DISSERTATION

SOIL MICROBIAL COMMUNITY DYNAMICS ASSOCIATED WITH AGRICULTURAL CROPS

Submitted by

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ABSTRACT

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It could be argued that the sustainability of agriculture hinges on our ability to understand and manage the interactions that occur between agricultural crops and microbial communities that reside in the soil. Soil microbes regulate the decomposition of organic matter, the cycling of nutrients to crops and can induce systemic resistance. They also drive global biogeochemical cycles that influence the climate, which in turn determine the growing environment for crops. Soil microbes also interact with crop plants directly via neutral, pathogenic, or beneficial symbioses that can influence plant health as well as resistance and resilience to pests and abiotic stress. Conversely, crop plants, via their growth, root exudation and litter production, are critical to the maintenance and growth of soil microbe populations. Despite their close association and importance to global agriculture, our understanding of the interactions between crop plants and their associated soil microbial communities remains poorly understood.

In this dissertation I conducted experiments in two agro-ecoregions (the US Northeast and the Southern mountains of Colorado) to better understand how crop plant composition and management influence soil microbial communities and populations of pathogenic microbes. Data from the first experiment are reported in Chapters 1 and 2. In this experiment I sampled soil microbial communities in a field experiment that was replicated across four locations in the Northeast US. The experimental treatments were plots of perennial ryegrass varying in perennial ryegrass genotypic composition and diversity. My objective was to determine whether the

genotypic composition and diversity of the perennial ryegrass stand influenced the structure (Chapter 1) and composition (Chapter 2) of the soil microbial community over the years of the experiment. I found that soil microbial community structure (measured as abundance of bacteria and fungi) and composition was not influenced by the cultivar mixtures, but rather by the year, location, and plant biomass.

In chapter 3 I present data from a survey of *Helminthosporium solani* and *Colletotrichum coccodes* and a controlled experiment on their effects on potato cultivars in post-harvest storage. The objective of these studies was to understand how specific fungal-fungal and fungal-plant interactions are important in a potato production system where the functional aspects of a specific microbial community is not well understood. Here we see an annual change in soil pathogen presence depending on crop plant in the field. My results show that microbial interactions are polymodal and dependent on host genotype, soil chemical and physical properties and management practices.

My final objective in this dissertation was to formally describe a fungal species, *Penicillium acequia* sp. nov. This fungal species was prevalent in agricultural soils and morphologically and genetically different from its closest relative. This new species has potential to be cultured and utilized as a biocontrol or for production of valuable secondary metabolites. Fungal antagonism as an option for crop plant disease control could reduce overall fungicide use. Inoculation of perennial cropping systems with beneficial microbes at planting over multiple years could harbor a soil microbiome that requires fewer inputs with reduced disease. We have a long way to go in describing the diversity of life in the soil that is critical to our food production system, and must characterize the presence and function of microbes within agricultural systems.

Collectively, the data from this dissertation suggests that there are important yet opaque outcomes of microbial interactions influenced by time and location among other variables. Host genetics, including resistance genes, allow for unique microbial interactions and nutrient exchange which may matter more than traditional phenotypic plant traits, though this requires more research. Microbial interactions continue to evolve, the importance of genetic quantity is not to be discounted, rather determining the biologic pathways mediating interactions will provide greater insight into the sustainability of agricultural ecosystems.

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Graduate school has been the culmination of many years of dedication and many personal sacrifices. Without my parents' dedication to my schooling from the beginning, I may not have succeeded in reading, writing, science and math. Without my parents urging I would never have gone to the Pennsylvania State University for a degree in biology. Dr. Roger Koide and Dr. Glenna Malcolm saw something in me to keep me working for three years in their fungal biology research, where I first began to appreciate the rewards of hard work and dedication to learning. Dr. Andrew Stephenson helped me to get my first bit of research funding to work on bacterial pathogens of cucurbits, giving me hope that financial support in the sciences is attainable. Kyle Loughlin's recommendation of me to work at the Walter Reed Army Institute of Research set me on my path as a professional scientist. Further urging from my WRAIR colleagues to go beyond the bench as a scientist came from many influential people—Major Kamau, Zac Tycz, Liana Sherrod, and Omely Marte. My then supervisor Dr. Evelina Angov showed me how dedicated someone can be to greater scientific goals and how to do it with integrity and precision. My PhD advisor Dr. Kirk Broders, keeping the lights on, coffee brewing, and the inspiration, from New Hampshire to Colorado, never gave up on me and my meandering path during the past six years. My thanks go to all of the research scientists, graduate students and faculty who have been encouraging and supportive. My fiancée, Anne H Howard, your support and guidance, clarity of thought and alternative views have buoyed this work beyond knowing. Those who have helped and supported me through this process of experimentation, successes, failures, learning, and wandering understand how humbling an experience it has been. I thank them all and appreciate their patience.

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Introduction

Plant species are influential in belowground energy flow, from recruiting mycorrhizae for extended hyphal nutrient acquisition to impeding neighboring plant growth or seedling emergence due to allelopathy [1]. Different plant species provide belowground niches for microbes which vary based on the environment, the life stage or tissue type, and their interactions. Plants supply soil microbes with varied energetic and structural support and may therefore influence soil microbial community composition and abundance [2, 3]. Rhizodeposition, including exudate production, is important not only in attracting or deterring microbes but also in root-root and root-fauna signaling [4, 5]. The same class of root exudate chemicals, for example, may attract both mutualistic and pathogenic microbes to the same plant [6]. Plant species differ in the quality and quantity of the root exudates they produce and also in functional traits that influence growth and reproduction of soil microbes [7, 8]. These inter-species differences influence the functionality and structure of the soil microbial community.

Greater plant species diversity results in increased soil carbon storage [9] and soil aggregate stability [10], both of which are important indicators of soil microbial community functionality. It is understood that temperate tree species produce widely different exudate profiles, particularly in the diversity of compounds which affect microbial respiration or recruitment [11, 12] as well as select for mycorrhizal mutualists [13]. Temperate grassland plant communities also produce diverse mixtures of organic and amino acids, carbohydrates, and signaling molecules which drive the abundance, diversity, and reproductive rate of soil microbes [14, 15]. A seminal study in *Agave*, a native desert plant of North America which has a history of cultivation, has shown unique microbial associations in the cultivated species compared to the native one. The authors also found a less diverse prokaryotic community in cultivated species and they identified a core microbial community associated with drought tolerance and plant growth [16]. Plant species exhibit different affinities for microbial interaction—some form close endophytic relationships with characterized stress tolerance implications [17–19] while others experience less well understood mutualisms or recently lost mutualisms due to domestication [20].

Genotypic diversity within the same plant species has also been shown to have a significant effect on the soil microbial community. This is likely due to variation of traits such as root architecture [21, 22], growth rate and phenology [23], and the quantity and quality of root exudation [1, 24], all of which may have a profound effect on structuring the microbial community. In agricultural systems where a domesticated genotype is planted near wild plant ancestors and in distinct ecotypes of *Arabidopsis thaliana,* effects of genotype on associated belowground microbes were observed [25]. These genotypic influences are usually small, and evidence is limited by the difficulty of a heterogeneous soil microbial community. Crops bred to have shallow root systems which maximize response to nutrient and water additions, and extensive inbreeding in *A. thaliana* have been shown to affect microbial recruitment [26]. Another perennial species, *Boechera stricta* exhibits genotype specific microbial associations which vary with plant age and environment, harboring distinct bacterial species in leaves more often than in roots. The authors found that in *B. stricta* 93% of detected prokaryotes present in roots and leaves were detected in the bulk soil where the plants were grown, suggesting bulk soil derivation of important plant microbe symbioses [27].

In agriculture, there is growing interest in the use of intra-specific diversity (i.e., mixing different genotypes/cultivars of a single species; also known as “blends”) as a cultural practice for managing crop disease and other pests and increasing the resilience of cropping systems against weather variability [28–31]. However, despite the increasing use of intraspecific mixtures in agriculture, few studies have been conducted to investigate how the composition or diversity of crop intraspecific mixtures may influence the composition and structure of the soil microbial community. Whether and how the composition and structure of the soil microbial community is influenced or managed through the choice of crop cultivar or strategic planting of intraspecific crop mixtures is therefore a critical question.

The composition and structure of the soil microbial community, particularly the relative abundance of fungi to bacteria, plays an important role in determining the functioning of agroecosystems. Fungi function as important decomposers of organic detritus while also exhibiting competition for belowground space and exchange of nutrients. Fungal mycorrhizal symbionts and their plant partners are both shown to select for ideal associations, which are dependent upon many factors including resource fluctuations, land use history, and the timing of the interaction [13]. Bacteria fill niches of symbiotic plant growth promotion and induce plant responses to pathogens. Plant growth promoting rhizobacteria (PGPR) protect against drought and promote growth in plants but also increase nutrient use efficiency which could lead to reduced agricultural fertilizer inputs [32]. In a perennial low input system an increase in the fungal to bacterial ratio coincides with greater fungal biomass [33] and decreased bacterial abundance [34]. The microbial community composition is not only crop dependent but environmentally dependent as well. The environmental conditions influencing plant productivity including seasonal precipitation, and mean annual temperature may be important in measuring belowground community effects.

The overall experimental objective of this study was to (i) measure the fungal and bacterial biomass in the bulk soil associated with 7 different perennial ryegrass (*Lolium perenne*) cultivar treatments in four locations over three years, to (ii) determine whether there is a treatment (cultivar mixture) effect on the microbial community biomass, measured as the ratio of fungi to bacteria in the associated soil, and to (iii) determine if there was an effect of aboveground plant biomass on belowground fungal and bacterial biomass.

Materials and Methods

Site description and selection

This study was conducted at four locations in the northeastern United States (Table 1.1). Locations of the sites from south to north are: Pennsylvania State University Rock Springs Agricultural Experiment Station (40.716996 N, 77.941813 W), Pennsylvania Furnace, PA., University of New Hampshire Organic Dairy Research Farm at Burley Demeritt (43.097257 N, 70.991599 W), Lee, NH., University of Vermont Wheeler Research Field (44.441892 N, 73.194520 W) Burlington, VT., and University of Maine Rogers Forage and Crops Research Farm (44.927842 N, 68.698811 W) Stillwater, ME (Figure 1.1). Study sites varied in mean annual temperature (MAT), seasonal precipitation, and soil characteristics. The MAT ranged from 5° C - 15° C in Pennsylvania to 0.8° C - 12.1° C in Maine, the winter hardiness zones ranged from 6b in Pennsylvania to 5a in Maine and Vermont (http://planthardiness.ars.usda.gov). Annual total precipitation across the four sites ranged from 281.9 cm in Burlington, Vermont to 218.4 cm in State College, Pennsylvania (National Climatic Data Center NCDC, National Oceanic and Atmospheric Administration NOAA). Mean annual temperature (MAT), mean annual precipitation (MAP), elevation and slope are reported in Table 1.1 as recorded at each experimental location. Data was collected from local weather stations monitored by (NCDC, NOAA) and utilization of the USDA National Resource Conservation Service (NRCS) database and Web Soil Survey [35].

The southernmost location, in Pennsylvania has a limestone derived soil type that is shallow yet well drained, characterized as a Hagerstown silt loam with 3-8% slope [36]. Pennsylvania soils are of clayey residuum weathered from limestone parent material and are fine, mixed, semiactive mesic Typic Hapludalfs. The New Hampshire sites soils are described by their characteristic inceptisol order, being well drained and sandy classified as loamy, mixed, superactive, mesic Lithic Dystrudepts (NRCS, USDA). They are characterized as a Hollis-Charlton very rocky fine sandy loam with 3-8% slope and its parent material classified as Till. The Vermont site soils are exceedingly sandy located near Lake Champlain, south of Burlington, Vermont. They are categorized as Adams and Windsor Loamy sands with 5-12% slope with sandy glaciofluvial deposits as parent material, with a taxonomic class of sandy, isotic, frigid Typic Haplorthods (NRCS, USDA). Soil at the Maine site was of fine silt with high organic matter and generally with low pH. The parent material is glaciomarine deposits categorized as fine-silty Pushaw-Swanville 0-8% slope taxonomically classified as mixed, semiactive, nonacidic, frigid Aeric Epiaquepts (NRCS, USDA).

Experimental sites were located on university experimental research stations where they were under no till prior to and for the duration of the study. The field sites were initially planted with winter rye in 2011 which was plowed under and seeded with red clover in spring of 2012 until being disced in late summer 2012. This study began when treatments germinated in September - October 2012 depending upon location. Treatment plots received no irrigation, fertilizer or chemical treatments, though simulated grazing was performed via mechanical mowing when aboveground biomass reached an average height of 25 cm it was clipped down to a height of 7.5 cm in all plots.

Treatment description

The experiment was a randomized complete block design (RCBD) with seven treatments replicated in five blocks constituting a total of 35 plots. A total of 15 *L. perenne* cultivars were used, comprising 7 different treatment mixtures (Table 1.2). The perennial ryegrass cultivar mixtures varied in their functional and genetic composition to mitigate common forage crop issues experienced in northeastern United States. The heading date (HD) indicates when the grass is physiologically maturing, producing maximum root exudates and requiring more nutrients from the soil and the microbial community [37]. Heading date varied in mixtures to ensure staggered maturation and sufficient aboveground biomass production during specific time periods. Winter hardiness (WH) composition varied to increase survival during severe winters which can dramatically reduce overwinter viability. These variable traits affect overall productivity and may reduce mid-summer stress due to heat and reduced soil moisture. For example, late heading forage (Treatment 5) contains later heading cultivars compared to early heading forage (Treatment 3). Ploidy of the individual cultivars varied within treatments, containing diploid and tetraploid cultivars (Treatment 2 and 3), as well as diploid (Treatment 4 and 7) and tetraploid (Treatment 1 and 5) only, cultivar mixtures. These treatments allow us to look at the influence of plant genotype and functionality on the microbial community composition, retention through time, and specific location by treatment interactions.

Each plot measured 5 meters (m) x 5 m with a 1m x 5m planted buffer strip of orchardgrass (*Dactylis glomerata* L.) between. Each replicated block was sown with an equal mass of seed with a walk-behind Carter small seed cone planter. Cultivars were provided by

Barenbrug USA (Tangent, Oregon) a grass seed supplier which also provided the trait ratings and genetic composition described in the previous paragraph (Table 1.2). For treatments with multiple cultivars the total seed weight was divided equally among individual cultivars. Treatment regimes in Pennsylvania and New Hampshire were different from Vermont and Maine treatments, due to the broad geographic scale – ME and VT sites were in a different winter hardiness zones from PA and NH sites. The two sets of treatments differed in that the most adapted cultivar for each hardiness zone was Mara for VT and ME and Remington for PA and NH

To assess treatment productivity during the second-year aboveground biomass was collected via mowing at the plot level, plant material was sorted, weighed, and identified by general composition including ‘perennial ryegrass’ and a composite of ‘all vegetation’. The first and last clipping for the growing season was recorded as “Initial” and “Final” respectively for each vegetation class. A single cultivar of white clover (*Trifolium repens*) was sown at a rate of 3.36 kg/hectare with the perennial ryegrass when treatments were established.

Soil sampling and analysis

In order to assess soil microbial biomass for each treatment, we collected multiple soil cores (2 cm wide and to a depth of 10-20 cm) at each location in September-October of each year. In each of the 35 treatment plots five soil cores were collected in a ‘W’ pattern. The five cores formed the composite soil sample for each plot. The composite samples were immediately placed on ice and transported to the lab in Durham, NH. Once at the lab, the composite soil sample was homogenized and a sub sample frozen in 15mL tubes at -80^{o}C, within 24 hours. A total DNA extraction was performed on 0.25 grams of soil that had all root and plant tissue removed. MoBio Power Soil (MoBio Labratories Carlsbad, CA) kits were used according to manufacturer's protocol. Extracted DNA was quantified with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE) and frozen at -80°C until qPCR analysis. A single composite soil sample was taken from each location when the treatments established for analysis of chemical and physical properties at the University of New Hampshire Soil Testing Service, Durham, NH.

Fungal and Bacterial Biomass Quantification

The total fungal (F) and bacterial (B) biomass and the F:B biomass ratio were determined based on the quantity of DNA, of each of these groups in the soil. Using the Roche 2.0 LightCycler (Roche, Basel, Switzerland) we optimized two separate protocols utilizing Taq-man primer probe pairs. The fungal protocol was used to quantify a 351 base-pair (bp) fungal sequence within the 18S rRNA gene [38]. We optimized this protocol using a total reaction volume of 20µL, with 10µL of 2x Promega qPCR master mix, 0.3µM of each primer, 0.1µM of probe, and adjusting to final volume with ddH_2O. Standard curves were created for both protocols--fungal and bacterial--where pure culture specimens of multiple individuals verified by sequence analysis were included. The bacterial quantification protocol amplified a 466 bp region of the V3-V4 16S rRNA gene [39]. This amplicon product has a broad species range and its design utilized 4,938 bacterial sequences. Similar qPCR master mixes were used in quantification of each product though the thermocycler profiles differed. A 10uL final reaction volume was chosen with 1uL of template DNA with the final reaction containing 1.8uM of both forward and reverse primers, 225 nM of the probe, 1X Promega qPCR master mix and molecular grade water. Standard curves in both bacterial and fungal qPCR protocols allowed for one standard being included within every run to anchor analysis to the standards (Standardization for more details). Cycle threshold (C_T) values were recorded for every DNA extract collected.

Triplicate reactions were conducted for each sample during the first year, with highly reproducible results. Single C_T values were recorded for each sample during years two and three. Fungal C_T (Fungi) and bacterial C_T (Bacteria) values were then used to create a single fungal to bacterial ratio (F:B).

Standardization

Extracted DNA was serially diluted for both known fungal and bacterial pure cultures whose sequences were verified via Sanger sequencing (GeneWiz, South Plainfield, New Jersey). The C_T values measured from the pure culture DNA amplified by qPCR were used to extrapolate the copy number of the targeted genomic region through serial dilutions. The importance of copy number variation and diluted total DNA for this analysis from edaphic community DNA was considered by using multiple known species and determining the most accurate value used for the samples in the study [40]. The bacterial standardization curve was based on the seven copies of the 16S rDNA in the *Escherichia coli* K-12 with a genome size of 4.64 Mbp [41] and the fungal curve based on quantitation of DNA extracted from *Fusarium oxysporum,* not including a copy number correction as similarly performed in other studies [42]. Standardized C_T values were reported as fungal and bacterial biomass for each sample.

Statistical analysis

Statistical analyses were performed on a mixed effects model through analysis of variance (ANOVA) in R for Mac OS X version 3.4.4 (R Core Development Team, 2018). The full mixed effects model was used separately for the three response variables (F:B, Fungi, and Bacteria) with a three way interaction for the fixed variables (Location, Treatment and Year), a random effect of Block, and a random effect of Block by Treatment to account for repeated measures at the plot level. The R packages used include ‘lme4’ and ‘emmeans’ which was implemented for pairwise comparisons within in the model [43]. Parameter estimation criterion used was restricted maximum likelihood. In the ANOVA analyses the “Kenward-Roger” degrees of freedom method was selected where t-tests and p-values are provided. The plotted residuals indicated linearity in the data and homogeneity of variance and normality of data distribution was checked with a QQ plot (Figures S1.1-1.2) [44]. Tukey’s multiple comparisons p-value adjustment method was for orthogonal contrast comparisons [45]. Including environmental covariates (MAT and seasonal precipitation) to the full mixed effects model, parameter estimation was limited to maximum likelihood and models were compared with AIC and BIC (-23.1 and 320) respectively, being equal for both models. We used a second mixed effects model to evaluate the effect of aboveground plant biomass on F:B including the fixed variables (location and treatment) and their additive interactions with plant biomass as covariates and the random effect variable of block. Two separate plant biomass values used in the model included the final measured weight of perennial ryegrass and the initial total plant biomass in each plot.

Results

Soil properties

The soils at the Vermont site had the least organic matter, calcium, magnesium, and potassium concentrations (Table 1.3). Soil at the Maine site was generally fine silt with high organic matter and the lowest pH of all treatments. The Pennsylvania site soils had the highest pH and concentration of calcium and the least amount of phosphorous. The New Hampshire site soils had the most organic matter out of all four locations as well as the highest estimated potassium saturation. The soil property measurements include a mean soil pH of 6.525 with a maximum of 7.1 in Pennsylvania and a minimum of 6.2 in Maine. Organic matter present in the soil averaged 3.05% with a maximum of 4.7% in the New Hampshire site and a minimum of

1.10% at Vermont site. The available and estimated saturation of select nutrients and other defining soil characteristics are present in Table 1.2.

Microbial response to host genotype, location and year

There was a significant effect of location, year and the year*location interaction (*P* <0.05) on the fungal (F), bacterial (B) and F:B biomass as determined by the ANOVA on treatment, year, location, all two-way, and three-way interactions in the full mixed effects model (Table 1.4). There was no significant effect of treatment on F:B, bacterial or fungal biomass at any location within a single year (*P* >0.05) (Table 1.4). There were within location significant differences in F:B response by treatment. Specifically, in the Maine and Pennsylvania sites the F:B biomass ratios decreased from year 1 to year 3 in 5 and 7 of the treatments, respectively. In the New Hampshire and Vermont sites 5 and 4 of their respective treatments exhibited increased F:B ratios from year 1 to 2 (Table 1.5). The fungal mean standardized biomass quantification was 11.2 across locations with a maximum of 14.42 at the Maine site year one and a minimum of 8.60 at the New Hampshire site in year three. The bacterial mean quantification was 4.17 with a maximum of 5.71 at the Maine site year three and a minimum of 3.09 in the Pennsylvania site during year two. The F:B biomass mean was 2.70 with a maximum of 3.64 at the Pennsylvania site during year two and a minimum of 1.84 at the New Hampshire site during year three (Figure 1.2).

Microbial response to variation in climate, aboveground biomass and soil

Using the full mixed effects model with environmental covariates (MAT and seasonal precipitation), F:B biomass increased significantly with increasing MAT and with fall and spring precipitation while decreased with winter and summer seasonal precipitation (*P* <0.05) (Table 1.6). The fungal biomass increased with increasing fall and spring precipitation (*P*<0.05). The bacterial biomass increased with increasing summer and winter precipitation while also decreasing with increasing fall and spring precipitation ($P<0.05$).

During the study the minimum MAT was in Maine (7.28ºC) and highest in Pennsylvania (10.12ºC), following the latitudinal gradient. Also following a latitudinal gradient, the MAP increased south to north with Pennsylvania having the least precipitation (218.4 cm) and Vermont having the most (281.9 cm). With seasonal precipitation as a covariate in the full mixed effects model there are opposite effects, where increasing winter and summer precipitation follow a decrease in F:B while increased spring and fall precipitation ($P<0.01$) coincide with increased F:B across all locations and years (Table 1.6).

Both final and initial ryegrass and total plant biomass were used in the mixed effects model to test the response of the soil microbial community (Table 1.7). There was a significant effect of location on the F:B response for the initial total plant biomass and both final ryegrass and final total plant biomass with the ANOVA of the mixed effects model ($P<0.05$). There was a single significant effect on the F:B response ($P=0.028$) to initial total plant biomass (Table 1.7). Performing pairwise comparisons within the model there was no significant difference of F:B between locations individual treatment level ryegrass production nor any within location differences by treatment production. The initial ryegrass biomass average was 133.92 grams per meter square (g/m^2) with a maximum of 384.1 g/m^2 and a minimum of 0.49 g/m^2 across all treatments. The final ryegrass biomass across locations had an average of 130.77 g/m^2 with a minimum of 17.00 g/m^2 in Pennsylvania and a maximum of 384.1 g/m^2 in Maine (Figure 1.4). There was a trend in Maine and New Hampshire for higher final ryegrass and total plant biomass across all treatments compared to the Pennsylvania and Vermont locations, where the Vermont and Pennsylvania growing season ended with much less aboveground plant biomass (Figure 1.5) at these experimental sites.

Discussion

Plant associated microbial community differences have been extensively investigated providing foundational evidence of unique communities detected with specific hosts, less understood is this association with genotypic mixtures of an individual plant species [46]. Individual cultivars or cultivar mixtures were not a significant factor of the fungal to bacterial biomass measured within any location or year in this study. We measured the biomass of bacteria and fungi, for fungi and bacteria both contain short and long-lived taxa which vary in quantifiable biomass and exhibit different functional roles. Oligotrophs are energy efficient microbes, and when compared to copiotrophs which are less efficient have a higher biomass yield for each unit of substrate consumed [47]. Oligotrophs traditionally succeed copiotrophs once the easily scavenged and metabolized energy substrates are depleted. Oligotrophic bacterial taxa (eg. Verrucomicrobia, Planctomycetes, and Chloroflexi) are ubiquitously found in undisturbed systems [48]. Accessibility of nutrients is an indicator of overall microbial biomass [49] and while fungal hyphae extend to exploit less accessible nutrients an increase in fungal to bacterial biomass is often observed [50]. The F:B biomass is a broad measure of the belowground community composition, neither discerning between disparate functional groups of microbes or changes within important taxonomic groups.

The functional roles of specific microbial taxa in the soil are not thoroughly understood, though progress has been made in discerning copiotrophic bacteria from other oligotrophic microbes [51]. The F:B significantly increased at the New Hampshire and Vermont sites in the first year of the study and then maintained a similar ratio across all treatments from year two to three (Figure 1.2). This trend was markedly different in the Pennsylvania and Maine sites where the F:B biomass increased, but not significantly, from year one to year two and decreased significantly (*P* <0.001) in year three. Interestingly, there was no significant F:B biomass difference between locations in the third year of the study. The F:B similarity shows that in three years across four regionally distinct locations, the belowground fungal and bacterial communities reach a similar ratio regardless of perennial ryegrass cultivar treatment. This suggests that the soil microbial community requires multiple years to reach an equilibrium in this perennial ryegrass system.

An increase in either fungal or bacterial community quantification belowground may correspond to shifts in aboveground plant productivity and health. We would expect with increasing *L. perenne* diversity the fungal community would increase over time, where more genetically diverse, tetraploid mixtures, would differ from lower genetic diversity mixtures. Here we show that in the second year, once the treatments establish, there is a correlation between increased early season plant biomass and decreased late season F:B biomass (*P*=0.03) but no effect of treatment. This may indicate that greater plant biomass, including annual weedy species, early in the growing season decreases fungal biomass compared to bacterial biomass measured later in the season where perennial ryegrass cultivars are planted. The Maine and New Hampshire locations experienced the most *L. perenne* productivity over the second sampling year while also incurring more total plant biomass compared to Pennsylvania and Vermont sites (Figure 1.4). The four experimental sites produced similar aboveground biomass across all treatments initially (Figure 1.5). Similar experiments have seen that plant species diversity, not plant biomass affect soil microbial biomass, usually bacterial biomass specifically [52, 53].

Soil microbial biomass is usually higher in natural undisturbed systems compared to similar managed ones [54, 55] regardless of soil properties. This functional dichotomy includes increased oligotrophic microbes in forests and grasslands compared to the microbes in agricultural soils. Having measured soil properties only once at the site level we are unable to relate soil characteristics to F:B biomass. There were significant effects of seasonal precipitation on fungal and bacterial quantification where increased spring and fall precipitation correlated to increased F:B and increased winter and summer precipitation correlated to decreased F:B biomass. Precipitation patterns affect soil microbes globally, specifically drying and rewetting events on certain microbial functional groups [56]. Ratios of fungi to bacteria are difficult to interpret due to the complexities of microbial communities in the soil, though an increased F:B biomass over time does imply increased fungal community membership while a decreased F:B corresponds to a reduced bacterial community biomass. There was a decrease in two of the locations (Pennsylvania and Maine) final F:B biomass which was equal to the final F:B biomass of New Hampshire and Vermont locations. This could mean that Pennsylvania and Maine locations both of which had similar soil organic matter generally had higher copiotrophic fungi and bacteria initially which were succeeded by fewer oligotrophic fungi and a similar biomass of bacteria.

Rapid soil microbial turnover often involves easily accessible nutrients which are present in recently disturbed soil or with development of root infrastructure [57]. Plant genotypes vary in their affinity to produce exudates and the development rate of roots, these are important for bacterial and fungal stress associated tolerances. Specific plant genotypes are bred to exhibiting unique phenotypes in natural systems, i.e. plant pathogen resistance within a specific region or early emergence and shoot development for resource acquisition [58]. F:B measure is broad therefore plant genotype specific microbial community membership is difficult to detect. Through pairwise comparisons we do see cultivar treatment specific differences within a location (Table 1.5). These differences mainly exhibit year to year variability in F:B biomass. Though the microbial associations are broadly quantified, this quantification is important for determining the microbial balance. In a skewed soil microbiome where one kingdom is overly abundant, the system is more susceptible to detrimental disease or abiotic stress [59, 60]. We know that microbial diversity is important for soil functioning and resilience as well as plant health. The annual change or rebalancing of the microbial community shows a system working and a system that is adapting to the changing weather and plant community requirements.

In this study, we demonstrated that the fungal and bacterial biomass fluctuates from year to year with two broad patterns of change under similar treatments. The two patterns include limited initial F:B change from year one to two followed by a significant decrease in year three (Pennsylvania and Maine), and a significant increase from year one to two followed by no significant change in year three (New Hampshire and Vermont). All locations and treatments result in no significant difference between the average F:B by the third year of the study. Genotypic plant diversity or “blends” of perennial ryegrass cultivars do not affect the F:B biomass across these four different locations over three years. This research should be further supported by amplicon sequence data to show the actual functional changes occurring within the system, specifically with different cultivar treatments.

Figures and Tables

Table 1.1. Physical location, environmental characteristics, and soil type. The mean annual temperature (MAT) in degrees Celsius, mean annual precipitation (MAP) in centimeters, elevation in meters (m), percent slope and soil type for the four locations described in this study. MAT and MAP curated from NCDC, NOAA data. Elevation, slope, and soil type data from NRCS.

	Pennsylvania	New Hampshire	Vermont	Maine
Coordinates	40.716996 N, 77.941813 W	43.097257 N, 70.991599 W	44.441892 N, 73.194520 W	44.927842 N, 68.698811 W
MAT (C)	10.12	9.62	8.87	7.28
MAP (cm)	218.4	236.2	281.9	261.6
Elevation (m)	371.85	57.91	60.96	38.1
Slope	3-8%	3-8%	5-12%	0-8%
Soil Type	Hagerstown Silt	Hollis-Charlton	Adams Windsor	Pushaw-Swanville

Table 1.2. Treatment mixtures of *L. perenne* at the Pennsylvania and New Hampshire field locations. The mixtures varied from Vermont and Maine sites by most adapted cultivar, where ever Remington was used in Pennsylvania and New Hampshire it was substituted with Mara in the northern Vermont and Maine sites. Winter hardiness (WH) is the average value of each cultivar in the mix rated on a numeric scale, the higher the value the hardier it is, heading date (HD) is the average value of each cultivar rated on a temporal maturation scale, information provided by Barenbrug USA. Ploidy is indicated by 2N (diploid) and 4N (tetraploid).

Treatment	**Cultivar**	**Heading Date**	**Ploidy**	**WH**	**HD**
T_1	Most adapted	Early	4N	6	5
T_2	3 Cultivars	Early and Late	Mixed	6.33	5
T_3	5 Cultivars	Earlier and Later	Mixed	6	5.2
T_4	5 Cultivars	Mixed	2N	6.6	5.2
T_5	5 Cultivars	Mixed	4N	6	5.4
T_6	Commercial Blend	Unknown	Unknown	NA	NA
T_7	5 Cultivars	Early	2N	5.8	5

Table 1.3. Soil chemical properties associated with each location measured during the second year of the study. Physical and chemical properties of soil sampled at each location in the study including, estimated (Est.) saturation (Sat.) of Base, Calcium, Magnesium, Potassium, and Phosphorous as well as organic matter from the UNH soil testing service.

	Pennsylvania	**New Hampshire**	**Vermont**	**Maine**
pH	7.1	6.3	6.5	6.2
Calcium (ppm)	1579	1057	815	992
Magnesium (ppm)	430	195	45	177
Potassium (ppm)	130	134	26	46
Phosphorous (ppm)	15	441	273	82
Est. CEC	11.8	11.2	5.2	11.2
Est. Base Sat.	100%	65%	86.60%	58.80%
Est. Ca Sat.	66.80 %	47.40%	78.10%	44.50%
Est. Mg Sat.	30.30 %	14.60%	7.20%	13.20%
Est. K Sat.	2.80%	3.10%	1.30%	1.10%
Est. P Sat.	1.40%	21.50%	23.60%	5.70%
Organic Matter	3.10%	4.70%	1.10%	3.30%

Table 1.4. Type III ANOVA table of mixed effects model of the three response variables (F:B, Fungi, and Bacteria) where all two and three way interactions are considered fixed and random variables of block and block : treatment interaction to account for repeated measures. Bold values indicate *P*<0.05, DF=degrees of freedom.

Response:		**F:B**	**Fungi**	**Bacteria**
	Df	F value	F value	F value
Location	3	**76.0200**	**99.3901**	**3.5078**
Treatment	6	0.6810	0.8963	1.1396
Year	2	**56.1797**	**26.0435**	**79.1859**
Location: Treatment	18	0.4423	0.6722	0.6979
Location: Year	6	**28.6340**	**20.1738**	**28.8629**
Treatment: Year	12	0.2141	0.4365	0.4818
Location: Treatment: Year	35	0.7089	0.9165	0.8405

Table 1.5. Pairwise comparison between years within location (Pennsylvania, New Hampshire, Vermont, Maine) by treatment (Treatment 1-Treatment 7) from full mixed effects model on F:B response, *P*-values provided indicate statistically different F:B between years within treatment, NA indicates not enough values to compare and '–' indicates no difference. Values in red represent significant decreases and black represent significant increases in F:B between years..

Pennsylvania	**T_1**	**T-2**	**T_3**	**T_4**	**T_5**	**T_6**	**T_7**
Year 1-2	-	-	-	-	-	-	-
Year 1-3	0.0005	0.0004	0.0040	0.0161	0.0285	0.0012	0.0128
Year 2-3	0.0341	0.0001	0.0052	-	0.0011	0.0011	0.0003
New Hampshire							
Year 1-2	-	0.0132	0.0165	0.0190	0.0128	0.0122	-
Year 1-3	-	0.0011	0.0575	-	0.0020	0.0583	-
Year 2-3	-	-	-	-	-	-	-
Vermont							
Year 1-2	0.0561	-	0.0120	-	0.0198	-	0.0436
Year 1-3	-	-	-	-	-	-	-
Year 2-3	-	-	-	-	-	-	-
Maine							
Year 1-2	-	-	-	-	-	-	NA
Year 1-3	0.0727	0.0003	0.0051	0.0429	0.0001	-	NA
Year 2-3	0.0004	0.0004	0.0016	0.0117	0.0001	0.0003	-

Table 1.6. Type III ANOVA table of mixed effects model showing effect of variable on F:B, Fungal, and Bacterial responses. The response variables (F:B) on the mixed effects model where location*treatment*year interactions are considered fixed and MAT and seasonal precipitation are additive covariates with the random variables of block and block:treatment. Bold values indicate *P*<0.05, Df=degrees of freedom.

Response:		**F:B**	**Fungi**	**Bacteria**
	Df	F value	F value	F value
Location	3	**84.3641**	**83.1061**	**10.9742**
Treatment	6	0.6810	0.8963	1.1396
Year	2	**89.9536**	**12.6881**	**82.2569**
Summer Precip	1	**22.7501**	2.7172	**20.8034**
Fall Precip	1	**10.6343**	**4.6366**	**5.5778**
Winter Precip	1	**11.2021**	0.2382	**12.4341**
Spring Precip	1	**15.0672**	**13.5436**	**4.7886**
MAT	1	**7.4384**	0.0050	**8.2008**
Location: Treatment	18	0.4423	0.6722	0.6979
Location: Year	1	**14.1252**	**11.3989**	**44.4875**
Treatment: Year	12	0.2141	0.4365	0.4818
Location: Treatment:Year	35	0.7089	0.9165	0.8405

Table 1.7. Type III ANOVA Table of Mixed Effects Model. The response variable (F:B) on the mixed effects model where the two way interactions are considered fixed and plant biomass is an additive covariate with the random variable of block. Bold values indicate $P<0.05$, Df=degrees of freedom.

Response: F:B		**Initial Veg**	**Final Veg**	**Initial Ryegrass**	**Final Ryegrass**
	Df	F value	F value	F value	F value
Treatment	6	1.0759	0.7742	0.9178	0.7852
Plant Biomass	1	**4.9025**	2.0289	2.0264	2.4386
Location	3	**2.9158**	**5.3217**	2.5174	**5.2213**
Treatment: Location	18	0.7184	0.6043	0.6879	0.7763
Plant Biomass: Location	3	1.7910	1.2111	1.4879	1.8264

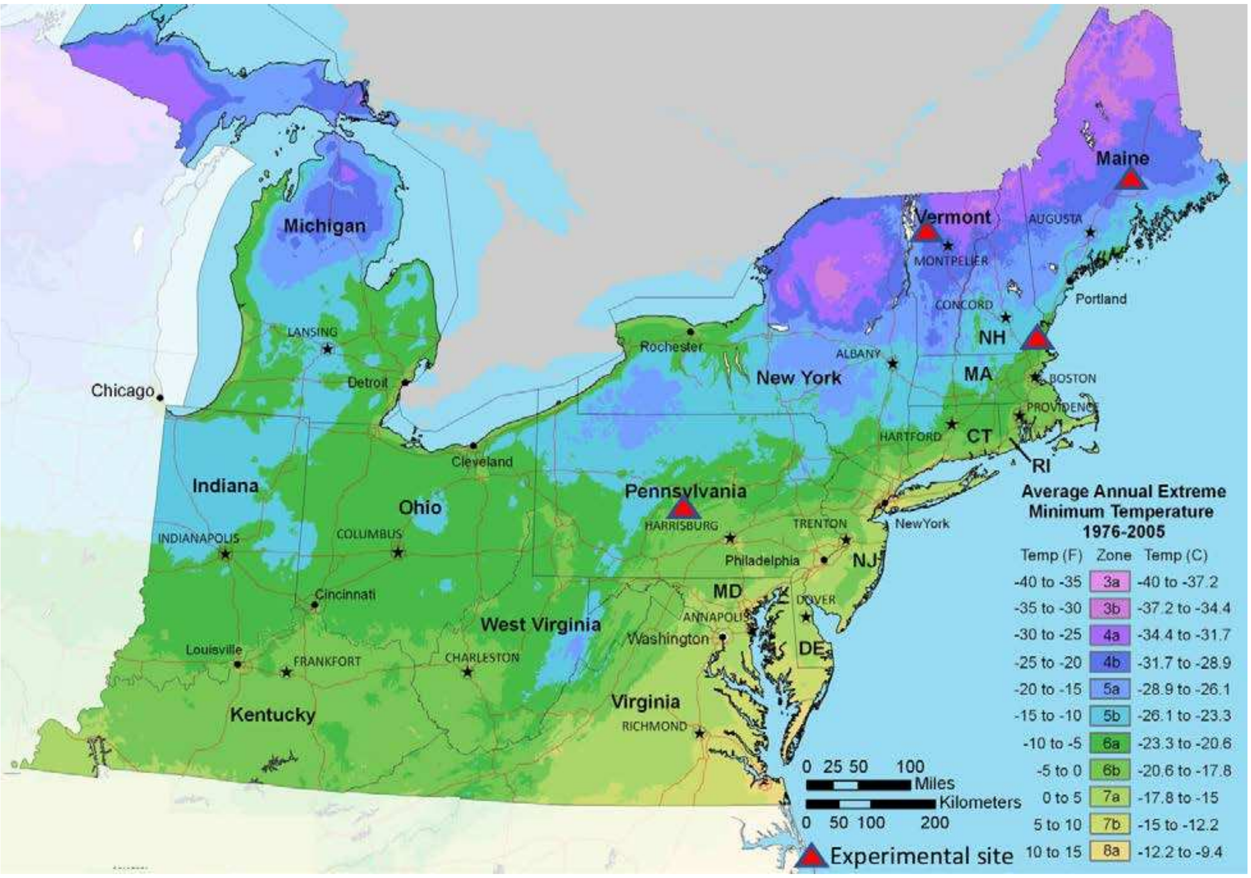

Figure 1.1. Cold hardiness zone map adapted from USDA ARS and Oregon State University PRISM Climate Group. Experimental sites are plotted with red triangles, zones separated by average annual extreme temperature are indicated by map color, Pennsylvania zone 6b, New Hampshire 5b, Maine 5a, and Vermont 5a.

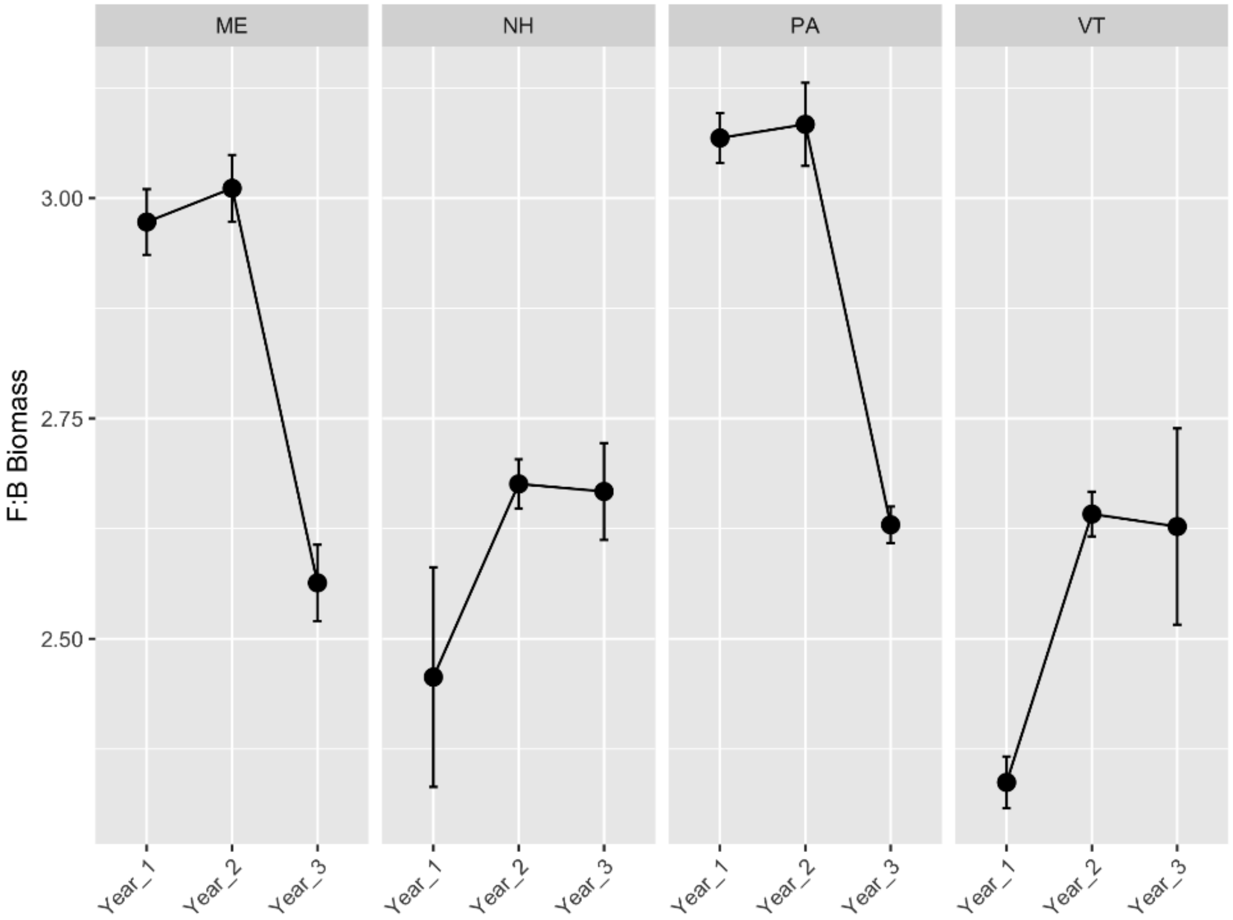

Figure 1.2. Mean (+/- SE) of F:B over time in Maine (ME), New Hampshire (NH), Pennsylvania (PA) and Vermont (VT), combining all *Lolium perenne* cultivar treatments. There was a significant effect ($P < 0.05$) of year at all locations, though no significant difference between locations in year 3.

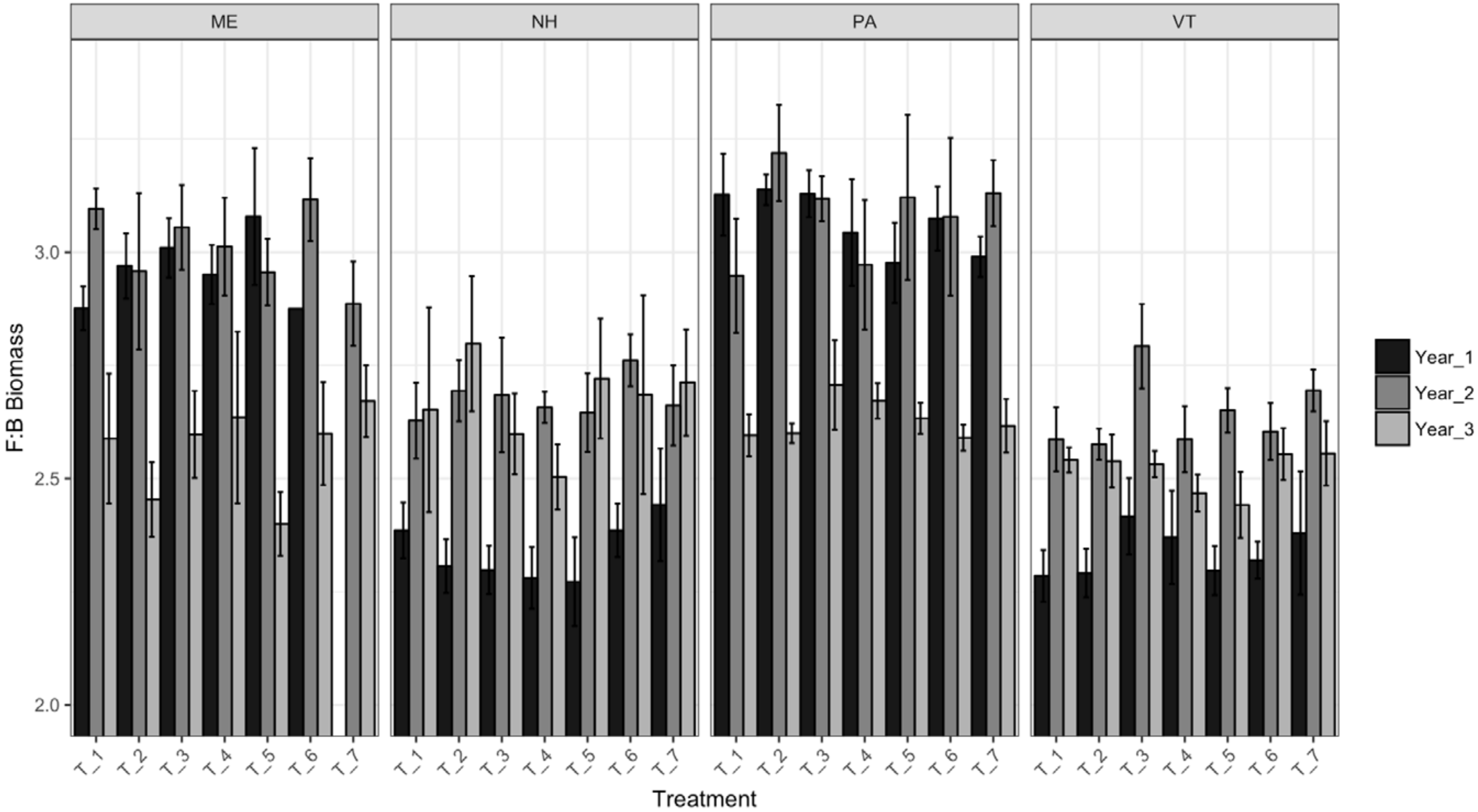

Figure 1.3. F:B biomass ratio (+/- SE) over time in Maine (ME), New Hampshire (NH), Pennsylvania (PA) and Vermont (VT), across all 7 *Lolium perenne* treatments (T_1-T_7). There was a significant difference ($P < 0.05$) between certain years and between certain treatments at all locations.

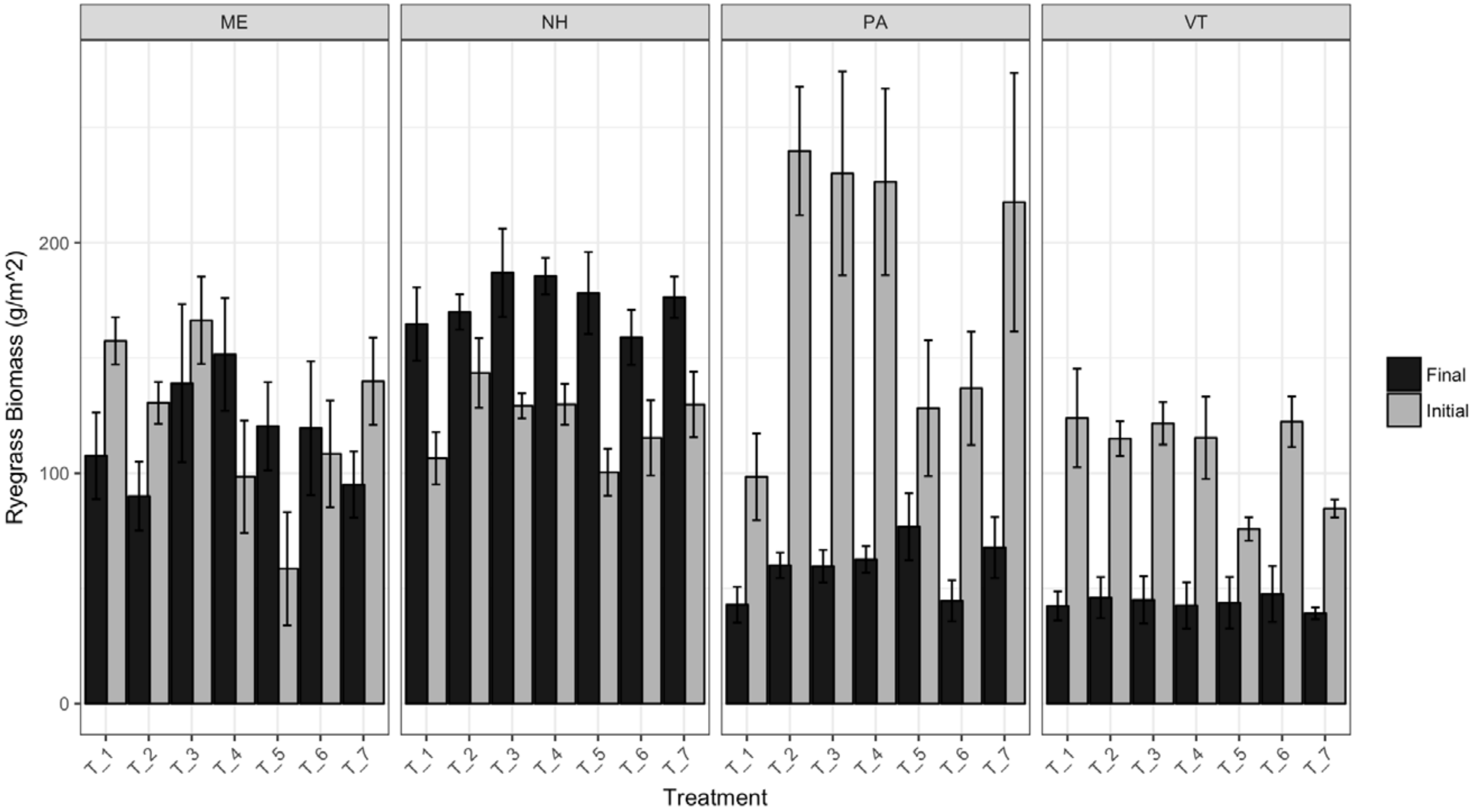

Figure 1.4. Ryegrass biomass g/m^2 (+/- SE) in Maine (ME), New Hampshire (NH), Pennsylvania (PA) and Vermont (VT), across all 7 *Lolium perenne* treatments (T_1-T_7). There was a significant difference ($P < 0.05$) between harvest times within treatments at all locations.

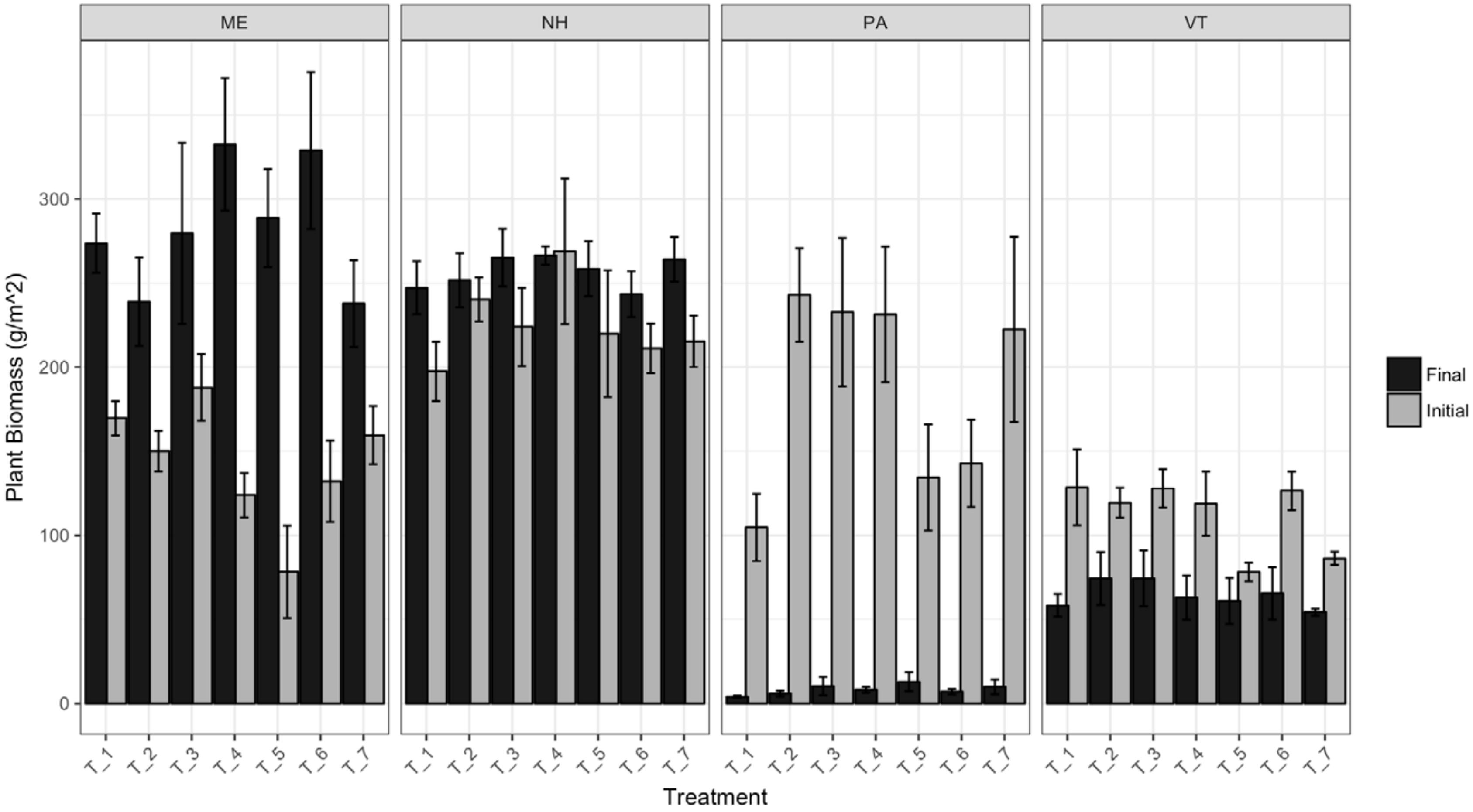

Figure 1.5. Total plant biomass g/m^2 (+/- SE) in Maine (ME), New Hampshire (NH), Pennsylvania (PA) and Vermont (VT), across all 7 *Lolium perenne* treatments (T_1-T_7). There was a significant difference ($P < 0.05$) between harvest times within treatments at all locations

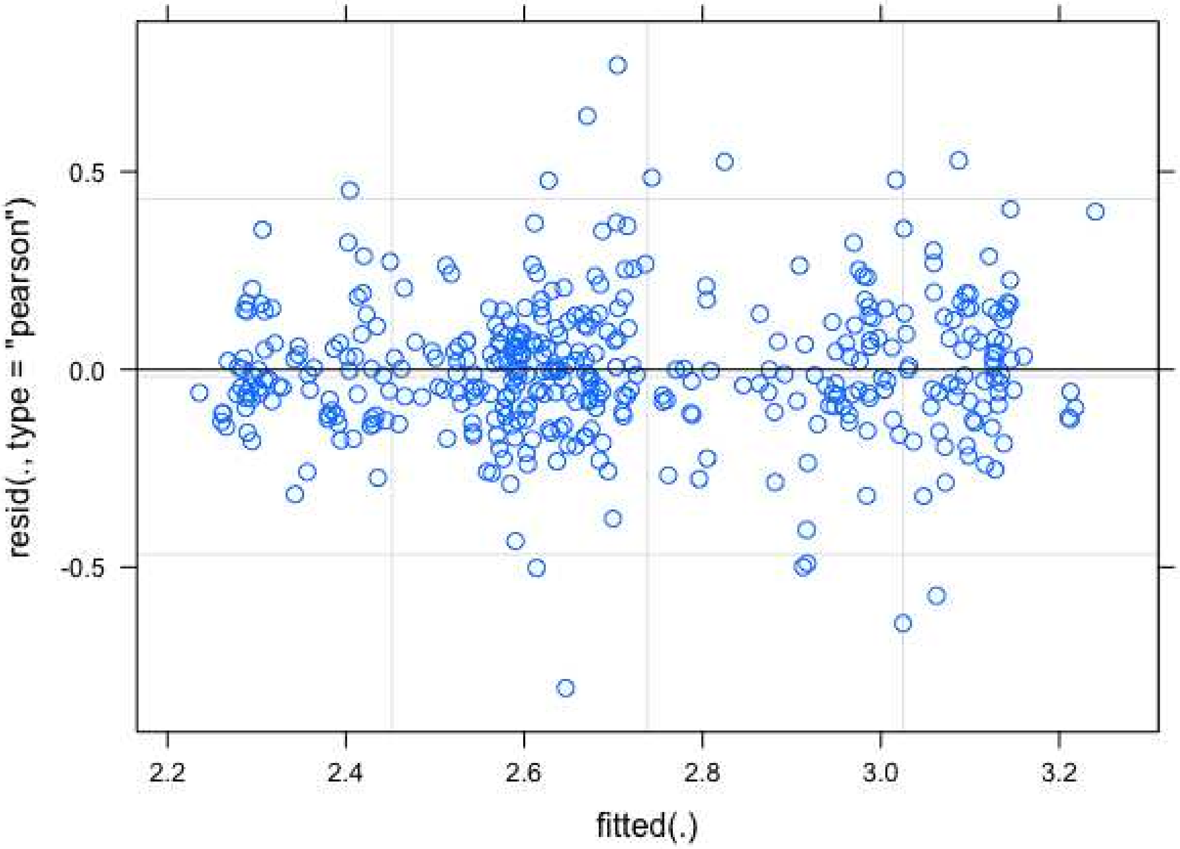

Figure S1.1. Residuals plot of the F:B biomass versus the fitted F:B biomass as a check for non-linearity, unequal variances and outliers.

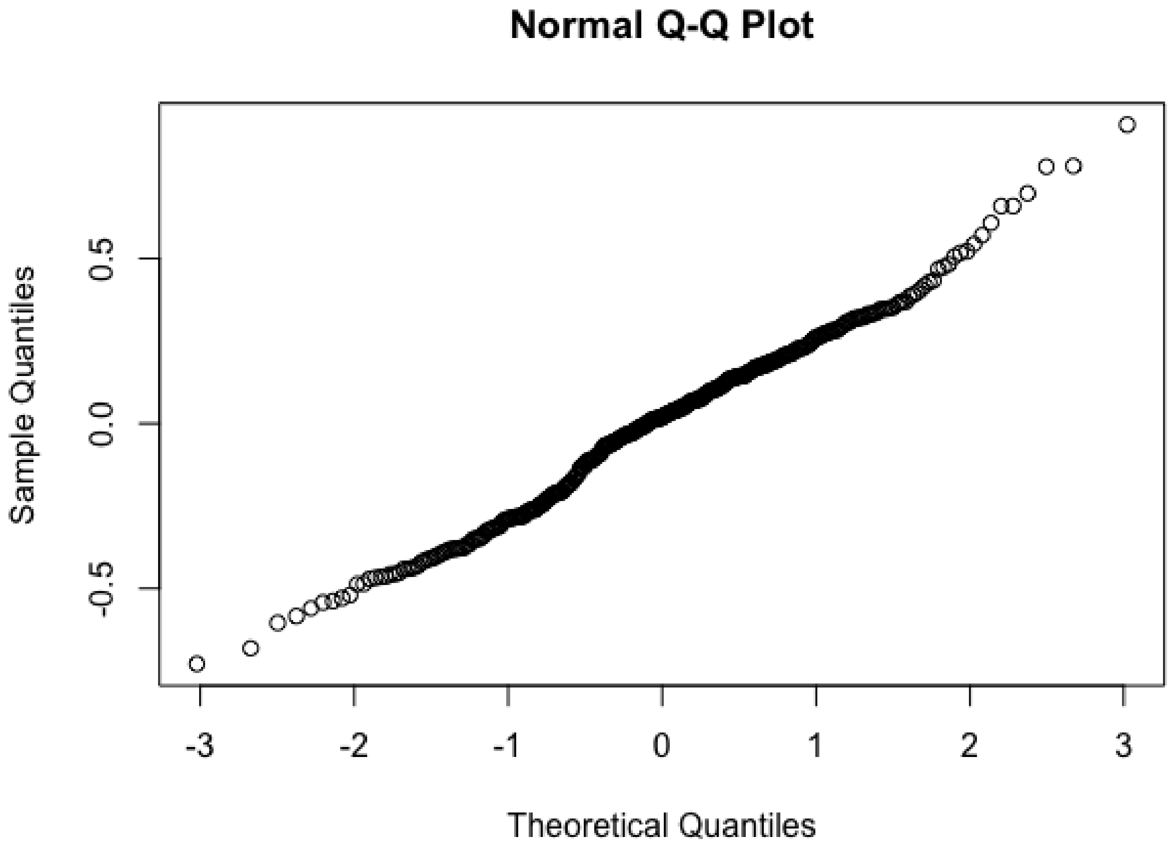

Figure S1.2. Normal Q-Q plot of the F:B biomass quantiles versus theoretical quantiles as a check for normality of data distribution.

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Chapter 2—Effects of perennial ryegrass mixtures on soil fungal and bacterial community composition: a multi-year study

Introduction

Relative abundance and diversity of the soil microbial community vary dependent upon differences in plant fitness and phenotype. In agricultural production systems, crop species and cultivar diversity can functionally influence the belowground microbial community throughout the production year, from seed germination through root elongation, grain fill, and senescence. These seasonal changes in the microbial community are a result of the alteration in the type and quantity of organic compounds released into the soil environment by the crop plant (1–3). The carbohydrates and niche availability provided by a plant community influences the soil microbial community composition and abundance (4,5). Microbes often utilize organic carbon from plant root exudates when available while others have developed intra cellular pathways of symbiosis throughout their evolutionary histories (4,6,7).

The use of intra-specific plant diversity (i.e., mixing different genotypes/cultivars of a single species) in agricultural production system has proven effective at managing crop disease and increasing the resilience of cropping systems against climate variability (8–11). Unfortunately, wide-scale use of the practice remains limited largely due to issues associated with harvesting cultivar and species mixtures. Even with these complications, there continues to be interest in cultivar and species mixtures due to increased nutrient requirements as well as management for increasing soil health due to over grazing and soil degradation (12). Despite the interest in the use of intraspecific mixtures in agriculture, few studies have been conducted to investigate how the diversity of intraspecific crop mixtures may influence the composition and structure of the soil microbial community. Evidence from both natural and agricultural systems suggest that the microbial communities associated with more genetically diverse plant systems tend to be more taxonomically diverse themselves. Specifically, it has been shown that genotypic diversity within a plant species has a significant effect on the soil microbial community (13–15). This is likely due to variation of traits such as root architecture (16,17), growth rate and phenology (18), and the quantity and quality of root exudation (14,19), all of which may have a profound effect on the structure of the microbial community. For example, genotypes of maize recruit unique microbial consortia when planted within the same soil (20), yet the individuals within genotypes do exhibit preferential recruitment. However, these genotypic influences remain difficult to quantify in a field setting. Incorporation of known genotypes with unique ploidy and functional traits into a production system may represent a useful strategy to understand these dynamics between resident microbial communities and cultivar mixtures of a plant species.

One system where plant cultivar and species mixtures may prove beneficial is in perennial pasture production used for livestock feed. As the plant biomass is harvested for feed, variation in plant growth stage is not a restraining factor. In addition, intra-species cultivar blends provide beneficial functional traits, for example maturation date, winter survival or hardiness variability, and specific resistance genes to potential pathogens improve crop productivity.

Small host genotypic differences may also have a large effect on the ability of a host to recruit and maintain symbiotic partnerships. Small changes in host specificity required for both signaling to microbes and receiving signals from microbes are important in recognition between a plant host and a potentially mutualistic microbe. These changes may alter the outcomes of the interactions with significant fitness consequences to both the host and symbiotic microbe (21).

Many microorganisms have evolved symbiotic relationships with plant root systems. The symbiotic relationships can range from mutualism to parasitism to commensalism. Mutualistic microbes, such as rhizobial (9) bacteria and mycorrhizal fungi, can provide otherwise inaccessible nutrients to the plant in exchange for the plants' sugar sources, and protect against parasitic microbes, including a whole suite of bacteria, fungi, and viruses (22). Finally, there are entire communities of commensalistic species of fungi and bacteria that interact with the plant host for reasons still unknown (23). These microbial communities and their plant hosts together must adapt to pressures exerted by the environment they inhabit on an annual basis.

Microbial species within a community can vary in abundance based on their interactions with plants and the soil, these microbes may become relatively more abundant or taxonomically diverse over time as environmental conditions fluctuate. As a perennial system diversifies the microbial community may also diversify and this may be important to the continued productivity of that system (24). The diversity of a soil microbial community is not only impacted by the associated plant community, but also by other factors such as soil type, precipitation regimes and plant productivity factors associated with the production system (25,26).

Previous studies investigating the microbial communities associated with diverse plant hosts have demonstrated that host species, geographic locations, and seasonality are all capable of influencing community composition, although the extent to which each plays a role has varied from study to study (18). Most previous studies have focused on the prokaryotic communities, providing limited information on the drivers of plant-associated eukaryotic diversity. While recent comparative studies (27,28) indicate that distinct factors can drive fungal and prokaryotic rhizosphere communities, it is unclear to what extent these differences extend to species or cultivar mixtures within a perennial species in varying soil systems over multiple years.

This is likely due to the fact that many plant hosts communicate with symbiotic or beneficial bacteria and fungi often considered the “core” microbiome. In order to understand these complex temporal interactions, it will be important to determine which microbial species represent the core microbiome as well as those that are more transient. Within the context of a perennial system the core microbiome represents those species that are not only associated with all the cultivar mixture treatments, but are also found in each year of the study. These represent those species less impacted by changes in the plant community. In contrast, the transient microbial communities represent those species that increase or decrease over time and may represent different trophic guilds and metabolic functions (29,30) that respond to influxes of different types of root exudates or increases in precipitation. For example certain bacterial genera, including *Arthrobacter* are metabolic substrate scavengers that occur in psychrophilic environments (31) and another *Kaistobacter* has exhibited plant disease suppressive behavior in agricultural soils (32). These genera may contribute to a unique microbial community composition (33) within specific environmental conditions experienced by plant communities.

As perennial systems mature and root systems expand, we also expect to see an increase in fungal species including both ecto- and endo-mycorrhizal fungi. The fungal species can provide nutrient exchange among plant species and can alleviate abiotic and biotic stress. Instances of bacterial antagonism and suppression of arbuscular mycorrhizal fungi (AMF) extending from plant roots (34), highlight the importance of understanding plant-microbe community dynamics on a multi-year timescale in a perennial agricultural system.

Given our lack of understanding on the impact of a perennial cultivar mixtures on the soil microbial community the objectives of this study were to 1) assess the effects of plant genotypic traits on soil microbial community diversity 2) characterize the “core” soil microbiome over multiple years; and 3) determine the relative microbial abundance associated with the perennial ryegrass cultivar mixtures between years. We hypothesize that location, time, and treatment (plant genotype) will influence microbial community diversity and relative abundance.

Methods and Materials

Site description and selection

This study was conducted at two locations in the northeastern United States, which represent distinct temperatures, annual precipitation, soil characteristics and geographical attributes (i.e. glacial and land use histories), Table 2.1 The two field sites were: Pennsylvania State University Rock Springs Agricultural Experiment Station (40.716996 N, 77.941813 W), Pennsylvania Furnace, Pennsylvania, University of New Hampshire Organic Dairy Research Farm at Burley Demeritt (43.097257 N, 70.991599 W), Lee, New Hampshire (Table 2.2). The distance from the most southern site to the most northern site is ~733 kilometers. Both locations are Eastern Temperate Forests at the Level I classification by the Environmental Protection Agency map of Ecoregions, but separate into Mixed Wood Plains and Ozark, Ouachita-Appalachian Forests at Level III for New Hampshire and Pennsylvania respectively (35). The temperature ranges and precipitation regimes varied between sites (Table 2.1). Located on agricultural research farms the soil types have broad class ranges, typically considered highly managed and having undergone multiple successional histories. Prior to this study the experimental sites were minimally managed pasture, composed of *Festuca rubra*, *Lolium multiflorum, Schizachyrium scoparium, Poa pratenis L., Dactylis glomerata L.* et al., for more than a decade prior to the start of this experiment. The southern site, in Pennsylvania has a limestone derived soil type that is shallow yet well drained, characterized as a Hagerstown silt loam with 3-8% slope (36). The Pennsylvania site soils are of clayey residuum weathered from limestone parent material and are fine, mixed, semiactive mesic Typic Hapludalfs. The New Hampshire site soils are described by their Inceptisol order, being well drained and sandy classified as loamy, mixed, superactive, mesic Lithic Dystrudepts (37). They are characterized as a Hollis-Charlton very rocky fine sandy loam with 3-8% slope and its parent material classified as Till (38). Soil from the sites had contrasting chemistries but similar texture.

These field sites were planted in winter rye in October 2011 which was then plowed under and seeded with red clover. The experimental sites were then disked and tilled in August 2012. This study began when treatments germinated in September - October 2012 depending upon location. Treatment plots received no irrigation, fertilizer or chemical treatments, though simulated grazing was performed via mechanical mowing when aboveground biomass reached an average height of 25 cm it was clipped down to a height of 7.5 cm in all plots. The experimental design was a randomized complete block with seven treatments and five replicates each, constituting a total of 35 blocks at each field site. Each plot measured 5 meters (m) x 5 m with a 1m x 5m planted buffer strip of orchardgrass (*Dactylis glomerata* L.) between plots. The soil sampling occurred once every September-October for four years.

Treatment description

Each replicated block was sown with an equal mass of seed with a walk-behind Carter small seed cone planter. Cultivars were provided by Barenbrug USA (Tangent, Oregon) a grass seed supplier which also provided the trait ratings and genetic composition described below (Table S2.1). For treatments with multiple cultivars the total seed weight was divided equally among individual cultivars. A total of 15 cultivars were used, comprising seven different treatment mixtures. The most adapted cultivar for each site within this regional winter hardiness zone was Remington (Table S2.1). Ploidy of the individual cultivars was controlled within treatments, containing diploid and tetraploid cultivars (T_2 and T_3), as well as separated by only diploid (T_4 and T_7) and tetraploid (T_1 and T_5) cultivars. This specific cultivar selection allows us to look at the influence of bulk plant genetic material on the microbial community recruitment, stability, and interaction. The perennial rye grass cultivars varied in mixture composition of heading date and winter hardiness to mitigate common forage issues, such as severe winters dramatically reducing viability, and productivity reduction mid-summer due to heat stress. Treatment regimes in Pennsylvania and New Hampshire were the same, despite the broad geographic distance – PA and NH are in the same winter hardiness zones. For example, late heading forage mixture (T_5) contains later heading cultivars compared to early heading forage (T_3). The heading date indicates when the grass is physiologically maturing, producing maximum root exudates and requiring more nutrients from the soil and its symbiotic organisms (39). Treatment 6 is the commercial cultivar blend (BG-24T). All treatment mixtures received an application of clover (*Trifolium repens*) at a rate of 3.36 kg/hectare in year two to enhance overall forage quality and nitrogen availability.

Data collection and analysis

Soil cores 2.5 cm wide taken at a depth of 20 cm were collected annually during September-October. Five cores were taken from each of 35 plots in a 'W' pattern per site and homogenized before being taken to the lab. DNA was extracted using MoBio PowerSoil Kit from 0.25g bulk soil sieve filtered (2 mm) of roots and gravel. DNA was eluted and quantified on a NanoDrop 8000 Spectrophotometer. An extraction of each replicated treatment plot of which there were five, were pooled to create a single composite DNA sample for each treatment at each location every year, totaling 38 for each location or 76 total samples. PCR amplification was performed following the Earth Microbiome Protocol (40) with quantification of PCR products on both Qubit and an Agilent 2200 TapeStation to ensure quality of libraries before pooling. Sequencing was performed at the University of New Hampshire Hubbard Genome Center for 16S rRNA V3-V4 region using 515f/806r primers (41) on the Illumina Hiseq 2x250 platform. The ITS region was sequenced using primers previously developed (5.8SR' and ITS4_Nextera) with Illumina MiSeq 2 x 300 read technology at the University of Minnesota Genome Core Facility (Minneapolis, MN). All sequences were deposited in the NCBI Short Read Archive. The accession numbers are shown in supplementary Table S2.2.

For both fungal and prokaryote DNA, cutadapt trimming software (43) was used in processing raw reads, cutting adapters and primers from sequence data with use of a sliding window of 15bp for end trimming. Data was then analyzed in R (R Core Team, 2017) for quality processing where reads were trimmed at 250bp for the forward and 225bp for the reverse reads. Error learning, chimera detection, and taxonomy calling were completed using the Ribosomal Database Project (RDP) naïve Bayesian classifier (44) with UNITE (45) and Greengenes (46) databases. Inference learning was conducted on a training set of total sequence reads with the Divisive Amplicon Denoising Algorithm (DADA) (47) executed within R through the 'dada2' package (48). Dada2 contains multiple functions to complete a full work flow on amplicon data including filtering the forward and reverse reads of sequences containing Ns and setting a maximum expected error as well as filtering poor quality reads. The dereplication step involves combining all exact matches to reduce computational requirements and an abundance value is given for that amplicon sequence variant (ASV). The Dada2 dereplication step is unique in that the quality score assigned to each sequence is retained for further downstream processing. A consensus quality score is assigned to each dereplicated variant to aid in true variant selection. Sample inference occurs when the DADA algorithm is used to infer true variants from the dereplicated sequences. Chimera detection and merging of paired end reads is implemented to remove spurious variants by utilizing the forward and reverse reads which overlap to provide longer reads for aligning to respective amplicon data bases (49).

Removal of chloroplast, cyanobacteria, and Archaea sequences from 16S ASVs was conducted along with variants detected below the minimum threshold mean read abundance of 10^{-5}. Fungal ASVs were assigned to functional guilds and trophic groups based on their taxonomic placement using FUNguild after removal of sequence variants not assigned to the kingdom Fungi and which met the minimum read abundance. This quantitative resolution is important in understanding the true assembly of microbial communities with limited bias of assumed sequence clustering methods which reduce or underestimate ecological richness (50).

Statistical Analysis

This experiment consisted of two experimental sites sampled over four years where seven treatments were sampled at each location for three years and three of those treatments were sampled at each location for a fourth year totaling 48 16S and ITS communities sequenced. The groups were analyzed separately, where the seven treatments (T_1-T_7) over the first three years create the ‘Complete’ dataset while the three treatments (T_3-T_5) over four years were grouped as ‘Ploidy’ dataset to isolate the effect of ploidy within other functional traits over four years. We sampled the Ploidy group for a fourth year due to the treatment range consisting of diploid cultivars (T_4) with greater winter hardiness, tetraploid cultivars (T_5) with slightly less but similar winter hardiness, and mixed (diploid and tetraploid) ploidy (T_3) which had the same winter hardiness average as T_5 (Table S2.1). The data set included 22 meta data variables, a subset of which were: seasonal precipitation, soil pH, number of cultivars in the treatment mixture, ploidy, winter hardiness, heading date, and annual average fungal:bacterial ratio as determined by qPCR.

The Shannon metric was used for quantifying alpha diversity of bacterial and fungal communities (51–53) to consider both evenness and richness. The Simpson evenness index was calculated and implemented as an evenness metric (54). The Bray-Curtis dissimilarity metric was used to compare sampling points which determines the compositional diversity based on counts between two sampling points (55). Community variation was measured across sampling points with treatment as a random factor in the model as well as between samples within each time point. Permutational multivariate analysis of variation (PERMANOVA) on the distance measures was performed through the adonis function in the *vegan* R package (56,57). This analysis tested the multivariate effect of treatment, location, year, seasonal precipitation and soil characteristics. Linear mixed-effects model fit by restricted maximum likelihood (REML) was conducted with 'nlme' package where model choice is based on Akaike information criterion (AIC) and Bayesian information criterion (BIC) (58).

A permutation test for homogeneity of multivariate dispersions with the *vegan* function 'BETADISPER' was used to ensure that the data met the appropriate dispersion assumptions. When comparing specific taxonomic ranks Tukeys honest significant difference (HSD) test was performed on the Bray-Curtis index (59,60) between groups when taxa are agglomerated at next lowest rank using 'tax_glom' in phyloseq and the 'TukeyHSD' function in R. Data points with missing meta data were adjusted for any necessary transformations or distribution tests of observations.

Rank comparisons between groups were conducted on distances within groups for analysis of variance (ANOVA). Principle coordinate analysis (PCoA) of distance measures along with visualization was performed in R for auto correlated analyses. Constrained ordination was performed with 'cap_ord' function of vegan to test environmental variables influence on the model (57). Heat map visualizations of most relatively abundant genera within the systems were produced with *phyloseq* and *NeatMap* (61,62) while Venn diagrams were produced with the function 'VennDiagram' in R.

Partial least squares discriminant analysis (PLS-DA) was performed to classify the specific taxa discriminating communities between years utilizing the *mixOmics* R package (63). This supervised multivariate analysis provides the frame work for discerning molecular signatures (ASVs) in our case, which contribute to the separation of data sets, here we used the repeated measure (year) as the grouping variable. The input requires total sum scaling (TSS) of the observational units prior to implementation where individual read counts are divided by total sum of reads in each sample, we kept ASVs which reads summed to more than 0.001 percent of the total sum of reads. The minimum number of components required to encompass the most variation in the data set are chosen before performing the sparse PLS-DA (sPLS-DA) which evaluates the ASV significantly contributing to the groups Euclidean distance separation of the data set.

Results

A total of 16,748 16S taxa and 3,678 ITS taxa were identified between the 24 samples in the two locations of the Ploidy dataset. The Complete group consisted of 22,678 16S taxa and 3,786 ITS taxa uniquely identified in 42 samples. In both the Complete and Ploidy groups the fungal and prokaryote alpha diversity as measured by the Bray-Curtis dissimilarity or weighted UniFrac distance, were independently affected by location and year (Table 2.3).

Linear mixed-effects model selection by restricted maximum likelihood was conducted where the model choice based on AIC, BIC, and Log likelihood varied depending on the data set (Table 2.5). Briefly, the fungal Ploidy group model for alpha diversity prediction was dependent on treatment (AIC=18.56), while the complete model was dependent on year (AIC=-14.98). Within the prokaryote Ploidy group model testing of alpha diversity associations, treatment was most significant in predicting community diversity (AIC=-14.53) while in the complete group year alone had the lowest AIC (-41.08) (Table 2.4).

Location and year in the Complete and Ploidy group were significant effects on the UniFrac distance metric of the bacterial communities (P <0.01), though treatment was not (P =0.43) as determined by our adonis test (Table 2.3). At the Pennsylvania and New Hampshire sites separately, both the Complete and Ploidy bacterial community dissimilarities were not different between treatments or years. The Shannon diversity between sites was different in the Ploidy group, with Pennsylvania being less diverse (μ =7.18, t =3.62) than New Hampshire (μ =7.32). The Shannon diversity in the Complete group was also different with Pennsylvania being less diverse (μ = 7.14, t = 7.70) than New Hampshire (μ =7.34). The Simpson evenness was not different (t =1.16) between locations in the Ploidy group but was in the Complete group (t =3.46) (Table 2.5).

Again, location and year in the Complete and Ploidy group were significant effects on the Bray-Curtis distance metric of the fungal communities (P <0.01), though treatment was not (P =0.50) as determined by our adonis test (Table 2.3). At the Pennsylvania and New Hampshire sites separately, both the Complete and Ploidy fungal community dissimilarities were not different between treatments or years. The Shannon diversity between sites in the Complete group was different with Pennsylvania being more diverse (μ=5.303, t= -3.759) than New

Hampshire (μ =5.1) though the Simpson evenness was not (*t* = -0.33) (Table 2.5). The fungal Ploidy group showed no difference between locations in Shannon diversity or Simpson evenness.

At the Pennsylvania site, the fungal community in T_7 (winter hardiness within early heading date) and T_3 (tetraploid mix) were separate from other treatments in year three with reduced Shannon values compared to the other 5 treatments (Figure 2.1a). At the New Hampshire site, there was a reduced Shannon alpha diversity in T_5 and T_6 compared to the other treatments in the complete dataset. There was a significant reduction in the bacterial diversity from year one to two across all treatments and there was a significant increase in all treatments from year two to three. The bacterial community in T_7 at the Pennsylvania site generally had significantly smaller increase in alpha diversity compared to the other 6 treatments (Figure 2.1b). Richness as measured by the Shannon metric shows an increasing trajectory for fungal richness compared to decreasing richness for prokaryotes over the study period (Figure 2.1c).

Bacterial Community Composition

The two prokaryote communities differed in taxonomic composition at each location and during each year. Bacterial diversity in the Ploidy group was greatest in the fourth year under T_3 in Pennsylvania and under T_5 in New Hampshire. The most abundant bacterial phylum, measured by relative abundance, in each location was Proteobacteria. The second most abundant phyla differed between communities, with the Bacteroidetes, followed by Verrucomicrobia being the second and third most abundant in at the Pennsylvania site. The second most prevalent phylum at the New Hampshire site was Actinobacteria followed by Bacteroidetes. Though each location contained the same top five phyla four years post disturbance, the relative abundance rank of the phyla differed (Figure 2.4). The PCoA of weighted UniFrac distance metric of each complete and Ploidy groups bacterial communities showed the change in phylogenetic distance over time in each treatment at each location (Figure 2.5).

We next investigated relative abundance patterns in bacterial communities over time. As literature has supported a more diverse community arose over time (51,64). Core bacterial ASVs, those found in all years represented 38.4% and 35.6% of total observations in all three years for the Complete group in Pennsylvania and New Hampshire sites (Figure 2.2a-b). Unique taxa shared between years one and three were markedly different with 10.3% shared ASVs at the Pennsylvania site versus 4.42% at the New Hampshire site (Figure 2.2b). In the Ploidy group analysis, the core bacterial community was 24.8% of ASVs in Pennsylvania and 29.7% of ASVs in New Hampshire sites. The largest difference in core ASVs was with 15.8% of the community in Pennsylvania and 6.5% in New Hampshire sites unique to year four (Figure 2.2c-d).

The results of the sPLS-DA in the Ploidy group provide specific ASVs which contribute to the group (year) separation. Within the Pennsylvania site the first component separation is dominated by year four ASVs in the order Acidobacteriales, Cytophagales, and Xanthomonadales. The New Hampshire sites first component separation is also dominated by year four ASVs contributing to the variation including Myxococcales, Pedosphaerales, and Pirellulales with factor loadings >-0.4 (Figure S2.1a-b).

Bacterial Richness and Abundance

Treatment 3 (mixed ploidy) in year two at the New Hampshire site exhibits a unique pattern of lower abundance of the top 20 genera compared to the other two treatments (Figure 2.4). The Burkholdaeriales in the New Hampshire Ploidy group community displayed an increase in abundance from year one to two (*P* =0.36) followed by a reduction in abundance in years three and four. There was a significant decrease (*P* =0.035) in the abundance of

Burkholderia spp. from year two to year three at the Pennsylvania site as measured by the Bray-Curtis distance measure.

There was a significant increase in the abundance of *Psuedomonas* spp. between years one and two (*P* <0.01) and a decrease in abundance between years two and three (*P* =0.03) at the New Hampshire site. In the Pennsylvania site *Pseudomonas* community membership showed no significant difference between years in either the Complete or Ploidy groups, though abundance did increase from zero reads in year one to >300 reads per ASV in year four. *Pseudomonas* abundance in the 'Ploidy' group increased in relative abundance from almost none in the first year to >4000 reads in the fourth year of the study in the New Hampshire site (Figure 2.4).

The abundance of Flavobacteriales increased from the initial year post disturbance in both years two and three (*P* =0.01) but was significantly lower in the fourth-year post disturbance from years two and three (*P* <0.01). Rhizobiales, an important nitrogen fixing gram negative Alphaproteobacteria decreased significantly in abundance over time (*P* <0.01), between year one and four (*P* =0.017) and between year two and four (*P* <0.01) respectively across both locations. The sPLS-DA results indicate Flavobacteriales as an order contributing in both the second and third year to the second component of the Ploidy group with comparably large factor loadings, >0.4 and >0.2 respectively.

Fungal Community Composition

By year four at the New Hampshire site the treatment with greater genetic plant diversity T_5 had higher fungal diversity compared to a similar mixed ploidy treatment T_3. At the Pennsylvania site T_3 had significantly lower Shannon diversity compared to T_4 and T_5. Compared to the New Hampshire Ploidy group analysis where the dominate symbiont orders were Mortierellales, Eurotiales, Agaricales, and Pleosporales (Figure S2.3) the Pennsylvania site community membership ranking differs in composition and order; NA (unknown), Mortierellales, Agaricales, Chantharellales, (Figure S2.3). There was clear dominance of Mortierellales, Eurotiales, and Pleosporales across all years in the New Hampshire site, while there was less dominance and more diversification in Pennsylvania with few orders exhibiting greater relative abundance by the fourth year (Figure 2.6).

The dominant order in all of the Pennsylvania site years classified as pathotrophic fungi was Hypocreales. The relative abundance of Hypocreales decreased from year one to year three at the Pennsylvania site from 2.96% to 2.33% of ASVs in the complete data set (Figure 2.6a). While the most dominant pathotrophic order in New Hampshire, Pezizales increased from 2.83% in year one to 3.01% in year three with T_1 contributing over 1.4% of those ASVs alone. The second most dominant order in New Hampshire Hypocreales decreased from year one (1.47%) to year three (1.30%) (Figure 2.6b).

Fungal Richness and Abundance

Fungal phylotypes were assigned using FUNguild (29) which assigns both a trophic mode as well as a functional guild to ASVs. Some of the prevalent taxa assigned to ‘pathotroph’ were Hypocreales, Sordariales, Pezizales, Morteirellales, and Hypocreomycetidea ord Incertae sedis (Figure S2.3) These orders include most of the soil borne pathogens associated with temperate grasslands, including species of *Alternaria*, *Magnaporthe*, *Chaetomium*, *Acremorium*, *Rhizophylyctis*, *Epicoccum*, *Gibberella*, *Verticillium*, *Olpidium*, and *Gaeumannomyces*. The assignment of ‘symbiotroph’ was also conducted allowing for visualization of the functional groups representation at each location over time. The ‘symbiotroph’ order Cantharellales had the greatest relative abundance at the Pennsylvania location in the fourth year and was detected in low abundance during the third year while being the fourth most relatively abundant group across all years in the Ploidy analysis (Figure S2.3b). Within the Ploidy group the Bray-Curtis metric of dissimilarity significantly differentiated location and year for taxa within the fungal pathogen phylotype ($P < 0.001$).

Core fungal ASVs, those found in all years represented 54.9% and 59.1% of total observations in all three years for the complete analysis in Pennsylvania and New Hampshire sites (Figure 2.3a-b). Unique taxa shared between years one and three were different with 9.55% shared ASVs in Pennsylvania versus 8% in New Hampshire (Figure 2.3a-b). In the Ploidy group analysis, the core fungal community composed 33.9% of shared ASVs in Pennsylvania and 40.2% of shared ASVs in New Hampshire. The percentage of unique non-core ASVs in the fourth year was 7.39% in the Pennsylvania site and 5.06% in the New Hampshire site (Figure 2.3c-d), with the Pennsylvania site exhibiting the highest percentage of unique ASVs in this year compared to any other year.

The results of the sPLS-DA in the Ploidy group provide specific ASVs which contribute to the group (year) separation. Within the Pennsylvania site the first component separation is dominated by year four ASVs in the order Orbilliales and Pezizomycotina with factor loadings >0.5. The New Hampshire sites first component separation includes a single ASV in year four in the order Sporidiobolales with a loading of >0.3, and two ASVs in the orders Pleosporales and Microascales with loadings >-0.5 (Figure S2.1c-d).

Discussion

This study demonstrates that at two locations, there are differences in both broad and specific soil microbial taxa across perennial ryegrass treatments. Furthermore, certain microbial communities transitions towards a more diverse community over the course of the 4-year experiment. The seven perennial grass treatments, varying in genetic and phenotypic traits, exhibited unique associations with both fungal and bacterial species. Putatively symbiotic microbial taxa often increased, with a reciprocal decrease in putative pathogenic taxa, the nature of these microbial classifications is based on the currently available trait database. The taxonomic abundance and diversification of both fungal and bacterial communities is evident though difficult to quantify beyond relative measures. The unique core fungal ASVs were comprised of taxa belonging to diverse lineages implying they are a phylogenetically broad groups of saprotrophs including *Mortierella spp.* and *Fusarium spp.* We show distinct membership in fungal and bacterial communities between locations where plant genotypic diversity interacts with the soil community diversity over time. The quantified microbial community from this experiment demonstrates that a perennial ryegrass system can include both pathogenic and saprophytic microbes which fluctuate temporally regardless of species genotype.

The soil microbiome contains numerous taxonomical diverse species that provide functional redundancy to the soil ecosystem. Species of Actinobacteria, Firmicutes, and Acidobacteria have been shown to suppress soil-borne plant pathogens (65) through the ability to produce volatile chemicals (66), weapon-like antibiotics (67) and siderophores with antifungal properties (68) all contributing to an environment unfavorable to bacterial and fungal pathogens. Though few functional traits are known within Flavobacteriales as an order, research suggests this group of bacteria plays an important role in suppressing fungi and nematodes in the soil through secretion of chitinases (69,70). These taxonomic groups were all observed in this study, in association with individual genotypes as well as mixtures, and highlight the complexity of the soil microbial interactions beyond the phylum level.

While broad phylum level interactions are important, finer taxonomic resolution often reveals details of the community composition and ecology. The *Pseudomonas* genus increased seven fold at the New Hampshire site in year one to year four. At the Pennsylvania site there was a four-fold difference in the same genus between year one and four. Numerous studies have described the genetic basis of both negative and positive interactions between *Pseudomonas* spp. and their plant hosts (71–73) demonstrating the importance of this genus in cropping systems. Specifically, secondary metabolites produced or systemic resistance induced by *Pseudomonas spp.* aid in soil suppressiveness (73) as well as having a direct biocontrol effect (74–76). In this study, none of the usual species were observed, rather *P. alcaligenes*, *P. veronii*, *P. fragi*, *P. viridiflava*, and *P. umsongensis* constituted some of the most prevalent species in the genus. These species have been shown to play important roles in soil bioremediation, dairy spoilage, and bacterial blight of kiwifruit respectively, with 16S reclassification grouping them with species such as *P. fluorescens* or *P. syringae*. The research conducted on *Pseudomonas spp.* as plant-growth promoting rhizobacteria (PGPR) (77–79) supports the role of pseudomonads and the species observed here offer opportunities for further PGPR investigation.

The slow-growing, ubiquitous soil bacterium *Candidatus* Udaeobacter copiosus, a member of Verrucomicrobia, which is associated with temperate grasslands (80), was also a common and persistent member across treatments and years in this study (Figure 2.4). In both the Pennsylvania and New Hampshire sites there was a ~30% reduction in *Ca*. U. copiosus relative abundance by year four. The presence of *Ca*. U. copiosus and reduction over the four years of this study agrees with what has been observed in other natural systems where management practices influence their abundance (81). This is likely the result of the fact the *Ca*. U. copiosus requires an undisturbed, mature soil community where its metabolically efficient lifestyle and minimalistic genome require consistent access to amino acids it must acquire from its environment and other soil biota (82).

The *Arthrobacter* genus increased two-fold in abundance in the Ploidy group during the second year at both locations though the New Hampshire site across all years retained twice the relative abundance and greater diversity compared to the Pennsylvania site (Figure S2.2). There is only one ASV, *Arthrobacter psychrolactophilus,* contributing to the *Arthrobacter* community membership at the Pennsylvania site, while three *Arthrobacter* ASVs observed at the New Hampshire site. *A. psychrolactophilus* is a psychrotrophic bacterium that grows at 0-30°C and has the ability to use lactose as its sole carbon source (83). A metabolically unique individual in one location and three similar ASVs in another location with twice the abundance may suggest that multiple variants of a species is supported in specific locations, which contribute to community diversity in an abundance related manner.

Several groups of fungi also experienced either an increase or decrease in abundance during the course of the 4-year study. The fungal communities between the two locations retained unique profiles despite having similar soil type, pH and land use history and identical plant treatments for four years. We observed that the dominant fungal groups at the Pennsylvania site shifted from being dominated by members of the Hypocreales in year one to members of the Pleosporales being most abundant in year four. A different trend was observed in New Hampshire where members of the Pezizales largely dominated in year one and Mortierellales became the most abundant group by year four. In both locations, we saw an increase in abundance of these four distinct genera, Paraphoma, Plectosphaerella, Gibellulopsis, and Ascochyta over four years. These genera mainly function as saprophytes, but also contain host-specific (cereals) and generalist plant pathogens that infect multiple hosts. This suggests that several plant pathogen species capable of infecting roots and crowns of perennial ryegrass were capable of increasing in abundance as a function of increases in susceptible host tissue over the course of the 4-year study. There is evidence suggesting polyploid plants have greater resistance to fungal pathogens compared to diploids (84,85), but this was not observed in our study, as pathogenic genera were equally distributed in both diploid and polyploid treatments.

Overall there was greater fungal diversity at the Pennsylvania site including an increase in the relative abundance of members in the orders Chantharellales and Agaricales, toward the end of the experiment during the fourth year of the study. The Chantharellales and Agaricales include many ectomycorrhizal species, which are symbiotic fungi associated with mineral scavenging and exchange of nutrients with plants during both short term post disturbance events (86) as well as long term successional histories (87). Plant successional stage is directly related to mycorrhizal responsiveness, and late successional plants are more responsive to mycorrhizal interactions (88). The mycorrhizal community abundance did not following a linear or exponential increase by year four and the inconsistent annual relative abundance may have been affected by plant productivity occurring in the Pennsylvania site, where as in the New Hampshire site there was a reduction in fungal diversity from year three to year four, both trends exhibited in other studies (89).

The concept of the core microbiome, while subject to debate, suggests that the microbial community is predetermined by soil characteristics and historical evolution or one that is in flux adapting to environmental change and seasonal shifts regarding plant productivity. Here we demonstrate that an edaphic microbiome associated with perennial forage cropping systems changes, increasing in diversity dependent on plant genotype with variability in diversity and abundance between years. The treatment with very early and very late heading ryegrass at the Pennsylvania site exhibited a significant decrease in fungal diversity in the fourth year with a significant increase in bacterial diversity at the same time. The combination of very early and very late heading cultivars may have resulted in less continuous maturation of above ground biomass with early belowground priming followed by an early summer decrease in available nutrients. This may have resulted in a resource inconsistency that the dominate fungal community could not adapt to as quickly as the bacterial community. This suggests that there is a core microbiome which adapts to annual resource availability, with certain functional and/or taxonomic groups increasing or decreasing depending on the environmental conditions, but rarely completely disappear or take over the soil ecosystem.

We observed soil microbial community shifts, some that were apparent immediately and others that were not observed until the fourth year. There was no clear increase in microbial diversity based on cultivar mixture ploidy, though maintaining the exact plant community structure over the course of the study was not conducted. The bacterial diversity varied between the two locations, and after four years we observed an increase in the 2N and 4N treatments compared to the mixed ploidy treatment. We also observed an increase in Pennsylvania site fungal diversity but a decrease at the New Hampshire site across all treatments in the fourth year. This annual flux in microbial diversity dependent upon host genotype implies single temporal snapshots of a soil community do not accurately reflect the plasticity contained in the environment. Natural organismal diversity often occurs in gradient stratification or in a stochastic patchwork distribution across a landscape, requiring multiple exploratory and analytical statistical methods to understand their temporal and spatial dynamics (90). By replicating this type of study, where multiple plant genotypic mixtures are planted in spatially distant locations and observing changes to the microbial community over temporal and spatial scales we can begin to shed light onto the dynamic nature of plant-microbiome interaction in perennial agricultural ecosystems, and how biotic and abiotic factors can impact the productivity of the entire system.

Figures and Tables

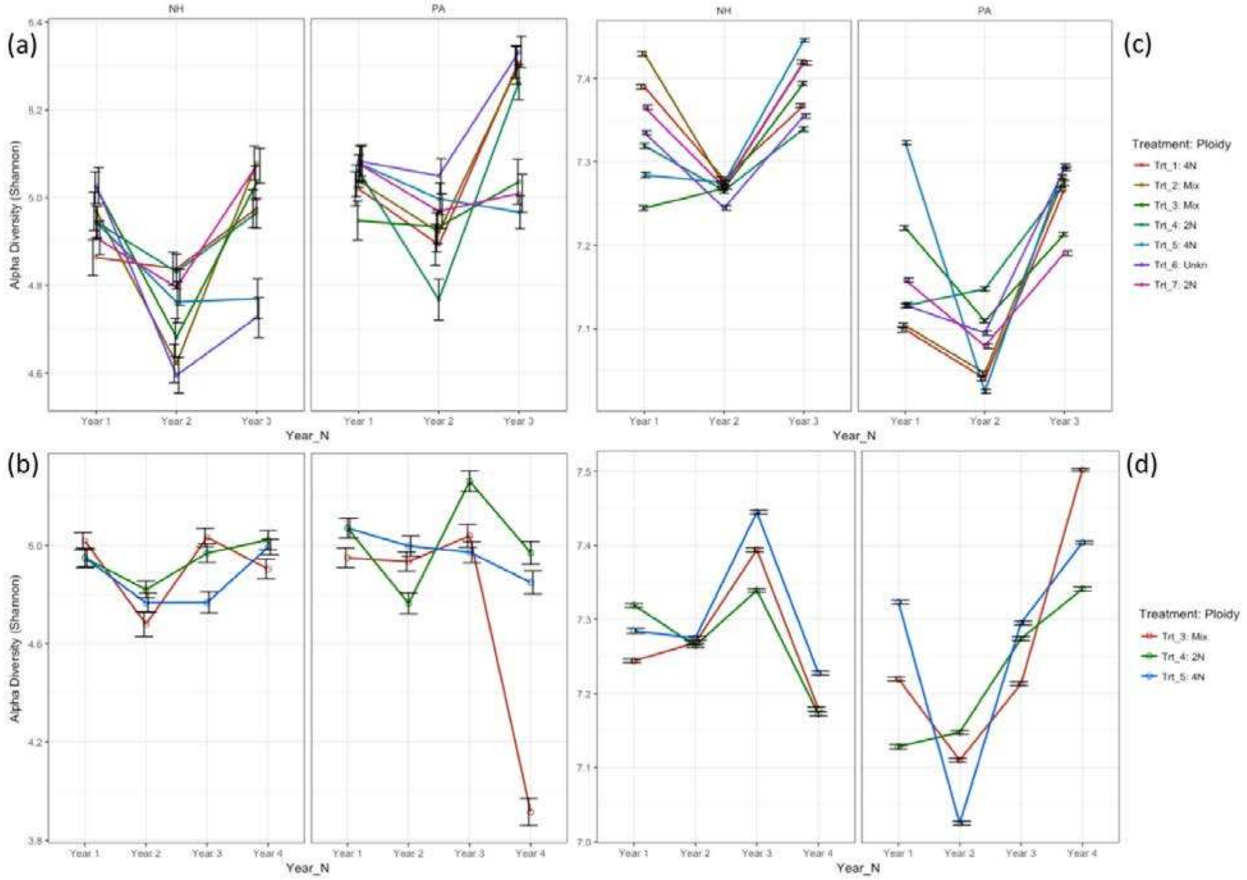

Figure 2.1. Temporal variation in soil fungal (A-B) and bacterial (C-D) communities under different perennial ryegrass cultivar mixtures in two sites (NH & PA) in the northeastern U.S. Alpha diversity was measured by the Shannon diversity index for the ‘Complete’ (A & C) and ‘Ploidy’ (B & D) treatments described in Table S1. Treatments are defined by colored lines and each point has error bars +/- standard error of the mean, the treatment ploidy is defined at 2N, 4N, unknown (Unkn) or mixed.

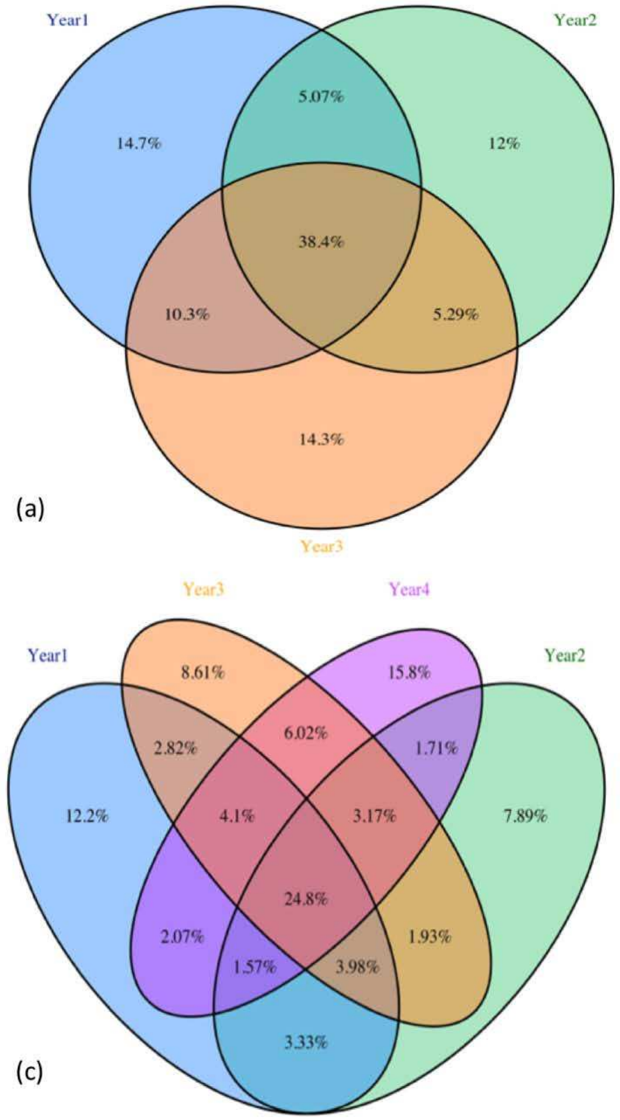

Figure 2.2. Temporal variation in the core soil bacterial ASVs associated with perennial ryegrass cultivar mixtures at sites in (a & c) Pennsylvania and (b & d) New Hampshire. The experiment was divided into two data sets the ‘Complete’ dataset consisted of 7 ryegrass mixture treatments over three years and the ‘Ploidy’ dataset consisted of 3 ryegrass mixture treatments over 4 years. Treatment details can be found in table S1. Each year is encircled and represents the percent unique and shared ASVs.

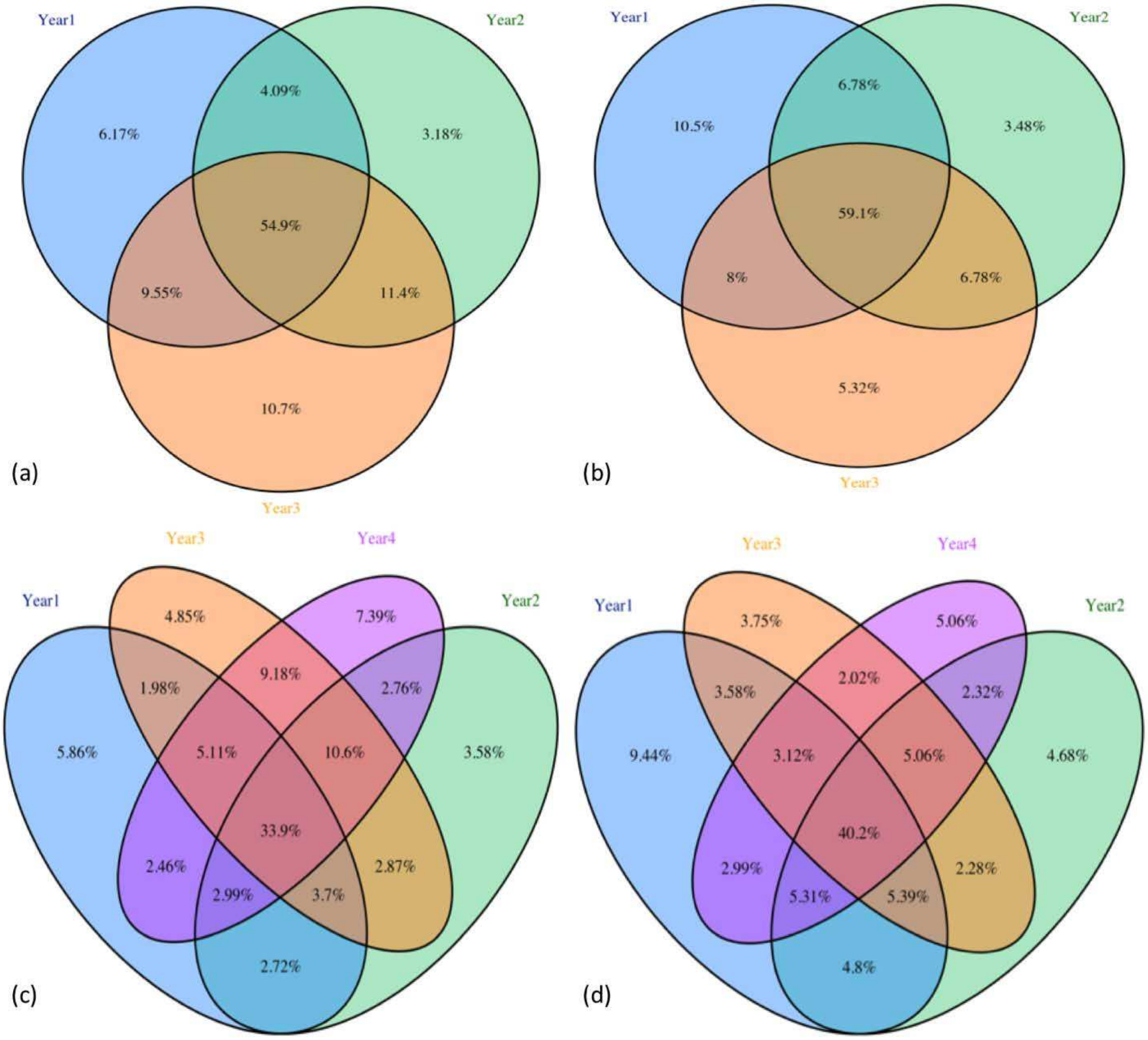

Figure 2.3. Temporal variation in the core soil fungal ASVs associated with perennial ryegrass cultivar mixtures at sites in (a & c) Pennsylvania and (b & d) New Hampshire. The experiment was divided into two data sets the ‘Complete’ dataset consisted of 7 ryegrass mixture treatments over three years and the ‘Ploidy’ dataset consisted of 3 ryegrass mixture treatments over 4 years. Treatment details can be found in table S1.1. Each year is encircled and represents the percent unique and shared ASVs.

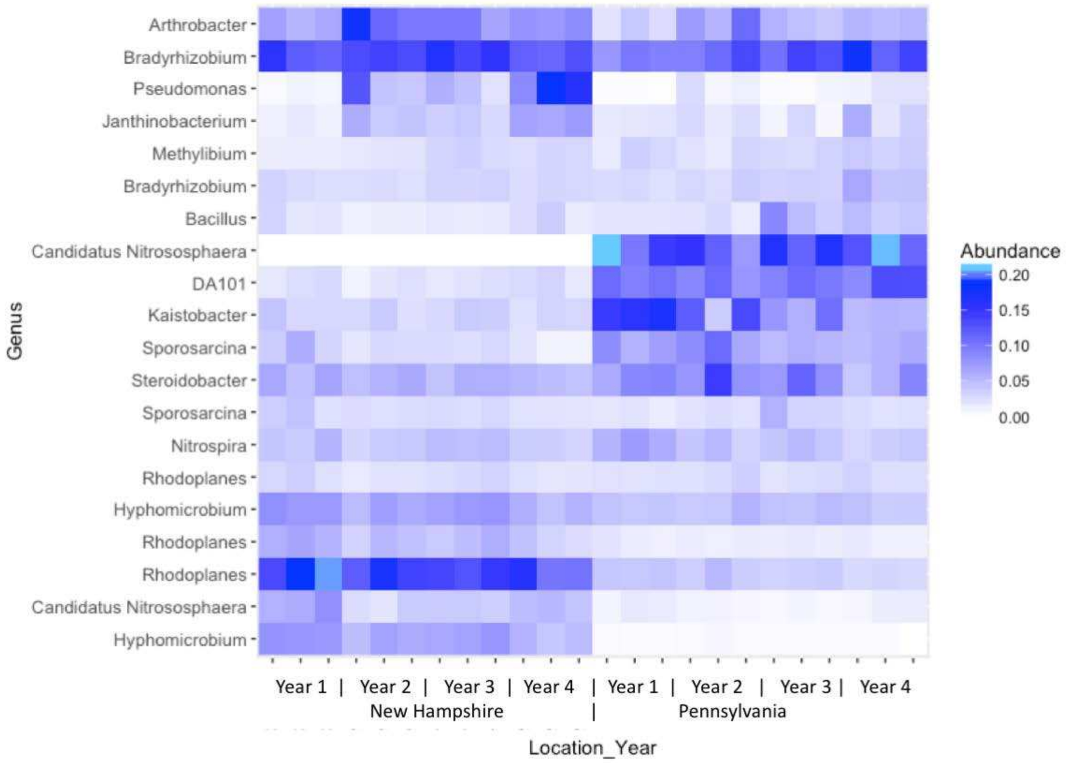

Figure 2.4. Top 20 bacterial genera observed in the ‘Ploidy’ analysis ordered by treatment (T_3-T_5) year and abundance. Relative abundance is indicated by color intensity, greater abundance is blue and lower abundance is white. New Hampshire on the left and Pennsylvania on the right with the year increasing from left to right for each site.

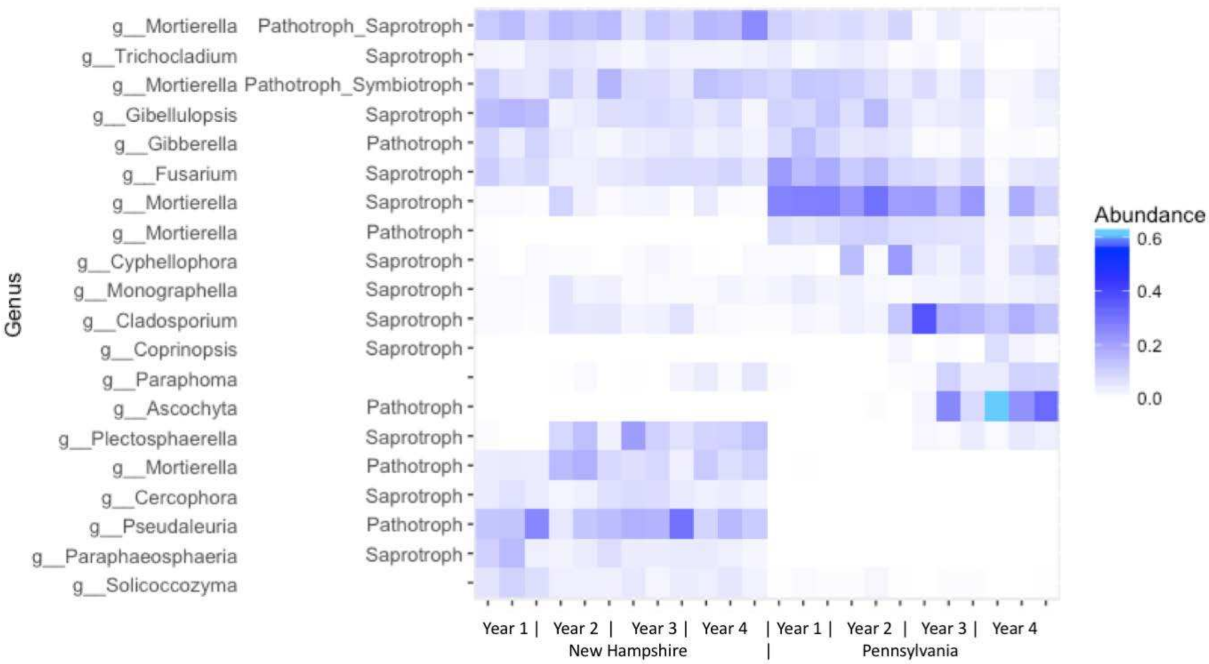

Figure 2.5. Genus and trophic mode of top 20 fungal genera observed in the 'Ploidy' analysis ordered by treatment (T_3-T_5) year and abundance. Relative abundance is indicated by color intensity, greater abundance is blue and lower abundance is white. New Hampshire on the left and Pennsylvania on the right with the year increasing from left to right for each site.

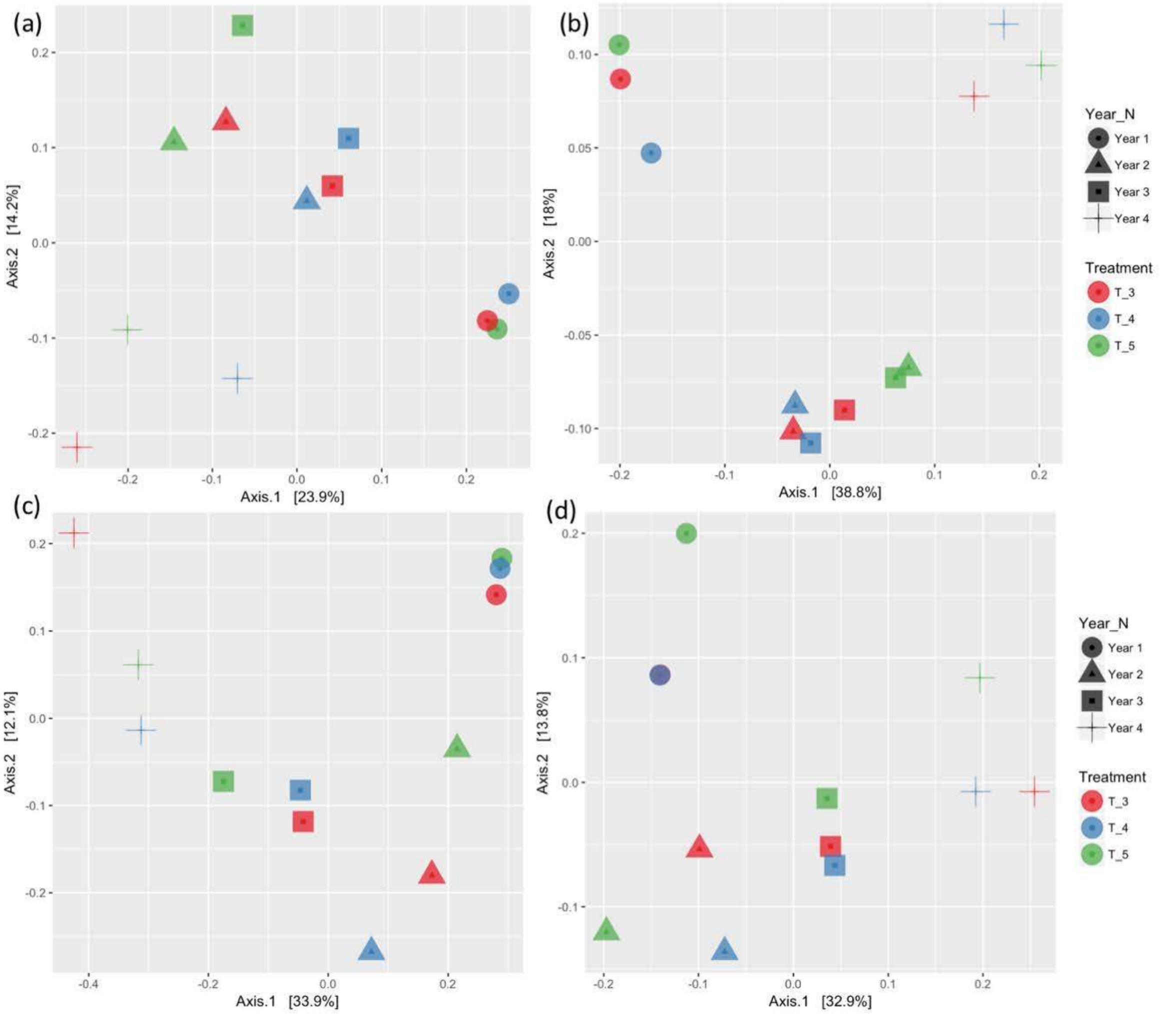

Figure 2.6. Principle Coordinate analysis (PCoA) of fungal (A&C) and bacteria (B&D) communities in New Hampshire (A-B) and Pennsylvania (C-D) measure using the Bray-Curtis dissimilarity measure of the microbial communities over four years (Year 1- 4) under three treatments in the 'Ploidy' dataset.

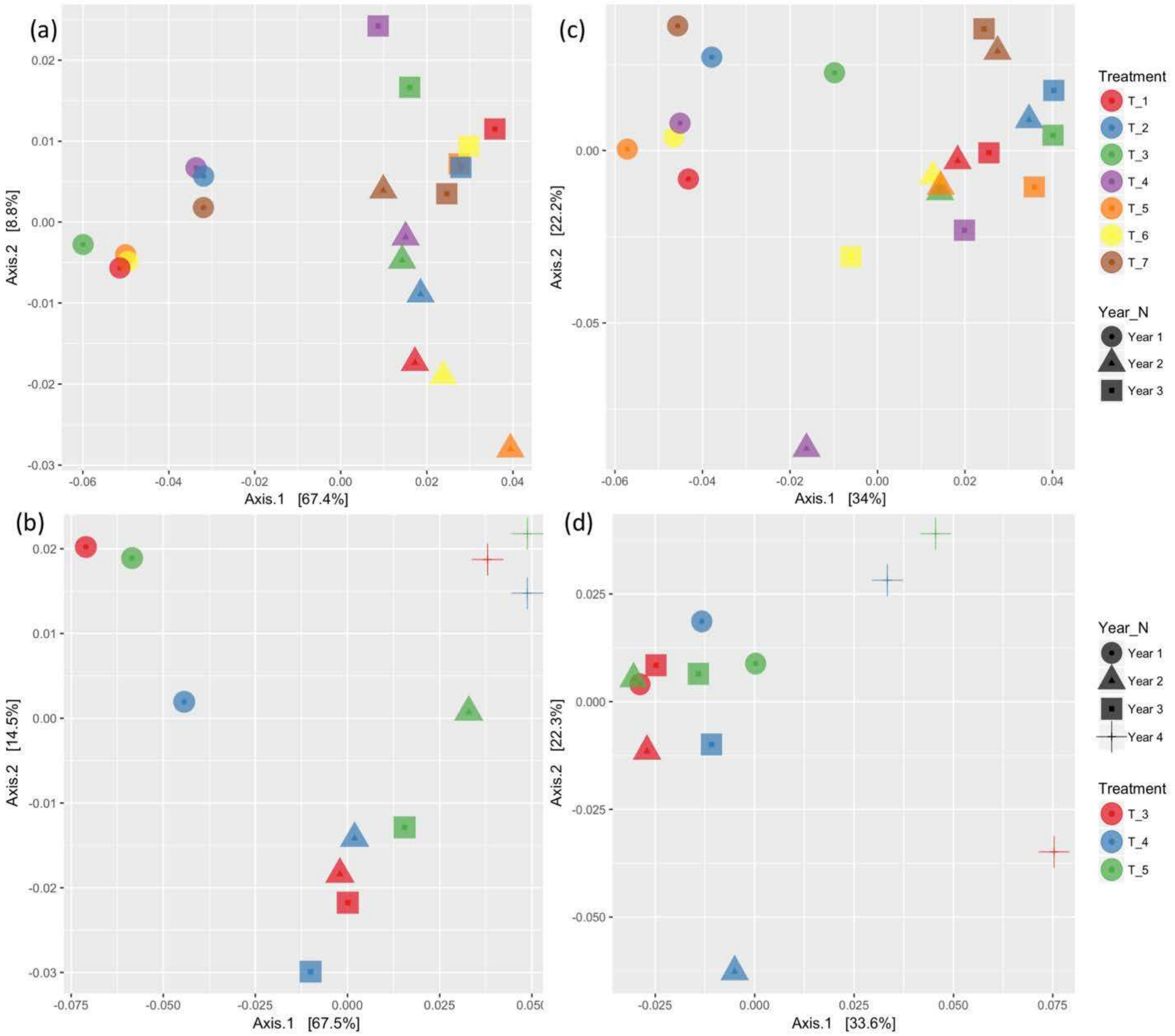

Figure 2.7. The weighted UniFrac distance metric which considers quantitative abundance of the bacterial communities over years studied for all treatments. Principle Coordinate analysis (PCoA) of Complete (A&C) and Ploidy (B&D) datasets in New Hampshire (A-B) and Pennsylvania (C-D) sites measured in the respective datasets.

Table 2.1. The mean annual temperature (MAT) in degrees C, mean annual precipitation (MAP) in millimeters, elevation in meters (m), percent slope and soil type for the four locations described in this study. Location, environmental characteristics, and soil type of the Pennsylvania and New Hampshire sites.

	Pennsylvania	New Hampshire
Coordinates	40.716996 N, 77.941813 W	43.097257 N, 70.991599 W
MAT (^{o}C)	10.12	9.62
MAP (cm)	218.4	236.2
Elevation (m)	371.85	57.91
Slope	3-8%	3-8%
Soil Type	Hagerstown Silt	Hollis-Charlton

Table 2.2. Important physical and chemical properties of soil sampled at each location in the study, estimated (Est.) saturation (Sat.) of Base, Calcium, Magnesium, Potassium, and Phosphorous as well as organic matter. Soil chemical properties associated with each location measured during the second year of the study.

	Pennsylvania	New Hampshire
pH	7.1	6.3
Calcium (ppm)	1579	1057
Magnesium (ppm)	430	195
Potassium (ppm)	130	134
Phosphorous (ppm)	15	441
Est. CEC	11.8	11.2
Est. Base Sat.	100%	65%
Est. Ca Sat.	66.80%	47.40%
Est. Mg Sat.	30.30%	14.60%
Est. K Sat.	2.80%	3.10%
Est. P Sat.	1.40%	21.50%
Organic Matter	3.10%	4.70%

Table 2.3. Unrarefied distance measure mixed effects model blocking by year for the Ploidy group fungal and bacterial community alpha diversity. Values in bold indicate $p < 0.05$.

'Ploidy': ITS		**'Ploidy': 16S**
Treatment	R^2=0.055, F=1.06, P=0.38$_{(2,23)}$	R^2=0.051, F=1.09, P=0.36 $_{(2,23)}$
Year	**R^2=0.159, F=2.05, P=0.001$_{(3,23)}$**	**R^2= 0.15, F=2.17, P=0.001$_{(3,23)}$**
Location	**R^2=0.39, F=13.14, P=0.001$_{(1,23)}$**	**R^2=0.40, F=16.9, P=0.001$_{(1,23)}$**
Treatment:Location	R^2=0.05, F=0.995, P=0.38$_{(2,23)}$	R^2=0.046, F=0.995, P=0.391$_{(2,23)}$
'Complete': ITS		**'Complete': 16S**
Treatment	R^2=0.07, F=0.952, P=0.52 $_{(6,41)}$	R^2=0.08, F=1.012, P=0.43$_{(6,41)}$
Year	**R^2=0.107, F=4.25, P=0.001$_{(2,41)}$**	**R^2=0.09, F=3.55, P=0.001$_{(2,41)}$**
Location	**R^2=0.39, F=33.31, P=0.001$_{(2,41)}$**	**R^2=0.44, F=35.31, P=0.001$_{(2,41)}$**
Treatment:Location	R^2=0.067, F=0.878, P=0.63 $_{(6,41)}$	R^2=0.074, F=0.99, P=0.455$_{(6,41)}$

Table 2.4. The model with the lowest value in bold is regarded as best given all variables available. Linear mixed-effects model selection for alpha diversity response in the complete group and Ploidy group for both 16S and ITS data sets. The Akaike information criterion (AIC), Bayesian information criterion (BIC) and log likelihood values represent values assigned to the quality of the model including the variables listed.

ITS ‘Ploidy’	**AIC**	**BIC**	**Log Likelihood**	**16S ‘Ploidy’**	**AIC**	**BIC**	**Log Likelihood**
~Treatment	**18.56**	**23.78**	-4.28	~Treatment	**-14.53**	**-9.3**	12.26
~Year	20.05	26.02	-4.027	~Year	-13.88	-7.91	12.94
~Treatment + Year	35.42	42.21	-3.71	~Treatment*Year	20.77	27.56	3.61
~Treatment + pH	26.30	33.42	-5.15	~Treatment*pH	-1.99	5.12	8.99
ITS ‘Complete’				**16S ‘Complete’**			
~Treatment	18.32	32.32	-0.16	~Treatment	-9.98	4.01	13.99
~Year	**-14.98**	**-6.66**	12.49	~Year	**-41.08**	**-32.76**	25.54
~Treatment + Year	49.74	73.76	-1.87	~Treatment*Year	40.56	64.59	2.71
~Treatment + pH	29.29	50.60	1.35	~Treatment*pH	-11.81	9.49	21.90

Table 2.5. Location differential metrics of mean observed, Shannon diversity, Simpson evenness, and Fishers exact across all years in each data set “Ploidy’ and ‘Complete’. T-test table based amplicon sequence variants (ASVs). Bold values indicate *P*<0.005 between locations.

16S ‘Ploidy’	Observed (ASVs)	Shannon	Simpson	Fisher
PA	**x̄= 3599.2, t= 3.567**	**μ=7.18, t= 3.62**	μ=0.99, t= 1.163	**536.91, t= 3.96**
NH	x̄= 4139.88	μ=7.32	μ=0.99	619.83
16S ‘Complete’				
PA	**x̄=3532.04, t= 8.26**	**μ=7.14, t= 7.70**	**μ=0.99, t= 3.46**	**525.71, t= 8.90**
NH	x̄= 4219.71	μ=7.34	μ=0.99	628.20
ITS ‘Complete’	Observed	Shannon	Simpson	Fisher
PA	x̄=1100.95, t= 0.115	**μ=5.303, t= -3.759**	μ=0.98, t= -0.33	200.90, t= -1.044
NH	x̄=1108.857	μ=5.11	μ=0.984	190.37
ITS ‘Ploidy’				
PA	x̄=1072.11, t= 0.67	μ=5.25, t= -1.86	μ=0.984, t= -0.08	202.52, t= -0.58
NH	x̄=1150.77	μ=5.12	μ=0.98	195.71

Table S2.1. Treatment mixtures of *L. perenne* at the Pennsylvania and New Hampshire field locations. The mixtures varied from Vermont and Maine only by most adapted cultivar, wherever Remington was used in PA and NH it was substituted with Mara in the northern VT and ME locations. Winter hardiness (WH) is the average value of each cultivar in the mix rated on a numeric scale, the higher the value the more winter hardy. Heading date (HD) is the average value of each cultivar rated on a temporal maturation scale, information provided by Barenbrug USA. Ploidy is indicated by 2N (diploid) and 4N (tetraploid).

Treatment	**Cultivar**	**Heading Date**	**Ploidy**	**WH**	**HD**
T_1	Most adapted	Early	4N	6	5
T_2	3 Cultivars	Early and Late	Mixed	6.33	5
T_3	5 Cultivars	Earlier and Later	Mixed	6	5.2
T_4	5 Cultivars	Mixed	2N	6.6	5.2
T_5	5 Cultivars	Mixed	4N	6	5.4
T_6	Commercial Blend	Unknown	Unknown	NA	NA
T_7	5 Cultivars	Early	2N	5.8	5

Table S2.2. NCBI Short Read Archive accession numbers and online location of data.

NCBI Short Read Archive	Accession Number	Location
16S amplicon data	SUB4275816	https://ncbi.nlm.nih.gov/subs/sra/
ITS amplicon data	SUB4275796	https://ncbi.nlm.nih.gov/subs/sra/

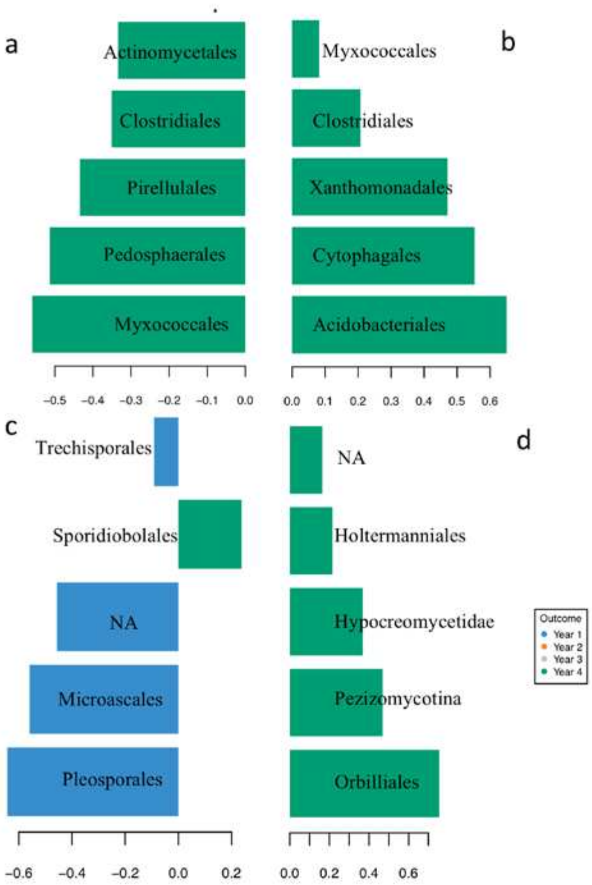

Figure S2.1. Discriminating ASV taxonomic orders which contribute the most to the first component of the sPLS-DA for bacterial (a-b) communities in Pennsylvania (b) and New Hampshire (a) sites, as well as for the fungal (c-d) communities in New Hampshire (c) and Pennsylvania (d) sites. Axis scale is for factor loading values.

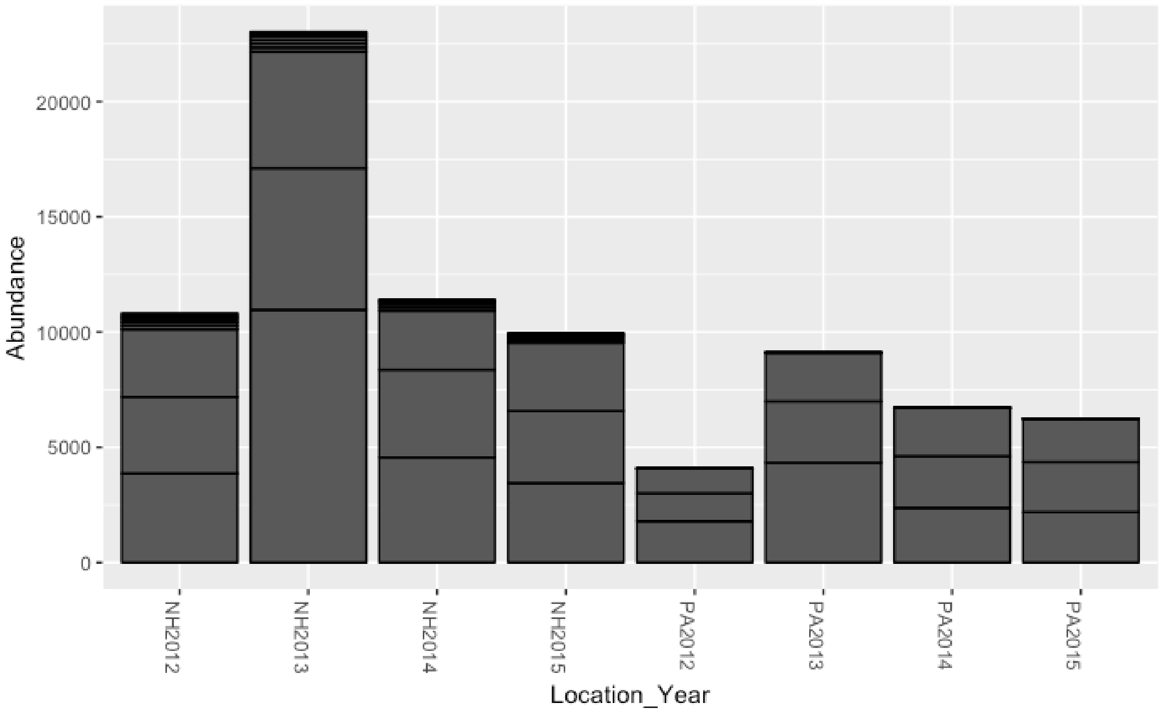

Figure S2.2. The abundance (reads) of the *Arthobacter* genus ASVs in the 'Ploidy' group, combining treatments three, four, and five during each year in both the New Hampshire and Pennsylvania sites after removing low abundance variants.

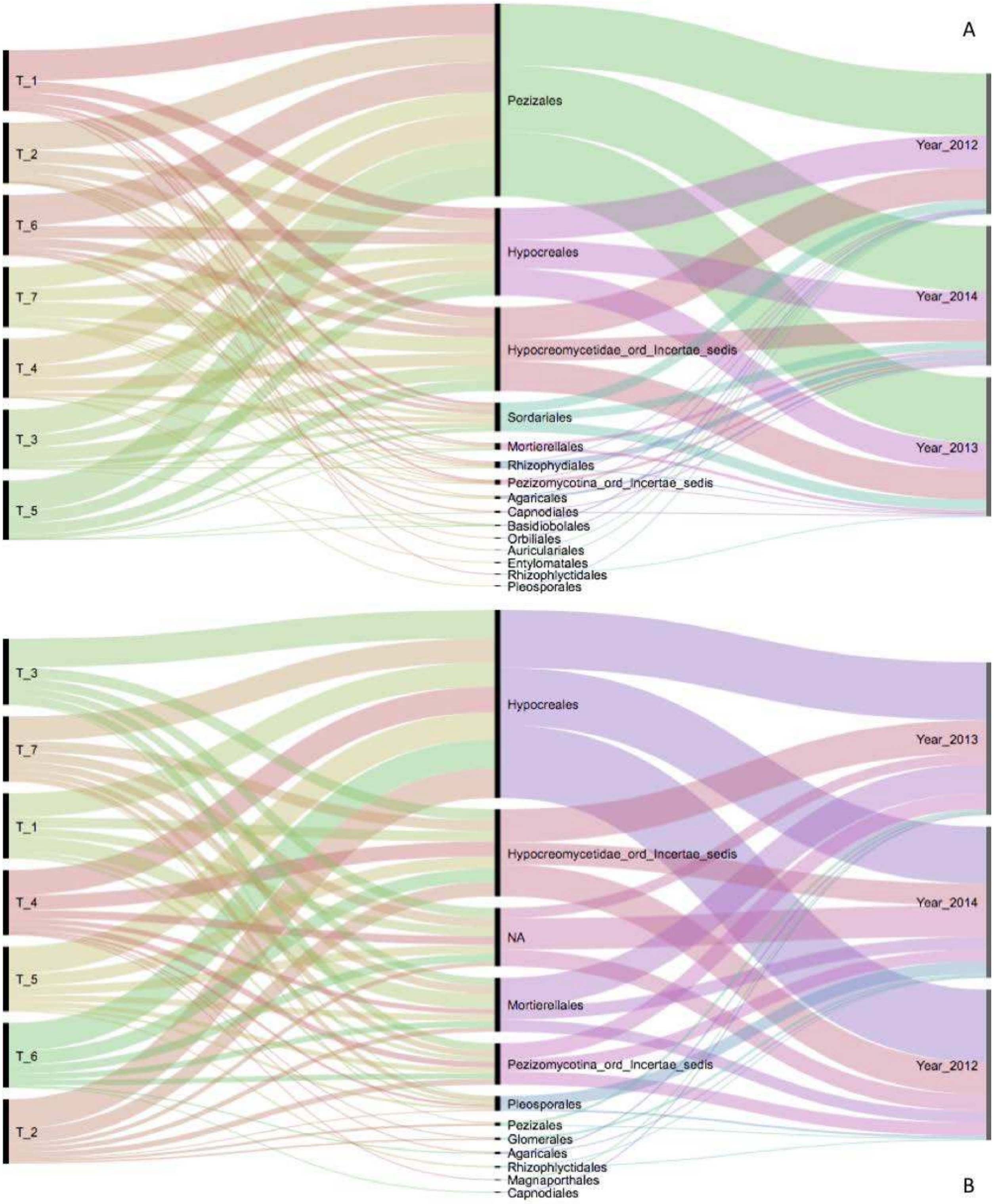

Figure S2.3. Fungal pathotrophs by taxonomic order observed in ‘Complete’ analysis ranked by relative abundance from highest (thick alluvium) to lowest (thin alluvium) in (a) New Hampshire and (b) Pennsylvania.

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Chapter 3—Ecology and epidemiology of silver scurf *(Helminthosporium solani*) and black dot (*Colletotrichum coccodes*) on potato in southern Colorado

Introduction

The fungal pathogens *Helminthosporium solani* Durieu & Montagne and *Colletotrichum coccodes* (Wallr.) S.J. Hughes continue to have a negative economic impact on potato production globally (1–4) and increasingly in regions where fresh packaging quality is important. Both of these soil-borne fungal pathogens leave unsightly blemishes that reduce economic value to light skinned or fresh packaged potatoes, but do not consistently reduce yields (5). The diseases caused by *H. solani* and *C. coccodes* are commonly referred to as silver scurf and black dot (6,7), respectively. These fungal pathogens exhibit unique life cycles while utilizing the same plant host, presenting an annual challenge to potato production.

Understanding the ecology of these pathogens in the soil and the impact of management practices such as crop rotation and cultivar selection in reducing persistence and spread in potato production systems (6) is important to grower profitability. However, due to the similarity of visual symptoms on the tuber, the two pathogens are often misdiagnosed (8). This misdiagnosis can potentially lead to incorrect application of fungicides during production and insufficient treatment during post-harvest storage. For example, if the silvering of skin, an indicative post-harvest symptom of both pathogens is treated with the fungicide thiabendazole, which *H. solani* has developed resistance to, disease symptoms may continue to spread in storage (9). On the other hand *C. coccodes* produces persistent sclerotia which are not entirely susceptible to soil fumigants used for control of *H. solani*, contributing to the pathogen’s longevity in the soil (10). At soil depths greater than 10-20 cm, fungal sclerotia of *C. coccodes* are recoverable for up to 8 years (11).

The lifecycles of these pathogens differ from one another in that *C. coccodes* can infect multiple hosts and the tubers, haulms, and roots of the plant, and is considered to have an endophytic life stage (12) but can also be a necrotrophic pathogen depending on host phenology (1,13). In contrast, *H. solani* has a very limited host range and infects only the tuber. *H. solani* has little ability to persist in soil without the potato host (14). It is believed that *H. solani* is more ephemeral in the soil and unlike *C. coccodes,* unable to survive or persist without its host. However, soil borne inoculum of *H. solani* has recently been suggested as a source of disease (9). This idea was recently discounted though (15), and *H. solani* was described as being weakly competitive (16) and the barley stubble tilled into the soil preceding a potato planting decreases the pathogens survival (17).

Typical management practices for both silver scurf and black dot include coating the seed piece with desiccant and fungicide following cutting to encourage suberization, reducing fungal infection upon planting and therefore reducing disease in storage. Previous studies have shown that traditional seed piece fungicide coating and foliar applications were not sufficient in reducing the pathogen inoculum in the soil once *C. coccodes* established in a field (18). Crop rotation as a management option for *C. coccodes* control may succeed if the duration is long enough to reduce viable sclerotia and the rotational crop is not a pathogen host.

Chemical control alone is also not a sufficient disease management solution and is recommended in conjunction with other environmentally dependent biological and cultural control methods (19). In the case of biological control, fungal antagonism has been shown to reduce disease severity and virulence of *C. coccodes* when found in conjunction with other soil borne pathogens such as *Verticillium dahliae* Kleb. (20,21). Multi-year persistence was reduced when found in conjunction with *Fusarium spp*. (11) but not when found with *Mucor spp,* a ubiquitous soil borne fungus. It is unclear if soil properties play a role in favoring antagonistic fungi over pathogenic fungi, such as *C. coccodes,* in potato production systems. Though this may be the case in the San Luis Valley potato growing region of Colorado, as the soil is classified as porous gravelly sand and is very low in organic matter (0.6%-2.7%), highly tilled, nutrient poor, and receives significant synthetic fertilizer and pesticides. This environment is characteristically less hospitable to fungi and bacteria used for biological control of pathogens (22). Whether there are fungal antagonists of potato pathogens in these soils is not well known and determining antagonistic or symbiotic microbial relationships with these specific cultivars is an understudied area.

Potato cultivar specific resistance may not have a substantial effect at reducing disease when multiple pathogens or a complex of pathogens are involved (23). Though there is cultivar specific resistance to black dot, limited resistance exists for silver scurf (16). Breeding programs have incorporated wild *Solanum spp*. into potato cultivars in an effort to reduce susceptibility (24), but there are no highly resistant cultivars to *H. solani* reported as far as we know. Breeding for resistance of black dot continues to be successful though mainly on russet type cultivars (25), here we test industry standard russet cultivars but also fair skinned cultivars which tend to be more susceptible to black dot and silver scurf. Knowledge of cultivar susceptibility in this production region, which produces specialty fresh packaged potatoes will assist growers in making cultivar choices in fields with a history of silver scurf and/or black dot.

The management of potato production involves nutrient additions, importantly nitrogen, and this fertilizer may affect the physiological state of the plant and its ability to resist fungal infection (42). The soil environment most hospitable to these fungal pathogens provides further insight to the ecology and epidemiology of silver scurf and black dot. The cosmopolitan range of

C. coccodes is due to its ability to survive on many hosts both living and dead in soil types ranging from high organic matter to high percent sand and a range of soil pH (1). The lifecycle of *H. solani* is less understood, as the sexual stage of this organism is unknown it reproduces primarily asexually via conidia and it rarely if ever produces sexual reproductive structures (38). Soil physical and chemical properties influence soil pathogen reproduction, persistence, and survival in a myriad of ways (39–41). We applied three different nitrogen fertilizer rates to two industry standard potato varieties inoculated with *H. solani* and *C. coccodes* to test the effect of nitrogen application on cultivar susceptibility Further understanding of the pathogens dependence upon management practices as well soil type will help fill a gap in our understanding of these plant pathogen interactions.

The knowledge gaps presented here on the persistence of these pathogens and their impact on potato storage, particularly in the San Luis Valley of Colorado, need to be understood in order to make disease management recommendations to reduce annual loss to silver scurf and black dot. The primary objective for the regional survey is to determine if the soil properties and crop species impact the incidence and severity of *C. coccodes* and *H. solani* in the soil and on tubers at harvest. Secondarily, post-harvest control of these fungal pathogens could reduce economic losses by mitigating symptom development in storage (43–46) through efficacy testing of fresh packaging treatment or chemical fog infiltration. This research would provide a modern reference of quantitative presence and persistence for these two important pathogens.

In addition, to understand the abundance and distribution of *C. coccodes* and *H. solani* in the San Luis Valley, we wanted to evaluate potential management strategies that would reduce disease severity in an inoculated field trial. Our goal was to evaluate the effect cultivar, fungal biocontrol, and nitrogen application on disease severity in field, and evaluate the effect of multiple post-harvest treatments on disease severity in storage. This included the following objectives: 1) determine the effect of cultivar and a single fungal antagonist, a novel *Penicillium sp*. recovered from the soil in the San Luis Valley in in field trials inoculated with *H. solani* and *C. coccodes*; 2) evaluate the effect of nitrogen application rate on two standard potato varieties in a *C. coccodes* and *H. solani* inoculated field trial; and 3) evaluated the effect multiple commercial fungicides and fungicide alternatives such as hydrogen peroxide, alcohol and bacterial biocontrol as post-harvest applications to reduce weight loss and disease severity in storage.

Materials and Methods

Experiment 1: Incidence and severity of C. coccodes and H. solani in the San Luis Valley of Colorado

A field survey was conducted during the growing seasons of 2016 and 2017 in order to determine the geographic distribution of *C. coccodes* and *H. solani* in the potato production region of the San Luis Valley, located in southcentral Colorado. The San Luis Valley is an arid region receiving on average just 35.5 cm of rain annually in the past 4 years (NOAA, NCDC), therefore overhead center pivot irrigation is commonly used on 64.7 hectare plots of land, which represents an entire pivot. The crops grown in this system are usually managed in one of three ways: one crop is grown under a full-pivot, two crops grown on half-pivot each, or four crops grown each on a quarter-pivot. Crops may include cash crops such as potato and barley, but also cover crops and green manure crops grown for improving soil health and reducing pathogen pressure. In the quarter pivot systems, some quarters are also left fallow. Management practices on the surveyed pivots focus on improving soil health by various methods including increasing organic matter and decreasing pathogen abundance by planting non-host crops such as sudan grass (Sorghum x drummondii), growing Brassica mustards and tilling them in the soil as a green manure. The two years of sampling included pivots in 8 different non-potato crops and multiple potato varieties. Sample locations were classified as in potato production or not in potato production, as well as which crop was currently being grown. Therefore, sampling was performed within a quarter pivot (~16 ha) irrespective of its current management state. Soil samples, three per site, were collected and composited along a 15 meter transect from the outer edge of the quarter towards the center of the pivot to account for within pivot variance. In the 2016 survey, composite soil samples were collected from 56 sites across 19 irrigation center pivots. Soil sampling occurred in each quarter of a pivot to account for variable cropping history and rotation pattern. Repeated sampling in 2016 and 2017 was complete at 50 sites under 15 pivots during this survey (Figure 3.1) in order to determine the effect of rotating to a non-potato crop on the abundance of *C. coccodes* and *H. solani* in the soil (Table 3.1). Global positioning system (GPS) coordinates were recorded for every sample site and mapped via ArcGIS v 10.6 software by Esri.

Sample processing and DNA Extraction

Soil samples and potato tubers collected during 2016 and 2017 growing seasons were immediately placed in a cooler and subsampled for further processing within 12 hours in the laboratory. One composite soil sample was collected from three points at each site to a depth of 25 cm, homogenized, and frozen at -20°C in 15mL tubes. From 68 sites within 15 pivots ~50g of soil was air dried and sent to the Colorado State Soil Testing Lab, Fort Collins, CO for chemical and physical texture analysis. Potato tubers were collected post-harvest from seven growers' storage facilities to determine the molecular based quantification of disease acquired during growth. Knowing if the tubers were infected before storage would provide a relationship between soil inoculum and tuber disease. Once in the lab, peels 2.5 cm x 10 cm were collected and lyophilized for 24 hours. Sample preparation for DNA extraction involved 0.25 g of soil stored at -20°C or lyophilized potato tuber peel. DNA extraction was conducted with Qiagen DNeasy PowerSoil HTP 96-well kit and the Zymo Quick-DNA Plant 96 kit, both on the Thermo Scientific KingFisher Flex Purification system—a robotic magnetic-bead based platform. Extracted DNA was eluted into a 96-well plate containing 100uL of pure H_2O and transferred to individual tubes for storage at -20°C. Tubers used in post-harvest experiments were stored in a humidity and temperature controlled (>90% relative humidity and 10°C) walk in cooler.

Molecular quantification of *H. solani* and *C. coccodes*

Nested PCR was successfully conducted on *C. coccodes* and *H. solani* samples to confirm detection in soil DNA extractions prior to performing quantitative PCR. To amplify *H. solani* and *C. coccodes* from soil and tuber peel, a modified DNA extraction protocol based on previously published methods by Cullen et al. (47,48) was used. The modification of the protocol included reducing reaction volume from 50 µL to 10 µL. The master mix (MM) volume containing the reagents necessary for the reaction was 9 µL, with an addition of 1 µL of normalized DNA diluted to 0.25 ng/µL template. Master mixes were composed of 0.38 µL of forward and reverse primers at a concentration of 10 µM, 0.13uL of Taq-Man TAMRA/VIC probe at 10uM concentration, 6.25 µL of Promega qPCR Master Mix, and 2.98 µL of purified DNA/RNA free H_2O. The profile on the Applied Biosystems QuantStudio 3 Real Time PCR System was 50°C for 2 minutes during Stage 1, 95°C for 10 minutes during Stage 2, and 95°C 15 seconds followed by 60°C for 60 seconds repeated for 45 cycles during Stage 3.

Experiment 2: Field trial of fungicide and biocontrol efficacy against *C. coccodes* and *H. solani*

The field trials were set up at the Colorado State University, San Luis Valley Research Center in Center, Colorado. The first trial was designed to test the effect of cultivar on the two pathogens. The experiment was a complete randomized design, with 6 potato cultivars and 6 treatments including the pathogens (*C. coccodes* & *H. solani*), pathogens plus biocontrol (*Penicillium* sp.), and biocontrol alone including an untreated control with each combination replicated 5 times. In each replicated plot 12 seed pieces were planted in two 25 cm wide rows with 6 seed pieces in each 2-meter-long row. Cultivars selected for this field trial included six varieties Canella Russet, Purple Majesty, CO99045 1 W/Y, CO00277-2R, AC03433-1W, and AC05175-3 P/Y. The numbered cultivar names are advanced breeding lines selected through the Colorado State University San Luis Valley Research Center potato breeding program. Seed pieces were cut on a modified Milestone cutter with propane heat sterilized blades to reduce disease spread and was completed on May 17th 2017. Cut potato seed were not treated with any fungicide or drying agent, but could suberize for approximately two weeks prior to planting. Potato seed pieces were planted on June 1st 2017. Potato plants were killed via mechanical chopping of haulms two weeks prior to tuber harvest on October 3-5th 2017 total plot yield was recorded and subsampled to 10 ~200 g tubers per plot. Tubers were immediately stored in a humidity and temperature controlled (>90% relative humidity and 10°C) walk-in cooler for the five months that the data collection occurred.

Preparation of fungal inoculum

Fungal cultures of *C. coccodes* and *Penicillium sp.* for use in the post-harvest experiment were isolated from soil, and the *H. solani* culture used was acquired from Dr. Gevens at the

University of Wisconsin. The ITS1-5.8s-ITS2 region of each isolate were sequenced to verify identification. The 7-day old cultures on PDA agar plates were transferred to liquid culture by inoculating 50 milliliters (mL) potato dextrose broth with multiple 1.0 cm plugs taken from the leading edge of agar plates. The 50mL falcon tubes were shaker incubated for 14 days at 25°C and 150 rpm. Field inoculum of fungi was created with sterilized hull-less barley incubated with 100 mL liquid culture of each pathogen in paraffin sealed sterilized metal trays at room temperature for 3-5 weeks. The 6 fungal treatments, pathogens alone, pathogens plus biocontrol, and biocontrol alone were sown in furrow (15 cm depth) at potato planting with ~25 grams of inoculated hull-less barley per seed piece before being mechanically covered with soil.

Experiment 3: Nitrogen application rate field trial

The second field trial was a randomized complete block design with three nitrogen levels on two potato varieties under two pathogen treatments with an untreated control. There were two blocks and three replicates of each treatment by cultivar by nitrogen rate combination. In each replicated plot 8 seed pieces were planted in two 25 cm wide rows 1.5 meters long with 58 cm spacing between plots. The tubers used in this trial were prepared in the same manner as those in Experiment 2 and planted at the same time. The fungal inoculum used was also prepared as described in Experiment 2.

The nitrogen fertilization experiment was conducted with three nitrogen rates (low, normal and high) applied to two different cultivars (CO00277-2R and Canella Russet) under three treatments: *H. solani* inoculum, *C. coccodes* inoculum, and an untreated control. Nitrogen rate was based off annual soil requirements with "normal" rate of 190.5 kg/hectare, "low" a 25% reduction (142.8 kg/ha), and "high" was a 25% increase (238 kg/ha) from normal soil requirements. This total nitrogen rate includes residual N in soil, dry broadcast fertilizer, in furrow applications as well as fertigations in season. Potato plants were mechanically chopped and tubers harvested as in Experiment 1. Tubers were collected and tagged by plot at harvest, subsampled and stored as described above.

Experiment 4: Post-Harvest fungicide efficacy

We tested eight post-harvest treatments including both novel and traditional formulas over multiple months in controlled storage for commercial and pre-breeding potato cultivars. The post-harvest experiment included four of the potato cultivars mentioned above—Purple Majesty, Canella Russet, CO99045 1 W/Y, and CO00277-2R—replicated five times for each of the nine treatments tested. The treatments included an untreated control (UTC), two ethanol concentrations (High: 40% and Low: 10%), two hydrogen peroxide concentrations (High: 3% & Low: 2%), Phostrol (thiabendazole), Stadium (fludioxonil, azoxystrobin, difenoconazole), Quadris (azoxystrobin) and BioSave 11LP (commercial *Pseudomonas sp*. biocontrol from JetAg Harvest). Each post-harvest treatment was mixed in a large plastic container and nylon mesh bags containing ten equally sized (~200 g) tubers were washed and dried before being dipped into the corresponding treatment mixture for 30 seconds. Each experimental unit was weighed for initial measurement after being washed, dried, and the treatment application air dried. Tubers were stored as described above.

The untreated control (UTC) was 4 L of deionized water; every other treatment also totaled 4 L. Ethanol (EtOH) from Sigma Aldrich was diluted at two concentrations for two treatments. The "high" concentration treatment was composed of 1.6 L of 95% EtOH with 2.4 L of H_2O for a 4:10 v/v ratio. The "low" ethanol treatment was at a 1:10 v/v ratio with 0.4 L 95% EtOH and 3.6 L H_2O. Hydrogen peroxide (H_2O_2) at 30% concentration from Sigma Aldrich was also diluted at two concentrations. The "high" treatment was at a 1:30 v/v ratio with 0.13 L H_2O_2

and 3.87 L H_2O. The low concentration was at a ratio of 1:50 v/v with 0.08 L H_2O_2 and 3.92 L H_2O. Phostrol was applied at the suggested rate 0.015 L to 3.99 L H_2O for the conventional treatment. Stadium fungicide by Syngenta contains three active ingredients, fludioxonil, azoxystrobin, and difenoconazole was applied at the rate of 29.5 mL per 907 kg potatoes. Quadris an azoxystrobin class of fungicide was applied at a rate of 25mL per 907 kg potatoes. The commercial biocontrol treatment was a lyophilized powder containing *Pseudomonas sp.* mixed with 4 L H_2O at the labeled rate of application (6.61 g/L).

Visual disease symptoms and percent weight loss were calculated for all three trials every 30 days over 3-5 months in storage and this experiment was repeated twice in two years. Measurement of weight loss with visual rating of disease severity on a scale of 1-5 are recognized to be accurate and efficient methods for quantifying post-harvest disease on potato (17).

Statistical analysis

Statistical analyses were conducted in the R v. 3.4.1 environment (R Core Development, 2018) using 'lme4' for mixed effects models and 'emmeans' for pairwise comparisons (49,50) as well as 'MuMIn' for model selection. One statistical method used for the disease survey was a generalized linear model to run a logistic regression with presence and absence for the *H. solani* detection on the associated data. Model selection was performed using 'dredge' for AIC subsets selection of the "top" model this performs a global model call including all soil properties and meta data, accounting for collinearity ($r > 0.5$) with the subset option invoked. Quantification of *C. coccodes* in soil produced few zeros therefore a multiple regression mixed model analysis was conducted including year for repeated measures blocking by "Pivot ID". When significant effects were observed through ANOVA with the "Kenward-Roger" degrees of freedom approximation, multiple comparisons of means were conducted with Tukey's multiple comparison p-value correction. When performing t test comparisons Shapiro-Wilk normality test and F-test for variance comparison was conducted. An alpha value of 0.05 was implemented for significance testing. Principle components analysis was conducted to display factors contributing to pathogen presence in the survey using 'FactoMineR' and 'factoextra' packages (51).

Weight loss and disease incidence were analyzed with mixed effects models to test for effects of cultivar, nitrogen fertilization, post-harvest treatment, and pathogen inoculation. Experimental block and time (month) were considered random effects as well as to account for repeated measures of disease rating and weight measurements. Spearman rank correlations were checked for nonlinear monotonic relationships between time and percent weight loss.

Results

Prevalence of *C. coccodes* and *H. solani* in the San Luis Valley of Colorado

While quantitative PCR methodologies have been developed for *C. coccodes* and *H. solani*, they have not been deployed in concert for quantification of both pathogens in the soil and on the potato tuber peel. Here, using previously published primers, we have detected levels of both pathogens on potato tubers as well as in the soil where they are grown and not being grown.

We collected soil from 137 sites over the two years for the incidence of *H. solani* and *C. coccodes*, to determine the effect of soil properties and current crop on the incidence and abundance of *H. solani* and *C. coccodes* in the San Luis Valley of Colorado (Table 3.1 and S3.1). Of the 137 sites sampled 60 and 100 were positive by qPCR for *H. solani* and *C. coccodes*, respectively (Figure 3.2). Detected abundance of *C. coccodes* in the soil ranged from 0.13 – 7580.6 femtograms/gram soil (fg/g). Quantification of *H. solani* ranged from 0.12 - 685.9 fg/g soil. There was a nearly complete reduction in *H. solani* detected in the soil in year two compared to year one (Figure 3.2b). When *C. coccodes* was present there was significantly greater quantities of *C. coccodes* than *H. solani* in the soil; often 10 times the amount of *H. solani* detected. *H. solani* was analyzed in a binary fashion due to uniform low quantification as either present or absent in a sample. Comparing the average quantification of *C. coccodes* in the soil where potatoes were being grown (976.1 fg/g) versus not grown (693.9 fg/g) the mean values were not significantly different due to high variability across sites (W=0.6, F=0.87, P = 0.33). In order to correlate soil-borne inoculum of *C. coccodes* and *H. solani* with tuber infection in storage we extracted DNA from 108 tubers from seven farms over two years. From the 108 tuber samples, qPCR detection of *H. solani* was positive for 35 tubers (0.01-14523 fg/g) and *C. coccodes* was detected on 85 tubers (0.11-8046 fg/g) (Figure 3.3).

The organic matter content and year were the best predictors for the presence of *H. solani* according to a generalized linear model selected with an AIC value of 8. Presence of *H. solani* was correlated with available soil nutrients and chemistry (Cu, NO_3-, P) while the incidence of *C. coccodes* was correlated with organic matter content and percent clay. Copper, nitrate, phosphorous and electrical conductivity all had positive relationships with *H. solani* abundance in the soil (Figure 3.4). Increased organic matter was correlated weakly (R_2=0.11) but significantly ($P_{adj.}$< 0.01) with increased detection of *C. coccodes* in the soils sampled (Figure 3.5).

Field Trial for Susceptibility and Nitrogen Rates

The greatest percent weight loss varied between cultivars and fungal pathogen inoculum, with the Red variety exhibiting the greatest weight loss when inoculated with both *H. solani* and *C. coccodes* and the White-Yellow variety with the biocontrol fungus. There was an effect of cultivar on weight loss after three months (P < 0.05) and at four months (P < 0.05) within the treatment cultivar interaction in the cultivar susceptibility experiment (Table 3.2). In this experiment cultivar CO00277-2R, a red skinned potato, exhibited a significant difference in percent loss over the five months depending on treatment. Potatoes inoculated with just *C. coccodes* had the most percent weight loss (3.04%) while the potatoes treated with the biocontrol alone showed the least weight loss (1.27%). The white yellow cultivar CO99045 1 W/Y had the greatest percent loss from the *Penicillium sp.* treatment. The lowest weight losses (1.18% & 1.02%), were observed when potatoes were treated with a combination of the biocontrol, *Penicillium sp.* and the pathogens *C. coccodes* or *H. solani*, respectively (Table 3.3). The other four cultivars exhibited no significant difference in storage percent weight loss regardless of treatment.

Percent weight loss and disease symptoms were influenced by nitrogen and pathogen treatments. Disease rating and percent weight loss under varied nitrogen application rate followed similar decreases over time as shown in the cultivar susceptibility experiment (Table 3.4). Disease severity was consistently greater for the red cultivar under the low nitrogen rate compared to normal and high applications over time (Table 3.4). Weight loss increased in tubers from high and low nitrogen addition plots relative to normal fertilizer additions in storage (Table 3.5). There was a negative effect of nitrogen rate on weight loss ($P < 0.003$), an effect of all two-way interactions and the three-way interaction of nitrogen rate, cultivar, and treatment ($P < 0.05$). Two of the six tested cultivars displayed different effects of percent weight loss in storage over the 5 months they were measured. Cultivar susceptibility results on both pathogens as well as the potential biocontrol were mixed, interactions of pathogens and the biocontrol were cultivar dependent. Certain cultivars, such as the purple skinned and the white yellow cultivars exhibited greater percent weight loss and show more symptoms of disease when inoculated in the field with the biocontrol and *H. solani* compared to all other treatments. The red and russet cultivars exhibited an opposite effect of the biocontrol and *H. solani*, experiencing less percent weight loss as well as fewer disease symptoms. The red cultivar did show the greatest susceptibility to *C. coccodes*, with high percent weight loss as well as greater disease symptoms (Figure 3.6).

Post-harvest disease and weight loss

Post-harvest treatments to control silver scurf and black dot reduced weight loss over the 3-month storage season starting in November when growers begin treating for post-harvest disease and preparing for fresh package shipping. There was an effect of post-harvest treatment, cultivar and treatment by cultivar interaction on weight loss and disease (Table 3.6). Hydrogen peroxide at the high rate was not significantly different from Phostrol in it's ability to prevent tuber weight loss for Purple Majesty, Colorado Rose, and CO99045 1 W/Y (Table 3.7). Canella Russet lost the least weight with both the commercial biological control (Bio-Save LP) and the common synthetic Phostrol treatment while tubers treated with Stadium and Quadris experienced greater weight loss than the untreated control. By the last month in storage the Canella Russett experience a weight loss of 24% compared to the untreated control. The CO99045 1 W/Y and Purple Majesty cultivars experienced the most significant weight loss with the biocontrol and UTC (Table 3.7). The white yellow cultivar had 46% more weight loss when treated with BioSave LP compared to Phostrol.

Discussion

Consumer demands on fresh market potatoes requires growers to produce blemish free fresh packaged potatoes. Our research into two blemish causing fungal pathogens has shown that with proper management practices, both weight loss and visual disease symptoms may be mitigated. Prior to this study, the prevalence and persistence of these pathogens, the susceptibility of new potato cultivars to *C. coccodes* and *H. solani*, and the associated post-harvest disease development were not well characterized in the potato growing region of southcentral Colorado. Over a two-year field survey, we determined that *C. coccodes* is persistent both in the soils of potato production fields and on potato tubers. We also determined that *H. solani* does not persist in the soil year to year. Susceptibility of new and commercial potato cultivars varies for these two pathogens based on percent weight loss and visual disease rating in storage. Post-harvest treatment options are numerous and effective in reducing weight loss and visual disease symptoms in storage for up to four months.

While crop rotation has proven to be an effective strategy against many soilborne pathogens, several important pathogens of potatoes, such as *V. dahlia* and *C. coccodes* can persist as sclerotia through multiple years of rotation crops. (52). Crop rotation regimes have a significant influence on the persistence and quantity of *C. coccodes* in the soil (6). In addition to crop history, infected seed potatoes along with inappropriate use of fungicides may contribute to pathogen persistence (53). This study demonstrates the rotation to a non-potato crop resulted in the reduction of soil borne pathogen inoculum. The results of the soil survey show that average levels of *C. coccodes* in the soil were not different whether potatoes were being grown in the soil or not. *H. solani* does not persist in the soil when fields are rotated out of potato regardless of crop. The results from this study suggest that *H. solani* detected in the soil is associated with infected potatoes planted at the start of the growing season since it is not able to persist in the soil following a crop rotation. In contrast, we found that *C. coccodes* is able to persist through a crop rotation out of potato as it was detected in the soil of several non-potato rotation crops in year two that followed an initial potato crop in year one.

The grower field survey implicates infected seed tubers as a contributor to soil borne *H. solani* presence in the soil, along with favorable soil nitrate, phosphorous, and electrical conductivity. Growers should ensure that they are planting disease free seed year over year, and if the pathogen is detected before or during harvest, enact strict control measures in storage to reduce pathogen build up during storage. Properly disinfecting storage facilities including air circulatory systems may reduce infection of tubers during storage as well as fog infiltrating or washing with appropriate post-harvest chemicals such as hydrogen peroxide or Phostrol. *C. coccodes* persists through crop rotations and is detectable in the same location after a potato crop is rotated out of the field, though in reduced levels indicating an effect of crop rotation. The negative correlation of certain soil properties e.g. pH and percent Iron (Figure S2.3) may provide avenues for further soil property pathogen interaction experimentation.

Another main objective with this study was to determine the susceptibility of potato cultivars during post-harvest storage to *C. coccodes* and *H. solani* alone and in conjunction with an inoculated fungal biocontrol (*Penicillium sp.*) that is endemic to local soils. We expected the biocontrol to compete for niche space both during the growing season as well as during storage which is a similar mode of action with other biocontrol organisms (28). Our results suggest that the biocontrol by cultivar interaction was highly variable, and this variability could be due to the ecology of the fungus. The physical structure and composition of potato periderm varies (54), this could also be a reason why the purple-white variety lost the most weight over time when inoculated with the biocontrol. Certain cultivars did exhibit greater susceptibility and an inability to defend against coinoculation of pathogen and biocontrol. Caution should be used when applying novel biocontrol organisms as the cultivar specific response varies, where the biocontrol may increase susceptibility in certain environments. Understanding the ecology of these pathogen-biocontrol-plant interactions will require *in vitro* experimentation and further regional surveying.

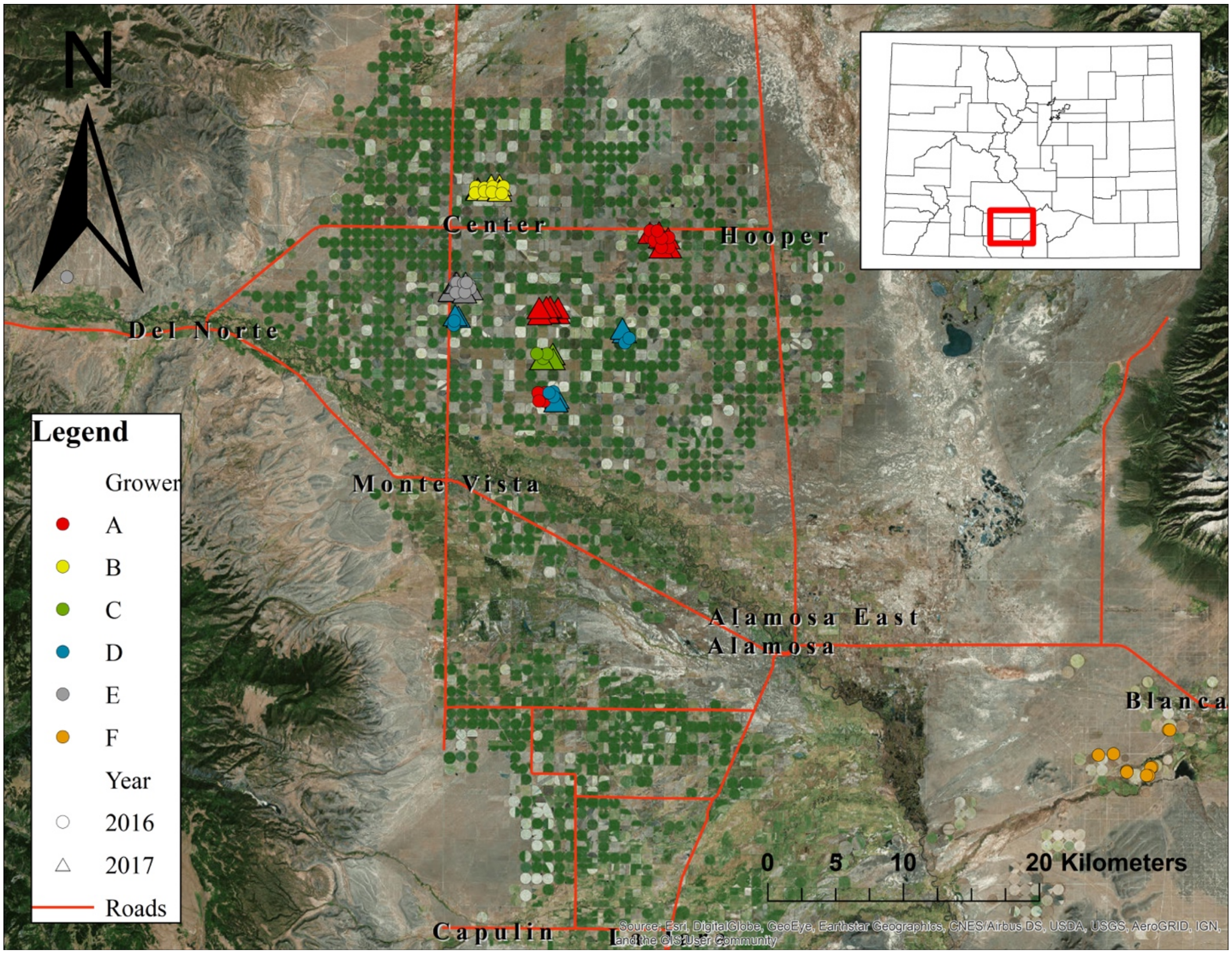
Field trials show that cultivar susceptibility varies and nutrient effects on pathological fungal organisms interact with one another. Certain cultivars, especially red skinned potatoes, experiencing nitrogen limitations are more susceptible to these soil-borne pathogens, especially when considering microbial complexes, than other cultivars. Edaphic interactions with endemic fungal biocontrol organisms may reduce tuber weight loss and decrease visual disease symptoms during long term cold storage.

In southern Colorado, post-harvest treatment options for potato are limited due to numerous confounding limitations, such as scales of implementation and variability in the processing of annual yields based on consumer demand and geopolitical economics. Post-harvest chemical treatments and storage directly influence weight loss and disease symptom development depending on the potato cultivar. Results of the post-harvest treatments on different cultivars also show cultivar dependent responses. Canella Russet lost the least weight with both the commercial biological control (Bio-Save LP) and the common synthetic Phostrol treatments. This suggests that an effective organic alternative to Phostrol is Bio-Save for russet cultivars. On cultivar CO 99045 1 W/Y, both Phostrol and the high concentration hydrogen peroxide treatments performed equally well in reducing percent weight loss. Colorado Rose and Purple Majesty responded equally well with two treatments, where Phostrol and the low concentration of hydrogen peroxide reduced weight loss equally (Table 3.8). This result suggests that the lower concentration of hydrogen peroxide may be necessary on specific cultivars of potatoes. Fresh pack potatoes must maintain high quality appearance during multiple months in cold storage. Treatment options are necessary for growers who detect disease in the field during the growing season to mitigate economic loss if possible. Ensuring the continued tuber quality and disease resistance in seed production regions is important to continued success and viability.

The post-harvest treatment experiment shows that alternative chemical options perform similarly and with certain cultivars better than commercial products, limiting losses during storage of diseased tubers. These treatment options tested on common commercial cultivars and advanced breeding lines of potatoes offer growers new options for reducing visual disease and weight loss. Further testing and economic scaling analyses should be performed to confirm the long-term potential usage of these post-harvest disease treatment methods.

These experiments should be useful for growers and potato breeders in similar agricultural environments. Though it is clear that both of these pathogens are detectable in the soil and on tubers, the annual fluctuation in presence and abundance is inconsistent. Crop rotations are helpful in reducing silver scurf but not black dot. Nitrogen rate, cultivar and fungal pathogen do interact and effect weight loss in storage while having less of an effect on disease severity. Finally, post-harvest chemical treatments are effective and on certain cultivars a biological control may be an effective alternative to synthetic fungicides.

Figures and Tables



Source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community

Figure 3.1. Regional map of southcentral Colorado where survey and experimental study too place, GPS marked survey sampling sites (o-2016 Δ-2017) each grower operation is highlighted by a different color.

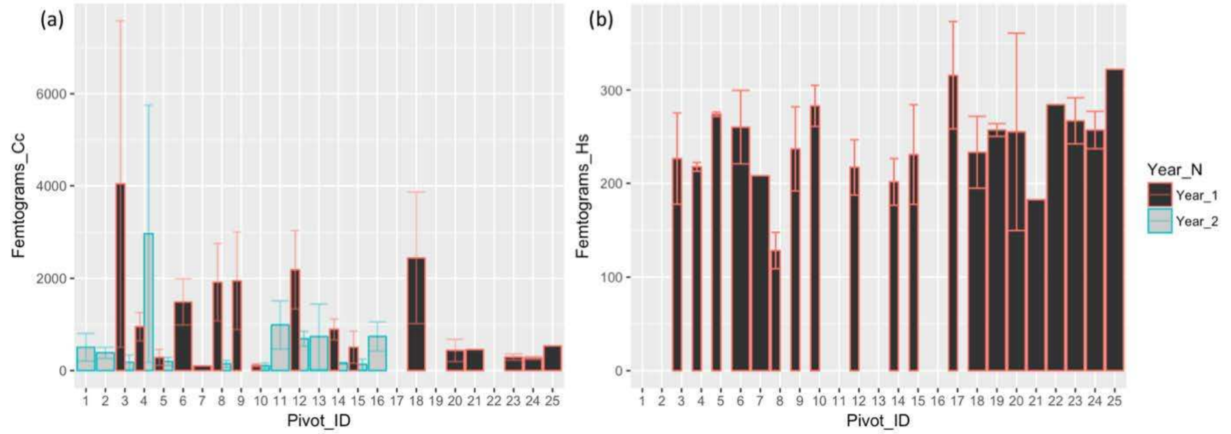


Figure 3.2. Quantity of A) *C. coccodes* and B) *H. solani* DNA (fg/g) in the soil at each pivot (1-25) for year 1 (black) and 2 (grey). Error bars are standard error of the mean. Note y-axis of (A) is 20x the y-axis of (B).

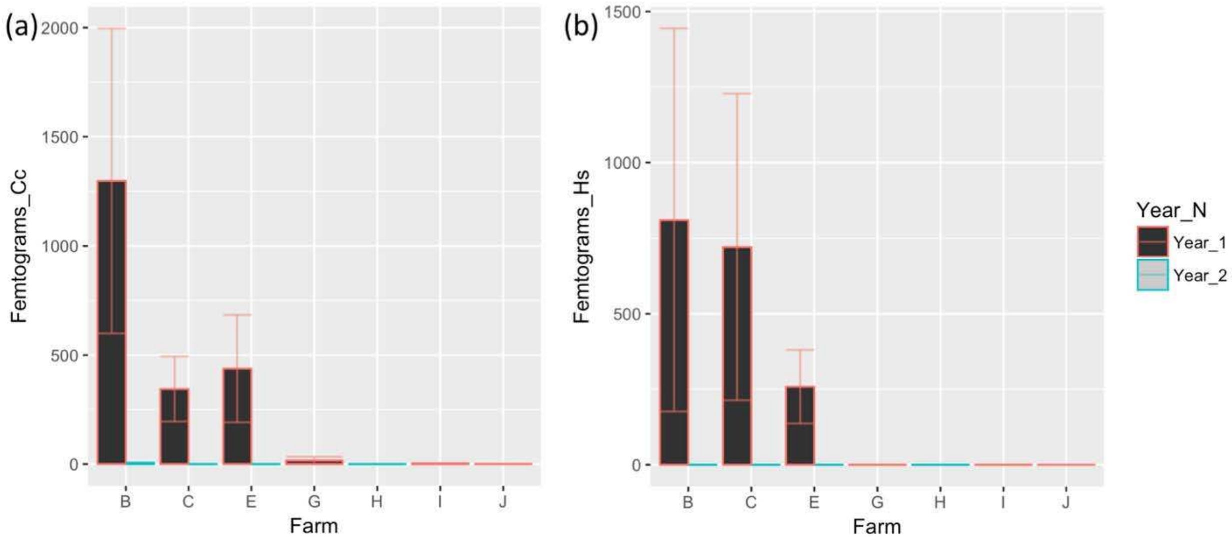


Figure 3.3. Quantity of A) *C. coccodes* and B) *H. solani* DNA (fg/g) detected in multiple tubers at each farm for year 1 (black) and 2 (grey). Error bars are standard error of the mean. Note x-axis relates to farms where tubers were harvested.

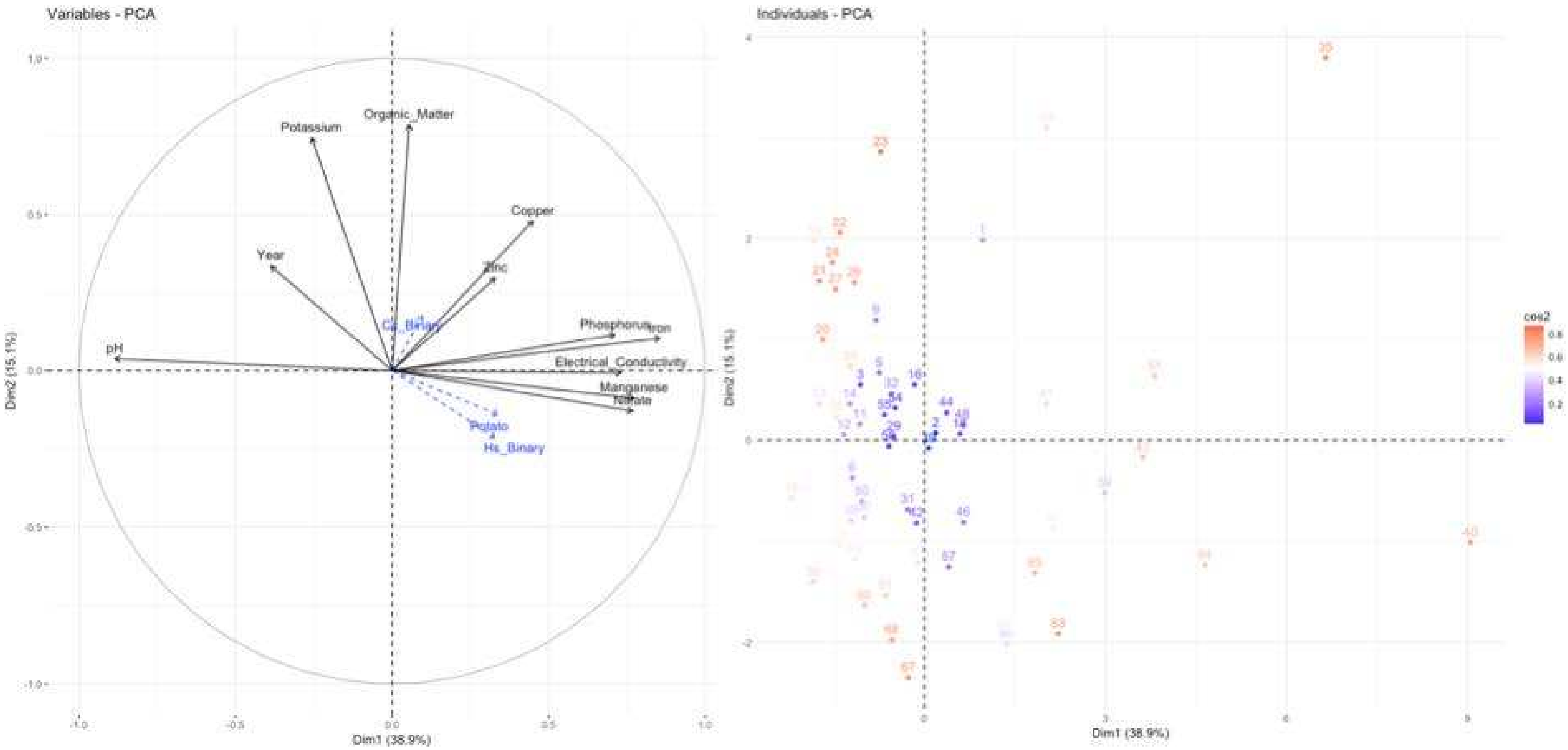


Figure 3.4. Influence of soil properties on *C. coccodes* (Cc) and *H. solani* (Hs) in potato production fields during the two years (2016-17). Principal component analysis (PCA) of the soil survey during year 2016-2017, the Variables-PCA is a loadings plot depicting the quantitative variables (soil properties) contributing to the variance in the data projected within the correlations circle. The blue dashed lines represent supplementary variables added to display the interaction presence or absence of Cc and Hs as well as potato crop have in the dataset. The Individuals – PCA presents the relative contribution (cos2) of each data point (soils sampled) on the overall variance, here the red color equals greater contribution, the blue color is less, and white is neutral.

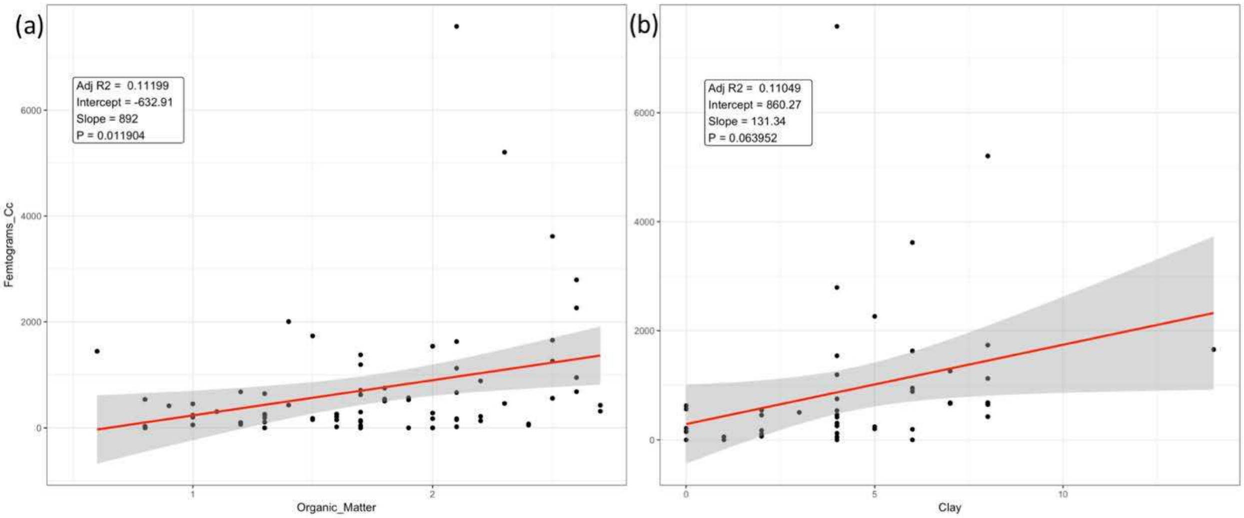


Figure 3.5. Linear model with confidence interval of the relationship between *C. coccodes* (fg/g) detected in the soil with significant soil physical properties, a) organic matter and b) percent clay. Adjusted R^2 and P values for each model are in upper left corner of graph. Independent variables include the respective soil property and farm where sample was taken.

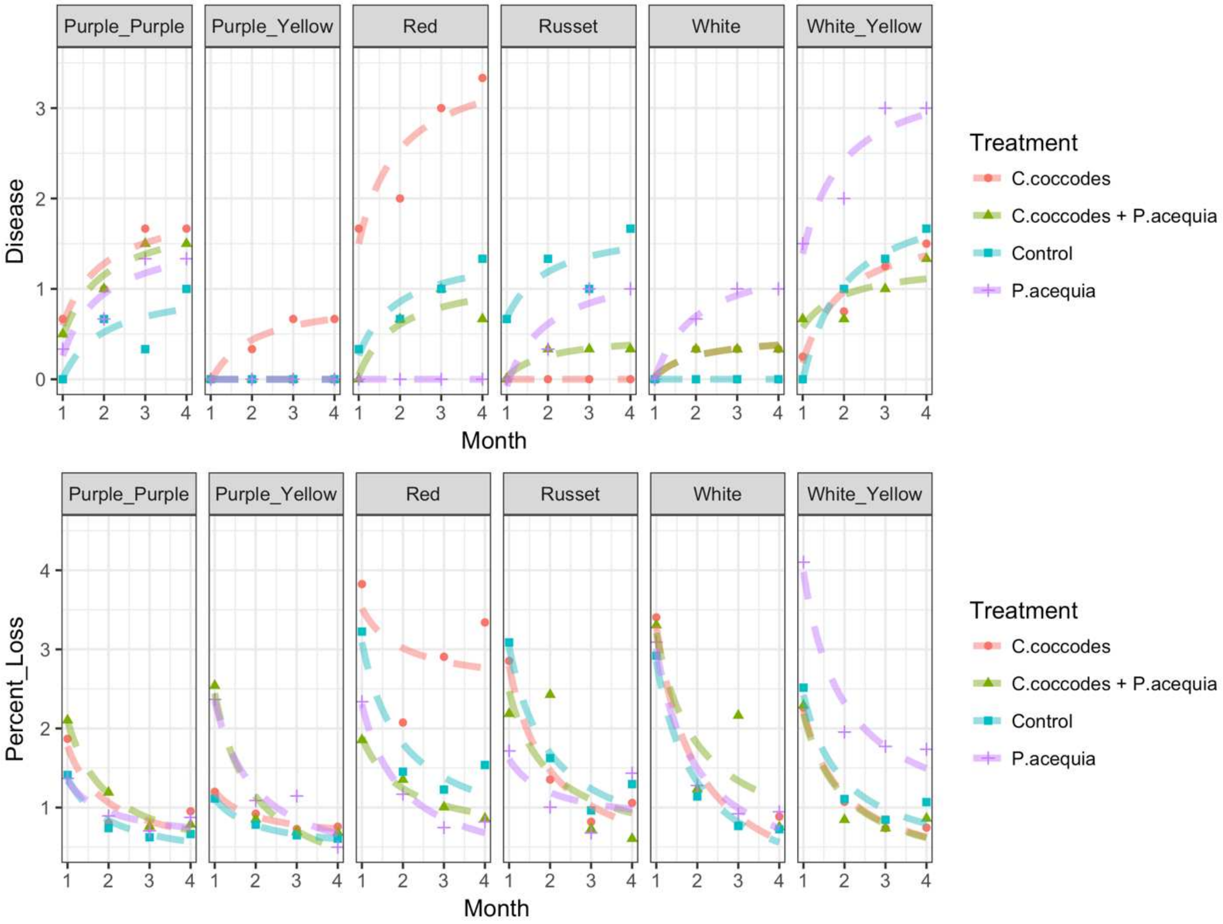


Figure 3.6. Disease severity and percent weight loss of six potato cultivars inoculated in the field with *C. coccodes*, *Penicillium sp.*, and the combination of these two fungi. Disease severity was measured as 1-5 and percent weight loss was measured as ((final-initial)/initial)*100 over the course of four months. Points are average of 3 blocks with 5 replicates in each block.

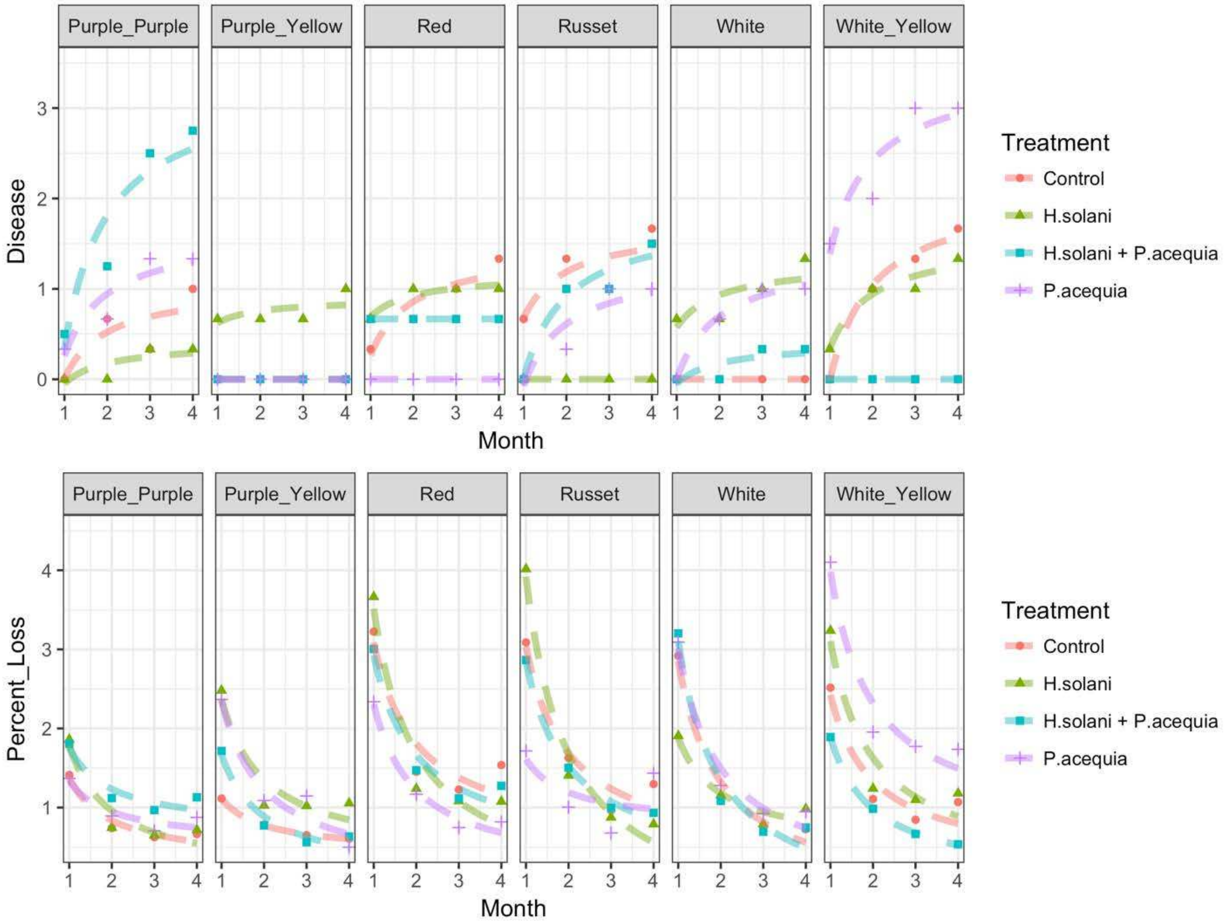


Figure 3.7. Disease severity and percent weight loss of six potato cultivars inoculated in the field with *H. solani*, *Penicillium sp.*, and the combination of these two fungi. Disease severity was measured as 1-5 and percent weight loss was measured as ((final-initial)/initial) *100 over the course of four months. Points are average of 3 blocks with 5 replicates in each block.

Table 3.1. Pivot ID and soil sampling survey results of soil chemical and physical properties averaged across first and second year.

Pivot_ID	**pH**	**Electrical Conductivity**	**Organic Matter**	**NO3-**	**P**	**K**	**Zn**	**Fe**	**Mn**	**Cu**	**Sand**	**Silt**	**Clay**
1, n=2	6.9	1.9	1.9	15.9	37.5	346.0	8.6	11.0	4.9	1.6	--	--	--
2, n=2	7.9	0.5	1.6	7.4	23.8	313.0	9.3	5.0	4.4	1.2	--	--	--
3, n=3	8.1	2.2	1.8	16.1	31.5	557.7	2.4	4.3	2.4	1.0	88.0	8.5	3.5
4, n=3	8.1	1.1	1.9	19.2	31.5	683.0	2.6	4.9	3.9	1.0	79.0	13.5	7.5
5, n=4	8.1	1.7	1.9	16.9	29.8	504.5	2.7	3.8	3.5	1.0	89.7	8.3	2.0
6, n=4	7.3	1.1	2.0	5.6	32.9	249.0	4.3	9.0	4.7	1.2	88.0	7.0	5.0
7, n=1	8.4	0.5	1.2	1.5	27.1	536.0	2.5	2.9	2.2	0.9	86.0	12.0	2.0
8, n=3	7.9	0.5	2.1	5.8	22.8	516.7	4.2	4.8	4.0	2.8	--	--	--
9, n=3	7.9	1.0	2.4	19.2	31.6	494.3	4.1	5.3	3.5	2.7	--	--	--
10, n=3	7.9	0.9	2.1	16.3	26.5	405.7	3.3	4.8	3.4	2.8	--	--	--
11, n=3	7.2	1.1	1.5	18.5	24.6	205.0	3.8	6.1	7.2	1.9	--	--	--
12, n=4	6.9	2.9	2.4	37.9	56.5	474.0	5.4	13.4	6.3	5.8	84.3	9.3	6.3
13, n=2	7.9	0.6	0.7	10.8	24.3	217.0	3.5	2.6	3.4	1.8	--	--	--
14, n=4	6.4	2.0	1.9	72.2	34.0	152.8	3.2	14.3	21.0	3.0	85.0	7.5	7.5
15, n=4	6.7	2.1	1.5	45.5	42.6	230.5	4.5	12.2	3.7	3.7	95.0	1.7	3.3
17, n=8	7.2	1.5	1.9	50.8	16.7	172.1	3.6	7.1	5.1	2.7	87.4	8.6	4.0
18, n=3	7.2	0.5	2.4	5.2	13.2	163.3	2.7	7.7	3.0	3.4	82.7	8.7	8.7
19, n=2	7.0	1.3	1.9	25.3	27.9	196.9	3.6	9.9	3.4	3.5	88.8	5.2	6.0
20, n=2	7.0	1.3	1.9	25.3	27.9	196.9	3.6	9.9	3.4	3.5	88.8	5.2	6.0
21, n=1	8.0	1.1	1.0	23.3	36.1	196.0	4.7	6.5	3.5	1.2	96.0	2.0	2.0
22, n=1	6.9	1.0	0.8	5.2	65.7	128.0	3.4	15.5	10.6	1.1	90.0	6.0	4.0
23, n=3	6.5	2.9	1.1	50.2	72.6	132.3	5.9	13.1	14.7	1.2	93.3	2.3	4.3
24, n=2	7.6	3.3	1.1	81.3	29.1	131.5	1.9	6.8	3.2	1.2	91.5	4.0	4.5
25, n=1	7.5	1.8	0.8	33.8	25.9	245.0	2.9	4.2	4.8	1.1	96.0	0.0	4.0

Table 3.2. Cultivar Susceptibility Trial mixed effects models. Including block and month as random effects the block month interaction was also used to account for repeated measures and both weight loss and disease were response variables. Significance is denoted in bold.

	Weight Loss	Disease
Cultivar	**F= 12.83, *P* = 1.557e-11**	**F= 14.00, *P*= 1.434e-12**
Treatment	F= 1.8212, *P* = 0.107	**F= 2.88, *P*= 0.0142**
Cultivar: Treatment	**F= 3.835, *P* = 6.256e-09**	**F= 6.57, *P*= < 2.2e-16**

Table 3.3. Pairwise comparisons of percent weight loss. Two potato cultivars (red and white yellow) with significantly different percent weight loss under treatments applied during storage over 5 months. Different letters indicate statistically different mean values taking into account multiple comparisons with Tukey's HSD.

CO00277-2R	Percent Loss		**CO99045 1 W/Y**	Percent Loss	
C. coccodes	3.04	a	Penicillium	2.39	a
Control	1.86	ab	H. solani	1.69	ab
H. solani	1.77	b	Control	1.38	ab
H. solani + Penicillium	1.72	b	C.coccodes	1.33	ab
C.coccodes + Penicillium	1.27	b	C.coccodes + Penicillium	1.19	ab
Penicillium	1.27	b	H.solani + Penicillium	1.02	b

Table 3.4. Nitrogen trial mixed effects models ANOVA. Here block and month are random effects. The block month interaction was also used to account for repeated measures and both weight loss and disease were response variables, significance is denoted in bold.

	Weight Loss	Disease
Cultivar	**F= 27.88 *P*= 2.018e-07**	F= 0.06 *P*= 0.802
Nitrogen	**F= 8.71 *P*= 0.00019**	**F= 5.77 *P*= 0.003**
Treatment	F= 0.12 *P*= 0.8787	F= 1.00 *P*= 0.368
Cultivar: Nitrogen	**F= 6.43 *P*= 0.0017**	F= 0.21 *P*= 0.807
Cultivar: Treatment	**F= 3.12 *P*= 0.0449**	F= 0.59 *P*= 0.549
Nitrogen: Treatment	**F= 7.74 *P*= 4.818e-06**	F= 1.97 *P*= 0.097
Cultivar: Nitrogen: Treatment	**F= 10.15 *P*= 7.133e-08**	**F= 4.29 *P*= 0.002**

Table 3.5. Pairwise comparisons of cultivar disease rating and weight loss at varied nitrogen rates. Tukey HSD test showing two potato cultivars (CO00277-2R and Canella Russet) with significantly different disease ratings under treatments with varied nitrogen application rates (Low, Normal, High). Different letters in a column signify significantly different values.

CO00277-2R	Disease	Weight Loss	**Canella Russet**	Disease	Weight Loss
Low	0.75 a	2.01 a	Low	0.71 a	1.62 a
Normal	0.34 b	2.09 a	Normal	0.43 a	1.91 a
High	0.15 b	1.47 a	High	0.63 a	1.67 a

Table 3.6. Post Harvest treatment trial mixed effects models results including block and month as random effects. The block month interaction was also used to account for repeated measures and both weight loss and disease were response variables, significance is denoted in bold.

	Weight Loss	Disease
Treatment	**F= 25.168 *P*= < 2.2e-16**	**F= 2.1672 *P*= 0.029**
Cultivar	**F= 90.961 *P*= < 2.2e-16**	**F= 25.3004 *P*= 4.194e-15**
Treatment: Cultivar	**F= 10.664 *P*= < 2.2e-16**	**F= 1.8137 *P*= 0.019**

Table 3.7. Effect of Post-Harvest treatment on mean weight loss. Experiment pairwise comparisons were made using Tukey HSD test, different letters next to average weight indicates a significant difference in weight (grams) of 5 replicates attributed to the treatment during the final month of cold storage. Different letters in a column signify significantly different values.

Treatment	**Canella Russet**	**Purple Maj**	**White Yellow**	**Red**
Phostrol	2275.47 a	2112.33 a	2471.29 a	1575.75 ab
Hydrogen Peroxide L	2040.72 ab	2127.46 a	2109.30 b	1442.24 bc
Hydrogen Peroxide H	2082.95 ab	2073.03 a	2543.82 a	1599.52 ab
Ethanol H	1995.88 ab	1689.87 b	2001.30 bc	1349.71 c
UTC	1923.63 b	1483.67 bc	1703.95 c	1361.33 c
Ethanol L	2064.49 ab	1745.57 b	2054.15 b	1439.58 bc
BioSave LP	2361.67 a	1311.81 c	1333.67 d	1669.05 e
Stadium	1539.12 c	NA	NA	1657.23 a
Quadris	1714.82 bc	NA	NA	1496.89 abc

Table S3.1. Center irrigated pivots identified by year the soil samples were collected, the crop currently growing or fallow if none, latitude and longitude in degrees and the femtograms of C. coccodes DNA per gram of soil.

Pivot_ID	**Year**	**Crop**	**Latitude**	**Longitude**	**Femtograms**
1	2017	Sudan Grass	37.692309	-106.058003	153.243
1	2017	Sudan Grass	37.695599	-106.058059	390.737
1	2017	Sudan Grass	37.696438	-106.064702	1377.28
1	2017	Sudan Grass	37.692259	-106.065683	94.188
2	2017	Barley	37.692451	-106.066852	429.589
2	2017	Barley	37.696157	-106.067827	42.715
2	2017	Barley	37.691431	-106.073307	438.368
2	2017	Barley	37.695443	-106.073894	620.206
3	2017	Sudan Grass	37.742674	-105.966678	18.688
3	2017	Sudan Grass	37.743106	-105.973018	335.677
3	2016	Potato	37.743	-105.9662778	7580.642
3	2016	Potato	37.74255556	-105.9721389	503.863
4	2017	Sudan Grass	37.736603	-105.965355	5755.2654
4	2017	Sudan Grass	37.736533	-105.97251	174.305
4	2016	Potato	37.73641667	-105.9658611	644.911
4	2016	Potato	37.73641667	-105.97225	1259.127
5	2017	Mustard Mix	37.746622	-105.974383	281.42
5	2017	Mustard Mix	37.746192	-105.981898	108.515

5	2016	Sudan Grass	37.74280556	-105.9815556	624.596
5	2016	Potato	37.74730556	-105.9816111	172.57
5	2016	Potato	37.74733333	-105.9758056	51.605
6	2016	Potato	37.633519	-106.068561	541.411
6	2016	Potato	37.639016	-106.067884	2791.275
6	2016	Potato	37.639024	-106.073774	1734.721
6	2016	Potato	37.633528	-106.072971	884.769
7	2016	Sudan Grass	37.74083333	-105.9781389	100.806
8	2017	Mustard Mix	37.774447	-106.126275	131.916
8	2017	Mustard Mix	37.773403	-106.126318	279.498
8	2017	Fallow	37.773445	-106.125825	20.431
8	2016	Potato	37.77519444	-106.1277778	1078.2
8	2016	Potato	37.77116667	-106.1283056	2752.429
9	2017	Potato	37.774581	-106.116866	313.509
9	2017	Potato	37.773376	-106.116855	557.522
9	2017	Potato	37.776716	-106.115138	---
9	2016	Potato	37.77461111	-106.1206944	710.842
9	2016	Potato	37.77241667	-106.1205	1076.192
9	2016	Potato	37.77538889	-106.1136389	887.157
9	2016	Potato	37.77180556	-106.1143333	5111.922
10	2017	Mustard Mix	37.773578	-106.108138	73.176
10	2017	Potato	37.776849	-106.108132	215.949
10	2017	Potato	37.772922	-106.107943	15.681
10	2016	Potato	37.77558333	-106.1048056	146.286
10	2016	Potato	37.77102778	-106.1061944	80.738
11	2017	Potato	37.661634	-106.061635	709.334
11	2017	Potato	37.664858	-106.061641	253.915
11	2017	Potato	37.665084	-106.06205	2004.202
12	2017	Quinoa	37.661609	-106.071116	527.061
12	2017	Quinoa	37.661607	-106.070981	852.697
12	2016	Potato	37.661837	-106.070006	681.871
12	2016	Potato	37.664894	-106.066777	2261.316
12	2016	Potato	37.664958	-106.075321	3614.369
13	2017	Mustard Mix	37.680105	-106.002367	28.811
13	2017	Mustard Mix	37.682324	-106.004081	1444.264
14	2017	Barley	37.688971	-106.14119	178.31
14	2017	Barley	37.689909	-106.143895	138.547
14	2016	Potato	37.683234	-106.144794	665.8
14	2016	Potato	37.685994	-106.14462	1124.54
15	2017	Quinoa	37.635999	-106.057881	244.946
15	2017	Quinoa	37.633746	-106.058827	17.175
15	2016	Potato	37.639771	-106.06112	65.487
15	2016	Potato	37.636512	-106.060758	1192.978
15	2016	Potato	37.639106	-106.064725	258.368
16	2017	Potato	37.705904	-106.147993	220.484
16	2017	Potato	37.706026	-106.14802	682.55
16	2017	Potato	37.706132	-106.148162	1315.1472
17	2017	Hemp	37.711589	-106.135484	751.394
17	2016	Mustard Mix	37.71161111	-106.1439167	211.43
17	2017	Mustard Mix	37.707449	-106.130842	1627.882
17	2017	Quinoa	37.705448	-106.136194	---
17	2017	Fallow	37.711584	-106.144036	44.731
17	2017	Fallow	37.711544	-106.142965	149.415

17	2016	Potato	37.71158333	-106.1428333	751.394
17	2016	Potato	37.71158333	-106.1428333	1627.882
17	2016	Potato	37.71158333	-106.1428333	---
17	2016	Potato	37.70491667	-106.1434444	---
17	2016	Potato	37.711581	-106.4686111	425.121
17	2017	Potato	37.705161	-106.136096	34.142
17	2016	Potato	37.71019444	-106.1325278	298.452
17	2016	Potato	37.71111111	-106.1369722	118.913
18	2016	Beans	37.70772222	-106.1314167	1653.905
18	2016	Hemp	37.71161111	-106.1347222	5205.388
18	2016	Potato	37.70511111	-106.1360833	460.156
19	2016	Potato	37.672767	-106.001684	55.182
19	2016	Potato	37.675921	-105.998201	---
20	2016	Potato	37.41883333	-105.5459167	192.87
20	2016	Potato	37.41880556	-105.5449167	678.714
21	2016	Sudan Grass	37.40261111	-105.5915556	452.478
22	2016	Potato	37.39405556	-105.5595	---
23	2016	Potato	37.39361111	-105.5604722	415.322
23	2016	Potato	37.38838889	-105.56225	201.239
23	2016	Potato	37.38852778	-105.5640278	255.517
24	2016	Potato	37.39127778	-105.5804167	305.877
24	2016	Potato	37.39066667	-105.5804167	239.727
25	2016	Potato	37.40177778	-105.6040833	536.403

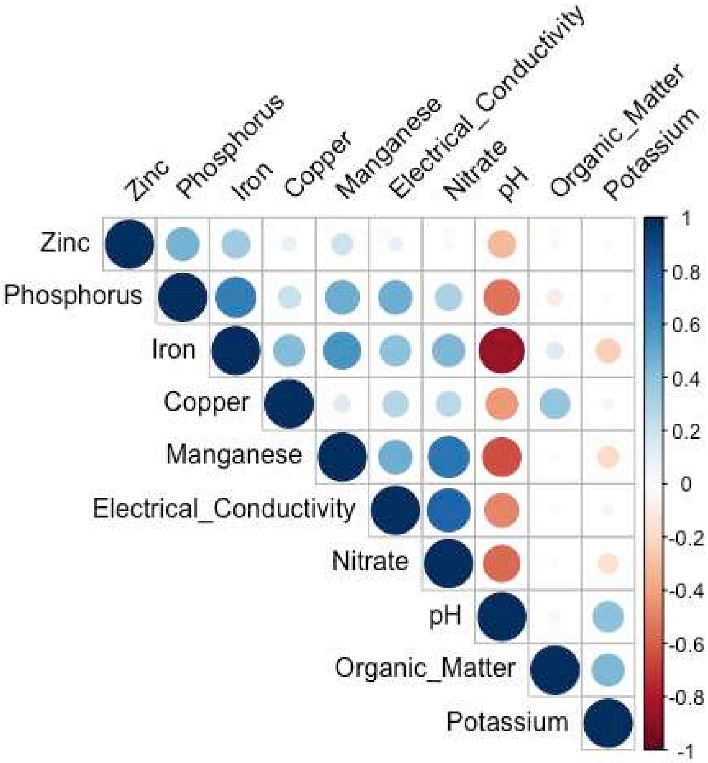


Figure S3.1. Correlation plot of all soil variables tested in the two-year soil sampling for *H. solani* and *C. coccodes*.

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Chapter 4—Phylogeny and morphology of a new *Penicillium species* from agricultural soil in southcentral Colorado

Introduction

Penicillium section *Lanata-Divaricata* has undergone extensive reorganization over the last four years, with five species added in 2015, fifteen in 2016 and one in 2017 (1–4). New species descriptions in this taxonomic group may be recently attributed to the current interest in soil microbiology and ecology (5). Visagie et al provide a recent review of the nomenclature and taxonomy of the *Penicillium* genus and defined useful approaches to identifying and describing species within each division (6). The historical expansion of the number of species within the genus began with the isolation and culturing of antibiotic producing *Penicillium* from the air (7). Recently, multi-locus sequence typing (MLST) has become more standardized between laboratories and cost effective. Yet not only has MLST become common and useful in delineating species of *Penicillium* but also the use of high quality microscopy and LC-MS-MS extrolite profiling (4).

Many *Penicillium* species have been the focus of bioprospecting studies (8,9), as they are known for producing a variety secondary metabolites with a diverse array of biological function. Novel sequencing, isolating and screening of environmental fungi has enabled further taxonomic classification of the previously unseen majority but also the discovery of their secondary metabolites and biochemical processes (10,11) which provide useful chemistries for medical and industrial applications (12,13). High resolution sequencing enables investigation of the organisms entire genetic composition and associated biochemical repertoire providing further understanding of how these organisms function within these ecosystems (14–16).

Penicillium species are important globally as contributors to saprophytic recycling of food and pathogenic mycotoxin production. Secondary metabolites produced by these species include the critical antibiotic penicillin, the harmful toxin patulin in apple products, ochratoxins in dried fruit and coffee, and citrinin in cereals (17,18). Species within *Lanata-Divaricata* are associated with detritus degradation and suggest potential sources of new cellulolytic or xylanolytic enzymes (8,19). The potential to produce biologically useful organic compounds by species within this section is understudied. Secondary metabolites of fungi have proven useful in pharmaceuticals and industrial fermentation and their toxic tendencies also offer an opportunity for alternative applications as biocontrols in agricultural systems.

Several new species within this section were described recently from a leaf litter and soil survey in the Columbian Amazon forest including *P. penarojense P. wotroi, P. araracuarense, P. elleniae,* and *P. vanderhammenii*. Specifically, these species were isolated from leaf litter degradation bags placed on the forest floor. These species had relatively high growth rates and produced both known and unknown extrolites e.g. penicillic acid, pulvilloric acid, and janthitrem. These five species could not be differentiated by micro or macro morphology yet were separated based on ITS and partial β-tubulin sequences and their extrolite profiles (20). These species are also closely related phylogenetically to another detritus degrading species *P. janthinellum*, which has been utilized recently for its production of cellulases for plant biomass conversion to sugars (21). The plant biomass (wheat straw) is often a byproduct of large scale cereal production and may be tilled into the soil or separately harvested for removal. Another species thoroughly studied in this section is *P. simplissicum* GP17-2, originally isolated from zoysia (*Zoysia tenuifolia*) and has been shown to activate multiple plant defenses in response to pathogenic interactions with bacteria and fungi (22). Plant growth promoting fungi represent a potential management option in agricultural settings where chemical controls have resulted in fungicide resistance or cannot be used in organic systems.

While studying the dynamics of soil borne pathogens of potatoes in the San Luis Valley (SLV) of Colorado, unknown isolates of *Penicillium* were routinely recovered from agricultural soils where barley is often utilized as a rotational crop. The SLV is a unique ago-ecosystem in that it is high elevation (~2300 meters) and receives on average 20-30 centimeters (cm) of precipitation annually. The relationship of these *Penicillium* isolates with the soil borne potato pathogens necessitated further characterization of this isolates and their role in the ecology of the agroecosystem in southcentral Colorado.

Here we describe a new species of *Penicillium* sect. *Lanata-Divaricata* as *Penicillium acequia* sp. nov., based on polyphasic analyses including multilocus phylogenetics, a morphologic description, and LC-MS extrolite profiling. We also evaluated the biocontrol capacity of *P. acequia* sp. nov., by observing the suppressive effect during co-inoculation of the fungal pathogen *Colletotrichum coccodes* (Wallr.) S.J. Hughes.

Methods and Materials

Sample collection, isolation and morphological examination

Soil samples were collected in late August from four overhead irrigated agricultural fields. One field location near Blanca, CO (SSS Farm) was under strict potato barley rotation, while the other three field locations in Center, CO (SLV Research Center and Mattive Farms) operate under diverse crop rotational schemes.

The putative species of *Penicillium* were recovered from soil samples which were collected from two locations near Center, CO (SLV 163 and SLV 2-532) and two locations near Blanca, CO (SSS 1-3 and SSS 1-1), which are approximately 80 kilometers (km) apart. The SSS soils are of Cosoa loamy sand and Fuertas sandy loam. The SLV soils are of Norte gravely sandy loam and San Arcacio sandy loam (NRCS Web Soil Survey) (23). The MAT isolates collected in 2018 were not used in the phylogenetic analysis but were confirmed morphologically similar to other isolates (Table 4.1). Isolates of *Penicillium* were recovered from the soil via soil dilution plating. Briefly, 1 gram (g) of sieved soil in 100mL sterile distilled H_2O and a 100 uL aliquot of soil solution was dispensed onto and potato dextrose agar amended with chloramphenicol (25.0 mg/L). Germinating spores were isolated after 24 hours of growth and transferred to plates of potato dextrose agar (PDA) amended with chloramphenicol. Isolates were stored on potato dextrose amended with chloramphenicol media slants at 4°C for long term storage. The type strain, isolate SLV 163 was deposited at the United States Department of Agriculture (USDA) fungal herbarium in Beltsville, Maryland and an ex-type deposited at the Westerdijk Fungal Biodiversity Institute formerly CBS-KNAW.

Initial internal transcribed spacer (ITS) sequencing of the isolates suggested no exact match in National Center for Bioinformatic Information (NCBI) libraries with only 97% nucleotide similarity and morphological characteristics were dissimilar to those of closely related species. The isolates recovered in this study aligned within the division *Lanata-Divaricata* and specifically within the clade containing *P. raperi* Smith.

Morphological Characterization

Strains were characterized and observed under the standard growth conditions most recently outlined in the literature (6). Media preparation, conidial inoculation, growth conditions and microscopic visualizations were carried out as suggested by Visagie et al (2014). Multiple medias used for this observation and identification include malt extract agar (MEA), Czapek yeast autolysate agar (CYA), yeast sucrose agar (YES) and oatmeal agar (OA). Oatmeal agar

(HiMedia + trace elements solution) was used to examine reproductive states in culture. Spore solutions contained 0.2% agar and 0.05% Tween80, where 1uL of spore solution was used for growth curve measurements, inoculations and subsequent morphologic description.

Spore solution containing 10^6-10^8 spores per uL in 0.2% agarose and 0.05% Tween 80 was inoculated in 3-point fashion on MEA, CYA, YES, and OA agar at temperatures from 25°C-33°C. The diameter in millimeters of each point was recorded at 7 days on each agar.

Microscopic observations were performed on Nikon SMZ18 dissecting light microscope and Nikon H550S compound light microscope. Slides were prepared using 60% lactic acid and excessive conidia were washed away with 90% Ethanol. Images were taken at 40x magnification and phase contrast microscope stage was used when necessary.

Phylogenetic analysis

Isolates were grown in pure culture on cellophane PDA. Mycelia were scraped from the cellophane and place in 2 mL tubes and lyophilized at -80°C before DNA was extracted with the Mo Bio PowerSoil DNA Isolation Kit (Qiagen Hilden, Germany). The ITS and β-tubulin loci were sequenced in the forward and reverse direction with ITS1f-ITS4 and Bt2a-Bt2b primers respectively (24,25). The calmodulin gene region was sequenced with both CAL228F and CAL737R (26) and the reverse complement was used to form a consensus sequence in the phylogenetic analysis (Table 4.3). Amplicons were sequenced using sanger sequencing technology at Quintara Biosciences (Denver, CO). Sequences were deposited in the Genbank database of the NCBI sequence read archive (SRA) under accession SUB4705398. To understand the phylogenetic placement of the *Penicillium* species recovered in this study, several available sequences within the section were downloaded from NCBI. Alignments, trimming and assembly of sequences was performed in Geneious v. 8.1.5 (BioMatters Ltd., Auckland, New

Zealand). Individual datasets were aligned with MAFFT plugin v. 1.3.5 in Geneious, ITS was aligned with L-INS-i while BenA, CaM, and concatenations of all three loci were aligned with E-INS-i algorithm. Alignments were subsequently used in MEGA7 to construct maximum likelihood (ML) trees using 1000 bootstraps with nucleotide substitutions on a General Time Reversible (GTR) model. Bayesian analysis of phylogeny was conducted via MrBayes v. 2.2.4 plugin to Geneious to measure posterior probability of multilocus concatenated sequences (27). MrBayes substitution model was HKY85, gamma rate variation, subsampling frequency of 200, and a burn in length of 100,000 using unconstrained branch lengths.

Secondary Metabolite Characterization

Agar plugs with *P. acequia* sp. nov. grown for 7 days of CYA and YES (6 samples) as well as from both blank medias (3 samples each) were frozen at -80 °C and lyophilized for 15 hours at 0.005 mbar and -80 °C before being weighed and transferred to glass 7 mL vials with bonded PTFE lids. 1.5 mL of 75% methanol 25% water (v/v) was added to each sample, vortexed for 30 seconds and sonicated in a bath sonicator for 30 minutes before the addition of 2 mL of 100% ethyl acetate followed by an additional 30 second vortex and 30-minute sonication. The samples were centrifuged at 3,000 rpm and 4°C for 15 minutes and the supernatant was transferred to new glass 7mL vials. To the original agar plugs, and additional 1 mL of ethyl acetate was added, vortexed and sonicated for 30 minutes followed by centrifugation and pooling of the supernatants. The agar plugs were extracted a third time with 1.2 mL of 50% ethyl acetate, 33% methylene hydrochloride, 17% methanol (v/v) for 30 minutes in a bath sonicator followed by centrifugation and pooling of the supernatant. Extracts were dried under nitrogen gas at room temperature and resuspended in 1mL of 100% methanol.

Liquid Chromatography - Mass Spectroscopy

For LC-MS analysis 3 µL of extract injected onto a Waters Acquity UPLC system in randomized order. Compounds were separated using a Waters Acquity UPLC CSH Phenyl Hexyl column (1.7 µM, 1.0 x 100 mm), using a gradient from solvent A (water, 0.1% formic acid) to solvent B (Acetonitrile, 0.1% formic acid). Injections were made in 100% A, held at 100% A for 1 min, ramped to 98% B over 12 minutes, held at 98% B for 3 minutes, and then returned to starting conditions over 0.05 minutes and allowed to re-equilibrate for 3.95 minutes, with a 200 µL/min constant flow rate. The column and samples were held at 65 °C and 6 °C, respectively. The column eluent was infused into a Waters Xevo G2 Q-TOF-MS with an electrospray source in either positive or negative ionization mode, scanning 50-2000 m/z at 0.2 seconds per scan, alternating between MS (6 V collision energy) and MSE mode (15-30 V ramp). Calibration was performed using sodium iodide with 1 ppm mass accuracy. The capillary voltage was held at 2200 V (2000 V in negative mode), source temp at 150 °C, and nitrogen desolvation temp at 350 °C with a flow rate of 800 L/hr.

Extrolite Analysis

For each sample, raw data files were converted to .cdf format, and matrix of molecular features as defined by retention time and mass (m/z) was generated using XCMS software in R (28) for feature detection and alignment. The matchedFilter algorithm was used for GC-MS data, and the centWave algorithm for LC-MS data. Features were grouped using RAMClustR (29), with options set as hmax=0.7, minModuleSize=2, linkage="average", and normalize="none" LC-MS data. Annotations were performed using a within RAMClustR through the findMain (30) function from the interpretMSSpectrum package to infer the molecular weight of each LC-MS compound and annotate the mass signals. The complete MS spectrum and a truncated MSE spectrum were written to a .mat format for import to MSFinder (31). The MSE spectrum was truncated to only include masses with values less than the inferred M plus its isotope, and the .mat file precursor ion is set to the M+H ion for the findMain inferred M value. These .mat spectra were analyzed to determine the most probable molecular formula and structure using MSFinder console. MSFinder was also used to perform a spectral search against the MassBank database. All results were imported into R and a collective annotation is derived with prioritization of MSFinder mssearch > MSFinder structure > MSFinder formula > findMain M. Annotation confidence is reported as described (32). Targeted searches for known extrolites were performed in R, followed by manual examination in RAMSearch (33) and comparison to published spectral signatures.

Statistical analysis of Extrolites

Abundances from blank media samples were subtracted from the fungal cultures grown on the same media to then calculate the fold change (FC) (CYA/YES abundance) and principle component analysis. Benjamini-Hochberg false discovery rate correction was applied to the probability obtained from the t-test performed between extrolites detected on the two medias. A volcano plot with the log2FC graphed with the 'ggplot2' package in R. The chemical taxonomy including Kingdom, Superclass, Class and Subclass was identified through batch compound classification utilizing ClassyFire (34) to show chemical classes upregulated during growth on each media.

Evaluation of potential biocontrol activity on a fungal pathogen

To evaluate the potential biocontrol activity of *P. acequia* sp. nov. a series of *in vitro* and *ex vitro* competition studies were conducted. Specifically, *P. acequia* sp. nov. and *C. coccodes* were grown on the same media to determine if *P. acequia* sp. nov. exhibited the capacity to suppress growth of *C. coccodes in vitro*. Spore solutions of each organism were placed ~15 cm apart from each other and allowed to grow for 2-4 weeks in the dark. P. acequia sp. nov. colonized C. coccodes and inhibited further growth on multiple medias at 25°C (Figure 4.2). Field trials including inoculation of both *P. acequia* sp. nov. and *C. coccodes* were conducted and reported (Lichtner, F. unpublished) where six potato cultivars were planted with both organisms together and separately to observe post-harvest disease weight loss and disease symptom development.

Results

Taxonomy

Penicillium acequia Lichtner and Broders, sp. nov. Figure S4.2. In section *Lanta-Divaricata* in subgenus *Aspergilloides*.

Etymology. Spanish, *acequia*, term for community operated watercourse, named for original irrigation method used for agriculture in the isolate's origin. The branching pattern of the divaricate conidiophores are like the branching patterns of the diversion canals from the main stem of the river.

Morphological description: Conidiophores monoverticillate stipes sometimes divaricate, stipes rough and grooved, 50-200 µm x 1.5-3.0 µm. Phialides ampulliform. Divaricate conidiophores with complex branching pattern observed including sub terminally formed branches. Conidia smooth walled 4-5 µm.

Type Strain SLV-163, isolated from potato soils in Alamosa and Rio Grande counties in southern Colorado. The culture is preserved in a glycerol stock at -80C where it is metabolically inactive.

Other specimens examined: SSS 1-3, SSS 1-1 (potato barley fields in Blanc, CO) and SLV 2-532 (agricultural soils, Center, CO).

Morphological and Phylogenetic analyses

The mean diameter on MEA at 25°C was 25.13 mm, 27.35 mm on CYA, 26.4 mm on YES, and 32.33 mm on OA. Growth diameter on CYA after seven days at 28°C averaged 35 mm, at 33°C 25.56 mm, and at 37°C 18.83 mm (Figure 4.1).

Colony morphology – Colony diam., 7 d, in mm: MEA 25-28; CYA 32-33; CYA 33°C 16-18; OA 25°C 30-34; YES 25° 25-26. CYA 25 °C, 7d: Colonies were cream to yellow moderately deep, lightly radially sulcate, mycelia white texture lanose, raised at center; dull green (Fig 4.3a.). Sporulation moderately dense present in all strains, conidia en masse dull green to dark green (Fig. 4.3g). Some exudates present on strains; other strains exudates absent. MEA 25°C, 7d: Colonies broad and low mycelia uniform sporulation radiating outward, dark green. Clear exudates present on CYA at 32°C. MEA 25°C, 7d: Colonies dark green with sparse mycelia radiating concentrically outward (Figure 4.3).

Conidiophores monoverticillate stipes sometimes divaricate, stipes rough and grooved, 50-200 µm x 1.5-3.0 µm. Phialides ampulliform. Divaricate conidiophores with complex branching pattern observed including sub terminally formed branches (Figure 4.4e). Conidia smooth walled 4-5 µm.

The phylogenetic results demonstrate that the isolates of *Penicillium* recovered from agricultural soils belong in the section *Lanata-Divaricata* and are most closely related to *P. raperi*. The aligned datasets for ITS, BenA, CaM, and concatenated loci were 622, 535, 513, and 2255 bp respectively. Average percent nucleotide identity between *P. acequia* sp. nov. and its closest neighbor species (*P. raperi*) varied, for ITS 97.6%-99.8%, BenA 99.04%-99.8%, CaM

98.7%, and 79.6%-86.2% for the concatenated sequences. ITS did not resolve species in this genus (Figure 4.5), but the phylogenetic tree of partial β-tubulin (BenA) sequences as well as calmodulin and the multilocus concatenation do resolve the individual species of *Penicillium* sect. *Lanata-Divaricata.* Maximum likelihood based phylogenetic trees of calmodulin (CaM) and concatenated (ITS, BenA, CaM) datasets for *Penicillium* sect. *Lanata-Divaricata* (Figure 4.6) resolves the separation from *P. raperi* with strong boot strap support (98% & 100%). A circularized phylogenetic tree based on the Bayesian alignment and tree supports sufficient resolution between species including *P. raperi* with 96.8% support (Figure S4.1). The Bayesian patristic distances for the three isolates concatenated sequences (SLV 163, SLV2532, and SSS 1-3) were 0.015, 0.011, and 0.017 respectively where the average patristic distance of all species within Lanata–Divaricata from *P. raperi* is 0.29 (Table S4.2).

The phylogenies consistently placed the isolates of *P. acequia* sp. nov. into a clade including the closely related *Penicillium* species *P. raperi*. The isolates from this clade are characterized by their matted floccose which is pale yellow eventually turning mauve colored. This species is associated with cultivated soil ecosystems particularly cabbage cultivation (35). Originally described as having very short conidiophores arising from aerial hyphae and irregular verticils of phialides and metulae. It was placed in proximity to the *P. janthinellum* series. Phylogenetic relationship derived from the single *BenA* gene or the *ITS* region did not resolve differences from *Penicillium raperi*, though the *Calmodulin* locus and concatenated (*ITS, BenA* and *CaM*) loci did distinguished it in a well-supported subclade with *P. raperi* sp. nov. The percent nucleotide similarity to the nearest neighbor, *P. raperi*, for the concatenated sequences is 79.68% - 86.23% depending upon the isolate when observing the entire section of *Lanata-*

Divaricata (Table S2.4) though this nucleotide difference ranges from 77.1% - 82.8% when considering the *P. raperi* clade. (Table 4.2).

Diagnostic features

Colonies were cream to yellow moderately deep, lightly radially sulcate, mycelia white texture lanose, raised at center; dull green on CYA. Sporulation moderately dense present in all strains, conidia en masse dull green to dark green on PDA. Some exudates present on strains; other strains exudates absent, not developing until ~10 days in culture. Colonies often broad and low mycelia uniform with sporulation radiating outward, dark green. Clear exudates present on CYA at 32°C. Colonies dark green with sparse mycelia radiating concentrically outward on MEA at 25°C.

Conidiophores appear to have monoverticillate stipes though sometimes divaricate, stipes rough and grooved, 50-200 µm x 1.5-3.0 µm often exhibiting wide variation between isolates. Phialides ampulliform. Divaricate conidiophores with complex branching pattern observed including sub terminally formed branches (Figure 4.3e) with conidia bearing phialides. Conidia smooth walled 4-5 µm on average.

Secondary Metabolite analysis

Significant differences in abundance were observed between the two medias, more benzenoids, organic acids and derivatives, and organic heterocyclic compounds on YES, while more lipids and lipid like molecules nucleosides, nucleotides and their analogues on CYA. Significantly greater diversity of organic acids, nucleosides and organic heterocyclic compounds were detected in YES compared to CYA (Figure 4.7). N-Undecylbenzenesulfonic acid was the most highly abundant compound produced when *P. acequia* sp. nov. was grown on YES compared to CYA (1.1 log2 FC). The extrolite 4-hydroxystyrene (-5.24 log2 FC) was one of the most abundant produced on YES compared to CYA, and is often found in fermented beverages and as a prohapten metabolized in the skin (36). Lecithin was also a highly abundant extrolite produced on YES media (-1.24 log2 FC) after 7 days. Papyriferic acid (-0.24 log2 FC) was another highly abundant compound with little biological significance currently understood (Table S4.1). None of the previously reported extrolites from other species in this division were detected by the same methods used here.

Competition assay

The competition assay did not result in a zone of inhibition, which is classified as the process by which one microbe reduces or limits growth of another. This is usually achieved through the production of a toxic secondary metabolite produced by one organism, which inhibits growth of the other. Instead what was observed of the colonization of *C. coccodes* by *P. acequia*. The mycelia of *P. acequia* grew into the mycelia of *C. coccodes* and produced prominent conidiophores in the leading mycelial edge of growth (Figure 4.2). However, it is unclear if this is a mycoparasitic response.

Discussion

The genus *Penicillium* sect. *Lanata-Divaricata* is undergoing continuous reorganization with 21 species being formally defined in the last three years. Here we describe with strong phylogenetic, morphological, and metabolomics support a closely related but significantly different species from a unique and isolated physical environment. This species is like its nearest phylogenetic neighbor *P. raperi* in both multi-locus sequence typing and environment of isolation (agricultural soils). This species is unique in its morphology, producing differently colored hyphae and exudates.

Morphologically this species varies from the most closely associated fungi with its mauve, yellow, or green floccose growth pattern which is not seen in any other published species in this section. The conidiophore branching pattern and length is divergent from *P. raperi*,

The San Luis Valley of Colorado is a unique agricultural environment, as the average altitude is 2,336 m above sea level and the area receives very little precipitation and is therefore classified as cold desert climate. However, it is also a depositional basin, which make the region ideal for agriculture with 95 frost free growing days on average annually—a seasonal maximum-minimum temperature of 25.5°C to 7.2°C. Winter weather is harsh with low night time temperatures—average seasonal maximum-minimum temperature is 3.3°C-$^{-}$13° C. Average annual precipitation is 23.49 cm, with 11.91 cm (51%) falling in June-September. Seasonal snowfall averages 78.48 cm and the ground is covered with >2.54 cm of snow for 35 days of each year on average. The SLV is the world's highest intensively irrigated agricultural valley of its size and was once an ancient lake during the Pliocene and Pleistocene currently referred to as Lake Alamosa (37), presenting a unique environment of biological isolation. The 4,267 m cirque surrounding the sandy dry basin creates a remote isolating geography and offers an opportunity for novel microbial interactions and adaptations due to its continental central location, proximity to major snowpack dependent headwaters and fast moving wind patterns.

It is with this unique environment, morphology and phylogenetic diversity that this *Penicillium* species should be described as a new addition to the division *Lanata-Divaricata*. This organism is endemic to the agricultural soils of south central Colorado from soils with a pH ranging from 5.5-7.2 and diverse nutrient profiles. During field trials in the San Luis Valley with *Penicillium acequia* sp. nov. co-inoculated with the fungal pathogen *C. coccodes* we observed cultivar dependent responses, where light skinned potatoes were negatively affected by the combination of fungi yet the russet varieties exhibited less disease on the skin during storage. This fungus has implications as a potentially important biocontrol for soil borne fungal pathogens of potato as a fast-growing niche occupier, 6 cm/day compared to 3.3 cm/day and producer of diverse organic acids.

Extrolite profile

Understanding the fungal metabolomics *in vivo* allows for testing unique hypotheses relating to functional uses. Performing semi-targeted metabolomics, where specific secondary metabolites are targeted provides reproducible methods for determining when and on what medias valuable compounds are produced. The extrolite data collected on this new species indicates *P. acequia* produces organic acids, benzenoids, and lipid like compounds which are useful in both the fermentation/biofuels industry and medicine. Though many of the most highly abundant compounds were not classified in great biological detail they provide a base for discovery and exploitation. The most highly abundant compound detected, 4-undecylbesylic acid, when *P. acequia* sp. nov. was grown on YES compared to CYA is an insoluble extremely strong acid based on its kPa (-1.8). Lecithin was also a highly abundant extrolite produced on YES media after 7 days indicating a novel alternative source of this essential fat compound. Papyriferic acid was another highly abundant compound detected that acts as an “antifeedant” triterpene which acts as a protectant from herbivory (38). Though none of previously reported extrolites from closely related species were detected by similar methods the technology and databases used may not yield the same results. The species are quite distant genetically with the nearest neighbor phylogenetically only sharing 77% of nucleotides in 3 conserved loci there is significant room for biochemical and metabolic diversification.

The comprehensive description of this novel organism should lead to further studies on the functional ecology within the system where it was recently found. *P. acequia* sp. nov. is recognized here as a new species within the *Lanata - Divaricata* section of *Penicillium* and is the first new species within the clade containing *P. raperi*, a similar soil borne fungi from agricultural soils. This organism has potential to be further investigated in its role as an organism associated with sandy soils where significant plant derived organic matter and subsequent detritus may be available annually.

Figures and Tables

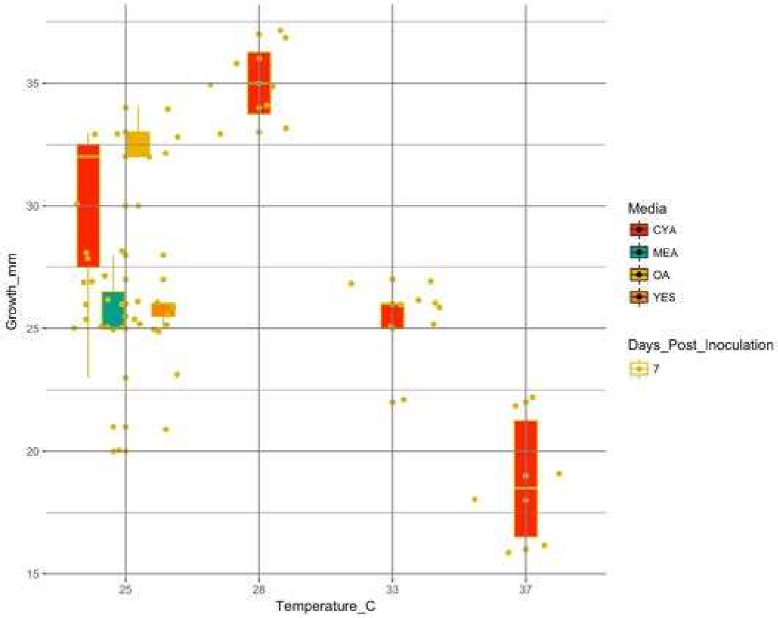

Figure 4.1. Growth rate of *P. acequia* sp. nov. on four different agar medias, Czapek yeast autolysate agar (CYA), malt extract agar (MEA), oatmeal agar (OA), and yeast sucrose agar (YES). The boxplots represent the variable diameter measured in millimeters grown in the dark under the four different temperatures on the y-axis. All measurements were taken seven days post inoculation.

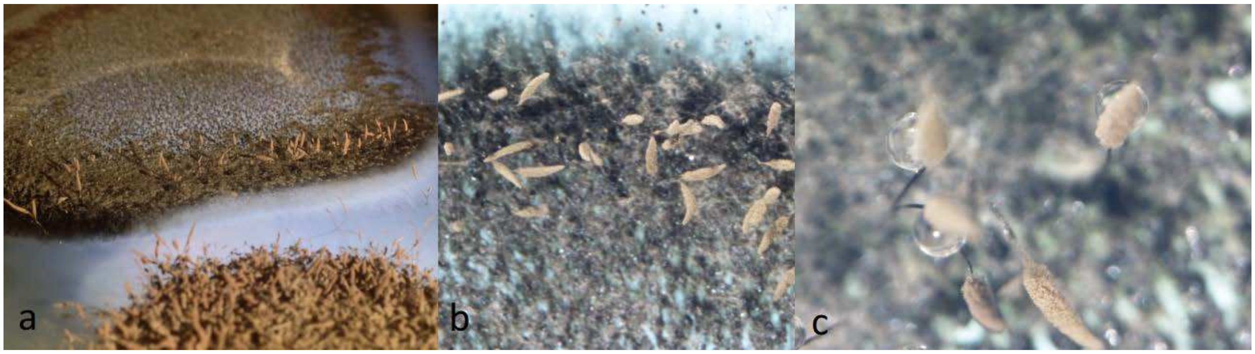

Figure 4.2. Interaction of *P. acequia* sp. nov. (light brown) growing in culture with *C. coccodes* (black) (a). Macroconidiophores (b & c) of *P. acequia* sp. nov. amongst the mycelia of *C. coccodes* (black) during the competition assay at 14 days post inoculation.

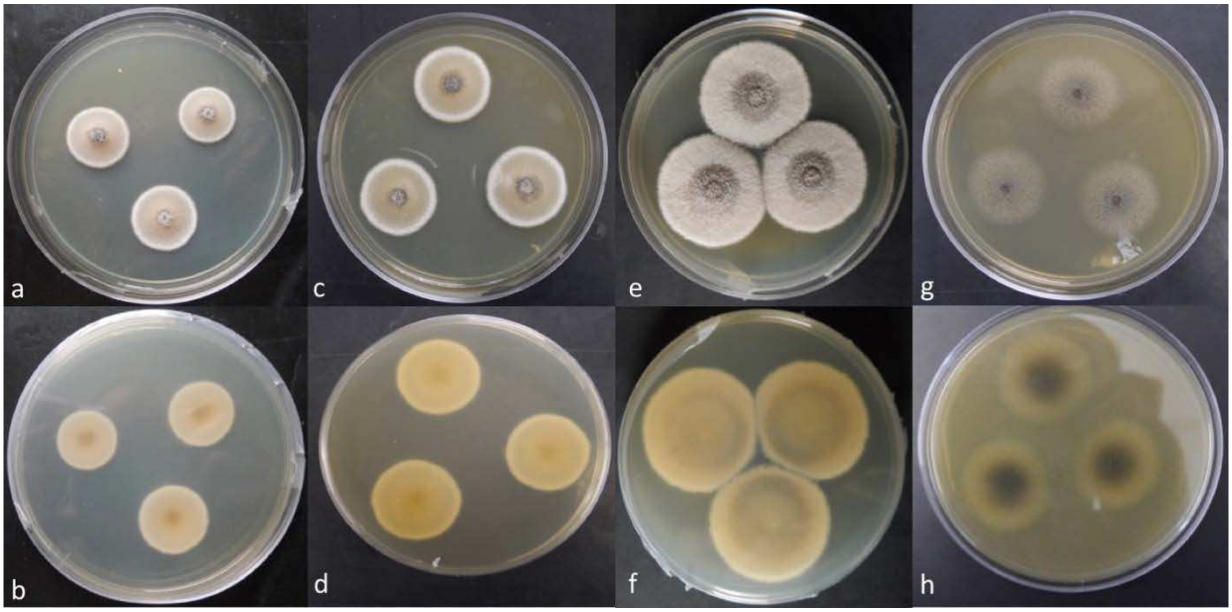

Figure 4.3. Colony characteristics of *Penicillium acequia* sp. nov. Colonies at 7 days post inoculation: a) CYA 37ºC, b) CYA 37ºC obverse, c) CYA 32ºC, d) CYA 32ºC obverse, e) CYA 28ºC, f) CYA 28ºC obverse, g) MEA 25ºC, h) MEA 25ºC obverse.

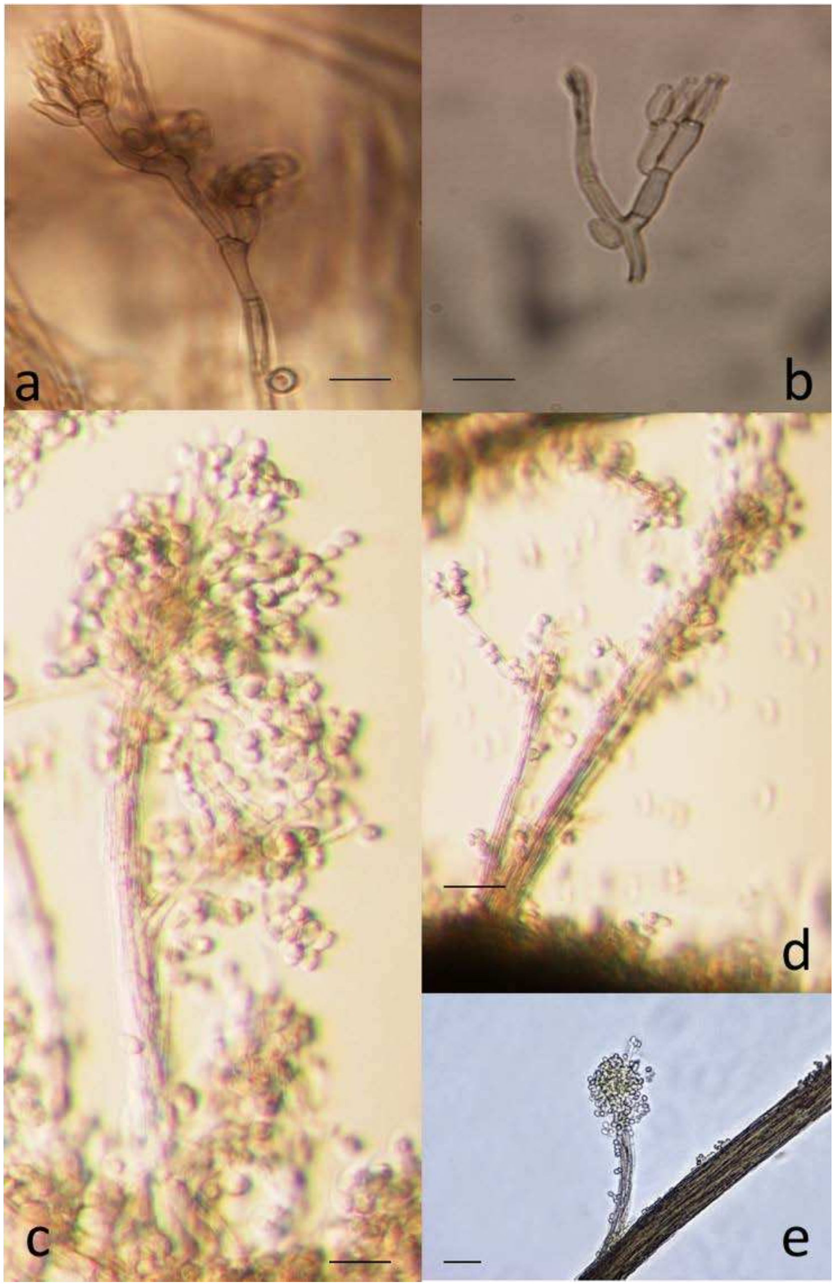

Figure 4.4. Conidia and conidiophores of *Penicillium acequia* sp. nov. (a-e). Fragments of phialides 100x (a-b), conidiophores at 7 days (c-d) 40x, non-terminally branching conidiophore at 14 days 40x (e). Scale bar 10 µm (a-d) 25 µm (e).

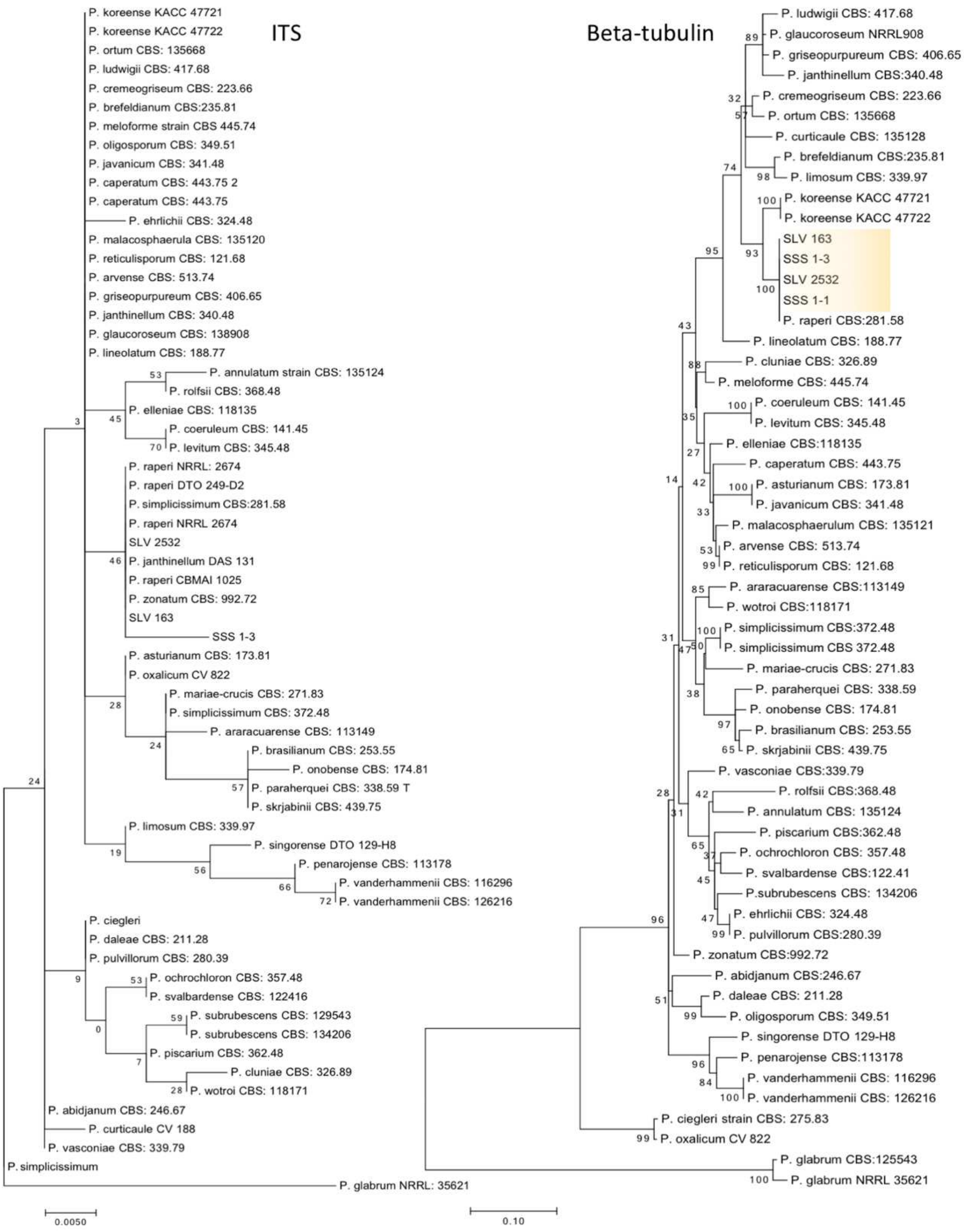

Figure 4.5. Phylogenetic tree of section Lanata-Divaricata including *Penicillium acequia* sp. nov. in yellow. The ITS maximum likelihood tree on the left and the β-tubulin on the right. Scale bar represents percent sequence similarity.

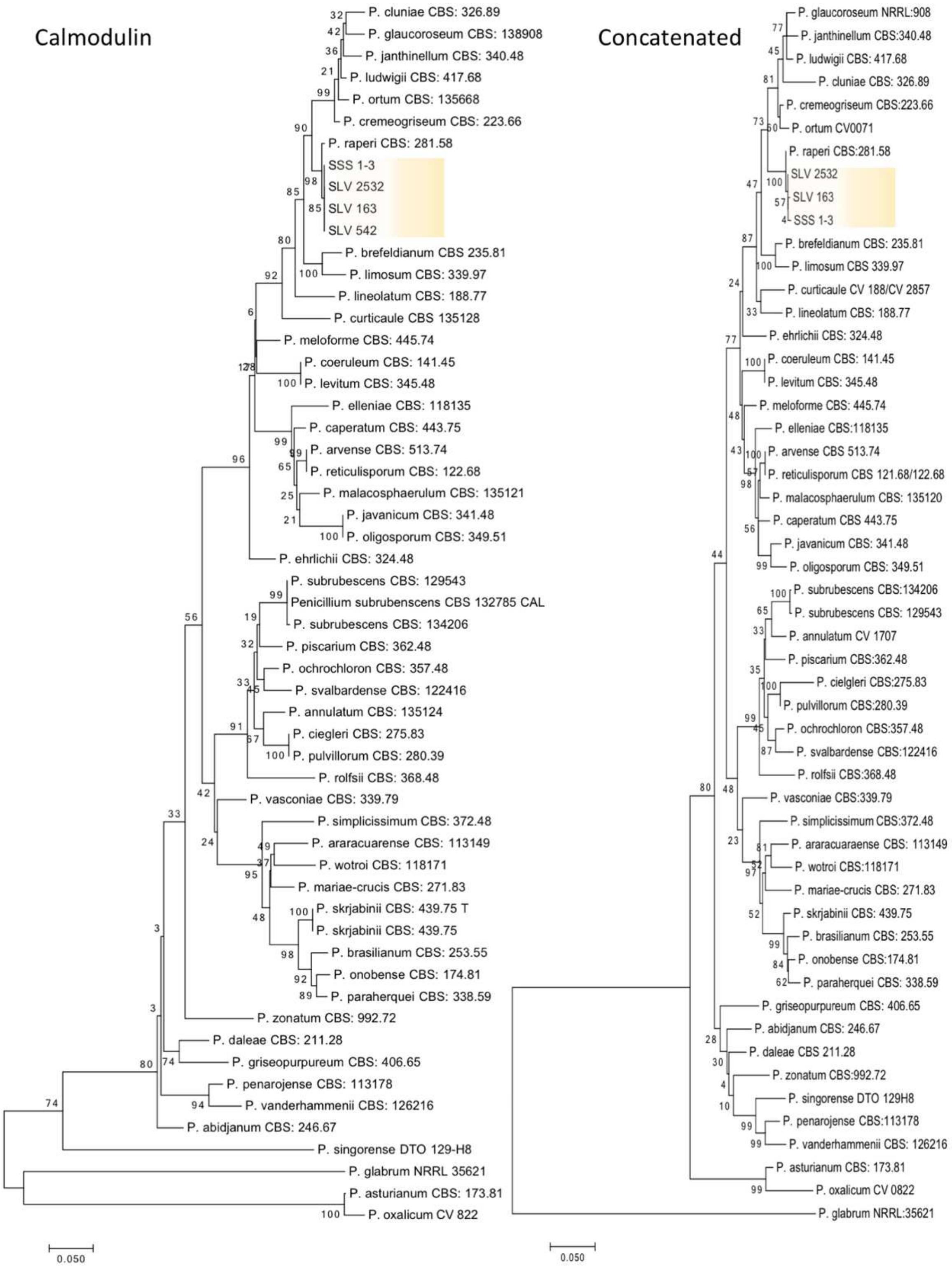

Figure 4.6. Phylogenetic tree of section Lanata-Divaricata including *Penicillium acequia* sp. nov. in yellow. The calmodulin maximum likelihood tree on the left and the concatenated sequence alignment on the right. Scale bar represents percent sequence similarity.

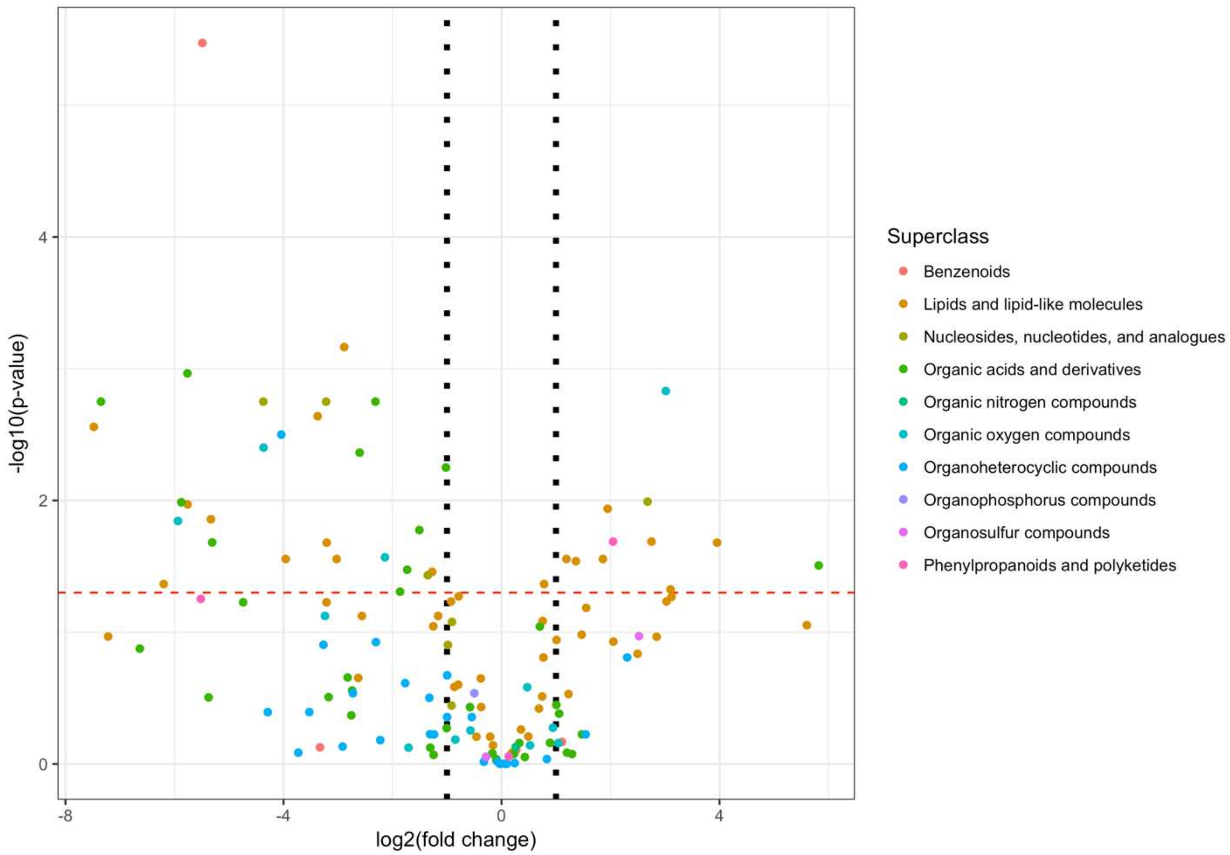

Figure 4.7. Extrolite profile of *P. acequia* sp. nov. on CYA and YES. The x-axis represents the log2(CYA/YES), metabolites with a positive value on the x-axis were produced more abundantly on CYA media than YES media, the two horizontal black dashed lines represent -1 and 1 to show extrolites produced greater than 2 fold on either media. The y-axis represents the -log10 (Benjamini-Hochberg adjusted p-value) to account for multiple comparisons, the red dashed line is at 1.3 which represents the –log10 of 0.05.

Table 4.1. The soil chemical and physical properties for each site sampled. At each location isolates of *Penicillium acequia* Lichtner Broders sp. nov. were collected.

Soil Samples	pH	Electrical Conductivity	Nitrate	Phosphorus	Potassium	Zinc	Iron	Copper	Sand %	Silt %	Clay %	Lime Estimate	Texture
SLV_1-6	5.9	3.2	148	31	288	5.7	14.8	2.2	82	10	8	Low	Sandy Loam
SLV-2-5	7.2	0.6	6.4	12.5	120	3	7.2	3.2	86	6	8	Low	Loamy Sand
SSS-1	6.6	1.9	9.6	100	135	9.87	12.4	1.47	90	4	6	Low	Loamy Sand
MAT1-3	5.5	2.8	58.6	112.9	526	6.49	29.9	9.14	87	7	6	Low	Loamy Sand
MAT14b	6.9	1	9	25.1	255	4.24	6.35	1.99				Low	Sandy Clay Loam

Table 4.2. Percent similarity of nucleotides in concatenated sequence MAFFT alignment using of 23 species including three isolates of *P. acequia* sp. nov. This represents 9 of all the recognized species in the Penicillium sect. Lanata – Divaricata.

	P. acequia 163	P. acequia 2532	P. acequia SSS13	P. raperi	P. ortum	P. cremeogriseum	P. ludwigii	P. glaucoroseum	P. janthinellum	P. limosum	P. brefeldianum	P. curticaule
P. acequia sp. nov. SLV163		91.8	93.2	77.1	82.1	82.8	79.2	78.6	78.9	78.6	80.7	80.5
P. acequia sp. nov. SLV2532	91.8		95.9	82.8	85.2	87.7	84.8	84.1	83.9	83.2	85.4	83.1
P. acequia sp. nov. SSS13	93.2	95.9		81.4	83.9	86.5	83.6	83.0	82.6	82.4	84.4	82.0
P. raperi CBS:281.58	77.2	82.8	81.4		83.5	85.7	86.9	86.1	86.2	85.5	84.5	81.2
P. ortum CV0071	82.1	85.2	83.9	83.5		96.1	90.2	89.3	91.3	88.0	89.8	92.4
P. cremeogriseum CBS:223.66	82.9	87.7	86.5	85.7	96.1		92.5	91.6	93.4	90.3	91.8	90.5
P. ludwigii CBS: 417.68	79.2	84.8	83.6	86.9	90.2	92.5		97.4	94.2	89.9	88.9	85.2
P. glaucoroseum NRRL:908	78.7	84.1	83.0	86.1	89.3	91.6	97.4		94.3	89.4	88.5	84.8
P. janthinellum CBS:340.48	79.0	83.9	82.6	86.2	91.3	93.4	94.2	94.3		91.4	90.3	86.4
P. limosum CBS 339.97	78.6	83.2	82.4	85.5	88.0	90.3	89.9	89.4	91.4		95.6	85.8
P. brefeldianum CBS: 235.81	80.7	85.4	84.4	84.5	89.8	91.8	88.9	88.5	90.3	95.6		87.7
P. curticaule CV 188/CV 2857	80.5	83.1	82.0	81.2	92.4	90.5	85.2	84.8	86.4	85.8	87.7	

Table 4.3. Primer combinations used in this study for genetic amplification and sequencing.

Locus	Primer	Primer sequence 5’ to 3’	Annealing temperature (°C)	Orientation	Reference
Internal transcribed spacer (ITS)	ITS1 f	TCCGTAGGTGAACCTGCGG	54	Forward	Gardes and Brunds 1993
	ITS4	TCCTCCGCTTATTGATATGC	54	Reverse	White et al 1990
B-tubulin	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	52	Forward	Glass and Donaldson 1995
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	52	Reverse	
Calmodulin	CAL 228F	GAGTTCAAGGAGGCCTTCTCCC	55	Forward	Carbone and Kohn 1999
	CAL 737R	CATCTTTCTGGCCATCATGG	55	Reverse	

Table S4.1. Annotated extrolites with the YES abundance >10,000 detected from the LC-MS analysis. Inferred chemical formula and the log2 fold change of abundance between the two medias. The negative log10 p-value is the Benjamini-Hochberg corrected p-value where significance is at *P*<1.3. Chemical classification from Kingdom, Super Class, class and sub class come from ClassyFire database.

Annotation	Inferred Formula	CYA Abundance	YES Abundance	Fold Change	-log10 p-value	Kingdom	Superclass	Class	Subclass
4-undecylbesylic acid	C17H28O3S	1882545.84	1491721.16	2.16	0.17	Organic compounds	Benzenoids	Benzene and substituted derivatives	Benzenesulfonic acids and derivatives
(4-Nitrophenyl) thiazol-5-ylmethyl carbonate	C11H8N2O5S	4056.08	661181.71	0.10	0.13	Organic compounds	Benzenoids	Benzene and substituted derivatives	Nitrobenzenes
Phenol, 4-ethenyl-	C8H8O	45997.27	654014.84	0.02	**5.47**	Organic compounds	Benzenoids	Benzene and substituted derivatives	Styrenes
3,5-Dinitro-4-hydroxyphenylpyruvate	C9H6N2O8	28670.87	451243.11	1.21	0.11	Organic compounds	Benzenoids	Phenols	Nitrophenols
Methyl (2Z)-3-[5-(1-propyn-1-yl)-2-thienyl]acrylate	C11H10O2S	5879.21	370479.12	1.12	0.07	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acid esters
(R)-2,3-Dihydroxy-isovalerate	C5H10O4	44816.10	317102.89	5.65	0.84	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
6-Hydroxypentadecanedioic acid	C15H28O5	15430.39	304458.39	48.59	1.05	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
9,10,12,13-Tetrahydroxyoctadecanoic acid	C18H36O6	6928.85	279029.84	7.20	0.97	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
(E)-2-tetradecenoic acid	C14H26O2	359043.95	258837.23	2.58	**1.54**	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
Soricin	C18H34O3	3799.45	233982.98	8.69	1.27	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates

(9Z)-11-[(2S,3R)-3-Pentyl-2-oxiranyl]-9-undecenoic acid	C18H32O3	-918.28	222635.64	8.15	1.23	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
9,10-Dihydroxyoctadecanoic acid	C18H36O4	13885.80	215984.67	4.15	0.93	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
9,12,13-TriHOME	C18H34O5	9481.82	203332.52	3.63	**1.56**	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
9,10,18-Trihydroxyoctadecanoic acid	C18H36O5	115289.44	172108.69	2.94	1.18	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
(12Z)-9,10-Dihydroxy-12-octadecenoic acid	C18H34O4	129880.09	159232.11	2.34	0.53	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
10-Undecenoic acid	C11H20O2	107735.19	152720.44	2.77	0.98	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
Tetracosanedioic acid	C24H46O4	13780.88	137603.95	1.71	0.81	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
UNPD218448	C21H36O8	2119.39	134493.21	0.58	0.60	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Fatty acyl glycosides
Linolate	C18H32O2	2864.70	128718.86	0.53	1.23	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Lineolic acids and derivatives
9HO	C18H32O3	352.94	124223.56	0.77	0.65	Organic compounds	Lipids and lipid-like molecules	Fatty Acyls	Lineolic acids and derivatives
(2S)-3-Hydroxy-2-{[(2E)-3-(methylsulfanyl)-2-propenoyl]oxy}propyl (2Z,4E,11E)-2,4,11-tridecatrienoate	C20H30O5S	-11405.64	114910.09	1.68	0.51	Organic compounds	Lipids and lipid-like molecules	Glycerolipids	Diradylglycerols
(2R)-3-(Phosphonooxy)-1,2-propanediyl (9Z,12Z,9'Z,12'Z)bis(-9,12-octadecadienoate)	C39H69O8P	5548.75	114766.38	0.16	0.65	Organic compounds	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphates

LPA(18:2)	C21H39O7P	5364.67	95199.36	0.73	0.21	Organic compounds	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphates
Lecithin	C46H86NO8P	10079.06	66765.33	0.01	**2.56**	Organic compounds	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines
LPE(16:0)	C21H44NO7P	6401.99	66349.60	0.55	0.58	Organic compounds	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines
PE(36:2)	C41H78NO8P	7088.88	65953.18	0.17	1.12	Organic compounds	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines
(4S,10R)-4-Amino-7,10-dihydroxy-7-oxido-3-oxo-2,6,8-trioxa-7lambda~5~-phosphaundecan-11-yl stearate	C25H50NO9P	1006.27	55653.30	0.14	**3.16**	Organic compounds	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoserines
MINEs-521437	C43H90O9P2	41299.59	52397.38	15.52	**1.68**	Organic compounds	Lipids and lipid-like molecules	Prenol lipids	Diterpenoids
(6R,7S,10E,14E)-16-(2-Hydroxy-5-methoxy-3-methylphenyl)-2,6,10,14-tetramethyl-2,10,14-hexadecatriene-6,7-diol	C28H44O4	8364.65	50854.42	0.42	1.04	Organic compounds	Lipids and lipid-like molecules	Prenol lipids	Diterpenoids
Ecabet	C20H28O5S	518.72	49680.57	0.77	0.43	Organic compounds	Lipids and lipid-like molecules	Prenol lipids	Diterpenoids
1,7,7-Trimethylbicyclo[2.2.1]hept-2-yl propionate	C13H22O2	1823.70	47201.65	1.04	0.00	Organic compounds	Lipids and lipid-like molecules	Prenol lipids	Monoterpenoids
MINEs-534178	C47H80O8P2	77584.03	44300.56	0.12	**1.56**	Organic compounds	Lipids and lipid-like molecules	Prenol lipids	Polyprenols
4-(5,6-Dihydroxy-6-methyl-1-hepten-2-yl)-1-methyl-1,2-cyclohexanediol	C15H28O4	314.97	43763.95	2.01	0.94	Organic compounds	Lipids and lipid-like molecules	Prenol lipids	Sesquiterpenoids
Papyriferic acid	C35H56O8	63471.47	41991.73	0.90	0.14	Organic compounds	Lipids and lipid-like molecules	Prenol lipids	Terpene glycosides

(1R,4aS,5R,6R,8S,8aR)-6,8-dihydroxy-1,4a,6-trimethyl-5-[(E)-3-methyl-5-[(2R)-3-methyl-5-oxo-2H-furan-2-yl]pent-3-enyl]decalin-1-carboxylic acid	C25H38O6	88409.34	41466.72	6.73	**1.69**	Organic compounds	Lipids and lipid-like molecules	Prenol lipids	Terpene lactones
(4S,5S)-5-[(1S,2R,4S,5R)-4-butyl-2-ethyl-2-hydroxy-5-methylcyclopentyl]-5-ethyl-4-hydroxyoxolan-2-one	C18H32O4	4212.93	39790.15	1.41	0.21	Organic compounds	Lipids and lipid-like molecules	Prenol lipids	Terpene lactones
4alpha-Methylzymosterol-4-carboxylate	C29H46O3	143.85	35753.06	0.87	0.21	Organic compounds	Lipids and lipid-like molecules	Prenol lipids	Triterpenoids
N-(1,3,4,5-Tetrahydroxy-2-tetracosanyl)octadecanamide	C42H85NO5	25941.31	33602.43	1.15	0.09	Organic compounds	Lipids and lipid-like molecules	Sphingolipids	Ceramides
phytosphingosine-18:0, ceramide	C36H73NO4	1250.64	33440.64	0.01	0.97	Organic compounds	Lipids and lipid-like molecules	Sphingolipids	Ceramides
N-(2-hydroxydocosanoyl)-phytosphingosine	C40H81NO5	542.98	33256.02	1.28	0.26	Organic compounds	Lipids and lipid-like molecules	Sphingolipids	Ceramides
Cer(t18:0/16:0)	C34H69NO4	17720.46	32245.78	1.69	1.08	Organic compounds	Lipids and lipid-like molecules	Sphingolipids	Ceramides
(2R)-N-[(2S,3R,4E,8E)-1-(beta-D-Glucopyranosyloxy)-3-hydroxy-4,8-octadecadien-2-yl]-2-hydroxyhexadecanamide	C40H75NO9	20549.78	32093.67	0.11	1.68	Organic compounds	Lipids and lipid-like molecules	Sphingolipids	Glycosphingolipids
(3beta,5alpha,6beta,22E)-Ergosta-7,22-diene-3,5,6,9-tetrol	C28H46O4	4685.13	31726.43	2.28	**1.56**	Organic compounds	Lipids and lipid-like molecules	Steroids and steroid derivatives	Ergostane steroids
(3beta,5alpha,16xi,22S,23S,25R,26S)-3,22-Dihydroxy-23,26-epoxyfurostan-26-yl beta-D-glucopyranoside	C33H54O10	13064.72	31045.82	3.86	**1.94**	Organic compounds	Lipids and lipid-like molecules	Steroids and steroid derivatives	Steroidal glycosides
[(2R,3S,4R,5R)-5-(2,4-dioxopyrimidin-1-yl)-3,4-dihydroxyoxolan-2-yl]methyl [(2R,3S,4R,5R)-5-(2,4-	C18H23N4O14P	16442.79	29344.46	0.05	**2.75**	Organic compounds	Nucleosides, nucleotides	(3'->5')-dinucleotides and analogues	(3'->5')-dinucleotides

dioxopyrimidin-1-yl)-4-hydroxy-2-(hydroxymethyl)oxolan-3-yl] hydrogen phosphate							, and analogues		
UD2	C17H27N3O17P2	1143.76	27059.93	0.39	**1.43**	Organic compounds	Nucleosides, nucleotides, and analogues	Pyrimidine nucleotides	Pyrimidine nucleotide sugars
S-{2-[Amino(dihydroxy)-lambda~4~-sulfanyl]ethyl}-L-cysteine	C5H14N2O4S2	2954.43	25758.25	2.01	0.45	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
S-{2-[Amino(dihydroxy)-lambda~4~-sulfanyl]ethyl}-L-cysteine	C5H14N2O4S2	-15354.58	25023.08	0.94	0.04	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
HMDB00159	C9H11NO2	35064.15	23849.59	0.42	0.07	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
D-Fructose, 1-[[(5S)-5-amino-5-carboxypentyl]amino]-1-deoxy-	C12H24N2O7	2885.94	23487.02	0.35	**1.78**	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
(4S,6R)-1,6-dimethyl-6-(1H-pyrrole-2-carbonyloxymethyl)-4,5-dihydropyrimidine-4-carboxylic acid	C13H17N3O4	17697.37	23026.20	1.17	0.08	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
(2S)-3-(2-{[(2S,3S)-3-Amino-3-carboxy-2-hydroxypropyl]sulfanyl}-1H-imidazol-5-yl)-2-(trimethylammonio)propanoate	C13H22N4O5S	510.05	22983.30	0.94	0.03	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
N-Acetyl-L-tyrosine	C11H13NO4	2042.65	22706.07	0.20	**2.75**	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
L-Lysine	C6H14N2O2	10905.44	22109.22	1.35	0.05	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
L-Glutamine	C5H10N2O3	11351.55	21306.41	2.78	0.22	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues

Leucenol	$C_8H_{10}N_2O_4$	18844.12	20994.78	0.15	0.56	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
5-Oxo-L-proline	$C_5H_7NO_3$	12765.13	20876.11	0.14	0.66	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
L-Prolyl-L-alanine	$C_8H_{14}N_2O_3$	15607.03	19175.60	1.85	0.16	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
5-Oxo-L-prolyl-L-leucine	$C_{11}H_{18}N_2O_4$	1983.21	16806.75	0.40	0.12	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
5-Oxo-L-prolyl-L-phenylalanine	$C_{14}H_{16}N_2O_4$	24959.11	15892.92	0.49	**2.25**	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
2,3-Dihydroxy-4-decynoic acid	$C_{10}H_{16}O_4$	13277.71	14868.39	1.63	1.04	Organic compounds	Organic acids and derivatives	Hydroxy acids and derivatives	Medium-chain hydroxy acids and derivatives
D-Gluconic acid	NA	657.31	14472.41	0.01	**2.75**	Organic compounds	Organic acids and derivatives	Hydroxy acids and derivatives	Medium-chain hydroxy acids and derivatives
2-Oxopentanoic acid	$C_5H_8O_3$	2113.05	14128.53	56.47	**1.51**	Organic compounds	Organic acids and derivatives	Keto acids and derivatives	Short-chain keto acids and derivatives
Ketoleucine	$C_6H_{10}O_3$	1047.77	14017.98	0.16	**2.36**	Organic compounds	Organic acids and derivatives	Keto acids and derivatives	Short-chain keto acids and derivatives
(5-Formyl-3-furyl)methyl dihydrogen phosphate	$C_6H_7O_6P$	20079.14	12472.67	0.50	0.27	Organic compounds	Organic acids and derivatives	Organic phosphoric acids and derivatives	Phosphate esters
Inositol cyclic phosphate	$C_6H_{11}O_8P$	2585.57	12166.84	0.03	**1.68**	Organic compounds	Organic acids and derivatives	Organic phosphoric acids and derivatives	
Dodecyl Sulfate	$C_{12}H_{26}O_4S$	26582.80	11570.51	2.45	0.08	Organic compounds	Organic acids and derivatives	Organic sulfuric acids and derivatives	Sulfuric acid esters

Table S4.2. Bayesian patristic distances between select species within Lanata - Divaricata as measured through MrBayes. Bold values are of *P. acequia* sp. nov.

	P. raperi CBS:281.58	**P. griseopurpureum CBS: 406.65**	**P. janthinellum CBS:340.48**	**P. curticaule CV 188/CV 2857**
P. araracuaraense CBS: 113149	0.351	0.362	0.294	0.269
P. wotroi CBS:118171	0.365	0.375	0.307	0.282
P. mariae-crucis CBS: 271.83	0.354	0.364	0.297	0.271
P. onobense CBS:174.81	0.389	0.399	0.332	0.306
P. paraherquei CBS: 338.59	0.392	0.403	0.335	0.31
P. brasilianum CBS: 253.55	0.393	0.403	0.336	0.31
P. skrjabinii CBS: 439.75	0.369	0.379	0.312	0.286
P. simplicissimum CBS:372.48	0.34	0.35	0.283	0.257
P. vasconiae CBS:339.79	0.302	0.312	0.245	0.22
P. ehrlichii CBS: 324.48	0.255	0.265	0.198	0.172
P. coeruleum CBS: 141.45	0.223	0.234	0.166	0.141
P. levitum CBS: 345.48	0.223	0.234	0.166	0.141
P. meloforme CBS: 445.74	0.222	0.233	0.165	0.14
P. malacosphaerulum CBS: 135120	0.238	0.249	0.181	0.156
P. caperatum CBS 443.75	0.23	0.241	0.173	0.148
P. elleniae CBS:118135	0.227	0.237	0.17	0.144
P. javanicum CBS: 341.48	0.231	0.242	0.174	0.149
P. oligosporum CBS: 349.51	0.257	0.267	0.2	0.175
P. brefeldianum CBS: 235.81	0.204	0.215	0.147	0.122
P. limosum CBS 339.97	0.21	0.221	0.153	0.128
P. lineolatum CBS: 188.77	0.196	0.206	0.139	0.114
P. cremeogriseum CBS:223.66	0.12	0.131	0.063	0.081
P. ortum CV0071	0.123	0.134	0.066	0.084
P. glaucoroseum NRRL:908	0.079	0.089	0.055	0.131
P. ludwigii CBS: 417.68	0.075	0.085	0.05	0.127
P. cluniae CBS: 326.89	0.105	0.116	0.081	0.157
P. acequia SLV 163	**0.015**	**0.131**	**0.097**	**0.173**
P. acequia SLV 2532	**0.011**	**0.131**	**0.097**	**0.173**
P. acequia SSS 1-3	**0.017**	**0.137**	**0.103**	**0.179**
P. raperi CBS:281.58		0.14	0.105	0.181
P. griseopurpureum CBS: 406.65	0.14		0.115	0.192
P. janthinellum CBS:340.48	0.105	0.115		0.124
P. curticaule CV 188/CV 2857	0.181	0.192	0.124	

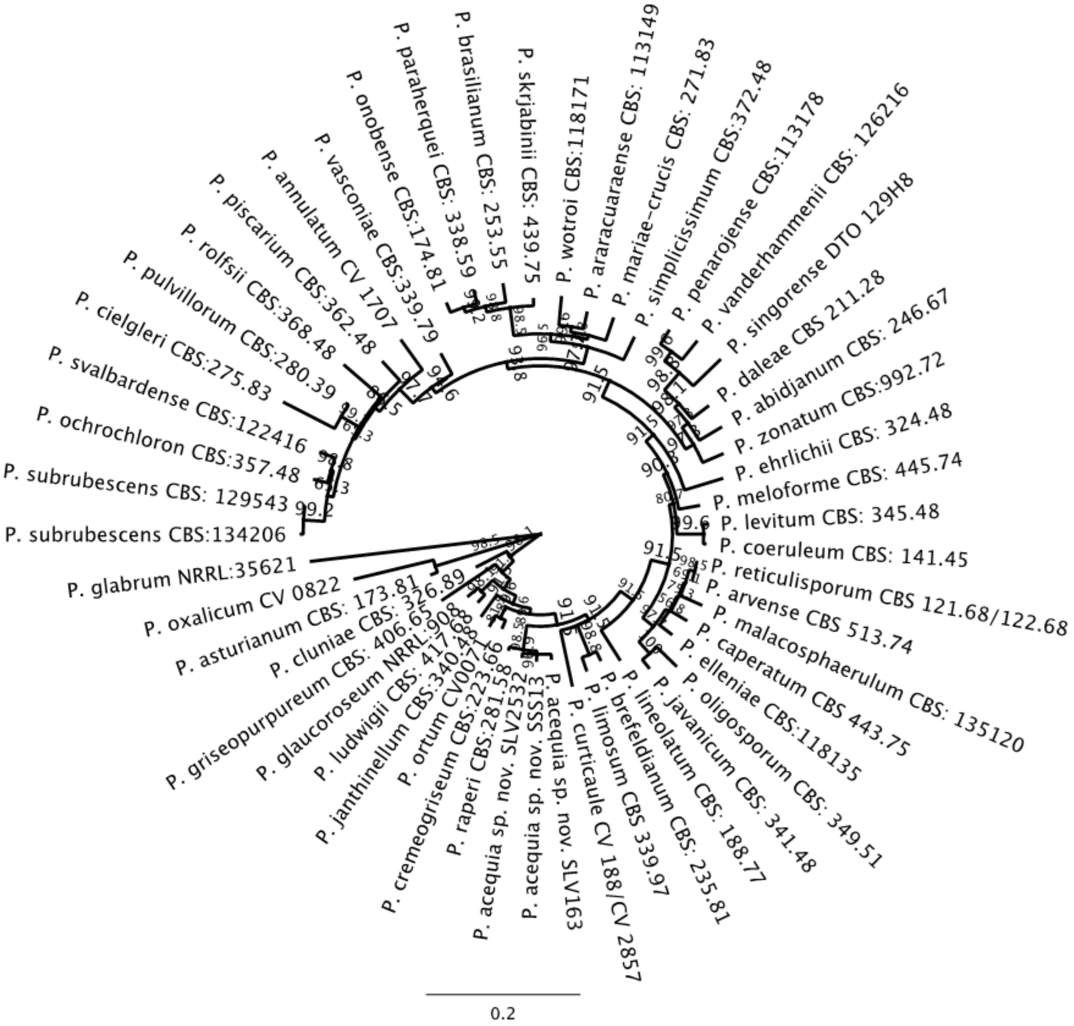

Figure S4.1 Circularized phylogenetic tree of Mr. Bayes posterior probability built based on Bayesian posterior distribution of model parameters. Each species represents the concatenated ITS, BenA and CaM loci from the CBS/NCBI databases including the type specimen (SLV 163) and 2 other isolates.

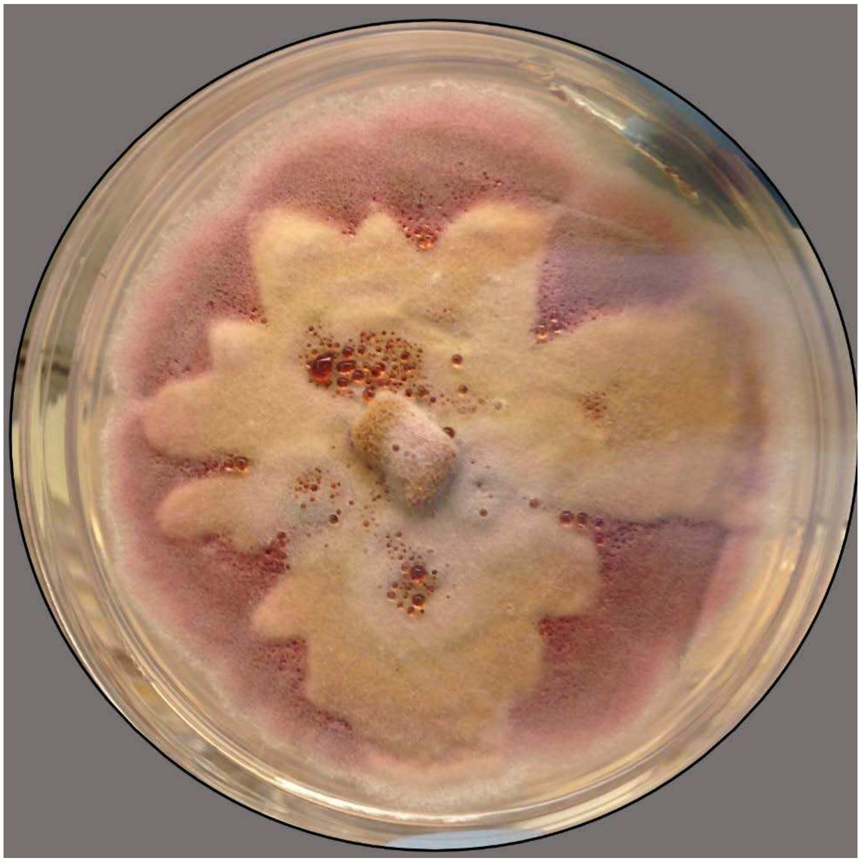

Figure S4.2 Type specimen of *P. acequia* sp. nov. after seven days of growth on potato dextrose agar amended with chloramphenicol grown in the dark at 25°C.

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