The Effect of Elevated Temperature on Salt Tolerance Mechanism in C$_4$ Xero-Halophyte *Kochia prostrata*

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Abstract—The study of effect of elevated temperature on the mechanisms of salt tolerance in plants are of great interest and perspective under global climate change. This study investigated the individual and combined effects of prolonged heat and four days moderate salinity on morphophysiological and biochemical parameters (biomass, photosystems I and II (F_v/F_m) efficiencies, apparent photosynthesis intensity, transpiration, dark respiration, water-use efficiency, contents of water, free proline, Na$^+$ and K$^+$ in aboveground parts of plants) in the C$_4$ xero-halophyte *Kochia prostrata*. The physiological processes and biochemical parameters actively involved during acclimation to stress under different treatments (control, heat, salinity, heat + salinity) were identified. A decrease in biomass and change in the K$^+$/Na$^+$ ratio was observed under all treatments. Acclimation to heat resulted in increased dark respiration intensity (Rd) and K$^+$ content. Under salinity conditions, an increase in Na$^+$ content, a decrease in PSI efficiency and transpiration intensity were observed. Combined stress (heat + salinity) resulted in increased proline and Na$^+$ contents in addition to high values of Rd and K$^+$. Principal component analysis showed that under combined stress, dark respiration, K$^+$ and proline are actively involved in acclimation. It was found that acclimation to elevated temperature affects the salt tolerance mechanisms in *K. prostrata,* since under combined stress, sodium ions accumulated 3-fold less than in plants under normal temperature and salinity. It is assumed that in *K. prostrata* plants grown at elevated temperature, K$^+$ and proline are more involved in the acclimation to salinity than Na$^+$. Dark respiration is likely the source of additional energy costs. We conclude that acclimation of C$_4$ halophytes to elevated temperature changes the importance of sodium and potassium ions, as well as proline, in the mechanisms of salt tolerance.

Keywords: *Kochia prostrata*, PSI, PSII, gas exchange, ions, proline, salinity stress, heat, combined heat and salinity stress

INTRODUCTION

Global climate changes and an increase in greenhouse gas concentrations lead to an increase in temperature [1, 2]. Elevated temperature affects many biological processes in plants, including morphogenesis, fluidity and composition of membrane lipids [3]; stimulates the enzymes' activity, primarily respiratory enzymes [1], inhibits growth and total productivity of plants [4]. According to many studies, photosynthesis is very sensitive to high temperature, and photosystem II (PSII) is considered as the most thermosensitive component of the photosynthetic apparatus [5]. High temperatures can damage various components of PSII, including the reaction center, oxygen-evolving and light-harvesting complexes [6]. Although there are other points of view about the relative thermal lability of the maximum quantum yield of PSII [7, 8].

The long-term effects of increased temperatures and decreased precipitation for 30 years showed a positive correlation with increased soil salinity in arid regions [9]. Halophytes are among the most successful plants growing in saline desert regions. Halophytes can survive and complete their life cycle at ≥ 200 mM NaCl [10, 11]. These plants have developed various strategies to live in high salt environments. Additionally, certain salinity levels have a beneficial effect on the vegetative growth of halophytes while inhibiting the growth of non-halophytes [11]. Therefore, it is important to understand the tolerance mechanisms of halophytes that act at different levels: molecular, phys-

iological and ecological. The long-term goal of such research is to use knowledge of halophytes to improve crop yields on saline soils. However, halophytes are not only valuable as scientific models but also have potential as crops in saline agriculture [12]; they can be used for food, fibre and industrial purposes, as well as green fertilisers and forage crops [10].

Halophytes use several strategies to survive under saline desert conditions [10]. The main constitutive tolerance mechanisms in halophytes are regulating the compartmentalisation of toxic Na^+, Cl^- ions in the vacuole and increase in the concentration of compatible osmolytes in the cytoplasm [10, 13]. Simultaneously, ions are an important factor in osmoregulation in halophyte cells; the plant strives not only to exclude Na^+ from the cytoplasm, but also to retain K^+ there, and to maintain ion homeostasis. The ratio of K^+/Na^+ in the cytoplasm is an important indicator of tolerance to salinity and is used for selective breeding for salt tolerance [14]. A feature of halophytes is that Na^+ can be used as a 'cheap osmolyte' when plants adapt to the low water potential of a saline environment [15]. Low-molecular organic solutes, the so-called compatible osmolytes, also play an important role in the salt tolerance mechanisms in halophytes [16]. They represent a broad group of compounds, such as amino acids, tertiary sulfonium and quaternary ammonium compounds, sugars and polyhydric alcohols. Compatible osmolytes accumulate in the cytoplasm to balance the osmotic potential of Na^+ and Cl^- accumulated in the vacuole. The advantages of compatible osmolytes are that they keep the basic physiological functions of the cell active; environmental signals control the induction of their biosynthesis, and they can be synthesised at all ontogenesis stage. Additionally, compatible osmolytes have important functions in protecting subcellular structures from oxidative stress [16]. Proline is one of the well-studied osmolytes. It has been shown that free proline increases the tolerance of various plant species to salt stress [17], positively affects growth parameters, photosynthesis, gas exchange and productivity under salinity [18]. These positive effects are mainly due to better nutrient acquisition, water uptake and biological nitrogen fixation. Proline also improves antioxidant activities and reduces Na^+ and Cl^- uptake and translocation while enhances K^+ assimilation by plants [19].

Some plants with C_4 type of photosynthesis are well adapted to arid habitats, i.e. to conditions of drought and high temperatures. There are many halophytes among C_4 species [20]. Under salinity, plants are exposed to ionic and osmotic stress due to low water potential values of saline soil. C_4 plants are much more efficient at carbon sequestration, water use and have higher rates of net photosynthesis, growth and dry biomass production than C_3 plants. However, the physiological response of C_4 plants to elevated temperatures

differs from that of C_3 species. The ambiguous and contradictory pattern of biochemical limitations is explained by the complexity of the biochemical model of C_4 photosynthesis in comparison with the C_3 type [21]. C_4 photosynthesis is an adaptation of the C_3 pathway to low CO_2 concentration, which is due to the presence of a carbon-concentrating mechanism (CCM), provides a decrease in the oxygenase function of Rubisco and photorespiration limitation, contributing to increased photosynthesis efficiency and decreased water loss in hot and dry environments [22]. According to our data and the opinion of some other researchers, the cyclic transport of electrons around PSI in C_4 plants also plays an important role in acclimation to arid climate and salinity [23, 24]. According to others, Rubisco inactivation was the primary constraint on the rate of photosynthesis in C_4 plants leaves as leaf temperature increased above $30°C$ [7]. At the same time, there is an opposite point of view: at high temperatures neither Rubisco content nor Rubisco activase activity limit C_4 photosynthesis [21, 25].

Climate change is the complex process, which accompanied by an increase in CO_2 concentration and temperature, drought and salinity. Therefore, studying and understanding combined effects of these factors on plants is of great importance in predicting the survival and distribution of plants in the future. Particularly, in the hot period, when daytime air temperature reaches the maximum, higher concentrations of salts in the soil are observed and plants are forced to adapt to the combined effect of these two harmful factors. Contrary to the expectation that the combined effects of high temperature and salinity will be more stressful than either of these factors alone, research results indicate that their combined effects cause less stress than salinity or high temperature alone [26, 27]. This is because effects of salinity and high temperature are not additive, but opposite, in other words, the higher the salt, the higher the temperature the plants will tolerate [26]. The data show that *Salicornia utahensis* respiratory metabolism is well adapted to these conditions, that is, to lower salt concentrations at low temperatures and higher salt concentrations at higher temperatures [26]. Furthermore, at different halophytes (*Atriplex, Centralasiatica, Suaeda salsa* and others), it was found that heat tolerance of PSII increases in plants adapted to salt [27]. In C_3 *Artemisia anethifolia* similar effect may be due to an improvement in the thermotolerance of the reaction centres, oxygen-evolving and light-harvesting complexes of PSII [27]. The enhanced heat resistance in C_4 sorghum can be expressed on all the components of PSII including reaction center, donor side and acceptor side [28]. Proline plays an important protective role in plant response to environmental stress, and its large accumulation in salt-treated sorghum might be the underlying reason leading to the enhanced heat tolerance [28]. Thus, an increase in heat tolerance of plants

adapted to salinity has been shown, however, the question remains about the effect of acclimation to elevated temperatures on the salt tolerance mechanisms, in particular, on the accumulation and role of Na^+ and K^+, and proline, dark respiration intensity and the of PSII efficiency, and cyclic electron transport around PSI which supplies additional ATP molecules for the functioning of CCM in C_4 plants. The aim of this study was to investigate the effect of acclimation to elevated temperature on mechanisms of salt tolerance in C_4 xero-halophyte *Kochia prostrata*.

MATERIALS AND METHODS

Plant material and growth conditions. Seeds of *Kochia prostrata* (L.) Schrad. subsp. *prostrata* (forage kochia, *K. prostrata* subsp. *virescens* in Ledeb. 1949, pro parte, *Bassia prostrata* (L.) A.J. Scott.) collected in foothill semi-deserts (Uzbekistan) were germinated on filter paper soaked in distilled water within 2—4 days. After that, the seedlings were transplanted to perlite in plastics pots of 24-cm length, 20-cm width and 10-cm depth, 20 seedlings per pot. Each plastic pot was placed on separate plastic tray. During next 30 days the seedlings were grown used the nutrient solution 50% Hoagland, which was added to each plastic tray. The seedlings were grown in two separated climate chambers under circadian illumination (using commercial luminescent white light tubes): 10 h dark/14 h light (200 μmol/m^2 s PAR, light meter LI-205A (Li-Cor, USA)) and two levels of temperature: in first chamber 40 plants were grown at $25 \pm 2°C$ and in second chamber others 40 plants were grown at $32 \pm 2°C$ [29]. When plants were 30 days old, we started treatment with solute of 200 mM NaCl (20 plants at 25°C and 20 plants at 32°C). Experimental NaCl solution was prepared on the basis of 50% Hoagland solution. 20 plants at 25°C and 20 plants at 32°C continued to grow on 50% Hoagland in solution, and then were used as control. All solutions were added to a plastic tray. Plants were treated with NaCl during 4 days. The physiological measurements were carried out on the plants at the end of the 4th day of treatment. In general, four experimental variants were used: 1) growing at 25°C without treatment (control); 2) growing at 25°C + 4 days treated with 200 mM NaCl (salinity); 3) growing at 32°C (heat); 4) growing at 32°C + 4 days treated with 200 mM NaCl (heat + salinity). Plant tolerance to osmotic stress was assessed by the decrease in its productivity compared with the control.

Dry biomass and water content. At the end of the experiment, water content (W, g H_2O/g dry wt) was assessed for the shoots in all the groups. Biomass was estimated for fresh (fr wt) and dry shoots (dry wt). Plant samples were dried at 80°C for 2 days until reaching a constant mass in order to measure quantitatively the dry shoot matter. The water content (W) in the shoots for each treatment and control group was calculated according to the following formula:

$$W = (fr\ wt - dry\ wt)/dry\ wt.$$

Proline, Na^+ and K^+ ions contents. Free proline was determined according to Bates et al. [30] with modifications. Dry shoot samples (0.2 g) from each group were homogenized in 2 mL of boiling distilled water, heated at 100°C for 10 min in a water bath and then the homogenates were centrifuged (5 min, 14000 *g*). One mL of homogenate was reacted with 1 mL acidic ninhydrin (ninhydrin 1% (w/v) in acetic acid 60% (v/v), 6 M orthophosphoric acid 40% (v/v)) and 1 mL glacial acetic acid in a tube for 1 hour at 100°C in a water bath, and the reaction terminated in an ice bath. The mixtures were read at 520 nm using a Genesis 10 UV Scanning spectrophotometer (Thermo Fisher Scientific, USA). Proline concentrations were determined using a calibration curve and expressed as mmol/ g dry wt. Na^+ and K^+ contents in the shoots was determined in water extracts from 100 mg dry samples by atomic absorption spectrometry (Hitachi 207, Japan), and expressed as mmol/ g dry wt.

CO_2/H_2O gas exchange. The CO_2/H_2O exchange was analyzed by placing a leaf segment into a temperature-controlled leaf chamber (25°C) where the sample was illuminated (1200 μE PAR) through a fiber-optic light guide from a KL 1500LCD light source (Schott, Germany) on acclimated plants to assess the potential of the photosynthetic apparatus. The steady-state CO_2/H_2O exchange rates at the leaf—air interface were measured with a single-channel LI-820 infrared gas analyzer (LI-COR, USA) in the open-circuit mode. Apparent photosynthesis (A) was expressed as μmol (CO_2)/(m^2 s). The leaf transpiration (*E,* mmol (H_2O)/(m^2 s)) was calculated from the difference in gas humidity at the inlet and outlet from the leaf chamber. In this experimental system, the humidity of gas flow at the entrance to the leaf chamber was kept constant at a known level using a LI-610 dew point generator (LI-COR). Humidity at the exit of the leaf chamber was determined with a HMP50 psychrometric sensor (Vaisala INTERCAP, Finland). Injected gas was atmospheric air preliminarily drawn into a 60-L polyethylene gasholder. Flow rate of 100 mL/min ensured continuous operation of the installation for 8 h. Mixing unit of the gas circuit made it possible to maintain a CO_2 concentration of 380 ppm in air flow supplied to the leaf chamber. After determining the photosynthetic CO_2/H_2O exchange, the light was switched off to measure the steady-state dark respiration (Rd, μmol (CO_2)/(m^2 s)) of leaves [31]. Water use efficiency (WUE) was calculated as the ratio of apparent photosynthetic assimilation to the transpiration rate (*A/E*).

Photosystem I. The redox potential changes of chlorophyll P700 were measured by monitoring the leaf absorbance at 820 nm using a dual-wavelength ED-P700DW pulse modulated system (Walz, Ger-

many) in combination with a PAM 101 fluorometer (Walz) [32]. The kinetics of P700 oxidation was measured under illumination with far-red light (720 nm, 17.2 W/m^2). The level of maximum P700 oxidation was determined by applying the flash from a xenon gas-discharge lamp (50 ms, 1500 W/m^2; Walz) in the presence of far-red light. PSI cyclic electron transport activity was measured as the P700 oxidation kinetics in response to far-red illumination by monitoring changes in leaf absorbance [23].

Photosystem II. The quantum yield of PSII photoreaction in dark adapted (20 min) leaf was determined with a pulse-amplitude-modulated chlorophyll fluorometer (PAM 101, Walz) [33]. The ratio of variable to maximum chlorophyll *a* fluorescence (F_v/F_m) was used as a measure of the maximum quantum yield of PSII reaction. During measurements, the sample was illuminated with weak modulated red light. The output signal of PAM 101 was processed with an analog–digital converter (PDA-100, Walz) and displayed on a computer. The potential photosynthetic efficiency of dark-adapted leaves was estimated from the values of minimal (F_0) and maximal (F_m) fluorescence using an expression:

$$F_v/F_m = (F_m - F_0)/F_m.$$

Statistical analyses. For the physiological measurements 3–5 biological and 3 analytical repetitions were used, and the means and standard errors (SEs) are calculated using Sigma Plot 12.5 statistical program. The effects of the factors and their interaction were assessed by using two-way ANOVA on analysis platform SigmaPlot 12.5. Mean separation was performed using Normality Test (Shapiro-Wilk) and all Pairwise Multiple Comparison Procedures (Bonferroni *t*-test). Differences were considered significant at $P < 0.05$. Statistical software package R version 3.6.1. was used to perform a multivariate statistical approach using a principal component analysis model (PCA).

RESULTS

Effect of Heat, Salinity and Their Combined Effects on Growth Parameters, Photosystems and CO$_2$/H$_2$O Gas Exchange

The studied *K. prostrata* plants were sensitive to the individual effects of heat and salinity. A decrease in fresh biomass during acclimation of plants to heat (1.6-fold) and under salinity (2.2-fold) was observed. However, under heat + salinity stress, fresh biomass remained the same as plants acclimatised to heat (Fig. 1a). The water content in the tissues of the aboveground parts of plants did not change under all treatments (Fig. 1b). Two-way analysis of variance (ANOVA) revealed the absence of significant differences in this parameter (Table 1).

A study of the efficiency of the photosystems of electron transport chain in the *K. prostrata* leaves

showed that the oxidation time of P700 (PSI) under illumination with far-red light was significantly ($P = 0.036$) decreased only under salinity (by 1.3-fold) (Fig. 1c, Table 1). The efficiency of PSII was assessed by the maximum quantum efficiency (yield) photochemistry (F_v/F_m). This parameter did not change significantly under all treatments (Fig. 1d, Table 1).

The CO$_2$/H$_2$O gas exchange measurement showed that the apparent photosynthetic rate (A) did not change significantly under all treatments (Fig. 2a). This indicates the preservation of functional activity of the photosynthetic apparatus under stressful conditions. Transpiration intensity (E) was significantly decreased only under salinity (2.3-fold) (Fig. 2b). The intensity of dark mitochondrial respiration (Rd) was significantly increased because of acclimation to heat by 69% (from control) and remained elevated under heat + salinity (Fig. 2c). There is a statistically significant effect of heat and combined stress (heat + salinity) to Rd ($P = 0.007$ and 0.047, respectively) (Table 1). Water use efficiency (WUE) did not significantly change under all treatments, demonstrating a stable water balance in *K. prostrata* shoots. (Fig. 2d, Table 1).

Effect of Heat, Salinity and Their Combined Effects on Proline and Ion Contents

Free proline content increased by 1.3-fold than the control under combined stress (heat + salinity) (Fig. 3a). There is a statistically significant effect of combined stress ($P < 0.001$) (Table 1).

Significant accumulation of Na$^+$ (5.5-fold higher than in that in control) was observed in *K. prostrata* shoots under salinity (Fig. 3b). Heat acclimation affected Na$^+$ accumulation under salinity. Na$^+$ content was 3-fold less under combined stress (heat + salinity) than under salinity (Fig. 3b). Two-way ANOVA revealed that there was a statistically significant interaction between heat and salinity stress ($P \leq 0.001$) (Table 1). Under salinity, K$^+$ accumulation was the same as in control plants. K$^+$ content was significantly higher (by 25%) during acclimation to heat than in control plants (Fig. 3c, Table 1). The addition of salt stress (heat + salinity) did not change potassium concentration (Fig. 3c) in acclimated plants. However, there is a statistically significant interaction between heat + salinity stress ($P = 0.042$) (Table 1).

The K$^+$/Na$^+$ ratio increased by 20% under heat and decreased by 5.6-fold under salinity (Fig. 3d). Under combined stress (heat + salinity), K$^+$/Na$^+$ decreased by 1.8-fold than heat and by 1.6-fold than the control. Thus, there is a statistically significant interaction between heat and salinity stress ($P = 0.006$) (Table 1).
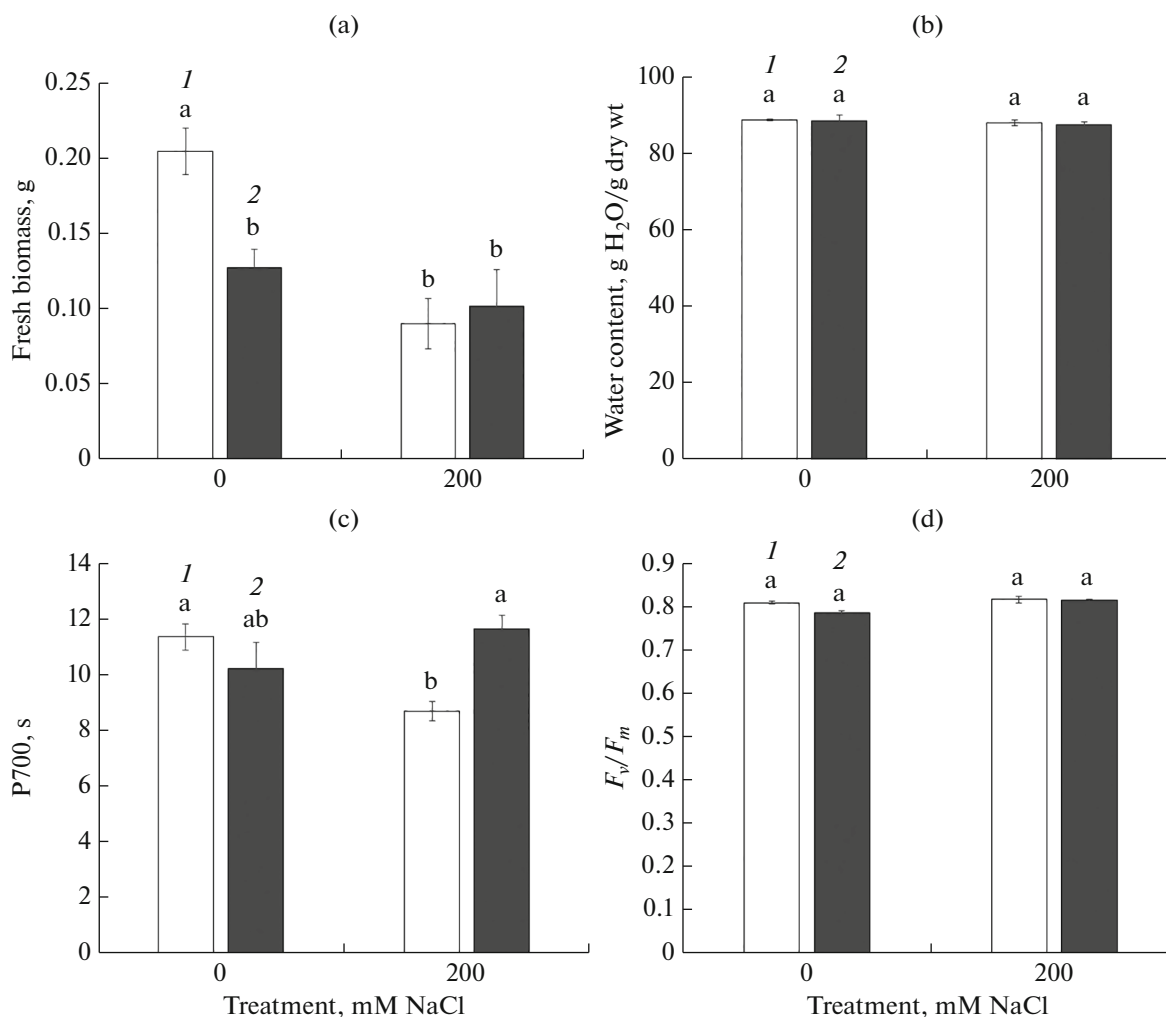
Fig. 1. Fresh biomass (a), water content (b), time required to reach the maximum P700 oxidation level under far-red light (PSI) (c) and maximum quantum yield of PSII reaction (d) of C_4-species *Kochia prostrata* growth at different temperature 25°C (*1*) and 32°C (*2*) without salinity (0 NaCl) and under moderate salinity (200 mM NaCl). Fluorescence was measured at 25°C. The values are means (± SE) of seven replicates. Different letters above the bars represent significant differences at the $P < 0.05$ (Bonferroni t-test pairwise comparison).

Principal Component Analysis (PCA) of the Effect of Heat, Salinity and Their Combined Effects on K. prostrata

Principal component analysis (PCA) for identifying factors (variables) influencing the heat, salinity and heat + salinity acclimation demonstrated differences in the response of *K. prostrata* plants. Three groups: control, salinity and heat + salinity are distinguished in Fig. 4. Two groups (control and salinity) were distinguished by PC1, which represented 30.87% of the total variation. The ratio of K^+/Na^+, Na^+ content, fresh biomass and PSI cyclic electron transport were the main factors that contributed to PC1. These parameters had the highest values of factor loading (Table 2). The second principal component (PC2) separated plants under heat + salinity from control and plants under salinity. K^+ concentration, Rd intensity and proline content were major contributors to PC2.

The first two principal components (PC1 and PC2) were enough to explain 50% of the pattern variation. Thus, PC1 divided the plants into groups by exposure of salinity and PC2 by exposure to heat + salinity. Plants acclimated to heat occupy an intermediate position and overlap with the control and heat + salinity groups.

DISCUSSION

One of promising fodder species is the xero-halophyte or facultative halophyte *Kochia prostrata* (Chenopodiaceae), which is characterised by moderate salt and drought tolerance [34, 35]. Recently, the mechanisms of salt tolerance in halophytes have been actively studied, and some progress has been made in this

Table 1. Two-way ANOVA showing the effect of the main factors (heat and salinity) and their interaction on growth, photosystems and gas exchange parameters, water, ions and proline contents of *Kochia prostrata*

Variable and source of variation	df	F	P	Variable and source of variation	df	F	P
Fresh weight				*Respiration*			
Heat	1	11.013	**0.003**	Heat	1	8.644	**0.007**
Salinity	1	50.161	**<0.001**	Salinity	1	3.370	0.079
Heat + Salinity	1	19.470	**<0.001**	Heat + Salinity	1	0.0848	**0.047**
Water content				*Water use efficiency*			
Heat	1	0.0789	0.781	Heat	1	0.884	0.356
Salinity	1	0.110	0.743	Salinity	1	0.398	0.534
Heat + Salinity	1	0.00114	0.973	Heat + Salinity	1	1.822	0.190
Photosystem I				*Na$^+$ concentration*			
Heat	1	1.817	0.191	Heat	1	4.556	0.055
Salinity	1	0.846	**0.036**	Salinity	1	181.291	**<0.001**
Heat + Salinity	1	9.050	0.056	Heat + Salinity	1	64.875	**<0.001**
Photosystem II				*K$^+$ concentration*			
Heat	1	0.422	0.522	Heat	1	44.287	**<0.001**
Salinity	1	1.033	0.320	Salinity	1	2.475	0.142
Heat + Salinity	1	0.362	0.553	Heat + Salinity	1	4.642	**0.042**
Photosynthesis rate				*K$^+$/Na$^+$ ratio*			
Heat	1	2.060	0.164	Heat	1	62.458	**<0.001**
Salinity	1	0.119	0.733	Salinity	1	242.285	**<0.001**
Heat + Salinity	1	1.382	0.251	Heat + Salinity	1	10.813	**0.006**
Transpiration rate				*Proline content*			
Heat	1	0.126	0.726	Heat	1	0.357	0.567
Salinity	1	1.307	**0.021**	Salinity	1	1.994	0.171
Heat + Salinity	1	5.553	0.057	Heat + Salinity	1	30.732	**<0.001**

The main significant factors are bold.

Table 2. Factor loading of morphophysiological and biochemical parameters on axes 1 and 2 of the principal component analysis on Fig. 4

Parameters	PC1	PC2
Fresh biomass	**−0.340**	−0.317
Water content	−0.021	−0.131
Proline content	0.079	**0.409**
Na$^+$ content	**0.488**	0.131
K$^+$ content	−0.192	**0.445**
K$^+$/Na$^+$ ratio	**−0.494**	−0.018
Photosystem I	**−0.319**	0.244
Photosystem II	0.202	−0.129
Apparent photosynthesis	−0.219	0.298
Dark respiration	−0.008	**0.444**
Transpiration	−0.242	0.220

The main significant factors are bold.

direction [11]. However, much less work is devoted to studying the combined effect of several stress factors, particularly; elevated temperature and salinity, and these studies mainly investigated the tolerance to high temperatures in plants adapted to salinity [27]. In our experiments, the plants were acclimated to elevated temperature, after which they were treated with moderate salinity during 4 days (heat + salinity). Such model experiments in climatic chambers allow controlling the effect of 1−2 factors, in contrast to natural conditions, where a large number of various factors act on plants. Controllability of the effect of factors make it possible to study the physiological processes and biochemical parameters involved in the acclimation to specific combined stress. In our experiments the fresh biomass of the aboveground part of *K. prostrata* plants decreased under both salinity and heat relative to control plants, but the water content and WUE did not significantly change (Figs. 1a, 1b and 2d). The action of combined stress (heat + salinity) led to a similar decrease in biomass, as in the case of effects of individ-
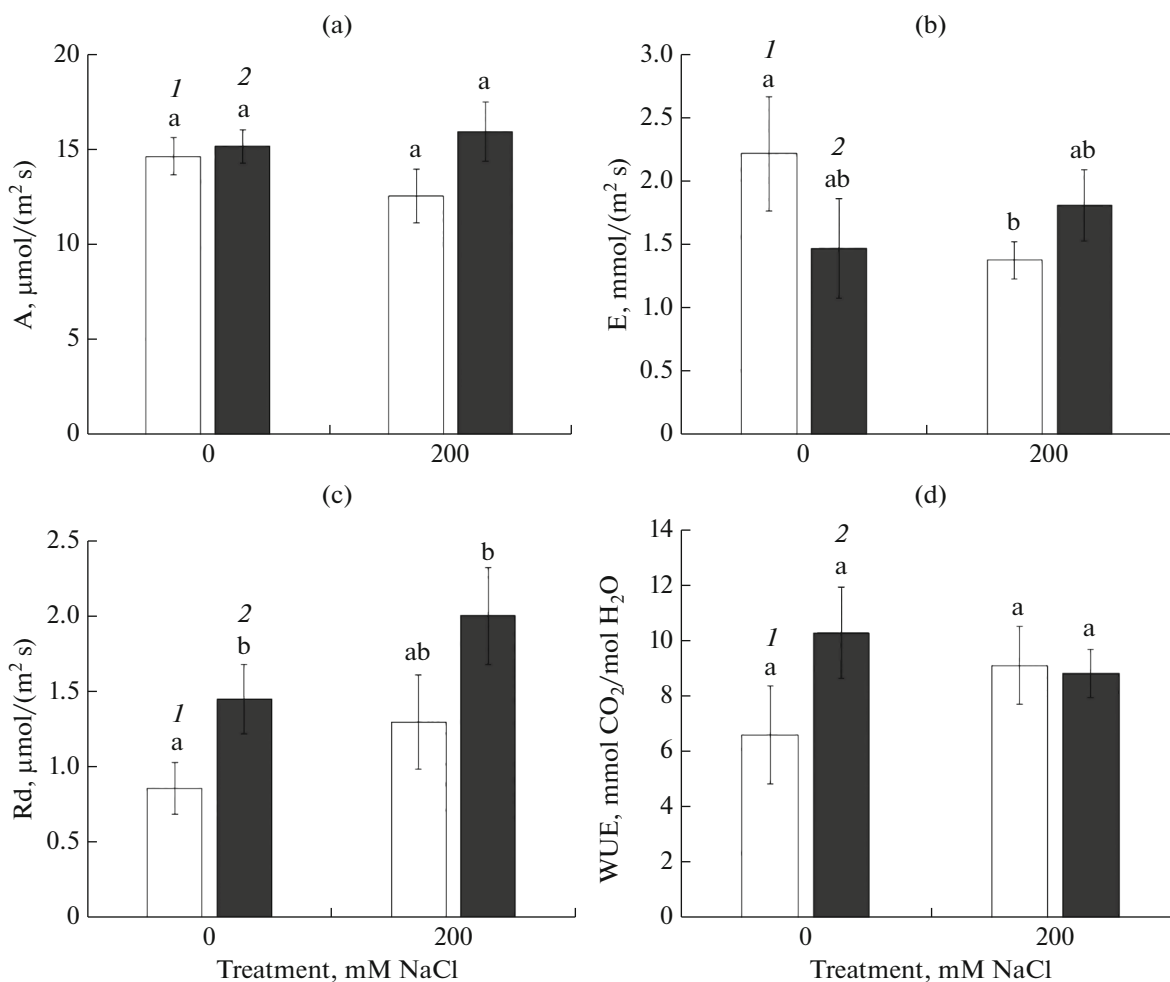
Fig. 2. Apparent photosynthesis (a), transpiration (b), dark respiration (c), and water use efficiency (d) in plant leaves of C$_4$-species *Kochia prostrata* growth at different temperature 25°C (*1*) and 32°C (*2*) without salinity (0 NaCl) and under moderate salinity (200 mM NaCl). CO$_2$/H$_2$O gas exchange was measured at 25°C and 1200 µE PAR. The values are means ($\pm$ SE) of seven replicates. Different letters above the bars represent significant differences at the $P < 0.05$ (Bonferroni t-test pairwise comparison).

ual stresses (Fig. 1a). Our results show the nonadditive effect of combined stress on this parameter.

It is known the effect of heat on carbon fluxes is associated with the stimulation of the enzymes' activity, primarily respiratory enzymes [1]. In our experiments, during the acclimation of plants to elevated temperature, increased intensity of dark respiration was also observed (Fig. 2c), also increased K$^+$ content and the K$^+$/Na$^+$ ratio (Figs. 3c, 3d). Since the increase in dark respiration was accompanied by a decrease in growth parameters, it can be assumed that the increased respiration is associated with adaptation and dissipation costs [31, 36]. The increase in potassium content revealed by us is probably associated with its important role in combating temperature stress. As is known, potassium helps to activate various physiological and metabolic processes, such as photosynthesis, respiration, stabilises ionic homeostasis, which helps

maintain tissue water potential, contributing to tolerance to extreme temperatures. Under high-temperature stress, potassium can act as an osmolyte and help maintain stomatal conductance and WUE [37]. Potassium also works in plant signalling systems involved in defence against various types of stress, and activates antioxidant defence systems. Thus, potassium is an important element in increasing plant tolerance to various stressors, including elevated temperatures [38].

Under salinity, there was a significant (5.5-fold compared with the control plants) Na$^+$ accumulation and a decrease in the K$^+$/Na$^+$ ratio (Figs. 3b, 3d), as well as a decrease in PSI efficiency and transpiration intensity (Figs. 1c, 2b). Halophytes use Na$^+$ as a 'cheap osmolyte' for adaptation to the low water potential of the [15], which decreases the K$^+$/Na$^+$ ratio. However, the decrease in transpiration intensity observed under salinity was not accompanied by sig-
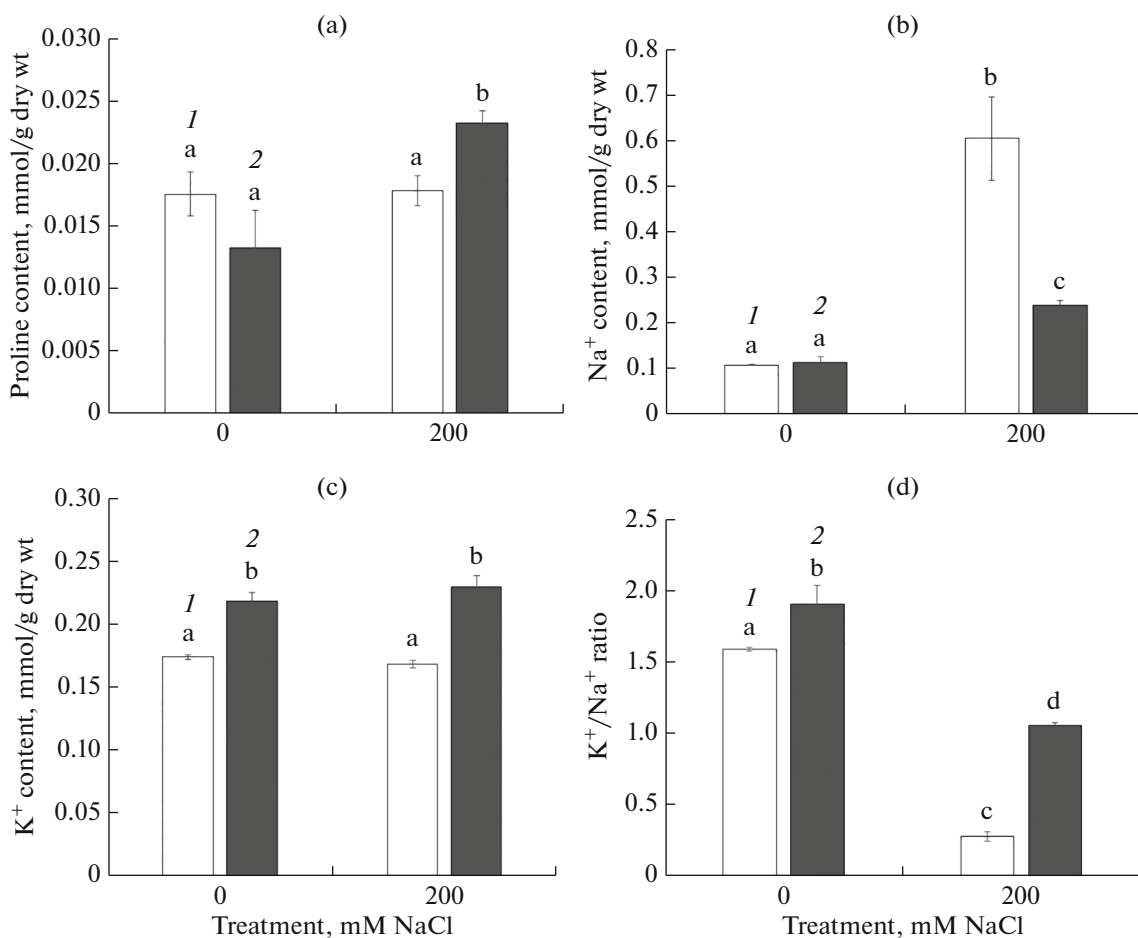
Fig. 3. Contents of free proline (a), Na^+ (b), K^+ (c) and the ration of Na^+/K^+ in plant leaves of C_4-species *Kochia prostrata* at different temperature 25°C (*1*) and 32°C (*2*) without salinity (0 NaCl) and under moderate salinity (200 mM NaCl). The values are means (± SE) of seven replicates. Different letters above the bars represent significant differences at the $P < 0.05$ (Bonferroni *t*-test pairwise comparison).

nificant changes in water content and WUE, i.e. stomatal closure, was not significant for plant water metabolism (Figs. 2b, 2d).

A feature of C_4 plants is the high functional activity of C_3 and C_4 cycles of photosynthesis. Consequently, energy consumption for assimilation of CO_2 increases compared to C_3 plants. Therefore, two additional ATP molecules are needed for each CO_2 molecule fixed to the CCM in C_4 plants. It is assumed that additional ATP is produced due to the cyclic transport of electrons around PSI, contributing to the creation of a pH gradient across the thylakoid membrane without the formation of nicotinamide adenine dinucleotide phosphate (NADPH) [23]. Comparative analysis within the genus *Flaveria* showed that C_4 plants show a higher expression of the genes of proteins involved in cyclic electron transport around PSI and changes in thylakoids structure that contribute to increased activity of cyclic electron flow [23]. In these experiments, *K. prostrata* plants grown under salinity showed a

decrease in the time required to reach the maximum P700 oxidation level (PSI) under far-red light, i.e. a decrease in the cyclic electron transport intensity was observed, which indirectly indicates a decrease in the C_4 CCM activity. Thus, the decrease in plant biomass under salinity can be associated with a decrease in PSI cyclic electron transport intensity and, possibly, with a less efficient CCM.

The combined effect of heat and salinity (heat + salinity) resulted in an increase in the contents of proline and sodium ions, as well as a decrease in the K^+/Na^+ ratio compared with the control (Figs. 3a, 3b), on the background of increased K^+ content (Fig. 3c) and dark respiration (Fig. 2c) caused by acclimation of plants to heat. On the one side we can assume that there is an additive effect in the action of tolerance mechanisms under combined stress: an increase of Rd intensity and K^+ content was observed at elevated temperature, and an increase in Na^+ content and a decrease in the K^+/Na^+ ratio under salinity was
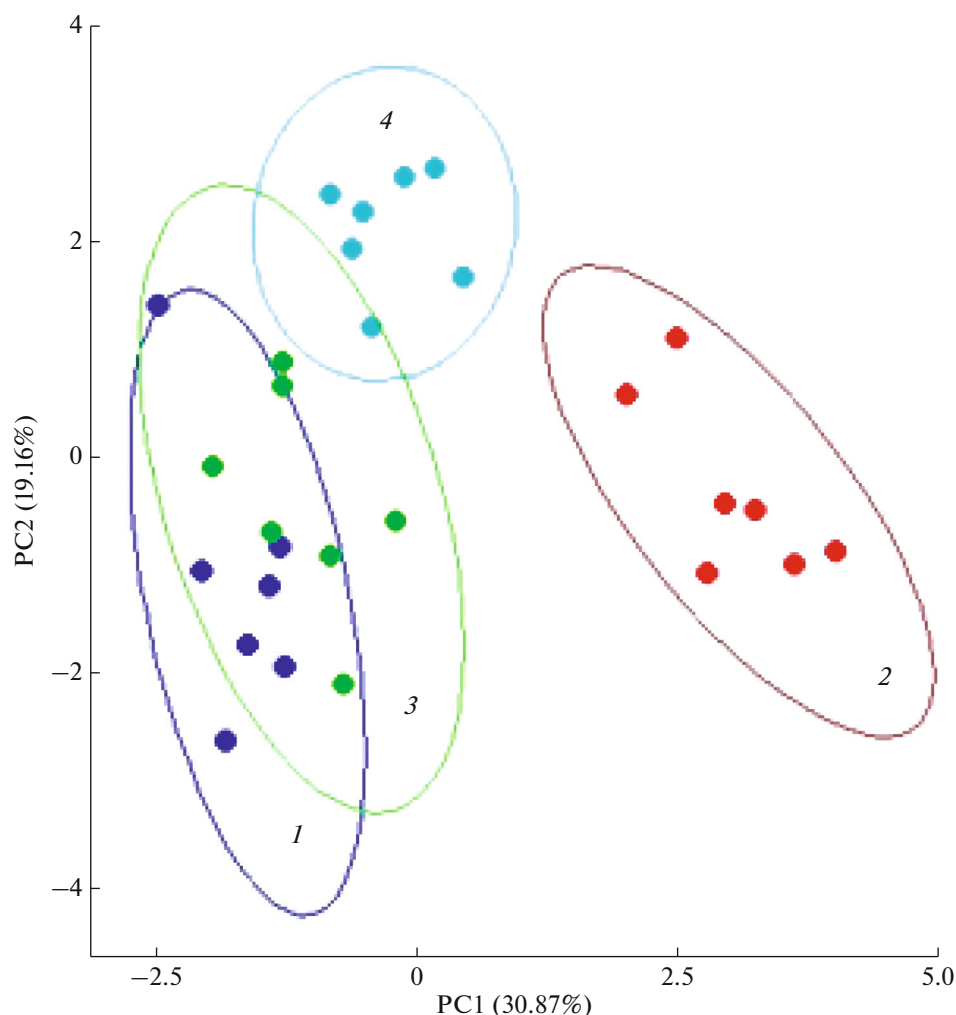
Fig. 4. Principle component analysis (PCA) score plot of the morphophysiological and biochemical parameters of C_4-species *Kochia prostrata* at different temperature 25 and 32°C under moderate salinity (200 mM NaCl). (*1*) 25°C, 0 mM NaCl (control); (*2*) 25°C, 200 mM NaCl; (*3*) 32°C, 0 mM NaCl; (*4*) 32°C, 200 mM NaCl. Parameters are listed in Table 2.

observed (as compared with control). On another side the action of combined stress caused a fundamentally different response of plants than the action of individual factors, namely, an increase in proline content under heat + salinity (Fig. 3a). Under combined stress, Na^+ content increased (as compared with control), but this increase was three times less than that under salinity (Fig. 3b). It is known that at 25°C the absorption of ions (Na, K, Ca and Mg) was higher than at higher (30−40°C) temperatures by plants *Amaranth gangeticus* [39]. Possible elevated temperature negatively affects the operation of sodium ion channels. The decrease in sodium ion content at elevated temperature was compensated by an increase in the content of K^+ and proline (Figs. 3a, 3c). The additional energy costs associated with the transport of potassium ions and proline biosynthesis were probably covered by an increase in the intensity of the dark mitochondrial respiration (Fig. 2c).

Photosynthesis is considered the most sensitive to high-temperature damage, especially when combined with salinity, and that PSII appears to be the most thermally sensitive part of the photosynthetic apparatus [27]. According to other authors, photosynthesis can function without harm at temperatures from 0 to 30°C [8], especially when the temperature does not rise rapidly, but gradually [7]. So, the maximum quantum yield of photosystem II (F_v/F_m) was less sensitive to leaf temperatures up to 45°C as compare with CO_2 assimilation in C_4 corn (*Zea mays*) [7]. The activation state of phosphoenolpyruvate carboxylase slightly decreased at a leaf temperature only above 40°C, whereas Rubisco activation state significantly decreased at a temperature of 32.5°C, i.e. inactivation Rubisco was the main limiter of apparent photosynthesis in maize leaves, at leaf temperatures above 30°C. Simultaneously, with a gradual increase in temperature, the acclimatisation of Rubisco and apparent

Table 3. Morphophysiological and biochemical parameters of C_4 xero-halophyte *Kochia prostrata* involved in the acclimation to heat, salinity and combined heat + salinity stress and revealed using multivariate methods of statistical analysis: two-way ANOVA and principal component analysis (PCA)

Treatments	Two-way ANOVA	PCA total
All treatments (heat, salinity, heat + salinity)	fr wt K^+/Na^+	fr wt PC1, PC2
Heat	Rd K^+	–
Salinity	PSI E Na^+	K^+/Na^+ (PC1) Na^+ (PC1) PSI (PC1)
Heat + Salinity	Rd Na^+ K^+ Pro	Rd (PC2) K^+ (PC2) Pro (PC2)

fr wt—fresh weight; Rd—dark respiration; E—transpiration; Pro—proline content; PSI—time required to reach the maximum P700 oxidation level under far-red light.

photosynthesis intensity to stress is observed [7]. In our experiments, no changes in PSII efficiency and apparent photosynthesis intensity were observed under any treatment (Figs. 1d and 2a). However, a decrease in the transpiration intensity was observed under salinity (Fig. 2b). This is consistent with the results of Munns and Tester [40], who showed that the apparent photosynthesis rate, calculated per unit leaf area, often remains unchanged under salinity, even if the stomatal conductance and transpiration rates are reduced.

To identify the physiological processes and biochemical parameters that are most actively involved in the acclimation to the individual and combined effects of stress factors, we used several statistical approaches. A comparative analysis of the results from two-way ANOVA and PCA is shown in Table 3. Two-way ANOVA made it possible to characterise the morphophysiological and biochemical parameters involved in acclimation to each variant of stress effects (heat, salinity and combined heat + salinity). Two-way ANOVA showed that the changes in biomass and the K^+/Na^+ ratio were common in all treatments' tolerance mechanisms (Tables 1, 3). In addition to these changes, an increase in the dark respiration intensity and K^+ content was observed under heat. When salt stress is applied to plants acclimated to heat (combined effect), there was an increase in proline and Na^+ contents in addition to high values of Rd and K^+. PCA influenced the parameters of *K. prostrata* and to char-

acterise the most significant parameters for acclimation to these stressors (Table 2, 3). The results obtained using different methods overlap and complement each other. PCA showed that dark respiration, potassium and proline are actively involved in the acclimation to combined stress (heat + salinity). Thus, under this condition, there was not a summation of the actions of factors and not an increase in tolerance, but a qualitatively new response was formed following acclimation to elevated temperature.

CONCLUSION

The C_4 xero-halophyte *K. prostrata* has constitutive features of tolerance to arid climate and salinity. However, our experiments showed that even a slight increase in temperature and moderate salinity significantly reduced plant growth. The water content in plant tissues and WUE did not change under all treatments (heat, salinity and heat + salinity). The stability of water status, maintained by the salt tolerance mechanisms (with sodium and potassium ions and compatible osmolyte proline) and stomatal closure, created conditions for PSII stabilisation. The intensity of apparent photosynthesis did not change probably due to the presence of C_4 CCM. During acclimation to elevated temperature, vital potassium ions and mitochondrial respiration played a special role. From the obtained experimental and statistical analysis data, it follows that the elevated temperature affects the physiological mechanisms of *K. prostrata* salt tolerance. Thus, in *K. prostrata* grown at elevated temperatures, K^+ and proline play a greater role than Na^+ in adaptation to salinity; additional energy costs are provided by dark respiration.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. This article does not contain any studies involving animals or human participants performed by any of the authors.

AUTHOR CONTRIBUTIONS

Authors ZFR and EVS designed the experiments. ZFR, EVS, MYuP, KNT, NYa, and PYuV collected samples and performed the experiments. ZFR drafted the manuscript and all authors revised it.

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