SZENT ISTVÁN UNIVERSITY
PhD SCHOOL OF ENVIRONMENTAL SCIENCES

PhD THESES

DEVELOPMENT OF A RAPID BIOTEST ADAPTABLE TO PLANTS TO CHARACTERIZE HEAVY METAL CONTENTS OF SOILS

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I. BACKGROUND AND GOALS

Chemical pollution of the environment increases sharply globally, and one of the most significant indicator of this increasing pollution is the accumulation of heavy metals in the soil. In mobile forms they can enter the food chain, damaging the environment seriously and posing a risk to human health.

In Hungary, the protection of underground waters and geologic media is regulated by the joint ministerial decree 10/2000. (VI. 2.) KöM–EüM–FVM–KHVM, wherein pollution levels (B) are relevant. Note, however, that current limit levels were set by using human risk analyzing softwares.

Comparing measured total soluble heavy metal contents of soils to environmental soil limit levels does not represent alone the adverse effect of heavy metals to the ecosystem. In the future, results of human risk assessments and environmental studies shall be harmonized when setting limit levels for soils.

The subject of my dissertation is justified by the fact, that a plant biotest method representing the toxicity of heavy metals to the ecosystem, characterizing the heavy metal pollution of soils and applying plant parameters is not available.

Thus, I aimed to develop a laboratory plant biotest method and assessing system suitable to characterize heavy metal polluted areas, on test soils contaminated by cadmium, lead and copper, applying winter wheat (Triticum aestivum), perennial rye-grass (Lolium perenne), white mustard (Sinapis alba) and coleseed (Brassica napus ssp. Oleifera) as test plants.

Furthermore, I compared the sensitivities of my test plants to the sensitivities to heavy metal loading of *Azotobacter agile* and *Pseudomonas fluorescens* soil bacteria, as well as of *Glomus intraradices* mykorrhiza fungus used generally in ecotoxicological tests.

In my researchwork our aim was to answer the questions listed below of theoretical and practical importance, for the development of a rapid plant biotest to characterize heavy metal polluted soils:

1. What test plant(s) and which of their plant physiological parameters are the best selections to be applied in our rapid plant biotest method to characterize the acute toxic effects of cadmium, lead and copper polluted soils?

2. In ecotoxicological testing of heavy metal loaded soils, what is the optimal

period of heavy metal loading of plant(s), during which test results are significantly differentiated?

3. What are the optimal evaluation methods for processing test results of plant physiological responses induced by increasing heavy metal loading?

4. What are the differences between the sensitivities of the test plants used in our biotest and the sensitivities of *Azotobacter agile-, Pseudomonas fluorescens-,* as well as the *Experimental mycorrhiza* test selected from currently used ecotoxicological test microorganisms, to heavy metal loading?

II. MATERIALS AND METHODS

The basis for a plant biotest method suitable to characterize heavy metal polluted soils was the rapid seedling biotest method developed by NOOMAN and FÜLEKY (1989, 1991/1992), with experimental parameters as described below.

Test plants were first precultivated in cultivation vessels of 500 ml volume, on 2g of household cotton wools soaked with 50 ml distilled water for each vessel, ensuring water *ad libitum*.
At the start, 4g/vessel of winter wheat, 2g/vessel of perennial rye-grass 3g/vessel of white mustard and 3g/vessel of coleseed were added as unpelletized seeds onto the cotton wool soaked with distilled water.

When the roots of the test plants have well interlaced their cotton wool cushions, they were removed and placed, together with the cotton wool, onto the soil samples in the cultivation vessels contaminated with cadmium, lead and copper at various levels.

200g/vessel test soil screened on a 2 mm hole size sieve was mixed with cadmium acetate, lead acetate or cupric sulfate solutions according to the loading level. 1× loading levels were identical for all three heavy metals with "B" (pollution) limit levels, calculated for air dried soil. A 0× loading means unloaded soil with natural heavy metal content.

Following the heavy metal loading period we have studied the acute toxicity of heavy metal loading on plant physiological parameters that are sensitive to heavy metal contaminations, and analyzed the heavy metal contents of the plants and the quantity of heavy metals taken up.

Plant samples were prepared to heavy metal analysis by hydrolyzing with hydrochloric acid, the heavy metal content was determined by an ICP spectrometer (ICAP 61E tip.)

As the first step to develop a plant biotest suitable to characterize heavy metal polluted areas, *preliminary experiments in cultivation vessels* have been performed. In the preliminary experiments, the test plant was winter wheat *(Triticum aestivum* variety *Lupus),* Ramann type brown forest soil was used with 0x, 1x, 2x, and 4x loadings. The test soil was taken at the Gödöllő-Szárítópuszta Experimental Area of the Szent István University, from level "A" of the control experimental parcel [K_A: 23; organic content: 2,03%; pH(H_2O): 6,8]
The preliminary experiments were aimed to select the optimal duration of heavy metal loading by applying 7, 10 and 14 days of heavy metal loading periods, then the responses of shoot height, as one of the plant physiological parameters of winter wheat, to heavy metal loading were tested.

In the second step in developing a rapid plant biotest suitable to assess heavy metal pollution of soils a *basic series of experiments* has been performed.

Test plants in the basic series of experiments were perennial rye-grass (Lolium perenne;variety: Juventus), white mustard (Sinapis alba; variety: Albatros) and coleseed (Brassica napus ssp. oleifera; variety: Danubia). Brown forest soil with alternating thin layers of clay substance ("kovárvány") on sand rock bed from the microheight at Inke was selected as test soil [K_A: 25; organic content: 2,02%; pH(H_2O): 5,7; pH(KCl): 4,89]
In the basic series of experiments the number of heavy metal loading levels was increased, applying 0-, 0,75-, 1-, 2- 4× loading levels. In addition to shoot heights other plant physiological parameters were tested as well, i.e. green and dry masses and humidity of the shoots, the mass of the roots and heavy metal content taken up by the shoots and the roots were measured.

Table 1 Heavy metal loading of test soil in he basic series of experiments

Element	**Heavy metal loading (mg /kg soil)**				
	0 x	**0,75x**	**1 x**	**2 x**	**4 x**
Cd	0	0,75	1	2	4
Pb	0	75	100	200	400
Cu	0	56,2	75	150	300

Test results were ecotoxicologically *evaluated* concerning the effective concentration values representing acute toxicities of heavy metal loading, which resulted in a 10, 20 and/or 50% reduction of the plant physiological parameters selected for testing. Furthermore, trends in inhibition of plant physiological parameters as a function of increasing heavy metal loading were determined.

Significant differences and regression analysis as biometric methods were used in the evaluation of test results.

In the *comparative ecotoxicologycal experiments,* aqueous extracts of the test soil were tested by standard Azotobacter agile- (MSZ 21978-30:1988.) and Pseudomonas fluorescens (MSZ 21470-88:1993) methods. Furthermore, in our experimental mycorrhiza test we have detected 3 vegetative parameters of mycorrhiza development on the roots of herbs treated with Glomus sp.

III. NEW SCIENTIFIC RESULTS

1. Based on the rapid seedling biotest (NOOMAN and FÜLEKY) a biotest method and assessment system has been developed to characterize heavy metal contaminated areas.

2. I have shown, that the most significant change in the test plants used [perennial rye-grass, (Lolium perenne); white mustard (Sinapis alba); and coleseed (Brassica napus ssp. oleifera)] induced by increasing the heavy metal loading was in the heavy metal content taken up by the shoots and roots. Increasing heavy metal content depends unambiguously upon heavy metal loading applied. Heavy metal contents taken up by the shoot could be much more sensitive indicators of soil contamination, than the limit level values for soil.

3. Loading test soils by Cd, Pb and Cu I have found, that shoot height reduction of perennial rye-grass (Lolium perenne) can be a sensitive, well measurable indicator of heavy metal contamination level of soils, as it can characterize the tendency as well as the extent of the growth inhibition effect of heavy metal loading.

4. I have shown, that acute toxicities of heavy metal loading can be well traced by monitoring the reduction of green mass of the test plants.

5. Comparative ecotoxicological test results with *Azotobacter agile* and *Pseudomonas fluorescens* soil bacteria as well as with *Glomus intraradices* mykorrhiza fungus on heavy metal contaminated soils have shown, that there is a difference in the sensitivities of plants and microorganisms generally used in ecotoxicological tests, respectively, to heavy metal pollution of soils. Thus, environmental and health risk assessments of heavy metal polluted areas shall be based on a complex treatment of ecotoxicological test results with various organisms.

IV. CONCLUSIONS

IV.1. Conclusions of test result of preliminary experiments in cultivation vessels

Evaluating preliminary tests with winter wheat we have found, that Cd and Pb contents of started test plants showed a significant difference according to the loading levels on the *10. day of the loading period* in both cases, thus, 10 days were defined as the duration of the heavy metal loading period in our plant biotest.

Natural Cd and Pb contents of the soil were also detectable in plants grown on unloaded soils, therefore 0,75x loading, below the limit level, was selected as the first loading level in the basic series of experiments.

Results concerning shoot height reduction of test plants grown on heavy metal loaded soils were contradictory. First, no correlation could be observed between plant height reduction and increasing heavy metal loading, and second, toxicities of the heavy metal loading series in the responses of plant physiological parameters tested have hardly reached the EC_{10} threshold value Based on the test results, we concluded as follows:

- The seeds of the winter wheat have a high nutrient content, and utilizes only a part of soil elements as a seedling, thus, test plants with low nutrient contents were selected for the rapid plant biotest in the basic series of experiments.

- Physico-chemical properties of Ramann-type brown forest soil used in the preliminary tests affect adversely the uptake of heavy metals, therefore it was replaced by sand in the basic series of experiments to minimize the occurrence of experimental errors.

- Literature data show that a number of plant physiological properties are sensitive to the toxic effects of heavy metal loading, thus, inclusion of monitoring further plant physiological parameters with a sensitivity to heavy metal loading (such as the green mass of the shoot, the dry mass of the shoot, comparison of root masses) was deemed necessary for the basic series of experiments.

IV.2. Conclusions of the basic series of experiments in developing a cultivation vessel biotest method

The rapid seedling biotest method described by NOOMAN and FÜLEKY offered a sound basis to develop a rapid plant biotest to characterize heavy metal polluted soils.

Adverse effects of heavy metal polluted soils could be well assessed through the responses of plant physiological parameters, when our results were compared to the EC_{10}, EC_{20}, as well as EC_{50} values of acute toxicities. Regression coefficients (b-value) and determination coefficients (R^2) provided useful supplementary information to the statistical analysis of our results.

In the test plants used, the increase of the heavy metal content could be undoubtedly correlated to heavy metal loading levels.

The greatest impact of increased heavy metal loading were observed in the heavy metal content taken up by the shoots and the roots. Heavy metal contents taken up by the shoots can be much more sensitive indicators of heavy metal pollution of soils, than the numerical values of soil limit levels.

Our results with Cd, Pb and Cu loading of test soils have shown, that shoot height reduction of perennial rye-grass (Lolium perenne) can be a well treatable and sensitive indicator of heavy metal pollution level of soils, as it is characteristic for the inhibiting effect of heavy metal pollution and for the acute toxicity level as well. (Figure 1)

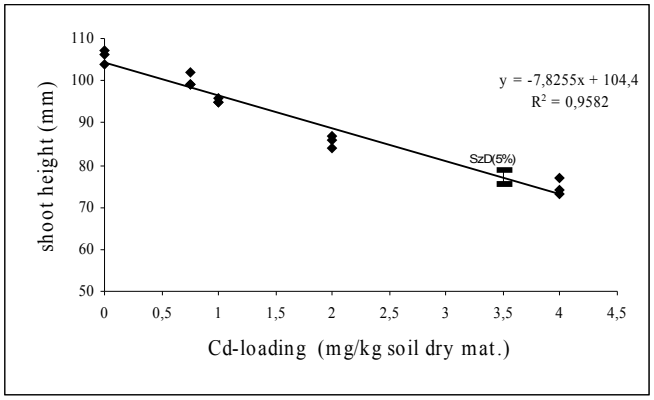

Figure 1. Shoot height of perennial rye-grass as a function of Cd loading

Acute toxicities of heavy metal loading could be easily monitored by the decreasing of green mass. However, as an inherent feature of the rapid plant test, observable changes in the dry mass of young test plants do not represent the differences in the damaging effects of heavy metal loading levels, as they covered a

rather low measuring range as a function of heavy metal loading levels, neither the differences of the results were significant, nor ecotoxicological differences could be assigned to the results.

In my soil loading experiments using copper I have found, that the toxicity of copper triggered the most sensitive responses, indicating the environmental health hazards of excessive accumulations of this essential micro-element.

IV.3. Conclusions of comparative ecotoxicological tests with microorganisms

Results of toxicological tests with bacteria correlated very well with plant biotesting results, as they indicated ecotoxicity at the soil limit level or slightly above. Among the two test bacteria used, Pseudomonas fluorescens showed higher sensitivity to Cd and Pb loaded soils, the highest inhibiting effect was observed at Cu loading for both strains.

A general conclusion of mycorrhiza tests is, that among the three vegetative parameters of mycorrhiza fungi used to assess the ecotoxicological effect of heavy metal pollution of soils in rapid biotests, colonization frequency (F%) was the best applicable indicator, as it provided appraisable results even in the second week of the loading period. Arbuscularity (A%) was also a sensitive indicator, however, it provided appraisable results only in the 6.-8. weeks of the loading period, and furthermore, based on F% values, the sensitivity of colonization frequency was by 20% higher.

V. PROPOSALS

Based on my test results in developing a rapid plant biotest suitable to characterize heavy metal loaded areas as well as in comparative ecotoxicological testing, **I propose:**

- The rapid plant biotest suitable to characterize heavy metal polluted soils shall be included to the ecotoxicological test methods, when monitoring areas exposed to heavy metal pollution for the assessment of ecological risks of heavy metals.

- Application of perennial rye-grass (Lolium perenne) as test plant in the ecotoxicological testing of heavy metal polluted areas.

- Including of unpolluted soil samples as control samples in the rapid plant biotest method suitable to characterize heavy metal polluted areas, as well as taking three

parallel samples from tested areas for the ecotoxicological assessment of heavy metal pollution.

- Shoot heights of test plants grown on soil samples tested related to shoot heights of plants grown on control soil shall be considered as a parameter of evaluation in the rapid plant biotest method suitable to characterize heavy metal polluted areas.

- The soil of the tested area shall be considered as contaminated by Cd, Pb and/or Cu at a level equal to the soil limit value on the basis of the rapid plant biotest method suitable to characterize heavy metal polluted areas, whenever shoot heights of test plants grown on two of three parallel samples of the tested area are reduced, as compared to plants grown on control samples, by the value of EC_{10} or more.

- The tendency to reduction of green masses of test plants shall be analyzed by the rapid plant biotest, whenever rapid informative data on the complex contamination of a polluted area and the environmental effects of contaminations are needed.

- Our experiments have shown that the sensitivities of test plants and bacteria or mycorrhiza fungi, respectively, to heavy metal contaminated soils are different, thus, when assessing the heavy metal pollution of an area, both the rapid plant test suitable to characterize heavy metal polluted areas and ecotoxicological tests with microorganisms applied recently shall be performed, for a complex assessing of health and environmental risks of the area tested.

- Finally I propose, that the rapid plant biotest method suitable to characterize heavy metal polluted areas shall be extended to characterize organic contaminations of soils and ecotoxicological effects of waste extracts.

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