processes that maintain the redox balance and protect PSA is far from being fully understood. This is especially true for the interaction of chloroplast and mitochondrial energy-dissipating systems during plant adaptation to stress [12]. It should be also pointed out that the influence of light on AOX activity was mainly examined in short-term experiments in which the seedlings or young plants were transferred from low to high light [13, 14].

The aim of this work was to conduct comparative studies of the diurnal dynamics of photosynthesis, respiration, thermal energy dissipation, and the pro-/antioxidant status of *Plantago media* L. leaves in habitats with contrasting light regimes in order to reveal the role of physiological mechanisms in long-term adaptation to growing conditions.

MATERIALS AND METHODS

Plant material and characteristics of plant habitats. *Plantago media* L. (hoary plantain) is a widely distributed perennial herbaceous plant with a taproot, which belongs to the Plantaginaceae family. In the European northeast of Russia, it occurs in sparse forests, floodplain meadows, on waste grounds, sandy soils, and on limestone outcrops. It should be noted that the species of the genus *Plantago* are characterized by high genetic and morphophysiological plasticity. We previously showed the role of environmental factors in the adaptive differentiation of phenotypes and in the manifestation of genetic polymorphism in *Plantago media* L. plants under natural conditions [15]. On this basis, we have chosen *P. media* as the model object of this study.

Field studies were performed in the South Timan, in the valley of the middle reaches of the Soiva River (62°45′ N, 55°49′ E), in the first half of July 2010–2014. South Timan belongs to the Atlantic–Arctic region of the temperate climatic zone. It is characterized by a temperate continental climate with long winters and short relatively warm summers. The average annual temperature is −1.5°C; the average temperature of the warmest month July is +16°C. The length of a growing season is approximately 130 days. The amount of precipitation during the active growing season is approximately 250 mm. The weather conditions during the research period (according to the data of the open weather archive www.rp5.ru) generally corresponded to the long-term average values of the regional climate.

Cenopopulations of *P. media* were located on (1) an open, scarcely overgrown talus slope from the watershed to the floodplain terrace (sun plants) and (2) at the foot of the slope in dense herbage (shade plants). The soils in the plant habitats were formed on a gravel-fine-earth layer with a shallow underlayment of large fragments of carbonate rocks. The humus-accumulative soil horizon was continuous at the foot of the slope, while it was poorly developed and discontinuous on the slope.

The microclimatic conditions in the habitats were measured using an multipurpose datalogger LI-1400 equipped with light, temperature and relative humidity sensors (LI-COR, United States). The intensity of photosynthetically active radiation (PAR), temperature, and air humidity were recorded close to leaves. On cloudless days, the solar radiation incident on plants on an open slope was almost an order of magnitude higher than that received by plants in the herbage (Fig. 1a). Differences in the light regime of habitats were noted in all years of research, irrespective of weather conditions during the growing season. It should also be noted that high light on the slope led to stronger heating of air and a noticeable decrease in air humidity (Figs. 1b, 1c).

Parameters of induced photosystem II chlorophyll *a* fluorescence were measured using a PAM-2100 fluorometer (Walz, Germany).The minimum (F_0) and maximum (F_m) fluorescence yields were measured after preliminarily dark adaptation of leaves for 30–40 min. In leaves adapted to actinic light, we determined the stationary (F_t), minimal (F_0'), and maximum (F_m') fluorescence yields. All measurements were performed in 20–30 replicates. Parameters characterizing the functional status of PSII were calculated and interpreted according to Goltsev et al. [16]. The maximum (F_v/F_m) and effective (Φ_{PSII}) quantum yields of PSII photochemical activity and the values of nonphotochemical quenching (NPQ) were calculated from the formulas given below and expressed in relative units:

$$F_v/F_m = (F_m - F_0)/F_m, \quad (1)$$

$$\Phi_{PSII} = (F_m' - F_t)/F_m', \quad (2)$$

$$NPQ = (F_m - F_m')/F_m'. \quad (3)$$

The electron transport rate (ETR) through PSII (expressed in μmol/m^2 s) was calculated as

$$\mathrm{ETR} = \Phi_{\mathrm{PSII}} \times \mathrm{PPFD} \times 0.5 \times 0.84, \quad (4)$$

where PPFD is the photosynthetic photon flux density (μmol/m^2 s), 0.84 is the coefficient of absorption of incident light by the leaf, and 0.5 is the coefficient corresponding to even distribution of excitation energy between PSI and PSII.

CO_2 exchange and leaf transpiration. The measurements were carried out using an ADC LCPro+ portable photosynthesis system (ADC BioScientific, United Kingdom) under natural lighting and temperature. Leaves were placed in a clamp chamber for 2–3 min. According to readings of the built-in sensor, the leaf temperature in the chamber at noon was 5 and 3°C higher on average than air temperature on the open slope and in the herbage, respectively. The rates of net CO_2 uptake (Pn) and transpiration (E) were calculated automatically from the difference between the instrument readings in the presence and in the absence of leaf in the chamber.

The rate of CO_2 net uptake was calculated from the formula

$$\mathrm{Pn} = u_s \times \Delta c, \quad (5)$$

where u_s is the rate of air flow in the leaf chamber, mol/m^2 s, and Δc is the difference in CO_2 concentrations in the chamber without a leaf and with a clamped leaf, μmol/mol.

The leaf transpiration rate (E) was determined by the formula

$$\mathrm{E} = (\Delta e \times u_s)/p, \quad (6)$$

where Δe is the difference between water vapor pressures in the chamber with and without a leaf, expressed in mbar; u_s is the rate of air flow in the leaf chamber, mol/m^2 s; and p is atmospheric pressure, mbar.

The rate of water vapor flow through the stomata (stomatal conductance, gs) was calculated as the reciprocal of the stomatal resistance (gs = 1/rs). Stomatal resistance, in turn, was found by the formula

$$\mathrm{rs} = ((\mathrm{w} - \mathrm{wm})/E) - \mathrm{rb}, \quad (7)$$

where w and wm are concentrations of saturated water vapor in the chamber in the presence and in the absence of leaf, mol/mol; E is the rate of leaf transpiration, mol H_2O/m^2 s; and rb is the resistance of the boundary layer for water vapor, m^2 s/mol H_2O.

The determinations were made in four to five biological replicates.

Respiration rate and contributions of individual respiratory pathways. The rate of leaf respiration was determined polarographically from O_2 uptake at 20°C using an Oxytherm system (Hansatech Instruments, United Kingdom). Leaf cuts from the middle part of freshly harvested leaf blades were placed in the reaction vessel containing HEPES buffer (50 mM, pH 7.2,

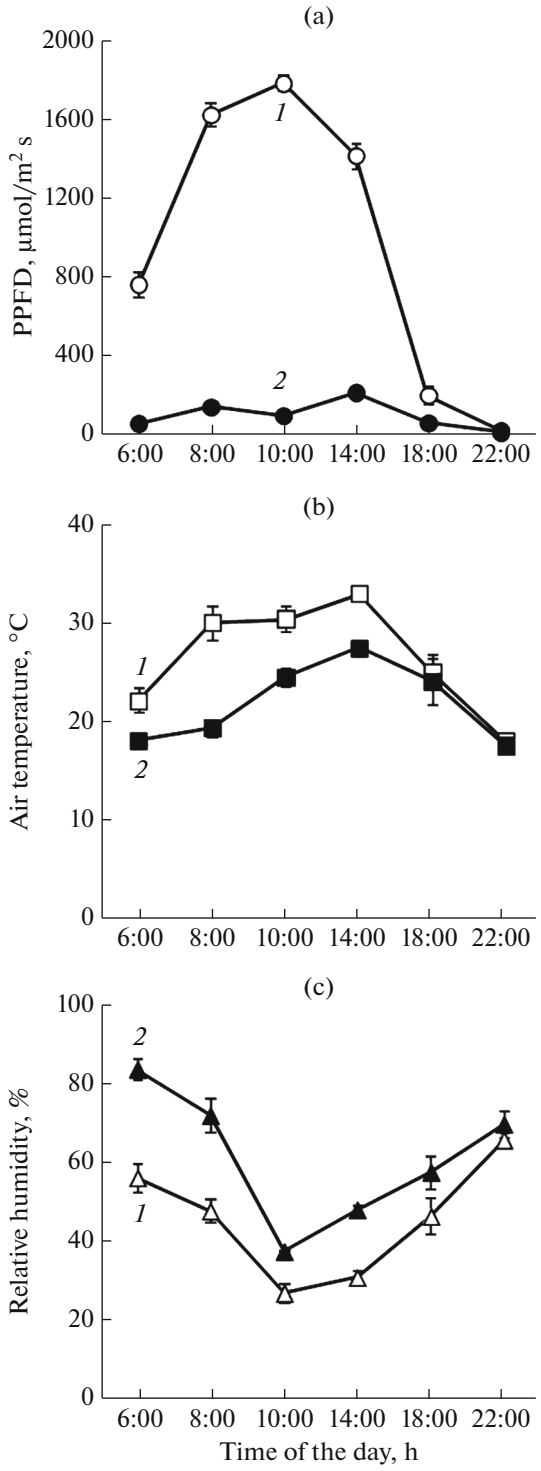


Fig. 1. Diurnal dynamics of (a) photosynthetic photon flux density (PPFD), (b) air temperature, and (c) relative humidity in *Plantago media* distinct habitats: (*1*) an open slope and (*2*) under a grass canopy. Average data represent the results of multiyear measurements for the warmest cloudless days in July (2010–2014).

Helicon, Russia). The samples were constantly stirred during the measurements. Activities of individual respiratory pathways were determined with the use of specific inhibitors, whose optimal concentrations were chosen in preliminary experiments. An 8 mM solution of salicylhydroxamic acid (SHAM) (Lancaster, United Kingdom) was used as an inhibitor of alternative oxidase (AOX). Cytochrome oxidase activity was inhibited with 2 mM KCN solution (Sigma, United States). The total rate of oxygen uptake was expressed as the sum of individual components and expressed in nmol O_2/g DW min:

$$V_t = V_{alt} + V_{cyt} + V_{res}, \quad (8)$$

where V_t is total respiration; V_{alt} is alternative respiration sensitive to SHAM, an inhibitor of alternative oxidase; V_{cyt} is cyanide-sensitive (cytochrome-dependent) respiration; and V_{res} is the residual respiration recorded in the presence of inhibitors for the alternative pathway (AP) and the cytochrome pathway (CP). The determinations were carried out in five to eight biological replicates.

The energy efficiency of leaf respiration was quantified with the coefficient $Y_{ATP/glucose}$ that characterizes the efficiency of ATP yield during glucose oxidation. For these estimates, we took into account the contribution of CP and AP to total respiration using the coefficients given in [17]. Provisionally, the amount of O_2 consumed in respiration was expressed in equivalents of the respiratory substrate. According to the balance equation, the respiratory uptake of 1 mg of O_2 is equivalent to the oxidation of 0.937 mg of glucose.

Sampling of plant material for biochemical analyses. To determine the content of carbohydrates, lipid peroxidation products, and the activity of antioxidant enzymes, functionally mature leaves were selected from the middle part of the rosette of 15–20 plants. The samples were fixed in liquid nitrogen and stored at –78°C.

Determining the content of soluble carbohydrates and starch. Soluble carbohydrates were extracted with 70% ethanol. The extracts were evaporated to an aqueous residue and purified from contaminations by solid-phase extraction using Diapak-amine concentrating cartridges (BioChemMak, Russia). Chromatographic analysis of carbohydrates was carried out with a 250 × 4 mm Diasfer-130-AMIN column, grain size 6 μm (BioKhimMak, Russia), using a refractometer as a detector. Acetonitrile/water mixture (70/30, v/v) was used as an eluent; the elution rate was 2.0 cm^3/min. The calibration curve was plotted using a commercial glucose sample as a standard (Sigma, United States). The starch content was determined by spectrophotometry after completing the reaction of an aqueous solution of polysaccharide with iodine in the presence of potassium iodide.

Evaluation of the pro-/antioxidant status of leaves. The activity of lipid peroxidation was assessed by the content of thiobarbituric acid reactive substances (TBARS) [18]. The amount of TBARS was calculated on the basis of the extinction coefficient after subtracting nonspecific absorbance at 600 nm. The H_2O_2 content was determined using xylenol orange [19]. The specificity was tested by the inhibition of H_2O_2 formation upon the addition of catalase (Sigma, United States). The content of superoxide anion radical ($O_2^{\bullet-}$) was estimated from its ability to reduce nitroblue tetrazolium (NBT) [20]. To test the specificity of $O_2^{\bullet-}$ generation, the samples were supplemented with a commercial preparation of superoxide dismutase (Sigma, United States) that inhibits superoxide generation by 80–90%.

The activity of superoxide dismutase (SOD) was assayed from the ability of the enzyme to suppress the photochemical reduction of NBT [21]. The activity of guaiacol peroxidase (GPX) was determined by a method based on the oxidative conversion of guaiacol to the colored tetraguaiacol compound [22]. The content of soluble protein was assayed according to Bradford [23] using BSA as a standard. Protein isolation procedures were carried out at 4°C.

Anatomical and morphological parameters were determined on cross sections from the middle part of the blade of functionally mature freshly harvested leaves (ten leaves, one leaf per plant). Sections were immersed in glycerol and examined under an Axiovert 200 M microscope (Carl Zeiss, Germany). Morphometric parameters of tissues and cells were assessed by image analysis using the Carl Zeiss Vision program (Carl Zeiss, Germany). The specific leaf weight (SLW)—dry weight per unit leaf surface area—was determined with leaf blades sampled from 30 plants in each habitat.

Statistical data processing. Statistical data processing was performed using a Statistica 10 program (StatSoft Inc., United States). Significant differences between the mean values of the measured parameters were revealed using a one-way analysis of variance, ANOVA (Duncan's test). The accordance of data distribution to normality was assessed using the Shapiro-Wilks test. Calculations were performed at a given significance level of $P \leq 0.05$. Tables and figures show mean values and their standard errors.

RESULTS

Anatomical and Morphological Characteristics of Leaves

At the time of research (the first ten days of July), *P. media* plants had a well-formed rosette with five to eight leaves and were in the flowering phase. The plants growing on an open well-lit slope had thicker leaf blades. The thickness of palisade mesophyll in leaves of sun plants was 112 μm on average, which is one-quarter thicker than in shade plants (Table 1). The leaves of plants growing in dense herbage were

Table 1. Morpho-physiological parameters of *Plantago media* leaves in habitats with high (sun plants) and low (shade plants) light

Index	Sun plants	Shade plants
Number of palisade mesophyll layers	2.5 ± 0.1^{a}	2.1 ± 0.1^{b}
Height of palisade mesophyll cells, μm	53.9 ± 1.6^{a}	45.2 ± 2.0^{b}
Width of palisade mesophyll cells, μm	29.2 ± 1.1^{a}	34.1 ± 1.3^{b}
Thickness of palisade mesophyll, μm	112 ± 5^{a}	83 ± 3^{b}
Leaf thickness, μm	258 ± 3^{a}	238 ± 3^{b}
Specific leaf weight (SLW), g/dm^2	0.81 ± 0.02^{a}	0.51 ± 0.02^{b}

Different superscript symbols indicate statistically significant differences between the sun and shade plants (ANOVA, Duncan's test, $P \le 0.05$).

Table 2. Diurnal dynamics of soluble carbohydrates and starch in leaves of *Plantago media* growing in habitats with high (sun plants) and low (shade plants) light

Time of the day, h	Monosaccharides, mg/g	Disaccharides, mg/g	Glucose, mg/g	Starch, mg/g	Starch/glucose ratio
Sun plants					
6:00	85.7 ± 2.0^{b}*	7.0 ± 2.3^{a}	83.5 ± 4.5^{b}*	27.5 ± 2.6^{a}*	0.33
14:00	95.1 ± 3.8^{c}*	9.2 ± 0.3^{a}	93.5 ± 4.9^{c}*	35.2 ± 5.2^{a}*	0.38
18:00	104.7 ± 2.8^{a}*	8.4 ± 0.6^{a}	103.8 ± 3.7^{a}*	24.1 ± 6.0^{a}*	0.23
23:00	104.0 ± 1.2^{a}*	6.5 ± 0.4^{a}*	103.7 ± 2.2^{a}*	26.5 ± 5.7^{a}	0.26
Shade plants					
6:00	119.9 ± 4.3^{a}	7.1 ± 0.7^{a}	116.6 ± 9.6^{a}	14.1 ± 0.6^{a}	0.12
14:00	111.5 ± 2.3^{a}	7.8 ± 0.8^{a}	108.8 ± 3.6^{a}	12.8 ± 3.2^{a}	0.12
18:00	172.6 ± 9.5^{c}	10.4 ± 0.6^{b}	166.9 ± 21.7^{c}	50.8 ± 7.6^{c}	0.31
23:00	138.7 ± 1.2^{b}	7.9 ± 0.1^{a}	133.8 ± 1.0^{b}	21.3 ± 3.1^{b}	0.16

Different superscript symbols denote statistically significant differences in the diurnal cycle; asterisks indicate significant differences between the sun and shade plants (ANOVA, Duncan's test, $P \le 0.05$).

thinner and characterized by a lower specific leaf weight (SLW).

Content of Soluble Carbohydrates and Starch

In the composition of soluble carbohydrates in leaves of *P. media,* monosaccharides (glucose, fructose) and disaccharides (sucrose and maltose) were identified. Monosaccharides accounted for 90–95% of the soluble carbohydrate pool (Table 2). Glucose dominated among monosaccharides, accounting for approximately 95% of their total content. The content of glucose in leaves of shade plants was higher than in leaves of sun plants. The amount of glucose in plant leaves from both habitats increased by the evening. The concentration of starch in leaves of sun plants did not change significantly during the day and was 30 mg/g DW average. In the first half of the day, the starch content in leaves of shade plants was two times lower than in leaves of sun plants. It increased by more than 3.5 times by the evening and reached 5% of the leaf dry weight but decreased rapidly during the night. The starch to glucose ratio in leaves of sun plants was significantly higher than in leaves of shade plants, especially in the morning and night hours.

Pro-/Antioxidant Status of Leaves

The content of H_2O_2 and $O_2^{\bullet-}$ in leaves of sun plants was 15–30% higher on average than in shade plant leaves (Fig. 2). These differences in ROS content became already noticeable in the morning hours and persisted until the night. The highest accumulation of ROS was observed in the midday hours. The content of lipid peroxidation products changed during the day in a similar manner. The leaves of sun plants accumulated 10% more TBARS on average than the leaves of shade plants.

The levels of SOD and GPX activity were significantly (by 15–20% on average) higher in sun plants than in shade plants (Fig. 3). The maximum activity of antioxidant enzymes in sun plants was observed in the daytime. In leaves of shade plants, the diurnal dynamics of SOD and GPX activities was weakly pronounced.

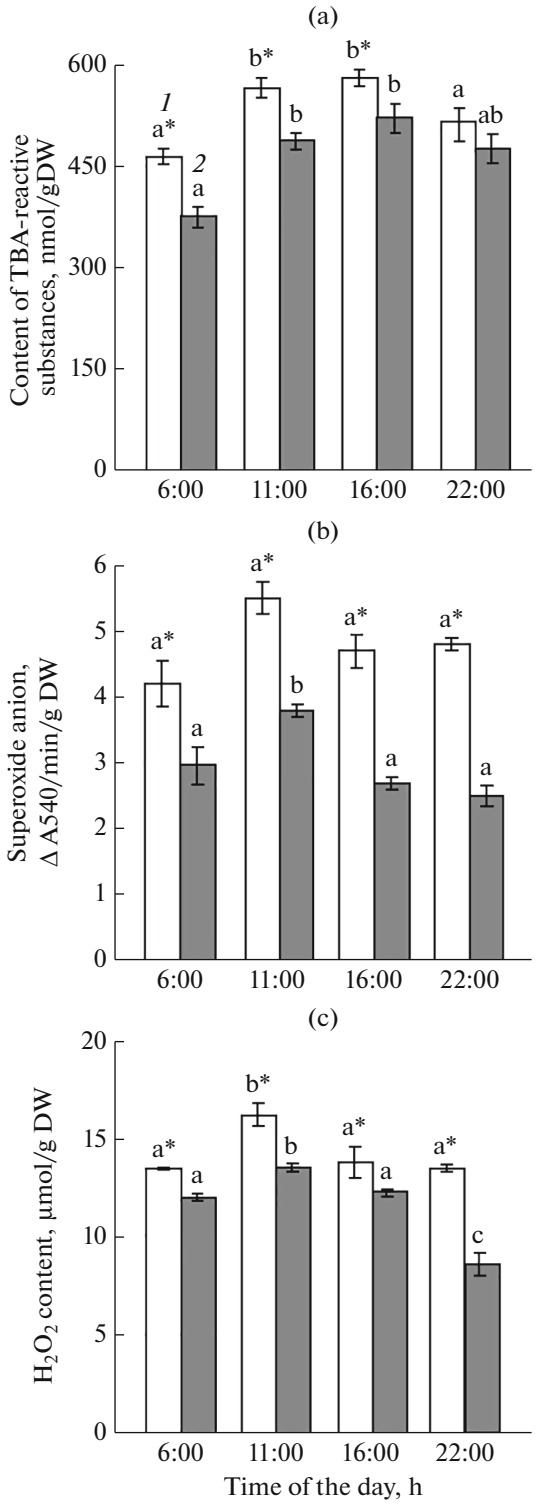


Fig. 2. Diurnal dynamics in the content of (a) lipid peroxidation products, (b) superoxide anion radical, and (c) H_2O_2 in *Plantago media* leaves. (*1*) sun plants; (*2*) shade plants. Letters indicate statistically significant differences throughout the day; asterisks indicate significant differences between the sun and shade plants at different times of the day (ANOVA, Duncan's test, $n = 3$, $P \le 0.05$).

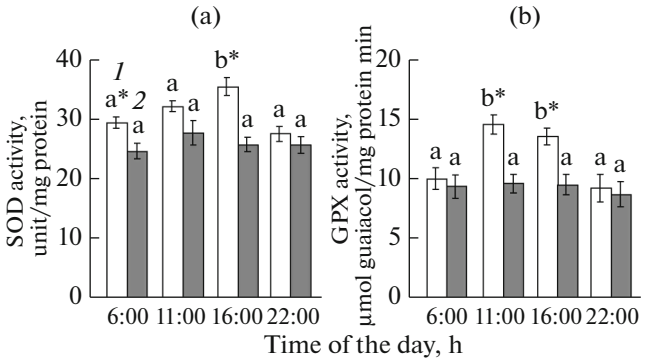


Fig. 3. Daily dynamics of activity of (a) superoxide dismutase and (b) guaiacol peroxidase in leaves of *Plantago media*. (*1*) sun plants; (*2*) shade plants. Letters indicate statistically significant activity changes during the day; asterisks indicate significant differences between the sun and shade plants at different times of the day (ANOVA, Duncan's test, $n = 3$, $P \le 0.05$).

CO_2 Exchange and Leaf Transpiration

The field assays of CO_2 exchange in *P. media* leaves allowed us to compare the plants from different habitats in terms of CO_2 assimilation and transpiration activities (Table 3). The highest rate of net photosynthesis (Pn) in sun plant leaves was observed in early morning hours at moderate light intensity and temperature. The Pn rate decreased threefold at noon, despite the increase in photon flux density. The low Pn rate also persisted in the afternoon. In the evening hours, the Pn rate declined further, along with the decrease in natural light. Following sunset (after 20:00 solar time), the apparent CO_2 uptake stopped completely. The rate of Pn in 1eaves of shade plants in morning hours was three to four times lower than in sun plants, remained stable in the first half of the day, and decreased later on along with the attenuation in irradiance. The transition from CO_2 uptake to CO_2 release was observed 1–1.5 h earlier in shade plants than in sun plants because the lighting reduced faster under the grass canopy than on an open slope.

In the daytime, the leaf transpiration was 2.5 times larger in sun plants than in shade plants; these differences were smoothed out when the evaporation rate decreased by night. The highest evaporation rates in both habitats were noted around noon (Table 3) when the solar irradiance and temperature were high and relative humidity was low (Fig. 1). In early morning hours, the stomatal conductance (g_s) for water vapor was 0.2 and 0.3 mol H_2O/m^2 s in shade- and sun plants, respectively; it decreased substantially throughout the daylight hours. When the apparent uptake of CO_2 ceased after sunset, the conductance g_s tended to increase. The leaves of sun- and shade plants differed in photosynthetic water use efficiency (WUE). In early morning hours, the ratio of net photosynthesis and evaporation rate, i.e., WUE, was 45% higher in leaves

Table 3. Diurnal dynamics of CO_2 net uptake (Pn), transpiration (E), stomatal conductance (g_s), and water use efficiency (WUE) in *Plantago media* leaves from habitats with high (sun plants) and low (shade plants) light

Time of the day, h	Pn, μmol CO_2/m^2 s	E, mmol H_2O/m^2 s	g_s, mol H_2O/m^2 s	WUE, mmol CO_2/mol H_2O
		Sun plants		
4–6	$9.23 \pm 0.36^{c*}$	$4.34 \pm 0.23^{b*}$	$0.30 \pm 0.01^{c*}$	$2.92 \pm 0.20^{a*}$
10–12	$3.11 \pm 0.34^{a*}$	$6.55 \pm 0.27^{c*}$	$0.15 \pm 0.01^{b*}$	0.70 ± 0.10^{b}
15–17	$2.60 \pm 0.30^{a*}$	$1.02 \pm 0.04^{a*}$	$0.07 \pm 0.003^{a*}$	2.53 ± 0.30^{a}
20–22	$-1.45 \pm 0.21^{b*}$	$0.60 \pm 0.07^{a*}$	$0.09 \pm 0.01^{a*}$	–
		Shade plants		
4–6	2.62 ± 0.27^{a}	1.67 ± 0.08^{b}	0.21 ± 0.02^{c}	1.57 ± 0.3^{a}
10–12	2.22 ± 0.22^{a}	2.55 ± 0.18^{c}	0.08 ± 0.01^{b}	0.93 ± 0.5^{a}
15–17	1.13 ± 0.35^{c}	0.37 ± 0.02^{a}	0.03 ± 0.002^{a}	3.39 ± 1.3^{b}
20–22	-1.2 ± 0.19^{b}	0.45 ± 0.06^{a}	0.07 ± 0.004^{ab}	–

Different superscript symbols denote statistically significant differences in the diurnal cycle; asterisks indicate significant differences between the sun and shade plants (ANOVA, Duncan's test, $P \leq 0.05$).

of sun plants than in shade plants. These distinctions primarily reflected the different photosynthetic activities of sun and shade leaves rather than variations in transpiration rates. In the midday, when net photosynthesis became depressed and evaporation increased, the WUE value decreased fourfold in sun plant leaves and by a factor of 1.7 in leaves of shade plants that had more stable photosynthesis. In the afternoon, a 3.6-fold increase in WUE was noted in both plant types, which was largely due to the decrease in transpiration rate rather than to changes in Pn.

Chlorophyll Fluorescence Parameters

Field measurements of maximum photochemical efficiency of PSII in leaves of sun- and shade *P. media* plants showed that the F_v/F_m ratiowas nearly 0.8 in early morning hours (Fig. 4). During the day, the F_v/F_m values decreased by 10–25% and equaled 0.62 and 0.71 in the sun and shade plants, respectively. After sunset, the F_v/F_m value increased to 0.8.

In the daytime, at high light and air temperature, a significant part of the light energy absorbed by sun plant leaves was dissipated in the form of thermal radiation. The parameter of nonphotochemical quenching (NPQ) was as high as 2.3–2.6. In this case, the effective quantum yield (Φ_{PSII}) did not exceed 0.22. In shade plant leaves where PPFD was five to ten times lower, the Φ_{PSII} was threetimes higher and NPQ was five times smaller than the respective parameters in sun plant leaves. The electron transport rate (ETR) through PSII in sun plants in the daytime at full irradiance was threetimes higher than in shade plants and amounted to 80–100 μmol/m^2 s.

Respiration and Involvement of Energetically Inefficient Alternative Pathway

The respiration in shade leaves was more intense than in sun leaves, especially in the first half of the day (Table 4). By the end of the day, the difference in O_2 uptake rates in plant leaves grown under different conditions reduced noticeably but remained statistically significant. The decrease in leaf respiration was more strongly pronounced in shade plants. The rate of O_2 consumption by shade plant leaves at night was twofold lower than in the morning.

The rate of respiration through the cytochrome pathway (V_{cyt}) in leaves of sun plants during daytime was 1.5 times on average lower than in the morning and evening; the contribution of the cytochrome pathway (CP) to total respiration in daytime did not exceed 25% (Fig. 5). The diurnal changes in the rate of alternative respiration (V_{alt}) were characterized by the opposite dynamics. The V_{alt} value increased in daytime, so that the contribution of the alternative pathway (AP) to total respiration reached 60%. In the shade plant leaves, the dynamics of V_{cyt} was similar to the pattern of total respiration V_t. The contribution of the cytochrome pathway to the total O_2 uptake was near 45%. The rates of V_{alt} changed in a similar manner, and the contribution of the alternative pathway to total respiration was 35% on average. Irrespective of the plant growing conditions, the contribution of residual respiration (V_{res}) was 15–17% of the total O_2 uptakeon average.

Despite significant changes in respiration rates, the V_{cyt}/V_{alt} ratio in leaves of shade plants remained almost constant in the diurnal cycle and amounted to 1.3

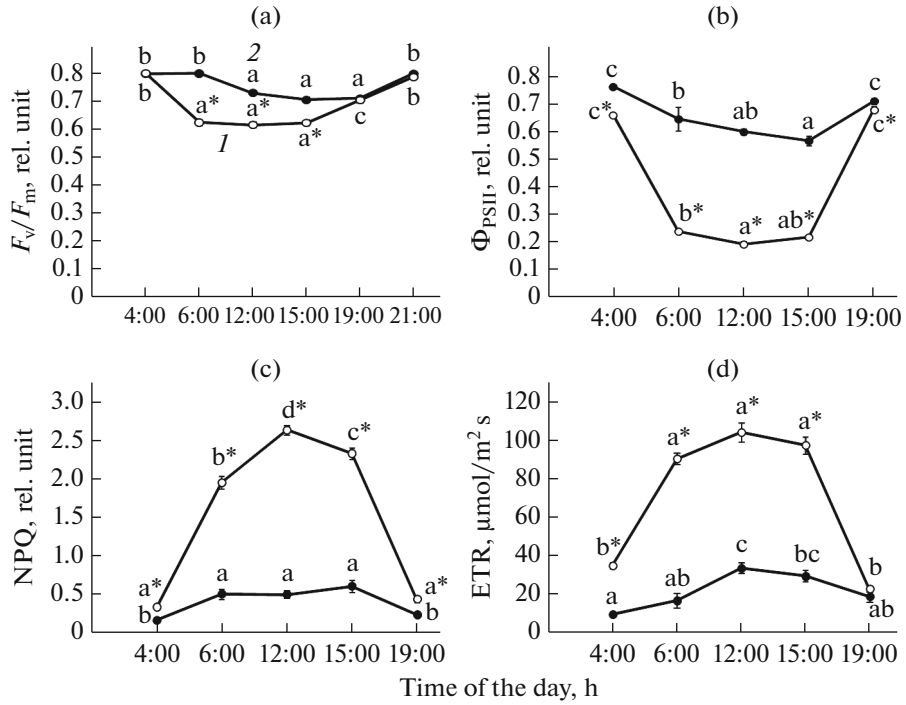


Fig. 4. Diurnal dynamics of (a) maximum quantum yield, (b) effective quantum yield of PSII, (c) nonphotochemical quenching, and (d) electron transport rate through PSII in *Plantago media* leaves. (*1*) sun plants; (*2*) shade plants. Different letters indicate statistically significant differences in the analyzed parameters during the day; asterisks indicate statistically significant differences in parameters between the sun and shade plants at different times of the day (ANOVA, Duncan's test, $n = 30–50$, $P \le 0.05$).

(Table 4). In sun plants, the cytochrome respiration was suppressed in the daytime, while the alternative respiration increased; therefore, the V_{cyt}/V_{alt} ratio decreased to 0.4 during the day and became 2.5 times lower than in the morning and evening hours.

DISCUSSION

We examined microclimatic conditions in habitats (Fig. 1) as well as the functional parameters of energy and carbon metabolism in *P. media* plants that were long-time adapted to different light levels. Under

Table 4. Diurnal dynamics of total (V_t), cytochrome (V_{cyt}), alternative (V_{alt}), and residual respiration (V_{res}) of *Plantago media* leaves from habitats with high (sun plants) and low (shade plants) light, nmol O_2/g DW min

Parameter	Time of the day, h			
	6:00	10:00	14:00	22:00
	Sun plants			
V_t	$1399 \pm 126^{b*}$	$1483 \pm 95^{b*}$	$1116 \pm 33^{a*}$	$1035 \pm 36^{a*}$
V_{cyt}	$606 \pm 111^{b*}$	$364 \pm 71^{a*}$	$276 \pm 27^{a*}$	$421 \pm 41^{ab*}$
V_{alt}	$582 \pm 51^{ab*}$	865 ± 49^{c}	$704 \pm 32^{b*}$	445 ± 79^{a}
V_{res}	$211 \pm 42^{bc*}$	$254 \pm 9^{c*}$	$135 \pm 8^{a*}$	169 ± 22^{ab}
V_{cyt}/V_{alt}	1.1 ± 0.3^{b}	$0.4 \pm 0.1^{a*}$	$0.4 \pm 0.1^{a*}$	1.1 ± 0.3^{b}
	Shade plants			
V_t	2413 ± 190^{b}	2533 ± 148^{b}	1534 ± 62^{a}	1275 ± 49^{a}
V_{cyt}	1137 ± 192^{b}	1159 ± 123^{b}	689 ± 44^{a}	594 ± 36^{a}
V_{alt}	857 ± 78^{b}	887 ± 96^{b}	536 ± 13^{a}	471 ± 30^{a}
V_{res}	419 ± 47^{bc}	486 ± 57^{c}	309 ± 17^{ab}	209 ± 20^{a}
V_{cyt}/V_{alt}	1.4 ± 0.4^{a}	1.4 ± 0.3^{a}	1.3 ± 0.1^{a}	1.3 ± 0.1^{a}

Different superscript symbols denote statistically significant differences in the diurnal cycle; asterisks indicate significant differences between the sun and shade plants (ANOVA, Duncan's test, $P \le 0.05$).

direct solar radiation of the open slope of southeastern exposure, the day air temperature rose to higher levels and relative humidity was noticeably lower than under the grass canopy, in the habitat of shade plants.

The light intensity close to leaves of sun plants was an order of magnitude higher than in shade plants; these plants differed in the diurnal dynamics of photosynthesis (Table 3). The highest values of net CO_2 uptake in sun plant leaves were noted in the morning at moderate lighting, temperature, and comparatively high relative humidity. A substantial suppression of Pn in the daytime at high photon flux density and elevated temperature was due to the decrease in stomatal conductance, which is confirmed by a statistically significant positive relationship between the above parameters ($r = 0.68$, $P < 0.01$). Apparently, the leaves of sun plants could not fully utilize available light energy for the photosynthesis because of the need to save water. Under these conditions, the closing of stomata prevents water losses during transpiration and restricts the CO_2 diffusion into mesophyll cells. We suppose that the temporary rise in transpiration around noon (10:00–12:00 solar time) was caused by elevated lighting and leaf temperature. Analysis of multiple diurnal patterns showed that the dependence of evaporation rate E on the stomatal conductance g_s was weak but statistically significant ($r = 0.32$, $P < 0.05$).

The leaves of shade plants actively photosynthesized before the noon; however, their Pn rate was significantly lower than that of sun plants, apparently due to insufficiency of available PAR. Analyses showed that the dependence of Pn on g_s in the leaves of shade plants was rather weak ($r = 0.29$, $P < 0.01$). At the same time, there was a correlation between E and g_s ($r = 0.46$, $P < 0.05$).

On the whole, field studies showed that the stomatal conductance of *P. media* leaves decreased during the daylight hours and was 1.5–2 times higher in sun plants than in shade plants. Changes in the rate of apparent photosynthesis and transpiration correlated to some extent with g_s. The values of g_s, E, and Pn found in this study and the ranges of their changes under the influence of environmental factors are consistent with the literature data for *Plantago* species [24, 25]. For example, the Pn rate of *P. major* plants was shown to decline from 8 to 1.5 μmol CO_2/m^2 s upon the decrease in soil water potential from –0.05 to –0.2 MPa [24]. Under normal water supply, the maximum Pn rate was observed at nearly 20°C. An increase in temperature to 35°C led to a significant suppression of photosynthesis, from 8 to 2 μmol CO_2/m^2 s. In experiments with *P. depressa* plants grown under controlled conditions, the g_s, E, and Pn values for leaves varied, depending on water supply, in the ranges of 0.13–0.19 mol H_2O/m^2 s, 2.7–4.1 mmol H_2O/m^2 s, and 4.1–7.4 μmol CO_2/m^2 s, respectively [25].

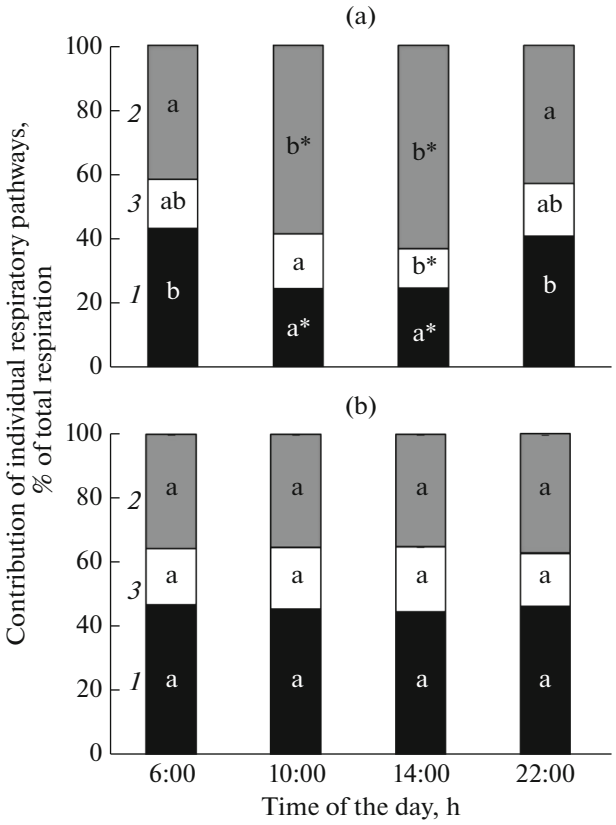


Fig. 5. Diurnal dynamics for the relative contributions of (*1*) cytochrome respiration, (*2*) alternative respiration, and (*3*) residual respiration to the total O_2 uptake in leaves of (a) sun plants and (b) shade plants of *Plantago media*. Different letters indicate statistically significant changes of the analyzed parameter during the day; asterisks indicate significant differences between the sun and shade plants at different times of the day (ANOVA, Duncan's test, $n = 4–6$, $P \le 0.05$).

The midday reduction in the potential and actual photochemical efficiencies of PSII in sun plants, paralleled by the suppression of CO_2 assimilation, suggests that elevated lighting combined with supraoptimal temperature exert a stressful effect on PSA [4, 26]. The diurnal changes of these parameters were much less pronounced in shaded plants. The effects of excessive insolation on photochemical efficiency of PSA (Fig. 4) and CO_2 assimilation (Table 3) were reversible and did not cause chronic photoinhibition in sun plant leaves, thus indicating the presence of an effective system for the protection and repair of PSII protein structures [27, 28].

Nonphotochemical quenching of excitation energy is one of the main mechanisms of plant protection against the overreduction of the photosynthetic ETC and excessive ROS generation [29, 30]. According to our data, the NPQ values were four to five times higher in sun plant leaves than in shade plants. There are several components in the development of NPQ,

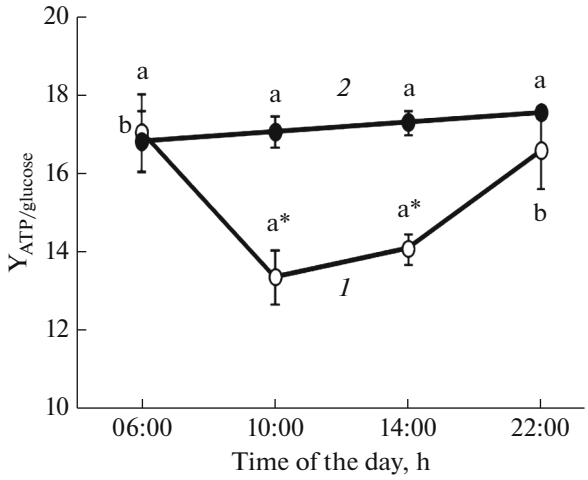


Fig. 6. Energy efficiency of respiration (Y, mol ATP/mol glucose) in (*1*) sun plant leaves and (*2*) shade plant leaves of *Plantago media*. Different letters indicate statistically significant changes of index Y during the day; asterisks indicate significant differences between the sun and shade plants at different times of the day (ANOVA, Duncan's test, $n = 4–6$, $P \le 0.05$).

the most significant component reflecting the involvement of the violaxanthin cycle (VXC) [31]. The deepoxidation state (DEPS) of the cycle pigments serve as an indicator of VXC activity. We previously showed that DEPS values in leaves of sun plants reached 70% at noon, while this index was 1.5 times lower in shade plants [32]. This implies the role of thermal dissipation of absorbed light energy in preventing the development of photooxidative stress and PSA destruction in sun plants exposed to high PAR and thermal radiation. The use of PAR energy during photosynthetic CO_2 fixation is associated with linear electron transport in the chloroplast ETC, which produces NADPH and ATP. A significant suppression of net CO_2 assimilation in the sun leaves of *P. media* plants during the daytime (Table 3) restricts the consumption of ATP and the reducing power in the Calvin–Benson cycle, which, according to the feedback principle, can also affect the efficiency of light energy use in primary photosynthetic processes. The data in Fig. 4b indicate the low photochemical efficiency of PSII in the daytime.

The comparison of net CO_2 uptake rate (Table 3) and the rate of electron transport through PSII (Fig. 4d) shows that a threefold increase in ETR in sun plants at 12:00 solar time (compared to early morning) is accompanied by a decrease in Pn over the same period by 65%. Our data suggest that the ratio of linear (coupled with CO_2 fixation) electron transport and electron flows to alternative acceptors shifted in sun plants in favor of alternative pathways (cyclic electron transport in PSII, pseudocyclic electron transport, photorespiration). A similar, although less pronounced trend was noted for shade plants.

The comparison of gas exchange parameters, chlorophyll fluorescence, and carbohydrate status of leaves suggests a possible biochemical limitation of photosynthesis. An increase in the starch to glucose ratio is known to serve as a signal for the suppression of photosynthesis [33]. According to our data (Table 2), this ratio was two to three times higher in sun plants than in shade plants. The increased accumulation of starch in leaves of sun plants is likely to result from the decrease in carbohydrates export under the influence of external conditions (high insolation combined with elevated temperature and moisture deficiency).

We found that the leaves of shade plants were characterized by a higher respiratory activity and a higher V_{cyt}/V_{alt} ratio, especially in the daytime. The contribution of the alternative pathway to respiration of shade plant leaves did not exceed 35%, which is typical for plants under normal conditions, whereas this contribution in sun plants was as high as 65% (Fig. 5). The energetically low-efficient AP alleviates the overreduction of the ubiquinone pool when the CP is saturated, thus regulating the balance between carbohydrate metabolism and electron transport. In strong light, the AP facilitates the unloading of chloroplast ETC by oxidizing the reducing equivalents delivered from chloroplasts to mitochondria under conditions of limited CO_2 assimilation [9, 10, 12, 34, 35]. It is reasonable to assume that the AP-mediated respiration during daytime depression of net CO_2 uptake in sun leaves of *P. media* plants optimizes the chloroplast energy pools (ATP, NADPH), improves the carbon balance in photosynthesizing cells, and reduces the threat of excessive ROS generation.

However, the involvement of AP diminishes the energy efficiency of respiration (EER), because the AOX-mediated electron flow bypasses the two energy-coupling sites where the generation of membrane potential and ATP synthesis take place [10]. According to the calculations of Amthor [17], the ATP yield during complete oxidation of 1 mole of glucose ($Y_{ATP/glucose}$) in respiration via the main (cytochrome) pathway is 29 moles (rather than 36 moles supposed previously). By contrast, the respiration through the alternative pathway generates 11 moles ATP. We used this information to estimate the EER of *P. media* leaves. After taking into account the contribution of AP in respiration, the $Y_{ATP/glucose}$ ratio for shade plant leaves was approximately 18 and did not change during the day (Fig. 6). The $Y_{ATP/glucose}$ ratio for sun plant leaves early in the morning and late in the evening was close to that for shade plants, whereas it was 25% lower during the daytime. The calculated absolute values should be regarded as approximate, already because the total O_2 uptake assayed routinely at 20°C comprised the residual component unrelated to mitochondrial respiration. Nevertheless, the obtained values

reflect the actual differences in EER for sun and shade plants. Apparently, the decrease in leaf EER is not critical for viability of *P. media* sun plants adapted to incidence of excess light. This is consistent with the opinion of Semikhatova [36] that the evolutionary changes in bioenergetics of living organisms aim at achieving the maximum energy efficiency, while temporary reductions in energy efficiency are needed to maintain homeostasis under the influence of adverse environmental factors.

It should be pointed out that the decrease in leaf EER of sun plants during the daytime (Fig. 6) occurred concurrently with the increase in NPQ (Fig. 4). This finding indicates synchronous changes in thermal energy dissipation occurring in chloroplasts and mitochondria. The interrelations between photosynthesis and respiration were repeatedly discussed by Semikhatova [36, 37]. Considering close interactions between photosynthesis and respiration, she emphasized that both processes are capable of supplying ATP, NAD(P)H, and metabolites required for plant cells. From the standpoint of current knowledge, the meaning of the above statement can be broadened by the notion that respiration is the process that protects PSA and provides favorable conditions for the functioning of photosynthesis.

The chloroplast and mitochondrial ETCs are the main source of ROS in phototrophic cells. Compared to shade plants, the leaves of sun plants contained higher levels of $O_2^{\bullet-}$ and H_2O_2. Under conditions of high irradiance and limited CO_2 assimilation, NAD(P)H recycling in the Calvin-Benson cycle decreases, which leads to overreduction of photosynthetic ETC and enhances electron leakage to O_2 with the formation of $O_2^{\bullet-}$ in the Mehler reaction. The superoxide anion radical dismutates into H_2O_2 either spontaneously or by means of SOD [11]. High concentrations of $O_2^{\bullet-}$ and H_2O_2 can inhibit the enzymes of Calvin–Benson cycle in chloroplasts and the Krebs cycle enzymes in mitochondria; they can also give rise to a powerful oxidizing agent, the hydroxyl radical ($HO^{\bullet}$) [11, 38]. The main target of $HO^{\bullet}$ in the cell is polyunsaturated fatty acids. Therefore, it is not surprising that the leaves of sun plants were characterized by higher rates of lipid peroxidation compared to shade plants leaves. On the other hand, ROS and lipid peroxidation products are important components of cell signaling and can modulate the transcriptional responses at the cell and tissue levels to mobilize protective mechanisms and increase plant resistance [38, 39]. Plant cells possess the antioxidant system to maintain ROS at the level that ensures efficient retrograde signaling. In our studies, the activity dynamics of antioxidant enzymes (SOD and GPX) that control the accumulation of $O_2^{\bullet-}$ and H_2O_2 coincided with the diurnal time course of ROS accumulation and was higher in the leaves of sun plants than in the shade plant leaves.

Thus, the long-term adaptation of *P. media* to different ecological and cenotic conditions results in the formation of phenotypes with definite physiological and biochemical properties serving to maintain the energy and cell redox balance in photosynthesizing tissues and organs. The plant PSA is well integrated into intracellular processes; it responds adequately to changes in environmental factors and is reliably protected from photooxidative stress. Synchronous activation of energy dissipation mechanisms in chloroplasts and mitochondria reduces the energy use efficiency, prevents the excessive generation of active oxygen radicals in the ETC, and counteracts the development of photooxidative stress. Our results show that a temporary decrease in the energy efficiency of respiration is not critical for plants and contributes to the increase in functional plasticity and stability.

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies with human participants or animals performed by any of the authors.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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