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Basic Concepts of High throughput Metabolomics in Plants

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Review Article

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ABSTRACT

Each cell contains many different metabolites and chemical molecules which are generated during cellular process. All the metabolites present in a cell at a particular time is called metabolome. The study of all the metabolites and their modification in a particular condition is called metabolomics. Metabolome is closely linked with genotype, physiology and environment. So,in a nutshell, metabolomics is the study of substrates and products of metabolism which are influenced by the genetic and environmental factors. In plants, metabolomics has now been frequently developed and studied in biotic and abiotic stress resistance. High throughput metabolomics includes time efficient and effective metabolite profiling techniques. These techniques are chromatography based and chromatography free methods. Chromatographic methods are NMR, GC-MS and LC-MS . Chromatographv free techniques include DI-MS,FI-MS,MALDI and Ambient MS . This paper will give an idea about how metabolomics work in elucidating plants phenotype, how sample is prepared for metabolite profiling, different techniques of metabolite profiling and various metabolomic databases.

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1. INTRODUCTION

The "metabolome" refers to a plant's or any other organism's whole complement of tiny molecules. It denotes the final phenotype of cells, as determined by gene expression and protein modifications. The phenotypic expressions are influenced by environmental stimuli and genetic mutation [1]. A complete metabolome of a single plant thus represent highly active component which is influenced by spatial and temporal components [2]. Metabolome may be further spatially characterized as a component of single plant which may be single plant organs, tissues, and a single cell with easily distinct chemical profiles [3,4].

Plants are thought to be most dynamic biochemical machinery [5], and hence constitute vast naturally occuring chemical libraries that have been inadequately used to yet [1]. There are many plant species which has never been studied for biochemical insight and even if biochemical studies has been done then those biochemicals are not structurally defined. The diversity of these naturally available resources have valuable utilization in the coming era in both scientific study (allowing for a more comprehensive knowledge of plant based metabolites) and practical research (e.g., as a medicinal component, flavoring agent compound development).

Utility of Metabolomics: Plant scientists have been able to go through a deep knowledge of cell biochemistry thanks to metabolomics and related technique that has swiftly developed a solid foothold in research as suggested by Hall (2007). These techniques have now been used to a wide range of taxa, both cultivated and wild, to address a wide range of biological issues of scientific and industrial importance. Many earlier studies have dealt with main metabolites, for which the synthesis pathways are well defined along with their genetic background [6]. Authentic standards are also widely accessible, allowing for both conclusive identification and complete quantification. However, extensive researches have been undertaken in the very complex and inadequately understood secondary metabolite pathways. These secondary metabolite pathways biologically important since they are linked to abiotic or biotic stress tolerance, as well as other environmental and organism interactions, food quality attributes like taste, organoleptic qualities and Use in medicine field and other things. Polyphenolics [7], alkaloids [8], brassinosteroid, and glucosinolate [9] studies have all made significant progress in the field of metabolomics.

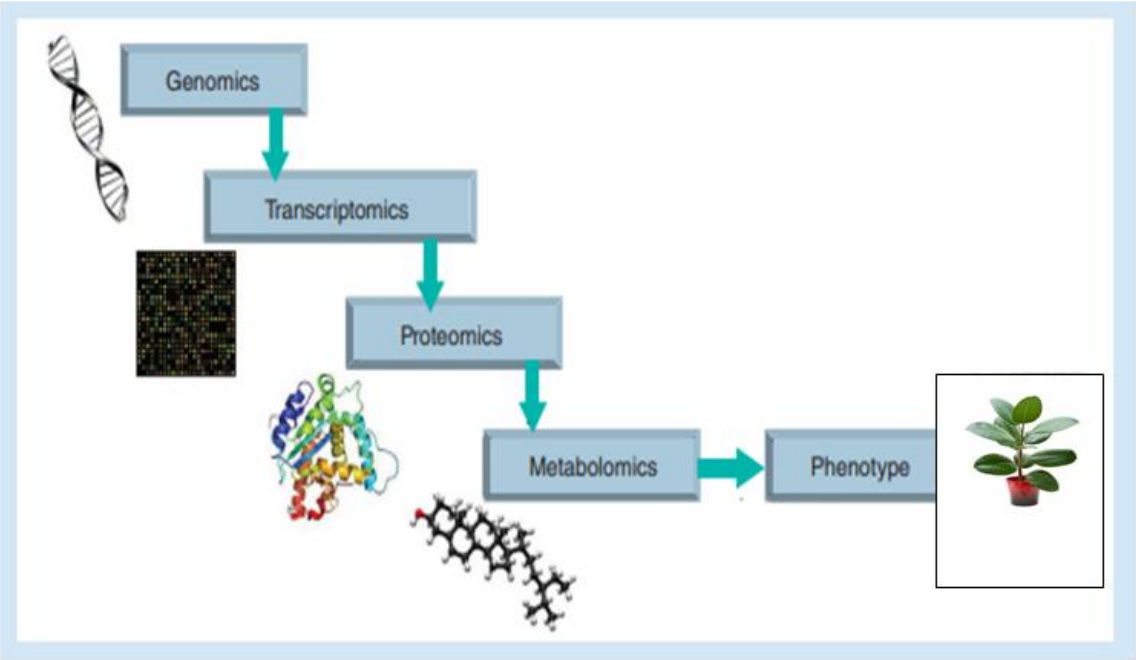

Fig. 1. The Cascade of Omics

General Procedure of metabolite profiling: Researches in plant science field require variability in at least one genetic or enviromnmental variation in order to get information about response due to change. For a successful research, before going on to chemical studies, all the prior steps should be well planned like from which tissue the sampling should be done, how sample will be taken etc.

Experimental design and plant growth: Plant metabolomics studies are based on thousands of metabolites that vastly outnumber the sample quantity, raising the possibility of false discovery. For minimizing the errors, it is very important to know the possible cause of error at various fundamental stages from plant growth to sample storage and dissemination in order to reduce experimental error. Knowing the cause of error will help in establishing "experimental design," as well as for making strategy in such a way that errors can be minimized to the possible extent [10]. It can be achieved through small-scale preparatory experiments before moving on to larger-scale trials. Adopting an appropriate statistical design for an experimental research along with proper labeling can also help in reducing non assignable variations [11]. The environment must be managed during culture, and changes in certain main environmental variables (such as fertiliser, temperature, and/or light) must be recorded. Even in regulated conditions like greenhouses or growth chambers, slight deviations might produce biochemical status changes. Data documentation and storage are also essential for data analysis [12]. As a result, the metadata format for plant growth should be simple to record and share. If all else fails due to high analytical costs, biological sample replication is essential and should be preferred to extraction and analytical. It's necessary to strike a balance between sample size and throughput. The experimental design must specify the number and composition of biological replicates. Replication is necessary for incorporating and measuring biological differences, as well as providing a sample.

Harvest: For the metabolite studies, the harvesting techniques of plant or plant part need to include several major points like at what age ,from which tissue and at what time the plant or plant part should be removed. If there is no standard technique of harvesting then there can be uncontrolled variability due to above factors. These factors become more significant when there are multiple pickings within single experiment season or when each session requires several operators to keep its time to a minimum. Whenever available, dedicated ontologies or reference articles (e.g., [13,14] should be used to define the age, or preferably the developmental stage, of the plants or their organs in relation to standardised growth conditions and/or phenology de Hours for seedlings in controlled environments. If the age of the organ is uncertain, sample homogeneity can be improved by using some other defined characteristics like organ aspect, colour, weight and size. Even for an organ with well defined growth stages, the sampling time and procedure need to be exact [15]. Because the occurrence of specific chemical activity of metabolite in leaf [8] and also in fruit [16] is time specific, the harvest time must be accurately set and the length limited. The organ's location (for example, fruit position) must be determined. Similar climatic conditions for each harvest on an open field are preferred. Plant parts should be treated with care and preserved in appropriate to avoid any damage and oxidation of metabolite. For keeping the metabolite intact in the sample, the enzyme activity should be prevented [17]. This can be done by keeping the sample at the freezing temperature or by keeping it in liquid nitrogen.

Sample Collection: An appropriate technique of sample collection is necessary for metabolite analysis, as well as samples that are representative of the overall population to be studied. Various plant material need further caution (e.g., different plant parts are made of different tissues). Each sample must be specified before storag, because the sample must fully represent the total tissue in the organ. Working as quickly as possible is important since the use of blades and scalpels produces wound stressors that influence chemical activity metabolite. Surfaces uncovered after incision should ideally be kept to a minimum. Microdissection necessitates the use of specialised equipment and processes. The pieces can be ground right away after being completely frozen in liquid nitrogen, or put into labeled nonreactive containers and held at 80 degree Celsius until grinding is practicable, before being distributed into tubes and stored at 80 degree Celsius. Extraction of metabolite components for all samples for a specific experiment must follow the similar procedure. Sample grinding is normally necessary to maximise solvent extraction and to homogenise the sample material [18]; nevertheless, contamination or volatilization of some chemicals of interest [18] need to be

reduced. Despite the possibility of volatile component alteration during freezing, samples are generally ground frozen for metabolomics [19]. Robotized grinding automation must be considered for HTP metabolomics research. Quality assurance and Quality Control (QC) are critical throughout the sample preparation process, right from growing the sample in field to its storage in the laboratory and distribution to chemical analyzers. The samples should be properly identifiable. This can be achieved by barcoding or by cataloguing the information in the databases.

Sample Storage: An experiment's samples should all be stored in the same way. Sample storage conditions and duration must be monitored and recorded. Plant samples are rarely studied for the influence of long-term preservation on their metabolites [20]. As a result, while dealing with several families of compounds utilising a mix of extractions and analytical procedures, it is worthful to suggest a few compounds as "markers" of excellent storage conditions and duration. Solid phase micro extraction trapping of volatile chemicals [21] may be conducted on full organs which can be kept as tissue pieces, or powdered tissue at 80 degree Celsius. Tissue samples can be preserved as raw or lyophilized samples, depending on the required analysis. Freeze-drying will result in the loss of volatiles, hence fresh-frozen samples are required for analytical evaluation of highly volatile chemicals [22]. It should be kept in mind that freeze-drying might result in the permanent loss of certain metabolites due to irreversible attachment to cell walls or membranes, despite the fact that freezing disrupts enzymatic reactions and microbial degradation during storage (Dunn et al., 2005a). Stability during sample storage is an important component that is seldom assessed, hence storage conditions must be regulated. Fruit that has been frozen for a long period has been found to change several aromatic components [23]. Typically, metabolite samples are kept at 80 degrees Celsius. It is advised that lyophilized samples be maintained at 20°C and in dry circumstances [24]. Tissue samples and extracts may need to be preserved in tubes filled with nitrogen or argon gas to prevent oxidation for certain reasons [25].

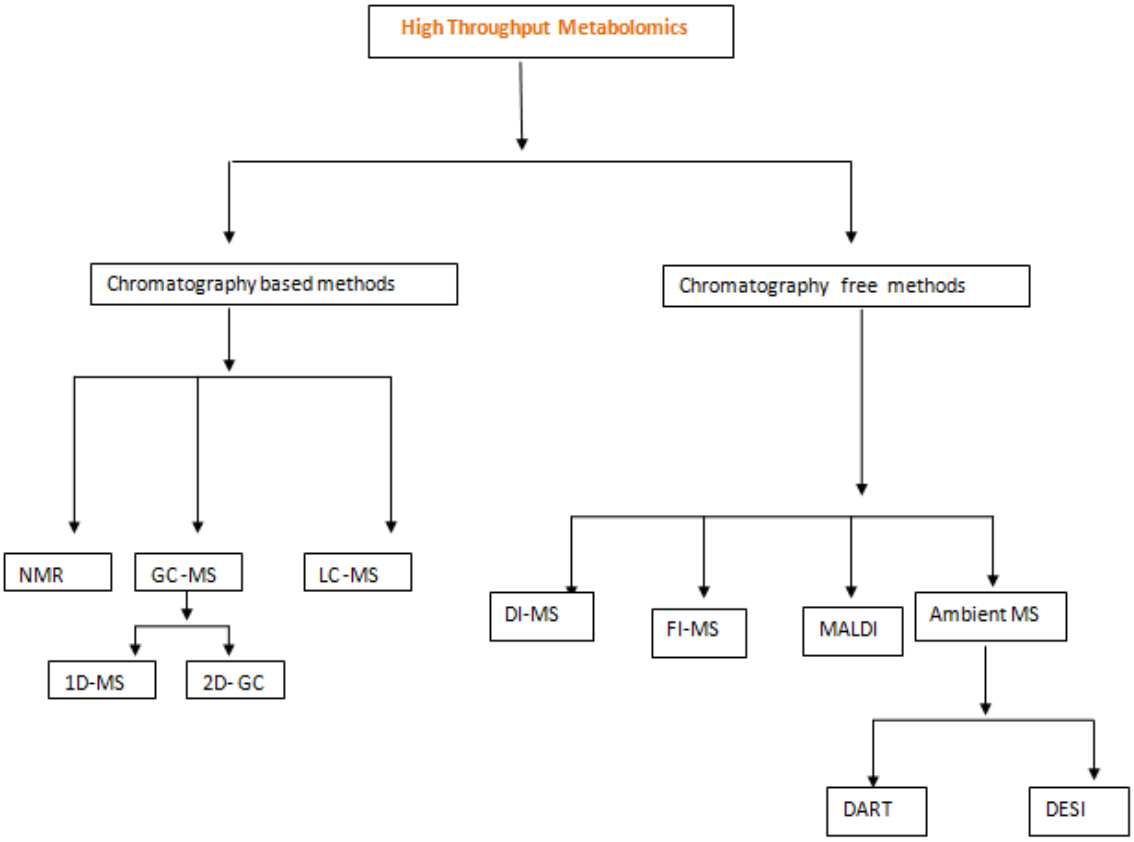

Chart 1. Some important high throughput metabolomics techniques

A) Chromatography based Metabolomics:-

1 .GC- MS Metabolite Profiling: Phenotyping through metabolic profiling using Gas chromatography Mass spectrometer has now become an important part of functional genomic analysis [26] and is about to become a regular technical practice to generate useful data [27]. This finding adds significantly towards growth of metabolomics [28]. Six general steps are involved in metabolite profiling using GC–MS:

1) Extraction of metabolite from the plant or organ.
2) Derivation of metabolite in the form which can be used for gas chromatography.
3) GC separation- Because it uses automated sample insertion robots, highly standardised gas flow settings, temperature programming, and uniform capillary column material, GC may be extremely reliable.
4) Ionization of compound- Electron Impact (EI) method is widely used as it is highly reproducible.
5) Detection of molecule and fragment ions with time resolution. TOF detectors that can be set to rapid scanning speeds are preferred for mass separation and detection.
6) GC–MS data file acquisition and assessment

Data preprocessing in GC-MS: In order to construct metabolite or chemical feature matrices for statistical computations, automated data analysis tools are required. Wagner et al., 2003 suggested retention indices and mass spectra in GC–MS profiling investigations for statistical computation of metabolite expression. Many tool have been created till now for compound recognition along with different aspects of metabolite profiling. In academia, many data preparation procedures have been used. One basic technique employs mass spectrum deconvolution first, then quantifies based on deconvoluted (mass fragment) abundances. If the goal is to conduct extensive metabolic profiling investigation, one should first detect entire metabolic characteristics, which necessitates the requirement of whole data set deconvolution through Chroma Time of flight technique [29]. But if the goal is to find metabolite differences in the samples, a more rapid approach would be to use generic software like TagFinder [30] or MetAlign. The present more pressing problem is identifying metabolites in complicated combinations in an automated and precise manner. Even today, human judgements are crucial for harmonizing and categorization of mass spectra or mass spectral tags (MSTs). Many software tools like NIST or GMD (Golm Metabolome Database) are available that sustain both MST recognition and interpretation of unknown mass spectral features [31]. A laboratory undertaking routine metabolic profiling, on the other hand, need to have their own pure reference library that are examined following being subjected to the same processing and analysis parameters as the profiled samples for unambiguous identification.

2. LC–MS Metabolite Profiling: For metabolic profiling of semi-polar secondary metabolites such as phenols, flavonoids, and glucosinolates, HPLC together with MS (in short LC–MS) is the recommended approach. These chemicals can be extracted with methanol solutions and evaluated without the need for previous derivatization. In LC–MS, ionization is commonly done through soft ionisation methods, ike electrospray ionisation (ESI) or atmospheric pressure chemical ionisation, which either produce positive or negative molecular ions. Modern high-resolution mass spectrometers, such as TOF/MS, ion cyclotron FT-MS, or Orbitrap FT-MS, can profile many compounds from plant extracts while also calculating elemental formulas from the discovered masses [32]. A comprehensive information about the relative abundance of hundreds of known and undiscovered semi-polar metabolites may be acquired through an essentially unbiased technique that undertakes all metabolite mass signals from the LC–MS raw data files [33]. C18-based reversed phase columns are often used in LC–MS-based metabolomics techniques to achieve optimum separation of large quantity of semi-polar chemicals that can be there in raw plant extracts [34]. This method has been used for secondary metabolite analysis in a variety of species, including Arabidopsis, crucifers, solanaceae family as well as plant tissues [35].

Data preprocessing in LC–MS profiling: MetAlign is a software which is freely available tool for untargeted processing of raw data files from LC–MS and GC–MS instruments from a variety of vendors (download at www.metalign.wur.nl). For up to hundreds of samples, it conducts various operations like correction of local baseline, selection of unbiased peak, and alignment of mass peak, which altogether results in mass intensity signals. This

software is ideal in case where mass spectrometry instruments have limited mass precision, such as the QTOF Ultima, since it allows you to choose window for precise mass computation. The information obtained from quality control samples can be used for consistent peak selection and grouping after processing. For recognised chemicals, around 70–80% of all peaks should be present in all quality control samples, with less than 20% variation and less than 3 ppm mass deviation. Thus the resulted peak table can be further utilized to remove signals that are inconsistent, low (noisy), or saturated. By adopting a mass spectra reconstruction technique, mass signals arising from the same sample can be classified as per their related retention duration and relative intensities across samples [7]. LC–MS signals from the same metabolite are grouped and replaced by a single representative metabolite signals.. To identify the important secondary metabolites, data-dependent tandem-MS/MS and MSn fragmentation studies can be used [33].

3. ^{1}H Nuclear Magnetic Resonance (NMR): Despite the fact that NMR spectroscopy has been a prominent technology in metabolomics since 2000 [36], it is underutilised in plant metabolomics compared to mammalian metabolomics. NMR can be either monodimensional ormay have several dimensions. HSQC (heteronuclear single quantum coherence spectroscopy) and 2D JRES (2D 1 H 1 J-resolved) spectroscopy are dominant and frequently crucial methods for metabolite identification, although 1D 1 H NMR has mostly been used in metabolomics until recently. Rapid spectrum collection and the ability to perform quantification are key factors in 1D 1 H NMR's success in metabolomics. Furthermore, NMR gives comprehensive molecule structure information and does not degrade the sample during the measurement.

Plant sample preparation for NMR usually involves many procedures. The technique of extraction has a significant impact on the subsequent identification of metabolites, and samples are always tested promptly after extraction. Although the processing of sap or fruit juice samples is faster than that of tissues, proteins present in extracted compounds need to be neutralized , either with alcohol like methanol or by high temperature treatment (Biais et al., 2009).The amount of sample needed in NMR method is quite higher (200–500 mg fresh weight) than that chromatographic procedures with or without mass spectrometry(60 mg fresh weight) .Since water is the most component of plant tissue and is made up of two atoms of hydrogen that may be detected using NMR, it is critical to remove it using lyophilization or cryodessication. This step should be conducted after cryogrinding for maximum efficiency, since fine plant powder aids in the freeze-drying and extraction processes [8].

For NMR based metabolite profiling, high-field devices with superconducting magnet coils operating at liquid helium temperature should be used. The chief commercially accessible field is currently 23.5 T, which corresponds to a 1 H frequency of 1 GHz. The ordinary fields generally used for plant metabolite profiling have a 1 H frequency of 400–600 MHz. A console (high-frequency channels for excitation and acquisition, gradient amplifier unit), a probe head, and an autosampler are also part of an NMR spectrometer. Tunable broadband inverse gradient probe heads (e.g., Bruker's ATMA-BBi and Varian's ProTune Auto-X ID) flushed with nitrogen gas (from a nitrogen separator enriching N2 to roughly 98 percent of compressed air) and temperature regulated are advised automatically. Water's chemical change is temperature dependent. For 1 H detection, inverse or indirect detection probe heads give the best signal-to-noise ratio. Standard probe heads have a diameter of 5 mm and need a sample volume of 500–600 mL [37]. HTP sample handling is made possible by autosamplers or robotic sample changers, which allow for the mechanical changing of NMR tubes according to a predefined programme. In terms of tubes, it is advised to always use same quality tube and cap from the same vendor for the whole series of tests.

B) Chromatography Free Metabolomics:-

Given that chromatography tends to be the most time-consuming aspect of quick analysis and smooth peak identification, omitting chromatographic separation entirely in metabolomics seems like a natural way to boost sample throughput. Direct infusion and flow injection are two methods for introducing liquid samples into mass spectrometers.

1. Flow injection: It involves passing a sample in continuous stream of organic phase running at a rate of 100–1000 L/min to electrospray interface. Peak girth varies depending on flow rate, sample concentration and electrospray

source but it usually falls between 5 and 15 seconds. With a conventional LC–MS equipment, this enables for the injection of 50–100 samples each hour, resulting in 1000–2000 injections per day [38]. Despite the great sensitivity of current mass spectrometers, such efficiency may be maintained for long periods of time by performing routine electrospray maintenance [38].

Flow injection techniques have coverage and sensitivity that are equivalent to rapid chromatography-based approaches. Thousands of m/z signals were detected in yeast extracts with 400 being detected after intensive filtering [39]. Sévin et al. [40] discovered more than 1000 chemicals in E. coli by matching a substantially higher number of characteristics to theoretical m/z within 0.001 Da tolerance. It shows that state-of-the-art devices may provide deep coverage of important pathways in primary metabolism even at very high throughput. Samples with low quantity or wide chromatographic elution, which are difficult to distinguish in, for example, LC–MS, partially compensates for the loss of detectable characteristics induced by the removal of chromatography. Because there is less noise in peak integration, flow-injection analysis has a competitive repeatability [41]. Furthermore, due to the short cycle duration and high frequency of analysis, most signal, background, and noise drifts that are prevalent in electrospray mass spectrometry of complex materials may be detected and corrected efficiently [42].

2. Direct Infusion (DI): It is the technique where samples are directly injected mass spectrometer's ionisation source devoid of previous chromatographic separation is known as direct injection analysis. This method is frequently used in conjunction with atmospheric pressure ionisation methods. Direct infusion is an efficient method that may handle a sample in a matter of minutes. The rapid analysis time improves cluster analysis accuracy and inter-sample repeatability. ESI and nominal mass resolution mass analyzers were used for direct injection analyses. For metabolic fingerprinting in crude fungal extracts, Smedsgaard et al. 1997 employed ESI in positive mode and a single-stage quadruple apparatus. Castrillo et al. [43] used direct injection ESI in positive mode and triple quadruple MS to examine yeast intracellular metabolites.

3. MALDI-MS: MALDI, or matrix-assisted laser desorption/ionization, a mass spectrometry which may be used to examine biomolecules (proteins, peptides, and sugars) as well as bigger chemical compounds (polymers and dendrimers). These compounds are ionised using MALDI because they are delicate and will fragment if ionised using other, more traditional ionisation techniques. A matrix protects the biomolecule from harm while also allowing for optimal vaporisation and ionisation utilising a laser beam (Fig. 2). The most frequent three crystallised molecules employed in MALDI-MS are 3,5-dimethoxy-4-hydroxycinnamic acid, alpha-cyano-4-hydroxycinnamic acid, and 2,5-dihydroxybenzoic acid (DHB). To form a solution, purified water and an organic solvent like ethanol or acetonitrile are mixed together.

Liu et al. [44] tested Citrus Huanglongbing (HLB) infected citrus leaves caused by the phloem bacteria *Candidatus Liberibacter.* Since this disease is asymptomatic, its phenotypic detection is not easy. Thus, they developed the fast detection method by testing metabolite differences between healthy and unhealthy leaves using matrix-assisted laser desorption /ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The result showed the infected and noninfected leaves were distinguishable on the basis of MALDI-TOF-MS along with multivariate analysis. It was found that 32 MS peaks of metabolite showed the clear cut differences among healthy and unhealthy plants. Among these peaks, in 9 substances the upward trend was observed and for 19 metabolites, downward trend was observed which included a significant decrease in malate content of infected leave regardless the symptom appeared or not. Thus, regulation pattern of metabolites can be used as biomarkers for early detection of infected plant.

3. Ambient mass spectrometry: Ambient ionization/sampling mass spectrometry (ambient mass spectrometry) is a kind of mass spectrometry that allows for high-throughput surface examination of native materials. Direct analysis in real time (DART) and desorption electrospray ionisation (DESI), two flagship ambient mass spectrometry methods have not only enabled previously impossible experiments, but have also been surrounded by a plethora of other techniques, each with their own advantages and applications. This chapter discusses the kind of studies that are at the heart of ambient mass spectrometry and includes a number of specific examples to help readers unfamiliar with the topic have a better understanding of it.

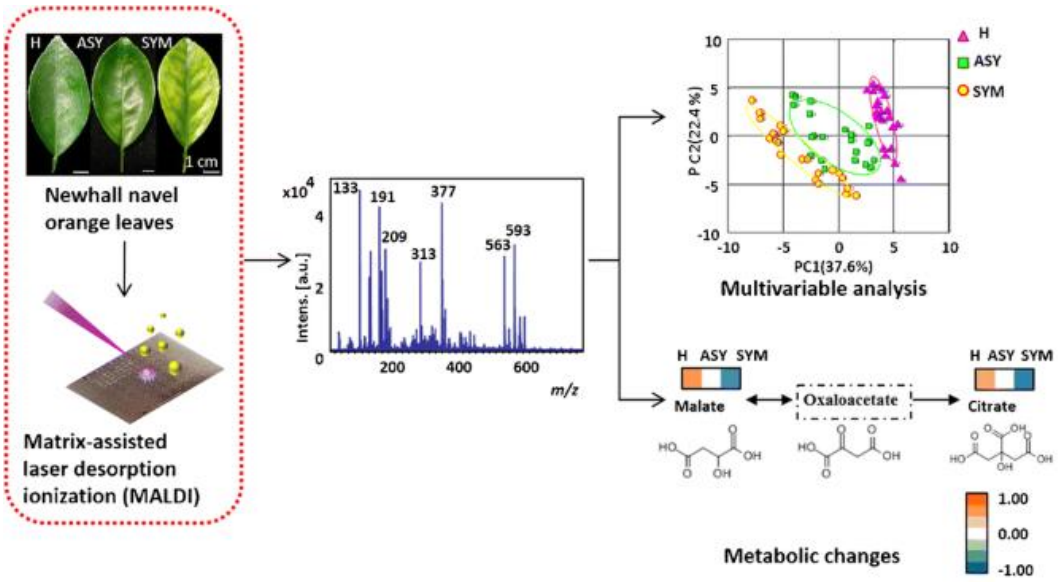

Fig. 2. MALDI analysis result by Youangquan et al. 2020

DART: DART-MS (direct analysis in real time mass spectrometry) is a well-known method for quickly analysing mass spectra of a wide range of compounds. DARTMS can analyse a sample under atmospheric pressure, allowing it to be analysed in an open lab environment. Chemicals that have been deposited or adsorbed on surfaces, as well as substances that are being desorbed into the environment, may be analysed using DART MS. This makes DART-MS excellent for forensic and safety applications, such as screening for explosives, warfare chemicals, or illicit compounds on baggage, clothes, or bank notes, and so on. DART may also be used to quickly analyse solid or liquid bulk materials for quality control. DART-MS can also be utilised on live animals. The DART ionisation source and interface may be customised in a variety of geometries and with a variety of accessories to alter the setup as needed, depending on the needs of analytical practise. DART-MS analyses data using a gas-phase ionisation method. In DART, the ionising species are initially created via a corona discharge in a pure helium environment, which produces excited helium atoms that, when released into the atmosphere, initiate a cascade of gas-phase processes. Finally, reagent ions generated at the surface from atmospheric water or (solvent) vapour are analysed, causing a chemical ionisation process.

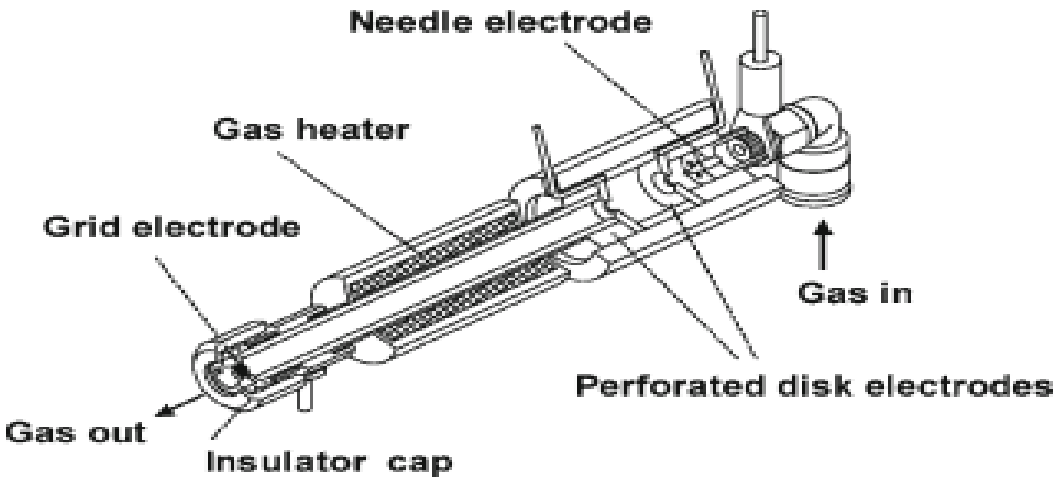

Fig. 3. DART ionization source (Cody et al. 2005)

Table 1. Available metabolome databases (Source: Han et al. [50])

Name	Content
Compound and species specific Databases	
PubChem	A collection of chemical structure and biological properties of small molecules
HMDB	A comprehensive data of more than 6800 human metabolites
LIPID MAPS	Structure and annotation of more than 9000 lipids
KNApSAcK	A cross-species metabolite database with around 28500 chemicals and over 1000 mass numbers, largely from plants and microorganisms.
Reference spectral Library	
NIST08	There are 14,802 positive ion spectra and 1410 negative ion spectra in a commercial collection of 2,20000 EI mass spectra from more than 190,000 pure chemical substances.
GMD	GC-TOF-MS analysis of mass spectra and retention indices of known plant metabolites
METLIN	A database of LC-MS mass spectra of metabolites from human and microbial species
MassBank	A high resolution MS/MS database standard chemical substances
MS2T	A MS/MS spectral tag library of phytochemical compounds
MMCD	A NMR and LC-MS spectral library of metabolite standard
Pathway specific Databases	
KEGG	Pathway maps, genes, species, enzymes, and ligands are all part of this composite database.
BioCyc	A collection of 506 pathway/genome databases, as well as a database of metabolite chemicals, for species having fully or partly sequenced geno
EcoCyc	A scientific database from E.coliK-12 MG1655
MetaCyc	A database of experimentally identified nonredundant metabolite path

DESI: Traditional electrospray ionisation (ESI) is the source of DESI [45,46]. To create an electrically charged aerosol, DESI uses a compressed air regulated electrospray of a solvent having low concentration of an electrolyte [47]. In contrast to ESI, the spray capillary in DESI is pushed back from the API interface's aperture, and the aerosol (devoid of analyte species) is directed at a 45° angle toward the item to be examined. Sprayer, object, and aperture together forms a compact V-alignment. The highly charged aerosol droplets are then propelled by the intense pneumatic spray onto the surface of the item to be studied, resulting in a thin liquid coating covering the surface. DESI makes a surface "moist." The solvent will be able to remove analytes from the item in this manner. The API interface samples having charged droplets released from the surface by the combined effect of the persistent aerosol stream and the electrostatic charging of the surface. The same mechanisms as in ESI are used to liberate isolated gas phase ions. DESI, like ESI, can analyse substances in the ultra-low to ultra-high mass range while keeping the molecules intact (Watson et al.2002).

2. CONCLUSION

Various model species have been used to achieve a substantial percentage of plant metabolomics success. However, improving the applicability of metabolomics in agricultural species has a lot of demands, possibilities, and obstacles. Metabolomics, has been effectively employed to improve the breeding of vital crops. The identification of genomic areas and genes linked with metabolic quantitative loci (mQTL) or the production of specialised metabolites has been done with key crops using current genetic resources as a foundation for future breeding initiatives. With multiple examples, metabolomics has considerably increased our understanding of plant specialised metabolism and natural product production at the molecular and biochemical levels. Metabolomics is also allowing researchers to gain a better knowledge of medicinal plants and to identify key metabolic QTLs for improved

breeding. Despite the fact that metabolomics has demonstrated its worth, it still confronts significant hurdles, such as large-scale metabolite identifications. The discipline of metabolomics can only improve as improved technology continue to advance. This shows that metabolomics has a bright future ahead of it [49-58].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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