

# Chapter 2

## Aspects of Experimental Design for Plant Metabolomics Experiments and Guidelines for Growth of Plant Material

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### Abstract

Experiments involve the deliberate variation of one or more factors in order to provoke responses, the identification of which then provides the first step towards functional knowledge. Because environmental, biological, and/or technical noise is unavoidable, biological experiments usually need to be designed. Thus, once the major sources of experimental noise have been identified, individual samples can be grouped, randomised, and/or pooled. Like other 'omics approaches, metabolomics is characterised by the numbers of analytes largely exceeding sample number. While this unprecedented singularity in biology dramatically increases false discovery, experimental error can nevertheless be decreased in plant metabolomics experiments. For this, each step from plant cultivation to data acquisition needs to be evaluated in order to identify the major sources of error and then an appropriate design can be produced, as with any other experimental approach. The choice of technology, the time at which tissues are harvested, and the way metabolism is quenched also need to be taken into consideration, as they decide which metabolites can be studied. A further recommendation is to document data and metadata in a machine readable way. The latter should also describe every aspect of the experiment. This should provide valuable hints for future experimental design and ultimately give metabolomic data a second life. To facilitate the identification of critical steps, a list of items to be considered before embarking on time-consuming and costly metabolomic experiments is proposed.

**Key words:** Biological error, Technical error, Experimental noise, Blocking, Pooling, Replication, Quenching of metabolism, Metadata

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### 1. Introduction

The ultimate goal of biology is to understand living systems in sufficient detail to enable accurate quantitative predictions about their behaviour (1). In the second part of the twentieth century, progress in biological research was mainly driven by the revolutionary concepts and technologies of molecular biology, which links

information about genetic traits to physical entities such as DNA or proteins. Strikingly, this led biologists to think “molecular”, eventually promoting reductionist approaches, which resulted in the attribution of biological phenomena to the actions of one or a few genes. Although reductionism is powerful in building logically simple hypotheses that are rather easy to test, it is very difficult to reconstitute a model for a whole biological system by combining the pieces of information it generates. Thus, from “functional genomics” biologists are moving to “systems biology”, in order to identify and integrate at the functional level all gene products present in a given biological system (2, 3). This move, which is characterised by the development of multiparallel technologies, called ‘omics, that produce massive data sets, now brings biologists to consider living systems as a whole again (4). However, this abundance of multiplexed information also presents many hurdles, starting with the major challenge of setting up the right experimental design. In particular, we may ask ourselves whether the unfocused nature of metabolomics conciliates the concept of *planning*. Have we set up the right experimental design? Have we the right number of samples for statistical analysis knowing that the number of metabolite peaks per sample usually exceeds the number of data points from an experiment? Do we need statistics to design valid experiments? Do we need pilot experiments before planning full-scale metabolomics analyses?

In this article, we briefly introduce the notions of experiment and experimental design; we then discuss some issues in designing ‘omics experiments, before embarking on a checklist for the design of experiments in the field of plant metabolomics.

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## 2. What Is Experimental Design?

In 400 BC, the philosophers Socrates, Plato, and Aristotle investigated the meaning of knowledge and the methods to obtain it, using a rational-deductive process. Later, scientists Ptolemy and Copernicus developed empirical-inductive methods that focused on precise observations and explanation of the stars. These early scientists were not experimenters. It is only when later scientists began to investigate earthly objects rather than the heavens, that they uncovered a new paradigm for increasing knowledge. In the early 1600s, Francis Bacon introduced the term “experiment” (5). The basis of this new paradigm called *experimentation* was a simple question, “If I do this, what will happen?” The key to understanding experimentation, and the characteristic that separates experimentation from all other research methods, is manipulating factors to see what happens. Explanations involve identifying the causes of what has been described and this involves finding out what factors influence

the variables. The scientific aspect of experimentation is the manipulation of variables under controlled conditions while taking precise measurements. Today, especially for metabolomic approaches, the key feature is still the deliberate variation of something so as to discover what happens to something else, and later to uncover the effects of presumed causes. But the real hurdle is that biological systems are complex, and many possible variables could be implicated, from genotype variability to fluctuations in growth conditions.

The design of experiments can be defined as a procedure aimed at planning experiments in the most efficient way to obtain data that describe the relationship between the different factors/variables affecting a process and its outputs (6). Traditionally, plant biology experiments have been performed by changing variables one by one, but it became evident that it is difficult to exactly reproduce measured results (7). In the 1920s, Ronald A. Fisher, a renowned mathematician and geneticist, developed the concept of “factorial design”, a powerful approach to deal with experimental error (8). An experiment with a factorial design can be defined as an experiment in which the effects of at least two factors are studied by testing all possible combinations. Environmental variables such as light intensity, temperature, moisture, or the availability of nutrients may vary across a field, a greenhouse, or even a growth chamber. In order to cope with such unavoidable but identifiable sources of variation, the arrangement of experimental units into groups (*blocking*) can be used. Then, in order to get an estimate of the errors that cannot be eliminated, multiple measurements (*replication*) can be performed randomly (*randomisation*) within each block. Factorial experiment design has proven efficient to evaluate the effects and possible interactions of several factors (9). This approach was built on the foundation of the *analysis of variance*, a collection of models in which the observed variance is partitioned into components due to different factors which are tested.

When a full factorial design requires too many samples to be processed, an “optimal design” can be used instead, given that combinations of various factor levels that are relevant have been identified. This is best achieved with the help of an algorithm (e.g. <http://www.optimal-design.org>). Optimal design is being used widely in all science and technology domains. For example, biotechnology companies always need to optimise production systems based on cell cultures, thus dealing with sometimes more than 50 variables (various nutrients, temperature, pH, speed of agitation, etc.). Running experiments one-factor-at-a-time would be extremely expensive, and would require months or even years. Recently, a complex cell-culture medium has been optimised by testing eight factors at five concentrations with 192 runs and within 8 days. By contrast, a full 5-level factorial experimental design would have required 390,625 runs (<http://www.statease.com/pubs/invitrogen.pdf>).

### 3. The Challenge of Designing ‘Omics Experiments

The advent of the “‘omics revolution” has forced us to re-evaluate our ability to acquire, measure, and handle data sets. In particular, many of us have had to realise that advanced statistics were inescapable.

#### 3.1. Throughput

‘Omics technologies provide unprecedentedly rich information about DNA, messenger RNA, proteins, and metabolites from complex biological systems. This is enabled by the development of a large variety of analytical platforms (e.g. DNA sequencers, microarrays, mass and nuclear magnetic resonance spectrometers), and by conceptual efforts in the areas of data management, biostatistics, data integration, computational modelling, and knowledge assembly protocols. However, ‘omics face technical difficulties, high costs, and time-consuming data analysis, which dramatically limit the number of samples that can be processed within experiments. Such difficulties actually favour poor experimental design and there is a widespread idea that the large number of measurements obtained in gene expression array, protein or metabolite identifications would somehow make up for small sample sizes (10). This idea is reinforced by some confusion about the meaning of high throughput in the literature. Originally used in biology in the context of screens, this expression has drifted to qualify technologies capturing large numbers of analytes per sample. But unless the behaviour of groups of analytes is studied, as can be done for example in PageMan (11), an analyte is definitely not a replicate. We would actually tend to consider ‘omics experiments as being rather low throughput, the first consequence of this being low replication, which appeals for great care during the design and analysis of experiments.

#### 3.2. False Discovery

The very notion that measuring every possible output variable is desirable has been seen as a big delusion surrounding the ‘omics, as system-wide measurements may violate statistical norms and have little precedent with respect to feasibility in analytical chemistry literature (12). ‘Omics experiments typically involve comparing a group of control samples with one or more groups of treated samples, with data often being expressed in a “semi-quantitative” way, which means that “fold-changes” are evaluated by calculating a ratio between the data obtained in treated and control samples. Replication (typically around five replicates) then allows checking whether the fold-changes are significant, generally by performing a  $t$ -test. However, methods based on  $t$ -tests depend on strong parametric assumptions (e.g. normality, homogeneity of variance, and independent errors), which are often invalidated by the restricted number of replicates (13).

A further striking problem is that the larger the number of analytes being measured, the easier it is to find rare events and therefore the easier it is to make the mistake of thinking that there is an effect when there is none. This is intimately bound to the multiple testing nature of ‘omics approaches and is called false discovery and requires multiplicity control (14). A range of methods and tools dedicated to the reduction of false discovery rate have been developed (e.g. a number of dedicated R-scripts can be found at <http://strimmerlab.org/notes/fdr.html>).

### 3.3. Significance

With respect to experimental design, we are tempted to put side by side ‘omics and experimentation on animals. Indeed, both suffer from low replication, the one because of technological issues, the other for obvious ethical reasons. An interesting article published in the journal *Laboratory Animals* reports a survey of three experiments performed with dogs or mice, which reveals that better experimental design could have resulted in the use of fewer animals (15). Furthermore, it demonstrates that factorial experimental design would have resulted in better precision. The same reasoning is valid for ‘omics experiments, as depicted below with a simple example.

Studies of metabolism usually face a large number of potential sources of variation. They can be biological (e.g. environmental, positional, temporal) or technical (e.g. experimenter, batch effect), some of them being unavoidable. To a certain extent, such interfering covariates can nevertheless be included in the analysis to adjust for their influences. For example, consider an experiment (see Table 1) in which two genotypes submitted to two treatments were grown in blocks corresponding to two shelves in a growth chamber (each shelf was characterised by slightly different growth conditions). A first option would be to perform a Student’s *t*-test by grouping replicates from different blocks. Because, Student’s *t*-test can only compare two treatments, it would also be necessary to transform the data into fold-changes. We chose to calculate treatment versus control ratios, by dividing each “treated” datum by averaged “control” data. However, such transformations imply the loss of two levels of information, eventually increasing the number of false positives or negatives. Indeed, we obtain a *p*-value of 0.16 (in Excel), which suggest that the response to the treatment was not significantly different between the two genotypes, or that sample size was too small. A more powerful option would be to perform a multifactorial analysis of variance (see Table 2). This time, we obtain a *p*-value of 6.52E-03, which indicates that there actually is a significant difference. A further interesting point is that a significant interaction is also found between treatment and shelf (*p*-value = 0.01), reinforcing the idea that the investigation of multiple factors at the same time can be more efficient and effective than a series of experiments aimed at each factor alone.

**Table 1**

**Fake experiment, in which two genotypes were grown under two treatments, on two different shelves, and in which one variable was measured**

Genotype	Treatment	Shelf	Variable
1	1	1	50
1	1	1	49
1	1	2	52
1	1	2	54
1	2	1	38
1	2	1	35
1	2	2	21
1	2	2	23
2	1	1	90
2	1	1	65
2	1	2	78
2	1	2	95
2	2	1	45
2	2	1	41
2	2	2	23
2	2	2	15

**Table 2**

**Analysis of variance performed on the fake experiment shown in Table 1 using the functions “factor”, “lm”, and “anova” in R (<http://www.r-project.org>)**

	<i>p</i> -Value
Genotype	3.50E-03**
Treatment	1.60E-05***
Shelf	0.14
Genotype × treatment	6.52E-03**
Genotype × shelf	0.81
Treatment × shelf	0.01*
Genotype × treatment × shelf	0.37

Only *p*-values are shown. Significance codes: “\*\*\*”, <0.001; “\*\*”, <0.01; “\*”, <0.05

## 4. A Checklist for the Design of Plant Metabolomics Experiments

Plants cannot escape their environment, but they have evolved a wide range of mechanisms to face sometimes highly fluctuating growth conditions. They make a variety of organs (leaves, roots, stems, tubers, etc.) composed of multiple specialised cell types (epidermis, guard cells, parenchyma, glandular hairs, etc.), each of them having a dedicated metabolism. In addition, abiotic (light, UV, water) and biotic (herbivore, parasitism, and pathogen attack) stress factors continually have to be dealt with and, for this, plants have developed a complex metabolic arsenal of compounds. Some of them are common, but many are restricted to one genus or perhaps even to one species. In addition to its high diversity, plant metabolism is also characterised by considerable robustness (e.g. metabolism operates under a wide range of temperatures), elasticity (e.g. metabolic fluxes can drop and recover within seconds when light fluctuates), and plasticity (e.g. plants are able to reprogram metabolism in response to many developmental, biotic, or abiotic challenges). The purpose of plant metabolomics is to capture instant pictures of this diversity, and integrate them into functional information (16). A major challenge is that such estimates must represent the amounts of the metabolites that were actually present in the harvested tissues when these were metabolising under the specified growth conditions (17).

One initial goal for metabolomics was to avoid exclusion of any metabolite by using well conceived sample preparation procedures and analytical techniques, thus allowing a comprehensive analysis of biological systems (18). However, unlike transcriptomics, and to a certain extent proteomics, technologies that are available to metabolomics are far from such comprehensiveness. Considerable progress has been achieved recently (19), but there is still no unique solution to extract and then determine every single metabolite simultaneously. This is further complicated by plant metabolomes being extremely diverse and complex (16). These limitations need to be taken into account so that the experimental design can be tuned to the biological question of interest (20).

### 4.1. Choose the Methodology

Globally, there are two types of data that can be generated in a metabolomics experiment, fingerprints and profiles. Fingerprinting, which is typically performed using FT-IR or <sup>1</sup>H-NMR, ignores time-consuming signal assignment and can thus be used to rapidly compare or classify samples in an unbiased way. It has been used to study the impact of environmental factors (21), cadmium toxicity (22), herbicide treatments (23) as well as to compare wild-type and transgenic plants (24, 25). Profiling, which is usually performed with MS- or NMR-based technology (see ref. 26 for

review), provides quantitative data. Its success strongly depends on the “quality” of the biological material, sample preparation, and sample extraction. Gullberg et al. (27) have detailed a useful report on appropriate strategies for the design of metabolomic experiments with GC-MS technology. Several classes of metabolites are nevertheless unsuited to GC-MS or NMR metabolomics, thus requiring dedicated experimental strategies. In particular, most intermediates of primary metabolism or coenzymes are present at low concentration and are usually very unstable. Their analysis may necessitate specific extraction protocols and instrumentation, e.g. LC-MS/MS (28), in addition to careful sampling procedures (*see below*). The analysis of several classes of secondary metabolites (e.g. phenylpropanoids, terpenoids) also requires dedicated LC-MS methodologies. In brief, experimenters should, at first, be aware of the possibilities offered by available or future equipment, as there is no unique and universal methodology so far.

#### **4.2. Evaluate Experimental Error**

Experimental error results from both biological and technical variability. Evaluating them can make a major contribution to the experimental design, by giving hints to reduce experimental error and/or by helping in the choice of replication strategy.

As discussed above, ‘omics approaches generalised the problem of making multiple hypotheses in a limited number of samples, eventually leading to new statistical concepts, or to the rediscovery of old ones (29), and tools dedicated to the optimisation of sample size were developed (30–32). False discovery has been considered as less challenging in metabolomics than in transcriptomics or proteomics because measurable metabolites are currently by far less numerous than measurable transcripts or proteins, and because variations in the metabolome are expected to be of much larger amplitude (33). However, this is counterbalanced by the high chemical diversity of metabolites which may cause experimental error. Indeed, nucleic acids consist of polymers of four nucleotides and share identical physico-chemical properties, and proteins are essentially made out of 22 primary amino acids, resulting in much lower chemical complexity than a metabolome (16). Accordingly, the chemical diversity of metabolites leads to unequal stability, matrix effects, differences in detection limits and linearity ranges. This is further complicated by the untargeted nature of metabolomics (18) because technical error cannot be defined for unexpected analytes.

To assess *technical variability*, pure chemicals can be used alone, but this is likely to be invalid due to matrix effects resulting from the high complexity of plant extracts. Recovery experiments, in which known metabolites of interest are mixed with plant extracts, are recommended instead (20). Then, the use of a range of concentrations of standards can be very helpful in determining the detection limit, which can be defined as the lowest level of a



spiked metabolite that is statistically significant (34). Furthermore, spiking different amounts provides an idea about the linearity of the dose–response curve for a given metabolite. Another useful procedure involves spiking extracts with suitable isotopomers (28). However, spiking is usually restricted to a few known metabolites. A simple and useful additional strategy is to use reference biological material, which would be similar to the material under investigation, and ideally “isotopomerised” (35, 36). Such material could also be obtained by mixing samples obtained under various conditions (environment, phenology, and/or genotype), in order to maximise the diversity of the resulting mixed metabolome. Dilution gradients can be made to evaluate linearity for each analyte under study. Then, recovery experiments can be performed by mixing samples of interest and reference material. Finally, this material can be used as an additional reference to the usual “control” samples that are grown in each experiment, offering the possibility to integrate data from many experiments (37).

Assessing *biological variability* is much more difficult, as it can vary depending on the genotype, the developmental stage, and/or the growth conditions. It is, however, useful to evaluate it within a large number of biological replicates obtained under standard conditions. Furthermore, biological variability should be documented and made accessible.

#### **4.3. Handle the Experimental Error**

What counts is whether the differences between conditions are larger than can be explained by experimental variability, and determining this requires statistically valid analyses. The precision of an experiment depends critically on the size of the experiment and the homogeneity of the experimental material. Even quite a small reduction in the within replicates standard deviation can lead to a dramatic increase in precision (15). Apart from working carefully, there are ways to decrease experimental error.

First, given technical error and/or biological variability have been evaluated, the most *adequate number of replicates* can be defined in relation to the aim of the experiment; it will have a major influence on reliability and reproducibility. A range of methods allowing the estimation of sample size have been developed for microarray experiments comparing two or more conditions (32). We assume that such methods should prove useful to metabolomics, which to a certain extent, face the same problem of having the number of analytes greatly exceeding the number of samples (33). A further point to consider is that, whenever possible, biological replication should be preferred to technical replication. In fact, technical variability, which is generated alternatively by experimenters, techniques, and/or equipment, is usually small (less than 10%) in comparison to biological variability. When considering that the number of samples that can be processed is limited by the technology, biological samples should always be preferred.

*Pooling samples* corresponding to different individuals grown under identical conditions can prove very useful in decreasing biological variability given that costs per sample are relatively low and costs per analysis high, which is generally the case in metabolomics. As shown by Rocke (10), pooling samples can dramatically reduce costs: A study that would cost 33,600 US\$ in which 56 samples are planned, using a simple experimental design with a standard deviation of 0.3 for biological replicates and 0.1 for analytical replicates, can be carried out for less than 10,000 US\$ by pooling seven samples per array and using only eight arrays for the whole study. In general, the largest gains from pooling are obtained when the cost per sample is low and the cost per analysis high. However, pooling assumes that information will be lost, as variations in metabolite levels always have a biological significance (16). A further disadvantage is that a single bad or unusual sample can ruin a pool or even an entire experiment.

When experimental noise cannot be avoided, *blocking* should be used, as discussed above. For example, if an experiment requires the use of two growth chambers with theoretically identical growth conditions, replicates (e.g. corresponding to genotypes) should be equally distributed between the two chambers because growth conditions will be practically different. Blocking should, however, be restricted to major sources of noise as a too complex experimental design would require too many samples and result in difficult statistical analysis.

*Randomisation* is a further way to cope with experimental error. It can be used throughout the experimental pipeline. Fields, greenhouses, and even controlled growth chambers never deliver uniform growth conditions; there are always gradients and border effects, even within blocks. When various genotypes are compared under one theoretical growth condition, they must be randomised and when time-course experiments are performed, plants grown under theoretically identical conditions should be harvested randomly. However, randomisation can lead to fastidious harvesting procedures. For example, when randomly planted individuals are pooled, extra time will be required to move from one individual to the other. Randomisation should also be used throughout the analytical procedure, from sample storage to detection. An article of Scholz and colleagues (38) illustrates very nicely how useful such precautions can be.

#### **4.4. Strike the Balance Between Sample Number and Throughput**

The rather low throughput offered by metabolomic technologies still limits the number of samples that can be processed and thus the scale of experiments. Based on literature and on our own experience, we estimate that in academic research, current plant metabolomic experiments do not usually represent more than several hundreds of samples. Although such size is considerable, it is merely adequate

for experiments in which a limited number of growth conditions are compared, but it is not suited for experiments in which effects of a number of potentially interacting environmental variables would be studied. For example, an experiment as simple as a time course with 10 harvest points, 6 replicates per harvest point, 2 growth scenarios and repeated once would already represent 240 samples.

Next, throughput of hundreds of samples is adequate for quantitative trait loci- (39–41) or association mapping studies (42), as they typically involve populations of hundreds of individuals. But again, this assumes that only one or two growth scenarios or harvest points would be used. Furthermore, the emergence of huge mapping populations such as the recently developed Maize nested association mapping population of 5,000 genotypes (43) poses an interesting challenge for ‘omics approaches.

Nevertheless, the balance between sample number and throughput should always be optimised to decrease the risk of performing costly but badly designed experiments. In addition to literature and database searches, it can be very useful to perform preliminary experiments, in which visual or biochemical diagnostic markers are evaluated to identify the most appropriate set-up to reach the desired physiological and/or developmental state (44). For example, given a detailed time-course experiment has been performed, it might be easier to define the time of harvest that would be the most relevant to the biological question of interest. As an example, glucose-6-phosphate, which is relatively easy and cheap to measure in high throughput (34), has been suggested as a marker for carbon status in leaves of *Arabidopsis thaliana*. In response to carbon starvation, this metabolite drops and recovers partially within hours, indicating a time window that is also characterised by a dramatic response at the level of the transcriptome (45).

#### **4.5. How to Grow Plants**

There are no recommendations about how to grow plants that would be specific for metabolomics. However, because plant metabolomes reflect short- to long-term interactions between genotypes and their environments, variables that are the most likely to affect metabolism should be controlled in a reproducible way and/or monitored. As already mentioned, randomisation within an experiment is essential to cope with unavoidable “local” effects associated with gradients in essential variables such as light intensity, temperature, and air humidity that are typical for greenhouses but also frequent in controlled growth chambers.

Possible interactions between environmental variables should also be foreseen. For example, under high light intensities, plants will tend to grow faster, thus consuming more water and nutrients (46). If these variables are not controlled, plants growing the

fastest might deplete resources quicker under high light, eventually running into limitations in water or nutrients. It is obvious that this would lead to erroneous results in situations where a mutant with stunted growth is studied.

Optimised labelling of individuals also belongs to a good experimental design. Bar codes are increasingly used in plant research, dramatically facilitating documentation and traceability of experiments.

#### 4.6. When to Harvest

The metabolic composition of plants or plant organs varies throughout their lifecycle. However, *age* is a rather imprecise criterion to define maturity of plants, as dynamics of traits such as phenology and sex expression (47) or metabolic composition (48) can vary dramatically in response to the environment. Furthermore, such responses may vary depending on the genotype (mutant, transformant, ecotype, or cultivar), eventually leading to apparent metabolic phenotypes that would be indirect, and thus very difficult to explain at the functional level. It might therefore be very useful to search for diagnostic markers that are specific for the desired developmental or physiological stage. Ideally, such markers would be visual and/or very easy and cheap to determine.

The next issue is to define the most appropriate *time of the day* to harvest plant tissues, as many metabolites can show strong diurnal variations. A widespread habit involves taking samples in the middle of the day, assuming that everything is “on” or at steady-state. While this might be true for fluxes and levels of intermediates through pathways connected to photosynthesis, it is wrong for many metabolite levels. It is indeed well known that in leaves a range of metabolites such as major carbohydrates including starch (44, 49), amino acids (50), fatty acids (37), or organic acids (51) accumulate during the day in leaves. Although less marked, diurnal fluctuations in metabolite contents have also been reported in developing fruits (52).

Harvest should be as quick as possible, posing again the problem of the size of the experiment (the more samples the longer it takes to harvest them). It might be useful to estimate how much time one sample would require and thus predict harvest duration. For example, if one sample requires 1 minute, an experiment with 300 samples would require 5 h, which would be likely to introduce considerable variation into the experiment, unless logistics have been adequately tuned.

#### 4.7. How to Harvest

Specific harvest and extraction protocols are available for plant metabolomics (see Chapter 4 for more details). However, some issues need to be taken into account at the level of the experimental design, mainly in terms of feasibility.

A major issue is that many metabolites are unstable due to particular chemical properties, or simply when the inactivation of

particular enzymes proves ineffective during harvest and/or extraction. The latter is particularly problematic for metabolites with high in vivo turnover rates, such as many intermediates in the primary metabolism as well as coenzymes. It has for example been reported that ATP pools turn over within seconds in leaves (53). For such metabolites, harvested tissues have to be inactivated as quickly as possible and under the growth conditions of interest, e.g. under ambient light if photosynthetic tissues are to be harvested during the photoperiod. Freeze-clamping is probably the most appropriate method for such purpose (54), but it is rather time-consuming, thus limiting the number of samples than can be taken.

Plants produce a large and diverse panel of naturally volatile compounds, which are also technically challenging to study, especially when they are collected from the headspace of leaves, flowers, or fruits, thus requiring sophisticated experimental set-ups (55–57).

#### **4.8. Giving Metabolomic Data a Second Life**

Because they are rather expensive and slow, ‘omics faces the paradox of measuring too many things in too few samples. When experiments have been thoroughly designed and described, it nevertheless becomes possible to perform meta-analyses with very large data sets. The implementation of public repositories further increases the amount of data that can be accessed to extract new information without needing to perform additional experiments, and to support and extend the interpretation of new data sets. The use of standardised conceptualisations with explicit specifications to report data and metadata (i.e. data about data) will be decisive. MIAME (Minimum Information About a Microarray Experiment) was the first initiative to impose the use of a controlled system to describe ‘omics experiments (58). Quickly, major scientific journals then started to require publications describing microarray experiments to comply with the MIAME guidelines, thus greatly improving accessibility to transcriptomics data. This initiative inspired the emergence of a range of minimum information checklists for reporting diverse biological experiments ([http://www.mibbi.org/index.php/MIBBI\\_portal](http://www.mibbi.org/index.php/MIBBI_portal)). Thus, standardisation efforts aiming at obtaining metabolomic data that support evaluation, repetition, and/or extension of experiments and ultimately enable data mining are ongoing (59, 60), resulting in guidelines that cover almost every aspect of the experimentation, ranging from growth conditions to technical details of the analysis. Importantly, minimal information checklists standardise the data content, as they impose what terms have to be described. However, they do not necessarily constrain the format, as terms can usually be described using free text. This is probably better achieved using ontologies (61) dedicated to specific aspects of the biological experimentation (e.g. genotypes, phenology, or abiotic growth

conditions), each of them providing structured terminologies that are implemented with precise specifications about the terms and their use (e.g. <http://www.gramene.org>; <http://www.codeplex.com/XeO>). The use of ontologies to generate metadata about experiments should ultimately prove more advantageous for data mining approaches, as they better constrain the format. Furthermore, given an experiment has been described using dedicated ontologies, the generation of the relevant minimum information checklist should be facilitated. Indeed, generating checklists can be time-consuming and frustrating, favouring mistakes as pointed out recently (62). Fortunately, tools dedicated to the planning of experiments and the generation of checklists and/or ontology-based descriptions have already been developed (45, 63), paving the way for a more efficient sharing of data and metadata. Recently, Xeml Lab, a platform that helps plant biologists to plan experiments, from setting environmental history to defining sampling strategy, and concomitantly generate machine-readable and ontology-based metadata files has been proposed (45). Thus, while filling in checklists is likely to become unavoidable for publication, it is worth considering the use of such tools at the very beginning of an experiment.

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## 5. Conclusions

In the last decade, great efforts and energy have been invested in advancing technologies as well as dedicated bioinformatics (see Chapters in Parts 2 and 3 in this book for more details). However, without care we will continue to generate data while running the risk of losing sight of the primary goal of the production of knowledge. We need to identify and understand the limitations of the methods we are using at each step of the experimentation, and then formulate the most appropriate experimental design.

We propose a list of items to be considered for experimental design in the field of plant metabolomics (see Fig. 1). Typically, once the biological question has been clearly formulated, one needs to choose the most appropriate biological resource and analytical technology. Which factor(s) can be varied to reveal a metabolic response, and how can this response be monitored? There are usually a number of valid options at this stage, but there are probably even more non-valid ones. Then, in order to cope with biological error, it is important to decide how to grow plant material and when and how to harvest samples. There are no recommendations about how to grow plants, but there is a real and urgent need to document the history of growth conditions for each sample, in

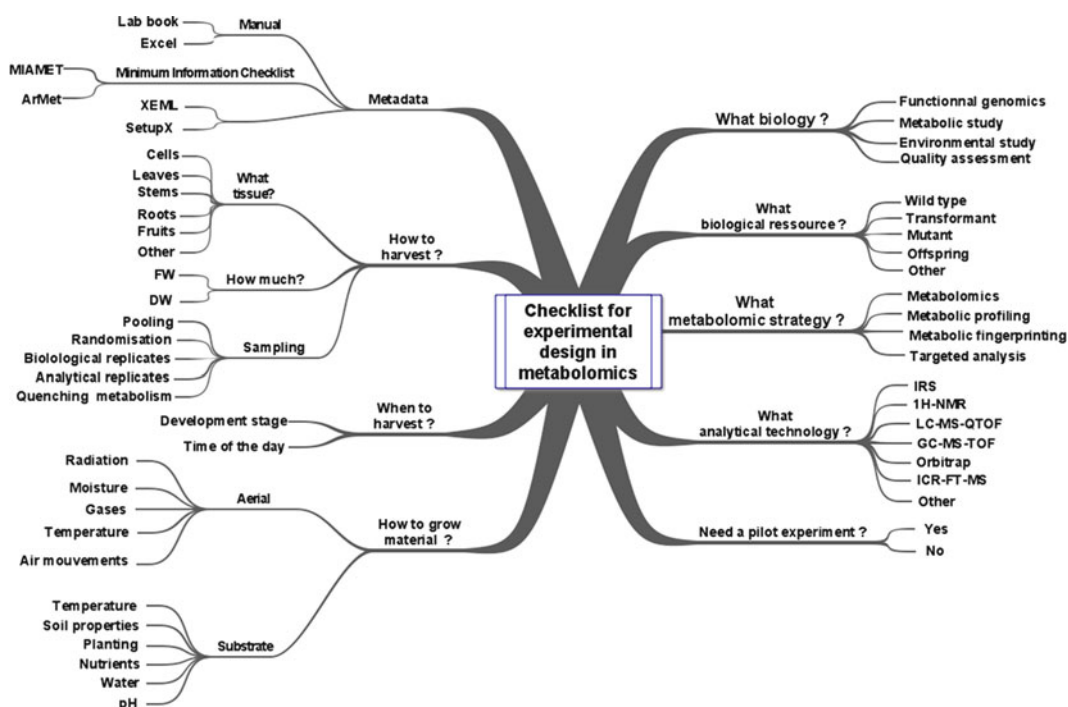


Fig. 1. List of items to be considered before starting a plant metabolomic experiment.

order to be able to compute metadata and analytical data in the near future. For this, minimum information checklists, ontologies, and tools such as Xeml Lab (45) are already available. Optimal reproducibility might be obtained by considering the following. First, it is essential to know how many samples are necessary to perform the analysis. Second, it is important to control both developmental stage and the time of the day at which samples are harvested. Third, the most adequate sampling procedure needs to be decided, mostly depending on how stable the metabolites of interest are. Experimental error needs to be evaluated and documented so that decisions about blocking and randomisation, pooling samples, and biological and technical replication may be made. It is also recommended that as many plants as possible are grown and that plant samples are as large as possible. Before embarking on time-consuming and costly experiments, it might be useful to perform a preliminary experiment verifying that all operational parameters are appropriate. Another good option would be to use tools dedicated to the planning of experiments and the generation of checklists and/or ontology-based descriptions. These could contribute to the success of plant metabolomics by enabling us to link environmental metadata with analytical data and to perform reliable and comparable analyses.



## Acknowledgements

This work was supported by the EU META-PHOR Project (FOOD-CT-2006-036220).

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Plant Metabolomics

Methods and Protocols

Hardy, N.W.; Hall, R.D. (Eds.)

2012, XIII, 340 p., Hardcover

ISBN: 978-1-61779-593-0

A product of Humana Press