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published in
The Plant Microbiome
2020

DOI (link to publisher)
10.1007/978-1-0716-1040-4_19

document version
Publisher's PDF, also known as Version of record

Chapter 19

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Abstract

Here we describe a suite of methods to identify potential taxonomic and functional soil microbial indicators of soil quality and plant health in biofuel crops in various areas and land types. This approach draws on tools to assess microbial diversity, greenhouse gas fluxes, and soil physicochemical properties in bioenergy cropping systems. Integrative statistical models are then used to identify potential microbial indicators for sustainable management of bioenergy crops.

Key words Soil quality, Molecular analysis, Conventional analyses in soil microbiology, Biogeochemical analysis, Statistics

1 Introduction

Soil microorganisms are critical to the maintenance of terrestrial ecosystem functions due to their involvement in key processes, such as soil structure formation, decomposition of organic matter, toxin removal, and the cycling of carbon, nitrogen, phosphorus, and sulfur [1]. In addition, microorganisms play key roles in suppressing soil-borne plant diseases, promoting plant growth, and in vegetation changes [2]. Because of the increased economic importance of bioenergy crops, the requirements for large-scale production in an environmentally sustainable manner have also increased. However, massive planting of the crop brings environmental risks that include a potential impact on tropical soil ecosystem sustainability, which is still an open question for soil microorganisms and microbial-mediated processes that lead to greenhouse gas emissions. The measuring and monitoring of the sustainability of bioenergy crop production are difficult and require some standardized

Lilia C. Carvalhais and Paul G. Dennis (eds.), *The Plant Microbiome: Methods and Protocols*, Methods in Molecular Biology, vol. 2232, https://doi.org/10.1007/978-1-0716-1040-4_19,

metrics, like those related to soil biological, physical, and chemical parameters, which are important indicators of soil quality.

According to Soil Science Society of America, soil quality can be defined as "the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation" [3]. In this sense, defining and maintaining soil quality and plant health parameters is key for sustainable primary production of raw materials for bio-based industrial processes. For this purpose, microbiologically relevant methods are needed to assess the potential impacts of bio-based production processes and waste materials on soil function and plant health.

Considering that microorganisms mediate fundamental biogeochemical processes required for the global cycling of nutrients and the sustainability of ecosystems [4], understanding the impacts of bioenergy cropping systems on these communities and their functions in the soil is of utmost practical and scientific importance. The inability to culture most microorganisms from environmental samples is a fundamental obstacle to understand microbial ecology and diversity. However, the development of molecular tools and methods based on direct isolation and analysis of nucleic acids, proteins, and lipids from environmental samples have revealed structural and functional information about microbial communities [5–15]. Molecular approaches such as genetic fingerprinting, metagenomics, metaproteomics, metatranscriptomics, and proteogenomics are vital for discovering and characterizing the vast microbial diversity and functions for understanding their interactions with biotic and abiotic environmental factors [16].

Improved knowledge of biogeochemistry is being integrated into ecological studies of soil microorganisms to better resolve the abiotic drivers of taxonomic and functional diversity [17–20]. Conventional methods in soil microbiology, such as potential enzyme activity and microbial biomass, commonly used to assess soil quality, have also been applied in combination with molecular and chemical approaches to compare the responses of specific microbial taxa as potential bioindicators for impact of soil management practices on microbial communities in sugarcane production fields [18]. Through the prism of the soil-plant-microbe interactions, there are examples of recruitment of beneficial microbial traits in the rhizosphere [20–24]. Souza et al. [25] showed that microbial communities that originate primarily from native soil around sugarcane plants colonize plant organs in distinct patterns. In turn, different studies highlighted the role of the untapped soil microbial diversity for modulating plant growth, development, pathogen defense, nutrient acquisition, and stress resistance [26–31]. Once the recruitment of beneficial taxa is identified, these microbial groups can be assessed as potential soil microbial indicators for

plant health [32]. Plant genotype strategy, quantitative loci mapping, and genome-wide association approaches can help to identify specific regions in the genome where the recruitment traits may be located [33]. Also, considering the importance of plant exudates to the recruitment of microbial groups, analyses of plant exudate composition could help to identify specific metabolites linked to the selection of beneficial taxa [30].

Taken together, these different methods can provide an important tool based on an integrative approach for screening of potential soil microbial indicators for sustainable management of bioenergy crops. In this context, potential microbial indicators can be defined as microbial taxa or microbial functional genes that are likely to respond promptly and accurately to soil perturbation (e.g., changes due to soil agricultural management) and plant stress (e.g., growth in non-ideal conditions) backed by traditional microbiological and chemical analyses in ecological sustainability evaluation. Here we provide an overview of conventional and molecular techniques in soil microbiology and soil-plant-microbe interactions, soil processes and properties as well as statistical analyses for potential bioindicators.

2 Integrative Approach

This section covers a range of analyses representing our integrative approach (Fig. 1). The molecular analyses encompass analysis of soil microbial community and plant-microbe interactions, focusing on taxa or functional genes. We combine culture-independent molecular as well as traditional methods to assess the taxonomic and functional ecological aspects of soil microbial communities. Soil processes and properties able to reflect the effects of agricultural management practices on soil functioning are also components of this approach.

2.1 Molecular Analyses

Molecular analyses are used to provide taxonomic and functional information about microbial communities in soil and rhizosphere. These analyses are adopted in the integrative approach because the vast majority of microbial cells that can be seen in a microscope and shown to be living with various staining procedures is unable to be grown in lab. It is estimated that only 0.1–1.0% of the living bacteria present in soils can be cultured under standard conditions. Given the evidence that many microorganisms resist being cultured, culture-independent methods for identifying and enumerating microorganisms in the environment have come to play a larger and larger role over the last several decades.

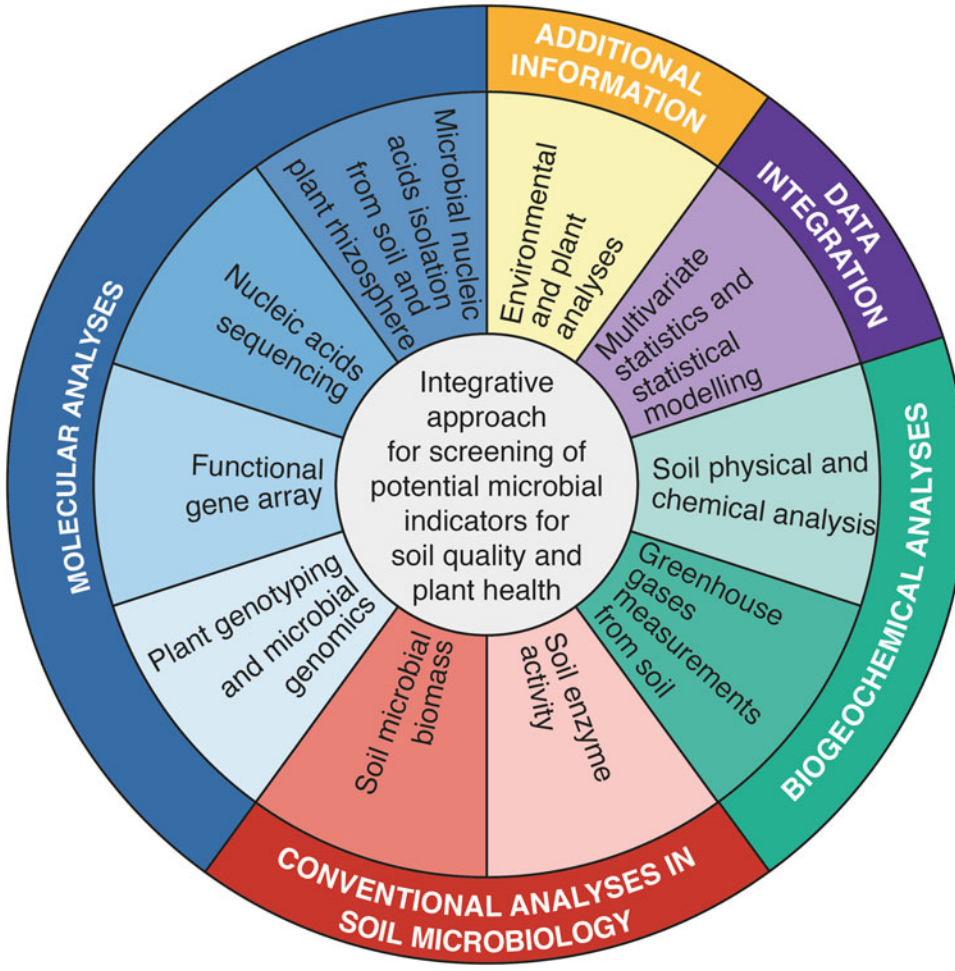

Fig. 1 Illustrative scheme of the integrative approach for screening of potential microbial indicators for soil quality and plant health based on molecular analyses, conventional analyses in soil microbiology, soil processes and properties analyses, and multivariate statistics

2.1.1 Microbial Nucleic Acids Isolation from soil and Plant Rhizosphere

McPherson et al. [34] provide details for soil and rhizosphere samples collection. Two strategies can be used to extract nucleic acids from soil (bulk soil and rhizosphere) [35]. The first approach consists of the direct extraction of nucleic acids from the soil through in situ cell lysis followed by their purification. An alternative approach is based on the separation of microorganisms from the soil particles followed by cell lysis and then nucleic acids purification. Direct extraction of nucleic acids from cells within the soil usually recovers greater quantities of DNA and RNA than microbial fractioning, and is considered to be more representative of the soil microbial community. Based on the first approach, many commercial nucleic acids extraction kits have been developed to simplify and accelerate DNA and RNA isolation and purification from environmental samples. After isolation, DNA and RNA extracts are

checked for quality and quantified. Nucleic acids yield can be assessed using various methods including absorbance (optical density), agarose gel electrophoresis, or use of fluorescent DNA-binding dyes. All three methods are convenient. Different technologies use the nucleic acids to identify and assess microbial genes in the soil or plant rhizosphere samples, which constitute taxonomic and functional components of the integrative approach here described.

2.1.2 Methods Focusing on Taxonomic and Functional Microbial Genes that Are Already Known

Amplicon sequencing is a targeted approach that allows the genetic variation to be analyzed in specific genomic regions. High throughput amplicon sequencing technology has been widely used in soil microbiome studies. Using different DNA and RNA oligonucleotides (primers) is possible to taxonomically and functionally characterize microorganisms previously described and not-yet-cultivated.

Amplicon Sequencing

Amplicon-based approaches targeting variable regions of specific phylogenetic markers (e.g., 16S rRNA and 18S rRNA genes, and ITS region) are widely used to describe semi-quantitatively bacterial, archaeal, and fungal community composition. This approach was transposed for functional studies—for example, targeting enzyme-coding genes catalyzing carbon, nitrogen, and phosphorus cycles—and in both cases it requires downstream bioinformatics analysis.

Microarray

Microarray technology facilitates the detection and quantification of genetic sequences or expressed microbial genes from particular samples in a high throughput format [36]. A microarray is made up of thousands of spots on a slide with each spot containing multiple copies of unique nucleic acid sequences that correspond to a single gene. While the GeoChip is a glass slide containing oligonucleotide probes targeting genes that confer specific function to microorganism, the PhyloChip microarray contains oligonucleotide probes of the 16S rRNA genes, which allows monitoring microbial populations. The GeoChip containing probes from genes involved in key microbially mediated biogeochemical processes, such as carbon, nitrogen and sulfur cycling, phosphorus utilization, organic contaminant degradation and metal resistance and reduction. It is particularly powerful for identification of genes with potential use as management-indicators of agricultural practices based on specialized functioning of the soil conducted by microbial groups [13, 19, 37].

2.1.3 Methods for Discovery and High Throughput of Taxonomic and Functional Microbial Genes

Metagenomics

Metagenomics is the study of genetic material recovered directly from environmental samples. This approach allows accessing the potential reservoir of novel genes in soil and plant hosts, offering possibilities to discover novel genes and novel biomolecules through the expression of genes from uncultivated and unknown microorganisms. The discovery and high throughput profiling of taxonomic and functional microbial genes by metagenomics comprise the following steps: collection, processing, and sequencing of samples; pre-processing of sequencing reads; sequence analysis to profile taxonomic, functional, and genomic features of the microbiome; and statistical and biological post-processing analysis. The metagenome bioinformatics tools allow translating raw reads into meaningful microbial features such as genomes, species abundances, and functional potential profiles. These last two features are especially important to reveal the community of microorganisms present in soil and their particular functions in this environment, without the necessity of obtaining pure cultures.

Metatranscriptomics and Metaproteomics

Metatranscriptomics and metaproteomics complement metagenomic analyses, by providing insight into gene expression at the level of transcripts and proteins, respectively. Metatranscriptomics is the study of the function and activity of the complete set of transcripts from environmental samples. In turn, metaproteomics is emerging as a complementary approach to metagenomics, which aims to characterize the proteins from the microbiota. The integrative omics approach leads to a comprehensive information describing the community from genes to RNA to proteins and metabolites. This additional information can refine the capability of the integrative approach to define potential microbial indicators for sustainable management of bioenergy crops by increasing the ecological resolution for the soil microbial communities.

2.1.4 Linking Microbial Genomics with Plant Genotyping

In a survey for microbial indicators is important to identify and characterize the microbial genes and functions that help the microorganisms to thrive in the plant environment (e.g. rhizosphere and endosphere). Methods for identification of genomic features include the whole-genome sequencing from cultivated microorganisms, and single-cell sequencing and metagenome-assembled genomes (MAGs) for the non-cultivated groups. Due to the limitation of microbial cultivation, the assembly and binning of shotgun metagenome approach may complement the single-cell approach to expand the genomic information of non-cultivated microbial groups associated with plants. The complete genome information helps in the genome-wide annotations of all the genes, proteins, enzymes, and metabolites related to microbial adaptation to plants. Based on the genomic data, a screening with

culture-dependent approaches can be performed by targeting the isolation of those beneficial microbial genera that are associated with a specific plant host. Thus, beneficial roles can be evaluated such as nutrient solubilization, antagonistic activity against pathogens, and improved tolerance to environmental stress. Once the recruitment of a beneficial particular taxon is confirmed, plant genotyping strategies can help the identification of genome traits linked to microbial recruitment in the plant microbiome. Using a sufficient number of plant genotypes, quantitative trait locus (QTL) mapping and genome-wide association (GWAS) can be performed aiming to identify specific regions of the plant genome where the recruitment traits of specific microbial groups are located. This link between microbial genomics and plant genotyping could be useful to drive molecular breeding towards the improvement of beneficial plant-microbe interactions in crop systems [33].

2.2 Conventional Analyses in Soil Microbiology

Microbiological parameters such as soil microbial biomass and enzymatic activity are used in the integrative approach to compare with the responses of specific-taxa and functional microbial genes as potential bioindicators for the impact of soil management practices on soil microbial communities.

2.2.1 Soil Microbial Absolute Abundance

The soil microbial absolute abundance, that is, the absolute microbial cell numbers in soil, can be analyzed directly or indirectly by multiple culture-independent measurements, including quantitative real-time PCR, phospholipid fatty acids (PLFA) and microbial biomass. Fluorescence-based quantitative real-time PCR (qRT-PCR) is a widely and commonly used technology to quantify DNA and RNA products. The qRT-PCR allows the quantification of microorganisms targeting conserved genes and through a functional gene approach. Through the prism of the integrative approach here described, qRT-PCR technologies are useful to validate the differential response of the soil bacterial, archaeal, fungal total community, specific-taxa and -functional microbial gene abundances due to soil agricultural management revealed semi-quantitatively by DNA and RNA sequencing approaches. Soil PLFA and microbial biomass analyses are widely used techniques for estimation of the total biomass and to observe broad changes in the soil microbiota composition due to land management and other environmental stressors. The standard method for PLFA analysis encompasses lipid extraction from soil samples with a single-phase chloroform mixture, and fatty acid methyl esters (FAMEs) analysis by capillary gas chromatography using a flame ionization detector (GC-FID). In turn, soil microbial biomass is a process that causes the death and total disintegration of the soil microorganisms. After disintegration, some of the cell constituents, such as carbon,

nitrogen, and phosphorus, can be extracted by extractors and determined by titration or colorimetry [38–40]. Differences in soil microbial quantity can be contrasted with differences in the abundance of microbial taxa in the soil in order to couple soil microbial abundances with microbial quantities to detect disturbance of soil management practices.

2.2.2 Soil Enzyme Activity

Many different classes of soil extracellular enzyme activity can be quantified in laboratory assays using a variety of synthetic substrates. Some protocols utilize substrates in assays that are coupled to a colorimetric reaction that can be detected with a spectrophotometer, while others utilize substrates that are bound to a fluorescent moiety [41]. Fluorescence extracellular enzyme assays are typically more sensitive (by an order of magnitude) than colorimetric assays, but can suffer from interference caused by impurities and the instability of many fluorescent compounds when exposed to light. The abundance of different carbon-, nitrogen-, and phosphorus-degrading enzymes (e.g. amylase, arylsulphatase, beta-glucosidase, cellulose, chitinase, dehydrogenase, phosphatase, protease, urease, and urease) in soils is controlled by numerous factors, including microbial biomass and community composition. The enzymatic activity in the soil is mainly of microbial origin, being derived from intracellular, cell-associated, or free enzymes. A unique balance of and biological, chemical, and physical (including microbial especially enzyme activities) components contributes to maintaining soil quality.

2.3 Soil Processes and Properties Analyses

2.3.1 Greenhouse Gases Measurements from Soil

Soil plays significant roles in the cycling of three major greenhouse gases: carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O). Agricultural soil is a dynamic biological system that both stores and releases these gases. In sugarcane production fields, the emissions of these gases mainly depend on crop management practices like fertilizer application, and organic additives [42]; therefore, changes in these management practices also offer possibilities for mitigation. This biological system is dependent on the physical and chemical properties of soils [43]. Of the range of potential indicators used to infer soil quality status, soil carbon is particularly important. Carbon within the terrestrial biosphere can behave as either a source or sink for atmospheric CO_2 depending on land management, thus potentially mitigating or accelerating the greenhouse effect. There are multiple techniques available for soil gas analysis: active, passive, and flux chamber [44]. Static chambers have been commonly used in bioenergy crops production areas in conjunction with gas chromatography [45–48], and they are suitable to feed the integrative approach with information about greenhouse gases from the soil.

2.3.2 Soil Physical and Chemical Analysis

The concept of soil quality includes assessment of soil properties and processes as they relate to ability of soil to function effectively as a component of a healthy ecosystem. Specific functions and subsequent values provided by agroecosystems are variable and rely on numerous soil physical and chemical, and biological properties and processes. The soil physical properties can be grouped into static (texture, depth, water holding capacity, roughness, soil loss and strength, porosity, aggregate stability and size distribution, tilth) and dynamic (least limiting water range, trafficability, leaching and erosion potential) indicator. In turn, the soil chemical properties can be grouped into parameters related to soil carbon status (e.g., organic carbon and organic matter), soil acidity (e.g., pH and Al saturation), and measures of nutrient availability (e.g., fertility, soil nitrogen, phosphorus and nitrogen, cation-exchange capacity).

The status of fertility and physical characteristics of soils can be analyzed based on different methods and laboratory protocols. Carter and Gregorich [49] described soil sampling and methods for the analysis of containing material, reagents, and procedures in details. These procedures can be adopted for physical and chemical analysis of the agricultural soil to provide information for the integrative approach.

2.4 Statistical Methods for Data Analysis and Integration (see Note 1)

2.4.1 Univariate Approach

Univariate analysis takes data, summarizes these data, and finds patterns in the data. Through the prism of experimental datasets, univariate statistics consist of determining whether a treatment group produces a significant difference against the controls or between different treatments altogether. When two groups are used, multicomparison tests are used such as the t test. When more than two groups are used, analysis of variance (ANOVA) is used first, followed by a multicomparison test. In addition, regression analysis is used to analyze data consisting of a dependent variable (or response variable) and one or more independent variables (or explanatory variables). In this case, the dependent variable is modelled as a function of independent variables. For example, these univariate analyses allow understand how soil management influences microbial total community composition, specific-taxa and -functional microbial gene abundances, and soil processes and properties in bioenergy cropping systems.

2.4.2 Multivariate Approach

Statistical techniques of multivariate analysis are suited to analyze, integrate, and interpret large amounts of data. This integrative statistical approach facilitates the identification of potential microbial indicators for sustainable management of bioenergy crops through analysis of the heterogeneous datasets and associations among the various results [42, 44–47]. Although there are many different multivariate techniques, the permutational multivariate analysis of variance (PERMANOVA) and multivariate generalized linear model (GLM) are commonly used to compare community

composition from a taxonomic and functional perspective [46, 47]. The PERMANOVA is a geometric partitioning of multivariate variation in the space of a chosen dissimilatory measure according to a given ANOVA, with *p*-values obtained using appropriate distribution-free permutation techniques. In turn, the multivariate GLMs deal with more than one dependent variable and one or more independent variables. It involves analyses such as the MANOVA and MANCOVA, which are the extended forms of the ANOVA and the ANCOVA, and regression models.

To integrate and visualize connections and theoretical relationships among soil microbiological, physical, and chemical properties, the heterogeneous datasets may be added to a networks analysis using specialized software [50]. All networks consist of sets of interacting nodes (e.g., microbial taxa, microbial functional genes, soil physical and chemical factors, greenhouse gases fluxes) whose relationships are represented by edges (e.g., nutrient or energy transfers, movement of individuals). Network-level metrics integrate information over the entire set of nodes and edges. For example, the number of nodes and the density of connections or connectance are both network-level statistics used to describe the overall complexity of a network. Networks can be constructed using CoNet [51], a robust ensemble-based network inference tool tailored to taxon and functional gene count data. In addition to taxonomic and functional microbial community information, CoNet accepts sample metadata as input, which allows associating molecular data to others datasets. Cytoscape [52] is used for the graphic representation of the networks. Taken together, the connections and theoretical relationships between microbial groups and functional genes in soil or rhizosphere community with the others' environmental information allow identifying taxa or functional genes in soil and rhizosphere microbial community able to act as potential microbial indicators for sustainable management of bioenergy crops.

3 Notes

1. This section does not exhaust the possibilities of analyses for the integrative approach presented here, but it reports analyses used by previous works for the purpose to identify potential microbial indicators for impacts of soil agricultural management. This integrative approach opens the possibilities to explore plant analyses as additional information to feed it and allow more accurate identification of potential microbial indicators for plant health.

Acknowledgments

This study was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil CAPES—23038.006927/2014-35/Premium 116/2017, Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP - 2011/51749-6), and BE-Basic 008.002.005, NWO-Fapesp 729.004.003, NWO-CNPq 729.004.013-456420/2013-4. AAN was supported by FAPESP 2017/03575-5.

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