

Letter to Editor

Effects of Salinity Stress on the Activity of Glutamine Synthetase and Glutamate Dehydrogenase in Triticale Seedlings

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Abstract

The effect of salinity on the activity of glutamine synthetase (GS EC 6.3.1.2.) and glutamate dehydrogenase (NAD(P)H-GDH EC 1.4.1.2-4) in shoots and roots of triticale seedlings was investigated. Stressed plants were cultured hydroponically on the 100 mM NaCl-enriched medium for 7 days and the physiological responses were measured. Apart from changes in morphology of plants, alterations in GS and NAD(P)H-GDH activity were reported. GS activity in roots of stressed plants grew slightly and in shoots it decreased by approx. 30% as compared to control plants. In shoots of both control and stressed plants two GS isoforms were detected: cytoplasmic (GS₁) and chloroplastic (GS₂). A drop in total GS activity in shoots of NaCl-treated plants was due to the drop in activity of GS₂ isoform. The activity of GS₁ grew slightly under saline stress. After staining of gels for GDH activity, no changes in isoforms were noted as compared with the control plants.

Keywords: salt stress, glutamate dehydrogenase, glutamine synthetase, isoenzymes, triticale

Introduction

Soil salinity is a limiting factor in plant cultivation in many areas around the world – in dry and temperate climates in particular. In recent years NaCl concentration in soil has increased as a result of human activity such as employment of chemical compounds for clearing snow [1], irrigation of soils with saline groundwater [2], uncontrolled disposal of communal sewage and industrial waste or incorrect securement of waste disposal sites.

Salt stress leads to unfavourable functional changes and damage to plant tissues. Among other symptoms, changes in properties of membranes and disorganisation in ion and metabolite transport are observed. Many metabolic pathways, such as photosynthesis, glycolysis and tricarboxylic acid cycle; are subject to modifications.

Under salinity the process of ammonium ion (NH₄⁺) incorporation into plant metabolism is also disturbed. It is commonly considered that the reactions catalyzed by glutamine synthetase (GS EC 6.3.1.2.) and cooperating glutamate synthase (GOGAT EC 1.4.1.13 or EC 1.4.7.1. respectively of the electron donor) constitute the major mechanism of NH₄⁺ assimilation in higher plants, and the activity of glutamate dehydrogenase (NAD(P)H-GDH EC 1.4.1.2-4) is an alternative pathway [3, 4]. Meanwhile, some authors argue that the physiological role of GDH-catalyzed reductive amination of 2-oxoglutarate increases significantly under abiotic stress [5, 6, 7]. Cereal plants are the main source of nutritional protein. The activity of GS and GDH - the enzymes participating in essential amino acid assimilation - is a major factor in protein yield. A decrease in this enzyme activity may limit to a large extent the protein yield in cereals. Therefore, the investigation of GS and GDH in plants

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exposed to high concentrations of NaCl in the medium is of great importance. The choice of research material fell on triticale seedlings (*Triticale* cv. Malno) because of the relatively high nutritional value of triticale grain and continuously enlarging acreage of this crop.

The investigations presented in this paper focused on the determination of changes in the activity and amount of GS and GDH isoforms in roots and shoots of triticale seedlings and the enzyme role in NH_4^+ ion assimilation in stress conditions.

Materials and Methods

Plant Material and Growth Conditions

Roots and shoots of 21-day-old winter triticale seedlings cv. Malno were investigated. The seedlings were cultured hydroponically for 14 days with root system aeration, on a mineral medium containing 5 mM NO_3^- as a nitrogen source. Then the seedlings were treated with 100 mM NaCl. The material was collected for assays after 7 days of salinity stress.

GS Extraction and Activity Assays

The plant material was homogenized in a buffer composed of 0.1 M Tris-HCl of pH 7.9, 20 mM MgCl_2 , 1 mM 2-mercaptoethanol (2-ME) and 0.05% TRITON X-100. 5 ml of the buffer was used per 1 g of plant material. The slurry was centrifuged for 15 min at 10,000 x g and 4°C. The sediment was discarded. The enzyme activities were measured in the supernatant after its dialysis in the buffer containing 0.05 mM Tris-HCl of pH 7.9, 1 mM 2-ME, 20 mM MgCl_2 . The activity of glutamine synthetase was assayed using the spectrophotometric method according to O'Neal [8] by measuring formed 4-Glutamyl hydroxamate. Total activity of the investigated enzyme was defined in μmols of 4-glutamyl hydroxamate formed $\text{min}^{-1} \text{g}^{-1}$ dry weight (DW) of seedling roots or shoots.

GDH Extraction and Activity Assays

The plant material was homogenized in a buffer composed of 0.1 M Tris-HCl of pH 7.8, 5 mM 2-ME, 20 μM . PMSF and 0.01% Triton X-100. 5 ml of the buffer was used per 1 g of plant material. The slurry was centrifuged for 15 min at 10,000 x g and 4°C. The sediment was discarded. The supernatant was dialyzed in a buffer composed of 0.025 M Tris-HCl of pH 7.8, and 5 mM 2-ME. The enzymatic extract thus obtained was used for GDH activity assay. The activity of glutamate dehydrogenase was determined using spectrophotometric method by Barash *et al.* [9] based on the measurement of decrease or increase in absorbance of samples (respective of the direction of the reaction) at 340 nm wavelength, in photometer Pharmacia Novo Spec. Total activity of the investigated enzyme was defined in μmols of oxidized or reduced coenzyme formed $\text{min}^{-1} \text{g}^{-1}$ DW of seedling roots or shoots.

Analytical Electrophoresis and Visualisation of GDH Isoform Activity on Polyacrylamide Gel

In order to determine the amount of GDH isoforms in shoots and roots of salt-stressed plants, analytic electrophoresis by the procedure of Laemmli [10] was performed. GDH was separated on 7.5% polyacrylamide gel. Approx. 20 μg protein in enzymatic extract was applied on gel. Separation was carried out in Tris-glycine buffer (0.05 M Tris and 0.192 M glycine) of pH 8.6 at constant voltage 120 V maintained throughout the electrophoresis.

On gels, after electrophoresis, the enzymatic activity was induced using the modified method by Czosnowski [11]. The gels were incubated for approx. 40 min. at 35°C with an incubation mixture composed of 0.1 M Tris-HCl pH 8.3, 0.26 mM phenazine metasulphate, 0.4 mM nitro-tetrazolium chloride, 0.75 mM NADP(P)⁺, 100 mM glutamate. Control gels were incubated in the mixture without 100 mM glutamic acid.

Separation of Glutamine Synthetase Isoforms

Isoforms of glutamine synthetase were separated using ion exchange chromatography on DEAE-Sepharose Cl-6B column. The enzymatic extract prepared as described earlier was concentrated in Amicon equipment using membrane 100,000 NMWL and then dialysed for 2 h in 0.025 M Tris-HCl buffer of pH 7.9 containing 1 mM 2-ME and 20 mM MgCl_2 . The prepared extract was placed on the column. The column with ion exchanger was equilibrated with 0.025 M Tris-HCl buffer of pH 7.9 containing 1 mM EDTA, 1 mM 2-ME and 10 mM MgCl_2 . Elution combined with GS₁ and GS₂ separation was performed at linear gradient NaCl (0 → 0.4 M) at 15 ml per 1 h. Fractions of 2.5 ml were collected and GS activity was determined therein.

Other Assays

For dry weight (DW) determination the roots and shoots were dried for 48 h at 80°C.

Protein was determined by the method of Lowry *et al.* [12]. Bovine serum albumin fraction V was used as a standard.

Nitrate content in plant material was determined by the method of Cataldo *et al.* [13].

Na⁺ content was determined using flame absorption atomic spectrometry FAAS. **Cl⁻ content** was determined by high performance liquid chromatography with conductometric detection HPLC.

Values are means \pm S.E. (n=4).

Results

Triticale seedlings, during their growth on the medium containing 100 mM NaCl, took up and accumulated toxic Na⁺ cations and Cl⁻ anions. After 7 days Na⁺ content was 3.56% in roots and 4.98% in shoots, whereas Cl⁻ content was 2.88% and 8.78%, respectively. In roots of control

Table 1. Na⁺ and Cl⁻ ion contents in control and stressed plants.

Seedling part	Na ⁺ content [% D.W]	Cl ⁻ content [% D.W.]
Shoot – control	0.0302	1.54
Shoot 100 mM NaCl	4.98	8.78
Root – control	0.064	1.18
Root 100 mM NaCl	3.56	2.88

Table 2. Nitrate content in stressed and control plants.

Seedling part	Nitrate content [μmole NO ₃ ⁻ g ⁻¹ DW]
Shoot – control	443.5 ± 22.2
Shoot – 100 mM NaCl	163.5 ± 8.2
Root – control	390.9 ± 19.5
Root – 100 mM NaCl	335.7 ± 16.8

plants sodium ion levels were as low as 0.064%, and chloride ions: 1.18%. In shoots of control plants likewise – the contents of Na⁺ and Cl⁻ were determined as low: 0.03% and 1.54%, respectively (Table 1). The ion contents were expressed in % DW.

Determination of NO₃⁻ levels allowed to note how in plants under salinity stress nitrate ion content changed significantly (Table 2). Both in shoots and roots of the plants treated with NaCl a decrease in nitrate content was reported. In shoots the reduction was considerable (approx. 3-fold), whereas in roots a negligible drop in NO₃⁻ ion content was reported.

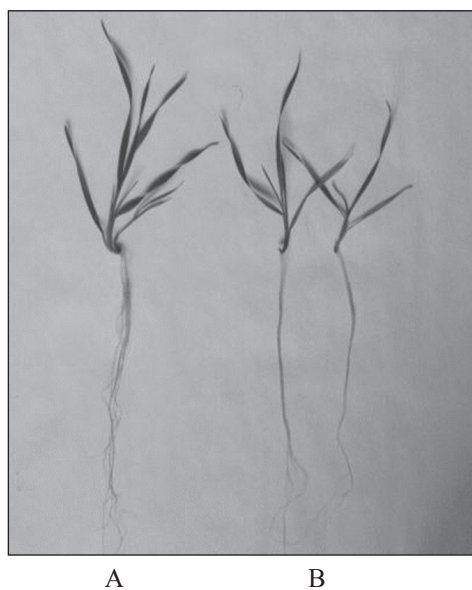


Fig. 1. Morphology of triticale seedlings: A – control plant, B – plants treated with 100 mM NaCl.

The presence of NaCl in the medium had an unfavourable effect on plant development. A decrease in biomass production of stressed triticale seedlings in comparison with control plants was observed. Shoots of NaCl-treated plants were less developed. They lodged more prominently than the control plants and were withered. Differences in root morphology between stressed and control plants were also noted. The root system in the stressed plants proved to be less developed, there were fewer roots in the bundle in the stressed plants than in the control plants (Fig. 1).

The presence of NaCl modified the activity of both glutamine synthetase (GS) and glutamate dehydrogenase (GDH). The effect of salinity stress on GS was 27% activity decrease in shoots only. In roots GS activity slightly increased (by 16%) (Fig. 2). Ion exchange chromatography on DEAE Sepharose C1-6B was used for separation of GS isoforms in triticale shoots. Two separate activity peaks both in stressed and control plants were obtained, one corresponding to GS₁ (cytoplasmic isoform) and the other to GS₂ (chloroplastic isoform). In control plants GS₁/GS₂ activities ratio was 1/8 and in plants grown on 100 mM NaCl it was 1/5 (Fig. 3).

The analysis of cytoplasmic and chloroplastic isoform activity revealed that the decrease in GS activity was mainly due to the unfavourable effect of NaCl on GS₂. No decrease in GS₁ activity in plants under salinity stress was reported as compared with control plants, but even a 15% growth, whereas in GS₂ there was a 26% decrease in activity.

The investigations also revealed the effect of NaCl on GDH activity. In seedlings under 100 mM NaCl nutrition, a marked increase in the enzyme aminating and deaminating activity was observed both in shoots and roots. NADH-dependent GDH activity rose by 50% on average in those parts of the seedlings, while NADPH-dependent activity increased by 200% in roots, and by as much as 290% in shoots as compared with the controls (Fig. 4). In order to determine GDH isoforms in NaCl-treated seedlings, analytic electrophoresis of enzymatic

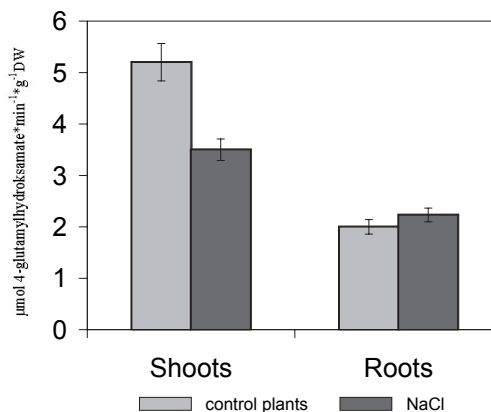


Fig. 2. Changes in total glutamine synthetase activity in triticale seedlings after 7-day stress caused by 100 mM NaCl and control plants.

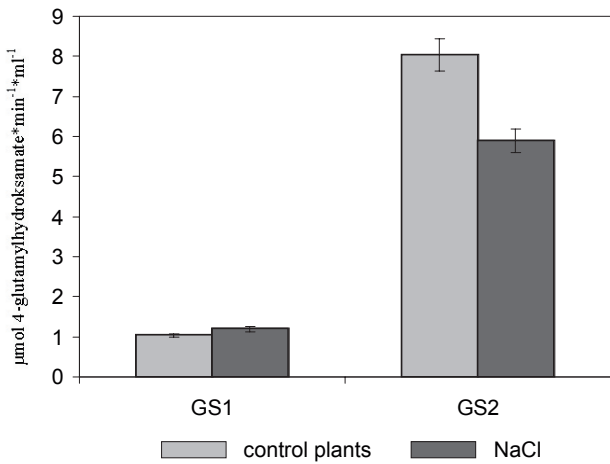


Fig. 3. Changes in activity of glutamine synthetase isoforms: cytoplasmic (GS1) and chloroplastic (GS2) in shoots of triticale seedlings after 7-day stress caused by 100 mM NaCl and control plants.

preparations from shoots and roots was performed. After staining of gels for GDH activity, no changes in isoforms were noted as compared with the control plants (Figs. 5 and 6).

Discussion

Soil salinity hampers agriculture in many regions of the world, also in temperate climate. Plant growth on saline soils is inhibited due to a limited osmotic potential, accumulation of toxic ions, disruption in uptake and assimilation of inorganic nitrogen. In triticale seedlings grown on NaCl-enriched medium an accumulation of Na⁺ and Cl⁻ ions was observed. This led to a limited growth of plants and their more prominent lodging related to the lowering of water potential. A reduction in the number of roots growing from the bundle was also reported. Moreover, as in Brzustowicz and Musiał [14], a decrease in biomass production was observed. The conducted investigations indicated that salinity modi-

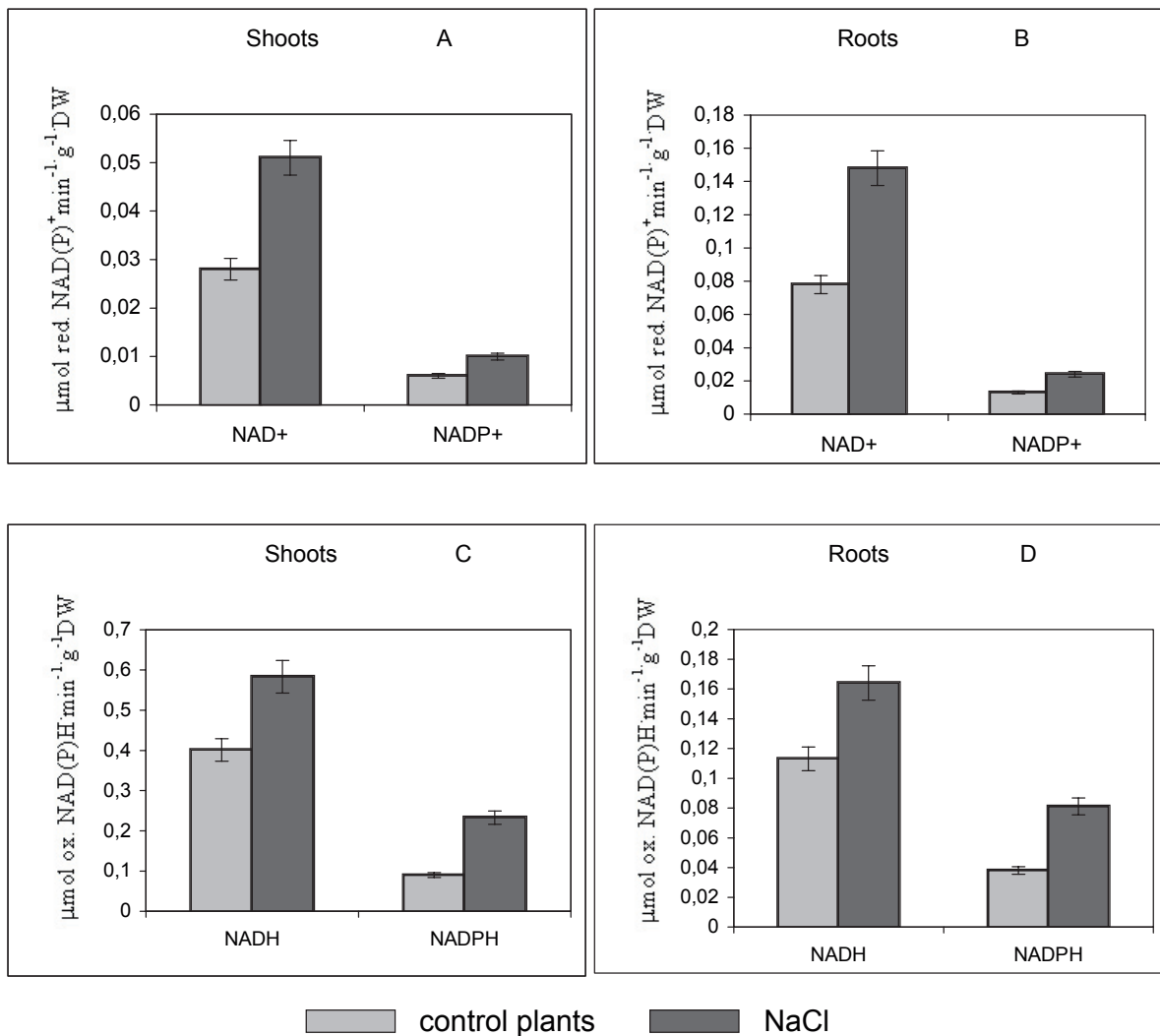


Fig.4. Changes in deaminating (A, B) and aminating (C, D) GDH activity in shoots and roots of triticale seedlings after 7-day 100 mM NaCl-induced stress and control plants.

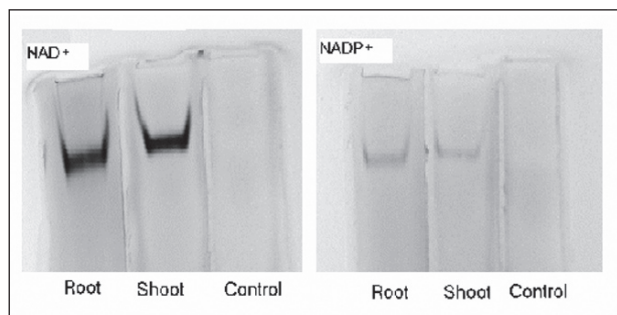


Fig. 5. Visualisation of GDH activity in control triticale seedlings. The zymogrames shown in this figure were stained as described in Materials and Methods.

fied the processes of nitrogen uptake and assimilation. In plants grown on NaCl-enriched medium NO_3^- ions transport from roots to shoots was notably inhibited, which resulted in a 3-fold decrease in nitrate content in shoots. These observations remain in accordance with the results obtained by Silveira *et al.* [15] for cowpea plants (*Vigna unguiculata*).

Soil salinity affected the activity of both GS and GDH – the major enzymes responsible for ammonia assimilation in plants. It could be concluded that the chloroplastic GS - (GS_2) was more salinity-sensitive of the two isoforms and the less stable one. Hence (probably under salt stress) the assimilating role was assumed mainly by GS_1 – the isoform also present in roots [16, 17]. There is a correlation between these results and changes in GS total activity, which decreased mainly in shoots, where the presence of chloroplastic GS isoform was detected. Further on, as Suarez *et al.* [18] investigating the woody plants report, in aging plants and those subjected to biotic and abiotic stresses, the major role in NH_4^+ assimilation is played by GS cytoplasmic isoform.

A rise in the aminating and deaminating GDH activity in both shoots and roots of triticale seedlings grown in the presence of 100 mM NaCl was observed. This suggested that under salinity stress an alternative pathway of ammonium ion assimilation was launched. GDH may be the alternative enzyme for the GS/GOGAT cycle in plants during abiotic stresses [7]. Authors Billard and Boucaud [19] reported that soil salinity impaired the functioning of the main ammonia assimilation pathway in beans (*Phaseolus vulgaris*) leading to the accumulation of NH_4^+ ions in plant cells, which in turn stimulated GDH aminating activity. Hence it can be postulated that under physiological stress GDH plays an important detoxicating role [20]. On the other hand, the increasing deaminating GDH activity in plants developing under salinity stress indicated that the enzyme, through catalysis of glutamic acid breakdown into 2-oxoglutarate, sustained carbohydrate metabolism by supplying an intermediate to the tricarboxylic acid cycle [21, 22]. GDH deaminating activity is also a source of reduction force in the shape of NAD(P)H, which plays a role in many metabolic processes [7, 4].

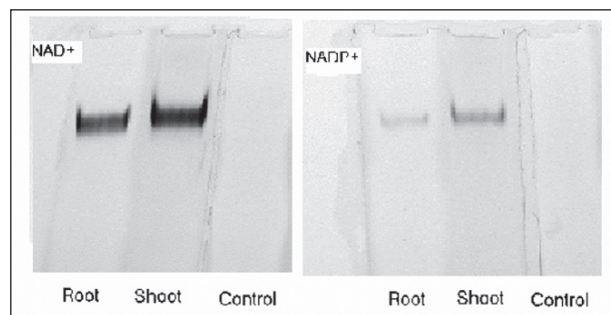


Fig. 6. Visualisation of GDH activity in 100 mM NaCl-treated triticale seedlings. The zymogrames shown in this figure were stained as described in Materials and Methods.

The presented results indicate that GDH complements the assimilating activity of GS. This is supported by the considerable increase in the aminating GDH activity. In the presence of NADPH as a coenzyme the rise was approximately 200% in roots and as much as 290% in shoots, as compared with the control plants. A concurrent increase in the deaminating GDH activity in the tested seedlings testified to its importance in carbohydrate catabolism, through the supply of 2-oxoglutarate for the tricarboxylic acid cycle as well as of reduction force in the shape of NAD(P)H.

In order to exclude possible effects of NaCl on GS and GDH activity *in vitro*, additional determinations were carried out. The activity of the enzymes isolated from roots and shoots of control plants was assayed in standard reaction mixture additionally enriched with 100 mM NaCl. The results thus obtained showed that NaCl had no effect on GS and GDH activity *in vitro*, because no alterations in these enzymes' activity were observed either in shoots or in roots. Therefore, all the changes presented as results ensued from *in vivo* abiotic stress.

The results obtained allowed us to conclude that despite the unquestionably unfavourable effect that NaCl had on triticale seedlings (morphology), the plants were able to adjust the mechanism of ammonia incorporation into organic compounds. This adaptational ability helped avoid shortage of the essential amino acids in plant cells.

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