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Metabolomic analysis of the consequences of cadmium exposure in *Silene cucubalus* cell cultures via ^{1}H NMR spectroscopy and chemometrics

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Abstract

Several essential and non-essential metals (typically those from periods 4, 5 and 6 in groups 11–15 in the periodic table) are commonly detoxified in higher plants by complexation with phytochelatin. The genetic and gross metabolic basis of metal tolerance in plants is, however, poorly understood. Here, we have analyzed plant cell extracts using ^{1}H NMR spectroscopy combined with multivariate statistical analysis of the data to investigate the biochemical consequences of Cd^{2+} exposure in *Silene cucubalus* cell cultures. Principal components analysis of ^{1}H NMR spectra showed clear discrimination between control and Cd^{2+} dosed groups, demonstrating the metabolic effects of Cd^{2+} and thus allowing the identification of increases in malic acid and acetate, and decreases in glutamine and branched chain amino acids as consequences of Cd^{2+} exposure. This work shows the value of NMR-based metabolomic approaches to the determination of biochemical effects of pollutants in naturally selected populations.

Keywords: Cadmium; Metabolomics; NMR spectroscopy; *Silene cucubalus*; Metabolite

1. Introduction

The development of novel analytical strategies for deriving information on differential gene function in relation to environmental stressors is essential in order to advance the molecular basis of metal tolerance. Whereas genomics and proteomics can provide insights into the potential of a biological system to interact with external perturbations (pharmaceutical/agrochemical compounds, pollutants, environmental effects), it is the resulting changes in the metabolic profile of the system that are potentially more use for the understanding of the biochemical reaction to stress. This is because it is changes in the metabolic profile that are the ultimate result of such external influences. '*Metabonomics*', defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" (Nicholson et al., 1999, 2002; Lindon et al., 2001), is increasingly being used for the analysis of a range of biological problems including toxicological assessment (Holmes et al., 2001), differentiation between genetic strains (Gavaghan et al., 2000), comparative mammalian biochemistry (Griffin et al., 2000) and natural product characterization (Bailey et al., 2002; Belton et al., 1998). In parallel, there have been developments in '*Metabolomics*', which broadly encompasses the study of the metabolic response in isolated systems as opposed to the whole system approach

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described by metabonomics. Metabolomic studies have been reported on the analysis of the consequences of genetic manipulation and strain differentiation at the cellular level, for example in the characterization of phenotypic differences in strains of yeast (Raamsdonk et al., 2001). While the application of NMR spectroscopy to metabonomic investigations has gained momentum, relatively little data have hitherto been published on the application of high resolution NMR spectroscopy in plant metabolomics. It has been reported, however, that a combination of off-line HPLC–NMR spectroscopy with rudimentary data analysis has been employed for the evaluation of metabolic changes in transgenic food crops (Noteborn et al., 2000). Several recent studies have shown the application of metabolomic-type analyses using GC–MS for the analysis of transgenic potato tubers (Roessner et al., 2000, 2001) and *Arabidopsis* genotypes (Fiehn et al., 2000). While MS-based detection techniques typically display greater analytical sensitivity than NMR spectroscopic detection, there is an inherent necessity for the analyte of interest to ionize in the mass spectrometer along with requirements for pre-analysis derivatization. This means that the non-selective, yet highly specific approach of NMR spectroscopy, where no pre-judgement of the sample is required, offers several advantages with respect to the development of an analytical methodology that is readily transferable between samples from differing applications. Here we demonstrate the value of NMR based metabolomics in the investigation of metal tolerance and toxicity in plants, specifically, the effects of cadmium on *Silene cucubalus*.

Cadmium is a putatively non-essential and potentially highly toxic element to all classes of living organisms. Soils and water may be contaminated with Cd^{2+} as a result of mining or industrial activities, use of phosphorus containing fertilizers, land applications of sewage sludge, and atmospheric deposition (di Toppi and Gabbrielli, 1999). Soil contamination of Cd^{2+} presents a significant concern as increased Cd^{2+} bioavailability may harm ecosystem functions, or result in an unacceptable level of transfer of Cd^{2+} to the food chain. Cadmium exposure results in lesions in the kidneys of higher vertebrates and man (Nicholson et al., 1983; Nicholson and Osborn, 1983). Recent research (Lombi et al., 2000) has shown that several plant species may be Cd-tolerant and indeed, one plant species (*Thlaspi caerulescens*, a Brassicaceae) has been identified as being a Cd^{2+} hyperaccumulator (defined as storing >100 mg Cd^{2+} kg^{-1} in the shoot dry matter). *S. cucubalus* is known to respond to cadmium exposure through the chelation of metal ions by a family of peptide ligands, the phytochelatins, which consist of repetitions of γ-Glu-Cys sequences with a terminal Gly (Grill et al., 1985; Zenk, 1996; Cobbett, 2000). However, despite the evidence for phytochelatin involvement, little is known about the gross changes in biochemical status in *S. cucubalus* cultures as a result of Cd^{2+} exposure. The aim of this work was to apply an NMR-based metabolomic approach to investigate the metabolic responses of *S. cucubalus* following Cd^{2+} exposure in vitro.

2. Results and discussion

2.1. ^1H NMR spectroscopic analysis of the samples

The ^1H NMR spectra for the predose (samples obtained on day 0, at the time of transfer into fresh media), control (samples obtained on day 3 at same time as dosed samples were obtained) and dosed (samples obtained on day 3 following exposure to 150 μM Cd^{2+}) are shown in Figs. 1a–c, respectively. It was possible to observe clear differences between these spectra, indicating changes in biochemical status with respect to time, i.e. between predose (a) and control (b) samples, where there is a time difference of three days and following exposure to the cadmium i.e. between control (b) and dosed (c) samples. Although differences between the spectra were readily observed, it was important to derive metabolic differences between sample classes based on the mathematical variance in the matrix rather than solely through visual inspection, hence the use of principal components analysis (PCA) to reduce the dimensionality of the data thus allowing easier interpretation of the results.

2.2. Pattern recognition analysis of the ^1H NMR spectra

PCA is an unsupervized method, i.e. analysis is performed without use of knowledge of sample class, which reduces the dimensionality of the data input whilst expressing much of the original *n*-dimensional variance in a 2- or 3-D map (Eriksson et al., 1999). By producing new linear combinations of the original variables, i.e. the integrated NMR spectral regions, it is possible to plot such data in order to indicate relationships between samples in the multidimensional space. The result is a diagram known as a scores plot that can be used to determine the similarities and differences between many samples Fig. 2). This dataset of NMR spectra from the cell culture extracts displayed good discrimination between the three classes analyzed, in that the classes were easily differentiated from one another. Further, this separation took place in the first two principal components (PCs[2]) which cumulatively account for 96.5% of the variance in the dataset, indicating that it is

[2] The abbreviation PC is in common usage to refer to both principal components and phytochelatins. PC is used to refer to principal components only throughout this work.

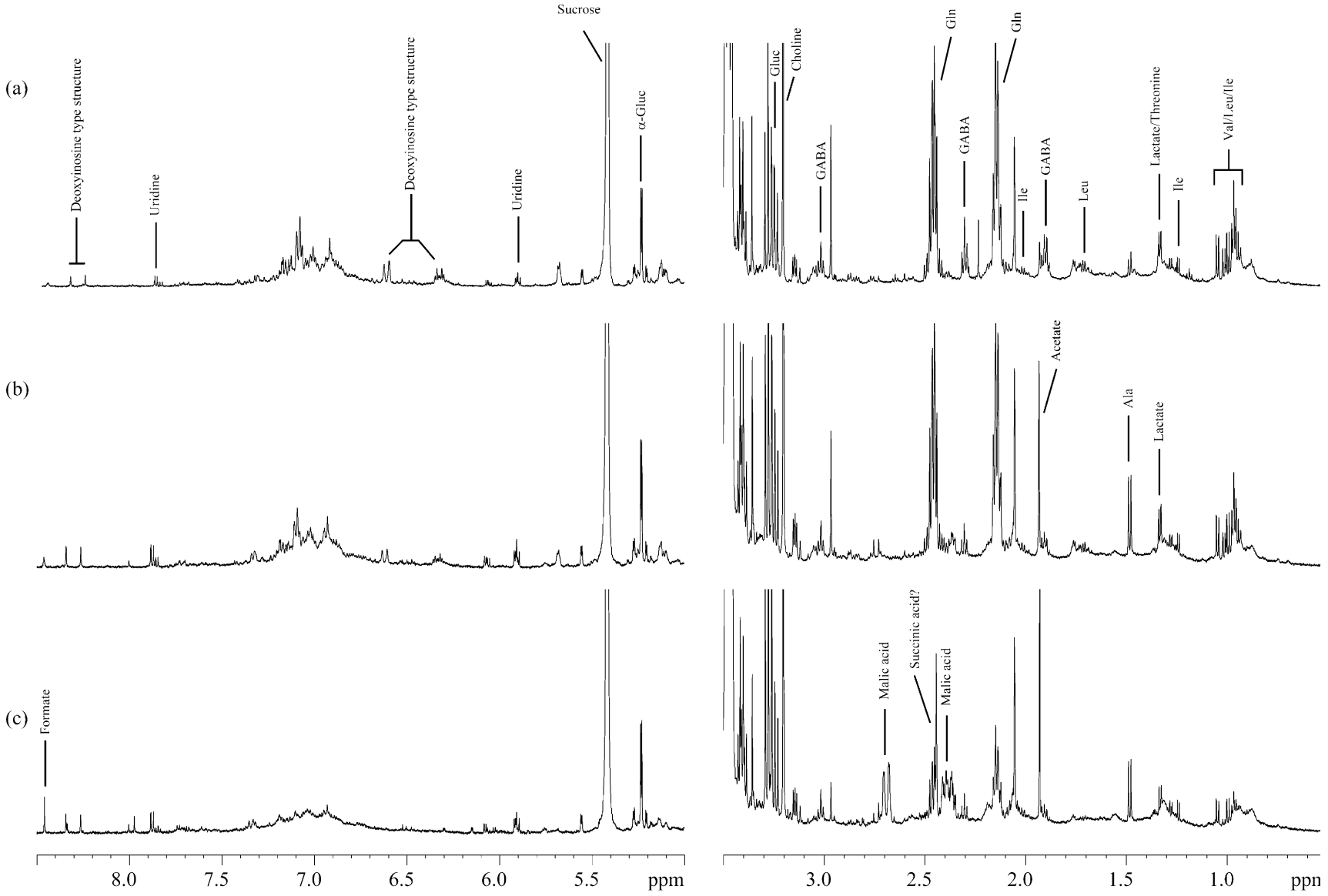

Fig. 1. NMR spectra for all three classes (a) predose (day 0 after new growing media was added), (b) control (day 3), (c) dosed (day 3 after addition of 150 μM Cd^{2+}). Region containing residual HOD and sucrose resonances (present in the media solution) are removed for clarity.

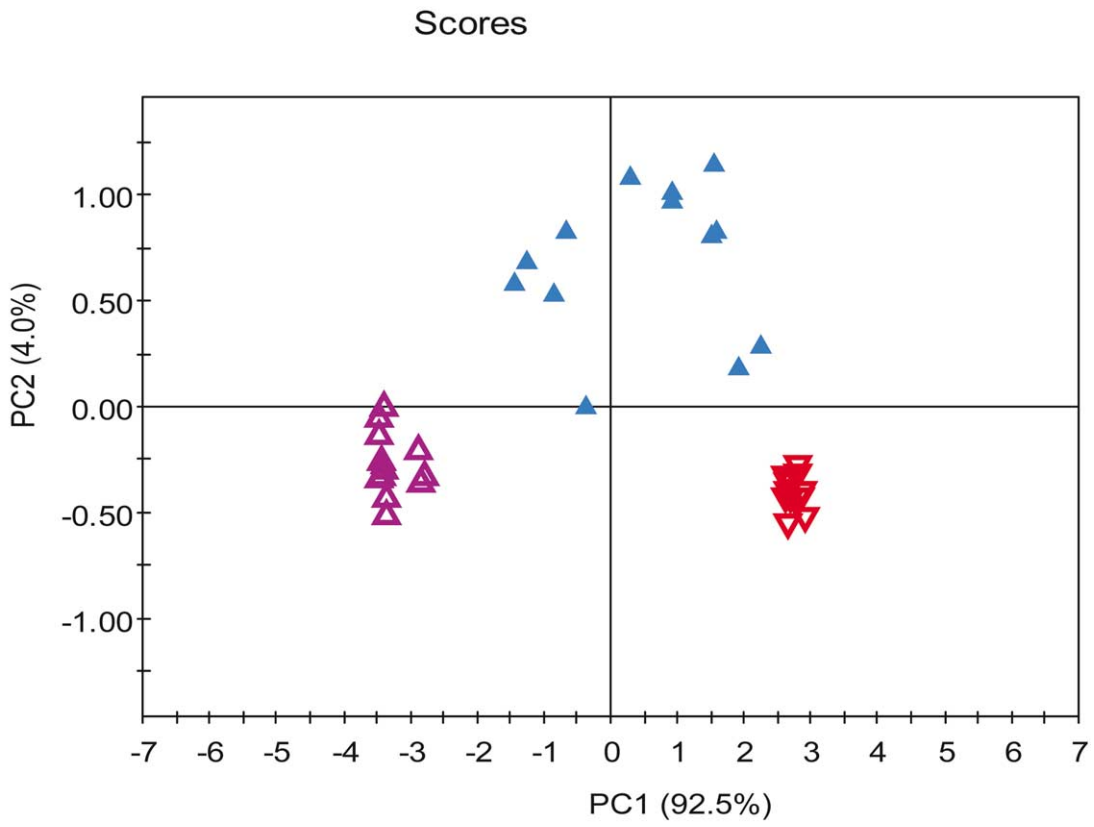

Fig. 2. Scores plot (PC1 v PC2) for predose (open red triangles), control (closed blue triangles) and dosed (open purple triangles) sample groups following PC analysis. The plot displays clear discrimination between the three groups, accounting for nearly 97% of the variance within the dataset.

the difference between the three classes analyzed that is the major discriminating factor between samples rather than any other unrelated variation between samples. There are differences in metabolic profile due to both dosing and incubation time in the absence of Cd^{2+}. The time-related changes reflect adaptation to the new growth/nutrient conditions in the culture flasks. Both the predose and dosed sample classes were tightly grouped together within their classes (Figs. 2 and 3), whereas the control data were much more diffuse (standard deviations for predose and dosed samples in PC1 were 0.1 and 0.2 respectively, while for control samples it was 1.3. For PC2, the values were 0.1, 0.2 and 0.4 respectively). It can be seen that at the start of the study the samples in the predose class are biochemically similar to each other (relative to the samples in the control group). After 3 days of growth the controls separate from the predose condition and the samples have also biochemically diverged with respect to each other, resulting in the larger standard deviations indicated above. The effects of the Cd^{2+}-exposure on the cellular metabolic profiles were markedly larger than the differences caused by the 'natural' divergence of the control and predose groups. The Cd^{2+} dosed group formed a tighter cluster than the controls, thus a 'metabolic lensing' effect is a result of the stressor (Cd^{2+}) having the largest overall effect on metabolism within the culture system.

The primary aim of this work was to explore the biochemical differences between control and dosed sample groups of *S. cucubalus* cell cultures following exposure to Cd^{2+}. A PCA scores plot following re-analysis using the control and dosed sample groups only is shown in Fig. 3. It can be seen that the groups are readily discriminated in PC1. Having obtained a model that is capable of discriminating between the two sample classes of interest, the dataset was interrogated in order to determine those variables, (and in turn NMR regions, and ultimately biochemical entities) that were most important in class separation. PCA produces a series of new variables (PCs) based on linear combinations of the original variables. By analyzing the weighting given to each of the original variables, i.e. the degree of correlation between the variables and the direction of the new model, it is possible to determine their importance, known as the variable loadings. As seen in Fig. 3, the separation between the control and dosed groups was achieved in PC1. It was, therefore, possible to determine variable importance by analyzing the correlation of each variable with PC1, Fig. 4. A positive value in the loadings plot shown in Fig. 4 implies a positive correlation with the scores in PC1. Thus all variables with positive values in Fig. 4 are positively correlated with the control group, whilst the variables with negative values are correlated with the dosed group. When the

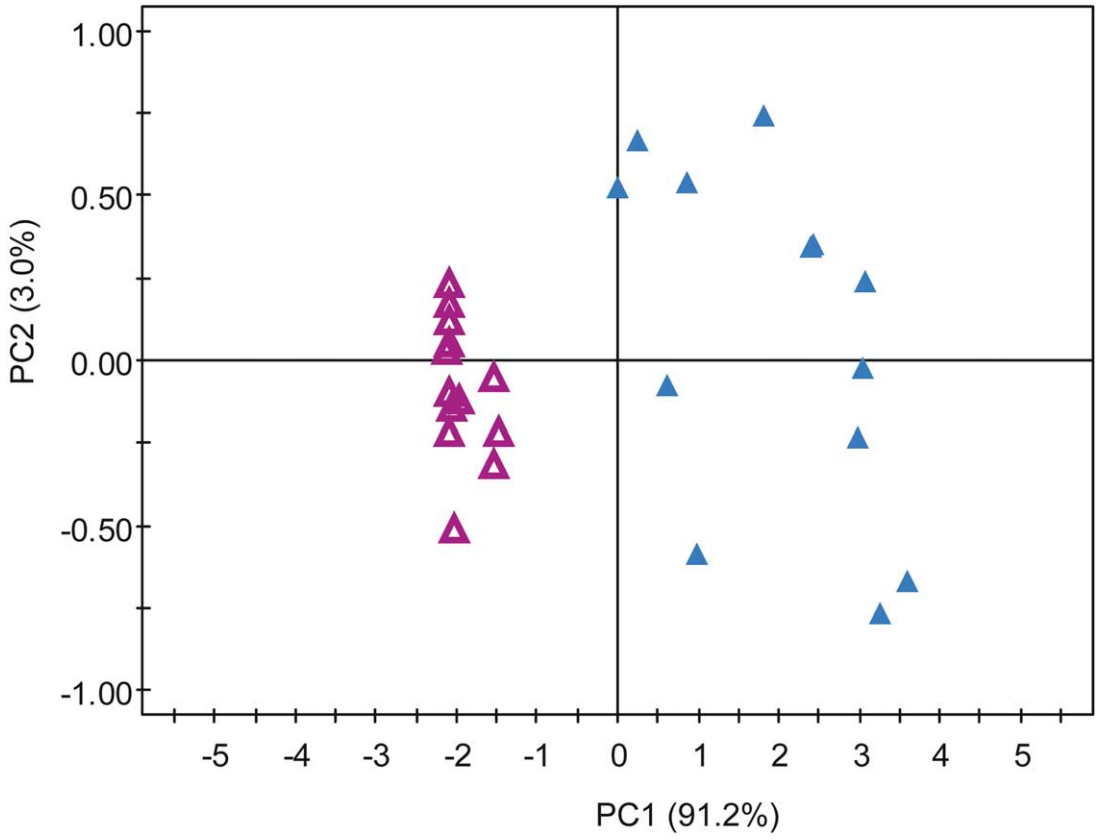

Fig. 3. Scores plot (PC1 v PC2) for dosed (open purple triangles) and control (closed blue triangles) samples groups following PC analysis. 94.2% of the variance within the dataset are explained in this plot.

variable loadings are plotted on the NMR frequency scale, it is apparent which NMR spectral regions are important. Hence by reference to established NMR assignments for small molecules and in certain cases 2-D NMR experiments (not shown), it is possible to identify the metabolite patterns that discriminated between the two groups.

The change that had the most influence on the discrimination between control and dosed groups was in the concentration of glutamine, which was substantially reduced between control and dosed groups, i.e. it has a large positive value in Fig. 4, indicating high levels in the control group, and lower levels in the dosed group. The major regions showing changes between control and dosed groups are summarized along with their metabolite assignments in Table 1. In general, the metabolites that were shown to be important are linked to the TCA cycle. Increased glucose levels suggests that utilization of glucose is reduced in Cd^{2+} exposed plants, while the presence of acetate may indicate either increased lipid metabolism or reduced utilisation of acetyl CoA in the TCA cycle. In addition, changes in levels of glutamate and malate may be related to changes in TCA intermediates. Although the anticipated presence of phytochelatins was not observed, this is due to the fact that they are present at too low a level for direct observation by ^{1}H NMR spectroscopy. This is particularly the case for a complex matrix like plant extracts where the dynamic range imposed by other metabolites places restrictions on otherwise observable species. The total amount of phytochelatins present in the dosed group, as determined by HPLC assay was approximately 1.5 μmol g^{-1} lyophilized material, with each phytochelatin present in the 50–1220 nmol g^{-1} lyophilized material range (data not shown; phytochelatins were not detected in either control or predose groups).

This work demonstrates that the combination of high resolution ^{1}H NMR spectroscopy with multivariate data analysis is readily amenable to the rapid screening of biological samples in order to produce a metabolic profile, which at its most basic level can allow metabolic fingerprints to be generated. Further, the implementation of chemometric approaches to interrogate the resulting complex data allows significant biochemical changes to be readily extracted from the data. By virtue of the NMR spectra already obtained, it is then possible to elucidate the nature of the metabolites that are key in the separation between sample groups.

While the more conventional analytical approach using GC–MS allows the detection and quantitation of many compounds during the execution of the chromatographic run, pre-analysis derivatization and thus pre-selection of the 'expected' metabolites prior to analysis poses an

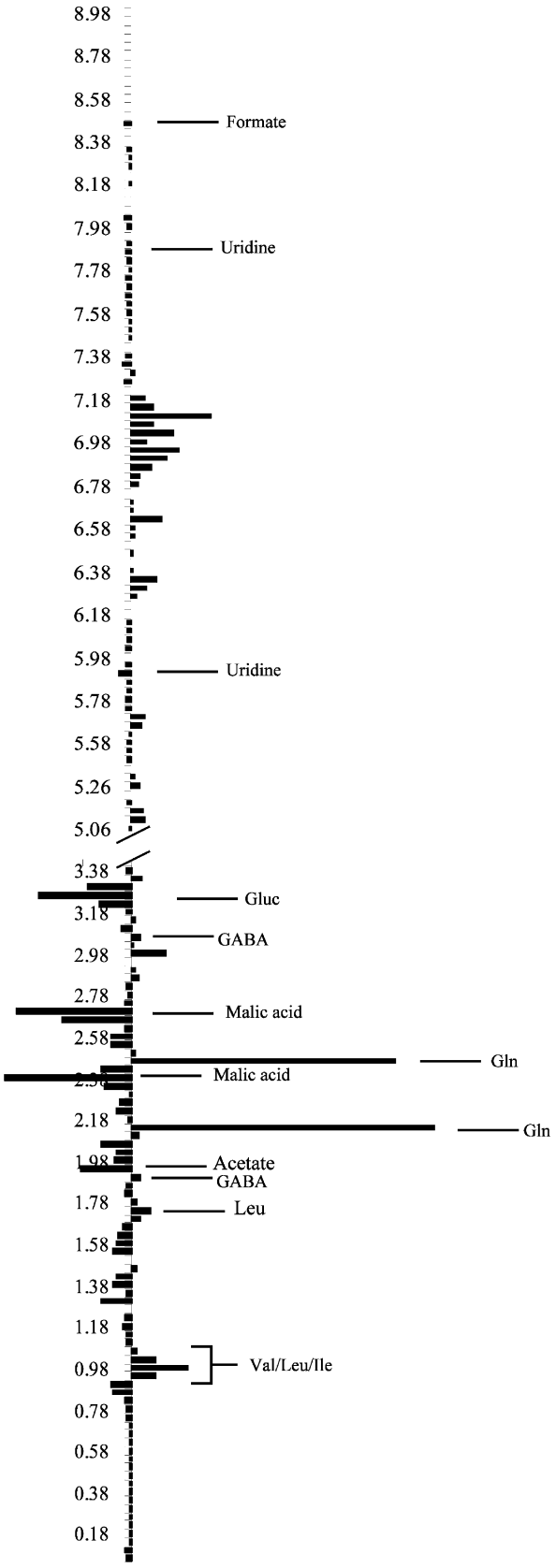

Fig. 4. Loadings column plot for dosed and control showing PC1 only. This plot allows elucidation of the chemical entities that are key in separating the control and dosed groups following PCA. Variables with a large positive value are positively correlated with the control group, whilst those with negative values are positively correlated with the dosed group.

obvious limitation of the methodology, as many non-derivatized chemical classes will be lost to the analysis. ^{1}H NMR spectroscopic approaches on the other hand, benefit from the non-selective nature of the technique, which means that no prior knowledge or judgement of the samples is required. NMR spectroscopy is an information rich technique providing key information for the structural identification of the metabolites detected. At the same time, this technique is not impeded by problems of differential detection between compound classes displaying differing chemical properties (such as ionisation in the case of mass spectrometric detection or UV absorbance in the case of HPLC for example). This means that NMR based approaches to metabolomics and metabonomics offer clear analytical advantages over alternative techniques, although NMR and MS approaches may also be considered in many applications to be complementary. In addition, the limited sample pre-treatment/derivatizations necessary, and the relatively short acquisition times mean that NMR spectroscopy may be utilized as a high throughput technique capable of rapidly analyzing the sample numbers required for statistically relevant studies.

With regards to this current work, it has been demonstrated that exposure of *S. cucubalus* cells to Cd^{2+} results in biochemical changes relating to energy production and the TCA cycle. There are indications however that lipid metabolism is also altered, perhaps in response to the down regulation of glucose metabolism. It may be hypothesized that it is this ability to switch the method of energy metabolism that imparts the Cd^{2+} tolerance to *S. cucubalus* whilst exposure to Cd^{2+} in highly sensitive species such as barley (*Hordeum vulgare*) results in reduced plant growth (Vassilev et al., 1998). In addition, while non-tolerant species show an increase in the levels of the stress biomarker proline (Vassilev et al., 1998), no increase in proline was observed in this study. Finally, this approach to metabolomic analysis has allowed the demonstration of the concept of 'metabolic lensing' with the variation within sample classes reduced between control and dosed classes as a result of the xenobiotic effect being greater than the inherent variance within a sample population. This suggests that it is important to obtain sufficient data points within a study to allow this phenomenon to be clearly identified as such, and also that biochemical variation is a factor that must be considered when planning metabolomic analyses.

3. Experimental

3.1. S. cucubalus suspension cell cultivation and sample preparation

Sterile *S. cucubalus* suspension cells (7 day old culture, obtained from existing cultures at the Institute of Plant

Table 1
Summary of the major changes between Cd^{2+} dosed and control sample groups

NMR spectral region (and intensity change between control and dosed)	Assignment	Concentration[a]/μmol/g dry weight (average, $n=3$)	
		Control	Dosed
Region 0.94–1.02 (decrease)	Valine	1.22±0.04	1.1±0.2
	Isoleucine	0.53±0.19	0.5±0.1
	Leucine	1.9±0.2	2.4±0.3
Region 1.94 (increase)	Acetate	14±2	17±4
Regions 2.14, 2.46 (decrease)	Glutamine	16.7±0.8	9.3±0.9
Region 2.38, 2.70, 2.66 (increase)	Malic acid	17±8	26±7
Region 3.26 3.22 4.34 (increase)	Glucose	[b]	[b]
Region 6.94–7.10 (decrease)	Unknown aromatic compounds	N/A	N/A

[a] Levels given are approximate only due to the overlap of resonances within the spectra, and the inherent errors associated with low level quantitation.
[b] Figures not given due to overlap of the glucose resonances.

Biochemistry, Halle, Germany) were vacuum filtered and washed with sterile water. A representative sample was flash frozen, lyophilized and taken as predose sample.

Two 1 l Erlenmeyer flasks with 250 ml fresh Linsmaier–Skoog growing media (Linsmaier and Skoog, 1965) were prepared, and 40 g (fr. wt.) cells added to each flask. In addition, one flask contained 3 ml sterile water (control flask), whilst the other flask contained 3 ml 12.5 mM $CdCl_2$ (final Cd^{2+} concentration 150 μM, dosed flask). Both flasks were cultivated under sterile conditions for 3 days (gyratory shaker 100 rpm, diffuse light 650 lux, 22 °C). After 3 days, cells from both flasks were vacuum filtered and washed with sterile water. Filtered cells were then flash frozen with liquid nitrogen and lyophilized.

Phytochelatin content of the cells was determined by HPLC with dithio-bis-nitrobenzoic acid postcolumn derivatization as described previously (Oven et al., 2002).

Replicates (approx 20 mg, $n=13$) of lyophilized cells from each flask were weighed out and added to D_2O (1 ml, containing 0.05% w/v 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid (sodium salt) (TSP) as NMR reference). Samples were agitated and then centrifuged at 13,000 rpm for 15 min. Supernatant (700 μl) was taken for NMR analysis.

3.2. ^1H NMR spectroscopy

NMR spectra were run on a Bruker (Bruker GmbH, Rheinstetten, Germany) DRX 600 Spectrometer, operating at 600.22 MHz for the ^1H frequency, fitted with a broadband inverse geometry probe. Spectra were the result of the summation of 64 free induction decays, with data collected into 32k datapoints, a spectral width of δ 14 and an acquisition time of 1.95 s. The water signal was suppressed using a standard 1D-presaturation pulse sequence (Nicholson et al., 1995). Prior to Fourier transformation, an exponential line broadening equivalent to 0.3 Hz was applied to the free induction decays and spectra were referenced to TSP at δ 0.00.

Quantitation was performed using a delay between pulses of 30 s to ensure full longitudinal relaxation. Concentrations were then calculated for each metabolite based on a known concentration of TSP.

3.3. Multivariate data analysis

One dimensional 600 MHz ^1H NMR spectra were reduced to 252 discrete chemical shift regions by digitisation to produce a series of sequentially integrated regions δ 0.04 in width between δ −0.02 and 9.98, using Bruker AMIX software (version 2.0, Bruker GmbH, Germany). The resulting data matrix was exported into Microsoft® Excel and selected regions removed, i.e. around the residual water signal (δ 4.54–4.98), sucrose (from the media solution, δ 5.46–5.38, 4.30–4.18, 4.10–3.42) and TSP (δ −0.02 to 0.02). The remaining 212 integral regions were normalized to the whole spectrum for subsequent Principal Components Analysis (PCA) (Eriksson et al., 1999).

PCA was performed using SIMCA-P 8.0 multivariate data analysis software (Umetrics, Sweden), with mean centring of the data preceding PCA. The output from the PCA analysis consisted of scores plots (giving an indication of the differentiation of the classes in terms of biochemical similarity), and loadings plots, which give an indication as to which NMR spectral regions were important with respect to the classification obtained in the scores plots.

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