CHANGES IN ACID PHOSPHATASE ACTIVITY AND ISOFORM PATTERNS IN ROOT CYTOPLASMIC AND MEMBRANE BOUND PROTEIN FRACTIONS OF *PISUM SATIVUM* L. SEEDLINGS EXPOSED TO CADMIUM APPLICATION

MODIFICĂRILE ACTIVITĂŢII FOSFATAZEI ACIDE ŞI A PATERNULUI IZOENZIMATIC ÎN FRACŢIILE PROTEICE DIN CITOPLASMA ŞI MEMBRANELE RĂDĂCINILOR PLANTULELOR DE *PISUM SATIVUM* L EXPUSE APLICĂRII CADMIULUI

***CAUŞ Maria*[1]**
e-mail: mcausmcv@yahoo.com

Abstract. *Changes in pea seedlings biomass and height, acid phosphatase (AcP) activity as well as isozymes of AcP patterns were analyzed in root cytoplasmic and membrane bound protein fractions of root seedlings grown under 4 and 40 µM $CdCl_2$ for 10 days. Significant decrease has been shown in growth and development of pea seedlings under cadmium (Cd) treatments. In pea root seedlings considerable increase in total cytoplasmic and covalently bound AcP activity was observed under Cd treatment at 4 and 40 µM $CdCl_2$. While both Cd concentrations notably declined the level of total root ionically bound AcP activity. Changes in total AcP activity from protein fractions separated from roots of 10 day old pea seedlings grown at the both concentrations of Cd were accompanied by the changes in the activity of AcP isozymes. Cd induced activity of five cytoplasmic AcP isozymes and two covalently bound AcP, but inhibited the activity of three ionically bound AcP.*

Key words: pea, seedlings, roots, acid phosphatases, isoforms, cytoplasmic, membrane bound, cadmium

Rezumat. *Au fost analizate modificările biomasei şi înălţimii plantulelor, activitatea fosfatazei acide (FA), precum şi spectrele izoenzimatice ale FA din citoplasma şi fracţiile proteice legate de membranele celulare radiculare ale plantulelor de mazăre, crescute timp de 10 zile în prezenţa a 4 şi 40 µM $CdCl_2$. A fost demonstrată o diminuare semnificativă în creşterea şi dezvoltarea plantulelor de mazăre sub influenţa tratamentelor cu Cd. În rădăcinile plantulelor de mazăre s-a observat o majorare semnificativă în activitatea totală a FA citoplasmice şi legate covalent in membranele radiculare ale plantulelor de mazăre la tratarea cu 4 şi 40 µM $CdCl_2$. Tot odată, ambele concentraţii de Cd au diminuat în mod semnificativ nivelul activităţii totale a FA legate prin legături ionice în membranele celulelor radiculare. Schimbările în nivelul activităţii totale a FA din fracţiile proteice separate din rădăcinile plantulelor cu vârsta de 10 zile, crescute în prezenţa ambelor concentraţii de Cd, au fost însoţite de modificări în activităţile izoenzimelor FA. Cd a indus*

[1] Institute of Genetics, Plant Physiology and Protection, Academy of Sciences of Moldova, Chisinau, Republic of Moldova

activitatea a cinci izoenzime FA din citoplasmă şi a două izoforme FA legate prin legături covalente în membranele celulare, dar a inhibat activitatea a trei izoenzime FA legate prin legături ionice în membranele celulelor radiculare.

Cuvinte cheie: mazăre, plantule, rădăcini, fosfataza acidă, izoforme, citoplasmă, membrane, cadmium

INTRODUCTION

Heavy metals, including Cd compounds, represent a serious stress factors due to their widespread in the environment (Benavides, 2005). The main sources of Cd compounds in the nature ecosystems may be the consequences of various human activities, including production and intensive use of phosphorus fertilizers and poor wastewater management (Jensen and Bro-Rasmussen, 1992). Most plants are sensitive to even low concentrations of Cd, which inhibits the vegetative growth in consequence of disturbances in various physiological and biochemical processes (Rodríguez-Serrano *et al.*, 2009). To deal with toxic concentrations of Cd, plants have developed a range of protective mechanisms, including induction of hydrolytic enzyme activities (Rodrıguez-Serrano *et al*., 2009).

Acid phosphatases (AcP; E.C. 3.1.3.2) are enzymes which hydrolyze a wide variety of phosphate esters with the release of orthophosphate anion (P_i) and energy, that are very important for diverse physiological processes of plant metabolism (Duff *et al*., 1994). In plants there are non-specific AcP, that show little or no substrate specify and AcP, which display a relative definite substrate specificity (Duff *et al*., 1994). Non specific AcP can exist in both soluble and membrane-bound forms and to have an important role in metabolism of seed germination and seedling growth (Asaduzzaman *et al*., 2011; Murtaza and Rehana Asghar, 2013).

The aim of this study was to establish the effect of different concentrations of cadmium on total activity and multiple forms of cytoplasmic and wall-bound acid phosphatases in protein fractions, separated from roots of pea seedlings.

MATERIAL AND METHOD

Plant Materials and cadmium treatment. Pea seeds (*Pisum sativum* L.) were surface sterilized with 10% H_2O_2 for 10 min and then rinsed several times with distilled water. Seeds were germinated on filter paper in dark for 10 days at 25°C. Germinated seeds were transferred and raised in plastic sinks in hydroponic culture of half strength Hoagland's nutrient solution in a growth chamber with a photoperiod of 16h/8h (light/darkness) at 24°C and 18ºC. Irradiance of 80 µmol m^{-1} s^{-1} was provided by white fluorescent tubes. The nutrient solution was replaced with fresh solution every day. Cadmium treatments were applied using Hoagland's nutrient solution with 4 and 40 µM $CdCl_2$. Control seedlings were grown on nutrient solution without cadmium application. Ten day old root seedlings were collected for separation of protein fractions and enzyme activity analyses.

Separation of cytosol and membrane-bound protein fractions from seedling roots. Pea roots (5 g) were cut in small pieces and homogenized with

pestle and mortar with 10 ml of homogenization media consisted of 50 mM Tris/Mes, 2 mM EGTA, 0,5 mM PMSF, 2 mM DTT, 0,1 % BSA, 0,125 M sorbitol, pH=7,4. After filtration through six layers of muslin cloth the homogenate was centrifuged 5 min, 800 g, 4ºC. Upper layers of extracts were centrifuged 20 min at 9000 g. Then supernatants were centrifuged 30 min at 42 000 g and 4ºC to obtain cytosolic and membrane fractions of proteins. Obtained supernatants were used as cytoplasmic fraction of AcP activity. Resulting pellets represented membrane protein fractions (MPF), which were subsequently three times washed with cold deionized water. Washed pellets of MPF have been dissolved in 10 mM Tris/Mes, 1 mM EGTA, 2 mM DTT, 1 mM MgCl2, 20% glycerol, pH=7,4 (buffer A) and subsequently were used to obtain ionically and covalently membrane bound protein fractions. 200 **µ**l of membrane fractions dissolved in buffer A were incubated on ice with media consisted of 1 % Triton X100, 1 M $CaCl_2$, 1 M NaCl, 0,1 mM Tris/Mes, pH=7,4. After 25 min incubated MPF were centrifuge 1 h at 20800 g and 4^0C. Resulting supernatants were designated as ionically bound protein fractions. Pellets were dissolved in 0.1 M sodium acetate buffer, pH=5,0 with 0.8 % cellulase and 0.6% pectinase, kept during of 20 h at 30^0C and then centrifuged 1 h at 20800 g and 4^0C. Supernatants were used as covalently bound protein fractions.

Acid phosphatase (AcP; EC 3.1.3.2) activity determination. A mixture of 15 µl enzyme extract, 24 mM sodium acetate buffer (pH=4.7), 5 mM p-nitrophenyl phosphate (PNP), as substrate, was incubated 20 min at 30ºC. The reaction was stopped with 0.5 M NaOH and absorbance was measured spectrophotometrically at 400 nm against the control reaction with boiled enzyme extracts. Changes in enzyme activities were expressed as a percentage of control.

Electrophoresis was carried out by the method (Laemmli U.K., 1970) on 7.5 % (w/v) polyacrylamide gel (PAGE) under non-denaturing conditions. Electrophoretic separation was effectuated during of about 2.5 hours at 4ºC. Following the electrophoretic separation the gels were stained for AcP activity using a solution consisting of 0.2M sodium acetate buffer (pH 5.0), 0.1 % alpha-Naphthyl phosphate, 5 mM $MgCl_2$ and 0.1% Fast Black for 1 h at 30°C. Differences between variants were documented by ANOVA analysis ("Statistics 7").

RESULTS AND DISCUSSIONS

Application of $CdCl_2$ to nutrient media had affected significantly growth rate as fresh biomass, root length and stems height of pea seedlings (tab.1). Both $CdCl_2$ concentrations (4 and 40 µM) inhibited the growth of pea seedlings, but the inhibition level of Cd at 40 µM was much higher than at 4 µM. It was observed a reduction of about 7 % and 32 % in the roots biomass, and of 21 % and 26 % in the upper parts biomass relative to control plants at Cd concentration of 4 and 40µM (tab.1). Administration of $CaCl_2$ at 40 µM to nutrient media reduced significantly the length of roots, which constituted about 33 % of control variant (tab.1). Root growth inhibition it is considered a general symptom of Cd toxicity (Siroka *et al.*, 2004). The reduction in biomass of Cd treated pea plants (4 and 40 µM) for 7 days in hydroponic culture was accompanied by the maximum accumulation of Cd in roots, followed by stems and leaves (Dixit *et a*l., 2001).

Table 1

Growth parameters of pea seedlings grown under $CdCl_2$ application in the nutrient media during of 10 days.

Treatments	Fresh weight (FW), g^{-1}		Length, cm^{-1}	
	root	upper part	root	upper part
Control	0.503 ± 0.092	0.864 ± 0.127	19.31 ± 0.129	8.25 ± 0.059
Cd_1 – 4 µM	0.467 ± 0.029	0.682 ± 0.042	17.95 ± 0.401	7.8 ± 0.064
Cd_2 – 40 µM	0.339 ± 0.209	0.624 ± 0.003	12.96 ± 0.085	6.05 ± 0.210

Studies with cell culture demonstrated that tomato cells can adapt to excessive amount of Cd^{2+} by accumulating Cd into the cytoplasm as Cd-binding peptides (Nouhe *et al*., 1991).

Our studies effectuated to establish the level of total AcP activity in different protein fractions, separated from pea root seedlings grown under 4 and 40 µM $CdCl_2$ in the nutrient media indicated an increase in total root cytoplasmic AcP activity by 20% and respectively 30% compared to control samples (fig.1). Also, an enhance of about 10% in total covalently bound AcP activity was observed in Cd treated root seedlings, the enzyme activity being approximately at the same level under the both cadmium treatments (4 and 40 µM) compared to the activity level in control (fig.1). Analysis of AsP activity in ionically bound protein fractions separated from root seedlings demonstrated a decline in enzyme activity. Seedling growth during of 10 days with 4 µM CdCl2 led to a decrease of total ionically bound AcP activity in roots of about 20 % and a decline of about 50 % in root seedlings with 40 µM CdCl2 (fig.1).

Cadmium induced increase of total cytoplasmic AcP activity (fig.1) was accompanied by the increase in the intensity of four acid phosphatase isoforms in soluble protein fractions from cytosol of root seedlings (fig.2A).

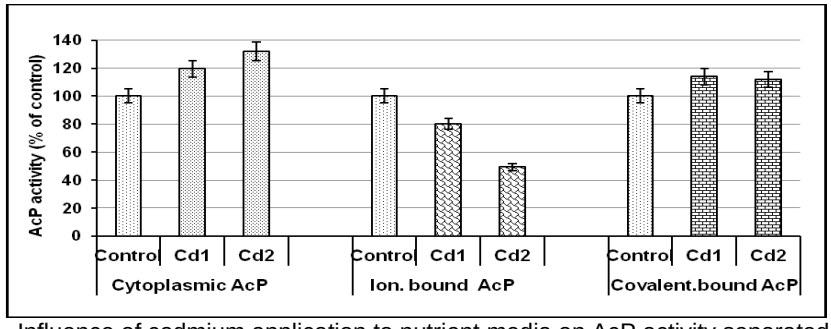

Fig.1 Influence of cadmium application to nutrient media on AcP activity separated from soluble protein fractions of root cytosol (cytoplasmic AcP), and from root cell membrane fractions – ionically (ion.) and covalently (covalent.) bound AcP. Control – Cd untreated seedlings; Cd1 – 4 µM CdCl2 and Cd2 – 40 µM CdCl2.

It suggests that the increase in total soluble enzyme activity under the both cadmium treatments (4 and 40 µM) compared to the activity level in control cytoplasmic AcP from pea root seedling (fig. 1) is due the increase of activity of existing AcP isoforms (fig. 2A).

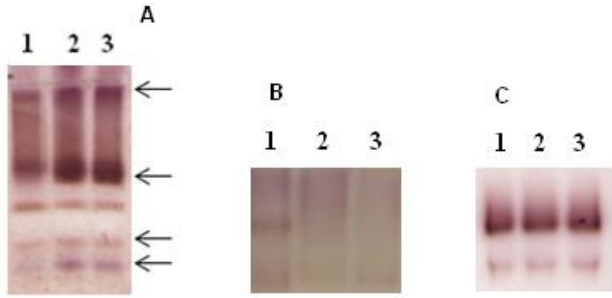

Fig. 2 Analysis of AcP isozymes separated from root soluble protein fractions: A – cytoplasmic AcP, from membrane protein fractions: B - ionically bound AcP and C – covalently bound AcP. 1 – Control, Cd untreated seedlings; 2 – 4 µM CdCl2 and 3 – 40 µM CdCl2

The results showed also that the application of Cd to nutrient media resulted in no markedly differences in number and activity of covalently bound AcP isoforms (fig. 2C). But, as it can be observed from the results presented in figure 2B the three ionically bound to membrane AcP isoenzimes present in control root seedlings are inhibited by the both (4 and 40 µM) Cd concentrations. Isozyme composition of acid phosphatase from different cell compartments are depended on the plant species, type of vegetative organ, plant development stages, nutrient composition of cultivation media and influence of stress factors (Johnson, 1973). Root phosphatases, particularly root surface phosphatases are important for plant phosphorous nutrition, as they can participate in the providing of plants with available forms of phosphorous compounds (Duff *et al.,* 1994). Previous studies (Dixit *et al.,* 2001) have shown that roots and leaves of pea (*Pisum sativum* L. cv. Azad) exposed to 4 and 40 µM CdCl2 performed a differential antioxidative responses to Cd application. Predominantly accumulation of Cd in roots was accompanied by an induction of antioxidant enzyme activities. So, along with an enhanced Cd accumulation in roots, that causes oxidative stress, which results in enzymatic antioxidative responses (Rodrıguez-Serrano *et al*., 2009; Dixit *et al.,* 2001; Pandey *et al*., 2012) also an induction or inhibition of total AcP activity as well as isozymes of AcP from soluble and membrane bound protein fractions of root cell cytosol occurs. In addition, it has been reported (Nouhe *et al.,* 1991) that tomato cells are capable to develop tolerance to Cd^{2+} ions up to 1 mM due to the synthesis of Cd-binding peptides in the cytoplasm and to retain smaller amounts of Cd^{2+} ions in the cell wall. We suppose that the energy necessary in the processes of Cd-binding peptides synthesis might be provided by the hydrolytic reactions of phosphorous compounds with involvement of acid phosphatase isoforms, localized in cytosol. On the other hand, our results on the differences in the level of acid phosphatase activity of the isoenzymes bound either ionically or covalently to the cell membrane under Cd treatments indicate on the different physiological mechanism of their regulation.

CONCLUSIONS

1. Inhibition of pea seedlings growth by $CdCl_2$ application to nutrient media do not correlate with total activity and intensity of acid phosphatase isoforms of soluble and covalently bound to membrane protein fractions in pea roots.

2. The increase in total AcP activity from root cytosol and covalently bound protein fractions occurred mostly due to the induction of existing AcP isoforms, since no new AcP isoenzymes appeared.

3. The presence of Cd^{2+} ions in the nutrient media caused an inhibition of total AcP activity as well as suppression of AcP isoenzymes in ionically bound protein fractions from pea seedling roots.

REFERENCES

1. **Asaduzzaman A.K.M., Habibur Rahman M., Yeasmin Tanzima, 2011** - *Purification and characterization of acid phosphatase from a germinating black gram (Vigna mungo L.) seedling*. Arch. Biol. Sci., Belgrade, 63 (3), p. 747-756.
2. **Benavides M.P., Gallego S.M., Tomaro M., 2005** -*Cadmium toxicity in plants*. Braz J Plant Physiol., 17, p. 21–34.
3. **Dixit V., Pandey V., Shyam R., 2001** - Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L., cv. Azad. J. Exp. Bot., 52 (358), p. 1101-1109.
4. **Duff S. M. G., Plaxton W. C., Sarath G. C., 1994**- *The role of acid phosphatases in plant phosphorus metabolism*. Physiol. Plant., 90, p. 791-800.
5. **Jensen A., Bro-Rasmussen F., 1992** - *Environmental cadmium in Europe*. Rev. Environ. Contam. Toxicol., 125, p.101-181.
6. **Johnson C.B., Holloway B. R., Smith H. et al., 1973** - *Isoenzymes of acid phosphatase in germinating peas*. Planta, 115, p. 1-10.
7. **Laemmli U.K., 1970** - *Cleavage of structural proteins during the assembly of the head of bacteriophage Tu*. Nature, 227, p. 680-685.
8. **Murtaza G., Rehana Asghar, 2013** - *Effect of salicylic acid on acid phosphatase activity during development and germination in pea (Pisum sativum).* Intern. J. Agric.Biology, 15 (3), p. 493-498.
9 **Nouhe M., Mitsumune M., Tohoyama H. et al., 1991** - *Contribution of cell wall and metal-binding peptide to Cd- and Cu tolerances in suspension-cultured cells of tomato.* Bot. Mag. Tokyo, 104, p. 217-229.
10. **Pandey N., Singh G.K., 2012** - *Studies on antioxidative enzymes induced by cadmium in pea plants (Pisum sativum)*. J. Environ. Biol., 33, p.201-206.
11. **Rodrıguez-Serrano M., Romero-Puertas M. C., Pazmino D.M. et al., 2009** - *Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium.* Plant Physiology, 150, p. 229 – 243.
12. **Siroka B., Huttova J., Tamas L. et al. 2004** - Effect *of cadmium on hydrolytic enzymes in maize root and coleoptile*. Biologia, Bratislava, 59 (4), p.513-517.