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FINAL REPORT

on the sub-project

"Pilot experiments for phytoremediation to depollute heavy metal and pesticide contamination"

carried out in 1999-2000

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1. Introduction

Central and Eastern European countries have to remediate large areas of land that have been contaminated with toxic chemicals, particularly with heavy metals and herbicides. One of the new technologies presently being developed worldwide for soil reclamation purposes is phytoremediation, which uses plants to clean up soils by absorbing toxic heavy metals or other pollutants. The present sub-project was intended to investigate the technical and economic feasibility of phytoremediation techniques designed to depollute heavy metal and pesticide contaminations by using poplar and willow plants. Before practical field experiments biochemical and physiological research was carried out in order to study the range of compounds decontaminated and the effects of the metabolites produced on the plants and the environment.

Laboratory and greenhouse experiments were carried out at the Plant Protection Institute of the Hungarian Academy of Sciences in Budapest, Hungary. In the second phase of the sub-project an *in situ* phytoremediation field experiment was carried out at a polluted site of Nitrokemia Ltd. at Fuzfogyartelep, close to the lake Balaton in Hungary.

2. Laboratory and greenhouse experiments (stress tolerance studies)

2.1. Experimental design

As test plants for our experiments, poplar and willow tree species were obtained from the Forest Research Institute at Sarvar, Hungary. As model polluting herbicides the chloroacetanilide herbicides acetochlor and metolachlor were selected. These herbicides are common containants in agricultural settings and phytoremediation seems to be an attractive option to reduce soil levels of these pesticides. In addition to the chloroacetanilide chemicals acetochlor and metolachlor, the important heavy metal pollutant mercury(II)-chloride and the herbicide 2,4-dichlorophenoxy acetic acid were selected.

Particular attention was paid in our investigations to the tripeptide thiol compound glutathione, which is ubiquitous in plant tissues. Glutathione is a principal component of the antioxidative and detoxification defense reactions in plant tissues. Glutathione has a pivotal role in the detoxification of numerous herbicides, including chloroacetanilide herbicides. These herbicides are detoxified by conjugation reactions between the herbicide molecules and glutathione catalyzed by the enzyme glutathione S-transferase. Glutathione, as the metabolic precursor of the heavy metal chelating phytochelatins, plays an important role also in the heavy metal detoxification in plants. For these reasons, changes in the foliar amount of glutathione and in the activity of glutathione S-transferase were measured in poplar and willow leaves. The conjugate of acetochlor and glutathione was prepared by chemical synthesis.

The inducibility of glutathione-related reactions was tested by the herbicide acifluorfen.

2.2. Biochemical investigations

a) Six poplar and three willow species were cultivated under greenhouse conditions.
b) Leaves were removed from the plants and, via the leaf petiole, were treated with solutions of the various pollutants.
c) Treated leaves were evaluated for phytotoxiciy symptoms after 24, 48, and 96 h of exposure.
d) Glutathione levels were determined in the poplar and willow tree leaves by an enzymatic recycling technique.
e) Foliar glutathione S-transferase activities were measured speoctrophotometrically in all species in order to select plants with high phytoremediation capacities.

f) Glutathione levels and glutathione S-transferase activities were measured in poplar and willow leaves treated by the herbicide acifluorfen, which is a well-known inducer of glutathione-related plant defense reactions.
g) The phytotoxic effects of acetochlor and metolachlor on a poplar hybrid (*Populus tremula* x *P. alba*) were determined using artificially contaminated soils.

2.3. Chemical syntheses and analytical methods

a) The glutathione conjugate of acetochlor was prepared by chemical synthesis.
b) An analytical method was worked out for the detection of the acetochlor-glutathione conjugate in plant tissues.

2.4. Results

We found that detached leaves of poplar and willow plants were only slightly susceptible to exposure to aqueous solutions (10^{-4} M) of herbicides (acetochlor and metolachlor) and of the heavy metal salt mercury(II)-chloride. Phytotoxic effects of different concentrations of these pollutants were studied on a poplar hybrid. The poplar hybrid (*Populus tremula* x *P. alba*) showed a high degree of tolerance against heavy metal salt, as well as chloroacetanilide and phenoxy acid herbicides.

The results of these studies also showed that leaves of the poplar and willow tree species investigated contained considerable amounts of glutathione (Fig. 1).

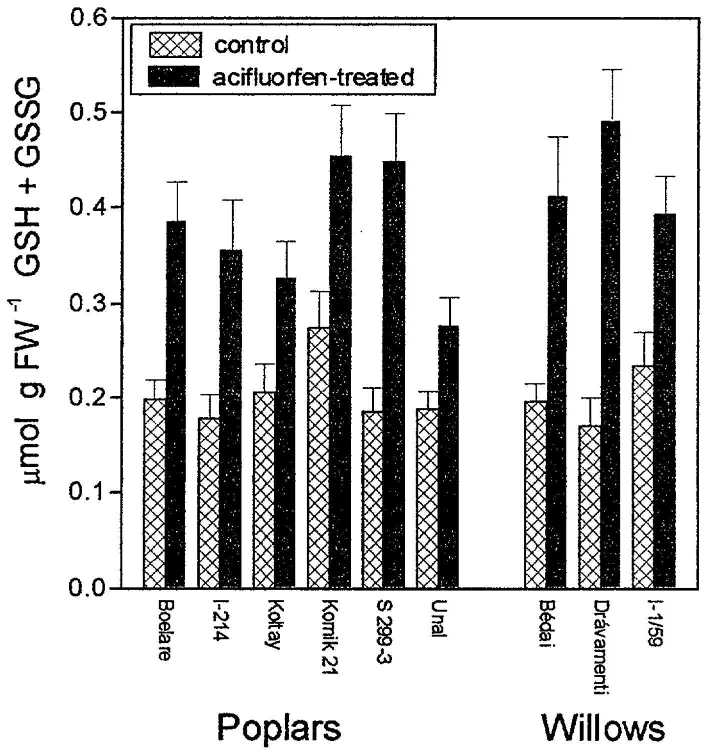

Figure 1. Changes of glutathione levels in detached poplar and willow leaves exposed to 50 µM acifluorfen for 2 days at 25 °C under constant illumination (150 µmol m^{-2} sec^{-1}). Leaves were taken from middle leaf positions of approx. 2-month-old plants and were put by their petioles into acifluorfen solutions or tap water. Means of three independent experiments ± SD are shown.

Poplar leaf extracts showed unusually high activities of glutathione S-transferase, which suggest the high detoxification capacity of these leaves (Fig. 2). Glutathione S-transferase activities in willow leaves were much smaller than in poplar leaves (Fig. 2).

The herbicide acifluorfen is a well-known inducer of the foliar glutathione level and glutathione S-transferase activity. In order to characterize the inducibility of glutathione-related detoxification reactions, which are often determining in herbicide resistance, glutathione levels and glutathione S-transferase activities were measured also in acifluorfen-treated poplar and willow leaves. Both the glutathione levels (Fig. 1) and glutathione S-transferase activities (Fig. 2) were considerably induced by acifluorfen. These results demonstrate the high detoxification capacity of poplar and willow plants.

As glutathione and the glutathione S-transferase enzyme play a crucial role in the detoxification of chloroacetanilide herbicides, a high tolerance of these trees against acetochlor and metolachlor could be predicted. Indeed, the poplar hybrid (*Populus tremula* x *P. alba*) showed a high degree of tolerance against the chloroacetanilide herbicide mixed into the soil. A number of soil concentrations of acetochlor and metolachlor were tested by investigating the biometric parameters and toxicity symptoms of 2-month-old poplar plants. Treatments with as high as 20-60 μg (g soil)$^{-1}$ herbicide concentrations caused only slight growth retardation and no visible toxicity symptoms on the poplar leaves. Under this concentration range no significant phytotoxic symptoms were observed on the poplar plants. These experimental findings also confirms that poplar species are good candidates for field experiments aimed at the removal of these herbicides from soils.

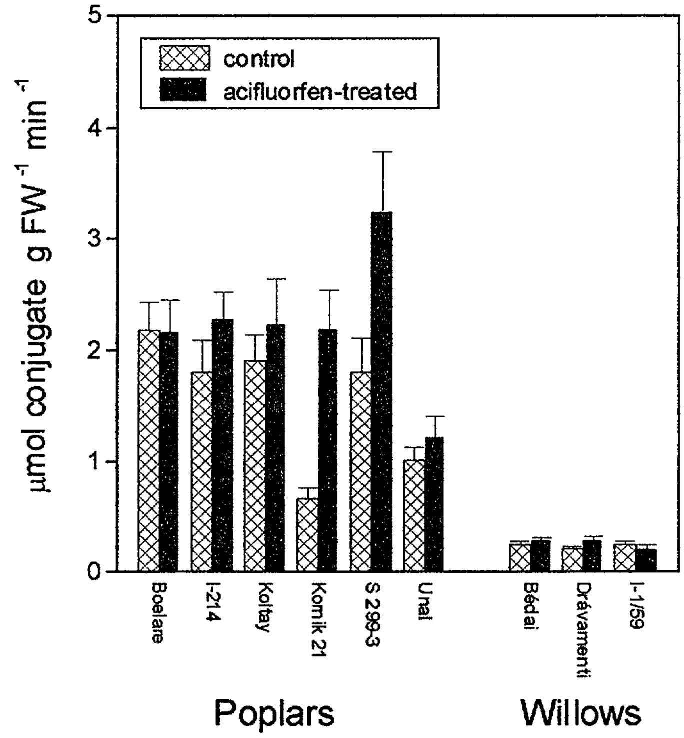

Figure 2. Changes of glutathione S-transferase activities in detached poplar and willow leaves exposed to 50 μM acifluorfen for 2 days at 25 °C under constant illumination (150 μmol m^{-2} sec^{-1}). Leaves were taken from middle leaf positions of approx. 2-month-old plants and were put by their petioles into acifluorfen solutions or tap water. Means of three independent experiments ± SD are shown.

In order to investigate the metabolism of the herbicide acetochlor in poplar leaves, the glutathione conjugate of acetochlor was chemically synthesized. A HPLC method was developed for the analysis of the glutathione conjugate of acetochlor in plant leaf tissues. The metabolism (degradation) of this acetochlor-glutathione conjugate in poplar leaf tissues is still being investigated.

3. Field experiments

3.1. Designing and setting up a field study

Based on the above results of laboratory and greenhouse studies, a field experiment was designed and set up, involving the planting of 1600 trees, belonging to 16 clones on poplar and willow obtained from the Forest Research Institute at Sarvar, Hungary. Sites were selected for the planting of these wild-type poplar and willow plants at a polluted industrial waste-dump area of approx. 40 hectars of the Nitrokemia Chemical Inc. Co. at Fuzfogyartelep in Western Hungary, in the close vicinity of the resort area of Lake Balaton (the largest freshwater lake in Central Europe). The Nitrokemia Company (H-8184 Fuzfogyartelep, Hungary) is a state owned enterprise. Its main activities include the manufacturing of organic intermediates, plant protection products (herbicides) and fine chemicals, distribution of chemicals, and power generation and supply. The company was interested in solving the problems of its strongly heavy metal- and pesticide-polluted sites and was willing to participate in the present phytoremediation project.

The waste-dump site is shown before and after the planting of poplar and willow trees on Figs. 3 and 4, respectively.

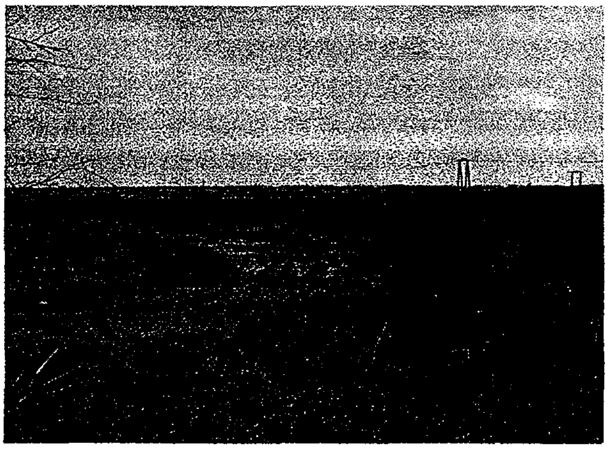

Fig. 3. The polluted site before planting the poplar and willow trees (April 6, 2000)

Fig. 4. The polluted site after planting the poplar and willow trees (April 21, 2000)

The groundwater and the soil at the selected phytoremediation site has been thoroughly analysed by the Environmental Directorate of the Company. The main pollutants are the following: heavy metals (Cd, Cr, Cu, Hg, Pb, Zn) and herbicides and herbicide intermediates (acetochlor, phthalic acid, phenoxyacetic acid, propisochlor, triazine), The groundwater contains considerable amounts of inorganic salts (chloride, sulphate) and has a marked electric conductivity.

Characteristic pollutant levels in the soil (depending on the site and the depth of the sampling in the soil): Cd: 0.28 – 1.9 mg / kg; Cr: 18 – 25 mg / kg; Cu: 10 – 19 mg / kg; Hg: 0.92 – 2.4 mg / kg; Pb: 15 – 200 mg/kg and acetochlor: 6.5-75 mg / kg.

At the present phase of our field studies the soils of polluted sites in the vicinity of the planted poplar and willow trees are extensively analyzed in cooperation with the Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Budapest. The level of various contaminants (acetochlor and various heavy metals) are determined. The effects of heavy metal and pesticide stress on the growth of poplar trees planted in contaminated soils as well as the heavy metal and pesticide uptake and partitioning and the detoxification capacity of wild type poplar and willow trees have been investigated. These studies are in progress at present, because the development of poplar and willow plants is still in an early phase.

During April and May of 2000 the poplar and willow plants developed well in spite of the strongly polluted environment. The developmental status of poplar plants at the end of May 2000 is demonstrated on Fig. 5.

Figure 5. Developmental status of poplar plants at May 26, 2000 in the polluted soil of the industrial waste-dump site of Nitrokemia at Fuzfogyartelep, Hungary.

The drought at the experimental sites during June-July in 2000 caused considerable problems in the development of plants. Several plants died (approx. 20 % of the total) due the soil dryness and many plants had to be severely cut back (Fig. 6).

Figure 6. Cutting-back of poplar trees because of the drought during June-July 2000 at the experimental site near Fuzfogyartelep, Hungary.

4. Testing of transgenic poplars for the phytoremediation of herbicide-polluted soils

Summary of the work carried out by G.Gullner in the laboratory of Prof. Rennenberg, University of Freiburg, Germany as consultant in the present phytoremediation project (Post US/HUN/96/206/17-51). For detailed results see the separate final report of this consultant.

A wild type poplar hybrid and its two transgenic clones (poplars overexpressing the bacterial gene encoding the γ-glutamylcysteine synthetase enzyme in the cytosol and in the chloroplasts of poplar leaves) have been tested for the detoxification of herbicides in soils. The γ-glutamylcysteine synthetase is the rate-limiting regulatory enzyme of the glutathione biosynthesis. Previous studies showed that the transgenic plants overexpressing this enzyme contain higher γ-glutamylcysteine and glutathione levels than wild type poplars. It was supposed that these transgenic plants are more tolerant than wild type poplars against such herbicides, which are detoxified in glutathione dependent reactions (e.g. atrazine and chloroacetanilide herbicides).

Poplar lines were micropropagated and grown in the greenhouse. The plants were exposed to the chloroacetanilide herbicides acetochlor and metolachlor mixed into the soil. The concentrations of the herbicides were selected in preliminary experiments by using wild-type poplar plants. The phytotoxic effects of the herbicides were characterized by biometric parameters (plant height, growth rate, shoot, and root fresh weight). The growth of all poplar lines was significantly reduced by the two-chloroacetanilide herbicides. However, the growth rate and the shoot and root fresh weights of the transgenic lines were significantly higher than those of the wild type plants were. The toxic effects of the two herbicides were nearly identical in all poplar lines studied. The exposure to the herbicides markedly increased the foliar cysteine, γ-glutamylcysteine and glutathione contents both in detached leaves immersed in herbicide solutions and in intact plants elevated in herbicide-exposure to the herbicides markedly increased the foliar cysteine, γ-glutamylcysteine and glutathione contents both in detached leaves immersed in herbicide solutions and in intact plants elevated in herbicide-treated soils. The elevation of the levels of these small molecular weight thiol compounds was higher in the transgenic than in wild type plants. The glutathione S-transferase enzyme also plays an important role in the detoxification of chloroacetanilide herbicides by catalyzing the conjugate formation between the herbicide molecules and glutathione. Considerable glutathione S-transferase activities were detected in poplar leaves. The exposure of poplar plants to chloroacetanilide herbicides mixed into the soil resulted in a marked induction of glutathione S-transferase activity in the upper leaf position but not in middle and lower leaf positions. The extent of the enzyme induction did not differ significantly between transgenic and wild type poplar plants.

The effect of the photooxidative herbicide acifluorfen was also investigated on the thiol composition of transgenic and wild type poplar leaves. Detached leaves were exposed to acifluorfen solutions both under illumination and in darkness. The acifluorfen treatments markedly increased the foliar cysteine, γ-glutamylcysteine and glutathione contents. The dramatic accumulation of γ-glutamylcysteine was observed only in the transgenic lines, particularly in darkness.

5. Conclusions

The sub-project resulted in successful laboratory, greenhouse and field investigations on the feasibility of phytoremediation methods to remediate soil contaminants. Poplar plants showed considerable herbicide and heavy metal tolerance, particularly against chloroacetanilide herbicides probably due to their high glutathione S-transferase activities. Our results showed that poplar plants are promising candidates for practical phytoremediation purposes at herbicide and heavy metal contaminated sites.

6. Scientific publications originating from the sub-project

a) Kömives,T. and Gullner,G. (2000) Phytoremediation. In: Plant-Environment Interactions (Ed. Wilkinson,R.E.), Marcel Dekker, New York, USA, pp. 437-452.

b) Gullner,G., Kömives T. and Rennenberg,H. Enhanced tolerance of transgenic poplar plants overexpressing γ-glutamylcysteine synthetase towards chloroacetanilide herbicides. Journal of Experimental Botany (submitted).

Budapest, July 28, 2000

ADDENDUM

to the Final Report on the sub-project "Pilot experiments for phytoremediation to depollute heavy metal and pesticide contamination"
Project No.: US/HUN/96/206

Our research and development project has followed two goals:

a) laboratory and greenhouse studies to identify poplar lines that are resistant to herbicides (particularly to chloroacetanilide herbicides) and heavy metals in order to use them in phytoremediation of soils polluted with such agrochemicals,

b) field experiments to test the most resistant lines for potency to take up and detoxify chloroacetanilide herbicides and heavy metals under field conditions in a heavily polluted industrial wasteland at Fűzfőgyártelep, near to the Lake Balaton in Hungary.

Both parts of the project were highly successful: we have identified stress-tolerant poplar lines with excellent potency for use in phytoremediation of chloroacetanilide herbicide polluted soils. We have launched a large-scale field experiment that involved the planting and growing of 1600 poplar trees belonging to the above lines.

Future tasks:

Short-term:

1) Soil and groundwater pollutant levels at the Fűzfőgyártelep (Lake Balaton) industrial site, as well as the physiological status of the 1600 poplar trees planted there will be closely monitored in the future.

2) Heavy metal levels will be measured and the metabolism of chloroacetanilide herbicides will be studied in leaves of poplar trees elevated at at the Fűzfőgyártelep (Lake Balaton) industrial site.

Medium-term:

1) further research is necessary to identify the biochemical and physiological properties of the herbicide-tolerant poplar lines that make them capable of taking up and detoxifying chloroacetanilide chemicals. Based on findings of this research, new lines will be designed for phytoremediation applications. Characterization of these lines for ability to take up and accumulate/detoxify a range of known pollutants with widely different chemical and physical properties will also identify structures that are difficult to phytoremediate with existing poplar biochemistry and physiology. Therefore, this project will also contribute to research and development targeting the design and production of transgenic poplar lines engineered for enhanced remediation.

Dr. Tamás Kömives
August 16, 2000