STUDIECENTRUM VOOR KERNENERGIE
CENTRE D'ETUDE DE L'ENERGIE NUCLEAIRE

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Geachte Heer Francis Swennen
Geachte Heer Bernard Delhausse,

Gelieve in bijlage het finaal rapport te vinden in het kader van het Belspo post-doc mandaat van Dharmendra Gupta voor het onderzoek '"Investigation of uranium and arsenic accumulation potential and resulting biological responses in selected plants" in samenwerking met de eenheid Biosfeer Impactstudies (BIS) van het SCK•CEN.

Het document in bijlage bevat

1. Het rapport
2. De samenvatting van het onderzoek en keywords
3. De evaluatie

Mocht u nog opmerkingen hebben of mocht dit verslag niet conform zijn dan zou ik dat graag van u vernemen.

Met vriendelijke groeten

Dr. Hildegarde Vandenhove
Head Biosphere Impact Studies
Deputy Institute Director Environment Health & Safety
SCK•CEN

ANNEX 1

BELSPO final Post-Doc Research Report for Dr. Dharmendra Kumar Gupta

BESPO Post-Doc: Dr. Dharmendra Kumar Gupta

Title: Investigation of arsenic and uranium accumulation potential and biochemical responses in selected plants.

Host Institute: Belgian Nuclear Research Centre

SCK•CEN mentor: Dr. Hildegarde Vandenhove

Period: 1 October 2011 - 31 March 2013

Purpose of proposed Research Plan

When crops are grown on contaminated areas for cultivation purposes or phytoextraction purposes, plants themselves will be affected by the contamination. They can counteract the effects of the contamination by decreasing the uptake of the contaminant or by transforming the contaminant in a non-hazardous form. For example, it is known that the arsenic (As)-resistant plants achieve arsenic tolerance mainly through reduced uptake of arsenic by suppression of a high affinity phosphate/arsenate uptake system. Arsenate may be detoxified through reduction to arsenite inside plant cells, which subsequently makes complexes with a class of thiol rich peptides known as Phytochelatins (PCs). For uranium (U), it is not known if phytochelatins or metallothioneins impact U uptake and sequestration within the plants. U-induced PC and MT production and the possible influence of PC and/or MT on U uptake will be evaluated.

Another way of plants to defend themselves to external stress factors is through the plant's oxidative defence. Cells have developed several antioxidant defence mechanisms in order to control the redox state of the cell in changing environmental situations. Various antioxidants of plants are broadly divided into two general classes; (1) low molecular weight antioxidants, [e.g. glutathione (GSH) and ascorbate (ASC)]; and (2) enzymatic antioxidants.

The chief objective of the project is to understand the mechanism for As and U metal accumulation potential and detoxification in selected model plants, e.g. *Lemna minor* and *Arabidopsis* and to evaluate a selection of oxidative stress biomarkers.

Actual objectives

Following the execution of the work, the initially proposed activities to reach the objectives have been changed. Actual activities to achieve the proposed objectives are

1. **Experiment**
   To evaluate if and how phytochelatins play a role in uranium detoxification in the selected model plant *Arabidopsis thaliana* by studying phytochelatin production and the oxidative defense mechanism upon U exposure (study includes antioxidative enzymes and metabolites, RT-PCR of genes encoding for enzymes involved in oxidative defense mechanism and phytochelatin synthesis). Compare with observations following Cd and As exposure.

2. **Experiment**
   Evaluate if, plants with different U accumulation potential have different U translocation from root to shoots, and phytochelatin production patterns are different. Therefore 6 different plant species were evaluated: *Arabidopsis thaliana*, Zea *mays, Brassica juncea, Pisum sativum, Phaseolus vulgaris* and *Nicotiana tabacum*, which can accumulate and bind uranium in there tissues and with emphasis on the role of cysteine, glutathione and phytochelatin were evaluate.

Achievements: experimental approach and results

Experiment 1

Arabidopsis thaliana wild type plants (Col. 0) were grown for three weeks in controlled conditions with amended Hoagland medium. After three weeks of plant growth, plants were exposed for 3 days to 4 different concentrations of uranium (2, 5, 10 and 25 µM) and one set any treatment served as control (0). Also treatment with Arsenic (10 µM) and Cadmium (2 µM) was prepared to compare the results with uranium phytochelatin initiations and other biochemical parameters including gene expression. In uranium treated plants, we estimated biomass, metal accumulation potential, glutathione reductase activity, non-protein thiol concentration, cysteine content, glutathione concentration (GSH, GSSG, GSH+GSSG) and Ascorbate concentration (ASA, DHA, ASA+DHA). On the other hand in arsenic, cadmium and uranium treated samples phytochelatin concentration (PC_2, PC_3, PC_4 and PC_5) and gene expression of PC_1, GSH_1 (At4G23100) and GSH_2 (At5G2738) were monitored.

Biomass was reduced with uranium treatment after 3 days. Metal accumulation potential was increased with treatment; it was always higher in roots compared to shoots after three days. Glutathione reductase activity was decreased in roots but was increased in shoots compared to control. Total ascorbate increased in all treatments in both roots and shoots following uranium treatment compared to control. Total glutathione increased in roots but decreased in shoots compared to control. Very interesting results were obtained related to phytochelatin induction with different treatments of As, Cd and U. For roots, we found, for example, that for roots PC_2 was recorded in all treatment after 3 days of contaminant treatment. PC_3 was only recorded in the treatment of U 25 µM, and with Cd and As treatment. PC_4 was not noticed in any treatment either with As, Cd or U. PC_5 was observed only in case of Cd. In shoots, PC_2 was recorded in Cd treatment and PC_4 is also noticed in Cd treatment after three days, in case of PC_5 it was noticed in 2, 5, 10, 25 µM uranium treatment as well as in As treatment. Manuscript writing is finished and ready for communication for publication.

<u>Experiment 2</u>

Arabidopsis thaliana (Col.0), *Zea mays, Brassica juncea, Pisum sativum, Phaseolus vulgaris and Nicotiana tabacum* seeds were grown for three weeks in controlled conditions with amended Hoagland medium. After three weeks of plant growth, plants were exposed for 3 days to 25 µM U and one set without ammendment served as control. Different plant compartments were sampled: for *Zea mays, Pisum sativum and Phaseolus vulgaris* (root tip, shoot base and leaf) and for *Arabidopsis thaliana, Brassica juncea, and Nicotiana tabacum* (only root and shoot) were harvested separately. We monitored uranium accumulation potential in different plant parts, phytochelatin initiation and production patterns, and we put emphasis on the role of cysteine, glutathione and phytochelatin in detoxification of uranium.

Uranium inhibited the growth of all tested plants, affecting mainly the roots, presumably because they constitute the first point of contact with the metal. Uranium exposure caused an enhancement in cysteine (except in Bean and Pea) and glutathione in root compared to the control. In case of leaves, GSH levels were low compared to the control. This is the first time that induction of PCs following U expsoure is going to be reported and it is interesting to note that in shoots production of PCs (PC4) is more pronounced than in roots despite the limited translocation of U from root to shoots. Our results strongly suggest that U plays a role in the induction of GSH biosynthesis. All tested plants show a different defense approach following uranium treatment and phytochelatin production pattern.

A manuscript describing the research results is finished and ready for communication for publication.

Problems/issues, if any:

Initially, we were phased with a lot of problems in standardization of phytochelatin standards, while running on HPLC, but after some time we were successful to run our samples and got nice results. It's a good experience to work with different instruments.

Scientific output:

1. Horemans, N., **Gupta, D.K.,** Nauts, R., Vandenhove, H. (2013) Role of phytochelatin and glutathione in detoxification of uranium under hydroponic conditions in seedlings of *Arabidopsis thaliana*. (Ready for communication).
2. **Gupta, D.K.,** VanhoudtN. Nauts, R., Horemans, N., Vandenhove, H. (2013) Phytochelatin production patterns in plants of different U accumulation potential (Ready for communication).

ANNEX 2- One-page summary of the research work

Contamination of the biosphere by heavy metals poses major environmental and human health problems worldwide. Uranium (U) is a non-essential but generally highly toxic element for plants. Plants have different strategies to defend themselves to metal-induced stress. For different metals it has been shown that glutathione and cysteine play a central role in the plant response both as an antioxidant and as a precursor of metal-complexing molecules such as phytochelatin (PC) or metallothioneins. However, for U, it is to date not known if PCs impact U uptake and sequestration within the plant. The main aim of this project was therefore to understand the mechanism for U metal accumulation potential and detoxification in selected model plants. Six plants were exposed to U for three days namely Arabidopsis (*Arabidopsis thaliana),* bean *(Pisum sativum), Indian mustard (Brassica juncea), maize (Zea mays),* pea *(Phaseolus vulgaris)* and tobacco *(Nicotiana tabacum).* U-induced PC production, changes in glutathione metabolism and the possible influence of PC on U uptake were evaluated.

1. Optimisation of PC extraction and quantification in plant samples

Phytochelatins are only present in relatively low concentrations in plants, even in metal-challenged plants. It are olygomers of glutathione with a general structure of (γ-Glu-Cys)n-Gly whereby n can vary between 2 and 11. Like glutathione, PCs can easily breakdown once extracted from plant samples due to oxidation. As existing methods for PC analysis were not satisfactory to obtain reliable quantification of low levels of PCs, the analysis method needed to be optimised. Major improvements included (i) optimisation of the extraction buffer, (ii) addition of an internal standard N-acetyl cysteine to the plant tissue just prior to extraction that enabled estimation of possible loss in thiols and (iii) use of different standards to enable calculation of the exact PC concentrations rather than expressing them as glutathione equivalents.

2. U uptake and internal redistribution in different plant species

As has been reported before (Vanhoudt et al., 2008) U mainly accumulates in root tissue. Root to shoot transfer did not differ greatly between tested plant species ranging from 0,00016 for Arabidopsis to maximum 0,00065 for maize. These factors are in the same order of magnitude as those reported by (Vanhoudt et al., 2008). Remarkably, however, in the study of Vandenhove et al. (2006) higher root to shoot transfer for beans were reported ranging from 0,01 for 0,1 µM U-exposed plants to 0.001 for 1000 µM U-exposed plants. Further studies are necessary to be able to understand this discrepancy.

3. Possible role for PCs in U-induced stress in plants

Levels of cysteine, glutathione and higher PCs were studied in roots and shoots of all plant species exposed to 25 µM U for 3 days and compared to control plants. Not all PCs could be retrieved and not all PCs were present in all tested plant species or tissues. Highest induction in U treated plants was found for PC3 or PC5 and for Arabidopsis. For PC3 a significant U-induced increase was also found in leaves of bean and tobacco. For *Arabidopsis* the glutathione metabolism was further investigated studying the gene expression of relevant genes involved in glutathione and PCs biosynthesis. No major differences in gene expression were observed however.

In conclusion these are the first data reporting on PC induction in U-treated plants. However, due to the small increase compared to Cd or As treated plants, PCs production and sequestering of U by PCs is probably not a major defence pathway plants use to cope with U excess.
Results will be assembled in two international, peer-reviewed papers one on the comparison of the different plant species and one on thiol metabolism in *Arabidopsis* specifically.

Keywords: Uranium, plant stress response, glutathione, phytochelatins, U uptake and translocation

References: Vandenhove H, et al. 2006. *Plant Physiology and Biochemistry* 44, 795-805. Vanhoudt N et al. 2008. *Plant Physiology and Biochemistry* 46, 987-96.

ANNEX 3 Evaluatie Post-Doc Dharmendra Gupta

Ondanks het feit dat we redelijk wat resultaat bereikt hebben (zie ANNEX 1) en er ook wat publicaties op stapel staan, ondanks het mooie CV van Dharmendra Gupta, is de samenwerking niet positief verlopen.
Dharmendra Gupta' capaciteiten die tot expressie kwamen tijdens zijn stage staan in schril contrast met zijn sterke CV dat ons vertelt dat hij een sterke expertise heeft in ao onderzoek naar fytochelatineproductie als detoxificant na metaalblootstelling, het hoge aantal publicaties o.a. ook in onderzoeksthema's die voor ons aantrekkelijk waren/zijn en verschillende publicaties in tijdschriften met een hoge impactfactor voor het betreffende onderzoeksdomein.

Ik verklaar me nader:

1. Dharmendra Gupta had blijkbaar geen doorgedreven ervaring met fytochelatineanalyse, hij was niet in staat om de analysemethode te optimaliseren, was niet in staat een een ijklijn op te stellen en constante controle op labowerkzaamheden was nodig
2. Beperkte kennis statistiek
    - Bovenstaande punten werden volledig opgevangen door het technisch personeel van SCK
3. Beperkte creativiteit: experimenten werden voornamelijk door ons uitgedacht; redelijk eenvoudige experimenten werden voorgesteld want tot meer was hij niet in staat. Nieuwe analyses (e.g. RT-PCR) leerde hij niet aan; Er was steeds controle nodig of alles OK werd uitgevoerd. Hij was niet te beschaamd om zichzelf achteraf alle verwezenlijkingen toe te eigenen.
4. Manuscripten – alle papers moeten door ons (her)(ge)schreven worden. Om de belasting op mijn groep enigszins te beperken voro wat het experimenteel werk betreft had ik Dharmendra gevraagd een reviewpaper te schrijven over mechanismen van U-opname door planten. Ik gaf eerst suggesties voor de paperopbouw en verbeterde vervolgens twee versies maar gaf het toen op: het was hopeloos.

Ik kan echt niet begrijpen dat Dharmandra Gupta in staat was zulk een CV op te bouwen. Waarschijnlijk is hij telkens bij onderzoekers terechtgekomen zoals in mijn groep die hem vooruit hielpen of het werk voor hem deden. Zelf wil ik met mijn groep niet bijdragen tot het bestendigen van de mythe en heb met de groep en Dharmendra besloten dat hij van 1 paper (volledig herschreven door N. Vanhoudt – van nul herbegonnen **Gupta, D.K.,** VanhoudtN. Nauts, R., Horemans, N., Vandenhove, H. (2013) Phytochelatin production patterns in plants of different U accumulation potential) toch eerste auteur kan zijn, dat hij tweede auteur is van paper 2 (Horemans, N., **Gupta, D.K.,** Nauts, R., Vandenhove, H. (2013) Role of phytochelatin and glutathione in detoxification of uranium under hydroponic conditions in seedlings of *Arabidopsis thaliana*) en de U-review paper kan en zal niet door hem geschreven worden. Deze review wordt bij tijd en wijlen geschreven door iemadn van mijn onderzoeksgroep.

Gegeven bovenstaande kan u begrijpen dat we niet wensen verder te werken met Dharmendra Gupta en zijn voormalig thuisinstituut. Het is uiterst jammer dat deze samenwerking zo verlopen is. We hebben met belangrijke inzet van het SCK personeel toch voor een behoorlijke output gezorgd voor BELSPO (2 journal articles).
Ik kan u tevens verzekeren dat onze samenwerking met Rajesh Tewari, Belspo post-doc die 1/12/12 startte, wel heel goed verloopt.

Met vriendelijke groeten

Hildegarde Vandenhove