

NADP-dependent enzymes are involved in response to salt and hypoosmotic stress in cucumber plants

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Abstract. Salt stress is one of the most damaging plant stressors, whereas hypoosmotic stress is not considered to be a dangerous type of stress in plants and has been less extensively studied. This study was performed to compare the metabolism of cucumber plants grown in soil with plants transferred to distilled water and to a 100 mM NaCl solution. Even though hypoosmotic stress caused by distilled water did not cause such significant changes in the relative water content, Na^+/K^+ ratio and Rubisco content as those caused by salt stress, it was accompanied by more pronounced changes in the specific activities of NADP-dependent enzymes. After 3 days, the specific activities of NADP-isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, NADP-malic enzyme and non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase in leaves were highest under hypoosmotic stress, and lowest in plants grown in soil. In roots, salt stress caused a decrease in the specific activities of major NADP-enzymes. However, at the beginning of salt stress, NADP-galactose-1-dehydrogenase and ribose-1-dehydrogenase were involved in a plant defense response in both roots and leaves. Therefore, the enhanced demands of NADPH in stress can be replenished by a wide range of NADP-dependent enzymes.

Key words: *Cucumis sativa* L. — Hypoosmotic stress — NADP-dependent enzymes — NADPH — Salt stress

Abbreviations: AAS, atomic absorption spectrometry; AES, atomic emission spectrometry; DH, dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; HSP, heat shock protein; NADP-ICDH, NADP-isocitrate dehydrogenase; NADP-ME, NADP-dependent malic enzyme; NP-GAPDH, non-phosphorylating glyceraldehyde-phosphate dehydrogenase; PVP, poly(vinylpyrrolidone); ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SDH, shikimate dehydrogenase.

Introduction

Whereas hyperosmotic stress caused by high salt concentration (mostly Na^+ and Cl^- and also other ions such as Ca^{2+} , K^+ , HCO_3^- , CO_3^{2-} , NO_3^- etc.) is well studied as one

of the most common abiotic stress, much less attention is devoted to plant hypoosmotic stress. The high salt concentration results in a decrease of soil water potential and thus a decrease water uptake by roots, a decreased turgor, closed stomata and therefore a decreased availability of CO_2 for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and reduced photosynthesis (Kosova et al. 2011; Isayenkov 2012; Tang et al. 2014). Salt stress causes substantial transcriptomic and metabolic reprogramming, which may in turn affect growth and development (Pétriaccq et al. 2013; Deinlein et al. 2014). High salinity increases respiration and reactive oxygen species (ROS) generation, which cause oxidative stress. Besides antioxidant ascorbate-glutathione cycle, plant phenolic compounds such as flavonoids are

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regarded as crucial compounds that are accumulated under stress and that can eliminate toxic effect of harmful ROS. In addition to oxidative stress, salt ions invoke osmotic stress and an imbalance in intracellular ion and redox homeostasis (Nawaz et al. 2010; Bartwal et al. 2013; Abbasi et al. 2016). Osmotic stress can be alleviated by synthesis of osmotically active compounds (Gzik 1996; Garg et al. 2002; Abebe et al. 2003; Pandolfi et al. 2010) or specific proteins (e.g. heat shock proteins, HSPs) (Wang et al. 2003; Doubnerova and Ryslava 2014). Nevertheless, Na⁺ ions in saline soils are toxic due to their unfavorable effect on K⁺ nutrition and cytosolic enzyme activities (Shi et al. 2000). The enormous negative membrane potential across the plasma membrane of plant cells favors the passive transport of Na⁺ into cells (Zhu 2003). Non-selective cation channels (NSSCs) catalyzing passive fluxes of cations through plasma membranes were proposed to form a major pathway for Na⁺ entry into plants (Demidchik and Maathuis 2007). However avoidance of Na⁺ toxicity and maintenance of high K⁺/Na⁺ ratio in cytoplasm is crucial for the normal function of cellular metabolism (Conde et al. 2011; Pitann et al. 2013). Main mechanisms, which prevents accumulation of Na⁺ ions in cytoplasm, are active Na⁺ efflux, restriction of Na⁺ influx and sequestration of Na⁺ ions in the vacuole (Shi et al. 2000; Abbasi et al. 2016).

On the other hand, hypoosmotic stress in plants causes less severe consequences than hyperionic stress and also than hypoosmotic stress in animal cells due to the presence of the cell walls. The metabolic changes accompanying hypoosmotic stress-induced ROS generation mediated by NADPH-oxidases (Kurusu et al. 2012), hypoosmotic stress-induced increase in membrane fluidity, increased tonoplast proton pumps activity and possible inhibition of aerobic metabolism (Ozolina et al. 2011) have not been fully clarified yet.

It was shown that 25–100 mM concentration of NaCl in the rooting medium of a cucumber resulted in marked depression of the net photosynthesis (Drew et al. 1990; Badawi et al. 2004). Decreased photosynthesis is related to lower photosynthetic yield in the form of NADPH. However, reduction equivalent NADPH is indispensable coenzyme in antioxidant systems, biosynthetic processes and plant defense responses. Therefore, we wanted to find out if NADP-dependent enzymes producing NADPH could compensate photosynthetic insufficiency in a salt stress plants. In addition to major NADP-enzymes: glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), NADP-isocitrate dehydrogenase (NADP-ICDH, EC 1.1.1.42), NADP-malic enzyme (NADP-ME, EC 1.1.1.40), non-phosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (NP-GAPDH, EC 1.2.1.9) and NADP-shikimate dehydrogenase (SDH, EC 1.1.1.25), which rather catalyzes reaction in which NADPH is metabolized, also less abundant enzymes were studied: NADP-ribose-1-dehydrogenase (ribose-1-DH, EC 1.1.1.115), NADP-

glucose-1-dehydrogenase (glucose-1-DH, EC 1.1.1.119), NADP-galactose-1-dehydrogenase (galactose-1-DH, EC 1.1.1.120), NADP-gluconate 2-dehydrogenase (gluconate-2-DH, EC 1.1.1.215) and NADP-glycerol-2-dehydrogenase (glycerol-2-DH, EC 1.1.1.156), with substrates in addition to NADP⁺: D-ribose, D-glucose, D-galactose, D-gluconate and D-glycerol, respectively.

The aim of our study was to show how salt stress (100 mM NaCl) and hypoosmotic stress in cucumber seedlings (*Cucumis sativa* L.) affect the activity of both the main and the less abundant NADP-dependent enzymes producing NADPH and the ratio of NADPH/NADP⁺. Furthermore, other parameters characterizing stress such as relative water content (RWC), the Na⁺/K⁺ ratios, the amount of compounds with antioxidative properties, the amount of Rubisco and HSP70 protein were compared. The salt stress was carried out in NaCl solution, hypoosmotic in distilled water. Both treatments involve also mechanical stress, changed oxygen availability and possible nutrition stress, therefore the comparison enables to study the only effect of NaCl. Plants grown in soil were used to evaluate the differences from real conditions.

Materials and Methods

Plant material

Cucumis sativa L. cv. Jogger F1 plants were grown in a greenhouse under 14-h photoperiod [overall integrated mid-values were *ca* 500 μmol (quantum) m⁻²s⁻¹], 22/18°C day/night temperature and relative humidity *ca* 80%. Cucumber seeds were allowed germinated and developed onto wet cotton wool for 1 week. Seedlings (21) were transferred into 0.5 l pots with soil (commercial horticultural substrate) and were grown in a greenhouse for next 4–5 weeks. One third of plants serves as group of control plants, which persevere in the soil, whereas remaining plants were extracted from the soil, their roots were washed thoroughly and one third was transferred to 1-liter bucket with 100 mM NaCl solution (group of 100 mM NaCl stressed plants) and one third to 1-liter bucket with distilled water (group of hypoosmotically stressed plants). The samples were collected 2 h (day zero), 1, 2 and 3 days after salt application from upper leaves and from roots. Four different biological experiments were done.

Determination of relative water content (RWC)

Determination of RWC was done according to Sharp et al. (1990) and calculated as (FW – DW)/(TFW – DW) × 100, where FW is fresh weight, DW drought weight and TFW fully turgid fresh weight.

Determination of Na^+ and K^+ ions by AAS

The real concentration of Na^+ and K^+ ions in leaves and roots was determined using atomic absorption spectrometry (AAS), spectrometer AAS 3 (Carl Zeiss Jena, Germany) in atomic emission spectrometry (AES) mode at 589.0 nm and 766.5 nm with spectral width of 0.3 nm and 0.1 nm, respectively, after atomization/excitation in flame. Samples for Na^+ determination were prepared by extraction of 1 g of plant material in 3 ml of distilled water and by centrifugation of extracts at $16,600 \times g$ and 4°C for 15 min. Supernatants were stored at -25°C until measurements.

Determination of total phenolics and flavonoids content

Cucumber leaves (2.5 g) were extracted with 50 ml of 80°C distilled water for 20 min, extracts were filtrated through 4-layer mull, centrifuged at $9,400 \times g$ and 4°C for 15 min and supernatants filtrated through syringe Schleicher&Schuell membrane filters with pore diameter of 0.45 μm . Final sample was used for determination of total phenolics using Folin reagent according to Cai et al. (2004) and of flavonoids according to Liu et al. (2002).

SDS-PAGE and quantification of Rubisco and HSP70

The soluble proteins in leaves extracts were separated by SDS-PAGE (Laemmli 1970). Rubisco (large subunit with relative molecular mass about 55 000) was detected by Coomassie Brilliant Blue staining and quantified. Protein HSP70 was detected immunochemically using specific rabbit polyclonal antibody (Agrisera, Sweden) on nitrocellulose membrane after transfer of proteins from gel. The visualization was carried out with goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma, USA) and 5-bromo-4-chloro-3-indolyl phosphate/Nitroblue tetrazolium tablet (Sigma, USA) incubation solution. The results were evaluated with program ImageJ.

Determination of soluble proteins

Soluble proteins were determined by Bradford reagent (Sigma, USA) with bovine serum albumin as the standard (Bradford 1976).

Estimation of NADP^+ and NADPH content

The amount of 1 g of plant material was inactivated either in 3 ml of 1 M HClO_4 at laboratory temperature (for NADP^+ estimation) or in 3 ml of 0.1 M NaOH at 100°C for 2–5 min (for NADPH estimation). Consequently, 0.02 g of poly(vinylpyrrolidone) (PVP) was added to these mixtures and extracts were centrifuged at $16,600 \times g$ and 4°C

for 15 min. Then the supernatants were neutralized with 5 M K_2CO_3 and 0.1 M HCl , respectively (Mateos et al. 2009).

NADP^+ was determined enzymatically in 1 ml of reaction mixture containing 100 mM Tris-HCl, pH 7.4; 5 mM D-glucose-6-phosphate, 2 mM MgCl_2 , 1 U of G6PDH from *Leuconostoc mesenteroides* and 100 μl of neutralized supernatant. After 30 min, 200 μl of detection solution (5 mg/ml iodinitrotetrazolium violet and 5 $\mu\text{g}/\text{ml}$ of phenazine methosulfate) was added and absorbance at 500 nm was measured in comparison with blank, in which G6PDH was replaced by distilled water.

NADPH was determined using 1 ml of neutralized supernatant after alkaline inactivation and 200 μl of above mentioned detection solution. Absorbance was measured immediately at 500 nm.

Enzyme activity assays

Approximately 0.5 g of the plant sample was homogenized with 1.5 ml of extraction buffer containing 100 mM Tris-HCl pH 7.8; 5 mM MgCl_2 ; 1 mM EDTA; 1 mM dithiothreitol; 1% (v/v) protease inhibitor cocktail for tissue and plant extracts (Sigma, USA). Then 0.02 g of PVP was added and the homogenate was centrifuged at $16,600 \times g$ and 4°C for 15 min. The activities of major NADP-dependent enzymes were determined in supernatant (further assigned as plant extract) by monitoring of increase in absorbance at 340 nm, which corresponds to liberating product NADPH. One unit of enzyme activity is defined as μmol of liberating product per min at 20°C .

The reaction mixture for determination of G6PDH activity contains: 100 mM Tris-HCl, pH 7.6; 5 mM D-glucose-6-phosphate; 2 mM MgCl_2 and 0.2 mM NADP^+ . Determination of NADP-ICDH activity was carried out in 100 mM Tris-HCl, pH 7.4; 2 mM isocitrate; 2 mM MgCl_2 and 0.2 mM NADP^+ . NADP-ME activity was determined in 100 mM Tris-HCl, pH 7.4; 10 mM L-malate; 2 mM MgCl_2 and 0.2 mM NADP^+ and SDH in reaction mixture containing 100 mM 2-amino-2-methyl-1-propanol- NaOH , pH 9; 3 mM shikimate and 0.2 mM NADP^+ . NP-GAPDH was determined using coupled reaction: firstly 1 U/ml of aldolase was incubated with 10 mM D-fructose-1,6-bisphosphate, 100 mM Tris HCl, pH 7.4; 2 mM MgCl_2 ; and 0.2 mM NADP^+ for 1 h and after that the plant extract was added. The glucose-1-DH, galactose-1-DH, ribose-1-DH, gluconate-2-DH and glycerol-2-DH were determined in 100 mM Tris-HCl, pH 7.4; 0.2 mM NADP^+ and particular substrate in 10 mM concentration (D-glucose, D-galactose, D-ribose, D-gluconate and D-glycerol, respectively). The volume of all reaction mixtures was 1 ml and reactions were started with 50–100 μl of the plant extract. Together with reaction mixtures also blanks without NADP^+ or substrates were processed.

Statistical analysis

The enzyme activity measurements, RWC and Na^+ and K^+ determination were processed in 3 samples in individual experimental day and were presented as mean \pm SD. At least 4 biological experiments were performed. Statistically significant differences in the mean values were tested by Student's *t*-test at $p \leq 0.05$.

Results and Discussion

The significant effect of salt stress on RWC and ion balance is only mild under hypoosmotic conditions

The consequences of salt or hypoosmotic stress in cucumber plants was followed by determination of RWC (Fig. 1) and the Na^+ and K^+ ion contents by AES (Fig. 2). After 3 days of salt stress, RWC in leaves decreased up to 60% compared to 92% RWC in controls grown in soil with regular watering. Hypoosmotic stress caused by exposure of cucumber plants to distilled water did not affect RWC at the beginning of stress but a slight decrease was found after 3 days of stress (Fig. 1). Hypoosmotic stress could affect the plant metabolism, the plant cells were not able to maintain turgor and water in the cells and thus RWC was decreased (but not as much as in salt stress). In salt-stressed plants the concentration of Na^+ significantly increased in both the leaves (up to 8-fold compared to controls grown in soil) and the roots (up to 15-fold compared to controls grown in soil) (Fig. 2A,B). In contrast, the concentration of K^+ ions kept decreasing in salt-stressed plants. Especially in the roots, the concentration of K^+ was almost undetectable after 3 days of salt stress (Fig. 2C,D). Hypoosmotic stress mostly did

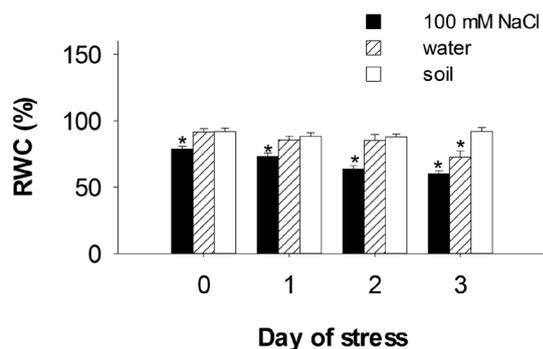


Figure 1. The relative water content (RWC) in leaves of cucumber plants stressed by salt (100 mM NaCl) and hypoosmotic stress caused by distilled water in comparison with plants grown in soil. * The significant differences from controls grown in soil with $p \leq 0.05$ calculated by *t*-test.

not significantly affect the Na^+ and K^+ concentrations in leaves and roots (Fig. 2).

The significant decrease in RWC (Fig. 1), sharply increased content of Na^+ ions in salt-stressed leaves and roots and decrease in the content of K^+ ions, especially in the roots (Fig. 2), are consistent with previous studies

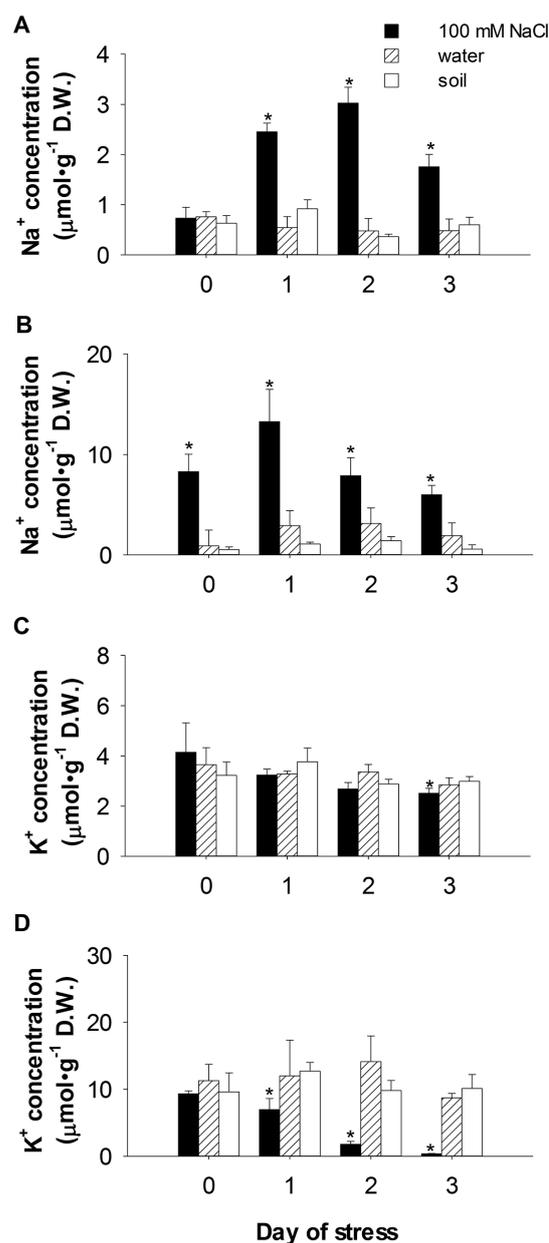


Figure 2. The concentration of Na^+ and K^+ ions in leaves (A, C) and roots (B, D) of cucumber plants stressed by salt (100 mM NaCl) and hypoosmotic stress caused by distilled water in comparison with plants grown in soil. * The significant differences from controls grown in soil with $p \leq 0.05$ calculated by *t*-test. The concentration is related to dry weight (D.W.).

dealing with other plant species under salt stress (Aleman et al. 2011; Abbasi et al. 2016). These changes in the K^+ / Na^+ ratio under salt stress have already been characterized. This phenomenon, which can be used to ameliorate the toxicity of Na^+ ions, consists in an increase in the K^+ content in the extrinsic environment. However, the maintenance of a high K^+ / Na^+ ratio in shoots is correlated with salinity tolerance in glycophytes (such as cucumber) (Hauser and Horie 2010). Na^+ sequestration in the vacuole in possible combination with Na^+ exclusion from the roots might be a crucial strategy for the viability of plants under severe salinity stress (Zhu 2003; Hauser and Horie 2010). Moreover, vacuolar sequestration not only lowers the Na^+ concentration in the cytoplasm but also contributes to osmotic adjustment (Zhu 2003). It was shown in *Arabidopsis* that two genes encoding plasma membrane NADPH oxidase are involved in ROS-dependent modulation of Na^+ / K^+ homeostasis through activation of plasma membrane Ca^{2+} -permeable channels resulting in increases in the cytoplasmic Ca^{2+} levels (Ma et al. 2012). It is known that Ca^{2+} ions activated the salt-stress signaling pathway (SOS, salt overly sensitive); the SOS1 gene encodes a Na^+ / H^+ antiporter eliminating toxic ions (Ramezani et al. 2013).

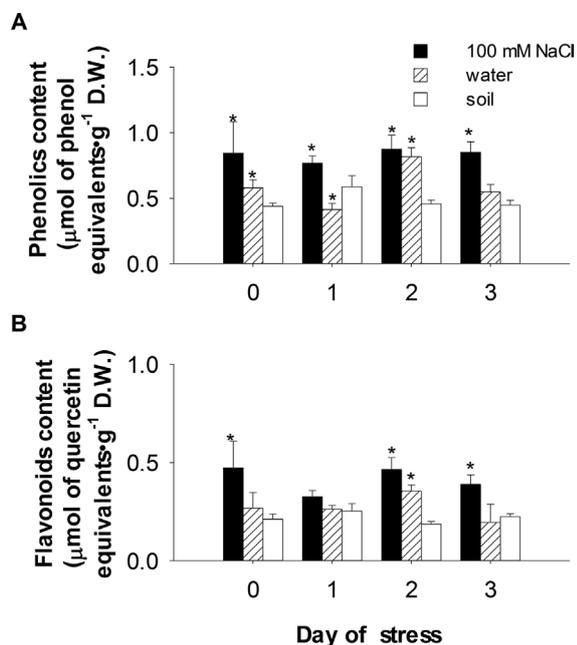


Figure 3. The content of total phenolics (A) and flavonoids (B) in leaves of cucumber plants stressed by salt (100 mM NaCl) and hypoosmotic stress caused by distilled water in comparison with plants grown in soil. * The significant differences from controls grown in soil with $p \leq 0.05$ calculated by *t*-test. The content of phenolics and flavonoids is related to dry weight (D.W.).

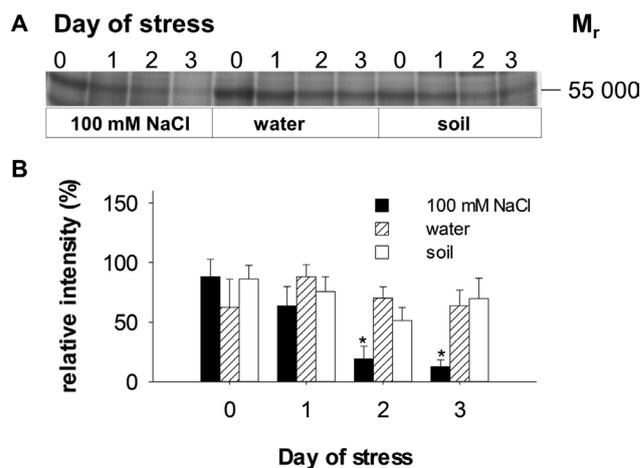


Figure 4. Detection of Rubisco protein in 10% polyacrylamide gel after SDS-electrophoresis (A) followed by densitometric quantification using ImageJ program (B). The equal amount of soluble proteins (50 μg) was applied to each line. * The significant differences from controls grown in soil with $p \leq 0.05$ calculated by *t*-test. 100% corresponds to the highest value.

Both stresses stimulated increased content of total phenolics and flavonoids

Stress is often followed by the synthesis of compounds with antioxidant properties, such as phenolics including flavonoids. Salt and hypoosmotic stress induced a higher leaf content of total phenolic compounds (Fig. 3A) and content of flavonoids (Fig. 3B). Particularly after 2 days of stress, compared to plants grown in soil, the content of phenolics was increased by salt and hypoosmotic stress 1.9-fold and 1.8-fold, respectively, and the content of flavonoids was increased 2.5-fold and 1.9-fold, respectively (Fig. 3A,B). In plants, phenolics such as flavonoids and other phenylpropanoids are generally regarded as crucial defense compounds that act as scavengers of harmful ROS. The antioxidant action of phenolic compounds is related to their ability to donate electrons or hydrogen atoms and to the nucleophilic character of the aromatic rings, which increases their chelating ability. In addition, the hydroxyl groups of phenolics are able to bind iron ions and thus suppress the superoxide-driven Fenton reaction (Bartwal et al. 2013).

Salt stress decreased the content of Rubisco but increased the amount of HSP70

The content of total soluble proteins was determined in both groups of stressed plants. As a consequence of the stress, photosynthetic proteins can be degraded, especially the most abundant protein Rubisco (Galmes et al. 2013). SDS electrophoresis followed by densitometric analysis of the

band corresponding to the large Rubisco subunit revealed a reduced amount of this protein in salt-stressed plants, in contrast to hypoosmotically stressed plants and plants grown in soil (Fig. 4). However, the content of soluble proteins in salt-stressed leaves was slightly enhanced during the first two days (Fig. 5), probably due to increased expression of defense proteins including chaperons HSP70. In the course of salt stress, the amount of protein HSP70 increased in the leaves (Fig. 6). A number of functions in abiotic stress were proposed for plant HSP70, e.g. fine-tuned regulation of the stomatal aperture and modulation of the transcriptional and physiological responses to abscisic acid (Clement et al. 2011), participation in the photosystem II D1 protein repair cycle (Yokthongwattana et al. 2001) and the maturation of chloroplast proteins in higher plants (Mulo et al. 2012), the function in stress signaling, the cooperation with osmolytes and, of course, the function in protein folding and in preventing aggregation and denaturation (Doubnerova and Ryslava 2014).

NADPH cycling in stressed plants

In general, stresses cause high energy consumption and enhancement of respiration with linked production of ROS

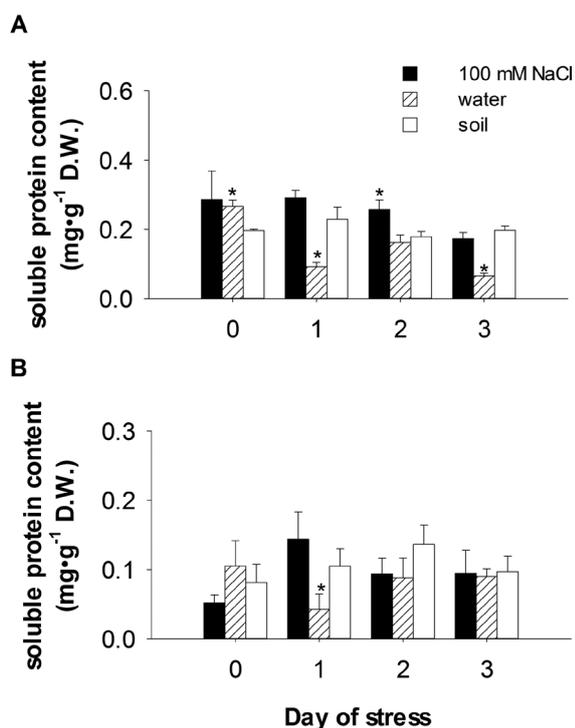


Figure 5. The concentration of soluble proteins in leaves (A) and roots (B) of cucumber plants stressed by salt (100 mM NaCl) and hypoosmotic stress caused by distilled water in comparison with plants grown in soil. * The significant differences from controls grown in soil with $p \leq 0.05$ calculated by t -test. The concentration is related to dry weight (D.W.).

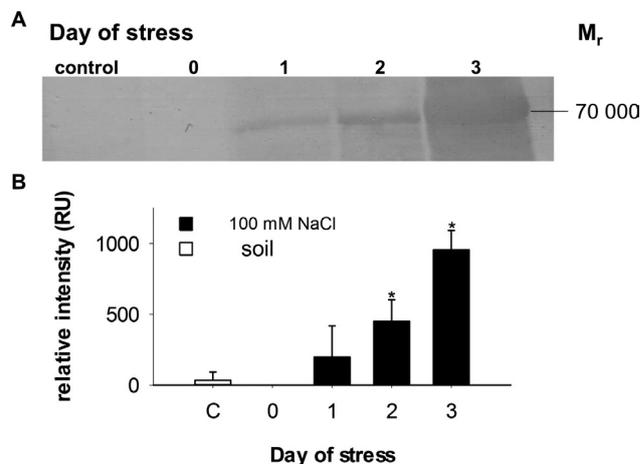


Figure 6. Immunochemical detection (A) of the protein HSP70 in leaves samples of plants exposed to 100 mM salt stress in course of stress compared to control C grown in soil. The equal amount of the soluble proteins (50 μ g) was applied to each line. Three representative blots were analyzed using ImageJ program (B). R.U. means relative units corresponding to peak area after conversion of bands into lane profile plots. * The significant differences in relative intensity from controls grown in soil with $p \leq 0.05$ calculated by t -test.

(De Block et al. 2005). Plasma membrane NADPH-oxidase is considered to be an important producer of ROS (Ma et al. 2012). The depletion of NADPH pools and decreased supplementation of NADPH to antioxidant systems is related not only to an increase in the ROS content but also to protein and lipid peroxidation and to DNA damage (Pétriaccq et al. 2013; Jakubowska et al. 2015).

The main source of NADPH and ATP in plants is light-driven photosynthetic flow. However, the salt stress leads to insufficient electron flow and reduced activity of photosynthetic enzymes (Bartwal et al. 2013).

Estimation of the NADP⁺ content indicated its increase in salt-stressed leaves, whereas the concentration of NADP⁺ in roots tended to decrease, although insignificantly (Fig. 7A,C). Salt stress did not affect the content of NADPH in either leaves or roots (Fig. 7B,D). The decrease in the NADPH/NADP⁺ ratio was also documented for other systems of plant-salt stressor, e. g. for glycophyte plants *Brassica juncea*, for halophyte plants *Sesuvium portulacastrum* L. (Srivastava et al. 2015), for olive plants (Valderrama et al. 2006) and rice suspension cells (Zhang et al. 2013). Hypoosmotic stress induced enhancement of the NADPH content in the roots (Fig. 7D) and, on the other hand, a decrease in NADP⁺ in the leaves (Fig. 7A).

Since salt stress and hypoosmotic stress affected the NADP⁺/NADPH ratio, the activity of NADPH-providing enzymes was studied.

Hypoosmotic stress enhanced the activities of major NADP-dependent enzymes more than salt stress

Immediately following application of hypoosmotic stress, the activity of NADP-dependent enzymes in leaves decreased compared to plants grown in soil. However, after 3 days, a defense response appeared in the form of substantially increased specific activities in hypoosmotically stressed leaves:

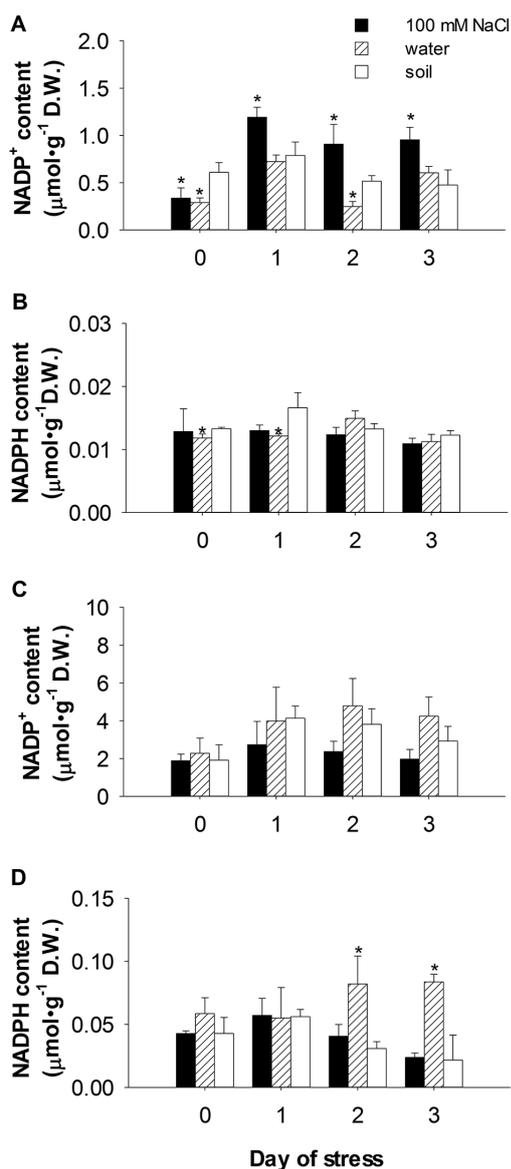


Figure 7. The estimation of NADP⁺ and NADPH concentration in leaves (A, B) and roots (C, D) of cucumber plants stressed by salt (100 mM NaCl) and hypoosmotic stress caused by distilled water in comparison with plants grown in soil. * The significant differences from controls grown in soil with $p \leq 0.05$ calculated by t -test. The concentration is related to dry weight (D.W.).

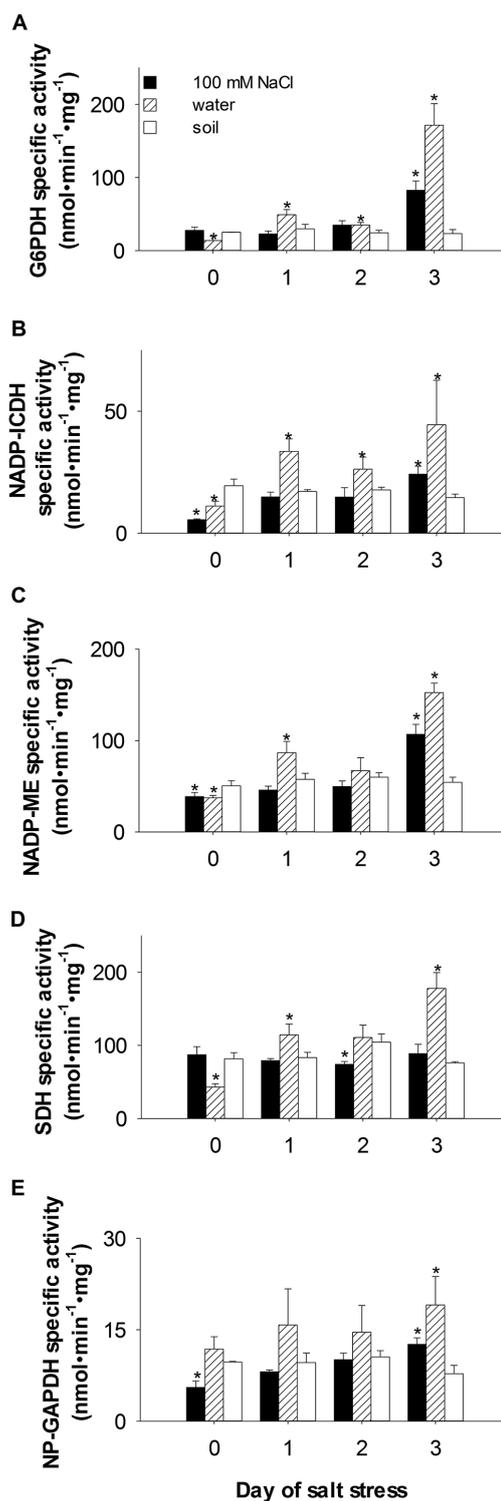


Figure 8. Specific activity of major NADP-dependent enzymes: G6PDH (A), NADP-ICDH (B), NADP-ME (C), SDH (D) and NP-GAPDH (E) in leaves of cucumber plants stressed by salt (100 mM NaCl) and hypoosmotic stress caused by distilled water in comparison with plants grown in soil. * The significant differences from controls grown in soil with $p \leq 0.05$ calculated by t -test.

increases in the activities of G6PDH (7.4-fold), NADP-ME (2.8-fold), NADP-ICDH (3.1-fold), NP-GAPDH (2.5-fold) and SDH (2.3-fold) were observed (Fig. 8). Plants exposed to salt stress for three days exhibited significantly increased specific activities of major NADPH-providing enzymes in leaves compared to non-stressed controls grown in soil, specifically G6PDH (3.5-fold), NADP-ME (2-fold), NADP-ICDH (1.7-fold) and NP-GAPDH (1.6-fold) (Fig. 8). Thus, compared to plants treated with distilled water, 100 mM

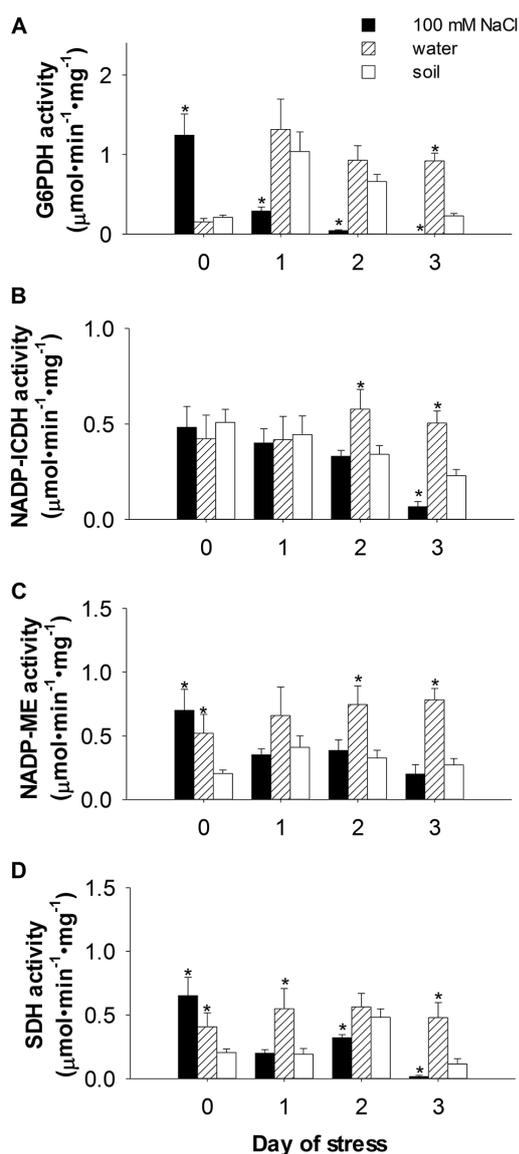


Figure 9. Specific activity of major NADP-dependent enzymes: G6PDH (A), NADP-ICDH (B), NADP-ME (C) and SDH (D) in roots of cucumber plants stressed by salt (100 mM NaCl) and hypoosmotic stress caused by distilled water in comparison with plants grown in soil. * The significant differences from controls grown in soil with $p \leq 0.05$ calculated by t -test.

NaCl caused significantly reduced activities of the above-mentioned NADP enzymes, especially after 3 days of stress (Fig. 8, Table S1 in Supplementary material). The reduced activities of the main NADP-enzymes in the leaves due to salt stress are consistent with increased unutilized NADP^+ content (Fig. 7A). Nevertheless, higher level of NADP^+ can act as an activator of NADP-enzymes such as G6PDH. The specific activity of SDH (enzyme which prefers NADPH to NADP) was mainly unchanged in salt-stressed plants (Fig. 8). However, SDH activities calculated *per* fresh weight increased at the beginning of stress (data not shown). SDH forms shikimate in the shikimate pathway preceding the phenylpropanoid pathway leading to biosynthesis of a wide range of phenylpropanoid compounds with protective and antioxidative properties (Tzin and Galili 2010). It probably participates in the increased content of total phenolics and flavonoids in the course of salt stress (Fig. 3). On the other hand, the pathway from shikimate to phenolic compounds through aromatic amino acids is very complex with different regulation points and thus directing the metabolic flow can cause the accumulation of phenolics.

It could be concluded from previous studies mostly concerning only one enzyme that particular NADP-dependent enzymes such as G6PDH, NADP-ICDH or NADP-ME are related to plant defense responses against abiotic stress (Sun et al. 2003; Chi et al. 2004; Valderrama et al. 2006; Letierrier et al. 2007; Liu et al. 2007a, 2007b; Li et al. 2011; Molina et al. 2011; Cardi et al. 2014; Hyskova Doubnerova et al. 2014; Bouthour et al. 2015). However, a comprehensive study concerning a broader spectrum of NADP-dependent enzymes is still lacking. Various functions of NADP-dependent enzymes during abiotic stress have been suggested (Doubnerova and Ryslava 2012): e.g. the involvement of G6PDH in the regulation of Na^+/H^+ antiporter through providing NADPH for plasma membrane NADPH-oxidase as an adaptation to salt stress (Li et al. 2011), the participation of NADP-ICDH in supplying carbon skeletons for nitrogen assimilation in rice exposed to cold (Lu et al. 2005) or the involvement of NP-GAPDH in anaerobic tolerance under conditions of submergence or in phosphorus deficiency stress (Pillai et al. 2002; Shenoy and Kalagudi 2005). The activity of some of these enzymes could be stabilized by chaperons, such as HSP70, which was demonstrated for NADP-ME (Lara et al. 2005). The significantly higher content of HSP70 in salt-stressed cucumber leaves documented the enhanced demand of this chaperon (Fig. 6).

The increased activities of G6PDH, NADP-ICDH, NADP-ME and SDH were also evident in hypoosmotically stressed roots (Fig. 9). The NADPH content in these roots was pronouncedly increased after 2 and 3 days (Fig. 7D). Compared to both groups of plants grown in soil and in distilled water, the specific activity of major NADP-dependent enzymes in roots is progressively decreased by salt stress

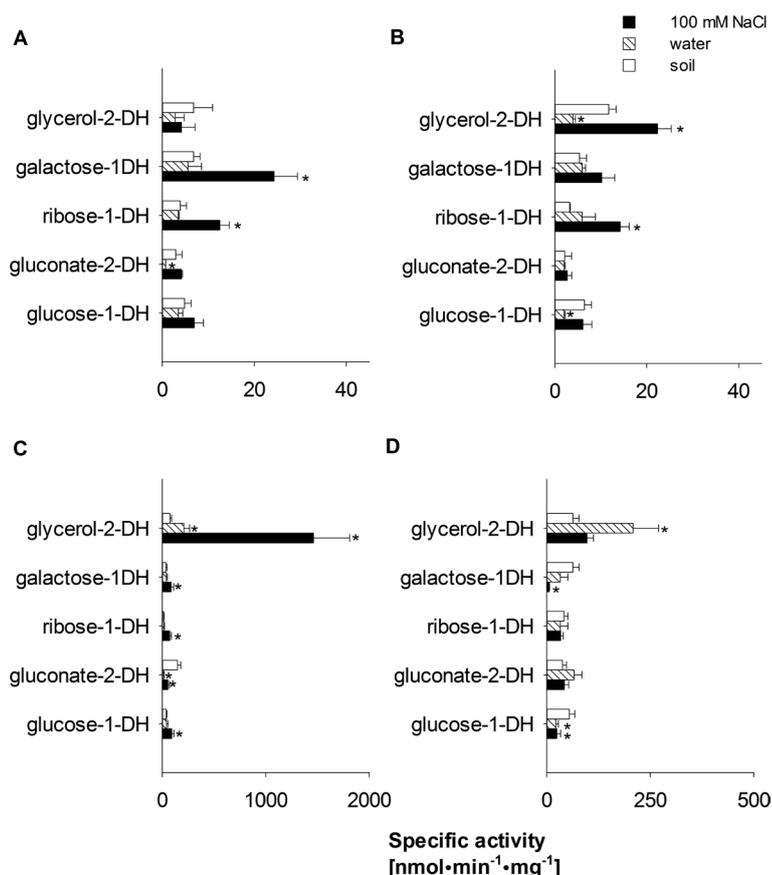


Figure 10. Specific activity of minor NADP-dependent enzymes in leaves (A, B) and roots (C, D) of cucumber plants stressed by salt (100 mM NaCl) and hypoosmotic stress caused by distilled water in comparison with plants grown in soil at the beginning of experiment (A, C) and after 1 day (B, D). * The significant differences from controls grown in soil with $p \leq 0.05$ calculated by *t*-test.

(Fig. 9, Table S1). It seems that the ratio of Na^+/K^+ could be readjusted in leaves in which the metabolic response in the form of increased activity of NADP-dependent enzymes was induced (Figs. 2, 8). The ratios correlate with a study concerned with 17 diverse genotypes of cucumbers under salt stress (Tiwari et al. 2010). On the other hand, strong salt stress exhibits a constantly increasing Na^+/K^+ ratio and a decrease of the activity of NADP-enzymes in roots (Figs. 2, 9). Furthermore, for example, G6PDH and maybe also some other enzymes are differently regulated by thioredoxin in roots and in leaves.

Involvement of less abundant NADP-enzymes in salt and hypoosmotic stress

In addition to major NADP-enzymes, the activity of glucose-1-DH, gluconate-2-DH, ribose-1-DH, galactose-1-DH and glycerol-2-DH was also detectable in cucumber leaves and roots (Fig. 10). Hence the effect of salt and hypoosmotic stress on these enzymes further called minor NADP-dependent enzymes could also be monitored. Whereas the activity of major NADP-dependent enzymes in leaves was increased in a later phase of salt stress, the activity of some

minor NADP-dependent enzymes increased at the beginning of stress, namely ribose-1-DH (3.2- and 4.4-fold higher specific activity than controls grown in soil at the beginning of stress and after 1 day, respectively), galactose-1-DH (up to 3.6-fold increase) and glycerol-2-DH (1.9-fold increase) (Fig. 10A,B). The product of galactose-1-DH, galactonolactone, could be a precursor for the glycolysis intermediate – glyceraldehyde-3-phosphate.

Hypoosmotic stress did not induce the activity of minor NADP-dependent enzymes in leaves (Fig. 10A,B) and roots (Fig. 10C,D) except for glycerol-2-DH. The activities of other minor NADP-enzymes were comparable to plants grown in soil. In cucumber roots, the specific activity of glycerol-2-DH increased in both stressed groups (Fig. 10C,D). This enzyme has been studied in yeasts and filamentous fungi, where it fulfills an important function especially during osmotic stress (Morales et al. 2010). Glycerol-2-DH could be part of a “glycerol cycle”, which is able to regulate the intracellular concentration of glycerol and generate NADPH at the expense of NADH and ATP (Belmans and Vanlaere 1987; Morales et al. 2010).

NADP-dependent enzymes can be an alternative source of NADPH in case of insufficient electron photosynthetic

flow (Corpas and Barroso 2014). With regard to implication of NADP-enzymes, it seems that production of NADPH is part of the defense strategy against hypoosmotic stress in cucumber leaves. In addition to the fact that reducing equivalent NADPH serves as a substrate for NADPH-oxidase and antioxidant systems, it is also an indispensable coenzyme in biosynthetic processes, including biosynthesis of osmotically active compounds, important amino acids, isoprenoids, flavonoids, lignins and fatty acids (Doubnerova and Ryslava 2012). Simultaneously, NADPH is required for NADPH-thioredoxin reductase, the regulatory enzyme that affects numerous cell processes and oxidative stress-response (Marty et al. 2009).

It can be concluded from this study that hypoosmotic stress is milder than salt stress according to the contents of RWC, Na⁺, K⁺, phenolics and Rubisco. On the other hand, hypoosmotic stress caused more enhanced activities of most NADP-dependent enzymes in leaves and roots than salt stress. However, some minor NADP-enzymes such as galactose-1-DH and ribose-1-DH were involved in defense at the beginning of salt stress. NADP-enzymes could participate in NADPH recycling, but these enzyme activities depend on the experimental design, type of stress and plant tissue.

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Supplementary Material

NADP-dependent enzymes are involved in response to salt and hypoosmotic stress in cucumber plants

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Supplementary Table S1. Statistical comparison of plants exposed to 100 mM NaCl (salt) with plants in distilled water (water) and with plants grown in soil (soil)

	Day of stress											
	0			1			2			3		
	Salt/soil	Salt/water	Water/soil	Salt/soil	Salt/water	Water/soil	Salt/soil	Salt/water	Water/soil	Salt/soil	Salt/water	Water/soil
RWC	↓0.002	↓0.002	N0.905	↓0.002	↓0.005	N0.300	↓≤0.001	↓0.002	N0.420	↓≤0.001	↓0.017	↓0.004
Na ⁺ leaf	N0.548	N0.850	N0.297	↑≤0.001	↑≤0.001	N0.082	↑≤0.001	↑≤0.001	N0.469	↑0.002	↑0.003	N0.497
Na ⁺ root	↑0.001	↑0.005	N0.706	↑0.003	↑0.007	N0.101	↑0.004	↑0.026	N0.132	↑≤0.001	↑0.011	N0.154
K ⁺ leaf	N0.284	N0.560	N0.445	N0.196	N0.814	N0.200	N0.347	N0.041	N0.077	↓0.038	N0.168	N0.504
K ⁺ root	N0.875	N0.244	N0.483	↓0.009	N0.194	N0.833	↓≤0.001	↓0.005	N0.141	↓0.001	↓≤0.001	N0.329
Phenolics	↑0.043	N0.137	↑0.021	↑0.036	↑≤0.001	↓0.038	↑0.003	N0.475	↑0.001	↑0.001	↑0.006	N0.064
Flavonoids	↑0.031	N0.089	N0.317	N0.062	↑0.042	N0.725	↑0.002	N0.051	↑0.001	↑0.005	↑0.033	N0.614
Rubisco	N0.865	N0.184	N0.191	N0.365	N0.091	N0.250	↓0.023	↓0.004	N0.089	↓0.005	↓0.003	N0.652
Proteins leaf	N0.132	N0.705	↑0.003	N0.060	↑≤0.001	↓0.003	↑0.012	↑0.009	N0.344	N0.130	↑≤0.001	↓≤0.001
Proteins root	N0.148	N0.072	N0.409	N0.218	↑0.017	↓0.032	N0.108	N0.791	N0.103	N0.925	N0.824	N0.652
NADP leaf	↓0.035	N0.536	↓0.008	↑0.017	↑0.003	N0.523	↑0.035	↑0.006	↓0.004	↑0.016	↑0.015	N0.267
NADPH leaf	N0.844	N0.638	↓0.017	N0.069	N0.209	↓0.034	N0.330	N0.061	N0.130	N0.109	N0.650	N0.267
NADP root	N0.962	N0.484	N0.609	N0.156	N0.372	N0.900	N0.065	N0.053	N0.365	N0.151	↓0.026	N0.148
NADPH root	N0.998	N0.099	N0.201	N0.912	N0.897	N0.938	N0.186	↓0.040	↑0.018	N0.858	↓≤0.001	↑0.007
G6PDH leaf	N0.370	↑0.030	↓0.029	N0.179	↓0.005	↑0.022	N0.056	N0.998	↑0.022	↑0.002	↓0.009	↑0.001
NADP-ICDH leaf	↓≤0.001	↓0.009	↓0.013	N0.149	↓0.004	↑0.005	N0.271	↓0.034	↑0.044	↑0.009	N0.130	↑0.047
NADP-ME leaf	↓0.046	N0.681	↓0.021	N0.061	↓0.006	↑0.023	N0.087	N0.126	N0.456	↑0.002	↓0.006	↑≤0.001
SDH leaf	N0.511	↑0.003	↓0.002	N0.409	↓0.015	↑0.030	↓0.012	↓0.021	N0.611	N0.158	↓0.004	↑0.001
NP-GAPDH leaf	↓0.002	↓0.009	N0.149	N0.182	N0.089	N0.157	N0.623	N0.161	N0.196	↑0.009	N0.080	↑0.016
G6PDH root	↑0.003	↑0.002	N0.118	↓0.007	↓0.010	N0.346	↓≤0.001	↓0.001	N0.084	↓≤0.001	↓≤0.001	↑0.001
NADP-ICDH root	N0.750	N0.563	N0.354	N0.569	N0.848	N0.780	N0.773	↓0.017	↑0.023	↓0.003	↓≤0.001	↑0.003
NADP-ME root	↑0.007	N0.233	↑0.022	N0.373	N0.081	N0.150	N0.366	↓0.020	↑0.010	N0.238	↓≤0.001	↑≤0.001
SDH root	↑0.006	N0.079	↑0.037	N0.817	↓0.020	↑0.020	↓0.017	↓0.019	N0.327	↓0.018	↓0.002	↑0.007
Glucose-1-DH leaf	N0.213	N0.055	N0.230	N0.836	↑0.021	↓0.008	ND	ND	ND	ND	ND	ND
Gluconate-2-DH leaf	N0.197	↑≤0.001	↓0.049	N0.610	N0.269	N0.863	ND	ND	ND	ND	ND	ND
Ribose-1-DH leaf	↑0.004	↑0.002	N0.631	↑≤0.001	↑0.015	N0.180	ND	ND	ND	ND	ND	ND
Galactose-1-DH leaf	↑0.005	↑0.005	N0.544	N0.063	N0.070	N0.588	ND	ND	ND	ND	ND	ND
Glycerol-2-DH leaf	N0.418	N0.537	N0.202	↑0.006	↑≤0.001	↓0.001	ND	ND	ND	ND	ND	ND
Glucose-1-DH root	↑0.014	↑0.031	N0.727	↓0.031	N0.737	↓0.018	ND	ND	ND	ND	ND	ND
Gluconate-2-DH root	↓0.008	↑0.010	↓0.002	N0.600	N0.130	N0.083	ND	ND	ND	ND	ND	ND
Ribose-1-DH root	↑0.003	↑0.004	N0.407	N0.251	N0.971	N0.487	ND	ND	ND	ND	ND	ND
Galactose-1-DH root	↑0.032	↑0.039	N0.713	↓0.003	N0.062	N0.092	ND	ND	ND	ND	ND	ND
Glycerol-2-DH root	↑0.002	↑0.004	↑0.016	N0.052	↓0.038	↑0.016	ND	ND	ND	ND	ND	ND

Pearson's coefficients, which correspond to data in Fig. 1-10, are presented. Symbols ↑, ↓ and N indicate a decrease, an increase or no change of particular parameter. DH, dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; NADP-ICDH, NADP-isocitrate dehydrogenase; NADP-ME, NADP-dependent malic enzyme; NP-GAPDH, non-phosphorylating glyceraldehyde-phosphate dehydrogenase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RWC, relative water content; SDH, shikimate dehydrogenase. ND means not determined.