Effect of exogenous lead on growth and carbon metabolism of pea (*Pisum sativum* L) seedlings

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Abstract The present study investigated the effect of exogenous lead (Pb) on seedling growth, carbohydrate composition and vital enzymes of sucrose metabolism, starch degradation, pentose phosphate pathway and glycolysis in pea seedlings. With 0.5 mM Pb, reduction of about 50 % in shoot and 80 % in root lengths was observed. At 5 and 7 days of seedling growth, cotyledons of Pb-stressed seedlings had about 25–50 % lower α - and β -amylase activities resulting in their higher starch content. Low starch content in the cotyledons of control seedlings at days 1, 3, 5 and 7 may be due to higher investment of carbon for seedling growth. Seedlings exposed to Pb showed significant inhibition of about 30–50 % in acid invertase activity in the growing tissues i.e. roots and shoots. Sucrose content increased by 10–20 % in shoots with much larger increase in cotyledons at 5–7 days of growth in Pb-stressed seedlings. In stressed seedlings, sucrose synthase (SS) and sucrose-6-phosphate synthase (SPS) enzymes were down regulated in the roots but SS activity was up regulated in the cotyledons leading to increased sucrose content. Exogenous Pb increased the activities of glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) in the cotyledons. Down regulation of G6PDH and up regulation of hexokinase (HXK) in the roots and shoots of stressed seedlings indicated that hexoses could be utilised preferably for glycolysis rather than pentose phosphate pathway in these tissues. Due to limited supply of sugars to growing tissues in the stressed seedlings, increased HXK may play a role in sugar sensing. Phosphoglucomutase (PGM) activity was maximum in the cotyledons and minimum in roots showing its importance in the conversion of glucose-1-phosphate into glucose-6-phosphate. Reduced seedling growth observed in the presence of exogenous Pb was mainly due to

the decrease in the activities of amylases and invertases in the cotyledons and growing tissues respectively. Further biosynthetic capacity of the roots and shoots was down regulated in the pea seedlings due to reduced efficiency of pentose phosphate pathway under Pb toxicity.

Keywords Pb toxicity · *Pisum sativum* · Carbon metabolism · Glycolysis · Pentose phosphate pathway

Abbreviations

G6PDH	Glucose-6-phosphate dehydrogenase
6PGDH	6-phosphogluconate dehydrogenase
HXK	Hexokinase
LSD	Least significant difference
PGM	Phosphoglucomutase
SS	Sucrose synthase
SPS	Sucrose-6-phosphate synthase

Introduction

Heavy metals are commonly defined as those having a specific gravity of more than 5 g/cm³. Main threats from heavy metals are associated with exposure to lead (Pb), cadmium (Cd), mercury (Hg) and arsenic (As). Among heavy metals, Pb with a specific gravity of 11.34 g/cm³, is one of the heaviest non-essential metal released into the natural environment from a range of anthropogenic activities i.e. mining, smelting, paints, gasoline, explosives and industrial wastes (Sharma and Dubey 2005). Singh and Kansal (1983) observed increased accumulation of Pb in soils receiving waste water from industrial towns of Punjab. It cannot be degraded chemically or biologically by microorganisms and so tends to accumulate in soil (Zaier et al. 2010). Affected soils contain Pb in the range of 400–500 mg Kg⁻¹ soil (Angelone and Bini 1992). Moreover, it tends to accumulate in the surface ground

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layer and its concentration decreases with soil depth (De Abreu et al. 1998).

There are many reports in literature which showed that Pb negatively affects the growth and metabolism in *Lycopersicon esculentum* (Kratovalieva and Cvetanowska 2001), *Pisum sativum* (Malicka et al. 2009), *Brassica juncea* (Meyers et al. 2008) and *Oryza sativa* (Verma and Dubey 2001). It also inhibited early root growth in *Triticum aestivum* seedlings (Kaur et al. 2010). Even very low concentrations of Pb can inhibit some vital processes such as photosynthesis, mitosis and water absorption with toxic symptoms of dark leaves, wilting of older leaves, stunted foliage and brown short roots (Patra et al. 2004). Toxicity of heavy metal ions is mainly due to their interference with electron transport in respiration and photosynthesis. Particularly Pb, Cd and Hg show strong affinities for ligands such as phosphates, cysteinyl and histidyl side chains of proteins, pteridines and porphyrins (Fodor 2002). These heavy metals can act on a large number of enzymes having sulfhydryl groups and affect conformation of nucleic acids and disrupt pathways of oxidative phosphorylation (Fodor 2002). Furthermore, when seeds are grown on heavy metal contaminated soil, the first event which faces the toxic effects of metal is germination.

Enzymes associated with starch degradation, sucrose synthesis and utilization of glucose play a key role in seedling growth which further determine the final yield of the crop. α -Amylase (EC 3.2.1.1) catalyses hydrolytic cleavage of internal α -1,4-glucan bonds of starch releasing fragments that can be further broken down by β -amylase (Yang et al. 2001). Maltose, the product of β -amylase (EC 3.2.1.2), is further degraded to glucose by α -glucosidase. Sucrose phosphate synthase (SPS, EC 2.4.1.14) converts glucose to sucrose in the cotyledons. This enzyme transfers glucose from UDP-glucose to fructose-6-phosphate to form sucrose-6-phosphate which is immediately converted to sucrose by irreversible action of sucrose-6-phosphate phosphatase. The sucrose is then transported to growing tissues where it is cleaved by invertase and sucrose synthase (SS, cleavage direction) (Hirose et al. 2002; Baroja-Fernandez et al. 2003). Invertase catalyses the hydrolytic cleavage of sucrose to glucose and fructose whereas sucrose synthase acts on sucrose in the presence of UDP to form UDP-glucose and fructose. Fructose is phosphorylated to fructose-6-phosphate and UDP-glucose acts as a donor of glucose moiety to various acceptors and is also involved in cellulose synthesis and starch synthesis by its conversion to glucose-1-phosphate and ADP-glucose. The glucose formed from sucrose cleavage is phosphorylated to glucose-6- PO_4 by hexokinase (HXK, EC 2.7.1.1) which is a major regulatory enzyme in sugar metabolism and sugar-sensing (Rolland et al. 2006). Fructokinase (EC 2.7.1.4, FRK) performs the same catalytic function as HXK, but using fructose as substrate. Glucose-6- PO_4 can be metabolized either through glycolysis or via pentose phosphate pathway which is

responsible either for meeting the NADPH requirement for various biosynthetic reactions or for providing substrates for energy yielding metabolic pathways. Glucose-6- PO_4 dehydrogenase (G6PDH, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44) are the key enzymes of this pathway where the former one catalyses the first regulatory reaction and controls the metabolism of glucose-6- PO_4 by this pathway. Although several studies had been conducted in the past showing the effect of various abiotic stresses on carbohydrate metabolism in seedlings of different plants (Kaur et al. 2003a; Jha and Dubey 2004; Devi et al. 2007) but there is little information on the effect of Pb on carbon metabolism in pea seedlings. Therefore keeping in view the above mentioned points, effect of exogenous Pb on carbohydrate contents and vital enzymes of starch degradation, sucrose metabolism, pentose phosphate pathway and glycolysis has been studied.

Materials and methods

Plant material and seedling growth

Pea (*Pisum sativum* L) cv PB 88 seeds were dipped in 0.1 % HgCl_2 for 5–10 min and washed thoroughly with sterilized water under aseptic conditions. Seeds were germinated in the conical flasks on 0.9 % agar containing water (control) and different concentrations (0.25–1 mM) of $\text{Pb}(\text{NO}_3)_2$. Flasks were kept in the incubator at $25 \pm 1^\circ\text{C}$ under dark. Growth data was taken at 7th day of growth. Samples in triplicate were taken randomly from different flasks.

Extraction and estimation of soluble sugars and starch

Soluble sugars were extracted from 500 mg of roots, shoots and cotyledons by the method described previously (Devi et al. 2007). Reducing sugars were determined colorimetrically using reaction with arsenomolybdate (Nelson 1944). Sucrose was determined after its complete hydrolysis with invertase and then estimating glucose by glucose oxidase (Kaur et al. 2003b). Starch was estimated in the residue left after the extraction of soluble sugars by hydrolysing it with excess of amyloglucosidase and then estimating glucose as reducing sugars (Kaur et al. 2003b). Total sugars were estimated using phenol-sulphuric acid method of Dubois et al. (1956).

Extraction and assays of enzymes

Enzymes were extracted at 4°C . Polyvinylpyrrolidone (100 mg/g tissue) was also added to inhibit oxidation of phenolic compounds while extracting the enzymes.

Invertases were extracted by crushing the roots, shoots and cotyledons (500 mg) with chilled 20 mM sodium phosphate buffer (Dey 1986). The contents were centrifuged at

10,000×g for 10 min. The supernatant was passed through a sephadex G-25 column to remove reducing sugars. Assay system for acid invertase consisted of 160 mM of sodium acetate buffer (pH 5.0), 100 mM of sucrose and 0.1 ml of enzyme extract in total volume of 1 ml. Reducing sugars formed after sucrose hydrolysis were estimated by the method of Nelson (1944). The assay system for alkaline invertase (EC 3.2.1.27) was same as that described for acid invertase except that sodium acetate buffer was replaced by sodium phosphate buffer (pH 8.0).

Sucrose phosphate synthase (EC 2.4.1.14) and sucrose synthase (EC 2.4.1.13) were extracted from different plant tissues with the buffer (pH 8.2) containing 100 mM HEPES (N-[2-hydroxymethyl] piperazine ethane sulphonic acid), 10 mM EDTA, 15 mM KCl, 5 mM MgCl₂, 2 mM sodium diethyl dithiocarbamate and 5 mM β-mercaptoethanol. Extracted material was centrifuged at 10,000×g for 15 min. The supernatant was passed through sephadex G-25 column using 10 mM HEPES buffer (pH 7.0). Reaction mixture for SPS consisted of 7.5 mM of UDP-glucose, 30 mM of fructose-6-phosphate, 0.1 M of HEPES (pH 8.2) containing 20 mM of MgCl₂, 15 mM of NaF and 0.1 ml of enzyme extract in a total volume of 140 μl. The assay system for SS was similar to SPS except that fructose-6-phosphate was replaced with fructose and NaF was not added in the assay system.

Amylases were extracted from the roots, shoots and cotyledons by crushing them with 50 mM sodium acetate buffer (pH 5.0) containing 1 mM CaCl₂ (Devi et al. 2007). α-Amylase activity was determined after destroying the β-amylase by heating the enzyme at 70 °C for 20 min and estimating reducing sugars formed from 2 % starch in 50 mM sodium acetate buffer (pH 5.0) in presence of 1 mM CaCl₂. β-Amylase activity was determined by estimating the reducing sugars formed after the enzyme action on 1 % starch prepared in 50 mM sodium acetate buffer pH 5.0 containing 1 mM EDTA.

Important glycolytic and related enzymes were extracted and assayed by the procedure described earlier (Devi et al. 2007). The roots, shoots and cotyledons were crushed with extraction buffer consisting of 20 mM HEPES buffer (pH 8.0) containing 1 mM EDTA, 5 mM MgCl₂ and 5 mM β-mercaptoethanol. Homogenate was passed through four layers of cheese cloth and filtrate was centrifuged at 10,000×g for 15 min. Supernatant was used for estimation of hexokinase (EC 2.7.1.1), fructokinase (EC 2.7.1.4), phospho glucomutase (EC 2.7.5.1), phospho hexose isomerase (EC 5.3.1.9) and fructose 1, 6 biphosphatase (EC 3.1.3.11).

Key enzymes of pentose phosphate pathway including glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were extracted and assayed by the method of Yuan and Anderson (1987). The reaction mixture for G6PDH contained 50 mM HEPES buffer (pH 7.5), 0.2 mM NADP,

5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 0.03 % triton X-100 and enzyme. The reaction was started by adding 2.5 mM glucose-6-phosphate. The method for assaying 6PGDH was same as that of G6PDH except that instead of glucose-6-phosphate, 6-phosphogluconate was used as a substrate.

Protein contents of various enzyme extracts were estimated by using Folin-phenol's reagent (Lowry et al. 1951).

Statistical analysis

Growth data has been presented as mean ± SD of three samples of 10 seedlings each. It was statistically analysed by applying one-way analysis of variance (ANOVA) followed by post hoc analysis, the LSD (Least significant difference) test. Data for other biochemical parameters given in Tables 1 and 2 was analysed by applying two-way ANOVA with interaction. LSD-A, LSD-B and LSD-AB represent the values for stress, days and interaction between these two factors.

Results

Effect of varying concentrations of exogenous Pb on seedling growth

Exogenous Pb ions severely repressed the seedling growth and inhibition was concentration dependent in the range of 0.25 mM to 1 mM of Pb. With increased Pb ion concentration, the reduction of seedling growth was accompanied by decreased water content of cotyledons, shoots and roots (Fig. 1). With 0.5 mM Pb, reduction of about 50 % in shoot and 80 % in root lengths were observed (Fig. 1). Since with 0.5 mM of Pb, reasonable growth and biomass partitioning into the roots and shoots of pea seedlings were observed, therefore this concentration was selected for further biochemical studies. Further, a preliminary experiment was conducted with 0.5 mM concentration each of Pb, polyethylene glycol-6000 (PEG-6000), NaCl and mannitol alongwith the control to see whether the adverse effects obtained are due to Pb or osmotic stress. It was found that except Pb, this much concentration of all these osmotic agents had hardly any effect on the seedling growth (Fig. 2). Thus the adverse effects obtained in this investigation are due to Pb toxicity only.

Effect of Pb toxicity on carbohydrate composition of pea seedlings

In general, total sugars, reducing sugars and starch contents were in higher amounts in cotyledons as compared to growing tissues. In comparison with control seedlings, sugar content decreased by 23 and 17 % at days 3 and 7 respectively in the roots of Pb-stressed seedlings. In comparison to 3rd day of

Table 1 Effect of Pb (0.5 mM) on carbohydrate composition (mg/g FW) of different tissues of pea seedlings at different days of growth

Tissue	Days	Total sugars		Reducing sugars		Sucrose		Starch	
		Control	Pb-stressed	Control	Pb-stressed	Control	Pb-stressed	Control	Pb-stressed
Roots	3	13.2±0.6	10.2±0.9 ^a	8.7±1.1	10.2±0.3 ^a	2.6±0.2	2.8±0.5	3.4±0.1	3.0±0.1 ^a
	5	7.3±1.1 ^b	7.0±0.4 ^b	6.5±0.1 ^b	8.8±0.2 ^{a,b}	3.6±0.4 ^b	2.8±0.3	2.4±0.1 ^b	1.9±0.2 ^{a,b}
	7	11.1±0.3 ^b	9.2±0.5 ^{a,b}	7.5±0.9 ^b	8.0±1.0 ^b	3.2±0.3 ^b	3.4±0.4 ^b	2.5±0.2 ^b	1.5±0.2 ^{a,b}
LSD (5 %)		A-0.71, B-0.87, AB-1.23		A-0.75, B-0.91, AB-NS		A-NS, B-0.46, AB-NS		A-0.16, B-0.20, AB-0.28	
Shoots	5	8.5±0.3	7.1±0.5 ^a	8.7±0.7	6.8±0.7 ^a	3.2±0.5	4.0±0.5 ^a	1.6±0.4	1.3±0.1
	7	9.9±0.7 ^b	8.3±0.3 ^{a,b}	9.9±0.7 ^b	8.3±0.3 ^{a,b}	3.0±0.3	3.4±0.4 ^a	1.4±0.3	1.5±0.2
LSD (5 %)		A-0.64, B-0.64, AB-NS		A-0.83, B-0.83, AB-NS		A-0.58, B-NS, AB-NS		A-NS, B-NS, AB-NS	
Cotyledons	1	48.6±2.0	45.7±3.4	3.0±0.1	2.8±0.1	34.0±8.5	32.0±5.6	78.1±2.0	88.1±4.0 ^a
	3	34.0±0.7 ^b	35.6±2.7 ^b	2.4±0.1 ^b	2.6±0.2	26.0±2.8 ^b	23.0±1.4 ^b	55.0±1.0 ^b	79.2±4 ^{a,b}
	5	26.0±2.7 ^b	42.3±6.8 ^a	2.7±0.4	2.0±0.7 ^{a,b}	22.0±2.4 ^b	32.0±3.1 ^a	37.2±2.0 ^b	43.2±4.0 ^{a,b}
	7	22.0±1.4 ^b	36.0±0.7 ^{a,b}	2.0±0.4 ^b	1.4±0.2 ^{a,b}	11.0±0.9 ^b	21.0±2.3 ^{a,b}	39.1±2.0 ^b	49.1±3.0 ^{a,b}
LSD (5 %)		A-3.61, B-5.11, AB-7.23		A-0.29, B-0.42, AB-NS		A-3.53, B-4.99, AB-7.05		A-2.56, B-3.62, AB-5.12	

Values are mean ± SD of data obtained from triplicate samples

Least significant difference (LSD) at 5 % probability level- A-Stress, B-Days, AB-Interaction between stress and days

^a significant differences from respective controls

^b significant differences from respective initial day (1st day in cotyledons, 3rd day in roots and 5th day in shoots)

seedling growth, at 7th day, contents of total and reducing sugars reduced significantly in the roots of control and Pb-stressed seedlings. In the stressed roots, reducing sugar content was 17 and 35 % higher at 3 and 5 days of seedling growth respectively. With the progress of seedling growth, starch content declined in the roots however % decrease was more in stressed roots (Table 1). In comparison with control, total sugars and reducing sugars were less in the shoots of Pb-stressed seedlings, but sucrose content was about 10–20 % more in the shoots of seedlings grown in the presence of Pb. With the progress of seedling growth, total sugars, reducing sugars, sucrose and starch contents declined significantly in the normal and stressed cotyledons (Table 1). Cotyledons of Pb-stressed seedlings showed about 25–30 % lower contents of reducing sugars at 5 and 7 days of growth. Starch content in cotyledons of stressed seedlings was about 11, 30, 14 and 20 % higher at 1, 3, 5 and 7 days of growth respectively as compared to that of control seedlings (Table 1).

Effect of exogenous Pb on the activities of amylases and sucrose metabolizing enzymes

Exogenous Pb induced reduction in α -amylase activity was about 40–60 % in the roots, 55–60 % in the shoots and 20–40 % in the cotyledons. With the progress of seedling growth, α -amylase activity increased in the roots and cotyledons of control and stressed seedlings. Although with Pb ions in the medium, β -amylase activity was not significantly affected in the roots at different days of growth but reduction of about 10–20 % was observed in the shoots of Pb-stressed seedlings at 5

and 7 days of growth (Table 2). With the progress of seedling growth, relative increase in β -amylase activity was more in the cotyledons, followed by roots and shoots. In comparison with control, cotyledons of stressed seedlings showed about 16, 51 and 35 % reduction in β -amylase activity at 3, 5 and 7 days of growth respectively.

In general, invertase activity was maximum in the growing tissues and minimum in the cotyledons (Table 2). Pb-stress induced decrease in acid and alkaline invertase activities was about 30–50 % in the roots and shoots. With the progress of seedling growth, acid invertase activity increased in the roots of control as well as stressed seedlings but decreased in the shoots. In the shoots of control as well as Pb-stressed seedlings, alkaline invertase activity increased from 5th to 7th day of growth. However, invertases did not show any significant trend in the cotyledons (Table 2).

Exogenous Pb also resulted in 25–40 % reduction of sucrose phosphate synthase (SPS) activity in the roots but had no significant effect in the shoots and cotyledons. In general, with the progress of growth, SPS activity decreased in the roots and shoots but reverse trend was obtained in the cotyledons. At 5th day of growth, sucrose synthase (SS) activity exhibited about 65 % decrease in the roots of Pb-stressed seedlings. Exogenous Pb caused 70 % reduction of SS activity in the shoots at 7th day of growth however it increased in the stressed cotyledons at 3, 5 and 7 days of growth (Table 2). At 7th day of seedling growth, SS activity was significantly more in the normal and stressed cotyledons as compared to 3rd day of growth.

Table 2 Effect of Pb (0.5 mM) on amylases and sucrose metabolizing enzymes in pea seedlings at different days of growth

Tissue	Days	α -amylase		β -amylase		Acid invertase		Alkaline invertase		SPS		SS	
		Control	Pb-stressed	Control	Pb-stressed	Control	Pb-stressed	Control	Pb-stressed	Control	Pb-stressed	Control	Pb-stressed
Roots	3	18.6±0.3	11.1±0.9 ^a	159±2.1	149±10	48.6±3.3	22.2±3.3 ^a	31.1±3.0	15.1±0.5 ^a	5±0.4	3.8±0.5 ^a	6.1±0.4	6.0±0.1
	5	40.9±0.7 ^b	15.8±1.6 ^{ab}	260±5.9 ^b	256±10 ^b	58.2±0.8 ^b	26.3±3.8 ^a	31.2±0.6	17.3±2.1 ^a	7±0.1 ^b	4±0.9 ^a	4.8±0.5 ^b	1.7±0.1 ^a
	7	85.1±2.5 ^b	40.6±6.5 ^{ab}	380±2.8 ^b	406±4.1 ^b	84.4±6.2 ^b	61.8±8.4 ^{ab}	25.9±3.2 ^b	15.5±2.1 ^a	3±0.8 ^b	2.7±0.4 ^b	6.6±0.3 ^b	6.2±0.7 ^a
LSD (5 %)		A-3.04, B-3.72, AB-5.26		A-NS, B-7.98, AB-11.28		A-5.09, B-6.23, AB-NS		A-2.25, B-2.75, AB-NS		A-0.60, B-0.73, AB-1.04		A-0.42, B-0.52, AB-0.73	
Shoots	5	23.7±0.5	10.8±1.3 ^a	271±8.8	246±3.2 ^a	29.5±0.8	20.8±2.9	10.5±0.4	7.2±0.1	3±0.6	3.6±0.6	1.9±0.7	2.1±0.2
	7	17.9±2.6	7.2±0.3 ^a	344±8.1 ^b	280±5.2 ^{ab}	24.5±1.0	13.7±2.0	20±0.6 ^b	21±1.6 ^b	1.7±0.3 ^b	0.8±0.3 ^b	4.2±1	1.3±0.4 ^a
LSD (5 %)		A-5.46, B-NS, AB-NS		A-8.96, B-8.96, AB-12.68		A-NS, B-NS, AB-NS		A-NS, B-1.17, AB-1.66		A-NS, B-0.66, AB-0.93		A-0.87, B-NS, AB-1.22	
Cotyledons	1	Traces	Traces	1.0±0.6	0.4±0.1	0.25±0.1	0.21±0.1	4±0.8	6.9±0.4 ^a	13±0.7	11±0.9	10±0.6	9.4±0.5
	3	1.0±0.04	1.0±0.01 ^b	6.1±0.9 ^b	5.1±1.0 ^{ab}	0.11±0.02 ^b	0.2±0.02	3±0.6 ^b	2±0.1 ^{ab}	23.0±1.4 ^b	28±1 ^b	24.9±3 ^b	28.9±2 ^{ab}
	5	7.7±1.4	6.2±0.4 ^{ab}	8.2±0.4 ^b	4.0±0.8 ^{ab}	0.29±0.04	0.18±0.01	1.5±0.3 ^b	1.3±0.2 ^b	28±3 ^b	34±6 ^b	25.2±1 ^b	29.3±2 ^{ab}
	7	8.8±0.1	5.4±1.7 ^{ab}	14.2±1 ^b	9.2±0.9 ^{ab}	0.37±0.05 ^b	0.33±0.1	4.18±0.1	5.2±0.1 ^{ab}	25±2 ^b	21.0±2 ^b	24.6±3 ^b	30.6±2 ^{ab}
LSD (5 %)		A-0.69, B-0.97, AB-1.37		A-0.67, B-0.95, AB-1.34		A-NS, B-0.08, AB-NS		A-0.35, B-0.50, AB-0.70		A-NS, B-3.24, AB-4.59		A-1.74, B-2.46, AB-NS	

Specific activity has been expressed as nmole of product formed/min/mg of protein. Values are mean ± SD of data obtained from triplicate samples

LSD (5 %)- A-Stress, B-Days, AB-Interaction between stress and days

SPS Sucrose phosphate synthase and SS Sucrose synthase

^a significant differences from respective controls

^b significant differences from respective initial day (1st day in cotyledons, 3rd day in roots and 5th day in shoots)

Fig. 1 Effect of increasing concentrations of Pb on % water content and seedling growth at 7th day. Growth data are mean $\pm$ SD of three samples of 10 seedlings each

Fig. 2 Effect of 0.5 mM concentration of Pb, NaCl, PEG-6000 and mannitol on seedling growth at 7th day

Effect of exogenous Pb on key enzymes of pentose phosphate pathway

Enzymes of pentose phosphate pathway were studied at 7th day of seedling growth. Glucose-6-phosphate dehydrogenase (G6PDH) activity decreased by about 80 and 60 % in the roots and shoots of Pb-stressed seedlings respectively however it increased many folds in the stressed cotyledons (Table 3). Exogenous Pb caused significant up regulation of 6-phosphogluconate dehydrogenase (6PGDH) activity in the roots and cotyledons but had no significant effect in the shoots (Table 3).

Effect of Pb on important enzymes of glycolytic pathway

Effect of exogenous Pb on enzymes of glycolytic pathway was also investigated at 7th day of seedling growth. Although hexokinase (HXK) activity was up regulated in roots and shoots of stressed seedlings but it remained unchanged in the cotyledons (Table 3). It was about 50 and 70 % more in the roots and shoots of stressed seedlings respectively. Fructokinase (FRK) activity decreased in the roots and cotyledons of Pb-stressed seedlings but remained unaffected in the shoots. Phosphoglucomutase (PGM) activity was not affected in the shoots and cotyledons under Pb toxicity but increased in the roots of stressed seedlings. Exogenous Pb decreased phosphoglucosomerase (PGI) activity in shoots but had no effect in the roots and cotyledons. Fructose 1, 6-bisphosphatase (FBPase) activity did not show any significant differences in the control and Pb-stressed seedlings (Table 3).

Discussion

The reduction of seedling growth in the presence of exogenous Pb was accompanied by relative decrease in % water content of roots, shoots and cotyledons (Fig. 1). Choudhury et al. (2010) observed that rice seedlings in the presence of

Table 3 Effect of Pb (0.5 mM) on specific activities of enzymes of pentose phosphate pathway and glycolysis in pea seedlings at 7 days of growth

Enzymes	Roots		Shoots		Cotyledons	
	Control	Pb-stressed	Control	Pb-stressed	Control	Pb-stressed
G6PDH	3.1±0.9	0.6±0.2 ^a	7.5±1.3	2.9±1.2 ^a	0.4±0.1	8.4±1.5 ^a
6PGDH	3.4±1.1	6.2±1.0 ^a	9.6±1.2	8.7±1.8	5.2±0.7	13.4±4.2 ^a
H XK	2.9±0.2	4.3±0.8	2.8±0.2	4.8±1.1	4.2±0.2	2.9±1.1
FRK	13.2±2.1	8.14±1.2 ^a	12.3±1.3	13.1±1.7	5.9±0.6	4.0±1.3
PGM	4.3±0.6	6.9±1.7	16.9±3.9	11.7±2.2	48.2±10.9	36.2±5.3
PGI	205.6±10.3	188.9±14.4	199.5±16.9	139.9±9.6 ^a	71.1±11.1	87.7±12.3
F1,6BPase	5.7±0.6	6.5±1.2	13.9±2.4	11.0±2.0	4.1±0.7	5.9±2.9

Specific activity has been expressed as nmole of product formed/min/mg of protein. Values are mean ± SD of data obtained from triplicate samples
G6PDH Glucose-6-phosphate dehydrogenase, *6PGDH* 6-phosphogluconate dehydrogenase, *H XK* Hexokinase, *FRK* Fructokinase, *PGM* Phosphoglucomutase, *PGI* Phosphoglucoisomerase, *F1,6BPase* Fructose 1, 6-bisphosphatase

^a Differences significant in comparison with respective control at ≤0.05 (Student's t-test)

arsenate also showed lowered water content in the roots and shoots. Pb could possibly interfere with the water uptake capacity of seedlings which might have resulted in reduced seedling growth by affecting the carbohydrate status of different tissues. Thus metabolic effect of Pb will be due to both heavy metal toxicity and water deficit conditions. Earlier it has been reported that higher levels of Pb inhibited the growth of higher plants such as vegetable crops (Moftah 2000) and corn seedlings (Malkowski et al. 2002). Reduced seedling growth under heavy metal toxicity had also been correlated with decreased water absorption (Devi et al. 2007; Kuriakose and Prasad 2008).

In cotyledons of control seedlings, total sugars and sucrose content decreased with the progress of seedling growth indicating that sucrose is rapidly transported to growing shoots and roots and is not accumulated in the cotyledons. Although in Pb-stressed seedlings, decrease in sucrose content with the progress of seedling growth was arrested indicating slowing down of the sucrose transport from cotyledons to the growing tissues (Table 1). Reduced mobilization of sucrose from cotyledons had also been reported in Cd stressed pea seedlings resulting in elevation of level of this disaccharide (Devi et al. 2007). Although starch content was significantly less in the roots of Pb-stressed seedlings at days 3, 5 and 7 days of growth, it was significantly more in the cotyledons of stressed seedlings. Such restriction in starch mobilization has also been observed in germinating seeds of *Vicia faba* under cadmium toxicity (Rahoui et al. 2008).

In comparison with control, in Pb-stressed pea seedlings α -amylase activity was less in all tissues (Table 2). A recent study has also shown that Pb (50–500 μ M) inhibited α -amylase activity in mustard (Singh et al. 2011). It appeared that lower starch content in the roots of stressed seedlings was not due to rapid hydrolysis of starch but due to lower rate of starch synthesis because of less availability of carbon due to

decreased rate of starch hydrolysis in cotyledons. Reduced starch mobilization from reserve tissues was also observed in germinating *Phaseolus vulgaris* L. seedlings under cadmium (Sfazi-Bousbih et al. 2010a) and copper toxicities (Sfazi-Bousbih et al. 2010b) which was attributed to reduced α -amylase activities. Acid invertase is the enzyme localised in vacuoles and apoplastic space and is responsible for seedling growth. Reduced activities of invertases in the growing tissues could be one of the important factors responsible for decreased seedling growth. In our earlier studies, reduced activities of acid and alkaline invertases were also reported in the growing tissues of the cadmium stressed seedlings (Devi et al. 2007). Jha and Dubey (2004) had also reported a reduced acid invertase activity in rice seedlings under arsenic toxicity. Low activity of SPS in the roots and shoots of stressed seedlings showed that sucrose synthesizing capacity is down regulated in these tissues under Pb toxicity. With the progress of seedling growth, Pb caused elevation of SS activity in the cotyledons. Verma and Dubey (2001) also reported enhanced sucrose synthase activity in rice seedlings in the presence of 100–500 μ M Cd (NO₃)₂.

The glucose formed from sucrose cleavage by the action of invertase in the growing tissues, is phosphorylated to glucose-6-phosphate. This glucose-6-phosphate could be metabolized either through glycolysis or via pentose phosphate pathway which is primarily responsible for meeting the NADPH requirement for various biosynthetic pathways. Exogenous Pb inhibited glucose-6-phosphate dehydrogenase (G6PDH) activity in the roots and shoots but enhanced in the cotyledons of stressed seedlings. In contrast to this, Smiri et al. (2009) observed decreased activity of G6PDH in the cotyledons of cadmium stressed seedlings. 6-phosphogluconate dehydrogenase was up regulated in the roots and cotyledons of stressed seedlings but remained unaffected in the shoots (Table 3). In general these enzymes determine the fate of pentose phosphate

pathway. Therefore, decrease of G6PDH and 6PGDH activities in the shoots of stressed seedlings indicates that pentose phosphate pathway is hampered under Pb toxicity leading to lesser production of NADPH. However in contrast to this, Mocquot et al. (1996) observed increased G6PDH activity in roots of 14 day old maize seedlings grown in copper containing solution. Thus response of particular crop to each heavy metal may also vary.

Exogenous Pb induced HXK activity in the roots and shoots will lead to efficient conversion of sucrose hydrolysed hexoses to their phosphorylated forms for entering into the glycolytic pathway as observed in pea seedlings under cadmium toxicity (Devi et al 2007). Anoxia and chilling stress also induced HXK activity in the roots and shoots of *Echinochloa crus-galli* (Fox et al. 1998). HXK is the sugar sensor which can induce or repress variety of genes based upon the status of soluble sugars (Rosa et al. 2009). Fructokinase (FRK) bypasses HXK dependent pathway and thus it may be additional sensor for sugars (Gupta and Kaur 2005). Moreover, its activity decreased in the roots and cotyledons of Pb-stressed seedlings. Under stress conditions, supply of hexoses is reduced. Under limited supply of hexoses, HXK has a limited role metabolically and possibly acts as a signalling molecule (Kakumanu et al. 2012). Phosphoglucumutase (PGM) converts glucose-1-phosphate, formed by starch phosphorylase, into glucose-6-phosphate and its activity was maximum in the cotyledons and minimum in roots. Although, fructose 1, 6-bisphosphatase (FBPase) is an important irreversible enzyme which converts fructose-1, 6-bisphosphate to fructose-6-phosphate in gluconeogenesis and Calvin cycle which are both anabolic pathways. However, its activity did not vary significantly in control and Pb-stressed seedlings. Phosphoglucoisomerase (PGI) is involved in the conversion of glucose-6-phosphate into fructose-6-phosphate which is converted into sucrose by the activities of sucrose-6-phosphate synthase and sucrose-6-phosphatase. Down regulation of PGI and other enzymes associated with sucrose synthesis, in the shoots of stressed seedlings showed that sucrose accumulation in the shoots may not be due to its increased synthesis but it could be due to reduced activity of invertases. Phosphoglucoisomerase and phosphoglucumutase activities were not significantly affected in the cotyledons under Pb toxicity which further corroborate the fact that reduced seedling growth in the presence of exogenous Pb was mainly due to reduced activities of amylases and invertases in the cotyledons and growing tissues respectively. Proteomic studies have also shown that heavy metals caused general shutdown of carbon metabolism (Rodriguez-Celma et al. 2010).

The presence of thiol groups has been shown in the active site of yeast hexokinase and 6PGDH (Gray et al. 1983; Dallochio et al. 1983). But activities of these enzymes were not down regulated in the Pb-stressed seedlings. However

invertase which contains cysteine residue in its catalytic domain was down regulated in the growing parts of the stressed seedlings (Sturm 1999). Therefore effect of Pb is not solely based on its reaction with sulfhydryl groups. These results do not depict the exact mechanism by which Pb impairs seed germination and seedling growth, but give an important piece of information about the regulation of carbon metabolism by exogenous Pb which can be further exploited in reducing the adverse effects of Pb toxicity.

References

- Angelone M, Bini C (1992) Trace elements concentrations in soils and plants of Western Europe. In: Biogeochemistry of trace metals. Lewis publishers, Boca Raton, pp 19–60
- Baroja-Fernandez E, Munoz FJ, Saikusa T, Rodriguez-Lopez M, Akazawa T, Pozueta-Romero J (2003) Sucrose synthase catalyzes the de novo synthesis of ADPGlucose linked to starch biosynthesis in heterotrophic tissues of plants. *Plant Cell Physiol* 44:500–509
- Choudhury B, Mitra S, Biswas AK (2010) Regulation of sugar metabolism in rice (*Oryza sativa* L.) seedlings under arsenate toxicity and its improvement by phosphate. *Physiol Mol Biol Plants* 16:59–68
- Dallochio F, Matteuzzi M, Bellini T (1983) Evidence for the proximity of cysteine and a lysine residue in the active site of 6-phosphogluconate dehydrogenase. *Ital J Biochem* 32:124–130
- De Abreu CA, De Abreu MF, de Andrade JC (1998) Distribution of lead in the soil profile evaluated by DPTA and Mehlich-3 solutions. *Bragantia* 57:185–192
- Devi R, Munjal N, Gupta Anil K, Kaur N (2007) Cadmium induced changes in carbohydrate status and enzymes of carbohydrate metabolism, glycolysis and pentose phosphate pathway in pea. *Environ Expt Bot* 61:167–174
- Dey PM (1986) Change in the forms of invertases during germination of mungbean seeds. *Phytochem* 25:51–53
- Dubois M, Gilles KA, Hamilton JK, Rebers RA, Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* 28:350–356
- Fodor F (2002) Physiological responses of vascular plants to heavy metals. In: Prasad MNV, Strazalka K (eds) Physiology and biochemistry of metal toxicity and tolerance in plants. Kluwer Academic Publishers, Dordrecht, pp 149–177
- Fox TC, Green BJ, Kennedy RA, Rumpho ME (1998) Changes in hexokinase activity in *Echinochloa phyllipogon* and *Echinochloa crus-galli* in response to abiotic stress. *Plant Physiol* 118:1403–1409
- Gray AJW, Barron NM, Barnard EA (1983) Location in the yeast hexokinase structure of residues related to the enzyme activity. *Biosci Rep* 3:963–971
- Gupta AK, Kaur N (2005) Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. *J Biosci* 30:761–776
- Hirose T, Takano M, Terao T (2002) Cell wall invertase in developing rice caryopsis: molecular cloning of OsCIN1 and analysis of its expression in relation to its role in grain filling. *Plant Cell Physiol* 43:452–459
- Jha AB, Dubey RS (2004) Carbohydrate metabolism in growing rice seedlings under arsenic toxicity. *J Plant Physiol* 161:867–872
- Kakumanu A, Ambavaram MM, Klumas CM, Krishnan A, Batlang U, Myers E, Grene R, Pereira A (2012) Effects of drought on gene expression in maize reproductive and leaf meristem tissue revealed by RNA-seq. *Plant Physiol*. doi:10.1104/pp.112.200444

- Kaur S, Gupta AK, Kaur N (2003a) Effect of kinetin on starch and sucrose metabolising enzymes in salt stressed chickpea seedlings. *Biol Plant* 46:67–72
- Kaur S, Gupta AK, Kaur N (2003b) Indole acetic acid mimics the effect of salt stress in relation to enzymes of carbohydrate metabolism in chickpea seedlings. *Plant Growth Regul* 39:91–98
- Kaur G, Singh HP, Batish DR, Kohli RK (2010) Lead (Pb)-inhibited early root growth in wheat involves alterations in associated biochemical processes. *Bioscan* 5:433–435
- Kratovalieva S, Cvetanowska L (2001) Influence of different lead concentrations to some morpho-physiological parameters at tomato (*Lycopersicon esculentum* Mill.) in experimental conditions. *Maced Agric Rev* 48:35–41
- Kuriakose SV, Prasad MNV (2008) Cadmium stress affects seed germination and seedling growth in *Sorghum bicolor* L. Moench by changing the activities of hydrolyzing enzymes. *Plant Growth Regul* 54:143–156
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275
- Malicka A, Piechalak A, Tomaszewska B (2009) Reactive oxygen species production and antioxidative defense system in pea root tissues with lead ions: the whole roots level. *Acta Physiol Plant* 31:1053–1063
- Malkowski E, Kita A, Galas W, Karcz W, Kuperberg JM (2002) Lead distribution in corn seedlings (*Zea mays* L.) and its effect on growth and the concentrations of potassium and calcium. *Plant Growth Regul* 37:69–76
- Meyers DER, Auchterlonie GJ, Webb RI, Wood B (2008) Uptake and localisation of lead in the root system of *Brassica juncea*. *Environ Pollut* 153:323–332
- Mocquot B, Vangronsveld J, Clijsters H, Mecnh M (1996) Copper toxicity in young maize (*Zea mays* L.) plants: effects on growth, mineral and chlorophyll contents, and enzyme activities. *Plant Soil* 182:287–300
- Moftah AE (2000) Physiological responses of lead polluted tomato and egg plant to antioxidation ethylendiurea. *Menufiya Agric Res* 25:933–955
- Nelson N (1944) A photometric adaptation of Somogyi method of determination of glucose. *J Biol Chem* 153:375–380
- Patra M, Bhowmik N, Bandopadhyay B, Sharma A (2004) Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance. *Environ Exp Bot* 52:199–223
- Rahoui S, Chaoui A, Ferjani EE (2008) Differential sensitivity to cadmium in germinating seeds of three cultivars of faba bean (*Vicia faba* L.). *Acta Physiol Plant* 30:451–456
- Rodriguez-Celma J, Rellan-Alvarez R, Abadia A, Abadia J, Lopez-Millan A-F (2010) Changes induced by two levels of cadmium toxicity in the 2-DE protein profile of tomato roots. *J Proteomics* 73:1694–1706
- Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. *Ann Rev Plant Biol* 57:675–709
- Rosa M, Prado C, Podazza G, Interdonato R, Gonzalez JA, Hilal M, Prado FE (2009) Soluble sugars-metabolism, sensing and abiotic stresses. *Plant Signal Behav* 4:388–393
- Sfafi-Bousbih A, Chaoui A, El Ferjani E (2010a) Cadmium impairs mineral and carbohydrate mobilization during germination of bean seeds. *Ecotoxicol Environ Safety* 73:1123–1129
- Sfafi-Bousbih A, Chaoui A, El Ferjani E (2010b) Copper affects the cotyledonary carbohydrate status during the germination of bean seed. *Biol Trace Elem Res* 137:110–116
- Sharma P, Dubey S (2005) Lead toxicity in plants. *Braz J Plant Physiol* 17:35–52
- Singh I, Kansal BD (1983) The accumulation of Cu, Zn, Cd and Pb in sewage and tubewell-irrigated soils in Punjab. National conference on lead, zinc, cadmium at work place: environment and health care. New Delhi 14–15 Dec. 1982. Indian Lead Zinc Information Centre, New Delhi
- Singh HP, Kaur G, Batish DR, Kohli RK (2011) Lead (Pb)-inhibited radicle emergence in *Brassica campestris* involves alterations in starch-metabolizing enzymes. *Biol Trace Elem Res* 144:1295–1301
- Smiri M, Chaoui A, Ferjani EE (2009) Respiratory metabolism in the embryonic axis of germinating pea seed exposed to cadmium. *J Plant Physiol* 166:259–269
- Sturm A (1999) Invertases: Primary structures, functions and roles in plant development and sucrose partitioning. *Plant Physiol* 121:1–7
- Verma S, Dubey RS (2001) Effect of cadmium on soluble sugars and enzymes of their metabolism in rice. *Biol Plant* 44:117–123
- Yang J, Zhang J, Wang Z, Zhu Q (2001) Activities of starch hydrolytic enzymes and sucrose-phosphate synthase in the stems of rice subjected to water stress during grain filling. *J Exp Bot* 52:2169–2179
- Yuan XH, Anderson IE (1987) Changing activity of glucose-6-phosphate dehydrogenase from pea chloroplasts during photosynthetic induction. *Plant Physiol* 85:598–600
- Zaier H, Mudarra A, Kutscher D, Fernandez de la Campa MR, Abdelly C, Sanz-Medel A (2010) Induced lead binding phytochelators in *Brassica juncea* and *Sesuvium portulacastrum* investigated by orthogonal chromatography inductively coupled plasma-mass spectrometry and matrix assisted laser desorption ionization-time of flight-mass spectrometry. *Anal Chim Acta* 671:48–54