APPLYING METABOLIC FINGERPRINTING TO ECOLOGY: THE USE OF FOURIER-TRANSFORM INFRARED SPECTROSCOPY FOR THE RAPID SCREENING OF PLANT RESPONSES TO N DEPOSITION

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Abstract. The potential for metabolic fingerprinting via Fourier-transform infrared (FT-IR) spectroscopy to provide a novel approach for the detection of plant biochemical responses to N deposition is examined. An example of spectral analysis using shoot samples taken from an open top chamber (OTC) experiment simulating wet ammonium deposition is given. Sample preparation involved oven drying and homogenisation via mill grinding. Slurries of a consistent dilution were then prepared prior to FT-IR analysis. Spectra from control, 8 and 16 kg N ha^{-1} yr^{-1} treatments were then subjected to cross-validated discriminant function analysis. Ordination diagrams showed clear separation between the three N treatments examined. The potential for using *Calluna vulgaris* (L.) Hull as a bioindicator of N deposition is further evident from these results. The results also clearly demonstrate the power of FT-IR in discriminating between subtle phenotypic alterations in overall plant biochemistry as affected by ammonium pollution.

Keywords: ammonium deposition, *Calluna vulgaris*, chemometrics, critical load, metabolic fingerprinting, plant response

1. Introduction

Concern over deposition of atmospheric NH_3 and NH_4^+ (collectively NH_y) converting nutrient-poor ecosystems to those favouring nitrophilic plant species has been widely reported (Krupa, 2003). Particular interest has focused on *Calluna vulgaris* (L.) Hull because of its reported sensitivity to NH_y (Van Der Eerden *et al*., 1991; Pitcairn *et al*., 1995; Lee and Caporn, 1998; Carroll *et al*., 1999). Most published studies to date either concentrate on morphological responses (Carroll *et al*., 1999), alterations in physiology (Caporn *et al*., 1994) or simple aspects of shoot chemistry such as total N (Kirkham, 2001). Among other plant responses a positive correlation between shoot N and increasing N deposition has been observed (Pitcairn *et al*., 1995; Hicks *et al*., 2000). This relationship has led to foliar N concentrations in *C. vulgaris* being considered as a bio-indicator of N deposition and heath land

Water, Air, and Soil Pollution: Focus **4:** 251–258, 2004.

health (Pitcairn *et al*., 1995; Hicks *et al*., 2000). However, there is a suggestion that other factors influence foliar N in *C. vulgaris* (Hicks *et al*., 2000). Identification of a particular shoot chemistry response to N deposition, especially relating to exceedance of this species critical load, would therefore be extremely useful both to identifying early symptoms of critical load exceedance, and therefore damage, and in furthering its potential as a bioindicator. However, detection of responses around the critical loading of 10–15 kg N ha^{-1} y^{-1} (Ashmore *et al*., 2003) would require a more sensitive technique than those detailed above.

Metabolic fingerprinting allows rapid classification of a sample according to its origin or biochemical relevance (Fiehn, 2001; Johnson *et al*., 2003). The discipline has been described as obtaining "enough information to unravel (otherwise hidden) metabolic alterations without aiming to get quantitative data for all biochemical pathways" (Fiehn, 2001). Essentially this technique screens and produces a spectral description of a sample's entire biochemistry. Attempts can then be made to categorise samples based on potentially subtle alterations in their metabolic fingerprint. Hence, metabolic fingerprinting is considered 'hypothesis generating' (Kell and Oliver, 2004) because of its unbiased and holistic nature.

The techniques of nuclear magnetic resonance (NMR) (Lindon *et al*., 2000), Fourier-transform infrared spectroscopy (FT-IR) (Timmins *et al*., 1998; Gidman *et al*., 2003; Johnson *et al*., 2003), electrospray ionisation mass spectrometry (ESI-MS) (Vaidyanathan *et al*., 2001, 2002; Goodacre *et al*., 2002) and Fourier-transform ion cyclotron mass spectrometry (FT-MS) (Aharoni *et al*., 2002) are all examples of rapid analytical methods that are capable of performing this form of analysis. The highly complex 'fingerprints' obtained by these methods (see Figure 1 for example) are interpreted using chemometric methods (Massart *et al*., 1988; Martens and Næs, 1989) including principal components analysis (PCA) (Jolliffe, 1986) and discriminant function analysis (DFA) (Manly, 1994; Timmins *et al*., 1998). The particular metabolic fingerprinting method that we have examined and subsequently report on within this paper is FT-IR.

FT-IR is a high-throughput and relatively inexpensive metabolic fingerprinting technique that measures molecular functional group vibrations (Goodacre *et al*., 1998; Timmins *et al*., 1998; Ellis *et al*., 2003). In this capacity, as opposed to conventional techniques like total N assays, measurements are not constricted to particular regions of chemistry or compounds. Instead FT-IR essentially allows a 'snap-shot' of a sample's entire chemistry at a given point in time to be taken. FT-IR has already been proved to be a successful technique in the differentiation of bacterial and fungal species (Helm *et al*., 1991; Goodacre *et al*., 1998; Timmins *et al*., 1998), detection of meat spoilage (Ellis *et al*., 2002), examination of salinity effects on tomato fruit (Johnson *et al*., 2003), investigation of intracellular macromolecular pools in marine microalgae (Giordano *et al*., 2001) and the investigation of plant-plant interference (Gidman *et al*., 2003). Here we investigate whether FT-IR can detect changes in *C. vulgaris* biochemistry induced by NH_4^+ increases over a deposition range that crosses the critical load for *C. vulgaris* heathlands.

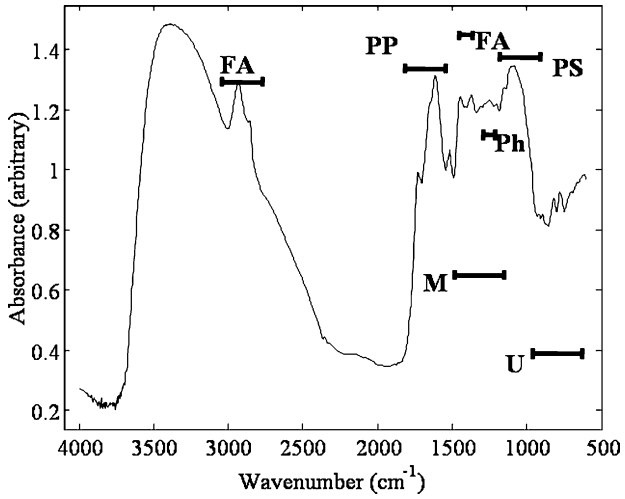

Figure 1. Typical raw FT-IR spectra of *C. vulgaris* oven-dried shoot tissue. Regions that experience functional group vibrations that relate to particular molecular species are highlighted; FA = fatty acids, PH = phosphates, PP = peptides, PS = polysaccharides, U = unassigned and M = mixed region of proteins, fatty acids, phosphate rich.

2. Materials and Methods

2.1. Experimental design and sample acquisition

C. vulgaris samples were collected from the Centre for Ecology and Hydrology's (CEH) OTC site (18 km SW of Edinburgh). During the summer of 1998, a central square in each OTC was excavated to 3 m^2 by 0.5 m deep. This excavation was then back-filled with peat and individual young (<3 y) plants of seven species, taken from a nearby ombrotrophic bog (Leith *et al.*, 2001, 2002). Overall six NH_4^+–N deposition treatments, applied as NH_4Cl mist, and five gaseous NH_3 deposition treatments exist. However, for the purpose of this paper, only three of the misting treatments are discussed (de-ionised water control, 8 and 16 kg N ha^{-1} y^{-1}). A full description of the experimental design can be found in Leith *et al.* (2001) and a description of the OTCs can be found in Fowler *et al.* (1989).

C. vulgaris samples were taken during October 2002. Four shoots, each about 4 cm in length, of current years growth were selected per plant and these samples were transported on ice. Samples were then oven-dried at 80 °C for 48 h before being sent to University of Wales, Aberystwyth (UWA) for FT-IR analysis.

2.2. Fourier-transform infrared spectroscopy

Samples were oven-dried upon arrival at UWA at 60–80 °C for a period of 48 h and then allowed to cool inside desiccators. Sample homogenisation was

then performed by ball milling at 30 Hz for 30 s. $\sim$ 20 mg of powder per sample was placed in 2 ml Eppendorf tubes before addition to the FT-IR plate. Immediately prior to analysis, samples were diluted with MilliQ H_2O to a constant factor of 100 mg ml^{-1}, the resultant slurries were applied to a 100 well aluminium plate and oven-dried for 30 min at 50 °C. The FT-IR instrument used was a Bruker IFS28 FT-IR spectrometer (Bruker Spectrospin Ltd., Banner Lane, Coventry, UK) equipped with a mercury-cadmium-telluride detector (more detail on the FT-IR equipment can be found in Timmins *et al*., 1998). Each sample, once analyzed, was represented by a spectrum containing 882 points (Figure 1) 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)).

2.3. Spectral processing and chemometric analysis

Spectra were processed prior to analysis in order to minimise spectral baseline shifts (Timmins *et al*., 1998; Gidman *et al*., 2003). This involved replacing CO_2 peaks (wavenumbers 2403–2272 cm^{-1} and 683–656 cm^{-1}) with a smooth trend, normalising the spectra so that the minimum and maximum absorbancies were set to 0 and +1 respectively, and finally detrending the minimum and maximum wavenumbers of the spectra to 0 (Timmins *et al*., 1998; Gidman *et al*., 2003). Initially, cluster analysis involved the reduction of the multidimensional FT-IR data by PCA (Jolliffe, 1986) according to the NIPALS algorithm (Wold, 1966). DFA, also known as canonical variates analysis (CVA), then discriminated between spectra using the retained principal components and the *a priori* knowledge of spectral treatment grouping. In order to assess whether the analysis was biased due to the high level of *a priori* knowledge an independent test set, taken prior to analysis from the data set in question, was generated randomly and projected into the DFA space (Radovic *et al*., 2001). Succesful validation, arising from test set spectra projecting into their relavant clusters, confirms real separation. Thus, DFA analysis was repeated on the entire data set, comprising both training and test sets whereas otherwise no differences between groups would have been assumed. These cluster analysis methods were implemented using Matlab version 5 (The Math Works, Inc., 24 Prime Par Way, Natick, MA, U.S.A.), which runs under Microsoft Windows NT on an IBM-compatible PC.

3. Results

The analysis by DFA, shown in Figure 2, provided good clustering between all three treatments (control, 8 and 16 kg N ha^{-1} y^{-1}). Two effects appear discernible from the DFA plot. Examination of the first discriminant function (DF) shows apparent separation between the control and N treated spectra. The second DF appears to

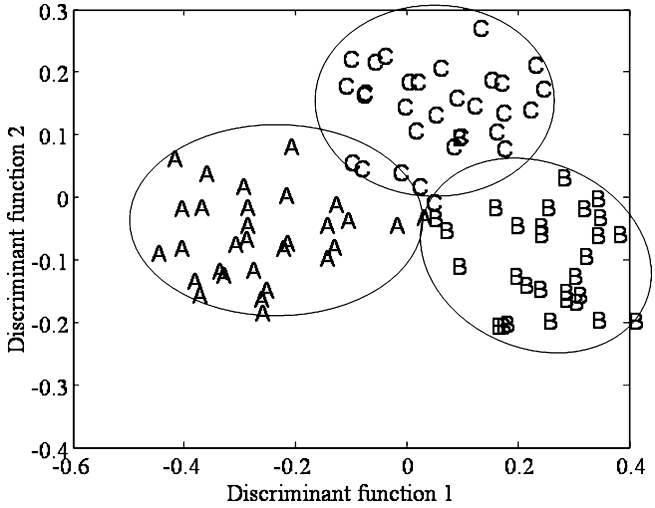

Figure 2. DFA ordination plot of *C. vulgaris* spectra using 17 principal components (explaining 99.74% of the total variance). Separation shown for plants subjected to 0 (A), 8 (B) and 16 (C) kg NH_4 ha^{-1} yr^{-1}. DFA *a priori* class structure based on grouping all spectra per treatment (one *a priori* class per treatment). Previous cross-validation ($n = 7$ for training, $n = 3$ for testing) validated this analysis (results not shown).

describe increases in N addition, from 8 to 16 kg N ha^{-1} y^{-1}, although here the control spectra cluster overlaps with both of the two N treatment clusters. Considering both DFs share a similar scale (from about -0.4 to $+0.4$) both contain equally important effects in terms of the model. Previous cross-validation of this model confirmed that the observed separation is not simply an artefact of the analytical procedure.

4. Discussion

Our results clearly show that wet application of NH_4Cl alters *C. vulgaris* shoot chemistry. Additionally, the FT-IR method can discriminate between effects caused by simulated depositions of 8 and 16 kg N ha^{-1} y^{-1}. This is important as this relatively small range crosses the critical load for N for UK upland *Calluna* heaths (Ashmore *et al.*, 2003). This strongly suggests that metabolic fingerprinting can detect shoot biochemistry fluctuations arising from NH_4^+ deposition in the field and may be useful in identifying pre-symptomatic changes. Furthermore, considering the technique used oven-dried whole shoot tissue, no initial assumption concerning shoot biochemistry fluctuations was required. This underlines the techniques 'hypothesis generating' power, and thus allows further exploration through more advanced metabolomic methods and chemometrics to target specific metabolites.

Further analysis of the data using more powerful machine learning methods such as artificial neural networks (Warner and Misra, 1996; Goodacre *et al*., 1998) and genetic algorithms (Davies *et al*., 2000; Johnson *et al*., 2003) may be able to narrow the difference seen down to a particular region of the metabolome. This knowledge would allow targeted metabolite analyses to elucidate the particular changes in metabolic chemistry due to NH_y treatment.

FT-IR has lower resolution compared with other fingerprinting methods such as NMR, GC-MS or ESI-MS, meaning its ability to identify specific metabolites is relatively poor. This could hamper the sensitivity of the technique when compounds at trace levels are involved in plant responses, especially considering the complexity of the resultant data obtained from whole oven-dried plant tissue. Chemometric data mining techniques are therefore still under development, and here the aim is to determine how much useful information can be derived from FT-IR spectra. However, it does have the benefit of being high-throughput.

A trade-off between ability to successfully identify metabolites and speed exists. Hence, analysis times of about 10 s per sample are typical of FT-IR, whereas with NMR and MS techniques a single sample takes several minutes to analyse. Not only does FT-IR have relative rapidity, it is also cost effective and not biased to any chemical species unlike NMR and MS. Additionally, as shown here, the results from the OTC experiment clearly illustrate the technique's potential for the future as a primary screening method before targeted analysis.

Overall, FT-IR has been shown to be a powerful tool in the high-throughput screening of plant tissue. Further development may prove that FT-IR is a valuable addition in understanding plant biochemistry responses to environmental factors, especially alongside conventional chemical analyses and more powerful metabolomic techniques, such as MS (Fiehn, 2001). Current work is assessing the techniques potential to determine NH_y response detection using *C. vulgaris* plant tissue obtained from the field, and to determine if the N signal is masked by other sources of variability such as climate, age, grazing pressure and N form.

Acknowledgements

The authors are grateful to the NERC for funding a CASE PhD under the GANE consortium for Ed Gidman. The staff of CEH Bush are thanked for their assistance in sample collection from their OTC experiment and the UK Department of the Environment, Transport and the Regions (Contract no. EPG 1/3/94) and NERC are thanked for their financial support towards this project. David I. Ellis is thanked for his technical assistance with FT-IR operation. Finally, we would like to show our continued appreciation to Dr. Helen Johnson for her assistance in chemometric analysis and current maintenance of the FT-IR apparatus.

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