Chapter 3
Rhizosphere Metabolomics: Methods and Applications

Sheela Reuben, V.S. Bhinu, and Sanjay Swarup(✉)

3.1 Introduction

The emerging field of rhizosphere metabolomics involves analysis of entire metabolite complement (metabolome), in an unbiased way to understand complex physiological, pathological, symbiotic and other relationships among the inhabitants of the rhizosphere. Metabolomic studies of the rhizosphere are quite challenging since the rhizosphere is a complex as well as a dynamic microenvironment. Metabolite composition in the rhizosphere is primarily governed by the nature of root exudates, secretions from rhizobacteria, fungi and other soil organisms. Conversely, the nature of these root exudates also directly or indirectly affects microbial growth in the rhizosphere. While some compounds enhance growth, others have antimicrobial activities. Apart from the diverse roles of compounds present, the complexity of the rhizosphere also stems from competition among rhizosphere microbes. Some of them are growth-promoting, while others are pathogenic. These effects are not only confined to the microbes but also extend to the plants growing in the rhizosphere. Hence, gaining knowledge of these rhizosphere metabolites as well as the effect of the biota will help us better understand this ecological niche.

The field of metabolomics utilizes analytical techniques such as chromatography, mass spectrometry (MS), nuclear magnetic resonance (NMR) and spectroscopy to profile, identify and estimate the relative abundance of metabolites at a given time. Various methods involving gene expression studies, enzymatic studies and biochemical techniques have been used to understand the events that occur in the rhizosphere. However, it is often observed that the metabolite levels do not coincide with the activity of the biosynthetic enzymes and their end products. Effects of the metabolites on the system, therefore, cannot be easily studied using solely RNA or enzyme-based techniques. Metabolomics helps to overcome

Sanjay Swarup
Small Molecule Biology Laboratory, Department of Biological Sciences,
National University of Singapore, Singapore 117543
e-mail: sanjay@nus.edu.sg

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these pitfalls and provides a comprehensive approach to provide a biochemical status report.

This chapter focuses on metabolomics in relation to the rhizosphere. A brief overview of the components of the rhizosphere, interaction between the different species and competition among rhizobacteria is provided. Many technologies used for metabolic profiling and their role in rhizosphere metabolomics are discussed in some detail. The sole of bioinformatics and data visualization methods are summarized. Finally, this chapter ends with a brief view of the applications of selected metabolomic studies.

3.2 The Rhizosphere—Chemical and Biological Components

The rhizosphere is a complex, dynamic and highly interactive microenvironment. It has an ecological advantage for those organisms that are exclusively associated with the roots of plants. Here, we provide a brief description of the rhizosphere components especially in relation to the origin and nature of metabolites and their effects on the rhizosphere biota and microbiota.

3.2.1 Composition of the Rhizosphere

3.2.1.1 Root Exudates

Root exudates play a key role in the rhizosphere, as their composition and abundance affect the growth and characteristics of the organisms thriving in the rhizosphere. Excellent reviews and books are available on this topic where details of exudate composition are provided (Bais et al. 2006; Mukerji et al. 2006; Pinton et al. 2000). The list of low molecular weight compounds identified in the rhizosphere is very long and broadly consists of amino acids, organic acids, sugars, phenolics and various other secondary metabolites. Exudates vary with respect to signals of biotic or abiotic origins. Allelochemicals in the root exudates govern the type of organisms that grow in the region. Allelochemicals are secondary metabolites that influence the growth of other organisms. Allelopathy, a phenomenon that refers to the role of allelochemicals, is exploited in the control of insects and weed plants. Some of the allelochemicals include tannins, cyanogenic glycosides, benzoquinones, flavonoids and phenolic acids. The biological and physiological mechanisms of allelochemicals have been reviewed (Weir et al. 2004; Inderjit and Duke 2003). Benzoxazinones are an important class of allelochemicals whose sample extraction and separation methods have been reviewed by Eljarrat and Barcelo (2001) and Bonnington et al. (2003), respectively. These compounds are easily hydrolyzed and hence care needs to be taken during their sample preparation.

3.2.1.2 Rhizobacteria

Rhizobacteria form an integral part of the rhizosphere. They include the microorganisms that are both beneficial as well as pathogenic. The term "rhizoengineering" refers to the engineering of rhizobacterial populations in order to improve the interactions and outcomes within the soil environment. Several beneficial microorganisms are known to cause breakdown of natural products or even degrade them to simple sugars that are recycled for other anabolic reactions in the rhizosphere biota. Many of these plant products are terminal metabolites of biosynthetic events in plants and it is not uncommon to find rhizobacteria that utilize these end products for energy generation. In the case of the phenylpropanoid compounds, they are exuded in the rhizosphere and some microbes degrade these compounds through specific metabolic pathways. Two examples are the phenylpropanoid catabolic pathway in plant growth promoting rhizobacterial strains of *Pseudomonas putida* (Pillai and Swarup 2002) and the fluorophenol degradation pathway in different species of *Rhodococcus* (Boersma et al. 2001).

3.2.1.3 Soil Fungi

These organisms could be pathogenic or symbiotic, such as in the case of the mycorrhizae and contribute to the complex biotic interactions in the rhizosphere. Fungal development is often stimulated in the presence of roots especially owing to the nitrogen released by the roots. The presence of some rhizobacteria may cause the inhibition of mycorrhizal growth. For example, the growth of ectomycorrhizae is inhibited by the presence of selected isolates of *Pseudomonas* and *Serratia* in the early infection stage of the fungi (Bending et al. 2002). Such growth inhibition is mostly mediated by the secretion of antibiotics or antimicrobial compounds in the rhizosphere such as phenazines or selected flavonoids.

3.2.1.4 Soil Nematodes

Soil nematodes play an important part in the rhizosphere. Nematodes influence the nature of root exudation, which affects the physiological functioning of microorganisms in the rhizosphere. These exudates may serve as signal molecules for nematode antagonists and parasites (Kerry 2000). Matrix assisted laser desorption ionization (MALDI) time-of-flight (TOF) analysis has been used in analyzing the metabolites from plant nematodes (Perera et al. 2005). These nematodes are often difficult to identify and the technique provides a good opportunity for a rapid and simple identification of plant parasitic nematodes. More details regarding MALDI TOF analysis are provided in Sect. 3.3.4.5.

3.3 Rhizosphere Metabolomics

3.3.1 Metabolomics: an Overview

"Metabolome" refers to the sum total of all the nonproteinaceous small molecules (metabolites) present in an organism. Metabolites are the small molecules that are the end products of enzymatic reactions. "Metabolomics" is unbiased identification and quantification of all metabolites present in a sample from an organism grown under defined conditions. Another term, "metabonomics," is used frequently in the biomedical (toxicology) literature and for methods involving NMR spectroscopy. However, "metabolomics" is a preferred term for unbiased metabolite analyses (Bhalla et al. 2005). Metabolomics is not restricted to *metabolic profiling*; it also encompasses a much broader study including identification of metabolites (to understand the range of metabolites produced by the organism), their quantitation (to detect the abundance of metabolites), comparisons (to understand the differences arising from perturbations in metabolic pathways), data analysis (chemoinformatics) and development of metabolic models. "Metabolic profiling" refers to obtaining a listing of the entire range of the metabolites present in the organism. Such complete profiles are not unattainable in practice, because one single method of extraction or analysis covers only a partial spectrum of the metabolome, as mentioned earlier. Various analytical methods alone, or in conjunction with others, have therefore been used for comprehensive metabolic profiling. Some of these techniques are discussed in Sect. 3.3.4.

Metabolomics has many applications, including but not restricted to (1) understanding the enzyme fluxes, (2) uncovering novel metabolic pathways, (3) unraveling cryptic pathways, (4) identifying biomarkers and (5) metabolic engineering of novel products that are industrially and biomedically relevant. Metabolomics, in conjunction with other "omics" such as functional genomics, proteomics and transcriptomics, has helped in better understanding the biological systems. Integration of data from the various fields has helped in painting a holistic picture of the biological system using the systems approach. Many excellent reviews are available for metabolomic studies (Sumner et al. 2003; Ryals et al. 2004; Villas-Boas et al. 2005; Bhalla et al. 2005; Dunn and Ellis 2005).

The rhizosphere is a constantly changing microenvironment, where there is a flux of energy, nutrients and molecular signals between the plant roots and microbes that affects their mutual interactions. Metabolites exuded from plants as well as the metabolites released or secreted by the microbiota present in the rhizosphere have a considerable effect on this microenvironment. Hence, metabolic profiling constitutes a powerful technique to understand the underlying phenomenon of such exudations and the effects of metabolites on soil ecological relationships, plant–microbe interactions and other soil organisms. The use of improved analytical techniques has helped in the characterization of microorganisms from soil. One example is the characterization of *Bacillus* and *Brevibacillus* strains using UV resonance Raman spectroscopy (López-Díez and Goodacre 2004). Other applications include understanding biotic

interactions. For example, gas chromatography (GC)/MS has been used to study symbiotic nitrogen fixation in legume roots and in understanding plant–microbe interactions (Desbrosses et al. 2005). Several techniques have been used in structural elucidation of metabolites, like NMR, IR spectroscopy, Fourier transform (FT) MS and so on. Tandem MS has also been used in such studies especially for studying metabolites from roots or root exudates. It is usually helpful in providing an initial partial structure that can be fully elucidated by NMR spectroscopy. For example, structural elucidation of montecristin, a key metabolite in biogenesis of acetogenins from the roots of *Annona muricata*, was performed using tandem MS and NMR (Gleye et al. 1997). Another application is the identification of allelochemicals (Eljarrat and Barcelo 2001).

3.3.2 Root Exudates Profiling

Root exudates form a major component of the rhizosphere. While numerous reports are available on identification of selected classes of root exudates as mentioned in Sect. 3.2.1.1, we briefly describe here some studies that have employed unbiased analytical methods. High-performance liquid chromatography (HPLC) and NMR spectroscopy have led to the identification and quantification of a number of metabolites in the root exudates of *Arabidopsis thaliana* (Walker et al. 2003). Nearly 289 possible secondary metabolites were quantified and chemical structures of ten compounds were elucidated. The authors conducted a time-course study of root exudates from plants treated with salicylic acid, jasmonic acid, chitosan and two fungal cell wall elicitors. Plants treated with salicylic acid had the maximum number of compounds in their exudates, while elicitation with jasmonic acid had the least effect on exudates. This method of root exudates profiling could identify differences in root exudation with respect to plant stress. Such types of studies can provide indirect reference to the metabolic pathways during the different stress conditions. Some of the compounds reported in the study had previously not been reported from *Arabidopsis*. The authors also tested the antibacterial as well as the antifungal activities of several of these compounds. This study also highlights the constant change in the exudation, which directly affects the microbial populations.

Root exudates profiling in graminaceous plants has been used to understand the acquisition of metal ions from soil. Root exudates profiling in graminaceous plants was conducted using multinuclear and two-dimensional NMR with GC/MS and coupled with high-resolution MS for metabolite identification (Fan et al. 2001). The root profiling method was used to examine the role of exudate metal ion ligands (MILs) in the acquisition of Cd and transition metals in barley and wheat. The change in the root exudate profile was studied in wheat, barley and rice grown on Fe- and Cd-deficient soils. MILs such as 3-epihydroxymugineic acid, mugineic acid, 2′deoxymugineic acid and malate in barley were elevated in Fe-deficient conditions, which in turn increased the Fe-mobilizing substances. The results suggest that enhanced exudation of murigenic acids and malate may be involved in

acquisition of transition metals but not of Cd, and also that the mechanisms of acquisition for essential and toxic metal ions may be different.

3.3.3 Current Limitations of Rhizosphere Metabolomics

While metabolomics is highly powerful, the field as such has certain limitations. Four general limitations are described here. One bottleneck at present is that it is technically challenging and expensive to detect a wide range of metabolites often at low concentrations; hence, combinations of techniques need to be used. This not only makes it an expensive approach but also demands high levels of technical expertise in analytical chemistry as well as "chemometrics" (analysis of chemical data), which are described in some detail in Sect. 3.3.4. Each technique has its own set of limitations, owing to which the entire profile is often not obtained. For example, with an ionization-based mass spectrometer such as an electrospray ionization (ESI) mass spectrometer the metabolites detected are often the highly ionizable ones under the particular buffer system and conditions used in specific experiments. Ones that do not ionize in such a buffer system are often not detected. The second challenge lies is the choice of sample extraction procedure. There is no universal extraction method that is suitable for all types of compounds. Often one needs to use different sample extraction techniques and buffer systems depending on the type of target compounds. Thirdly, metabolomics is limited by the requirement of an accurate and well-curated database for the spectra of compounds. Such databases are required for comparisons of established signatures or fingerprints of various metabolites. As more and more laboratories are beginning to use metabolomics, the future of a comprehensive database looks bright. At present, many laboratories use standards for various compounds to establish the identity of a metabolite. Some of the compounds are not commercially available and these need to be isolated from natural sources. Hence, a large proportion of the compounds detected remain unidentified. A major development in solving this problem is the increasing adoption of GC/MS by many metabolomics researchers. As GC/MS generates spectra for fully ionized compounds, such spectra are highly reproducible between laboratories and can be used as references and exchanged between researchers. The fourth bottleneck in this field is the limited availability of chemoinformatics tools that expedite analysis of a large amount of data and convert it to an interpretable form with respect to the biological characteristics of the various systems. There has been considerable progress in the computational biology field in recent years and more tools are being developed. Some of the bioinformatics tools are discussed in Sect. 3.3.5.1.

In addition to the current limitations in metabolomics, the field of rhizosphere metabolomics in particular faces two additional challenges. One is due to the different collection procedures required for root exudates. These are collected by growing plants hydroponically, aeroponically or even in soil; hence, specialized techniques and care are required for collection purposes. Secondly, it is difficult to

separate the exudations from various interacting biotic agents in the rhizosphere such as plants and their associated microbes. Further advances in these areas are required to bridge the gap in the knowledge base of gnotobiotic and field-based systems. Spectroscopic methods used for the identification of soil bacteria are becoming increasingly popular as they provide information on nonculturable microbes as well as on the relative abundance of various microbial species. For example, Raman microscopy analysis gives the spectral profile from a single cell, which helps in bacterial species differentiation (Huang et al. 2004).

3.3.4 Rhizosphere Metabolomics Methods

As exemplified by the root exudate profiling studies already described, they rely largely on a highly sophisticated suite of analytical techniques. We briefly describe the major groups of techniques used in metabolomic studies. Some of these include the chromatography techniques, MS, NMR and spectroscopy of various types. The utility of analytical techniques in metabolomics has been more extensively reviewed by Dunn et al. (2005).

3.3.4.1 Chromatography Techniques

Chromatography techniques help in separation and analysis of the metabolites. Different types of such techniques are available for metabolite analyses (Table 3.1). Here, we give a few examples that represent how these techniques can help in understanding the nature of root exudates or interactions in the rhizosphere:

- Thin layer chromatography. This technique involves the separation of metabolites on the basis of differential partitioning between the components of a mixture and the stationary solid phase. This is a very simple and inexpensive analytical method. Reverse-phase thin layer chromatography (TLC), along with some other techniques, has been useful in understanding fungal-bacterial interactions in the rhizosphere. The rhizobacteria *Pseudomonas chlororaphis* PCL1391 produces an antifungal metabolite phenazine-L-carboxamide, which is a crucial trait in its competition with the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* in the rhizosphere (Chin-A-Woeng et al. 2005). In this study, TLC was used to identify autoinducer compounds that were released during the expression of sigma factor psrA in different quorum-sensing gene mutants. In another application, TLC was used in studying the nodulation signaling metabolites that are secreted into the growth medium produced due to the *nod ABC* genes of *Rhizobium* and *Bradyrhizobium* strains (Spaink et al. 1992). TLC can be used to separate polar metabolites and fatty acids as well as to test the purity of compounds.
- Reverse-phase HPLC. In this technique the metabolites are separated on the basis of their hydrophobicity and they can be identified by comparing the retention

Table 3.1 Summary of the different available analytical techniques that have proven valuable in soil biology studies

Techniques	Special features	Examples from soil biology studies
Chromatography		
Thin layer chromatography	Simple and inexpensive	Study of nod metabolites (Spaink et al. 1992)
Anion-exchange chromatography	Effective separation	Study of soluble carbohydrates (Cataldi et al. 2000)
Mass spectrometry		
Matrix-assisted laser desorption ionization	Useful in identification of metabolites	Study of aconitum alkaloids from aconite roots (Sun et al. 1998)
Proton transfer reaction mass spectrometry	Rapid and real-time analysis	Study of rhizosphere volatile organic compounds and their induction by biotic stresses (Steeghs et al. 2004)
Spectroscopy		
Nuclear magnetic resonance	Less sensitive but powerful for chemical structure identification	Building flux maps and metabolic network modeling. (Ratcliffe and Shachar-Hill 2005)
Tandem analysis methods		
Liquid chromatography/electrospray ionization mass spectrometry	Analysis of even labile compounds as there is no need to derivatize	Identification of isoflavone conjugates from roots of lupine species (Kachlicki et al. 2005)
Gas chromatography/mass spectrometry	Low-mass volatile compound can be identified	Identification of signaling molecules during ectomycorrhizae formation (Menotta et al. 2004)
Gas chromatography/combustion/isotope-ratio mass spectrometry	Helps to study dynamic nature of metabolites	To study the nature and dynamics of plant sugars in the rhizosphere (Derrien et al. 2003)

times with those of standard compounds. This method has been used in comparing the root exudates from different cultivars. For example, root exudates from seven accessions were evaluated using HPLC (Czarnota et al. 2003). Another application includes the use of HPLC in quantifying the amount of sorgoleone, a photosynthetic inhibitor in the rhizosphere of sorghum plants (Weidenhamer 2005). Polydimethylsiloxane (PDMS) was used for the study. The amounts of sorgoleone retained on the PDMS increased with time, which could be shown using HPLC methods. The use of HPLC in root exudates profiling has already been mentioned in Sect. 3.3.2 (Walker et al. 2003).

- Anion-exchange chromatography. Another form of chromatography, this technique is based on charge-to-charge interactions between the target compounds and the

charges immobilized on the column resin. In anion-exchange chromatography the binding ions are negative and the immobilized functional group is positive. It has been used to determine the composition of soluble carbohydrates in plant tissues such as olive roots (Cataldi et al. 2000). The authors have used the technique for efficient separation of carbohydrates. Such studies can be extended to understand the movement of sugars and the types of sugars that are available in the soil for the rhizobacteria.

Chromatography techniques are powerful tools when used in conjunction with other techniques such as MS. Liquid chromatography (LC) and GC techniques have been used with different types of mass spectrometers as described in the context of rhizosphere metabolomics here.

3.3.4.2 Mass Spectrometry

MS analysis has come a long way since 1954, when John Beynon from Imperial Chemical Industries, UK, first suggested that the spectra could be correlated to structure and outlined the basic rules of MS. MS has now developed into a powerful analytical tool with applications in chemical analysis, drug development, natural products analysis and biomedical applications to name a few. Computer interfacing has added an additional software-driven component, which has brought the instrument within the reach of biologists. In a mass spectrometer, the samples are ionized by different methods. This is usually done in the source part of the mass spectrometer. There are different ionization methods, like electron impact, chemical ionization, ESI, fast atom bombardment, field ionization, field desorption and laser desorption. In electron impact ionization, the samples are ionized by the bombardment of electrons. The ionization is caused by the interaction of the fields of the bombarded electron and the molecule, resulting in the emission of an electron. In an ESI mass spectrometer the sample is sprayed as a fine liquid aerosol. A strong electric field is applied under atmospheric pressure to the liquid passing through a capillary tube, which induces charge accumulation at the liquid surface, which then breaks up to form highly charged droplets. As the solvent evaporates, the droplets explode to give ions. The spectra obtained are usually those of mutiply charged molecular ions owing to protonation. In laser desorption ionization, a laser pulse is focused onto the surface of the sample, some part of the compound gets desorbed and reactions among the molecules in the vapor-phase region result in ions. An extension of this method is the MALDI method. In this technique, samples are mixed with a suitable matrix and allowed to crystallize on grid surfaces. Samples are then irradiated with laser pulses to induce ionization. Most of the energy of the laser pulse is absorbed by the matrix, so unwanted fragmentation of the biomolecule is avoided. Chemical ionization is considered to be “soft ionization” technique as the number of fragment ions produced is less. In this method a reactant gas like methane is passed through the sample and the interaction of the ions with neutral molecules produces new ions. The ions formed by any of the methods are then accelerated through a column

and deflected in a magnetic field. In the mass analyzer the ions are separated according to their mass-to-charge ratio (*m*/*z*) and finally they are detected by an ion detector. In a triple-quadrupole mass analyzer, the ions from the source are passed between four parallel rods. The motion of the ions depends on the electric field, which allows only ions of the same *m*/*z* to be in resonance and to the detector at the same time. Triple-quadrupole MS is most often used for quantification purposes. In the case of an ion-trap mass analyzer, the ions are focused using an electrostatic lensing system into the ion trap. An ion will be stably trapped depending upon the values for the mass and the charge of the ion. A mass spectrum is obtained by changing the electrode voltages to eject the ions from the trap. In a TOF detector the molecules are detected on the basis of the time that each molecule takes to reach the detector.

A schematic representation of the acquisition of mass-spectral data is provided in Fig. 3.1.

Chromatographic separations followed by mass-spectral analysis provide additional separation. This is because the metabolites are first separated on a chromatographic column which partitions the metabolites into different fractions and each of the fractions is further analyzed by a mass spectrometer. The separation of the metabolites into fractions helps in reducing the ion suppression effect and enhances detection and therefore more metabolites can be analyzed from samples. The metabolites can be fragmented for identification purposes using a tandem mass spectrometer. A Tandem mass spectrometer can be considered as two mass spectrometers in series with a collision cell in-between. This type of instrument

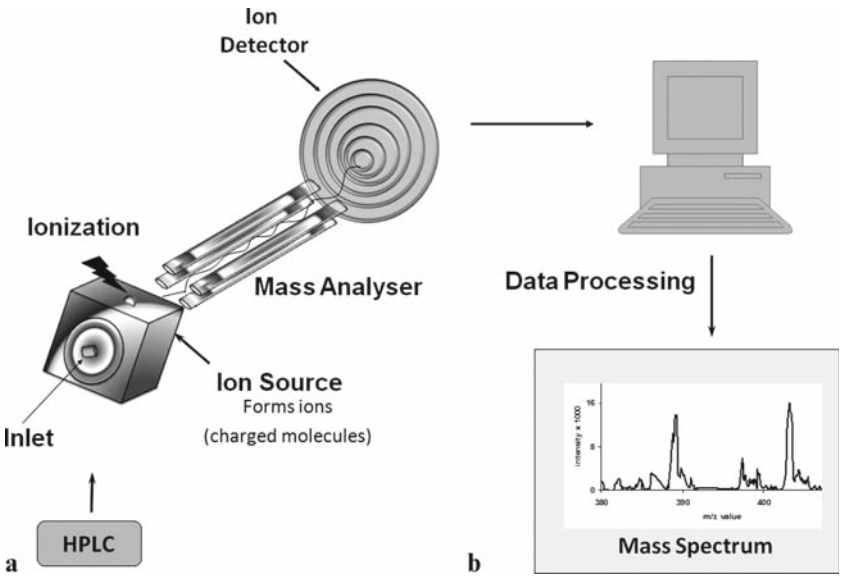

Fig. 3.1 The various steps in acquiring a mass spectrum

helps in fragmenting targeted ions that give rise to daughter ions. These daughter ions form a fingerprint that can then be compared with fingerprints of standards or databases. Mass spectrometers have been used in conjunction with various chromatographic methods. The two commonly used chromatographic methods are LC in conjunction with MS and GC in conjunction with MS. Some of these analytical techniques have been described recently (Dunn et al. 2005; Sumner et al. 2003). MS can also be used to optimize the chromatographic separations. The separations are best if the metabolites are limited to the least number of fractions. Conversely, if the same metabolite is present in more fractions then the separations by HPLC are not very efficient (Fig. 3.2). In this example, each of the metabolites is in just one fraction (no more than one) and 80% belong to this category, indicating an effective HPLC-based separation.

3.3.4.3 Gas Chromatography/Mass Spectrometry

This technique is mostly used to study volatile compounds. As GC/MS relies on the hard ionization methods, ion spectra are highly uniform and reproducible between experiments. Owing to this advantage, standard databases can be created and shared between laboratories. Several examples of GC/MS use are available in the plant metabolomics literature. For example, the GC/MS technique has been used to study the differences in plants of different developmental stages with respect to their day length (Jonsson et al. 2004). GC/MS has been useful in identifying molecules such as those involved in signaling during ectomycorrhizae formation (Menotta et al. 2004). These molecules are exuded during the presymbiotic interaction between *Tuber borchii* (ectomycorrhiza) and the host plant *Tilia americana*. Seventy-three volatile organic compounds (VOCs) could be identified and 29 of these were produced during interaction between the fungi and the host and; therefore, they could possibly be signaling molecules. The technique thus assists in increasing our understanding of rhizosphere signaling.

GC–combustion–isotope-ratio MS (GC/C/IRMS) is another useful technique that has been adopted in rhizosphere metabolomics. An Isotope-ratio mass spectrometer accurately determines the elemental isotope ratios very precisely and accurately. Single focusing magnetic sector mass spectrometers with multiple detectors are used in this technology. The principle of IRMS is that the ratio of isotopes in a compound varies according to its source and forms an isotopic fingerprint, which can be detected using a mass spectrometer. The advent of IRMS has helped in evaluating the interactions between organisms and the environment by studying the variability of the natural abundance of stable isotopes. Stable isotope mass-spectrometric approaches are also useful in understanding biotic interactions in complex ecosystems. Different phenomena involving soil microorganisms and soil invertebrates were recently determined by the $\delta^{13}C$ values of individual compounds (Evans and Evershed 2003). GC/C/IRMS was used in conjunction with ^{13}C labeling to study the nature and dynamics of plant sugars in the

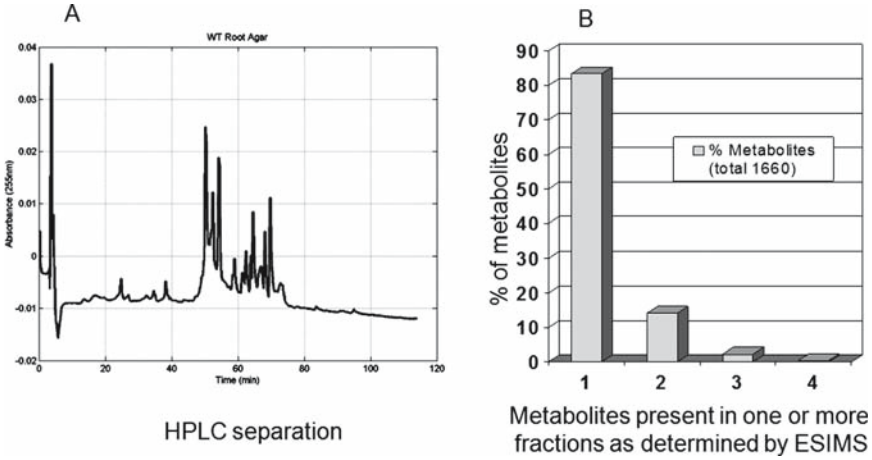

Fig. 3.2 Use of a mass spectrometer to optimize high-performance liquid chromatography (HPLC) methods. **a** Offline analytical HPLC chromatogram of *Arabidopsis* Columbia plant roots grown on agar plates. Metabolites present in plant roots were collected into 16 major reverse-phase HPLC fractions and each fraction is separated by electrospray ionization mass spectrometry. **b** The frequency of each mass/charge (*m*/*z*) value in the different fractions. The frequency helps in determining the efficiency of the HPLC run

rhizosphere (Derrien et al. 2003). In this study, the sugars were hydrolyzed and trimethylsilylated (addition of several carbon atoms per sugar) to derivatize the carbohydrates prior to GC analysis. The polar hydroxyl groups were replaced by nonpolar groups that contain carbon. The isotope excess of each sample was determined using calibration of the number of analyzed added carbon atoms in terms of the ratio of ^{13}C to normal C of individual sugars. The study highlighted the use of this technique and discussed the derivatization aspects and proposed further use of the technique in understanding the sugar dynamics in soil. IRMS has been coupled with the continuous flow mode to understand the C cycling in forest soil (Formanek and Ambus 2004). The efflux of CO_2 is a combination of respiratory activity of roots and associated rhizosphere organisms, soil fauna and soil microorganisms. The contribution of the CO_2 from each group can be analyzed to understand C cycling and sequestration.

3.3.4.4 Liquid Chromatography/Mass Spectrometry

Although numerous metabolites can be identified in a single run using GC/MS, the technique may not always prove useful especially in the case of metabolites that are sequestered in compartments and are labile or degraded in high-temperature regimes; hence, such metabolites are difficult to derivatize. In such cases, LC/MS

may be the technique of choice. This technique is very commonly used as it is a very convenient platform especially when used in conjunction with ESI MS. Nearly 13–20 isomeric isoflavone conjugates have been identified from roots of lupine species using ESI MS (Kachlicki et al. 2005). In that study, a comparative analysis of triple-quadruple and ion-trap analyzers was conducted. The study highlighted the utility of these techniques in analyzing metabolites in biological samples. Such techniques can be used to study the role of metabolite conjugations in root–microbe interactions since flavonoids play a major role in plant–microbe interactions as discussed in Sect. 3.4.2.

3.3.4.5 Matrix-Assisted Laser Desorption Ionization Time of Flight

This is a very sensitive method and quantities as low as 10^{-15}–10^{-18} mol can be detected. This method has been useful in the study of aconitum alkaloids from aconite roots (Sun et al. 1998). This kind of analysis often leads to the identification of new metabolites as in this case where three new alkaloids were identified. MALDI TOF is most useful for determining the mass accurately.

3.3.4.6 Proton Transfer Reaction Mass Spectrometry

This new technology allows rapid and real-time analysis of most biogenic VOCs without preconcentration or chromatography. Compounds are ionized by a chemical ionization method using H_3O^+ ions. The H_3O^+ ions transfer their protons to the VOCs, which have higher proton affinities than water. The process is referred to as "soft ionization" as it avoids excessive fragmentation of the biomolecules and allows real-time analysis. The detection limit in proton transfer reaction MS is as low as a few parts per trillion. This technique has been used to study rhizosphere VOCs and their induction by biotic stresses (Steeghs et al. 2004). The VOCs can be analyzed without previous separation by chromatography. VOCs induced specifically as a result of interactions between microbes and insects and *Arabidopsis* roots could be detected. For example, in the abovementioned study, compatible interactions of *Pseudomonas syringae* DC3000 and *Diuraphis noxia* with *Arabidopsis* roots showed rapid release of 1,8-cineole, a monoterpene that was not previously reported in *Arabidopsis*.

3.3.4.7 Spectroscopy Methods

Spectroscopic techniques have been increasingly used in studying and identifying metabolites. FTIR spectroscopy is a technique that is useful in identifying

organic and inorganic chemicals. The chemical bonds in a molecule can be determined by interpreting the IR absorption spectrum. Molecular bonds vibrate at various frequencies depending on the elements and the type of bonds and therefore give a specific absorption spectrum. FTIR spectra of pure compounds are so unique that they are like a molecular "fingerprint." Raman spectroscopy is another technique where the observed spectrum is based on the vibration of a scattering molecule. When a photon is incident on a molecule, it interacts with the electric dipole of the molecule. The interaction can be viewed as a perturbation of the molecule's electric field. Both FTIR and Raman spectroscopy are effective in the rapid identification of bacteria and fungi (Goodacre et al. 2000).

3.3.4.8 Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy is a less sensitive technique than MS; however, it is highly powerful in identification of small molecules and is one of the most used forms of spectroscopy. It helps in accurately determining the structure of a metabolite. Any molecule containing one or more atoms with nonzero moment is detectable by NMR. Biologically important atoms such as ^{1}H, ^{13}C, ^{14}N, ^{15}N and ^{31}P are all detectable by NMR. All biologically important metabolites provide NMR signals. NMR spectra are characterized by the chemical shifts, intensity and fine structure of the signals. These signals help in identification and quantification of the metabolites. The use of NMR in metabolic fingerprinting and profiling of plants has recently been reviewed by Krishnan et al. (2005). These authors reviewed NMR profiling and multivariate data analysis with respect to the effect of stress on wild-type, mutant and transgenic plants. This highlights the potential of NMR in plant metabolomics. NMR has also been used in investigating the operation of networks in plants. Labeling with isotopes in conjunction with metabolite analysis by NMR can help in building flux maps that can be useful in metabolic network modeling (Ratcliffe and Shachar-Hill 2005). ^{133}Cs NMR has been used to examine the intracellular and extracellular pools of Cs^{+}-containing, and CsCl-perfused, excised maize seedling roots. Hence, further insight was gained into the ion transport and subcompartmentalization in the root tissues (Pfeffer et al. 1992). Quantitative NMR is used for quantitative measurements. Further, solid-state NMR has been used in studying plant nitrogen metabolism (Mesnard and Ratcliffe 2005). Solid-state ^{1}H NMR is useful in elucidating the structure as well as the dynamic nature in the solid phase. In this technique, the structure is derived on the basis of the number of H atoms, the neighboring H atoms and the environment of the H atoms. These techniques give a wealth of information on the metabolites in plants and can also be useful in identifying and quantitating root exudation metabolites. The utility of NMR in metabolomic studies has been reviewed by Griffin (2004) and Reo (2002).

3.3.5 Methods and Tools for Metabolomics Data Handling and Analysis

Metabolomic studies often lead to huge data sets that need to be analyzed and stored. In typical HPLC-based separation followed by offline MS such as ESI MS of each fraction, ions can be detected per fraction in positive mode and a slightly lesser number in the negative mode. Hence, one sample from a single injection can yield 10,000–12,000 data points. With replications and various samples, the number increases considerably, generating several hundred megabytes of data per experiment.

Databases to store such data as well as for the identification of the metabolites, therefore, become essential. Raw data obtained from the instruments have to be preprocessed by several methods to minimize effects of machine variations or experimental errors during weighing of samples or injections into the instruments. Examples of preprocessing include data normalization, baseline correction and alignment of spectra. Following preprocessing, the data need to be converted to useful biological information by a variety of data analysis techniques. Lastly, the analysis of these data can lead to verification of an original hypothesis or the discovery of new associations, which can be experimentally validated. The following criteria have been proposed for creating robust and interpretable multivariate models for comparison of many samples (Jonsson et al. 2005):

1. Each sample is characterized by the same number of variables.
2. Each of these variables is represented across all observations.
3. A variable in one sample has the same biological meaning or represents the same metabolite in all other samples.

Increasing attention is recently being given to metabolic modeling, which leads to development of metabolic networks. Such networks help in understanding the biochemical behavior at the whole-cell level. Evolutionary computation-based methods such as genetic algorithms and genetic programming are ideal strategies for mining such high-dimensional data to generate useful relationships, rules and predictions (Goodacre 2005).

3.3.5.1 Tools for Data Analysis

Several tools for data analysis have been developed by computational biologists for the preprocessing and analysis of data. One such recent tool is MZmine (Katajamaa and Oresic 2005), which is useful for data generated via LC/MS studies. It contains algorithms useful in data preprocessing such as spectral filtration, peak detection, alignment and normalization. The visualization tools enable comparative viewing of the data across multiple samples and peak lists can be exported to other tools for data analysis. Data obtained from metabolomic studies often are very complex as multiple dimensions may be involved. For example, various dimensions due to

different treatments such as doses or time-point-based data may need to be handled. Specific types of biostatistical tools are required to make meaningful conclusions from such data. One such statistical tool is ASCA analysis of variance–simultaneous component analysis) (Smilde et al. 2005). It is a direct generalization of analysis of variance from univariate to multivariate data. This tool helps in analyzing complex data generated by LC/MS involving different parameters such as time and dose factors. Other multivariate data analysis techniques such as principal component analysis (PCA) and partial least squares regression (Martens and Naes 1993) can also be used to analyze metabolomics data. The technique can reduce the number of dimensions to two or three, which can be represented graphically. These representations allow the user to visualize the patterns or clusters in the data sets as hierarchical plots or scatter plots. PCA helps in visualizing the data in a simplified way and helps in extracting meaningful biological interpretations. A variation of PCA is the weighted PCA where spectra of repeated measurements are converted to weights describing the experimental error and it adds interpretation to the metabolomics data (Jansen et al. 2004). Multivariate data analysis has been reviewed by van der Werf et al. (2005).

MSFACT is another metabolite data analysis tool, and consists of spectral formatting, alignment and conversion tools (Duran et al. 2003). This tool helps in reformatting, alignment and export of large chromatographic data sets to allow more rapid visualization and interrogation of metabolomics data. Applications of the tool were illustrated using GC/MS profiles from *Medicago truncatula*. Metabolites from various tissues such as roots, stem and leaves from the same plant were easily differentiated on the basis of metabolite profiles. The tool uses hierarchical clustering, two-dimensional PCA and three-dimensional PCA as visualization tools.

Another recent tool is XCMS, which is suitable for analyses of LC/MS data. It is able to filter and identify relevant peaks and match the peaks in different samples. The tool can also calculate retention time deviations. It is capable of simultaneously preprocessing, analyzing and visualizing the raw data from hundreds of samples. Statistical data analysis can also be performed and it includes functionality for peak picking, nonlinear retention time alignment and relative quantitation. It is freely available at http://metlin.scripps.edu/download/.

A more recent and comprehensive online tool for preprocessing, chemometrics and analysis of LC and MS data is Metabolomics Data Analysis Tool (MetDAT). This tool (http://smbl.nus.edu.sg/METDAT/) is available free online for researchers from academic and nonprofit organizations. MetDAT performs alignment, baseline correction and normalization of data using a number of algorithms. It can calculate log ratios of different treatments with respect to a reference data set. It also allows generation of Venn diagrams that identify common as well as unique molecules in two to four data sets. MetDAT includes chemometric methods like PCA and hierarchal and K-means clustering as well as biostatistical methods such as analysis of variance. The online tool deals with small data sets for rapid analysis. In its offline complete software package, this tool allows user-provided databases to store the data as well as analysis of the data to enable extraction of meaningful biological interpretations. Users are able to upload their data, analyze and store the data,

which can later be recalled. The data are also organized according to the project, subproject and experiments. The sample extraction methods, LC methods, buffer system used, the type of instrument and the instrument settings can be input into the database. Hence, this software package can be used for managing large metabolomics projects involving several resources and experiments. Though there are other tools for MS data analysis and HPLC data analysis, MetDAT provides a complete set of programs for data preprocessing and analysis, unique algorithms and programs for data analysis, and a user database in a single package.

Upon metabolite data analysis, novel compounds and metabolic pathway features are frequently discovered. It then becomes essential to mine the literature for reports on such compounds or their enzymes or pathways. Searching databases can often lead to a large number of publications. For example, a simple search for plant sugars yields 43,926 abstracts in the PubMed database. Analysis and integration of knowledge from such abstracts becomes highly cumbersome to an extent such that it takes enormous effort, manpower and time to assimilate and interpret the information. This problem can be minimized by using knowledge mining tools such as Dragon Plant Biology Explorer (DPBE; http://research.i2r.a-star.edu.sg/DRAGON/ME2/) (Bajic et al. 2005). This tool allows plant biologists to mine existing literature and visualize the interconnectedness. DPBE is a system which integrates information on genes from PubMed abstracts with gene functions based on standard gene ontologies and biochemical entity vocabularies, and presents the associations as interactive networks. DPBE complements the existing biological resources for systems biology by identifying potentially novel associations using text analysis between cellular entities based on genome annotation terms. One of the most useful aspects of DPBE to biologists is that it condenses information from a large volume of documents for easy inspection and analysis, thus making it feasible for individual users. Two modules of the explorer, the Metabolome Explorer and the Pharmacology Explorer, are especially relevant to metabolomics researchers (Bhalla et al. 2005). Interconnections among cellular entities such as metabolites, enzymes, genes, mutants, plant anatomical features, cellular components and function can be visualized as networks. Nodes in the networks are hyperlinked to the original abstracts in color-coded forms. Hence, DPBE can be used to interpret novel information generated from metabolomics projects as well as to research new topics by beginners or experienced scientists.

3.3.5.2 Databases

Different kinds of databases are required to efficiently analyze metabolomics data. Some such databases are outlined here briefly:

1. Databases for storing experimental data. These helps to recall data for comparison or different types of analyses and usually such databases are created in-house. Data are stored as flat files for smaller data sets, while for larger complex data, relational databases are used. Relational databases also have functions “built in”

that help them to retrieve, sort and edit the data in many different ways. A universal database for the input of all metabolomics data would be helpful for comparison of results from different experiments by different people. Such databases are at present available for microarray data. These data can then be used as a starting point for experiments by other scientists. The MetDAT software package described previously provides one such option as it incorporates a project and experiment management system.

2. Databases for comparing with other standard or data sets. One of the biggest challenges in MS is to generate reproducible fragmentation patterns especially using soft ionization methods such as ESI MS. Databases then have to include parent, precursor and daughter ion information. Currently, there is very little understanding of the fragmentation patterns of various metabolites; hence, individual laboratories have to generate their own databases based on their methods and instrument settings. Spectral data are available in the public domain for only some metabolites. For example, the National Institute of Standards and Technology (NIST), USA, has a chemistry Web book that provides information on the molecular weight, formula, structure as well as mass spectra. Chemical information on several parameters is available for over 40,000 compounds and mass spectra of 15,000 compounds are available (http://webbook.nist.gov/). Another freely available database for drugs and metabolites is provided by the Mass Spectrometry Database Committee (http://www.ualberta.ca/~gjones/mslib.htm). In addition, a number of commercial databases are available for users. Some of these are the NIST/EPA/NIH Mass Spectral Library for electron impact (ESI) spectra (http://www.nist.gov/srd/nist1a.htm) and the Wiley Registry of Mass Spectral Data (http://www.wileyregistry.com/). Some of the tools and databases available are compiled in Table 3.2.
3. Databases of biochemical reactions and pathways. To understand the role of various metabolites in biological processes it is imperative to understand the biochemical reactions in which such metabolites are involved and the pathways to which they belong. This helps to predict the molecular mechanisms that govern the various processes taking place in the cell as a whole. Consequently several attempts are under way to create large-scale databases on gene-regulatory and biochemical networks. Such databases provide a comprehensive coverage of the chemical reactions. Such database can therefore, be helpful in deducing the reactions that are affected upon treatment or in transgenic, mutant, knockout or knockdown RNA interference plants.

A summary of some of the available databases is provided in Table 3.2 All these databases contain features that make them unique, but none of them singly fulfills all the requirements for a good reference for metabolic pathway studies (Mendes 2002; Wittig and De Beuckelaer 2001). Despite this, such databases provide a wealth of knowledge and play an important role in understanding the complexities and the interrelationships among the genes, proteins and metabolites. Selected tools and databases from this category are described here briefly:

- MetaCyc. This is a database of experimentally elucidated pathways. It has around 700 pathways from 600 organisms. It stores pathways involved in primary

Table 3.2 Summary of tools and databases available for metabolite studies

	Application	Reference and Web site
Tools		
MZmine	Data preprocessing such as spectral filtering, peak detection, alignment and normalization	Katajamaa and Oresic (2005), http://mzmine.sourceforge.net/
ANOVA–simultaneous component analysis (ASCA)	Analysis of complex data generated by liquid chromatography/mass spectrometry involving different parameters like time and dose factors	Smilde et al. (2005), http://www.bdagroup.nl/
Metabolomics Data Analysis Tool (MetDAT)	Data processing such as alignment, baseline correction and normalization of data. It also includes chemometric methods such as principal component analysis and hierarchal clustering and biostatistical methods such as ANOVA	http://smbl.nus.edu.sg/METDAT/
MSFACTs	Includse spectral formatting, aligning and conversion tools	Duran et al. (2003), http://www.noble.org/PlantBio/MS/MSFACTs/MSFACTs.html
XCMS	Incorporates data preprocessing such as nonlinear retention time alignment, matched filtration, peak detection and peak matching	http://metlin.scripps.edu/
Dragon Plant Biology Explorer (DPBE)	A knowledge-mining tool that extracts information and organizes it for easy interpretation	Bajic et al. (2005), http://research.i2r.a-star.edu.sg/DRAGON/ME2/
Databases		
National Institute of Standards and Technology (NIST)	Provides information on the molecular weight, formula, structure as well as mass spectrum	http://webbook.nist.gov
Kyoto Encyclopedia of Genes and Genomes (KEGG)	Molecular interaction networks in biological processes	Kanehisa et al. (2004), http://www.genome.ad.jp/kegg/
MetaCyc	Elucidated pathways from 600 organisms	Zhang et al. (2005), http://metacyc.org/
AraCyc	Biochemical pathways of *Arabidopsis* developed at The *Arabidopsis* Information Resource	Mueller et al. (2003), http://www.arabidopsis.org

(continued)

Table 3.2 (continued)

	Application	Reference and Web site
BioPathAt	Knowledge-based analysis of genome-scale data by integrating biochemical pathway maps	Lange and Ghassemian (2005)
LIGAND	Consists of the following databases: COMPOUND, GLYCAN, REACTION and ENZYME	Goto et al. (2002), http://www.genome.ad.jp/dbget/ligand.html
BRITE	Biomolecular relations in information transmission and expression database	Kanehisa et al. (2006), http://www.genome.ad.jp/kegg/brite.html
Alliance for Cellular Signalling (AFCS)	Provides information on signal transduction	http://www.signaling-gateway.org/
PathDB	A metabolic pathway database	http://www.ncgr.org/pathdb/

ANOVA analysis of variance

metabolism (including photosynthesis), secondary metabolism, as well as associated compounds, enzymes and genes. It is available at http://metacyc.org/.

- AraCyc. This is a database containing biochemical pathways of *Arabidopsis* developed at The *Arabidopsis* Information Resource (http://www.arabidopsis.org) with the aim of representing *Arabidopsis* metabolism using a Web-based interface (Mueller et al. 2003). This database now contains 197 pathways that include information on compounds, metabolic intermediates, cofactors, reactions, genes, proteins and protein subcellular locations. The Web site also has an "omics viewer" that allows users to upload the data onto a pathway chart and then visualize the variations in the data and map the pathway changes in a visual form.
- The Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.ad.jp/kegg/) is a complex set of databases, and includes knowledge on molecular interaction networks in biological processes (PATHWAY database), knowledge of genes and proteins (GENES/SSDB/KO databases) and knowledge of the chemical compounds and reactions (COMPOUND/GLYCAN/REACTION databases). KEGG currently covers 15,037 pathways, of which 229 are reference pathways. It has genome information of 181 organisms and catalogs 646,192 genes with ortholog clusters known for 33,305 of the genes. It describes 6,000 chemical reactions and links to 10,000 chemical compounds.
- BioPathAt. This newly developed visual interface allows the knowledge-based analysis of genome-scale data by integrating biochemical pathway maps (BioPathAtMAPS module) with a manually scrutinized gene-function database (BioPathAtDB) for the model plant *Arabidopsis thaliana* (Lange and Ghassemian 2005).

Often there is great discord in the data generation and analysis methods of different laboratories or even during different experiments, which may lead to misinterpretation of the data or irreproducible data. In order to have uniformity in the data published and also for efficient analysis and recording, The Standard Metabolic Reporting Structures (SMRS) group (http://www.smrsgroup.org/) has been set up to provide guidelines for reporting metabolomic studies. The guidelines pertain to three main areas: (1) origin of the biological sample, (2) analytical methods used in the analysis of the material and (3) the multivariate statistical methods (chemometrics) used to retrieve information from the sample data (The Standard Metabolic Reporting Structures Working Group 2005). Further, to enhance the accuracy and descriptions of the methods and experiments, a framework for plant metabolomics called ArMet (Architecture for Metabolomics) has been created (Jenkins et al. 2004). It provides the entire experimental timeline from the sample preparation to data analysis. Such data models will help in comparison of data sets, allow proper interpretation of the results and repetition of results. It gives a basis for storage and transmission of data.

We have provided a brief introduction to the available analytical methods, the bioinformatics tools and the databases available for metabolomics with examples related to rhizosphere metabolomics. We shall now describe the different events in the rhizosphere such as bioconversions that have been deduced with the help of these techniques and tools.

3.4 Bioconversions of Rhizosphere Metabolites

Bioconversion helps in the generation of energy and also signaling molecules for intracellular and intercellular functions. How soil bacteria transform these molecules (bioconversion) from the environment to generate energy and use it for growth and other purposes is very interesting. Bacteria take most of the starting materials from the living environment by involving processes such as biotransformation, biocatalysis or biodegradation. While these terms seem different, they refer to the same group of processes, namely, bioconversion or microbial metabolism. The use of the term depends on what is being studied and more often is based on the intended focus of the study. "Microbial metabolism" from an industrial application viewpoint refers to the process as biotransformation or employing biocatalysis. If the study concerns degrading environmental pollutants or organic compounds, it is commonly referred to as "biodegradation." In this section, we shall refer to the process collectively as "bioconversion" for it may involve one of the abovementioned process or a combination of them, depending on the environment and its inhabitants. Bioconversions have been known since the days of Louis Pasteur (1857); however, a renewed interest in biotransformation was witnessed only in the late twentieth century. This is partly due to interests in developing a sustainable environment coupled with a healthier life.

Plants release chemicals into the rhizosphere; they can positively or negatively regulate growth and development of the microenvironment, including the rhizosphere (Rice 1984). Aromatic hydrocarbons, including many plant phenolics, are ubiquitous in nature. Plant phenolics, including quinines, are the most common class of subterranean allelochemicals (Inderjit 1996). Indeed, next to glucosyl residues, the benzene ring is the most widely distributed chemical structure in nature (Dagley 1981) and therefore they are the second largest group of natural products, including many plant metabolites released into the rhizosphere. Some of the common aromatic hydrocarbons released into the rhizosphere are flavonoids (Narasimhan et al. 2003), members of the phenylpropanoid group of compounds; additionally contamination of vegetated soils can also expose rhizobacterial populations to pollutants such as naphthalene, phenanthrene, fluoranthene and benzo[*a*]pyrene. Although the signature benzene ring of aromatic hydrocarbons is commonly found in many of these compounds, their breakdown involves several complex metabolic networks. These compounds, therefore, signal developmental events in the roots of some plants that enhance their growth, indicating some active events that occur in the rhizosphere. These events are centered on the process of bioconversion/biotransformation. Interestingly, to cope with the complexity of metabolizing these aromatic compounds, microorganisms have adopted two simple but fundamentally different strategies: aerobic and anaerobic mechanisms. Under aerobic conditions, aromatic compounds are generally transformed by monooxygenases and dioxygenases into a few central intermediates such as catechol, protocatechuate and gentisate. This phenomenon is commonly referred to as "funneling pathways." Under anoxic (anaerobic) conditions, aromatic compounds need to be transformed by means other than by oxygenases, more so owing to the absence of oxygen, thus reductively attacking the ring structure via an ATP-dependent mechanism.

3.4.1 Metabolite Conversion Pathways in Bacteria

Bacteria possess the unique ability to break down ring-containing compounds that are resistant to degradation. The first representation of the metabolic pathway and the enzymatic reactions resulting in the mineralization of aromatic compounds (naphthalene in this study) was by Davies and Evans (1964). Since then, metabolite conversion by microbes, including bacteria, fungi and algae, has been extensively studied (Kuiper et al. 2004). Conversion of metabolites, for instance, compounds with more than three fused rings, is less ubiquitous, but the number of organisms found to degrade these aromatics is increasing (Kanaly and Harayama 2000). For some of the aromatic and complex compounds with more than three fused rings, "cometabolism" often serves as the main route for degradation. Another notable aspect of most aromatics that could influence bioconversion includes their hydrophobicity, which in turn depends on the number of fused rings, and their relative water solubility is low. Generally, the uptake of aromatic compounds by bacteria proceeds via the water phase and hence their water solubility may also be an important aspect, at least in the context of bioavailability of metabolites. Some microbes are able to produce biosurfactants that may be involved in enhanced bioavailability of metabolites for further conversion. Bioconversion involves the breakdown of metabolites through one of the abovementioned processes. Metabolites and/or often-recalcitrant compounds are transformed into less complex metabolites or through mineralization into inorganic minerals, water and carbon dioxide (in the case of aerobic pathways) or methane (in anaerobic pathways). The general ability of bacteria to use aromatic or ring-containing compounds such as the plant phenolics, halogenated hydrocarbons and others is related to the fact that most of these compounds are commonly present in the environment as a result of plant-derived material (Harwood and Parales 1996). By contrast, man-made compounds have been in contact for over 100 years only, and as a result their breakdown and properties are less well characterized. Two major routes of bioconversion, i.e., aerobic and anaerobic, and the peripheral and central pathways are discussed in the following sections.

Aerobic and anaerobic pathways have similarities and yet significant differences (Table 3.3). In the aerobic catabolic funnel, most peripheral pathways involve oxygenation reactions carried out by monooxygenases and/or hydroxylating dioxygenases that generate dihydroxy aromatic compounds (such as catechol, protocatechuate, gentisate, homoprotocatechuate, homogentisate, hydroquinone and hydroxyquinol) (Gibson and Subramanian 1984). These intermediate compounds are the substrates of ring-cleavage enzymes that use molecular oxygen to open the aromatic ring between the two hydroxyl groups (*ortho* cleavage, catalyzed by intradiol dioxygenases) or proximal to one of the two hydroxyl groups (*meta* cleavage, catalyzed by extradiol dioxygenases) (Harayama and Timmis 1992). Central pathways, therefore, involve a series of reactions leading to the formation of Krebs cycle intermediates (central metabolism) that are further easily converted to tricarboxylic acid (TCA) cycle intermediates (ven der Meer et al. 1992). In the

Table 3.3 Comparison of aerobic and anaerobic aromatic metabolism pathways

Features	Aerobic	Anaerobic
Channeling reactions	+ O_2	+H_2O, +2[H], −2[H] + H_2O + CO_2, + CoA + ATP
Central intermediates	Catechol, gentisate, protocatechuate	Benzoyl-CoA, resorcinol, phloroglucinol
Properties of central intermediates	Easy to oxidize (cleave)	Easy to reduce (hydrate)
Attack at the ring	O_2	2 or 4[H] (H_2O)
Ring cleavage	Oxygenolysis of aromatic	Hydrolysis of 3-oxo compound
Pathway to central metabolites	3-Oxoadipate pathway, e.g., → succinate + acetyl-CoA	Oxidation, e.g., → glutaryl-CoA → acetyl-CoA

CoA Coenzyme A

anaerobic catabolism of aromatic compounds, the peripheral pathways converge mostly to benzoylcoenzyme A and occasionally to resorcinol and phloroglucinol, which become dearomatized by a specific multicomponent reductase that requires energy in the form of ATP (Gibson and Harwood 2002). At times, the rhizosphere can have partially to completely anaerobic (anoxic) conditions, depending on the soil characteristics (such as compactness and waterlogging conditions). Under anoxic (anaerobic) conditions, aromatic compounds need to be transformed by means other than by oxygenases, more so owing to the absence of oxygen (early studies by Tarvin and Buswell 1934; Dutton and Evans 1967), implying that the aromatic-ring structures are reductively attacked (Dutton and Evans 1969; Evans and Fuchs 1988). It should be noted, however, that there is limited knowledge on anaerobic degradation of polymeric high molecular weight aromatics such as lignins, which could represent probably more than half of the aromatic compounds (Young and Frazer 1987).

The lignin pathway branches out from the initial steps of the phenylpropanoid biosynthesis pathways, which is well known for the production of flavonoids. These groups of compounds are released as rhizosecretion and they influence rhizobacterial populations and competition. The bioconversions of these rhizosecretions are briefly discussed here. Rhizobia and *Agrobacterium* that are capable of degrading *nod* gene-inducing flavonoids have been reported (Rao and Cooper 1994) A *Rhodococcus rhodochrous* strain has been described as being capable of styrene degradation (Warhurst et al. 1994). Microbial enzymes with wide substrate specificity are certain to provide better survival benefits to those harboring the enzymes than those that do not.

3.4.2 Case Study: Bioconversion of Flavonoids

Flavonoids are ubiquitous in the plant kingdom and in the rhizosphere and are also an integral part of the human diet (Hollman et al. 1997). Understandably,

several microbial genera are known to participate in the breakdown of aromatic compounds, including phenylpropanoids. These have been reported from two ecological niches, viz., soil and intestines, and include rhizobia, *Agrobacterium tumefaciens*, a thermophilic *Bacillius* sp., *Pseudomonas* sp., a *Rhodococcus* strain and a strain of the fungus *Aspergillus niger*. Other degraders are from the anoxic environment of the intestine and include *Clostridium* strains, *Eubacterium* species and *Butyrivibrio* species. Flavonoid degradation pathways have been well studied in the intestinal flora. As in most other cases involving degradation of aromatics, studies on uptake and detection of intermediates and accumulation of end products have generally lead to the elucidation of the pathway (Chang and Zylstra 1998; Bode et al. 2000).

Several rhizobia, including the lotus rhizobia, can degrade quercetin via a novel form of ring cleavage, yielding phloroglucinol and protocatechuic acid (Rao et al. 1991; Rao and Cooper 1994). Hopper and Mahadevan (1991) reported the degradation of catechin by *Bradyrhizobium japonicum*, which was cleaved through an inducible catechin oxygenase to yield phloroglucinolcarboxylic acid and protocatechuic acid as the initial products that were further decarboxylated to phloroglucinol and dehydrated to resorcinol. Phenylpropanoid degradation by a soil pseudomonad and the presence of new oxygenases in the degradation of flavones and flavonones by *Pseudomonas putida* suggests that degradation occurred by a fission in the A-ring, via hydroxylation at C-8 (Shultz et al. 1974). A common pathway for the degradation of flavones and flavonones by *Pseudomonas putida* is generally accepted, where it is converted to protocatechuate and/or catechol, which is further cleaved via the β-ketoadipate pathway, resulting in the formation of oxaloacetic acid. Oxaloacetate could be routed though the TCA cycle for further metabolism and energy generation. A more detailed flavonoid mineralization pathway in a plant-growth-promoting rhizobacterial strain was recently described by Pillai and Swarup (2002) (Fig. 3.3). Using a comparative metabolomics approach with wild-type and flavonoid auxotrophic strains, they elucidated the metabolic pathway. The archetypal flavonoid quercetin was used in the study to monitor the degradation events in the soil pseudomonad. Quercetin was converted to naringenin and then to dihydroxy aromatic compounds that could be cleaved by ring-cleavage oxygenases and led to formation of single-ring compounds such as protocatechuate. As seen from Fig. 3.3, at least two intermediate compounds are produced during the flavonoid bioconversion before production of compounds that enter the TCA cycle. In addition to flavonoids, various intermediates have been identified with different phenolics as carbon sources in the environment. Phenolic degradation pathways produce acids that are partly subjected to further degradation and the phenolics detected over time may not be consistent. Jeffrey et al. (1972a) have reported degradation of taxifolin involving hydroxylation of its A-ring in a pseudomonad, while in another study, also involving a soil pseudomonad, they reported the oxidative fission of the A-ring of dihydrogossypetin (Jeffrey et al. 1972b). This shows the existence of a variety of catabolic metabolisms for different compounds, all in a single bacterial species. Such versatility of soil microbes allows speculation of the existence of novel metabolic regulatory pathways in these strains.

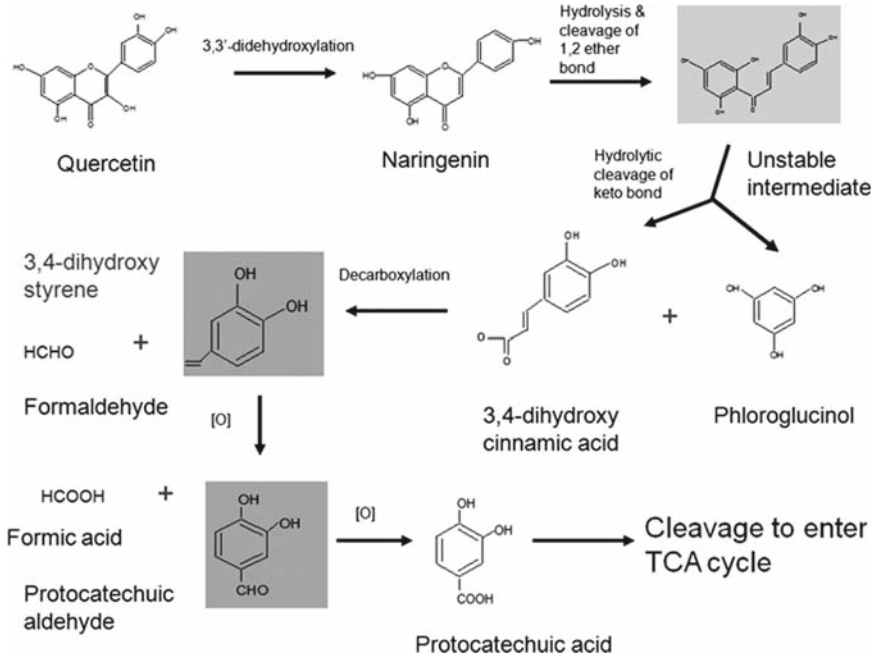

Fig. 3.3 Phenylpropanoid degradation pathway in phenylpropanoid utilizing strains of *Pseudomomas putida*. Quercetin degradation pathway in *P. putida* strain PML2. The identities of all compounds except compounds III, V and VI were confirmed by NMR spectroscopy. All compounds are stably formed except for compound III. Hydrolysis and cleavage of ether and keto bonds and the presence of an unstable intermediate (compound III) were inferred on the basis of the structures of compounds II and IV

3.5 Applications of Rhizosphere Metabolomics

Rhizoremediation involves the use of plants as well as rhizobacteria to clean up contaminated soil and water. Two processes have been described to constitute rhizoremediation, namely, *phytoremediation* and *bioaugmentation* (Kuiper et al. 2004). "Bioaugmentation" refers to enhanced availability of a substrate using specific microbes. Microbial degradation of the pollutants is enhanced owing to stimulation of root exudates. The root system of plants aid in spreading the rhizobacteria through soil and help to penetrate otherwise impermeable soil layers. Pollutant-degrading bacteria can be inoculated on plant seeds to improve the efficiency of phytoremediation or bioaugmentation.

Phytoremediation of polyaromatic hydrocarbons (PAHs) is driven by root–microbe interactions (Rugh et al. 2005). Bacterial degradation has been shown to be the dominant pathway for environmental PAH dissipation. The authors tested various plant species and the efficacy in degrading PAHs. It was found that in soils that were planted, there was an increase in heterotrophic and biodegradative cell numbers compared with the situation in unplanted soils. The study showed that the expanded

metabolic range of the rhizosphere bacterial community would contribute more to effective degradation of PAHs.

Plants can be genetically engineered so as to create a biased rhizosphere. This is possible by enhancing the growth of selected microbial species which can help in increasing the biodegradation capacity of the soil. Plants can be engineered such that they are resistant to soil-borne pathogens, are better hosts to beneficial microorganisms, can remediate toxic waste or can attract communities of soil microorganisms that enhance plant health (O'Connel et al. 1996). Engineering plants which exudate specific nutrients that enhance the growth of specific microorganisms helps in creating a biased rhizosphere that is more efficient in biodegradation (Fig. 3.4). For example, the genetically engineered plants that produce opines could change the bacterial populations in soil (Oger et al. 1997). Degradation of environmental pollutants can be enhanced in the rhizosphere by microorganisms that can utilise root exudates as carbon source. For example, biodegradation of polychlorinated biphenyls (PCBs) can be enhanced by growth of PCB-degrading rhizobacteria along with plants that can exude phenylpropanoids. The rhizobacteria are able to utilize phenylpropanoids and hence are able to grow better in the rhizosphere as there is less competition for compounds like phenylpropanoids from other

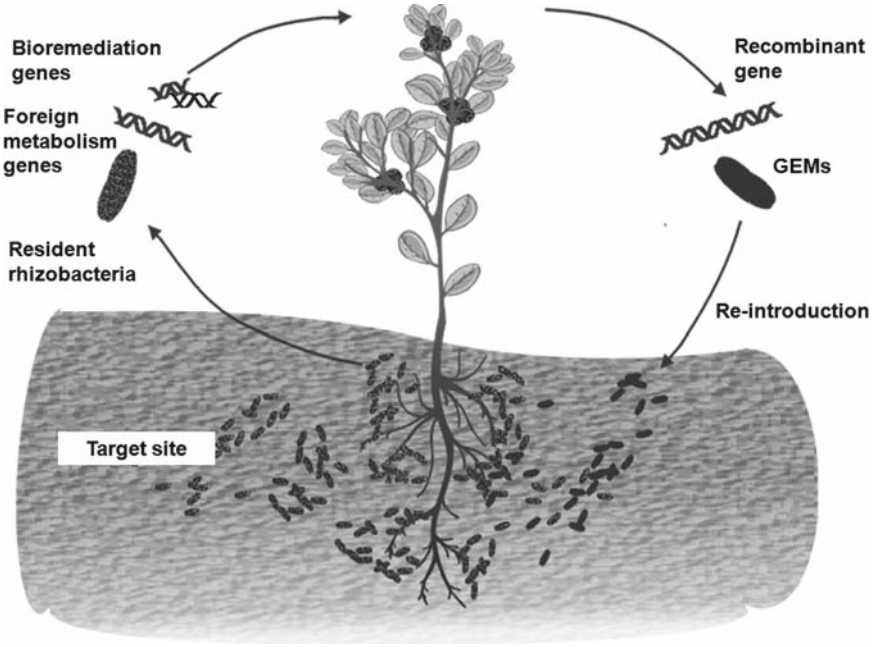

Fig. 3.4 Rhizoengineering approach to improve bioremediation efficiency. Resident rhizobacteria that can mineralize phenylpropanoids for their growth can be isolated from polluted site. They can be modified by transfer of bioremediation genes/pathway. The genetically modified microorganisms (*GEMs*) thus produced can be reintroduced to the rhizosphere, where they will be competitive for growth and perform bioremediation efficiently

microorganisms. Since they are able to grow better, the efficiency of PCB degradation is increased (Narasimhan et al. 2003). This method has the advantage that it does not rely on genetic engineering of plants as in the case of the previous methods. Plant-assisted rhizoremediation in the long run will turn into an effective mode for rhizoremediation of toxic organic pollutants. At petroleum hydrocarbon contaminated sites, two genes encoding hydrocarbon degradation, alkane monooxygenase (*alkB*) and naphthalene dioxygenase (*ndoB*), were 2 and 4 times more prevalent in bacteria extracted from the root interior (endophytic) than from the bulk soil and sediment, respectively (Siliciano et al. 2001). These results indicate that the enrichment of catabolic genotypes in the root interior is both plant-dependent and contaminant-dependent.

3.6 Conclusions

Metabolomics has emerged as the final frontier in functional genomics. The field has broad applications in understanding the composition and interactions of the rhizosphere. Although there are certain limitations in rhizosphere metabolomics in its present state, these are likely to be addressed as the field becomes more widely appreciated. Some of the techniques used for studying plant or animal metabolism can be extended to the rhizosphere as well. A number of analytical tools for the separation of metabolites are available for metabolomics researchers. However, metabolite databases and tools for processing and analyzing the data need further improvement. Future directions in this field are likely to be (1) in methods development, (2) identifying signaling molecules that originate from both plants as well as rhizosphere microbiota and (3) understanding the role of rhizosphere metabolites in affecting plant growth and physiological functioning.

References

Bajic VB, Veronika M, Veladandi PS, Meka A, Heng M-W, Rajaraman K, Pan H, Swarup S (2005) Dragon Plant Biology Explorer. A text-mining tool for integrating associations between genetic and biochemical entities with genome annotation and biochemical terms lists. Plant Physiol 138:1914–1925

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57:233–266

Bending GD, Poole EJ, Whipps JM, Read DJ (2002) Characterisation of bacteria from Pinus sylvestris-Suillus luteus mycorrhizas and their effects on root-fungus interactions and plant growth. FEMS Microbiol Ecol 39:219–227

Bhalla R, Narasimhan K, Swarup S (2005) Metabolomics and its role in understanding cellular responses in plants. Plant Cell Rep 24:562–571

Bode HB, Zeeck A, Plückhahn K, Jendrossek D (2000). Physiological and chemical investigtions into microbial degradation of synthetic poly (cis-1,4-isoprene). Appl Environ Microbiol 66:3680–3685

Boersma MG, Solyanikova IP, Van Berkel WJ, Vervoort J, Golovleva LA, Rietjens IM (2001) 19F NMR metabolomics for the elucidation of microbial degradation pathways of fluorophenols. J Ind Microbiol Biotechnol 26:22–34

Bonnington L, Eljarrat E, Guillamón M, Eichhorn P, Taberner A, Barceló D (2003) Development of a liquid chromatography-electrospray-tandem mass spectrometry method for the quantitative determination of benzoxazinone derivatives in plants. Anal Chem 75:3128–3136

Cataldi TRI, Margiotta G, Iasi L, Di Chio B, Xiloyannis C, Bufo SA (2000) Determination of Sugar Compounds in olive plant extracts by anion-exchange chromatography with pulsed amperometric detection. Anal Chem 72:3902–3907

Chang H-K, Zylstra GJ (1998) Novel organization of the genes for phthalate degradation from *Burkholderia cepacia* DBO1. J Bacteriol 180:6529–6537

Chin-A-Woeng TFC, van den Broek D, Lugtenberg BJJ, Bloemberg GV (2005) The *Pseudomonas* chlororaphis PCL1391 sigma regulator psrA represses the production of the antifungal metabolite phenazine-1-carboxamide. Mol Plant Microbe Interact 18:244–253

Czarnota MA, Rimando AM, Weston LA (2003). Evaluation of root exudates of seven sorghum accessions. J Chem Ecol 29(9):2073–2083

Dagley S (1981) New perspectives in aromatic catabolism. In: Leisinger T, Cook AM, Hütter R, Nüesch J (eds) Microbial degradation of xenobiotics and recalcitrant compounds. Academic, New York, pp 181–186

Davies JI, Evans WC (1964) Oxidative metabolism of naphthalene by soil pseudomonads. Biochem J 91:251–261

Derrien D, Balesdent J, Marol, Santaella C (2003) Measurement of the 13C/12C ratio of soil-plant individual sugars by gas chromatography/combustion/isotope-ratio mass spectrometry of silylated derivatives. Rapid Commun Mass Spectrom 17:2626–2631

Desbrosses GG, Kopka J, Udvardi MK (2005) Lotus japonicus metabolic profiling. Development of gas chromatography-mass spectrometry resources for the study of plant-microbe interactions. Plant Physiol 137:1302–1318

Dunn WB, Ellis DI (2005) Metabolomics: current analytical platforms and methodologies. Trends Anal Chem 24:285–294

Dunn WB, Bailey NJ, Johnson HE (2005) Measuring the metabolome: current analytical technologies. Analyst 130:606–625

Duran AL, Yang J, Wang L, Sumner LW (2003) Metabolomics spectral formatting, alignment and conversion tools (MSFACTs). Bioinformatics 19:2283–2293

Dutton PL, Evans WC (1967). Dissimilation of aromatic substrates by *Rhodopseudomonas palustris*. Biochem J 104:30–31

Dutton PL, Evans WC (1969) The metabolism of aromatic compounds by *Rhodopseudomonas palustris*: a new reductive method of aromatic ring metabolism. Biochem J 113:525–536

Eljarrat E, Barcelo D (2001) Sample handling and analysis of allelochemical compounds in plants. Trends Anal Chem 20:584–590

Evans CJ, Evershed RP (2003) Compound-specific stable isotope analysis of soil mesofauna using thermally assisted hydrolysis and methylation for ecological investigations Anal Chem 75:6056–6062

Evans WC, Fuchs G (1988) Anaerobic degradation of aromatic compounds. Annu Rev Microbiol 42:289–317

Fan TWM, Lane AN, Shenker M, Bartley JP, Crowley D, Higashi RM (2001) Comprehensive chemical profiling of gramineous plant root exudates using high resolution NMR and MS. Phytochemistry 57:209–221

Formanek P, Ambus P (2004) Assessing the use of delta C-13 natural abundance in separation of root and microbial respiration in a Danish beech (*Fagus sylvatica* L.) forest. Rapid Commun Mass Spectrom 18:897–902

Gibson DT, Subramanian V (1984) Microbial degradation of aromatic hydrocarbons. In Gibson DT (ed) Microbial degradation of organic compounds. Dekker, New York, pp 181–252

Gibson J, Harwood CS (2002) Metabolic diversity in aromatic compound utilization by anerobic microbes. Annu Rev Microbiol 56:345–369

Gleye C, Laurens A, Hocquemiller R, Cave A, Laprevote O, Serani L (1997) Isolation of montecristin, a key metabolite in biogenesis of acetogenins from *Annona muricata* and its structure elucidation by using tandem mass spectrometry. J Org Chem 62:510–513

Goodacre R (2005) Making sense of the metabolome using evolutionary computation: seeing the wood with the trees. J Exp Bot 56:245–254

Goodacre R, Shann B, Gilbert RJ, Timmins EM, McGovern AC, Alsberg BK, Kell DB, Logan NA (2000) Detection of the dipicolinic acid biomarker in Bacillus spores using Curie-point pyrolysis mass spectrometry and Fourier transform infrared spectroscopy. Anal Chem 72:119–127

Goto S, Okuno Y, Hattori M, Nishioka T, Kanehisa M (2002) LIGAND: database of chemivcal compounds and reactions in biological pathways. Nucleic Acids Res 30:402–404

Griffin JL (2004) Metabolic profiles to define the genome: can we hear the phenotypes? Philos Trans R Soc Lond B Biol Sci 359:857–871

Harayama S, Timmis KN (1992) Aerobic biodegradation of aromatic hydrocarbons by bacteria. In: Sigel H, Sigel A (eds) Metal ions in biological systems, vol 28. Dekker, New York, pp 99–156

Harwood CS, Parales RE (1996) The B-ketoadipate pathway and the biology of self-identity. Annu Rev Microbiol 50:553–590

Hollman PCH, van Trijp JMP, Buysman MNCP, Gaga MSVD, Mengelers MJB, Vries JHM, Katan MB (1997) Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. FEBS Lett 418:152–156

Hopper W, Mahadevan A (1991) Utilization of catechin and its metabolites by *Bradyrhizobium japonicum*. Appl Microbiol Biotechnol 35:411–415

Huang WE, Griffiths RI, Thompson IP, Bailey MJ, Whiteley AS (2004) Raman microscopic analysis of single microbial cells. Anal Chem 76:4452–4458

Inderjit (1996) Plant phenolics in allelopathy. Bot Rev 62:186–202

Inderjit, Duke SO (2003) Ecophysiological aspects of allelopathy. Planta 217:529–539

Jansen JJ, Hoefsloot HCJ, Boelens HFM, van der Greef J, Smilde AK (2004) Analysis of longitudinal metabolomics data. Bioinformatics 20:2438–2446

Jeffrey AM, Knight M, Evans WC (1972a) The bacterial degradation of flavonoids: hydroxylation of the A-ring of taxifolin by a soil pseudomonad. Biochem J 130:373–380

Jeffrey AM, Jerina DM, Self R, Evans WC (1972b) The bacterial degradation of flavonoids: oxidative fission of the A-ring of dihydrogossypetin by a *Pseudomonas* sp. Biochem J 130:383–390

Jenkins H, Hardy N, Beckmann M, Draper J, Smith A, Taylor J et al. (2004) A proposed framework for the description of plant metyabolomics experiments and their results. Nat Biotechnol 22:1601–1607

Jonsson P, Gullberg J, Nordstrom A, Kusano M, Kowalczyk M, Sjostrom M, Moritz T (2004) A strategy for identifying differences in large series of metabolomic samples analyzed by GC/MS. Anal Chem 76:1738–1745

Jonsson P, Bruce SJ, Moritz T, Trygg J, Sjöström M, Plumb R, Granger J, Maibaum J, Nicholson JK, Holmes E, Antti H (2005) Extraction, interpretation and validation of information for comparing samples in metabolic LC/MS data sets. Analyst 130:701–707

Kachlicki P, Marczak L, Kerhoas L, Einhorn J, Stobiecki M (2005) Profiling isoflavone conjugates in root extracts of lupine species with LC/ESI/MSn systems. J Mass Spectrom 40:1088–1103

Kanaly RA, Harayama S (2000) Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol 182:2059–2067

Kanehisa M, Goto S, Kawashima S, Okuno Y, Hattori M (2004) The KEGG resource for deciphering the genome Nucleic Acids Rese 32:D277–D280

Kanehisa M, Goto S, Hattori M, Aoki-Kinoshita KF, Itoh M, Kawashima S, Katayama T, Araki M, Hirakawa M (2006) From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res 34:D354–D357

Katajamaa M, Oresic M (2005) Processing methods for differential analysis of LC/MS profile data. BMC Bioinformatics 6:179

Kerry BR (2000) Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant parasitic nematodes. Annu Rev Phytopathol 38:423–441
Krishnan P, Kruger NJ, Ratcliffe RG (2005) Metabolite fingerprinting and profiling in plants using NMR. J Exp Bot 56:255–265
Kuiper I, Lagendijk EL, Bloemberg GV, Lugtenberg BJJ (2004) Rhizoremediation: a beneficial plant-microbe interaction. Mol Plant Microbe Interact 17 6–15
Lange BM, Ghassemian M (2005) Comprehensive post-genomic data analysis approaches integrating biochemical pathway maps. Phytochemistry 66:413–451
López-Díez EC, Goodacre R (2004) Characterization of microorganisms using UV resonance raman spectroscopy and chemometrics. Anal Chem 76:585–591
Martens H, Naes T (1989) Multivariate calibration. Wiley, New York
Mendes P (2002) Emerging bioinformatics for the metabolome. Brief Bioinformatics 3:134–145
Menotta M, Gioacchini AM, Amicucci A, Buffalini M, Sisti D, Stocchi V (2004) Headspace solid-phase microextraction with gas chromatography and mass spectrometry in the investigation of volatile organic compounds in an ectomycorrhizae synthesis system. Rapid Commun Mass Spectrom 18:206–210
Mesnard F, Ratcliffe RG (2005) NMR analysis of plant nitrogen metabolism. Photosynth Res 83:163–180
Mueller LA, Zhang P, Rhee SY (2003) AraCyc: a biochemical pathway database for *Arabidopsis*. Plant Physiol 132:453–460
Mukerji KG, Manoharachary C, Singh J (eds) (2006) Microbial activity in the rhizosphere. Soil biology, vol 7, Springer, Heidelberg
Narasimhan K, Basheer C, Bajic VB, Swarup S (2003) Enhancement of plant-microbe interactions using a rhizosphere metabolomics-driven approach and its application in the removal of polychlorinated biphenyls. Plant Physiol 132:146–153
O'Connell KP, Goodman RM, Handelsman J (1996) Engineering the rhizosphere: expressing a bias. Trends Biotechnol 14:83–88
Oger P, Petit A, Dessaux Y (1997) Genetically engineered plants producing opines alter their biological environment. Nature biotechnology 15:369–372
Pasteur L (1857) Mémoire sur la fermentation appelée lactique. Mém Soc Sci Agric Arts 5:13–37
Perera MR, Vanstone VA, Jones MGK (2005) A novel approach to identify plant parasitic nematodes using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 19:1454–1460
Pfeffer PE, Rolin DB, Schimdt JH, Tu SI, Kumosinski TF, Douds DD (1992) Ion transport and subcellular compartmentation in maize root tissue as examined by in vivo CS-133 NMR spectroscopy. J Plant Nutr 15:913–927
Pillai BVS, Swarup S (2002) Elucidation of the flavonoid catabolism pathway in *Pseudomonas putida* PML2 strain by comparative metabolic profiling. Appl Environ Microbiol 68:143–151
Pinton R, Varanni Z, Nannipier Pi, Willig W (eds) (2000) The rhizosphere: biochemistry and organic dubstance at the soil-plant interface. Dekker, New York
Rao RJ, Cooper JE (1994) *Rhizobia* catabolize nod gene-inducing flavonoids via C-ring fission mechanisms. J Bacteriol 176:5409–5413
Rao RJ, Sharma ND, Hamilton JTG, Boyd DR, Cooper JE (1991) Biotransformation of the pentahydroxy flavone quercetin by *Rhizobium loti* and *Bradyrhizobium* strains (Lotus). Appl Environ Microbiol 57:1563–1565
Ratcliffe RG, Shachar-Hill Y (2005) Revealing metabolic phenotypes in plants: inputs from NMR analysis. Biol Rev Camb Philos Soc 80:27–43
Reo NV (2002) NMR-based metabolomics. Drug Chem Toxicol 25:375–382
Rice EL (1984) Allelopathy 2nd edn. Academic, Orlando
Rugh CL, Susilawati E, Kravchenko AN, Thomas JC (2005) Biodegrader metabolic expansion during polyaromatic hydrocarbons rhizoremediation. Z Naturforsch C 60:331–339
Ryals J (2004) Drug discovery metabolomics. Metabolomics–an important emerging science. Business briefing: Pharmatech 51–54

Shultz E, Engle FE, Wood JM (1974) New oxygenases in the degradation of flavones and flavonones by *Pseudomonas putida*. Biochemistry 13:1768–1776

Siciliano SD, Fortin N, Mihoc A, Wisse G, Labelle S, Beaumier D, Ouellette D, Roy R, Whyte LG, Banks MK, Schwab P, Lee K, Greer CW (2001) Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Appl Environ Microbiol 67:2469–2475

Smilde AK, Jansen JJ, Hoefsloot HCJ, Lamers R-J AN, van der Greef J, Timmerman ME (2005) ANOVA-Simultaneous component analysis (ASCA): a new tool for analyzing designed metabolomics data. Bioinformatics 21:3043–3048

Spaink HP, Wijfjes AHM, Vanvliet TB, Kijne JW, Lugtenberg BJJ (1993) Rhizobial lipo-oligosaccharide signals and their role in plant morphogenesis are analogous lipophilic chitin derivatives produced by the plant. Aust J Plant Physiol 20:381–392

Steeghs M, Bais HP, de Gouw J et al. (2004) Proton-transfer-reaction mass spectrometry as a new tool for real time analysis of root-secreted volatile organic compounds in *Arabidopsis*. Plant Physiol 135:47–58

Sumner LW, Mendes P, Dixon RA (2003) Plant metabolomics: large-scale phytochemistry in the functional genomics era Phytochemistry 62:817–836

Tarvin D, Buswell AM (1934) The methane fermentation of organic acids and carbohydrates. J Am Chem Soc 56:1751–1755

The Standard Metabolic Reporting Structures Working Group (2005) Summary Recommendations for standardization and reporting of metabolic analyses. Nat Biotechnol 23:833–839

van der Meer JR, de Vos WM, Harayama S, Zehnder AJB (1992) Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol Rev 56:677–694

van der Werf MJ, Jellema RH, Hankemeier T (2005) Microbial metabolomics: replacing trial-and-error by the unbiased selection and ranking of targets. J Ind Microbiol Biotechnol 32:234–252

Villas-Boas SG, Rasmussen S, Lane GA (2005) Metabolomics or metabolite profiles? Trends Biotechnol 23:385–386

Walker TS, Bais HP, Halligan KM, Stermitz FR, Vivanco JM (2003) Metabolic profiling of root exudates of *Arabidopsis thaliana*. J Agric Food Chem 51:2548–2554

Warhurst AM, Clarke KF, Hill RA, Holt RA, Fewson CA (1994) Metabolism of styrene by *Rhodococcus rhodochrous* NCIMB 13259. Appl Environ Microbiol 60:1137–1145

Weir TL, Park SW, Vivanco JM (2004) Biochemical and physiological mechanisms mediated by allelochemicals. Curr Opin Plant Biol 7:472–479

Weidenhamer JD (2005). Biomimetic measurement of allelochemical dynamics in the rhizosphere. J Chem Ecol 31(2):221–236

Sun W, Liu S, Liu Z, Song F, Fang S (1998) A study of *Aconitum* alkaloids from aconite roots in *Aconitum carmichaeli* Debx using matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom 12:821–824

Wittig U, De Beuckelaer A (2001) Analysis and comparison of metabolic pathway databases. Brief Bioinformatics 2:126–142

Young LY, Frazer AC (1987) The fate of lignin and lignin-derived compounds in anaerobic ecosystems. Geomicrobiol J 5:261–293

Zhang P, Foerster H, Tissier CP, Mueller L, Paley S, Karp PD, Rhee SY (2005) MetaCyc and AraCyc. Metabolic pathway databases for plant research. Plant Physiol 138:27–37