Gene Expression of *Medicago sativa* Inoculated with *Sinorhizobium meliloti* as Modulated by the Xenobiotics Cadmium and Fluoranthene

Heike Neumann* and Dietrich Werner

Fachgebiet Zellbiologie und Angewandte Botanik, Fachbereich Biologie der Philipps-Universität Marburg, Karl-von-Frisch-Str., D-35032 Marburg, Germany.
Fax: ++49–06421–2828997. E-mail: werner@mailer.uni-marburg.de

* Author for correspondence and reprint requests

Z. Naturforsch. **55c,** 222–232 (2000); received October 14/November 22, 1999

Medicago sativa (Alfalfa), *Sinorhizobium meliloti*, Xenobiotics

Alfalfa plants (*Medicago sativa* cv. Europe) inoculated with *Sinorhizobium meliloti* 2011 (formerly *Rhizobium meliloti,* de Lajudie *et al.*, 1994) were cultivated for 14 days under standardized growth conditions in mineral medium with addition of the heavy metal cadmium or the polycyclic aromatic hydrocarbon fluoranthene. These xenobiotics significantly reduced the numbers of root nodules before any visible damage to the plant could be detected. EC10, EC50, and EC90 (effective concentrations reducing nodulation, shoot and root fresh weight by 10, 50, or 90% compared to the control without pollutant) were calculated. EC50 for cadmium ranged from 5.8 μM (nodulation) to more than 20 μM (root fresh weight). Testing fluoranthene resulted in an EC50 of 2.5 μg cm^{-2} for nodulation, and EC50 values of more than 35 μg cm^{-2} for shoot and root biomass production, indicating that the effect parameter nodulation is 10-fold more sensitive than shoot and root fresh weight. With mRNA differential display techniques the effects of both xenobiotics on gene expression in alfalfa root systems were studied. 37 differentially displayed transcripts were detected. Two of them, called DDMs1 and DDMs2, were confirmed by northern hybridization to be down-regulated in the presence of the xenobiotics. The expression of transcript DDMs1 was enhanced in alfalfa control plants inoculated with rhizobia, the transcript level was increased 2.5–3-fold compared to non-inoculated plants. This positive effect of nodulation was suppressed, partly by 35 μg cm^{-2} fluoranthene and totally by 20 μM cadmium. The decrease in DDMs1 transcription was highly affected by the cadmium concentration with an EC50 of 5.9 μM. Compared to nodulation, almost identical EC10, EC50, and EC90 values were found for DDMs1 expression. Sequence analysis of DDMs1 revealed a significant overall homology (50% identity) to a hypothetical protein from *Arabidopsis thaliana* with high similarity to a copper transporting ATPase. High levels of transcript DDMs2 were observed in control plants with a 50% decrease in the xenobiotic-treated plants. DDMs2 gave a strong homology (82% identity) to the cytoplasmatic 60S ribosomal protein L18 from *Arabidopsis thaliana.*

Introduction

In the last decades significant amounts of potentially toxic chemicals have been introduced anthropogenically into the biosphere. These so-called xenobiotics can accumulate in the soil, affecting the soil biota. Therefore, there is a still increasing demand to detect effects of xenobiotics at the lowest possible level. In this study, we chose two chemicals – fluoranthene as a hydrophobic aromatic compound and cadmium as a water soluble heavy metal – to evaluate their phytotoxic effect and their influence on plant gene expression.

Polycyclic aromatic hydrocarbons (PAH), including fluoranthene, are present at high concentrations in the soils of many industrially contaminated sites (Wilson and Jones, 1993). They are frequently found at levels from 1 to 10 mg kg^{-1} of soil. Levels in uncontaminated soil range from 0.2 to 1 mg kg^{-1} (Lewandowski *et al.*, 1997). Prominent sources of PAH-contamination are leakage from petrol stations, and former gasworks. They are also generated by combustion of fossil fuels and wood-preserving industries (Wetzel, 1998). The fate of PAHs as ubiquitous pollutants is of great environmental concern due to their toxic, mutagenic, and carcinogenic potential (Lewandowski *et al.*, 1997). These properties increase with the number of benzene rings. They are rather insoluble in water and are associated with lipid containing fractions. Proposed mechanisms that mediate PAH toxicity to organisms are (a) binding of

0939–5075/2000/0300–0222 $ 06.00 © 2000 Verlag der Zeitschrift für Naturforschung, Tübingen · www.znaturforsch.com ·

Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung") beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition "no derivative works"). This is to allow reuse in the area of future scientific usage.

the molecule to hydrophobic sites, causing disturbances in normal function of membranes and cells; (b) binding of PAH metabolites to macromolecules such as proteins and nucleic acids with molecular and cellular damages (for details see Ayrton *et al.,* 1990; Molven and Goksoyr, 1993).

The heavy metal cadmium is a serious environmental pollutant. It was ranked as number 7 on the „Top 20 Hazardous Substances Priority List" of the Agency for Toxic Substances and Disease Registry/Environmental Protection Agency in 1997 (Fay and Mumtaz, 1997). Cadmium is primarily used in metal coatings, nickel-cadmium batteries, and pigments. It may also be introduced to the atmosphere as a by-product from the smelting of ores, the burning of fossil fuels, and the incineration of municipal waste materials. Consequently, there is a high anthropogenic emission of heavy metals into the biosphere (Zenk, 1996). According to the „Niederländische Liste" (1988) soils containing more than 20 mg Cd kg^{-1} have to be treated by e.g. phytosanitation, the removal of heavy metals by (hyper-)accumulating crops (Leendertse and Pak, 1998). Uptake of cadmium by plants primarily takes place from the soil solution through the roots and leads to a decrease in photosynthesis and transpiration (Streit, 1991). The strong affinity of Cd ions for cysteine residues and phosphate groups might lead to the denaturation of proteins involved in plant metabolism, explaining its great toxicity (Balsberg Pahlsson, 1989). Other main mechanisms of cadmium damage involve the generation of reactive oxygen species and DNA strand breaks (Hsiu-Chuan Liao and Freedman, 1998).

There are only a few standardized and widely used bioassays with higher plants for toxicity testing of xenobiotics such as heavy metals and PAHs. They use root and shoot biomass of monocotyledonous and dicotyledonous plants (ISO 11269–1, 1993; ISO/DIS 11269–2, 1995).

We used the symbiosis of legumes and rhizobia (van Rhijn and Vanderleyden, 1995) with the formation of nitrogen fixing root nodules to develop a biotest with the nodulation of *Medicago sativa* by *Sinorhizobium meliloti* as a more sensitive process than root growth or plant biomass production.

After optimization and standardization (Wetzel and Werner, 1995; Neumann *et al.*, 1998a), this test system was used to determine the effects of cadmium and fluoranthene on phenotypic parameters such as nodulation and biomass production. Furthermore, mRNA differential display (Liang and Pardee, 1992) was used to identify transcripts in alfalfa plants that are differentially expressed by the addition of cadmium or fluoranthene to the growth medium. Using DDRT (differential display reverse transcription) as a tool for the analysis of gene expression, it was possible to detect quantitative changes in gene transcription. Two out of 37 differentially displayed cDNA fragments were confirmed by northern hybridization experiments as being differentially expressed, and were further cloned and sequenced.

Materials and Methods

Growth and test medium

A 500 μM stock solution of $CdCl_2$ (grade: 98%, Sigma, Deisenhofen, Germany) was prepared in distilled water (pH 7.5) and aliquots were added to an autoclaved mineral medium solidified with Gelrite® by sterile filtration, as previously described (Neumann *et al.,* 1998a). The final cadmium concentrations were 0, 0.1, 0.5, 1, 5, 10 and 20 μM. The medium was poured into petri dishes, holding them in a 45° position to produce a sloping surface to provide more space for the plant shoots in the upper part of the dish.

Fluoranthene (grade: 99%, Serva, Heidelberg, Germany), due to its lower solubility in water, was sprayed as a diethylether solution onto the solidified mineral medium. An etheral stock solution (5 mg ml^{-1}) was prepared, and different dilutions were applicated by airbrush onto the surface of the medium. The amounts were 0, 1, 10, 100, 500 and 1000 μg per dish, resulting in 0; 0.04; 0.35; 3.5; 17.5 and 35 μg cm^{-2} fluoranthene.

Plant growth and plant inoculation

Germination of *Medicago sativa* cv. Europe (alfalfa) seeds and cultivation of *Sinorhizobium meliloti* 2011 were as described by Wetzel and Werner (1995). Rhizobial cultures were diluted to 10^8 cfu ml^{-1} and 50 μl of the resulting suspensions were plated onto the surface of the mineral medium. For plates treated with fluoranthene, the rhizobial inoculation was carried out 24 h before the application of the xenobiotic. The petri dishes were in-

cubated for 24 h at 28 °C in the dark before transplanting the pregerminated alfalfa seedlings (three seedlings per dish). The plants were grown for 14 days in a phytotron with the following standardized growth conditions: 16 h, 25 °C light period / 8 h, 20 °C dark period, 75% relative humidity. The petri dishes were placed upright and illuminated from above with sodium lamps. The photosynthetically effective radiation was 190 μmol $s^{-1}m^{-2}$. For both chemicals, each concentration level was tested in eight replicates. Each test series contained a control without toxicant. In tests with fluoranthene contaminated medium, an additional ether control was included.

Nodulation and growth parameters

Root nodules per plant were counted, and plants were harvested and separated just below the hypocotyl into roots and shoots. Shoot and root fresh weight of each plant was determined.

Statistic analysis of the effect parameters

Significance of variation at the $P \leq 0.05$ level was analyzed by the Kruskal-Wallis and Mann-Whitney procedure, using the SPSS statistic analysis package (SPSS Inc., Chicago, USA). Error bars with the same letter in Fig. 1 indicate that the means (n = 24) were not significantly different. EC50 were calculated using the curve fit procedure of the computer program SIGMAplot (Jandel Scientific, Corte Madera, USA).

RNA Isolation

Table I shows the different plant growth treatments chosen for total RNA isolation. After 14 days of cultivation frozen plant roots (treatments A – F, see Table I) were ground into a fine powder using a liquid nitrogen-cooled mortar and pestle, before total RNA extraction was carried out using the TRIzol reagent (Life Technologies Inc., Eggenstein, Germany) according to the manufacturer's instructions.

mRNA Differential Display

DDRT-PCR was carried out with total RNA extracted from roots of *M. sativa* plants of treatments A – F (see Table I) following the protocol of Liang & Pardee (1992).

Table I. Plant growth treatments used for mRNA differential display analysis.
Plants were grown in mineral medium for 14 days under the following conditions: 16 h, 25 °C light period / 8 h, 20 °C dark period, 75% relative humidity. The photosynthetically effective radiation was 190 μmol $s^{-1}m^{-2}$.

Treatment	Plants *M. sativa*	Inoculation with *S. meliloti*	Application of xenobiotic[1]
A	control plants	–	none
B	control plants	+	none
C	test plants	–	35 μg cm^{-2} fluoranthene
D	test plants	+	35 μg cm^{-2} fluoranthene
E	test plants	–	20 μM cadmium ($CdCl_2$)
F	test plants	+	20 μM cadmium ($CdCl_2$)

[1] The levels of the Cd and fluoranthene treatments correspond to the highest concentration level tested in the bioassay resulting in more than 80% decrease in nodulation; –: without rhizobial inoculum; +: with rhizobial inoculum.

DNase treatment: 50 μg of total RNA was treated with 3 units of DNase RQ 1 (Promega, Mannheim, Germany) in the presence of a RNase inhibitor (40 U) in 40 mM Tris-(hydroxymethyl)aminomethan-HCl buffer, pH 7.9, containing 10 mM NaCl, 10 mM $CaCl_2$, and 6 mM $MgCl_2$. The DNA-free RNA preparations were precipitated with ethanol and dissolved in DEPC (diethyl pyrocarbonate) -treated water.

Reverse transcription: First strand cDNA synthesis was performed with the SUPERSCRIPT™II reverse transcriptase (Life Technologies Inc.) using 2.5 μg total RNA in the presence of four oligo$(dT)_{11}$MN primers (MN = AT, AC, GC, GT, CG, CA, GA, CC) in a volume of 20 μl, using the buffer provided by the enzyme supplier.

Amplification of cDNA fragments: A 0.3 μl aliquot of the first strand reaction was used as a template for PCR-amplification, in a total volume of 20 μl containing 2.5 μM of the same oligo$(dT)_{11}$MN primer used in the reverse transcription reactions, 1 μM of eight random decamer oligonucleotide primers (see Bauer *et al.*, 1993: 5′ primers no. 1, 2, 3, 4, 5, 12, 16, and 26), 1 μM dNTPs, 37 kBq α^{32}P dATP (ICN), 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 1.8 μg bovine serum albumin, and 0.5 units Taq polymerase (Life Technologies Inc.) After a denaturation step for 5 min at 95 °C, amplification was carried out for 30 cycles of 30 s at 95 °C, 2 min at 40 °C, and 30 s at 72 °C with a final extension period of 5 min at 72 °C. All PCRs were performed in duplicate. The duplicate PCR products

were separated on 6% denaturating polyacrylamide gels, which were transferred onto Whatman paper, dried under vacuum, and exposed overnight on X-ray films (Kodak Biomax MR) for fragment detection. Differentially displayed fragments were cut out of the gel, and the DNA eluted out of the gel slice by boiling. The cDNA fragments were then reamplified with the same primer sets. PCR parameters were similar to those described above, except that the concentration of the dNTPs was increased to 20 μM and the α^{32}P dATP was omitted. Amplified cDNA fragments were resolved in 1.4% agarose gels and purified using a DNA purification kit (NucleoSpin Extract 2 in 1, Machery-Nagel, Düren, Germany).

Cloning and sequencing of cDNA fragments

The gel-purified cDNA fragments were ligated to the T-A cloning vector pGEM-T (Promega) with T4-Ligase (Promega), and the ligation mixture was used to transform $CaCl_2$ -competent *E. coli* DH5α cells (Sambrook *et al.*, 1989). Recombinant plasmids from positive clones were isolated with the Wizard™ Plus Miniprep DNA Purification System (Promega). Cycle sequencing of both DNA strands was performed in a Gene Amp 2400 thermocycler (Perkin Elmer, Weiterstadt, Germany), using IRD800-labelled universal M13 forward- and M13 reverse-primers and the thermosequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (USB-Amersham, Braunschweig, Germany). Products were resolved on an automatic sequencer (LICOR, MWG-Biotech, München, Germany). Remote sequence searches were performed with different BLAST programs at the National Center of Biotechnology Information server (Altschul *et al.*, 1990 and 1994).

Northern blot analyses

Samples of 20 μg total RNA were separated on a denaturating 1.2% agarose gel containing 2.2 M formaldehyde. To verify that similar amounts of RNA were loaded onto the gels, these were stained with ethidium bromide and photographed under UV light prior to blotting. The RNA was blotted by capillary transfer (Sambrook *et al.*, 1989) onto Hybond-N nylon membranes (Amersham, Braunschweig, Germany) and fixed by UV-crosslinking. Probes were labelled by PCR amplification of the cloned cDNA fragments using the same PCR conditions as described for the reamplification of the differentially expressed fragments, except that 72 kBq α^{32}P dCTP were added. After 1 h prehybridization, membranes were hybridized in the hybridization buffer (0.5 M NaP_i, pH 7.0, 1 mM EDTA, 7% (w/v) SDS, 1% (w/v) BSA) with the heat denatured DNA-probe at 62 °C for 16 h. Membranes were washed twice for 10 min at room temperature in 2 × SSC (saline sodium citrate), 0.1% (w/v) SDS, and twice for 10 min at 62 °C in 0.1 × SSC, 0.1% (w/v) SDS. Blots were exposed to X-ray films at −70 °C for 1 or 2 days for signal detection. Northern hybridization experiments were conducted in two replicates.

Results

Dose responses

The EC10, EC50, and EC90 values are the effective concentrations reducing biomass production (shoot and root fresh weight) or nodulation after 14 days cultivation by 10, 50, or 90% compared to the control without pollutant. An exponential curve-fitting model was used for calculation of these values. Functions and curve-fit coefficients for cadmium and fluoranthene effects are given in Table II. In all tests, nodulation was the most affected parameter.

Cadmium concentrations ranging from 0.1 to 1 μM did not reveal any inhibitory effect on nodulation. Higher concentrations resulted in a significant exponential decrease of nodule numbers per plant (Fig. 1a). At 5.8 μM, a 50% inhibition of nodule formation was observed (Table II). A cadmium concentration of 20 μM gave a complete inhibition of nodule formation. Biomass production determined as shoot and root fresh weight was less affected by cadmium. EC50 values higher than 20 μM indicate a lower sensitivity of biomass production than nodulation as effect parameter.

The dose response plot for fluoranthene resulted in an EC50 of 2.5 μg cm^{-2} for the nodulation as effect parameter (Table II). At concentrations up to 0.35 μg cm^{-2}, no inhibition of nodule formation was detected. At 35 μg cm^{-2}, about 80% less nodules were produced compared to control plants (Fig. 1b). EC50 values for shoot and root fresh weight were higher than 35 μg cm^{-2}, in-

Table II. Effective concentrations EC10, EC50, and EC90 of cadmium ($CdCl_2$) and fluoranthene for nodulation, shoot, and root fresh weight of *M. sativa* infected by *S. meliloti*.
EC10, EC50, and EC90 values are given as molarity (μM) for cadmium and as weight per area (μg cm^{-2}) for fluoranthene. EC values were calculated by an exponential curve fitting model, the exponential curve can be described with the equation $y = a \cdot e^{-bx}$, whereas y represents the effect parameter NOD, SFW or RFW, x the xenobiotic concentration and bx the exponent.

Compound	Statistic	Parameter	EC10	EC50	EC90
			(μM)	(μM)	(μM)
	$y = a \cdot e^{-bx}$	NOD	0.5	5.8	19.6
$CdCl_2$	$a = 94.9$	SFW	5.5	>20	>20
	$b = 0.115$	RFW	4.4	19.1	>20
			(μg cm^{-2})	(μg cm^{-2})	(μg cm^{-2})
	$y = a \cdot e^{-bx}$	NOD	0.3	2.5	>35
Fluoranthene	$a = 98.3$	SFW	4.1	>35	>35
	$b = 0.286$	RFW	8.5	>35	>35

NOD: nodulation; SFW: shoot fresh weight; RFW: root fresh weight.

dicating a 10-fold higher effect of fluoranthene on nodulation as effect parameter.

mRNA Differential display

Total RNA extracted from alfalfa plants grown under the conditions indicated in Table 1 was used for DDRT-PCR experiments. Comparison of the banding patterns on the DDRT-polyacrylamide gels obtained using 64 combinations of oligo$(dT)_{11}$MN and random primers revealed numerous types of differences. Some cDNA bands were detected only in inoculated alfalfa plants, and the expression of some of these symbiosis-specific genes might be down-regulated by cadmium or fluoranthene contamination (data not shown). The intensity of other cDNA fragments was either enhanced or reduced in inoculated and not inoculated plants, either by the cadmium or fluoranthene treatments, whereas the intensity of other bands seemed to be affected (down- or up-regulated) by both cadmium and fluoranthene.

About 6400 bands were obtained with the 64 primer sets, and differences were observed in 37 of them (0.5%). The 37 differentially represented bands were cut out of the gels. Thirty four of them could be reamplified with the same primer pairs as used for the original DDRT-PCR.

In this study, we focussed our attention on two differentially displayed cDNA fragments. The intensity of the cDNA band called DDMs1 was increased in plants infected with *S. meliloti*. This effect was observed in control plants and plants grown in fluoranthene supplemented mineral medium, but it was not detectable in alfalfa grown in cadmium contaminated substrate (data not shown). The expression of DDMs2 seemed to be reduced, whenever alfalfa plants were grown in contaminated mineral medium (data not shown).

Expression of the genes corresponding to DDMs1 and DDMs2

The expression patterns of the genes corresponding to DDMs1 and DDMs2 are shown in northern blots with total RNA (Fig. 2). RNA extracted from roots of alfalfa plants (treatments A – F, Table I) was hybridized with the cloned and radiolabelled fragments DDMs1 or DDMs2. The results of DDRT analyses were confirmed by northern experiments. The size of the mRNA corresponding to DDMs1 was 1.9 kb, and that of the DDMs2-mRNA was 0.8 kb.

About equal loading of the northern blots was confirmed by the ethidium bromide fluorescence of the gels prior to blotting. In order to quantify the transcript levels, the intensity of northern signals of each sample was measured using the computer program SIGMAgel (Jandel Scientific, Corte Madera, USA) and normalized with reference to the intensity of the 28S and 18S rRNA bands on the northern gels.

As shown in Table III, the transcript level of DDMs1 in inoculated plant treatments B and D

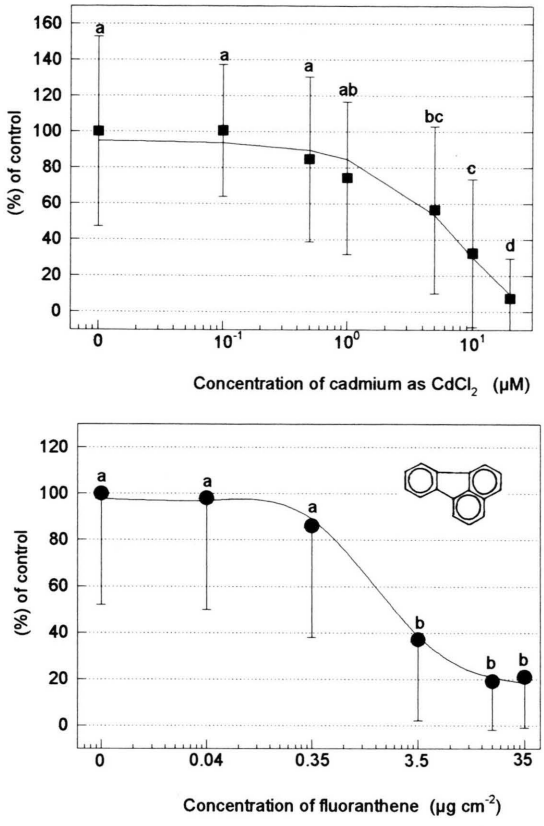

Fig. 1. Dose response plot of (a) cadmium-toxicity and (b) fluoranthene-toxicity on nodulation of *M. sativa* by *S. meliloti*. Error bars with different letters (a or b) indicate significant differences of the means (n = 24), whereas error bars with the letter combination ab indicate no significant difference to means characterized by the letter a or b.

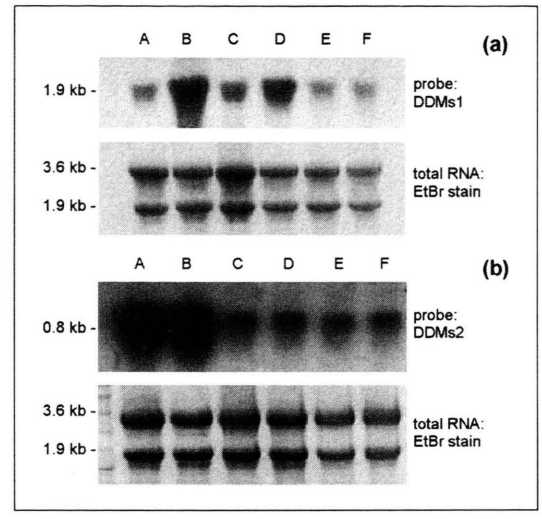

Fig. 2. Northern blot analyses with the differential display fragments DDMs1 and DDMs2. Total RNA (20 µg per lane) from inoculated roots of alfalfa plants (treatments A – F as indicated in Table I) was separated by gel electrophoresis, blotted, and hybridized to the radiolabelled cDNA fragments DDMs1 (panel a) or DDMs2 (panel b). (a): The intensity of DDMs1 was increased in plants infected with *S. meliloti*. This effect was observed in control plants and in plants grown in fluoranthene contaminated medium (lane B and D), it was not detectable in alfalfa grown in cadmium contaminated medium (lane F). (b): The expression of DDMs2 was reduced, whenever alfalfa plants were grown in contaminated medium (reduced signal in lane C, D, E, and F). Equal loading of the northern blots was shown by ethidium bromide fluorescence.

(see Table I) was increased 2.5- to 3-fold compared to plants grown in cadmium supplemented medium (treatment F: I = 1.26). A 50% reduction of the DDMs2 gene expression was observed compared to control plants, when alfalfa was cultivated in fluoranthene or cadmium contaminated substrate.

Cadmium concentrations affect the gene expression of DDMs1

Corresponding to the nodulation inhibiting effect of $CdCl_2$ at different doses (Fig. 1a), we were interested in the effect of different cadmium concentrations on gene expression of DDMs1. Alfalfa plants inoculated with *S. meliloti* were grown for 14 days in a mineral medium supplemented with cadmium at different concentrations. After RNA isolation from the roots of the different test plants, northern experiments were carried out with total RNA using the cloned fragment DDMs1 as probe.

As shown by the expression pattern in Fig. 3, increasing cadmium concentration (0, 0.5, 1, 5, 10, 20, and 40 µM Cd) in the mineral growth medium led to a dose responsive reduction of the DDMs1 transcript level. At 5 µM cadmium, a significantly decreased gene expression was found, about 40% lower than the control. At 10 µM, a Cd induced transcript reduction of 75 % was observed. With higher cadmium concentration no further decrease in gene expression of DDMs1 did occur in our experiments.

This dose response of the DDMs1 transcription allowed the calculation of EC-concentrations for the effect parameter gene expression of DDMs1

Table III. Relative changes in levels of mRNA of the genes corresponding to DDMs1 and DDMs2 caused by cadmium and fluoranthene.

Probe	Plants[1]	Relative amount of total RNA[2]	Relative gene expression[2]
DDMs1	A	1.00[3]	1.00
	B	1.10	3.03
	C	1.27	1.06
	D	0.98	2.47
	E	0.92	1.13
	F	0.78	1.26
DDMs2	A	1.00	1.00
	B	0.93	1.09
	C	1.03	0.45
	D	0.95	0.45
	E	0.77	0.52
	F	0.75	0.52

[1] Plant treatments A – F are indicated in Table I.
[2] In order to quantify the transcript levels, the intensity of northern signals of each sample A – F was measured using the computer program SIGMAgel and normalized with reference to the intensity of the 28S and 18S rRNA bands on the northern gel.
[3] The intensity of control plants (treatment A) was defined as I = 1.0.

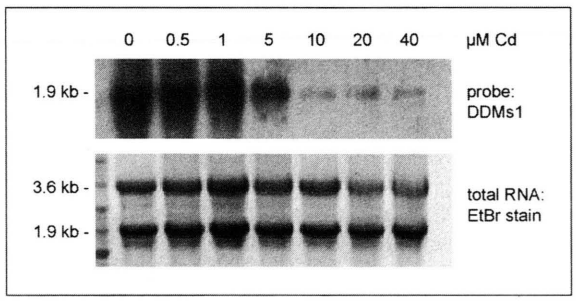

Fig. 3. Effect of the cadmium concentration on gene expression of DDMs1. Total RNA (10 µg per lane) from inoculated roots of alfalfa plants grown in mineral medium contaminated with different doses of $CdCl_2$ was separated by gel electrophoresis, blotted, and hybridized to the radiolabelled cDNA fragment DDMs1 (upper panel). Equal loading of the northern blots was shown by ethidium bromide fluorescence (lower panel) of the gel prior to blotting. Increasing Cd concentration led to a dose responsive reduction of the DDMs1 transcript level. At 5 µM Cd, a significantly reduced gene expression was found. This effect was enhanced at 10 µM Cd.

(Table IV). Compared to the effect parameter nodulation, almost identical EC10, EC50, and EC90 values were found.

Table IV. EC10, EC50, and EC90 concentrations of the chemical $CdCl_2$ for the effect parameters nodulation and gene expression of DDMs1. Values for nodulation are also listed in Table II, values for gene expression were determined after quantification of hybridization signals using the computer program SIGMAgel. The EC values were calculated using the curve fit procedure of SIGMAplot.

Effect parameters:			
Nodulation (µM)		Gene expression of DDMs1 (µM)	
EC10	0.5	EC10	0.4
EC50	5.8	EC50	5.9
EC90	19.6	EC90	20.0

Sequence analyses

The two differentially expressed cDNA fragments DDMs1 and DDMs2 confirmed by northern hybridization to be differentially transcribed were analyzed by sequence analysis. Sequencing of plasmids derived from different clones of each construct gave the same sequence, demonstrating that the differentially displayed bands corresponded to single transcripts. In both cases, the random primer was localized at the 3′ end, the anchored oligo$(dT)_{11}$ primer at the 5′ end of the cDNA fragment.

Fig. 4a shows the alignment of the derived amino acid sequence encoded by DDMs1 with a hypothetical protein from *Arabidopsis thaliana* (accession number Z99707). The 317 bp fragment DDMs1 gave 50% identity and 63% similarity over 93 amino acids to this 173 amino acids long hypothetical protein revealing a similarity to a copper transporting ATPase (see Fig. 4a).

The 294 bp fragment DDMs2 turned out to encode a 187 amino acids long peptide with 82% identities and 93% similarity over 98 amino acids to the cytoplasmatic 60S ribosomal protein L18 from *Arabidopsis thaliana* (accession number U15741) and numerous other eukaryotic organisms. The alignment of these sequences is shown in Fig. 4b.

Discussion

Criteria for the choice of xenobiotics

Cadmium ($CdCl_2$) and fluoranthene were chosen as xenobiotics, in order to investigate the influ-

(a)

```
identities: 47/93 (50%)
positives:  60/93 (63%)

DDMs1      281  EEQIKITNQIKAQFDALTPKRPIKPNRSEPESQQNPVDSTFSSRNI   142
                |+|||  |||+| ||+| |||| || ||||         ++    +
CAB16754.1 16   EQQIKNANQIRADFDSLAPKRPTKPTRSEP-GFPGSFSASDKITDH    60

DDMs1      143  PELEKLQSLQSNSQTILSSKGLVDA-QDEFVETKYYQELFSIDKQHHT   3
                || +| ||||| +   +  +|   | ||||+||+||  | +|||||||
CAB16754.1 61   PEADKFQSLQSQTHGKVLGEGDSSAVQDEFLETEYYSNLTAIDKQHHT 108
```

(b)

```
identities: 81/98 (82%)
positives:  92/98 (93%)

DDMs2      294  YTEGKEGKIAVVVGAVTDDIRVYEVPAIKVTALRFTEKARARIEK  130
                +  ||| ||||+|| +|||+||+|+||+|||||||||+|||||||
RL18_ARATH  70  FMTGKEDKIAVLVGTITDDLRVHEIPAMKVTALRFTERARARIEK  114

DDMs2      129  AGGECLTFDQLALRAPLGQNTVLLRGTKNAREAVKHFGRAPG      35
                |||||||||||||||||||||||||| ||+|||||||| |||
RL18_ARATH 115  AGGECLTFDQLALRAPLGQNTVLLRGPKNSREAVKHFGPAPG     156

DDMs2       34  VPHSNTKPYVR  2
                ||||++|||||
RL18_ATRAH 157  VPHSHSKPYVR  167
```

Fig. 4. (a): Alignment of the deduced amino acid sequence encoded by the 317 bp fragment DDMs1 with a hypothetical protein from *Arabidopsis thaliana* (CAB16754.1, accession number Z99707); (b): Alignment of the deduced amino acid sequence encoded by the 294 bp fragment DDMs2 with the cytoplasmatic 60S ribosomal protein L18 from *Arabidopsis thaliana* (RL18_ARATH, accession number U15741) |: identical residues; +: similar residues; positives: summarized identical and similar residues; the sequence flanking numbers of DDMs1 and DDMs2 refer to the number and position of nucleotides; these of *A. th.* refer to the number and position of amino acids.

ence of pollutants on the gene expression of alfalfa plants. These compounds belong to different chemical groups: The heavy metal cadmium is found in contaminated soils as Cd^{2+} ion. Its water solubility and consequently its toxicity is influenced by the pH of the soil solution. On the other hand, PAHs, including fluoranthene, behave hydrophobically.

Cadmium is easily taken up by the roots from the soil solution via ion channels and active transport mechanisms, when plants are grown in acidic soils. Soil properties like ion exchange capacity and adsorption onto organic soil colloids determine the bioavailability (Gisi, 1997; Lewandowski *et al.*, 1997).

Fluoranthene uptake mainly takes place through the plant shoot: hydrophobic chemicals are transported through the wax layers of the cuticula into the plant tissue (Paterson *et al.*, 1990). Due to their hydrophobicity, their minimal uptake by plant roots is influenced by the amount and nature of humic substances.

Biotic processes can transform the chemicals once absorbed from the environment, thus altering their phytotoxicity and reactivity (Nannipieri *et al.*, 1997). Ideally, the degradation of organic compounds leads to the formation of CO_2 and H_2O. However, toxic intermediate metabolites can be produced during catabolism, like the transformation of benzo(a)pyrene to epoxides in *Chenopodium rubrum* callus cultures (Harms *et al.*, 1977). On the other hand, most heavy metals can not be metabolized, and accumulate in plant tissues. Possible mechanisms of tolerance involve the complexation by phytochelatins or metallothioneins, the binding to cell wall compounds, or their accumulation in vacuoles (Lozano-Rodriguez *et al.*, 1997).

The widespread presence in many contaminated soils and the different physico-chemical properties

of cadmium and fluoranthene with different uptake mechanisms led to the choice of these xenobiotics for our investigations.

Effects of xenobiotics on plant growth

High sensitivity of the plant growth and nodulation was found for both cadmium and fluoranthene. The established plant test (for details see Neumann *et al.*, 1998a) was applicable for both substances. As shown by the EC values in Table I, nodulation was reduced before visible damage to the plant and effects on shoot and root weight could be detected.

A cadmium concentration of 20 μM (2 mg l^{-1}) resulted in a complete inhibition of nodule formation. Porter and Sheridan (1981) observed a 100% loss of the activity of the nitrogen fixing enzyme nitrogenase in four week-old alfalfa plants incubated for 72 hours in a nutrient solution supplemented with 10 mg Cd l^{-1}. Seed germination assays with *Trifolium pratense* (Miller *et al.*, 1985) gave an EC50 value for Cd of 57 mg l^{-1}. The EC50 for the nodulation determined in this study was significantly lower with 5.8 μM (0.6 mg l^{-1}). These experiments indicate a 100-fold higher effect of cadmium on nodulation and emphasize the high sensitivity of symbiotic processes in response to xenobiotics.

Data for shoot and root growth had almost the same sensitivity as those for *Pisum sativum* towards $CdCl_2$ reported by Landberg and Greger (1994). They found a 30 % growth reduction after 15 days with 16 μM $CdCl_2$ treatment.

The EC50 value of fluoranthene was determined as 2.5 μg cm^{-2} for nodulation as the effect parameter. Further plant tests on a soil substrate resulted in an EC50 value of 13 mg kg^{-1} for the nodulation parameter (Neumann, unpublished).

The studies reported by Rippen (1995) using *Avena sativa* or *Brassica napa* cultivated in fluoranthene contaminated solid substrates resulted in EC50 values for the biomass production > 1000 mg kg^{-1}. In our experiments, EC50 values for shoot and root growth were determined as > 35 μg cm^{-2}. Nodulation was a 10-fold more sensitive indicator for the presence of fluoranthene than shoot or root fresh weight. Similar results were obtained with the PAHs acenaphtene, fluorene, and phenanthrene (Neumann, in preparation). These results indicate the high sensitivity of the symbiotic interaction between alfalfa and its microsymbiont S. *meliloti* also against PAHs.

First results using a soil substrate confirm the higher sensitivity of nodule formation compared to the biomass production as effect parameter to monitor the biological effect of xenobiotics (Neumann *et al.*, 1998b).

Effects on gene expression

To investigate at the molecular level the phytotoxic, nodulation-inhibiting effects of xenobiotics on gene expression, we used the mRNA differential display method which was established with mammalian tissues and has not been widely used in plant systems (Callard *et al*, 1994; Wilkinson *et al.*, 1995; Goormachtig *et al.*, 1995; Martin-Laurent *et al.*, 1997; Seehaus and Tenhaken, 1998).

Two out of 37 differentially displayed cDNA fragments were confirmed by northern hybridization to be differentially transcribed.

As indicated by the expression pattern of DDMs1, the decrease in DDMs1-transcription was highly affected by the amount of cadmium present in the growth medium. The exponential dose response of DDMs1 expression was used to calculate EC values. Compared to the effect parameter nodulation, almost identical EC10, EC50, and EC90 values were achieved revealing the same sensitivity of both nodulation and DDMs1 gene expression.

DDMs1 gave an overall similarity of 63% to a hypothetical protein from *Arabidopsis thaliana* with similarity to a copper transporting ATPase being the only match with significant overall similarity. Cadmium and copper are both heavy metals transported as bivalent cations. As many other metal cations which act as important cofactors of enzymes, and are toxic at high concentrations, they are bound by phytochelatins, metal-induced peptides of the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n=2–11) that have been found in algae and higher plants, and some fungi (Cervantes and Gutierrez-Corona, 1994). Another mechanism for metal tolerance is the complexation by metallothioneins, inducible low-molecular, cysteine-rich, metal-binding proteins. They are widely distributed throughout living organisms such as mammals, plants, fungi, and cyanobacteria (Cervantes and Gutierrez-Corona, 1994; Zenk, 1996).

The similar properties of cadmium and copper are pointed out by Cervantes and Gutierrez-Corona (1994). The authors found a metallothionein-encoding gene in yeast used for copper homoeostasis. It has also been observed that high transcript levels of this gene lead to cadmium resistance in yeast cells (Jeyaprakash *et al.*, 1991).

In this respect, DDMs1 expression and sequence data make it plausible that this transcript might indeed encode for a putative ATPase involved in the transport of bivalent heavy metal cations.

Cadmium might influence the expression of this enzyme by various mechanisms:

(a) By binding to reduced cysteine residues, cadmium can directly denature proteins which function as activators of the transcription of the copper transporting ATPase encoding gene.

(b) Cadmium competes with copper for binding sites of a regulatory protein, thereby the transcription of the copper transporting ATPase is inhibited by negative feedback.

As reported by Hsiu-Chuan Liao and Freedman (1998), several enzymes such as ATPases, superoxide dismutase, glutathione peroxidase, and ubiquitin are known to be influenced by cadmium. Damage of active transport mechanisms in higher animals is reported by Theede and Scholz (1982).

Sequence analysis of the DDMs2-fragment revealed a 82% identity and 93% similarity to the cytoplasmic 60S ribosomal protein L18 found in *A. thaliana* and in many other eukaryotic organisms such as yeast, chick pea and rat. The transcript levels of the corresponding gene were decreased in alfalfa plants grown in pollutant-supplemented medium. This might indicate a general inhibition of metabolic activity in the stressed plants, reflected in a lower production of ribosomal proteins and protein synthesis.

Acknowledgements

We thank the Umweltbundesamt in Berlin, Germany, for support in the project „Testguideline für Wurzelsymbiosen als Indikator für bodenbelastende Altstoffe und Umweltchemikalien". We also thank the "Fond der Chemischen Industrie" for support.

Altschul S. F., Gish W., Miller W., Myers E. W. and Lipman D. J. (1990), Basic local alignment search tool. J. Mol. Biol. **215,** 403–410.

Altschul S. F., Boguski M. S., Gish W. and Wootton J. C. (1994), Issues in searching molecular sequence databases. Nature Genetics **6,** 119–129.

Ayrton A. D., McFarlane M., Walker R., Neville S., Coombs M. M. and Ioannides C. (1990), Induction of a P-450 I family of proteins by polycyclic aromatic hydrocarbons: possible relationship to their carcinogenicity. Toxicology **60,** 173–186.

Balsberg Pahlsson A. M. (1989), Toxicity of heavy metals (Zn, Cu, Cd, Pb) to vascular plants. Water, Air, and Soil Poll. **47,** 287–319.

Bauer D., Müller H., Reich J., Riedel H., Ahrenkiel V., Warthoe P. and Strauss M. (1993), Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). Nucl. Acids Res. **21,** 4272–4280.

Callard D., Lescure B. and Mazzolini L. (1994), A method for the elimination of false positives generated by the mRNA differential display technique. BioTechniques **16,** 1096–1103.

Cervantes C. and Gutierrez-Corona F. (1994), Copper resistance mechanisms in bacteria and fungi. FEMS Microb. Rev. **14,** 121–138.

Fay R. M. and Mumtaz M. M. (1997), Food Chem. Toxicol. **34**, 1163–1165.

Gisi U., Schenker R., Schulin R., Stadelmann F. X. and Sticher H. (1997), Bodenökologie. Thieme Publ., Stuttgart, New York.

Goormachtig S., Valerio-Lepiniec M. K. S., Van Montagu M., Holsters M. and de Bruijn F. J. (1995), Use of differential display to identify novel *Sesbania rostrata* genes enhanced by *Azorhizobium caulinodans* infection. Mol. Plant Microbe Interact. **8,** 816–824.

Harms H., Dehnen W. and Mönch W. (1977), Benzo(a)-pyrene metabolites formed by plant cells. Z. Naturforsch. **32c,** 321–326.

Hsiu-Chuan Liao V. and Freedman H. (1998), Cadmium-regulated genes from the nematode *Caenorhabditis elegans.* J. Biol. Chem. **273,** 31962–31970.

ISO 11269–1 (1993), Soil Quality – Determination of the Effects of Pollutants on Soil Flora – Part 1. Method for the Measurement of Root Growth. VCH Publ., Weinheim, New York, Basel, Cambridge.

ISO/DIS 11269–2 (1995), Soil Quality – Determination of the Effects of Pollutants on Soil Flora – Part 2. Effects of Chemicals on the Emergence and Growth of Higher Plants. VCH Publ., Weinheim, New York, Basel, Cambridge.

Jeyaprakash A., Welch J. W. and Fogel S. (1991), Multicopy *CUP1* plasmids enhance cadmium and copper resistance levels in yeast. Mol. Gen. Genet. **225,** 363–368.

de Lajudie P., Willems A., Pot B., Dewettinck D, Maestrojuan G., Neyra M., Collins M. D., Dreyfus B., Kersters K. and Gillis M. (1994), Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of the genus *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. Int. J. Sys. Bacteriol. **44,** 715–733.

Landberg T. and Greger M. (1994), Influence of selenium on uptake and toxicity of copper and cadmium in pea (*Pisum sativum*) and wheat (*Triticum aestivum*). Physiol. Plant. **90,** 637–644.

Leendertse P. C. and Pak G. A. (1998), Green soil cleanup by farmers: a challenge? In: Contaminated Soil '98 – Proceedings of the Sixth International FZK/TNO Conference on Contaminated Soil (Telford T. ed.). Scottish Enterprise, Edinburgh, UK, London, 1105–1106.

Lewandowski J., Leitschuh S. and Koss V. (1997), Schadstoffe in Böden: Eine Einführung in Analytik und Bewertung. Springer Publ., Berlin, Heidelberg, New York, Wien, Barcelona, Budapest, Paris, Singapur, Tokyo.

Liang P. and Pardee A. B. (1992), Differential display of eukaryotic messenger RNA by means of polymerase chain reaction. Science **257,** 967–971.

Lozano-Rodriguez E., Hernandez L. E., Bonay P. and Carpena-Ruiz R. O. (1997), Distribution of cadmium in shoot and root tissues of maize and pea plants: physiological disturbances. J. Exp. Bot. **48,** 123–128.

Martin-Laurent F., van Tuinen D., Dumas-Gaudot E., Gianinazzi-Pearson V., Gianinazzi S. and Franken P. (1997), Differential display analysis of RNA accumulation in arbuscular mycorrhiza of pea and isolation of a novel symbiosis regulated plant gene. Mol. Gen. Genet. **256,** 37–44.

Miller W. E., Peterson S. A., Greene J. C. and Callahan C. A. (1985), Comparative toxicology of laboratory organisms for assessing hazardous waste sites. J. Environ. Qual. **14,** 569–574.

Molven A. and Goksoyr A. (1993), Biological effects and biomonitoring of organochlorines and polycyclic aromatic hydrocarbons in the marine environment. In: Ecotoxicology Monitoring (Richardson M., ed.). VCH Publ., Weinheim, 1137–162.

Nannipieri P., Badalucco L., Landi L. and Pietramellora G. (1997), Measurement in assessing the risk of chemicals to the soil ecosystem. In: Ecotoxicology: Responses, Biomarkers and Risk Assessment, an OECD workshop (Zelikoff J. T. ed.). SOS Publ., Fair Haven NY, USA, 507–534.

Neumann H., Bode-Kirchhoff A., Madeheim A. and Wetzel A. (1998a), Toxicity testing of heavy metals with the *Rhizobium*-Legume symbiosis: high sensitivity to cadmium and arsenic compounds. ESPR – Environ. Sci. Pollut. Res. **5 (1),** 28–36.

Neumann H., Bode-Kirchhoff A., Wetzel A. and Werner D. (1998b), Toxicity testing of polycyclic aromatic hydrocarbons with the *Rhizobium*-Legume symbiosis. In: Contaminated Soil '98 – Proceedings of the Sixth International FZK/TNO Conference on Contaminated Soil (Telford T., ed.). Scottish Enterprise, Edinburgh, UK, London, 1041–1042.

Niederländische Liste (1988), Leitfaden Bodensanierung Niederlande. Leitraad Bodemsanering, Staatuitgeverij. 's-Gravenhage, Netherlands.

Paterson S., Mackay D., Tam D. and Shiu W. (1990), Uptake of organic chemicals by plants: a review of processes, correlations, and models. Chemosphere **21,** 297–331.

Porter J. R. and Sheridan R. P. (1981), Inhibition of nitrogen fixation in alfalfa by arsenate, heavy metals, fluoride, and simulated acid rain. Plant Physiol. **68,** 143–148.

Rippen G. (1997), Handbuch Umweltchemikalien: Stoffdaten – Prüfverfahren – Vorschriften. 41. Ergänzungslieferung, ecomed Publ., Landsberg München.

Sambrook J., Fritsch E. F. and Maniatis T. (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor. Lab. Press, Plainview, New York, USA.

Seehaus K. and Tenhaken R. (1998), Cloning of genes by mRNA differential display induced during the hypersensitive reaction of soybean after inoculation with *Pseudomonas syringae pv. glycinea*. Plant Mol. Biol. **38,** 1225–1234.

Streit B. (1991), Lexikon Ökotoxikologie. VCH Publ., Weinheim, New York, Basel, Cambridge.

Theede H. and Scholz N. (1982), Anreicherung und Schadwirkung von Cadmium bei Meerestieren. Naturw. Rdsch. **35 (7)**, 286–292.

van Rhijn P. and Vanderleyden J. (1995), The *Rhizobium*-plant symbiosis. Microbiol. Rev. **59,** 124–142.

Wetzel A. and Werner D. (1995), Ecotoxicological evaluation of contaminated soil using the legume root nodule symbiosis as effect parameter. Environmental Toxicology and Water Quality: An Intern. J. **10**, 127–133.

Wetzel A. (1998), Advances in biomonitoring: sensitivity and reliability in PAH-contaminated soil. In: Environmental Biomonitoring: The Biotechnology Ecotoxicology Interface (Lynch J. M. and Wiseman A., eds.). Cambridge University Press, Cambridge, pp. 27–45.

Wilkinson J. Q., Lanahan M. B., Conner T. W. and Klee H. J. (1995), Identification of mRNAs with enhanced expression in ripening strawberry fruit using polymerase chain reaction differential display. Plant Mol. Biol. **27,** 1097–1108.

Wilson S. C., Jones K. C. (1993), Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. Environ. Poll. **81,** 229–249.

Zenk M. H. (1996), Heavy metal detoxification in higher plants – a review. Gene **179,** 21–30.