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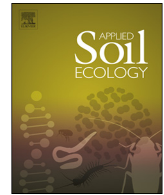

Reanalysis of microbiomes in soils affected by apple replant disease (ARD): Old foes and novel suspects lead to the proposal of extended model of disease development

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ABSTRACT

The aim of this study was to identify the presumed microbiological drivers in soils affected by apple replant disease (ARD) based on reanalysis of a larger cohort of molecular data. A literature search was conducted to identify the relevant deep-sequencing datasets from ARD-affected soil microbiomes next to the data on environmental variables and molecular techniques. The sequencing datasets of bacteria and fungi were analyzed using a taxonomic approach in mothur, using SILVA and UNITE databases, respectively. Variation partitioning and network analysis were used to identify the extent of variability related to environmental, spatial, spatially structured environmental or methodological sources.

The structure of bacterial and fungal microbial communities in ARD-affected differed significantly from healthy soils and revealed a pool of microbial OTUs co-occurring in ARD-affected soils. Available meta-data explained most of the genetic variability in bacterial communities, in addition to the different molecular methods that accounted for 25% of the variability. Most of the variability in fungal communities remained unexplained and was not related to the differences in molecular methods among the studies. The variables that affected most both bacterial and fungal microbial communities were the presence of ARD, the types of soil treatments and the plant rootstock. In addition, the lack of congruency in reporting standards of measured soil characteristics precluded the inclusion of numerous measured soil parameters recorded in published literature, hence hampering the depth of reanalysis.

In conclusion, this large-scale reanalysis indicated that ARD can be defined as an opportunistic microbial infectious disease, created by certain prevailing environmental conditions affecting microbial metabolism and their interaction with the plant host. A call for a united standard for reporting soil parameters is also issued.

1. Introduction

Soil is one of the most complex and diverse habitats as one gram of soil can contain from 10^3 to 10^7 bacterial species (Schloss and Handelsman, 2006). To provide insight into complex microbial networks in these systems, studies over the past ten years have routinely adopted amplicon and shot-gun Next-Generation Sequencing (NGS) approaches. This has led to the accumulation of studies presenting data produced with different platforms (e.g. Ion torrent, Roche 454 and Illumina), using various DNA extraction procedures and experimental conditions (Bonilla-Rosso et al., 2012; Claesson et al., 2010; Inceoglu et al., 2010). In addition, distinct bioinformatic approaches (e.g. MG-RAST, QIIME, mother, custom made pipelines) with their inherent assumptions, myriad of settings, algorithmic approximations and biases, distinct levels of congruency and benchmarking lead to contrasting results and interpretations (D'Argenio et al., 2014). These limitations make the comparison and generalization of the results published in unrelated studies difficult.

Apple replant disease is a complex syndrome that causes reduced growth and diminished production in apple trees that are replanted in the soil of previous orchard. Moreover, plants show significantly shortened internodes, discolored roots, root tip necrosis and a reduction in root biomass, which can ultimately lead to plant death within the first growing season (Mazzola and Manici, 2012). The reduction in growth

Abbreviations: ARD, Apple Replant Disease; AMOVA, analysis of molecular variance; H, healthy; HOMOVA, homogeneity of molecular variance; NGS, Next Generation Sequencing; OUT, operational taxonomic unit; RG, reduction of growth; SAN, soil association network

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and production caused by ARD may decrease profitability up to 50% throughout the life cycle of the orchard (van Schoor et al., 2009).

Due to the complexity the aetiology of the disease is not yet clear, but the most endorsed hypothesis is a change in soil microbial communities (Mazzola and Manici, 2012) within the four hypotheses: First, the "specific ARD hypothesis", that states that only a small part of the microbiota found in the soil is involved in diseases. However, problems of this hypothesis are diseases which also arise in the absence of these pathogenic microorganisms or that no diseases occur despite the presence of microorganisms. For example, fungal species belonging to the *Cylindrocarpon*, *Rhizoctonia*, *Phytophthora* and *Pythium* genera are frequently found in ARD-affected soils, but their presence and frequency can vary from soil to soil (Tewoldemedhin et al., 2011a,b). Second, the "non-specific ARD hypothesis" states that many different microorganisms in soil are responsible for the development of ARD. In analogy with the dental plaques hypothesis (Marsh, 1994) ARD cases result from the interaction between the microorganisms in the soil and the host itself. Third, the "ecological ARD hypothesis" proposes an equilibrium-shift within (currently unknown) key factors towards the development of the disease. The fact that microbial pathogens represent only a minor part of the resident microbiota and that the disease can progress in absence of the specific pathogens, support this hypothesis. Fourth, the recent "keystone-pathogen-hypothesis" (Rosier et al., 2014) proposed that interaction of one key microbial player with the host can trigger a specific response of the host that makes it more susceptible to a number of other pathogens.

In order to understand the interactions between ARD and the microbiome of soils in ARD apple orchards, microbial communities of fungi and bacteria were routinely targeted within experiments testing the effects of different soil treatments on ARD severity (Mazzola et al., 2015; Nicola et al., 2017; Yim et al., 2015). However, direct comparison of these studies is difficult, since different chemical approaches and analytical pipelines were used in data generation and analysis. In this re-analysis, we collected primary data and integrated all the studies to elucidate the main microbiological drivers involved in ARD-development. In addition to the re-analysis of molecular data, the role of different environmental conditions, geographical locations and that of molecular methods were taken into account through the analysis of the variability of microbial communities in these soil.

2. Materials and methods

2.1. Search and selection of relevant literature and data extraction

A literature search was performed on Web of Knowledge database, looking for all the studies on apple replant disease that analyzed the soil microbiome through metagenomics approaches. The combinations of key words used for the search were: "apple replant disease" AND "soil" AND "microbiome", "apple replant disease" AND "soil" AND "454", "apple replant disease" AND "soil" AND "Illumina" (August 30, 2017). Studies in the form of abstracts from conferences, studies on plants other than apple and studies that did not report on amplicon-metagenomic datasets were omitted. The acceptable studies (Sun et al., 2014; Franke-Whittle et al., 2015; Mazzola et al., 2015; Yim et al., 2015; Nicola et al., 2017; Peruzzi et al., 2017) were retained and the primary data retrieved from public repositories reported by the publication authors. This resulted in 140 and 72 datasets for Bacteria and Fungi, respectively (n = 212 datasets). The following information was extracted from these studies: soil niche (bulk soil or rhizosphere), study type, rootstock, soil treatments, number of orchards sampled, number of samples, duration of the experiment, health of the plant, year and season of sampling, location, global positioning system coordinates, altitude, soil texture, pH, organic matter content, soil nutrients. In addition, the characteristics of the analytical approaches were mapped, such as DNA extraction method, the gene region amplified, primers used, sequencing technique, sequencing depth, software used for data analysis and number of reads after denoising. The samples were also categorized as healthy or diseased based on the reported values for the significant reduction of growth (RG).

2.2. Sequence analysis

Sequence files from past publications were obtained from the NCBI Sequence Read Archive database (SRA). The 64-bit version of the source code SRA Toolkit for Ubuntu Linux version 2.6.0 were downloaded from NCBI and used to programmatically access data housed within SRA and convert it from the SRA format to fastq or sff formats. The SRA Toolkit contains a series of independent data-"dump" utilities that allow for conversion of SRA data into different file formats. Fastq-dump was used to convert data to fastq and fasta format.

Some of the datasets resulted in an interleaved distribution of sequences. In order to be able to parse the mixed datasets into sample specific data files described in published papers (Franke-Whittle et al., 2015; Mazzola et al., 2015; Sun et al., 2014; Yim et al., 2015), authors were asked to submit a design file of their experiment. The lack of design file prevented the use of interleaved sequence mix, therefore the study by Sun et al. (2014) had to be omitted from downstream analyses. A tab-delimited mapping file describing experimental conditions of each study was created from the published literature.

Bacterial and fungal datasets were analyzed using mothur (Schloss et al., 2009) with the SILVA (Quast et al., 2013) and UNITE (Koljalg et al., 2013) databases, respectively. Phylotype approach was used in order to minimize the bias in alignment or binning steps of bacterial and fungal sequences due to different length or gene section. The mothur standard operating procedure approach (Kozich et al., 2013) was adopted and sample analysis scripts were generalized between studies in order to minimize differences in bioinformatics approaches. The resulting *.taxonomy and *.count_table files were merged and served as basis for generation of *.shared files specific for bacteria and fungi.

Bacterial and fungal samples were subsampled to 2500 sequences, omitting the samples with fewer sequences ($n_B = 132$, $n_F = 68$). To cover the developed approach a novel utility was suggested to the developers of the mothur program that was successfully integrated into the novel version of the program (mothur v.1.39.1 – the February 2017 release of mothur).

2.3. Statistical analysis

Bacterial and fungal datasets were analyzed separately in mothur. Initially, alpha diversity estimates (rarefaction curves, various parametric and non-parametric diversity indices) were calculated in order to compare microbial diversity within samples and to check whether the provided sequencing depth was sufficient.

The beta (between-sample) diversity analyses were used to test for differences in the structure of microbial communities in terms of phylogenetic composition (parsimony, unweighted UNIFRAC), abundance of particular clades (weighted UNIFRAC), analysis of molecular variance (AMOVA), homogeneity of molecular variance (HOMOVA). Lefse, random forest and other approaches were used to test for the congruency in sample assignment, i.e. classification efficiency of samples to healthy-diseased classes. Metastats, instead, was used to identify the taxa that were differentially abundant between different H-RG classes ($p < 0.05$, after False Discovery Rate correction).

The OTU association network analysis (nonlinear Spearman association, $p < 0.005$, and $R^2 > 0.8$) was used to identify taxa that were positively or negatively associated with each other across groups of samples and to identify the most dense network clusters suitable for more detailed taxonomic characterization. This resulted in four tables of tightly associated microbial taxa (Bacteria-H, Bacteria-RG; Fungi-H, Fungi-RG) that were further analyzed in Cytoscape (V3.2.0) and the characteristics of their co-occurrence networks were recorded to disentangle the difference between healthy-diseased classes.

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