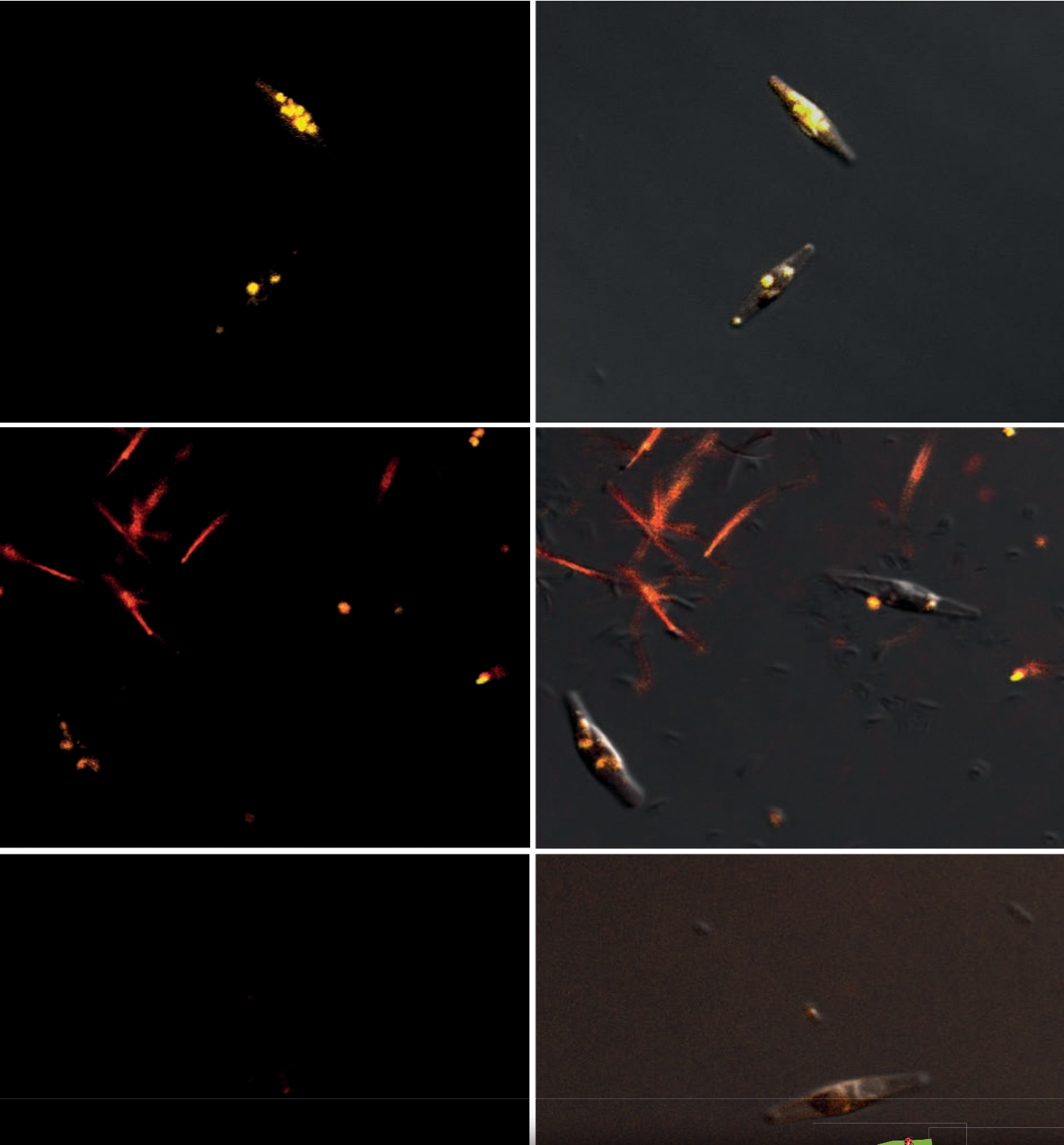


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Confocal microscopy images of Nile Red (NR) stained *Phaeodactylum tricornutum* after 6 days of growth in the presence of (A) 0 μ M, (B) 18 μ M and (C) 75 μ M rosmarinic acid. Images on the left are overlay images of red and yellow channels while images on the right are brightfield images. Yellow drops indicate neutral lipids while red color corresponds to polar lipids.

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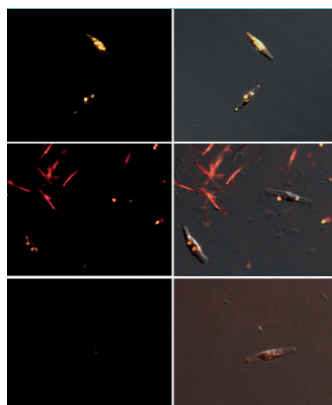
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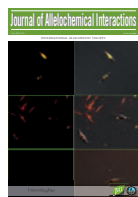
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INDEX

- 9-14 Alberto Oliveros-Bastidas, Pedro L.C.A. Alves, Nuria Chinchilla, José M.G. Molinillo and Francisco A. Macías**
Soil Microbial Respiration According to the Wheat Culture History.
- 15-27 Leslie A. Weston, Dominik Skoneczny, Paul A. Weston and Jeffrey D. Weidenhamer**
Metabolic Profiling: An Overview – New Approaches for the Detection and Functional Analysis of Biologically Active Secondary Plant Products.
- 29-38 Ana Paula Ferro, Rogério Marchiosi, Rita de Cássia Siqueira-Soares, Edicléia Aparecida Bonini, Maria de Lourdes Lucio Ferrarese and Osvaldo Ferrarese-Filho**
Effects of cinnamic and ferulic acids on growth and lignification of maize roots.
- 39-56 J.I. Fritz and D. Schneider**
Transformation and Activity Change of Selected Allelochemicals by Microbial Metabolisation.
- 57-70 J.I. Fritz and D. Schneider**
Transformation and Activity Change of Selected Allelochemicals by Microbial Metabolisation. Supplementary data.





Soil Microbial Respiration According to the Wheat Culture History

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ABSTRACT

Soil micrganisms represent one of the largest reservoirs of biodiversity in the world. Soil microorganisms are very important as almost every chemical transformation taking place in soil involves their active contributions. The study of soil dynamics in presence of phytotoxic agents is of great importance because of the role that microorganisms have in modifying the phytotoxic compounds and consequently affecting their biological properties. Possible biotransformation developed by soil microorganisms could yield compounds with modified biological properties. The aim of the present work is to study microbial respiration in soil cultivated with different varieties of *Triticum aestivum* L. by measuring the emission of carbon dioxide (CO₂), and by correlating the results to the ability of the soil to degrade allelochemicals and to release CO₂.

Keywords: Benzoxazinones, degradation, CO₂ emission, microflora, allelopathy.

Introduction

Benzoxazinoids have been described as important allelochemicals from Gramineae, Acanthaceae, Rannunculaceae and Scrophulariaceae plants (Niemeyer, 1988; Pratt *et al.*, 1995), with a biological activity including fungistatic (Bravo and Lazo, 1996; Friebe *et al.*, 1998), antifeedant (Klun *et al.*, 1967; Argandoña *et al.*, 1983) and phytotoxic (Barnes and Putnam, 1987; Perez and Ormeno-Nunez, 1991) activities. As part of the ongoing studies into allelochemicals as natural herbicide models, the description of soil dynamics in presence of phytotoxic agents is of great importance because of soil microorganisms have the ability to biotransform them in the compounds with modified biological properties, affecting their allelopathic capability.

Microorganisms in soil are one of the largest reservoirs of biodiversity in the world, where the number of species can reach 10³ and 10⁴ (Foster,

1988). One gram of soil typically contains about 10⁹ to 10¹⁰ bacteria and several hundred meters of fungal hyphae (Clegg and Murray, 2002). Given this high biological activity, it is not surprising that these microorganisms are considered to be a crucial variable in the functioning of an ecosystem and the phenomena that occur within it. As a result, in the study of allelopathy, processes that occur are continually being revised in the search for a better understanding of this phenomenon.

The root system of a plant has a high capacity to exude compounds with a wide variety of physical and chemical properties into the rhizosphere. This exudation is a response to stress or abiotic and biotic factors from the area in which the plant is located. The rhizosphere is defined as the volume of soil that is affected by the presence of the roots of growing plants (Nicholas, 2007). It has been found that the soil zone in which the most marked effects are caused by compounds released by the roots is from the root

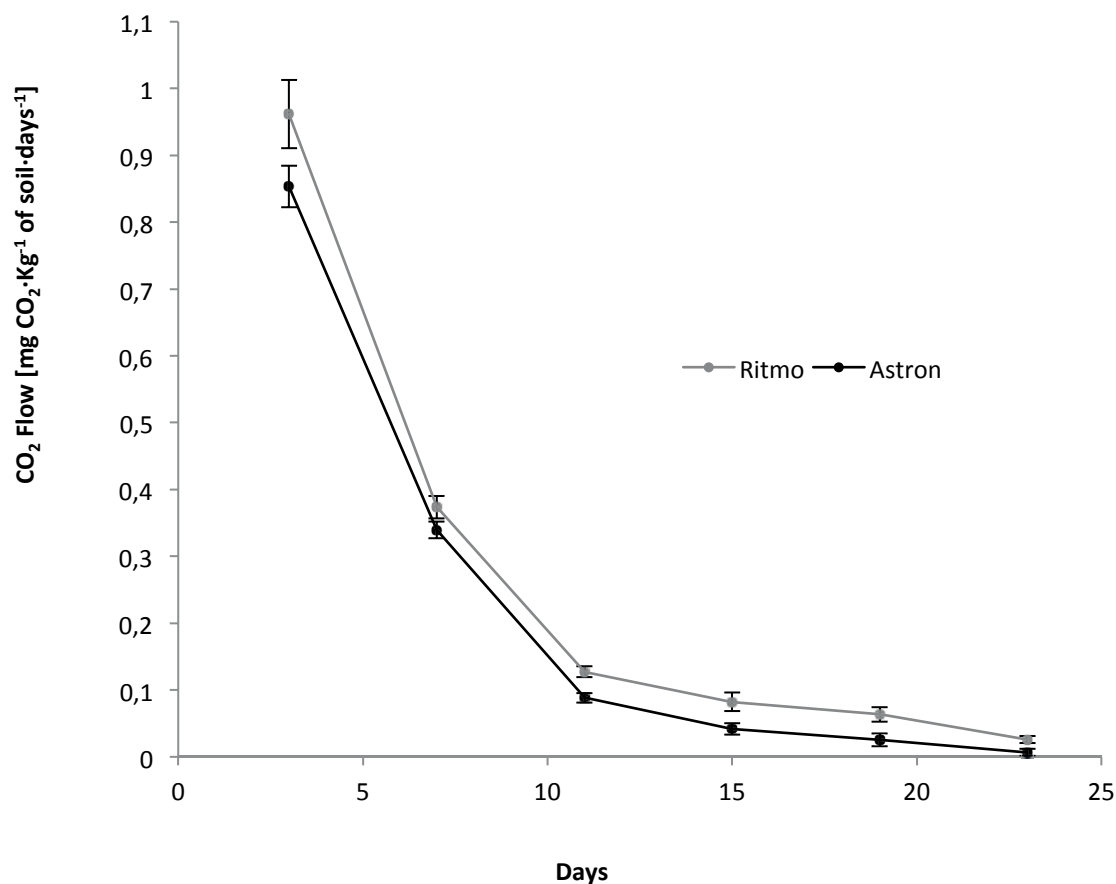


Figure 1. CO₂ flow measured for the soil extracts of two wheat varieties (Ritmo and Astron) during 23 days of incubation. Each point corresponds to the amount of CO₂ emitted by independent soil samples. Error bars represent standard deviation of the mean for n = 3.

surface to a distance of 2 mm. This distance is relative and depends on the soil properties (particle size, water content, pH buffer capacity, etc.) (Nye, 1984; Albrecht, 2002). Thus, most phytochemicals released from plants are founding the rhizosphere and this is also where one would expect the maximum activity to be shown by the compounds or reactions to occur with them, as in the case of biodegradation.

The most widely used methods are measurement of enzymatic activity (Lechevalier, 1977; Bååth *et al.*, 1998) and the emission of carbon dioxide (CO₂). The latter process is the result of the decomposition of organic substrates to obtain energy for growth and microbial functions (Wang *et al.*, 2003). In the work described here we measured the amounts of CO₂ released in order to estimate microbial activity in the soil under investigation.

It is of interest to characterize the biotic characteristics of each soil and to correlate these results with the ability of the soil to degrade allelochemicals and to release CO₂. However, the microbial characterization of soil samples (including

bacteria, fungi, actinomycetes and invertebrates) is very complex, mainly due to its heterogeneity (Macfadyen, 1970; Swift *et al.*, 1979; Lee, 1985; Binet *et al.*, 1998; Williamson *et al.*, 2005).

These work reported here concerned a study of the microbial respiration in different cultivated varieties of *Triticum aestivum* L. soils. In selecting the soils it was important to consider the microbial community in the rhizosphere, which depends on species, variety and stage of development of the growing plant. Therefore, several criteria were applied: (i) the phytotoxic activity of the seedling leachates, (ii) content of hydroxamic acids and (iii) their backgrounding the biotransformation of allelochemicals.

Materials and methods

Selection of Wheat Varieties and Soil Study of the Allelochemical Release

The soils selected were those in which plants that release the greatest quantities of compounds

into the rizosphere were grown, both on the surface (epidermis) and by root exudation. According to this hypothesis, soil microbial communities from different cultures (potentially allelopathic) can be differentiated as a result of their ability to release compounds to the environment. The contents of DIBOA and DIMBOA were determined in six wheat varieties (*Triticum aestivum* cv. Hill, Portal, Ritmo, Astron, Stakado, and Solist). Also, the wheat coleoptile bioassay was used as a protocol for the general evaluation of the bioactivity of wheat extracts (Hancock *et al.*, 1964). Under these two criteria, soils in which Astron and Ritmo varieties had been grown were selected for this study (Macias *et al.*, 2005).

Soil collection

The selected parcels for the cultivation had similar physicochemical characteristics during their cultivation (Table 1) and they did not contain any other plant species in growth. After harvest, samples of soil from the vicinities of the plants were taken to get the maximum density of the microbial associated with the plant underground parts. The samples were taken at a depth of 15–20 cm in a radius of 3 cm around the plant lines. One hundred random soil samples were collected for each crop (0.014 m³). The soil was sieved to a size of 2 mm and the samples were placed in plastic bags and stored at –20 °C prior to study. Before the degradation studies, dry plant material and calcareous stones were removed from the samples using a sieve that gave a distribution of soil particles of less than 1 mm.

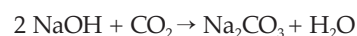
Analysis of hydroxamic acids in soil

All samples were analyzed on a Merck Hitachi HPLC system equipped with a LACHROM L-7100 Quaternary gradient pump, an L-7455 LACHROM Diode Array Detector and an L-7200 LACHROM autoinjector. Data were collected and processed using an HPLC data system (Merck Hitachi D7000). Instrumental conditions for the analysis of hydroxamic acid derivatives were Lichrospher 100 RP-18 (250×4.0 mm, 5 µm) reversed-phase column at 25 °C. Mobile phases were water:1% AcOH (a) and methanol:1% AcOH (b) at a flow rate of 1 mL min⁻¹. The injection volume was 50 µL. The following gradient was used for separation: at 0 min, 30% b; 2 min, 30% b; 19 min, 60% b; 21 min, 100% b. Under these conditions, the following retention times were obtained for each compound: DIBOA 9.01 min; DIMBOA 10.69 min; BOA 12.56 min; MBOA, 16.56 min; AMPO, 19.74 min and APO 24.03 min. The detection was carried out at the following wavelengths: 254, 253, 263, 271,

and 280 nm for DIBOA, DIMBOA, BOA, MBOA, AMPO and APO. For quantitative analysis, stock solutions (1 mg/mL) of each individual standard were prepared by dissolving accurate amounts of pure standard in acidified MeOH (1% AcOH). Working standard solutions were obtained by further dilution of stock solutions with MeOH:acidified H₂O (1% AcOH) (70:30). These solutions were used to generate the external standard response calibration curves for subsequent measurement of quality parameters and concentrations of hydroxamic acid derivatives in soils at different times.

Measurement of soil respiration

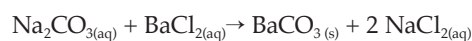
Measurements of microbial biomass in soil were performed according to the method of Jenkinson and Powlson, wherein the microbial populations is determined by recording the amount of CO₂ emitted (Jenkinson and Powlson, 1976). This method is based on the measurement of CO₂ released during the microbial activity in the soil and trapping it in a solution of NaOH, in which the following reaction takes place:



The determination of the initial and final amounts of NaOH enables the amount of NaOH that has reacted with carbon dioxide to be calculated (2 mol NaOH:1 mol CO₂). In this method, the determination of these amounts is carried out by titration with hydrochloric acid, using phenolphthalein to indicate the end point of the titration.

For these measurements, 24 g of each dry and moist soil was used with 7.16 mL of distilled water, representing 55% of imbibitions (100% point imbibition = 0.5428 mL.g⁻¹ soil). The moistened soil was placed in a 2.5 liter bottle and two 25 mL beakers, one containing 20 mL of distilled water and the other 20 mL of 0.5N NaOH, were prepared. The system was closed and the samples were incubated at 25 °C for different times.

At the end of the incubation time a 30% (w/v) solution of BaCl₂ (2.0mL) was added to each of the two beakers to precipitate the CaCO₃ formed by the reaction of NaOH with CO₂. This step is necessary because CaCO₃ may interfere in the titration of free NaOH with HCl. The reaction is:



Once the CaCl₂ had been added, the two samples were titrated with 0.5 N HCl in the presence of phenolphthalein to determine the end point. The

Table 1. Physicochemical Analysis of Wheat Crop Soil.

Exchange capacity (mequiv/100g)	33.91
Exchangeable cations (mequiv/100g)	
Ca ²⁺	28.96
Mg ²⁺	3.08
Na ⁺	0.29
K ⁺	1.58
Carbonates (%w/w)	18.25
Active limestone (%w/w)	8.81
Asimilable phosphorus (Olsen, ppm)	8.7
Total organic content (ppm)	1.35
Organic nitrogen (%w/w)	0.08
pH (H ₂ O:1/2.5)	8.33
pH (KCl)	7.31
Asimilable potassium (ppm)	635
Clay (%w/w)	52.6
Sand (%w/w)	15.4
Slime (%w/w)	32.0
Textural classification	clay

determination of free NaOH (which had not reacted with CO₂) allowed the reacted amount to be calculated (corrected to the amount given by the control, which is the amount of CO₂ in the atmosphere). With this value, the amount of CO₂ trapped was determined (along with that generated by the ground) by titration with NaOH.

Statistic

Assays for the measurement of the release of CO₂ were carried out in triplicate and calculated the corresponding standard deviations. To compare the difference between the two data series we used the Tukey’s test. These calculations were performed using the Statistica 10.0 software (Statsoft, 2012).

Results and discussion

The study of microorganisms by conventional methods includes culture processes, isolation and *in vitro* classification techniques. These techniques can be very laborious and, in some cases, the use of culture media can only promote the growth of certain groups of microorganisms (Bakken, 1985). Moreover, the separation of microorganisms from the soil particles is unreliable (Schallenberg *et al.*, 1989).

Thus, indirect measures of microbial activity are used to estimate the microbial community in soils.

The results for the two types of soil are shown in Figure 1, in which the flows of CO₂ for different incubation times for the two soil types are indicated. The behavior is similar to that usually described for these measurements, i.e., a high initial CO₂ evolution that gradually decreases to almost zero after 23 days of incubation.

As the nature of the microorganisms in the soil is unknown, CO₂ measurements were recorded for different incubation times. This ensures that there is sufficient time for the growth of the various colonies of microorganisms that may exist in the soil samples.

As can be observed in the figure, on the third day of incubation there is a high release of CO₂ from both soils, with values of 2.885 mg CO₂.kg⁻¹ for the Ritmo variety soil and 2.560 mg CO₂.kg⁻¹ for the Astron variety soil. A marked decrease then occurred to levels of 1.398 and 0.973 mg CO₂.kg⁻¹ for the respective soils on the eleventh day of incubation. Thereafter, the CO₂ emission continued to decrease smoothly to values of 0.595 and 0.151 mg CO₂.kg⁻¹, respectively.

For all of the times studied, the soil associated with the growth of *T. aestivum* cv. Ritmo showed significant differences from the soil planted with the variety Astron (Tukey’s test, α=0.01). The first

Table 1. DIBOA and DIMBOA production in root exudates of wheat varieties Astron and Ritmo. Conversion of BOA and MBOA in their associated soils and CO₂ levels generated by the soil.

Soil*	Root exudates** (mmol kg ⁻¹ Fresh Weight)		Respiration [‡] [mg CO ₂ ·kg ⁻¹ of soil·days ⁻¹]	% Conversion [§]	
	DIMBOA	DIBOA	CO ₂	BOA	MBOA
Astron	58.89 ± 1.06	18.21 ± 0.41	0.853 ± 0.031	84 ± 3	34 ± 2
Ritmo	51.33 ± 2.31	13.90 ± 0.87	0.962 ± 0.051	99 ± 2	39 ± 1

* Soil according to variety of cultivated wheat. ** Root exudate values measured in a hydroponic medium for 50 seedlings and six days of growth after germination. [‡]CO₂flow [mg CO₂·kg⁻¹ of soil·days⁻¹] during two days. [§]Maximum values for the conversion in soil after 10 days of incubation. Dose: BOA: 2 mg·g⁻¹ of soil. MBOA: 0.25 mg·g⁻¹ of soil.

soil showed higher emission of CO₂, which indicates larger populations of microorganisms in this soil. This finding can be correlated with the ability of these microorganisms to convert the allelochemicals into BOA and MBOA. Previously, it has been reported that DIBOA and DIMBOA degrades to BOA and MBOA, followed by conversion of BOA to APO, and MBOA to AMPO (Macias *et al.*, 2004;2005). BOA and MBOA degraded rapidly in both soils, with a loss of 70-99% after 10 days. However, the soil cultivated with wheat variety Ritmo had higher activity at some initial doses of BOA and MBOA. Thus, the Ritmo variety soil showed a greater degradation capacity (Table 2). In other words, the higher populations levels of microorganisms, the greater the amount of chemicals degraded.

The soil that contains greater quantities of allelochemicals has lower microbial populations. Astron soil, which has the highest amounts of DIMBOA and DIBOA in root exudates, had lower microbial populations in the soil. This situation reflects the possible antibiotic effect of these compounds in the environment close to the plant, which causes a decrease in the biotransformation processes of the allelochemicals released.

These results show that the exudation of antibiotic compounds could be crucial in the allelopathic activity. It is important that allelochemicals and their degradation products have two properties: phytotoxic activity and toxicity to microorganisms.

The results described above are consistent with those published previously, where a plant influences the populations type of the microorganisms present in soil and, consequently, its ability to transform allelochemicals (Hashimoto and Shudo, 1996; Grayston *et al.*, 1998; Glenn *et al.*, 2003). Thus, the microflora associated with the root system of plants depends on the variety of wheat and the compounds released into

the soil. The populations can be increased for those microorganisms that are able to metabolize the toxins released by the plant or use them as a carbon source (Voloshchuk *et al.*, 2007).

Conclusions

The rhizosphere of the Astron wheat variety showed inhibitory effects and reduced the emission of CO₂ may be due to the higher amounts of DIMBOA and DIBOA in the root exudates.

Acknowledgments

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Metabolic Profiling: An Overview – New Approaches for the Detection and Functional Analysis of Biologically Active Secondary Plant Products

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ABSTRACT

The study of the diverse roles of plant metabolites or secondary plant products involved in plant defence has made rapid advancements in recent years, partly due to the use of metabolomics or targeted metabolic profiling of plant extracts. Metabolomics refers to the use of various analytical techniques to quantitate the suite of primary and secondary metabolites in complex sample matrices collected from organisms; metabolic profiling refers to metabolomic studies that focus on quantifying a subset of the metabolomic components. The metabolites present in plant extracts reflect the end products of gene expression at a particular point in time and often include both plant and microbial metabolites, corresponding to complex regulatory systems in the genome of a living plant. Metabolic profiling is a critical tool in understanding how a living system responds to environmental conditions to which it was subjected, including biotic stress. It also facilitates the use of functional gene annotation through the use of data mining tools for the comprehensive characterization of a plant genotype. In addition, metabolic profiling can also be useful for determination of complex pathways of secondary product biosynthesis in higher plants and a broader understanding of biological activity and function associated with the presence or absence of key secondary products in the metabolome. Metabolic profiling of organisms involved in trophic interactions can provide valuable information on joint metabolic networks and assist in broader understanding of a system. Here, we explore examples of applications of metabolic profiling used to better understand the complex role of secondary products in plant growth, development, defence, and the chemistry of food crops.

Keywords: metabolomics, chromatography, QToF, mass spectrometry, secondary plant products, allelochemicals.

Introduction

Metabolomics is defined as the systematic study of unique chemical fingerprints associated with cellular processes in living organisms (Breitling *et al.*, 2013; Rochfort, 2005). It frequently includes the separation, detection and quantification of all metabolites in a sample by analytical techniques involving gas and/or liquid chromatography (GC or LC) coupled with mass spectrometry (MS) (Roessner and Bacic, 2009), but may be accomplished using nuclear magnetic resonance (NMR) spectroscopy techniques applied to complex mixtures (Kim *et al.*, 2010). Metabolic profiling refers to the focused or targeted study of metabolites in biological systems in response to a particular treatment or state. In most instances, metabolic profiling focuses on small molecules or metabolites present in an extract that reflect the metabolome at a particular point in time

(Kim and Verpoorte, 2010). The metabolome itself consists of the larger collection of all metabolites in a biological system including both primary and secondary metabolites; metabolomics can therefore be performed on a cell, a tissue, an organ or an organism, as well as on matrices such as blood, urine, soil or culture media. Metabolomics also has the potential to aid in pharmaceuticals, natural product drug discovery and functional food analysis (Rochfort, 2005); understanding mechanisms of toxicity (Clarke and Haselden, 2008) as well as in plant phenotyping and quantitative trait analysis (Kiambi *et al.*, 2008; Roessner and Bacic, 2009).

Metabolic profiling of allelochemicals in the rhizosphere could also provide strong insight into the resulting decomposition following incorporation of plant material into the soil (Krogh *et al.*, 2006) and the complex interplay between plants and their associated rhizosphere microorganisms, an area which

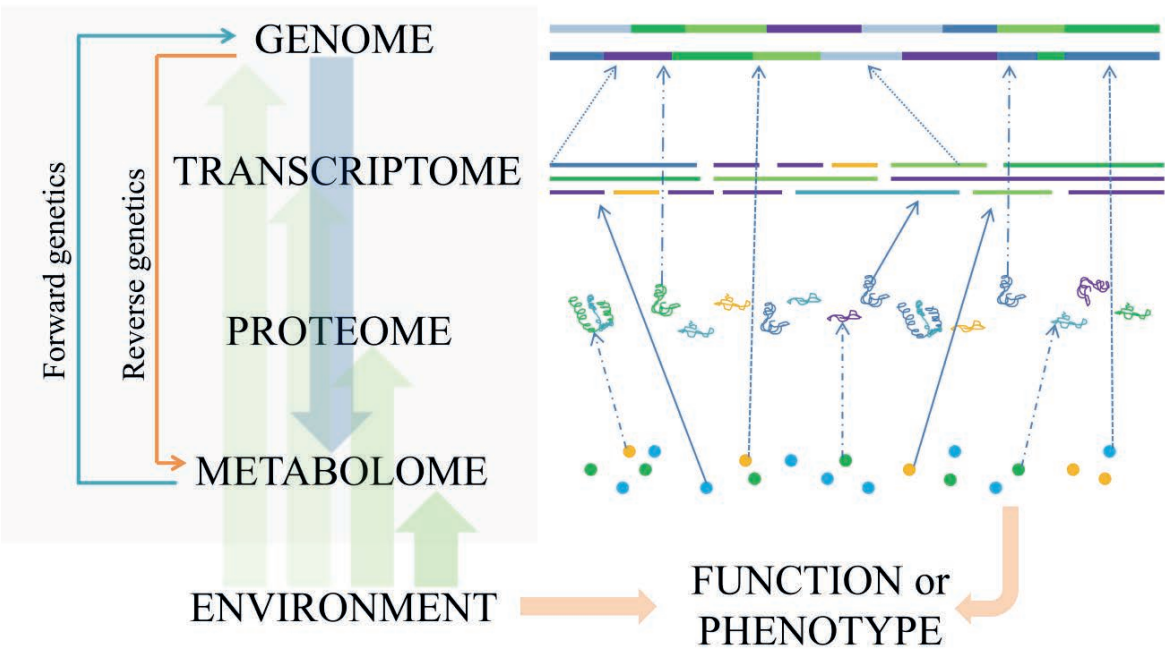


Figure 1. The metabolome is a result of the interaction of a plant's genome with its environment. Metabolites present in a system are the end products of gene expression and can interact with the proteome, transcriptome and genome. Metabolomics can provide an instantaneous reflection of the metabolome at a particular point in time and complements genomic, transcriptomic and proteomic approaches to the study of living systems. New bioinformatics techniques for data analysis allow data integration across the “omics” and contribute to the study of biological systems using two common strategies; forward genetics and reverse genetics approaches.

is relatively understudied. Today, plant metabolomics is still considered to be an emerging field; its progress has been associated with the development of suitable platforms for the isolation and identification of secondary plant products, and pinpointing their roles in complex interactions. However, there are currently many useful reviews on the subject of metabolomics and instrumentation in general (Ellis and Goodacre, 2006; Nguyen *et al.*, 2012; Tugizimana *et al.*, 2013).

From the plant research perspective, recent advances in genomics, transcriptomics and proteomics have led to an improved understanding of the role of specific genes in the regulation of cellular processes in higher plants (Roessner *et al.*, 2001). However, these techniques often do not present the complete story regarding the processes of metabolism and catabolism in an organism or a cell at a particular point in time. Metabolomics complements both genomic and proteomic approaches to the study of living systems (Fig. 1) as it does provide a reflection of the chemistry of a living organism at a given point in time. Metabolomics is therefore a powerful tool for use in systems biology or functional genomics, both of which attempt to integrate genomic, transcriptomic, proteomic and metabolomic information to present a more complete image of the processes occurring in a living organism (Breitling *et al.*, 2013; Rochfort, 2005).

Metabolomic studies of animal systems are relatively advanced, in comparison to the plant sciences, as exemplified by The Human Metabolome project led by Wishart in Canada; in 2007 a database of nearly 2500 metabolites, 1200 drugs and 3500 food constituents making up common components of a human metabolome was released (Wishart *et al.*, 2007). Similar plant projects have been underway in *Medicago trunculata* and *Arabidopsis thaliana* and are now closer to completion, despite the surprisingly greater complexity of metabolites in plant/bacterial systems (Farag *et al.*, 2008). The complexity of all metabolomics data generated, whether plant, bacterial or mammalian, has required new bioinformatics techniques and software for data analysis, including the use of unsupervised, multivariate data analysis and heat maps to detect patterns in complex data (Morgenthal *et al.*, 2006). One finds that metabolic profiling can be performed with routine ease, but data handling represents the bulk of time and effort in obtaining useful results.

In this review, we focus on the use of metabolomics and metabolic profiling to study plant metabolites involved in plant defence, using both above- and below-ground examples of metabolic profiling, in an effort to understand the role of primary and secondary metabolites in plant function and as a

part of broader studies in plant genomics biology. We also point out how these techniques may have application to the study of plant/plant or plant/microbial interactions and allelopathy.

Metabolic Profiling: Benefits and Utilisation

There are several advantages of the study of metabolites in plants and their extracts through metabolomics. Firstly, when used in concert with transcriptomics and proteomics for study of plant biological systems, metabolomics can help elucidate details of plant natural product biosynthesis (Dixon and Sumner, 2003). Secondly, because the number of detectable key metabolites is often significantly reduced compared to the number of genes in an organism, metabolomic analyses can be performed by smaller teams of researchers than other “omics” studies. Thirdly, one can focus on chemistry which is not yet well understood, and evaluate and identify metabolites for which we have limited knowledge (Breitling *et al.*, 2013). This is critically important in developing fields such as plant toxicology and allelopathy. Fourthly, since the metabolome directly reflects the current functional state of a system, metabolite concentrations may change markedly during biochemical reactions. These changes can be detected and quantified, while in comparison, enzyme flux associated with proteomic analyses might be limited over time, resulting in less sensitivity in detection of systems change. Last but not least is the potentially reduced cost of metabolite analysis when compared to costs incurred for transcriptomic and proteomic studies (Dunn and Ellis, 2005).

Metabolic profiling is now becoming an important tool in understanding the systematic responses of an organism to changing environmental conditions (Carrari *et al.*, 2006; Dixon and Sumner, 2003; Rochfort, 2005; Roessner and Bacic, 2009), and facilitates functional gene annotation (Schauer and Fernie, 2006). The term “metabolic profile” was first introduced by Horning and collaborators in 1971 after they demonstrated that gas chromatography-mass spectrometry (GC/MS) could be used to measure compounds present in human urine and tissue extracts. The Horning group and those of L. Pauling and A. Robinson further advanced GC-MS methods to monitor the metabolites present in urine in the 1970s. This was the first clear example of metabolic profiling (Robinson and Robinson, 2011). Analytical techniques used in metabolomic studies (Fig. 2) (MS coupled with GC or LC, as well as NMR) provide both structural and quantitative data and can be used in a “global” or “targeted” manner to either determine structures of hundreds/thousands of compounds

or detect biologically active metabolites at part per billion levels (Griffiths and Wang, 2009). Today, plant scientists have adapted these concepts and techniques to study the role of plant secondary products (PSMs) in the function of the organism, including roles in regulation and plant defence (Dixon and Sumner, 2003; Kim *et al.*, 2010; Leiss *et al.*, 2009; Roessner and Bacic, 2009).

Sample Preparation and Analytical Tools

Since sample preparation and clean-up have a profound impact on the selection of compounds to be analysed, the use of metabolic profiling allows one to focus on a specific group of chemicals or particular pathway and thus minimizes the effects of matrix complexity. Matrix complexity can be decreased by post-extraction processing (such as solid phase extraction or SPE) or by selection of a specific organs or tissues for processing (Fiehn, 2002). Metabolic profiling in our laboratory has included extracts of periderm peels (less complex than the whole root) and shoot extracts which were concentrated and purified by SPE. These processes enriched our matrices for certain chemical constituents such as naphthoquinones, N-containing compounds, or pyrrolizidine alkaloids, and provided advantages by simplification of the matrix and concentration of key compounds of interest (Fig. 3) (Weston *et al.*, 2013).

Roessner and Bacic (2009) indicated that the particular conditions under which a plant is growing can strongly influence the levels of metabolites produced by plants, and thus sample collection. In particular, light-dark photoperiod, light intensity and time of day are factors which impact the concentration of PSMs and may seriously influence metabolic profiling studies. This suggests that investigators must pay attention to the time of plant collection, and thus attempt to minimize variation over time by harvesting at discrete intervals. Samples must also be carefully stored to minimize enzymatic degradation or decomposition of natural products due to heat or light exposure. As plant tissues are often difficult to extract due to presence of waxy cuticles or lignins and contain both hydrophilic and lipophilic compounds of interest, it is critical that the optimal strategies for uniform extraction of small molecules of interest should be determined at the outset of a study (Roessner and Bacic, 2009).

Extraction of freshwater or seawater samples remains a challenge for investigators working with aquatic plants, however several techniques have been used to extract metabolites from seawater matrices. Most common is the use of liquid/liquid extraction, but this is time and solvent consuming.

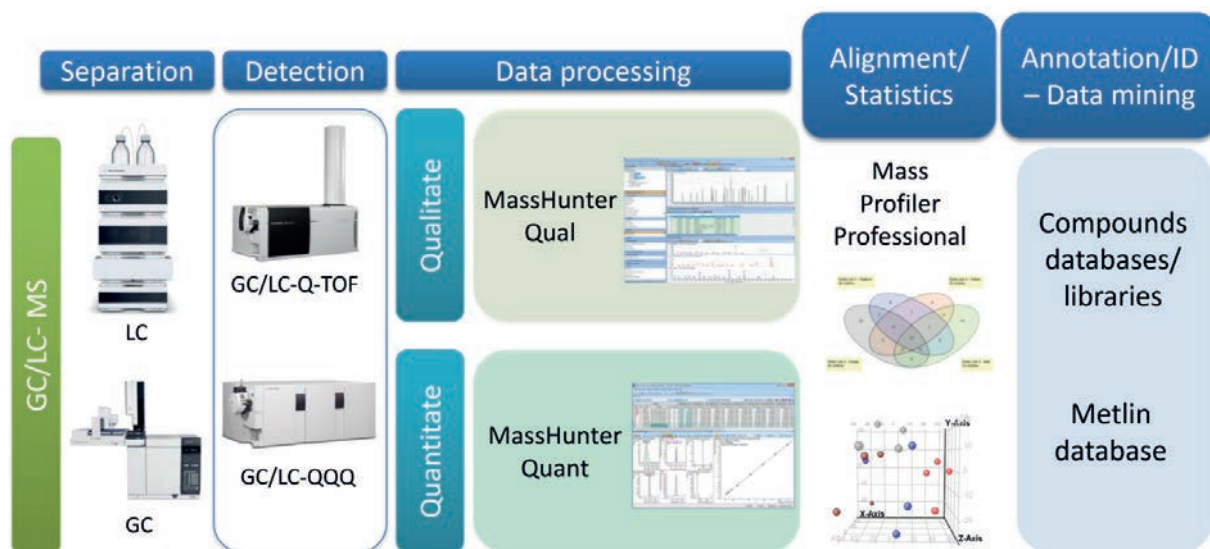


Figure 2. The typical work flow for performing metabolic profiling of secondary plant products. Analytical techniques used in these studies generally include mass spectrometry coupled with gas and/or liquid chromatography, which provides both limited structural, qualitative and quantitative data. Profiling can be performed in a “global” or “targeted” manner. Data may be analysed in a variety of ways, using several platforms. For more information on selected workflow see www.agilent.com/labs/features/2010_metabolomics.html.

A wide range of polar and non-polar compounds have been successfully extracted from aqueous media using C_{18} SPE (Goultquier *et al.*, 2012). An organotin metabolite was previously extracted from seawater by headspace solid phase micro-extraction (HS-SPME) and later quantified by GC/MS (Békri *et al.*, 2006). This methodology was also applied to extract iodinated and brominated compounds exuded by diatoms (Vanelslender *et al.*, 2012). Alternatives to SPME extraction include the stir bar sorptive extraction (SBSE) where passive sorbent-polymethyldimethylsiloxane (PDMS) is used. In this methodology high concentrations of sea salt increase the extraction range of metabolites (Goultquier *et al.*, 2012).

Small quantities of plant tissue are often frozen in liquid nitrogen as a technique of choice for immediate processing upon collection, but freeze-drying or immediate storage of plant tissues at $-80\text{ }^{\circ}\text{C}$ upon collection, or fresh processing are alternative approaches, especially with plant products that may be particularly labile or photosensitive. Whole leaf tissues or roots can also be freeze-dried and then homogenized to a powder for full extraction (Roessner and Bacic, 2009). We often utilize a temperature- and pressure-controlled speed extraction unit (Buchi Corporation, Flawil, Switzerland) that processes multiple freshly collected plant samples uniformly. This solves several problems 1) it minimizes human error; 2) it optimizes and speeds up the extraction process; and 3) provides a high level of uniformity

within sample extracts as well as an improved yield of compounds of interest. In particular, the use of high pressure often results in maximal extraction of secondary products present in exceedingly small concentrations in a complex matrix, such as a field-collected soil. Both fresh and dried materials can be extracted by subjecting them to programmed temperature and pressure elution; plant, soil and rhizosphere samples have also been successfully extracted.

Discrete plant tissues often contain high concentrations of specialized natural products in specific locations, such as root periderm or root hairs or glandular trichomes on the leaf surface. In this case, root periderm tissue can be thinly peeled, cut, dissected or analysed by use of microprobes and processed by direct solvent extraction or centrifugation for further extraction (Weston *et al.*, 2012) or leaf tissue can be processed rapidly by dipping or performing leaf peel extractions (Ryan, 2014). The use of microprobes formed by coating stainless steel wires with silicone tubing for insertion into soil has also been optimized for soil extraction and successfully used for the study of plant metabolites in complex soil matrices, including the study of thiophenes released by the living roots of *Tagetes* spp. (Weidenhamer *et al.*, 2009) or sorgoleone released by living roots of *Sorghum* spp. (Weston *et al.*, 2013a). We have also successfully used this technique, along with direct extraction of root periderm, to study naphthoquinones and other potentially active allelochemicals in soils infested

with *Echium plantagineum*, or Paterson's curse, a noxious invasive weed in Australia (Weidenhamer *et al.*, 2014; Weston *et al.*, 2013). In addition, use of SPME fibres can facilitate trapping of both volatiles (Loi *et al.*, 2008) and non-volatiles (Dayan *et al.*, 2009) for evaluation by GC-MS.

Most recently, investigators have used laser micro-sectioning technology to excise particular cell types, which are then extracted and analysed by conventional metabolic profiling, or *in situ* mass spectrometry for imaging in and across planes of various tissues. This approach generally uses Matrix Assisted Laser Desorption/Ionisation, Time of Flight (MALDI ToF) spectrometry and involves mounting tissues on a MALDI plate and exposing them to laser pulses, which frees and ionizes molecules in the matrix for additional analysis by MS (Roessner and Bacić, 2009).

Today, most plant investigators use a combination of LC and GC techniques coupled with high resolution mass spectrometry (LC/MS and GC/MS, respectively) or NMR for metabolite profiling. The use of both triple quadrupole MS (QQQ) or sensitive ion traps for quantification, along with quadrupole time of flight mass spectrometry (Q-ToF) for accurate and precise profiling of complex matrices, has resulted in major advancements and putative annotation of PSMs present at low abundance in plant extracts (Fig. 2). These instruments are used routinely by many laboratories for metabolic profiling of plant extracts. A variety of MS techniques can be used, depending on the sensitivity, mass resolution and dynamic range required (Griffiths and Wang, 2009; Obata and Fernie, 2012). In 2005, the first metabolomics web database, METLIN, was developed as a library for use in characterization of human metabolites at the Scripps Research Institute. Originally containing mass spectral data for >10,000 metabolites, METLIN now contains data for well over 65,000 metabolites, including tandem mass spectrometry data, representing the largest dataset in metabolomics. By using available databases and compound libraries, plus the creation of one's own personal compound library based on comparison with known standards, one can routinely identify or annotate low concentrations of PSMs (Smith *et al.*, 2005).

High field NMR, which can be used alone on complex mixtures or coupled to LC, offers a different approach to compound identification based on nuclear interaction (Kim *et al.*, 2010; Obata and Fernie, 2012). Although often less sensitive than MS techniques, structural information content and reproducibility can sometimes be higher, allowing NMR to play a critical role in structural elucidation of key metabolites (Obata and Fernie, 2012). One of the

main advantages of metabolomics analyses utilising NMR is the possibility of identifying metabolites by comparing NMR data with references or by use of two-dimensional NMR for structural elucidation. Two-dimensional NMR is often well-suited for the analysis of phenolics in plant extracts and for primary metabolites such as sugars and amino acids. NMR is also relatively rapid; sample preparation requires about 30 minutes and spectral acquisition can occur in as little as 10 minutes (Kim *et al.*, 2010).

Secondary Metabolite Profiling and Functional Genomics

PSMs are often species-specific and play important regulatory roles or assist in biological functions such as plant defence (Weston *et al.*, 2012; Quinn *et al.*, 2014). Plants produce numerous compounds which participate in growth and development (primary metabolites) and diverse groups of constituents whose biological significance is often unknown (secondary metabolites) (Croteau *et al.*, 2000; Quinn *et al.*, 2014; Wink, 1999). Although knowledge of secondary metabolism in plants has improved significantly in the last decade, the role of the vast majority of PSMs remains unknown. It is estimated that more than 100,000 PSMs have been characterized in higher plants (Croteau *et al.*, 2000; Wink, 1999). PSMs play important roles in plant interactions with the environment (Hartmann, 2007), defence against microbes, pathogens and herbivores, or as chemical attractants (Ober and Hartmann, 2000; Quinn *et al.*, 2014). Their targeted study by metabolic profiling has resulted in major advances in our understanding of their roles in plant defence, nitrogen utilisation, plant/microbial interactions and chemical signalling (Lau *et al.*, 2014; Weston and Mathesius, 2013).

Two common strategies are utilised to determine the function of PSMs through metabolic profiling. The first is the use of forward genetics where phenotypic plant features are the starting point in studying gene activity and resulting metabolites, produced by a series of unknown or unidentified genes. In contrast, a reverse genetics approach works backwards from a known specific gene or protein sequence towards identification of corresponding metabolites and the phenotype of specific organisms (Fig. 1). Both techniques are useful tools for understanding more complex molecular interactions in biological systems (Kiambi *et al.*, 2008). For example, metabolic profiling allows identification of the rapid production of phytoalexins by plant tissue in response to elicitors (Bednarek *et al.*, 2001); this approach could be used to further screen for unknown phytoalexins and the genes involved in their production, as well as the

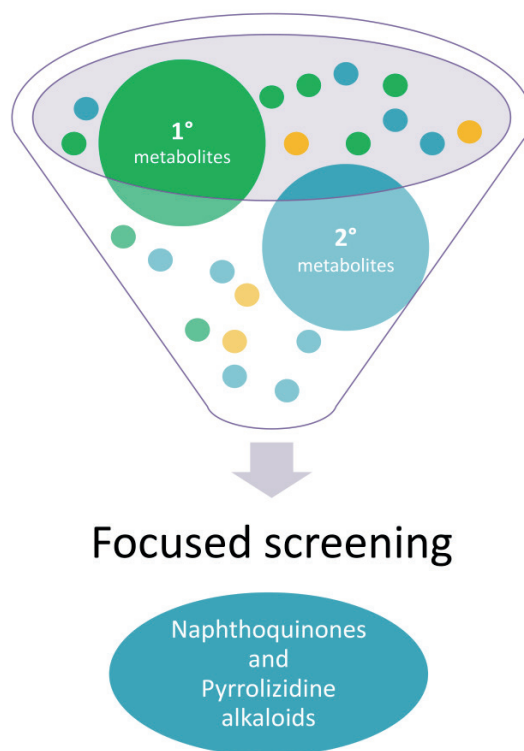


Figure 3. Plant extracts of *Echium plantagineum* were prepared using techniques to enrich for bioactive secondary products of interest, specifically naphthoquinones and pyrrolizidine alkaloids. Targeted metabolic profiling was then performed on compounds of interest. This simplifies the sample matrix and also increases the concentrations of the compounds of interest (Skoneczny *et al.*, 2014; Weston *et al.*, 2013).

targeted study of known phytoalexin systems.

Metabolic profiling was successfully employed to investigate the plant-microbe interactions using rice (*Oryza sativa*) and the pathogen *Magnaporthe grisea* as a model system. In this case, the authors identified 93 compounds of interest in plant extracts using NMR and GC/MS techniques, and additionally profiled samples with LC/MS to study numerous biochemical changes in rice plants 24 h after inoculation (Jones *et al.*, 2011).

Metabolomics is also a powerful tool for transgenic organism risk assessment. When plant breeders introduce new genes into breeding lines for trait enhancement, both proteomics and metabolomics can play an important role in assessing the impact of the new genes at the molecular level. Gene expression profiles coupled to metabolic profiles can provide key information regarding safety of food crops, for example, or excessive production of toxic secondary metabolites. In a study performed by Urbanczyk-Wochniak *et al.* (2005) evaluating wild type and transgenic potato tubers, strong correlations were found between presence of nutritionally important metabolites and incorporation of key transgenes into potatoes. As another example, targeted introduction

of structural genes into transgenic tomatoes has also resulted in production of tomato fruit yielding high levels of stilbenes, deoxychalcones or flavones, important in plant defence and also anti-oxidant activity contributing to human health (Schijlen *et al.*, 2006; Weston and Mathesius, 2013). There are now many examples of using metabolic engineering to produce bioplastics, vaccines and derivatives of natural plant products for drug design (Lau *et al.*, 2014). In the case of golden rice, higher levels of vitamin A have been incorporated into rice through transgenes for carotenoid production. In many instances, the production of toxic secondary products has been suppressed by the use of selective breeding coupled with metabolic profiling to detect the presence of undesirable metabolites in the transgenic plants, such as the case of solanine in potato tubers (Dixon and Sumner, 2003; Lau *et al.*, 2014). This technique could be employed to select plant forages for reduced toxicity to grazing livestock or ornamentals and turfgrasses for reduced hypoallergenic activity.

The plant metabolome also generally contains a significant number of volatile compounds, which are typically analysed by GC/MS or systems employing cryo-SPE. Volatiles clearly play important roles

in plant/plant, plant/insect and plant/microbial interactions and act as signalling mediators. Tikunov *et al.* (2005) analysed 94 commercial varieties of tomato for volatile compounds exuded from tomato pulp. Novel approaches in data analysis, including spectral alignment, multivariate data analysis and MS spectral reconstruction, allowed the authors to identify 322 volatiles, representing 80% of all volatiles previously identified in tomato using multiple techniques for detection (Tikunov *et al.*, 2005). Another interesting study was performed by Eom *et al.* (2006) to study volatile allelochemicals emitted by common ornamental groundcovers in an attempt to identify weed-suppressive plants for use along roadsides. Groundcovers grow over the soil surface and often suppress weed growth by successfully competing for resources with other plants, or by emitting significant quantities of PSMs which may be associated with plant defense, insect attraction, herbivore repellency, or allelopathic activity. The growth-inhibiting properties associated with the presence of PSMs of several species including *Alchemilla mollis*, *Nepeta × faassenii*, *Phlox subulata*, *Sedum acre*, *Solidago cutleri* and *Thymus praecox* were investigated. *Nepeta × faassenii* and *S. cutleri* proved to be suppressive, likely due to the production of bioactive volatiles. In this case, the authors profiled volatile foliar constituents and identified 21 compounds in the volatile mixture extracted from *Nepeta × faassenii* (ornamental catmint) using HS-SPME to collect plant volatiles for GC/MS (Eom *et al.*, 2006).

Marine organisms also produce a wide variety of unusual chemicals that are often structurally unique and difficult to identify. Researchers have investigated the genomics of selected marine ecosystems and often concentrated on unique metabolic pathways present in marine organisms (Goulitquer *et al.*, 2012). Although GC-ToF analysis is used for identification of unique volatile metabolites, LC-MS based techniques are best suited for the analysis of marine organisms and ecosystems due to high salinity associated with these samples (Ferne *et al.*, 2012).

Metabolomics, in conjunction with transcriptomics, also further revealed the adaptation strategies to limited Fe use by marine plankton. Low abundance of Fe is often growth limiting for plants and phytoplankton, however it was discovered that marine organisms employ unique strategies to overcome this limitation. *Phaeodactylum tricornutum* responds to Fe availability by reducing the quantity of Fe-chelating glucose derivatives. Moreover, cellular processes including photosynthesis, mitochondrial electron transport and nitrate assimilation can be downregulated at low Fe levels due to high Fe requirements by specific enzyme complexes in these processes (Ferne *et al.*, 2012). Using

LC/MS, algal-produced chemicals were investigated; in this case changes in biosynthesis of secondary metabolites in other marine organisms were observed as a direct result of products produced by algal blooms. An additional study evaluated volatiles produced by diatom cell cultures, collected by HS-SPME, and identified 18 iodinated and brominated volatiles including cyanogen bromide, a potent allelochemical (Goulitquer *et al.*, 2012; Vanelislander *et al.*, 2012). Genomics data in diatoms has revealed several novel biosynthetic pathways previously unknown; however, their functions and roles remain unclear at this time. Metabolomics of primary and secondary metabolites appears to be a very promising tool for provision of additional data for these understudied marine organisms and genetic × environmental interactions (Ferne *et al.*, 2012).

Impact of Plant Stress as Assessed by Metabolic Profiling

One approach for the novel use of plant metabolic profiling is the study of the impact of environmental stress on plant performance. This screening is of particular importance to plant breeders and stress physiologists who are interested in comparing commercial cultivars of crop species with those of wild species or landraces, which might exhibit tolerance to particular plant stressors. Roessner and Bacic (2009) described the use of metabolic profiling to compare salt-tolerant barley genotypes with less tolerant genotypes when cultivated under high salt (sodium ion) conditions. Their results showed that a less tolerant genotype contained higher levels of amino acids, which were correlated with increased levels of necrosis. In contrast, a stress-tolerant genotype exhibited increased levels of organic acids and sugars, which are thought to be involved in protection against stress in barley leaf tissues (Widodo *et al.*, 2009). Metabolic profiling can also be used to evaluate the impact of water stress on plant growth. In water-stressed plant leaves, increases in proline and decreases in aspartate are often noted in metabolite profiles comparing stressed and unstressed plant leaves. Interestingly, plants of different maturity stages at various levels of water stress respond differently in terms of their metabolic profiles. Changes in branched chain amino acids were also noted in the leaves of water-stressed wheat, barley and tomato (Obata and Ferne, 2012).

A unique experiment to assess the impact of plant community diversity (species number and composition) on the metabolome of plants of varying architectural traits (tall *versus* short) was performed to gain insight into mechanisms used by plants which

impact performance and competition in biodiverse communities, including competition for resources such as C and N. Less competitive, small-statured plants showed metabolic signatures correlated with increasing diversity and species richness, indicating both C and N limitation in corresponding metabolites. In comparison, taller species which became dominant in mixed communities did not show altered metabolic profiles in response to altered resource availability with increasing plant diversity. In this case, both LC/MS QToF and GC/MS QToF evaluation of metabolites were performed to detect and quantify most analytes of importance (Scherling *et al.*, 2010).

Metabolic profiling can also be coupled in broad scale studies on population genetics of plant species to obtain key information on impact of environment or plant stress on plant genotype and gene expression of PSMs. In a recent study performed with geographically distinct populations of Paterson's curse (*Echium plantagineum* L., Boraginaceae), plants found near roadsides across Australia were surveyed along three distinct longitudinal transects for presence of pyrrolizidine alkaloids, important secondary products involved in plant defence against grazing herbivores and toxicity (Quinn *et al.*, 2014; Weston *et al.*, 2013). Composite samples were collected from each of 45 sites; metabolic profiling using LC-MS found 14 alkaloids and their N-oxide derivatives in all plants, with significantly greater levels in warm, dry sites compared to cooler, moister sites. Toxic pyrrolizidine alkaloids consistently occurred in all shoot extracts, with lephantine-N-oxide, echimidine-N-oxide and echiumine-N-oxide predominant. The patterns of abundance of secondary metabolites in *E. plantagineum* suggest that climate change resulting in warmer, drier conditions might result in greater production of these defensive compounds, making this noxious weed even more toxic to livestock, and also potentially more allelopathic due to the elevated production of root-produced naphthoquinones (Weston *et al.*, 2013).

Use of metabolic profiling to evaluate secondary plant products in the soil rhizosphere

Metabolic profiling can also be used to evaluate plant growth responses belowground to associations formed with microbes, other plants, insects or macrobiota in the soil rhizosphere. Experimentation belowground has often proven difficult due to inability to access the soil rhizosphere and the living plant roots it harbors, the complexity of the soil matrix and the sheer numbers of organisms present in the soil (Bertin *et al.*, 2003; Weston and Mathesius, 2013). Given our recent ability to detect and analyse soil constituents at very low concentrations through

targeted MS techniques, metabolic profiling of soil extracts has resulted in considerable information regarding the flux in secondary metabolites produced by root exudation, as well as degradation and transformation of both primary and secondary metabolites over time (Carlsen *et al.*, 2012).

In a field study investigating the fate of flavonoids released from legume stands over time, sensitive LC-MS/MS techniques were used to profile a diverse group of over 20 flavonoids released from living and decomposing white clover (*Trifolium repens* L.) stands in Denmark *in situ* and after soil incorporation of the clover as a green manure (Carlsen *et al.*, 2012). As the authors report, numerous studies have implicated allelochemicals produced by white clover in weed suppression, as well as negative interactions associated with allelopathy or replant/pathogenesis problems following white clover establishment. This ground-breaking study evaluated the pattern of flavonoid release from living clover grown under field conditions and also from leachates following incorporation of green cover crops into field soil. The investigators found that the flavonoid aglycones formononetin, medicarpin, and kaempferol predominated in soil analyses, with glycosides of kaempferol and quercetin also present at relatively high concentrations. Kaempferol persisted for days in field soil surrounding living or incorporated clover stands. These aglycones and related constituents have specifically been reported to possess substantial phytoinhibitory activity (Rice, 1984) and thus these findings may help to explain the potential for allelopathy and autoallelopathic interactions associated with established white clover stands (Weston and Mathesius, 2013). Carlsen *et al.* (2012) also noted that highest flavonoid concentrations in clover crops were associated with presence of clover flowers, in comparison to leaves, stems or roots in soil degradation studies. Several of the flavonoids identified are also known inhibitors of fungal growth, while others are associated with stimulation of microbial growth in the rhizosphere (Mandal *et al.*, 2010).

Krogh *et al.* (2006) performed metabolic profiling of allelochemicals (benzoxazinones) of wheat and rye in the soil. GC-MS and NMR techniques were employed for the identification of allelochemicals and LC-MS was later utilized for targeted analyses of soils for PSMs. The dynamics of leaching, transformation and metabolism of selected PSMs in the soil was evaluated through the use of focused studies (Krogh *et al.*, 2006). The effects of allelochemicals and their degradation intermediates were also studied with respect to non-target aquatic organisms. Others have used targeted

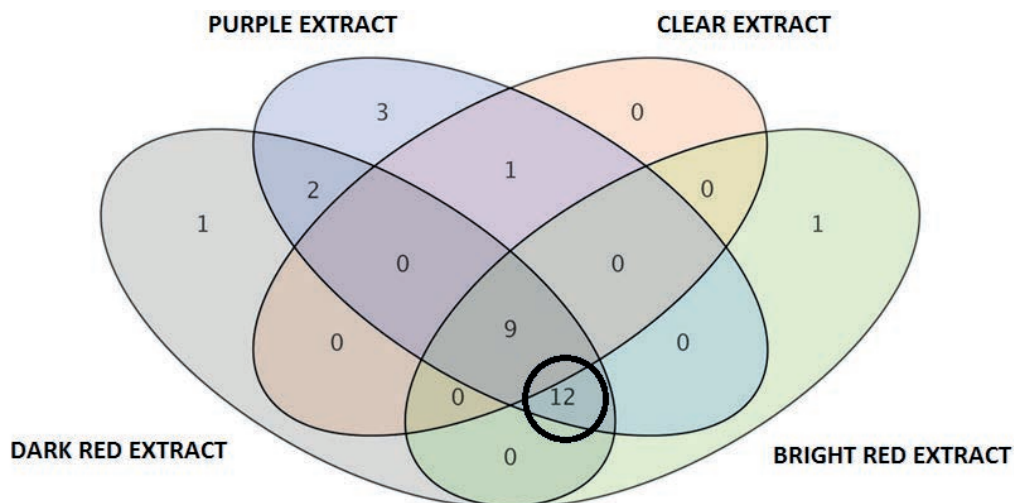


Figure 4. Coloured root extracts of *Echium plantagineum* were separated by their unique colouration into samples that ranged from purple, dark red and bright red to clear. Targeted metabolic profiling resulted in detection of numerous naphthoquinones and other related compounds; coloured samples varied qualitatively in their composition. Only 12 annotated compounds were in common in all coloured root extracts and 9 of them were shikonin-related compounds including α -methylbutyrylshikonin, benzoylshikonin, β,β -dimethacrylshikonin, deoxyshikonin, isobutyrylshikonin, teracrylshikonin and 1-methoxyacetylshikonin. Venn diagrams such as this one can be used to clearly visualise profiling results for samples that vary qualitatively and/or quantitatively, and indicate overlap of common constituents (Skoneczny *et al.*, 2014).

approaches to study the activity of secondary metabolites on aquatic organisms in an attempt to study the biosynthetic degradation pathways of these compounds in aquatic environments (Fritz and Braun, 2006). Metabolic profiling can enable the study of catabolism and bioactivity of natural products in a variety of environments, in association with bioassay-directed isolation, and may reveal novel metabolites implicated in organismal interactions.

An interesting approach to the study of PSMs in roots was performed using Paterson's curse (*Echium plantagineum*) roots, which produce high concentrations of red-coloured naphthoquinones (NQs) in both the root periderm and as exudates by living root hairs. These shikonin derivatives have strong antimicrobial and antifungal properties, and are also suppressive to annual ryegrass germination (Weston *et al.*, 2013). Numerous NQs have been identified in Paterson's curse root extracts using metabolic profiling in an attempt to study the biosynthetic pathway(s) responsible for their production, and to assess their relative activity as allelochemicals. When coloured root extracts were separated and combined by shade of colouring from red to clear, profiling and subsequent principal component analysis showed clear differences in content of related NQs, based on extract colouration.

Red- coloured extracts were highest in levels of shikonin, acetylshikonin and deoxyshikonin, with lower levels in pink and yellow extracts and non-detectable levels of NQs in clear extracts. For data generated in the afore-mentioned experiment, a Venn diagram was designed to present naphthoquinone constituents present in distinctly coloured samples (Fig. 4) (Skoneczny *et al.*, 2014). In addition, silicone microprobes placed in soil around roots of pot-grown *E. plantagineum* plants revealed the presence of several NQs in soil containing *Echium* roots, and upon removal of soil probes, the probes exhibited a pink or red color. Probe extracts were analysed using LC-QToF/MS and contained detectable concentrations of NQs, indicating that plant roots might exude or release significant levels of bioactive secondary products in surrounding rhizosphere (Weidenhamer *et al.*, 2014). The ability to profile the dynamics of these metabolites in soil provides a tool for further investigation of their ecological role in the rhizosphere. The fate of root-produced NQs in soil ecosystems is now under additional investigation.

Current Challenges in the Use of Metabolic Profiling – the case of *Medicago truncatula*

Although we are currently able to elucidate

many intermediates in biosynthetic pathways through the use of metabolic profiling, there are some key challenges to overcome to fully utilise and integrate this technology into plant physiological and biochemical studies. The first challenge is presented by the sheer number of PSMs and their related metabolites of interest in plants. *Arabidopsis* has been reported to encode 25% of its genome as metabolism enzymes, and this percentage may be even higher in legumes (Dixon and Sumner, 2003). With over 18,000 species of legumes, the diversity of secondary products produced—including alkaloids, non-protein amino acids, isoflavonoids, saponins and triterpenoids—can be staggering. Some PSMs are as yet unidentified, requiring focused attempts to purify and perform NMR and other spectrometry for final structural characterization to discriminate between isomers with the same molecular formula (Kim *et al.*, 2010; Weston and Mathesius, 2013). Recent research on legumes performed at the Noble Foundation in Oklahoma, USA has demonstrated that metabolic profiling can be successfully used to study both novel PSMs including isoflavonoids and phenylpropanoids and their biosynthetic pathways (Frag *et al.*, 2008). By performing exogenous and endogenous profiling of cell suspension cultures of *M. truncatula* in the presence and absence of methyl jasmonate and fungal elicitors, Frag and co-workers (2008) found novel and flexible methods of biosynthesis of isoflavonoids in these legume systems, along with three novel products. Their results also suggested differential methods for synthesis of the phytoalexin medicarpin, depending on the nature of elicitation.

The use of multiple methods for analysis of secondary metabolites is critical when evaluating the broader metabolome of plants. Frag and co-workers, along with many earlier researchers, used primarily LC-MS for profiling, but today the use of both LC and GC coupled to MS, NMR or other spectrometric techniques allows for the detection and analysis of a wider range of secondary constituents.

Summary

Metabolomics is a rapidly emerging technology that evaluates global metabolite profiles in a plant system under a specific set of conditions or at a particular point in time, to study impact of environment upon a biological system. The metabolome consists of both primary and secondary plant products and can be particularly challenging to analyze due to its inherent chemical diversity. Metabolic profiling, in contrast, is applied more specifically for analysis of a portion of the plant

metabolome, and is often used by investigators to assess the current state of the plant, its tissues, or the specific matrix in which it is growing, for a more targeted understanding of metabolites of interest. Although there are many ways to perform metabolic profiling, the use of LC or GC coupled to MS is most common. In most cases, more than one analytical approach is required to study the full complement of constituents of interest. Metabolic profiling has proven most useful in facilitating functional gene annotation through the use of data mining tools for the comprehensive characterization of a plant genotype. In addition, metabolic profiling can also be useful for determination of complex pathways of secondary product biosynthesis in higher plants as well as a broader understanding of biological activity and function associated with the presence or absence of key secondary products in the metabolome. This provides an opportunity to give biological meaning to differences and activity observed in living systems.

Investigators have recently employed metabolic profiling to study impacts of plant stress upon secondary product production, role of secondary products in plant toxicity and human health, and complex interactions between plants and rhizosphere organisms. In addition, metabolic profiling, in combination with transcriptomics and proteomics, has and will contribute to a broader understanding of plant physiology and function, and the ecological roles of diverse PSMs. These tools can be successfully employed for the study of joint metabolic networks of interacting organisms, leading to a broader understanding of ecological interactions at multi-trophic levels. The use of multiple instrumental platforms for chemical analysis is often most effective for identification and role of PSMs involved in plant interactions such as allelopathy. The study of plants in mixed stands in the field or under controlled environmental conditions will soon be facilitated by metabolic profiling. Our own laboratory along with those of I. Fomsgaard and others are currently attempting such studies. In the future, new developments and reductions in the cost of instrumentation will continue to allow enhanced detection of PSMs at ultralow concentrations, enabling greater precision in identification of metabolites of interest and their roles in allelopathic interference. We believe that metabolomics will also result in further advances in the regulation of plant growth and development in the next decade, as more focused studies of the complete plant metabolome, including metabolites produced by the plant and those of associated micro- and macrobiota, are routinely performed.

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Effects of cinnamic and ferulic acids on growth and lignification of maize roots

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ABSTRACT

Cinnamic acid and its hydroxylated derivative, ferulic acid, are known allelochemicals that affect the root growth of several plant species. Some studies have indicated that the impairment of root growth by allelochemicals is associated with lignification of the cell walls. However, this hypothesis has not been tested in monocot plants. Here, we evaluated the effects of these two allelochemicals on growth, activities of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL), lignin content and the composition of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) monomers in maize roots. Cinnamic acid reduced root growth, TAL activity and lignin (H+G+S) content. When applied in conjunction with piperonylic acid, cinnamic acid reduced total lignin and H, G and S content. Ferulic acid reduced root growth and PAL/TAL activities, but it increased G monomer and lignin (H+G+S). When applied in conjunction with 3,4-(methylenedioxy)cinnamic acid, ferulic acid reduced total lignin and G and S content. The results indicated that decreased maize root growth by the cinnamic acid was not related to the lignin production while exogenous ferulic acid could be channeled into the phenylpropanoid pathway and resulted in an excessive production of lignin, thus solidifying the cell wall and restricting the plant growth.

Keywords: Allelochemical, lignin, monolignol, phenylpropanoid pathway, root growth, *Zea mays* L.

Introduction

Allelochemicals are secondary metabolites regularly released by higher plants into the surrounding soil which can influence the growth and development of neighboring plants, both positively and negatively. This phenomenon is known as allelopathy, defined as the ability of plants to protect themselves as well as chemical communication between microbes, plant/microbe, plant/insect or plant/herbivore (Weir *et al.*, 2004). A large number of allelochemicals are commonly found in soils, affecting plant growth at concentrations of up to 10 mM (Siqueira *et al.*, 1991; Macias, 1995) and due to their high metabolic activity, plant roots are quite susceptible to the stress induced by allelochemicals. After uptake by the roots, allelochemicals alter physiological and biochemical processes such as water utilization, mineral uptake, photosynthesis, amino acid metabolism, protein synthesis, glycolysis, mitochondrial respiration and ATP synthesis, among countless others (Weir *et al.*, 2004). However, conclusive modes of allelochemical action on plants are far from being understood.

One of the most striking events in the development of higher plants is the lignification of cell walls, a dynamic and flexible process important for many specialized functions such as regulation of water flow, hydrophobicity, rigidity and protection against biotic and abiotic stresses (Harrington *et al.*, 2012). Lignification, which refers to the anchorage of lignin in the plant cell wall, is also a crucial step for the root growth. Furthermore, the wall of the root cell is the first tissue that is affected by stress signals (Komatsu *et al.*, 2010).

Lignin is a heterogeneous polymeric end-product of the phenylpropanoid pathway generated by combinatorial radical coupling of *p*-hydroxycinnamyl alcohols (called monolignols) (Vanholme *et al.*, 2012). In the first two steps of this metabolic route, the non-oxidative deamination of L-phenylalanine by phenylalanine ammonia-lyase (PAL) yields *t*-cinnamate, followed by hydroxylation of the aromatic ring catalyzed by cinnamate 4-hydroxylase (C4H) to generate *p*-coumarate. The coupling of these enzymes establishes a rate-limiting step in the pathway. PAL is found in dicots and monocots, but a

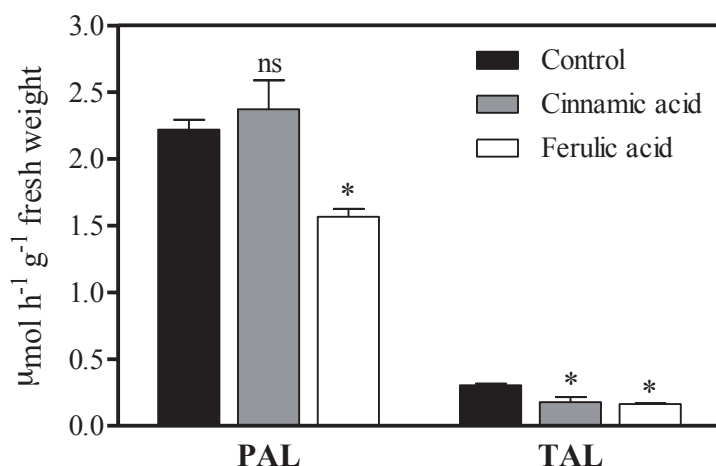


Figure 1. Effects of 1.0 mM cinnamic and ferulic acids on phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activities of maize roots. *Means ($n = 6 \pm \text{SE}$) significantly smaller than the control (Dunnett's multiple comparison test, $P \leq 0.05$). ns, not significant.

secondary tyrosine ammonia-lyase (TAL) activity is associated with the enzyme from monocots, including maize (Rosler *et al.*, 1997). TAL is highly selective for L-tyrosine, which is directly converted to *p*-coumarate, reducing the number of enzymes necessary for the production of this metabolite from two to one (Ferrer *et al.*, 2008). The next step is the activation of the acid to a thioester by action of 4-coumarate: CoA ligase (4CL) which yields *p*-coumaroyl-CoA. The *p*-coumaroyl-CoA is transesterified and hydroxylated by action of 4-hydroxycinnamoyl-CoA: shikimate/quinic acid 4-hydroxycinnamoyltransferase and *p*-coumarate 3-hydroxylase to generate caffeoyl-CoA. A methoxylation reaction catalyzed by caffeoyl-CoA *O*-methyltransferase produces feruloyl-CoA from caffeoyl-CoA. By the sequential action of cinnamoyl-CoA reductase, ferulate 5-hydroxylase, caffeic acid 3-*O*-methyltransferase and cinnamyl alcohol dehydrogenase (CAD) the CoA thioesters are converted in monolignols (*p*-coumaryl, coniferyl and sinapyl alcohols). Finally, oxidative polymerization of these three monolignols, by action of cell wall-bound peroxidase (POD), generates the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of the lignin.

Cinnamic and ferulic acids are known allelochemicals that affect the growth of several plant species (Cheng, 1995; Panteli and Voutsas, 2010). Some studies have suggested that a correlation between a reduction in plant growth and increased root lignin content is the consequence of the plant defenses acting against allelochemical stress (Devi and Prasad, 1996; Politycka, 1999; dos Santos *et al.*, 2004; Bohm *et al.*, 2006; Soares *et al.*, 2011). Stress-induced lignification can limit cell expansion, the capacity for nutrient uptake and the ability to sustain

plant growth. In previous reports, ferulic (dos Santos *et al.*, 2008), *p*-coumaric (Zanardo *et al.*, 2009), caffeic (Bubna *et al.*, 2011) and cinnamic (Salvador *et al.*, 2013) acid are shown to impair soybean root growth which was associated with changes in the key enzymes of the phenylpropanoid pathway, resulting in premature cell wall lignification. These data suggest that allelochemicals could be directly channeled into the phenylpropanoid pathway, increasing the lignin content, solidifying the cell wall and reducing soybean root growth.

All of the previously cited studies have evaluated the effects of allelochemicals in dicot plants. To extend these studies, the effects of cinnamic and ferulic acids in maize as a monocot plant model were evaluated on root growth, PAL and TAL activities, total lignin content and the composition of H, G and S monomers. To evaluate the possible entry of the exogenous allelochemicals into the phenylpropanoid metabolic route, experiments were performed using two selective enzymatic inhibitors of this pathway: piperonylic acid (PIP, a *quasi*-irreversible inhibitor of the enzyme C4H) and 3,4-(methylenedioxy)cinnamic acid (MDCA, a competitive inhibitor of the 4CL enzyme).

Material and Methods

General procedures

Maize (*Zea mays* L. cv. IPR-114) seeds were surface-sanitized with 2 % sodium hypochlorite for 5 min, rinsed extensively with deionized water and dark-germinated at 25 °C on two sheets of moistened filter paper. Twenty-five 3-d-old seedlings of uniform size

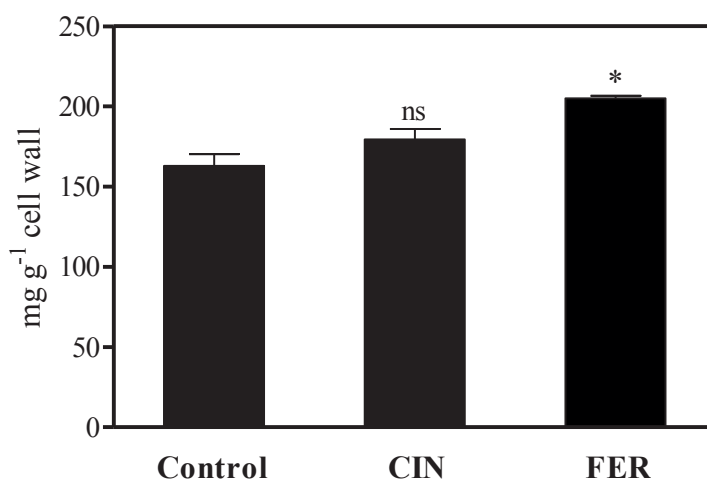


Figure 2. Effects of 1.0 mM cinnamic (CIN) and ferulic (FER) acids on lignin content of maize roots. *Mean ($n = 5 \pm \text{SE}$) significantly higher than the control (Dunnett's multiple comparison test, $P \leq 0.05$). ns, not significant.

were supported by an adjustable acrylic plate and dipped into a 10×16 cm glass container filled with 200 mL of half-strength Hoagland's solution (pH 6.0), with or without 1.0 mM cinnamic or ferulic acids. All nutrient solutions were buffered with 67 mM potassium phosphate buffer to eliminate the effects of very low pH. The concentrations of the allelochemicals were based on a survey of the literature (Siqueira *et al.*, 1991; Macias, 1995; Ng *et al.*, 2003; Batish *et al.*, 2008; Singh *et al.*, 2009; Zanardo *et al.*, 2009; Bubna *et al.*, 2011; Salvador *et al.*, 2013; Lima *et al.*, 2013). Additional experiments with 0.1 mM PIP or 1.0 mM MDCA were conducted, as indicated in the figure legends. The containers were kept in a growth chamber at 25 °C, with a light/dark photoperiod of 12/12 h and a photon flux density of $280 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 24 h. The roots were measured before incubation and at the end of experiments, and the difference in the lengths were calculated for all of the samples. Where indicated, the fresh root weight was determined immediately after incubation and the dry weight was estimated after oven-drying at 80 °C to a constant weight. Cinnamic and ferulic acids, PIP and MDCA were purchased from Sigma-Aldrich (St Louis, MO, USA) and all other reagents used were of the purest grade available or chromatographic grade.

Enzymatic assays

After incubation, all treated and untreated seedling roots were detached and enzymes were extracted. Phenylalanine ammonia-lyase (PAL) was extracted as described by Ferrarese *et al.* (2000). Fresh roots (1.0 g) were ground at 4 °C in 0.1 M sodium borate buffer (pH 8.8), homogenates were centrifuged (2200 g, 15 min)

and the supernatant used as the enzyme preparation. The reaction mixture (100 μmoles sodium borate buffer, pH 8.7, and a suitable amount of enzyme extract in a final volume of 1.55 mL) was incubated (40 °C, 5 min) for the PAL activity assay. L-Phenylalanine (15 μmoles) was added to start the reaction, which was stopped after 1 h of incubation by the addition of 50 μL of 5 M HCl. Samples were filtered through a 0.45- μm disposable syringe filter (Hamilton® Co., Nevada, USA) and 20 μL aliquots were analyzed in a high performance liquid chromatography (HPLC) system (Shimadzu® 10AVP, Tokyo, Japan) equipped with a LC-10AD pump, a Rheodyne® injector, an SPD-10A UV detector, a CBM-101 Communications Bus Module, and a Class-CR10 workstation system. A reversed-phase Shimpack® CLC-ODS column (150 mm $\times$ 4.6 mm, 5 μm) protected with an equivalent pre-column, was used at 30 °C. The mobile phase was 70 % methanol (v/v) with a flow rate of 0.8 mL min^{-1} for an isocratic run of 10 min and UV detection was carried out at 275 nm. Data collection and integration were performed with Class-CR10 software (Shimadzu®, Tokyo, Japan). *t*-Cinnamate, the product of PAL, was identified by comparing its retention time with a standard compound. Parallel controls without L-phenylalanine or with *t*-cinnamate (added as an internal standard in the reaction mixture) were performed as described elsewhere (Ferrarese *et al.*, 2000). PAL activity was expressed as $\mu\text{mol t-cinnamate h}^{-1} \text{g}^{-1}$ of fresh weight.

Tyrosine ammonia-lyase (TAL) was extracted as described by Khan *et al.*, (2003) with modifications. Fresh roots (1 g) were ground at 4 °C in 50 mM Tris-HCl buffer (pH 8.5), homogenates were centrifuged (2200 g, 10 min) and the supernatant used as the enzyme

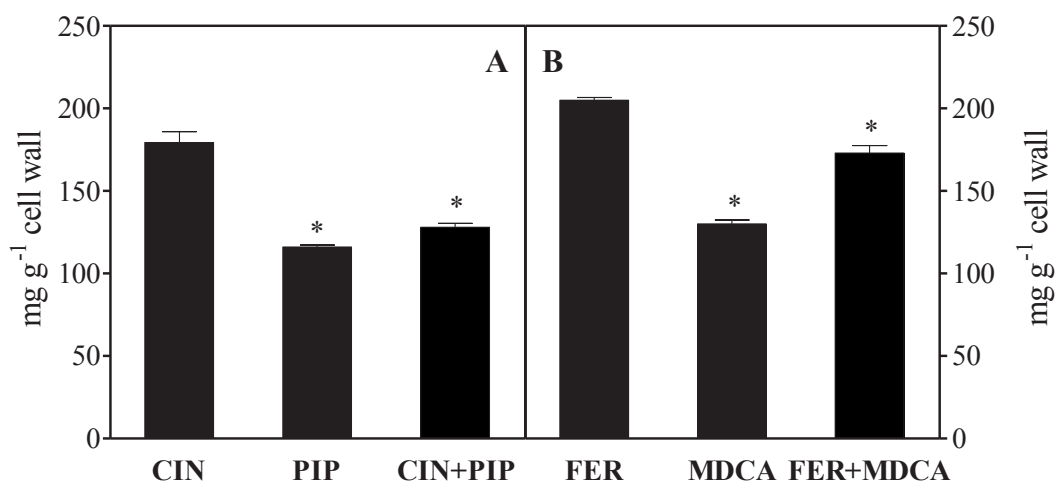


Figure 3. Lignin content in maize roots treated with 1.0 mM cinnamic acid (CIN), 0.1 mM piperonylic acid (PIP), 1.0 mM cinnamic acid plus 0.1 mM piperonylic acid (CIN+PIP), 1.0 mM ferulic acid (FER), 1.0 mM 3,4 (methylenedioxy)cinnamic acid (MDCA) and 1.0 mM ferulic acid plus 1.0 mM 3,4 (methylenedioxy)cinnamic acid (FER+MDCA). *Means ($n = 5 \pm SE$) significantly smaller than CIN (A) or FER (B) treatments (Dunnett's multiple comparison test, $P \leq 0.05$).

preparation. The reaction mixture (100 μ moles Tris-HCl buffer, pH 8.0, and a suitable amount of enzyme extract in a final volume of 0.95 mL) was incubated (40 °C, 5 min) for the TAL activity assay. L-Tyrosine (5.5 μ moles) was added to start the reaction, which was stopped after 1 h of incubation by the addition of 50 μ L of 5 M HCl. Samples were filtered through a 0.45- μ m disposable syringe filter (Hamilton® Co., Nevada, USA) and 20 μ L aliquots were analyzed by HPLC as described earlier. The mobile phase was methanol:4 % acetic acid (30 %:70 %) with a flow rate of 1.0 mL min⁻¹ for an isocratic run of 15 min and UV detection was carried out at 320 nm. *p*-Coumarate, the product of the TAL reaction, was identified by comparing its retention time with a standard compound. Parallel controls without L-tyrosine or with *p*-coumarate (added as an internal standard in the reaction mixture) were performed. Since cinnamic acid is converted into *p*-coumarate by the action of C4H, controls with and without L-tyrosine were made to eliminate any endogenous *p*-coumarate. TAL activity was expressed as μ mol *p*-coumarate h⁻¹ g⁻¹ of fresh weight.

Quantification of lignin and monomer composition

After the incubation period, dry roots (0.3 g) were homogenized in 50 mM potassium phosphate buffer (7 mL, pH 7.0) with a mortar and pestle and transferred into a centrifuge tube (Ferrarese *et al.*, 2002). The homogenate was centrifuged (1400 *g*, 6 min) and the pellet washed by successive washing and centrifugation as follows: twice with phosphate

buffer pH 7.0 (7 mL); 3× with 1 % (v/v) Triton® X-100 in pH 7.0 buffer (7 mL); 2× with 1 M NaCl in pH 7.0 buffer (7 mL); 2× with distilled water (7 mL); and 2× with acetone (5 mL). The pellet was dried in an oven (80 °C, 24 h) and cooled in a vacuum desiccator. The dry matter obtained was defined as a protein-free cell wall fraction. Lignin was assayed by the acetyl bromide method (Morrison, 1972). Samples (20 mg) of the protein-free cell wall fraction were placed in a screw-cap centrifuge tube containing 0.5 mL of 25 % acetyl bromide (v/v in glacial acetic acid) and incubated at 70 °C for 30 min. After complete digestion, the samples were quickly cooled on ice, mixed with 0.9 mL 2 M NaOH, 0.1 mL 7.5 M hydroxylamine-HCl and 2 mL glacial acetic acid. After centrifugation (1000 *g*, 5 min), the absorbance of the supernatant was measured at 280 nm and lignin content expressed as mg g⁻¹ cell wall.

The alkaline nitrobenzene technique was used to oxidize lignin and determine its monomeric composition (Zanardo *et al.*, 2009). The protein-free cell wall fraction (50 mg) was sealed in a Pyrex® ampule containing 1 mL 2 M NaOH and 0.1 mL nitrobenzene and heated to 170 °C for 150 min with occasional shaking. The sample was then cooled at room temperature, washed twice with chloroform, acidified to pH 2 with 5 M HCl and extracted twice with chloroform. The organic extracts were combined, dried, resuspended in 1 mL methanol and diluted in methanol/4 % acetic acid in water (20:80, v/v). All the samples were filtered through a 0.45- μ m disposable syringe filter (Hamilton® Co., Nevada, USA) and 20 μ L aliquots analyzed by HPLC as previously

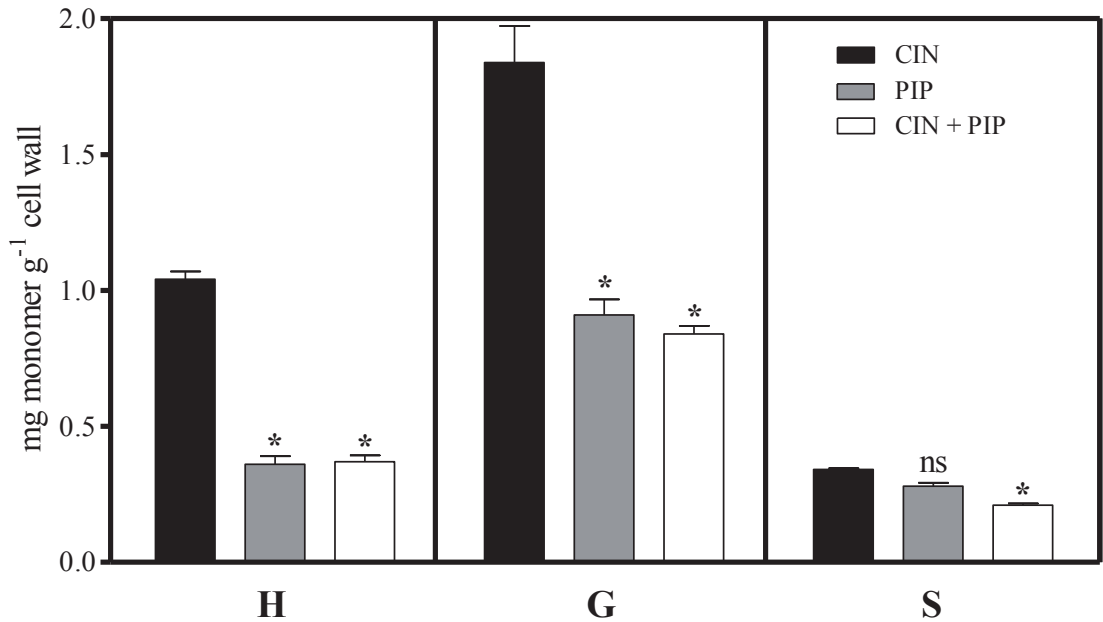


Figure 4. Lignin monomer composition in maize roots treated with 1.0 mM cinnamic acid (CIN), 0.1 mM piperonylic acid (PIP) and 1.0 mM cinnamic acid plus 0.1 mM piperonylic acid (CIN+PIP). H, *p*-hydroxyphenyl; G, guaiacyl; and S, syringyl monomers. *Means ($n = 3 \pm \text{SE}$) significantly smaller than CIN treatment (Dunnett's multiple comparison test, $P \leq 0.05$) ns, not significant.

described. The mobile phase consisted of a mixture of methanol and 4 % acetic acid in water (20:80, v/v) with a flow rate of 1.2 mL min⁻¹ for an isocratic run of 20 min. Quantification of the monomer aldehyde products (*p*-hydroxybenzaldehyde, vanillin and syringaldehyde) released by nitrobenzene oxidation was performed at 290 nm using the corresponding standards and results are expressed as mg monomer g⁻¹ cell wall.

Statistical analyses

The experimental design was randomized and each plot represented one glass container with 25 seedlings. The data were expressed as the mean of three to six independent experiments $\pm$ SE. An analysis of variance was used to test the significance of differences (Prism 5, GraphPad Software Inc., San Diego, CA). The differences between the parameters were evaluated using Dunnett's multiple comparison test. P values ≤ 0.05 were considered to be statistically significant.

Results

• Effects on root growth

Table 1 summarizes the effects of allelochemicals on maize root growth (lengths and weights). Cinnamic

acid was the most effective inhibitor of root length, *i.e.*, 93.7 % reduction compared with the control, while ferulic acid reduced root growth by 77.9 %. The effects of both compounds were also evident for root fresh weights, which significantly decreased after cinnamic (27.6 %) and ferulic (22.5 %) acid treatments when compared with the controls. Similarly, root dry weights were reduced by cinnamic (25.2 %) and ferulic (19.6 %) acids.

• Effects on PAL and TAL activities

In agreement with the effects observed on root growth, phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) enzyme activities in seedlings treated with cinnamic and ferulic acids were different from those of controls (Fig. 1). Roots exposed to ferulic acid had significantly decreased PAL activity by 29 % in comparison to the control (2.21 $\mu\text{mol h}^{-1} \text{g}^{-1}$ fresh weight). Compared with the control (0.31 $\mu\text{mol h}^{-1} \text{g}^{-1}$ fresh weight), cinnamic and ferulic acids treatments decreased TAL activities by 42 % and 46 %, respectively.

• Effects on lignin content and its monomeric composition

Cinnamic acid (CIN) did not affect the lignin content in comparison with the control (163 mg g⁻¹ cell

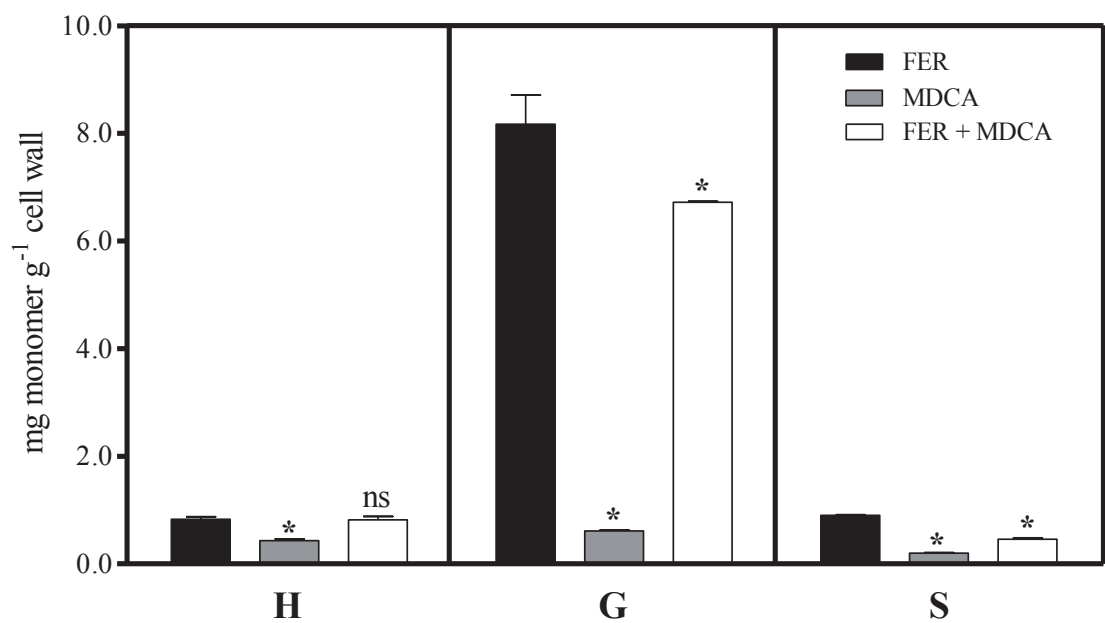


Figure 5. Lignin monomer composition in maize roots treated with 1.0 mM ferulic acid (FER), 1.0 mM 3,4 (methylenedioxy) cinnamic acid (MDCA) and 1.0 mM ferulic acid plus 1.0 mM 3,4 (methylenedioxy)cinnamic acid (FER+MDCA). H, *p*-hydroxyphenyl; G, guaiacyl; and S, syringyl monomers. *Means ($n = 3 \pm SE$) significantly smaller than CIN treatment (Dunnett's multiple comparison test, $P \leq 0.05$) ns, not significant.

wall) (Fig. 2). By contrary, the exposure of maize roots to ferulic acid (FER) increased lignin content by 25.8 %.

Table 2 shows the effects of allelochemicals on lignin monomer composition. Compared with the corresponding controls, cinnamic acid decreased the contents of G (50.9 %) and S (67.9 %) monomers. The analysis of monomers revealed an increase (117.9 %) in the content of G and a slight decrease (15 %) in the S monomer after exposure to ferulic acid. Table 2 also summarizes the results of lignin (referred to as the sum of H+G+S) measurements. While cinnamic acid decreased the H+G+S contents by 43.8 %, ferulic acid increased it by 72.8 % in comparison to the controls. The H:G:S ratio was altered by the action of allelochemicals as follows: cinnamic acid increased H and ferulic acid increased G, compared with the control ratio.

- Effects of cinnamic and ferulic acids and enzyme inhibitors on lignin content and its monomeric composition

Subsequent analyses were conducted to evaluate the changes in lignin content and monomeric composition in response to these allelochemicals when selective inhibitors of phenylpropanoid pathway were used. PIP (a *quasi*-irreversible inhibitor of the enzyme C4H) reduced lignin content by 35 % in comparison to the cinnamic acid (CIN) alone (Fig. 3A). Similarly, a reduction (29 %) in the lignin content was noted in roots exposed to cinnamic acid plus inhibitor (CIN+PIP) compared with the cinnamic acid (CIN) treatment alone. Treatment of roots with MDCA

Table 1.

Changes in the root length and fresh and dry weights of maize treated for 24 h with 1.0 mM cinnamic and ferulic acids.

Condition	Root length (cm)	%	Fresh weight (g)	%	Dry weight (g)	%
Control	3.03 ± 0.02		2.75 ± 0.13		0.194 ± 0.002	
Cinnamic acid	0.19 ± 0.02*	-93.7	1.99 ± 0.04*	-27.6	0.145 ± 0.001*	-25.2
Ferulic acid	0.67 ± 0.04*	-77.9	2.13 ± 0.07*	-22.5	0.156 ± 0.004*	-19.6

Means ($n = 4 \pm SE$) significantly smaller than the control (Dunnett's multiple comparison test) are marked with an asterisk (*). The column % represents inhibition of statistically significant means in comparison to control (0 mM).

Table 2. Changes in the lignin monomer composition of maize treated for 24 h with 1.0 mM cinnamic and ferulic acids. The results are expressed as mg monomer g⁻¹ cell wall.

Monomer	Control	Cinnamic acid	Ferulic acid
H	0.92 ± 0.03	1.04 ± 0.03 ^{ns}	0.83 ± 0.05 ^{ns}
G	3.75 ± 0.05	1.84 ± 0.13*	8.17 ± 0.54*
S	1.06 ± 0.01	0.34 ± 0.05*	0.90 ± 0.02*
H + G + S	5.73 ± 0.08	3.22 ± 0.18*	9.90 ± 0.60*
H : G : S	16 : 65 : 19	32 : 57 : 11	8 : 83 : 9

H, *p*-hydroxyphenyl; G, guaiacyl and S, syringyl. Means ($n = 3 \pm \text{SE}$) significantly smaller than the control (Dunnett's multiple comparison test) are marked with an asterisk (*). ns, not significant.

(a competitive inhibitor of the enzyme 4CL) and ferulic acid plus MDCA (FER+MDCA) reduced lignin content by 37 % and 16 %, respectively, in comparison to the ferulic acid (FER) alone (Fig. 3B).

Lignin monomer composition was evaluated by alkaline nitrobenzene oxidation (Fig. 4). Following treatment with PIP, the H and G monomer contents decreased by 65 % and 51 %, respectively, compared with the cinnamic acid (CIN) alone. The exposure of roots to the allelochemical plus inhibitor (CIN+PIP) significantly reduced H (64 %), G (54 %) and S (38 %) contents compared with cinnamic acid (CIN) treatment. The analysis of monomer content revealed significant decreases in H (48 %), G (93 %) and S (78 %) after exposure to MDCA compared with the allelochemical (FER) treatment (Fig. 5). In conjunction with ferulic acid, MDCA reduced the content of G (18 %) and S (19 %) monomers compared with the allelochemical treatment.

Discussion

Plant roots are susceptible to allelochemical stress during early seedling growth (Inderjit and Duke, 2003; Weir *et al.*, 2004) due to high metabolic activity and the start of lignification at this stage (Donaldson, 2001). Studies with cinnamic and ferulic acids have shown inhibitory effects on root growth (length and weight) in different plants species (Baleroni *et al.*, 2000a; Ng *et al.*, 2003; Ding *et al.*, 2007; Macias *et al.*, 2007; Politycka and Mielcarz, 2007; Reigosa and Pazos-Malvido, 2007; Salvador *et al.*, 2013) including maize (Janovicek *et al.*, 1997; Prasad and Devi, 2002; Gelsomino *et al.*, 2015). This study further confirmed the susceptibility of maize for both compounds, which reduced the length as well as the fresh and dry weights of roots.

Different modes of action have been proposed for the inhibition of plant growth by allelochemicals

(Dayan *et al.*, 2000). Disruption and depolarization of the cell membrane, increases in lipid peroxidation and reactive oxygen species (ROS), decreased H-ATPase activity and loss of viability (Onnerud, 2002; Yu *et al.*, 2003; Ding *et al.*, 2007). As a cascade, many other metabolic processes such as photosynthesis and cellular respiration (Weir *et al.*, 2004), protein synthesis (Baziramakenga *et al.*, 1997), lipid mobilization (Baleroni *et al.*, 2000b), glucose metabolism (Ferrarese *et al.*, 2001) and enzyme activities (Doblinski *et al.*, 2003; Salvador *et al.*, 2013) can be affected. Despite the relevance of the cell membrane as a possible site of allelochemical action, the cell wall can be considered one of the first tissues affected by stress signals, which are then transmitted to the cell interior and influence additional processes (Komatsu *et al.*, 2010). It is also known that changes in plant growth can be attributed to changes in the conductive tissues, such as phloem and xylem (Chen *et al.*, 2006). Previous work suggests that the impairment of root growth by allelochemicals is associated with premature lignification of the cell walls (Komatsu *et al.*, 2010; Bubna *et al.*, 2011; Salvador *et al.*, 2013) as well as structural changes in root cells consistent with enhanced lignin production (dos Santos *et al.*, 2008). However, all these reports have addressed the effect of allelochemicals on soybean, a dicotyledonous plant. In the current work, experiments were conducted with maize, a monocotyledonous plant, to test whether phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activities and the production and composition of lignin were affected by allelochemicals.

As described earlier, PAL and TAL reactions form cinnamate derivatives which form monolignols that are polymerized within lignin (Vanholme *et al.*, 2012). After ferulic acid treatment, PAL and TAL activities decreased in spite of lignin production and there was enhanced formation of G monomer and a high

H+G+S value. Effects of phenolic allelochemicals on PAL activity are contradictory and can be stimulatory (Politycka, 1999; dos Santos *et al.*, 2004), have no effect (Shann and Blum, 1987) or be inhibitory (Sato *et al.*, 1982; Zanardo *et al.*, 2009; Bubna *et al.*, 2011). While the action of allelochemicals on PAL activity have been reported, their effects on TAL have not been described to date. It was hypothesized that the high influx of exogenous ferulic acid into the phenylpropanoid pathway, via the 4CL reaction, inhibited PAL/TAL, presumably by a feedback process (Blount *et al.*, 2000). As a consequence, the low levels of phenylpropanoid derivatives could be supplied by exogenous ferulic acid with subsequent lignin production. In soybean roots, other hydroxylated cinnamic acid derivatives such as *p*-coumaric and caffeic acids decreased PAL activity but increased lignin production (Zanardo *et al.*, 2009; Bubna *et al.*, 2011). This study showed that cinnamic acid decreased TAL activity, the levels of G and S monomers as well as lignin (sum of H+G+S). Upon initial observation, there were no clear explanations for these findings. However, as the phenylpropanoid pathway was subject to complex control, it was possible that cinnamic acid inhibited other enzymes such as CAD which catalyzed the final step in a branch of phenylpropanoid synthesis specific for the production of lignin monomers. Another possibility was that cinnamic acid itself could induce potential side effects, affecting maize root lignification.

Since exogenous allelochemicals can be incorporated into the structure of lignin by means of the phenylpropanoid pathway (dos Santos *et al.*, 2008; Zanardo *et al.*, 2009; Bubna *et al.*, 2011; Salvador *et al.*, 2013), changes in lignin monomer composition caused by specific inhibitors could be enlightening. Because cinnamic acid forms *p*-coumaric acid through the action of the C4H enzyme, maize roots were stressed by the allelochemical, alone or jointly with PIP, a *quasi*-irreversible inhibitor of the enzyme (Schoch, 2002). As expected, the exposure of maize roots to the PIP reduced the total lignin content and the amount of H and G monomers in comparison to the ferulic acid treatment alone. Jointly with cinnamic acid, PIP reduced total lignin content and the content of all monomers. These findings indicated that the C4H inhibitor prevented either the entry of the allelochemical into the metabolic pathway or the formation of lignin and lignin monomers. These observations were supported by reductions in the H+G+S sum and alterations of the H:G:S ratio (32:57:11) which differed from controls (16:65:19). These findings clearly indicated that exogenously applied cinnamic acid entered into the phenylpropanoid pathway via the C4H reaction and reduced the production of lignin

and its monomers. Based on these data, decreased maize root growth caused by cinnamic acid was not related to enhanced lignification, as hypothesized for soybean (Salvador *et al.*, 2013).

To examine whether exogenous ferulic acid can be channeled into the phenylpropanoid metabolic route, experiments were performed using MDCA, a competitive inhibitor of the 4CL enzyme which converts hydroxycinnamic acids to hydroxycinnamoyl-CoA thioesters (Chakraborty *et al.*, 2009). Results showed that MDCA reduced the total lignin and lignin monomers compared to the ferulic acid treatment while treatment with ferulic acid and MDCA reduced the content of total lignin and G/S monomers. As MDCA inhibited 4CL, the access of exogenous ferulic acid to the phenylpropanoid pathway was blocked. In contrast with the results for cinnamic acid, ferulic acid increased the sum of H+G+S and changed the H:G:S ratio (8:83:9) primarily due to the enhanced formation of the G monomer. These findings strengthened the hypothesis that ferulic acid could be channeled into the phenylpropanoid pathway via the 4CL reaction. Furthermore, increased lignin production was associated with decreased maize root growth, which was similar to effects reported for soybeans (dos Santos *et al.*, 2004; 2008). Supporting evidence for this hypothesis comes from studies in which the incorporation of exogenous ferulic acid leads to the formation of feruloyl-CoA and further to coniferyl and sinapyl alcohols in poplar (Hamada *et al.*, 2003) and that labeled ferulic acid is incorporated into G and S monomers followed by an increase in the cell-wall lignification in robinia (Yamauchi and Fukushima, 2004).

Conclusions

The results obtained in the present research suggested that 1) cinnamic acid reduced maize root growth, TAL activity and lignin (H+G+S) content. The use of PIP as a selective inhibitor of the C4H reaction indicated the specific entry point of this allelochemical into the phenylpropanoid pathway. However, for this study there was no direct link between the reduction of root growth and the lignification process. 2) Ferulic acid reduced maize root growth and PAL/TAL activities but increased G monomer production and lignin (H+G+S) levels. The use of MDCA as a specific inhibitor of the 4CL reaction indicated that ferulic acid could be channeled into the phenylpropanoid pathway. In summary, inhibition of maize root growth may be attributed to the excessive production of lignin due to exogenously applied ferulic acid, as enhanced lignin polymerization solidified the cell wall and restricted plant growth.

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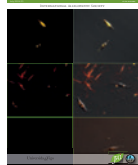
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Transformation and Activity Change of Selected Allelochemicals by Microbial Metabolisation

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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

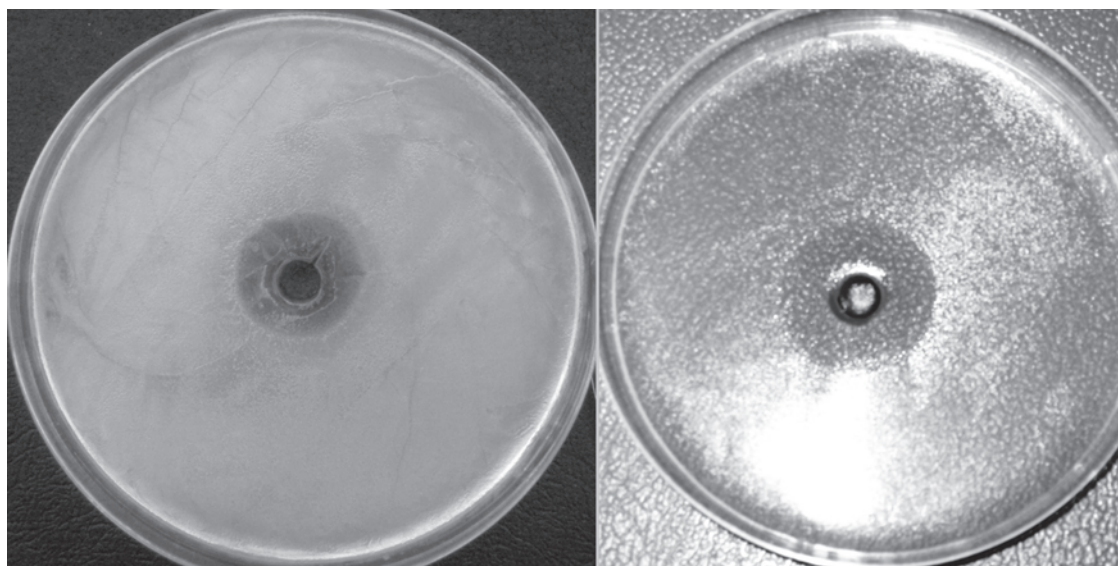


Figure 1. Inhibition zones of *Pseudomonas* sp. (left) and *Athrospira* sp. (right) with extract from Himalayan Balsam leaves placed in the centre hole.

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-

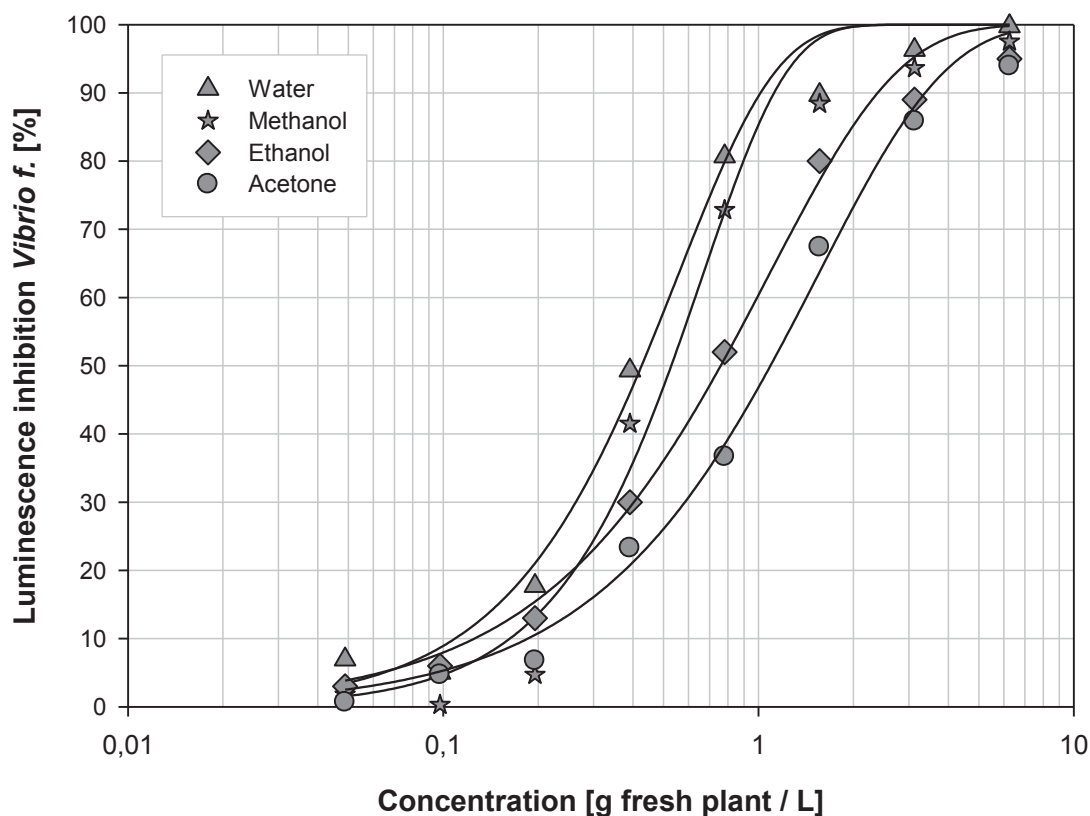


Figure 2. Dose-response-relations of *Impatiens glandulifera* leaf extracts made with different solvents for luminescent bacteria. Each data point in the graph represents the mean of double determinations, the lines are the curve fitted functions, according to Weibull.

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.

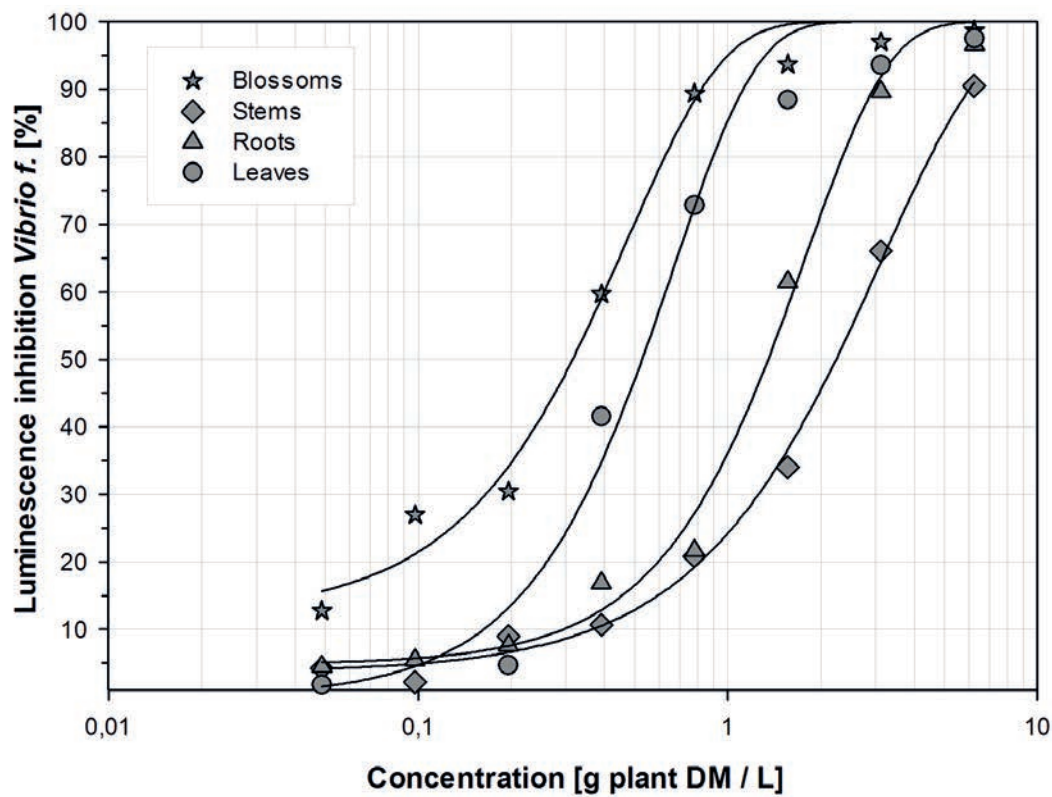


Figure 3. Dose-response-relations for methanol extracts produced from different compartments of *Impatiens glandulifera* demonstrating the effect against luminescent bacteria (*Vibrio fischeri*). Each data point in the graph represents the mean of double determinations, the lines are the curve fitted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

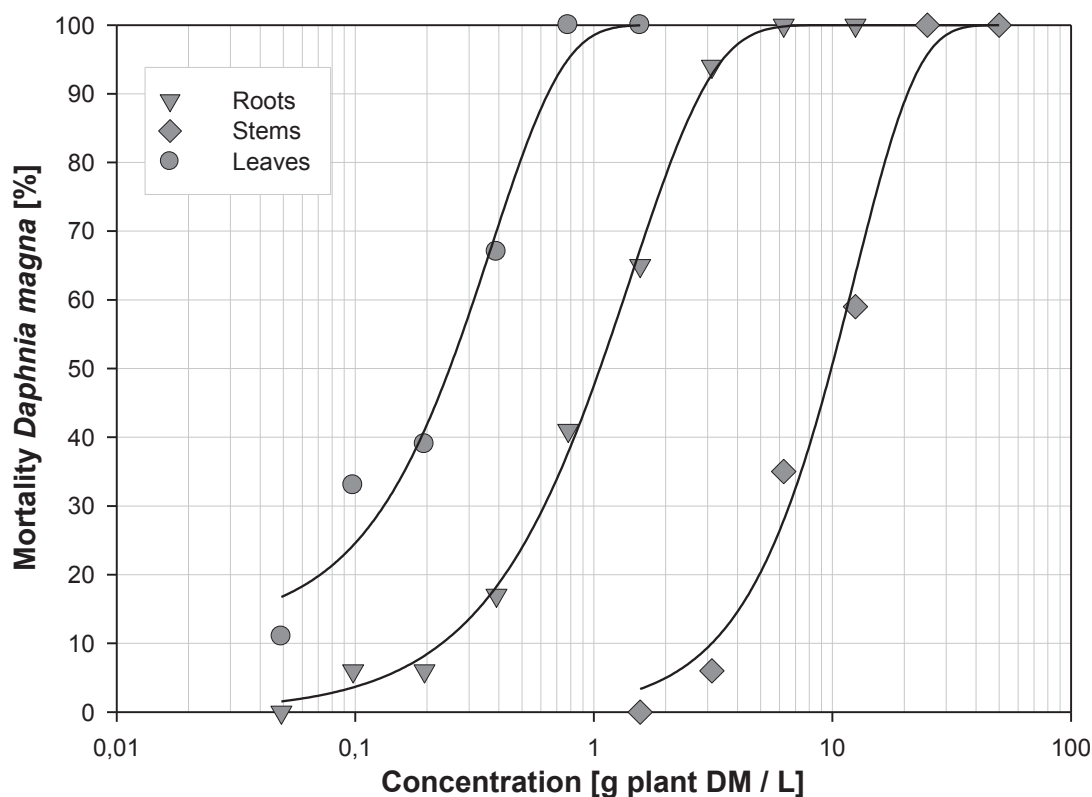


Figure 4. Dose-response-relations of extracts made from different compartments of *Fallopia japonica* to demonstrate the effect against water flea (*Daphnia magna*). Each data point in the graph represents the mortality of 18 animals, the lines are the curve fitted functions, according to Weibull.

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.

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

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

legend to Equation 1:

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

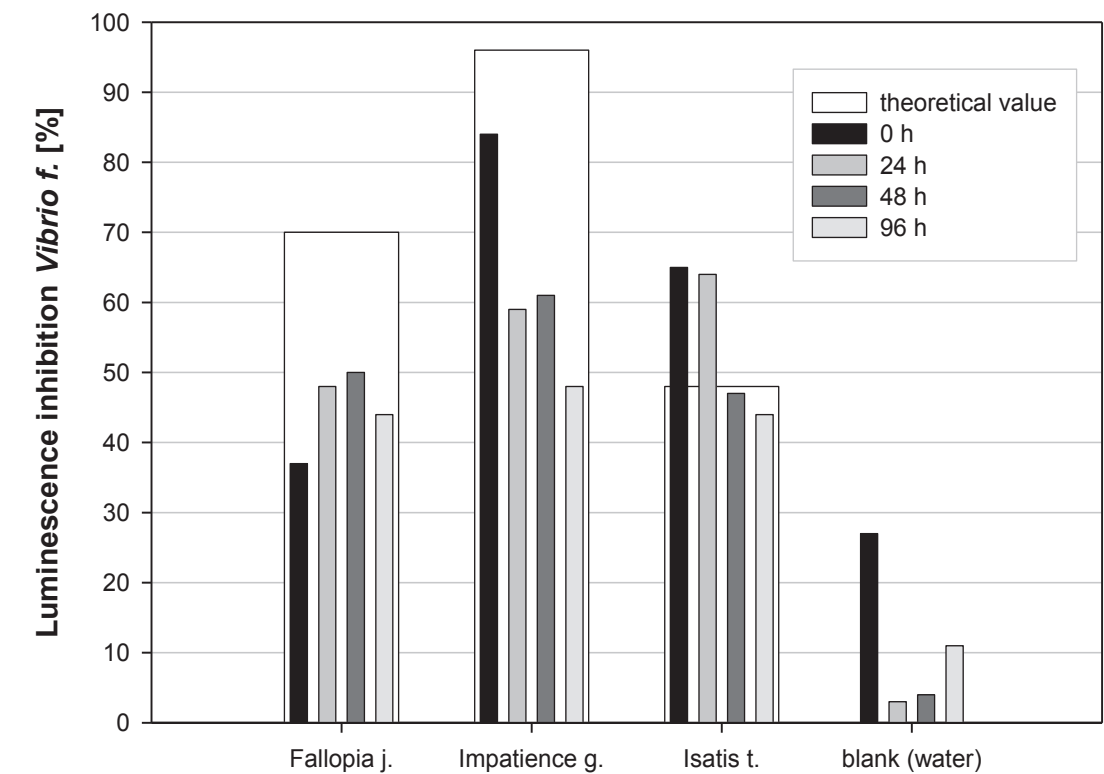
y = inhibition [%]

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

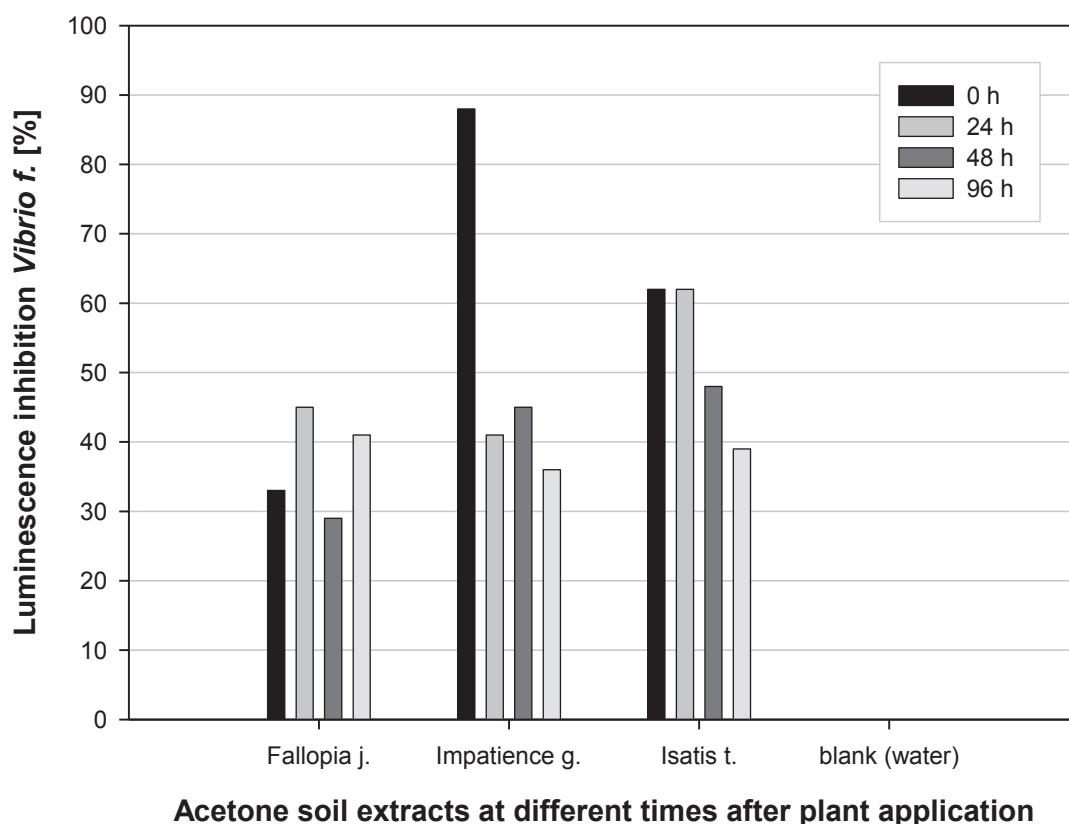


Figure 6. Inhibition dynamics of plant extracts for luminescent bacteria in the third soil contact experiment, with samplings at 0, 24, 48 and 96 hours. The soil extracts were prepared using acetone as solvent.

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.

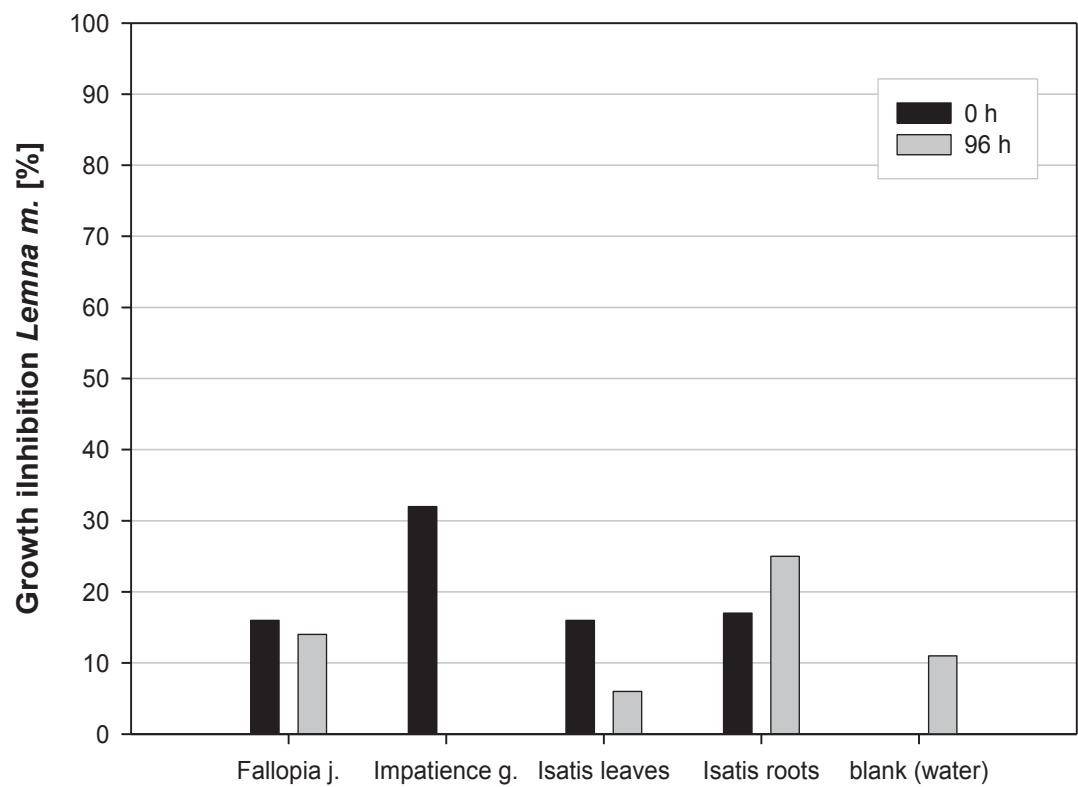


Figure 7. Growth inhibition dynamics of plant extracts for duckweed in the second soil contact experiment, with samplings at 0 and 96 hours. The soil extracts were prepared using water as solvent.

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

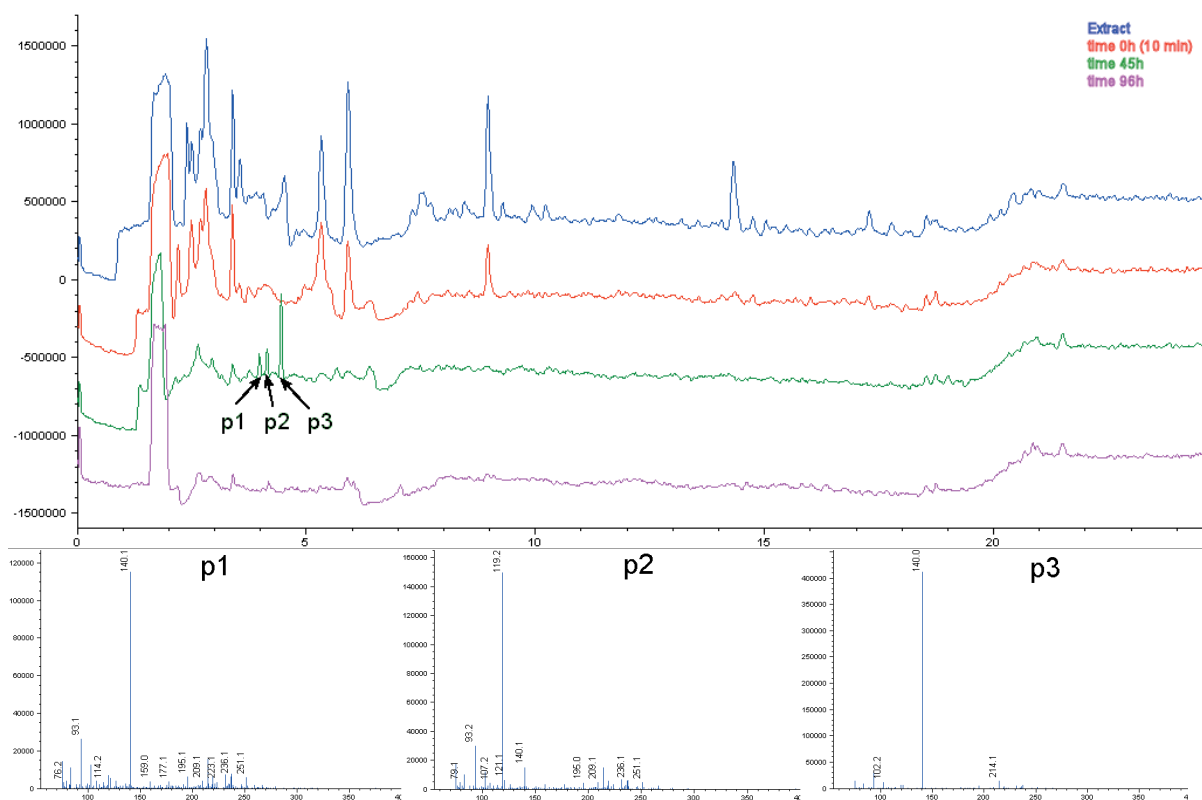


Figure 8. HPLC-MS chromatograms of aqueous extracts of the second soil contact experiment with *Isatis t.* Graph details as described in Figure Fehler: Referenz nicht gefunden. Mass spectra of peaks at retention times 3:55 (p1), 4:15 (p2) and 4:30 min (p3) in the soil extract after 45 hours.

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

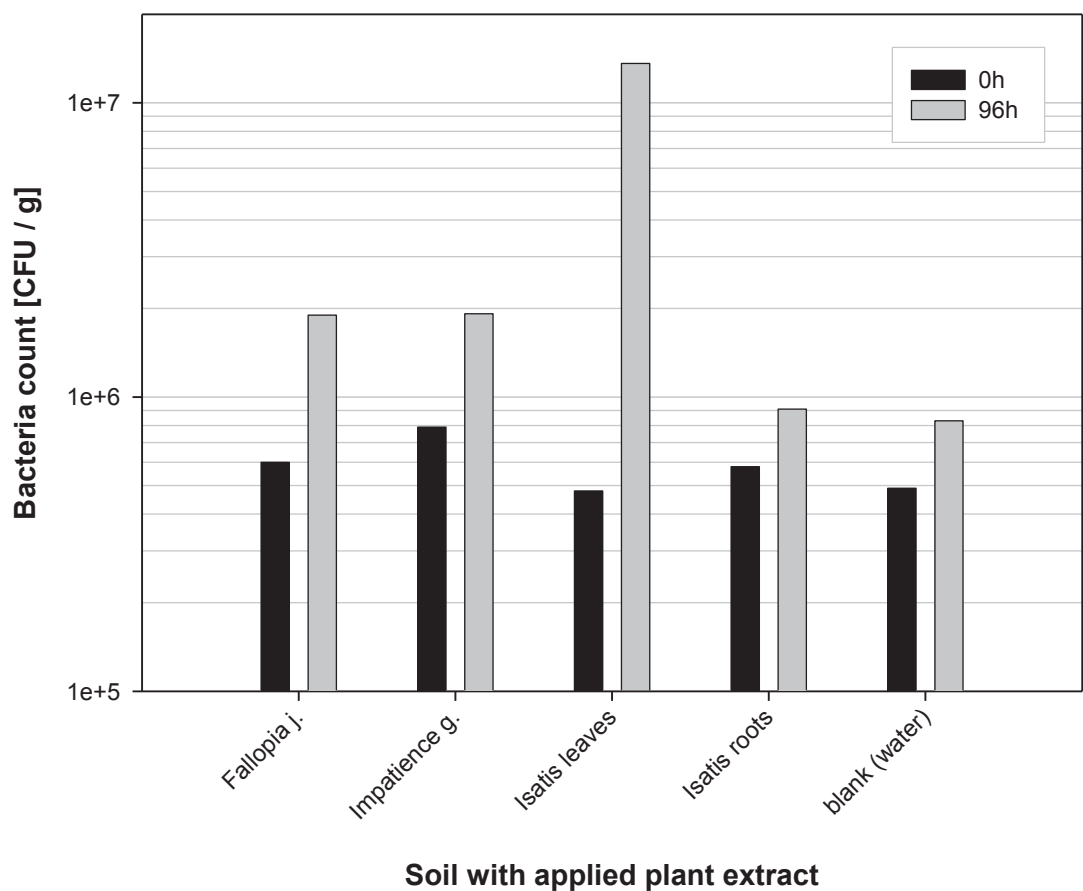


Figure 9. Colony forming units (CFU) for bacteria of the second soil contact experiment at the beginning and after 96 hours.

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

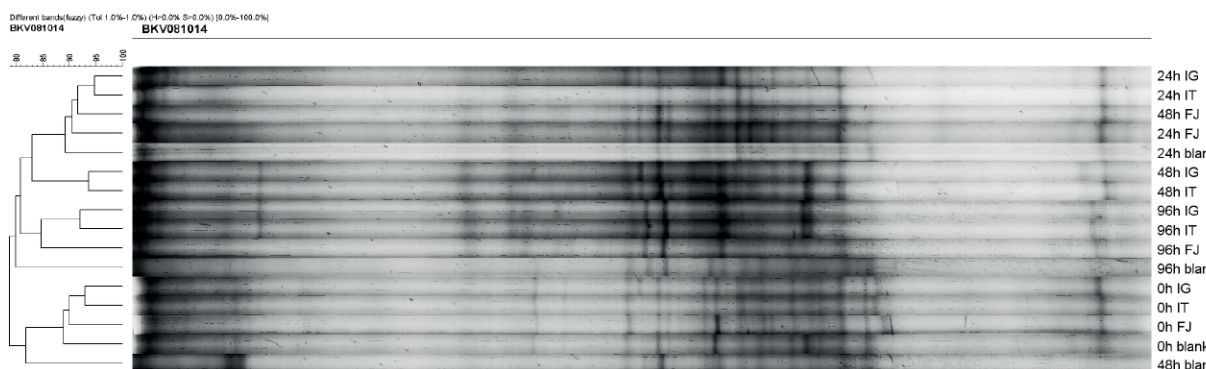


Figure 10. Dendrogram for bacteria populations in soil at different times (0, 24, 48, 96 hours) after plant extract application in experiment No. 3 (left). The band patterns were obtained by DGGE (right), the cluster analysis was based on UPGMA with band appearances and Pearson coefficient. IG = *Impatiens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

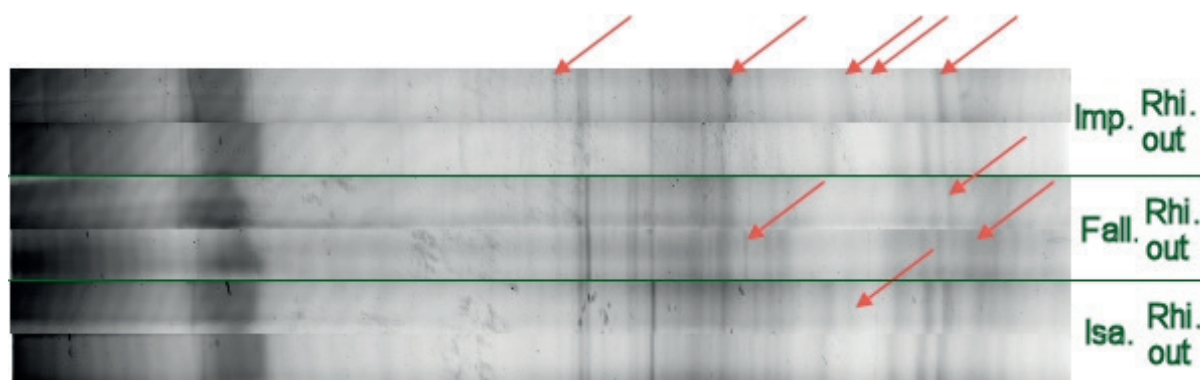


Figure 12. DGGE band patterns of bacteria populations in soil within and outside the root zone of the three selected plants: Imp. = *Impatiens g.*, Fall. = *Fallopia j.*, Isa. = *Isatis t.*, Rhi. = within root zone (rhizosphere + some adhering soil), out = outside root zone. The most obvious differences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

Supplementary data

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Table S1.

Analysis parameters of the soil from the IFA-site used for degradation experiments

Parameter	Result	Unit
Fraction 0-2 mm	100	%
Fraction >2 mm	0	%
DM (dry matter)	90.2	%
VS (volatile solids)	4.3	%
WHC (Water Holding Capacity)	0.52	g H ₂ O / g DM
pH-value	7.5	-
Basic respiration	0.11	mg CO ₂ / g DM*24 h
Glucose-induced respiration	0.13	mg CO ₂ / g DM*24 h

Table S2.

Method parameters for HPLC separation of aqueous plant and soil extracts, intended for comparing fingerprint analysis and mass identification of components and metabolites. Method based on (Eljarrat and Barceló, 2000; Eljarrat *et.al.*, 2004), modified for higher resolution of polar compounds.

Instrument part	Type / Parameter
Instrument	hewlett packard HPLC-MS Series 1100
Column	Phenomenex Synergy 4µm MAX-RP 80A; 35°C
Mobile phase	solvent A: 0.05% acetic acid in water; solvent B: methanol; flow: 1.0 ml / minute
Gradient separation	0-4 min: 5% B; 4-17 min: linear to 50% B; 17-22 min: linear to 95% B; 22-27 min: 95% B; 27-30 min linear to 5% B; post-run time: 4 minutes
Sample injection	25 µl aqueous solution; filtered through 0.22 µm pore size
Detection	mass selective detection (Quadrupol), spray chamber 350°C, 12 L/min N ₂ ; API-ES ionisation, positive mode, fragmentor voltage: 70 V; mass detection window: 75-500 Da

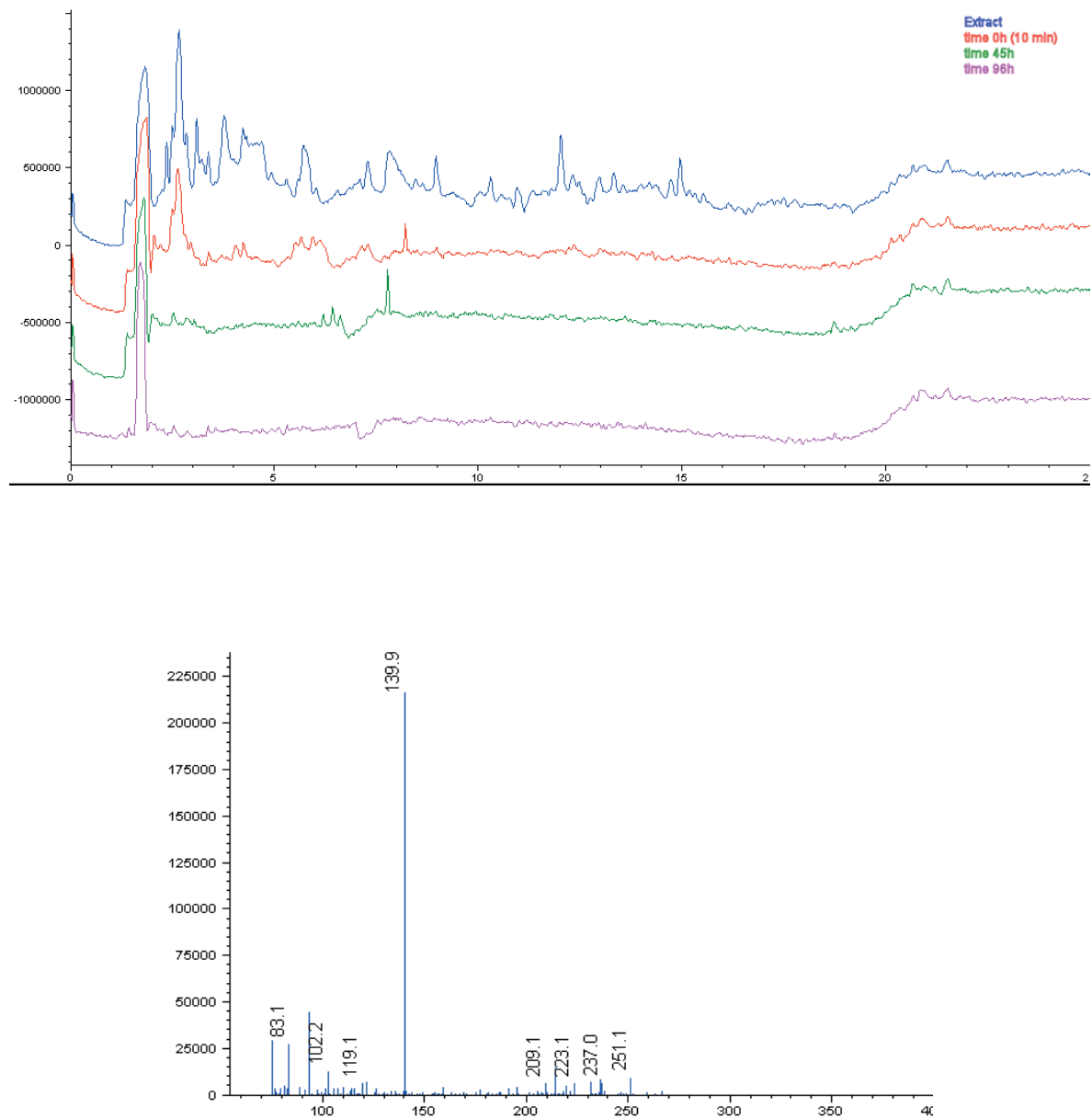


Figure S1.

HPLC-MS chromatograms of aqueous extracts of the second soil contact experiment with *Fallopia j.* Experiment times are (from top to bottom): plant extract before application, at 0, 45 and 96 hours. All extracts were analysed at the same dilution rate. Mass spectrum of peak at retention time 7:50 min (arrow) in the soil extract after 45 hours.

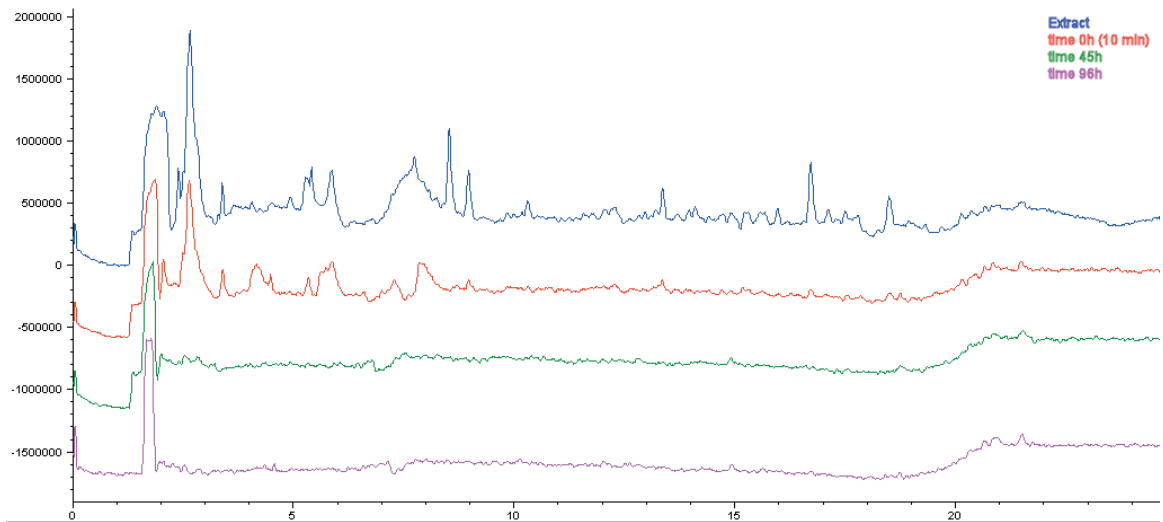


Figure S2.

HPLC-MS chromatograms of aqueous extracts of the second soil contact experiment with *Impatiens g.* Graph details as described in Figure S1.

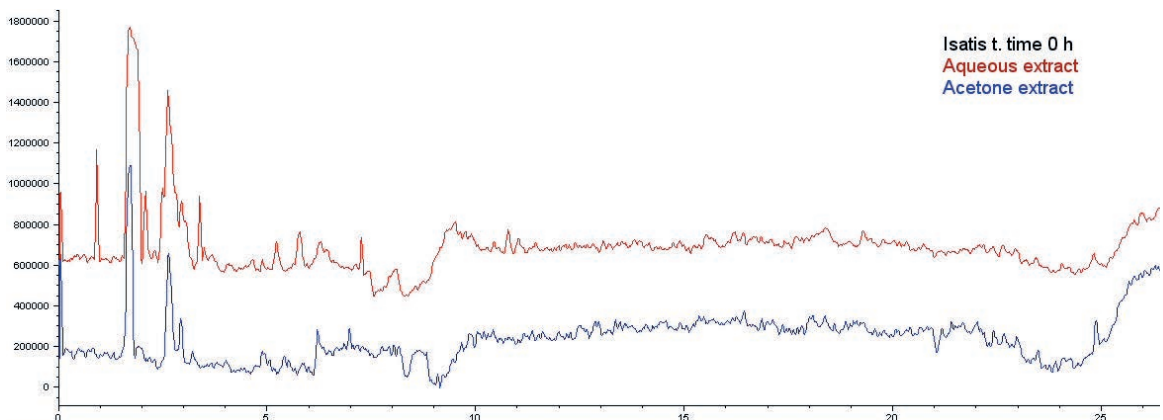


Figure S3.

HPLC chromatograms of aqueous (upper) and acetone (lower) extracts of the third soil contact experiment with *Isatis t.* at 0 hours. Both extracts were analysed at the same dilution.

Sequence data (identified by NCBI-blast)

The sample identifiers correspond to the band numbering in Figure 11 of the main article, provided here as a smaller copy for quick reference.

NCBI-blast results, 16S-rDNA 341f-907r, threshold 99%:



>1-1f well A01 UT080626 Run01 Cimarron 4.00 Even Spacing 428
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TAATCATTGCTCGTAGCCTTTGACGGTACCTAATCCAAGATAACGCCACC
GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTACCGTGTGCAAGCGTT
GTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGNGGTCCTTTAAGTCTGA
TGTGAAAGCCCCACGGCTCAACCGTGGANGGTCATTGGAACTGGGGGAC
TTGAGTGCAGAAAGGAGAGTTGGAATCCCCGTGTTAACCGTTGAAAAAT
GGCGTAAANATTTTGGAGGAAACACCCAGTTGGCGAAAGGCCACTTTTC
CTGGTCTNTTTAACCTTGAANCCCTAAGGCCCAAAAACCNGTTGGGGGAG
CAAAACCAAGGAATTTAATACCCCTGTTATCCACCCCTAAACAATTATTG
CTATTNTTGGGGGGTTTCTGCCCTTTTGTCTACTTACGCTTTTAACAC
TCCGCCTGGGGATTCTGGGCCCGCANNCTAACTCAAGAAATTTTACGTCGG

ID: Bacillus cereus

>1-1r well E01 UT080626 Run01 Cimarron 4.00 Even Spacing 511
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AGGATATCTAATCCTGTTTGCTCCCCACGCTTTCGCTGCCCTCAGTGTGAG
TTACAGACCAGAAAGTCTGCCTTCGCCACTGGTGTTCCTCCATATCTCTA
CGCATTTACCCGCATACACGTGGAATTCCACTCTCCTCTTCATGCACTCA
AGTCCCCCAGTTTCCAAGTGACCCTCCACGGTTGAGCCGGGGGCTTTTAC
ATACAGACTTAAAGGACCGCTGCGCGCGCTTTAACGCCCAATAATTCCG
GAAAACGCTTGCCACCTACTGTATTACCCGGGGGTTGGTGGCACGTAGT
TAGCCGTTGGCTTTCTNGTTAGGTACCGTCAAGGTACCAGCAGTTTACTCT
CGTACTTTTTCTCCCTAACAACAACCTTACACCCGAAGCCTTCTTCCTC
ACGCGGTTTGTCTCTCAACTTCCCCCATTCNAAAAATCCCCACNTCTC
GCCCCCCCCA

ID: Bacillus cereus

>1-3f well A02 UT080626 Run01 Cimarron 4.00 Even Spacing 476

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AACAACCTACCAGTACTGTAATCTTGCTACGCTACCTTGACGTGTACCTA
ATCAAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAC
GTGTGCAAGCGCTTGTCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGG
TCCTTTAAGTCTGATGTGAAAGCCCCCGCTCAACCGTGGAGGGTCATTG
GAAACTGGGGGACTTGAGTGCAGAAAAGGAGAGTGGAAATTCCACCGTGT
ACGGTAAATGCGTAATATTTGGGAGGAAACACCAAGTGGGCGAAAGGGCA
ACTTTCCTTGGGTCCTTTTAACTTGAACACTCAGGCGCGGAAAAGCGTT
GGGGAGCAAACCATGATTAATAACCTGGTATCCACGCGTAAACGATAT
GCTAATTGTAGGGGGGTTTCTGCCNTTATGCTGCTAAACGCATTA
ACACTCCGCTGGGGATTACGGGGCCTGNAAGNCTAAACTCAAGNAATTT
TAGT

ID: Bacillus sp. SG-1 or Bacillus cereus

>1-3r well E02 UT080626 Run01 Cimarron 4.00 Even Spacing 509

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CTGATGGACTACCAGAGATATACTAATTTCTGTTTCGCTCCCCACGCTT
TCGCTGCCTCAGTGTACAGACCAGAGAAGTCTGCCTTACGCCACT
GTGTGTTCTCACATATCTCTACTGCATTTACCGCTACACTGTGNGAAT
TCCACTCTCTCTTCTTGCACTCAAGTCCCCAGTTTCCAAATGACCCTC
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CGCGCTTACCGCCCAATAATTCCGGAACGCTTGCCACCTACTTAAT
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CCGTCAAGTACCACATTTACTCTCGTACTTGTCTTCCCTAACACAGAC
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TCCNCCATTGCGAAAAAATCCCCATGTCGCCCCCA

ID: Bacillus sp. SG-1

>2-1f well A03 UT080626 Run01 Cimarron 4.00 Even Spacing 439

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AACGTTAATCATTTGCTCGTAGCCTTGACGTGTACCTAATCACAAGAAACG
CCACGGCTCAACTACGTGCCACCAGCCGCGGTCAATACGTACGTGTGCAA
TGCGCTTGTCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTNCTTTA
AGTCTGATGTGAAAGCCCCCGCTCAACCGTGGAGGGTCATTGGAACTG
GGGACTTGAGTGCAGGAAAGGAGAGTGGAAATCCACTTGTACGGTGAAA
TGCGTATATATTTGGAGGAACACCAGTTGGCGAAGGCCACTTTCTGGTCT
GTAACTGACACTCAGCGGAAAGCGTGGGGGAGCAAACATGGATTAATA
CCCTGGTTNTCCACCCTAACATACTTGCTATTGTTGGGGGTTTCGCCCTT
TTGCTCTACCTTTTACCTCCGCTGGGATTGGCAAGCTAAACCTCAAG
GAAATTTTACGACG

ID: Bacillus sp. SG-1, Bacillus cereus

>2-1r well E03 UT080626 Run01 Cimarron 4.00 Even Spacing 600
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TACTAATTTCTGTTTCGCTCCCCACGCTTTCGCTGCCTCGATGTGTACG
TTACTATGACCGAGGAAAGTCTGCCTTCGCCACTGTGTGTTCCATCACTA
ATATCATCTACTGCATTTACCTAGCATACTACTTGTGTGAATTCCAAC
NTCCTCTGTCATGCACTCAAGTCCCCCATGTTTACCNAGTGAGCCCATCC
GACGGATTGAGCCNNGGGGGCTTTCCTATACCAGACTTAAGAGGACCTGA
CCTGACGCGCGCTNTACGCCCAATAATTCTTGGAACGCTTTGCCAC
CTTATGTTANTTAACCCGGGGGGTGGCTGGGCAACGTAAGGTTAGCG
CCGTGGACTTTTCTTNNTTAGGTACCGTCTAAGAGTTACACAGTCAGTTN
ACTCTCGTTACTTGTCTTCCCTAAACAACAGAGNTTACGGACCCGAAG
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ID: Bacillus sp. SG-1

>2-3f well A04 UT080626 Run01 Cimarron 4.00 Even Spacing 357
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TCAAGAAAGCCACGGCTAACTACGTGCCACCAGCCGCGGTAATACGTACG
TGGCAAGCGTTGTCCGGAATTATTGGGCGGTAAAGCGCGCGCAGGGCCGG
TTTCCTTTAAAGTCTTGNATGTTGAAAGCCCCCGGCCTCCAACCCGTGG
GAAGGTCAATTTGGAAGTGGGGGAACTTTATTGGCGAAAAGGNAGANG
TTGAATTTCCACTGTTAGGTAAATTGCCTTAAAAATTTTGAAGGAAAC
CCCCCTGGGGGAAGGGCACACCCTTCTTCTCGTGGGTTTCTCTTTAA
CNTGTCTACCCCTCTCTAAAGGGGGCCCCAAAAAAGNGCTGTGGGGGGG
AACACAAAAACACAGGGAATTTAATTCCCTGTATTATCCCCCCCCCTAA
ACAATTATTGCTTATTGTTTGGGGGGNTTCCCGCCCTTATGCTGCATC
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ID: Anoxybacillus flavithermus WK1

>2-3r well E04 UT080626 Run01 Cimarron 4.00 Even Spacing 509
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AGACAACCCCACTGAACACTTANCACTTCATCGTTGTACGTGTGTAGGAC
TACGCAGAGATATCTAATTTCTGTTTGTCTCCCCACGCTTTCGCTGCCTC
ATGTGTCAAGTTACAGACCAGANAGTCTGCCTTCGCCACTGGTGTTCCTCA
CATATCTCTACTGCATTTACCAGCATACACTGTGTGAATTTCACTCTCC
TCTTCATGCACTCAAGTCCCCCATTTTCCAAGTGACCCTCCACGGATTGA
GCCGGGGGCTTTCACATACTAAAGGACCGACTGCGCGCNCTTACG
CCCAATAATTCCGAAAAACGCTTGCCCCCTATTANTACCGGGGGGTGG
TGGCAGTAGTTGCCGTGTGTTTCTGGTTAGGTACCGTCAAGGTACCAGC
AGTACTCTGTAAGTTCTTCCCTAAACAACAGAGCTTAACGACCCGAAG
GCCTTCTTCGCTACACGCGGGTGTGNGTCCTTCAGACTTGCGCCATTGC
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ID: Anoxybacillus flavithermus WK1

>3-1f well A05 UT080626 Run01 Cimarron 4.00 Even Spacing 477

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TTAATCATTGTCTACGTTACCTTGACGGGTAACCTAATCAGAATACGCCN
CGGCTCACACTACCTGCCACCCAGGCCGCGGTAAGTACGTNCCGTGTGCG
AATGCGCTTGTACTGGAATTAGTTGGGCGTAGANGCGNGCGCAGGCGGT
CCTTTAAGTCTGANGTGAAAGCCCCCGCTCAACCGTGGAGNGTCATTGG
AACTGGGGGACTTCAGTGCGGAATAGGAGAGTGGAANTCCACTTGTAGC
GAGTNAATGCATATATATTTGGAGGAACACCCGTGGCGAAGGCCACTTT
CTGGTCTGTACTGACACTCAGGCCGAAANGCGTGGGGAGCCAACATGATT
AATACCCGTGTACTCCACCCTAACATACTGCTTTTGTGGGGCTTTCGCCT
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CAAGNGTTTACGA

ID: Bacillus sp. SG-1

>3-1r well E05 UT080626 Run01 Cimarron 4.00 Even Spacing 418

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ACGGTGGATGNACTACCGATGTGTATCTCACATTCGCTGTTTCGCTCACC
GCACGCTGTTGCGTGCCTCGAGTAGTCAGTTGACAGACCGAAGANAGTC
TGCCGTTACAGCCGAGTGGTGTGTTCCATCACTAATATTCATCAGTACTGC
TATTCGACCGCTTACACTGTGTGAATTCCTACTTCCCTCTGTGCATTG
CACTCAAGTCCCCATCTATATACCGATGAGCCCATCCGTGGATTGAGCC
GGGGGGCTTTCACATACAGAACTTAAGAGGACCTGACTGGCGCGCGCATT
NTACGCCCCAAATAATTGCCTGGAAAGAAACGCTATGCCCCACCCCTTAT
GTATTGANCGCNGGGGGGTTGGGTTGGCGAAGTTTAGTTAGGCCCGGTGT
GATTTTCTTGTTAGGTACAGTCTAAGGTAACCAGCAATTTTAACGTCTC
GTTACTTGGTCTTCCCTAACAAACANAGCTTAAAGNACCCGGAAGGGT
CNTTCTTCGCTACACGGCCGGGTTTGGTCTCTCCAAGACTTTTNCCCC
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A

ID: ID: Anoxybacillus flavithermus WK1

(or: Bacillus sp. SG-1)

>3-2f well A06 UT080626 Run01 Cimarron 4.00 Even Spacing 495

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CGNGCGCAGGCGGTCTCTTTAAGTCTTGATGTGAAAGCCCCCGGTCAA
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ACCAGTGGCGAAGGCCACTTTCTTGGTCTTTAACTGACCCTTCAGGCNCC
GAAAGCGTGGGGAGCAAACATGATTAATACCTGGTTATCCACGCCGTAA
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ID: none

>3-2r well E06 UT080626 Run01 Cimarron 4.00 Even Spacing 592
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ID: none

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ATGACGTTAATCATTGCTACGCTTGCCTTGACGTGTACCTAATACAAGGA
ATACGCCACGGCTCACACTACGTGCCACCAGGCCGCGGTCAATACGTACC
GTGTGCGAATGCGCTTGCTCTGGAATTATTGGGCGTAGAAGCGCTGCGCA
GGCGGTCTTTCAAGTCTGATGTGAAAGCCCCNGCTCAACCGTGACGG
GTCATTGGAAACTGGGGGACTTGAGTGCGAAAGGAGAGTGGAANTTCCAC
TTGTACCGNGTTAAATGCGTAATATATTGGAAGGAACACCCGTGGGCG
AAAGGCCACCTTCTCCTGGTCTTTTAACTGACACCTCAGCACCAAAAAG
CGTGGGGAAACAAACAATGATTTATTCCTGTATCCACCCCTAACATAC
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CCACTCCGCTGGGAGTACGGCGAAANGCTAAANCCCAAGGAAAGNTGTT
CG

ID: none

>3-3r well F01 UT080626 Run01 Cimarron 4.00 Even Spacing 574
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CTCTGGTTTGCTCCCCATCTGCTTTGCGCTGCCTCGATGTAGTCAAGTT
ACTAGACCGAGGAATAGTCTGTCTGTTGCGCGAGCTGGTGTTCATCTACTA
ATATCATCTACTGCATTTACCAGCTTAGCTACTGTAGGAATTCCAACCT
NTCCTCTGTGCATGTCACTCAAGTCCACCCATCTTATCCGAAGTGACCCA
TCGCGACGGATTGAGCCGGGGGTCTTTTACCTAATACAGACTTAAGAGG
ACCGACCTGGCGCGCGCTTTACCGCCAAAATAAATTCCATGGAAAAACGC
CTTGGCCACCTTAATGTATTACCCGCGGGTTGGCTGGGCAACGTAAGTT
TGGCCCGGTGGTTTCTGCGTGTAGGTACCCGTCAAAAGTTACCAGTCAG
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GTCTTCTTCGCTCACGCGGTTTTGGTCCTTCAGAATTCGCCCCATTGCN
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ID: none

>4-1f well B02 UT080626 Run01 Cimarron 4.00 Even Spacing 528

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AAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTG
CGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCTGTTTTGT
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AATGCGTAAGATGTGGAGGAATACCAATGGCCGAAAGGCAGCCCCCTGG
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ID: none

>4-1r well F02 UT080626 Run01 Cimarron 4.00 Even Spacing 526

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ID: Beta proteobacterium KB13 ctg_1106380163073

>4-2f well B03 UT080626 Run01 Cimarron 4.00 Even Spacing 481

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TAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGC
GCAGGCTGTTTTGTAAGTCAGATGTGAAATCCCCGAGCTCAACTTGGGAA
CTGCGTTTGAAACTACAAGACTAGAATATGTCAGAGGGGGGTAGAATTCC
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GCAGCCCCCCTGGGGANTNAATATTTGGACGCCTCATTTGGCCAAAAAC
CGTTTGGGGGGGNCCCCAAACACGGGGATTTTAAAAANCCCCCTTGGGT
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ID: none

>4-2r well F03 UT080626 Run01 Cimarron 4.00 Even Spacing 46
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ID: none

>4-3f well B04 UT080626 Run01 Cimarron 4.00 Even Spacing 456
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TATAGGGTGAGAGTTGACGGTACCTTGATAAGAAGCACCGGCTAACTACG
TGCCAGCAGCCGCGGTAAATACGTAGGGTGCGAGCGTTAATCGGAATTACT
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ID: Beta proteobacterium KB13 ctg_1106380163073

>4-3r well F04 UT080626 Run01 Cimarron 4.00 Even Spacing 495
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GGTATTCCTCCACATCTCTACGCATTTCACTGCTACACGTGGAATTCTAC
CCCCCTCTGACATATTCTAGTCTTGTAGTTTCAAACGCAGTTCCCAAGTT
GAGCTCGGGGATTTACATCTGACTTACAAAACAGCCTGCGCACGCTTTA
CGCCCAGTAATTCCGATTAACGCTCGCACCCCTACGTATTACCGCGGCTGC
TGGCACGTAGTTGCCGGTGCTTCTTATCAAGGTACCGTCAACCTCACCCCT
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ID: Beta proteobacterium KB13 ctg_1106380163073

>5-1f well B05 UT080626 Run01 Cimarron 4.00 Even Spacing 380

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 TAAGAAGCACCGGCTAACTACGTGCCACGCAGCCGCGGTAATACGTAGGG
 TGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCTGTTTT
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 ACCCTGGTATCCACGCCCTAAACGATGTCTACTATTTGTGGTGGAGTAA
 AATCCCTGAGTAACGCAGCTAACTGCGTCGAACGTAGACCGCCTGGGGAG
 TACGGTCGCAAAAATTAACACCCAGG

ID: none

>5-1r well F05 UT080626 Run01 Cimarron 4.00 Even Spacing 291

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 CCCCNGTNCCTCCCCNAGA

ID: none

>5-2f well B06 UT080626 Run01 Cimarron 4.00 Even Spacing 330

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 GCGTGTAATACGTAGTGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAA
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 AATTCCACGTGTAGCAGTGAAATGCGTAAGATGTGGAGGAATACCACATG
 GCGAAGGCAGCCCCCTGGGATAATATTTGACGCTCATGCACCAAAAAGCGT
 TGGGGGAACAAAACAGGATTNAAAATTACCCCTTGGTATTCCACCCCTA
 AACGATGTCTACTATTGTTGGTGGAGTAAAATCCCTGATAACGCACTAAC
 GCGTGAAATAACGCCTGGGGAGTACGGTCGCAAGATTAATACTCAATGAT
 TAGTGAGGG

ID: none

>5-2r well F06 UT080626 Run01 Cimarron 4.00 Even Spacing 446
GTAGTTCATCCNANTGATCAGTNTGACNTCNCGACGAAATAACGTGACTT
ANACATCAGTGTAGGGCGATGTGACCTAGCCAGAGGTA CTCTCACAGTCA
CTGGTTTGCTCCCCACGCTTTCGTGCATGAGCGTCAATATTATCCCAGGG
GCTGCCTTCGCCATTGGTATTCCCTCCACATCTCTACTGCATTTCACTAGC
TACACGTGGAATTCTACCCCCCTCTGACATATTCTAGTCTTGTAGTATTC
AAACGCAGTTCCCAAGTTGAGCTCGGGGATTTCACATCTGACTTACAAAA
CAGCTGCGCACGCTTACGCCCAGTAATCCGATTAACGCTTCGCACCC
CTACGTATTTACCGGGGGGTGGTTGGAACGTTAGTTAGGCCGGGTGNTT
CTTATCAAGGTACCGTCAACCTCACCTATATTAGAAGGTAAGTTTCTT
CCCTTGCGAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCACGCGGAA
TGGCTGGATCAGGCTTGCGCCCATTGTCCAAAATTCCCCACTGTGCTCCC
CGA

ID: none

>5-3f well C01 UT080626 Run01 Cimarron 4.00 Even Spacing 497
TTCCATGCCGCGTGGAATGATAGAAGGCTTCGGNATTGTAAAGCTCTTTC
GACAAGGGAAGAAAAC TTATCTCTCTAATCATCAGCGGTGAGAGTTGACG
GTACCTATGTATAAGAAGCACCGGCTCAACTCACGTGCCACGCAGCCGCG
GTCAATACGTAGGGTGCGAGCGTTAATCGCGAATTACTGGGCGTAATAGC
GTGCGCAGGCTGTTTTGTAAATCATGATAGTGAAATCCCCGAGCTCAACT
TGGAAGTGCCTTTGAAACTACAAGACTAAATATGTCAGAGGGGGGTAGA
ATTCCATCGTGTAGGCAGTGAAATGCGTATAATGTGGAAGGAATACCAAT
GGCGAAGGCCGCCCCCTGGGAATATATTGACGCTCATGCACACAAAAGG
GTTGGGNGACAAAAAAGGATTTCTCCCTGGTTTCATCCCCGCCCTAAA
CGATGTCTACTATTTGTTGGTGGAGTAAAATCCTTGAGTAACGCTTAACG
CTTCACTAGACCGCTGGGGAGTACGGTCCGCAAGATTAAACTCCNCGGT
ACGGGGG

ID: none

>5-3r well G01 UT080626 Run01 Cimarron 4.00 Even Spacing 381
TAACGNTTNTGGTACTNAACTCCNTACGNTNCGNAGTACTNACACANN CN
NGTAGACTGAGGATCACTACGATGGAGTTATNCTCCGAACCGAACAAGCT
AGCTATGACGATCGATGTGAGGGCGATGTGAACTAGCCAGAGCTATCTAA
TTCCTGTTNGCTCCCCATCGCTTGCGTGCATGATGCGTCAATATTATCCC
AGGTGGCTGCCTTCGCCGAGTTGGGTATTCTTCCACTATCATCGTACTG
CATTCTCGACTAGCATACACGNNGAATTCTACCCCCATCTGACATATTC
ATAGNCATGNAGTATCGAGAACGCAGCTTCCCAAGTTGAGCTCGGGGATT
CACAAGTGA CTTACAAAAACAGCCCTGCGCACGCTTTTACCGCCCCAAGT
AAATTTACCAAAATTAANGGCTCCCCCCCCCTAATATTTTATTTCCC
GGGGGGGGTGGGGGGGGGCAGTTAATGTGGGCCGGGTGTTTCTTTAATG
AAAGGTACCGGTCAACCTCTACCTATATTAGAAGNTAAGTTTCTATA
CCCTTGCGAAAGAGCTTAANAACCCGAAGGCCTTCTTCACTCNCGCGGAA
TGGCTGGATCAGGCTTGCGCCCATTGTCCAAAATTCCCCACTTCGCTCTG

ID: none

>6-1f well C02 UT080626 Run01 Cimarron 4.00 Even Spacing 176

CTNCCGNAGCCGCGTTGNAGATGANGAAAGGTCTTCGGGATTGTAAAGCC
GCTTTCGAGCAGGGAACGAAAACGTGTCTCGGAGCTAATACTCACTCGAC
GGTTCATATGACTGGTACCTGCANAAAGTAAGCACCGGCTTAACCTAC
GTGGCCAGCAGCGCCGCGGGTAATAACGTAGGGTGCGAAAGACGTTAATC
GGAATTACTGGGCGTAAAGCGTACGCAGAGCGGCTTTGCAAGTACAGGTG
TGAAATCCCCGGGCCTAACCTGGGAACTGCATTTGNGACTGGATGGCTTG
AGTTTGTGAGAGGGGGGTGGAATCCCCGTGTAAACAGTGAAAATTGGC
GTAAATATTTGGAAGGAACCAACCGATTGGGGGAAAAGGGGGGGGCCCCC
CCTTGGGACCTTTTAAATTTGGAACCCCCCAAGGTTCCCAAAAACCGTT
GGGGAGCAAACAGGATTAATAACCCCTTGGTATCCACGCCCTCAAACGAT
GTCNATCTATGTTGTCTGGAGGGTTTCTCCTCTCTGTACNCCCTAAAC
TCGTTAATTCTANCGCCTGNGGAGTACGTCCCANAAATTAATACTCAAAGA
AANTTTTA

ID: Sideroxydans lithotrophicus ES-1

>6-1r well G02 UT080626 Run01 Cimarron 4.00 Even Spacing 155

ATTCAGTANCGGACTAAGAGGAAGTTAGCACCATGCCAGAGACAACTCA
AGATCTCGATCATACTGNCGNATTAGGGGGTTGAGAGCTAATCNCAACN
AGGGGTATATANTATACTCTGCTGTTGCTNTCTCCACCGTCGTCTTC
TGTCTGCACTGATCGACGGTGATGANATNTATTCGCGCACGAGGAGAGCT
TCGCCGTCTCTTCCACTAGGGTGTCTCTCTCCTACATATATACTACAT
ATTTTACCATGCGTATACGACTGGTGAGAAATCTCTAACCACCACTCCT
CTTCTACCAAAAACATAAATGAGCAGTTTCATAAATGACGGTTCCC
CNCGGTTTTTTGGAGCCCGCGGGGGGATTATTTACACCTGTGTATTTAA
ANGCCGCCCCGTGTACGCCNTCTTTTTTCCCCCCCCCAAAATTTTCCC
AAAAAATAAATTTTTTCCCCCCCCCCTTTTTTTTAAATCCCCCGCG
GGGGGGGTGGTTTGGGGGGGCGAGAATAAGGATTTATTCGGCGGGGGG
GTTTATTTTCTGTAGGGGTACCAGCTCGATATAATCGCGAGAGTATTAT
AAAATCAGCGCACAGTTTCCTTCCCTGCCGAAAAAAAGGTTTATAACA
CCACGAAGGGGCGTTTTCCCTCACGCCGGGAGTGGGTTGNATAATCTGGN
TNTNCCNTTGTACAAAAAAATTTCCACATGGTGCCTCCCCCCTAGCCT
GAGGG

ID: Sideroxydans lithotrophicus ES-1 (98.5%)

>6-2f well C03 UT080626 Run01 Cimarron 4.00 Even Spacing 170

ACTCAAGATGGANTTCGACTGTGATACCTGCTCANAAGTAAGCANCCGTG
GCTTATAACTATCGGTGGCCACGTACACGCCCTGCGGTCGTAAATACTGG
TAGTGGGTGCGAAAGCGTTAAGTCTGGAACCTTACTGGGCGTAAAGCGTAC
GCAGGCGGCNNTTGCAAAGACNNGGTGTGAAATCCCCGGGCCTTAACCT
GGGAACCTGCCATTTGTNGACTGCAATGGCTTGAAGTTGTCAAAAAGGGG
GGTGGAATTTCCACTGTGTATGCCAAGTTGAAAATGCGGTAAAAATTATC
NTGGGAAAGGAAAACACCCCAAGTTGCGAAAACGGNNGGNACCCCTCTC
GGCAACACTTTTATATACCCCTTCTCACAAAAAACAACCGTTGGGGA
GCCAAAACCAGGGGAATTTAAAATTACACCCCTTGGTAGTTCCCACTG
CCCCCTAAAACGAATGTCAAACCTAATTGTTGTTGGGGAGGGGGTTTAT
TTCCCTCTCGCGCTAACCCACCTAACTCGTTANNTAGACGCCTTCGGGAG
TAAAAGGGCCCCAAG

ID: none

>6-2r well G03 UT080626 Run01 Cimarron 4.00 Even Spacing 104
TTTGATGGCAATATTTAACCCCAACNTANGGCTAGTCCTTCNTCACNGCT
AGGTGATATGCATGCCTCCACCCGTCGTCTTCTGCTAGCACTNTCGATCG
ACGGTCGAATCGATGCATCATTCGCGCACGCAGGGAGAGCTGTCGCCGTN
TCTCTGTCCACTCAGCGGTGGTTCTCTCTCCCCTCACAGTATCATAACTG
ACGAACTTTCTACCTATGCGTACTAACGACTGGGAATNACTCTCACCCCA
CTCTTCCTTCATCACACAAACATCTACAACCACCTAGCAGATCATCATAC
AAGATAGGCAGGGGATTCTCTCCCNAGGGTGTATAGGACGCCNGGGGGGG
AGTTAATTAACACCTGGTTCGNATGTTAAAAAAGAGCCGGCCCGTTGCTG
ATAACGGCCTTTTTAATAACCCCCCNACAAAAATAAANCNAATAAAAAA
AAACGTTTGGTACCCCCACCCCTTAATTNAATTCNCGCGGGGGGGGGGG
TTGTTGGGGGGGGGGGAAAAAAATNTATATTTTCGGGGGGGGGGGGTT
TATTTTCTTTTTTATANGGGGTTACCCAGCCGGATTAAAAACCGCCCGGA
AGGATAATTTGATCGCGGGCCAGGTATTTTCGTTTCCCCTGGGCCCCGAA
AAAAAAAGGGGGGTGTTTATTAATAAACCCCGCGANAGAGAGGGGCGTAT
CTTTCCCCTGCCACCGCGCGGGGAGGTGGTGGGTGGGAATACGAGCCTT
GTTGNCCCCNTCAATTTCAAAAAAATTATCCCCCTGNCTGC

ID: none

RECEIVED April, 23, 2015
ACCEPTED May, 11, 2015



INTERNATIONAL ALLELOPATHY SOCIETY