The effect of potentially toxic elements and sewage sludge on the activity of regulatory enzyme glutamate kinase

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ABSTRACT

The glutamate kinase activity was investigated as a plant stress response to Cd, Zn, As or sewage sludge application to soil in the pot and field experiments with spinach (*Spinacia oleracea* L.). Allosteric regulation of glutamate kinase activity by free proline creates a possibility for an increase in glutamic acid content due to the synthesis of glutathione and phytochelatines in plant cells. For this reason the high rates of As, Cd and Zn applied into soil strongly decreased the glutamate kinase activity. Allosteric regulation of the glutamate kinase activity did not inhibit the synthesis of proline and hydroxyproline under stress condition caused by organic pollutants after application of sewage sludge. Formed proline was bound to stress proteins and therefore glutamate kinase activity was not inhibited.

Keywords: proline regulation; toxic elements; plant stress metabolism; chronic stress

Potentially toxic elements are widely distributed throughout the environment. The adaptation of plants to toxic concentrations of these elements depends upon various mechanisms operating at both intra and intercellular levels. The molecular mechanism proposed for explanation of the plant metal tolerance through the detoxification resulted in cell wall binding, compartmentalization of elements in vacuoles and adaptive mechanisms of a metabolic and enzymatic nature (Sanita di Toppi and Gabbrielli 1999, Schützendübel and Polle 2002).

Metabolisms of glutathione, organic acids, peroxidases, stress proteins, proline and related amino acids, as well as mechanisms of compartmentalization, lignification, and root development are affected by the presence of the toxic elements and many other stress agents, such as salinity, heat, drought (Ramachandra Reddy et al. 2004, Demiral and Türkan 2005). Accumulation of free proline in plants has been often reported as a response to a wide range of environmental stresses (Curtis et al. 2004, Verslues and Bray 2004, Klotke et al. 2004) including presence of heavy metals (Shaw and Rout 2002). The proteinogenic amino acid proline functions as an osmolyte, radical scavenger, electron sink, stabilizer of macromolecules, and a cell wall component (Matysik et al. 2002).

In higher plants, proline is synthesized in cytosol either from L-glutamic acid or from L-ornithine. On the other hand, proline is metabolized in the mitochondria to L-glutamic acid via proline dehydrogenase (Di Martino and Pizzuto 2006).

Exposure to toxic metals induces the synthesis of phytochelatins in plants. Phytochelatins are synthesized from glutathione by the influence of the enzymes γ-glutamylcysteine synthetase (this enzyme catalyzes the formation of a peptide bond between the γ-carboxyl group of glutamate and the α-amino group of cysteine) and glutathione synthetase. After induction of this synthesis plants prefer phytochelatin production pathway (the use

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Supported by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. MSM 6046070901, and by the Academy of Sciences of the Czech Republic, Project No. AVOZ 50380511.

Table 1. The total content of toxic elements in tested soil and sewage sludge (the average of two experiment years)

	As	Cd	Zn
	(mg/kg)		
Chernozem	18.0 ± 1.0	0.416 ± 0.073	87.1 ± 4.4
Sewage sludge	7.1 ± 0.7	3.76 ± 0.21	1105 ± 16

Table 2. Experimental design

	Treatment	Fertilization	Application of risk elements
Field experiment	1	NPK	0
	2	NPK	toxic elements I
	3	NPK	toxic elements II
	4	NPK	sewage sludge
Pot experiment	1	NPK	0
	2	NPK	As
	3	NPK	Cd
	4	NPK	Zn

of glutamate for bond between glutamate and cysteine) to proline synthesis. Glutamyl kinase and glutamylphosphate reductase have been proposed to convert glutamate to Δ^1-pyrroline-5-carboxylate. There are few convincing reports introducing these two enzymes in plants (Štefl and Vašáková 1982, GarcíaRíos et al. 1997, Girija et al. 2002). Hu et al. (1992) isolated a bifunctional enzyme, Δ^1-pyrroline-5-carboxylate synthetase, with both glutamate kinase and glutamylphosphate reductase activities, that catalyzed the first two steps in the proline biosynthesis. The glutamate kinase enzyme catalyzed the first step of proline biosynthesis, i.e. the conversion of glutamic acid to γ-glutamyl phosphate. The glutamate kinase activity responds to abiotic stress in this way (Táborský and Vašáková 1994).

This study was focused on the investigation and explanation of changes in the plant metabolism under a chronic stress caused by contaminants. The glutamate kinase activity [E.C.2.7.2.11] was investigated as a plant response to stress caused by Cd, Zn, As or sewage sludge application into soil.

MATERIAL AND METHODS

Spinach (*Spinacia oleracea* L. cv. Matador) was cultivated on Chernozem soil (pH_{KCl} = 7.2, C_{ox} = 1.83%, CEC = 258 mval/kg, soil density 1.65 kg/dm^3) in a precise field experiment and a model pot experiment. Spinach was used as the test plant in this study due to its high toxic element accumulation capacity and short growth period.

Field experiment

Soil was treated with two rates of toxic elements (Cd, Zn, As) or with sewage sludge in a precise field experiment. Fresh homogeneous sewage sludge (26.4 g/kg soil) with a mean 34% of dry matter was used as the source of toxic elements in this experiment (Table 1). The first rate of toxic elements was set according to their content in sewage sludge; the second rate was ten times higher (Tables 2 and 3). All treatments were tested in four replications (2 m^2 each). Spinach was planted up to full leaves development.

Average month temperatures and rainfall during the spinach growing period are described in Table 4.

Table 3. The rates of toxic elements

	Element	Rate of toxic elements (mg/kg soil) I	II	Source of elements
Field experiment	As	0.06	0.6	$Na_2HAsO_4.7\ H_2O$
	Cd	0.03	0.3	$CdCl_2$
	Zn	10.0	100.0	$Zn(CH_3COO)_2.2\ H_2O$
Pot experiment	As	60		$Na_2HAsO_4.7\ H_2O$
	Cd	30		$CdCl_2$
	Zn	250		$Zn(CH_3COO)_2.2\ H_2O$

Table 4. Average month temperatures and rainfall during the spinach growing period

Month	Temperature (°C)		Rainfall (mm)	
	2005	2006	2005	2006
April	10.1	8.9	13.8	58.3
May	14.0	13.5	82.6	97.0
June	16.5	17.7	65.2	58.9

Pot experiment

For the verification of the effect of toxic elements on glutamate kinase activity a pot experiment was set up using high rates of individual toxic elements. Five kg of soil was thoroughly mixed with 0.5 g N, 0.16 g P, and 0.4 g K, applied in ammonium nitrate and potassium hydrogen phosphate in the control treatment and with the same amount of nutrients plus a toxic element (As or Cd or Zn) in the observed treatments (Tables 2 and 3). Soil mixture was filled into plastic pots and sown with spinach seeds. Soil moisture was regularly controlled and kept at 60% of MWHC. All treatments were prepared with four replications. Spinach was planted up to full leaves development.

Duration of field and pot experiments was 44 and 46 days in two experiment years.

Analyses of toxic elements

Dry above-ground spinach biomass was used for determination of the total As, Cd and Zn contents. The plant material (1 g of dried and powdered biomass) was decomposed in a dry ashing procedure performed in a mixture of oxidizing gases (O_2 + O_3 + NO_x) using the Apion Dry Mode Mineralizer (Tessek, CZ). The ash was dissolved in 1.5% HNO_3 (Miholová et al. 1993). Each sample was decomposed and analysed three times.

Varian SpectrAA-400 (Australia) atomic absorption spectrometer with a GTA-96 graphite tube atomizer was used to determine cadmium. A pyrolytically-coated tube with a L'vov platform was used for all of the measurements.

Flame atomization (air-acetylene flame) was applied (Varian SpectrAA-300 atomic absorption spectrometer, Australia) for zinc determinations.

Arsenic was determined by a continual hydride generation technique using the Varian SpectrAA-300 (Australia) atomic absorption spectrometer equipped with a VGA-76 hydride generator. The quality of the plant analyses was verified using the RM 12-02-03 Lucerne reference material (Table 5).

Analysis of the glutamate kinase activity

Glutamate kinase was isolated from acetone-dried spinach fresh above-ground biomass. The hydroxamate method was used for analysis and the determination of its activity was described by Vašáková and Štefl (1982). Each sample (1 g of acetone-dried powder) was analysed two times. The results of glutamate kinase analyses from both years were averaged.

RESULTS AND DISCUSSION

Application of toxic elements or sewage sludge into the soil influenced the content of toxic elements in the spinach biomass (*Spinacia oleracea* L.) (Table 6). The lowest difference between the control treatment and treatments with toxic elements I and sewage sludge application was determined for Cd content. Application of toxic elements I rate and sewage sludge did not increase Cd content in spinach. Only the addition of toxic elements II rate increased the Cd content in spinach by 28.6% compared with the control. High sorption capacity of Chernozem in the field experiment limited

Table 5. Quality control of plant, soil and sewage sludge analyses

Element	Reference material					
	RM 12-02-3 lucerne		RM7001 light sandy soil		RM 12-03-12 sludge	
	certified content	obtained content	certified content	obtained content	certified content	obtained content
As	0.263 ± 0.007	0.253 ± 0.047	12.3 ± 1.1	16.5 ± 0.8	8.87 ± 1.08	7.49
Cd	0.136 ± 0.003	0.134 ± 0.008	0.32 ± 0.05	0.38 ± 0.04	1.97 ± 0.21	1.75 ± 0.02
Zn	33.2 ± 0.5	31.0 ± 0.7	120 ± 7	119 ± 33	1310 ± 40	1371 ± 13

Table 6. Total content of As, Cd and Zn in dry above ground spinach biomass (mg/kg)

	Treatment	As	Cd	Zn
Field experiment	0	0.075 ± 0.009	0.63 ± 0.06	68.4 ± 8.8
	toxic elements I	0.150 ± 0.031	0.65 ± 0.04	119.0 ± 5.6
	toxic elements II	0.168 ± 0.009	0.81 ± 0.10	215.0 ± 9.1
	sewage sludge	0.148 ± 0.017	0.69 ± 0.06	75.7 ± 1.9
Pot experiment	0	0.073 ± 0.065	0.44 ± 0.03	63.8 ± 2.2
	As	3.387 ± 0.286	–	–
	Cd	–	39.80 ± 2.60	–
	Zn	–	–	285.7 ± 6.7

the cadmium availability for plants. The limited Cd and Zn accumulation in plant biomass after application of sewage sludge to the soil was also confirmed by Balík et al. (2000) and Tlustoš et al. (2001). Arsenic and zinc contents significantly increased in the spinach biomass compared to the control. As content increased by 100% in the plants grown on the soil with toxic elements I and with the sewage sludge treatments, and by 124% in the toxic elements II treatment. The highest increase of zinc content was also determined in spinach grown on toxic elements II treatment (by 214%), increase of Zn content in other treatments was 74% on toxic elements I and 11% on sewage sludge treatment in contrast to control treatment. A non-significant increase of Zn content in plant biomass grown in sewage sludge treatment was caused by limited Zn availability in the sewage sludge for plants. The yield of the above-ground biomass was not affected by addition of toxic elements and sewage sludge (data are not shown).

Application of high rates of toxic elements led to the increase of all toxic elements contents in plant biomass (Table 6) in the pot experiment in contrast to the field trial. Arsenic and cadmium rates significantly decreased the yield of the above-ground spinach biomass (Figure 1). Arsenic rate was toxic for plants; therefore the biomass yield of this treatment formed only 10% of the yield of control. The decrease of spinach yield caused by Cd application was 65% compared with the control treatment. Application of zinc did not significantly affect the biomass yield. Tlustoš et al. (2006) also stated that a high content of toxic elements in soil inhibited plant growth due to the phytotoxicity of elements to plants.

Proline is an important amino acid for plant adaptation to stress condition. It is intensively incorporated into proteins, and later accumulated as free proline. The decisive role in the intensity of proline biosynthesis under stress conditions is obviously in the regulation of the first enzyme of proline biosynthesis from L-glutamate – proline-inhibitable enzyme glutamate kinase [E.C.2.7.2.11] *via* a feedback mechanism, which is governed by the content of proline (Štefl and Vašáková 1982,

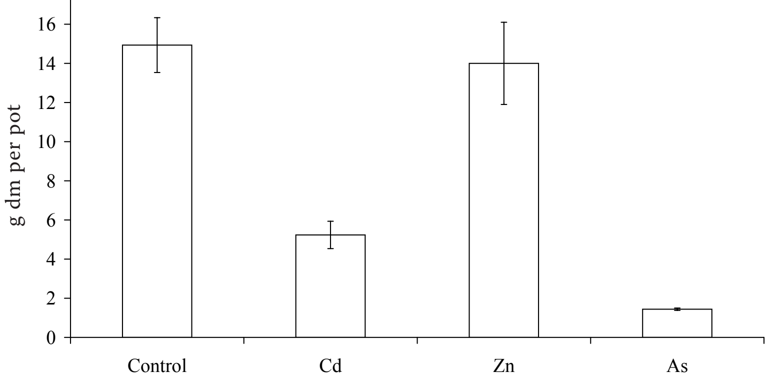

Figure 1. Yield of aboveground biomass of spinach growing in pot experiment (g dm per pot)

1984). Free proline decreases the activity of glutamate kinase. Glutamic acid not transformed to proline can be used for synthesis of gluthatione. Phytochelatins are synthesized from glutathione and increase the tolerance of plants against toxic elements. This result was also confirmed by our experiments.

Both rates of toxic elements in field experiment did not significantly influence glutamate kinase activity (Table 7). Our results showed a significant increase of the glutamate kinase activity only in the spinach biomass growing on sewage sludge treatment. Pollutants of organic origin contained in the sewage sludge could be the source of the metabolic stress for plants. Results of the analysis of used sewage sludge determined in the Laboratory of Waste-Water Treatment in Prague showed a high content of organic pollutants (447 μg/kg monoaromatic hydrocarbons, mainly xylene; 270 μg/kg polychlorinated biphenyls; 15 770 μg/kg polyaromatic hydrocarbons, mainly fluoranthene, phenanthrene and naphthalene). Baran and Oleszezuk (2003) found also polychlorinated terphenyls, naphthalene, phenols, chlorophenols, phthalates, polychlorinated biphenyls and polyaromatic hydrocarbons in sewage sludge and effects of these substances on plants. These substances can induce plant metabolic stress mechanisms. Proteins containing proline have a significant role in these mechanisms; for this reason the effect of allosteric inhibition of glutamate kinase activity was low and the activity of glutamate kinase increased. Formed proline is bound to proteins and therefore glutamate kinase activity is not inhibited.

For the verification of the toxic element effect on glutamate kinase activity a pot experiment using high rates of individual toxic elements was set up. Results of the glutamate kinase activity showed a strong effect of all three elements (Table 7), mainly of As. Arsenic decreased the glutamate kinase activity by 87.7%. Effect of zinc as an essential element in the glutamate kinase activity was significantly lower compared to both Cd and As.

Allosteric regulation of glutamate kinase activity by free proline makes it possible to increase glutamic acid content using in synthesis of glutathione and phytochelatines in plant cell. Increased content of free proline inhibited biosynthesis of further proline. Increased inhibition of proline biosynthesis resulted in the preference of metabolic way of phytochelatin synthesis, glutamate kinase activity therefore decreased in As, Cd and Zn treatments of the pot experiment. According to our previous results of sequential extraction of spinach biomass (Pavlíková et al. 2004, 2005) the mass portion of phytochelatins fractions increased in spinach cultivated on soil contaminated with toxic elements as well as with sewage sludge. The role of proline accumulation in plants stressed by toxic elements has not been fully elucidated. Sharma et al. (1998) and Shaw and Rout (2002) reported accumulation of free proline in plants in response to toxic element stress. According to Shaw and Rout (2002) the accumulation of proline has not provided a protective mechanism against toxic elements but probably a physiological reaction inducing plant ontogenetic cycle. Schat et al. (1997) suggested that accumulation of proline was a result of water stress induced by toxic elements, however it has not acted as a protective mechanism.

Table 7. Relative changes of glutamate kinase activity

	Treatment	Activity of glutamate kinase (%)
Field experiment	0	100
	toxic elements I	103.7 ± 2.3
	toxic elements II	94.1 ± 4.5
	sewage sludge	166.6 ± 1.9
Pot experiment	0	100
	As	12.3 ± 3.1
	Cd	39.6 ± 2.8
	Zn	61.9 ± 2.1

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Received on February 6, 2007

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