



Metabolomics: an unexplored tool for allelopathy studies

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ABSTRACT

Allelopathy is a very complex phenomenon, influenced by many inherent and environmental factors. Due to this complexity, its elucidation is a challenging task, requiring expertises from several fields.

A valuable approach to study these interactions is systems biology, a holistic approach giving a broad view of biological systems and relying on omics technologies. Among omics sciences, metabolomics could be very powerful, but despite its potential it has been seldom applied to allelopathy studies.

This review aims to introduce metabolomics approach to researchers involved in the study of allelopathy: to this end, an overview of this approach is given along with examples of application of metabolomics to other ecological issues (effects of abiotic and biotic stresses). Furthermore, the scarce examples of application of metabolomics or metabolomics-related approaches to allelopathy studies are reported.

Keywords: Allelopathy, Ecometabolomics, Metabolomics.

Abbreviations

CE-MS: Capillary Electrophoresis – Mass Spectrometry
DFA: Detrended Fluctuation Analysis
DIMS: Direct Infusion Mass Spectrometry
ESI: ElectroSpray Ionization
FT-ICR-MS: Fourier Transform – Ionic Cyclotron Resonance – Mass Spectrometry
FT-IR: Fourier Transform-Infrared
GC-MS: Gas Chromatography – Mass Spectrometry
HCA: Hierarchical Cluster Analysis
HR-MS: High Resolution – Mass Spectrometry
LC-MS: Liquid Chromatography – Mass Spectrometry
MAS-NMR: magic-angle spinning – Nuclear Magnetic Resonance
MS: Mass Spectrometry

MS-MS (MSn): TANDEM Mass Spectrometry
NMR: Nuclear Magnetic Resonance
PCA: Principal Component Analysis
PCO: Principal Coordinates Analysis
PLS-DA: Partial Least Squares – Discriminant Analysis
QTOF: Quadrupole Mass Analyzer – Time Of Flight
ROS: Reactive Oxygen Species
SOM: Self Organizing Maps
TOF-MS: Time Of Flight – Mass Spectrometry
UPLC-DAD: Ultra-Performance Liquid Chromatography-Diode Array Detector
1D, 2D: one dimensional, two dimensional

Introduction

A small, but growing body of evidence suggests that plants may be capable of recognizing and responding to neighbouring plants in a species specific manner (Broz *et al.* 2010).

Among biotic plant interactions, allelopathy represents an important factor in determining species distribution and abundance as well as in regulating dynamic processes within plant communities (Reigosa *et al.* 2006). The study of this phenomenon is very challenging because of several reasons. Firstly, it is governed by different factors such as individual chemical structure, antagonistic, agonistic, additive, sequential, or synergistic effects. Secondly, the fate of allelochemicals in the soil, their interaction with

the microorganisms in the rhizosphere, biochemical, chemical and photochemical transformations, and many other factors need to be assessed. Moreover, the production of allelochemicals is influenced by abiotic (e.g. temperature, light, soil characteristics, rainfall) and biotic factors (e.g. life cycle, competition, pathogens, pests, parasites, herbivores), with a resulting unequal distribution of putative allelochemicals in a plant in different seasons and in different environmental conditions (Granéli & Salomo, 2010; Inderjit & Duke, 2003).

This complex scenario requires a number of different approaches to assess each distinct process (Scognamiglio *et al.* 2013). Thanks to its holistic view, systems biology could be a valuable tool in allelopathy. The combined use of all omics sciences

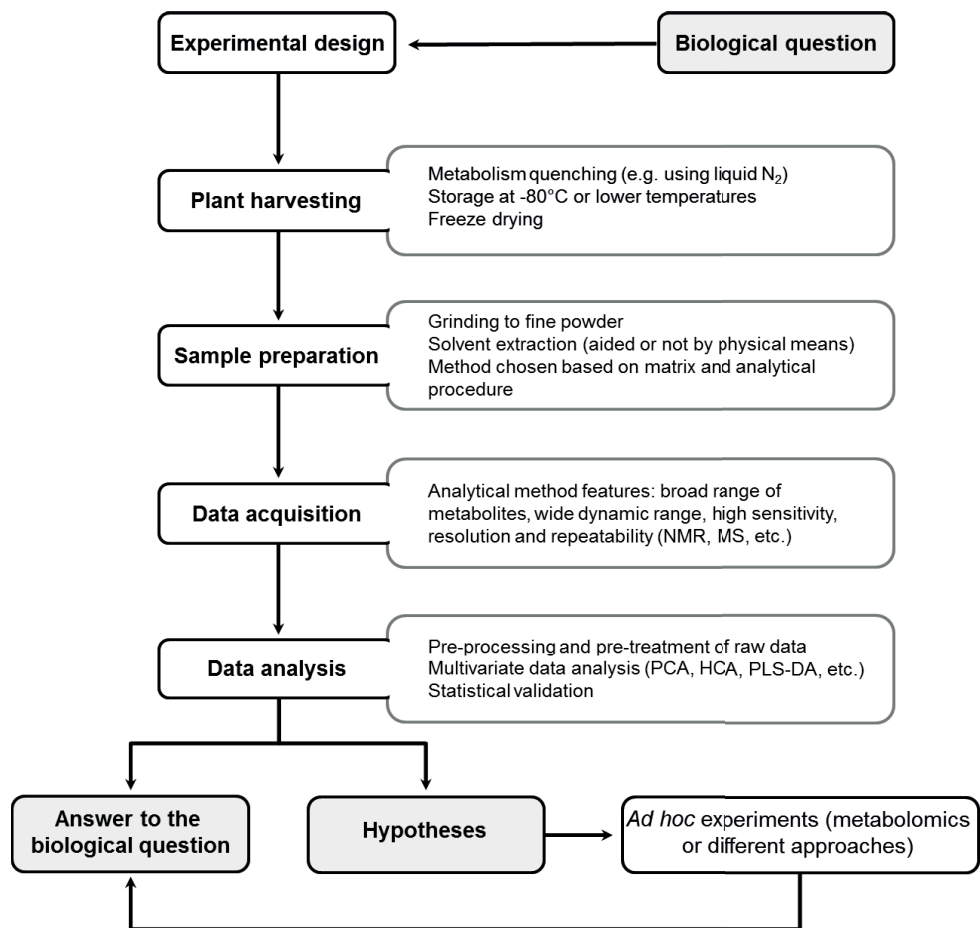


Figure 1. Metabolomics approach workflow.

can be very useful and helpful in this field, enabling scientists to have a full view of the organisms and of the complex processes (Moreno-Risueno *et al.* 2010; Urano *et al.* 2010). Among the omics sciences, metabolomics has several advantages in chemical ecology: because all metabolites are measured at once, also unstable compounds can be detected, additive and synergistic effects are also more likely to be evidenced, since metabolites are analysed together (Forseth and Schroeder 2011). Furthermore, it is a high throughput approach, thus allowing the analysis of many different samples in relatively short times. Finally, it gives a broad view of the biochemical status of the organism: the metabolome represents the end result of all processes in the cell from the transcriptional, translational and post-translational cellular events, defining cells' biochemical phenotype (Macel *et al.* 2010).

Based on these considerations metabolomics could be applied to allelopathy studies. Herewith the utility of metabolomics in the study of allelopathy is presented, and examples of application in several fields of chemical ecology are reported.

Metabolomics

Metabolomics is the non-targeted qualitative and quantitative analysis of all low molecular weight metabolites (<1500 Da) in a biological sample (Kim *et al.* 2011; Verpoorte *et al.* 2007). It is a component of systems biology, along with the other omics technologies. These analyses, based on the comprehensive monitoring of global metabolite networks and their fluctuation in response to various stimuli, give a broad view of the biochemical status of an organism, or more broadly, of a biological system. The metabolome, in particular, provides a snapshot of the end results of all processes in the cell from the transcriptional, translational and post-translational events, giving clues about what is going on in the system and defining its biochemical phenotype (Sumner *et al.* 2003). This one-time-point image might not be enough for studying dynamic processes, but this problem can be overcome by taking many subsequent pictures and integrating all the information, or by using fluxomics (Kim *et al.* 2011; Ratcliffe and Sachar-Hill 2006). It can be fairly stated that while genomic, transcriptomic and proteomic approaches predict what is expected to happen

in an organism, metabolomics portrays what is really happening (Aliferis and Jabaji 2011).

Metabolomics stands at the interface of biology and chemistry: its object is the entire complement of small molecules, but the subject is the study of biological phenomena. Noteworthy, due to its untargeted nature, it is a data-driven, hypotheses generating approach.

This field is relatively new: the first publication about metabolomics date back to 1998 (Oliver *et al.* 1998). It is slowly growing and not completely standardized, due to technical problems: as a consequence of its highly variable target, metabolomics does not have well defined methods, contrary to the other omics sciences.

Besides the application of metabolomics in different fields of research and analyses (Sumner *et al.* 2003, Verpoorte *et al.* 2007), it is usefully applied to the study of the responses to several stresses and some kind of interactions (Kim *et al.* 2011): this issue will be further examined in the present paper.

The use and contribution of metabolomics and related approaches in chemical ecology is thoroughly exemplified by Prince and Pohnert (2010), who reported two examples of how metabolomics could be useful, describing the available approaches and advantages (*viz.* detection of unstable compounds, detection of additive or synergistic effects, identification of chemical signals and definition of their effects at the phenotype level) and disadvantages (*viz.* problems mainly related to the inherent noisiness of biological and ecological systems). However, although it has been applied to several fields, its full potential in chemical ecology, and especially in plant-plant interactions is yet to be understood (Jones *et al.* 2013).

Besides metabolomics, other related technologies have been introduced, to date: metabonomics, metabolic profiling, target metabolite analysis, metabolite fingerprinting, and metabolite footprinting. The literature is somehow confusing when defining what the aim is of each of these, but the readers are invited to refer to the definition reported in the glossary (Box 1).

A true metabolomic study has the aim to investigate a problem and search for answers to one or more biological questions: it is not a simple report of the metabolite content of a biological system (Kim *et al.* 2011).

Metabolomics workflow: from experimental design to data analysis

Metabolomics requires the analysis of a wide range of different compounds, which differ in many aspects: chemical nature, solubility, and concentration (Schripsema, 2009).

An important premise: a single extraction technique and a single analytical platform do not provide the isolation and the detection of the complete metabolome (Fukusaki and Kobayashi 2005; Wolfender *et al.* 2013).

A broad literature base exists on the analytical and data analysis techniques used in metabolomics, and the readers are invited to refer to this literature for more in-depth understanding of the practical operations and for the analytical approaches (Table 1), as well as to the recent comprehensive review by Wolfender *et al.* (2013) and to the recommendations for reporting metabolite data (Fernie *et al.* 2011; Goodacre *et al.* 2007; Sumner *et al.* 2007). The entire metabolomic experimental scheme from the experimental design, to data mining and biological interpretation is accurately explained in the paper by Wolfender *et al.* (2013). However, in order to introduce metabolomics approach to non-expert readers, the typical workflow is illustrated in Figure 1, and the most relevant aspects are discussed below.

The first step is the experimental design, which will be based on the biological question underpinning the research. As metabolomics usually takes into account a large number of samples, and hence a great work load, the design of suitable experiments is a crucial point (Brown *et al.* 2005). Inherent biological variability has to be taken into account from an early stage of the research, for plants grown in both natural and in controlled conditions.

The second step is plant harvesting. The method used will depend on the study, but it is generally recommended to quench plant metabolism as soon as the plant is harvested in order to avoid unwanted enzymatic reactions (Fernie *et al.* 2011). Freeze-drying process is usually performed prior to extraction, in order to remove water for practical purpose (it may interfere with some extraction and analytical techniques) and to avoid reactivation of enzymes. An important issue is timing: if samples are to be compared, it is recommended to collect samples at the same moment of the day and at the same growth stage. It is also important to monitor all environmental conditions and/or annotate all metadata (Wolfender *et al.* 2013).

Sample extraction and preparation methods highly depend on the matrix to be analysed and on the analytical platform (Kim and Verpoorte 2010). In this perspective, NMR-based metabolomics has the advantage of minimum sample preparation: the extraction can be carried out directly in deuterated solvents, often with a mixture of phosphate buffer in D₂O and MeOD (1:1). This represents a great advantage in light of the need for standardization of procedure.

Once extracted, metabolites need to be analysed, and several techniques can be used. A list of these

Table 1. Reviews on analytical techniques used in metabolomics (gray box indicates the reviewed methodology).

Review paper	Analytical technique							
	NMR	MAS-NMR	LC-MS	GC-MS	CE-MS	DIMS	FT-ICR-MS	FT-IR
Allwood et al. 2008								
Dunn 2005								
Fukusaki and Kobayashi 2005								
Goodacre et al. 2004								
Hall 2006								
Kim et al. 2011								
McGhie et al. 2012								
Schripsema 2009								
Sumner et al. 2003								
Verpoorte et al. 2007								
Wishart 2008								
Wolfender et al. 2013								
Xiao et al. 2012								

techniques and of viewpoints on their advantages and disadvantages in metabolomics application go beyond the aim of the present review, as these topics have been extensively discussed in the literature (Table 1).

Among the techniques, NMR and MS seem to be the most powerful tools, for many reasons. NMR is a physical measurement of the resonances of magnetic nuclei: an external magnetic field is used to align the nuclear spins. Then, they are irradiated with specific frequencies in order to be promoted from a low to a high energy state and the radiation emitted during relaxation is detected. The frequency at which each nucleus resonates depends on its chemical environment. As a consequence, each compound has a highly specific spectrum, which is an information-rich graph. ¹H NMR is commonly used in metabolomics thanks to several advantages: first of all, easy sample preparation, ease of standardization and high reproducibility, being the solvent used and the magnetic field strength the only variables (Verpoorte *et al.* 2007). Furthermore, as the majority of metabolites contain hydrogen, ¹H NMR can be considered as unbiased compared to other platforms (Allwood *et al.* 2008), a desirable characteristic for metabolomics: it implies that in NMR based metabolomics the only bias are introduced by the solvent choice. Also, quantitative analysis is possible (Kim *et al.* 2011; Wishart 2008). The combined use of 1D and 2D NMR techniques allows the rapid identification of all of the compounds present in a mixture.

NMR metabolomics appears to be highly reproducible, it allows the contemporary identification and quantification of a large number of compounds, and needs short times of analysis (including the extraction procedures). The only limitation of NMR is its low sensitivity when compared to mass spectrometry, although sensitivity has been drastically increased with recent advances like the introduction of higher magnetic fields and microcryoprobes (Forseth and Schroeder 2011; Kim *et al.* 2011). A further drawback of NMR is that some classes of lipids can only be identified as total groups and not as individual compounds by means of 1D NMR. On the other hand, NMR, allows the identification of unknown compounds in the analysed mixtures, as it gives important structural information: recent advances in this framework have been registered lately (Forseth and Schroeder 2011).

In MS, ions are generated, separated by their mass-to-charge ratio and analysed by a detector. A number of ion sources and of analysers is available (Xiao *et al.* 2012). The main advantage of MS based techniques is sensitivity, but, on the other hand the identification of metabolites can be tricky, especially for the unknown compounds. The structural elucidation of compound structures is possible only by tandem MS (MS-MS), preferably HR(MS)ⁿ. However, for unknown metabolites, they should be isolated and characterized by NMR for definite structural elucidation (Sumner *et al.* 2007).

MS is usually combined with chromatography (GC or LC-MS): the hyphenated MS approach is useful for quantification and the retention time can be considered as a further hint of metabolite identity (pure standards are needed). However, the quantification requires suitable external calibration (calibration curves) with the use of chemical standards (Allwood *et al.* 2008). Furthermore, GC-MS of plant extracts (if not volatile) requires derivatization, which introduces bias, while LC-MS success mainly depends on the separation step efficiency. The newest LC-MS/MS approaches offer a useful tool for metabolite identification and quantification (Xiao *et al.* 2012), although it is recommended to follow published guidelines (Fernie *et al.* 2011; Goodacre *et al.* 2007; Sumner *et al.* 2007).

The principal drawback of MS-based approaches is their difficult standardization, as a consequence of a number of combinations of ion sources and analysers that strongly impact the analysis output.

It is worth to underline that the choice of the analytical platform and strategy largely depends on the aim of the study, and it is generally accepted that the best results can be achieved by a combination of extraction and analytical measurements (Allwood *et al.* 2008; Kim and Verpoorte 2010).

Raw data are processed and large data sets coming out from metabolomic studies are usually analysed by multivariate data analysis, with the aim to obtain as much information as possible from the samples with the aid of biostatistical softwares. Both unsupervised (PCA, HCA, SOM independent component analysis) and supervised algorithms (PLS-DA, hierarchical clustering, DFA) are used (Allwood *et al.* 2008; Fukusaki and Kobayashi 2005; Sumner *et al.* 2003). Once again, a detailed survey of the approaches is available in the literature (Brown *et al.* 2005; Goodacre *et al.* 2004; Wolfender *et al.* 2013), and dedicated papers deal with this particular topic (Boccard and Rudaz 2014; Broadhurst and Kell 2006; Jansen *et al.* 2010; Van den Berg *et al.* 2006).

Micrometabolomics

The great improvements in analytical methods, especially with reference to sensitivity, prompted the birth of micrometabolomics and single cell metabolomics. Each tissue or cell in an organism has its own metabolome and each “cellular metabolome” can be differently affected by inherent or external stimuli: the analysis at this level can allow the detection of possible key metabolites, but also a better understanding of the response to biotic and abiotic interference (Moco *et al.* 2009).

This approach is possible thanks to laser-assisted

microdissection application, which is able to separate cells or tissue pieces. These samples are then analysed by MS and/or NMR. Clearly, MS (paired with chromatographic techniques) is the method of choice in this framework, due to its high sensitivity, but also NMR techniques are used thanks to the latest improvements in hardware, pulse sequences and spectral acquisition protocols (Moco *et al.* 2009; Schneider and Holscher 2007). Nonetheless, MS- and NMR-based imaging approaches have successfully been used in this field (Moco *et al.* 2009; Zenobi 2013).

Metabolomics in plant environmental responses - Ecometabolomics

The application of metabolomics in the study of interactions of organisms with their environment is known as ecometabolomics, or more generally, as environmental metabolomics (Sardans *et al.* 2011). This approach has many advantages for studying biotic and abiotic interactions between the organism and the environment, as well as for assessing organism function and fitness at molecular level (Bundy *et al.* 2009).

The emergence of environmental metabolomics represents one of the most important developments in plant physiology and ecology. Recently, the application of metabolomics and related techniques (Box 1) for profiling of plants in terms of small molecule content has been reviewed (Brunetti *et al.* 2013). Any change in growth conditions that modifies the metabolic homeostasis is defined as stress. Following a stress, plants adjust their metabolic pathways (acclimation) to face external solicitations. The plant responses to different abiotic and biotic stresses are also examined (Shulaev *et al.* 2008).

Metabolomics for abiotic plant stress responses

Plants regularly encounter adverse growth conditions. Climatic factors, such as extreme temperatures (heat, cold, freezing), drought (deficient precipitation, winds), irradiations, and contamination of soils (salt, heavy metals, pesticides) are the main abiotic plant stressors (Table 2). The metabolic network of plants is perturbed by these abiotic factors and must be reconfigured in order to allow both the maintenance of metabolic homeostasis and the production of compounds that ameliorate the stress resilience (Krasensky & Jonak 2012).

Based on metabolomics approaches, stress compounds such as proline, γ -amino butyrate and polyamines are reviewed (Obata & Fernie 2012). Specific reviews on plant responses to salinity or temperature stresses are available (Sanchez *et al.* 2008; Guy *et al.* 2008).

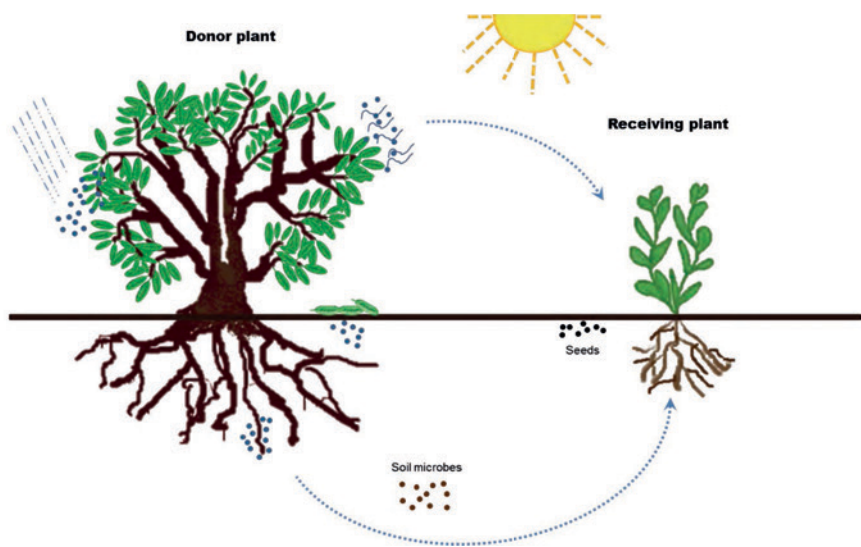


Figure 2. Schematic representation of the allelopathic phenomenon. The donor plant synthesizes and releases allelochemicals (blue circles). Virtually all plant organs can do it and the release pathways are variable. Once released, allelochemicals might be modified (or not) by biotic and/or abiotic factors and, finally, they can reach the receiving plant, affecting it in a direct or indirect manner.

Metabolomics for biotic plant stress responses

The high sensitivity and potentiality of metabolomic techniques in detecting molecular responses of plants to biotic interactions are reported in the literature (Sardans *et al.* 2011; Bundy *et al.* 2009; Jansen *et al.* 2009; Weingart *et al.* 2013). However, the studies available in this field are very scarce and carried out mainly on the basis of metabolic profiling analyses or on the modification of specific classes of secondary metabolites involved in specific processes. Some recent examples of studies on plant responses to biotic interactions are reported in Table 3. The most studied interactions are between plants and microorganisms, especially fungi and bacteria.

Potential for applying metabolomics approaches in allelopathy studies

Plant-plant interactions

Allelopathy is defined as the beneficial or harmful influence of chemical substances (belonging to diverse chemical groups) produced mainly from the secondary metabolism of plants, microorganism, and fungi that affect the growth and development of nearby plants or microorganisms, and interfere with the dynamical processes of agricultural and natural ecosystems (De Albuquerque *et al.* 2011). According to the definition of the International Allelopathy Society (IAS), it involves interactions between plants and other organisms, excluding animals (Torres *et al.* 1996), while according to other sources, allelo-

chemicals may be involved not only in plant-plant and plant-microorganisms interactions, but also in plant-insect or plant-herbivore communication (Weir *et al.* 2004).

Here plant-plant interactions will be considered. Two main actors are involved in this interaction: donor plant, which produces and releases the allelochemical, and the receiving plant that is somehow sensitive to it (Fig. 2).

Concerning the donor plant, allelochemicals are present in virtually all organs (leaves, stems, roots, rhizomes, seeds, flowers, pollen, fruits, etc.), which produce or just store them, and they can be found in different concentration in each compartment (Gatti *et al.* 2010). The quantity and emission pathway vary within species (Friedman 1995). Most allelochemicals are released into the environment by root exudation, vaporization, leaching (lixiviation) or decomposition of plant organs (Fernandez *et al.* 2009), depending on their physico-chemical properties and on the specific site of accumulation and/or production. To further complicate this picture, there is a plethora of abiotic (e.g. temperature, light, soil characteristics, rainfall) and biotic (e.g. life cycle, competition, pathogens, pests, parasites or herbivores) factors that may influence the production, release, and nature of allelochemicals (Rivoal *et al.* 2011).

Finally, allelochemicals have to be transported from the donor plant to the receiving plant (Fig. 2): besides the physical distance, the existing ecosystem (made up by microorganisms, fungi, soil organic matter, etc.) could contribute in modifying these molecules.

Table 2. Examples of metabolomics studies for abiotic plant stress responses.

Investigated species	Stress	An. technique	Reference
<i>Oryza sativa</i>	Cold	HPLC	Morsy et al. 2007
<i>Arabidopsis thaliana</i>	Cold	CG-TOF-MS	Cook et al. 2004
<i>Arabidopsis thaliana</i>	Cold	FT-ICR-MS	Gray & Heath 2005
<i>Arabidopsis thaliana</i>	Drought	GC-TOF-MS	Urano et al. 2009
		CE-MS	
<i>Arabidopsis thaliana</i>	Drought/Heat	GC-MS	Rizhsky et al. 2004
<i>Arabidopsis thaliana</i>	Heat/Cold	CG-MS	Kaplan et al. 2004
<i>Arabidopsis thaliana</i> *	Heavy metals	NMR	Le Lay et al. 2006
<i>Silene cucubalus</i> *	Heavy metals	NMR	Bailey et al. 2003
<i>Oryza sativa</i>	Oxidative stress	CE-MS	Ishikawa et al. 2010
<i>Arabidopsis thaliana</i> *	Oxidative stress	CG-MS	Baxter et al. 2007
<i>Oryza sativa</i>	Ozone	CE-MS	Cho et al. 2008
<i>Brassica napus</i>	Pesticides	UPLC-DAD	Petersen et al. 2011
<i>Phaseolus vulgaris</i>	Phosphorus deficiency	GC-TOF-MS	Hernandez et al. 2007
<i>Lycopersicon esculentum</i>	Salt	FT-IR	Johnson et al. 2003
<i>Arabidopsis thaliana</i> *	Salt	GC-MS	Kim et al. 2007
		LC-MS	
<i>Thellungiella halophila</i>	Salt	GC-MS	Gong et al. 2005
<i>Oryza sativa</i>	Salt	GC-MS	Zuther et al. 2007
<i>Arabidopsis thaliana</i>	Sulfur deficiency	GC-MS	Nikiforova et al. 2005
		LC-MS	
<i>Arabidopsis thaliana</i>	UV-B light	LC-QTOF-MS	Kusano et al. 2011
		GC-TOF-MS	
<i>Arabidopsis thaliana</i>	UV-B light	LC-QTOF-MS	Lake et al. 2009
<i>Vitis vinifera</i>	Water/Salt	GC-MS	Cramer et al. 2007
<i>Arabidopsis thaliana</i>	Water/Salt	LC-QTOF-MS	Arbona et al. 2010
<i>Thellungiella halophila</i>			

*cells cultures

Once allelochemicals have reached the receiving plant, they can affect physiological functions like respiration, photosynthesis, ion uptake, enzyme activity, water status, transpiration, stomata opening, hormone levels, cell division and differentiation, signal transduction, gene expression and structure and permeability of cell membranes and walls, etc. (Chon and Nelson 2010; Zhuo and Yu 2006; Reigosa *et al.* 1999). Allelochemicals can also elicit the production of reactive oxygen species (Weir *et al.* 2004; Scognamiglio *et al.* submitted) or of secondary metabolites. The biochemical and metabolic alterations produce morphological changes in receiving plants: seeds appear darkened and swollen, the roots or radicles and shoots or coleoptiles can be reduced, root tips swollen or affected by necrosis, root axes can be curled, root hairs number reduced or increased, etc. (Badhoria 2011). Many allelochemicals seem to influence seed germination. Allelochemicals can also indirectly influence neighbouring species by modifying biotic and abiotic interactions, such as nutrient uptake and bioavailability (Tharayil *et al.* 2008; Tharayil 2009), or positively or negatively affect microorganism populations in the soil (Castaldi *et al.*, 2009).

This two-character picture is absolutely not exhaustive as the biological activities are governed by a number of different factors: individual chemi-

cal structure, antagonistic, additive, sequential, or synergistic effects (An *et al.* 2001). This is probably the situation in field conditions where the amount of allelochemicals is often too low to explain allelopathic activity, justified on the contrary, by interactions among allelochemicals. It has also been demonstrated that the specific mixture of allelochemicals and their specific ratio is more important than mere additive effects (An *et al.* 2001).

Allelopathy studies

Allelopathic interactions can be considered elucidated when many evidences, here discussed, are available (Box 2). Firstly, the existence of chemicals (or their mixtures) able to influence other plant performance should be proven. From the donor plant point of view, beyond the identification of active compounds, also the identification of production and storage sites are very important, along with the identification of emission pathways and release mechanisms. It should be demonstrated that metabolites are released into the soil (or, more generally, into the environment) and can reach the receiving plant. Then it should also be demonstrated that this receiving plant takes up or “senses” these compounds and responds to them. Finally, the modes of action and the possible molecular mechanisms involved should be established.

Table 3. Examples of metabolomics studies for abiotic plant stress responses.

Plant species	Organism species	An. technique	Reference
<u>Plant-fungus interaction:</u>			
<i>Brassica rapa</i>	<i>Leptosphaeria maculans</i>	¹ H NMR	Abdel-Farid et al. 2009
	<i>Aspergillus niger</i>		
	<i>Fusarium oxysporum</i>		
<i>Vitis vinifera</i>	<i>Uncinula nector</i>	¹ H NMR	Figueiredo et al. 2008
	<i>Plasmopara viticola</i>		
<i>Vitis vinifera</i>	<i>Phaeomoniella spp</i>	¹ H NMR	Lima et al. 2010
	<i>Fomitiporia spp.</i>		
<i>Triticum aestivum</i>	<i>Fusarium graminearum</i>	GC-MS	Paranidharan et al. 2008
<i>Horeum vulgare</i>	<i>Fusarium graminearum</i>	LC-ESI-LTQ-Orbitrap	Bollina et al. 2010
<i>Lupinus angustifolius</i>	<i>Colletotrichum lupini</i>	HPLC-DAD	Muth et al. 2009
		LC-MS	
<i>Solanum tuberosum</i>	<i>Rhizoctonia solani</i>	FT-ICR-MS	Aliferis and Jabaji 2012
		GC-MS	
<i>Oryza sativa</i>	<i>Magnaporthe grisea</i>	GC-MS	Jones et al. 2011
		LC-MS	
		¹ H-NMR	
<u>Plant-bacterium interaction:</u>			
<i>Arabidopsis thaliana</i>	<i>Pseudomonas syringae</i>	FT-IR	Allwood et al. 2010
<i>Medicago sativa</i>	<i>Sinorhizobium meliloti</i>	¹ H NMR	Barsch et al. 2006
<i>Citrus sinensis</i> ,	<i>Candidatus liberibacter</i>	CE-DAD	Cavillos et al. 2009
<i>Solanum lycopersicum</i>	<i>Pseudomanas syringae</i>	¹ H NMR	López-Gresa et al. 2010
		LC-MS	
<i>Catharanthus roseus</i>	<i>Phytoplasma</i>	¹ H NMR	Choi et al. 2004
<i>Chicorium intybus</i> <i>Solanum tuberosum</i>	<i>Dickeya dadantii</i>	¹³ C NMR	Effantin et al. 2011
<i>Brassica rapa</i>	<i>Bacillus subtilis</i>	¹ H NMR	Jahangir et al. 2008
	<i>Staphylococcus aureus</i>		
	<i>Escherichia coli</i>		
<i>Arabidopsis thaliana</i>	Soil microbiome	GC-MS	Badri et al. 2013
<u>Plant-virus interaction:</u>			
<i>Nicotiana tabacum</i>	tobacco mosaic virus	¹ H NMR	Choi et al. 2006
<i>Brassica oleracea</i>	<i>Pieris brassicae</i>	HPLC-DAD, HPLC-MS-MS	Ferreres et al. 2007
<i>Brassica oleracea</i>	<i>Pieris rapae</i>	UPLC-MS	Jansen et al. 2009
<i>Prunus persica</i>	<i>Myzus persicae</i>	¹ H NMR	Poëssel et al. 2006
<i>Solanum lycopersicum</i>	tobacco mosaic virus	¹ H NMR	Lopez-Gresa et al. 2012
<u>Plant-insect interaction:</u>			
<i>Brassica oleracea</i>	<i>Pieris rapae</i>	UPLC-MS	Jansen et al. 2009
<i>Brassica rapa</i>	<i>Plutella xylostella</i>	¹ H-NMR	Widarto et al. 2006
	<i>Spodoptera exigua</i> Hubner		
<i>Vitis berlandier.</i> × <i>V. riparia</i>	<i>Daktulosphaira vitifoliae</i>	GC-MS	Lawo et al. 2011
<u>Plant-herbivore interaction:</u>			
<i>Brassica rapa</i>	<i>Plutella xylostella</i>	¹ H NMR	Schroeder et al. 2006
	<i>Spodoptera exigua</i>		

The demonstration of existence of active chemicals is not a problem as phytotoxicity is easy to check, and isolation and structural elucidation of natural products is well established (Bucar *et al.* 2013). Traditional approaches to discover and assess allelochemicals are based on bioassay-guided fractionation. This approach relies on analytical procedures to separate and characterize chemicals and on suitable bioassays: many successful examples have been reported and recently reviewed (De Albuquerque *et al.* 2011; Macías *et al.* 2007; Scognamiglio *et al.* 2013).

Far less literature is reported dealing with the definitive elucidation of the other phenomena, especially the mode of action. Up to now, the study of the mode of action of allelochemicals lacks reporting of established methods, with highly differing experimental designs being used (Macías *et al.* 2007). The mode of action of most allelochemicals is unknown (Duke 2013), and also the molecular mechanisms are rarely known, although the availability of new techniques and approaches offers useful tools to learn more (Golisz *et al.* 2008; Inderjit *et al.* 2009).

Box 1: Glossary*

Metabolome: the entire complement of small molecules (<1500 Da) in a biological system (organism, organ, tissue, cell).

Metabolomics: untargeted, comprehensive qualitative and quantitative analysis of the metabolome under a given set of conditions.

Metabonomics: measures of the changes (biochemical perturbations) induced by pathophysiological stimuli or genetic modification in biofluids.

Metabolic profiling: identification and quantification of a group of related compounds (frequently belonging to a specific metabolic pathway or sharing common chemical features).

Metabolic fingerprinting: screening tool to differentiate among samples of different origin or undergone different treatments. It is a high throughput qualitative screening of the metabolic composition of the system (no identification or quantitation).

Metabolic footprinting: measurements of metabolites secreted into extracellular growth medium.

Target metabolite analysis: analysis of few metabolites related to a specific metabolic reaction by using optimized extraction and separation/detection techniques.

*Sources: Allwood *et al.* 2008; Dunn *et al.* 2005; Fiehn 2001; Goodacre 2004; Hall 2006; Kim *et al.* 2011

Although the phenomena hitherto analysed are very complex, there is something even more difficult to study: the cosmos existing between the donor and the receiving plant. Secondary metabolites undergo degradation and/or transformation processes activated by biotic (e.g. soil microbes) and/or physicochemical processes (e.g. binding to organic matter, leaching, etc.), phenomena which affect their bioavailability, or more drastically, their nature and activity (Macías *et al.* 2007; De Albuquerque *et al.* 2011).

Based on these considerations, it is clear that greater understanding of allelopathy calls for new methods and new approaches, mainly based on systems biology. Up to now, notwithstanding the need of a global overview in order to elucidate the complicated allelopathic interactions, few papers deal with a system biology approach in this field.

The combined use of all “omics” sciences can be very useful and helpful in chemical ecology, enabling scientists to have a full view of the organisms and of the complex processes related to allelopathy.

Metabolomics and related approaches in allelopathy

Few studies reported a metabolomics approach in allelopathy investigation. A method for the study of plant-plant interference by metabolic fingerprinting has

been reported (Gidman *et al.* 2003). Two species were chosen in order to study their chemical interactions: *Brachypodium distachyon* and *Arabidopsis thaliana*. Plants of the two species were grown solitary and mixed. Samples were harvested at three time points and analysed by FT-IR and PCA. The study demonstrated the power of this approach in detecting changes in the total metabolic profiles of plants in response to biotic interactions, with FT-IR as an appropriate rapid screening prior to targeted metabolite analyses.

Recently, a co-culturing method/metabolomics approach was introduced to study chemically mediated interactions of planktonic organisms (Paul *et al.* 2013). The authors set up a device where different organisms could be grown, with no direct contact, but allowing metabolite exchanges. Diatoms *Thalassiosira pseudonana* and the bacterium *Dinoroseobacter shibae* were grown in this system and metabolic profiling of the diatoms was carried out. Diatoms were extracted, the extracts were derivatized using methoxamine hydrochloride followed by silylation and analysis by GC-MS. Data were analysed by principal coordinate analysis (PCO) and canonical analysis of principal coordinates. MS data and retention times were used to tentatively identify metabolites. The authors found that many metabolites were increased in diatoms co-cultured with bacteria.

Box 2: Evidences needed for allelopathic phenomenon elucidation

Donor plant:

- Existence of chemicals (or their mixtures) able to influence other plant performance.
- Identification of production and storage sites.
- Identification of emission pathways and release mechanisms.

Fate of emitted allelochemicals.

Receiving plant:

- Uptake of allelochemicals or their interaction with receptors (or different indirect effects).
- Mechanisms of action.
- Modes of action.

Very recently, a new approach has been developed for allelochemical studies (D'Ambrosia *et al.* 2013; Scognamiglio *et al.* 2014). In this approach, NMR-based metabolomics is applied to analyse donor plants, the extracts of the donor plants are screened for their activity on a test species, and the receiving plant's morphological and metabolic changes are measured.

From the donor plant point of view, by using multivariate data analysis, plant extract components that correlate with growth inhibition activity can be identified and also synergistic and additive effects can be found.

On the other hand, from the analysis of the receiving plant, the molecular changes induced by the allelochemicals can be observed and their fate in the receiving plant can be studied. This information from a single experimental design that also gives clues about the mode of action to be further explored.

The method has been standardized on a test species: *Aegilops geniculata*. A first experiment was carried out with a known allelochemical (coumarin) in order to understand the best stage of the plant life cycle at which to perform treatment and harvesting. This was achieved by daily sampling of plants and multivariate data analysis of non-treated plants and of plants treated with coumarin. From this first study, it was able to observe how dynamic the response was: harvesting at different time points gave important clues. However,

for practical purposes, a one-time point sampling was chosen for further studies.

At this point, the method was standardized with known allelochemicals (besides coumarin, catechol, p-hydroxybenzoic acid, p-coumaric acid and juglone). From metabolomics analysis of treated plants, the molecular changes upon treatment were detected and were found to be mainly in accordance with the allelochemicals' known mode of action.

The method has been applied to study the effects of *Phillyrea angustifolia* plant extracts prepared at different phenological stages (Scognamiglio *et al.* 2013). Different plant extracts produced a very different response in the target plant metabolome. The most active were those of the cold season (from November to March), characterized by the highest amount of oleuropein and inducing a decrease of *cis*-aconitate and an increase of citrate and malate. This indicated a perturbation in the tricarboxylic acids cycle, suggesting the inhibition of *cis*-aconitase. Based on this and on further observations, as this enzyme is considered an indicator of oxidative stress, the induction of ROS formation was proposed (Baxter *et al.* 2007; Zhao *et al.* 2005).

Hence, the method allowed the observation of responses specific for each extract composition, which depended on the collection time and made it possible to study how phenology could impact plant allelopathic potential.

Recently, the same approach has been applied to a set of plants from the Mediterranean region (Scognamiglio *et al.* submitted).

Metabolomics and allelopathy: a look forward

Metabolomics and metabolic profiling of donor plant can offer useful insights in the identification of active compounds. Plant extracts assayed for their biological activity can be characterized by metabolomics analysis, in order to identify putatively active compounds. Besides the methods discussed in the previous sections, it has never been applied in allelopathy, although many examples exist using metabolomics based techniques to uncover compounds involved in bioactivity from complex natural sources (e.g. plant extracts) (Yuliana *et al.* 2013).

Once the allelochemical has been identified, its production and/or storage tissues could be studied by subsequent restriction of the scale of analysis: from whole plant, to plant organ, to plant tissue, to plant cell. Hence, micrometabolomics and single cell metabolomics could be very helpful. Once these sites are defined, it follows that the way by which these metabolites are released into the environment can be elucidated, as this will depend both on the physicochemical features of the metabolites and on the sites where they are produced or accumulated. While metabolites present in roots are likely to be released by exudation, leaf allelochemicals can follow different pathways for their release. Obviously, volatilization will depend on the volatility of allelochemicals. Furthermore, the study of this emission pathway is easy as the analysis of plant volatiles is well established and maybe the oldest example of a metabolomics-like approach (Beck *et al.* 2013). Leaching will be possible, on the other hand, only if metabolites are deposited on the leaf surface. Otherwise, compounds are likely to be released into the environment by decomposition (Fig. 2). Ad hoc experiments could be useful to validate this hypothesis.

From metabolomic analysis of receiving plant and its organs, it is possible to study the fate of allelochemicals and the metabolic changes induced in the plant.

Concerning the mode of action, metabolomics has been proposed as a tool to explore the mechanism (molecular target site) and mode of action (complete sequence of processes: viz. how a compound is taken up, distributed, metabolized, binds to the target site, and results in changes in the regulation of pathways and in a specific phenotype) of phytotoxic compounds and pesticides (Aliferis and Jabaji 2011; Tresch 2013). Metabolomics can, indeed provide invaluable information on the biochemical status of the organisms

after exposure to bioactive compounds. In this context, plant metabolomics and plant fingerprinting technologies could also be used in the discovery of mode of actions of newly discovered candidate herbicides of natural origin (Aliferis *et al.* 2009).

The potential of metabolomics in the field of allelopathy is also well-supported by many applications in plant stress-response to biotic and abiotic stressors (Tables 2 and 3).

References

- Abdel-Farid IB, Jahangir M, van den Hondel C, Kim HK, Choi YH, Verpoorte R (2009) Fungal infection-induced metabolites in *Brassica rapa*. *Plant Sci* 176:608–615
- Aliferis KA, Jabaji S (2012) FT-ICR/MS and GC-EI/MS metabolomics networking unravels global potato sprout's responses to *Rhizoctonia solani* infection. *PLoS One*. 7(8):e42576. doi: 10.1371/journal.pone.0042576.
- Aliferis KA, Jabaji S (2011) Metabolomics—A robust bioanalytical approach for the discovery of the modes-of-action of pesticides: A review. *Pestic Biochem Phys* 100(2):105–117.
- Aliferis KA, Materzok S, Pazioutou GN, Chrysaitokousbalides M. (2009). *Lemna minor* L. as a model organism for ecotoxicological studies performing 1H NMR fingerprinting. *Chemosphere*, 76(7):967–973.
- Allwood JW, Clarke A, Goodacre R, Mur LAJ (2010) Dual metabolomics: a novel approach to understanding plant-pathogen interactions. *Phytochemistry* 71:590–597.
- Allwood JW, Ellis DI, Goodacre R (2008) Metabolomic technologies and their application to the study of plants and plant–host interactions. *Physiol Plant*, 132(2):117–135.
- An M, Prateley JE, Haig T (2001) Phytotoxicity of *Vulpia* residues: III: Biological activity of identifies allelochemicals from *Vulpia myuros*. *J Chem Ecol*, 27(2):383–394.
- Arbona V, Argamasilla R, Gómez-Cadenas A (2010) Common and divergent physiological, hormonal and metabolic responses of *Arabidopsis thaliana* and *Thellungiella halophila* to water and salt stress. *J Plant Physiol* 167:1342–1350.
- Bhadoria PBS (2011) Allelopathy: A natural way towards weed management. *Am J Exp Agric* 1:7–20.
- Badri DV, Zolla G, Bakker MG, Manter DK, Vivanco JM (2013) Potential impact of soil microbiomes on the leaf metabolome and on herbivore feeding behavior. *New Phytol*. 198:264–273.
- Bailey NJ, Oven M, Holmes E, Nicholson JK, Zenk MH (2003) Metabolomic analysis of the consequences of cadmium exposure in *Silene cucubalus* cell cultures via 1H NMR spectroscopy and chemometrics. *Phytochemistry* 62:851–858.
- Barsch A, Tellstrom V, Patschkowski T, Kuster H, Niehaus K (2006) Metabolite profiles of nodulated alfalfa plants indicate that distinct stages of nodule organogenesis are accompanied by global physiological adaptations. *Mol Plant Microbe Interact* 19:998–1013.

- Baxter CJ, Redestig H, Schauer N, Repsilber D, Patil KR, Nielsen J, Selbig J, Liu J, Fernie AR, Sweetlove LJ (2007) The metabolic response of heterotrophic *Arabidopsis* cells to oxidative stress. *Plant Physiol* 143:312–325.
- Beck JJ, Smith L, Baig N (2013) An overview of plant volatile metabolomics, sample treatment and reporting considerations with emphasis on mechanical damage and biological control of weeds. *Phytochem Anal*. doi: 10.1002/pca.2486
- Boccard J, Rudaz S (2014) Harnessing the complexity of metabolomic data with chemometrics. *J Chemometr*, 28(1):1–9.
- Bollina V, Kumaraswamy GK, Kushalappa AC, Choo TM, Dion Y, Rioux S, Faubert D, Hamzehzarghani H (2010) Mass spectrometry-based metabolomics application to identify quantitative resistance-related metabolites in barley against *Fusarium* head blight. *Mol Plant Pathol* 11:769–782.
- Broadhurst DI, Kell DB (2006) Statistical strategies for avoiding false discoveries in metabolomics and related experiments. *Metabolomics*, 2(4):171–196.
- Brown M, Dunn WB, Ellis DI, Goodacre R, Handl J, Knowles JD, O'Hagan S, Spasić I, Kell DB (2005) A metabolome pipeline: from concept to data to knowledge. *Metabolomics* 1(1):39–51.
- Broz AK, Corey DC, De-la-Peña C, Lewis MR, Greene E, Callaway RM, Sumner L, Vivanco JM (2010). Plant neighbor identity influences plant biochemistry and physiology relate to defense. *BMC Plant Biol*. 10:115–129.
- Brunetti C, George RM, Tattini M, Field K, Davey MP (2013) Metabolomics in plant environmental physiology. *J Exp Bot* 64(13):4011–4020.
- Bucar F, Wube A, Schmid M (2013) Natural product isolation—how to get from biological material to pure compounds. *Nat Prod Rep*, 30(4):525–545.
- Bundy JG, Davey MP, Viant MR (2009) Environmental metabolomics: a critical review and future perspectives. *Metabolomics* 5:3–21.
- Castaldi S, Carfora A, Fiorentino A, Natale A, Messere A, Miglietta F, Cotrufo MF (2009) Inhibition of net nitrification activity in a Mediterranean woodland: possible role of chemicals produced by *Arbutus unedo*. *Plant Soil* 315:273–283.
- Cevallos–Cevallos JM, Rouseff R, Reyes-De-Corcuera J (2009) Untargeted metabolite analysis of healthy and Huanglongbing infected orange leaves by CE-DAD. *Electrophoresis* 30:1240–1247.
- Cho K, Shibato J, Agrawal GK, Jung YH, Kubo A, Jwa NS, Tamogami S, Satoh K, Kikuchi S, Higashi T, Kimura S, Saji H, Tanaka Y, Iwahashi H, Masuo Y, Rakwal R (2008) Integrated transcriptomics, proteomics, and metabolomics analyses to survey ozone responses in the leaves of rice seedling. *J Proteome* 7(7):2980–2998.
- Choi YH, Kim HK, Linthorst HJM, Hollander JG, Lefeber AWM, Erkelens C, Nuzillard JM, Verpoorte R (2006) NMR metabolomics to revisit the tobacco mosaic virus infection in *Nicotiana tabacum* leaves. *J Nat Prod* 69:742–748.
- Choi YH, Tapias EC, Kim HK, Lefeber AWM, Erkelens C, Verhoeven JIJ, Brzin J, Zel J, Verpoorte R (2004) Metabolic discrimination of *Catharanthus roseus* leaves infected by phytoplasma using ¹H NMR spectroscopy and multivariate data analysis. *Plant Physiol* 135:2398–2410.
- Chon SU, Nelson CJ, (2010) Allelopathy in Compositae plants. *Agron Sustain Dev*, 30:349–358.
- Cook D, Fowler S, Fiehn O, Thomashow MF (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of *Arabidopsis*. *Proc Natl Acad Sci USA* 101:15243–15248.
- Cramer GR, Ergul A, Grimplet J, Tillett RL, Tattersall EA, Bohlman MC, Vincent D, Sonderegger J, Evans J, Osborne C, Quilici D, Schlauch KA, Schooley DA, Cushman JC (2007) Water and salinity stress in grapevines: early and late changes in transcript and metabolite profiles. *Funct Integr Genomics* 7:111–134.
- D'Ambrosia B, Scognamiglio M, Fiumano V, Esposito A, Choi YH, Verpoorte R, Fiorentino A (2013). Plant bioassay to assess the effects of allelochemicals on the metabolome of the target species *Aegilops geniculata* by an NMR-based approach. *Phytochemistry*, 93:27–40.
- De Albuquerque MB, Dos Santos RC, Lima LM, Melo Filho PA, Nogueira RJMC, Da Camara CAG, Ramos AR (2011) Allelopathy, an alternative tool to improve cropping systems. A review. *Agron Sustain Dev* 31:379–395.
- Duke SO, Bajsa J, Pan Z (2013) Omics methods for probing the mode of action of natural and synthetic phytotoxins. *J Chem Ecol*, 39(2):333–347.
- Dunn WB, Ellis DI (2005) Metabolomics: current analytical platforms and methodologies. *TrAC Trend Anal Chem*, 24(4):285–294.
- Effantin G, Rivasseau C, Gromova M, Bligny R, Hugouvieux-Cotte-Pattat N (2011) Massive production of butanediol during plant infection by phytopathogenic bacteria of the genera *Dickeya* and *Pectobacterium*. *Mol Microbiol* 82:988–997.
- Fernandez C, Monnier Y, Ormeño E, Baldy V, Greff S, Pasqualini V, Mévy JP, Bousquet-Mélou A (2009) Variations in allelochemical composition of leachates of different organs and maturity stages of *Pinus halepensis*. *J Chem Ecol*, 35:970–979.
- Fernie AR, Aharoni A, Willmitzer L, Stitt M, Tohge T, Kopka J, Carroll AJ, Saito K, Fraser PD, DeLuca V (2011) Recommendations for reporting metabolite data. *Plant Cell* 23(7):2477–2482.
- Ferreres F, Sousa C, Valentao P, Pereira JA, Seabra RM, Andrade PB (2007) Tronchuda cabbage flavonoids uptake by *Pieris brassicae*. *Phytochemistry* 68:361–367.
- Fiehn O (2001) Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. *Comp Funct Genomics* 2(3):155–168.
- Figueiredo A, Fortes AM, Ferreira S, Sebastiana M, Choi YH,

- Sousa L, Acioli-Santos B, Pessoa F, Verpoorte R, Pais MS (2008) Transcriptional and metabolic profiling of grape (*Vitis vinifera* L.) leaves unravel possible innate resistance against pathogenic fungi. *J Exp Bot* 59:3371–3381.
- Forseth RR, Schroeder FC (2011) NMR-spectroscopic analysis of mixtures: from structure to function. *Curr Opin Chem Biol*, 15(1):38–47.
- Friedman J (1995) Allelopathy, autotoxicity, and germination. In: Kigel J Galili G (eds.) *Seed development and germination*, pp. 629–644. New York, Marcel Dekker.
- Fukusaki E, Kobayashi A (2005) Plant metabolomics: potential for practical operation. *J Biosci Bioeng* 4:347–354.
- Gatti AB, Ferreira AG, Arduin M, Gualtieri de Andrade Perez SC, (2010) Allelopathic effects of aqueous extracts of *Artisotlochia esperanzae* O. Kuntze on development of *Sesamum indicum* L. seedlings. *Acta Bot Bras* 24:454–461.
- Gidman E, Goodacre R, Emmett B, Smith AR, Gwynn-Jones D (2003) Investigating plant–plant interference by metabolic fingerprinting. *Phytochemistry*, 63(6):705–710.
- Golisz A, Sugano M, Fuji Y (2008) Microarray expression profiling of *Arabidopsis thaliana* L. in response to allelochemicals identified in buckwheat. *J Exp Bot* 59:3099–3109.
- Gong Q, Li P, Ma S, Indu Rupassara S, Bohnert HJ (2005) Salinity stress adaptation competence in the extremophile *Thellungiella halophila* in comparison with its relative *Arabidopsis thaliana*. *Plant J* 44: 826–839.
- Goodacre R Broadhurst D Smilde A. K Kristal, B. S Baker J D Beger R Bessant C, Connor S, Capuani G, Craig A, Ebbels T, Kell DB, Manetti C, Newton J, Paternostro G, Somorjai R, Sjöström M, Trygg J, Wulfert, F (2007) Proposed minimum reporting standards for data analysis in metabolomics. *Metabolomics*, 3(3):231–241.
- Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB (2004) Metabolomics by numbers: acquiring and understanding global metabolite data. *Trends Biotechnol* 22 (5): 245–252.
- Granéli E, Salomo PS. (2010) Factors Influencing Allelopathy and Toxicity in *Prymnesium parvum*. *J Am Water Resour As* 46 (1):108–120.
- Gray GR, Heath D (2005) A global reorganization of the metabolome in *Arabidopsis* during cold acclimation is revealed by metabolic fingerprinting. *Physiol Plant* 124: 236–248.
- Guy C, Kaplan F, Kopka J, Selbig J, Hinch DK. (2008) Metabolomics of temperature stress *Physiol Plant* 132:220–235.
- Hall R D (2006) Plant metabolomics: from holistic hope, to hype, to hot topic. *New phytol* 169(3):453–468.
- Hernandez G, Ramirez M, Valdes-Lopez O, Tesfaye M, Graham MA, Czechowski T, Schlereth A, Wandrey M, Erban A, Cheung F, Wu HC, Lara M, Town CD, Kopka J, Udvardi MK, Vance CP (2007) Phosphorus stress in common bean: root transcript and metabolic responses. *Plant Physiol* 144: 752–767.
- Inderjit, Dahl CC, Baldwin IT (2009) Use of silenced plants in allelopathy bioassays: a novel approach. *Planta* 229:569–575.
- Inderjit, Duke SO (2003) Ecophysiological aspects of allelopathy. *Planta* 217: 529–539.
- Ishikawa T, Takahara K, Hirabayashi T, Matsumura H, Fujisawa S, Terauchi R, Uchimiya H, Kawai-Yamada M (2010) Metabolome analysis of response to oxidative stress in rice suspension cells overexpressing cell death suppressor Bax Inhibitor-1 *Plant Cell Physiol*. 51(1): 9–20.
- Jahangir M, Kim HK, Choi YH, Verpoorte R (2008) Metabolomic response of *Brassica rapa* submitted to pre-harvest bacterial contamination *Food Chem* 107:362–368
- Jansen JJ, Allwood JW, Marsden-Edwards E, van der Putten W H, Goodacre R, N M van Dam (2009) Metabolomic analysis of the interaction between plants and herbivores. *Metabolomics* 5:150–161
- Jansen JJ, Smit S, Hoefsloot HC, Smilde AK (2010) The photographer and the greenhouse: how to analyse plant metabolomics data. *Phytochem Anal*, 21(1):48–60.
- Johnson HE, Broadhurst D, Goodacre R, Smith AR (2003) Metabolic fingerprinting of salt-stressed tomatoes. *Phytochemistry* 62: 919–928.
- Jones OAH, Maguire ML, Griffin JL, Jung Y-H, Shibato J, Rakwal R, Agrawal G K, Jwa N-S (2011) Using metabolic profiling to assess plant-pathogen interactions: an example using rice (*Oryza sativa*) and the blast pathogen *Magnaporthe grisea*. *Eur J Plant Pathol* 129:539–554.
- Jones OA, Maguire ML, Griffin JL, Dias DA, Spurgeon, D J Svendsen, C (2013) Metabolomics and its use in ecology. *Austral Ec*, 38(6):713–720.
- Kaplan F, Kopka J, Haskell DW, Zhao W, Schiller KC, Gatzke N, Sung DY, Guy CL (2004) Exploring the temperature stress metabolome of *Arabidopsis*. *Plant Physiol* 136: 4159–4168.
- Kim HK, Choi YH, Verpoorte R (2011) NMR-based metabolomics: where do we stand, where do we go? *Trends Biotechnol* 29:267–275.
- Kim JK, Bamba T, Harada K, Fukusaki E, Kobayashi A (2007) Time-course metabolic profiling in *Arabidopsis thaliana* cell cultures after salt stress treatment. *J Exp Bot* 58: 415–424.
- Kim, HK, Verpoorte, R (2010) Sample preparation for plant metabolomics. *Phytochem Anal* 21(1):4–13.
- Krasensky J, Jonak C (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *J Exp Bot* . 63(4): 1593–1608.
- Kusano M, Tohge T, Fukushima A, Kobayashi M, Hayashi N, Otsuki H, Kondo Y, Goto H, Kawashima M, Matsuda F, Niida R, Matsui M, Saito K, Fernie AR (2011) Metabolomics reveals comprehensive reprogramming involving two independent metabolic responses of *Arabidopsis* to UV-B light *Plant J* 67: 354–369.
- Lake JA, Field KJ, Davey MP, Beerling DJ, Lomax BH. (2009) Metabolomic and physiological responses reveal multi-

- phasic acclimation of *Arabidopsis thaliana* to chronic UV radiation. *Plant Cell Environ* 32(10): 1377-1389.
- Lawo NC, Weingart GJ, Schuhmacher R, Forneck A (2011) The volatile metabolome of grapevine roots: first insights into the metabolic response upon phylloxera attack. *Plant Physiol Biochem* 49:1059-1063.
- Le Lay P, Isaure MP, Sarry JE, Kuhn L, Fayard B, Le Bail JL, Bastien O, Garin J, Roby C, Bourguignon J (2006) Metabolomic, proteomic and biophysical analyses of *Arabidopsis thaliana* cells exposed to a caesium stress. Influence of potassium supply. *Biochimie* 88: 1533-1547
- Lima MRM, Felgueiras ML, Graca G, Rodrigues JEA, Barros A, Gil AM, Dias ACP (2010) NMR metabolomics of esca diseased *Vitis vinifera* cv. Alvarinho leaves. *J Exp Bot* 61:4033-4042.
- López-Gresa MP, Lisón P, Kim HK, Choi YH, Verpoorte R, Rodrigo I, Conejero V, Bellés JM (2012) Metabolic fingerprinting of tomato mosaic virus infected *Solanum lycopersicum*. *J Plant Physiol* 69:1586-1596.
- López-Gresa MP, Maltese F, Belle's JM, Conejero V, Kim HK, Choi YH, Verpoorte R (2010) Metabolic response of tomato leaves upon different plant-pathogen interactions. *Phytochem Anal* 21:89-94.
- Macel M, vanDam NM, Keurentjes JJB (2010) Metabolomics: the chemistry between ecology and genetics. *Mol Ecol Resour* 10: 583-593.
- Macías FA, Galindo JL, Galindo JC (2007) Evolution and current status of ecological phytochemistry. *Phytochemistry*, 68(22):2917-2936.
- McGhie TK, Rowan DD (2012) Metabolomics for measuring phytochemicals, and assessing human and animal responses to phytochemicals, in food science. *Mol Nutr Food Res*, 56(1):147-158.
- Moco S, Schneider B, Vervoort J (2009) Plant micrometabolomics: the analysis of endogenous metabolites present in a plant cell or tissue. *J Proteome Res* 8(4):1694-1703.
- Moreno-Risueno MA, Busch W, Benfey PN (2010) Omic meet networks – using system approaches to infer regulatory networks in plants. *Curr Op Plant Biol* 13: 126-131.
- Morsy MR, Jouve L, Hausman JF, Hoffmann L, Stewart JM (2007) Alteration of oxidative and carbohydrate metabolism under abiotic stress in two rice (*Oryza sativa* L.) genotypes contrasting in chilling tolerance. *J Plant Physiol* 164: 157-167.
- Muth D, Kachlicki P, Krajewski P, Przystalski M, Stobiecki M (2009) Differential metabolic response of narrow leafed lupine (*Lupinus angustifolius*) leaves to infection with *Colletotrichum lupini*. *Metabolomics* 5:354-362.
- Nikiforova VJ, Kopka J, Tolstikov V, Fiehn O, Hopkins L, Hawkesford MJ, Hesse H, Hoefgen R (2005) Systems rebalancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of *Arabidopsis* plants. *Plant Physiol* 138: 304-318.
- Obata T, Fernie A R (2012) The use of metabolomics to dissect plant responses to abiotic stresses. *Cell Mol Life Sci* 69:3225-3243.
- Oliver SG, Winson MK, Kell DB, Baganzn F (1998) Systematic functional analysis of the yeast genome. *Trends Biotechnol*, 16:373-378.
- Paranidharan V, Abu-Nada Y, Hamzehzarghani H, Kushalappa AC, Mamer O, Dion Y, Rioux S, Comeau A, Choiniere L (2008) Resistance-related metabolites in wheat against *Fusarium graminearum* and the virulence factor deoxynivalenol (DON). *Botany* 86:1168-1179.
- Paul C, Mausz MA, Pohnert G (2013) A co-culturing/metabolomics approach to investigate chemically mediated interactions of planktonic organisms reveals influence of bacteria on diatom metabolism. *Metabolomics* 9(2):349-359.
- Petersen IL, Tomasi G, Sørensen H, Boll ES, Hansen HC, Christensen JH. (2011) The use of environmental metabolomics to determine glyphosate level of exposure in rapeseed (*Brassica napus* L.) seedlings. *Environ Pollut* 159(10): 3071-3017.
- Poëssel JL, Sauge MH, Corre MN, Renaud C, Gaudillère M, Maucourt M, Deborde C, Dufour C, Loonis M, Lacroze JP, Pascal T, Moing A (2006) Metabolic profiling of shoot apices infested by the peach-potato aphid. In: Ward JL, Beale MH (eds) *Proceedings from the 4th international plant metabolomics conference*, pp 296. *Metabolomics* 2:269-334.
- Prince EK, Pohnert G (2010) Searching for signals in the noise: metabolomics in chemical ecology. *Anal Bioanal Chem*, 396(1):193-197.
- Ratcliffe RG, Shachar-Hill Y (2006) Measuring multiple fluxes through plant metabolic networks. *Plant J*, 45(4):490-511.
- Reigosa MJ, Pedrol N, González L (2006) Allelopathy. A physiological process with ecological implications. Springer.
- Reigosa MJ, Sánchez-Moreiras A, Gonzáles L, (1999) Ecophysiological approach in allelopathy. *Crit Rev Plant Sci*, 18:577-608.
- Rivoal A, Fernandez C, Greff S, Montes N, Vila B (2011) Does competition stress decrease allelopathic potential? *Biochem Sys Ecol*, 39:401-407.
- Rizhsky L, Liang H, Shuman J, Shulaev V, Davletova S, Mittler R (2004) When defense pathways collide. The response of *Arabidopsis* to a combination of drought and heat stress. *Plant Physiol* 34: 1683-1696.
- Sanchez DH, Siahpoosh MR, Roessner U, Udvardi M, Kopka J (2008) Plant metabolomics reveals conserved and divergent metabolic responses to salinity. *Physiol Plant* 132: 209-219.
- Sardans J, Peñuelas J, Rivas-Ubach A (2011) Ecological metabolomics: overview of current developments and future challenges. *Chemoecology* 21:191-225.
- Schneider B, Hölscher D (2007) Laser microdissection and cryogenic nuclear magnetic resonance spectroscopy: an alliance for cell type-specific metabolite profiling. *Planta*, 225(3):763-770.
- Schripsema J (2009) Application on NMR in plant metabo-

- lomics: techniques, problems and prospects. *Phytochem Anal*, 21:14-21.
- Schroeder FC, del Campo ML, Grant JB, Weibel DB, Smedley SR, Bolton KL, Meinwald J, Eisner T (2006) Pinoresinol: a lignol of plant origin serving for defense in a caterpillar. *Proc Natl Acad Sci USA* 103:15497–15501
- Scognamiglio M, D'Abrosca B, Esposito A, Pacifico S, Monaco P, Fiorentino A (2013) Plant growth inhibitors: allelopathic role or phytotoxic effects? Focus on Mediterranean biomes. *Phytochem Rev* 12(4):803-830.
- Scognamiglio M, D'Abrosca B, Fiumano V, Golino M, Esposito A, Fiorentino A (2014) Seasonal phytochemical changes in *Phillyrea angustifolia* L.: Metabolomic analysis and phytotoxicity assessment. *Phytochem Lett*, 8:163-170.
- Scognamiglio M, Fiumano V, D'Abrosca B, Esposito A, Choi YH, Verpoorte R, Fiorentino A. Chemical interactions between plants in Mediterranean vegetation: the influence of selected plant extracts on *Aegilops geniculata* metabolome. *Phytochemistry*, submitted.
- Shulaev V, Cortes D, Miller G, Mittler R (2008) Metabolomics for plant stress response. *Physiol Plant* 132: 199–208.
- Sumner LW, Mendes P, Dixon RA (2003) Plant metabolomics: large-scale phytochemistry in the functional genomics era. *Phytochemistry* 62:817-836.
- Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, Fan T W-M, Fiehn O, Goodacre R, Griffin JL, Hankemeier T, Hardy N, Harnly J, Higashi R, Kopka J., Lane AN, Lindon JC, Marriott P, Nicholls AW, Reilly MD, Thaden JJ, Viant MR (2007) Proposed minimum reporting standards for chemical analysis. *Metabolomics*, 3(3):211-221.
- Tharayil N (2009) To survive or to slay. *Plant Signal Behav* 4:580-583.
- Tharayil N, Bhowmik PC, Xing B (2008) Bioavailability of allelochemicals as affected by companion compounds in soil matrices. *J Agric Food Chem*, 56:3706-3713.
- Torres A, Oliva RM, Castellano D, Cross P (1996) In: *First World Congress on Allelopathy. A science of the Future*, SAI, University of Cadiz, Spain p.278.
- Tresch S (2013) Strategies and future trends to identify the mode of action of phytotoxic compounds. *Plant Sci* 212:60-71.
- Urano K, Kurihara Y, Seki M, Shinozaki K (2010) 'Omic' analyses of regulatory networks in plant abiotic stress responses. *Curr Op Plant Biol* 13: 132-138.
- Urano K, Maruyama K, Ogata Y, Morishita Y, Takeda M, Sakurai N, Suzuki H, Saito K, Shibata D, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2009) Characterization of the ABA-regulated global responses to dehydration in *Arabidopsis* by metabolomics. *Plant J*, 57:1065-1078.
- van den Berg RA, Hoefsloot HC, Westerhuis JA, Smilde AK, van der Werf MJ (2006). Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC genomics*, 7(1):142.
- Verpoorte R, Choi YH, Kim HK (2007) NMR-based metabolomics at work in phytochemistry. *Phytochem Rev* 6:3-14.
- Weingart GJF, Lawo NC, Fornek A, Krska R (2013) Study of the volatile metabolome in plant-insect interactions. In *The Handbook of Plant Metabolomics*. Eds Weckwerth W, Khal G: Wiley- VCH Verlag. Pag 127-153.
- Weir TL, Park SW, Vivanco JM (2004) Biochemical and physiological mechanisms mediated by allelochemicals. *Curr Op Plant Biol* 7:472-479.
- Widarto HT, Van Der Meijden E, Lefeber AW, Erkelens C, Kim HK, Choi YH, Verpoorte R (2006) Metabolomic differentiation of *Brassica rapa* following herbivory by different insect instars using two-dimensional nuclear magnetic resonance spectroscopy. *J Chem Ecol* 32:2417-2428.
- Wishart DS (2008) Quantitative metabolomics using NMR. *TrAC Trends Anal Chem*, 27(3):228-237.
- Wolfender JL, Rudaz S, Choi YH, Kim HK (2013) Plant metabolomics: from holistic data to relevant biomarkers. *Curr Med Chem*, 20(8):1056-1090.
- Xiao JF, Zhou B, Ransom HW (2012) Metabolite identification and quantitation in LC-MS/MS-based metabolomics. *TrAC Trends Anal Chem*, 32:1-14.
- Yuliana ND, Jahangir M, Verpoorte R, Choi YH (2013) Metabolomics for the rapid dereplication of bioactive compounds from natural sources. *Phytochem Rev*, 12(2):293-304.
- Zenobi R (2013) Single-Cell Metabolomics: analytical and biological perspectives. *Science* 342(6163):1243259.
- Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol Adv* 23:83–333.
- Zhuo YH, Yu JQ (2006) Allelochemicals and photosynthesis. In: Reigosa, M. J., Pedrol, N., González, L. (eds.). *Allelopathy: A physiological process with ecological implications*. Kluwer Academic Publishers, Netherlands, pp. 127-139.
- Zuther E, Koehl K, Kopka J (2007) Comparative metabolome analysis of the salt response in breeding cultivars of rice. In: Jenks MA, Hasegawa PM, Jain SM (eds) *Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops*. Springer-Verlag, Berlin, Heidelberg, New York, pp 285–315.

RECEIVED March 7, 2014

ACCEPTED April 10, 2014
