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Investigating plant–plant interference by metabolic fingerprinting

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Abstract

New analytical developments in post-genomic technologies are being introduced to the field of plant ecology. FT–IR fingerprinting coupled with chemometrics via cluster analysis is proposed as a tool for correlating *global* metabolic changes with abiotic or biotic perturbation and/or interactions. The current study concentrates on detecting chemical responses by inter-species competition between a monocotyledon *Brachypodium distachyion* and a dicotyledon *Arabidopsis thaliana*. Growth analysis of 42 days old plants showed differences in both species under competition. Clear changes in the FT–IR metabolic fingerprints of *B. distachyion* in competition with *A. thaliana* were observed, whilst there were no apparent chemical differences in the *A. thaliana* plant tissues. This study demonstrates the power of this approach in detecting changes in the global metabolic profiles of plants in response to biotic interactions, and we believe FT–IR is appropriate for rapid screening (10 s per sample) prior to targeted metabolite analyses.

Keywords: Metabolic fingerprinting; Chemometrics; Inter-specific competition; Plant ecology

1. Introduction

Metabolomics is a term defined as the study of the total biochemical composition of a cell or a tissue sample (Oliver et al., 1998; Johnson et al., 2000). Metabolic fingerprinting involves obtaining "enough information to unravel (otherwise hidden) metabolic alterations, without aiming to get quantitative data for all biochemical pathways" (Fiehn, 2001) and can essentially be regarded as taking a rapid biochemical 'fingerprint' of such a sample. This is typically performed via rapid analytical methods such as nuclear magnetic resonance (NMR) (Lindon et al., 2000), Fourier transform–infrared spectroscopy (FT–IR) (Goodacre et al., 1998; Johnson et al., 2000) or electrospray ionisation mass spectrometry (Vaidyanathan et al., 2001, 2002; Goodacre et al., 2002). The resultant 'fingerprint' obtained by the method employed within this paper, that of FT–IR, is not interpretable by eye (see Fig. 1 for typical spectra) and like other metabolic fingerprinting methods is analysed using chemometric methods. Chemometrics is the discipline concerned with the application of statistical and mathematical methods to chemical data (Massart et al., 1988; Martens and Næs, 1989), and the most common ones are based on dimensionality reduction via cluster analysis using principal components analysis (PCA) (Jolliffe, 1986) and discriminant function analysis (DFA) (Manly, 1994; Timmins et al., 1998).

Using a combination of FT–IR with the most appropriate chemometrics metabolic fingerprinting has been successfully employed in the classification of olive oil (Lai et al., 1994), adulteration of cocoa butters (Goodacre and Anklam, 2001), examination of salinity effects on tomato fruit (Johnson et al., 2000) and in the differentiation of bacterial and fungal species (Helm et al., 1991; Goodacre et al., 1998; Timmins et al., 1998) including mutants (Oliver et al., 1998) and bacterial physiological parameters (Goodacre et al., 2000). However, applying metabolomic techniques to plants is a new and innovative field generally used as a complementary method to transcriptome and proteome analyses of the same plant material within functional genomics (Fiehn et al., 2000; Fiehn, 2001, 2002; Phelps et al., 2002).

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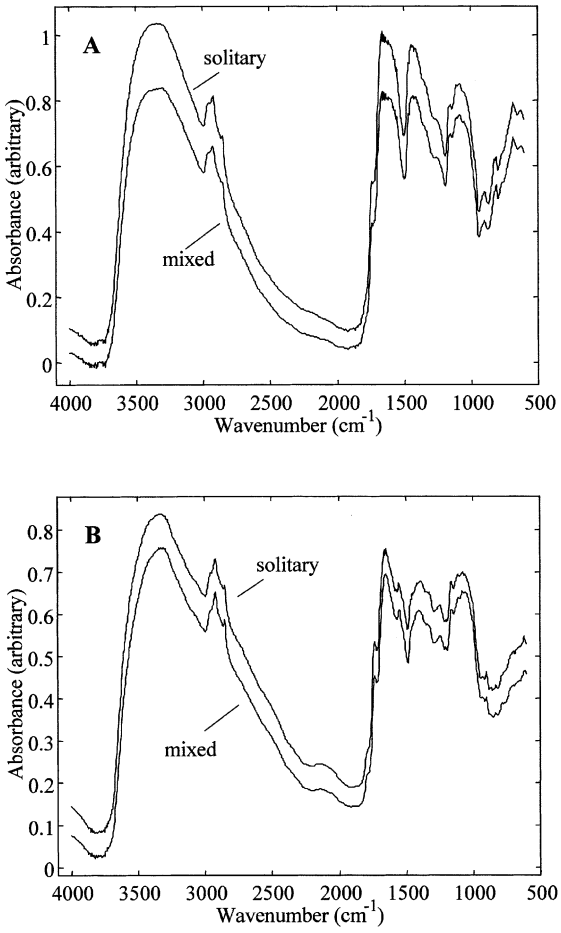

Fig. 1. Typical raw FT–IR spectra of (A) *A. thaliana* and (B) *B. distachyon* under solitary conditions and inter-specific co-existence (mixed).

Ecological systems are highly complex and variable, but importantly contain a multitude of chemicals and functions that are as yet undiscovered. Traditional chemical ecological approaches have been strongly hypothesis driven, testing logical chemicals (experimenter driven) in response to a perturbation or processes. However, such approaches are confining, constrained and often biased and *subjective* because they rely heavily on the experience of the researcher in guiding and selecting the chemical analysis. By contrast, metabolic fingerprinting (e.g., via FT–IR) takes a more global approach and detects whether there are any changes occurring in response to a particular factor. This route allows high throughput screening which is appropriate for application in large scale and highly variable ecological systems. Such a holistic approach is therefore more *objective* than traditional approaches. Thus it should be emphasised at this stage that such technologies are potentially hypothesis deriving, by allowing an indication of which metabolic changes are involved in a given situation, rather than strongly hypothesis driven, such as making an initial assumption as to how chemistry is affected and then analyzing a specific compound (Goodacre et al., 2000; Kell et al., 2001; Goodacre and Kell, 2003).

Inter-specific plant–plant interference is considered to be an obvious source of influence on the metabolic fingerprint of plants in the field and thus a simple glasshouse experiment was designed in order to test whether such influence could be discriminated by FT–IR. The mechanisms that affect plant growth in relation to interference can be split into two areas, resource competition and allelopathy (Grace and Tilman, 1990; Harper, 1997). Resource competition can be generally defined as implying a limitation of a factor required for plant growth, such as sunlight, water, or nutrients (Putnam and Tang, 1986; Rice, 1984). Allelopathy can be described as the effect of chemical compounds produced by plants or microbes on another plant, either directly or indirectly (Rice, 1984; Inderjit and delMoral, 1997). These compounds can arise from leaf leachates or extracts (Michelsen et al., 1995; Quayyum et al., 2000), decomposing material (Blackshaw et al., 2001) and/or root exudates (Inoue et al., 1992; Macharia and Peffley, 1995; Baghestani et al., 1999; Ridenour and Callaway, 2001).

Our aim was to discover if plant–plant interference could produce detectable changes at the level of the metabolic profile of a leaf. The two species selected for this were *Brachypodium distachyon* and *Arabidopsis thaliana* (Col-0). This choice was purely for their genetic background rather than as a real ecological system. *B. distachyon* is an important model species in the study of temperate cereals with its small genome and rapid life-cycle (Draper et al., 2001) while a wealth of information exists for *A. thaliana* due to its status as a classic model dicotelydon species in molecular biology.

2. Results and discussion

As expected all growth measurements for *A. thaliana* showed significant increases over time (Table 1). Co-existence with *B. distachyon* significantly reduced cauline leaf area (−14%; difference between means), cauline leaf biomass (−12%), root biomass (−40%) and total biomass (−10%). However, a significant interaction between plant age and competition was observed for root biomass. As plants became older, partitioning significantly altered with LWR, RWR, LAR and SLA being reduced, while SWR and IWR increased (Table 2, and see footnote within for definitions of partitioned growth parameters). Competition only appeared to affect SWR (6% increase) and RWR (22% reduction) although RGR and RWR were affected by an interaction between plant age and competition.

B. distachyon also showed significant increases for all growth measurements over time (Table 1). Competition

for this species significantly inhibited both leaf area (−21%) and biomass (−20%) while root biomass was significantly stimulated (+40%). Partitioned growth for this species exhibited plant age by competition interactions for the parameters LWR, RWR and LAR (Table 2). However SWR was significantly inhibited by competition alone (−14%). Unsurprisingly, all growth parameters were significantly increased over time, with the exception of RWR, which appeared to be inhibited by plant age.

The next stage was to analyse the same plant tissues by FT–IR as detailed in the experimental section below.

Table 1
Results of balanced two-factor ANOVAs for growth measurements taken from *A. thaliana* and *B. distachyon*[a]

Species	Growth measurement	Source of variation										
		Within and residual		Competition			Age			Competition * age/time		
		SS	df	SS	df	*F*	SS	df	*F*	SS	df	*F*
Arabidopsis thaliana (Col-0)	Rosette leaf area (mm^3)	42 042 037	28	2 873 181	1	*1.91*	174 594 934	2	*58.14***	5 450 418	2	*1.81*
	Rosette leaf biomass (g)	0.0164	28	0.0014	1	*2.43*	0.0886	2	*75.77***	0.0011	2	*0.96*
	Cauline leaf area (mm^3)	58 056 250	70	4 411 536	1	*5.32**	439 958 805	2	*265.24****	3 398 219	2	*2.05*
	Cauline leaf biomass (g)	0.0043	28	0.0012	1	*7.98**	0.1890	2	*617.15****	0.0007	2	*2.34*
	Inflorescence biomass (g)	54.72	70	0.58	1	*0.74*	271.99	2	*173.97****	0.10	2	*0.19*
	Stalk biomass (g)	1.85	28	0.05	1	*0.69*	196.13	2	*1483.52****	0.10	2	*0.77*
	Root biomass (g)	0.0060	28	0.0154	1	*71.81****	0.0502	2	*117.28****	0.0072	2	*16.85****
	Total biomass (g)	0.85	28	0.27	1	*8.93***	75.09	2	*1240.86****	0.11	2	*1.78*
Brachypodium distachyon (ABR1)	Leaf area	8.28	70	1.25	1	*10.61***	18.73	2	*79.18****	0.40	2	*1.68*
	Leaf biomass (g)	11.35	70	1.12	1	*6.91**	43.23	2	*133.27****	0.80	2	*2.45*
	Stalk biomass (g)	14.25	70	0.56	1	*2.73*	53.67	2	*131.80****	0.22	2	*0.54*
	Root biomass (g)	10.53	70	2.59	1	*17.24****	37.91	2	*126.04****	0.39	2	*1.30*
	Total biomass (g)	9.26	70	0.00	1	*0.00*	44.29	2	*167.26****	0.11	2	*0.40*

[a] Number of plants equals 90 for each species. This total is spread between the two main factors (Competition and Harvest) which, when crossed, give 6 overall factors ($n=15$). Sum of squares (SS), degrees of freedom (df) and *F*-ratios (*F*) given for competition (solitary and mixed), age ($t=1$, $t=2$ and $t=3$) and the interaction between them both as well as SS and df for the within and residual error. Significance levels for balanced ANOVA presented next to corresponding *F*-ratios where statistically significant differences arise (*where $P\geqslant 0.05$, **where $P\geqslant 0.01$ and ***where $P\geqslant 0.001$) (Sokal and Rohlf, 1969).

Table 2
Results of balanced two-factor ANOVAs for partitioned growth measurements[a,b]

Species	Partitioned growth measurement	Source of variation										
		Within and residual		Competition			Age/time			Competition* age/time		
		SS	df	SS	df	*F*	SS	df	*F*	SS	df	*F*
Arabidopsis thaliana (Col-0)	LWR	0.0694	28	0.0021	1	*0.86*	3.4372	2	*692.88****	0.0013	2	*0.26*
	RWR	0.0670	70	0.0268	1	*27.95****	0.1689	2	*88.17****	0.0066	2	*3.46**
	SWR	0.0346	28	0.0057	1	*4.59**	1.6330	2	*661.13****	0.0016	2	*0.66*
	IWR	0.0188	28	0.0021	1	*3.09*	0.5703	2	*425.20****	0.0005	2	*0.37*
	LAR ($mm^2\ mg^{-1}$)	430.50	28	56.44	1	*3.67*	13 651.97	2	*6825.99****	37.49	2	*1.22*
	SLA ($mm^2\ mg^{-1}$)	1.183	70	0.002	1	*0.14*	0.489	2	*14.48****	0.066	2	*1.95*
	RGR ($g.g^{-1}\ day^{-1}$)	0.0111	42	0.0010	1	*3.70*	0.0867	1	*328.98****	0.0037	1	*13.87****
Brachypodium distachyon (ABR1)	LWR	0.19	70	0.17	1	*62.01****	0.06	2	*1.33*	0.06	2	*10.51****
	RWR	0.26	70	0.32	1	*76.06****	0.05	2	*6.75***	0.09	2	*10.33****
	SWR	0.10	70	0.02	1	*16.02****	0.03	2	*11.32****	0.00	2	*0.91*
	LAR ($mm^2\ mg^{-1}$)	0.0030	70	0.0020	1	*46.24****	0.0066	2	*75.75****	0.0005	2	*5.98***
	SLA ($mm^2.mg^{-1}$)	0.00033010	70	0.00000054	1	*0.12*	0.00095612	2	*101.39****	0.00000552	2	*0.59*
	RGR ($g.g^{-1}\ day^{-1}$)	0.0484	42	0.0022	1	*1.87*	0.1029	1	*89.37****	0.0001	1	*0.06*

[a] Inflorescence weight ratio (IWR), leaf area ratio (LAR), leaf weight ratio (LWR), relative growth ratio (RGR), root weight ratio (RWR), specific leaf area (SLA), and shoot weight ratio (SWR) according to Hunt (1978) taken from *A. thaliana* and *B. distachyon*.

[b] Number of plants equals 90 for each species. This total is spread between the two main factors (Competition and Harvest) which, when crossed, give six overall factors ($n=15$). Sum of squares (SS), degrees of freedom (df) and *F*-ratios (*F*) given for competition (solitary and mixed), age ($t=1$, $t=2$ and $t=3$) and the interaction between them both as well as SS and df for the within and residual error. Significance levels for balanced ANOVA presented next to corresponding *F*-ratios where statistically significant differences arise (*where $P\geqslant 0.05$, **where $P\geqslant 0.01$ and ***where $P\geqslant 0.001$) (Sokal and Rohlf, 1969).

The resulting whole-tissue FT–IR metabolic fingerprints are complex and Fig. 1 shows typical FT–IR spectra of *B. distachyon* and *A. thaliana*. These spectra, and indeed all those collected (data not shown) show broad overlapping peaks with recognisable amide I (C=O at $\sim$1650 cm^{-1}) and amide II (NH and CN at $\sim$1550 cm^{-1}) protein vibrations, acyl CH_2 and CH_3 vibrations from fatty acids in the region 3050–2800 cm^{-1}, polysaccharides (C–O and ring vibrations) at $\sim$1090 cm^{-1} and P=O vibration from nucleic acids at 1320 cm^{-1} (Naumann et al., 1988; Stuart, 1997; Schmitt and Flemming, 1998). The complexity of these spectra means that they are essentially uninterpretable to the naked eye and thus multivariate analyses are needed to interpret these hyperspectral data.

Therefore DFA was used to analyse these FT–IR spectra and the pseudo 3 dimensional plot of the first three discriminant functions is shown in Fig. 2. It is clear that DFA can differentiate between 'solitary' and 'mixed' *B. distachyon* plants at 42 day of age, thus demonstrating the power of this cluster analysis method for the simplification of these metabolic fingerprint data. Additionally, cross-validation of the DFA was performed by splitting the data into a training and independent test set as described elsewhere (Radovic et al., 2001). Projection of the test-set variables into this resultant PC-DFA model showed satisfactory agreement, based on competition treatment, between the training and independent test-set (data not shown for brevity). This confirms that the separation noted is real and not simply an artefact of the analytical procedure. By contrast, there were no apparent differences in the metabolome of *A. thaliana* in any of the harvests even though this species clearly exhibited morphological responses to co-existence with *B. distachyon*. To answer the question of whether this plant–plant interference is attributable to allelopathy, shading or below ground resource competition is not possible (Inderjit and del-Moral, 1997), but this will be a matter for future study.

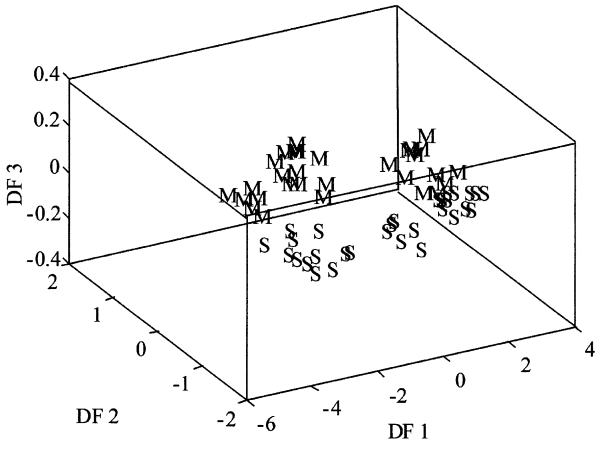

Fig. 2. Pseudo 3D DFA analysis ordination plot of *B. distachyon* FT–IR spectra using 20 principal components (explaining 99.817% of the total variance). Separation shown for plants at 42 days based on competition treatments (S = solitary; M = mixed with *A. thaliana*). DFA *a priori* class structure based on grouping machine replicates of two separate samples together (five a priori classes per treatment). Each point is based on an average score calculated from the scores for each samples machine replicates.

Most previous studies concerning plant–plant interference have concentrated on morphological responses alone (e.g. see Inoue et al., 1992; Macharia and Peffley, 1995; Baghestani et al., 1999; Ridenour and Callaway, 2001). One study has shown changes, both positive and negative, due to simulated plant–plant interference in chlorophyll a concentration when *Juncus effuses* and two sedge species were subjected to shoot extracts from *J. effuses* (Ervin and Wetzel, 2000). Thus we believe that the ability of FT–IR to detect alterations in the metabolic fingerprint of *B. distachyon* leaves as a result of plant–plant interference is highly exciting and worthy of further investigation.

By contrast to GC–MS and LC–MS which involve the preparation of specific plant extracts, FT–IR is not biased to any specific chemical species and thus generates a 'holistic' (bio)chemical fingerprint of the biological sample under investigation. The power of this approach in detecting unforeseen alterations in global metabolic profiles is valuable and could be used as a rapid screening method prior to any targeted metabolite analyses. Ultimately, the real challenge with reference to the current scenario will be to analyse plant–plant and other trophic interactions in natural ecosystems.

3. Experimental

3.1. Growth analysis and harvesting

The experiment involved two treatments for both *B. distachyon* and *A. thaliana* (Col-0), solitary (single plants in pots) and mixed (one of each species in a pot). Three harvests were performed during the duration of the experiment ($t = 1$ at 25 d, $t = 2$ at 34 d and $t = 3$ at 42 d). Overall, each combination of competition, species and harvest had a total of 10 replicates ($n = 120$) within a randomised block design. Plants were harvested for dry weight partitioning and leaf samples from each species (rosette leaves from *A. thaliana*) were collected for FT–IR analysis.

Balanced two-factor Analysis of Variance (ANOVA) was performed on growth measurements (ln transformed) and partitioning (arcsine transformed where necessary) in order to test for any significant interactions between competition treatment and plant age (for explanation of ANOVA see Sokal and Rohlf, 1969). Block effects were also incorporated within the ANOVAs. For the parameters where no other factors interacted with block the ANOVAs were recalculated accordingly.

3.2. Sample preparation and Fourier transform–infrared spectroscopy

Sample preparation for FT–IR analysis was optimised for the two plant species such that good spectra with high signal-to-noise ratio were obtained (that is to say maximum absorbance was between 0.6 and 1.2). It was found that the most optimal spectra were obtained from fresh leaf material with dry weight for *B. distachyon* of 65 mg ml^{-1} and *A. thaliana* of 25 mg ml^{-1}; fresh leaf moisture content was taken into account when calculating these concentrations. Leaves were weighed (typical dry weights were ~15 mg for *B. distachyon* and ~5 mg for *A. thaliana*) and immediately frozen in liquid N_2 (freeze clamping) to stop any inherent enzymatic activity which would alter the metabolite pool in the plants. All samples were stored at −80 °C and great care was taken to avoid thawing tissues before creating whole-tissue slurries by grinding with liquid N_2. Tissue suspensions were prepared in milli-Q water to the concentrations given above, vortexed and immediately applied into the wells of an aluminium plate FT–IR sample carrier.

Prior to analysis the samples were oven-dried at 50 °C for 60 min. Samples were run in triplicate and these pseudoreplicates are henceforth referred to as machine replicates. The FT–IR instrument used was a Bruker IFS28 FT–IR spectrometer (Bruker Spectrospin Ltd., Banner Lane, Coventry, UK) equipped with an MCT (mercury–cadmium–telluride) detector cooled with liquid N_2. The aluminium plate was then loaded onto the motorised stage of an adapted reflectance TLC accessory (Timmins et al., 1998). The PC used to control the IFS28, was also programmed (using OPUS version 2.1 software running under IBM O/S2 Warp provided by the manufacturers) to collect spectra over the wavenumber range 4000–600 cm^{-1}. Spectra were acquired at a rate of 20 s^{-1}. The spectral resolution used was 4 cm^{-1}. To improve the signal-to-noise ratio, 256 spectra were co-added and averaged. Each sample was thus represented by a spectrum containing 882 points (see Fig. 1 for typical examples) and spectra were displayed in terms of absorbance as calculated from the reflectance-absorbance spectra using the Opus software [which is based on the Kubelka–Munk theory (Griffiths and de Haseth, 1986)].

3.3. Spectral pre-processing

To minimize problems arising from baseline shifts three preprocessing steps were taken as detailed in Timmins et al. (1998). First CO_2 peaks were removed from the spectra, then spectra were normalised so that the smallest absorbance was set to 0 and the highest to +1 for each spectrum, and finally the minimum and maximum bins of the spectra were detrended to 0.

3.4. Cluster analysis

The initial stage involved the reduction of the multidimensional FT–IR data by PCA (Jolliffe, 1986). PCA is a well-known technique for reducing the dimensionality of multivariate data whilst preserving most of the variance. PCA was performed according to the NIPALS algorithm (Wold, 1966). DFA, also known as canonical variates analysis (CVA), then discriminated between groups on the basis of the retained PCs and the a priori knowledge of which spectra were replicates. Thus this process does not bias the analysis in any way (Manly, 1994). These cluster analysis methods were implemented using Matlab version 5 (The Math Works, Inc., 24 Prime Par Way, Natick, MA, USA), which runs under Microsoft Windows NT on an IBM-compatible PC.

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