Acta Physiol Plant (2016) 38:161
DOI 10.1007/s11738-016-2181-z

ORIGINAL ARTICLE

Humic acid protects barley against salinity

Markéta Jarošová[1] · Bořivoj Klejdus[1,2] · Jozef Kováčik[1,2] · Petr Babula[3] · Josef Hedbavny[1]

Received: 27 April 2016 / Revised: 12 May 2016 / Accepted: 25 May 2016 / Published online: 2 June 2016

Abstract Barley (*Hordeum vulgare*, cv. Radegast) plants cultured in Hoagland solution were exposed to NaCl and/or humic acid (HA) for 7 days. Plants revealed relatively high sensitivity to NaCl (100 mM), which was manifested by a considerable decline in growth, tissue water depletion, and high sodium accumulation. HA typically increased the content of organic metabolites (syringic acid, alanine, proline, ascorbic acid, glutathione, and phytochelatin 2), NaCl evoked the opposite effect (not for proline), and the combined treatment (NaCl + HA) showed mostly the positive impact of HA. However, these responses differed between shoot and root tissues. Salinity, but not HA, depleted the Krebs cycle acids (except for succinic acid). Salinity induced ROS formation, and HA reversed these symptoms, as evidenced by fluorescence microscopy. Changes of nitric oxide level were also detected. HA suppressed the NaCl-induced increase in Na, while the impact on other nutrients was not extensive. Moreover, foliar and hydroponic HA application revealed similar mitigating effects on NaCl stress. Overall, these data indicate the potential of HA to protect barley against NaCl stress by limiting Na uptake and positively impacting amount of some metabolites.

Keywords Cereals · HPLC–MS/MS · Osmotic stress · Phenolic acids · Salinization

Communicated by O. Ferrarese-Filho.

✉ Jozef Kováčik
jozkovacik@yahoo.com

[1] Institute of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University in Brno, Zemědělská 1, 613 00 Brno, Czech Republic

[2] CEITEC, Central European Institute of Technology, Mendel University in Brno, Zemědělská 1, 613 00 Brno, Czech Republic

[3] Department of Physiology, Faculty of Medicine, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic

Introduction

Salinity is one of the major abiotic stressors limiting agricultural production and plant distribution with NaCl as the predominant salt (Jin et al. 2009). Many crop species are sensitive to the presence of NaCl (Szalai and Janda 2009). The responses of plants to excess NaCl are very complex and involve changes in morphology and metabolism (Kováčik et al. 2009; Yousfi et al. 2010). Also, elevated NaCl evokes the formation of reactive oxygen species (ROS) depending not only on the developmental stage and plant species/organ (Jamal et al. 2011; Radl et al. 2013; Jabeen et al. 2015) but also on the mode of NaCl application (Spanò and Bottega 2016).

Barley (*H. vulgare* L.) is widely cultured in arid and semiarid regions. It is considered a moderately tolerant forage crop and a highly tolerant grain crop, but a great variability exists among cultivars in response to salinity (Jin et al. 2009; Kamboj et al. 2015).

Soil quality may be influenced by organic materials, which improves its chemical, physiological, and biological properties (Khaled and Fawy 2011). Humin, humic, and fulvic acids, commonly known as humic substances, are the main organic soil compounds.

Despite extensive research, the metabolic response of plants to either increased salinity or humic substances have only rarely been reported. The aim of our work was to study the impact of humic acid (HA) on the metabolic

response of barley exposed to salinity stress. To achieve this, selected metabolites were quantified by combined liquid chromatography/mass spectrometry, and fluorescence microscopy was used aimed at visualizing changes of ROS and nitric oxide in root tips. Foliar and hydroponic applications of HA were also compared.

Materials and methods

Plant culture and experimental design

Barley (*Hordeum vulgare* L., cv. Radegast) grains were surface-sterilized in 70 % ethanol, rinsed in deionised water, and germinated in Petri dishes on wet filter paper for 72 h in a growth chamber. Thereafter, uniform seedlings were placed in 1/10-strength Hoagland solution in dark plastic boxes (Kováčik et al. 2014a) and 7 days-old ones were exposed to either 100-mM NaCl, 6.0-mg HA/L (technical humic acid, Sigma-Aldrich, Cat. No. 53680) or a combination (NaCl + HA). Also, foliar application of HA (6.0 mg/L) to plants cultured with 100-mM NaCl was tested by applying 5 mL of test solution per shoot daily (Triton X-100, 0.1 mL/L, was added to the stock solution to increase solubility, and the hydroponics was protected from contamination by droplets). The dose of HA was selected based on the preliminary experiments (impact of HA on Na accumulation, data not shown). Control plants were cultured in Hoagland solution with no additions. Unless otherwise specified, plant material was harvested 7 days after treatment, but time dynamics of some antioxidants was monitored (1, 3 and 7 days of exposure), and selected metabolites were measured in the shoots only. All experiments were performed in a growth chamber under conditions as reported earlier (Kováčik et al. 2014a). Spectrophotometry was carried out with Agilent/HP DAD UV/Vis 8453 spectrophotometer. Metabolites were quantified by liquid chromatography with mass spectrometry detection (LC–MS/MS) using device Agilent 1200 Series Rapid Resolution LC system coupled on-line to Agilent 6460 Triple quadrupole detector with Agilent Jet Stream Technologies. Fluorescence microscopy was performed by Axioscop 40 (Carl Zeiss, Germany) equipped with appropriate set of excitation/emission filters (Kováčik et al. 2014a).

Measurement of growth, tissue water content, soluble proteins, and free amino acids

After 7 days of exposure to treatments, plants were harvested, separated into roots and shoots, and dried. Their length, fresh, and dry mass were also recorded. For parameters measured in fresh samples, whole shoots or roots were powdered using liquid N_2 and assayed as described below. Plant water content [100 − (dry mass × 100/fresh mass)] was determined to recalculate parameters measured in fresh samples. Dry samples (dried at 75 °C to constant weight) were ground to a fine powder and analyzed for mineral nutrients, free amino acids, and phenolic acids. Soluble proteins were quantified using Bradford method and bovine serum albumin as standard (in extracts prepared using 50-mM potassium phosphate buffer) and free amino acids by HPLC (Hewlett Packard, Waldbronn, Germany) with fluorometric detector FLD HP 1100 and precolumn derivatization as reported earlier (Kováčik et al. 2014a).

Assay of antioxidants and antioxidative enzymes

Extraction of antioxidants (ascorbic acid—AsA, reduced glutathione—GSH, phytochelatin 2—PC2) was done in 0.1 M HCl (fresh biomass) followed by centrifugation, and the supernatant from these crude extracts was analyzed by LC–MS/MS system at specific *m/z* values (Kováčik et al. 2014b). Activities of antioxidative enzymes were determined in potassium phosphate buffer extracts mentioned above. Ascorbate peroxidase (APX), glutathione reductase (GR), catalase (CAT), and superoxide dismutase (SOD) activities were evaluated spectrophotometrically (Kováčik et al. 2014a, b, 2015).

Determination of phenolic and organic acids

Selected cinnamic and benzoic acid derivatives from dried shoots only were extracted in 80 % methanol, while aliphatic organic acids were quantified in the 0.1-M HCl extracts from fresh samples mentioned above; identification and quantification was performed by LC–MS/MS system using specific *m/z* values and available standards (Kováčik et al. 2014a, 2015).

Quantification of mineral nutrients

Mineral nutrients (Na, K, Ca, Mg, Fe and Zn) were determined by atomic absorption spectrometer AA30 (Varian Ltd., Mulgrave, Australia) and the air-acetylene flame. Samples were prepared by digestion of dried samples in the mixture of concentrated ultrapure HNO_3 and water using microwave decomposition (Ethos Sel Microwave Extraction Labstation, Milestone Inc.) as noted earlier (Kováčik et al. 2014b, 2015).

Fluorescence microscopy

Only root tips were observed owing to their direct contact with the culture medium. They were excised from similar

positions and immediately stained. ROS were visualized using CellROX® Deep Red Reagent ($644_{ex}/665_{em}$) and nitric oxide (NO) using 2,3-diaminonaphthalene ($365_{ex}/415_{em}$). Stock solution of CellROX® Deep Red Reagent in DMSO was diluted by phosphate buffered saline (PBS) buffer (0.05 M, pH 6.8) to final concentration of 5 μM, while stock solution of 2,3-diaminonaphthalene in 0.62 M HCl was diluted by PBS buffer to the final concentration of 250 μM. Root tips were stained for 60 min at 37 °C in the dark, washed three times with PBS buffer, and observed (Kováčik et al. 2014a, b).

Statistical analyses

Homogeneity of variance was verified by Levene's test, and data were evaluated using ANOVA followed by Tukey's test (MINITAB Release 11, Minitab Inc.; State College, PA, USA) at $P < 0.05$. Number of replications (n) in tables/figures denotes individual plants measured for each parameter. At least three boxes were cultured for each treatment.

Results and discussion

Effect of HA and NaCl on physiology and mineral content of barley plants

In accordance with previous findings in *Triticum aestivum* grown for 55 days with 80- or 120-mM NaCl (Jamal et al. 2011), we found that 100-mM NaCl decreased shoot and root length compared with control plants (Table 1). Protective effect of humic acid has been demonstrated in many cereals, e.g., in maize and wheat under salt stress (Khaled and Fawy 2011; Aydin et al. 2012). Our results showed that HA was able to relieve the growth inhibition induced by NaCl in shoots but not in roots (Table 1). Only shoot water content was affected by salinity (Table 1) as previously reported in barley (Yildiz and Terzi 2013). HA altered the water content neither alone nor in combination with NaCl (Table 1), which could be related to a lower HA dose used in this experiment in comparison with other works (Khaled and Fawy 2011). Soluble protein content was unaffected by NaCl or HA, and only foliar HA was able to compensate

Table 1 Shoot and root length, water, soluble protein, and mineral nutrients content in barley (*H. vulgare*, cv. Radegast) plants after 7 days of treatments, as mentioned in Fig. 1

	Control	NaCl	HA	NaCl + HA	NaCl + HA/foliar
Shoots					
Length (cm)	19.37 ± 1.90ab	12.23 ± 0.42c	21.00 ± 0.66a	16.23 ± 0.66b	18.03 ± 0.45ab
Water content (%)	89.18 ± 0.27a	86.29 ± 0.49b	88.83 ± 1.04a	85.17 ± 0.70b	86.06 ± 1.33b
Soluble proteins (mg g^{-1} DW)	14.95 ± 0.71a	17.09 ± 0.99a	16.06 ± 1.38a	17.96 ± 0.50a	17.95 ± 2.12a
Na (mg g^{-1} DW)	0.409 ± 0.063d	48.88 ± 2.34a	0.838 ± 0.081c	22.71 ± 3.36b	22.90 ± 3.05b
K (mg g^{-1} DW)	4.07 ± 0.85a	2.03 ± 0.64b	3.65 ± 0.40a	1.94 ± 0.21b	2.01 ± 0.22b
Ca (mg g^{-1} DW)	0.516 ± 0.036a	0.317 ± 0.025b	0.625 ± 0.068a	0.260 ± 0.076b	0.275 ± 0.021b
Mg (mg g^{-1} DW)	2.17 ± 0.19ab	1.58 ± 0.10c	2.42 ± 0.21a	1.39 ± 0.48c	1.57 ± 0.45bc
Fe (mg g^{-1} DW)	0.101 ± 0.001a	0.066 ± 0.002b	0.084 ± 0.007ab	0.064 ± 0.003b	0.070 ± 0.002b
Zn (mg g^{-1} DW)	0.055 ± 0.002a	0.036 ± 0.008b	0.035 ± 0.009b	0.029 ± 0.002b	0.028 ± 0.006b
Roots					
Length (cm)	12.80 ± 0.36a	7.20 ± 0.60b	13.37 ± 0.35a	7.80 ± 0.40b	8.07 ± 0.42b
Water content (%)	92.96 ± 0.12a	90.01 ± 0.49a	93.58 ± 0.76a	93.21 ± 4.76a	90.37 ± 0.28a
Soluble proteins (mg g^{-1} DW)	4.34 ± 0.27ab	3.80 ± 0.61b	4.43 ± 0.70ab	4.64 ± 0.25ab	5.06 ± 0.27a
Na (mg g^{-1} DW)	3.28 ± 0.32c	20.35 ± 3.70a	2.93 ± 0.74c	11.47 ± 2.09b	14.85 ± 0.84b
K (mg g^{-1} DW)	2.99 ± 0.76a	1.24 ± 0.04b	4.16 ± 0.88a	0.393 ± 0.054c	0.434 ± 0.046c
Ca (mg g^{-1} DW)	0.749 ± 0.055a	0.561 ± 0.087b	0.565 ± 0.016b	0.588 ± 0.070b	0.335 ± 0.041c
Mg (mg g^{-1} DW)	1.54 ± 0.37a	0.439 ± 0.036c	1.96 ± 0.015a	0.737 ± 0.076b	0.587 ± 0.029bc
Fe (mg g^{-1} DW)	2.92 ± 0.74b	3.21 ± 0.52b	2.48 ± 0.29b	5.05 ± 0.25a	4.33 ± 0.51ab
Zn (mg g^{-1} DW)	0.046 ± 0.011c	0.048 ± 0.007bc	0.071 ± 0.010ab	0.067 ± 0.009bc	0.093 ± 0.011a

Data are means ± SDs ($n = 10$ for length and water content and $n = 6$ for other parameters). Values within rows, followed by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$)

for its decrease induced by NaCl in roots (Table 1). In comparison, more extensive depletion of soluble proteins was observed in salt-tolerant *T. aestivum* roots exposed to 80-mM NaCl over 16 days (Radl et al. 2013).

As might be expected, Na strongly accumulated in both shoots and roots of barley after the addition of NaCl (ca. 100- and 7-times more when compared with control; Table 1), which is in accordance with data from various barley cultivars exposed to 150-mM NaCl (Kamboj et al. 2015). It was an interesting finding that HA reduced Na accumulation in both above- and below-ground tissues (Table 1). This is in contrast with data from corn plants where the level of Na was elevated after exposure to 60-mM NaCl with foliar application of 0.1 % HA (Khaled and Fawy 2011).

The decrease in K^+ content under salinity detected in barley tissues (Table 1) may be related to the apparent restriction in elongation growth owing to K^+ importance for many biochemical processes. Besides, NaCl also negatively affected the accumulation of Ca, Fe, and Zn but decreased Mg content in barley roots only. This negative effect on mineral nutrient uptake was observed, e.g., in cultivars of *Triticum durum* exposed to 60-mM NaCl for 35 days (Aşik et al. 2009). Compared with accumulation of Na, the interaction between NaCl and HA did not affect majority of macro- and micronutrients in the present study (Table 1).

Effect of HA on metabolic responses in NaCl-exposed barley

Amount of free amino acids in barley plants exposed to NaCl revealed that NaCl significantly decreased the content of Asp, Gly, Thr, Arg, and Ala (Table 2). Consequently, NaCl depleted the total amino acid amount except for histidine and proline: strong decrease in total shoot amino acid content was also observed in barley plants at 100-mM NaCl (Yousfi et al. 2010). In the present study, HA has an elevating effect on the majority of monitored free amino acids except for His, Tyr, Val, Leu, and Ile (Table 2). Interaction between HA and NaCl elevated the accumulation of Asp, Glu, Gly, and Ala. Moreover, foliar unlike to hydroponic application enhanced the content of phenylalanine, which is a crucial precursor of the phenylpropanoid pathway. Proline is known to be involved in osmoregulation, acts as a free radical scavenger (Yildiz and Terzi 2013) and is typically elevated by NaCl in various species (Haddadi et al. 2016). In both combined treatments, amount of proline was higher compared with HA alone but lower in comparison with NaCl. These results indicate that

Table 2 Accumulation of free amino acids (μmol g^{-1} DW) in barley (*H. vulgare*, cv. Radegast) shoots after 7 days of treatments, as mentioned in Fig. 1

	Control	NaCl	HA	NaCl + HA	NaCl + HA/foliar
Aspartic acid	3.34 ± 0.14c	2.12 ± 0.09d	6.62 ± 0.27a	4.74 ± 0.20b	4.38 ± 0.12b
Glutamic acid	1.24 ± 0.22c	0.961 ± 0.001c	3.95 ± 0.10a	3.84 ± 0.09a	3.46 ± 0.05b
Serine	2.83 ± 0.32b	2.26 ± 0.22b	4.93 ± 0.32a	2.35 ± 0.27b	2.30 ± 0.16b
Histidine	0.083 ± 0.001b	0.138 ± 0.005a	0.086 ± 0.007b	0.127 ± 0.020a	0.115 ± 0.011ab
Glycine	0.505 ± 0.065c	0.331 ± 0.039d	0.843 ± 0.024a	0.644 ± 0.029b	0.629 ± 0.005b
Threonine	2.45 ± 0.08b	1.25 ± 0.11d	4.12 ± 0.20a	1.41 ± 0.13d	1.84 ± 0.05c
Arginine	0.475 ± 0.014b	0.407 ± 0.011c	0.574 ± 0.021a	0.447 ± 0.032bc	0.410 ± 0.003c
Alanine	12.6 ± 0.72d	9.20 ± 1.33e	22.91 ± 0.59a	16.81 ± 0.19c	19.06 ± 0.37b
Tyrosine	0.623 ± 0.034a	0.517 ± 0.046ab	0.612 ± 0.023a	0.517 ± 0.042ab	0.416 ± 0.061b
Cysteine	0.570 ± 0.070b	0.554 ± 0.029b	0.818 ± 0.038a	0.532 ± 0.046b	0.615 ± 0.014b
Valine	2.11 ± 0.23ab	1.87 ± 0.07b	2.38 ± 0.04a	1.91 ± 0.02b	2.23 ± 0.09a
Methionine	n.d.	0.050 ± 0.004a	0.052 ± 0.007a	0.046 ± 0.003a	0.052 ± 0.010a
Phenylalanine	0.571 ± 0.030b	0.559 ± 0.018b	0.734 ± 0.057a	0.557 ± 0.083b	0.807 ± 0.045a
Isoleucine	0.745 ± 0.059a	0.663 ± 0.132a	0.829 ± 0.100a	0.689 ± 0.053a	0.836 ± 0.080a
Leucine	1.28 ± 0.10ab	1.06 ± 0.09b	1.46 ± 0.17a	1.49 ± 0.13a	1.18 ± 0.08ab
Lysine	0.262 ± 0.043ab	0.221 ± 0.018b	0.352 ± 0.046a	0.324 ± 0.020a	0.361 ± 0.019a
Proline	0.962 ± 0.177d	18.4 ± 1.33a	2.87 ± 0.16c	10.60 ± 0.54b	11.00 ± 0.59b
Total	30.66 ± 0.42d	40.59 ± 1.71c	54.09 ± 0.58a	47.02 ± 0.43b	49.68 ± 0.60b

Data are means ± SDs ($n = 6$). Values within rows, followed by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$)

Table 3 Accumulation of phenolic acids (μg g^{-1} DW) and organic acids (mg g^{-1} DW) in barley (*H. vulgare*, cv. Radegast) shoots after 7 days of treatments, as mentioned in Fig. 1

	Control	NaCl	HA	NaCl + HA	NaCl + HA/foliar
Phenolic acids					
Ferulic acid	2.24 ± 0.33a	2.34 ± 0.31a	2.01 ± 0.16a	2.60 ± 0.38a	2.10 ± 0.10a
Galic acid	1.68 ± 0.17ab	1.07 ± 0.31b	2.11 ± 0.18a	1.46 ± 0.02b	1.85 ± 0.14ab
p-coumaric acid	2.78 ± 0.19a	1.42 ± 0.08b	2.32 ± 0.22ab	2.40 ± 0.09ab	2.65 ± 0.19ab
*p*OHbenzaldehyde	1.86 ± 0.06a	1.43 ± 0.10b	1.50 ± 0.16b	1.52 ± 0.15b	1.70 ± 0.05ab
*p*OHbenzoic acid	2.93 ± 0.21a	0.742 ± 0.023d	2.39 ± 0.17b	1.47 ± 0.12c	1.20 ± 0.11c
Protocatechuic acid	4.85 ± 0.56a	1.28 ± 0.39c	2.14 ± 0.21b	1.94 ± 0.11bc	2.05 ± 0.34b
Salicylic acid	1.92 ± 0.41a	0.894 ± 0.097c	1.57 ± 0.06ab	1.35 ± 0.27bc	1.35 ± 0.39ab
Sinapic acid	5.82 ± 0.85ab	1.32 ± 0.45c	6.02 ± 0.25a	4.86 ± 0.39b	4.95 ± 0.31b
Syringic acid	0.126 ± 0.025d	0.534 ± 0.078c	0.820 ± 0.043ab	0.977 ± 0.133a	0.701 ± 0.062b
Vanilic acid	7.45 ± 0.43a	4.18 ± 0.79c	6.29 ± 0.57ab	5.47 ± 0.48b	4.48 ± 0.18c
Vanilin	3.18 ± 0.63ab	3.26 ± 0.59ab	3.88 ± 0.68a	3.09 ± 0.22ab	2.64 ± 0.40b
Total phenolic acids	35.24 ± 1.19a	18.52 ± 1.10c	30.76 ± 1.61ab	27.11 ± 1.47b	25.84 ± 1.23b
Organic acids					
Citric acid	11.5 ± 1.17a	1.74 ± 0.11d	7.22 ± 0.56b	5.38 ± 0.24c	6.67 ± 0.40b
Fumaric acid	0.127 ± 0.003a	0.077 ± 0.004c	0.096 ± 0.005b	0.097 ± 0.015b	0.098 ± 0.003b
α-Ketoglutaric acid	n.d.	n.d.	n.d.	n.d.	n.d.
Lactic acid	0.030 ± 0.002c	0.026 ± 0.002c	0.106 ± 0.017a	0.048 ± 0.005b	0.058 ± 0.005b
Malic acid	2.11 ± 0.15a	1.13 ± 0.35b	1.20 ± 0.24b	1.35 ± 0.27b	1.26 ± 0.34b
Pyruvic acid	11.90 ± 0.84a	0.044 ± 0.004bc	0.053 ± 0.007b	0.035 ± 0.002c	0.056 ± 0.004b
Quinic acid	1.58 ± 0.44ab	0.241 ± 0.011c	2.19 ± 0.34a	1.73 ± 0.20ab	1.51 ± 0.08b
Succinic acid	0.106 ± 0.013b	0.423 ± 0.021a	0.107 ± 0.021b	0.383 ± 0.031a	0.127 ± 0.014b

Data are means ± SDs ($n = 6$). Values within rows, followed by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$)

n.d. not detected

proline appears to be related to salt tolerance as its content changed similarly to shoot Na amount (Table 1).

Among 11 monitored phenolic derivatives, NaCl depleted the content of *p*-hydroxybenzaldehyde, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, salicylic, sinapic, and vanilic acids in the shoot (Table 3). These data are in contrast with the results found in other plants, such as chamomile (Kováčik et al. 2009), in which phenolic acids rather increased. These differences might be related to given species and experimental conditions. HA coapplied with NaCl had a significant ameliorating effect on some acids and their sum when compared with NaCl-treated plants (Table 3): this could play a role in diminishing the oxidative stress. Other phenols may also be involved in the protection against salinity, for example, the expression of some flavonoid genes along with the accumulation of metabolites was detected in *Solanum nigrum* exposed up to 150-mM NaCl over 3 weeks (Ben Abdallah et al. 2016).

Salt treatment induced a decrease in the majority of organic acids, except for succinic acid, in shoot tissue (Table 3). Slight accumulation of succinic acid was also observed in NaCl-treated *Arabidopsis thaliana*: γ-aminobutyric acid metabolism seems to be a source of succinate mobilized under stress conditions to help in buffering the tricarboxylic acid cycle (Renault et al. 2013). Depletion of the majority of monitored organic acids, such as citric, fumaric, and malic acids, indicates a deleterious impact on respiration. It may lead to the inhibition of cellular respiration and, consequently, to disruption of shoot development. Addition of HA to the culture medium evoked a decrease in citric, malic, and pyruvic acids but enhanced lactic acid accumulation which is evidence of induction of fermentative processes. However, HA coapplied with NaCl stimulated mainly citric acid accumulation (compared with NaCl alone), which is further evidence supporting a positive effect of HA against salinity.

HA modulates NaCl-induced oxidative stress and antioxidants

The most intensive increase in ROS was observed under the NaCl treatment (Fig. 1). Application of HA alone had a

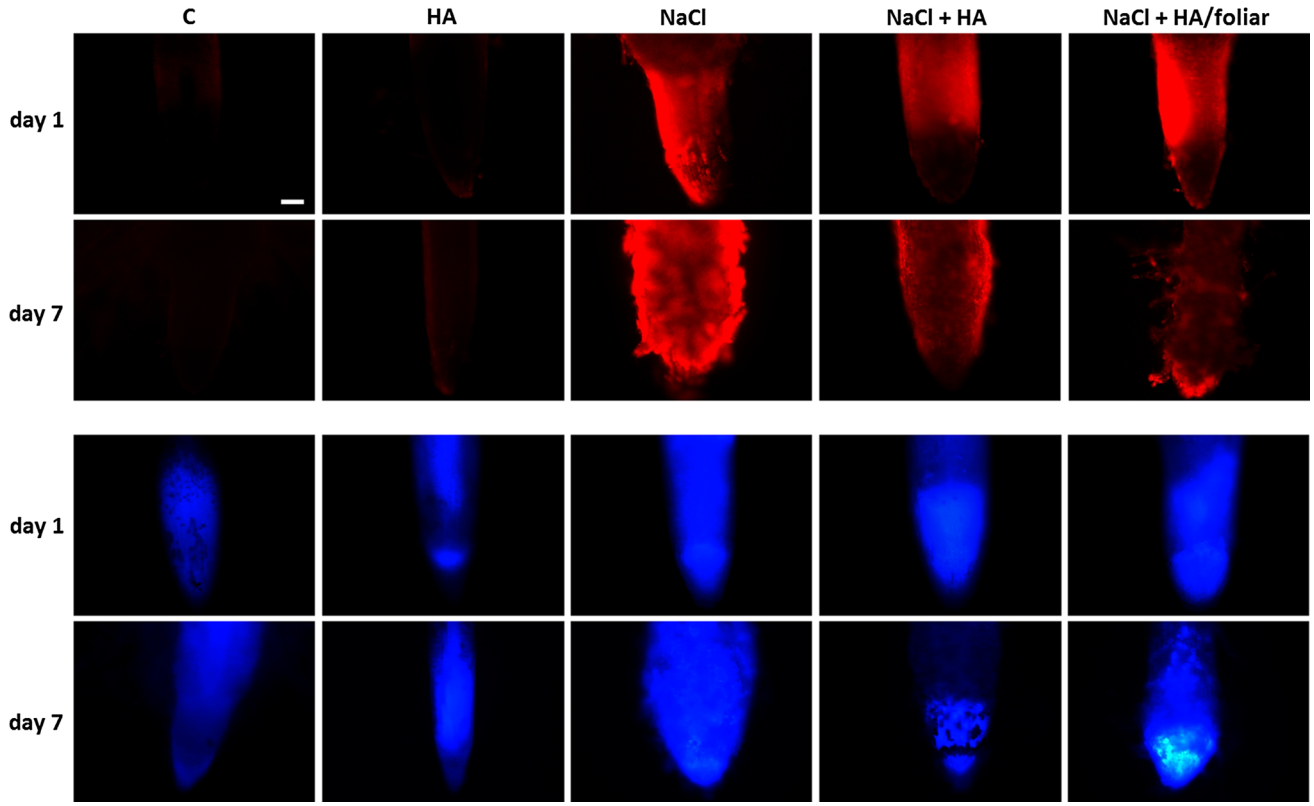

Fig. 1 Fluorescence microscopy of reactive oxygen species (CellROX® Deep Red Reagent, *red* signal) and nitric oxide (2,3-diaminonaphthalene, *blue* signal) in *H. vulgare* root tips cv. Radegast exposed to NaCl (100 mM), humic acid (HA, 6.0 mg/L), NaCl + humic acid applied hydroponically (NaCl + HA), or NaCl + foliar application of humic acid (NaCl + HA/foliar) over 1 or 7 days. *C* control, *bar* indicates 100 µm. *HA* technical humic acid (Sigma-Aldrich, Cat. No. 53680)

negligible impact on ROS generation, while HA in combination with NaCl attenuated ROS formation in particular in the treatment with foliar HA application (Fig. 1). NO formation was also visibly affected by HA coapplication mainly after 7 days of exposure and in the apical part of root tips—ROS were suppressed, but NO enhanced (Fig. 1). Changes of NO evoked by HA may, therefore, be involved in the protective effect of HA observed in our study, and further experiments with the manipulation of NO level are needed.

Salinity induced a time-dependent decrease in the GSH and AsA content in barley shoots (Fig. 2): this contrasts with report that NaCl-induced an increase in AsA content in barley leaves cultured under 160-mM NaCl (Pérez-López et al. 2010). In the roots, NaCl-induced depletion of AsA or GSH (if any) was less visible. HA alone had either no effect or stimulated an increase in AsA or GSH amount time-dependently. In the combined treatments, foliar and hydroponic HA coapplication (with NaCl) showed a typically similar impact in the shoots (Fig. 2a) and rather stimulated AsA or GSH accumulation (in comparison with NaCl alone) in the roots (Fig. 2b). A more pronounced impact of HA coapplication in the roots could be related to the above-mentioned attenuation of the ROS formation (see Fig. 1). Phytochelatin (PCs) accumulation in humate-treated plants has only rarely been reported (Figueroa et al. 2007). In the present study, NaCl caused a time-dependent decrease in PC2 content in barley shoots and roots (Fig. 2). This contradicts the data from *Cajanus cajan* roots, where the level of PC2 was not affected after 60 days with 60-mM NaCl (Garg and Chandel 2012). Surprisingly, HA applied alone to the culture medium induced the biosynthesis of PC2 in roots (Fig. 2), which could be related to the ability of HA to stimulate mineral nutrients uptake through eventual formation of PC2-metal complexes (Garg and Chandel 2012).

The activities of APX, GR, CAT, and SOD were significantly higher in barley roots after 7 days of exposure to NaCl (Fig. 3), which corresponds to the increase in ROS formation (Fig. 1). In accordance, the activities of APX, GR, CAT, and SOD increased very rapidly (within 1 day) under salt conditions (200-mM NaCl) in barley, and then variously elevated levels were maintained (Kim et al. 2005). Increase in SOD activity observed here was also detected in barley shoots exposed to 100-mM NaCl (Jin et al. 2009). NaCl depleted the activity of GR in the shoots

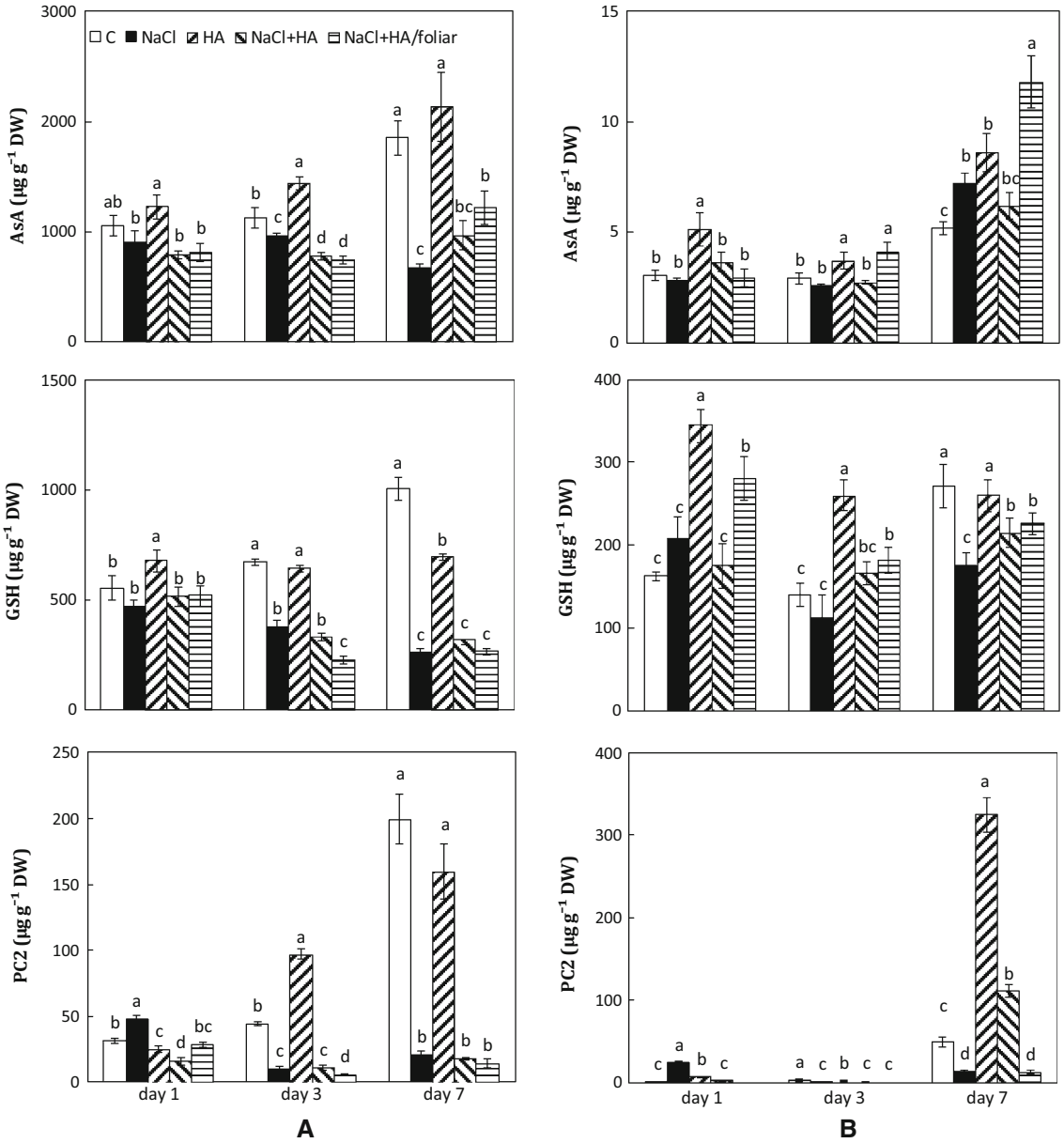

Fig. 2 Time course of quantitative changes of non-enzymatic antioxidants (*AsA* ascorbic acid, *GSH* reduced glutathione, *PC2* phytochelatin 2) in barley (*H. vulgare*, cv. Radegast) shoots (**a**) and roots (**b**) cultured in treatments as mentioned in Fig. 1. *C* control. Data are means ± SDs ($n = 6$). Values for each harvest day, followed by the *same letter*(*s*), are not significantly different according to Tukey's test ($P < 0.05$)

which may be related to the observed decline of GSH content. Changes in the activity of antioxidative enzymes under the influence of HA have only rarely been reported (García et al. 2012). HA alone elevated the activities of GR and CAT in shoots (Fig. 3). This is in accordance with data where HA (20–80 mg L^{-1}) increased CAT activity in rice (García et al. 2012). We also found that applied HA decreased the activities of APX, CAT, and SOD in the roots (in comparison with NaCl-treated roots), and this may be related to depleted signal of ROS (Fig. 1). It is concluded that HA reduces the oxidative damage in NaCl-treated roots by modulating oxidative balance; however, these effects are certainly dose-dependent (if compared present data with other works discussed above).

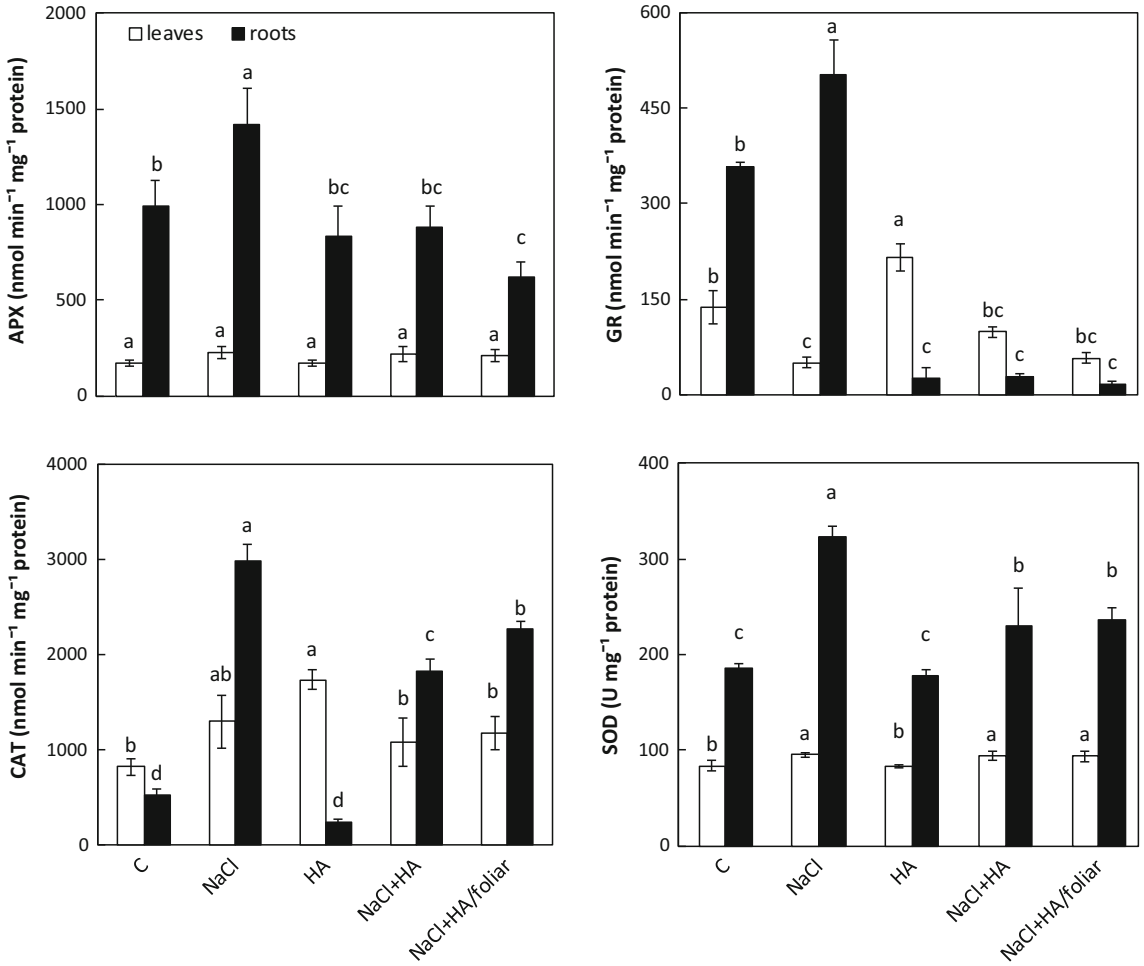

Fig. 3 Changes in the activity of antioxidative enzymes (*APX* ascorbate peroxidase, *GR* glutathione reductase, *CAT* catalase, *SOD* superoxide dismutase) in barley (*H. vulgare*, cv. Radegast) plants after 7 days of treatments as mentioned in Fig. 1. Data are means ± SDs ($n = 6$). Values for leaves (shoots) or roots, followed by the *same letter*(*s*), are not significantly different according to Tukey's test ($P < 0.05$)

Conclusions

Metabolic analyses of hydroponically cultured barley confirmed that low dose of humic acid (6.0 mg/L) may positively affect shoot growth and accumulation of selected metabolites including antioxidants (ascorbate, thiols), while the protective impact of HA in combination with NaCl was rather visible at the level of Na uptake and oxidative stress appearance. Foliar and hydroponic HA application revealed roughly similar efficiency. Our data suggest the potential usefulness of HA to protect the sensitive, but valuable barley cultivar Radegast against NaCl excess and urge for further field research.

Author contribution statement MJ and JK designed the experiments, performed enzymatic measurements, data analyses, and wrote the manuscript. BK, PB and JH performed HPLC, fluorescence microscopy and mineral nutrients assay, respectively.

Acknowledgments This work was mainly realized in CEITEC—Central European Institute of Technology with research infrastructure supported by the project CZ.1.05/1.1.00/02.0068 financed from European Regional Development Fund. The work was also supported by IGA project (No. IP 2/2014) provided by the dean of Faculty of Agronomy (Mendel University in Brno). The authors thank Dr. Silvia Mihaličová for proof reading the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

References

Aşik BB, Turan MA, Çelik H, Katkat AV (2009) Effect of humic substances on plant growth and mineral nutrients uptake of wheat (*Triticum durum* cv. Salihli) under conditions of salinity. Asian J Crop Sci 1:87–95

Aydin A, Kant C, Turan M (2012) Humic acid application alleviate salinity stress of bean (*Phaseolus vulgaris* L.) plants decreasing membrane leakage. Afr J Agric Res 7:1073–1086

Ben Abdallah S, Aung B, Amyot L, Lalin I, Lachaal M, Karray-Bouraoui N, Hannoufa A (2016) Salt stress (NaCl) affects plant growth and branch pathways of carotenoid and flavonoid biosyntheses in *Solanum nigrum*. Acta Physiol Plant 38:article no. 72

Figueroa JAL, Wrobel K, Afton S, Caruso JA, Corona JFG, Wrobel K (2007) Effect of some heavy metals and soil humic substances on the phytochelatin production in wild plants from silver mine areas of Guanajuato, Mexico. Chemosphere 70:2084–2091

García AG, Santos LA, Izquierdo FG, Loss Sperandio MV, Castro RN, Berbara LL (2012) Vermicompost humic acids as an ecological pathway to protect rice plant against oxidative stress. Ecol Eng 47:203–208

Garg N, Chandel S (2012) Role of arbuscular mycorrhizal (AM) fungi on growth, cadmium uptake, osmolyte, and phytochelatin synthesis in *Cajanus cajan* (L.) Millsp. under NaCl and Cd stresses. J Plant Growth Regul 31:292–298

Haddadi BS, Hassanpour H, Niknam V (2016) Effect of salinity and waterlogging on growth, anatomical and antioxidative responses *in Mentha aquatica* L. Acta Physiol Plant 38:article no. 119

Jabeen Z, Hussain N, Wu D, Han Y, Shamsi I, Wu F, Zhang G (2015) Difference in physiological and biochemical responses to salt stress between Tibetan wild and cultivated barleys. Acta Physiol Plant 37:article no. 180

Jamal Y, Shafi M, Bakht J (2011) Effect of seed priming on growth and biochemical traits of wheat under saline conditions. Afr J Biotechnol 10:17127–17133

Jin X, Huang Y, Zeng F, Zhou M, Zhang G (2009) Genotypic difference in response of peroxidase and superoxide dismutase isozymes and activities to salt stress in barley. Acta Physiol Plant 31:1103–1109

Kamboj A, Ziemann M, Bhave M (2015) Identification of salt-tolerant barley varieties by a consolidated physiological and molecular approach. Acta Physiol Plant 37:1716

Khaled H, Fawy HA (2011) Effect of different levels of humic acids on the nutrient content, plant growth, and soil properties under conditions of salinity. Soil Water Res 6:21–29

Kim SY, Lim JH, Park MR, Kim YJ, Park TI, Se YW, Choi KG, Yun SJ (2005) Enhanced antioxidant enzymes are associated with reduced hydrogen peroxide in barley roots under saline stress. J Biochem Mol Biol 38:218–224

Kováčik J, Klejdus B, Hedbavný J, Bačkor M (2009) Salicylic acid alleviates NaCl-induced changes in the metabolism of *Matricaria chamomilla* plants. Ecotoxicology 18:544–554

Kováčik J, Klejdus B, Babula P, Jarošová M (2014a) Variation of antioxidants and secondary metabolites in nitrogen-deficient barley plants. J Plant Physiol 171:260–268

Kováčik J, Babula P, Klejdus B, Hedbávný J, Jarošová M (2014b) Unexpected behavior of some nitric oxide modulators under cadmium excess in plant tissue. PLoS One 9:e91685

Kováčik J, Klejdus B, Babula P, Soares ME, Hedbavný J, Lourdes Bastos M (2015) Chromium speciation and biochemical changes vary in relation to plant ploidy. J Inorg Biochem 145:70–78

Pérez-López U, Robredo A, Lacuesta M, Sgherri C, Mena-Petite A, Navari-Izzo F, Muñoz-Rueda A (2010) Lipoic acid and redox status in barley plants subjected to salinity and elevated CO_2. Physiol Plant 139:256–268

Radl AA, Farghaly FA, Hamada AM (2013) Physiological and biochemical responses of salt-tolerant and salt-sensitive wheat and bean cultivars to salinity. J Biol Earth Sci 3:B73–B88

Renault H, El Amrani A, Berger A, Mouille G, Soubigou-Taconnat L, Bouchereau A, Deleu C (2013) γ-Aminobutyric acid transaminase deficiency impairs central carbon metabolism and leads to cell wall defects during salt stress in *Arabidopsis* roots. Plant Cell Environ 36:1009–1018

Spanò C, Bottega S (2016) Durum wheat seedlings in saline conditions: salt spray versus root zone salinity. Estuar Coast Shelf Sci 169:173–181

Szalai G, Janda T (2009) Effect of salt stress on the salicylic acid synthesis in young maize (*Zea mays* L.) plants. J Agron Crop Sci 195:165–171

Yildiz M, Terzi H (2013) Effect of NaCl stress on chlorophyll biosynthesis, proline, lipid peroxidation and antioxidative enzymes in leaves of salt-tolerant and salt-sensitive barley cultivars. J Agric Sci 19:79–88

Yousfi S, Rabhi M, Hessini K, Abdelly C, Gharsalli M (2010) Differences in efficient metabolite management and nutrient metabolic regulation between wild and cultivated barley grown at high salinity. Plant Biol 12:650–658