

Transformation and Activity Change of Selected Allelochemicals by Microbial Metabolisation

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ABSTRACT

The presented work focusses on methodology and screening for allelopathic effects in two European invaders and in one colour and medical plant. Himalayan Balsam (*Impatiens glandulifera*), Japanese Knotweed (*Fallopia japonica*) and Woad (*Isatis tinctoria*) were chosen and harvested as fresh plants during their growth period from places near Tulln, Austria. The search for bioactive compounds was not limited to those affecting higher plants (e.g. weeds), but we also screened the plants for effects against bacteria, fungi, algae and water flea. Advantages and limitations of the applied methods were discussed and procedures were optimised, if possible. Plant parts showing high activity were extracted and the solutions were applied to native soil. We monitored conventional microbiological parameters (e.g. CFU-counts) as well as changes of the microbial community structure (by molecular biological analysis) and degradation or bio-transformation of the introduced plant substances by HPLC-MS fingerprinting. This yielded an evaluated list of chemical analytical and biotest procedures, focussing on applied characterisation of interactions between secondary plant metabolites and soil microorganisms. Even though it was not possible to identify degrading or passively influenced microorganisms from the treated soil, a methodology for further research in this field could be developed.

Keywords: allelochemicals, activity screening, biotests, soil micro-organism population, transformation, HPLC-MS analysis.

Introduction

Higher plants interact and communicate with other organisms in their habitat by chemical signals. The resulting effect is commonly described by the term, and the science of allelopathy (Molisch, 1937; Rice, 1984). Many important studies of the past have focussed on discovering such interactions, identifying responsible substances, their biosynthesis as secondary plant metabolites, their modes of action in target plants and their biodegradation (Fujii and Hiradate, 2007; Hartmann and Schmidt, 2008; Macias *et al.*, 2004). Much less is known about their ecological role, about signalling pathways between producing plants and target organisms and about the role of soil microorganisms that are responsible for biotransformation (El-Shahawy *et al.*, 2012).

One of the surprising results of a comprehensive European research project called FateAllChem (EU-funded, QLK5-CT-2001-01967) was that degradation metabolites of wheat benzoxazinoids showed higher biological activity than the parent substances (Fritz and Braun, 2006; Idinger *et al.*, 2006). The metabolites were identified as phenoxazinones (Fomsgaard *et al.*, 2004, 2006; Kumar *et al.*, 1993; Zikmundova *et al.*, 2002) and synthetically produced for further inves-

tigations (Macías *et al.*, 2006), allowing the measurement of their physical and chemical properties. In the European research project biotests were applied, using a broad range of test organisms, such as primary producers, consumers and destruenters, represented by bacteria, fungi, algae, insects and higher plants. The authors of the project report concluded that more research would be necessary to discover the ecological meaning behind the interactions between secondary plant metabolites and degrading microorganisms.

The objective of this work was to develop a suitable methodology and to supply some dose-response relations for extracts made from three selected plants. To this end presumed target as well as non-target organisms were considered. In addition the kinetics of such activities on soil microorganisms were to be measured while the substances were applied to soil.

Materials and Methods

Collection of plant material

Himalayan Balsam (*Impatiens glandulifera*), Japanese Knotweed (*Fallopia japonica*) and Woad (*Isatis tinctoria*) were chosen for the experiment. The first

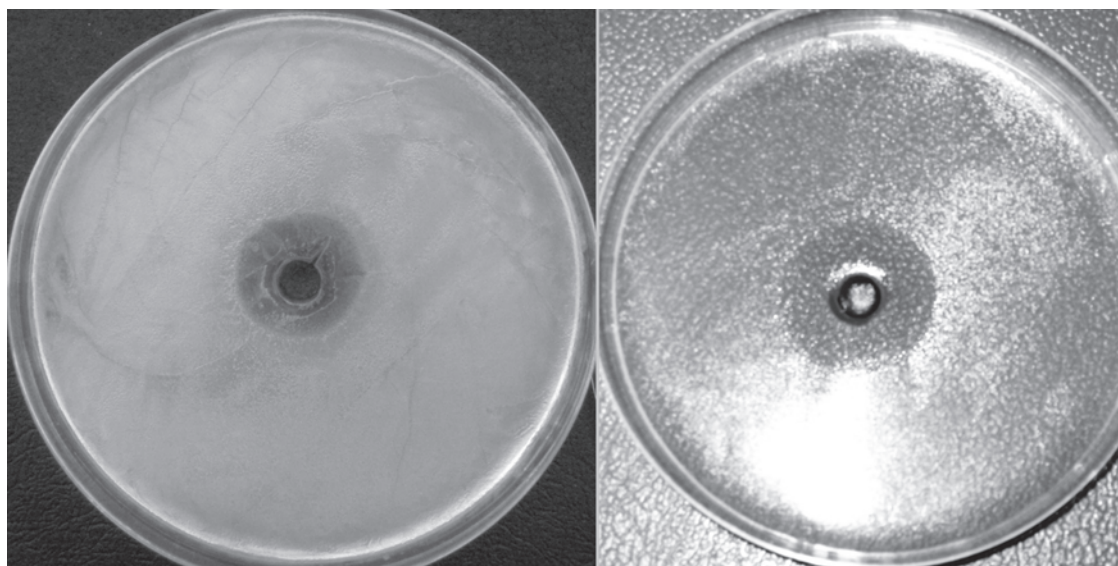


Figure 1. Inhibition zones of *Pseudomonas* sp. (left) and *Athrospira* sp. (right) with extract from Himalayan Balsam leaves placed in the centre hole.

two are well known European invaders, successful in their spreading and harmful to local ecosystems because of their strong allelopathic properties (Gerber *et al.*, 2008; Hulme and Bremner, 2006). Woad is a plant cultivated in Europe since the 9th century and widely known as colour and medical plant with bactericidal and fungicidal properties (Hamburger, 2006; Hancock, 1997; Oberthür, 1975; Vetter and Foltys de Garcia, 2003).

Knotweed leaves and the parts of Balsam plants *growing above ground* were collected at the Danube floodplain near Tulln, over two consecutive years.

Four-month-old (first growing stage) and two-year-old (second growing stage) plants of Woad were used in separate experiments. The leaves of the young Woad plants, cultivated at the Institute's own field in Tulln, Austria. The two-year-old plants were harvested at the Institute 'Arche Noah' in Schiltern, Austria, and separated into the above and below ground parts.

Conservation of fresh plant material

All freshly harvested plant materials were rinsed with tap water, and any foreign plant parts were disposed of. After separation into major plant compartments, such as roots, stems, leaves and blossoms, each was cut into pieces of roughly 2-3 cm size, filled separately into plastic bags and was frozen as fast as possible and stored at -20°C. The whole procedure, from harvesting to freezing, was done within a few hours to keep possible microbial metabolic activity as low as possible.

The frozen plant parts were freeze-dried at 0.94 mbar vacuum during 48-56 hours, at a plate temperature of +2°C. The dried tissue was ground to less than 0.5 mm particle size (Retsch ZM1000) and stored in airtight bottles at +4°C in the dark.

The woad plants were conserved by freezing, but some of them were dried at 45°C for 24 hours. This was done to verify the possible increase of bioactive compounds after a heat shock, as reported by Oberthür (1975) and Oberthür *et al.* (1974).

Dry matter (DM) was determined gravimetrically from all fresh and all freeze-dried plant material after heating to 105°C over night.

Extraction of plant materials

All aqueous extracts were produced by a DM to water ratio of 1:10, independent of which plant material (dried, frozen or fresh) was used. Freeze-dried powder was weighed into a glass bottle, water was added and the mixture was shaken overhead for 2 hours. For analytical purposes freeze-dried material was extracted in a ratio of 1:10 with methanol, ethanol or acetone, the mixture shaken overhead for 2 hours, centrifuged and paper-filtered; the solvent was evaporated to dryness in vacuum, and the residue was re-solved in the original volume of water. The extraction experiments started using ethanol; later methanol and acetone were used covering a wider range of solvent polarities. Solvent extraction was chosen in order to avoid disturbances caused by biological activities during the extraction time. Fresh and heat-dried plant material was filled into a commercial kitchen blender (Kenwood, no type print-

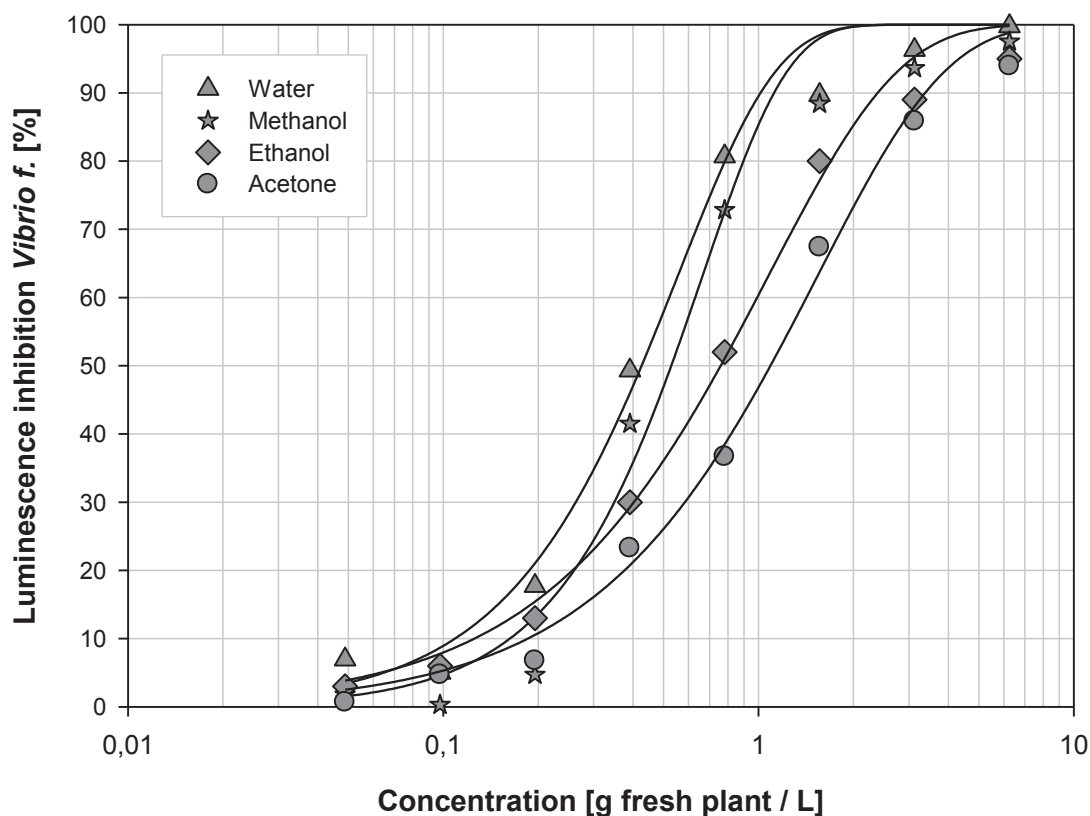


Figure 2. Dose-response-relations of *Impatiens glandulifera* leaf extracts made with different solvents for luminescent bacteria. Each data point in the graph represents the mean of double determinations, the lines are the curve fitted functions, according to Weibull.

ed) together with the necessary amount of water and mixed for about 5 minutes, starting slowly with increasing speed up to the maximum (switch positions 1 to 5).

All rough extracts were centrifuged at 14,300 G for 15 minutes. The supernatant was filtered through a sieve of 125 µm pore size (Retsch) to remove floating particles. 10 g Kieselgur (Merck, 1.08119) was mixed with 300 ml extract and paper-filtered (Schleicher & Schuell, Ø110 mm) by vacuum aid. Afterwards a second filtration step was carried out in the laminar air flow cabinet using a sterile filter (PALL Life Sciences, Ø47 mm, 0.2 µm, sterile). These final, now sterile, extracts were stored at -20°C.

Commercial Woad extracts

In addition to fresh plants, some raw Woad extracts were supplied by a partner company. Very little was known about the history and about the production details. Among the few data available, we found that sample No. 01 had been made only some weeks before delivery. The extract was dark green in colour and was declared to have been produced by pressing fresh plants without adding water. The samples Nos. 03 and 16 had been stored for years,

under anaerobic conditions with uncontrolled microbial activity before delivery to our laboratory. These extracts were of intensive brown-green colour, with typical Woad smell and were free from precipitates or turbidity. The pH-values of the extracts were: 3.61 for No. 01, 7.65 for No. 03 and 9.87 for No. 16.

Soil for degradation experiments

About 20 kg soil was collected from the Institute's own field in situ. Foreign plant residues were removed together with the upper three centimetre of the soil. The soil was sieved to a particle size below 2 mm (Retsch) and stored at +10°C under aerobic conditions. DM content was determined by drying at 105°C, water holding capacity was determined by a house method (Schinner *et al.*, 1995). For additional parameters see Table S1 in the supporting material.

Bioassays and endpoints

Biotests with bacteria, algae, water flea and duckweed were conducted according to strict standards, as given in Table 1. All these measurements were done in three replicates per dilution.

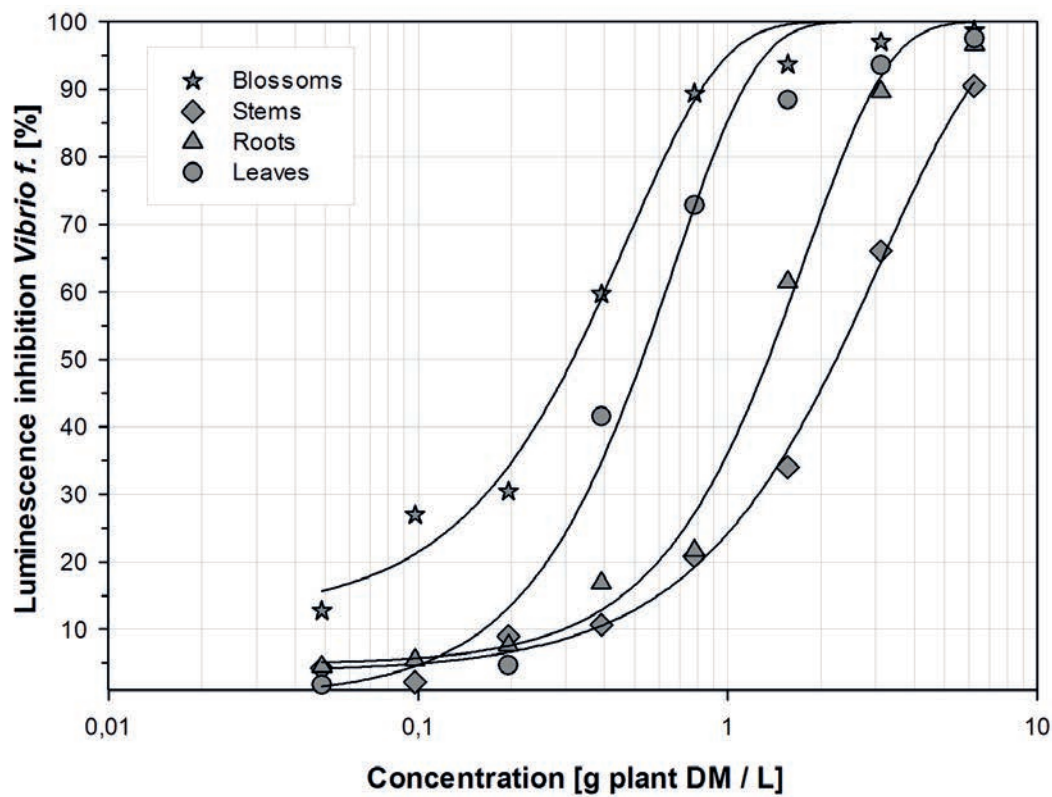


Figure 3. Dose-response-relations for methanol extracts produced from different compartments of *Impatiens glandulifera* demonstrating the effect against luminescent bacteria (*Vibrio fischeri*). Each data point in the graph represents the mean of double determinations, the lines are the curve fitted functions, according to Weibull.

The plant germination test was used as described by Fujii *et al.* (2003, 2004), modified by using homogeneous agar layers containing different plant extract concentrations. Each measurement was done in six replicates.

The agar diffusion test was developed at our Institute based on the principle of the Kirby-Bauer test, but using plant extract instead of antibiotics. *Pseudomonas sp.* was cast into plate count agar, and *Athrospira sp.* (Cyanobacterium) was cast into carbon free mineral-medium agar. 100 µl plant extract was poured into a hole of 6 mm diameter, which was punched into the centre of the agar layer. Cultivation was done at 37°C in the dark for *Pseudomonas sp.*, and in light for *Athrospira sp.* Each test was done in three replicates.

The fungi spore germination test was used as described by Lemmens (2008). For this test, 100 µl hot (55–60°C) potato dextrose agar (PDA, Merck, 1.10130) was poured into the wells of a 96 well titer plate. After cooling, 10 µl of a *Fusarium c.* spore suspension, containing 10 spores / µl, was added to each well. 2 hours later either 10 µl pure or diluted extract (sample), or 10 µl distilled water (blank), or 10 µl 2.5% solution of Akacid (POC, Austria), a broad-band fungicide as positive control, were added. Each test was done in

six replicates. If *Fusarium* germinated and grew, the colour of the PDA changed to ochre and later to red-brown. Additionally, the germination was monitored by visual inspection through a stereo microscope (80–120 fold magnification). The test was quantified for each well by noting the day on which the fungi colour was clearly seen, at least until day seven.

Inhibition values from each of these biotests were evaluated by comparing germination, growth or life activity of the organisms compared to those in the respective blanks, and were expressed as per cent (%) reduction. Inhibition values should be interpreted in the following way: the higher the value, the higher the negative effect to the exposed organism.

If the pure extract showed inhibition, dilutions were measured in the same way. Dilutions were made in geometrical distances (1:2, 1:4, 1:8 and so on) as far as necessary until no inhibition was observed. Data pairs consisting of concentration and respective inhibition value were plotted and fitted to the Weibull function (Cristensen and Nyholm, 1984, as given in Equation 1a) by non-linear regression analysis using SigmaPlot 9.0 and 12.0 (Systat software Inc.). The probability density function formulated by the Swedish Professor Waloddi Weibull (1887–1979) is a

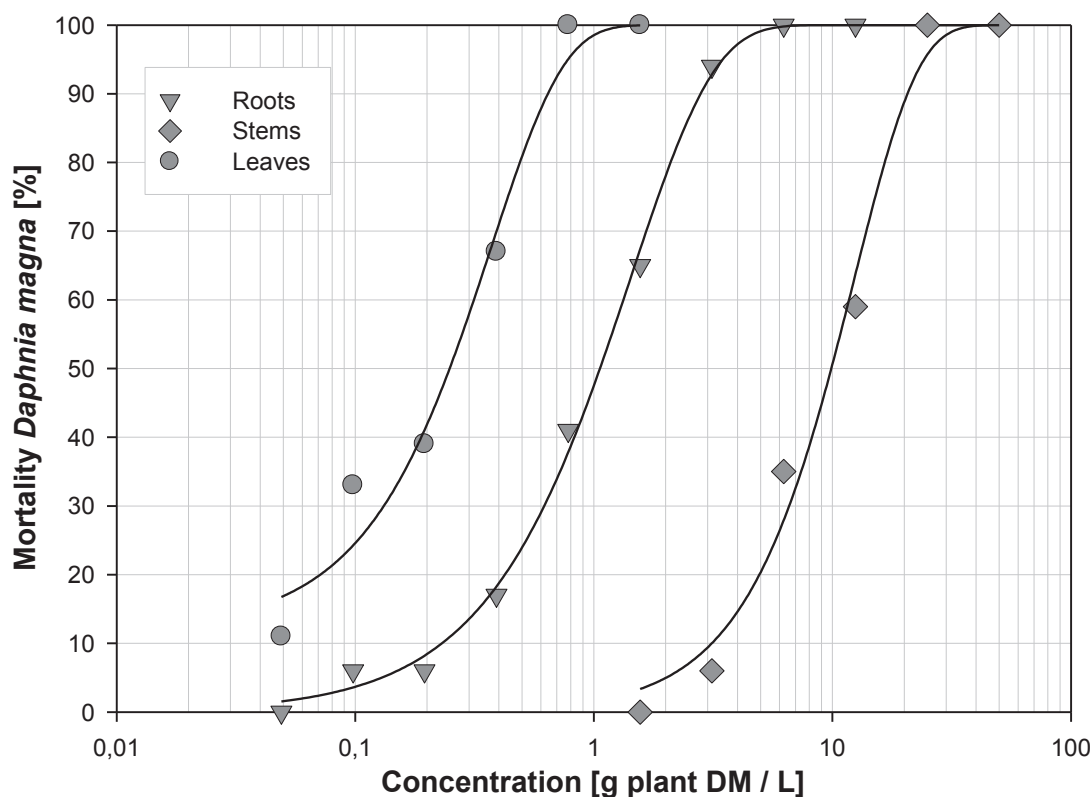


Figure 4. Dose-response-relations of extracts made from different compartments of *Fallopia japonica* to demonstrate the effect against water flea (*Daphnia magna*). Each data point in the graph represents the mortality of 18 animals, the lines are the curve fitted functions, according to Weibull.

popular and widely used mathematical function that describes the shape of distributed values, such as dose-response data. In accordance to the OECD rule (2003) curve fits were made from all available but at least from five data pairs in the range between 10 and 90% inhibition.

The function intercept at 50% inhibition (the EC_{50} -value) was calculated according to Equation 1b and was expressed as concentration of plant material per litre (compare with Figure 3). EC_{50} -data should be interpreted as: the lower the value, the less amount of substance was necessary to achieve the 50% inhibition effect, therefore the more active was the material. If the original extract showed less than 50% inhibition, no EC_{50} -value could be determined.

$$y = 100 * (a + (1-a) * (1 - \exp(-(x/b)^c))) \quad \text{Equation 1a}$$

$$EC_{50} = b * \ln 2^{1/c} \quad \text{Equation 1b}$$

legend to Equation 1:

the input coordinates are: x = concentration [mg / L];

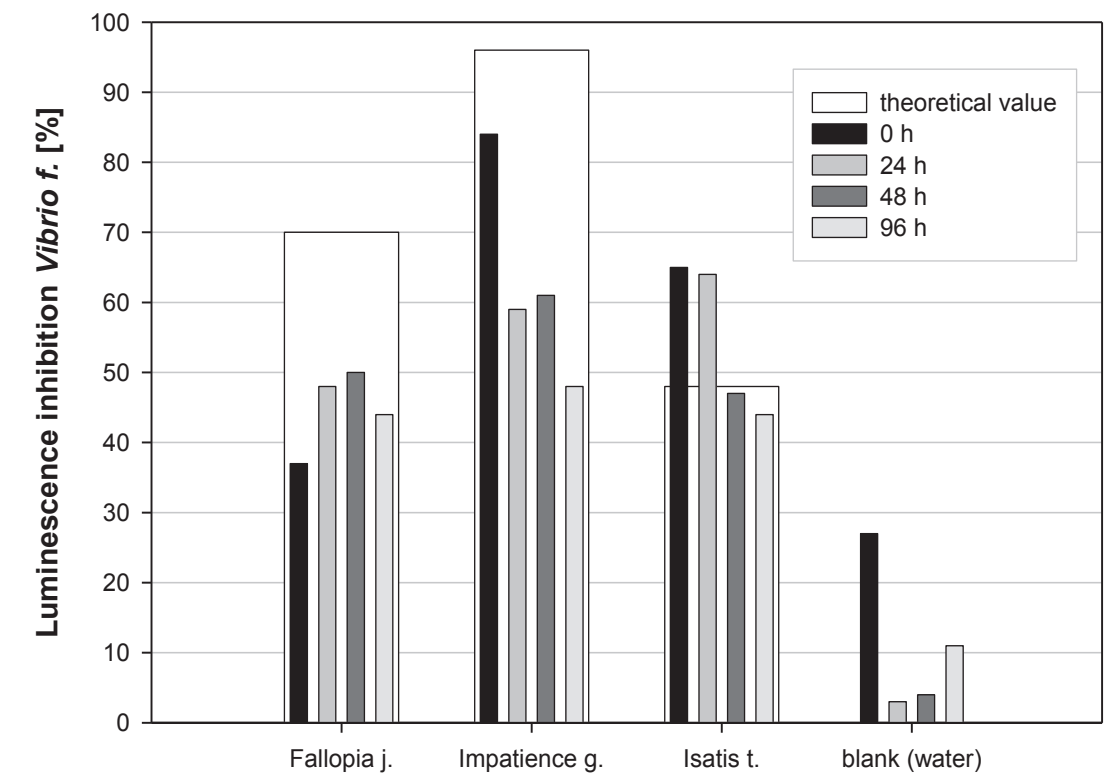
y = inhibition [%]

the function variables a , b and c are calculated by the curve fit algorithm

The Software provides standard deviations of the variables a , b and c which were used to calculate the standard deviations of the resulting EC_{50} -values by applying the principle of uncertainty propagation.

Collection of root soil

Soil from the root zone (wider distance than the rhizosphere) was collected from the places near the growing plants. The same three plant species were selected and soil was collected from two growing sites that were separated but close to each other. In each position, foreign plants, if any, that were growing within a range of less than 50 cm around the stems of the target plants were pulled out and removed. Soil adhering to their roots and an additional soil layer of at least 3-5 cm were removed carefully. The soil surface, too, was removed in small portions until the roots of the target plant became visible. Using a spade, a square-shaped soil block with the stem in the middle was cut out and transported to the laboratory. Soil adhering to and in between the target roots was collected by shaking and declared as 'influenced'. At a distance of 50 cm from the target plant, a second soil sample was taken from a similar depth, but well out-



Aqueous soil extracts at different times after plant application

Figure 5. Inhibition dynamics of plant extracts for luminescent bacteria in the third soil contact experiment, with samplings at 0, 24, 48 and 96 hours. The soil extracts were prepared using water as solvent. The broad bars in the rear are predicted (theoretical) inhibitions of the used extracts, considering a dilution of 1:4.2 according to experimental setup.

side the root zone, and declared as ‘not influenced’.

Soil contact test

60 g of soil and 18.55 g of either distilled water (blank) or aqueous extract (sample) were added to 100 ml glass beakers, resulting in a final water content equal to 90% water-holding capacity (WHC). Four beakers were filled with each sample and with blanks, which made it possible to remove one (undisturbed) baker for analytical purposes on each sampling date. All beakers were covered with aluminium foil, into which some holes were pricked to ensure aeration. The tests were incubated in the dark in an air-conditioned room (20°C, 50% relative humidity).

Three almost similar soil contact experiments were conducted in sequential order. The setups differed in the times at which samples were taken and in the number of analyses done with each. Screening experiment 1 was monitored only at the end for bioluminescence inhibition, bacteria CFU and molecular biology providing some provisional information. Experiment 2 was monitored at 0, 45 and 96 hours for bioluminescence inhibition and HPLC-MS analysis and at 0 and 96 for bacteria CFU and molecular biol-

ogy. Experiment 3 was monitored at 0, 24, 48 and 96 hours for molecular biology, and simultaneously for bioluminescence inhibition and HPLC-MS analysis from aqueous and acetone extracts.

All samplings were performed either immediately after initial mixing (after about 15 minutes, declared as 0 h) or at the given time. The soil in each beaker was carefully mixed and distributed into different tubes, in the amounts required for later analysis (12 g for CFU-counts, 32 g for duckweed bioassay, 3 g for DNA-extraction, 20 g combined for HPLC-analysis and luminescent bacteria bioassay). Analysis or further treatment was done within 30 minutes, or, if this was not possible, the respective sample tube was immediately frozen and stored at -20°C.

Aqueous and acetone extracts (for HPLC analysis and for bioassays) from soil samples were produced by mixing the soil with the same weight of solvent, shaking at room temperature for 30 minutes. Afterwards they were centrifuged at 1800 G for 20 minutes. All aqueous extracts were filtered immediately under sterile conditions (syringe filter, Gema Medical, Ø25 mm, 0.45 µm). Acetone extracts were evaporated to dryness in vacuum, re-solved in the same amount of distilled water, followed by sterile

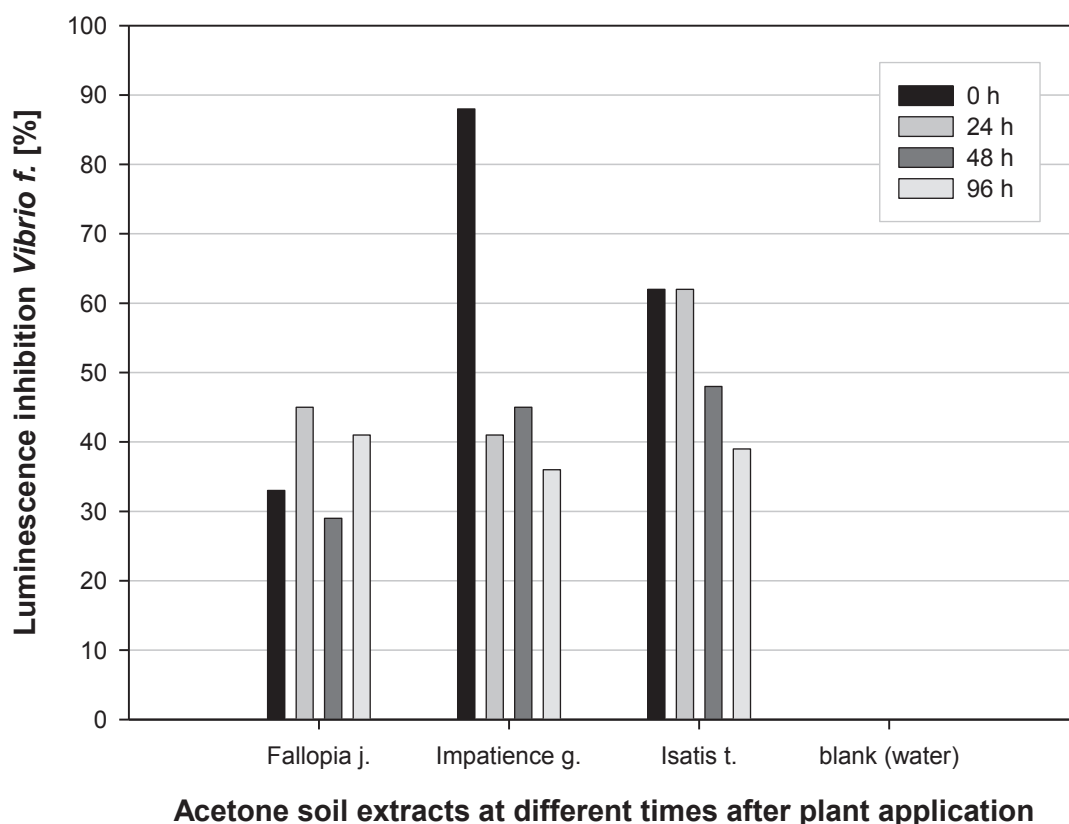


Figure 6. Inhibition dynamics of plant extracts for luminescent bacteria in the third soil contact experiment, with samplings at 0, 24, 48 and 96 hours. The soil extracts were prepared using acetone as solvent.

filtration. All those extracts were stored at -20°C until further use. The final dilution rate compared to the applied original plant extract was 1:4.2.

Determination of CFU (colony forming unit)

5 g of soil were filled into one compartment of a special filter-bag, 100 ml of physiological NaCl solution (0.9% NaCl, sterile) were added, the bag was closed and put into the 'Stomacher' extraction machine (IUL, Masticator), where the suspension was squeezed for 4 minutes (5 hits/min). The liquid sample on the bag's other compartment was either used directly or was diluted, if necessary, and put onto Petri dishes with plate count agar (OXOID, CM325, 17.5 g/L), conducted in three replicates. After incubation at 22°C for 72 h, the number of grown colonies (CFU) was counted.

DNA extraction and PCR amplification

For the isolation of genomic DNA the commercial kit 'PowerSoil™ DNA Isolation Kit' (MO BIO Laboratories Inc., USA) was used according to the instructions of the manufacturer. Root soil and degradation soil were both treated by the same procedure.

PCR amplification targeting bacterial 16S rDNA was performed by using the primer pair 341 f-GC and 907 r (Green, 2005). The forward primer had GC-rich sequences attached to the 5' end, as is usual for DGGE separation (Kirk *et al.*, 2004; Vainio and Hantula, 2000). The final volume of the PCR reaction mixture was 60 μl , consisting of 38.9 μl PCR-water, 6 μl MgCl_2 (25 mM, Fermentas Life Science), 6 μl 10x Taq buffer (Fermentas Life Science), 6 μl dNTP Mix (2 mM, Fermentas Life Science), 0.6 μl Bovine Serum Albumin (20 mg/ml, Fermentas Life Science), 0.6 μl of each primer (50 pmol/ μl , Thermo Scientific), 0.2 μl Tag DNA-polymerase (Fermentas Life Sciences), and 1 μl of template DNA. The used PCR-programme started with an initial denaturation at 95°C for 4 min, followed by 14 cycles of: 95°C for 30 sec, 58°C for 30 sec (every second cycle the annealing temperature was reduced by 0.5°C), 72°C for 45 sec; 21 cycles of: 95°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, and a final extension at 72°C for 10 min, followed by cooling at 4°C . The success of the PCR amplification was checked with a 0.8% agarose gel, ethidium bromide stained.

All extractions were done in two replicates and the two isolates were both amplified separately.

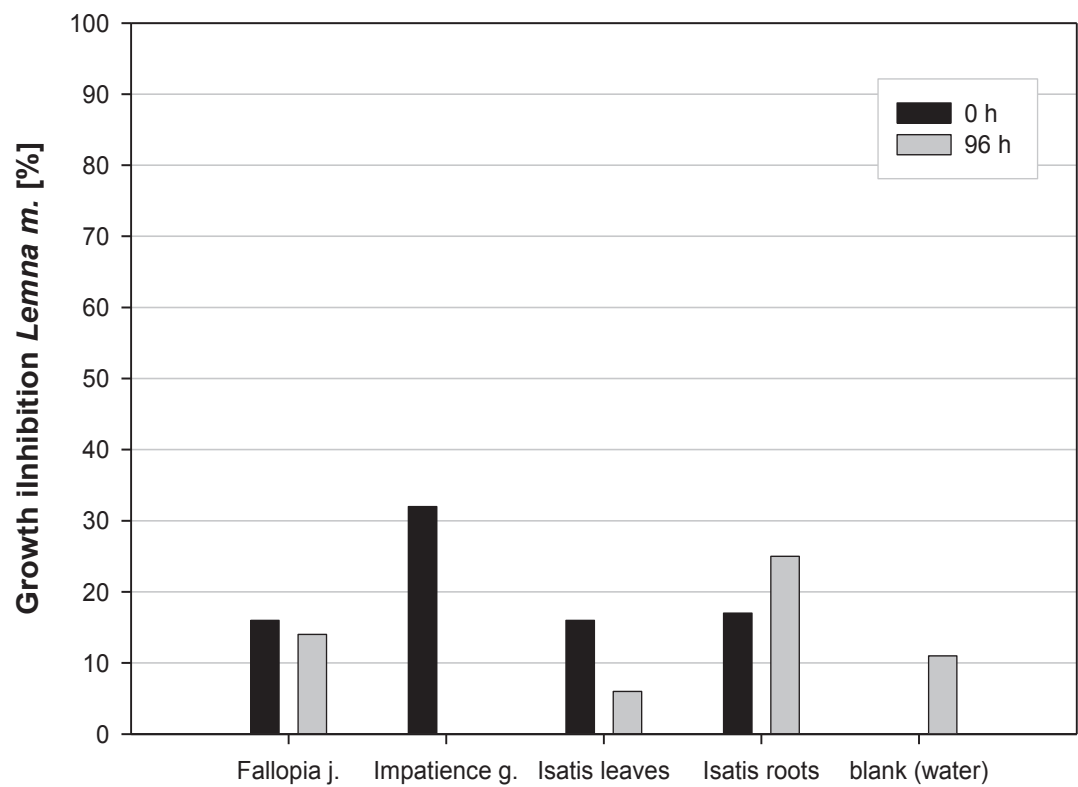


Figure 7. Growth inhibition dynamics of plant extracts for duckweed in the second soil contact experiment, with samplings at 0 and 96 hours. The soil extracts were prepared using water as solvent.

DGGE separation

DGGE analysis of the PCR products was carried out according to Muyzer *et al.* (1993, 2001) using a D-GENE™ DGGE-System (Bio-Rad Laboratories) with 6% acrylamide gel (acrylamide/bisacrylamide, 37.5:1) (7.5% for fungi). The samples containing the DNA fragments mixed with loading dye (6x, Fermentas Life Sciences) were loaded onto the gel and electrophoretically separated in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM Na₂EDTA) at 65°C and a constant voltage of 100 V for 16 hours. In order to separate PCR products of similar sizes, an initial denaturation gradient ranging from 35% to 60% was used. A 100% denaturing solution consists of 7 M Urea and 40% formamide.

DGGE bands were visualised by 1 hour staining with SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene OR) diluted 1:10,000 in 1 x TAE, destained for 20 min, and scanned with a Typhoon imager (pixel size: 50 microns, focal plane: +3 mm, acquisition mode: fluorescence, emission filter: 520 nm – BP 40). Scans were analysed using the computer programme GelCompare II v. 4.50 (Applied Maths, Belgium) to obtain band positions and band intensities. Mean values from the two replicate isolates were calculated for further comparison.

HPLC-MS analysis

Aqueous plant extracts as well as extracts from the soil contact experiments were analysed by HPLC-MS. The method as described by Eljarrat and Barceló (2000) and Eljarrat *et al.* (2004) was used, but modified to a more flat gradient for better resolution of polar components (see Table S2 in the supporting material). Chromatograms were used for qualitative fingerprint comparisons of extract compositions and degradation changes during the soil contact experiment. Because no standard substances were available, mass spectra of noticeable peaks (appearing or disappearing) could not be identified unequivocally, the identification using a gas chromatography mass library failed.

Results and Discussion

Evaluation of biotests

The algae growth inhibition test according to OECD guideline No. 201 was not suitable for routine measurement of dose-response relations of plant extracts. Because all extracts were coloured from dissolved chlorophyll, both extinction and fluorescence measurement were disturbed. Aqueous extracts from

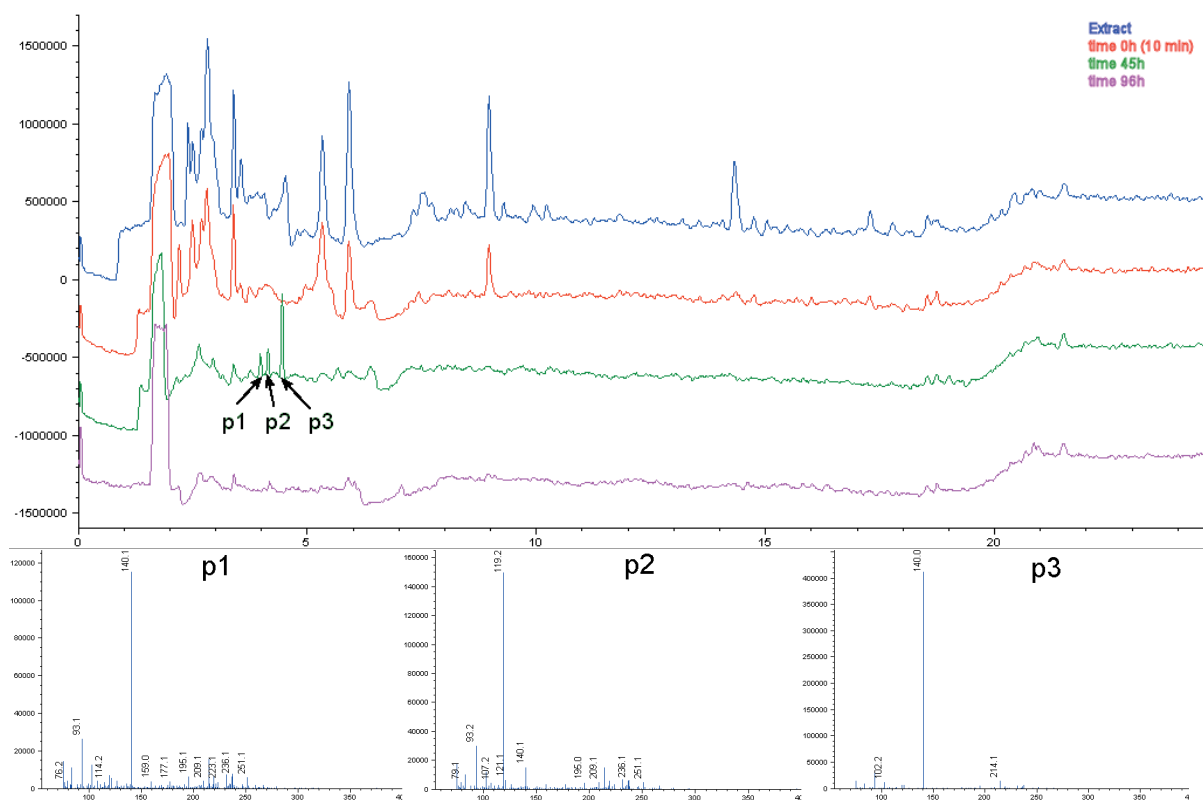


Figure 8. HPLC-MS chromatograms of aqueous extracts of the second soil contact experiment with *Isatis t.* Graph details as described in Figure Fehler: Referenz nicht gefunden. Mass spectra of peaks at retention times 3:55 (p1), 4:15 (p2) and 4:30 min (p3) in the soil extract after 45 hours.

leaves of all three plants were measured and algae cell counts were determined manually by using a Thoma chamber (results in Table 4). The manual counting was much too labour intensive to measure all necessary dilutions. Hence, no dose-response-relations and therefore no EC_{50} -values could be obtained from this test.

Some major problems were encountered applying the cress seed germination test. Even though the extracts from all plants and all their compartments were tested, not one dose-response relation could be calculated. The generation of quantitative results failed. We tried to scale the observed germination patterns by estimating the root length at days 2 and 4, and by using the value 0 for 'no germination'. The accuracy of the root length measurement was low, because different results were obtained depending on the observing person and the angle of view through the agar layer. In addition, the reproducibility of the seed germination rates, even in the blanks, was very weak. Neither a general rule nor a correlation to environmental factors was found to increase the quality of quantitative results. Success or failure often depended on random facts. However, a summary of the very few reliable data derived from two plant extracts is given in Table 2.

We tried to quantify the results of the agar diffusion tests (for both species) by measuring the diameter of the inhibition zones. This turned out to be difficult if not impossible, because the inhibition zones often were not bigger than one or two millimetres with an observed maximum of around 5 millimetres (see Figure 1); most of them appeared to have diffuse edges and were not symmetrical. After some preliminary trials it was decided to skip this test, because major optimisation work would be necessary before routine use. No quantitative data can be presented in this paper. As a side effect, it could be observed in almost all tests that the colour of the extract in the centre hole changed during the incubation time. In some cases orange or orange-brown precipitates were seen (see Figure 1, right). However, it was not possible to isolate and identify transformation products from these preliminary tests.

Biotests with luminescent bacteria (*Vibrio fischeri*), water flea (*Daphnia magna*), duckweed (*Lemna minor*) and fungi (*Fusarium culmorum*) proved to be reproducible and suitable for screening as well as for quantitative measurement of allelopathic effects from crude plant extracts. The data are presented and discussed in the following paragraphs (plant compartments and effect selectivity). However, not only a careful

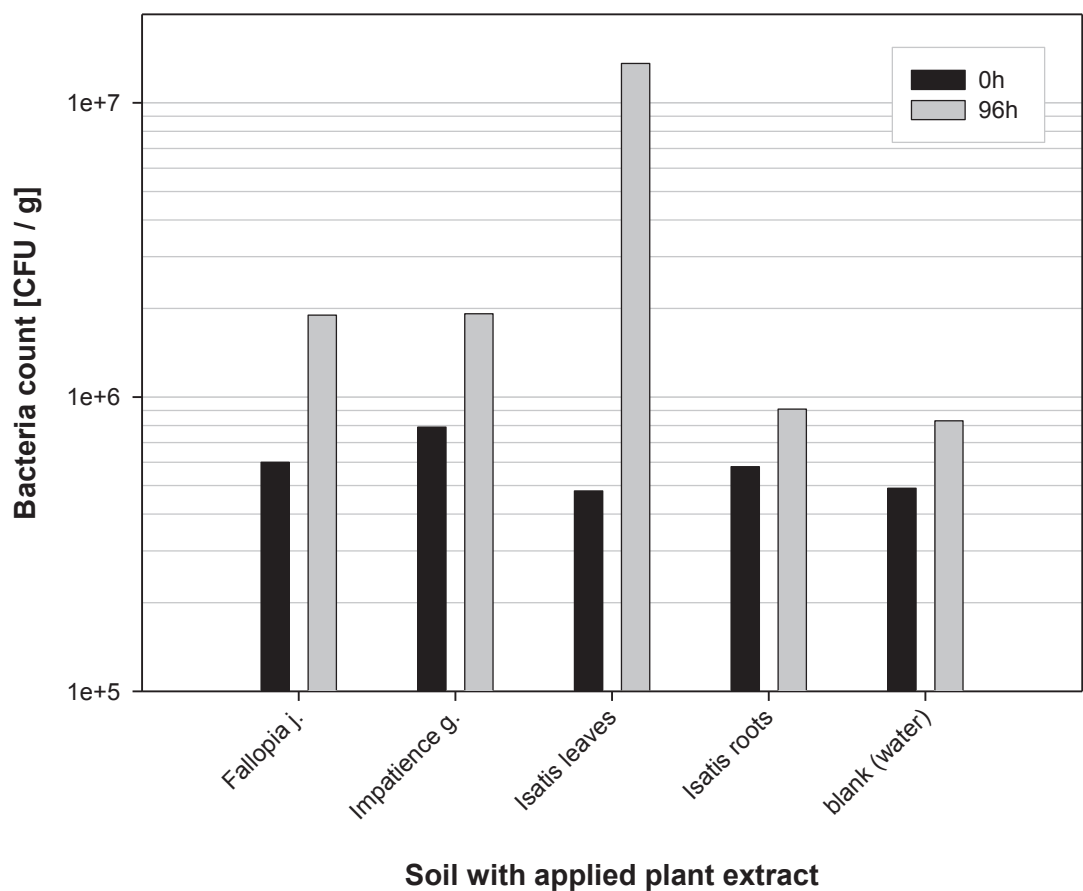


Figure 9. Colony forming units (CFU) for bacteria of the second soil contact experiment at the beginning and after 96 hours.

choice of biotests but also a critical review of biotest conditions should be done whenever quantitative allelopathic results are compared (Hartmann and Schmidt, 2008).

Sterile and non-sterile conditions

Because of the fast degradation of some allelochemicals which was observed in our laboratory as well as by many others (Fomsgaard *et al.*, 2004; Fujii and Hiradate, 2007; Macías *et al.*, 2004), it was one of the major and foremost goals to achieve best possible sterile conditions for extraction, storage and also during the biotest running time. The sterile/non-sterile test was one of the first after pre-selection of a set of biotests. The luminescent bacteria test was chosen for the comparison because it does not take longer than 30 minutes, a time too short for significant bio-degradation of most of the known allelochemicals. From a theoretical point of view, however, abiotic effects (such as adsorption or catalysis etc.) could neither be measured nor excluded.

Aqueous raw extracts from all three plants were produced under best possible sterile conditions, then divided into two portions: one was handled under the usual open laboratory conditions without any special precautions, and the other was immediately filtered and handled under sterile conditions, even during the biotest. This was compared with an ethanol extract produced at the same time, evaporated to dryness and re-solved in sterile water. All solvent extracts (ethanol, methanol, acetone) were assumed to be sterile, even during extraction time.

The results are presented in Table 3. The higher the EC₅₀-value, the more plant material was necessary to achieve 50% inhibition, and therefore the less active was the material. The activity loss due to non-sterile manipulation (preparation of dilutions, time during organisation of test preparation and test duration itself) was, in three of four cases, higher than 50% - for a test that lasted 30 minutes. It can be predicted that a higher loss would have been the result during a longer lasting biotest or during time needed for a more complicated test preparation. To sum up: under less sterile conditions activity testing must be done as

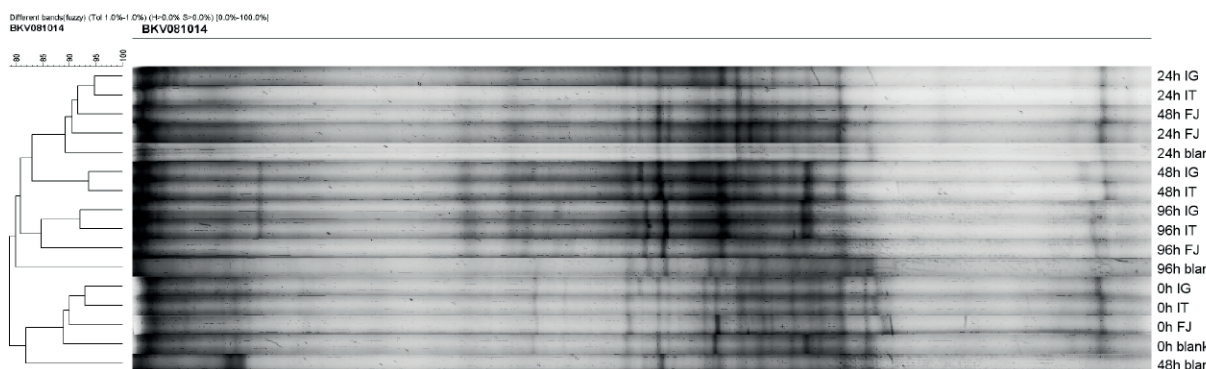


Figure 10. Dendrogram for bacteria populations in soil at different times (0, 24, 48, 96 hours) after plant extract application in experiment No. 3 (left). The band patterns were obtained by DGGE (right), the cluster analysis was based on UPGMA with band appearances and Pearson coefficient. IG = *Impatiens g.*, IT = *Isatis t.*, FJ = *Fallopia j.*

fast as possible, but it can never match the quality of results when proceeded under sterile conditions.

Solvent extraction of plants

Because allelochemicals were not identified during these experiments, important physico-chemical properties, such as polarity and solubility, were unknown. By simultaneously using water, methanol, ethanol and acetone for the extraction, we intended to learn empirically about extraction yields and stability. Freeze-dried *Impatiens* leaves were extracted using water and three solvents simultaneously, and dose-response relations were measured with luminescent bacteria. The results are shown in Figure 2. Methanol extracts showed almost the same EC_{50} -value as aqueous extracts did under sterile conditions. Ethanol and acetone extracts showed slightly lower inhibition activity.

These results can be interpreted in the following way:

- 1) Water extraction under sterile conditions did not lead to reduced allelopathy because significant biodegradation did not occur.
- 2) It was assumed that no biodegradation would happen in pure methanol or ethanol.
- 3) Methanol extraction could be used as replacement for water if sterile extraction conditions could not be achieved for some reason.
- 4) Ethanol and acetone led to reduced inhibition activity (half and one third of water extract), maybe because their polarity is lower than that of water and the bioactive components were strictly polar.
- 5) No general advice can be given on how to prepare an optimised extract. Polarity and solubility of allelochemicals cover a wide range (see Babulaa *et al.*, 2006; Berger and Schagerl, 2004; Li and Hu, 2005); therefore the choice of solvent has to be carefully considered.

Plant compartments and plant species

Freeze-dried plant compartments were extracted with methanol, transferred to aqueous solution and the activities were quantified by applying the luminescent bacteria test. Example results for *Impatiens g.* are given in Figure 3, and for *Fallopia j.*, in Figure 4. It can be seen that the highest activity was found in the blossoms, followed by the leaves, then the roots and finally the stems. The activity difference between stems and blossoms in *Impatiens* was almost tenfold, suggesting a high antibiotic activity for nectar defence and an efficient transport with low remaining concentrations in the stems. Unfortunately, no blossoms from the other two plants were available.

All EC_{50} -values of all plant parts against almost all biotest organisms are presented in Table 4. The effect selectivity differs clearly, even though no more than two biotest results were available for some plant parts.

Woad (*Isatis tinctoria*) did show significant inhibition effects against bacteria, if prepared from plants of the first growing period. Moderate effects were observed against water flea and duckweed and comparably low effects against fungi. The last observation was surprising, even in contrast to the results reported by others. Woad extracts are commonly advertised as being effective for wood protection against fungal attack (Hamburger, 2006; Hancock, 1997; Vetter and Foltys de Garcia, 2003). However, the *Fusarium sp.* used in this work was a typical plant pathogen (Friebe *et al.*, 1998) and seldom detected at or in wood. The Woad allelochemicals modes of action therefore may not be related to a common fungal organelle or to the general fungal metabolism. Maybe the activity is selective for pests that the Woad plant encounters during its growth period, which may have some random similarities to lignocellulose degrading species.



Figure 11. DGGE scan with samples from the first soil experiment, comparing bacteria population patterns in soil 96 hours after water (left three lanes) or Woad extract (right three lanes) had been added. The marked bands were cut out and were sequenced for bacteria identification.

Himalayan Balsam (*Impatiens glandulifera*) extracts had, except for the blossoms, higher activity against the micro-crustaceae *Daphnia* than against the *Vibrio* bacteria. This selectivity was up to tenfold for leaves, and about two to threefold for stems and roots. Since the accountable substances were not identified, no further interpretation is currently possible.

Japanese Knotweed (*Fallopia japonica*) extracts did show significant inhibition effects against both tested organisms but with lower intensity (lower yield) compared to the other two plants.

Soil contact experiment

The soil contact test was done three times in series, with an increasing number of analytical parameters or with increasing density of monitoring. Nevertheless, the test setup was the same in all three cases, and the effects measured in all three experiments appeared reproducible.

The different courses of bioactivity against luminescent bacteria and duckweed were followed in

aqueous extracts and, in experiment 3, also in acetone extracts. Luminescent bacteria inhibition differed in detail, but not in general, from each other (see Figures 5 and 6). Even the dynamics appeared similar. Therefore it could be assumed that nothing was lost due to microbial activity during the critical aqueous extraction procedure. The expected inhibition values concerning fresh extracts at a dilution rate of 1:4.2 (experimental setup) are shown in Figure 5. Inhibitions were measured during the whole running time of the experiment, for *Impatiens* rapidly decreasing, for *Isatis* more slowly, but for *Fallopia* increasing compared to the start (time 0 h). All of these results should be interpreted in comparison to chemical analysis by HPLC-MS (see below).

The dynamics for duckweed is shown in Figure 7 (aqueous extraction available only). It can be summarised as weak inhibition, not much different from the blank.

Inhibitions deriving from the soil matrix (in blanks) occurred after the soil was conditioned to a water content which re-activated microbial metabo-

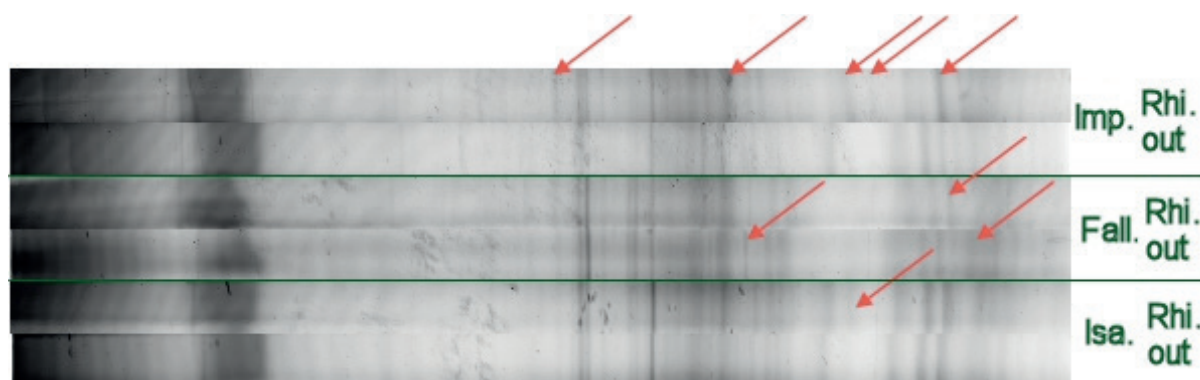


Figure 12. DGGE band patterns of bacteria populations in soil within and outside the root zone of the three selected plants: Imp. = *Impatiens g.*, Fall. = *Fallopia j.*, Isa. = *Isatis t.*, Rhi. = within root zone (rhizosphere + some adhering soil), out = outside root zone. The most obvious differences are marked by arrows.

lism. During storage of the soil at sub-optimal water content, especially bacteria survive in a resting stage. The consequences of such a re-activation were a short time inhibition of other organisms, the intensity depends on the amount of available substrate, which can be measured by sensitive biotests (Fritz, 1999).

All aqueous and all acetone extracts were analysed by HPLC-MS. Example chromatograms are given for *Isatis* in Figure 8 (aqueous), analysis results for *Fallopia*, for *Impatiens* and chromatograms from aqueous and acetone extracts are provided in the supporting material, Figures S1, S2 and S3 respectively. All acetone extracts resulted in much lower yield (lower peak heights in HPLC) compared with aqueous extraction. All soil contact tests showed a common behaviour: detectable substances disappeared rapidly. In most cases, none of the original substances was present after 48 to 96 hours. However, these results did not correlate strictly with the biotest results (see Figure 5) where inhibitions of luminescent bacteria did either slowly or not decrease. For the interpretation of this outcome, several conclusions are drawn:

- 1) Separation and detection via HPLC-MS may not have covered the allelochemicals present in plants or the metabolites present in soil extracts. As long as the accountable substances are not identified, this problem cannot be overcome. Combined semi-preparative HPLC with fraction collection and micro-scale biotests may be a solution, but was, unfortunately, not available for our experiments.
- 2) The extraction procedure may not have been suitable. Especially metabolites resulting from biodegradation may have been much less polar and therefore much less soluble in water. Following this hypothesis, we used acetone as an alternative solvent. However, metabolites may also have been adsorbed to the soil matrix and may not have been

extractable at all. A soil contact biotest (for example, similar to the *Pseudomonas* mutagenicity test) could be a possible solution. Such a biotest must be developed from scratch.

- 3) Biotests may have been disturbed by indirect effects of applying other plant components and inducing soil microbial activity (Fritz, 1999). This cannot be excluded by the test setup chosen here. However, the introduced amount of total solids was low (in the range of 1-3 g total soluble solids per kg soil), and the chosen soil had been in a condition where induction of microbiological activity was low (see glucose induced respiration in Table S1). Therefore the overall induction effect is considered to have been low and effects may have derived predominantly from secondary plant metabolites (see Cheng, 1992; Javaid *et al.*, 2006). Nevertheless, a carefully designed extract clean up should be considered to avoid the application of primary plant metabolites to a starving microbial soil community.

Bacteria cell counts were determined at the beginning and the end of the second experiment only; the results are shown in Figure 9. Within 96 hours the number of CFU was increased in all test variations, including the blank. But the highest increase, almost 10-fold, was observed for *Isatis* leaves extract. Bacterial growth could be classified as moderate for all other plant extracts and as very little when related to the unavoidable bacteria activation effect in the blank. When comparing the selective inhibition effects (see Table 5), none of the plant extracts reduced the bacteria counts, which must be interpreted either as selective inhibition or as selective growth support or as both at the same time.

The microorganism populations were analysed at every sampling time in the third experiment. DGGE band patterns were compared (UPGMA cluster analysis with Pearson correlation coefficient) and

Table 1. Overview of the applied biotests used for screening and dose-response relation measurements of plant extracts.

Test type	Organism	Response	Duration	Standard / Ref.
Luminescent bacteria	<i>Vibrio fischeri</i>	bioluminescence	30 minutes	EN ISO 11348-3
Water flea	<i>Daphnia magna</i>	acute mortality	24 hours	DIN 38412 L30
Duckweed	<i>Lemna minor</i>	growth	7 days	OECD draft 221
Algae	<i>Pseudokirchneriella subcapitata</i> , <i>Chlorella sp.</i>	growth	3 days	OECD 201
Bacteria	<i>Arthrospira maxima</i> , <i>Pseudomonas aeruginosa</i>	growth (diffusion zone)	24 hours	similar to Kirby-Bauer test
Fungi	<i>Fusarium culmorum</i>	spore germination	7 days	Lemmens, 2008
Higher plant	<i>Lepidium sativum</i>	seed germination	4 days	Fujii et al, 2003, 2004, modified

arranged in dendrogram style according to their similarities (see Figure 10). Three major clusters appeared: all samples including blank at the beginning (15 minutes after extract application), all extract samples at 24 hours, all at 96 hours contact time. The samples at 48 hours were not so clearly clustered. At the beginning the blank grouped together with the samples but it did not later in the experiment. We interpret this as a sign for influenced development of the bacteria populations due to the application of plant extracts. However, the final populations did not differ too much. Woad crude extract, product No. 16, was applied in the first soil experiment. The DGGE gel scan (Figure 11) shows a clear difference in the bacteria population compared to the blank. Some of the bacteria (marked bands cut out) could be identified by their DNA sequences when compared with entries in the NCBI-blast database (National Center for Biotechnology Information, 2009). The bacteria identified (threshold 99% similarity) were *Bacillus sp.* (*B. cereus*) from bands 1-1, 1-3, 2-1 and 3-1, *Anoxybacillus flavithermus* from bands 2-3 and 3-1, beta-Proteobacterium KB13 from bands 4-1 and 4-3, *Sideroxydans lithotrophicus* from band 6-1. Unfortunately all the other sequenced bands did not match with an entry in the database better than the threshold. Nevertheless, our results show that the application of Woad extract reduced the abundance of bacteria belonging to the family *Bacillaceae* and we identified at least one bacteria species (band 6-1) in the treated soil, which is usually not a soil bacterium.

Micro-organism populations in the root zone of growing plants

Bacteria populations from the soils inside and outside the root zone were analysed by DGGE and

band patterns were compared (see Figure 12). It must be considered that the PCR amplification was not done quantitatively, and therefore, total intensities cannot be compared. Nevertheless, intensity relations between bands of one lane may be compared with intensity relations of bands from another lane. Especially for *Impatiens g.*, and to a lesser extent, for *Fallopia j.*, differences in the form of additional and missing bands can be seen (marked by arrows in Figure 12). The samples from *Isatis t.* appeared to be almost equal. As it had not been possible to harvest rhizosphere soil only (a fraction of outside soil was unavoidable), all differences appeared weak. However, the results indicate an influence of root exudates to the bacteria populations. Both inhibition and support were observed, supporting the findings at the soil contact experiments. The data presented are first screening results. Influenced species or groups of bacteria could not be identified.

Conclusions

Conventional standardised biotests with *Vibrio fischeri*, *Daphnia magna* and *Lemna minor* are commonly known to be stable, reproducible and sensitive, and have been proved suitable for activity analysis in allelopathy.

The agar diffusion test using either Cyanobacteria or *Pseudomonas* was found to have potential for allelopathy analyses, even if major improvement work is necessary before it can be recommended for routine use. What makes the test promising is the fact that different microorganisms of choice can be used and that the procedure is simple.

The standard freshwater algae test cannot be recommended, neither for screening nor for quantitative

Table 2. Results of seed germination tests with cress. ¹⁾All figures are mean values from 18 seeds in the blank and from 9 seeds in each sample concentration.

Sample	Conc.	Root length 48 h ¹⁾	Root length 96 h ¹⁾	Inhibition 96 h
	[mg DM / L]	[mm]	[mm]	[%]
Blank	-	2.9	9.2	0
Himalayan Balsam leaves (<i>Impatiens glandulifera</i>)	16	1.0	2.7	71
	40	2.8	4.2	54
	160	0.7	6.6	29
	400	0.3	0.9	90
	1600	0.1	0.6	94
	4000	0.1	0.4	95
Japanese Knotweed leaves (<i>Fallopia japonica</i>)	16	5.1	15.9	0
	40	2.4	7.4	19
	160	1.2	10.0	0
	400	2.6	7.9	14
	1600	1.0	3.0	67
	4000	0.6	2.1	77

measurement of allelopathic effects of aqueous plant extracts. The terrestrial plant germination test using cress, however, was found to be a suitable test for screening purposes, because inhibition effects deriving from plant extracts could be detected.

It could be clearly demonstrated that sterile conditions are necessary to obtain reliable results from biotests characterising allelochemicals. The biotest organisms themselves, especially microorganisms may degrade or modify the allelochemicals during the biotest runtime. Any additional contamination in the sample or in the test set-up can cause unpredictable, non-reproducible results.

Extract production on a bigger scale should be done using water (as already demonstrated by Ribeiro *et al.*, 2012) or, if sterile conditions cannot be achieved, ethanol, at the risk of some activity loss. The use of methanol should be avoided for personnel health reasons.

Care should be taken when one is tempted to generalise a detected effect of a plant extract. For example, the historically documented wood protection effect of Woad does not mean that such an extract is an all-round fungicide, and observed bacteria inhibition of *Impatiens* does not necessarily exclude a growth support of some soil bacteria species.

The soil contact test confirmed the observations

made by our group in the past: Allelochemicals may be degraded or modified and may therefore either be detoxified or activated due to microorganism activity. The time needed may be very short, probably less than 24 hours. The outcome is not yet predictable from a theoretical point of view. Intensive measurements and sensitive instrumental analysis will be necessary to discover the path of released allelochemicals in soil. This goes far beyond the scope of the project presented here. However, more research should be done to identify the ecological role of allelochemicals and to describe the complex interaction between donor plants and receiving soil organisms.

The most crucial questions to be answered in future will probably be: How narrow do plants select a microorganism population in their rhizosphere beneficial for them? And what may be the benefit for those bacteria and fungi that transform, but do not fully degrade allelochemicals?

As the applied methodology revealed several minor and even some major uncertainties, upcoming research should focus on improved plant extract preparation to avoid the co-application of carbohydrates, proteins and other low molecular substances to soil. The collection of plant root exudates for activity screening and for instrumental analysis may give answers to some of the questions newly arose.

Table 3.

Biotest endpoints (EC₅₀-values) obtained with the luminescent bacteria test from freshly prepared plant extracts using water under sterile and non-sterile conditions and ethanol as solvents. Explanation in the text.

Plant	EC ₅₀ of plant extracts [g fresh plant / L] (± standard deviation; n)		
	H ₂ O non-sterile	H ₂ O sterile	EtOH
<i>Impatiens glandulifera</i> (leaves)	1.1 (±0.08; 30)	0.47 (±0.06; 30)	2.4 (±0.12; 27)
<i>Fallopia japonica</i> (leaves)	10.4 (±1.1; 30)	6.1 (±0.55; 30)	7.6 (±0.4; 30)
<i>Isatis tinctoria</i> (leaves)	15.2 (±1.4; 27)	11.7 (±1.0; 27)	11.6 (±0.9; 27)
<i>Isatis tinctoria</i> (roots)	5.0 (±0.25; 24)	2.2 (±0.13; 21)	2.6 (±0.16; 24)

Table 4. Effect selectivity of extracts made from three plants, expressed as EC50-values. 1)EC50-values for crude extracts of *Isatis t.* (obtained from the private partner) are shown as vol%; all self-made extracts are shown as g plant DM / L ± standard deviation (sd). 2)Only one concentration was measured. All correlation calculations done from 7 to 10 concentrations in triplicate each, except *Daphnia* with 18 animals per concentration. Explanation in the text.

Plant	Part / Product	EC ₅₀ -values ¹⁾ ±sd			
		<i>Vibrio</i>	<i>Daphnia</i>	<i>Lemna</i>	<i>Fusarium</i>
Woad (<i>Isatis tinctoria</i>)	Crude extr. No. 01	0.09 (±0.002)	1.4 (±0.26)	-	-
	Crude extr. No. 03	0.31 (±0.01)	1.5 (±0.3)	2.6 (±0.07)	25 (± 3.2)
	Crude extr. No. 16	0.69 (±0.05)	1.2 (±0.3)	5.4 (±0.11)	38 (±3.9)
	Leaves, 2 year plants	11.7 (±0.22)	2.5 (±0.46)	-	-
	Roots, 2 year plants	2.2 (±0.07)	0.4 (±0.13)	-	-
	Leaves, 1 year plants	1.8 (±0.04)	20 (±4.5)	-	-
Algae [% Inh.] ²⁾					
Himalayan Balsam (<i>Impatiens glandulifera</i>)	Blossoms	0.37 (±0.006)	0.53 (±0.09)		78
	Leaves	0.53 (±0.014)	0.04 (±0.01)		100
	Stems	2.3 (±0.05)	1.1 (±0.4)		-
	Roots	1.4 (±0.05)	0.53 (±0.08)		100
Japanese Knotweed (<i>Fallopia japonica</i>)	Leaves	1.7 (±0.025)	0.29 (±0.07)		76
	Stems	8.4 (±0.4)	9.9 (±1.1)		81
	Roots	2.0 (±0.07)	1.1 (±0.3)		100

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