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Using Next-Generation Sequencing Technology for Pathogen Discovery

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Abstract

Blueberries are woody perennials that, with proper care, can produce a crop each year for decades. In some fields in New Jersey, fruit yields and plant health are declining without definitive cause. Microbial communities in the soil can directly or indirectly affect plant health. Thus, determining what soil organisms are associated with decline, might provide clues for developing approaches to remediation. Soils were collected from the rhizosphere of blueberry plants in healthy and 'decline-associated' fields. DNA was extracted from the soils and target regions (16S for bacteria, ITS for fungi, and 18S for nematodes) were amplified by PCR and sequenced. Taxonomic determinations of the soil microorganisms were based on sequence similarity using BLAST. Preliminary data showed the bacterial community was dominated by the Proteobacteria, with no distinct differences between the soil types. FUNGuild was used to assign the fungal taxa to ecological guild. Most of the fungi detected were saprotrophs, but a substantial number of potential plant pathogens were also identified. We identified more than 70 nematode genera and of those genera detected, only three were classified by NEMAGuild to be plant parasites. Follow up studies are needed to confirm the presence and potential impact of bacterial, fungal, and nematode phyla that may be associated with decline.

Index Words: blueberry decline, replant disease, microbiome, 16S, ITS, 18S, FUNGuild

Introduction

Highbush blueberry (*Vaccinium corymbosum* L.) is a long lived woody perennial. Varieties are clonally propagated and rooted cuttings can take 3 to 5 years to grow into mature bushes. With proper care, mature bushes can yield a commercially acceptable crop for decades. However, yields in some fields are declining and the problem is not reliably alleviated through field replanting. This condition is commonly called 'blueberry decline' or 'replant disease'. The cause of the condition is thought to be due, at least in part, to a buildup of pathogenic organisms or parasites, such as nematodes, in the soil. Replant disease has been described in tree fruits and other crops (Mazzola, 1998, Yang et al., 2012, Larsen, 1995) and the best studied system is apple replant disease (ARD) (Tewoldemedhin et al., 2011, Mazzola, 1998). Nicola et al. (2018) conclude that ARD is an "opportunistic microbial infectious disease, created by certain prevailing environmental conditions affecting microbial metabolism and their interaction with the plant host. "A study in blueberries in North Carolina and Georgia suggested that the cause of replant to be nematodes, but other pathogens and/or parasites might be involved (Jagdale et al., 2013).

Thus, to begin addressing the replant problem in blueberry, we sought to determine what microorganisms inhabit the soils around healthy vs. decline-associated plants. Characterization of the soil microbial community is a daunting task. This is due to several causes: specific soil

microbes can reach high numbers in small amounts of soil, identified microbes vary widely in taxonomic class, and variation across even short distances can be extensive. These issues can at least be partly addressed using modern next-generation (massively parallel) sequencing techniques. Sequencing of millions of DNA fragments and bioinformatics tools can be used to not only identify what is in the soil, but also to determine the relative amounts of specific organisms in a given sample. Determining the functions of the soil inhabitants is the next hurdle. We used FUNGuild and NEMAGuild to assign the identified fungal and nematode taxa to ecological guilds (Nguyen et al., 2016). The next phase of this study will specifically determine which microbes are affecting the crop (positively or negatively).

Materials and Methods

Rhizosphere soils were collected from blueberry fields in Atlantic and Burlington Counties, NJ. An effort was made to collect paired samples from each farm where one sample was from a productive area of a field and the other was from a poor area. Each sample consisted of a mixture of soil from the root zone of 2-3 plants. Samples were collected from 5 farms (10 samples total). DNA was extracted from 0.5 g of each soil sample using the PowerLyzer PowerSoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's directions. Samples were sent to Molecular Research LP (Shallowater, TX, USA) for PCR amplification and sequencing. Portions of the 16S rDNA and ITS were amplified by PCR, targeting bacteria and eukaryotes (primarily fungi), respectively. Nematodes were targeted using primers described by Powers et al. (2011) that were designed to amplify a portion of the 18S rDNA from nematodes. All PCR fragments were sequenced using the Illumina MiSeq (Illumina, San Diego, CA) platform.

Reads were separated into bins by sequence similarity and these were clustered into operational taxonomic units (OTUs) at 97% similarity. Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes (DeSantis et al., 2006) and RDPII (http://rdp.cme.msu.edu) (for bacteria) and NCBI (www.ncbi.nlm.nih.gov) (for eukaryotes). To better characterize the possible functional contribution(s) of the identified fungi and nematodes, taxa were assigned to ecological guilds using FUNGuild and NEMAGuild (Nguyen et al., 2016).

Results

Over 20 million reads were generated for each sample that were binned into 1247 OTUs. Most OTUs could be identified by sequence similarity with classified organisms in one or more of the databases interrogated. Although we were primarily interested in bacteria, fungi and nematodes, some non-target organisms, such as algae, tardigrades and arthropods were also identified (data not shown).

The fungi were dominated by the phyla Ascomycota (57.1%) and Basidiomycota (21.2%). Although 585 fungal genera were identified, most were found at low and inconsistent levels across the samples. None were routinely found to be to be associated with either the healthy or replant soil categories. When FUNGuild was used to assign the taxa to ecological guild, most of the fungi detected were saprotrophs, but a substantial number of potential plant pathogens were also identified. Symbionts were also found including those known to be ericoid mycorrhizae (such as *Rhizoscyphus* and *Oidiodendron*). Many other genera of mycorrhizal fungi were detected that are not generally thought to be associated with ericoid plants. Putative

endophytes were also detected. Their presence as true endophytes, and possible function, needs to be confirmed.

Fungi

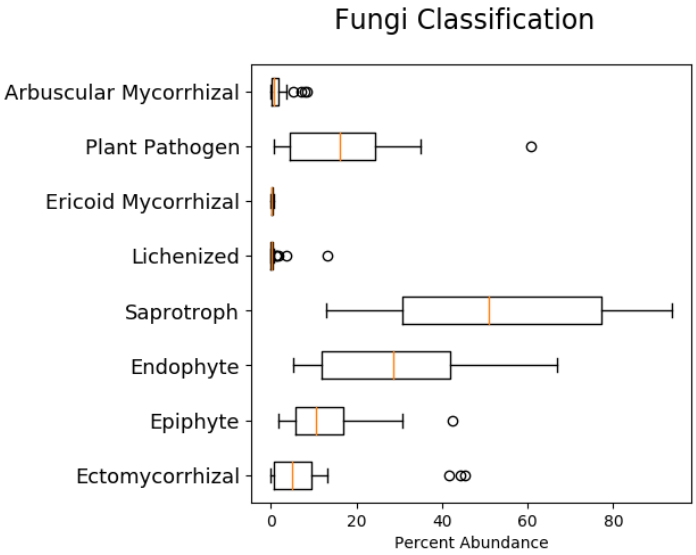

Figure 1. FUNGuild classification of blueberry rhizosphere OTUs. Yellow bars represent the median percent abundance of all samples. The box extends from the first through the third quartile, lines extend to the last sample within 1.5x the inter-quartile range. Outliers are plotted as circles.

Bacteria

Proteobacteria (41.0%) was the most prevalent phylum detected. High numbers of Acidobacteria (19.3%) were also detected as might be expected in acidic 'blueberry' soils. While there are few known bacterial diseases of blueberry, there is the potential that some bacteria are beneficial as plant growth promoters or as suppressors of other diseases (Compant et al., 2010, Lugtenberg & Kamilova, 2009, Weller et al., 2002).

Nematodes

Pathogenic nematodes have been shown to be an important component of replant disease in Georgia and North Carolina (Jagdale et al., 2013), however, primers for detection and subsequent identification of nematodes are not routine. Using a primer set described by Powers et al. (2011), we identified more than 70 nematode genera, but most were present at very low levels. Of those genera detected, only three- *Trichodorus*, *Paratrichodorus*, and *Ditylenchus*- were classified by NEMAGuild to be plant parasites. Nematodes can damage the roots of parasitized plants, resulting in stunted growth, but potential virus transmission by nematodes is also a concern (Martin et al., 2012).

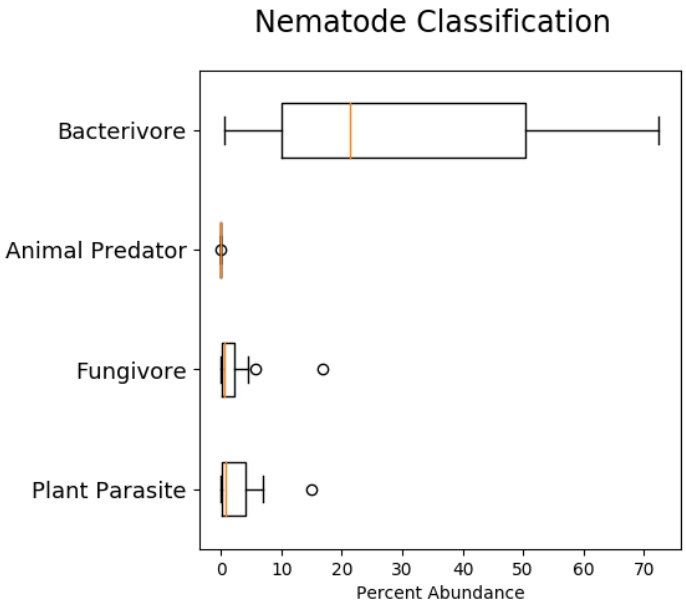

Figure 2. NEMAGuild classification of blueberry rhizosphere OTUs. Yellow bars represent the median percent abundance of all samples. The box extends from the first through the third quartile, lines extend to the last sample within 1.5x the inter-quartile range. Outliers are plotted as circles.

Some of the nematode species that were identified have never been reported in blueberry soils. This could be due to sensitivity of the methods, misidentification, or a combination thereof. This necessitates follow up studies to confirm the presence of at least those phyla that may be important to replant disease.

Discussion and Conclusions

It was unclear what organisms might be associated with blueberry replant disease. This was at least partly due to the high variation in the organisms detected and their relative quantities within and between locations. While FUNGuild successfully categorized the fungi into their ecological guilds, it was still difficult to identify those microorganisms that are playing some role in plant health and how their relative abundance might contribute to soil decline. One way to begin approaching this problem is to identify those organisms that respond rapidly to the presence of a blueberry plant. We are using stable isotope probing (SIP) to label the organisms that respond to the plant exudates. This should help narrow the number of taxa that need to be studied in more detail. Finally, taxonomic identification was sometimes problematic. This is due to several factors including the short sequences used and database quality. To address the read length problem, we have begun amplifying the entire rRNA operon from target groups and sequencing on the Oxford Nanopore MinIon (Oxford, UK).

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