Chapter 5
Plant Metabolites Involved in Plant–Pathogen Interactions

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Abstract Plants constantly confront different pathogens and undergo stress. To overcome such hurdles, plants produce primary and secondary metabolites. Primary metabolites are essential for the growth and development of plant and secondary metabolites are vital for plant survival by providing resistance against various pathogens and maintaining an elegant stability with the environment. Plants produce a huge number of metabolites, and many of such metabolites have yet to be identified. For the analysis of these wide range of highly complex metabolites synthesized by the plants, various tools and techniques are required for the study of metabolomics. Study of plant metabolomics comprises of sample preparation or extraction of bioactive molecules from the plants, detection and identification of the metabolites, and data processing and statistical analysis of the identified metabolites. Modern technologies used for the study of plant metabolomics includes metabolic fingerprinting, metabolite profiling and targeted and non-targeted detection analysis. Starting with the definition of primary and secondary metabolites, we aimed to focus on the behavior of different metabolites during plant–pathogen interaction and to finally concentrate on different tools and techniques, which are required for the identification and analysis of metabolites. With the help of current high-resolution mass spectrometers it has become quite feasible to identify low-molecular-mass metabolites. Efforts are made to develop computational tools for the identification of unknown metabolites and to develop mass spectral databases which will provide an authentic reference for the identified compounds.

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5.1 Introduction

Under natural conditions, plants are surrounded by many probable enemies. To defend against pathogen bout, plants create defense strategies mainly through chemical and mechanical defenses (Olivoto et al. 2017). The former includes structures such as trichrome, thick cuticle, spines, and smooth, sticky, or hard surfaces, which avert pathogens from laying eggs or food selection. Chemical defenses comprise a variety of constituents which are repellent, toxic, or which make plant tissues difficult to digest for animals. These chemical substances retaliate against abiotic or biotic stimuli, traditionally referred to as secondary metabolites which play a major role in plant defense mechanism (Goyal et al. 2012).

Plants are an unlimited source of phytochemicals in the form of primary and secondary metabolites. However, secondary metabolites corrugate leading interest because of their multifunctional activities extending from antimicrobial, insecticidal, antibiotic properties, to extremely important pharmaceutical activities (Stöckigt et al. 1995). Studies on the functions of these compounds for plant defense has increased in the last two decades (Rhodes et al. 1994).

5.2 Primary Metabolites

Primary metabolites are limited molecules derived from living cells. Primary metabolites are the intermediary or final products of the metabolic pathways of transitional metabolism (e.g., lipid metabolism, amino acid metabolism, carbohydrate metabolism), and these are the composition units for vital macromolecules, or can be altered into coenzymes (Demain 2000). Primary metabolites such as organic acids, phytosterols, amino acids, nucleotides, and acyl lipids are biomolecules required to perform basic metabolic processes. These are found throughout the plant kingdom, required for basal metabolic roles that are usually noticeable and are highly useful to plants. These are produced in plentiful masses and can be effortlessly extracted from different species of plants. Primary metabolites are a portion of a cell's basic molecular structure (Croteau et al. 2000).

Primary metabolites are concentrated in vegetative storage organs and seeds in higher plants and are required for physiological development to perform basic cell metabolism. Generally, primary metabolites are obtained for commercial use, which is high volume–low value bulk chemicals. However, there are exemptions to this rule. For example, β-carotene and myoinositol are exclusive primary metabolites as their extraction, isolation, and purification are strenuous (Balandrin et al. 1985). Primary metabolites are mainly used in the food industry which incorporates fatty acids (utilized for manufacturing soaps and detergents), vegetable oils, flavor nucleotides (5′-inosinic acid, 5′-guanylic acid), alcohols (ethanol), polyols (mannitol, glycerol, erythritol, xylitol), amino acids (monosodium glutamate, lysine, threonine, phenylalanine, tryptophan), organic acids (acetic, propionic, succinic, fumaric, lactic), sugars

(fructose, sorbose, ribose), vitamins [biotin, riboflavin (B2), cyanocobalamin (B12)], and polysaccharides (xanthan, gellan) (Demain 2000).

5.3 Secondary Metabolites

Secondary metabolites are derivatives of primary metabolites, synthesized by plants in a diverse array. Secondary metabolites are well known to act as chemical defenses that avert pests and pathogens; they have a key role in controlling plant growth and protecting plants from environmental pressures (Fraire-Velázquez and Balderas-Hernández 2013). They do not comprise basic molecular cell structure. These metabolites are produced in lesser amounts and their extraction from plants is tough. Classes of secondary metabolites are restricted to selected plant species or families; they are found at particular stages of development, with a limited role in the plant (Osbourn et al. 2003).

Plants collectively produce natural products of above 100,000 low molecular mass, i.e., secondary metabolites. These metabolites can be distinguished from the constituents of intermediary (primary) metabolic products in that they are generally nonessential for the chief metabolic processes of the plant. Most of them are derived from the phenylpropanoid, alkaloid or fatty acid/polyketide, and isoprenoid pathways. Such a rich diversity has made it difficult to apply conventional molecular and genetic techniques to know the functions of natural products, also to select the genotypes for improved defense against microbial infection or insect/animal predation in plant defense, or to increase plant disease resistance by using metabolic pathway engineering (Dixon 2001).

5.3.1 Major Classes of Secondary Metabolites

Secondary metabolites can be divided into two different chemical groups: nitrogen-containing compounds and nitrogen-free compounds.

5.3.1.1 Nitrogen-Containing Secondary Metabolites

Nitrogen-containing compounds include alkaloids, nonprotein amino acids, amines, glucosinolates, cyanogenic glycosides, protease inhibitors, and lectins.

5.3.1.1.1 Alkaloids

Alkaloids are biologically active compounds which contain a ring structure or a heterocyclic compound with a nitrogen atom connected to minimum two carbon

atoms and have some role in biological, ecological, pharmacological, chemical, and medicinal activity. Alkaloids are special compounds which participate in various biological processes of microorganisms, plants, and animals at different cellular levels in altered environments (Aniszewski 2015). Alkaloids are classified depending upon their physical and biological attributes to help their morphological identification and understand their technical and applied uses. Alkaloids can be classified according to their chemical structure, ecological, and biological action; biosynthetic pathway; and relation with chemical and technological innovations.

According to biological activity, alkaloids are cleaved into neutral or weakly basic molecules (e.g., indicine, ricinine), animal-derived alkaloids (e.g., arthropod, anuran, and mammalian alkaloids), moss alkaloids, nonnatural alkaloids, marine alkaloids, and fungal and bacterial alkaloids (Pelletier 1983). Nonnatural alkaloids are a part of bioorganic and stereochemistry research, which are used in pharmacological research. Due to constant change in the species of the pathogens and their infection ability, it has become necessary for plants to become resistant to medicines and antibiotics.

Based on their relationship in the branches of chemistry and technology, alkaloids can be divided into three groups: (1) natural, (2) biomimic and bionic, and (3) synthetic. Natural alkaloids are synthesized by living organisms and are naturally synthesizing molecules which exist in nature because of the progression of life on Earth. Biomimetic alkaloids are structurally identical to natural alkaloids and are copied artificially in laboratories. Bionic alkaloids are those biomimetic compounds which are synthesized artificially but are not similar analogues to natural alkaloids. Synthetic alkaloids are molecules which are artificially synthesized using high-level techniques and planned models, having the chemical characteristics of alkaloids.

Based on their chemical structures at alkaloid base, it can be divided into various types: bisindoles, indolizidines, carbolines, purines, pyrolidines, pyrrolizidines, steroids, terpenoids, diterpenes, triterpenes, pyridines, quinolozidines, quinolones, and quinolizolines (Eftekhari-Sis et al. 2013). Based on shape, structure, and the biological pathway used to create the molecules, alkaloids can be of three main type: true alkaloids, protoalkaloids, and pseudoalkaloids (Hegnauer 1988). True alkaloids are derived from amino acid with nitrogen in their heterocyclic ring. These are extremely reactive compounds with biological activities even in low doses. Except nicotine, all true alkaloids are bitter in taste. The primary precursors of true alkaloids are L-tryptophan, L-tyrosine/phenylalanine, L-ornithine, L-lysine, and L-histidine. These alkaloids can be natural, bionic, or synthetic, and some examples of true alkaloids are dopamine, cocaine, quinine, and morphine. In protoalkaloids, the *N*-atom acquired from an amino acid is not part of the heterocyclic ring. These compounds are derived from L-typtophan and L-tyrosine. These can be bionic, natural, or synthetic alkaloids, and mescaline, hordenine, and yohimbine are some examples. Pseudoalkaloids are not derived from amino acids, but from the precursors of amino acids from the amination and transamination reactions. They can also be obtained from non-amino acid precursors (Aniszewski 2015).

5.3.1.1.2 Amines

Amines are ammonia derivatives where one, two, or all three hydrogens of ammonia are replaced by organic groups. They play a significant metabolic and physiologic role in living organisms. Biologically active amines are cyclic, heterocyclic, and aliphatic and most of them are named after their precursor amino acids, e.g., tryptamine from tryptophan, tyramine from tyrosine. Bioactive or biologically active amines can be classified based on the number of amine groups as mono- (phenylethylamine, tyramine), di- (cadaverine, histamine, tryptamine, serotonin, putrescine), or polyamines (spermine, agmatine, spermidine). According to their chemical structure, amines can be aromatic (phenylethylamine, tyramine), aliphatic (putrescine, spermine, spermidine, cadaverine, agmatine), or heterocyclic (histamine, serotonin, tryptamine). Based on their biosynthetic pathway, they are classified as natural or biogenic (Glória 2005).

5.3.1.1.3 Nonprotein Amino Acids (NPAAs)

In nature, more than thousands of nonprotein amino acids are extracted from microorganisms, plants, and other sources (Barrett 2012). These amino acids are not formed in the main chains of protein, but some times they do get added in protein by post-translational modification. For these amino acids an exact transfer RNA and codon triplet is absent (Hunt 1985). Many nonprotein amino acids are considered as structural analogs of protein amino acids. For example, S-aminoethylcysteine is an analog to L-azetidine-2-carboxylic acid to L-proline, L-lysine, 3-cyanoalanine to L-alanine and L-indospicine, or L-canavanine to L-arginine (Wink 2003).

5.3.1.1.4 Cyanogenic Glycosides (CNglcs)

CNglcs are the source of HCN that occur extensively in the plant kingdom (Conn 1969) and are specialized bioactive plant products derived from amino acids characterized by α-hydroxynitriles (cyanohydrins) and oximes as key intermediates. Cyanogenic glycosides release ketones and toxic hydrogen cyanide (HCN) when hydrolyzed by α-hydroxynitrilases and β-glycosidases in a process referred to as cyanogenesis. Cyanogenesis is an effective defense against herbivores but is not effective against fungal pathogens because many fungi convert HCN into carbon dioxide and ammonia (Gleadow and Møller 2014).

5.3.1.1.5 Glucosinolates (GSLs)

Glucosinolates (GSLs), the precursors of isothiocyanates, are organic anions containing β-thioglucoside-*N*-hydroxysulfates, which represents an important and unique class of secondary metabolites found in seeds, roots, stem, and leaves of

plants (mainly in the Brassicaceae) (Fahey et al. 2001; Vig et al. 2009). Glucosinolates on hydrolyzation liberate D-glucose, sulfate, and an unstable aglycone, which converts to isothiocyanate (Mithen et al. 2000). There are more than 120 diverse glucosinolates identified till date, mainly belonging to the family Brassicaceae and other important crops. Glucosinolates represent a classical example of plant compounds which affect the plant–insect interactions (Hopkins et al. 2009). The defense activity of glucosinolates are increased upon hydrolysis by the enzyme myrosinase. In plants, myrosinase is stored in special myrosinase cells. Myrosinase is a thioglucosidase that transforms glucosinolates into toxic isothiocyanates (Rask et al. 2000). In damaged plant tissues, due to the myrosinase activity, the glucosinolates stowed in the vacuole come in contact with the myrosinase and result in the formation of various toxic products, such as nitriles, isothiocyanates, and oxazolidinethiones (Bones and Rossiter 2006).

5.3.1.2 Nitrogen-Free Secondary Metabolites

Nitrogen-free compounds are various terpenoids (mono-, di-, tri-, and tetraterpenes; saponins; and cardiac glycosides), polyketides (anthraquinones), polyacetylenes, and phenolics (phenolics acids, flavonoids, catechol tannins, anthocyanins, lignans, galloyl and lignins).

5.3.1.2.1 Terpenoids

Terpenoids are abundant in plants with more than 30,000 compounds (Aharoni et al. 2006). Among the myriad bioactive compounds produced by plants, terpenoids (isoprenoids) epitomize the largest and most varied group of chemicals. A majority of plant terpenoids are utilized for specialized chemical interactions and defense in abiotic and biotic stress environments (Tholl 2015). They play substantial roles in nature during plant–plant, plant–environment, plant–insect, and plant–animal interactions (Pichersky and Gershenzon 2002).

5.3.1.2.2 Phenolics

Plants phenolic compounds emerge as one of the main categories of secondary metabolites and are essential for the growth, development, resistance to pathogens, pigmentation, reproduction, and for many other functions in plants (Lattanzio et al. 2006). As stated by Harborne, the term “phenolic” embraces plant substances with an aromatic ring bearing one (phenol) or more (polyphenol) hydroxyl substituents in common (Harborne 1989). Phenolic substances are water soluble as they frequently occur homogenized with sugar as glycosides and are commonly located in the cell vacuole. Flavonoids form the largest group among the natural phenolic compounds whereas simple monocyclic phenols, phenolic quinones, and phenylpropanoids exist

in considerable amounts (Harborne 1984). The participation of phenolic compounds in the defense role of plant–animal and/or plant–microorganism interaction, is related to their antinutritional and antibiotic properties (Wink 1997). Flavonoids help plants to reside in soils rich in noxious metals, such as aluminum (Barcelo and Poschenrieder 2002). Several flavonoids oozing out from plant roots act as signaling molecules, which induce bacterial gene transcription and production of proteins are required for the infection method (Cooper 2004; Hungria and Stacey 1997; Kobayashi et al. 2004). Several flavonoids oozing out from plant roots function as signals, which induce bacterial gene transcription, and protein products are required for the infection process (Cooper 2004; Hungria and Stacey 1997; Kobayashi et al. 2004). Based on their defense role, flavonoids can be split into two groups, i.e., preformed and induced compounds. Preformed flavonoids are innate compounds that may play a signaling and/or a direct role in defense. These are synthesized during the regular development of plant tissue. Induced flavonoid compounds are produced by plants during physical injury, stress, or infection (Treutter 2006).

5.3.2 Responses of Secondary Metabolites During Biotic Stress in Plants

Plants, being sessile organisms, live in persistently changing environments which are often unfavorable or stressful for their growth and development (Zhu 2016). Adverse environmental conditions result in affected plant growth; metabolism is enormously involved in physiological regulation, signaling, and defense responses (Fraire-Velázquez and Balderas-Hernández 2013). Very often the wild-type species is resistant to microorganisms, and abiotic or biotic stress. To strengthen the resistance of a specialized species will be of no use. So, an addition of secondary metabolites to increase the resistance of a plant in which the species is adapted could be the solution (Wink 1988).

Higher plants often persuade the synthesis and hoarding of defense-related secondary metabolites upon biotic stress (e.g., herbivore or pathogen attack), referred to as phytoalexins (Mithöfer et al. 2004). Plants possessing these biochemical defense mechanisms help them to prevent or reduce further damage from pathogens (Eder and Cosio 1994). Plants, during infection or stress, accumulate phytoalexins which are low-molecular-weight antimicrobial compounds (Kuc 1995).

Defensive secondary compounds or metabolites become associated in response to both abiotic and biotic stress conditions (Akula and Ravishankar 2011). PhA (Phenylamides) play an important role in plant growth, development, and stress defense. PhA involved in plant defense have an antimicrobial activity that can protect plants against both abiotic and biotic stresses (Edreva et al. 2007). It is also reported that the active production of reactive oxygen species (ROS) in plants controls several different physiological processes, such as pathogen defense, abiotic and biotic stress response and systemic signaling. However, cells are provided with

outstanding antioxidant defensive machineries to detoxify the detrimental effects of ROS. These antioxidant defense mechanisms may be either nonenzymatic (e.g., carotenoids and flavonoids) or enzymatic (e.g., catalase glutathione peroxidase, superoxide dismutase) (Gill and Tuteja 2010).

5.3.3 *Behavior of Secondary Metabolites During Plant Defense Mechanisms*

5.3.3.1 Phenolic Compounds

Phenolic compounds are natural products which arise biogenetically from phenylalanine and tyrosine during shikimate, phenylpropanoids, and flavonoids pathways (Lattanzio et al. 2006). Tannins are a varied group of polyphenolics. Tannins may defend plant by reducing the digestibility of plants consumed by herbivores by binding with digestive enzymes, dietary proteins (Robbins et al. 1987). In 1970, it was reported by Feeny that tannins act as a defense compound to the Oak moth (*Opheropthera brumata*) larvae. He found that tannin level in the leaves increased significantly prior to cessation of feeding by the larvae. This limitation was assumed to be because of the reaction of tannins with digestive enzymes in the gut of the larvae and the complexation of tannins with host proteins (Feeny 1970). High tannin-containing 'bird-resistant' cultivars (sorghum plants) have also been recognized, which were compared to cultivars with low tannin which were severely damaged in field trials (Bennett and Wallsgrove 1994). Slugs are one of the major pests which attack several economically essential crops. *Deroceras reticulatum* is amongst the major slug pests of potato which leads to extensive crop damage. Some potato cultivars were found considerably less attractive to slugs owing to high levels of phenolics and polyphenoloxidase activities (Bennett and Wallsgrove 1994). Plants with high level of phenolics are very less palatable to herbivores, and polyphenolics like tannins are considered common antifeedants (Fahey and Jung 1989). More often, phenolics are noxious towards the fungal pathogens in vitro and are accumulated near the infection sites which lead to lignin deposition, necrosis, and resistance (Moran 1998). It was also reported that in sweet potato cultivars resistant to *Meloidogyne incognita* (root-knot nematode), the concentrations of soluble and wall-bound phenolics significantly increased after infection (Gapasin et al. 1988). It is well known that Rhizobia species utilizes phenolic acids as a carbon source (Irisarri et al. 1996). Plant phenolic compounds behave as potential candidates as signaling molecules in the establishment of arbuscular mycorrhizal symbioses, initiation of legume rhizobia symbioses, and can also act as a means in plant defense processes. Flavonoids are multifarious of polyphenolic compounds, act as signaling molecules in plant–microbe interactions. Flavonoids are released from different zones of the leguminous plant roots (Mandal et al. 2010). Flavonoids (luteolin) bind at the active transcriptional sites of Rhizobial *nod* genes that control root nodule organogenesis. This induction of the rhizobial *nod* genes leads to the production of

Nod (nodule-inducing) factors, lipochitooligosaccharides (LCOs), which are modified differently depending on the Rhizobium species (Schultze and Kondorosi 1998). Many phenolics and alkaloids released from roots or seeds function primarily as defense elements against soil-borne pathogens and root-feeding insects (Ndakidemi and Dakora 2003). Rapid accumulation of phenolic acids, mostly cinnamic, tannic, gallic and ferulic acids revealed the effectiveness of Rhizobia in inducing resistance in rice plants against the nectrotrophic soil-borne fungus *R. solani* (Mishra et al. 2006). Gallic acids provide antimicrobial activity (Binutu and Cordell 2000). Gallotannins are a derivative of gallic acid which get converted during accumulation and provide defense to plants against bacteria and fungi (Singh et al. 2002). Cinnamic and ferulic acids ascended from the shikimic acid pathway and are reported to be antioxidant and antifungal, respectively (Madhavi et al. 1997). Cinnamic acid serves as a precursor for the production of ferulic acid and is a key product of the phenylpropanoid pathway, which plays a crucial role in providing host resistance during pathogenic stress (Singh and Prithiviraj 1997).

5.3.3.2 Alkaloids

The majority of alkaloids are regarded as derivatives of certain amino acids, e.g., tryptophan, lysine, ornithine, phenylalanine, and tyrosine. Precursors of terpenoid and steroid are incorporated into the carbon skeletons of alkaloids. Till now, around 3000 different phytoalkaloids are known, which occur dissolved as cations in plant sap. They are mostly accumulated in the peripheral region of leaves, bark, or fruit, which can be shed (Levinson 1976). Common alkaloids can be found in the Liliaceae, the Leguminosae, the Amaryllidaceae, and the Solanaceae plant families which can be important resistance factors against herbivorous pests (Petterson et al. 1991). Because of their general toxicity and deterrence capability, alkaloids are believed to be key defensive elements against predators, especially mammals (Hartmann 1991; Robinson 1980). Death of a large number of animals in USA is reported due to the ingestion of plants containing alkaloids. A large number of grazing livestock is infected by consumption of alkaloids-containing plants such as lupines (*Lupinus*) and larkspur (*Delphinium*) (Keeler 1975).

5.4 Extraction and Isolation of Bioactive Compounds from Plant Extracts

It is an ancient thought that plant extracts have great healing power, and these phytocompounds have recently attracted interest because of their versatile applications (Bariş et al. 2006). Plant species contain various metabolites. However, only a small percentage of these phytochemicals have been investigated around the globe (Hostettmann and Wolfender 1997). Use of chemical pesticides induces

environmental hazards in agricultural systems. So, the use of phytocompounds as an antimicrobial agent can be the best biorational alternative today (Tiwari et al. 2007). As large number of plant species are available for future studies, it is vital to have effective methods to evaluate the efficacy of plant origin antimicrobial agent and the identification of biologically active principles involved in it (Tanaka et al. 2006). Plants contain a number of metabolites which expose only a very narrow range of their constituents. Thus, the potential of higher plants is still largely unexplored as sources of new drugs (Hamburger and Hostettmann 1991). The selection of plant species for screening for biologically active constituents is a crucial factor in the investigation.

During searching for active phytocompounds, it is essential to verify the plant varieties for the success of the study. Targeted grouping of plant material is based on considering chemotaxonomic interaction and utilization of current ethnomedical information. The use of different technologies has allowed rich isolation of various fungicidal, larvicidal, and molluscicidal products (Hostettmann and Wolfender 1997).

Thousands of bioactive compounds from plants are found to be safe and have less adverse effects due to their beneficial biological activity, e.g., antimicrobial, antioxidant, and wound-healing activity. The leading steps to exploit the phytochemicals from plant resources include extraction, isolation, screening, identification, and characterization of bioactive compounds. Plant extracts contains multicomponent mixtures of bioactive compounds with different polarities, which still poses problems in identifying and characterizing phytochemicals and their separation. Purification of phytochemicals most commonly includes member of chromatographic techniques and other different purification methods to identify phytochemicals (Sasidharan et al. 2011). To extract the desired phytochemical from a plant, sample preparation is a crucial leading step in the analysis of plant or herbs for further separation and characterization of those isolated bioactive compounds (Huie 2002).

5.5 Metabolomics Tools and Their Application in Plants and Plant–Host Interactions

Metabolomics is now a briskly developing technology. With the help of specialized bioinformatics tools and data mining tools, metabolomics, like transcriptomics and proteomics, generates a huge amount of data. Metabolomics tools can possibly lead to identifying many of the compounds in plants undergoing stress (Shulaev et al. 2008). Current methodologies used in plant metabolomics comprise metabolic fingerprinting, metabolite profiling, and targeted and nontargeted detection analysis (Halket et al. 2004; Bajad and Shulaev 2007; Fiehn 2002); these are described below.

5.5.1 Metabolic Profiling and Fingerprinting

Research in metabolomics includes metabolite profiling and fingerprinting approaches. "Metabolic profiling" was coined during the 1970s (Horning and Horning 1971), and it is used to identify and quantify metabolites associated with their certain metabolic pathways or similarities in their compound classes. It involves common chromatographic separation techniques like liquid chromatography coupled with MS (LC-MS) or gas chromatography coupled with MS (GC-MS) to detect, quantify, and, if at all possible, identify the metabolites in an extract. In metabolite fingerprinting, metabolite profiles are obtained from simple cellular extracts or crude samples through rapid and high-throughput methods. Metabolite fingerprinting involves techniques like NMR (nuclear magnetic resonance spectroscopy) (Krishnan et al. 2004), Raman spectroscopy, Fourier-transform infrared spectroscopy (FTIR) (Johnson et al. 2003), MS (Goodacre et al. 2003), and electrospray ionization (ESI)-MS to detect all the metabolites present in a sample irrespective of their identification (Allwood et al. 2008).

Liquid chromatography mass spectrometry (LC-MS) is preferred for the analysis of flavonoids, phenylpropanoids, and alkaloids. Using GC-MS, fatty acids were found as the key component to provide resistance to gall midge rice varieties (Agarrwal et al. 2014). By using liquid chromatography tandem mass spectrometry (LC-MS/MS), the identification and quantification of more than 90 flavonoids were reported. It also studies the occurrence of their biosynthesis in various rice tissues during different developmental stages (Dong et al. 2014). As compared with the common cultivars, it was found that tomatoes contain 70-fold higher flavonoids by using LC/photodiode array detection along with liquid chromatography, quadrupole time of flight mass spectrometer (LC-QTOF-MS), and direct infusion QTOF-MS (Hall et al. 2002).

Capillary electrophoresis–mass spectrometry (CE-MS) as well as liquid chromatography–mass spectrometry (LC-MS) offers a better alternative for nonvolatile compounds. Capillary electrophoresis–mass spectrometry (CE-MS) has now come into view as a powerful tool for the analysis of charged molecules. CE-MS provides separation of metabolites based on charge and size and then it is detected using MS by observing over a large range of m/z values. CE-MS provides very high resolution, and nearly any charged species can be able to infuse into MS (Soga et al. 2003). The application of LC-MS in metabolomics is gradually growing after its recent acceptance of the ultra-performance liquid chromatography technology, which helps it to increase the efficiency of separation and decreases the analysis time of metabolites (Giri et al. 2007; Granger et al. 2007).

GC-MS is biased in contrast to nonvolatile high molecular weight metabolites and is functional towards polar nonvolatile metabolites (e.g., organic acids, amino acids, and sugars) that are volatile up to 250 °C through chemical derivatization (Allwood et al. 2008). In 2000, Roessner et al. (2000) described two stages of derivatization process for the analysis of plant extracts using GC-MS. Firstly, O-alkylhydroxylamine transforms the carbonyl group of the sample to oximes for

thermal stabilization and then it is treated with a silylating compound, e.g., *N*-methyl-*N*-(trimethylsilyl) trifluoro-acetamide, which leads to the formation of volatile trimethyl-silyl esters (Roessner et al. 2000). Electron impact (EI), facilitating GC-MS ion formation, provides independent exclusion of the sample's solvent before vaporized sample being cleared into the ionization source which allows steady electron flow and thus ionizes the vaporized molecule (Gross 2006). Detection of mass is conducted by QTOF-MS or ion-trap-based mass analyzers. A single QTOF-mass analyzer requires an hour of chromatographic time to provide a standard separation of a complex metabolite (Dunn and Ellis 2005). Metabolites can be identified by tandem MS (MS-MS) which is responsible for metabolite fragmentation through collision with an inert gas like argon and causes collision-induced dissociation (Wysocki et al. 2005).

NMR (nuclear magnetic resonance) spectroscopy is a nondestructive technique which requires least sample preparation, and is presently also considered high throughput (hundreds of samples per day). NMR uses nuclei with odd mass or atomic numbers, which behave like magnets and intercommunicate with an external magnetic field by a method called nuclear spin (Kitayama and Hatada 2013). In particular, ^{1}H NMR has been extensively used for metabolites profiling in clinical samples (Holmes et al. 2000; Nicholson and Wilson 1989) and also has been functional towards complex compounds exuded out from the roots of cereals (Fan et al. 2001). Unlike GC-MS, which senses only volatilized compounds, ^{1}H NMR can instantaneously detect all compounds bearing proton in a sample. It covers mostly organic compounds, such as ethers, amino acids, fatty acids, carbohydrates, amines, and lipids esters, present in plant tissues. ^{1}H-NMR provide a nonbiased fingerprint in contrast with other metabolomics approaches, and therefore NMR is now evolving as one of the standard metabolic profiling platforms (Ward et al. 2003).

5.5.2 Targeted and Nontargeted Detection Analysis

Separation methods of numerous analytes from a particular sample have now been established. However, for effective application of such methods, it needs detectors which is accomplished of fast data-acquisition rates along with high specificity and sensitivity. Two major means of MS-based metabolomics are targeted and nontargeted detection analysis. Analytes in the targeted detection mode are predetermined, having a definite mass filter allotted to a specific analyte (Bajad and Shulaev 2007). Standard methods have been developed to identify specific members of a compound class while ignoring others. For example, polyamines thought to be involved in various important plant processes, e.g., drought stress, and quantification of polyamines in different plant species in response to various stimuli or environmental conditions has been developed (Bouchereau et al. 2000). Targeted analysis also results in comparative metabolite profiling of a huge number of identified metabolites. SRM (Selected reaction monitoring) provides high meticulosity and has been successfully used to quantitate a number of analytes at the same

time. For example, SRM can analyze more than 100 metabolites in a single chromatographic run, based on highly parallel targeted assays (Bajad and Shulaev 2007; Bajad et al. 2006). Target analysis will continue to be the most wide-spread system in different areas of biological research. However, in case of functional genomics studies it has restricted use, because the levels of target analytes may be changed by unexpected reasons which cannot be understood without comprehensive approaches. Thus, a wider analysis of metabolic modifications is required to limit overinterpretation of data (Fiehn 2001).

The problem with targeted detection is that it is not an actual beneficial approach as it is not quite practical to assemble SRMs for all molecules of interest present in the sample containing numerous analytes. Thus, it cannot detect analytes with no SRMs. Also, it is difficult to predict the compositions of the sample. Therefore, in such cases, nontargeted detection analysis is used to cover a broad array of analytes and used to detect and find unknown or novel molecules (Hong et al. 2003; Tohge et al. 2005).

In nontargeted detection analysis, to detect common molecules of specific molecular mass range, scanning of mass spectrometer is done over a set *m*/*z* 100–1000 in both positive and negative ionization modes. For comparative profiling, full-scan mode acquired data along with low-resolution instruments are most commonly used. Data obtained in full-scan mode along with automated MS to MS/MS switching provide added information about the elemental composition and arrangement of fragments of the analytes, as well as the unknown components by using an accurate mass instrument (e.g., Fourier transform MS or qTOF). Obtained data are then subjected to library (e.g., the NIST library) search to identify the unknown compounds (Bobeldijk et al. 2001). Nontargeted analysis provides an unbiased detection method of the chemical nature of the sample which results in a holistic approach to detect and identify unexpected or unknown metabolites which can be important in environmental and pharmaceutical analysis (Bajad and Shulaev 2007). There are some examples of a nontargeted analysis in pharmaceutical and environmental analysis. Ibanez et al., using SPE-LC/QTOF-MS along with data processing, identified six unknown compounds from environmental waters (Ibáñez et al. 2005). A successful study of drug metabolites in pharmaceutical has also been outlined using application of nontargeted analysis (Idborg et al. 2004).

5.5.3 Data Processing and Analysis

Regardless of any analytical technique used, data analysis forms an essential part. The raw data must be preprocessed to transform them to a readable format. The modified data can be subjected to data reduction to facilitate the use of only appropriate input variables in the succeeding data analysis (Brown et al. 2005). Before analyzing data from most analytical instruments statistically, consequential preprocessing is required, and standardization of techniques is necessary. A lot of researchers put emphasis on requirement of post-sampling techniques such as

deconvolution, noise reduction, internal standards reference, alignment of profile, and labeling of peak by using spectral libraries (Hall et al. 2002).

Large volumes of data produced from metabolome analysis are analyzed by the instruments to detect small signals with high resolution. For this metabolome analysis, programmed software is required to detect peaks of raw NMR or MS data to arrange the peaks in order amongst samples and to detect and measure the quantity of each metabolite (Fukushima et al. 2009; Fernie and Schauer 2009; Go 2010). Numerous statistical methods are used for metabolomics data. PCA (principal component analysis) is a multivariate analysis usually used in the study of metabolomics. PCA provides an outline of every sample or interpretation in a data and highlights the variance between the complex metabolites in each sample (Catchpole et al. 2005; Baker et al. 2006; Dixon 2003; Kim et al. 2006; Ku et al. 2009b). In addition to this, other statistical analytical methods are used to analyze metabolomic datasets, which are PLS-DA (partial least squares discriminant analysis) (Jonsson et al. 2004; Ku et al. 2009a; Kusano et al. 2007), HCA (hierarchical cluster analysis) (Grata et al. 2007; Parveen et al. 2007), and BL-SOM (batch-learning self-organizing map analysis) (Hirai et al. 2004).

Bioinformatics is the key supporter to gather information and make sense of the data. Currently, in the field of metabolomics, bioinformatics mainly focuses on the metabolic pathway simulation and construction of models (Fiehn et al. 2001). Schuster et al. (2000) tried to shed new light on the concept of identifying possible metabolic pathway leading to a given element. Databases like the Kyoto Encyclopedia of Genes and Genomes (KEGG) can serve as a model in providing information on the combination of simulated pathway with pathway databases. KEGG provides knowledge of systematic analysis of functions of gene based on the networks of genes and molecules. It develops and provides several computational tools for reforming biochemical pathways from a complete genomic sequence and predicting the regulatory networks of gene from the profiles of gene expression. The KEGG databases are updated on a daily basis and are available without restrictions (http://www.genome.ad.jp/kegg/) (Ogata et al. 1999).

Data handling and analysis of metabolomics using "omic" technologies have been improved dramatically in recent years. They help in the detection of specific metabolites in a biological sample in a nonbiased and nontargeted way. Compared to omics technology applied for the study of genomics, transcriptomics, and proteomics, metabolomics has numerous theoretical advantages over the other omics approaches (Horgan and Kenny 2011). The functional genomics databases include DOME (http://medicago.vbi.vt.edu), MetNetDB (http://www.metnetdb.org/MetNet_db.htm), data model for plant metabolomics research ArMet (http://www.armet.org/) (Shulaev et al. 2008), and pathway databases and pathway viewers like KEGG (http://www.genome.ad.jp/kegg/), KaPPA-View (http://kpv.kazusa.or.jp/kappa-view/) (Tokimatsu et al. 2005), MetaCyc (http://metacyc.org/) (Caspi et al. 2006), AraCyc (http://www.Arabidopsis.org/tools/aracyc/) (Zhang et al. 2005), BioCyc (http://biocyc.org) (Paley and Karp 2006), MapMan (http://gabi.rzpd.de/projects/MapMan/) (Thimm et al. 2004), BioPathAT (http://www.ibc.wsu.edu/research/lange/public%5Ffolder/) (Lange and Ghassemian 2005), and the Atomic Reconstruction of Metabolism database (http://www.metabolome.jp/) (Yamazaki et al. 2004).

Some of the selected open-access bioinformatics tools for multifarious LC-MS data analysis applied to environmental and pharmaceutical analysis are BL-SOM (http://prime.psc.riken.jp/?action=blsom_index) (Kanaya et al. 2001), MZmine (http://mzmine.sourceforge.net/) (Katajamaa and Orešič 2005), XCMS (http://metlin.scripps.edu/download/) (Smith 2013), MSFACTs (http://noble.org/) (Duran et al. 2003), MeMo (http://dbkgroup.org/memo/) (Spasić et al. 2006), and MET-IDEA (http://noble.org/) (Broeckling et al. 2006).

LC-MS is broadly used in both proteomics and metabolomics. Integrated non-target metabolomics (LC-MS/MS) and proteomics (2D gel electrophoresis) have been applied in wheat genotype Nyubai. Gunnaiah et al. (2012) found that Fusarium head blight resistance locus, *Fhb1*, provides resistance against the spread of *F. graminearum* within the spikes. The involvement of *Fhb1* in providing resistance in wheat, in response to *F. graminearum*, is mainly recognized due to the triggering of fatty acid, terpenoid, and phenylpropanoid metabolic pathways. This study used NILs (near isogenic lines) which set a good example to demonstrate that proteometabolomic studies are not delimited up to the genetics of a given QTL (Gunnaiah et al. 2012).

Biotechnological approaches require genetic modification to govern the assembly of specific metabolites in plants, to progress food quality, to increase their adaptation against environmental stress, and to increase crop yield. Unluckily, these approaches do not essentially lead to an estimated result because of the complex mechanisms required for the plants metabolic regulation. Metabolites such as inositol, salicylic acid, ethylene, and jasmonic acid have been linked to plant defense signaling pathways against biotic stress (Kushalappa and Gunnaiah 2013). Many resistances related (RR) metabolites which were identified based upon nontargeted analysis to possess antimicrobial properties (Ahuja et al. 2012; Ballester et al. 2013) are discussed in Table 5.1. Many bioinformatic tools are accessible for LC-MS data processing for mass spectral output processing and compound annotation mentioned in Table 5.2.

5.6 Conclusion and Future Aspects

Currently, plant–pathogen interactions exemplify the utmost biochemically complex and thought-provoking scenarios being evaluated by metabolomics approaches. For example, there is complication in identifying which metabolites are procured from the plant and which metabolites are interacting from the pathogen side. Phytocompounds are involved in resistance mechanisms of plant. Until now, there is much information on the mechanism of resistance of plants against invading pathogens, but very less is known about the pathogenicity of invading pathogens. Toxins certainly play a role in the pathogenicity factor during plant–pathogen interaction. Plant components which have a negative effect upon the growth and development or survival of another organism can be considered as toxins. The way plants store their toxins are often critical for their effectiveness. Some plant species

Table 5.1 List of resistance related (RR) metabolites identified in plants against biotic stresses, following nontarget metabolomics approaches

Plants	Disease/pathogens	Resistance related biochemicals	Chemical groups/pathways	Disease resistance mechanism	References
Wheat	*F. graminearum*	Sinapic acid, deoxyphodophyllotoxin, phyllanthusmin, linoleic acid, 13(S)hydroperoxylinolenic acid, deoxyloganate	Phenylpropanoids Fatty acids	Antimicrobial	Gunnaiah et al. (2012)
Arabidopsis	*Golovinomyces orontii*	Camalexin Indol-3-yl-methylamine	Indole group	Phytoanticipin/ phytoalexin	Consonni et al. (2010)
Citrus	Huanglongbing (*Candidatus* spp., *Liberibacter* spp.)	L-glycine Mannose	Amino acid Sugar	Antimicrobial	Cevallos-Cevallos et al. (2012)
Grape	Fungi	Caffeic acid Inositol Alanine, glutamine, and glutamate	Phenylpropanoid sugar Amino acids	Antimicrobial Signal transduction Ammonia recycling liberated by PAL	Figueiredo et al. (2008)
Barley	*Fusarium graminearum*	*p*-coumaric acid, sinapate Kaempferol-3-*O*-β-d-glucopyranosyl, kaempferol-*O*-rutinoside, Flavonoid glucosides, Capric acid, lauric acid	Phenylpropanoids Flavonoids Fatty acids	Signaling molecules Antimicrobial Cell wall strengthening	Bollina et al. (2010)

Referred from Kushalappa and Gunnaiah (2013)

Table 5.2 Open access databases for metabolite search and compound annotation

Database	Features	Weblink
PRIMe	Provide web-based service for metabolomics and transcriptomics tools. It measures standard metabolites through GC/MS, CE/MS, LC/MS, and multi-dimensional NMR spectroscopy, unique tools for transcriptomics, metabolomics, and integrated analysis of omics data	http://prime.psc.riken.jp/
PlantCyc	Plant metabolic pathway database for compounds, enzymes, genes, and pathways intricated in primary and secondary metabolism. Tools for BLAST, user input pathway generation and comparative analysis Downloadable reference pathways for rice, Arabidopsis, cassava corn, papaya, grape, poplar, potato	http://www.plantcyc.org/
METLIN	Over 64,000 structures, tandem mass spectra of more than about 10,000 metabolites, external link to other databases, batch search	http://metlin.scripps.edu/
ReSpect	Provides phytochemicals tandem mass spectral database	http://spectra.psc.riken.jp/
KNAPSACK	Database for metabolites-species relation, search options; organism name, organism taxonomic tree, metabolite name, molecular weight, formula, batch search	http://kanaya.aist-nara.ac.jp/KNApSAcK/
McGill-MD	Metabolites related to plant biotic stress resistance, *in planta* fragmentations using LC-LTQ-orbitrap and annotated with in silico fragmentation	http://metabolomics.mcgill.ca
ChEBI	A database, dictionary, and ontology of manually annotated small molecules	http://www.ebi.ac.uk/chebi/init.do

Referred from Kushalappa and Gunnaiah (2013)

store toxins in resin ducts, laticifers (Dussourd and Hoyle 2000), or glandular trichomes (Hallahan 2000) from where the toxins are released in huge amounts as soon as these structures are broken by pathogens. For instance, hydrogen cyanide released from cyanogenic glycosides inhibits cellular respiration (Jones et al. 2000), saponins disrupt cellular membranes (Osbourn 1996), and cardenolides are specific Na^+/K^+-ATPase inhibitors (Bramer et al. 2015). One substantial challenge for the study of plant metabolomics is it lacks fully defined and interpreted metabolome for any plant species. It is estimated that the plant kingdom produces around 90,000–200,000 diverse metabolites. Still, the definite number of metabolites present in independent species of a plant is unknown (Fiehn et al. 2001). A reiterate theme in all aspects of spheres of plant–pathogen interaction is the ability of each participant to recognize and respond to cues generated by the other. Still, understanding of molecular recognition and response systems, receptors involved in plant perception of pathogens in its infancy, and many other important questions remain unanswered. In future, research focusing on the identification of effector molecules from pathogens and their mechanism of action is likely to set a new stage in plant–pathogen interaction.

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