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Annual Report

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Phytoremediation of soils contaminated with heavy metals and radionuclides

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Executive Summary

The overall objective of this project is to optimize the use of plants to remove heavy metal contaminants from soils and water resources in affected areas of Kazakhstan, the former atomic testing ground of the ex-USRR. Phytoremediation of soils and water will decrease the health hazards to local population, much of which has been affected by radionuclides, making possible the use of vast tracks of contaminated land into useful agricultural areas.

Work has started with two types of plant species: (a) crops such as barley, pea, tomato and maize and (b) Some well known HM hyperaccumulators like Typha and Phragmites. During this first year of work, we have focused on the determination of methodologies and key enzymes involved in the production of phytochelatines in order to understand better the key metabolic systems which provide the plant with means to chelate and accumulate heavy metals. We have studied the following system:

- Regulation of the enzyme OASTL (O-acetylserine thiol lyase) or cysteine synthetase
- Regulation of the incorporation of cysteine into gluthatione
- Regulation of the incorporation of glutathione into phytochelatines
- Study of the relationship of heavy metal accumulation with enzyme induction and activity
- The effect of N source (nitrate and ammonium), HM and salinity on the activation of key enzymes of phytochelatine biosynthesis

All work carried out during this first year was in compliance with the objectives of the project. Collaboration between the two groups has been good and Dr. Vladimir Kuzovlev is working in Sede Boqer since August 1999. The Israeli investigators will visit the field experimental sites in Kazakhstan during the present year 2000.

Section I

A) Research Objectives

Overall aim and specific objectives

Overall aim: Removal heavy metals and radionuclides from contaminated soils by the use of selected annual crops capable of taking up and immobilizing in the plant biomass large amounts of the polluting agents.

Specific objectives:

1. Preliminary selection of appropriate endemic and/or non-endemic (a rare and endangered) annual plant populations with heavy metal accumulation potential, with anatomical characteristics which facilitate harvesting.

2. Study of the biosynthesis of metal-chelators and transporters in roots and shoots of the selected plants, and determination of optimal metal accumulation during the plant life cycle.

3. Application of simple agrotechniques such as fertilization with nitrate, sulfate and Mo to enhance biomass production and the chelating capacity of the selected plants.

4. Study of additional chelating mechanisms in the plants and determination of optimal conditions for heavy metal influx, transport and compartmentation.

This project is meant to select plant species capable of accumulating heavy and radioactive metals, to enhance their uptake and chelation in roots as well as their transport to the shoot and their storage in vacuoles. These studies will be carried out in parallel in field experiments with the selected plant species in order to devise new agrotechniques for the optimization of heavy metal removal. At a later stage of this project, production of transgenic plants reinforced by molecular insertion of foreign genes controlling heavy metal transport and chelate production is envisaged.

B) Research Accomplishments

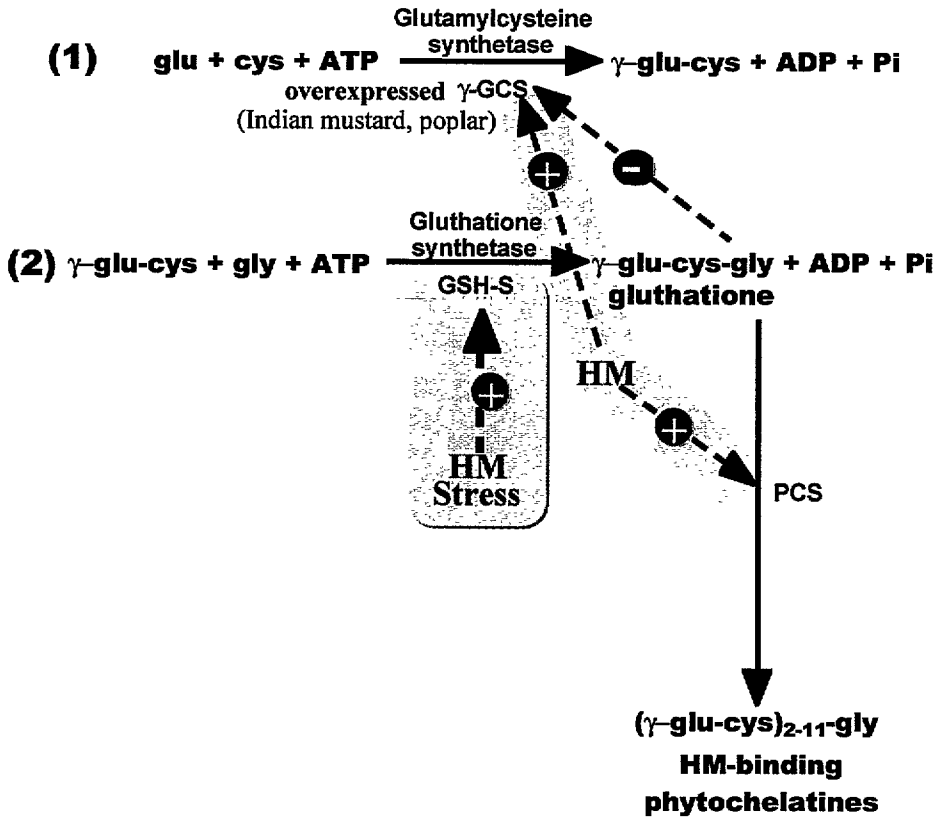
The first year of work has focused mainly on studies of the biosynthesis of heavy metal chelates by plant roots and some aspects of the regulation (induction and activation) of key enzymes of the biosynthetic pathways involved in the process. Two types of plant species were studied: (I) Crops (pea, barley, maize, tomato) and (II) known hyper-accumulators (typha and phragmites)

(a) Regulation of phytochelatin synthesis and accumulation in pea plants.

Soils contaminated with heavy metals often show increased levels of more than one metal. An excess of heavy metals such as Cd, Pb, Zn, Ni, Cu and others in the soil become extremely toxic to plants. Some plant species possess a unique ability to adapt rapidly and evolve tolerance to toxic or even lethal levels of heavy metals. Plants can detoxify heavy metals ions by means of a family of heavy metal-binding polypeptides called (MBPs), metallothionins (MTs) and phytochelatins (PCs) which bind metal ions through closely spaced cysteine thiolate groups. This polypeptides and glutathione have been implicated in heavy-metal homeostasis in plants. Phytochelatins are produced in plants upon exposure to heavy metals. As soon as free metal ions are chelated, PC production is terminated. After metal binding, PCs seem to be degraded. PCs may serve as a biomarker for heavy metal toxicity.

Glutathion (GSH) plays several important roles in defence strategies of plants against environmental stress. GSH is the precursor of phytochelatines, which allow the plant to withstand supra-optimal concentrations of heavy metals. GSH often constitutes the major pool of non-protein reduced sulfur. Factors that may control the rate of GSH synthesis in plants are (1) the availability

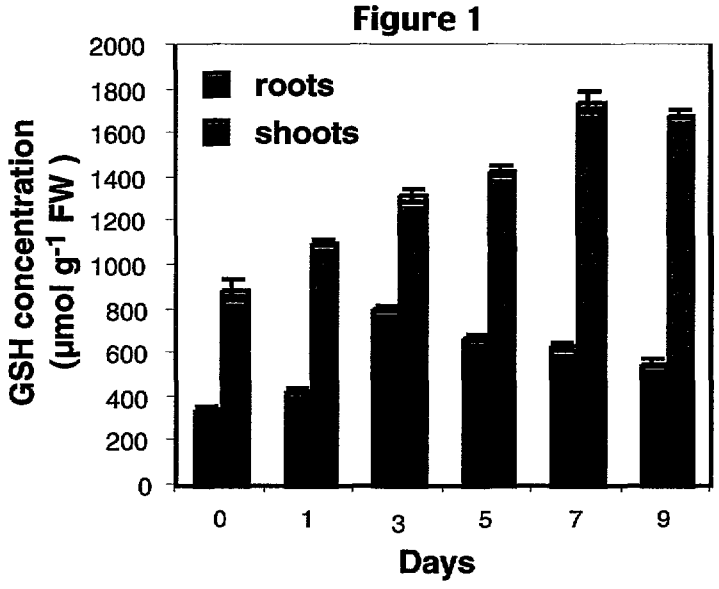
of cysteine, (2) feedback inhibition of glutamyl-cysteine synthetase (γ- ECS) by GSH and (3) the activity of glutathione synthetase (GS). A correlation has been observed between PC biosynthesis and the quantity of GSH in the tissue.



The chemical structure of PC bears close similarity to GSH. PC has the general structure (γ- Glu-Cys)n-Gly, with n=2-11. PCs are products of enzymatic biosynthesis. PC cannot be primary gene products that are synhesized on the ribosome because the γ-glutamyl linkage is not produced during translation and, in the case of iso-PC (β-Ala). Phytochelatin synthase (PS) adds the Glu-Cys from glutathione to another residue, resulting in the elongation of phytochelatin chain increasing the number of repeating units from n to n+1. Longer chain length seems to bind metals more tightly.

Plants vary in their response to stress factors and particularly to heavy metals. For inactivation of heavy metals plants synthesise phytochelatines. The increase of phytochelatin levels is usually preceded by enhanced levels of GSH in plant tissues.

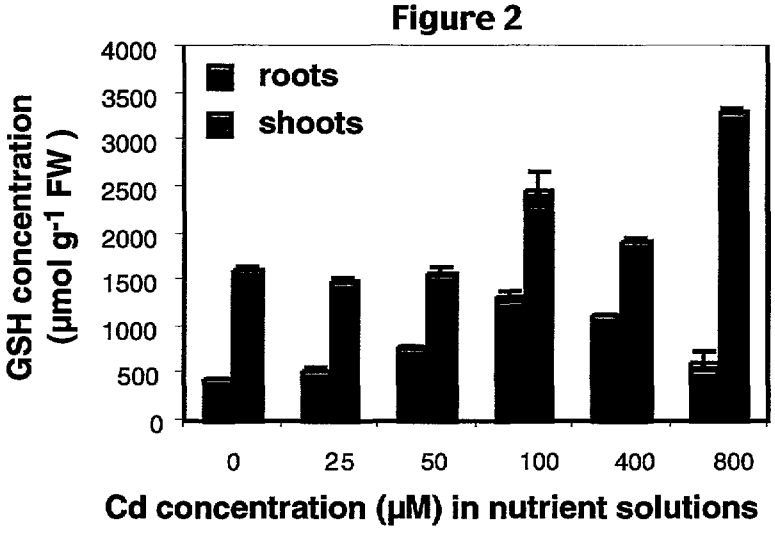
The highest level of GSH following heavy metal treatments was observed in pea plants after 7-9 days (Fig.1).



Time course of GSH accumulation in roots and shoots of Pea plants in response to Cd (50µM).

For seedlings GSH level was highest after seven days of treatment with Cd (Fig.1). The level of GSH in seedlings was significantly higher than in roots of plants.

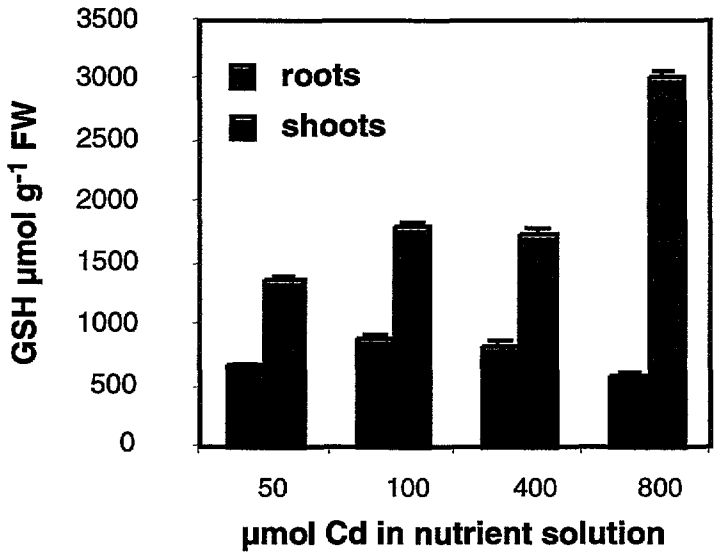
Cd at concentration between 100 - 800 µM effectively stimulated synthesis of GSH in roots of pea seedlings (Fig.2).



GSH pool in Pea plants as affected by Cd concentration for 5 days.

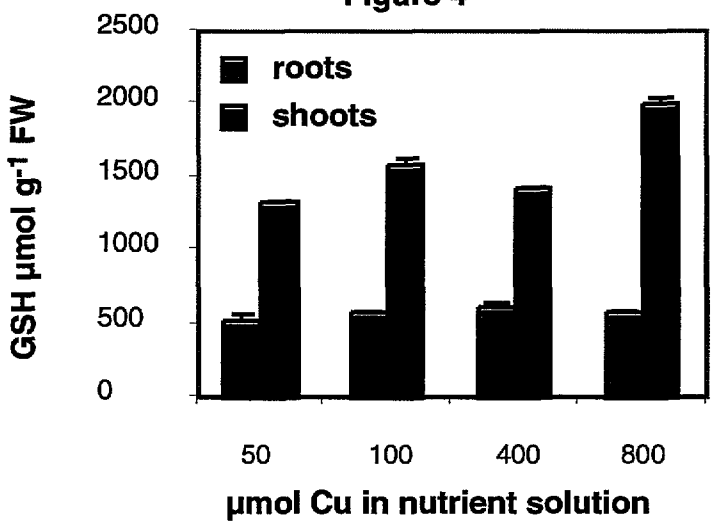
When Cu and Zn were used in nutrient medium no significant levels of GSH were observed in roots and seedlings of pea plants. Therefore Cu and Zn had less potent effect on GSH synthesis and accumulation in pea plants(Fig.3,4,5).

Figure 3



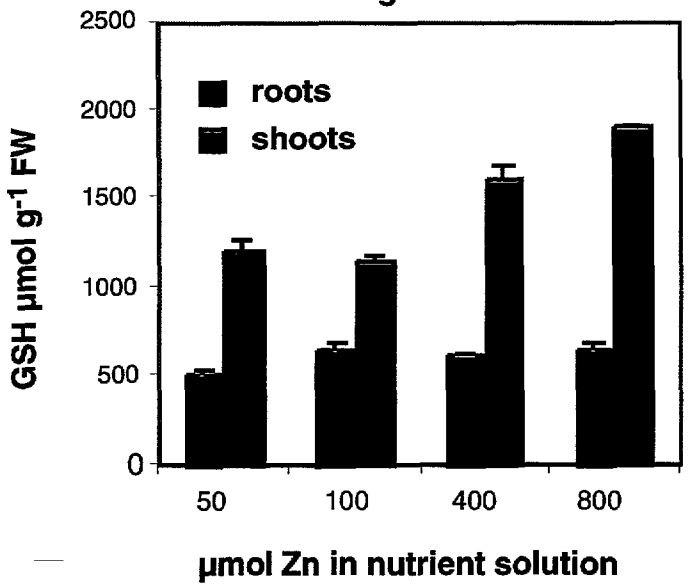
GSH pools in Pea seedlings as affected by growth in nutrient solution containing Cd for 3-5 days.

Figure 4



GSH pools in Pea seedlings as affected by growth in nutrient solution containing Cu for 3-5 days.

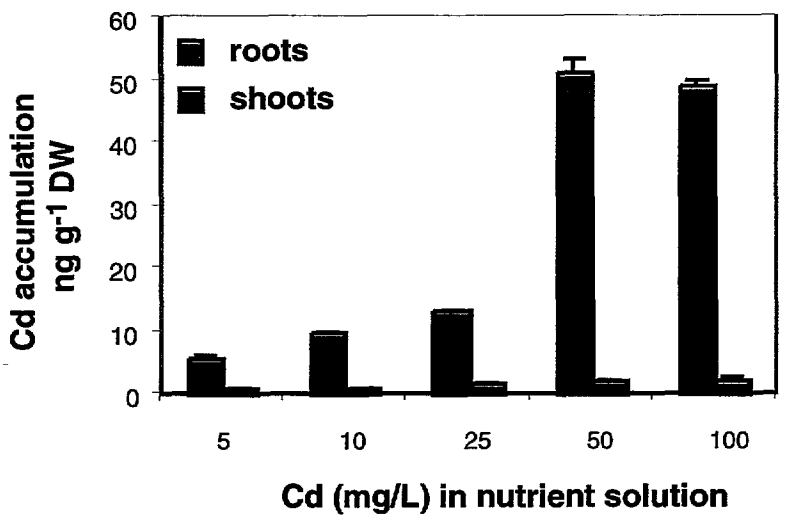
Figure 5



GSH pools in Pea seedlings as affected by growth in nutrient solution containing Zn for 3-5 days.

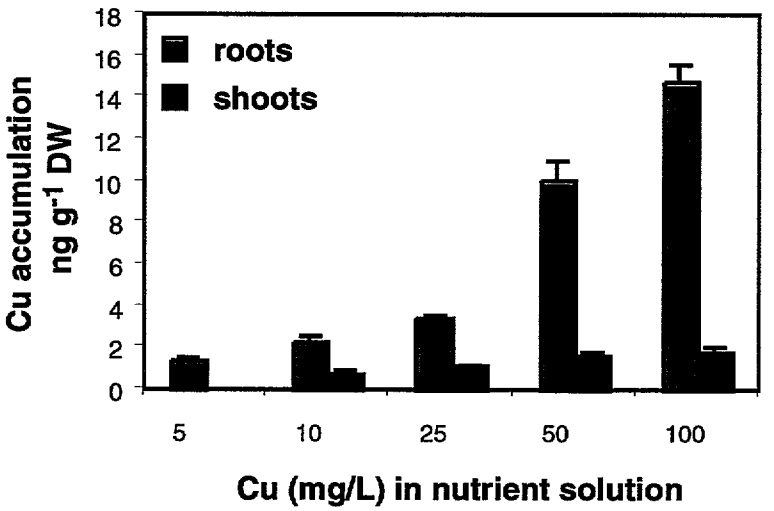
Similar concentrations of Cd, Cu and Zn in nutrient solution were used to monitor the amount of ions of heavy metals accumulated in roots and seedlings of pea. Enhanced concentration of heavy metals was detected in roots of plants compare to seedlings (Fig.6,7,8).

Figure 6



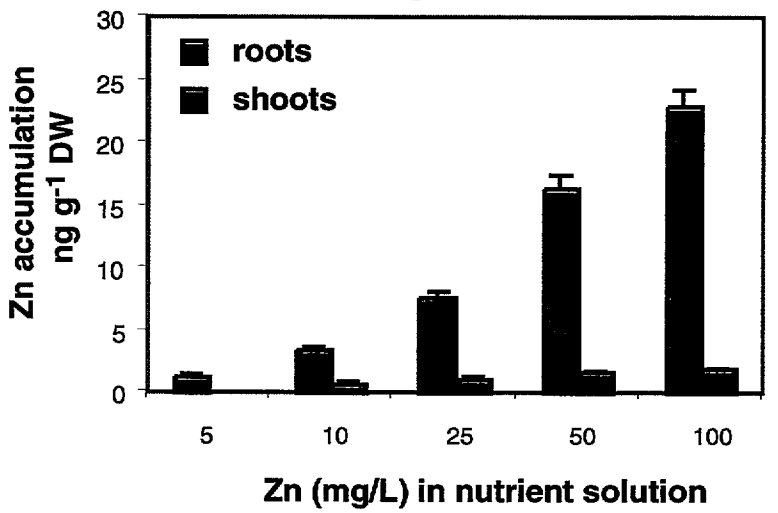
Accumulation of Cd in roots and shoots of Pea seedlings as affected by metal concentation in the nutrient meduim.

Figure 7



Accumulation of Cu in roots and shoots of Pea seedlings as affected by metal concentation in the nutrient meduim.

Figure 8

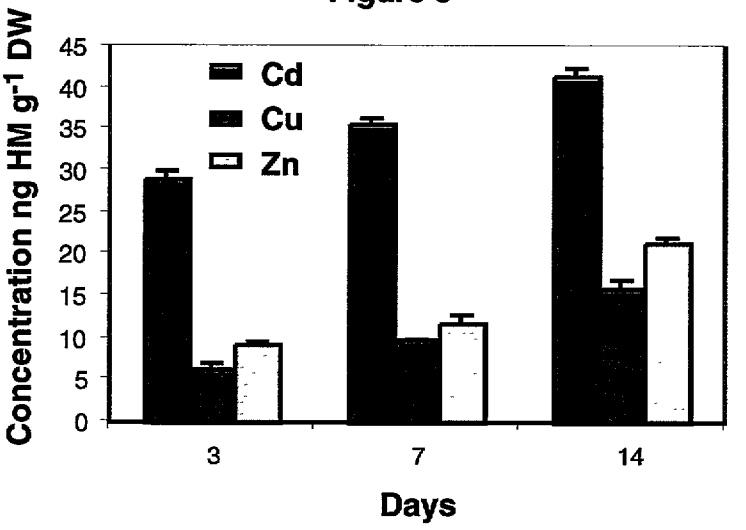


Accumulation of Zn in roots and shoots of Pea seedlings as affected by metal concentation in the nutrient meduim.

Correlation was observed between GSH synthesis and the ability of roots to accumulate heavy metals in pea plants. Pea seedlings did not show significant accumulation of metals while GSH concentration was higher than in roots. Cd ions were most readily (50 ng/g DW roots) accumulated in plants compare to Zn (25 ng/g DW roots) and Cu (15 ng/g DW roots).

Cd accumulation was observed after three days of exposure to heavy metal. For Cu and Zn this period was 14 days (Fig. 9). The reason may be a faster rate of uptake of Cd than Cu and Zn. This feature is presently being tested.

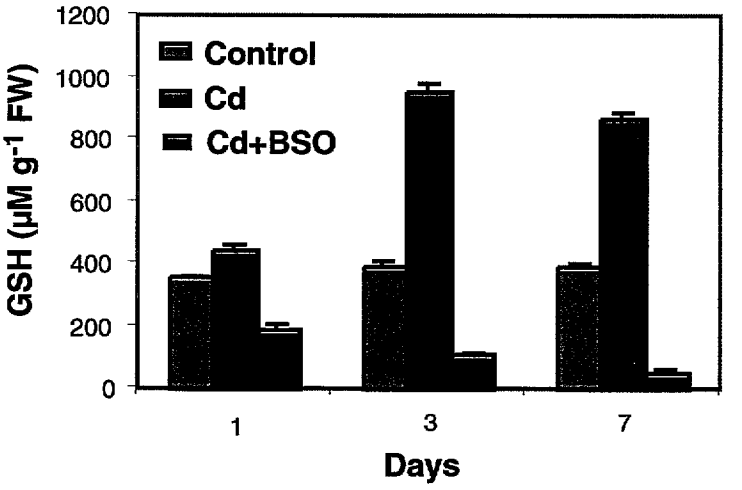
Figure 9



Accumulation of heavy metals in roots of pea plants cultivated in medium containing 50 mg/L of Cd, Cu and Zn for 3,7 and 14 days

The effect of BSO ((L-Buthionine- sulfoximine), a potent inhibitor of Glutamyl cysteine synthetase) on GSH levels and Cd accumulation in roots of plants was studied. BSO remarkably decreased level of GSH (Fig. 10).

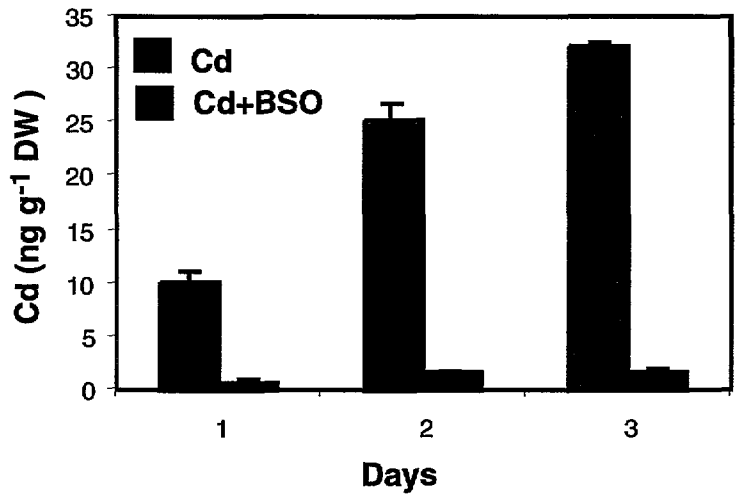
Figure 10



Concentration of GSH in roots of pea plants as affected by CD and BSO

Cd accumulation also decreased in roots in response on BSO treatment (Fig.11). Thus, GSH level in roots of plants in tightly connected with Cd accumulation while such correlation for seedlings was not observed in experiments.

Figure 11



Accumulation of Cd in roots of pea plants as affected by 1 mM BSO

Conclusions:

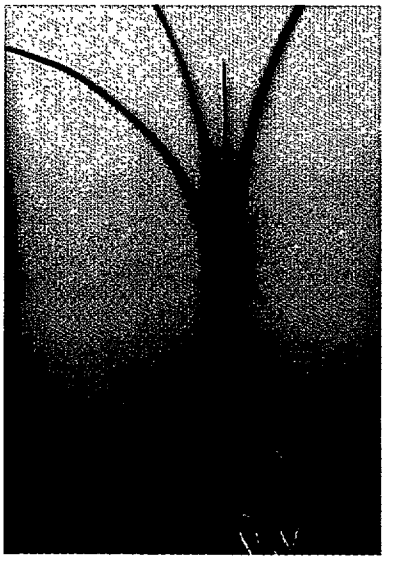
1) GSH level in pea seedlings was highest after seven days of the addition of Cd to the nutrient solution. Cd treatment stimulates synthesis of GSH, the precursor of heavy metal binding proteins, in roots of plants at concentrations from 100 to 800 μM.
2) Cu and Zn were less effective on the enhancement of GSH synthesis and accumulation in pea plants. It will be interest to test is addition of Cd could enhance the removal of Cu and Zn.
3) There is a direct correlation between GSH synthesis and the ability of pea roots to accumulate heavy metals. Cd accumulated most readily (50 ng/g DW roots) in plants as compared to Zn (25 ng/g DW roots) and Cu (15 ng/g DW roots).
4) L-Buthionine-sulfoximine, a potent inhibitor of Glutamyl cysteine synthetase, decreased the concentration of GSH in the tissues as well as the accumulation of Cd in pea roots.

Further research

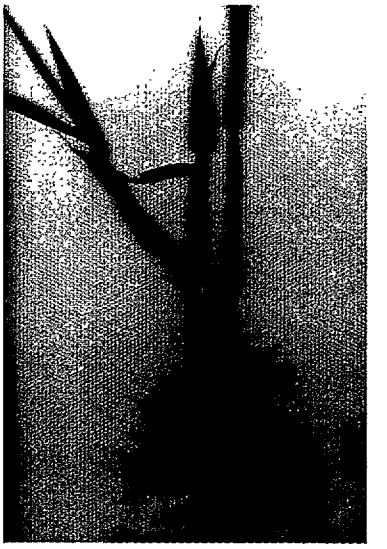
- To follow the heavy metals accumulating ability of pea plants under salinity stress (50-150 mM NaCl).
- The effect of nitrogen (nitrate, ammonium) supply on heavy metals accumulation in roots of pea plants. Activity of Glutamyl cysteine synthetase as affected by nitrogen sources and salinity in plant roots.
- To test chromatography methods such as gel-filtration and reverse phase HPLC to identify and isolate metal binding proteins in pea plants.

(b) Stress induced responses of two wetland plant species potentially useful in phytoremediation

Introduction



Typha latifolia L.
cattail



Phragmites australis (Cav.) Trin ex Steud.
Common reed

Two wetland plant species: *Typha latifolia* and *Phragmites australis* were used in our experiments which had the objective to study the effects of stress conditions on cysteine synthase (CS or o-acetylserine thyol lyase OASTL) activity and some possible related metabolic and defense pathways in plants (Hell, 1997). Thiol compounds are important in stress defense mechanisms. Glutathione (γ–Glu-Cys-Gly) is a major source of thiol groups, which are strong electron-donors and therefor ideally suited for biological redox processes and in antioxidative responses during stress (Leustek and Saito, 1999). glutathione is also the key element in phytochelatin synthesis, involved in detoxification and homeostasis of heavy metals in plants (Zenk, 1996). Overexpression of OASTL conferred to plants increased tolerance of oxidative stress and this suggest that it may become possible to engineer stress resistant plants by manipulating cysteine synthesis (Saito, 2000). Also genetic engineering can produce useful plants for phytoremediation, in order to clean up polluted soil, water and air (Meagher, 2000). Reed and cattail are promising plants for these kinds of biotechnologies, as in experiments with cadmium both plants accumulated the toxic metal in their roots and defended themselves by increased synthesis of thiolic compounds.

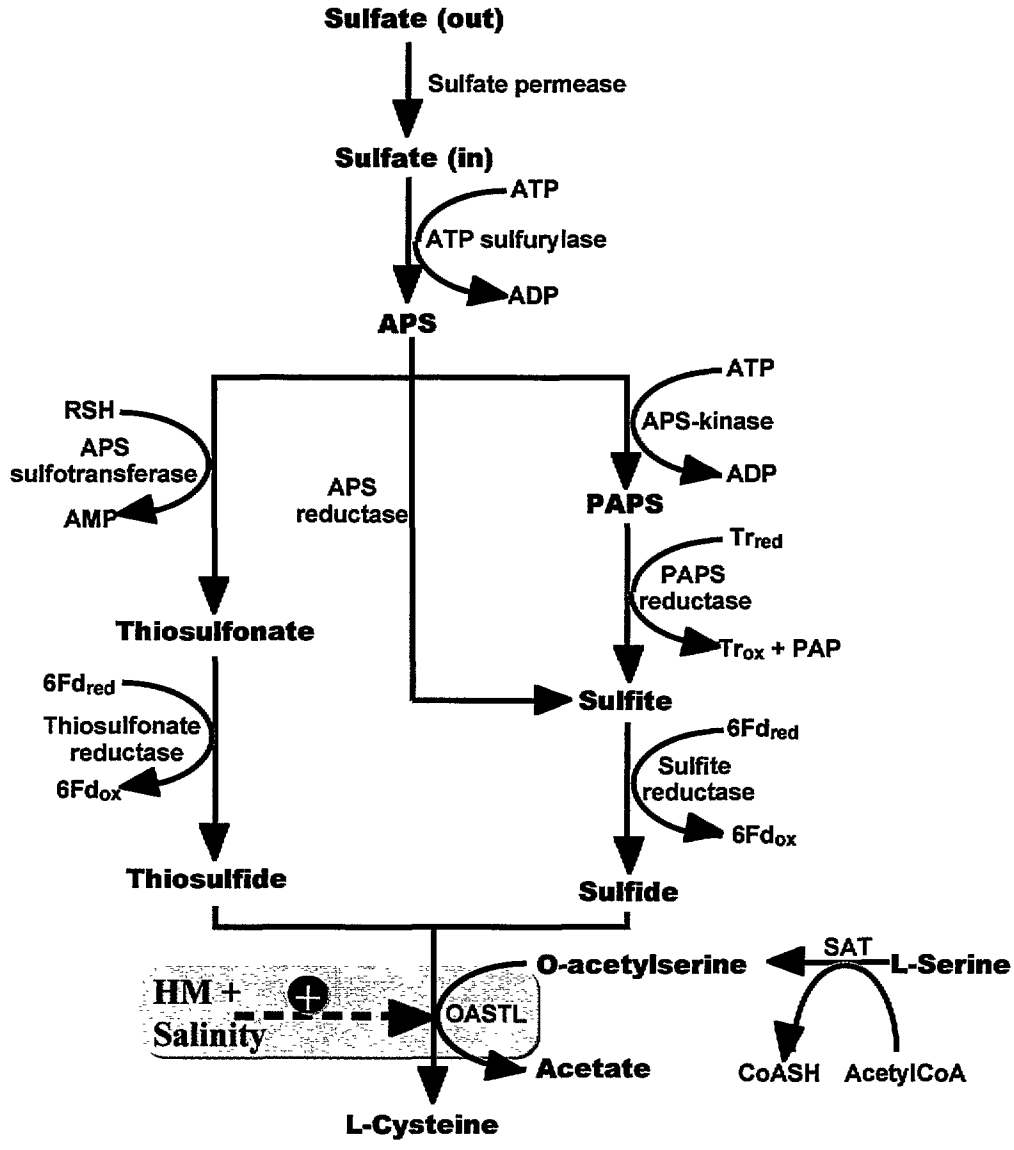
Materials and methods

Experiments were carried out with reed (*Phragmites australis* Cav. Trin. Ex. Steud) and cattail plants (*Typha latifolia* L.) grown under controlled conditions. Plants were obtained by regeneration from rhizomes collected from local populations as well as plant micropropagated through tissue cutures.

Selected plants for experiments were grown in hydroponics with half strength Hoagland nutrient solution. In the case of controls, the nitrogen source was 2mM NO_3^-. Treated plants were grown for 2 weeks with: 2mM NH_4^+, 2.5- 100 μM Cd (as CdSO4) and 100 mM NaCl. Samples were taken several times during the treatments. OASTL (o-acetylserine thiol lyase) activity was determined

spectrophotometrically, using the method described by Leon et al. (1988) and Barroso et al. (1998). In order to study the changes at transcriptional level of OASTL, the cDNA probe (kindly provided by Cecilia Gotor, Institute of Plant Biochemistry, Seville, Spain) was amplified in competent E.coli cells and the total RNA extracted from plants according to Brusslan and Tobin, (1992). The extracted RNA was transferred on nitrocellulose membranes for further hybridisation with the cDNA probe.

Changes in the glutathione pool were recorded according to Law et al., (1983). Aldehyde oxidase (AO) activity was detected by native gel electrophoresis (Omarov et al., 1999).



Results and discussion

OASTL activity was stimulated under different stress conditions mainly and firstly in roots in both experimental plants (fig.1, 2 and 3). Earlier results showed stimulatory effects in experiments carried out with detached leaves subjected to short Cd and salt treatments. Higher OASTL activities were recorded in Typha plants (fig.1). In different extend and different time periods NH_4^+, Cd and NaCl had the same stimulatory effect on OASTL activity.

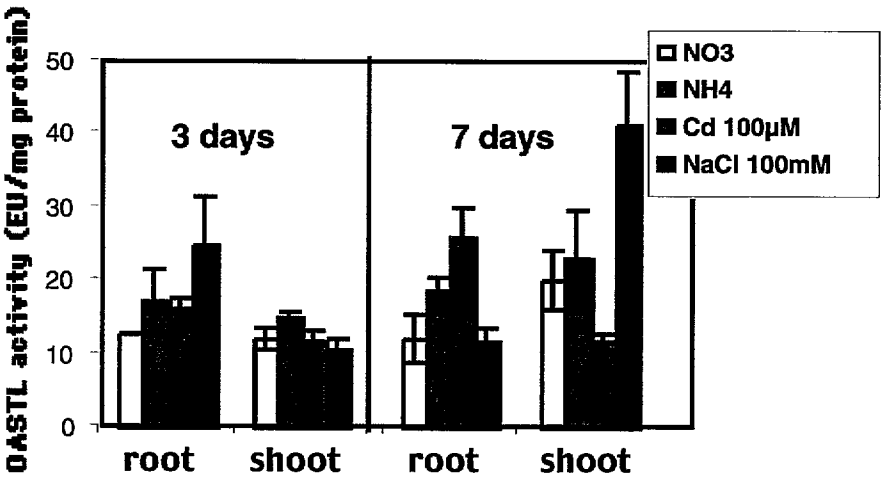


Figure 1:OASTL activity in Typha plants subjected to Cd stress

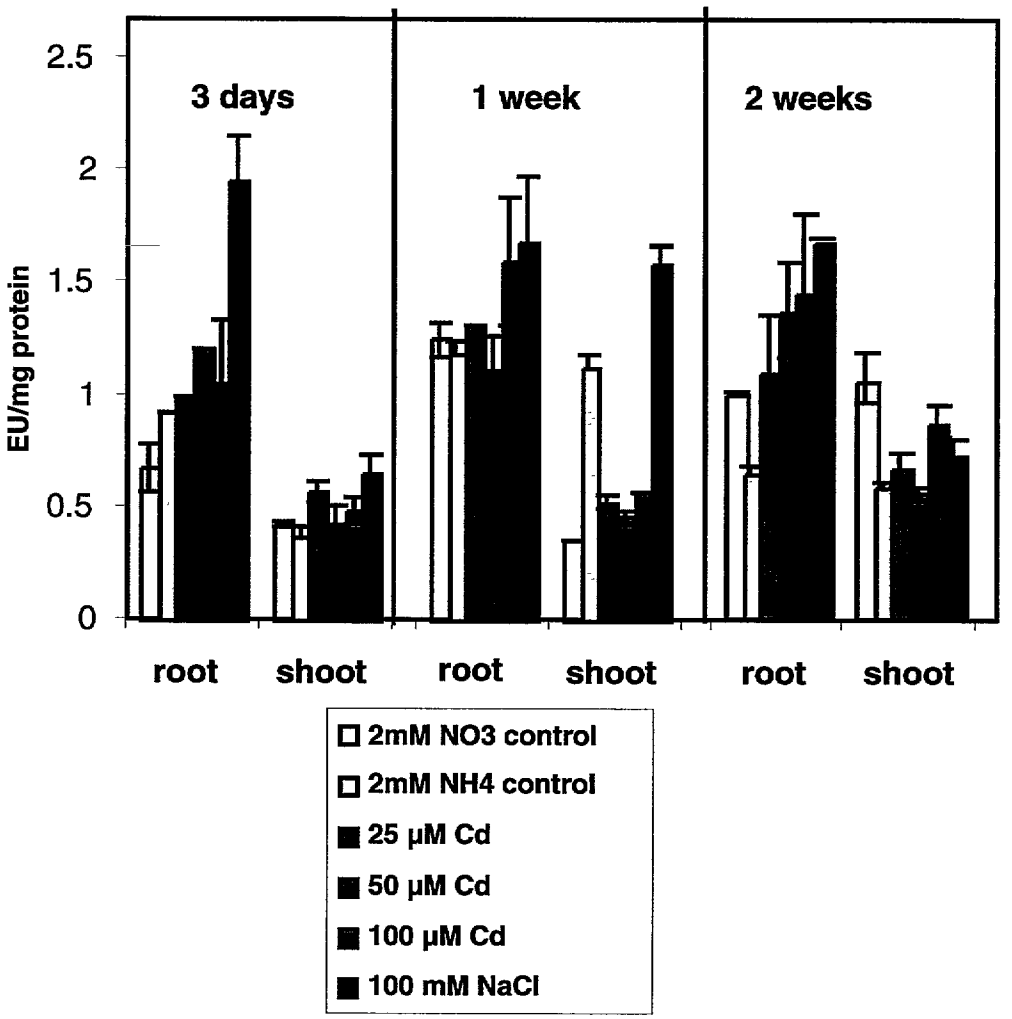


Figure 2: OASTL activity in Phragmites plants (regenerated from rhizomes) in different nutrient solutions and subjected to Cd and salt stress

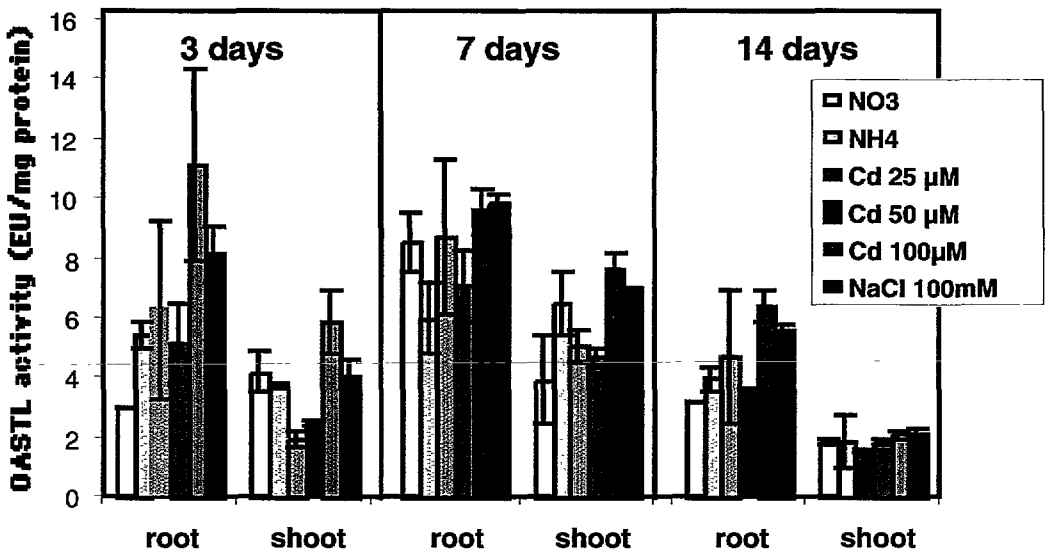


Figure.3: OASTL activity in micropropagated Phragmites subjected to NH4, Cd and NaCl

Total GSH content remained largely unchanged in shoots but showed considerable change in roots. The increases and decreases of glutathione pool depend on time and on the kind of the stress too (fig.4 and fig 5). The steady state of glutathione suggest that adaptive processes to stress conditions are functioning in the roots (May et al., 1998, Saito et al., 1994).

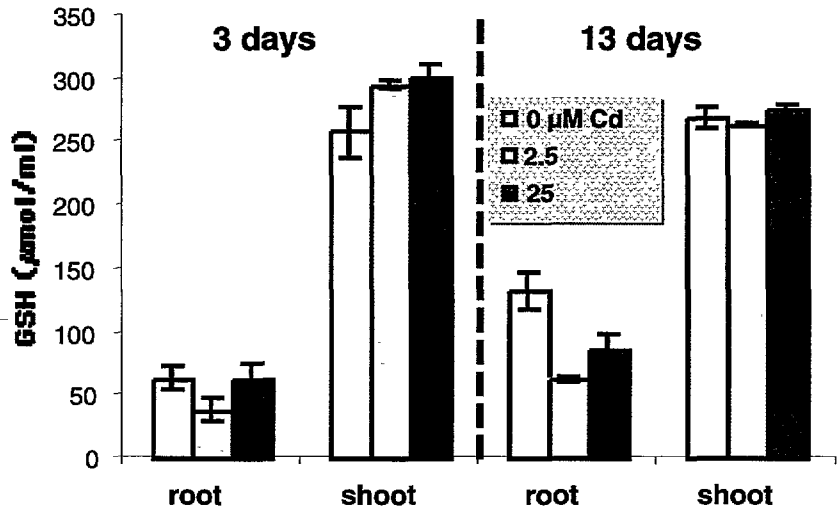


Figure 4:Total glutathione content of Typha plants subjected to Cd stress

The decrease of glutathione on Cd treated plants suggests that the incorporation of glutathione into phytochelatins, which bind the Cd and immobilizes the metal ions (Schafer et al., 1998). The increase showed the moment when, during stress conditions, glutathione is translocated from the shoots to the roots, to protect the plants against oxidative stress via the glutathione cycle. Previous measurements showed increase of total thiol pool in shoots shortly after treatments, while in the roots this increase appeared later.

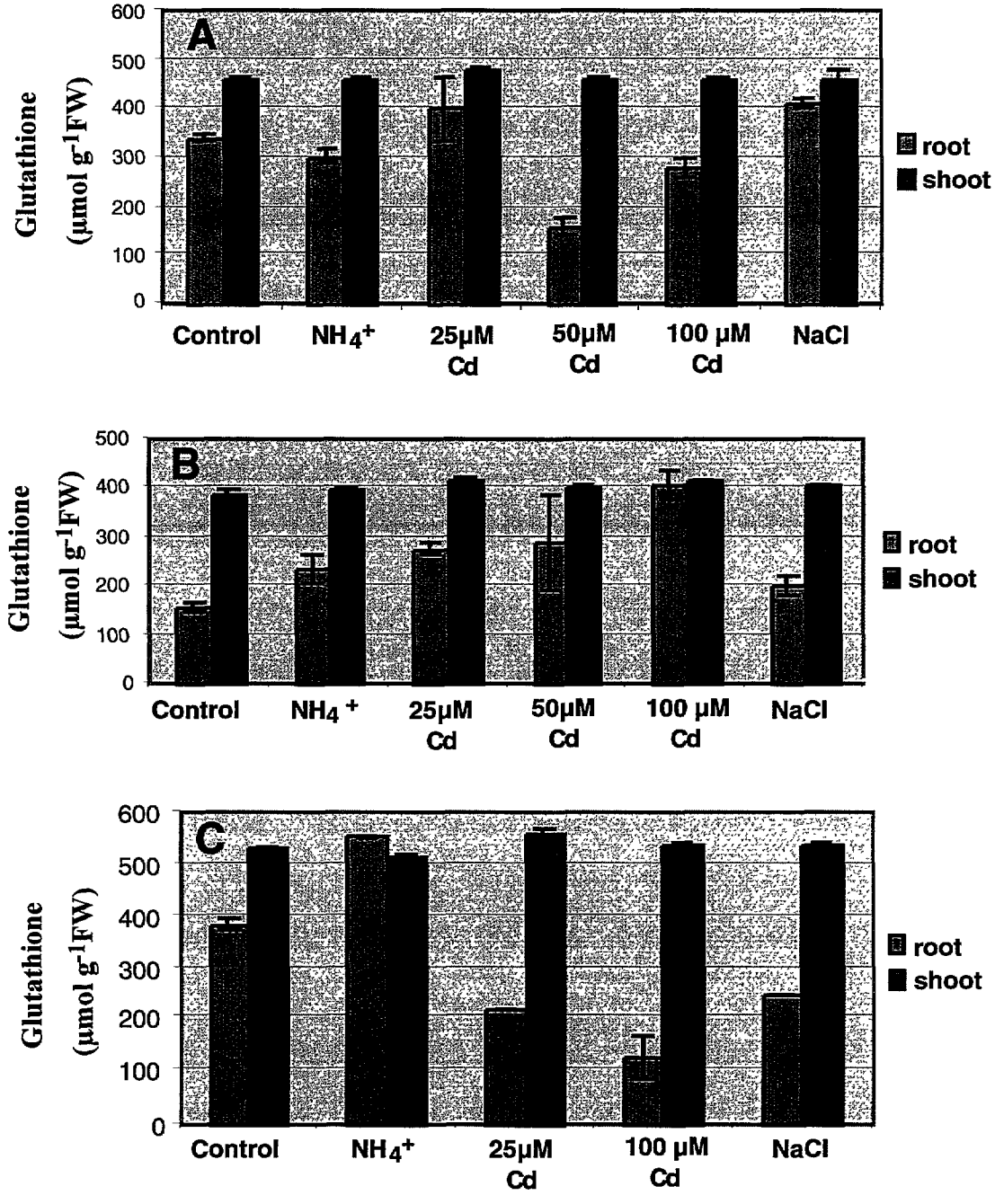


Figure 5. Glutathione pool changes in Phragmites plants after (A) 3 days , (B) 7 days and (C) 14 days treatments

Aldehyde oxidase activity increased in plants exposed to NH_4^+, Cd and NaCl (Fig.6, Tabel 1). This stimulation increases in paralel with OASTL. It seems that both both sulfurylating enzymes, Cysteine synthase and Mo-hydoxylase sulfurylase, are regulated by the same factors. There may exist a relation between the two enzymes: a) dioxo-AO requires a sulfurylase in order to become active while OASTL activation is mediated by abscisic and thus depends on the activity of AO. This last idea has been discussed in recent papers (Barroso et al., 1999, Saito, 2000).

General conclusions of the present stage of research:

- Key compounds in stress defense mechanisms such as cysteine synthase (OASTL), glutathione and total free thiol pool responded to increased levels of heavy metals in the nutrient solution of Typha and Phragmites plants subjected to stress conditions.
- OASTL activity varied with time and the following heavy metal concentrations in the nutrient solution: Cd (2.5-100 μM), NaCl (100 mM) and NH_4^+ (2 mM) as the only nitrogen source.
- AO activity correlated well with OASTL under stress conditions. They were regulated by the same factors (salinity, ammonium ions, heavy metals).

Short term plans

- Determination of ABA levels in plants subjected to the experimental conditions which enhanced AO and OASTL activity
- Enzymatic activities of AO and OASTL as affected by exogenous supplementation of ABA. e
- Western blot analysis of AO
- Completion of northern blot analysis of OASTL

Long term plans

- Immunoblot analysis of enzymes involved in the defense strategies in Cd and/or general stress (OASTL, glutathione synthase, gamma glutamyl cysteinil synthase)-depending on the possibilities to obtain antibodies and cDNA probes
- Cloning of the gene for cysteine synthase (OASTL)
- Genetic engineering of plants for hyperaccumulation of heavy metals and stress resistance (overexpression of OASTL can lead to the obtaining of stress resistant and efficient Cd-chelating plants)

C) Scientific Impact of Collaboration

Dr. Vladimir Kuzovlev from the Kazak laboratory has been working in Sede Boqer since August 1999. During this period he has learned a number of biochemical techniques and has become proficient in the use of HPLC and other equipment.

D) Description of Project Impact

We have been determining ways to enhance the HM accumulation capacity of plant species. During the second and third year of the program, field and pot experiments to test the regulation of HM removal from soils by selected plant species will be carried out. The project impact on HM removal from Kazak soils will be assessed only then. However, we can already see that the capacity

(practical and scientific) to achieve more effective technologies will be well developed with the Kazak group.

E) Strengthening of Developing Country Institutions

Dr. Zerekbay Alikulov has been recently appointed as Head of the Plant Physiology Department of the State University of Astana, the new capital of Kazakhstan. The present project will allow him to initiate the introduction of modern equipment and will contribute to the development of new scientific skills to solve many of the numerous and crop production problems produced by unfavourable environmental conditions.

F) Future Work

See experimental results above

Section II

A) Managerial Issues

None

B) Budget

No special problems

C) Special Concerns

None

D) Collaboration, Travel, Training and Publications

During the year 2000 Zerekbay Alikulov is expected to come for some time to Sede Boqer. During the same year, the Israeli scientists will travel to Kazakhstan. Preliminary material for publications is in preparation although further data is required.

E) Request for American Embassy Tel Aviv or A.T.D. Actions

None