



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

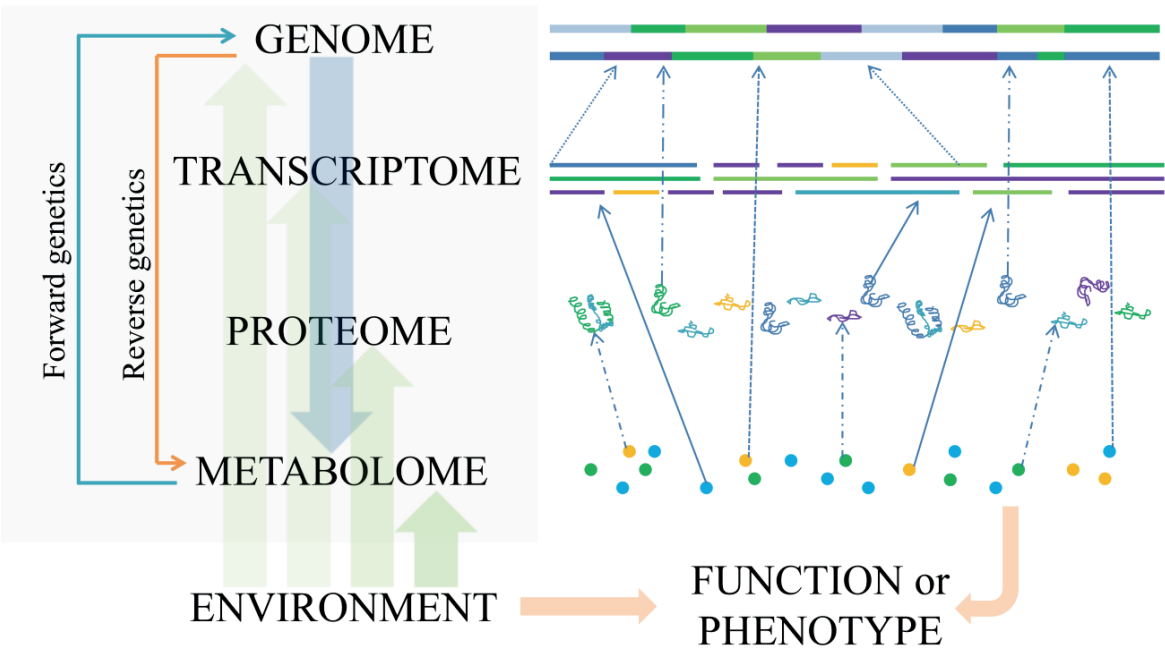


Figure 1. The metabolome is a result of the interaction of a plant's genome with its environment. Metabolites present in a system are the end products of gene expression and can interact with the proteome, transcriptome and genome. Metabolomics can provide an instantaneous reflection of the metabolome 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 truncatula* 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.

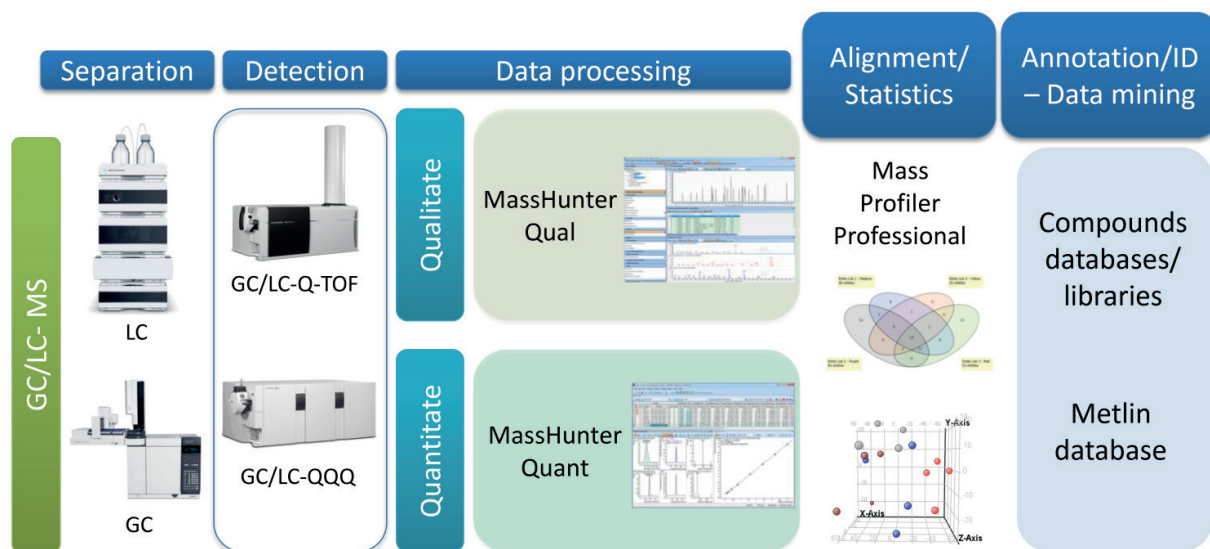


Figure 2. The typical work flow for performing metabolic profiling of secondary plant products. Analytical techniques used in these studies generally include mass spectrometry coupled with gas and/or liquid chromatography, which provides both limited structural, qualitative and quantitative data. Profiling 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 Baci, 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

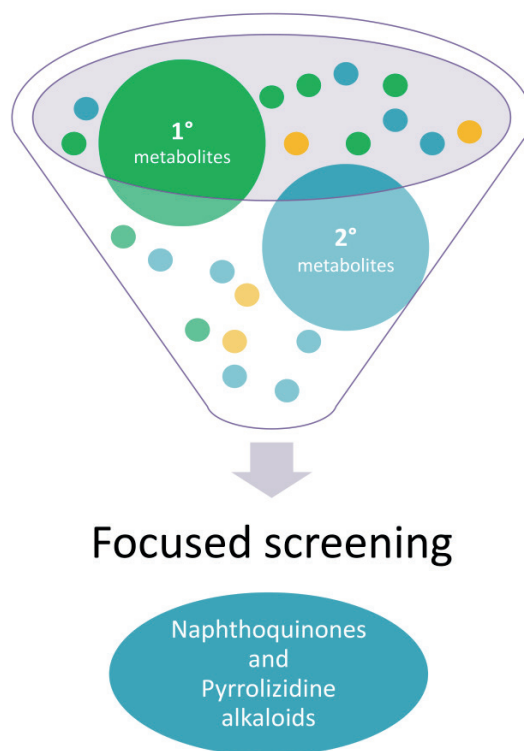


Figure 3. Plant extracts of *Echium plantagineum* were prepared using techniques to enrich for bioactive secondary products of interest, specifically naphthoquinones and pyrrolizidine alkaloids. Targeted metabolic profiling was then performed on compounds of interest. This simplifies the sample matrix 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; Vanellander *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 lephthantine-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

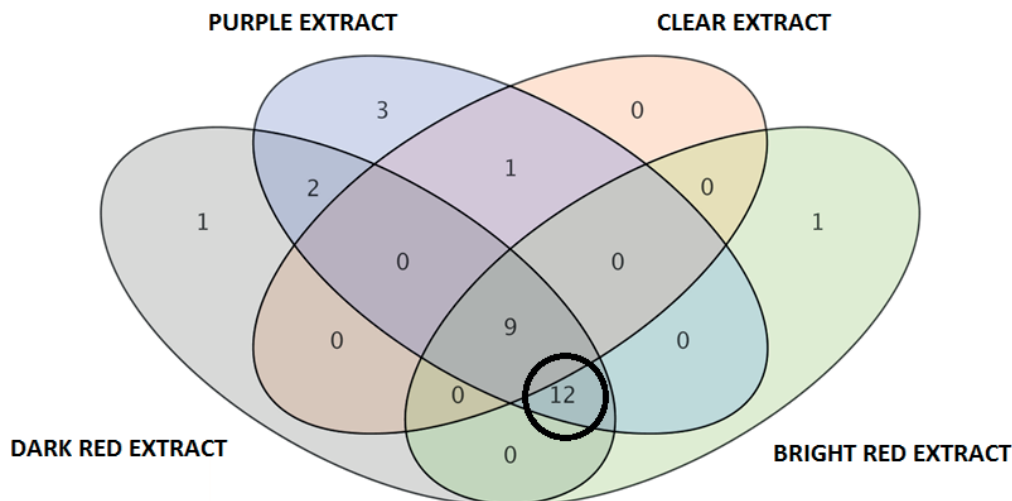


Figure 4. Coloured root extracts of *Echium plantagineum* were separated by their unique colouration into samples that ranged from purple, dark red and bright red to clear. Targeted metabolic profiling resulted in detection of numerous naphthoquinones and other related compounds; coloured samples varied 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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