REIMS (Rapid Evaporative Ionization Mass Spectrometry) and Multi-variant Statistics, Two Tools in Support of Weed Grass Speciation and Phenotype Characterization

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INTRODUCTION

The control of grass weeds in commercial wheat crop production represents a major challenge to sustainable intensification in arable agriculture in Europe and is a particular problem in the more western countries of England (Figure 1), France, Germany and parts of Belgium and the Netherlands[1]. Black-grass can seriously reduce crop yields through competition for nutrients, especially nitrogen. Consequently, there can be considerable variation in crop yield response to similar black-grass populations. On average, yield losses of 0.4–0.8 t wheat/ha can be expected at black-grass populations of 12–25 plants/m^2, but much higher losses of over 2 t wheat/ha at densities of over 100 plants/m^2 (based on weed free crop yielding 8 t/ha)[2].

Herbicides are often considered the primary method of control, but loss of existing herbicides through regulatory action, lack of new modes of action and increasing resistance means that non-chemical methods will become increasingly important. Post-emergence herbicides, such as chlorotoluron, clodinafop, fenoxaprop, pinoxaden, flupyrsulfuron, mesosulfuron+iodosulfuron and pyroxsulam have the potential to give high levels of control in cereals (>95%), but since the 1980's resistance to all these herbicides is increasing. As weed populations differ in their tolerance to herbicides, methods to discriminate between them using rapid phenotyping technologies are of increasing interest.

REIMS is an emerging direct analysis technique allowing rapid, *in situ* characterization of biological tissues[3]. Here we applied REIMS to provide direct analysis of weed grass leaves for the identification of species, populations and phenotypic traits based on profiling low molecular weight metabolites. To demonstrate the potential of this approach, we have shown that REIMS is able to rapidly differentiate between leaf material originating from seedlings of *Alopecurus myosuroides* (black-grass) or *Lollium rigidum* (annual ryegrass) and also the emergence of herbicide resistant populations (Figure 1) based on subtle differences in spectral features and ratios across the mass range 50-1200 *m/z* in negative ionization mode.

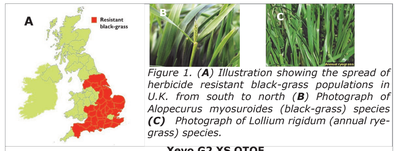

*Figure 1. (**A**) Illustration showing the spread of herbicide resistant black-grass populations in U.K. from south to north (**B**) Photograph of Alopecurus myosuroides (black-grass) species (**C**) Photograph of Lollium rigidum (annual rye-grass) species.*

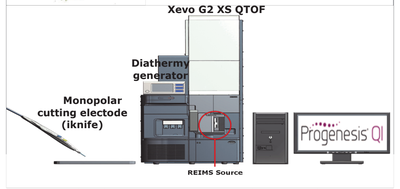

Figure 2. iknife with REIMS instrumentation configuration and MVA informatics software for data interpretation.

METHODS

Weed grasses (wild type black-grass and rye-grass and target site and multi-herbicide resistant populations of black-grass) were cultivated under controlled environmental conditions at the University of Newcastle.

Figure 3. Photograph of cultivated of black-grass wild-type and herbicide resistant populations.

Leaves removed from growing ca. 6 week old seedlings (Figure 3) and directly sampled using the monopolar cutting electrode iknife. The aerosol "smoke" generated from the grass was transferred to the mass spectrometer by a Venturi air jet pump-based ion transfer apparatus mounted in the orthogonal position relative to the atmospheric interface of a time of flight mass spectrometer.

A database of spectra was acquired in negative ion mode over the 50-1200 *m/z* range and MVA models created using Principal Component Analysis (PCA) for data reduction followed by orthogonal partial least squares discriminatory analysis (OPLS-DA).

Biological replicates from the same genetic line of least 5, and technical replicates of at least 3 were used to constructed the MVA models. As a validation of the model, spectra obtained from the test samples were compared to the model space to give a phenotype level classification.

Instrument parameters

Instrument: prototype REIMS source on Xevo G2-XS QTof
Sampling: Monopolar cutting electrode iknife
Cut time: 3–5 s
Acquisition mode: Tof MS in sensitivity mode
Scan rate: 1 scan / s
Polarity: negative
Mass range: 50–1200 m/z
Diathermy settings: autocut mode 20 w
Dopant solvent: Isopropanol (IPA) flow rate: 100 µl/ml

The data analysis was performed using Progenesis QI v2.2 (direct analysis workflow) and EZinfo v3.0.3 (MKS Data Analytics Solutions) to identify species specific and herbicide resistant candidate marker ions in the spectral profiles.

RESULTS AND DISCUSSION

The acquired REIMS spectra featured fatty acids and long chain fatty acids in the 150-500 *m/z* region, glycerophospholipids in the 600-900 *m/z* region and triglycerides in the 900-1000 *m/z* region in all cases. Several other small molecular weight compounds including members of the polyphenol (flavones-C-glycocides, anthocyanins and cyanidin compounds); fatty acid and aromatic acid classes previously characterized in conventional ESI HRMS based metabolomics studies have also been tentatively identified following REIMS with accurate mass precursor ion matching.

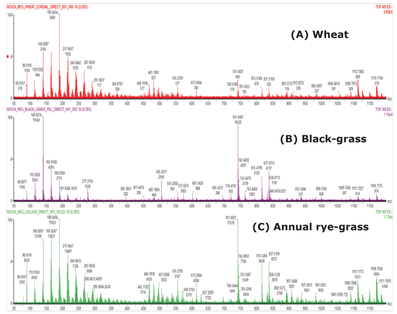

Figure 4. Typical REIMS TOF MS neg ion 50-1200 m/z spectra obtained for wild type wheat (A) and black-grass (B) and rye-grass (C) species.

Spectra obtained from wild type black-grass and rye-grass were modelled using EZinfo following Progenesis QI spectral alignment and peak detection to determine the significant biochemical differences between two weed grass species.

The most unique, species specific features were selected using an S-Plot model (Figure 5) and further work using database searching (PlantCyc, Chemspider and LipidMaps) and REIMS MS(Q)/MS(TOF) (Figure 6) was conducted to determine chemical identity of the candidate markers.

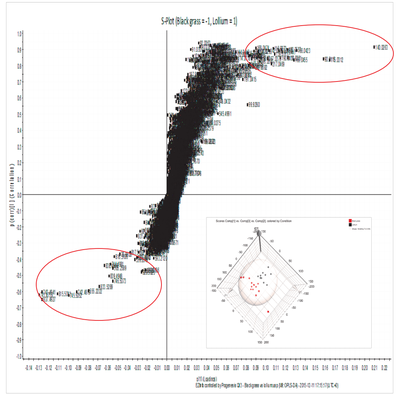

Figure 4. EZinfo S-Plot showing the covariance between wild type black-grass species (lower quadrant) and wild-type rye-grass (upper quadrant) weed grass species following REIMS profiling 50-1200 m/z. Inset shows a 3D OPLS pareto scaled model of wild type black-grass vs. rye-grass species. Red ovals indicate the approximate regions of unique markers that were selected for further characterisation.

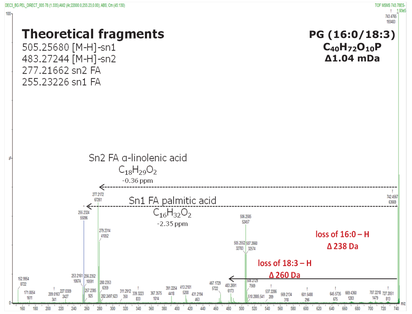

Figure 6. REIMS QTOF MS/MS spectra showing the observed fragmentation profile and tentative identification of PG (16:0/18:3) via theoretical fragment ion match.

As the next step, REIMS spectra from wild-type (1), target site resistant (1) and multi-herbicide resistant (3) populations of black-grass were acquired and modelled using an unsupervised algorithm, OPLS (Figure 7)

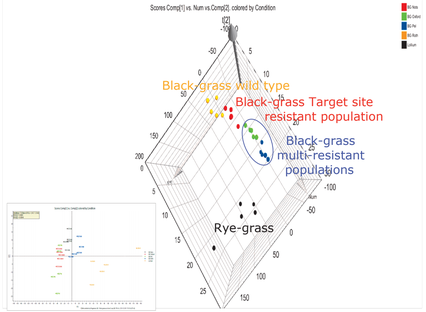

Figure 7. EZinfo 3D OPLS plot showing the different groups obtained following REIMS TOF MS profiling of wild type rye grass, wild type, target site resistant and multi-herbicide resistant populations of black-grass populations. Inset 2D OPLS plot with fit parameters.

In a further experiment, wild type black-grass cultivated from seeds originating from the same genetic line collected over different years were analysed in order to estimate the influence of "genetic drift" on the spectral feature differences.

Some evidence of a population change between 1997, 2004 and 2009 was observed as expressed by changes in the metabolite profile. The wild type populations from 2004 and 2009 model closely in PCA space whereas the population 1997 occupies a different region (Figure 8).

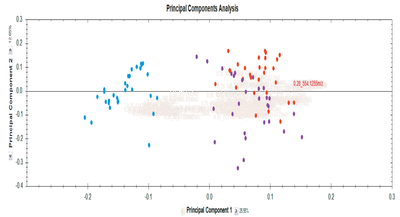

Figure 8. Progenesis QI PCA model showing the influence of "genetic drift" on the metabolite profile in populations (n=5) of wild-type black-grass originating from the same genetic line cultivated from seeds collected from 1997 (blue); 2004 (purple) and 2009 (orange)

A further experiment was conducted using a second cultivation of wild type and herbicide resistant black-grass populations excluding the 1997 population. The REIMS spectral profiles were modeled combining the multi-herbicide resistant populations collected from three different U.K. regions (coded Fen, Pen and Pend) and wild-type from 2 years (2004 and 2009) n=5 (Figure 9).

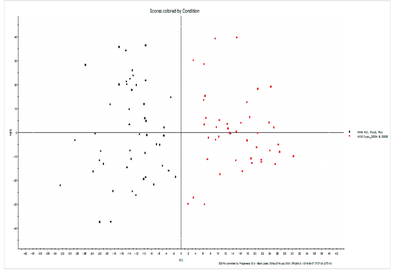

Figure 9. EZinfo OPLS-DA scores plot showing the grouping between 3 populations of multi-resistant black-grass (black) and 2 populations of wild type black-grass.

In order to determine the significance and relevance of the phospholipid class of compounds in the observed spectral profile differences between rye-grass, black-grass and multi-herbicide resistant populations a box and whisker plot was constructed showing 5 phospholipid compounds belonging to three classes whose identity had been tentatively confirmed using REIMS MS/MS previously. The results indicate that members of the phospholipid class represent good candidate markers for both species and herbicide resistance, with no significant overlap between between the 3 populations. Certain candidate phospholipids were consistently found to be down-regulated in the herbicide resistant populations (Figure 10).

The phosphatidic acid (PA) family have been previously reported to serve as second messengers in plants, triggered in response to various biotic and abiotic stresses, including pathogen infection, drought, salinity, wounding, cold, cell death and oxylipin production [4].

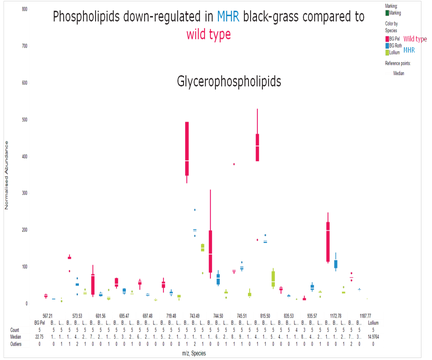

Figure 10. Box and whisker plot showing the significance of certain phospholipid compounds as candidate markers for weed grass speciation (rye-grass vs black-grass) and indicators of herbicide resistant populations of black-grass.

CONCLUSIONS

- We have demonstrated the applicability of iknife sampling with REIMS (QTOF-MS) and chemometric modelling as a tool for rapid speciation of weed grasses and differentiating between wild-type and herbicide resistant populations of the same species.
- The preliminary findings also established that the methodology could distinguish between different black grass populations, based on the profile of certain glycerophospholipid compounds. The significance of these alterations in membrane composition relative to herbicide tolerance traits are currently under investigation.
- Using iknife sampling and REIMS we have observed common marker compounds previously reported following conventional plant metabolomics studies using solvent extraction, UPLC separation and ESI QTof MS.

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