THESIS

SOIL BACTERIAL INFLUENCE ON ALFALFA GROWTH AND HEALTH

Submitted by

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ABSTRACT

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Soil microbial communities have demonstrated enormous potential for promotion of plant health and productivity. In particular, the diversity of the soil community may play an important role for increased plant growth. However, previous research has focused on soil fungal diversity and neglected the role that diversity of soil bacteria may play in influencing plant growth. Therefore, a greenhouse study was conducted to determine if soil bacterial community structure influences alfalfa productivity. Prior to setup, nine soils with varying physico-chemical and microbiological profiles were chemically and biologically characterized. Soil physico-chemical factors for experimental soils were quantified via standard methods of soil nutrient testing. In addition, soil microbiology was characterized using 454 pyrosequencing to determine soil diversity indices and taxonomic classification of the soil bacterial community. These microbial communities were extracted into soil suspensions and transplanted to alfalfa plants growing in a sterile substrate. Filtered (soil microorganisms removed) and non-filtered (soil microorganisms retained) soil suspensions were applied to separate soil chemical and microbiological effects. Alfalfa plants were grown in a greenhouse for 60 days post germination; then roots and shoots were harvested, dried and weighed. This experimental setup was used to answer two distinct research questions.

In the first study, alfalfa biomass was correlated with both soil physico-chemical and bacterial measures to determine which soil factors influenced plant productivity. For four soils, a biologically inactive (filtered) extract included unidentified chemical factors that had a negative

effect on plant biomass production. However, in two of these cases inclusion of soil microbes counteracted this negative effect and restored plant growth to a level equal to the non-amended control. Among bacterial classes, the relative abundance of Deltaproteobacteria in soils was significantly correlated with plant productivity. Correlations between plant productivity and soil bacterial richness, diversity and evenness were marginally significant and more highly correlated than soil physico-chemical factors. Results suggest that soil microbiology can compensate for negative effects on plant growth due to soil chemistry, potentially due to microbial remediation of organic soil chemical residues such as herbicides. Also, in this study, relative abundance of specific bacterial taxa was more highly correlated than bacterial diversity indices with improved plant productivity.

Many species of bacteria, referred to collectively as plant growth promoting rhizobacteria (PGPR), are known to be particularly beneficial to plant health and yield. However, inconsistency in establishment of PGPR inoculants has limited their practical use in the field. While PGPR inoculation failures have been partially attributed to competition with the indigenous soil community, studies focusing on the role that indigenous soil bacteria play on the establishment of PGPR inoculants are rare. Soil bacterial diversity is known to prevent establishment of fungal pathogens and may inhibit PGPR establishment as well. Therefore a second study was conducted using four of the nine original experimental soils, which were selected to represent the largest variety of US locale and management types from collected soils. Including four soils allowed for expansion beyond previous bacterial diversity research, which utilized only one soil type, while simultaneously including inoculation treatments of two different organisms. The same experimental setup was utilized except that either a PGPR (*Pseudomonas putida*) or a pathogenic microorganism (*Phytophthora medicaginis*) was

introduced for comparison to non-amended controls. Subsequently, effects on alfalfa biomass production and disease were measured. In addition, PGPR colonization by *P. putida* KT2440 was quantified using qPCR via detection of the *gfp* gene carried on the KT2440 plasmid.

Results from the second study showed increases in alfalfa productivity with added PGPR were significantly larger in soils with higher soil microbial diversity. However, no differences in PGPR root colonization were observed among non-filtered treatment groups. These results suggest that the increased effectiveness of the PGPR in high diversity communities was due to increased effectiveness of other beneficial soil microorganisms. Indeed, several native PGPR and N cycling species were correlated with shoot biomass increases when adding PGPR. Conversely, disease incidence and severity caused by *P. medicaginis* was not significantly associated with soil bacterial diversity. These results emphasize the role of soil microbial community composition and its functional relationship with the invading organism in predicting effects of an introduced PGPR inoculant or soil pathogen.

In conclusion, both soil chemical and biological qualities were evaluated to lend confidence that observed effects on alfalfa biomass and microbial invasion were due to biological rather than chemical influences. Soil bacteria were found to influence plant productivity by counteracting other soil factors with negative effects on plant growth. In addition, soil community diversity played a less consequential role in these experiments than the specific taxonomical and functional bacterial members. Furthermore, soil bacterial diversity significantly improved the beneficial effects of PGPR inoculants, but was not shown to significantly reduce disease incidence or severity.

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CHAPTER 1: LITERATURE REVIEW

Introduction

Recent information suggests that agricultural productivity rests on a foundation of soil microbial activity [1-6]. The soil harbors enormous microbial diversity [7], and contemporary research has reinforced this fact [8]. There is a growing appreciation of the genetic potential and the functional importance of the soil microbiome [9] in plant health and growth. The literature is full of studies that determine the mechanistic details of plant-microbe interactions [10-13]. However, in reality, these interactions are much more complex, involving a vast array of microbes and often producing synergistic and unexpected effects [14]. It is necessary to move away from the mechanistic but potentially simplistic view of individual plant–microbe interactions and take into account all the factors that influence this complex ecosystem. The plant, the soil, and the soil microbes all work together to mediate and influence the various exchanges (see Fig. 1.1) that contribute to plant health and productivity [6]. The goal of this thesis research was to add to our current understanding of soil biological influence on plant production and improve upon methods to manipulate soil bacterial communities for enhanced yield.

The role of broad microbiome characteristics in plant health and productivity

When describing the structure of the soil microbial community, mathematical measures of species richness, evenness and diversity are frequently referenced [15]. Species richness is a statistical estimate of the number of species present, evenness is a measure of the homogeneity of abundance, and diversity combines the two [15]. Increasing microbial richness may equate to

greater community-level trait diversity and/or functional redundancy, and leads to more consistent functioning across variable environments [16]. In support of this model, increasing soil microbial species richness has been shown to be an accurate predictor of plant health and productivity [17-20]. In addition, soil species richness may maximize overall microbial activity or niche saturation, which is important to competitive exclusion of pathogens. For example, increasing microbial biomass and/or diversity has been found to accompany enhanced pathogen- or disease-suppressiveness [21-23] and to limit successful invasion by introduced organisms [24]. In addition to community-level effects, other studies have focused on the disease-suppressive ability of particular taxons or groups of microbes [14, 25, 26].

Beyond reducing disease, soil microbial diversity has been shown to promote plant productivity, diversity and nutrient acquisition [19, 20, 27, 28]. Most of these studies center on the diversity of soil fungi alone, and similar research using soil bacteria alone [29] or in combination with soil fungi is limited. Other studies have indicated that the reduction of microbial richness does not result in decreased soil ecosystem functions or reduced plant productivity [30]. Soil functional diversity, rather than taxonomical diversity, may prove to be a better predictor of ecosystem productivity [30-32]. In support of this idea, one study found that plant productivity increased only when increased fungal diversity spanned a range of functional groups, not taxonomic groups [28].

Diversity is neither the only measure of soil community structure nor the only predictor of soil functioning. Evidence supports an important role for evenness in community functioning, particularly under stresses or perturbations [33]. For example, rare members of the microbiome may be unable to effectively perform important functions. In this case, evenness (relative abundance) of members of the microbiome becomes necessary for a complete set of ecological

functions [34]. For example, one field study showed that increasing evenness of predators and pathogens was critical to the successful biocontrol of potato insect pests [35]. Low soil microbial evenness has been associated with reduced plant productivity [36]. The richness and evenness of the soil microbiome are central to providing stability, resilience to stress and disease, and high levels of internal nutrient cycling [37]. Importantly, soil microbial evenness can be modified by agricultural management practices [38, 39]. In the near term, a more feasible way to harness beneficial microbial functions in agricultural systems would be to attempt to manipulate the soil microbiome directly (Fig. 1.2) through careful management or other means. As an example, inoculation of microorganisms has been used for some time in agriculture. However, new approaches may enhance the success of introducing novel microbes into soil. For instance, deliberately imposing disturbances may increase soil vulnerability to colonization and enhance the establishment of exogenous beneficial organisms. This might take the form of disruption by fungicide application, crop rotation or tilling prior to the application of plant growth promoting rhizobacteria [38], mycorrhizal fungi [40], or other beneficial microbes in the form of active compost or bio- preparations [41]. Future formulation of beneficial microbial inoculants should be optimized for ecological functions contained by species within the formulation and in the indigenous community. Such formulations rely on understanding the functions of specific plant-associated soil microorganisms and significant gaps in this knowledge pose an obstacle to realizing this goal.

The role of soil microbes in soil fertility and plant health

The beneficial effects of soil microbes on plant health and the associated mechanisms of action are of increasing interest [1, 4, 5, 12, 13]. Many recent advances have been made in this

rapidly developing field and emphasize potential practical applications for sustainable and integrated approaches to agriculture. For example, adding beneficial microorganisms to those already present in the soil can maximize plant nutrient uptake [42], increase plant growth [4, 43-45], confer resistance to abiotic stress [46], and suppress disease [47-50]. These benefits can be particularly pronounced with microorganisms called plant growth promoting rhizobacteria (PGPR) that effectively colonize the root soil interface, or rhizosphere. Plants interact with a variety of PGPRs that are capable of increasing photosynthetic capacity [51, 52], conferring drought and salt tolerance [51-54], and improving the effectiveness of the plant's own iron acquisition mechanisms [51]. Many PGPRs aid in disease suppression via the release of antimicrobial or antifungal compounds that deter plant pathogens [25, 55]. For example, fluorescent Pseudomonads produce the antibiotic 2, 4-Diacetylphloroglucinol (DAPG), which has been extensively studied as a protectant against soil-borne diseases and has been directly linked to disease suppression [56, 57]. *Bacillus subtilis* also releases the antibiotics surfactin and iturin into the rhizosphere, which play a major role in plant disease suppression [58], while also conferring increased plant growth promotion.

As an example of the potential of microbial inoculation, consider the outcome of a greenhouse study using tomato plants inoculated with PGPR and mycorrhizal fungi. It showed that inoculated plants that received less than 75% the full rate of fertilizer had yields identical to non-inoculated plants that received full fertilizer treatments [59]. This finding emphasizes that awareness of the existing soil fertility level is critical to maximizing PGPR benefits, since a diminishing effect is seen when starting N, P, and K levels are high [60]. Given the wide variety of effects and mechanisms of action, it's not surprising that multi-species PGPR formulations have been found to be even more effective in suppressing disease [41, 61-63]. Compost

formulations that include beneficial bacteria can be particularly effective in suppressing plant pathogens [63, 64].

These discoveries may offer potential for PGPR applications to improve agricultural production and sustainability. However, exploiting the immense benefits that PGPRs provide via rhizosphere inoculation will require additional work, as there is inconsistency in their performance at the field scale [65, 66]. PGPR success relies heavily on rhizosphere competence, and recent research has determined some of the bacterial traits necessary for this outcome [67]. One factor necessary for the colonization of PGPRs in the rhizosphere is effective competition with the indigenous soil microbial community [68]. One study determined that indigenous soil communities with higher microbial biomass showed reduced PGPR colonization after inoculation [38]. Other studies have determined the characteristics of soil communities that experience a lasting effect after beneficial microbial inoculation [69]. More research is necessary in this area to determine which aspects of the indigenous microbial communities are most influential when applying beneficial inoculants.

Direct microbiome manipulation

In the near term, a more feasible way of harnessing beneficial microbial functions in agricultural systems would be to attempt to manipulate the soil microbiome directly (Fig. 1.2). Inoculation of microorganisms has been used for some time in agriculture. However, new approaches may enhance the success of introducing novel microbes into soil. For instance, deliberately imposing disturbances may increase soil vulnerability to invasion and enhance the establishment of exogenous beneficial organisms. This might take the form of disruption by fungicide application, crop rotation or tilling prior to the application of plant growth promoting

rhizobacteria [38], mycorrhizal fungi [40], or other beneficial microbes in the form of active compost or bio-preparations [41].

Hybrid approaches are also possible, tweaking both plant and microbiome characteristics to achieve a better fit. Attempts have been made to create plant genotypes that are tailored to particular microbes, such as engineering plants to produce novel carbon sources that are readily available to an inoculant strain [70]. Alternatively, information on the identity of beneficial microbial taxa could be used to deduce probable substrates that could be supplied exogenously or via plant root exudation in order to enrich those particular taxa. For instance, recent work has identified microbial taxa and antibiotic biosynthetic genes associated with disease suppression [14]. Knowledge of the metabolic capacity of these organisms could be used to develop strategies for selective enrichment. At an even finer level, an understanding of the regulation of antibiotic biosynthetic gene expression in these organisms may allow for manipulation of relevant microbial functions through the introduction of appropriate signaling molecules.

Chemical pollution, a growing human population, and the depletion of resource and energy reserves have accentuated the need for sustainable agricultural practices. One path toward sustainability involves a greater reliance on the beneficial functions afforded by the soil microbiome. This thesis furthers research that is vital to our ability to more fully exploit the plant-associated microbiome in agricultural crop production. The first study was conducted with alfalfa (*Medicago sativa*) to investigate varying effects of nine different indigenous soil communities on plant productivity. Separating soil biological and chemical influences on plant productivity has historically proved difficult, but is vital for understanding the potential for soil microbial communities to promote plant health and productivity. The second segment investigates the effects of soil bacterial community diversity on pathogen invasion and PGPR

establishment. Specific membership of the soil bacterial community was also analyzed for effect and relative significance. Disease suppressive soils and their ability to suppress pathogen invasion have been well studied. From previous work we know that soil fungal [71] and bacterial diversity [24] as well as specific taxa [14] play a role in preventing pathogen invasions. This research seeks to determine the relevance of these same soil factors as potential reasons for PGPR not establishing well in field environments.

Figures

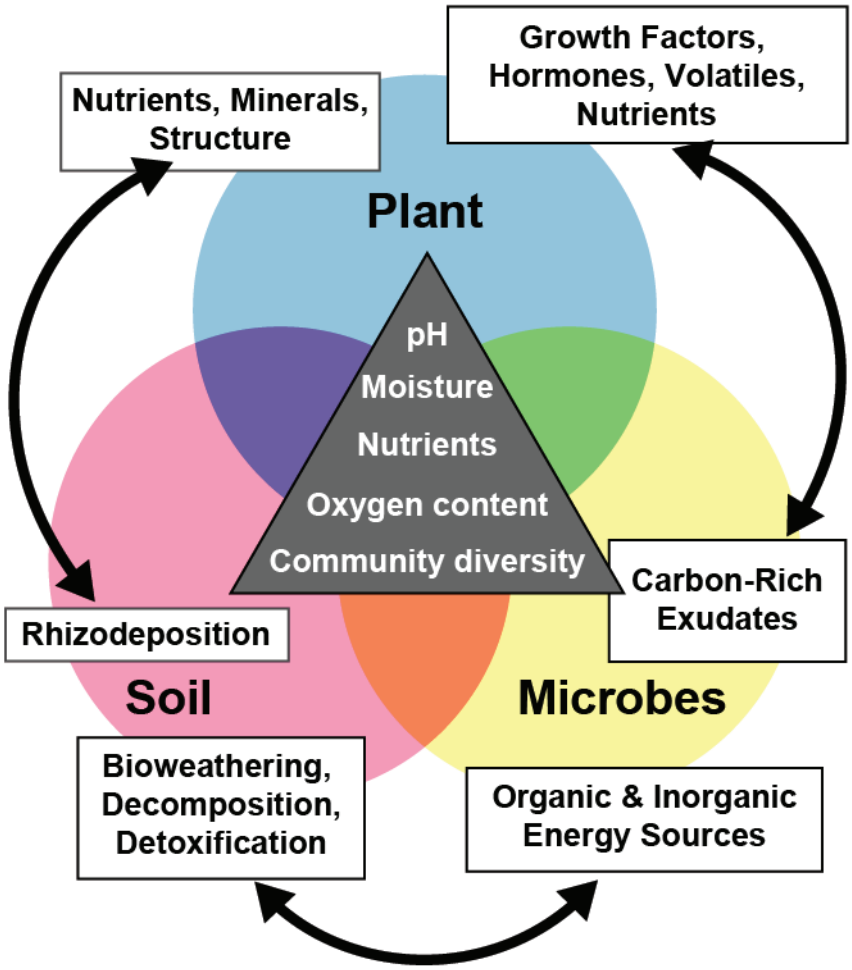


Figure 1.1 Belowground interactions affecting plant health and productivity. Plant, soil, and microbes interactively define the plant growth environment. Mechanisms are shown by boxes and arrows. Environmental characteristics impacting plant performance are shown in the central triangle.

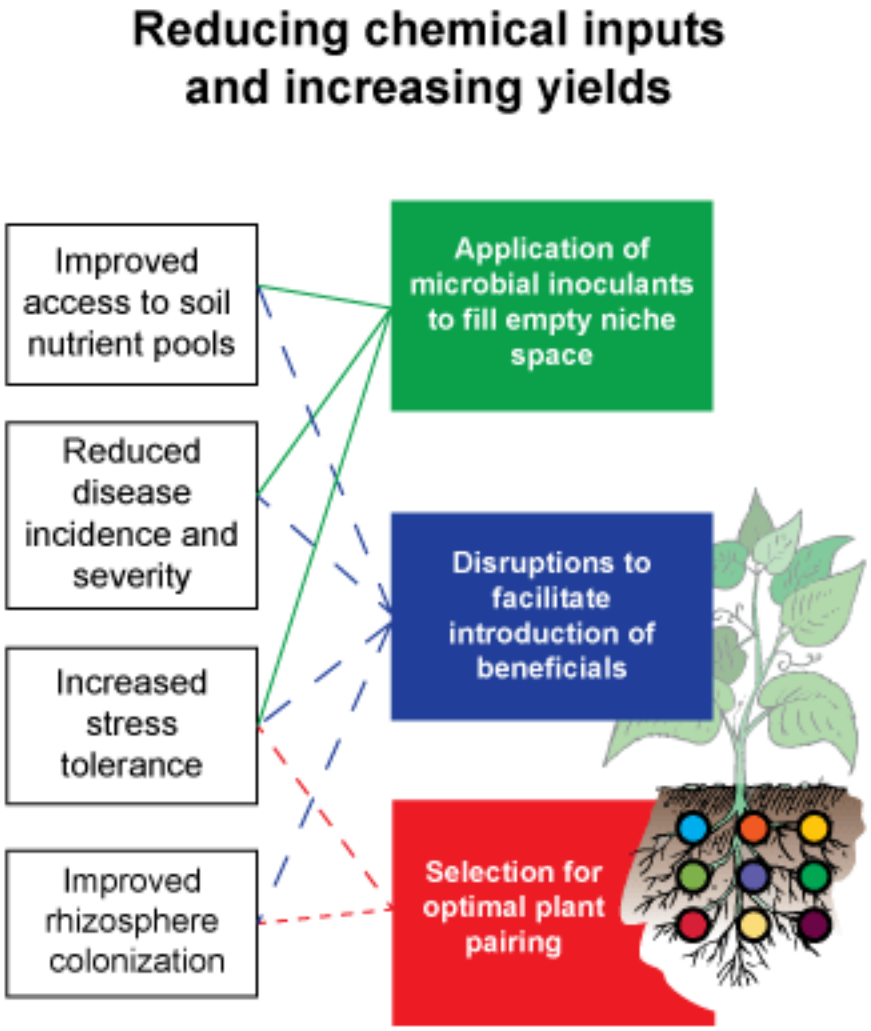


Figure 1.2 A variety of strategies could be used to promote beneficial services provided by soil microbial communities, with the aim of reducing chemical inputs while sustaining or improving crop yields. Manipulating soil microbial communities directly (right side), could improve conditions for plant productivity (left side).

CHAPTER 2: EFFECTS OF DIFFERENTIAL BACTERIAL DIVERSITY FROM NATURAL SOIL COMMUNITIES ON ALFALFA PRODUCTIVITY

Introduction

Identifying environmental factors that influence plant productivity, positively or negatively, is important for increasing crop yield for food and biofuel production. A plant's level of success in its soil environment is the outcome of a complex interplay between biological, chemical and environmental factors. Optimizing soil microbiology for increased agricultural productivity is gaining attention for its potential to improve yields without additional costly inputs [2]. However, separating plant responses to soil biology from responses to soil chemistry has proven difficult.

Many studies linking plant performance to soil biology have focused on the effects of soil microbial diversity. Early studies investigating effects of species diversity on ecosystem productivity focused on fungi, particularly arbuscular mycorrhizal fungi (AMF). Multiple studies showed increased plant productivity with increasing AMF species richness [72-76]. One proposed mechanism for this effect is niche complementarity [28, 77-79], which contends that higher diversity communities also represent a more diverse set of metabolic processes and more complete utilization of ecosystem resources [80]. In support of this idea, soil microbial diversity was demonstrated to have a stronger productivity effect in low nutrient environments [27], where full resource utilization is especially critical. Maherali and Klironomos [28] tested the niche complementarity hypothesis and found that richness of functional traits, varied in this case by altering phylogenetic diversity, was more important for increasing plant growth than species richness.

However, more diverse communities are also more likely to harbor the most productive species, both above ground and below [81] due to a selection effect. A selection effect, also called a sampling effect, occurs because high diversity communities have higher total species and thus higher probability of one being a super-producer. A selection effect occurs when these super-producers, rather than community diversity per se, drive the productivity increase (*reviewed in* [82]). Selection effects may simply be experimental artifacts, or a mechanism by which diversity effects are manifested in nature [83]. Selection effects could be manifested, for instance, in the inclusion of a particularly strong pathogen antagonist, or of a specialized mutualist such as a nitrogen fixer [84]. Recent research has revealed connections between increased pathogen success and both low plant diversity [71] and low AMF diversity in soil [85]. It is unlikely that any one mechanism fully explains plant productivity changes associated with soil microbial community interactions. Accordingly, the additive partitioning hypothesis contends that complementarity and selection effects function simultaneously to improve productivity, and provides a means to investigate the relative contribution of each [20].

Most of this work has been done with mycorrhizal fungi. Analogous studies involving rhizobacteria alone or in combination with fungi have been limited. There are, however, multiple mechanisms by which rhizobacteria can enhance plant productivity as well. These include: improved nutrient acquisition [59, 86], pathogen antagonism [47, 49, 50], increase in photosynthetic capacity ([51], [87]), osmotic stress tolerance [88] and plant hormone production [44, 88]. These services to the host plant are provided by specific rhizobacterial species, but soil bacterial community characteristics like evenness or diversity may also play a role. The same productivity-enhancing mechanisms proposed for soil fungi, including niche complementarity and selection effects, may also be at work in soil bacterial communities. For example, soils with

higher bacterial diversity were recently shown to reduce invasions of a bacterial pathogen [24] as was previously demonstrated with AMF soil diversity [85]. Another study found that the ratio of rhizobacteria with positive effects to those with negative effects is more important to plant productivity than total soil bacterial species richness [29].

A difficulty that unites investigations that focus on soil bacteria with those focusing on soil fungi is the need to separate soil biological and chemical productivity effects. Ultimately, chemical and biological influences combine to create the total picture of soil health and, in effect, crop health and yield [3]. Multiple experimental approaches have been developed to deal with this difficulty, and each has strengths and weaknesses. One approach utilizes pots of sterilized soil, sometimes mixed with an inert medium like sand, populated with artificially-assembled fungal or bacterial communities of varying richness, typically of 1 to 100 species [17, 24, 85]. Artificially-assembled microbial consortia have a strong advantage in providing careful control of community composition and species diversity. However, artificially-assembled consortia never approach the complexity of natural soil microbial communities, which contain thousands of species. As a result, sampling effects may be particularly problematic when simple consortia are used. Using naturally-assembled soil microbial communities present in field soil may elicit less concern over sampling effects and will include both bacterial and fungal species.

Due to difficulty in separating soil chemistry effects from soil biological effects, experimental designs utilizing field soil have primarily included only one soil. By limiting the number of field soils included in the experiment, soil chemistry effects can be held constant while other variables, such as soil community diversity, are varied. However, by including only one soil type, studies of this design are limited in ability to generalize across soil types or microbial communities. Standardizing the soil or growth medium also removes the possibility of

observing interactive effects that may result from combined chemical and biological properties of soil. To control for biological and non-biological effects, both autoclaved and non-autoclaved soils can be included to evaluate effects on plant traits [17, 24, 85]. However, autoclaving changes soil structure and chemistry [89]. Using gamma-irradiation for sterilization [20, 28]; may reduce, but does not eliminate, changes in soil chemistry [89]. In summary, previous research has been limited by inclusion of sampling effects, alteration of soil chemical effects due to sterilization methods, and lack of variety in soil physico-chemical and biological profiles.

The goal of this study was to determine whether soil bacterial diversity is related to plant productivity. Based upon results from previous studies using artificially assembled soil fungal communities, the hypothesis for this experiment was that natural soil suspensions with higher bacterial diversity would result in increased plant productivity not attributable to soil physico-chemical properties. Our experimental design offers an improved ability to separate soil biological and chemical influences on plant growth. We also test realistically complex microbial communities derived from a range of field soils. Soils were selected with the goal of optimizing variation in microbial community composition and diversity. To this end, nine soils with varying management histories were collected and characterized for soil physico-chemical and biological characteristics. Suspensions of soil microbes and corresponding filtered controls were prepared for each soil and applied to plants growing in a standard inert medium. Alfalfa (*Medicago sativa*) was chosen as the host plant because *Rhizobia spp.* are known to be important bacterial symbionts that increase alfalfa productivity. Relative soil abundance of *Rhizobia spp.,* as measured by 454 pyrosequencing technology, was used in addition to correlations with soil diversity indices to investigate relative influence of each on alfalfa biomass production. In summary, this experimental design allowed for screening of potential productivity effects caused

by differences in relative abundance of a primary mutualist versus a true diversity effect, while also considering the involvement of soil physico-chemical factors.

Materials and Methods

Soil descriptions and analyses

In order to represent microbial communities of differing composition and structure, soil was collected from nine sites with varying management histories (Table 2.1). Sites varied in geographic location as well as in predominant plant cover, and included natural, organic and conventional agricultural management histories. All soil samples were collected to a depth of 15 cm, from at least three locations in the same field or 25' by 25' area in the case of forests. Following collection, soils were bulked by site, homogenized and stored at 4°C. *Arabidopsis sp.* was naturally present in the soils collected from three agriculturally fallow sites. Potato fields were located in the San Luis Valley of Colorado and represented different farm management, rotation systems, potato cultivars, and pesticide usage. All potato fields had been in potato production for 3 years or more. Forest sites 1 and 3 were dominated by Ponderosa Pine (*Pinus ponderosa*) and under standard management of the US Forest Service. Forest site 2 was dominated by Douglas-fir (*Pseudotsuga menziesii*) and managed by Oregon State University.

Soils collected from each location were analyzed for pH, electric conductivity (EC), C(total):N(total) ratio, nitrate (NO_3-N) and various mineral nutrients by the Colorado State University Soil, Water, Plant Testing Laboratory.

Seedling establishment

Medicago sativa (alfalfa, cv. Saranac) seeds were surface-sterilized with an aqueous solution of 3% sodium hypochlorite for 10 min followed by three washes with sterile distilled water. Seeds were germinated singly in micro-centrifuge tubes containing 0.7% water agar in a growth chamber (37°C, 12h light/dark cycles). At 5 days post germination, seedlings were transplanted into 4 x 21 cm cone-shaped pots, in a randomized block design with each rack as a block. Prior to transplant, cones were filled with an inert medium comprised of sand and vermiculite (1:1, v:v; approximately 145 g per cone), which had been thrice steam sterilized in an autoclave for 30 minutes. Seedlings were placed on a greenhouse misting bench that provided consistent moisture and humidity. Seedlings were moved from the misting bench to a standard greenhouse bench at 18 days post germination. Thereafter, the plants were fertilized (15-5-15 NPK) every five days using a Dosatron fertilizer injector (Dosatron, Clearwater, FL, USA) to ensure a consistent application rate. Racks were rotated into new locations on the greenhouse bench twice during the growing period. Thirteen replicate plants were grown for each of 19 different treatments (9 soils x 2 preparations as described below, plus non-amended control).

Soil microbial community suspensions

Suspensions of soil microbes and corresponding filtered controls were prepared from each of nine different soils (Figure 2.1). Filtered suspensions were prepared the day before application by combining 10 grams of soil per 100 ml of millipore filtered water in flasks. Flasks were shaken for 30 minutes at 220 rpm at room temperature and allowed to settle for 30 minutes. The supernatant, a suspension of soil microorganisms and fine soil particles, was centrifuged for 30 minutes at 9000 rpm to remove remaining soil particles, and vacuumed through a 0.45 μm

pore filter (Merck, Billerica, MA, USA). Filtered suspensions were kept at 4º C overnight. Filtered treatments reflect non-biological differences in soil suspensions, largely chemical components except possibly for small amounts of viruses.

Non-filtered soil suspensions were prepared similarly, except that centrifugation was less intense (3 minutes, 3000 rpm) in order to remove soil particles but maintain soil microbes in suspension, and supernatants were not microfiltered. The resulting non-filtered supernatants contained both chemical and biological components of the soil suspension.

Filtered and non-filtered suspensions were applied to plants on the same date, at a rate of 15 ml soil suspension per cone. Two applications were made, the first at 11 days post germination and the second at 18 days post germination. Control plants received only water in place of soil suspension applications.

Soil microbial community characterization using 454 pyrosequencing

DNA was extracted from soil from each location using the UltraClean-htp 96-well soil DNA kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions, except for the addition of an extra ethanol wash and an additional purification step using AM-Pure Beads (Agencourt, Danvers, MA). Extracted DNA was quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) to verify a ratio of absorbance at 260:280 nm within the range of 1.7 and 1.9. PCR amplification and library preparation for pyrosequencing followed a previously published method [11]. Pyrosequencing was performed under contract with the University of Florida Genomics Facility.

MOTHUR version 1.24.1 [90], in combination with the Greengenes database [91], was used to classify Operational Taxonomic Units (OTUs) and their relative abundances using

previously outlined procedures [11]. Equalized subsampling resulted in 2119 reads per sample, and an average coverage of 73%. For diversity calculations, a genetic distance of 3% was used to provide the highest level of taxonomic information and to avoid possible pyrosequencing error. Microbial community diversity was evaluated using a variety of richness, diversity and evenness indices.

Analyses of soil properties

Multivariate analysis of variance based on dissimilarities [92] was conducted on soil physico-chemical data, soil bacterial taxonomic data, and soil community structure data to verify that soils varied with regard to these qualities. The analysis was conducted using the *adonis* function of the vegan package [93] using R 2.14.2 (www.r-project.org). The *adonis* function is a non-parametric method analogous to analysis of variance (ANOVA). *Adonis* was used rather than the more commonly used analysis of similarities [94] because *adonis* can accommodate both continuous and categorical predictors and their interactions [93]. *P*-values were based on 999 permutations.

Graphical visualization of differences between soils was accomplished via principal component analysis (PCA) for soil physico-chemical factors using R. To standardize for different units of measure, all variables were normalized to a mean of 1 by dividing each variable by its respective mean prior to PCA analysis. Differences in soil bacterial taxonomy were visualized via principal coordinate analysis (PCoA), using the Bray-Curtis distance matrix as calculated by the *labdsv* package [95].

Analyses of plant productivity

Plants were grown to 60 days of age to give adequate time for beneficial microorganisms to impact plant productivity [96]. At harvest, plant shoots were cut and collected for drying. Roots were collected for drying over the course of the next five days. Shoot and root tissues were dried at 70°C for 7 days and then moved to room temperature and humidity for at least three days before weighing.

Statistical analyses were performed on log-transformed biomass data. Mean dry biomass of plants from the filtered soil suspension treatments was compared individually to the non-amended control to assess effects due to chemistry. This comparison was done via a one-way ANOVA with Dunnett's *post-hoc* test. The same test was used to compare mean dry biomass of non-filtered controls and assess effects due to a combination of soil chemistry and soil biology. To isolate the soil biological effect on plant growth, two-way ANOVA tests were performed to compare non-filtered treatments with corresponding filtered controls. This comparison utilized an analysis of simple effects [97], which examines the effect of one factor (i.e., filtering) separately for each level of the other factor (i.e., soil). All results were expressed as back-transformed means ± standard error of the mean (S.E.M.).

A Pearson product-moment correlation coefficient (r) was computed to assess the relationship between plant biomass, soil physico-chemical factors, soil bacterial diversity and evenness, and the relative abundance of particular bacterial taxa.

Results

Soil physico-chemical properties

Soil properties varied widely amongst the nine collected soils (Table 2). Soil pH ranged from 4.7 to 7.6, with the highest pH in the agricultural potato soils. Soil electrical conductivity (EC) ranged from 0.2 to 0.9 mS, a four-fold difference between soils. Soil nitrogen (N) and carbon to nitrogen ratios (C:N) differed 3 to 4 fold across soils. Soil extractable phosphorus (P) and potassium (K) were also widely disparate, with values from less than 1 ranging to almost 40 ppm for P and more than 500 ppm for K. Zinc (Zn) and Copper (Cu) measurements showed similar ranges between soils of nearly 1 ppm to approximately 20 ppm. Manganese (Mn) ranged more widely from 2 ppm to over 250 ppm. Iron (Fe) showed the widest span of measurements ranging from less than 10 ppm to nearly 6500 ppm across soil samples. Analysis of the dissimilarity of soil physico-chemical properties using *adonis* revealed significant variation (r^2 = 0.515, p = 0.002) between the three management groups: potato, fallow, and forest. Principal component analysis was performed using all measured soil physico-chemical factors and separated the soils into three main groups (Figure 2.2a). These results demonstrate that a range of physico-chemical characteristics were represented across experimental soils and were strongly influenced by management type.

Soil microbiological community characteristics

Eleven bacterial classes were represented with abundance of 100 or more total reads across all soils. Averaged across all soils, the most abundant bacterial groups were Actinobacteria (33%) and Alphaproteobacteria (22%), and these classes consistently had the highest relative abundances across soils (Figure 2.3). The FAL2 soil differed from other soils

with high numbers of Synechococcophycideae, a subclass of Cyanobacteria. Similarly, high abundance of Oscillatoriophycideae was measured only for the FAL1 soil. Notably, the abundance of Synechococcophycideae and Oscillatoriophycideae were substantial enough in these soils to nearly equal levels of Alphaproteobacteria. Potato soils consistently contained higher numbers of Gammaproteobacteria (Figure 2.3), a microbial class that contains multiple species of known plant growth promoting rhizobacteria (PGPR).

Analysis of the similarity of soil bacterial community membership using *adonis* revealed significant ($r^2 = 0.641$, $p = 0.008$) variation between the three management groups. Compositional similarity of soil microbial communities across experimental soils was also evaluated via principal coordinate analysis (Figure 2.2b). Although the FAL3 soil microbial community was quite similar to the forest soil communities, the three management types showed non-overlapping confidence ellipses. Thus, a range of microbial communities was represented across the soils used in this experiment.

Analysis of soil microbial richness, evenness, and diversity

Alpha diversities were estimated for the various soil microbial communities, using a variety of indices including operational taxonomic unit (OTU) richness (approximation to the number of species present), evenness (relative abundance of species present), and diversity (a combination of richness and evenness). Complete analysis of the alpha diversity of the nine soils (OTUs at 3% genetic distance) is provided in Table 2.3. Microbial diversity (Shannon index) ranged from 5.47 to 6.35 across the nine experimental soils. The three potato soils had communities with the highest diversity levels, while the lowest diversity soil community was from an agriculturally fallow field (FAL2). Richness and evenness of the soil microbial

communities followed patterns of soil microbial diversity, with the highest values exhibited by potato soils and lowest in FAL2.

Analysis of the dissimilarity of soil bacterial community structure using *adonis* revealed a significant ($r = 0.795$, $p = 0.04$) variation between the three management groups. In summary, the soils used in this work spanned a range of microbial diversity and evenness.

Soil chemistry influences plant productivity independent of soil biology

The chemical components of each of the nine soil treatments were evaluated for effects on plant productivity. Significant differences (ANOVA; $p < 0.05$) in shoot biomass in filtered (F) treatment groups were observed when compared to the non-amended control, with POT1, POT2, FAL1, and FAL2 significantly reducing shoot biomass (Figure 2.4a). Roughly equal reduction in shoot biomass was measured for all four of these treatments, and did not correspond with any measured soil physico-chemical properties. Significant reduction of alfalfa root biomass was observed only for the POT1 filtered treatment (data not shown). None of the filtered soil suspension applications had a positive effect on plant productivity when compared to the non-amended control. Soil physico-chemical properties were poorly correlated with plant biomass ($r < 0.50$, $p > 0.10$; Table 2.4).

Soil microbiology influences plant biomass

Four non-filtered treatments, FAL2, FAL3, FOR1 and FOR3, showed significantly reduced shoot biomass compared to the non-amended control (one-way ANOVA with Dunnett's *post hoc*; $p < 0.05$). All other soil suspensions produced shoot biomass not statistically different

from the non-amended control (Figure 2.4b). Root biomass followed the same pattern as shoot biomass (data not shown).

Plants receiving non-filtered treatments were exposed to both chemical and biological fractions of the soil suspensions. Therefore, these plants were subject to the same chemical components that resulted in reduced biomass with filtered suspensions, but also included soil microbiological components. As such, each non-filtered suspension was also compared with its filtered counterpart. Inclusion of the biological components in the non-filtered soil suspensions were the sole difference between these two treatment groups, allowing for the separation of soil microbiological effects on plant biomass. Both positive and negative effects on plant biomass by soil microbial suspensions were observed (Figure 4). Statistically significant negative influences on alfalfa shoot and root biomass were observed for the non-filtered treatments from FAL3 (shoot only), FOR1 (root only), and FOR3 (root and shoot) soils (Figure 2.5). The negative effect on both root and shoot biomass was particularly pronounced in the non-filtered FOR3 treatment. Treatments POT1 and FAL1 showed a significantly positive effect on alfalfa shoot biomass ($p < 0.05$) relative to the filtered control (Figure 2.5a). Alfalfa root biomass was affected similarly, with significant increases in the POT1 treatment ($p < 0.05$), although increases in the FAL1 treatment were not statistically significant (Figure 2.5b).

Plant biomass measurements did not correlate with soil bacterial richness or diversity (Table 2.5). However, biomass was correlated with soil bacterial evenness, but only marginally significantly ($0.05 < p < 0.1$). Shoot biomass correlated more strongly than root biomass. Notably, correlations with bacterial richness and diversity were higher than for any of the measured soil physico-chemical properties, ranging upwards to 0.60. Among bacterial classes, the relative abundance of Deltaproteobacteria was significantly correlated with increased root

biomass ($r = 0.68$; $p = 0.04$; Table 2.6). Deltaproteobacteria was also the class with the highest correlation of relative abundance to shoot biomass, although this result was not statistically significant ($r = 0.53$; $p = 0.13$; Table 2.6). Correlations of shoot and root biomass with the relative abundance of known alfalfa mutualists (order *Rhizobiales*, family *Rhizobiaceae*, and genera *Rhizobium* and *Sinorhizobium* were not significant ($r < 0.50$, $p > 0.20$; data not shown).

Discussion

Soil physico-chemical properties influence plant biomass

Filtered soil treatments included all soil suspension components capable of passing through the 0.45 μm pore filter. Most fungal spores and average sized bacteria (typically 1 – 1.5 μm) would not pass through this filter. However, ultramicrobacteria, defined as bacteria smaller than 0.3 μm in size, may pass through [98] but are a very limited taxonomic group. Viruses would also be small enough to escape filtration. However, it is important to note that viruses cannot infect the plant host without a vector or injury to allow entry into the cytosol [99]. No symptoms of viral infection were observed on plants in this experiment. Therefore, filtered components largely represent the chemical fraction of the soil suspension, potentially including small amounts of herbicides, fungicides, secondary metabolites and sugars or their breakdown products. Each suspension treatment was applied only twice, for a total volume of 30 ml to each alfalfa plant. Despite this small volume, a significant reduction in plant biomass occurred in four filtered soil suspensions: the organic potato, one conventional potato, and fallow soils from Wisconsin and France (POT1, POT2, FAL1, FAL2). Because the non-amended control treatment was also deprived of beneficial microbial interactions, these biomass decreases were not due to the exclusion of any beneficial microorganisms by the filtration process. Interestingly,

agricultural soil suspensions were associated with the most significant reductions in biomass, which is the opposite of what might be expected from high fertility soils. Plants in our experiment were supplied with fertilizer, which may have made them insensitive to additional nutrients in soil filtrates. None of the measured soil physico-chemical factors were significantly correlated with plant biomass (Table 2.4). The identities of the factors in these soil suspensions that reduced biomass are unknown, but could include phytotoxic compounds produced by pathogens or traces of herbicides. Rhizobacteria have been shown to effectively degrade organic compounds in the soil [100]. Given the negative effect of the filtered suspensions on plant growth, it might be that bacteria present in non-filtered suspensions of organic potato soil (POT1) and Wisconsin fallow soil (FAL1) successfully degraded phytotoxic compounds, allowing plants to grow normally. This metabolic breakdown of phytotoxins could effectively neutralize the negative soil effects due to soil chemistry.

Soil microbial diversity differentially influences plant biomass

Four non-filtered soil suspensions: France fallow soil (FAL2), Sweden fallow soil (FAL3) and the two pine forest soils (FOR1 & FOR3), significantly reduced shoot and root biomass (Figure 3b). These effects support previous reports showing that soil microbial communities influence plant biomass [17, 20, 85, 101] and suggest that the degree and direction of influence varies among communities. Given that the observed effect on plant biomass was negative relative to the non-amended control, it is likely that the Sweden fallow (FAL2) and Oregon pine forest (FOR3) soil suspensions exerted pathogen pressure on alfalfa plants. Since no differences in bacterial pathogens populations were identified by soil sequencing analysis, the pathogen pressure was likely fungal in nature. Interestingly, the Sweden fallow (FAL2) and

Oregon pine forest (FOR3) soils also had the lowest diversity and evenness measures (Simpson indices) of the nine soils. Previous research has shown that increased microbial diversity results in improved pathogen exclusion [24], which suggests that these low diversity soils may have better allowed pathogen establishment. It is also notable that shoot biomass was more highly correlated with soil bacterial diversity and evenness than with soil physico-chemical factors (Table 2.5 and Table 2.4).

Relatively weak relationships between bacterial diversity and increased plant biomass in this work may be related to the use of realistically complex, naturally assembled microbial communities. For instance, particular taxa capable of strong effects may be less likely to achieve dominance or high abundance in these communities compared to dramatically simpler artificial consortia. One limitation of our sequence-based characterization of the microbial communities used to generate soil suspensions is that our analysis did not include fungi. The ITS primer sets typically used for identifying fungi have inherent biases toward particular taxonomic groups and result in shorter sequence lengths that do not allow classification to genus or species level [102, 103]. The relationship between plant biomass and soil microbial community diversity measures may have been stronger if the fungi were included. It is also possible that niche complementarity plays a more important role in fungal communities than bacterial communities, due to the ability of bacteria to share functional genes via horizontal gene transfer [104]. If so, bacterial diversity may not be as important as fungal diversity for plant biomass. The provision of adequate nutrients to the experimental plants may have also minimized the importance of bacterial diversity, since previous studies have shown that soil microbial diversity is more likely to increase productivity in low nutrient environments [27].

Given that soil microbial effects on plant biomass are a combination of diversity, evenness, total microbial biomass, and specific taxa present in the soil, diversity effects on biomass may also be masked by other soil factors that reduce total productivity. One recent study found that plant productivity was not a function of soil bacterial diversity, but rather the ratio of beneficial bacterial species to detrimental bacterial species [29]. These findings emphasize the complexity of relationships between bacterial communities and plant growth performance.

Soil microbiology can compensate for negative effects due to soil chemistry

Four filtered soil suspensions included unidentified physico-chemical factors that reduced plant biomass (Figure 2.4a). The corresponding non-filtered soil suspensions also contained the negative factors reducing biomass. However, two instances of counter-acting biological and chemical effects were observed in the organic potato (POT1) and Wisconsin fallow (FAL1) soil suspensions. Filtered soil suspensions inhibited biomass accumulation relative to the non-amended control (Figure 2.4), while non-filtered counterpart treatments reversed this effect (Figures 2.4 and 2.5). This suggests the possibility of soil biological factors counter-acting the effects of soil chemical factors. In addition to community level effects, promotion of plant growth can also be a direct or indirect effect of specific microbial members of the soil community [12]. Proposed mechanisms for microbial induced plant growth have included: nutrient acquisition [59, 86], pathogen antagonism [47, 49, 50], increase in photosynthetic capacity [51, 87], osmotic stress tolerance [88] and plant hormone production [44, 45]. Rhizobacteria present in the non-filtered soil suspensions could function to improve plant growth via any of these mechanisms. However, the improved growth may not have been detected due to counteracting negative effects due to soil chemistry.

Soil microbial community membership and plant biomass

Our study found a significant positive correlation between the relative abundance of Deltaproteobacteria and improved plant growth. Deltaproteobacteria are not well-documented as containing plant-growth promoting species, but are characterized by propagation via spores and syntrophic metabolism. Syntrophic metabolism refers to the communal breakdown of a wide variety of compounds, including saturated fatty acids, unsaturated fatty acids, alcohols and hydro-carbons [105]. This adaptive form of metabolism could allow Deltaproteobacteria to degrade a wide variety of chemical compounds not available as substrates for other organisms or in isolation. The ability to degrade potentially phytotoxic substrates offers one potential explanation for our results and suggests that the rhizosphere competence of Deltaproteobacteria should be investigated further. Further studies elucidating which bacterial species in soil suspensions successfully colonize the rhizosphere will be necessary to clarify the role of Deltaproteobacteria and to identify which other taxa are influential to plant growth.

The protocol outlined in this research could also be a valuable tool for exploring ecological functions of soil bacteria that are relevant to plant growth. Identifying bacteria present in rhizosphere soils, in combination with documented changes in plant traits, can expand our knowledge of agriculturally and ecologically relevant soil microorganisms. Soil suspensions provide an intriguing low-tech solution for transferring experimental results to a field environment.

Soil biology and soil chemistry were shown to have contrasting effects on plant growth, particularly shoots. There is a strong potential that such mutually counteracting soil chemical and biological effects on plant growth have gone unnoticed in other experimental systems. Our results suggest that higher soil bacterial diversity does not significantly increase plant biomass

production. However, plant biomass was more highly correlated with soil bacterial diversity than with soil physico-chemical factors. Further research is needed to confirm these findings and to account for the additional influence of fungi and soil fauna. Improved methods for studying naturally-assembled microbial communities will advance soil research done in greenhouses toward outcomes that more closely mimic field-production environments.

Figures

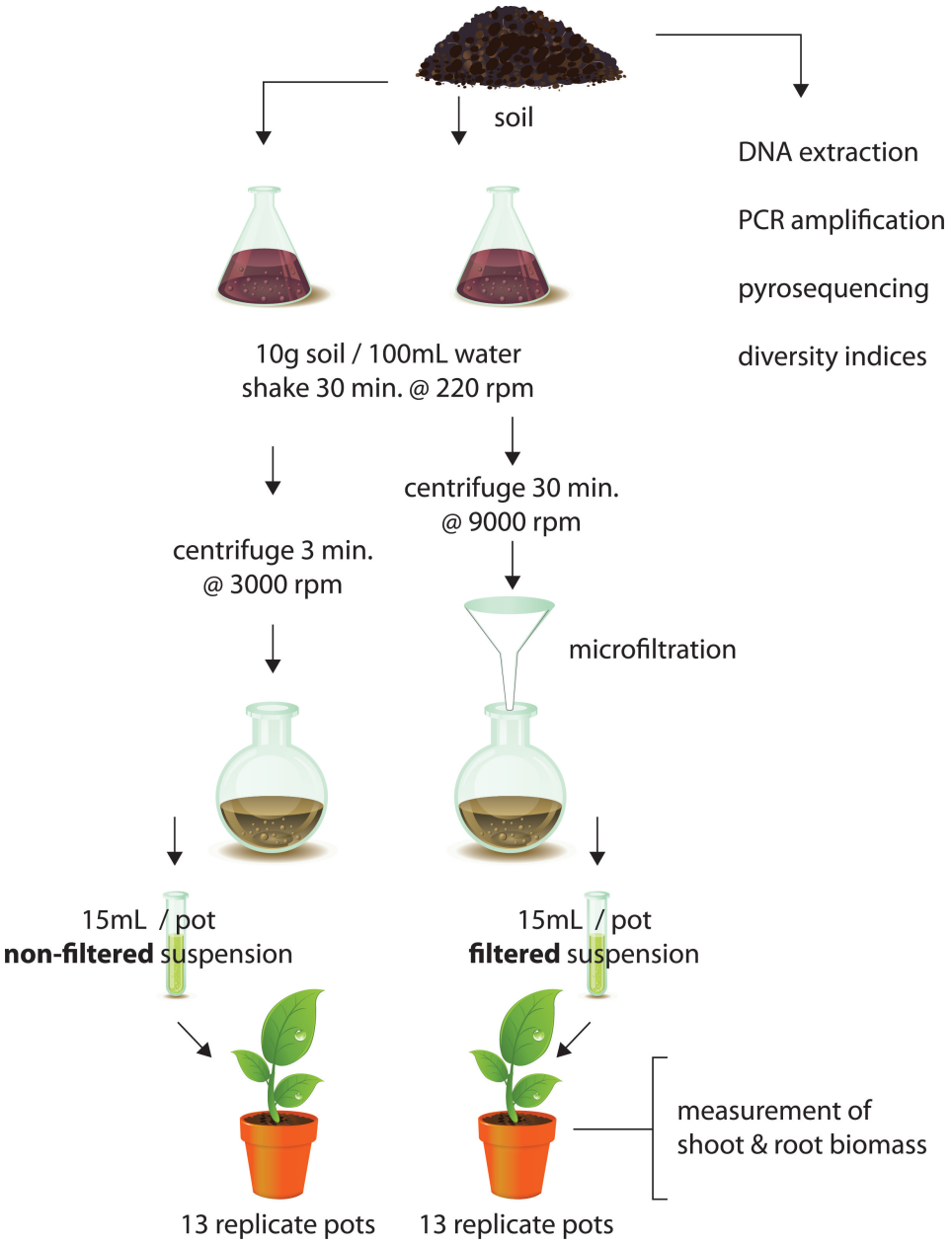
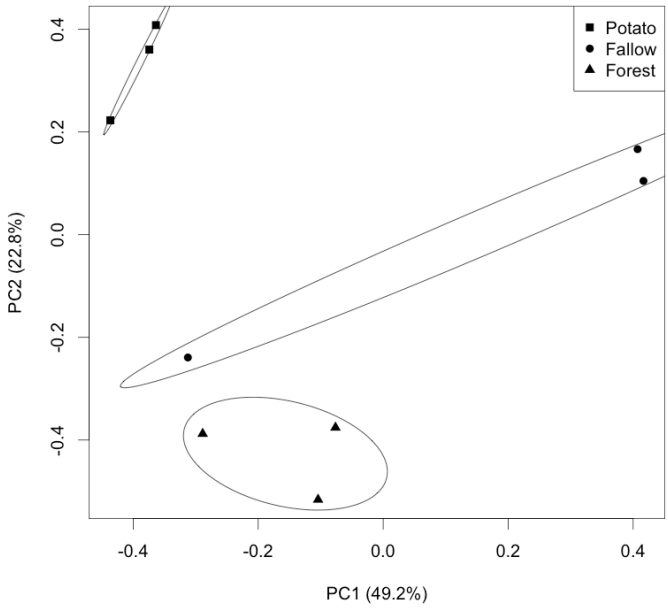


Figure 2.1 Schematic diagram of the experimental design described in the Materials and Methods. This procedure was performed for each of 9 different soils.

a)



b)

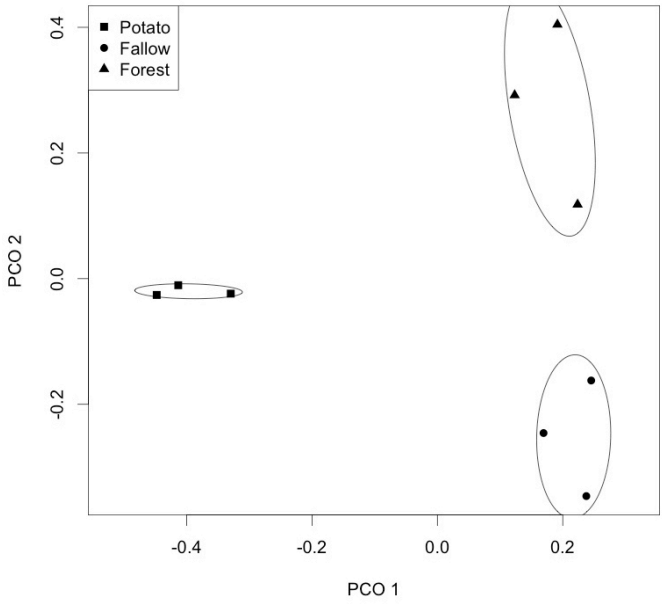


Figure 2.2 Principal Component Analysis (PCA) illustrates the clustering of soils for a) the soil physico-chemical factors (by management type) and b) Principal Coordinate Analysis (PCoA) illustrates clustering by management type of soils for soil bacterial composition. PCO utilized the Bray-Curtis distance matrix using OTU relative abundances at 3% genetic distance.

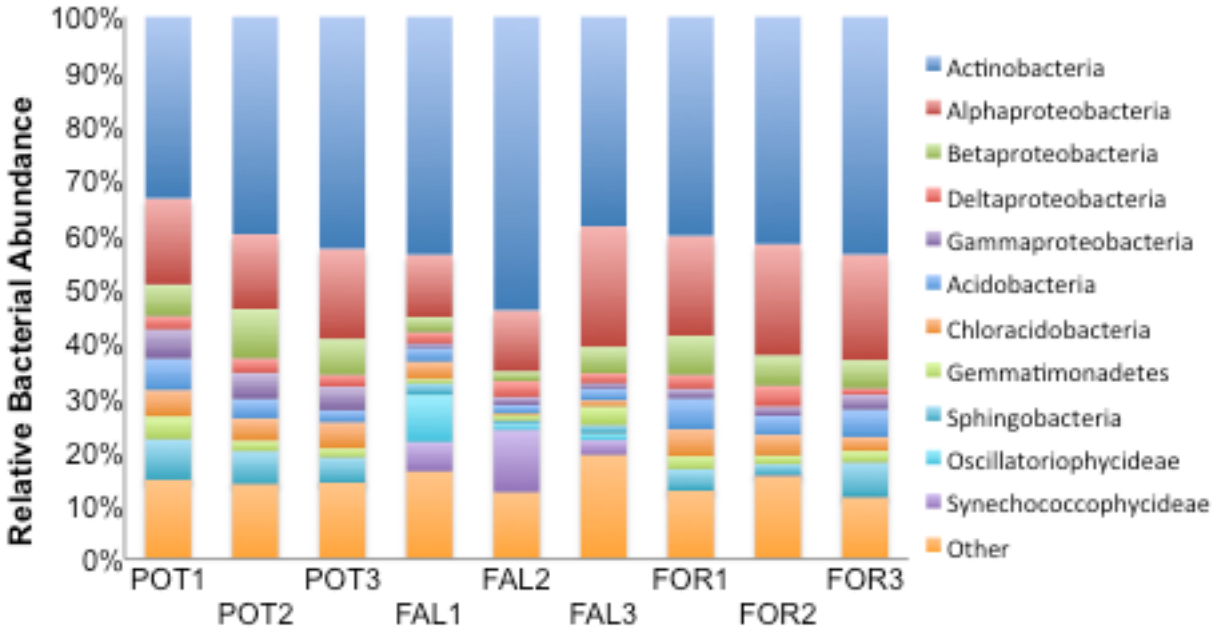
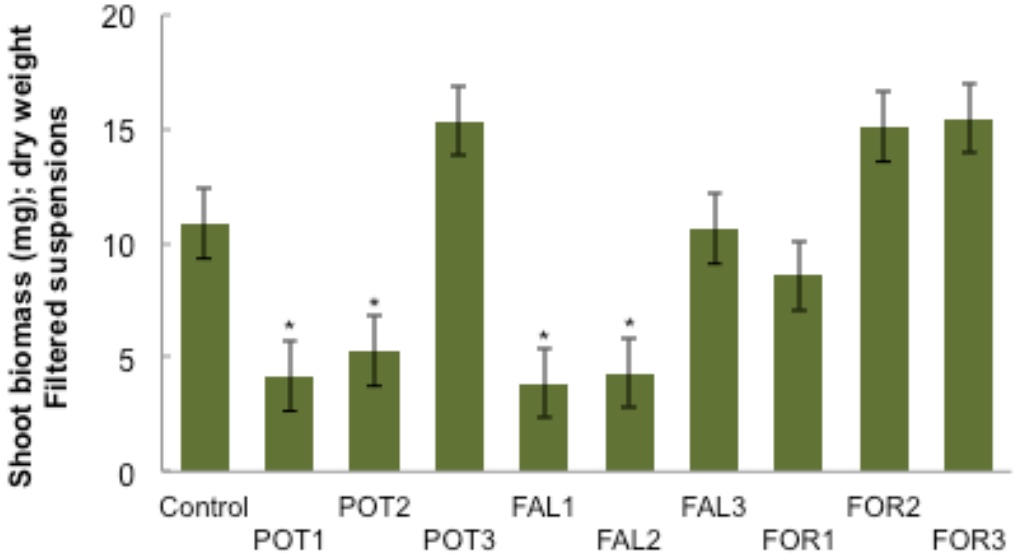


Figure 2.3 Relative abundance of bacterial taxa at the class level detected in 9 soils used in soil suspensions. Classes represented by fewer than 100 sequences were grouped into the 'Other' category for simplification of presentation.

a)



b)

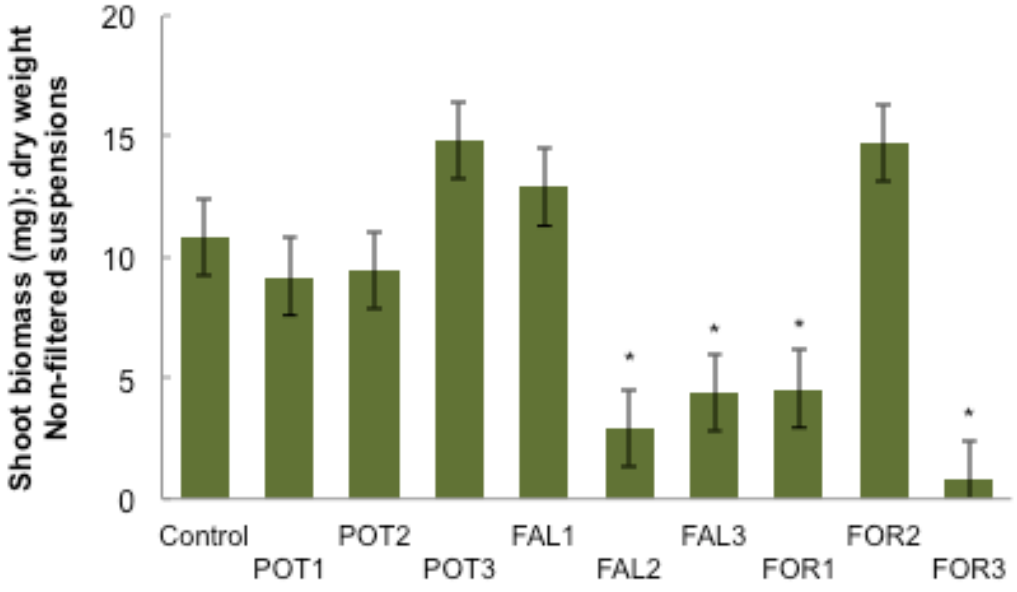
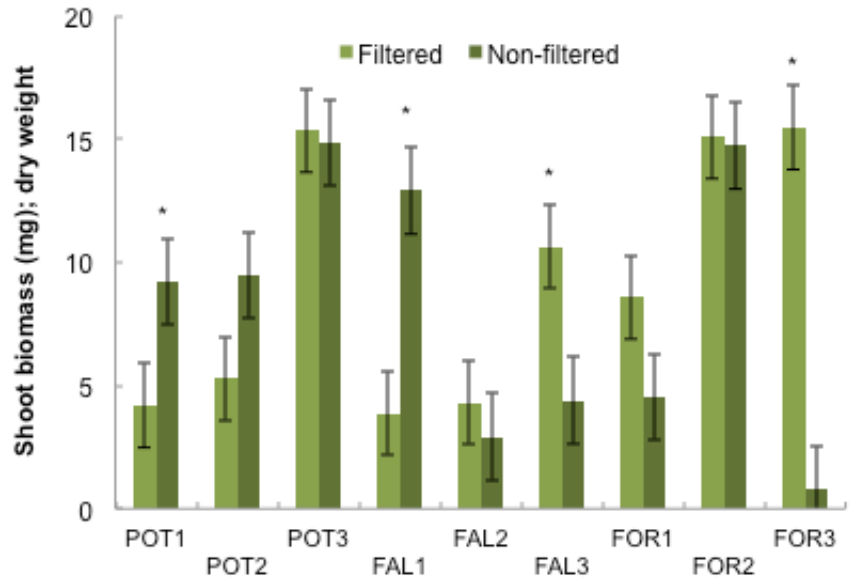


Figure 2.4 Average alfalfa shoot biomass (dry weight) for a) filtered soil treatments and b) non-filtered soil treatments as well as the non-amended control are shown. Error bars represent standard error. * indicates statistically significant differences compared to the non-amended control (ANOVA with post hoc Dunnett's test; $p < 0.05$).

a)



b)

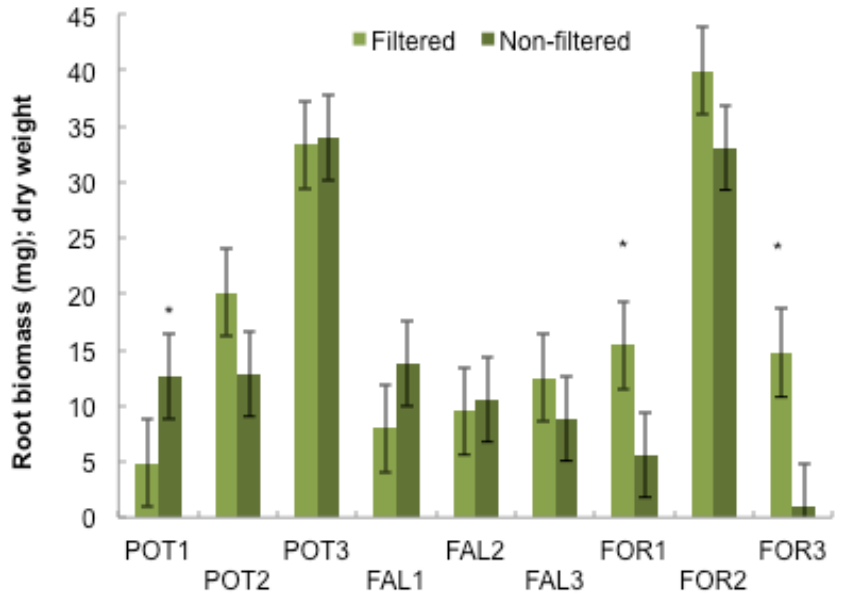


Figure 2.5 Average dry weight of a) shoot and b) root dry weight biomass for each non-filtered soil suspension relative to its filtered counterpart. Observed biomass differences shown here can be attributed to the presence of soil biota. Error bars represent standard error. * indicates that filtered and non-filtered treatments differed significantly (Two-way ANOVA, $p < 0.05$)

Table 2.1 Site descriptions for 9 soils used in soil suspension applications

Soil Label	Location	Description	Management
POT1	Colorado	Potato cropping	Organic
POT2	Colorado	Potato cropping	Conventional
POT3	Colorado	Potato cropping	Conventional
FAL1	Wisconsin	*Arabidopsis thaliana* dominated	Fallow
FAL2	France	*Arabidopsis thaliana* dominated	Fallow
FAL3	Sweden	*Arabidopsis thaliana* dominated	Fallow
FOR1	Colorado	Pine Forest	Natural Area
FOR2	Oregon	Douglas-Fir Forest	Natural Area
FOR3	Oregon	Pine Forest	Natural Area

Table 2.2 Soil physico-chemical properties of nine soils used in soil suspension applications.

	pH	EC (mS)	N (%)	C (%)	C:N	NO_3-N (ppm)	P (ppm)	K (ppm)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)
POT1	7.6	0.9	0.10		9.8	26.4	39.7	585	4	9	2.1	1.5
POT2	7.4	0.5	0.08		9.6	11.9	14.7	229	3	7	1.8	1.3
POT3	6.3	0.8	0.06		10.2	10.5	23.7	200	3	10	10.7	1.8
FAL1	5.8	0.3	0.07		13.5	8.3	3.7	42	1	21	2.7	2.5
FAL2	4.7	0.5	0.19		10.6	19.2	4.3	43	4	6429	199	7.5
FAL3	4.9	0.2	0.07		15.0	4.1	23.7	36	21	5326	269	5.7
FOR1	5.8	0.3	0.24		31.3	0.4	0.1	0.1	6	118	12.9	1.6
FOR2	5.5	0.4	0.61		9.5	22.0	2.5	452	6	106	10.3	27.8
FOR3	5.4	0.3	0.33		13.8	6.0	6.8	241	3	71	14.3	1.9

Table 2.3 Bacterial alpha diversity (OTUs, defined at 3% genetic distance) for nine soils used in soil suspension applications.

		Shannon		Simpson	
Soil	S_{chao}	H	E_H	E_D	S_D
POT1	2815	6.35	0.92	0.29	291
POT2	2731	6.23	0.91	0.22	219
POT3	2803	6.18	0.90	0.21	199
FAL1	1929	5.89	0.89	0.17	141
FAL2	1694	5.47	0.84	0.11	76
FAL3	2105	5.77	0.87	0.12	93
FOR1	1774	5.78	0.87	0.12	94
FOR2	2119	5.96	0.88	0.14	122
FOR3	2144	5.96	0.88	0.11	93

S_{chao} = estimated richness, H = diversity index, E_H and E_D = evenness, S_D = effective species or # of species at observed diversity index assuming complete equitability in abundance

Table 2.4 Soil physico-chemical properties correlated poorly with plant biomass (filtered treatments, dry biomass). Values shown are Pearson's correlation values relating soil physico-chemical factors and measured plant characteristics. No statistically significant correlations were revealed.

	Shoot biomass	**Root biomass**
pH	0.48 *(0.19)*	0.33 *(0.45)*
EC	0.40 *(0.29)*	0.49 *(0.19)*
N	-0.03 *(0.94)*	0.04 *(0.91)*
C	-0.07 *(0.86)*	-0.17 *(0.62)*
C:N	-0.34 *(0.37)*	-0.45 *(0.23)*
NO3-N	0.38 *(0.31)*	0.49 *(0.18)*
P	0.21 *(0.59)*	0.21 *(0.59)*
K	0.37 *(0.32)*	0.33 *(0.39)*
Zn	-0.24 *(0.53)*	-0.14 *(0.72)*
Fe	-0.46 *(0.22)*	-0.17 *(0.67)*
Mn	-0.44 *(0.24)*	-0.19 *(0.63)*
Cu	0.33 *(0.39)*	0.46 *(0.21)*

Table 2.5 Relationships between soil microbial diversity and plant biomass (Pearson's correlation coefficients). P-values are shown in parentheses. See Table 3 for definitions of diversity measures.

		Shannon		**Simpson**	
	S_{chao}	H	E_H	E_D	S_D
Shoot biomass	0.45 *(0.22)*	0.52 *(0.16)*	0.55 *(0.12)*	0.60 *(0.08)*	0.56 *(0.12)*
Root biomass	0.41 *(0.27)*	0.33 *(0.38)*	0.36 *(0.34)*	0.47 *(0.20)*	0.43 *(0.25)*

Table 2.6 Relationship between soil bacterial abundance and plant biomass (Pearson correlation coefficients). Values shown are Pearson's correlation values relating soil bacterial taxa abundance and measured plant characteristics. * indicates ($p < 0.05$).

	Shoot biomass	**Root biomass**
Actinobacteria	-0.34 *(0.37)*	-0.09 *(0.81)*
Alphaproteobacteria	-0.17 *(0.67)*	-0.14 *(0.72)*
Betaproteobacteria	0.22 *(0.55)*	0.16 *(0.68)*
Deltaproteobacteria	0.54 *(0.13)*	0.68* *(0.04)*
Gammaproteobacteria	0.24 *(0.54)*	0.23 *(0.56)*
Acidobacteria	-0.17 *(0.65)*	-0.40 *(0.28)*
Chloracidobacteria	0.56 *(0.12)*	0.36 *(0.35)*
Gemmatimonadetes	-0.13 *(0.73)*	-0.20 *(0.61)*
Sphingobacteria	-0.03 *(0.93)*	-0.20 *(0.60)*
Oscillatoriophycideae	0.28 *(0.47)*	0.02 *(0.95)*
Synechococcophycideae	-0.26 *(0.50)*	-0.08 (*0.83)*
Other	0.43 *(0.25)*	0.35 *(0.35)*

CHAPTER 3: SOIL MICROBIAL DIVERSITY DIFFERENTIALLY INFLUENCES INVASION BY PATHOGENIC AND BENEFICIAL MICROORGANISMS

Introduction

Plants and soil microorganisms (the soil microbiome) have evolved intimate relationships that can be either beneficial or detrimental to plant health and growth [1]. For instance, disease-suppressive soils provide a well-studied example of how the soil microbiome discourages community invasion by pathogenic organisms and in some cases improves productivity. Higher soil species richness [106], diversity [24], evenness [35] and microbial biomass [22, 23] have been credited with an increase in soil disease suppressiveness. In contrast, soils with lower biodiversity and evenness are more vulnerable to pathogen invasion and disease [18, 71].

Obviously, not all soil environments are naturally disease suppressive. In these cases, manipulating the soil microbial community for improved plant health and productivity could become an important tool [2]. One such option involves the use of beneficial microbial inoculants also known as plant growth promoting rhizobacteria (PGPR). These microorganisms could colonize the rhizosphere and provide functionalities to the plant such as exclusion of pathogens, antagonism of pathogens via antibiotics, and production of plant growth and stress alleviating hormones [1]. However, despite proven success in controlled experimental environments, evidence of inoculant success in the field is lacking.

The goal of this study was to determine whether soil bacterial diversity affects pathogen or PGPR colonization and effects. Previous research has shown that soil microbial communities

function in disease suppression. So, the hypothesis for this experiment was extended to include PGPR. Our hypothesis was that natural soil suspensions with higher bacterial diversity would result in reduced disease and reduced increases in plant biomass with PGPR application. Previous experiments focusing on effects of soil bacterial diversity have incorporated only one soil type [24, 29]. To expand upon previous methods, this study emphasizes a variety of soil types while incorporating two different inoculation treatment groups: an alfalfa pathogen or an alfalfa PGPR. Soils were selected with the goal of optimizing variation in microbial community composition and diversity. To this end, four soils with varying geography and management histories were collected and characterized for soil physico-chemical and biological characteristics. Realistically complex microbial communities were suspended in water using a range of field soils and used to populate alfalfa growing in a greenhouse environment. In addition, suspensions of soil microbes and corresponding filtered controls were prepared for each soil and applied to plants growing in a standard inert medium. Subsequently, either a PGPR or pathogen was introduced into the various soil communities and resulting alfalfa biomass was measured. In summary, this experimental design allowed for screening of potential differences in the ability of a pathogen or PGPR to establish in the presence of an indigenous soil community, while also considering the involvement of soil physico-chemical factors.

Materials and Methods

Soil descriptions and analyses

Four soils with varying locale and management were used in the experimental study (Table 1). Two soils were collected from agricultural soils in the San Luis Valley of Colorado:

one organic production field for potato and one conventional production field for potato. These two fields had been in production for at least three years and represent different rotation of crops, potato cultivars, and pesticide usage. The third soil was from an agriculturally fallow site where *Arabidopsis thaliana* was naturally present. The fourth soil was collected from a forest site managed by the US Forest Service and dominated by Ponderosa Pine (*Pinus ponderosa*). Soil samples were taken from at least three locations in each field from the top 15 cm of the soil profile. All three samples were combined, mixed thoroughly to homogenize and stored at 4°C.

Physico-chemical profiles for all soils were characterized by the Colorado State University Soil Testing Facility including soil pH, electric conductivity (EC), C:N ratio, nitrate (NO_3-N) and various mineral nutrients and represent a subset of data presented in Chapter 1.

Soil microbial community characterization using 454 pyrosequencing

Soil DNA was extracted according to the manufacturer's instructions using the UltraClean-htp 96-well soil DNA kit (MoBio, Carlsbad, CA), except for the addition of an extra ethanol wash and an additional purification step using AM- Pure Beads (Agencourt, Danvers, MA). Preparation for pyrosequencing, including PCR amplification and library preparation, was conducted according to the method published in [39]. University of Florida Genomics Facility performed pyrosequencing analysis using a 454 Life Sciences GS FLX system with standard chemistry.

Soil bacterial diversity measures and taxonomic classification were determined using MOTHUR version 1.24.1 [90]. Sequences were removed if they contained ambiguous bases, quality scores ≤ 25, or holopolymers ≥ 10. Remaining sequences were aligned using the SILVA alignment [107] and gaps were removed. Chimeras were detected using uchime [108] and also

removed. Finally, the resulting sequences were classified with the Greengenes database [91] using the MOTHUR-embedded naïve Bayesian classifier [109]. Any sequences identified as chloroplast were removed. Following classification, sequences were screened so that 95% of sequences had the same minimum length and endpoint (optimize=minlength-end, criteria=95). Next, so that all sequences covered the same genetic space, sequences were filtered again for vertical alignment and gap removal (vertical=T, trump=.). An average coverage of 73% was achieved with 2119 reads per sample after equalized subsampling. The typical genetic distance of 3% was used for defining Operational Taxonomic Units (OTUs) and reported OTU abundances are relative abundances. Relative abundance was determined by dividing the number of reads for any given OTU by the total number of reads obtained for each soil. Diversity indices including Chao, Shannon, Shannon Evenness, Simpson, and Inverse Simpson indices were also calculated using MOTHUR.

Seedling establishment

A sterile, inert growing media of sand and vermiculite (1:1, v:v) was thrice steam sterilized for 30 minutes in an autoclave and filled into cone-shaped pots. The cone-shaped pots, 4 cm in diameter x 20.7 cm long, were randomized in racks in a block design. The Saranac cultivar of *Medicago sativa* was chosen for its susceptibility to alfalfa root rot disease in order to provide adequate disease incidence rates in control plants. Alfalfa seeds were first surface-sterilized in 3% sodium hypochlorite solution for 10 minutes, then rinsed three times with sterilized distilled water. Micro-centrifuge tubes were filled with 0.7% water agar and populated with one alfalfa seed, then maintained at 37°C in a growth chamber with 12h light/dark cycles.

Seedlings were transplanted at 5 days post germination (dpg) into prepared cone-shaped pots and placed in a greenhouse on a misting bench that provided constant moisture and humidity. At 18 dpg, racks of cone-shaped containers were moved to a standard greenhouse bench. Fertilization using a Dosatron fertilizer injector (Dosatron, Clearwater, FL, USA) was done every 5 days with consistent 15-5-15 NPK fertilizer with added micronutrients.

Soil microbial community suspensions

Two types of microbial suspensions were prepared from the four soils, filtered and non-filtered. Each of the four soils was combined with Millipore filtered water in a flask at 1:10 (w:v). Consequently, flasks were shaken at room temperature for 30 minutes at 220 rpm and set aside for 30 minutes to settle. The supernatant was decanted into 500 mL centrifuge bottles and centrifuged for 3 minutes at 3000 rpm to remove soil particles. The remaining solution was left unfiltered and was used as a soil microbial suspension to populate sterile media with four different soil microbial communities. Filtered suspensions were prepared similarly the previous day. For filtered preparations, the supernatant was centrifuged for 30 minutes at 9000 rpm to remove remaining soil particles, then decanted and vacuumed through a 0.45 μm pore filter (Merck, Billerica, MA, USA). Filtered suspensions were kept at 4º C overnight. Most average-sized bacteria and fungal spores are larger than 0.45 μm and would not pass through this filter. Ultramicrobacteria, defined as bacteria smaller than 0.3 μm in size, may pass through at this level of filtration [98].

At 11 dpg, filtered and non-filtered suspensions were applied to at a rate of 15 mL soil suspension to each cone containing approximately 145 g of autoclaved sand and vermiculite (1:1, v:v) mixture. A second soil suspension application was made at 18 dpg.

Microbial inoculants

Treatments were further divided into three groups: a soil-suspension-only control, added plant growth promoting rhizobacteria (PGPR), or added pathogen groups. One-third of each rack received a different microbial treatment for a total of 24 unique treatment groups (Figure 3.1).

At 21 days post germination, the PGPR inoculum was applied to 13 of 39 cones for each soil suspension treatment at a rate of 15mL per cone (containing 143 g of soil media). Strain KT2440 of *Pseudomonas putida*, a plant growth promoting rhizobacteria (PGPR) with kanamycin resistance, was grown for 8 hours in LB broth with kanamycin (50 μg / L). The culture was pelleted via centrifugation for 10 minutes at 7000 rpm. The pellet was rinsed with sterile water three times and re-suspended in phosphate buffered saline (PBS) at a standardized optical density (OD) of 2 x 10^7 cfu/mL in preparation for application.

Alfalfa pathogen, *Phytophthora medicaginis* strain M2019, an oomycete which causes root rot in *Medicago sativa*, was obtained from Dr. Deborah Samac at University of Minnesota. *P. medicaginis* was cultured according to a previously published method [110] for 2 weeks prior to application to allow for complete growth over the V8 agar plate. Two days prior to inoculation, sporulation was induced according to the method described by Gray et. al. (1983). Inoculum was prepared by blending mycelial mats and zoospores from six 9mm v8 agar plates with 200mL sterile water. At 22 days old, a mixed inoculum of mycelium and oospores was applied to one-third of the replicates for each soil suspension treatment group. Inoculum was applied at a rate of 3mL per cone. In addition, one-third of each soil treatment group received no added inoculant bringing total treatment groups to twenty-four (4 soils x 2 preparations (filtered or non-filtered) x 3 inoculant types (PGPR, pathogen, or none). An additional thirteen plants

served as untreated controls and did not receive a soil suspension application. Untreated controls received 15 mL water in place of inoculation with PGPR or pathogen.

DNA extraction for qPCR and generation of standard curves

Root tissue from three alfalfa plants from each treatment group were collected for qPCR analysis and stored at -80°C until DNA was extracted. DNA was isolated from the roots using the PowerSoil DNA 96-well Kit (MoBio Laboratories, Inc. Solana Beach, CA) according to the manufacturer's instructions, except for the addition of an extra ethanol wash. For the q-PCR standard curves, duplicate cultures of *P. putida* KT2440 (GFP) were grown in LB broth containing 50 μg/mL kanamycin antibiotic at 30°C and 220 rpm. DNA was extracted and purified using the UltraClean® Microbial DNA Isolation kit (MoBio Laboratories, Inc. Solana Beach, CA), and 10-fold serial dilutions were made for qPCR analysis. Total DNA for serial dilutions of *P. putida* KT2440 (GFP) was quantified using a PicoGreen dsDNA Quantitation Kit (Invitrogen, Carlsbad, CA) using a modified protocol. Working PicoGreen reagent was prepared by diluting the concentrated DMSO-PicoGreen stock solution, provided in the PicoGreen kit, 1:200 with TE according to the kit instructions. The extracted *P. putida* KT2440 DNA sample was added in 2μL quantity to 198 μL of PicoGreen dsDNA working reagent and incubated for 5 minutes at room temperature. Fluorescence was determined using a calibrated TBS-380 Mini-Fluorometer, which quantified dsDNA. Standard dilutions of extracted KT2440 DNA were subsequently prepared at 25, 20, 15, 5, and 1 ng/μL with TE. Concentrations of KT2440 DNA dilutions were confirmed via the same protocol using lambda DNA standards included in the PicoGreen kit.

Primer pairs and qPCR analysis

The qPCR primers were designed for the green fluorescent protein gene (*gfp*) (Integrated DNA Technologies, Coralville, IA) (Table 3.2). Different forward and reverse primer concentrations were tested in order to obtain the minimum cycle threshold values and provide optimal amplification. Subsequently, a range of annealing temperatures was tested using serial dilutions of the standard and the optimal primer concentrations. An annealing temperature of 54°C was chosen based upon an absence of primer dimers and minimum cycle threshold values. Following optimization, the qPCR reactions for the experimental samples were performed in 96-well plates and contained 1000 nM of each forward and reverse primer, 10 μL Maxima SYBR Green Supermix (Thermo Scientific, Waltham, MA), and 2 μL of isolated soil DNA. The qPCR was initiated with a 8.5 min cycle at 95 °C and followed by 35 cycles of denaturing for 15 s at 95 °C, annealing for 30 sec at 54 °C, and extension for 60 sec at 72 °C. The qPCR was carried out in a spectrofluorimetric thermal cycler, iCycler IQ™ Real-Time PCR Detection System (BIO-RAD). In all qPCR reactions, to check for singular product, reaction products were analyzed by melt curve analysis and confimed on a 3% agarose gel. The amount of amplified *gfp* was calculated using cycle threshold values obtained from triplicate samples. Cycle threshold values were converted to ng DNA obtained from *P. putida* KT2440 using the four-point external standard curve generated from pure KT2440 DNA. Difference in levels of detected *gfp* for various soil suspension treatments was determined via the non-parametric Kruskal-Wallis analysis of variance in combination with Wilcoxon Rank Sum tests ($\alpha = 0.05$).

Analyses of plant biomass production and microbial effects

Previous research indicates that alfalfa biomass increases due to *P. putida* inoculation may not be significant until 8 weeks post inoculation [96]. Therefore, all plant shoots were cut and collected for drying at 60 dpg to maximize plant biomass increases due to PGPR inoculation. Soil was removed from roots and root surfaces were rinsed prior to collection. For biomass measurements, shoot and root tissues were dried at 70°C for 7 days. Prior to weighing, tissue samples were kept at room temperature and humidity for at least three days.

Separate comparisons were made of filtered and non-filtered soil suspension treatments. Pairwise comparisons of alfalfa shoot and root biomass both with and without added PGPR were performed using the Wilcoxon Rank Sum test ($\alpha = 0.05$). This comparison was used to determine if a significant change in root or shoot biomass resulted when adding PGPR with each of the four soil suspensions. All results were expressed as ± standard error of the mean (S.E.M.).

To determine pathogen effects, disease severity and disease incidence were both considered. Alfalfa Root Rot disease severity was rated according to a previously published scale [129]. Disease incidence was determined by counting plants with 25% or more disease affected roots as diseased and less than 25% as not affected by disease.

The change in alfalfa biomass with PGPR versus without was calculated for each non-filtered soil suspension. Correlations between plant biomass increases when adding PGPR and measured soil factors, e.g. physico-chemical qualities, bacterial diversity and evenness, were calculated using the Pearson product-moment correlation coefficient (r). Correlations between disease incidence and measured soil factors were calculated using the same method. Pearson correlation coefficients with values close to 1 indicate a strong relationship between the soil factor and plant biomass or disease, while values close to zero indicate a weak relationship.

Additionally, a chi-squared test using contingency tables of disease counts was utilized to determine if observed differences in disease incidence held significance.

Results

Soil physico-chemical and microbiological characteristics

The four soils varied in both physico-chemical and microbiological characteristics. Soil pH was similar in the two potato soils at 7.4 and 7.6, but the fallow and forest soils were more acidic with respective pH values of 5.8 and 5.4. Soil electrical conductance (EC) values ranged from 0.3 to 0.9, a threefold difference. Percentage of soil nitrogen (N) was in the 6-10% range with the potato and fallow agricultural soils, but a much higher 33% in the forest soil. The carbon to nitrogen ratio (C:N) was lower in the two potato soils (range 9.5 - 9.8) and higher in the fallow and forest soils (range 13.5 - 13.8). The two potato soils were similar in pH, percent nitrogen (N%), and C:N ratio, but the organic potato soil had more than twice as much NO3-N (N in the nitrate ion form) as the conventional potato soil and the largest value of all four soils. The organic potato soil also was substantially higher in soil phosphorus (P) (39.7 ppm) and potassium (K) (585 ppm) than the other three soils that ranged downward to 3.7 ppm for soil P and 41.6 ppm for soil K. The forest soil had the highest levels of iron (Fe) at 70.8 ppm and Manganese (Mn) at 14.3 ppm. The fallow soil had the lowest level of zinc (Zn) at 0.91 ppm and the highest level of copper (Cu) at 2.5 ppm. All measured soil physico-chemical characteristics are detailed in Table 3.3.

The four soils were analyzed via pyrosequencing to calculate Shannon's diversity, Shannon's evenness, Simpson and Inverse Simpson indices (Table 3.4). Simpson Index

(quantifying diversity) values ranged from 0.106 to 0.287 for the four experimental soils. Inverse Simpson Index (quantifying evenness) values ranged from 93 to 291. The two potato soils had communities with the highest diversity and evenness levels and the least diverse, and also least even, soil bacterial community was from the forest soil.

The taxonomic composition of the four soils also varied (Figure 3.2). Both potato soils had higher relative abundances of Actinobacteria compared to the other soils, but the organic potato soil had less Actinobacteria than conventional potato. Relative abundance of Gammaproteobacteria was higher while Actinobacteria was lower in both potato soils. Conventional potato soil had higher relative abundance of the Betaproteobacteria class. Fallow soil was the only soil type to present Synechococcophycideae and Oscillatoriophycideae, which were absent from all three other soils. Fallow soil had the highest number of OTUs that were present at numbers less than 100 and grouped into the category 'Other'. Fallow and Forest soils were similar in their high abundance of Actinobacteria (44%) and low abundance of Chloracidobacteria (3%). Forest soil had the highest relative abundance of Alphaproteobacteria, 3-8% higher than the other soils. The most striking difference between the four soils was in the most abundant bacterial class, Actinobacteria.

Phytophthora root rot disease incidence and severity

All alfalfa plants suffered significantly lower shoot and root biomass with added *Phythophthora* compared to without *Phytophthora,* regardless of which soil microbial suspension was applied. Wilcoxon rank sum tests showed no significant difference in alfalfa root or shoot biomass between filtered and non-filtered treatment groups with added pathogen (data not shown). Alfalfa roots were also assessed for disease incidence and severity. Filtered soil

suspensions did not show different incidence of disease relative to the control (Fig. 3.3a). Only the non-filtered organic potato treatment group exhibited borderline significance with regard to reduced disease incidence ($p = 0.053$) when compared to its filtered counterpart. All other non-filtered soil suspensions had disease incidence levels that were not statistically different from filtered applications. Disease severity when adding *Phytophthora* was equal to the non-amended control with all filtered and non-filtered treatment groups except for the filtered organic potato, which showed increased disease severity (Fig 3.3b). The forest soil was the only treatment group with lower disease incidence in filtered (soil microorganisms removed) versus non-filtered suspensions, a 20% reduction. Soils with higher Zn levels were associated with a lower disease incidence ($r^2 < -0.96$, $p = 0.04$). Soil microbiology also influenced disease incidence. Percent incidence of disease was negatively correlated with higher soil bacterial diversity as measured by both Shannon indices ($r^2 < -0.70$), an indication that higher soil bacterial diversity was correlated with lower disease incidence. The same correlation was observed with Simpson and Inverse Simpson diversity indices, but it was less pronounced. None of the correlations between soil bacterial diversity and disease incidence was statistically significant (Table 3.5).

PGPR root colonization

Using the DNA extracted from *P. putida* KT2440, the standard curve for the *gfp* gene ($y = -0.2686x + 9.7195$; $r^2 = 0.9776$) was linear over four orders of magnitude (Fig. 3.4). Amounts of amplifiable *P. putida* KT2440 DNA (*gfp)* are shown in Fig. 3.5 and are reflective of differences in PGPR root colonization. Significantly more PGPR were detected in the filtered organic potato treatment as compared to other treatments. A consistent effect of higher PGPR was found in all filtered treatments compared to non-filtered treatments, which contained soil

microorganisms. Detected PGPR was not statistically different between any non-filtered treatment groups. Calculation of the Pearson product-moment coefficient did not reveal any significant relationships between *gfp* (ng/g root tissue) and soil diversity, evenness, or richness measures. In addition, increases in alfalfa shoot biomass did not correlate with PGPR root colonization.

Soil chemical properties influence PGPR effects on plant biomass

The four filtered soil suspensions were evaluated for potential chemical effects on plant biomass increases with added PGPR. No alfalfa plants receiving filtered soil suspensions showed increased shoot or root biomass with added PGPR versus without (Figures 3.6a & 3.6b). However, plant biomass increases were observed in the non-amended control, which received PGPR inoculation but no soil suspension. Equal increases in shoot biomass were observed when PGPR were added after the two non-filtered potato soil suspensions, which contained both the chemical and microbiological components. Neither filtered nor non-filtered fallow and forest treatment groups had an increase in plant biomass with added PGPR. Three soil physico-chemical properties showed strong, significant correlation with percent increases in biomass with added PGPR: EC ($r^2 = 0.98$, $p = 0.02$), NO_3-N ($r^2 = 0.98$, $p = 0.02$), and P ($r^2 = 0.95$, $p = 0.05$). All of these properties had a positive effect on PGPR increases in plant biomass. A strong correlation between increased plant biomass ($r^2 = 0.90$, $p = 0.10$) and soil pH (range 5.4 – 7.6) was observed, but was not significant. Notably, plant biomass increases were only observed when these physico-chemical components were applied as a combination of chemical and biological factors in the form of non-filtered suspensions.

Soil microbial diversity increases PGPR effects on plant biomass

Soil microbes were added to alfalfa plants, along with soil chemical components, in the form of non-filtered soil suspensions. Two non-filtered suspensions, the organic and conventional potato, exhibited significantly higher shoot biomass with added PGPR compared to without (Figures 3.7a & 3.7b). These two potato soils also have the highest soil microbial diversity values as measured using Shannon and Inverse Simpson indices (Table 3.4). The organic potato soil suspension group was the only one to significantly increase root biomass with added PGPR versus without (Fig. 3.7b). Increased plant growth with added PGPR was strongly and significantly correlated with higher soil microbial diversity and evenness (Table 3.6). In alfalfa plants receiving PGPR, the strongest correlation to increases in shoot biomass were soil suspensions with high evenness as measured by the Simpson Index ($r^2 = 0.99$, $p = 0.01$). The Inverse Simpson Index, a measure of soil bacterial evenness, also exhibited a significantly strong correlation ($r^2 = 0.98$, $p = 0.01$).

Specific bacterial species correlate with plant biomass increases with added PGPR

Since previous research has found increases in shoot biomass, rather than root biomass, to be most indicative of *P. putida* plant growth increases [96], correlations between abundance of bacterial taxa were performed with percent increases in shoot biomass. Twelve species had a significant ($p < 0.05$) and strong correlation ($r^2 > 0.90$) with increased alfalfa shoot biomass in non-filtered soil suspensions when adding PGPR (Table 3.7). Four bacterial classes were represented*: Actinobacteria (4 species), Alphaproteobacteria (5 species), Betaproteobacteria (2 species), and Chloracidobacteria (1 species)*. Eight out of twelve of the genetic sequences were not classifiable to the species level, but every sequence except one was classified to genus. Half

of these genera contain species that have been characterized as N-fixing or carrying the *nifH* gene (*Agromyces, Kocuria, Microbacterium, Micromonospora, Achromobacter,* and *Azohydromonas*) [111-114]. Also related to the N cycle, *Lutibacterium spp.* functioned to reduce N [115] and showed positive correlation to biomass increases with PGPR. Two genera (*Kocuria* and *Micromonospora*) were previously isolated from N-fixing root nodules formed as part of rhizobia symbiosis [113, 116]. Four genera (*Micromonospora*, *Balneimonas*, *Agromyces* and *Achromobacter*) have been shown to exhibit biocontrol or plant growth promoting functions [117-121]. Other bacterial groups (*Paracoccus* and *Lutibacterium*) were previously characterized by degradation or bioremediation of chemical toxins [122-124]. Finally, two photosynthetic genera (*Chloracidobacteria* and *Rhodobiaceae*) were associated with increased shoot growth when adding PGPR [125, 126].

Two bacterial groups (*Plantomyces* and *Skermanella)* were associated with a lack of increase in shoot biomass with added PGPR ($r^2 > 0.95$, $p < 0.05$; Table 3.8), which was observed with non-filtered fallow and forest soil suspensions. The first, *Skermanella,* is a poorly characterized genus, but was isolated from coal mining soil and shows a high tolerance for the element antimony [127]. The second, *Actinoplanes,* are known root endophytes, produce chitin-degrading enzymes [120], and have been used to reduce root rot in controlled environments [128]. In addition, over 120 different antibiotics have been produced by various *Actinoplanes* strains [129].

Discussion

Soil chemical properties affect both disease and PGPR effects

Filtered soil suspensions included all soil suspension components that are soluble in water and pass through a 0.45 μm pore filter. Filtered components largely represent the chemical fraction of the soil suspension, potentially including small amounts of herbicides, fungicides, secondary metabolites, and sugars and breakdown products. Ultramicrobacteria, defined as smaller than 0.3 μm in size, may also pass through [98], but are a limited taxonomic group. Viruses are also small enough to pass filtration, however no symptoms of viral infection were observed during the experiment. In contrast, most fungal spores and average sized bacteria (roughly 1 – 1.5 μm) are removed by the microfiltration process [98]. A total of 30 ml of filtered soil suspension, split into two applications, was applied to each alfalfa plant. This small volume of soil chemical extract was enough to reduce disease incidence by half, compared to non-amended controls, in the forest treatment group. Similarly, soil chemical extracts reduced PGPR effects. Increases in plant biomass observed in non-amended controls were completed absent when adding PGPR to all filtered treatment groups.

Soils showing higher Zn levels were correlated with reduced disease incidence. Low Zn levels were correlated with increased Phytophthora Root Rot severity in alfalfa in previous studies [130] due to an improvement in plant function with adequate Zn. It is possible that soil Zn, particularly high in the fallow soil, dissolved in the soil suspension and passed through the filtration process and resulted in a decreased disease rate.

Attempts were also made to correlate soil physico-chemical factors with biomass increases when adding PGPR. None of the measured soil physico-chemical factors was significantly negatively correlated with biomass increases with added PGPR, which would

indicate a negative effect by a soil physico-chemical property on PGPR activity. However, since all four filtered suspensions exhibited a lack of effect with added PGPR, it is difficult to determine which component may be preventing the PGPR from increasing plant growth. Agrochemicals such as pesticides, herbicides, and fungicides from soil residues are potential components of filtered soil suspensions. Importantly, *Pseudomonas* strains are known to be sensitive to several agrochemicals including chlorophenoxy herbicides [131], copper oxychloride and other pesticides [132], and tebuconazole and other fungicides [61]. Such residues are water soluble, filterable, and could have affected PGPR activity or survival. In contrast to PGPR effects, PGPR root colonization was different across treatment groups as detected using qPCR quantification of *gfp* (Figure 3.5). However, calculation of Pearson's correlations did not reveal any relationships between soil physico-chemical differences and PGPR root colonization. It is interesting to note that while colonization was significantly higher in the filtered potato and forest treatments (Fig. 3.5), no difference in shoot biomass was observed in any of the filtered treatments (Fig. 3.6). This result suggests that higher PGPR colonization will not always result in increased plant biomass and should be carefully considered when determining appropriate use of PGPR. In this case, the lower biomass observed in filtered treatment groups suggests that filtered suspensions may be causing plant stress that the PGPR was not able alleviate. As such, adding a PGPR to the soil did not result in any improvements in plant growth.

Soil bacterial diversity differentially influences pathogen and PGPR

Non-filtered soil suspensions would include all of the chemical components as well as biological components such as soil bacteria, fungi, protozoa and other faunal predators. Non-filtered soil suspensions achieved reduced disease severity in all cases and three of the four

exhibited reduced disease incidence relative to their filtered counterparts. In calculating the Pearson's correlation coefficient, strong negative correlations ($r^2 \leq -0.70$) were observed for disease incidence and severity with soil bacterial diversity and evenness (Table 3.5). However, in contrast to previously reported research [24], the relationship between soil bacterial diversity and pathogen effects were not significant ($\alpha = 0.05$). However, this result may have been confounded by varying soil levels of Zn, which also affects Phytophthora root rot disease. Two non-filtered suspensions, the organic and conventional potato, restored the significant increases in shoot growth with applied PGPR versus without to the same level observed in non-amended controls. The organic and conventional potato soils also had the highest levels of soil bacterial diversity among the four experimental soils (Table 3.4).

While soil bacterial diversity did not significantly affect pathogen success, it improved plant biomass increases when adding PGPR. To better understand these contrasting outcomes, we must examine the ecosystem processes at work. Proposed mechanisms by which soil microbial diversity inhibits pathogens include: resource-based competitive exclusion and antagonism of the invading organisms [24]. Competition for resources increases with increasing soil bacterial diversity due to a greater rate of consumption and more resource types being consumed by the indigenous community [133, 134]. Competition for the remaining limited nutrients reduces the success of invasions in communities with higher diversity [77]. Hence, the higher the diversity of the soil bacterial community, the less likely an organism can successfully invade. However, other ecosystem processes must be influencing PGPR effects since we observe an increase in PGPR effects with increasing soil bacterial diversity.

When adding PGPR, the highest plant biomass was observed in plants receiving the two non-filtered potato suspensions, which are also the soils with the highest bacterial diversity. Plant

biomass in these groups was equal to control plants grown in the sterile sand and vermiculite mixture, which provided a competition-free environment for testing PGPR inoculations. Multiple explanations for an equal biomass effect despite unequal competition are possible. The PGPR may be relatively more resource competitive, lack antagonists, benefit from beneficial preparation of the environment by other organisms, or enhance positive effects of other organisms. Any of these ecological mechanisms potentially explain the contrasting effects of soil bacterial diversity on the PGPR versus the pathogen. To determine which is most influential, the levels of PGPR quantified via qPCR from root tissues must be considered. PGPR colonization of roots was unilaterally reduced across all non-filtered treatments relative to filtered treatments (Fig 3.5). These results suggest an inhibitory effect by the indigenous soil community, possibly due to niche and resource competition with added PGPR organisms. In addition, equivalent root colonization of the PGPR suggests that varying levels of competition or benefit from different soil communities was not the reason for differences in root colonization. Although, given the complexity of soil communities, mutually counteracting relationships between varying antagonists and commensals of the soil community cannot be ruled out. The more likely explanation for the differences observed in plant biomass increases is enhancement of positive effects of other soil organisms by the added PGPR. By enhancing the beneficial effects of other organisms, plant biomass production is increased, even when the PGPR level of colonization is not.

To further explore potential relationships between *P. putida* and other soil bacteria, the specific bacterial membership of the experimental soils is examined in the next section. *Pseudomonas putida* has been shown to enhance the beneficial effects of multiple other soil microorganisms. For example, adding siderophore-producing *Pseudomonas* strains, like *P.*

putida, in combination with microbial inducers of systemic resistance suppressed *Fusarium* wilt of radish better than the application of individual strains [135]. Addition of *P. putida* also amplifies beneficial effects of Vesicular Arbuscular Mycorrhizal (VAM) fungi, which improve plant biomass through improved acquisition of P and water [136, 137]. Root colonization by VAM fungi was increased four-fold when VAM were added with *P. putida* [96]. These are just a few examples of microbial communities functioning better together than alone; many others are likely to occur in complex soil communities. Competition with indigenous soil microorganisms has previously been proposed as a reason that PGPR inoculations sometimes fail in field applications [38]. As a consequence, it has been suggested that disrupting the soil community, for example by applying fungicide, might improve PGPR colonization [2]. These results suggest that such a strategy be considered very carefully, since the diversity of the indigenous soil microbial community may be critical to achieving desired increased in plant biomass with PGPR applications.

Specific bacterial species correlate with increases in plant biomass with added PGPR

Percent increase in dry shoot biomass with added PGPR was positively correlated with abundance of twelve species from four different bacterial classes (Table 3.7). The majority of these species were related to N cycling. Some are known to fix atmospheric N or carry the *nifH* gene, which codes for the enzyme necessary to perform that function (*Agromyces spp.*, *Kocuria rosea, Microbacterium flavescens*, *Micromonospora spp.*). Others function within N fixing nodules (*Kocuria rosea*, *Micromonospora spp.*) or by reducing N (*Paracoccus spp.)*. Since data on varying number of root nodules is lacking for this study, it is unclear if the positive association between *P. putida* and these bacterial species related to N-cycling increased alfalfa

biomass through increased nodulation or other means. Since *P. putida* has been shown to enhance the beneficial effects of other soil microorganisms, such as the nodule-forming symbiont, *Sinorhizobium meliloti* [68], it could have enhanced other N-cycling functions. In fact, PGPR have been found to increase in effectiveness when applied in formulations of multiple species [138]. One previously characterized PGPR had increased abundance in high productivity potato soils. *Belneimonas,* which has been correlated with improved growth in wheat plants, was also correlated with increased PGPR effects in this study. Therefore, the functions of other PGPR in the higher diversity potato soils could have been enhanced by *P. putida* and resulted in increased plant biomass in these treatments.

Several species were correlated with shoot biomass increases when adding PGPR that are known bacterial antagonists. These include: *Agromyces spp. and Micromonospora spp.* However, while these, two bacterial groups are known as general antagonists to other bacteria, they are less effective against *P. putida* One species of *Agromyces, Agromyces ramosus,* attacks and feeds on some species of bacteria, but was experimentally shown not to feed on *P. putida* [118]. *Micromonospora spp.* produce antibiotic and antifungal compounds [120], at least one of which was less effective on *Pseudomonas spp.* [139]. The decreased effects on *Pseudomonas* organisms could explain why the presence of these organisms did not decrease *P. putida* colonization in this research. As an alternative form of commensalism, three bacterial groups contain species previously characterized by their ability to degrade various environmental toxins. *Paracoccus sp.* Strain KT-5 was previously shown to degrade pyridines, which are used in herbicide synthesis and as pesticides (Qiao and Wang 2010). Similarly, *Lutibacterium spp.,* degrade polyaromatic hydrocarbons (PAHs) [122]. These bioremediating functions are of particular importance considering that filtered soil suspension applications contained an

unknown chemical component that eliminated all increases in plant growth with added PGPR. These organisms could allow better functioning of the PGPR or other beneficial bacteria in the two non-filtered potato soil treatment groups by removing chemicals, such as pesticides or fungicides, toxic to *P. putida* and other organisms.

A full list of species associated with improved PGPR effects along with their known ecological functions is detailed in Table 3.7. Further research is needed to identify specific bacterial species that promote plant growth to a greater degree in the presence of other PGPR like *P. putida*. Carefully planned multi-species PGPR formulations could significantly improve the practicality of using PGPR to increase agricultural production.

Bacterial species negatively correlated with shoot biomass when adding PGPR

Actinobacteria, taken as an entire bacterial class, was negatively correlated with an increase in shoot biomass with added PGPR. *Actinobacteria* have also been found to play an important role in preventing soil invasion by bacterial pathogens [24]. A full list of species negatively correlated with alfalfa biomass when adding PGPR along with their known ecological functions is detailed in Table 3.8. One specific unclassified species of genus *Actinoplanes* was correlated with reduction of beneficial PGPR effects ($r^2 = -0.99$; $p = 0.02$). *Actinoplanes* are well characterized for their ability to produce antibiotics, with over 120 different antibiotics identified to date [129]. If one or more the *Actinoplanes* antibiotics is effective against *P. putida,* a higher abundance of this unclassified *Actinoplanes spp.* in the fallow and forest soils could account for a failure of the PGPR to establish in these treatments. An unclassified species of the genus *Skermanella* was also correlated with reduced benefit when adding PGPR ($r^2 = -0.98$; $p = 0.02$). *Skermanella spp.* are not well characterized, but were isolated from coal-mining soil and

demonstrate high tolerance for the potentially toxic metal antimony [127]. Further research on other ecological roles and rhizosphere competence of *Skermanella spp.* is necessary to confirm this association.

These data consider only soil bacterial diversity and do not include measures of soil fungal diversity. Perhaps the fungal to bacterial ratio or the total soil fungi diversity might be more relevant to explaining the ability of this PGPR to increase plant biomass in some soil communities better than others. Further study comparing plant biomass production and PGPR colonization in combination with soil fungi measures would be valuable in determining the hierarchy of bacterial and fungal antagonism versus commensalism.

Conclusion

Soil bacterial diversity was shown to have contrasting effects on pathogen versus PGPR effects on plant health and growth. Pathogen-caused disease was not shown to change with increasing bacterial diversity. However, significantly greater PGPR effects on plant biomass were observed with more diverse soil bacterial communities. In contrast, PGPR colonization of alfalfa roots did not differ between varying soil microbial communities. These seemingly conflicting outcomes are possibly due to the ability of PGPR to enhance the activities of other beneficial microorganisms. In addition, more diverse soil bacterial communities are more likely to include these beneficial microorganisms, such as plant mutualists or other PGPRs, which increasingly provide plant benefit when PGPR are present. Further research is needed to confirm these findings and to account for the additional influence of fungi and soil fauna. Continued research in this area will identify bacterial species consortia that provide higher increases in plant

biomass together than individual members alone and improve success of field inoculation with PGPR organisms.

Table 3.1 Site descriptions for four soils used in soil suspension applications

Soil Label	Location	Description	Management
POTATO-ORG	Colorado	Potato cropping	Organic
POTATO-CONV	Colorado	Potato cropping	Conventional
FALLOW	Wisconsin	*Arabidopsis thaliana* dominated	Fallow
FOREST	Oregon	Pine Forest	Natural Area

Table 3.2 Sequences of primers used for q-PCR analysis

Primer	Sequence (5'-3')	Tm (°C)	Fragment (bp)
gfp forward	ATGCGTAAAGGAGAAGAACTTTTCA	54.9	717
gfp reverse	TTTGTATAGTTCATCCATGC	47.7	

Table 3.3 Soil physico-chemical qualities for four soils used in soil suspension applications.

	pH	EC (mS)	N (%)	C:N	NO_3-N (ppm)	P (ppm)	K (ppm)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)
POTATO-ORG	7.6	0.9	0.101	9.82	26.4	39.7	585	3.5	9.1	2.1	1.5
POTATO-CONV	7.4	0.5	0.077	9.55	11.9	14.7	229	2.9	7.4	1.8	1.3
FALLOW	5.8	0.3	0.066	13.5	8.3	3.7	41.6	0.91	20.7	2.7	2.5
FOREST	5.4	0.3	0.326	13.8	6	6.8	241	3.3	70.8	14.3	1.9

Table 3.4 Bacterial alpha diversity (OTUs, defined at 3% genetic distance) for four soils used in soil suspension applications.

Soil	S_{chao}	Shannon		Simpson	
		H	E_H	E_D	S_D
POTATO-ORG	2815	6.35	0.918	0.287	291
POTATO-CONV	2731	6.23	0.905	0.224	219
FALLOW	1929	5.89	0.878	0.172	141
FOREST	2144	5.96	0.879	0.106	93

H = diversity index, E_H and E_D = evenness, S_D = effective species or # of species at observed diversity index assuming complete equitability

Table 3.5 Relationships between Alfalfa Root Rot disease incidence and severity and soil bacterial diversity and evenness measures (Pearson's correlation coefficients). No statistically significant correlations were revealed. See Table 3 for definitions of diversity measures.

	Disease Severity	**pvalue**	**Disease incidence**	**pvalue**
Shannon Index	-0.77	*p = 0.23*	-0.70	*p = 0.30*
Shannon Evenness	-0.79	*p = 0.21*	-0.71	*p = 0.29*
Inverse Simpson	-0.65	*p = 0.34*	-0.57	*p = 0.42*
Simpson Index	-0.48	*p = 0.52*	-0.34	*p = 0.63*

Table 3.6 Relationships between soil bacterial abundance and percent increase in alfalfa biomass with added PGPR. All p-values are shown in parentheses below Pearson's correlations coefficients (r^2) values in the table.

			Shannon		**Simpson**	
Plant Trait	s_{obs}	**Chao**	***H***	E_H	E_D	S_D
Shoot Biomass	0.85 *(0.07)*	0.83 *(0.09)*	0.91 *(0.05)*	0.93 *(0.04)*	0.98 *(0.01)*	0.99 *(0.01)*
Root Biomass	0.80 *(0.10)*	0.85 *(0.07)*	0.80 *(0.10)*	0.77 *(0.12)*	0.75 *(0.13)*	0.72 *(0.14)*

H = diversity index, E_H and E_D = evenness, S_D = effective species or # of species at observed diversity index assuming complete equitability

Table 3.7 Bacterial species positively correlated with increases in alfalfa shoot biomass with added *P. putida*. Soil bacterial abundance (total sample reads for the bacterial species) correlated with percent increase in shoot biomass. P-values are shown in parentheses below Pearson's r^2.

Class	*Classification*	*r^2*	*Description*
Actinobacteria	***Agromyces sp.*** *(unclassified)*	**0.97** *(0.03)*	N-fixing [112] Endophyte associated with root nodules of legumes [140] Biocontrol, improved plant growth [118]
	Kocuria rosea	**0.96** *(0.04)*	N-fixing [113] Endophyte associated with wild grape roots [120] and nodules of legumes [113]
	Microbacterium flavescens	**0.96** *(0.04)*	N-fixing [112] Endophyte associated with aquatic plants [141]
	Micromonospora sp. *(unclassified)*	**0.96** *(0.04)*	N-fixing [112] Biocontrol, improved plant growth [120] Endophyte associated with aquatic plants [141]
Alphaproteobacteria	***Balneimonas sp.*** *(unclassified)*	**0.98** *(0.02)*	Associated with increased productivity in wheat [119]
	Kaistobacter sp. *(unclassified)*	**0.96** *(0.04)*	Poorly characterized, found in soils and one radioactive site [142]
	Lutibacterium sp. *(unclassified)*	**0.98** *(0.02)*	Includes hydrocarbon-degrading species [122]
	Paracoccus sp. *(unclassified)*	**0.98** *(0.02)*	Includes N-reducing [124] and pyridine degrading [143] species

	Rhodobiaceae sp. *(unclassified)*	**0.98** *(0.02)*	Grows phototrophically in light and chemotrophically in dark and requires NaCl for growth. [126]
Betaproteobacteria	***Achromobacter sp.*** *(unclassified)*	**0.95** *(0.05)*	Stimulates plant growth in sunflowers [117] Includes N-fixing species [114]
	Azohydromonas sp. *(unclassified)*	**0.97** *(0.03)*	Includes N-fixing species [111]
Chloracidobacteria	***unclassified***	**0.99** *(0.01)*	Photosynthetic thermophile [125]

Table 3.8 Bacterial species negatively correlated with changes in alfalfa shoot biomass with added *P. putida*. Soil bacterial abundance (total sample reads for the bacterial species) correlated with percent increase in shoot biomass. P-values are shown in parentheses below Pearson's r^2.

Class	*Classification*	*r^2*	*Description*
Actinobacteria	***Actinoplanes sp.*** *(unclassified)*	**0.99** *(0.01)*	Produces antibiotics[129] and chitinase [120] Biocontrol of root rot pathogens [128]
Alphaproteobacteria	***Skermanella sp.*** *(unclassified)*	**0.98** *(0.02)*	Antimony-resistant, isolated from coal-mining soil [127]

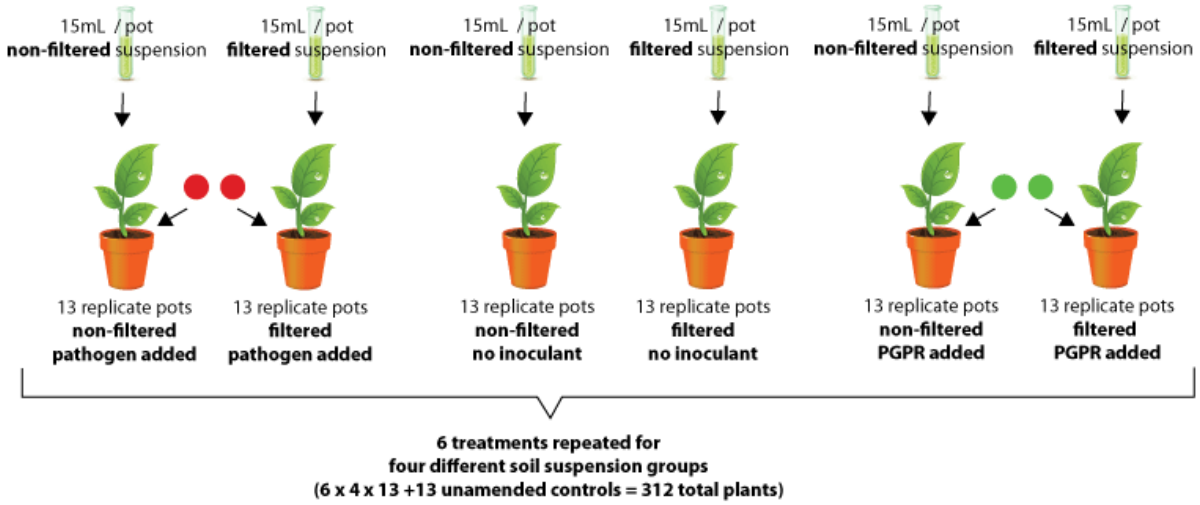


Figure 3.1 Schematic diagram of the 24 treatment groups including no added organism, added PGPR, or added pathogen for pots already treated with filtered and non-filtered soil suspensions of four different soils (Table 1). An non-amended control was also utilized (not shown).

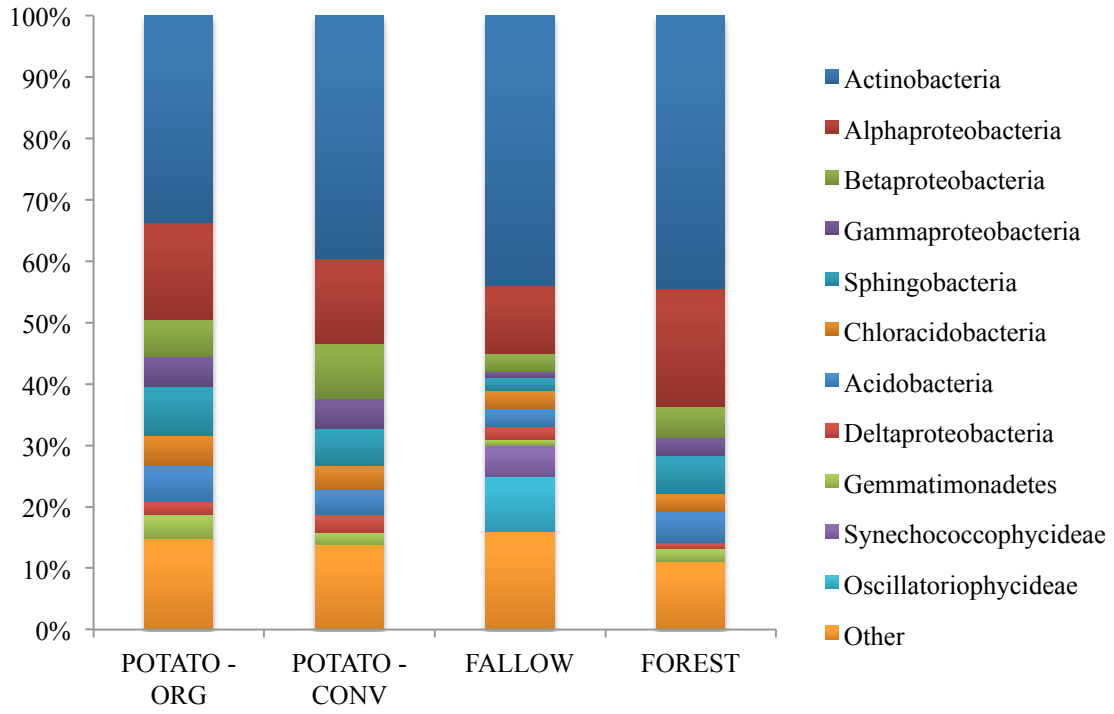
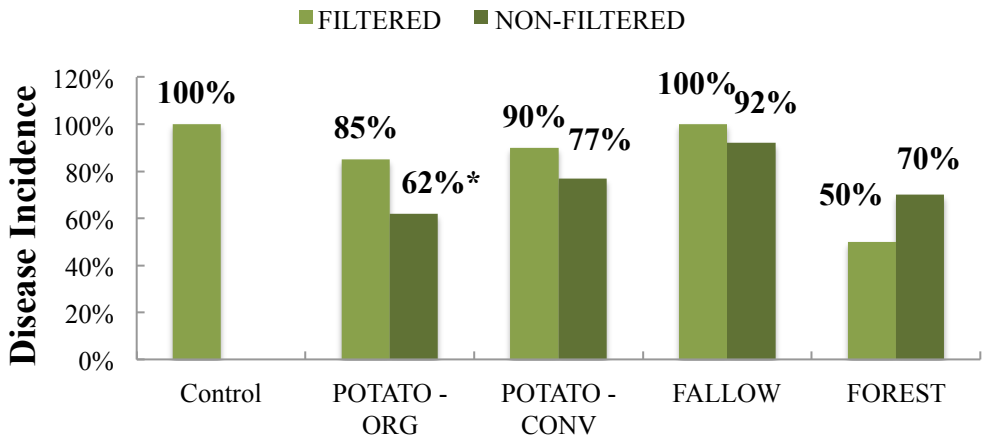


Fig 3.2 Relative abundance of bacterial taxa at the class level detected in 4 soils used in soil suspensions. Classes represented by fewer than 100 sequences were grouped into the 'Other' category for simplification of presentation.

(a)



(b)

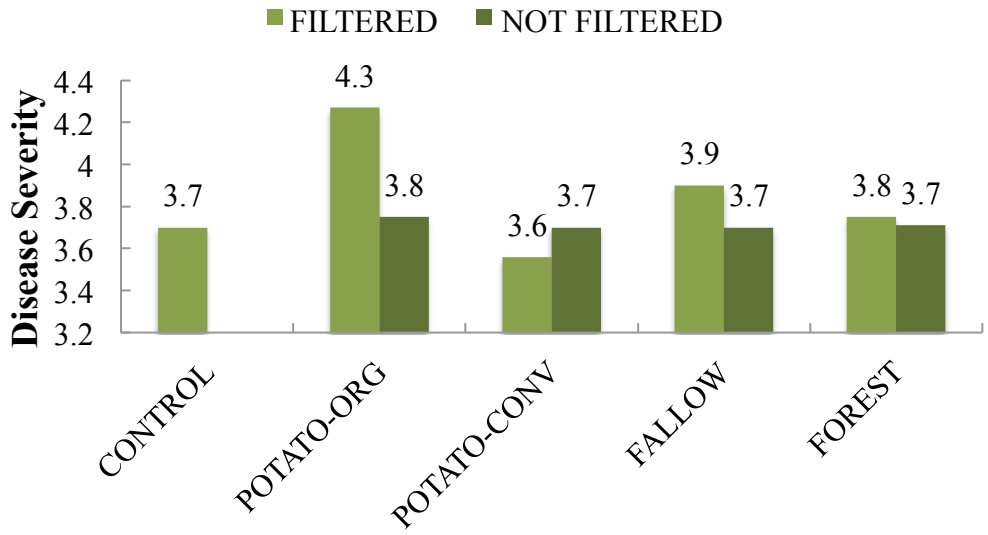


Fig 3.3 Alfalfa Root Rot disease incidence (a) and severity (b) are shown for each filtered and non-filtered soil microbial suspensions with an added pathogen, *Phytophthora medicaginis.* * indicates statistically significant differences in disease incidence with filtered versus non-filtered soil suspensions using chi-squared test with contingency tables ($p < 0.05$).

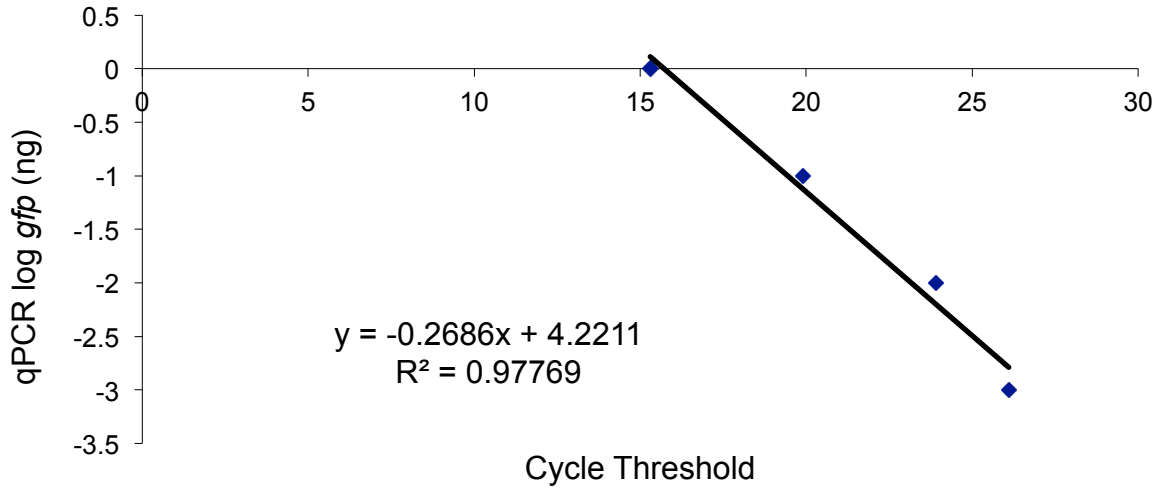


Fig. 3.4 Standard curve for the detection of Pseudomonas putida KT2440 in pure culture using the green fluorescent protein gene, *gfp,* generated using qPCR.

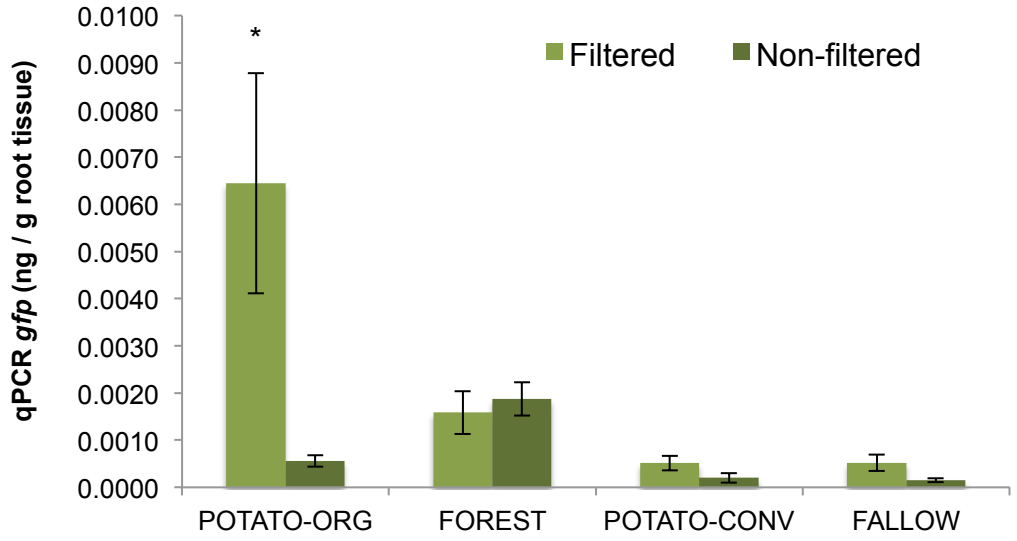
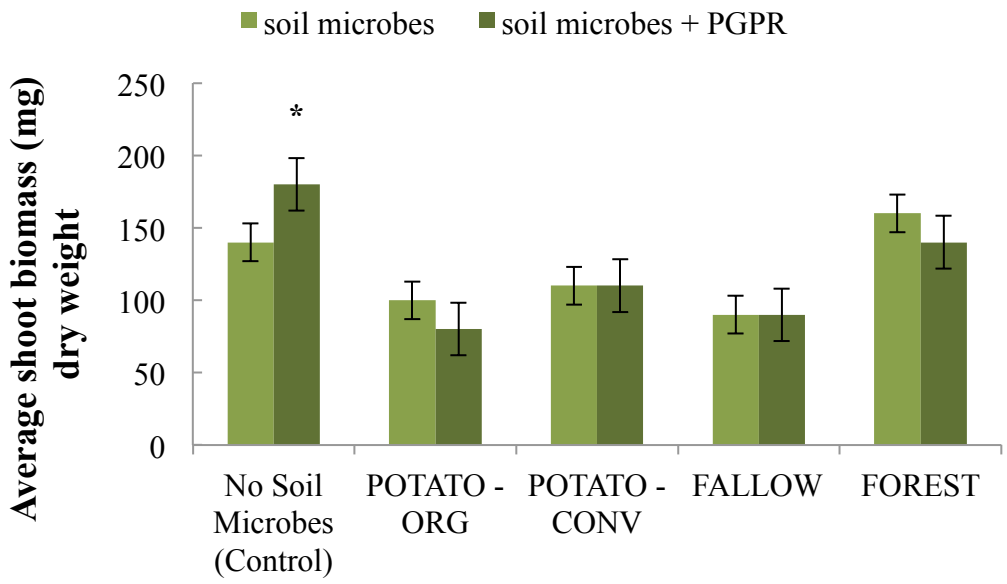


Fig. 3.5 Comparison of *Pseudomonas putida* KT2440 as detected by the green fluorescent protein gene, *gfp,* shown as ng per g root tissue for all filtered and non-filtered treatments with added PGPR. DNA utilized for qPCR experiment was extract from non-homogenized alfalfa roots. Asterisk (*) indicates significantly higher *gfp* detected using Kruskal-Wallis non-parametric test combined with Wilcoxon Rank Sum test.

(a)



b)

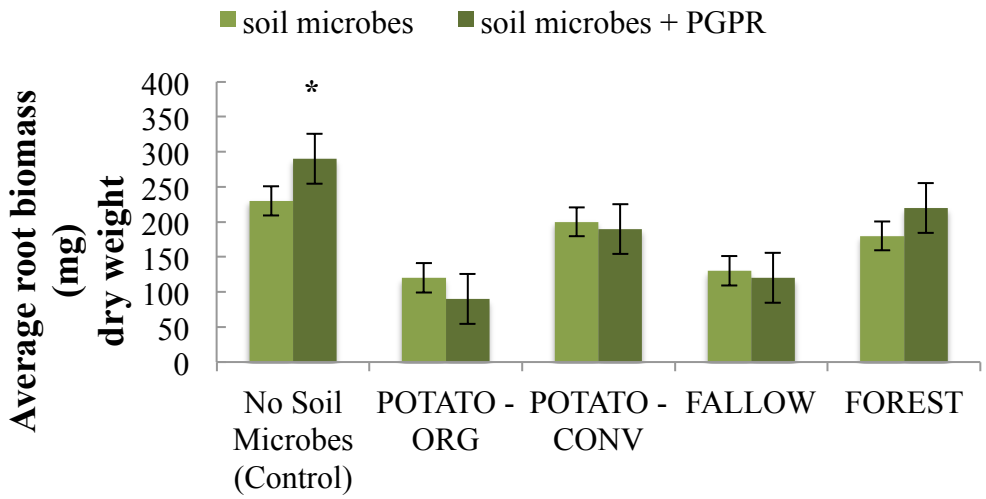
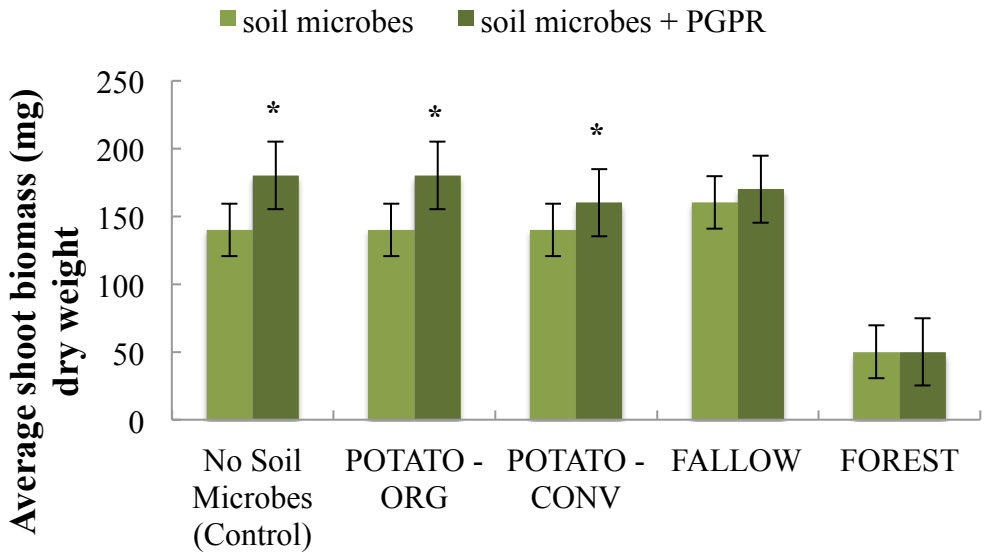


Fig 3.6 Average dry shoot (a) and root (b) biomass, measured in (mg), are shown with soil microbial suspensions (only) and with added PGPR for each of four soils. All displayed data is from filtered soil microbial suspensions, except for the control group, which did not receive any soil microbial suspension. Asterisk (*) indicates significant increase biomass with added PGPR versus without PGPR ($p < 0.05$).

(a)



(b)

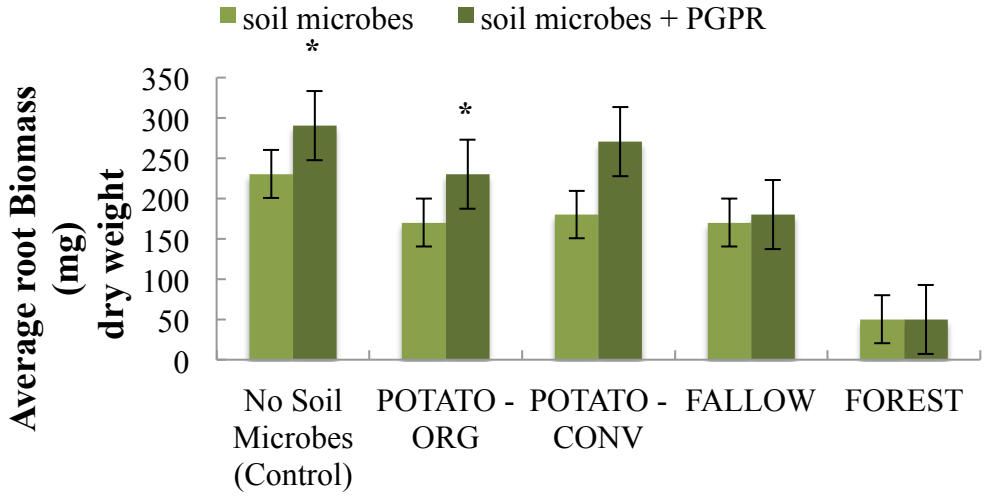


Figure 3.7 Average dry shoot (a) and root (b) biomass, measured in (mg), are shown with non-filtered soil microbial suspensions (only) and with added PGPR for each of four soils. The control group did not receive any soil microbial suspension, and is also shown both with and without added PGPR. * indicates significant increase in biomass with added PGPR versus without PGPR ($p < 0.05$).

CONCLUSION

Studies examining whether soil microbial diversity influences plant health and productivity are important for formulating methods for manipulating soil microbiology for improved yields. These studies examined how a diverse set of indigenous soil communities influenced both alfalfa biomass production and establishment of introduced beneficial or pathogenic microorganisms. Much research exists on the effects of soil fungal diversity on plant productivity [18, 20, 28, 71], but very few [29] look at the influence of bacterial diversity as this study does. Also, the ability of an indigenous soil microbial community to exclude pathogens has been well researched [24], but the effects on exclusion of beneficial microbial inoculants has not [38]. Furthermore, previous work has included microbial communities from a very limited number of soils, in most cases including a singular soil community for evaluation [18-20, 24, 29, 71]. The experiments for this thesis focus on bacterial communities and include multiple soil types with diverse microbial communities; nine in the first study and four in the second. Another important feature of these studies is utilization of naturally-assembled microbial communities. Naturally-assembled soil microbial communities are less subject to selection effects and more closely mimic the complexity of the natural environment [83]. It is important to study naturally assembled soil communities, because they are more complex and contain thousands of microbial species, while artificially assembled communities contain less than 100 [24]. Additionally, it is important to consider the separate effects of soil biology and chemistry on plant biomass. To separate biological from chemical effects on plant biomass production and microbial invasions, soil suspensions were applied in both microfiltered and non-microfiltered forms to a sterile substrate. This separation of soil chemical and biological influences on plant traits has

historically proved very difficult. Therefore, this experimental design was utilized in both studies for this thesis to provide a clear separation of chemical versus biological influences on experimental outcomes.

The first study investigated differences in alfalfa root and shoot biomass resulting from variation in the soil microbial community. Results of this study do not reinforce previous research showing that more diverse soil microbial communities encourage plant biomass production. This disparity is likely due to the use of naturally assembled soil communities and emphasis on soil bacteria, rather than fungi in this research. These results align closely with other studies utilizing naturally complex communities and focusing solely on soil bacteria [29]. Anderson and Habiger [29] report that the specific taxonomic membership of soil bacteria present had a stronger effect on plant productivity than community measures such as diversity. In addition, soil microbiology was shown to have a bioremediation function by compensating for negative effects on plant biomass due to soil chemistry. The neutralization of negative soil chemistry effects was highly correlated with the bacterial class *Deltaproteobacteria,* which was previously shown to demonstrate syntrophy by cooperating with other microorganisms to breakdown chemical substrates [105]. Overall, alfalfa biomass correlated more highly with soil bacterial richness, diversity, evenness and specific taxonomic groups than with soil physico-chemical factors such as pH, N, and C (total):N(total) ratio. Soil biology and soil chemistry were shown to have contrasting effects on plant growth, particularly shoots. There is a strong potential that such mutually counteracting soil chemical and biological effects on plant growth have gone unnoticed in other experimental systems. These results emphasize the complexity of soil influences on plant biomass production.

In the second study, diversity of the soil bacterial community was not shown to affect pathogen invasion as per previous findings [24], which may be due to the more complex natural soil communities utilized by this study. However, the opposite result was observed when introducing the plant growth promoting rhizobacteria (PGPR), *Pseudomonas putida.* Beneficial effects with added *P. putida,* as measured by a percent increase in alfalfa shoot biomass, were found to be significant and highly correlated with soil bacterial diversity. However, root colonization of *P. putida* was not found to differ between non-filtered treatment groups (soil microorganisms retained). These results suggest that the mechanism for increased plant biomass was improved benefit from other soil microorganisms in the presence of *P. putida.* In filtered treatments, where soil microorganisms were removed, root colonization by the PGPR was improved but did not increase plant biomass. This result suggests that higher levels of PGPR colonization will not always improve plant growth, especially in the absence of other beneficial soil organisms. Specific taxonomy of the soil bacterial community was also found to be important to PGPR effects. Increases in shoot biomass were significantly correlated with specific nitrogen fixing and plant growth promoting genera in experimental soils and negatively correlated with *Actinoplanes,* known to produce numerous antibiotics. In summary, other members of the soil microbial community, plant stressors and whether the PGPR will help alleviate those stressors must be considered before applying PGPR. The results of this study have broader implications for improving our ability to manipulate the soil microbial communities for improved plant health and growth.

Further experiments can be conducted on representatives of the genera that showed significant effect with added PGPR to confirm enhanced PGPR performance. Additional pyrosequencing analyses post-harvest could confirm the rhizosphere competence of identified

bacteria. Also, using carefully designed qPCR primers to detect quantities of soil fungi would help elucidate the relative importance of bacterial and fungal members of the soil community for optimal plant biomass production. While this study made use of multiple soils, future studies further expanding the variety of soils, environmental and cultural systems are needed to better establish generalized conclusions associated with increased plant biomass and PGPR effects. This work also uncovered two bacterial species that showed potential deleterious effects on PGPR establishment. Further research should be applied to investigate the possible prevention of PGPR activity with these specific bacteria. Finally, more research could develop specific microbial partners that work synergistically to enhance plant production, increasing overall health of the agroecosystem. Understanding how microorganisms antagonize or benefit each other will contribute greatly to improving plant biomass production when manipulating agricultural soil microbial communities.

REFERENCES

1. Babalola OO (2010) Beneficial bacteria of agricultural importance. Biotechnology letters 32: 1559-1570. doi: 10.1007/s10529-010-0347-0
2. Bakker MG, Manter DK, Sheflin AM, Weir TL, Vivanco JM (2012) Harnessing the rhizosphere microbiome through plant breeding and agricultural management. Plant and Soil 360: 1-13. doi: 10.1007/s11104-012-1361-x
3. Chaparro JM, Sheflin AM, Manter DK, Vivanco JM (2012) Manipulating the soil microbiome to increase soil health and plant fertility. Biology and Fertility of Soils 48: 489-499. doi: 10.1007/s00374-012-0691-4
4. Cummings SP (2009) The application of plant growth promoting rhizobacteria (PGPR) in low input and organic cultivation of graminaceous crops; potential and problems. Environ Biotechnol 5: 43-50.
5. Esitken A (2011) Use of Plant Growth Promoting Rhizobacteria in Horticultural Crops. Bacteria in Agrobiology: Crop Ecosystems: 189-235.
6. Zolla G BM, Badri DV, Chaparro JM, Sheflin AM, Manter DK, Vivanco JM (In press) Understanding root-microbiome interactions. . In: Bruijn, Fd (ed.) Molecular Microbial Ecology of the Rhizosphere. Springer-Verlag, Heidelberg
7. Torsvik V, Salte K, Sorheim R, Goksoyr J (1990) Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. Applied and environmental microbiology 56: 776.
8. Roesch LFW, Fulthorpe RR, Riva A, Casella G, Hadwin AKM, Kent AD, Daroub SH, Camargo FAO, Farmerie WG, Triplett EW (2007) Pyrosequencing enumerates and contrasts soil microbial diversity. The ISME journal 1: 283-290.
9. Morales SE, Holben WE (2011) Linking bacterial identities and ecosystem processes: can 'omic' analyses be more than the sum of their parts? FEMS microbiology ecology 75: 2-16.
10. Badri DV, Weir TL, van der Lelie D, Vivanco JM (2009) Rhizosphere chemical dialogues: plant–microbe interactions. Current opinion in Biotechnology 20: 642-650.
11. Badri DV, Zolla G, Bakker MG, Manter DK, Vivanco JM (2013) Potential impact of soil microbiomes on the leaf metabolome and on herbivore feeding behavior. New Phytologist.
12. Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. Annual review of microbiology 63: 541-556. doi: 10.1146/annurev.micro.62.081307.162918
13. Maheshwari DK (2010) Plant Growth and Health Promoting Bacteria. Springer
14. Mendes R, Kruijt M, de Bruijn I, Dekkers E, van der Voort M, Schneider JH, Piceno YM, DeSantis TZ, Andersen GL, Bakker PA, Raaijmakers JM (2011) Deciphering the rhizosphere microbiome for disease-suppressive bacteria. Science 332: 1097-1100. doi: 10.1126/science.1203980
15. Colwell RK (2009) Biodiversity: concepts, patterns, and measurement. The Princeton Guide to Ecology.
16. Loreau M, Naeem S, Inchausti P, Bengtsson J, Grime JP, Hector A, Hooper DU, Huston MA, Raffaelli D, Schmid B, Tilman D, Wardle DA (2001) Biodiversity and ecosystem functioning: current knowledge and future challenges. Science 294: 804-808.

17. Lau JA, Lennon JT (2011) Evolutionary ecology of plant-microbe interactions: soil microbial structure alters selection on plant traits. The New phytologist 192: 215-224. doi: 10.1111/j.1469-8137.2011.03790.x
18. Schnitzer SA, Klironomos JN, Hillerislambers J, Kinkel LL, Reich PB, Xiao K, Rillig MC, Sikes BA, Callaway RM, Mangan SA, van Nes EH, Scheffer M (2011) Soil microbes drive the classic plant diversity-productivity pattern. Ecology 92: 296-303.
19. van der Heijden MGA, Boller T, Wiemken A, Sanders IR (1998) Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79: 2082-2091.
20. Wagg C, Jansa J, Schmid B, van der Heijden MG (2011) Belowground biodiversity effects of plant symbionts support aboveground productivity. Ecol Lett 14: 1001-1009. doi: 10.1111/j.1461-0248.2011.01666.x
21. Larkin RP, Honeycutt CW (2006) Effects of different 3-year cropping systems on soil microbial communities and rhizoctonia diseases of potato. Phytopathology 96: 68-79.
22. Ochiai N, Powelson ML, Crowe FJ, Dick RP (2008) Green manure effects on soil quality in relation to suppression of Verticillium wilt of potatoes. Biology and Fertility of Soils 44: 1013-1023. doi: 10.1007/s00374-008-0289-z
23. Postma J, Schilder MT, Bloem J, van Leeuwen-Haagsma WK (2008) Soil suppressiveness and functional diversity of the soil microflora in organic farming systems. Soil Biology and Biochemistry 40: 2394-2406. doi: 10.1016/j.soilbio.2008.05.023
24. van Elsas JD, Chiurazzi M, Mallon CA, Elhottova D, Kristufek V, Salles JF (2012) Microbial diversity determines the invasion of soil by a bacterial pathogen. Proceedings of the National Academy of Sciences of the United States of America 109: 1159-1164. doi: 10.1073/pnas.1109326109
25. Garbeva P, van Veen JA, van Elsas JD (2004) Microbial diversity in soil: selection microbial populations by plant and soil type and implications for disease suppressiveness. Annual review of phytopathology 42: 243-270.
26. N M (1983) Microbial antagonism to Phytophthora. American Phytopathological Society, St. Paul, MN
27. Jonsson LM, Nilsson MC, Wardle DA, Zackrisson O (2001) Context dependent effects of ectomycorrhizal species richness on tree seedling productivity93: 353-364. doi: 10.1034/j.1600-0706.2001.930301.x
28. Maherali H, Klironomos JN (2007) Influence of phylogeny on fungal community assembly and ecosystem functioning. Science 316: 1746-1748. doi: 10.1126/science.1143082
29. Anderson M, Habiger J (2012) Characterization and identification of productivity-associated rhizobacteria in wheat. Applied and environmental microbiology 78: 4434-4446. doi: 10.1128/AEM.07466-11
30. Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G (2003) Microbial diversity and soil functions. European Journal of Soil Science 54: 655-670.
31. Andrén O, Balandreau J (1999) Biodiversity and soil functioning—from black box to can of worms? Applied Soil Ecology 13: 105-108.
32. Bardgett RD, Shine A (1999) Linkages between plant litter diversity, soil microbial biomass and ecosystem function in temperate grasslands. Soil Biology and Biochemistry v. 31(2) p. 317-321: 317-321. doi: doi/10.1016/S0038-0717(98)00121-7

33. Wittebolle L, Marzorati M, Clement L, Balloi A, Daffonchio D, Heylen K, De Vos P, Verstraete W, Boon N (2009) Initial community evenness favours functionality under selective stress. Nature 458: 623-626.
34. van Elsas JD, Costa R, Jansson J, Sjoling S, Bailey M, Nalin R, Vogel TM, van Overbeek L (2008) The metagenomics of disease-suppressive soils - experiences from the METACONTROL project. Trends in biotechnology 26: 591-601.
35. Crowder DW, Northfield TD, Strand MR, Snyder WE (2010) Organic agriculture promotes evenness and natural pest control. Nature 466: 109-112. doi: 10.1038/nature09183
36. Wilsey BJ, Potvin C (2000) Biodiversity and ecosystem functioning: importance of species evenness in an old field. Ecology 81: 887-892.
37. Elliott LF, Lynch JM (1994) Biodiversity and soil resilience, p. 353-364. *In* D. J. Greenland and I. Szabolcs (ed.), Soil resilience and sustainable land use. CAB International, Wallingford, United Kingdom.
38. Fliessbach A, Winkler M, Lutz MP, Oberholzer H-R, Mäder P (2009) Soil amendment with Pseudomonas fluorescens CHA0: lasting effects on soil biological properties in soils low in microbial biomass and activity. Microbial ecology 57: 611-623.
39. Sugiyama A, Vivanco JM, Jayanty SS, Manter DK (2010) Pyrosequencing Assessment of Soil Microbial Communities in Organic and Conventional Potato Farms. Plant Disease 94: 1329-1335. doi: 10.1094/pdis-02-10-0090
40. Gosling P, Hodge A, Goodlass G, Bending GD (2006) Arbuscular mycorrhizal fungi and organic farming. Agriculture, Ecosystems & Environment 113: 17-35.
41. Perez-Piqueres A, Edel-Hermann V, Alabouvette C, Steinberg C (2006) Response of soil microbial communities to compost amendments. Soil Biology and Biochemistry 38: 460-470.
42. Kirankumar R, Jagadeesh KS (2010) Enhanced growth promotion of tomato and nutrient uptake by plant growth promoting rhizobacterial isolates in presence of tobacco mosaic virus pathogen. Karnataka Journal of Agricultural Science 21: 309-311.
43. Guiñazú LB, Andrés JA, Del Papa MF, Pistorio M, Rosas SB (2010) Response of alfalfa (Medicago sativa L.) to single and mixed inoculation with phosphate-solubilizing bacteria and Sinorhizobium meliloti. Biology and Fertility of Soils 46: 185-190.
44. Hayat R, Ali S, Amara U, Khalid R, Ahmed I (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Annals of Microbiology 60: 579-598. doi: 10.1007/s13213-010-0117-1
45. Lim J-H, Kim S-D (2009) Synergistic Plant Growth Promotion by the Indigenous Auxins-producing PGPR Bacillus subtilis AH18 and Bacillus licheniforims K11. Journal of the Korean Society for Applied Biological Chemistry 52: 531-538.
46. Selvakumar G, Panneerselvam P (2012) Bacterial Mediated Alleviation of Abiotic Stress in Crops. Bacteria in Agrobiology: Stress Management.
47. Chithrashree, Udayashankar AC, Chandra Nayaka S, Reddy MS, Srinivas C (2011) Plant growth-promoting rhizobacteria mediate induced systemic resistance in rice against bacterial leaf blight caused by Xanthomonas oryzae pv. oryzae. Biological Control 59: 114-122. doi: 10.1016/j.biocontrol.2011.06.010
48. De Vleesschauwer D, Höfte M (2009) Advances in Botanical Research. Elsevier

49. Jetiyanon K, Kloepper JW (2002) Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. Biological Control 24: 285-291. doi: http://dx.doi.org/10.1016/S1049-9644(02)00022-1
50. Okubara PA, Bonsall RF (2008) Accumulation of Pseudomonas-derived 2,4-diacetylphloroglucinol on wheat seedling roots is influenced by host cultivar. Biological Control 46: 322-331. doi: 10.1016/j.biocontrol.2008.03.013
51. Xie X, Zhang H, Pare PW (2009) Sustained growth promotion in Arabidopsis with long-term exposure to the beneficial soil bacterium Bacillus subtilis (GB03). Plant signaling & behavior 4: 948-953.
52. Zhang H, Kim M-S, Sun Y, Dowd SE, Shi H, Paré PW (2008) Soil bacteria confer plant salt tolerance by tissue-specific regulation of the sodium transporter HKT1. Molecular plant-microbe interactions : MPMI 21: 737-744.
53. Dimkpa C, Weinand T (2009) Plant–rhizobacteria interactions alleviate abiotic stress conditions. Plant, Cell & Environment: 1682-1694.
54. Zhang H, Murzello C, Sun Y, Kim M-S, Xie X, Jeter RM, Zak JC, Dowd SE, Paré PW (2010) Choline and osmotic-stress tolerance induced in Arabidopsis by the soil microbe Bacillus subtilis (GB03). Molecular plant-microbe interactions : MPMI 23: 1097-1104.
55. Weller DM, Raaijmakers JM, Gardener BBM, Thomashow LS (2002) Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annual review of phytopathology 40: 309-348.
56. Raaijmakers JM, Weller DM (1998) Natural plant protection by 2, 4-diacetylphloroglucinol-producing Pseudomonas spp. in take-all decline soils. Molecular plant-microbe interactions : MPMI 11: 144-152.
57. Raaijmakers JM, Weller DM, Thomashow LS (1997) Frequency of Antibiotic-Producing Pseudomonas spp. in Natural Environments. Applied and environmental microbiology 63: 881-887.
58. Kinsella K, Schulthess CP, Morris TF, Stuart JD (2009) Rapid quantification of Bacillus subtilis antibiotics in the rhizosphere. Soil Biology and Biochemistry 41: 374-379.
59. Adesemoye AO, Torbert HA, Kloepper JW (2009) Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. Microbial ecology 58: 921-929. doi: 10.1007/s00248-009-9531-y
60. Shaharoona B, Naveed M, Arshad M, Zahir ZA (2008) Fertilizer-dependent efficiency of Pseudomonads for improving growth, yield, and nutrient use efficiency of wheat (Triticum aestivum L.). Applied microbiology and biotechnology 79: 147-155.
61. Ahemad M, Khan MS (2012) Effect of fungicides on plant growth promoting activities of phosphate solubilizing Pseudomonasputida isolated from mustard (Brassica compestris) rhizosphere. Chemosphere 86: 945-950. doi: 10.1016/j.chemosphere.2011.11.013
62. Berg G (2009) Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied microbiology and biotechnology 84: 11-18.
63. Yang X, Chen L, Yong X, Shen Q (2011) Formulations can affect rhizosphere colonization and biocontrol efficiency of Trichoderma harzianum SQR-T037 against Fusarium wilt of cucumbers. Biology and Fertility of Soils 47: 239-248.
64. Pugliese M, Liu BP, Gullino ML, Garibaldi A (2011) Microbial enrichment of compost with biological control agents to enhance suppressiveness to four soil-borne diseases in greenhouse. Journal of Plant Diseases and Plant Protection (JPDP) 2: 45-50.

65. Mark G, Morrissey JP, Higgins P, O'Gara F (2006) Molecular-based strategies to exploit Pseudomonas biocontrol strains for environmental biotechnology applications. FEMS microbiology ecology 56: 167-177. doi: 10.1111/j.1574-6941.2006.00056.x
66. Morrissey JP, Dow JM, Mark GL, O'Gara F (2004) Are microbes at the root of a solution to world food production? Rational exploitation of interactions between microbes and plants can help to transform agriculture. EMBO reports 5: 922-926.
67. Barret M, Morrissey JP, O'Gara F (2011) Functional genomics analysis of plant growth-promoting rhizobacterial traits involved in rhizosphere competence. Biology and Fertility of Soils 47: 729-743.
68. Dutta S, Podile AR (2010) Plant growth promoting rhizobacteria (PGPR): the bugs to debug the root zone. Critical reviews in microbiology 36: 232-244. doi: 10.3109/10408411003766806
69. Bernard E, Larkin RP, Tavantzis S, Erich MS, Alyokhin A, Sewell G, Lannan A, Gross SD (2012) Compost, rapeseed rotation, and biocontrol agents significantly impact soil microbial communities in organic and conventional potato production systems. Applied Soil Ecology 52: 29-41. doi: 10.1016/j.apsoil.2011.10.002
70. Savka MA, Dessaux Y, Oger P, Rossbach S (2002) Engineering bacterial competitiveness and persistence in the phytosphere. Molecular plant-microbe interactions : MPMI 15: 866-874.
71. Maron JL, Marler M, Klironomos JN, Cleveland CC (2011) Soil fungal pathogens and the relationship between plant diversity and productivity. Ecol Lett 14: 36-41. doi: 10.1111/j.1461-0248.2010.01547.x
72. Naeem S, Håkansson K, Lawton JH, Crawley MJ, Thompson LJ (1996) Biodiversity and plant productivity in a model assemblage of plant species: 259-264.
73. Naeem S, Thompson LJ, Lawler SP, Lawton JH, Woodfin RM (1994) Declining biodiversity can alter the performance of ecosystems. Nature 368: 734-737.
74. Tilman D, Downing JA (1994) Biodiversity and stability in grasslands. Nature 367: 363-365.
75. Tilman D, Wedin D, Knops J (1996) Productivity and sustainability influenced by biodiversity in grassland ecosystems. Nature 379: 718-720.
76. van der Heijden MG, Bardgett RD, van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol Lett 11: 296-310. doi: 10.1111/j.1461-0248.2007.01139.x
77. Fargione J, Tilman D, Dybzinski R, Lambers JH, Clark C, Harpole WS, Knops JM, Reich PB, Loreau M (2007) From selection to complementarity: shifts in the causes of biodiversity-productivity relationships in a long-term biodiversity experiment. Proceedings Biological sciences / The Royal Society 274: 871-876. doi: 10.1098/rspb.2006.0351
78. Hooper DU, Dukes JS (2003) Overyielding among plant functional groups in a long-term experiment. Ecology Letters 7: 95-105. doi: 10.1046/j.1461-0248.2003.00555.x
79. Tilman D, Fargione J, Wolff B, D'Antonio C, Dobson A, Howarth R, Schindler D, Schlesinger WH, Simberloff D, Swackhamer D (2001) Forecasting agriculturally driven global environmental change. Science 292: 281-284. doi: 10.1126/science.1057544
80. Loreau M (2001) Microbial diversity, producer-decomposer interactions and ecosystem processes: a theoretical model. Proceedings Biological sciences / The Royal Society 268: 303-309. doi: 10.1098/rspb.2000.1366

81. Huston MA (1997) Hidden treatments in ecological experiments: re-evaluating the ecosystem function of biodiversity110: 449-460. doi: 10.1007/s004420050180
82. Loreau M (1998) Biodiversity and ecosystem functioning: a mechanistic model.95: 5632-5636.
83. Hooper DU, Chapin Iii FS, Ewel JJ, Hector A, Inchausti P, Lavorel S, Lawton JH, Lodge DM, Loreau M, Naeem S (2005) Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. Ecological monographs 75: 3-35.
84. Loreau M, Hector A (2001) Partitioning selection and complementarity in biodiversity experiments. Nature 412: 72-76.
85. Schnitzer SA, Klironomos JN, HilleRisLambers J, Kinkel LL, Reich PB, Xiao K, Rillig MC, Sikes BA, Callaway RM, Mangan SA, van Nes EH, Scheffer M (2010) Soil microbes drive the classic plant diversity–productivity pattern. Ecology 92: 296-303. doi: 10.1890/10-0773.1
86. Zhang H, Sun Y, Xie X, Kim MS, Dowd SE, Pare PW (2009) A soil bacterium regulates plant acquisition of iron via deficiency-inducible mechanisms. The Plant journal : for cell and molecular biology 58: 568-577. doi: 10.1111/j.1365-313X.2009.03803.x
87. Zhang H, Xie X, Kim MS, Kornyeyev DA, Holaday S, Pare PW (2008) Soil bacteria augment Arabidopsis photosynthesis by decreasing glucose sensing and abscisic acid levels in planta. The Plant journal : for cell and molecular biology 56: 264-273. doi: 10.1111/j.1365-313X.2008.03593.x
88. Zhang H, Murzello C, Sun Y, Kim MS, Xie X, Jeter RM, Zak JC, Dowd SE, Pare PW (2010) Choline and osmotic-stress tolerance induced in Arabidopsis by the soil microbe Bacillus subtilis (GB03). Molecular plant-microbe interactions : MPMI 23: 1097-1104. doi: 10.1094/mpmi-23-8-1097
89. McNamara NP, Black HIJ, Beresford NA, Parekh NR (2003) Effects of acute gamma irradiation on chemical, physical and biological properties of soils. Applied Soil Ecology 24: 117-132. doi: 10.1016/s0929-1393(03)00073-8
90. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and environmental microbiology 75: 7537-7541. doi: 10.1128/AEM.01541-09
91. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and environmental microbiology 72: 5069-5072. doi: 10.1128/AEM.03006-05
92. Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Austral Ecology 26: 32-46. doi: 10.1111/j.1442-9993.2001.01070.pp.x
93. Oksanen J, Kindt R, Legendre P, O'Hara B (2007) Vegan: the community ecology package, v. 1.8-4.
94. Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology 18: 117-143. doi: 10.1111/j.1442-9993.1993.tb00438.x
95. Roberts D (2007) Labdsv: Ordination and multivariate analysis for ecology. R package version 1: 3-1.

96. Staley TE, Lawrence EG, Nance EL (1992) Influence of a plant growth-promoting pseudomonad and vesicular-arbuscular mycorrhizal fungus on alfalfa and birdsfoot trefoil growth and nodulation14: 175-180.
97. Winer BJ (1971) Statistical principles in experimental design.
98. Shirey JJ, Bissonnette GK (1991) Detection and identification of groundwater bacteria capable of escaping entrapment on 0.45-micron-pore-size membrane filters. Applied and environmental microbiology 57: 2251-2254.
99. Poranen MM, Daugelavicius R, Bamford DH (2002) Common principles in viral entry. Annual review of microbiology 56: 521-538. doi: 10.1146/annurev.micro.56.012302.160643
100. McGuinness M, Dowling D (2009) Plant-associated bacterial degradation of toxic organic compounds in soil. International journal of environmental research and public health 6: 2226-2247. doi: 10.3390/ijerph6082226
101. van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396: 69-72. doi: 10.1038/23932
102. Monard C, Gantner S, Stenlid J (2012) Utilising ITS1 and ITS2 to study environmental fungal diversity using pyrosequencing. FEMS microbiology ecology. doi: 10.1111/1574-6941.12046
103. Orgiazzi A, Lumini E, Nilsson RH, Girlanda M, Vizzini A, Bonfante P, Bianciotto V (2012) Unravelling soil fungal communities from different Mediterranean land-use backgrounds. PloS one 7: e34847. doi: 10.1371/journal.pone.0034847
104. Lorenz MG, Wackernagel W (1994) Bacterial gene transfer by natural genetic transformation in the environment. Microbiological reviews 58: 563-602.
105. McInerney MJ, Struchtemeyer CG, Sieber J, Mouttaki H, Stams AJ, Schink B, Rohlin L, Gunsalus RP (2008) Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. Annals of the New York Academy of Sciences 1125: 58-72. doi: 10.1196/annals.1419.005
106. Knops JMH, Tilman D, Haddad NM, Naeem S, Mitchell CE, Haarstad J, Ritchie ME, Howe KM, Reich PB, Siemann E (1999) Effects of plant species richness on invasion dynamics, disease outbreaks, insect abundances and diversity. Ecology Letters 2: 286-293.
107. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glockner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic acids research 35: 7188-7196. doi: 10.1093/nar/gkm864
108. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27: 2194-2200. doi: 10.1093/bioinformatics/btr381
109. Wang Y, Hammes F, Boon N, Egli T (2007) Quantification of the filterability of freshwater bacteria through 0.45, 0.22, and 0.1 μm pore size filters and shape-dependent enrichment of filterable bacterial communities. Environmental Science & Technology 41: 7080-7086.
110. Gray FA, Bohl WH, Abernethy RH (1983) Phytophthora root rot of alfalfa in Wyoming. Plant Disease 67: 291-294.

111. Coelho MR, de Vos M, Carneiro NP, Marriel IE, Paiva E, Seldin L (2008) Diversity of nifH gene pools in the rhizosphere of two cultivars of sorghum (Sorghum bicolor) treated with contrasting levels of nitrogen fertilizer. FEMS microbiology letters 279: 15-22. doi: 10.1111/j.1574-6968.2007.00975.x
112. Gupta VVSR (2012) Beneficial microorganisms for sustainable agriculture. MICROBIOLOGY 113. doi: papers2://publication/uuid/863F4F90-EE73-4BFE-97E5-3DEE494BED4B
113. Karagöz K, Ateş F, Karagöz H, Kotan R, Çakmakçı R (2012) Characterization of plant growth-promoting traits of bacteria isolated from the rhizosphere of grapevine grown in alkaline and acidic soils. European Journal of Soil Biology 50: 144-150. doi: 10.1016/j.ejsobi.2012.01.007
114. Taulé C, Mareque C, Barlocco C, Hackembruch F, Reis VM, Sicardi M, Battistoni F (2012) The contribution of nitrogen fixation to sugarcane (Saccharum officinarum L.), and the identification and characterization of part of the associated diazotrophic bacterial community. Plant and Soil 356: 35-49.
115. Qiao L, Wang J-L (2010) Microbial degradation of pyridine by Paracoccus sp. isolated from contaminated soil. Journal of hazardous materials 176: 220-225.
116. Trujillo ME, Alonso-Vega P, Rodriguez R, Carro L, Cerda E, Alonso P, Martinez-Molina E (2010) The genus Micromonospora is widespread in legume root nodules: the example of Lupinus angustifolius. The ISME journal 4: 1265-1281. doi: 10.1038/ismej.2010.55
117. Ambrosini A, Beneduzi A, Stefanski T, Pinheiro FG, Vargas LK, Passaglia LMP (2012) Screening of plant growth promoting Rhizobacteria isolated from sunflower (Helianthus annuus L.). Plant and Soil 356: 245-264.
118. Casida LE (1983) Interaction of Agromyces ramosus with Other Bacteria in Soil. Applied and environmental microbiology 46: 881-888.
119. Elghair SAA (2012) The effect of nitrogen on rhizobacteria associated with wheat shoot productivity. Master's Thesis: 73.
120. Hirsch AM, Valdés M (2010) Micromonospora: An important microbe for biomedicine and potentially for biocontrol and biofuels. Soil Biology and Biochemistry 42: 536-542. doi: 10.1016/j.soilbio.2009.11.023
121. You MP, Sivasithamparam K, Kurtböke DI (1996) Actinomycetes in organic mulch used in avocado plantations and their ability to suppress Phytophthora cinnamomi. Biology and Fertility of Soils 22: 237-242.
122. Chung WK, King GM (2001) Isolation, characterization, and polyaromatic hydrocarbon degradation potential of aerobic bacteria from marine macrofaunal burrow sediments and description of Lutibacterium anuloederans gen. nov., sp. nov., and Cycloclasticus spirillensus sp. nov. Applied and environmental microbiology 67: 5585-5592.
123. Ma Y, Prasad MN, Rajkumar M, Freitas H (2011) Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. Biotechnology advances 29: 248-258. doi: 10.1016/j.biotechadv.2010.12.001
124. Swaroop S, Sughosh P, Ramanathan G (2009) Biomineralization of N,N-dimethylformamide by Paracoccus sp. strain DMF. Journal of hazardous materials 171: 268-272. doi: 10.1016/j.jhazmat.2009.05.138
125. Bryant DA, Costas AMG, Maresca JA, Chew AGM, Klatt CG, Bateson MM, Tallon LJ, Hostetler J, Nelson WC, Heidelberg JF, Ward DM (2007) Candidatus

Chloracidobacterium thermophilum: An Aerobic Phototrophic Acidobacterium. Science 317: 523-526.
126. Garrity GM, Bell JA, Lilburn T (2005) Family Rhodobiaceae. Springer, New York
127. Luo G, Shi Z, Wang H, Wang G (2012) Skermanella stibiiresistens sp. nov., a highly antimony-resistant bacterium isolated from coal-mining soil, and emended description of the genus Skermanella. International journal of systematic and evolutionary microbiology 62: 1271-1276. doi: 10.1099/ijs.0.033746-0
128. El-Tarabily KA, St. J. Hardy GE, Sivasithamparam K (2010) Performance of three endophytic actinomycetes in relation to plant growth promotion and biological control of Pythium aphanidermatum, a pathogen of cucumber under commercial field production conditions in the United Arab Emirates. European Journal of Plant Pathology 128: 527-539. doi: 10.1007/s10658-010-9689-7
129. Lazzarini A, Cavaletti L, Toppo G, Marinelli F (2000) Rare genera of actinomycetes as potential producers of new antibiotics. Antonie van Leeuwenhoek 78: 399-405.
130. Singh Grewal H (2001) Zinc influences nodulation, disease severity, leaf drop and herbage yield of alfalfa cultivars. Plant and Soil 234: 47-59.
131. Benndorf D, Thiersch M, Loffhagen N, Kunath C, Harms H (2006) Pseudomonas putida KT2440 responds specifically to chlorophenoxy herbicides and their initial metabolites. Proteomics 6: 3319-3329. doi: 10.1002/pmic.200500781
132. Ingle RW, Deshmukh VV (2010) Antifungal activity of PGPR and sensitivity to Agrochemicals. Annals of Plant Protection Sciences 18: 451-457.
133. Tilman D (1982) Resource competition and community structure. Monographs in population biology 17: 1-296.
134. Tilman D (1999) The Ecological Consequences of Changes in Biodiversity: A Search for General Principals 101. Ecology 80: 1455-1474.
135. de Boer M, Bom P, Kindt F, Keurentjes JJB, van der Sluis I, van Loon LC, Bakker PAHM (2003) Control of Fusarium Wilt of Radish by Combining Pseudomonas putida Strains that have Different Disease-Suppressive Mechanisms. Phytopathology 93: 626-632.
136. Augé RM (2001) Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 11: 3-42.
137. Barea JM, Azcon-Aguilar C, Azcón R (1987) Vesicular−arbuscular mycorrhiza improve both symbiotic N2 fixation and N uptake from soil as assessed with a 15N technique under field conditions. New Phytologist 106: 717-725.
138. Nakkeeran S, Fernando W, Siddiqui Z (2006) Plant growth promoting rhizobacteria formulations and its scope in commercialization for the management of pests and diseases. PGPR: biocontrol and biofertilization: 257-296.
139. Waitz JA, Sabatelli F, Menzel F, Moss EL (1974) Biological Activity of Antibiotic G-418, a New Micromonospora-Produced Aminoglycoside with Activity Against Protozoa and Helminths. Antimicrobial Agents and Chemotherapy 6: 579-581. doi: 10.1128/aac.6.5.579
140. Zakhia F, Jeder H, Willems A, Gillis M, Dreyfus B, de Lajudie P (2006) Diverse Bacteria Associated with Root Nodules of Spontaneous Legumes in Tunisia and First Report for nifH-like Gene within the Genera Microbacterium and Starkeya. Microbial ecology 51: 375-393.

141. Chen WM, Tang YQ, Mori K, Wu XL (2012) Distribution of culturable endophytic bacteria in aquatic plants and their potential for bioremediation in polluted waters. Aquatic Biology 15: 99-110.
142. Valenzuela-Encinas C, Neria-González I, Alcántara-Hernández RJ, Estrada-Alvarado I, Zavala-Díaz de la Serna FJ, Dendooven L, Marsch R (2009) Changes in the bacterial populations of the highly alkaline saline soil of the former lake Texcoco (Mexico) following flooding. Extremophiles : life under extreme conditions 13: 609-621.
143. Lin Q, Donghui W, Jianlong W (2010) Biodegradation of pyridine by Paracoccus sp. KT-5 immobilized on bamboo-based activated carbon. Bioresource Technology 101: 5229-5234. doi: http://dx.doi.org/10.1016/j.biortech.2010.02.059