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## Review Article

# Metabolomics as an emerging tool to study plant–microbe interactions

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In natural environments, interaction between plant roots and microorganisms are common. These interactions between microbial species and plants inhabited by them are being studied using various techniques. Metabolomics research based on mass spectrometric techniques is one of the crucial approaches that underpins system biology and relies on precision instrument analysis. In the last decade, this emerging field has received extensive attention. It provides a qualitative and quantitative approach for determining the mechanisms of symbiosis of bacteria and fungi with plants and also helps to elucidate the tolerance mechanisms of host plants against various abiotic stresses. However, this -omics application and its tools in plant–microbe interaction studies is still underutilized compared with genomic and transcriptomic methods. Therefore, it is crucial to bring this field forward to bear on the study of plant resistance and susceptibility. This review describes the current status of methods and progress in metabolomics applications for plant–microbe interaction studies discussing current challenges and future prospects.

## Introduction

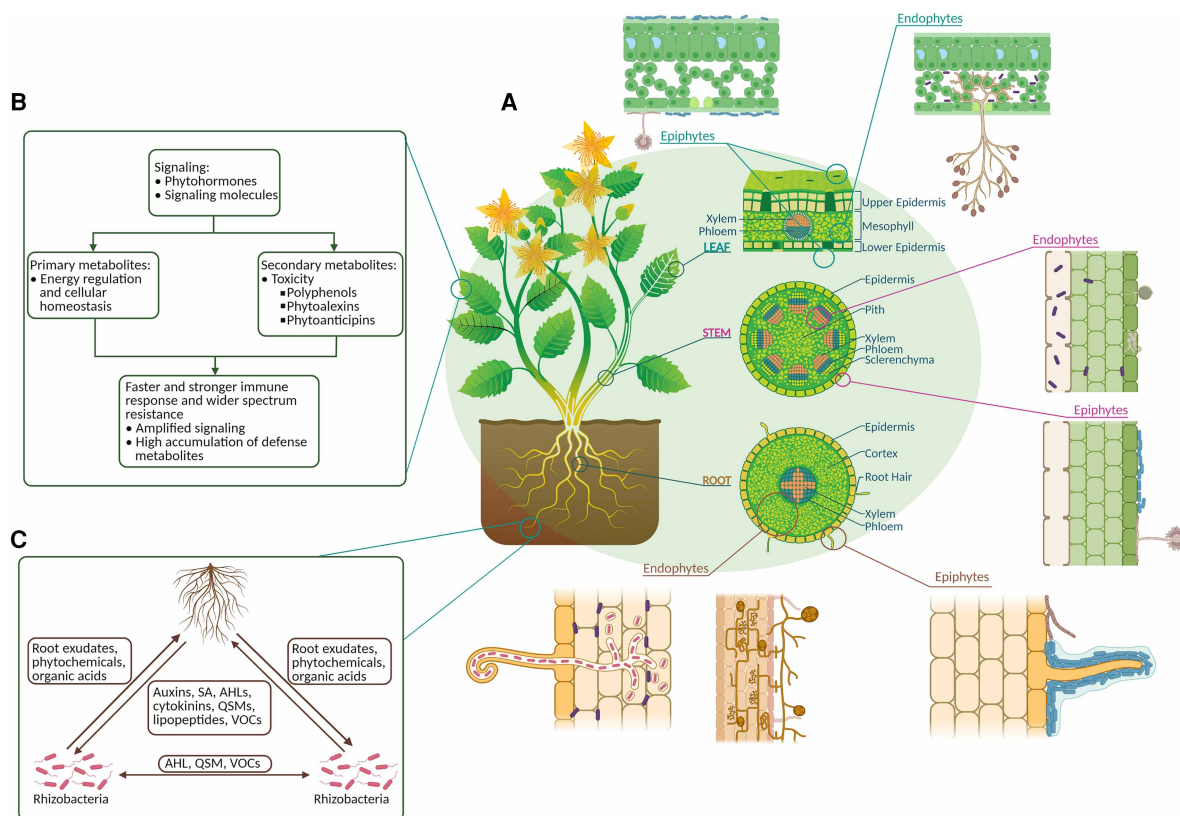
Metabolomics aims to qualitatively and quantitatively analyse the metabolites of living systems and their dynamic responses to changes in the environment [1–3]. Analysis, detection and identification of metabolites are the core of any metabolomics approach. The field of metabolomics originated from metabolite profiling in the 1970s [4]. Fiehn et al. [5] proposed the term ‘metabolomics’ and defined it as a ‘comprehensive and quantitative analysis of all metabolites in a biological system’. Metabolomics focuses on all small molecule components and the fluctuations in individual cells, cell types, tissue types or organs and is often used to study plant and microbial systems. Today, metabolomics is a growing field among omics sciences that is mainly concerned with high-throughput snapshots of metabolomes [6].

In the natural environment, plants are inhabited by a large number of microbiota that include fungi, bacteria, actinomycetes, algae, and protozoa [7]. Hence, it is difficult to generalize plant-physiology due to this diversity. Among these organisms, the majority of these are bacteria [8]. Plant growth promoting bacteria (PGPB) live mostly inside the rhizosphere and/or roots of plants and can be extremely beneficial for their growth and development. Similarly, fungi play an important role in natural and agricultural ecosystems. They act as important decomposers and recyclers of organic materials and they can interact with the belowground or with aboveground plant tissues [9]. These interactions between plants and fungi are complex and the outcomes are diverse, ranging from parasitism to mutualism. [Figure 1A](#) summarizes the sites where these interactions occur between microbiota and plant tissue.

To analyse the interactions between various species, the application of metabolomics not only provides a comprehensive picture of metabolic pathways that are involved but also explains the underlying mechanisms of host and microbial interactions. [Figure 1B,C](#) illustrates examples of compounds

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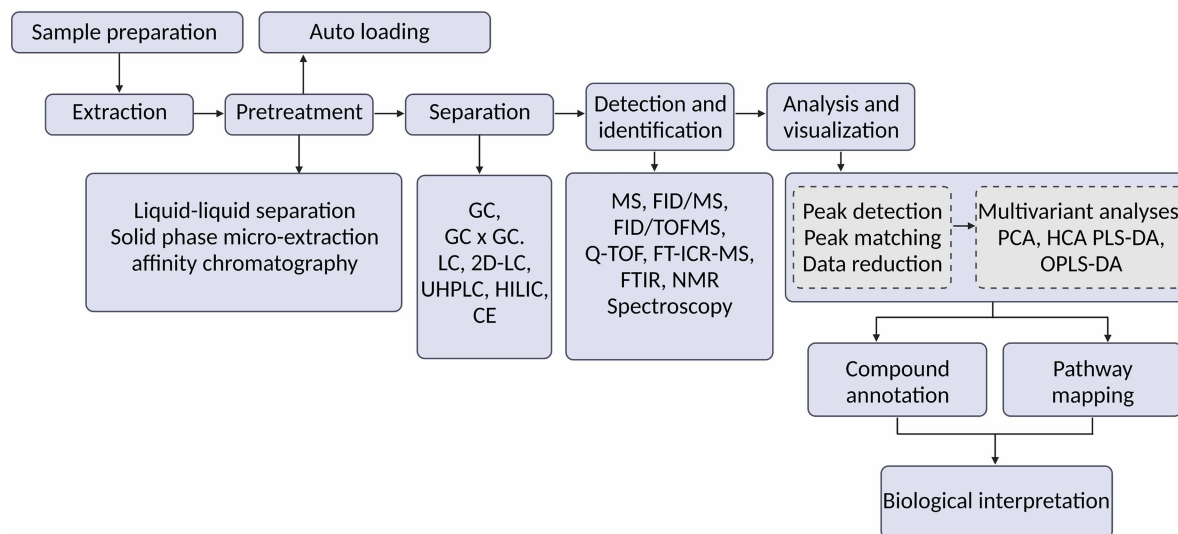
**Figure 1. Diagram showing possible sites of interactions between plants and microbiota (A) taken from Walker et al. [10] and compounds secreted by the microbes and metabolite profiles present in leaves (B) and root exudates (C).**

(A) Blue rods, bacterial epiphytes; dark purple rods, bacterial endophytes; red rods, root nodule bacteria; fungi shown in brown and grey. Not to scale. Abbreviations: volatile organic compounds (VOCs), quorum sensing molecules (QSM), N-acyl homoserine lactones (AHL), Salicylic acid (SA).

secreted during the beneficial relationships between plants and associated microbiota. Interactions between fungi and their hosts is an intriguing field comprising of the interactions of fungal species with plant, insect, animal or human hosts. In this review, we aim to summarize the advantages of metabolomics analysis platforms and their applications in investigating the interactions of different species while focusing on plant–bacterial and plant–fungal interactions.

## Metabolomics methods for plant–bacterial/fungal interactions

Metabolomics is a well-known technique for studying plant–microbe interactions. Several studies have been reported on important biotic interactions in plants, especially with mycorrhiza, PGPB, and many forms of filamentous fungi, including different *Trichoderma* strains. Numerous theoretical viewpoints have been shared on the advantageous relations such as nutrient intake, receptor recognition, and positive effect on growth and development. Metabolomics has played a key role in elucidating the connection and the differentiation between disease or symbiosis. The communications between *Arabidopsis*, PGPB and other microbes have been investigated together showing an effective illustration of the integration of different types of omics data [11]. Although there are ample of studies reported using metabolomics techniques, research in this field is still under rapid development with constantly evolving methodologies [12]. Figure 2 shows the compilation of techniques mentioned above and more, including experimental design methods, sample preparation, data acquisition, data processing, and biological interpretation relevant to metabolomics of plant–microbe interaction research.



**Figure 2. Metabolomics analysis flow for plant-microbe interaction research.**

GC, gas chromatography; LC, liquid chromatography; UHPLC, Ultra-High-Performance Liquid Chromatography; HILIC, hydrophilic interaction liquid chromatography; CE, capillary electrophoresis; FID/MS, flame ionization detector mass spectrometry; FID/TOFMS, flame ionization detector time of flight mass spectrometry; Q-TOF, quadrupole time of flight; FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrum; FTIR, fourier transform infrared; NMR, nuclear magnetic resonance; PCA, principal component analysis; HCA, hierarchical cluster analysis; PLS-DA, partial least squares discriminant analysis; OPLS-DA, orthogonal partial least squares discriminant analysis; HMDB: human metabolome database; KEGG, Kyoto encyclopedia of genes and genomes. Figure adapted from Chen et al. [4].

## Omics era and recent plant microbiome research expansion

Plants and microbes often communicate in the rhizosphere where plant roots secrete exudates. The rhizosphere communities i.e. the microorganisms surrounding the root regions are influenced by the chemical composition of these root exudates [13]. For example, Zhang et al. [14] showed that the exudates released by cucumber roots attracted *Bacillus amyloliquefaciens* SQR9, in which citric acid was identified, causing biofilm formation [14]. The host plant and the metabolism of microbes are affected by several bacterial genes such as nodulation genes. These genes are responsible for producing Nod factors during nodulation of roots [15]. Metabolomics has been employed for studying the chemical exudation process from grassroots during development, showing its effect on the assembly of the rhizosphere community [16]. Organic acids were the primary exuded metabolites consumed by the bacterial enrichment in the substrate [17]. Flavonoids are another class of compounds that are found in root exudates, which induce bacterial nod genes and initiate nodule formation in the root [18]. Another study by Negrel et al. [19] identified a number of lipid markers of *Plasmopara viticola* inoculation in grapevines through untargeted metabolomics studies.

Metabolomics has been useful in showing that the relationship among plants and PGPB can be quite specific. An example of this is given by Chamam et al. [20] where two *Azospirillum* strains improved the growth of two rice (*Oryza sativa*) cultivars, but affected their metabolome differently. Walker et al. [21] combined LC-MS and nuclear magnetic resonance (NMR) to study the metabolism of two maize cultivars inoculated with three different *Azospirillum* strains. The results showed that these bacteria were strain-dependent showing effects only on the secondary metabolism of inoculated plants without affecting the primary metabolism involving the physiological functions such as plant growth and development. Another metabolomic study on the interaction between different maize genotypes and two nitrogen fixing PGPB species or their mutants with impaired nitrogenase activity allowed the identification of plant compounds affected by bacterial nitrogen fixation [22].

A parallel protocol on the relationship among plants and symbionts to be specific has been shown extensively for arbuscular mycorrhiza (AM) where five distinct plant species were inhabited with a single AM and the metabolomic profiling indicated remarkably divergent and species-specific metabolic changes regardless of

a retained ‘core metabolome’ [23]. In another study, UHPLC combined with time-of-flight mass spectrometer Q-TOF/MS was used to detect putative mycorrhiza-associated metabolites in *Medicago truncatula* colonized roots with the AM fungus *Rhizophagus irregularis*. The study detected 71 mycorrhiza-associated metabolites with at least 10-fold more in mycorrhizal roots as compared with non-colonized roots [24]. Like investigating mycorrhizal fungi, metabolomics has been also extensively used to study endophytic fungi in many plant tissues.

Recently, Valette et al. [25] combined metabolite analysis using Ultra High Performance Liquid Chromatography-Diode Array Detector hyphenated to high resolution electrospray ionization time-of-flight mass spectrometer (UHPLC-DAD/ESI-QTOF) with gene expression data to obtain high resolution datasets of secondary metabolites affected by the inoculation with different PGPB species in rice roots and to obtain further insights into the role of specific affected metabolites. In another study by Mhlongo et al. [26], UHPLC coupled to a triple quadrupole mass spectrometer (UHPLC-QqQ-MS) techniques provided new insights into the plant defense mechanisms involved in the early interactions between tomato and various PGPB species.

Matrix-assisted laser desorption ionization mass spectrometric imaging (MALDI-MSI) is another technique that is recently used for gaining the spatial information of metabolites in inoculated roots and root nodules. Recently, the metabolite profiles of roots and nodules of *Medicago truncatula* associated with *Sinorhizobium meliloti* during nitrogen fixation was studied using MALDI-MSI [27]. This study detected various amino acids, organic acids, sugars, lipids and flavonoids, along with molecular ion images obtained from nitrogen-fixing and non-fixing nodules. MALDI-MSI has been employed to identify and solve the spatiotemporal production of antimicrobial compounds (surfactins, iturins, pliplastatin, streptorubin and fengycin) released by root-colonizing *Bacillus* (e.g. *B. subtilis* and *B. amyloliquefaciens*) [28,29]. Furthermore, MALDI MSI coupled with MS/MS analysis was used to study the spatiotemporal changes and identify antibiotic compounds released by roots of *Solanum lycopersicum* biofilmed with *B. amyloliquefaciens*. The results from this study identified a new variant of surfactins released at later time points post-inoculation [30].

In a study by Cao et al. [31], the metabolic effect of endophytic *Neotyphodium lolii* and its host perennial ryegrass (*Lolium perenne*) have been studied by direct infusion mass spectrometry (MS) in immature leaves, sheaths and blades, with data obtained from MS<sup>2</sup> and MS<sup>3</sup> product ion spectra. The results showed changes in the metabolome in inoculated plants, with key compounds such as mannitol, peramine and perloline. Another study by Waqas et al. (2012) [32] showed the production of hormone substances, such as auxin, gibberellin (GA), and cytokinin from endophytic fungi which directly promoted rice plant growth. Recently, the technique of UHPLC-Q-TOF/MS for the rapid identification and profiling of several structurally similar bioactive shikonins from the root periderm tissues of Australian annual weed *Echium plantagineum* and Australian perennial weed *E. vulgare* has been used by Skoneczny et al. [33]. The results showed that shikonin levels was 2.5 times higher in *E. vulgare* extracts as compared with *E. plantagineum*.

Endophytic fungi are known to produce antagonistic substances that not only help plants resist pathogenic microorganisms but can also indirectly induce and activate the plant defense system. This helps in improving the plant defense ability and adaptation to diseases and insect pests. The antagonistic substances produced by endophytic fungi mainly include small-molecular-weight active substances and antimicrobial peptides. In a recent study by Liu et al. [34], NMR and MS spectrum data were used to identify a strain of *Aspergillus racemose* from mangrove plants in Hainan, which produces the metabolites 22-epi-aflaquinolone B (used against wheat total erosion bacteria) and 14-epi-isochaetominine C, used against wheat scab in agricultural bacterial diseases.

In one of the few metabolomic studies on tripartite associations (plant–bacteria–fungi), Roupael et al. [35] applied a mixture of PGPB and beneficial fungi with or without a protein-based biostimulant to maize plants. UHPLC analysis of shoot and root samples displayed a distinct metabolic response in each of the treatments, particularly showing an effect on secondary metabolites.

There is growing interest in linking metabolomics with lipidomics [36]. This integrated approach could contribute to a better understanding of metabolism which metabolomics alone would otherwise lack in defining [37]. Several studies have been reported using this integrated approach to understand the rearrangements of metabolites and lipids during plant–microbe interactions. For example, metabolomics techniques such as untargeted GC–MS and untargeted QqTOF LC–MS were used to compare the root metabolic and lipid profiles of inoculated and non-inoculated *Brachypodium distachyon* Bd21–3 plants with *Azospirillum brasilense* Sp245 grown at low temperatures and supplied with insufficient phosphorus. It has been proposed that the nutritional status of the plant influenced the interaction of the plant with *Azospirillum*: bacteria were sensed as pathogens

despite sufficient phosphorus in plants, however, the interaction became beneficial for the plants as their phosphorus levels decreased [38]. Another study by Gupta et al. [39] used GC–MS to analyse polar metabolites and LC–MS to analyse lipids in roots of two barley cultivars (with contrasting salinity tolerance) during the early stages of interaction with *Trichoderma harzianum* T-22. They found that many of the metabolic changes in the inoculated salt treated sensitive cultivar show similar responses as the uninoculated tolerant cultivar, while a number of metabolites were changed in both cultivars following fungal inoculation only.

## Plant–microbe interactions metabolomics experimental setup

When research is mainly focused on root interactions with PGPB or fungi, seed preparation and inoculation play a major role in the successful establishment of the plant–microbe relationship. To isolate the effects of microbial inoculation on plant metabolism, it is essential to carefully sterilize the seed surface using appropriate protocols. While this step will not eliminate the possible seed endophytes vertically transmitted from the parental generation, it will surely decrease the microbial population. Seeds can then be germinated and transplanted to the growth substrate or directly planted. In the first case, seeds can be inoculated prior to the transplant by dipping their roots in a bacterial solution containing the inoculum at the desired strength. Alternatively, plants can also be inoculated in their substrate, by pouring the desired volume of bacterial solution on them. Similar protocols can be followed for seeds intended to be inoculated with fungus where seeds can either be submerged in a solution containing fungal spores or alternatively roots can be inoculated by pouring the desired volume with a specific number of spores. If the metabolomic study is the first to characterize the interaction between a specific plant and a PGP microorganism, it may be preferable to grow plants in a sterilized substrate and in a controlled environment (growth chamber, greenhouse), in order to minimize the noise that would come from a more complex setup. The hypotheses obtained from ‘reductionist’ experiments can then be tested in more natural environments, where conditions change continuously, and plants interact also with native microbiota. At harvest, plant metabolism should be quenched as soon as possible by shock-freezing the plant tissue.

## Prospects and challenges

To attain the goal of holistic analyses of the metabolome, a wide range of chemometrics methods, chemistries, advanced analytical instrumentation and novel computational approaches providing high degrees of sensitivity and reproducibility, are required in metabolomics. In contrast with other -omics methodologies, metabolomics encounters several unique challenges making the field particularly demanding. These challenges arise especially from the inherent characteristics of the metabolome such as: (i) being highly dynamic where metabolome is changing continuously at different rates, (ii) chemically diverse nature of metabolites with different physico-chemical properties, biological functions and highly diverse and dynamic stereo chemistries, (iii) wide range of metabolite levels and the bio-complexity of living systems involving several biological cycles and compartmentalization at the organism and cellular level) [40,41,42,43]. These challenges are more complex when plants constituting an enormous treasure of microbiome are explored. There has been a tremendous progress in understanding the plant microbiome. However, further understanding of the processes is still required that will help understand the microbiome community formation and function in plants.

- The early stages of contact between plants and microorganisms retain particular interest, as they usually define whether the latter will be perceived as beneficial or pathogenic [44]. Recent studies have also shown that the relationship between plants and microorganisms can change with the worsening of the environmental conditions [45], and determining what mechanisms take place in the interplay at those stages is of great importance. Future studies should combine frequent analysis of inoculated plants with newly developed methods such as MALDI-MSI, which allow to determine the location of the detected metabolites in the analyzed tissues. By doing so, they could precisely resolve into time and tissue distribution of the compounds being involved in the interaction, in order to better understand its underlying mechanisms.
- The metabolomic analysis of plant tissues inoculated with PGP bacteria seldom allows to determine whether the detected metabolites were synthesized by the plant or by the microorganisms [46], and this task is still difficult to achieve. The sole comparison with the metabolome of plants and microorganisms grown in axenic culture can provide some useful information, but does not give the full picture, as there still can be compounds produced by an organism only when interacting with another. Integrating metabolomics with the analysis of the transcriptome of plants and microorganisms alone and interacting with each other can

help in this task by ascribing the various metabolites to the organism which also show an increased expression of the genes related to their metabolism [38].

- As mentioned above, there has been little or no effort made in differentiating microbial metabolites from plant metabolites as the microbes are not removed from the metabolite extraction [47,48,49,50]. This becomes a problem especially, when species specific databases are available, as there can be cross-contamination between the plant and the microbial metabolome which is a serious issue. To solve this problem, culturing microbial cells in stable isotope media can be a powerful way to trace the origin of biomolecules. Stable isotope labeling can be applied for reference metabolite labeling (e.g. for accurate quantification), metabolic flux analysis and identification of metabolites in different organisms [51,52,53,54]. For example, a study performed by Pang et al. [55] used targeted metabolomic analysis on *Arabidopsis thaliana* epidermal peels with guard cells infected with a plant bacterial pathogen *Pseudomonas syringae* pv. tomato (*Pst*) DC3000 labeled with heavy isotopes. The results suggested that *Pst* DC3000 infection changed plant metabolites, including signaling and primary metabolites.
- High resolution mass spectrometry (HRMS) is another powerful technique that has undergone an exciting phase of technological evolution with application in plant–microbe interactions studies [56,57]. The HRMS instrumentation has increased mass resolution that help to distinguish between isotopic distributions and generate fragmentation patterns. This improves the accuracy of predicting the chemical formula and identifying compounds through library matching. However, HRMS instrumentation does not replace standard low-resolution mass spectrometers used in several research laboratories. For example, for nontargeted analyses where HRMS is advantageous, high resolution is not necessary for basic applications. HRMS generates highly complex data in large quantities, which is not necessary for routine, targeted analyses as these can be processed using low-resolution mass spectrometry. For such targeted analyses of a limited subset of compounds, single Da resolution methods can be used for detection and quantification [58].
- Metabolomics is an essential link between transcriptomics and phenomics, potentially allowing to identify the genes responsible for the phenotype differences observed in the organisms following different treatments. If the genome of such organisms has already been sequenced and annotated, the genes linked to the different metabolites could then be selected for further studies. In the case of plants inoculated with PGP microorganisms, metabolites responsible for an improved phenotype could thus be linked to the respective genes and then be researched in varieties that express them at higher levels. Alternatively, those genes could be edited to be constitutionally expressed in already profitable varieties, in order to further increase their fitness.
- Normally, metabolomic studies of plant–microbe interactions are first performed in controlled environments (growth chambers, greenhouses), which allow to isolate the different inputs in the system and clearly define the relations among them. If the final goal of metabolomic studies is to produce knowledge to improve future agriculture, the hypotheses drawn from such experiments should be validated in progressively less controlled environments, where plants are subjected to environmental fluctuations.
- In the past years, most of the metabolomic studies were performed on the response of the model plant *Arabidopsis* to external stimuli [59]. In the upcoming years, it will be necessary to extend the metabolomic studies to agriculturally relevant crops, which often differ strongly from the most studied model plant species.

## Summary

- Metabolomics is a data-driven, hypothesis-generating scientific approach which aims to detect and quantify 1000s of compounds per analysis. It provides a suitable approach to study complex biological interactions within the rhizosphere and reciprocal responses between plants and organisms.
- Metabolomics is an emerging field in the plant sciences, however its application to study beneficial plant microbe interactions is lagging compared with other omics approaches, providing many opportunities to broaden our understanding of the underlying mechanisms of beneficial plant microbe interactions

- Application of metabolomics to study plant microbe interactions comes with several challenges, such as the differentiation of the origin of metabolites analysed, uncovering the metabolic complexity of two or more organisms interacting and linking metabolome information with other omics data such as transcriptomics, proteomics or phenomics.

## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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## Author Contributions

S.G. designed the ideas presented in the article; M.S. assisted in the literature and participated in the writing; S.G., M.S. and U.R. wrote the manuscript.

## Abbreviations

AM, arbuscular mycorrhiza; HRMS, high resolution mass spectrometry; NMR, nuclear magnetic resonance; PGPB, plant growth promoting bacteria.

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