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BRIEF COMMUNICATION

Glutathione metabolism in soybean callus-cultures as affected by salinity

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Abstract

Induction and growth of soybean callus cultures were influenced by NaCl, especially at the highest concentration tested (150 mM). Protein content was raised as NaCl was increased in the Murashige and Skoog medium. Total sulfhydryl group (-SH) and glutathione (GSH) concentrations were also increased in NaCl treated cultures. The affinity (Km) of glutathione reductase (GR) for oxidized glutathione (GSSG) was gradually increased as NaCl level was raised in the medium. The GSH/GSSG ratio was raised significantly as the result of GR activity. The increase in GR activity may constitute an adaptive response of soybean callus to NaCl.

Additional key words: glutathione reductase, *Glycine max*, NaCl, proteins.

Glutathione is an important metabolite and has a number of functions in plants as an antioxidant, a co-factor of enzymatic processes, a protein reductant, in storage of reduced sulfur, detoxification by conjugation, and binding of heavy metals through phytochelatin complexes (Lamoureux and Rusness 1981, Rennenberg 1982, 1987, Halliwell 1984, Smith *et al.* 1990). It was suggested that glutathione may play a protective role in salt tolerance of plants (Strogonov 1974, De Kok and Oosterhuis 1983).

To obtain more insight into the role of glutathione and glutathione reductase as factors in tolerance of plants to salinity stress, we studied the effect of the different concentrations of NaCl in Murashige and Skoog (MS) medium on growth, protein content, oxidized and reduced glutathione content and glutathione reductase activity in soybean callus cultures after 21 d.

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Abbreviations: GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; Km - Michaelis constant.

Soybean (*Glycine max* L.) callus was cultured in aseptic conditions in 50 cm^3 conical flasks, containing 20 cm^3 of MS-medium, supplemented with 0.5 mg dm^{-3} α-naphthaleneacetic acid (NAA), 1.5 mg dm^{-3} benzyladenine (BA), 170 mg dm^{-3} $NaH_2PO_4 \cdot H_2O$, 100 mg dm^{-3} inositol, 0.1 mg dm^{-3} thiamine-HCl, 0.5 mg dm^{-3} nicotinic acid, 30 g dm^{-3} saccharose and 7 g dm^{-3} agar. Sodium chloride was incorporated into the MS-medium at concentrations 0, 25, 50, 100 and 150 mM. All treatments were given to three replicate cultures, pH was adjusted to 5.5 ± 0.2 before autoclaving. Cultures were kept in an incubator at temperature of 27 °C and continuous fluorescent white light (376.8 μmol m^{-2} s^{-1}).

Non-protein thiols were extracted (De Vos *et al.* 1989) by grinding 1 g fresh mass in 1 cm^3 5 % (m/v) 5-sulfosalicylic acid plus 6.3 mM EDTA at 0 °C in a mortar. The homogenate was centrifuged at 10.8 *g* for 15 min at 4 °C. The clear supernatant was immediately used for the determination of thiol with Ellman's reagent (Ellman 1959). 0.1 cm^3 supernatant was mixed with 0.7 cm^3 of 0.5 M K_2HPO_4 (pH 7.5) and 0.1 cm^3 of 10 mM 5,5-dithiobis(2-nitrobenzoic) acid. The absorbance was measured spectrophotometrically after 5 min, at wavelength 412 nm against blank with 0.1 cm^3 H_2O. Glutathione reduced (GSH) and oxidized (GSSG) were assayed by the GSSG-recycling method (Anderson 1985).

Enzyme extractions were prepared according to Halliwell and Foyer (1978) with some modifications. 1 g of callus was ground in a mortar with 1.0 cm^3 0.1 M KH_2PO_4-KOH buffer, pH 7.5, containing EDTA (1 mg cm^{-3}) at 0 °C. The homogenate was centrifuged at 10.8 *g* at 4 °C for 20 min. The supernatant was used for enzyme assay. Reaction mixtures contained 0.7 cm^3 of 1 M K-phosphate buffer, pH 7.6, 0 to 0.2 cm^3 of 5 mM GSSG (disodium salt, *Sigma*) in 5 % $NaHCO_3$ (m/v) and 0.1 cm^3 of enzyme extract in 0.1 M KH_2PO_4 buffer, pH 7.5, to brought a final volume 1.1 cm^3; 0.1 cm^3 of 10 mM DTNB [5.5'- dithiobis-(2-nitrobenzoic) acid. *Serva*] reagent was added. Reaction was started with the enzyme extract and kept in water bath at 30 °C for 10 min. The reaction was stopped by 5 % TCA. The activity was measured as the increase in the absorbance of DTNB at 412 nm against blank with GSSG. The protein content of the enzyme extract was measured according to Bradford (1976).

Glutathione reductase activity was measured at three or five levels of GSSG, between 0.05 to 1.0 mM. Activity was expressed as increase in change in DTNB absorbance at 412 nm g^{-1}(fresh mass). The data were transformed in a double reciprocal plot according to the procedure of Lineweaver and Burk (Palmer 1985). The data yield straight lines and linear regression varied from 0.98 to 0.99 ($P = 0.05$). The transformation of the data resulted in Km values, which represent the affinity of the enzyme for GSSG.

Soybean calli get brownish as NaCl increased in the MS-medium especially at higher concentrations (100 and 150 mM). The increase in salinity levels resulted in a reduction of growth (dry mass) especially at high salinity levels (150 mM), whereas the protein content significantly increased as NaCl increased, especially at moderate salinity levels (50 and 100 mM) (Table 1).

On the fresh mass basis, the salinity-stressed cultures grown at 25, 50 and 100 mM NaCl contained about 147, 170 and 124 % more of non-protein-SH than the control

cultures, while at the highest concentration of NaCl (150 mM) the amount of non-protein-SH compound was about of 87 % of the control (Table 2). The increase in GR activity per mg protein (Table 1) in salinity-stressed cultures is consistent with the trend observed when GR activity is expressed on fresh mass basis. The Km values was increased as NaCl increased in the MS-medium (Table 1). The increase in Km of GR means that the affinity of GR to GSSG was higher in NaCl treated cultures especially at 50 and 100 mM.

Table 1. Effect of NaCl salinity on callus growth, protein content, glutathione reductase (GR) activity and Km for GSSG. Culture period was 21 d.

NaCl [mM]	Protein content [mg g^{-1}(d.m.)]	Growth [g(d.m.)]	GR activity [U g^{-1}(f.m.) s^{-1}]	[U mg^{-1}(prot.) s^{-1}]	Km for GSSG [mM]
0	200.70 ± 3.95 c	0.36 ± 0.4 a	6.61 ± 0.4 e	0.030 ± 0.004 d	0.34 ± 0.001 e
25	240.00 ± 3.96 a	0.32 ± 0.03 ab	13.28 ± 0.49 c	0.050 ± 0.003 c	0.40 ± 0.002 bc
50	244.39 ± 3.05 a	0.29 ± 0.01 bc	15.29 ± 0.32 b	0.060 ± 0.002 b	0.41 ± 0.001 bc
100	248.65 ± 1.85 a	0.27 ± 0.02 d	17.32 ± 0.31 a	0.080 ± 0.005 a	0.76 ± 0.034 a
150	217.13 ± 9.99 b	0.17 ± 0.01 c	7.68 ± 0.33 a	0.026 ± 0.005 d	0.65 ± 0.003 b
LSD at 5 %	9.76	0.04	0.69	0.009	0.28
LSD at 1 %	13.88	0.06	0.99	0.014	0.40

Means of three determinations in three replicates. Those not significantly different from each other are followed by the same letter ($P = 0.05$).

Glutathione (GSH) content was increased significantly as salinity was raised in the MS-medium of callus cultures to 100 mM, but at the highest NaCl content, the amount of GSH was significantly lower. Changes in the concentrations of oxidized glutathione (GSSG), were opposite to those of GSH, while GSH increased, the GSSG

Table 2. Effect of NaCl salinity on the content of non-protein-SH, reduced glutathione (GSH), oxidized glutathione (GSSG) and GSH/GSSG ratio of soybean callus cultures. Culture period was 21 d.

NaCl [mM]	Non-protein -SH [μmol (SH) g^{-1}(f.m.)]	GSH [μmol (SH) g^{-1}(f.m.)]	GSSG [μmol (SH) g^{-1}(f.m.)]	GSH/GSSG
0	12.86 ± 0.63 d	9.30 ± 0.30 d	5.30 ± 0.20 a	1.85 ± 0.06 d
25	18.98 ± 0.47 b	10.35 ± 0.41 c	4.10 ± 0.21 b	2.51 ± 0.06 c
50	21.93 ± 1.47 a	11.60 ± 0.79 b	3.53 ± 0.30 c	3.31 ± 0.47 b
100	16.02 ± 0.53 c	17.52 ± 0.50 a	3.25 ± 0.25 c	5.41 ± 0.39 a
150	11.21 ± 0.27 e	7.92 ± 0.07 e	2.75 ± 0.25 d	2.89 ± 0.25 bc
LSD at 5 %	1.45	0.87	0.44	0.55
LSD at 1 %	2.05	1.24	0.63	0.78

Means of three determinations in three replicates. Those not significantly different from each other are followed by the same letter ($P = 0.05$).

decreased as salinity-stress was increased to 100 mM (Table 2). Consequently, the GSH/GSSG ratio was increased significantly as NaCl raised (Table 2).

In higher plant cells, glutathione (GSH) is believed to remove toxins generated during cellular metabolism (Salin 1987 and Foyer *et al.* 1991). Salinization with NaCl severely disturbed sulfur metabolism of mustard and horsebean (Strogonov 1974). It retarded sulfate reduction and caused shifts in the thiol/disulfide equilibrium. These shifts were mainly due to accumulation of non-protein disulfide compounds.

Our results show that sulfhydryl group and glutathione (GSH) concentrations were raised as salinity level increased. Similar results were observed during water stress (Gamble and Burke 1984) and cold stress (Steffen 1991, Shewfelt and Erickson 1991, Walker and McKersie 1993). Thus glutathione and glutathione reductase may protect chloroplasts against damaging oxygen products *via* an ascorbate-glutathione cycle in which ascorbate and glutathione are key enzymes (Halliwell 1982, Schoner and Krause 1990, Ranieri *et al.* 1993). It was proposed that GSH maintains cysteine, homocysteine, enzymatic protein, ascorbate in active form and may regulate the thiol/disulfide ratio in proteins and protect cell membrane against hydrogen peroxide and free radicals (Halliwell and Foyer 1978, Kosower and Kosower 1978).

It is important to maintain a high level of GSH, which may be achieved either by its biosynthesis *de novo* or by reduction of GSSG with glutathione reductase. This was apparently enhanced by means of an enhanced affinity of glutathione reductase for GSSG (Km). The GSH/GSSG ratio was increased significantly, presumably as a result of GR activity. Podhradsky *et al.* (1990) stated that a high GSH/GSSG ratio is necessary not only for the role of GSH as a reductant but also to achieve optimal protein synthesis. The GSH/GSSG status is sensitive to the redox state of a cell, rapid reduction of GSSG is predominantly caused by GR activity (De Kok and Oostehuis 1983). Fahey *et al.* (1980) found that the increase of GR activity is accompained by the increase of GSH/GSSG ratio which can be related to the increasing protein synthesis and/or reduction of GSSG.

In the present study, we have shown that elevated levels of non-protein-SH, GSH, GSSG, GSH/GSSG ratio and GR activity were produced due to salinity stress. The increase in glutathione reductase activity may constitute an adaptive response of soybean callus culture to NaCl salinity.

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