

2017

Effects of Root Isoflavonoids and Hairy Root Transformation on the Soybean Rhizosphere Bacterial Community Structure

Laura White
South Dakota State University

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EFFECTS OF ROOT ISOFLAVONOIDS AND HAIRY ROOT TRANSFORMATION
ON THE SOYBEAN RHIZOSPHERE BACTERIAL COMMUNITY STRUCTURE

BY

LAURA WHITE

A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

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Specialization in Microbiology

South Dakota State University

2017

EFFECTS OF ROOT ISOFLAVONOIDS AND HAIRY ROOT TRANSFORMATION
ON THE SOYBEAN RHIZOSPHERE BACTERIAL COMMUNITY STRUCTURE

This thesis is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidates are necessarily the conclusions of the major department.

~~Senthil Subramanián, Ph.D.~~
Dissertation Advisor

Date

~~Volker Brözel, Ph.D.~~
Head, Biology and Microbiology

Date

~~Kinchel Doerner, Ph.D.~~
Dean, Graduate School

Date

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ABBREVIATIONS

“	Inch
φ	Phi
°C	Degrees Celsius/centigrade
× g	g-force
A	Amp
BLAST	Basic Local Alignment Search Tool
CARD-FISH	Catalyzed reporter deposition-fluorescence <i>in situ</i> hybridization
CFU	Colony forming unit
cm	Centimeter
dH ₂ O	Distilled H ₂ O/water
DCA	Detrended correspondence analysis
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DS	Distal soil
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescence <i>in situ</i> hybridization
g	Gram
GFP	Green fluorescent protein
h	Hour
HPLC	High performance liquid chromatography
Hz	Hertz
IFS	Isoflavone synthase
IFSi	Isoflavone synthase interference

IFS-RNAi	Isoflavone synthase ribonucleic acid interference
in	Inch
kg	Kilogram
kHz	Kilohertz
mg	Milligrams
min	Minute
ml	Milliliter
MS	Middle soil
N	Nitrogen
ng	Nanogram
nmoles	Nanomoles
OTU	Operational taxonomic unit
<i>P</i>	P-value
PBST	Phosphate buffered saline Tween 20
PCR	Polymerase chain reaction
pH	Potential hydrogen
PPM	Parts per million
PS	Proximal soil
qPCR	Quantitative polymerase chain reaction
Ri	Root inducing
RISA	Ribosomal RNA intergenic spacer analysis
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RNAi	RNA interference
RT-qPCR	Quantitative reverse transcription polymerase chain reaction

s	Second
SDSU	South Dakota State University
SESOM	Soil extracted soluble organic matter
SFS	Bulk soil/soybean field soil
SYBR	Synergy Brands, Inc.
T-DNA	Transfer DNA
T _L	T-DNA left
T _R	T-DNA right
μL	Microliter
μM	Micromolar
UNR	Untransformed root
UV	Ultraviolet
V	Volt
V13	Variable regions V1-V3
V35	Variable regions V3-V5
VA	Volt-ampere
VAC	Volts of alternating current
VC	Vector control
W	Watt
wpp	Weeks post planting

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ABSTRACT

EFFECTS OF ROOT ISOFLAVONOIDS AND HAIRY ROOT TRANSFORMATION ON THE SOYBEAN RHIZOSPHERE BACTERIAL COMMUNITY STRUCTURE

LAURA WHITE

2017

Rhizodeposits play a key role in shaping rhizosphere microbial communities. In soybean, isoflavonoids are a key rhizodeposit component that aid in plant defense and enable symbiotic associations with rhizobia. However, it is uncertain if and how they influence rhizosphere microbial communities. Isoflavonoid biosynthesis was silenced via RNA interference of isoflavone synthase in soybean hairy root composite plants. Successive sonication was implemented to isolate soil fractions from 3 different rhizosphere zones at 1 and 3 weeks post planting. PCR amplicons from 16S rRNA gene variable regions V1-V3 and V3-V5 from these soil fractions were analyzed via denaturing gradient gel electrophoresis and/or Roche 454 sequencing profiles. Extensive diversity analysis of the denaturing gradient gel electrophoresis patterns indicated that, indeed, isoflavonoids significantly influenced soybean rhizosphere bacterial diversity. These results also suggested a temporal gradient effect of rhizodeposit isoflavonoids on the rhizosphere. Roche 454 sequencing data was resolved using *MOTHUR* and *vegan* to identify bacterial taxa and evaluate changes in rhizosphere bacterial communities. The soybean rhizosphere was enriched in Proteobacteria and Bacteroidetes, and had relatively lower levels of Actinobacteria and Acidobacteria compared to bulk soil. Isoflavonoids had a small effect on bacterial community structure, and in particular on the abundance of Xanthomonads and Comamonads. Additionally, isoflavonoids appeared to have a temporal gradient

effect on the rhizosphere. The effect of hairy root transformation on rhizosphere bacterial communities was largely similar to untransformed plant roots with ~74% of the bacterial families displaying similar colonization underscoring the suitability of this technique to evaluate the influence of plant roots on rhizosphere bacterial communities. However, hairy root transformation had notable influence on Sphingomonads and Acidobacteria.

INTRODUCTION

1. Rhizosphere microbiome and plant growth

Plant-microbe interactions have garnered increasing interest as researchers search for efficient methods to improve plant growth, health and yield (Busby et al., 2017). Various studies have shifted their focus to interactions between plant roots and microorganisms such as bacteria, fungi, and viruses residing within the soil (i.e. the soil microbiome). A soil region of particular interest is the rhizosphere, which consists of the soil surrounding and influenced by plant roots. Previous research has shown soil microbial density is highest within this rhizosphere region, showing upwards of a fivefold increase in colony forming units compared to soil farther from the plant roots (Clark, 1940; Hinsinger and Marschner, 2006). When they are not inducing diseases, members of the rhizosphere microbiome can positively affect the plants with regards to improving tolerance to abiotic (ex. flooding, drought, high salinity, etc.) and biotic stressors (ex. pathogens) as well as promoting plant health, growth and yield (Müller et al., 2016). These positive effects are accomplished in various ways. For example, plant-growth-promoting rhizobium can help alleviate oxidative damage to the plants in drought conditions by increasing proline accumulation and stimulating antioxidant enzyme activity (Kohler et al., 2008). They may also rescue the normal growth of plants in environments with high saline levels by degrading the ethylene precursor 1-aminocyclopropane-1-carboxylate (Mayak et al., 2004). Mutualistic soil microbes may also improve plant tolerance to pathogens through tactics such as producing lytic enzymes, antibiosis, inhibiting pathogen virulence, competing for nutrients, and inducing plant resistance (Braga et al., 2016;

Müller et al., 2016). For example, *Pseudomonas* spp. are noted to produce antibiotics such as pyoluteorin and pyrrolnitrin that target pathogenic fungi in crop plants (Haas and Keel, 2003). These same soil microbes may also help develop disease-suppressive soils where plants do not contract a disease, despite the presence of the pathogen. Soil microbes may also affect plant nutrient status both directly and indirectly. This may be accomplished through the formation of symbiotic associations with microbes like rhizobia and mycorrhizal fungi to gain access to nitrogen and phosphorous under limiting conditions (Smith and Smith, 2011; Udvardi and Poole, 2013). Compounds, like auxin, produced by some soil microbes may enhance nutrient acquisition by altering root system architecture. Alternatively, soil microbes may mobilize nutrients not readily available to plants through mineralization, solubilization, or excretion of siderophores (Braga et al., 2016; Müller et al., 2016).

2. Effect of plants on the rhizosphere microbiome

Though the soil microbiome can affect plants in multiple ways the plants, in turn, can affect the soil microbiome in both size and diversity. Studies in multiple plant species, such as maize and *Arabidopsis thaliana*, have shown differences between bacterial communities of bulk soil and rhizosphere soil at the phylum level (Lundberg et al., 2012; Peiffer et al., 2013). For example, one study showed Acidobacteria and Firmicutes decreased in rhizosphere soil whilst Acidobacteria increased and Actinobacteria decreased in bulk soil during soybean growth (Sugiyama et al., 2014). Plant community diversity, developmental stage, species and genotype are also noted to influence soil microbial community structure (Philippot et al., 2013). For example, one soybean study showed the rhizosphere bacterial community changed with the

reproductive growth stages R₁, R₃, R₄, R₅, R₆ and R₈ as well as between two genotypes, though to a limited extent (Xu et al., 2009). Another study in *A. thaliana* noted bacterial phyla such as Actinobacteria, Bacteroidetes, Acidobacteria and Cyanobacteria followed distinct patterns associated with plant's development (Chaparro et al., 2014). Comparing multiple studies highlights how different plant species can affect the soil microbiome. For example, the *Populus deltoids* rhizosphere is dominated by the Proteobacteria, Acidobacteria and Verrucomicrobia bacterial phyla whereas the maize rhizosphere is dominated by the Proteobacteria, Bacteroidetes and Actinobacteria phyla (Gottel et al., 2011; Peiffer et al., 2013).

The ability of plant roots to induce changes in the soil microbiome is attributed to factors that alter soil properties or directly affect the soil microbes. These factors include changing soil pH through the release and uptake of ions, altering soil oxygen pressure by water uptake, and modifying soil nutrient availability via plant uptake or secretion of rhizodeposits (Philippot et al., 2013). Soil pH exerts a strong effect and is considered an important, if not the best, predictor of soil microbial community composition compared to other factors like climate and soil moisture deficit, organic content, and carbon to nitrogen ratio. One study also noted soil bacterial diversity and richness was lowest in acidic soils compared to neutral soils, further demonstrating the impact of soil pH on the soil microbiome (Fierer and Jackson, 2006; Bru et al., 2011). Plants can help adjust this soil pH up to 1 or 2 units by releasing or taking up ions through their roots (Riley and Barber, 1971; Gahoonia et al., 1992). In addition to altering pH, plants can also affect soil microbes using water uptake to impact soil oxygen pressure. The level of soil moisture content alters gas diffusion rates to and

from microbial communities, which regulates the activities of aerobic against anaerobic microbes. For example, soil moisture content is noted to affect microbial activity for processes like nitrification, respiration and denitrification. Soils with low saturation only showed low levels of nitrification and respiration. Moderately saturated soils display little to no denitrification and higher levels of both nitrification and respiration. Highly saturated soils showed a high level of denitrification, a moderate level of nitrification, and little respiration (Linn and Doran, 1984; Young and Ritz, 2000). These shifts in microbial activity serve as indicators of changes in the microbial community.

3. Rhizodeposits

In addition to the aforementioned tactics, plants may also modify soil nutrient contact either by consuming said nutrients or secreting rhizodeposits. Rhizodeposits – organic compounds such as amino acids, sugars and vitamins – are of particular interest since they more directly influence soil microbes by providing carbon sources, altering soil chemistry and acting as signaling compounds (Philippot et al., 2013). The composition and concentrations of these rhizodeposits can be affected by the plant's growing environment (ex. soil properties), its interactions with symbiotic or pathogenic bacteria, and even its developmental stage (Rovira, 1969; Tang et al., 1995). Some plant-microbe interactions instigate the production of a specific rhizodeposit. For example, attacks by *Pseudomonas syringae* pv *tomato* – a foliar pathogen – induces the secretion of L-malic acid in *A. thaliana*. This secretion then signals and recruits *Bacillus subtilis* FB17 – a beneficial rhizobacterium – to form a protective biofilm on the plant roots (Rudrappa et al., 2008). Other factors, like plant

developmental stage, can affect the secretion of multiple rhizodeposits simultaneously. This was demonstrated in *Arabidopsis*, which produced a different rhizodeposit composition for the two leaf growth stage, 5 leaf rosette stage, bolting stage, and flowering stage. Sugar alcohol and sugar secretion levels were higher at earlier growth stages and gradually lowered at later growth stages. Phenolic and amino acid secretion levels showed the opposite trend, starting at low levels at earlier growth stages and gradually increasing at later growth stages (Chaparro et al., 2013). Plants may use rhizodeposits to attract beneficial microbes to improve stress tolerance, promote plant growth, improve nutrient acquisition through the establishment of symbiotic relationships (ex. root nodule formation for nitrogen fixation), and defend against pathogenic microbes via antibiotic production or protective biofilm formation. Soil microbes, both pathogenic and beneficial, are able to use these rhizodeposits as nutrient sources or chemoattractants (Bais et al., 2006; Lugtenberg and Kamilova, 2009). Rhizodeposits like (E)- β -Caryophyllene aid with plant defense. Maize roots exude this compound when attacked by *Diabrotica virgifera* larvae to attract an entomopathogenic nematode, a natural predator of said larvae (Rasmann et al., 2005; Köllner et al., 2008). Other rhizodeposits can play both beneficial and detrimental roles to the plant. For example, strigolactones serve as hyphal branching factors for symbiotic arbuscular mycorrhizal fungi, which improve plant and water nutrient uptake (Akiyama et al., 2005). However, they also serve as germination stimulants for root parasitic plants like *Striga* spp., which can cause severe crop yield losses (Ruyter-Spira et al., 2013). Since rhizodeposits serve many

different functions, and because plant-microbe interactions are rather complex, this study focused rhizodeposits that serve a more active role in a single plant species.

4. Hairy root transformation and rhizodeposits

When ascertaining the effects species-specific rhizodeposits exert on the microbial community, a major limitation is a lack of genetic mutants in all plant species. Implementing RNA interference (RNAi) in hairy-root composite plants is a useful tactic for surmounting this limitation, particularly because it is adaptable to many dicot species. One such approach uses the root-inducing (Ri)-plasmid of *Agrobacterium rhizogenes* to generate hairy-root composite plants with untransformed shoots and transgenic roots (Tzfira and Citovsky, 2006; Pitzschke, 2013). This is advantageous since the biosynthesis pathway remains active in the plant shoot and compounds transported from shoot to root for exudation or deposition will likely remain unaffected unless specific transporters are silenced. However, one should consider the hairy root transformation procedure alters the plant's genome. During the procedure *A. rhizogenes* transfers a segment of its Ri plasmid – referred to as transfer DNA (T-DNA) – into the plant's nuclear DNA to be transcribed into mRNA during infection. The aforementioned T-DNA possesses genes that control opines, which serve as nitrogen, carbon, and energy sources for the *A. rhizogenes* (Chilton et al., 1982; Willmitzer et al., 1982; Petit et al., 1983). Different opines are produced depending on the *A. rhizogenes* strain. Additionally, the T-DNA region of the Ri plasmid can differ depending on the opines involved. For example, the Ri plasmid of *A. rhizogenes* strains producing mannopine or cucumopine only possess one T-DNA region whereas strains producing agropine have a split T-DNA

consisting of two regions, noted as T-DNA left (T_L) and T-DNA right (T_R). In the case of agropine-type Ri plasmids, the T_R region controls the biosynthesis of auxin and opines whereas the T_L region induces neoplastic roots with faster growth rates compared to normal tissue. The single T-DNA fragment of mannopine- or cucumopine-type Ri plasmids has a function similar to the aforementioned T_L region and controls the biosynthesis of opines, but it does not control the biosynthesis of auxin (Veena and Taylor, 2007). For this study the *A. rhizogenes* strain K599, which contains a cucumopine-type Ri plasmid, was selected (Xiang et al., 2016). Regardless of which Ri plasmid is involved, the production of different opines or auxin levels could affect the rhizosphere by providing different mixtures of nutrients. Also, the plant roots may still produce inconsistent, rhizodeposit levels after successful hairy root transformation. For example, silencing isoflavonoid biosynthesis in roots was noted to affect the accumulation of *p*-hydroxybenzaldehyde, *p*-hydroxy benzoic acid, liquiritigenin, and coumaric acid, although the majority of phenylpropanoid pathway metabolites remained unaffected (Subramanian et al., 2006; Lozovaya et al., 2007). Additionally, hairy root cultures across multiple plant species are noted to steadily generate high amounts of secondary metabolites in response to damage by pathogens, such as the *A. rhizogenes* used for the transformation (Bulgakov, 2008; Chandra, 2012). Such changes in rhizodeposit levels will likely affect the rhizosphere bacterial communities, thus one should exercise caution when implementing this approach. Another possible approach uses RNAi to silence parts of the flavonoid exudation machinery as well as adsorbents to help ensure rhizodeposit isoflavonoids fail to reach the rhizosphere microbiome. However, the rhizodeposition of compounds like

isoflavonoids occurs through other mechanisms, such as root border cells, in addition to root exudation (Hawes et al., 2000). Thus silencing root exudation machinery components, like ABC transporters, may fail to efficiently deplete isoflavonoid rhizodeposits (Sugiyama et al., 2007; Brechenmacher et al., 2009). Additionally, utilizing adsorbents may interfere with quorum signaling between bacteria and induce nonspecific alterations within the root-surface microbiome. This may lead to indirect effects on nontarget organisms, including the plant generating the rhizodeposits (Hassan and Mathesius, 2012).

5. Isoflavonoids and plant-microbe interactions

The rhizodeposits of interest, isoflavonoids, were selected because they are legume-specific compounds that likely help signal bacterial communities rather than act as an energy source. Isoflavonoids are produced by a specialized branch of the phenylpropanoid pathway, which uses metabolic channeling to physically organize enzymes into complexes through which intermediates are channeled without diffusion into the majority of the cytosol (Srere, 1987). Such complexes enable efficient control of metabolic flux as well as protection for unstable intermediates from non-productive breakdown or access to enzymes from possibly competing pathways.

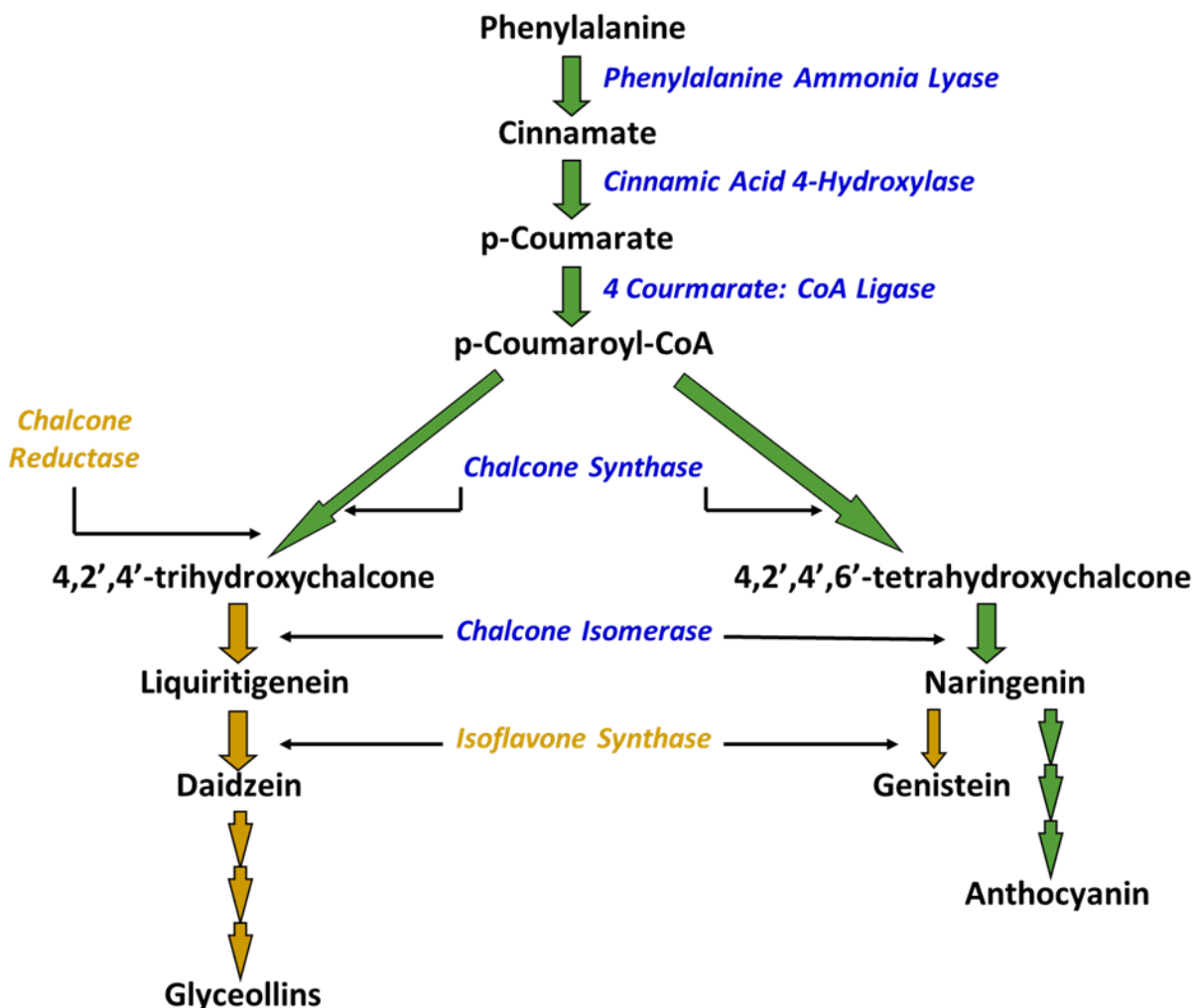


Figure 1. Partial diagram of the phenylpropanoid pathway in soybean.

A previous study confirmed the aforementioned metabolic channeling between isoflavone synthase and IOMT at the starting point for the isoflavonoid phytoalexin pathway. In this case, isoflavone synthase catalyzes the oxidation of the complexes naringenin or liquiritigenin to produce the isoflavonoids genistein or daidzein. Such channeling can impact plant defense responses. For example, intermediates designated to become a specific metabolic end product could be channeled in a certain way so they use different groups of metabolic enzymes than other products that may share a few of the same biosynthetic steps. Thus multiple genes for many

phenylpropanoid pathway enzymes could have both distinct and overlapping functions, which could help improve the efficiency of induced defenses (Liu and Dixon, 2001; Dixon et al., 2002). This is especially noteworthy since isoflavonoids are noted for their assistance with plant defense against pathogenic microbes in addition to their ability to regulate nodulation factors (Hassan and Mathesius, 2012). In particular, the isoflavonoids daidzein and genistein have been shown to inhibit *Sinorhizobium meliloti* nod genes and induce *Bradyrhizobium japonicum* nod genes in leguminous plants (Peck et al., 2006; Subramanian et al., 2006). The isoflavonoids coumestral and medicarpin serve as nod gene suppressors in *S. meliloti* (Zuanazzi et al., 1998). Coumestral is also noted to serve as an active stimulator of hyphal growth and, in a *Medicago truncatula* mutant, able to facilitate hyper-infection of the mycorrhizal symbiont *Glomus intraradices* (Morandi et al., 1984; Morandi et al., 2009). Other isoflavonoids play defensive roles to protect plants against pathogenic microbes. For example, one study showed that silencing of isoflavone synthase – a key enzyme for isoflavone formation – in soybean leads to greater susceptibility to the root rot pathogen *Phytophthora sojae* (Subramanian et al., 2005). Derivatives of isoflavonoids called pterocarpans also aid plant defense in legumes, mitigating damage from harmful fungi. Notably, pisatin production reduces damage caused by the *Nectria haematococca* fungus in *Pisum sativum* L. (Naoumkina et al., 2010). Isoflavonoids can also affect nutrient acquisition in *Medicago sativa* by dissolving ferric acid to make phosphate and iron readily available to the plant in iron deficient conditions (Ichihara, 1993). Other research implies isoflavonoids can break down auxin in white clover and modulate auxin transportation in soybean (Hassan and

Mathesius, 2012). Although isoflavonoids appear to play more active roles in the interactions between plants specific soil microbes, it is uncertain if or how they affect the soil microbial community as a whole. Additionally, knowledge of actual flavonoid concentrations, including isoflavonoids, in soil and how said concentrations change in space and time is limited.

6. Isoflavonoids in soybean

Glycine max (soybean) was the chosen plant species firstly, because isoflavonoids are legume-specific, and secondly, it serves an important role in biodiesel fuel, livestock feed, and biocomposite building material production (Singh, 2010). In particular, processed soybeans provide the world's largest source of animal protein feed as well as its second largest source of vegetable oil. The United States serves as the world's leading producer of soybean, producing ~106.86 million metric tons from 2015 to 2016 compared to the ~96.50 million metric tons produced by Brazil, the second largest producer. Nearly half of the soybeans produced in the United States (~52.68 million metric tons) were exported within that same year (United States Department of Agriculture, 2017). Within the United States, soybean is the second-most-planted field crop, with ~83 million acres planted in 2016 (USDA, 2016). Since soybean production and exportation are so prominent in the United States, improving soybean yield is an important endeavor. Studies that clarify how plant rhizodeposits, like isoflavonoids, affect plant-microbe interactions will help us reach that goal by enabling us to improve plant health and growth. In soybean, isoflavonoids are essential for root nodule formation because of their ability to induce nodulation genes in the symbiont *B. japonicum*. This was demonstrated in a previous study, which

showed a severe reduction in nodulation by *B. japonicum* in soybean with low isoflavonoid levels (Subramanian et al., 2006). Isoflavonoids are also noted to help protect soybean against the root rot pathogen *P. sojae*. A previous study revealed soybean with low isoflavonoid levels were more susceptible to *P. sojae* due to disruption of both nonrace-specific resistance in the cotyledon tissues as well as R gene-mediated race-specific resistance within the roots (Subramanian et al., 2005). With the aforementioned in mind, increased isoflavonoid levels could aid with nutrient acquisition (i.e. increased formation of nitrogen-fixing root nodules) as well as defense (i.e. inhibiting pathogens) to improve soybean health, growth and yield. To accomplish this, one could apply a seed coat treatment containing high isoflavonoid levels to provide an early advantage to soybean seedlings. Alternatively, one could use metabolic engineering of the phenylpropanoid biosynthesis pathway to increase soybean isoflavonoid production throughout the growing season.

7. Goals and approaches

The ultimate goal of our study was to determine the effect of isoflavonoids on soybean rhizosphere bacterial communities. In particular, we aimed to note any changes in the general community structure as well as how specific taxonomies were affected. To reach this goal, we first needed to generate soybean roots with drastically reduced isoflavonoid levels. Our next hurdle was acquiring rhizosphere soil samples in a reproducible fashion without damaging the soybean roots or lysing the bacteria. We then needed to determine the best approaches to acquire data regarding the overall bacterial community as well as its various taxonomies. Last of all, we had to select the most appropriate analyses for our datasets.

8. Root and rhizodeposit isoflavonoids and plant-microbe interactions

Previous isoflavonoid studies focused on successfully generating plant roots that produced low levels of isoflavonoids and how the reduced levels impacted specific microbes. These studies used RNA interference (RNAi) to silence the chalcone synthase or isoflavone synthase – key enzymes in isoflavone biosynthesis – to reduce isoflavonoid production. One study examined how effectively silencing either isoflavone synthase or chalcone synthase reduced isoflavonoid production and how the reduced isoflavonoid levels affected the susceptibility of 17 to 19 lines of 2 soybean genotypes to *Fusarium solani* f. sp. *glycines*. In this case isoflavonoid production was successfully reduced by silencing either enzyme, although the degree of success varied among the different soybean lines (Lozovaya et al., 2007). Another study silenced isoflavone synthase to ascertain how reduced isoflavonoid levels affected the establishment of a symbiotic relationship between soybean and *B. japonicum* (Subramanian et al., 2006). Unlike the previous studies, we wanted to determine how isoflavonoids affected a microbial community rather than a specific microbe. To that end, we chose to examine the bacterial diversity of 3 soybean rhizosphere soil samples from 3 root types at 1 and 3 weeks post planting. As with the other isoflavonoid studies, we decided to use RNAi to silence isoflavone synthase and drastically reduce root isoflavonoid production.

9. Methods for isolation of rhizosphere soil

We then needed to separate the rhizosphere soil from the soybean roots. Previous studies in soybean and potato acquired rhizosphere soil samples using sterile brushes after manually shaking off loosely adhering soil (İnceoğlu et al., 2010; Sugiyama et

al., 2014). Brushing to remove the rhizosphere soil can be problematic as one may damage the plant roots during the process (ex. breaking root segments, removing nodules, etc.). This can complicate subsequent root-related data acquisition processes such as root image analysis or nodule counting. The acquired rhizosphere soil samples may also vary from plant to plant due to the human factor, either because one person brushes the roots more vigorously than another or the same person uses inconsistent force from one day to the next. To avoid such problems, other studies in rice and *Arabidopsis* used sonication to acquire rhizosphere soil samples after removing the loosely adhered soil through sequential washes (Doi T, 2007; Bulgarelli et al., 2012). Although sonication is a better alternative to manual brushing, it may not be feasible for larger roots (ex. tree roots). Additionally, one may need to adjust the sonication time depending on the soil composition. For example, soil mainly composed of smaller particles, like clay, will stick to plant roots more tightly than soil mainly composed of larger particles, like peat. All the aforementioned techniques were used to isolate the rhizosphere as a whole. We took this one step further and used a successive sonication to reproducibly isolate microbial communities from 3 rhizosphere regions.

10. Methods for evaluation of rhizosphere microbial community

We then needed to choose the appropriate approach to examine the rhizosphere bacterial communities. Previous studies have used cultivation and microscopic examination (Hattori, 1976). However, microscopic examination is unable to distinguish between active and inactive microorganisms. Additionally, the majority of soil environmental bacteria are non-culturable, to the extent that only 1% are

detectable using the culture method (Amann et al., 1995). To avoid the aforementioned pitfalls, many studies conduct DNA sequence analyses of marker genes that are evolutionarily stable to examine the diversity and phylogeny of bacteria (Tringe and Hugenholtz, 2008). In bacteria, prime examples of conserved genes include the 5S, 16S and 23S rRNA genes (Maidak et al., 1997). The 16S rRNA gene is particularly favorable for several reasons. Firstly, it is present in all bacteria. Secondly, its function has not altered over time, indicating random sequence changes can serve as more accurate measurements of time (i.e. evolution). Thirdly, its highly conserved regions provide excellent candidates for primer design. Lastly, its large size of 1,500 base pairs and 9 hypervariable regions makes it suitable for identifying phylogenetic characteristics of bacteria (Woese, 1987; Tringe and Hugenholtz, 2008). Although each hypervariable region may be used to differentiate among multiple bacterial genera or species, they exhibit differing degrees of sequence diversity. Thus no single region can distinguish amongst all bacteria. For example, one study noted the V2 region was better at distinguishing among *Mycobacterium* species whereas the V3 region was better at distinguishing among *Haemophilus* species. This study also concluded the V2, V3 and V6 regions collectively contained the maximum nucleotide heterogeneity and best discriminatory power among the 110 bacterial species examined (Chakravorty et al., 2007). Therefore using multiple variable regions helps ensure the successful identification of a larger number of bacterial species or genera. With this in mind, we elected to amplify the variable regions V1-V3 and V3-V5 of the 16S rRNA gene to examine the rhizosphere bacterial community (see Fig. 2).

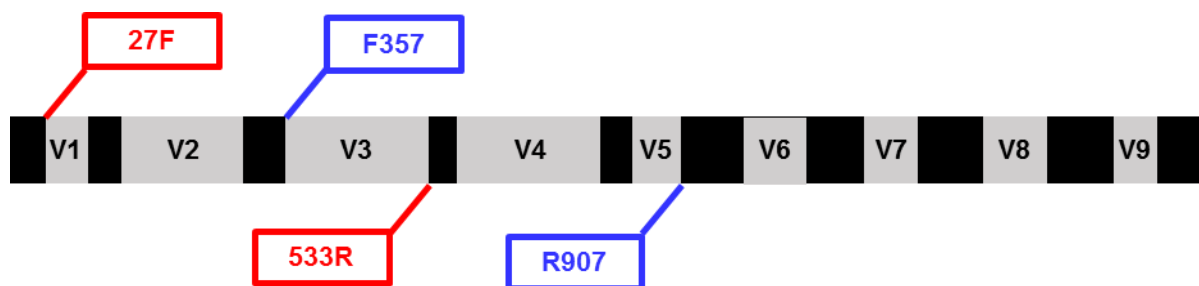


Figure 2. Diagram of the 16S rRNA hypervariable regions.

Positions of the forward primers 27F and F357 and reverse primers 533R and R907 used to amplify the V1-V3 (red) and V3-V5 (blue) 16S rRNA variable regions for this study are shown.

Two variable regions were chosen to identify a larger number of bacteria in case one variable region database contained information the other database lacked (i.e. if one variable region identified a bacterial species but the other did not). After amplifying our chosen variable regions of 16S rRNA gene, we needed to select the appropriate techniques to better define how isoflavonoids impacted the rhizosphere bacterial community diversity and the magnitude of said impact. Rhizosphere studies in rice and *Arabidopsis* used fluorescence *in situ* hybridization (FISH) and PCR-independent catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) to examine the rhizosphere microbiome. However, FISH and CARD-FISH can be limited by factors like soil particle autofluorescence and difficulty isolating target DNA/RNA fragments from soil (Doi T, 2007; Bulgarelli et al., 2012). These techniques can also only detect a few target bacteria simultaneously, making in-depth bacterial community analysis difficult and time-consuming. Other rhizosphere studies in *M. truncatula* and *A. thaliana* used rRNA intergenic spacer analysis (RISA) and denaturing gradient gel electrophoresis (DGGE) to examine the rhizosphere bacterial community (Mougel et al., 2006; Micallef et al., 2009). However, both techniques can suffer from PCR amplification biases and, for DGGE, one band can represent

multiple species (Fakruddin and Mannan, 2013). Such factors make detailed microbial community analysis difficult. To achieve a more in-depth analysis, many of the aforementioned rhizosphere studies also implemented pyrosequencing. This technique is particularly useful since it can process long read lengths with high accuracy, although it suffers from a high error rate when encountering poly-bases longer than 6 base pairs (Liu et al., 2012). For this study, we chose to first use DGGE to detect large shifts in the rhizosphere bacterial community structure. DGGE was chosen over techniques like FISH since our focus was on the bacterial community rather than a few target species. To limit PCR amplification bias, we stopped the process while sequence amplification was in the log phase. We then used pyrosequencing to clarify how isoflavonoids affected said community (i.e. enrichment or reduction of different bacterial taxa). After acquiring the sequencing data, we needed to process it using an appropriate program. Many rhizosphere studies have used the *MOTHUR* software to identify bacterial taxa within the sequencing data, although other programs such as BLAST were also utilized. However, while BLAST is limited to supplying bacterial taxonomy data, *MOTHUR* provides bacterial taxonomy data as well as operational taxonomic unit (OTU) data. This provides additional ways to view and analyze the bacterial community structure. With this in mind, we chose to process our sequencing data using the *MOTHUR* software. Many rhizosphere studies choose to examine both the OTU and bacterial taxonomy data, implementing statistical analyses such as hierarchical clustering, multivariate analysis of variance, and principal coordinate analysis (Doi T, 2007; Gottel et al., 2011; Bulgarelli et al., 2012; Peiffer et al., 2013; Sugiyama et al., 2014). With this in mind,

we chose to process our sequencing data via *MOTHUR* to acquire both the OTU and bacterial taxonomy data. Said data was then analyzed using various statistical analyses, including those previously listed, to ascertain how isoflavonoids impacted the soybean rhizosphere bacterial community diversity and the magnitude of said impact.

11. Specific aims

The specific aims in this study were to,

1. Generate and evaluate soybean roots with reduced isoflavonoid levels using RNAi in hairy root composite plants,
2. Optimize methods for isolation of rhizosphere fractions with varying affinities to soybean roots,
3. Evaluate changes in microbiome diversity of different rhizosphere fractions from isoflavonoid silenced roots using DGGE, and
4. Evaluate taxonomic changes in the microbiomes of isoflavonoid silenced roots using pyrosequencing of 16S amplicons.

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**Chapter 1: Spatio Temporal Influence of Isoflavonoids on Bacterial
Diversity in the Soybean Rhizosphere**

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1. Abstract

High bacterial density and diversity near plant roots has been attributed to rhizodeposit compounds that serve as both energy sources and signal molecules. However, it is unclear if and how specific rhizodeposit compounds influence bacterial diversity. We silenced the biosynthesis of isoflavonoids, a major component of soybean rhizodeposits, using RNA interference in hairy-root composite plants, and examined changes in rhizosphere bacteriome diversity. We used successive sonication to isolate soil fractions from different rhizosphere zones at two different time points and analyzed denaturing gradient gel electrophoresis profiles of 16S ribosomal RNA gene amplicons. Extensive diversity analysis of the resulting spatio-temporal profiles of soybean bacterial communities indicated that, indeed, isoflavonoids significantly influenced soybean rhizosphere bacterial diversity. Our results also suggested a temporal gradient effect of rhizodeposit isoflavonoids on the rhizosphere. However, the hairy-root transformation process itself significantly altered rhizosphere bacterial diversity, necessitating appropriate additional controls. Gene silencing in hairy-root composite plants combined with successive sonication is a useful tool to determine the spatio-temporal effect of specific rhizodeposit compounds on rhizosphere microbial communities.

2. Introduction

Pioneering microbiology studies by L. Hiltner in the early 1900s showed that the highest microbial density in soils occurs very close to plant roots (Hinsinger and Marschner 2006). For example, a four- to fivefold increase in colony forming units (CFUs) was observed in root-surface scrapings as compared with soil samples 0.5 cm

away from the roots (Clark 1940). Such changes are attributed to the rich carbon energy sources provided by the plant. Indeed, plants release, on average, 10 to 15% (Jones et al. 2009) of their photosynthetic assimilates into the rhizosphere, a process called rhizodeposition (Dennis et al. 2010). These rhizodeposits originate from sloughed off root border and root border-like cells from root tips, active root exudation, and cell lysis. Rhizodeposits are composed of sugars, amino acids, organic acids, fatty acids, proteins, ions, secondary metabolites, mucilage, water, and miscellaneous carbon-containing compounds (Bais et al. 2006; Dennis et al. 2010).

Significant evidence accumulated over the years indicates that the composition of root microbial communities is influenced, in large part, by the plant species and its developmental stage (Micallef et al. 2009; Mougel et al. 2006; Weisskopf et al. 2006). Indeed, an intricate coevolution of plants and rhizosphere microbial communities was suggested by the observation that resident plants or their root exudates are capable of maintaining the biomass and diversity of soil fungal communities to a much greater extent than nonresident or introduced plants (Broeckling et al. 2008). This is supported by the observation that invasive weeds have the ability to significantly influence native rhizosphere microbial communities to exert their dominance in new environments (Inderjit et al. 2006). Therefore, it is clear that components of rhizodeposits significantly influence the composition and activity of rhizosphere microbial communities.

It is not well-understood which rhizodeposit compounds recruit or influence which groups of microbes and how. An effective approach is to examine microbial

associations with plant mutants deficient in the biosynthesis and rhizodeposition of specific groups of compounds (Prithiviraj et al. 2005; Rudrappa et al. 2008). It is worth noting that composition of rhizodeposits varies substantially among different plant species (Czarnota et al. 2003; Warembourg et al. 2003). Therefore, studies using model plant species might not reveal the roles of species-specific rhizodeposit compounds (e.g., isoflavonoids that are legume-specific compounds). This demands the development of an efficient system to generate plant materials with altered rhizodeposit composition as well as reproducible methods to isolate and examine rhizosphere microbes. We and others have previously used RNA interference (RNAi) in hairy-root composite plants to elucidate the role of flavonoids in specific root-microbe interactions (Oger et al. 1997; Wasson et al. 2006; Zhang et al. 2009). For example, we identified that isoflavonoids in soybean are essential for interaction with the symbiont *Bradyrhizobium japonicum* (Subramanian et al. 2006) and resistance against the root-rot pathogen *Phytophthora sojae* (Subramanian et al. 2005). These results unequivocally demonstrated the crucial roles of isoflavonoids in the interaction of soybeans with these microbes and also established that RNAi in hairy-root composite plants can be used to effectively modify rhizodeposit compositions. We used RNAi in hairy-root composite plants to silence isoflavonoid biosynthesis, used successive sonication steps to reproducibly isolate microbial communities with different affinities to the roots, and demonstrated using denaturing gradient gel electrophoresis (DGGE) analyses that root isoflavonoids significantly influence soybean rhizosphere microbial communities.

3. Results

3.1. Root surface preparations and analysis of bacterial diversity

We used an RNAi construct against isoflavone synthase (IFS) to generate isoflavonoid-deficient hairy-root composite plants as previously described (Collier et al. 2005; Subramanian et al. 2006). Consistent silencing of IFS genes in these roots and a significant reduction in root isoflavonoids were confirmed by quantitative polymerase chain reaction (PCR) and high-performance liquid chromatography analyses, respectively (Fig. 1.1).

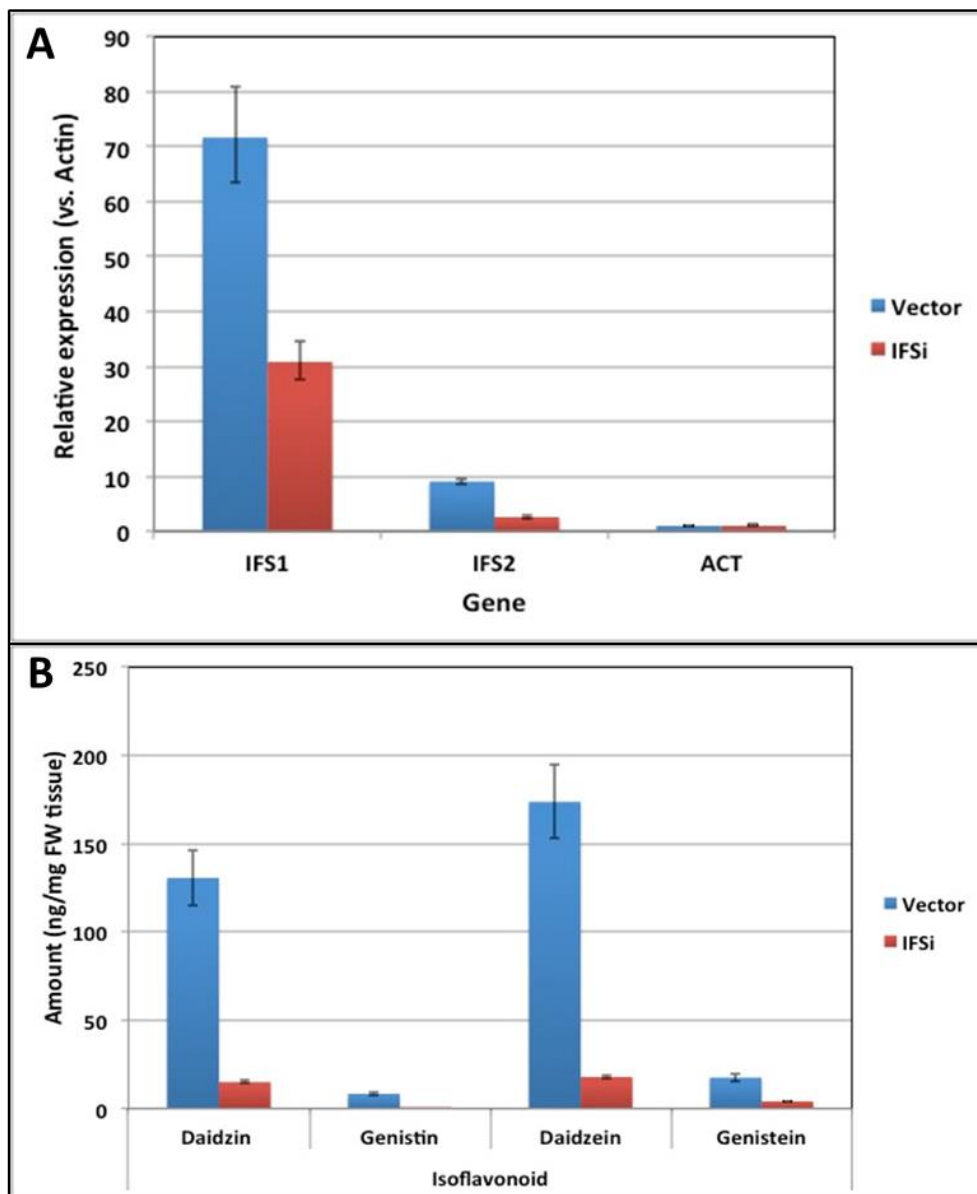


Figure 1.1. RT-qPCR and HPLC analysis to confirm silencing of isoflavone biosynthesis in IFS-RNAi roots.

(A) Relative expression levels of IFS1 and IFS2, two genes encoding isoflavone synthase in soybean assayed by RT-qPCR in vector control and IFS-RNAi roots. Data presented are expression levels normalized to that of Actin. (B) Root isoflavonoid content assayed by reversed phase HPLC. Data presented are the levels of Daidzin (+ other conjugates), Genistin (+ other conjugates), Daidzein and Genistein. qPCR and HPLC assays were performed as described previously (Subramanian et al. 2006. *Plant J.* 48:261-273).

We planted vector-transformed controls (VC) and IFS-RNAi (IFSi)-transformed composite plants in soil mixed from various soybean fields and

harvested roots at 1 and 3 weeks post planting (wpp) for root-surface preparations. These root-surface preparations, representing different rhizosphere zones, were collected through three successive sonication steps (Fig. 1.2).

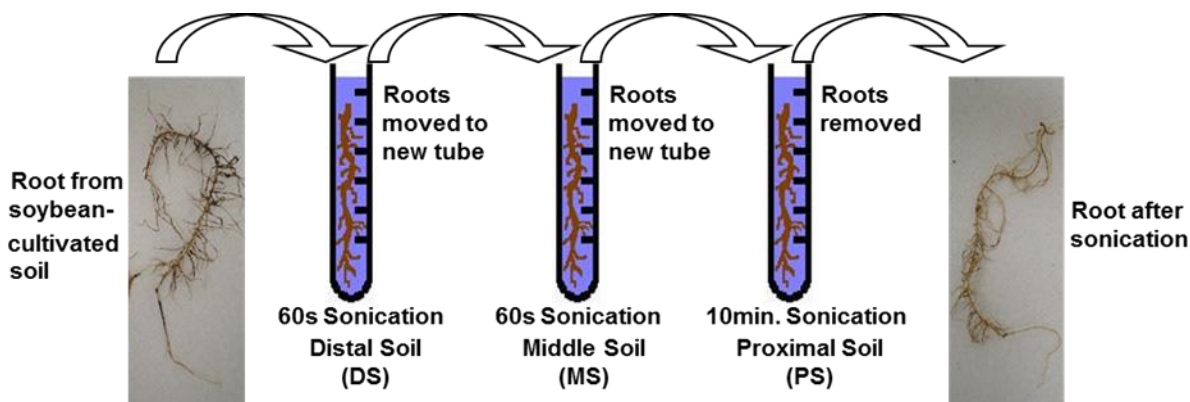


Figure 1.2. Schematic indicating successive sonication steps used to isolate distal, middle, and proximal soil samples from soybean roots.

Pictures of a soybean composite plant root before and after the three sonication steps are shown.

We hypothesized that the stronger the bacterial proximity or affinity to the roots, the stronger the physical force (i.e., sonication time) required to isolate them. Three successive sonication steps yielded the distal soil (DS), middle soil (MS), and proximal soil (PS) samples. We expected that the PS sample would represent the fraction that is very closely associated with the root surface, including bacterial biofilms. Bacterial communities in each of the samples were analyzed by DGGE profiling of 16S ribosomal (r)RNA gene amplicons (V3 to V5 region). Dissimilarities between samples from different rhizosphere regions, different time points after planting, and root isoflavonoid content were compared through rigorous population diversity and statistical analyses.

3.2. Distinct bacterial groups isolated using differential sonication

First, we tested to learn if bacterial communities obtained from different sonication times were reproducible, by comparing DS, MS, and PS samples from two independent experiments. Indeed, we obtained three distinct clusters of bacterial communities in a reproducible manner using different sonication times both at 1 and 3 wpp (Fig. 1.3, DS vs. MS vs. PS). Detrended correspondence analysis (DCA) using the *decorana* method in the R package *vegan* showed that the DS, MS, and PS samples had distinct profiles at both 1 and 3 wpp (Fig. 1.3). The first two DCA axes explained approximately 65 to 70% of the variance. The difference among the DS, MS, and PS samples was statistically significant based on *adonis*, a nonparametric multivariate analysis of variance test using distance matrices at both 1 and 3 wpp ($P < 0.05$, Bray-Curtis distance matrices).

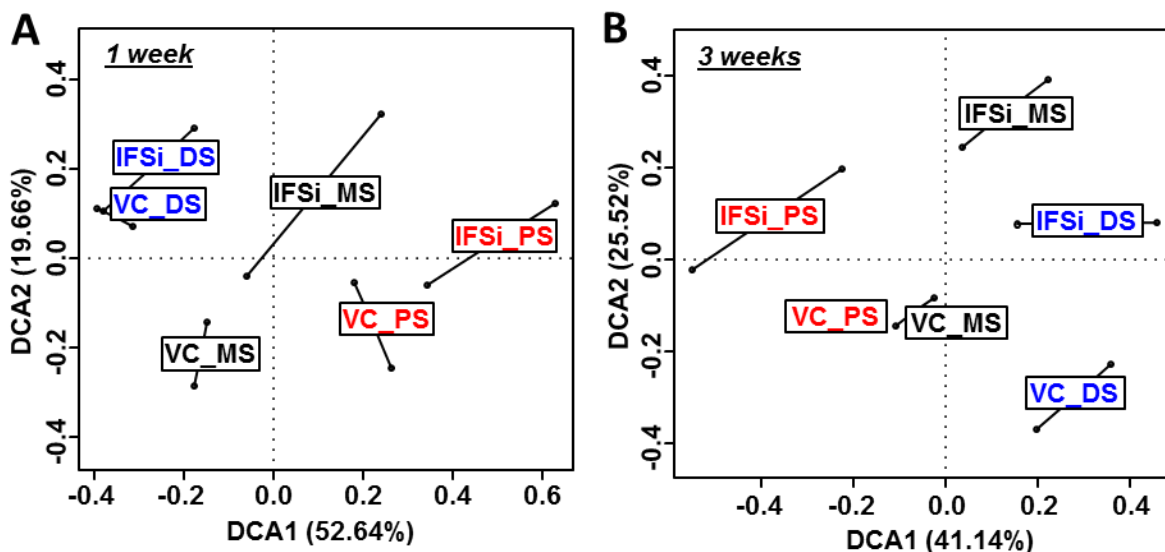


Figure 1.3. DCA plots showing separation of DS, MS and PS samples from VC and IFSi roots at 1 and 3 wpp.

Detrended correspondence analysis (DCA) plots showing the separation of distal, middle, and proximal soil (DS, MS, and PS) samples from vector-transformed control (VC) and isoflavone synthase-RNA interference (IFSi) roots at (A) 1 and (B) 3 weeks after planting. DCA1 and DCA2 indicate the major axes of dissimilarity. Data points of the same sample type from two independent experiments are shown connected by a line.

The same conclusion was obtained using constrained ordination analyses (*capscale* and constrained correspondence analysis; Fig. 1.4).

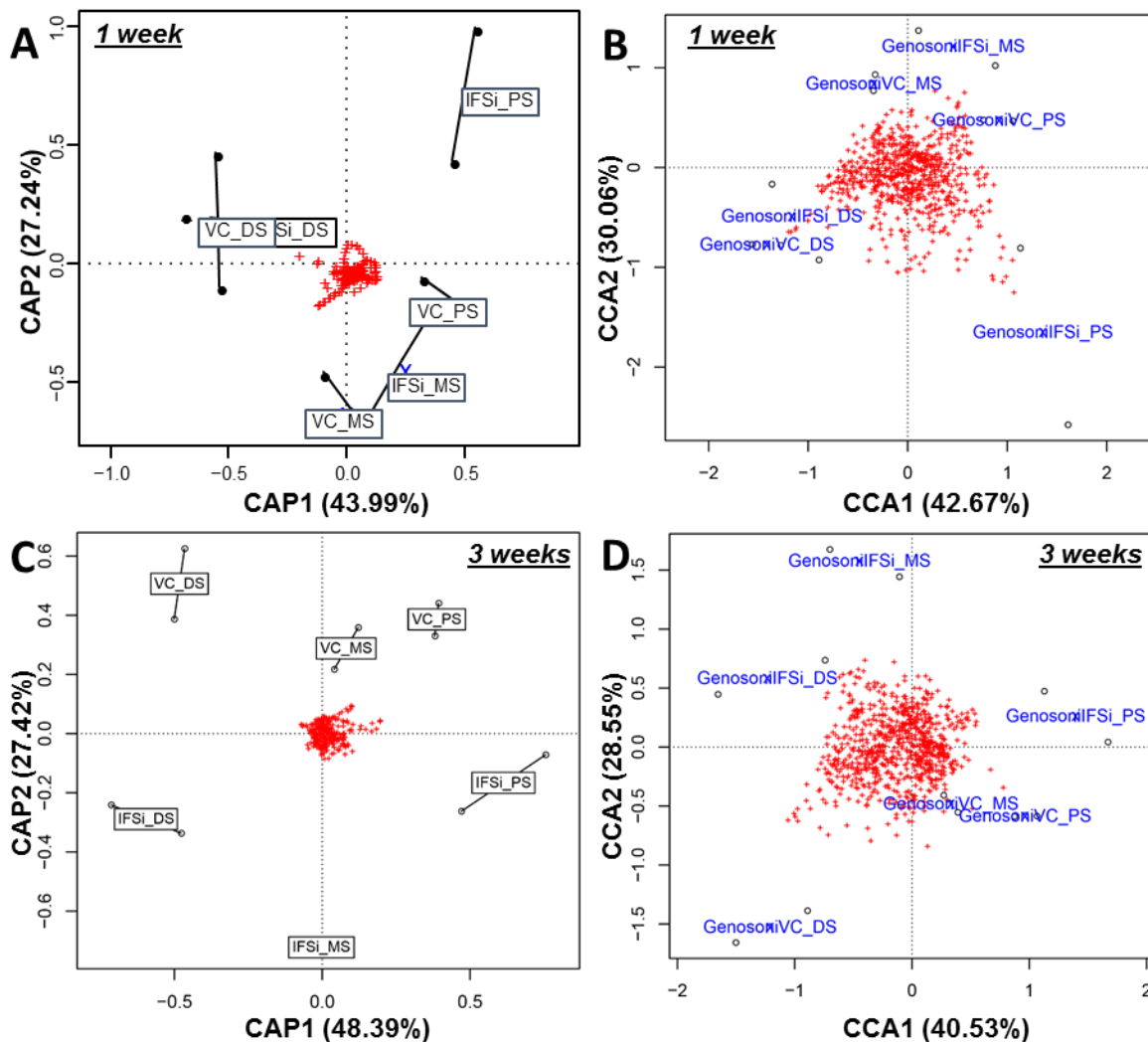


Figure 1.4. Capscale and CCA plots of DGGE profiles for DS, MS and PS fractions for VC and IFSi roots at 1 and 3 wpp.

(A, C) Capscale and (B, D) constrained correspondence analysis of denaturing gradient-gel electrophoresis profiles of samples from distal, middle, and proximal soil (DS, MS, and PS) fractions prepared from roots of vector control and isoflavone synthase-RNAi (IFSi) plants at 1 and 3 weeks after planting. Capscale significance values were $P < 0.01$ for the one and three week samples.

In agreement, hierarchical cluster analysis also placed samples from different rhizosphere zones into distinct branches (Fig. 1.5).

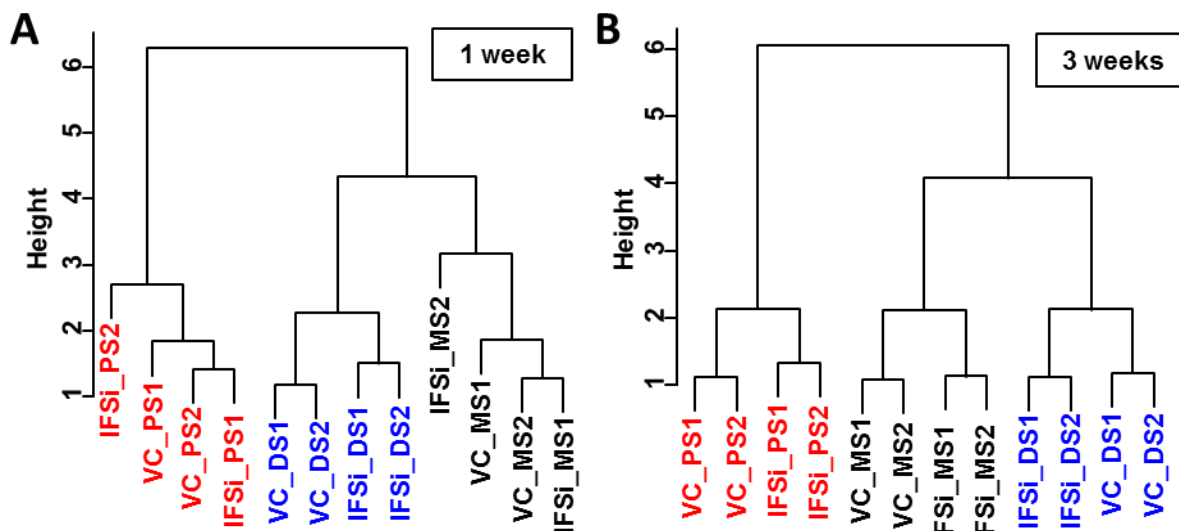


Figure 1.5. Dendrograms showing hierarchical clustering of DS, MS and PS samples from VC and IFSi roots at 1 and 3 wpp.

Dendrograms showing hierarchical clustering of distal, middle, and proximal soil (DS, MS, and PS) samples from vector-transformed control (VC) and isoflavone-synthase-RNA interference (IFSi) roots at (A) 1 and (B) 3 weeks after planting. Numbers following the samples indicate the experiment from which they were obtained.

It is worth noting that in all these analyses, the profiles of distal rhizosphere zone samples were very distinct from those of middle and proximal zone samples. Such distinct separation suggests that successive sonication can reproducibly isolate distinct bacterial communities with increasing affinity or proximity to plant roots.

3.3. *Effect of time-in-soil on bacterial composition*

Next, we examined if the length of time in the soybean field soil affected the composition of rhizosphere bacterial communities. We compared PS bacterial profiles between roots from 1 and 3 wpp plants in both VC and IFSi plants.

Results from DCA indicated that, regardless of root genotype, samples obtained from 1 and 3 wpp were clearly different from one another, at least in the PS (Fig. 1.6, E vs. L). The first two DCA axes explained approximately 73% of the

variance. The effect of time-in-soil was statistically significant in influencing bacterial community composition (*adonis* $P < 0.05$, Bray-Curtis distance matrix).

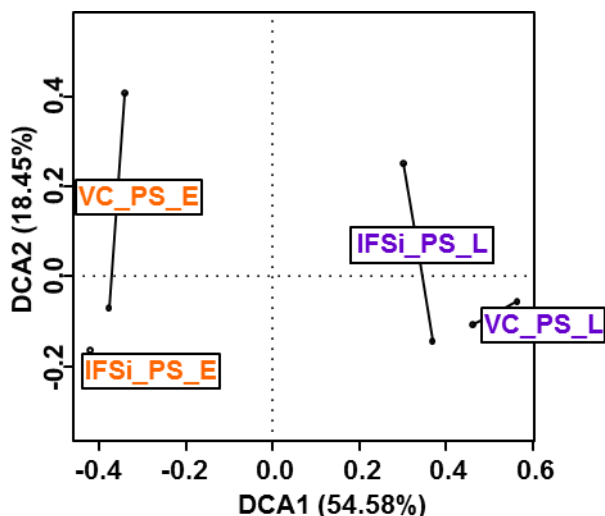


Figure 1.6. DCA plot showing separation of PS samples from VC and IFSi roots 1 and 3 wpp.

Detrended correspondence analysis (DCA) plot showing the separation of proximal soil (PS) samples from vector-transformed control (VC) and isoflavone synthase-RNA interference (IFSi) roots 1 and 3 weeks after planting (E and L). DCA1 and DCA2 indicate the major axes of dissimilarity. Data points of the same sample type from two independent experiments are shown connected by a line.

This conclusion was also supported by other constrained ordination analyses (Fig. 1.7). Constrained axes explained approximately 90 to 95% of the variance between the 1 and 3 wpp samples.

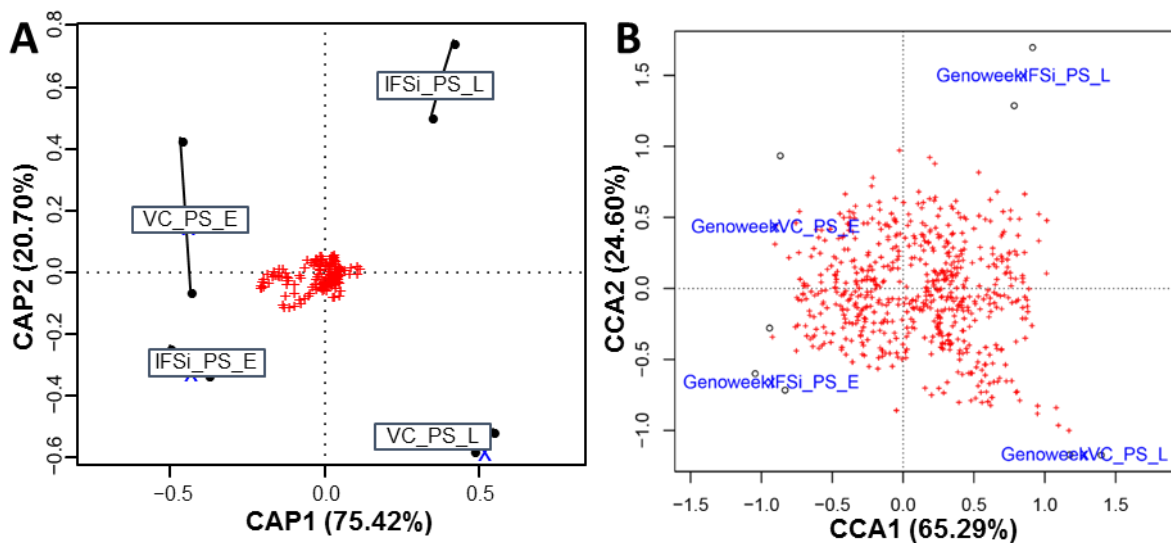


Figure 1.7. Capscale and CCA plots of DS, MS and PS samples from VC and IFSi roots 1 and 3 wpp.

(A) Capscale and (B) constrained correspondence analysis of distal, middle, and proximal soil (DS, MS, and PS, respectively) samples from roots of vector control and isoflavone synthase-RNAi (IFSi) plants at 1 (labeled _E) and 3 (labeled _L) weeks after planting. Capscale significance value was $P < 0.01$.

Consistently, hierarchical cluster analysis also showed that bacterial profiles of 1 and 3 wpp roots clustered in distinct branches (Fig. 1.8).

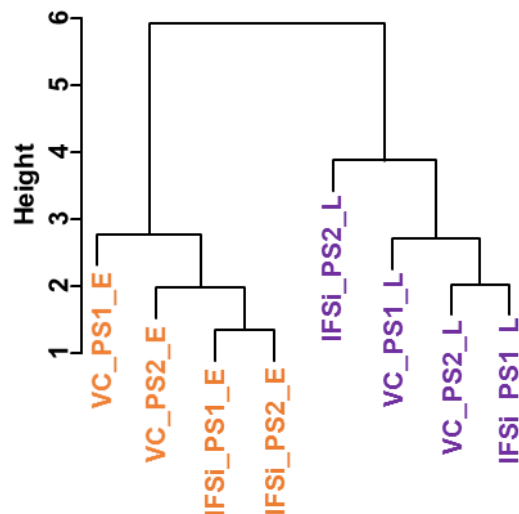


Figure 1.8. Dendrogram showing hierarchical clustering of PS samples from VC and IFSi roots 1 and 3 wpp.

Dendrogram showing the hierarchical clustering of proximal soil (PS) samples from vector-transformed control (VC) and isoflavone synthase-RNA interference (IFSi) roots 1 and 3 weeks after planting (E and L). Numbers following the sample labels indicate the experiment from which they were obtained.

General diversity indices (Shannon, Simpson, and Inverse Simpson) were generally higher for samples obtained 3 wpp compared with those obtained 1 wpp (Fig. 1.9). In addition, there was no obvious pattern among the general diversity indices in the different rhizosphere zones at 1 wpp.

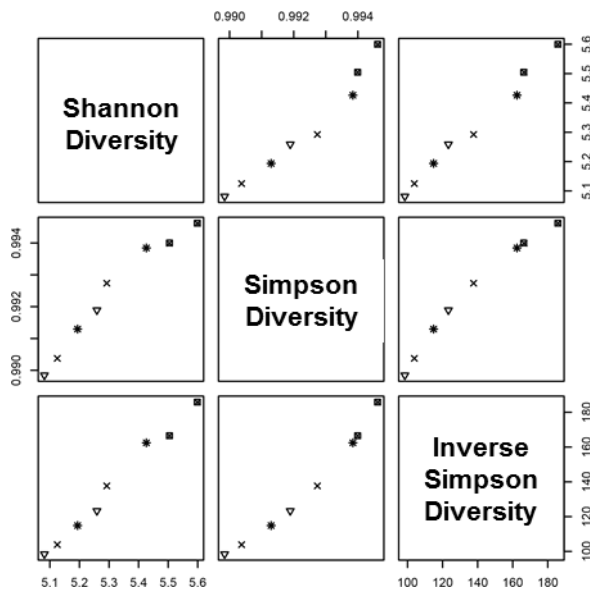


Figure 1.9. Comparison of Shannon, Simpson and inverse-Simpson diversity indices for PS fractions from VC and IFSi roots at 1 and 3 wpp.

Comparison of Shannon, Simpson, and inverse-Simpson diversity index plots for proximal soil (PS) fractions from vector control and isoflavone synthase RNAi (IFSi) roots at 1 and 3 weeks post planting (x - VC PS 1wpp; ∇ - IFSi PS 1wpp; ⊠ - VC PS 3wpp; * - IFSi PS 3wpp). Overall, these diversity indices were higher for the 3 wpp samples relative to the 1 wpp samples.

Interestingly, the proximal rhizosphere zones had less diversity than the distal and middle zones at 3 wpp (Fig. 1.10). It appears that the bacterial communities had established themselves at specific rhizosphere zones at 3 wpp as compared with 1 wpp. Some bacteria likely utilized the extra time to drive out competitors while others needed specific bacteria present before they could thrive.

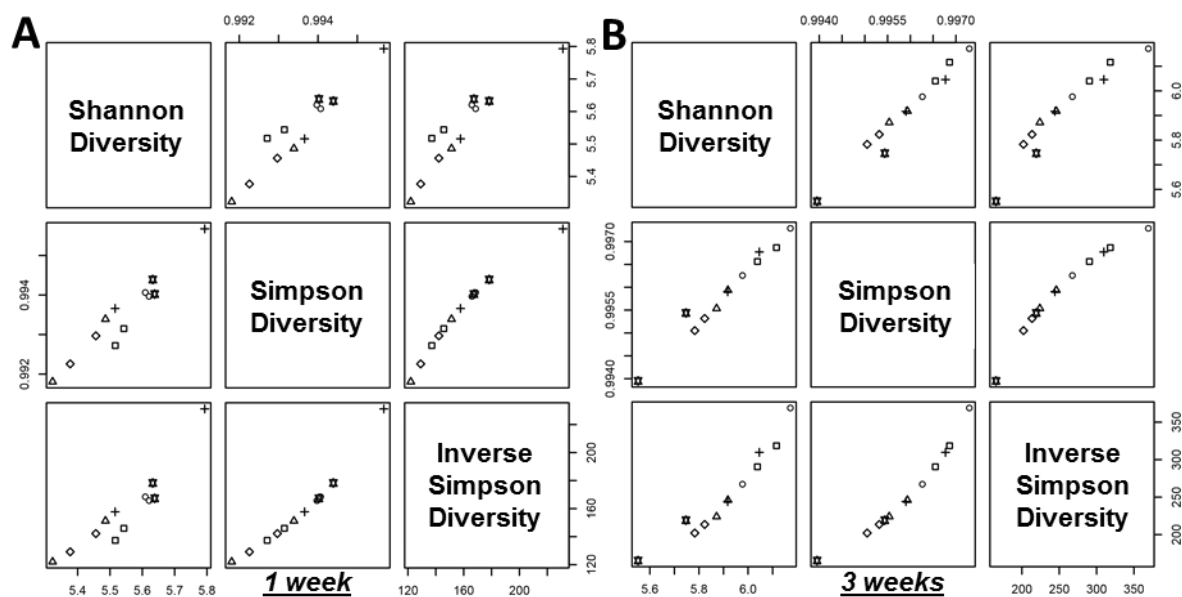


Figure 1.10. Comparison of Shannon, Simpson and inverse-Simpson diversity indices among DS, MS and PS samples from VC and IFSi roots at 1 and 3 wpp.

Comparison of Shannon, Simpson, and inverse-Simpson diversity index plots among distal, middle, and proximal soil (DS, MS, and PS, respectively) samples from vector control and isoflavone synthase RNAi (IFSi) roots at 1 and 3 weeks post planting (\square = VC DS; \circ = IFSi DS; \triangle = VC MS; $+$ = IFSi MS; \diamond = VC PS; \star = IFSi PS). No discernable pattern was detected among the rhizosphere zones for the 1 wpp samples. The proximal rhizosphere zones displayed lower diversity than the middle and distal zones for the 3 wpp samples.

3.4. Effect of isoflavonoids on bacterial composition in the rhizosphere

We also examined the effect of root isoflavonoid composition on bacterial community dissimilarities in the three rhizosphere zones. Results from DCA showed that VC and IFSi roots had clearly distinguishable bacterial profiles at both 1 and 3 wpp. However, at 1 wpp, the DS samples did not appear to show any significant difference between the two genotypes (Fig. 1.3A, VC DS vs. IFSi DS), whereas MS and PS samples from the two genotypes were well separated (Fig. 1.3A, VC PS vs. IFSi PS and VC MS vs. IFSi MS). In contrast, at 3 wpp, the two genotypes showed a significant difference in bacterial community composition in all three rhizosphere zones (Fig. 1.3B, VC DS vs. IFSi DS, VC MS vs. IFSi MS,

and VC PS vs. IFSi PS). As above, additional constrained coordinate analyses clearly supported these conclusions. Hierarchical cluster analysis, on the other hand, indicated that, at 3 wpp, there was a clear separation of samples from VC and IFSi roots in each rhizosphere zone but not at 1 wpp. For example, we observed clear sub-branching of VC and IFSi PS samples at 3 wpp (Fig. 1.5B), but no such separation was observed at 1 wpp. Therefore, by 3 wpp, isoflavonoids clearly exert a significant influence on microbial composition in all three rhizosphere zones examined. The effect of root genotype on bacterial community composition was statistically significant at 3 wpp (*adonis* $P < 0.05$, Bray-Curtis distance matrix).

3.5. Use of hairy-root composite plants for rhizosphere microbiome studies

Having established that, at 3 wpp, all three rhizosphere zones examined had significant differences in bacterial community composition between VC and IFSi roots, we performed a thorough analysis at this time point with multiple replicates obtained from at least three independent root-surface preparations. Since the hairy-root composite plant generation is known to alter the physiology of roots, we also used another control, in which we generated “composite plants” without *Agrobacterium rhizogenes* infection. These plants underwent the same “transformation” procedure but produced adventitious roots from stem explants instead of transgenic hairy roots. Comparison of bacterial community composition between untransformed (UNR) and VC roots suggested that the hairy-root transformation procedure itself altered the microbiome of all three rhizosphere zones at 3 wpp. The bacterial profiles of transgenic VC roots and nontransgenic

UNR roots were significantly different from each other in all three rhizosphere zones (Fig. 1.11, VC vs. UNR). Nevertheless, comparison of bacterial community composition between VC and IFSi roots indicated that reduced root isoflavone levels significantly influenced the microbiome of all three rhizosphere zones at 3 wpp (Fig. 1.11, VC vs. IFSi). The differences in each zone were statistically significant (*adonis* $P < 0.01$, Bray-Curtis distance matrix).

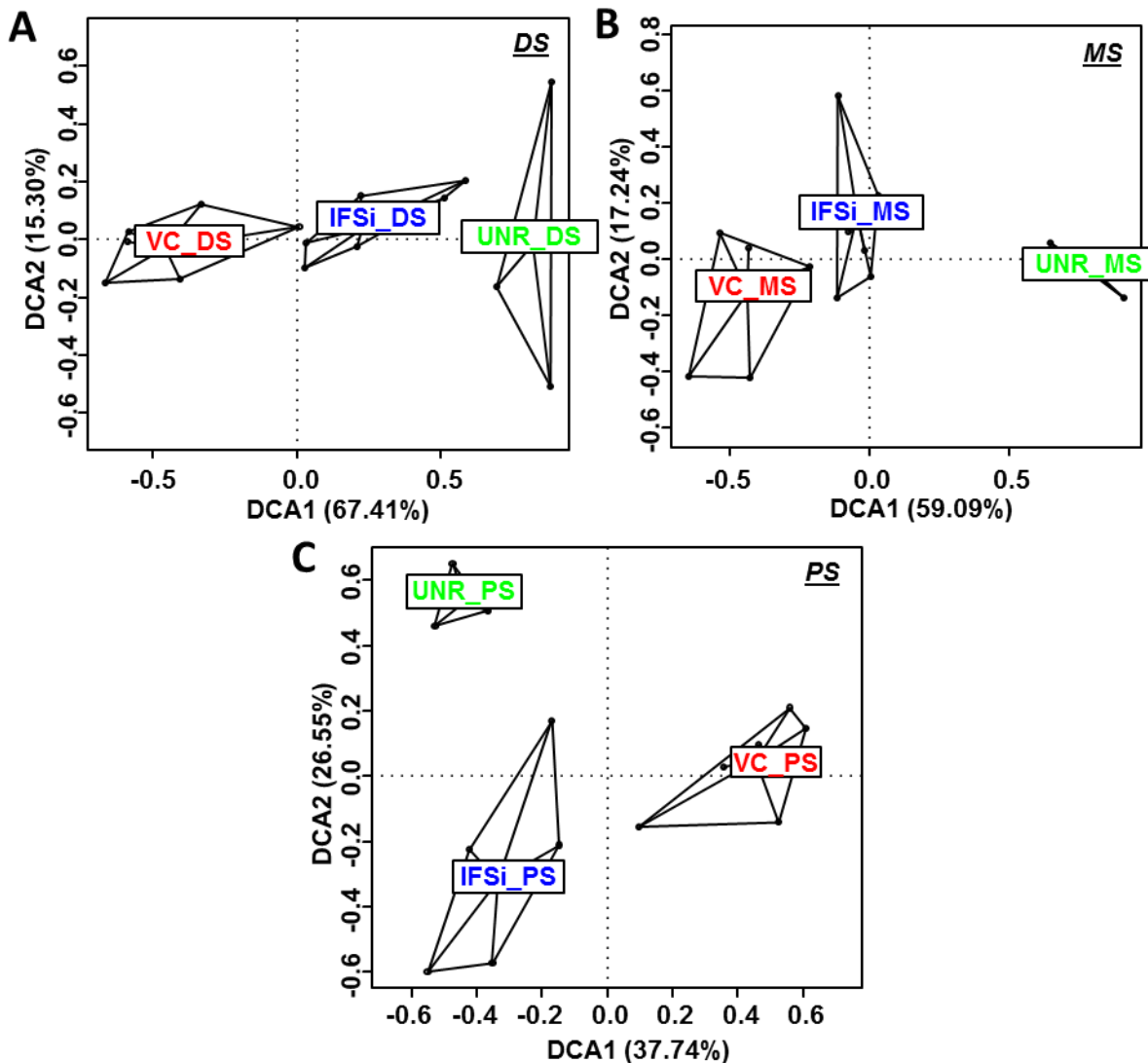


Figure 1.11. DCA plots showing separation of DS, MS and PS samples from VC, IFSi and UNR roots 3 wpp.

Detrended correspondence analysis (DCA) plots showing the separation of (A) distal, (B) middle, and (C) proximal soil (DS, MS, and PS, respectively) samples from vector-transformed control (VC), isoflavone synthase-RNA interference (IFSi), and untransformed (UNR) roots 3 weeks after planting. DCA1 and DCA2 indicate the major axes of dissimilarity. Data points of the same sample type from two independent experiments are shown connected by lines.

Similar to the above comparisons, additional constrained correspondence analyses also pointed to the same conclusion (Fig. 1.12).

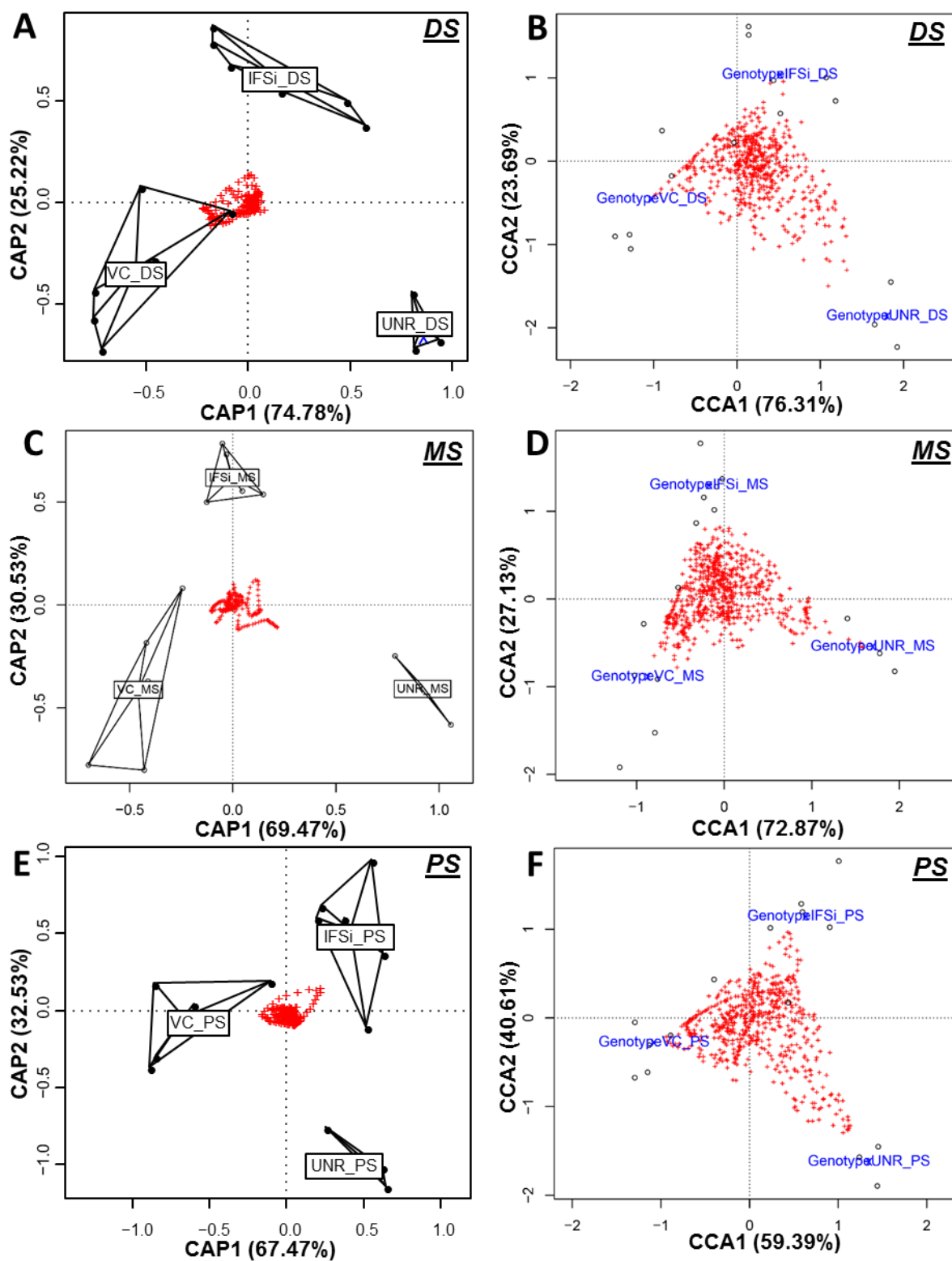


Figure 1.12. Capscale and CCA of DGGE profiles for DS, MS and PS samples from UNR, VC and IFSi roots at 3 wpp.

(A, C, E) Capscale and (B, D, F) constrained correspondence analysis of denaturing gradient-gel electrophoresis profiles of samples from distal, middle, and proximal soil (DS, MS, and PS, respectively) fractions prepared from roots of untransformed (UNR). Vector control (VC) and IFS-RNAi (IFSi) plants at three weeks after planting. Capscale significance values were $P < 0.01$ for DS, MS, and PS, samples.

Hierarchical clustering yielded varying branches across the three rhizosphere zones, presumably due to variation between independent root-surface preparations. The DS zone samples had two distinct branches, one with VC and the other with UNR and IFSi samples. The second branch had two major sub-branches enriched for either UNR or IFSi samples (Fig. 1.13).

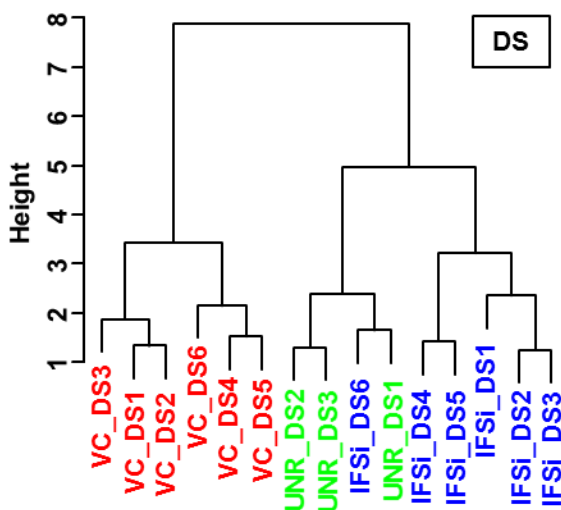


Figure 1.13. Dendrogram showing hierarchical clustering of DS samples from VC, IFSi and UNR roots 3 wpp.

Dendrogram showing the hierarchical clustering of distal soil (DS) samples from vector-transformed control (VC), isoflavone synthase-RNA interference (IFSi), and untransformed (UNR) roots 3 weeks after planting. Numbers following the sample labels indicate the experiment from which they were obtained.

The MS zone samples formed two distinct branches one for UNR and the other for VC. Four of the six IFSi samples had a distinct sub-branch closer to UNR samples, indicating clear differences between VC and IFSi samples (Fig. 1.14).

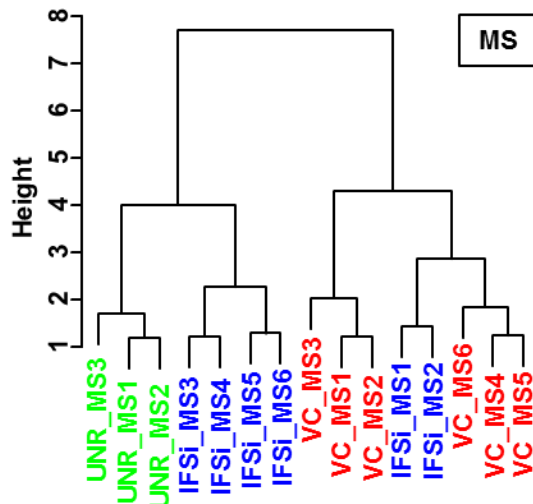


Figure 1.14. Dendrogram showing hierarchical clustering of MS samples from VC, IFSi and UNR roots 3 wpp.

Dendrogram showing the hierarchical clustering of middle (MS) samples from vector-transformed control (VC), isoflavone synthase-RNA interference (IFSi), and untransformed (UNR) roots 3 weeks after planting. Numbers following the sample labels indicate the experiment from which they were obtained.

The PS zone samples also had two distinct branches but one with VC and the other with UNR and IFSi samples. The second branch had two major sub-branches dividing close to the origin. Each of these branches were enriched for either UNR or IFSi samples (Fig. 1.15).

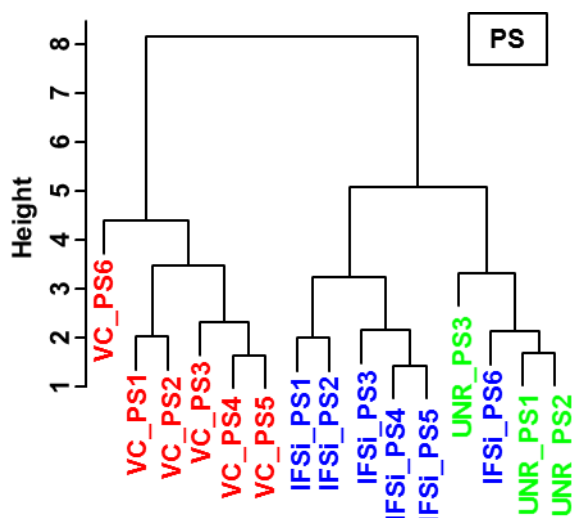


Figure 1.15. Dendrogram showing hierarchical clustering of PS samples from VC, IFSi and UNR roots 3 wpp.

Dendrogram showing the hierarchical clustering of proximal soil (PS) samples from vector-transformed control (VC), isoflavone synthase-RNA interference (IFSi), and untransformed (UNR) roots 3 weeks after planting. Numbers following the sample labels indicate the experiment from which they were obtained.

Our results indicate that i) isoflavonoid rhizodeposits significantly influence the microbiome of soybean rhizosphere, ii) differential sonication can be used to reproducibly isolate microbes in different rhizosphere zones, iii) a longer growth period of plants in the soil enables them to strongly influence the rhizosphere, and iv) the hairy-root composite process itself significantly influences the rhizosphere microbiome, necessitating additional controls when using this system to study the roles of specific rhizodeposit compounds in the rhizosphere.

4. Discussion

A number of studies have identified the influence of plant genotype and the environment on the composition and diversity of rhizosphere microbiota (Bulgarelli et al. 2012; Gottel et al. 2011; Peiffer et al. 2013; Philippot et al. 2013). In addition, either the roles of specific compounds in rhizodeposits, the roles of specific cellular

transport machinery on rhizosphere microbial diversity, or both have been investigated (Bais et al. 2006; Walker et al. 2003). In the latter case, the availability of genetic mutants impaired in biosynthesis and transport of specific rhizodeposit compounds has been crucial. For example, mutations in an *Arabidopsis thaliana* ABC-transporter gene, ABCG30 (resulting in increased exudation of phenolic acids and reduced exudation of sugars), caused significant changes in root microbial community structure as assayed by high-throughput sequencing of rRNA gene amplicons (Badri et al. 2008, 2009). This study revealed the association of a number of potentially beneficial bacteria with *abcg30* mutant roots. Some rhizodeposit compounds are produced by nearly all plant species and these primarily serve as carbon sources (e.g., amino acids, sugars, and polysaccharides). In addition to these, there are a number of species-specific compounds that are likely to attract specific microbes that have the capacity to metabolize them as a carbon source or that might serve as signal molecules to specific rhizosphere microbes (e.g., isoflavonoids in soybean). Therefore, determining the roles of species-specific rhizodeposit compounds in shaping the microbial community is crucial for rhizosphere engineering. A major bottleneck in such approaches is the lack of a comprehensive collection of genetic mutants in all plant species. We used RNAi in hairy-root composite plants to overcome this bottleneck and manipulate root isoflavonoid composition and, thus, rhizodeposit isoflavonoid composition. This method is adaptable to a wide variety of dicot species. The *A. rhizogenes* strain K599 has a broad host range, and composite plants ready to plant in soil can be obtained in 2 to 4 weeks, using the ex vitro composite plant generation method. It should, however, be

noted that the majority of monocots cannot be transformed using *A. rhizogenes*, which makes this approach limited to dicots. In addition, the method produces composite plants with transgenic roots and untransformed shoots. Therefore, shoots still have an active biosynthesis pathway and compounds that are transported from the shoot to the root and exuded or deposited are less likely to be affected unless specific transporters (if known) are silenced. Also, although RNAi silencing is a very successful procedure, plant roots may still produce inconsistent, though miniscule, levels of rhizodeposit compounds that may still impact the rhizosphere bacterial communities. This may result in additional variation amongst samples, as seen in the IFSi samples from all three rhizosphere zones (Fig. 4). Finally, we noticed that the hairy-root transformation process itself can alter the microbiome and, therefore, relevant controls are necessary to make proper conclusions and interpretations. Nevertheless, the method appears to be well-suited to study the effect of specific rhizodeposit compounds on rhizosphere microbes in many plant species that lack a comprehensive mutant collection. When combined with quantitative and high-resolution bacterial profiling methods such as pyrosequencing, some of these variations can be directly correlated to the level of silencing to better interpret the results.

Previous studies used sonication to either separate rhizosphere soil from nonrhizosphere soil or to isolate one region of the rhizosphere (Bulgarelli et al. 2012; Doi 2007). We used successive sonication to reproducibly isolate different soil fractions with specific bacterial composition. We defined these as distal, middle, and proximal soil fractions for convenience. Isolating cultivable bacterial species from

these fractions and subsequently examining their colonization will enable us to validate their spatial localization in the rhizosphere. Nevertheless, reproducible isolation of similar bacterial communities from these preparations suggested that the same strength of sonication isolated the same set of bacteria from the roots. In addition, longer presence of roots in the soil was required to influence bacterial communities in soils with least affinity to the roots. It is conceivable that rhizodeposits can attract or dissuade microorganisms but only at a limited distance. For example, rhizodeposit compounds might form a gradient merely due to physical diffusion or utilization by microbes in the proximal soil.

Finally, our results indicate that root flavonoids significantly influenced bacterial community composition in the rhizosphere in a spatio temporal manner. Isoflavonoids have been implicated in nonspecific defense against plant pathogens (Dixon 2001; Dixon et al. 2002). Isoflavonoids also regulate *nod* genes in rhizobia bacteria, specifically *B. japonicum* (Kosslak et al. 1987). Additionally, compared with sugars, amino acids, and organic acids present in rhizodeposits, isoflavonoids (and other secondary metabolites) are species-specific and are more likely to recruit unique microbial communities. Silencing of isoflavonoid biosynthesis in the roots did not significantly influence other metabolites in the phenylpropanoid pathway, except the accumulation of liquiritigenin (the substrate of IFS), *p*-hydroxy benzoic acid, coumaric acid, and *p*-hydroxybenzaldehyde (Lozovaya et al. 2007; Subramanian et al. 2006) (data not shown). We cannot exclude the possibility that a small proportion of the changes in root bacterial profiles could be due to these relatively small changes in nontarget phenylpropanoids. We also considered other approaches, such as the use of

RNAi to silence components of flavonoid exudation machinery and the use of adsorbents to prevent rhizodeposit isoflavonoids from reaching rhizosphere microbes. However, rhizodeposition of isoflavonoids occurs through mechanisms other than root exudation as well, e.g., root border cells (Hawes et al. 2000). Therefore, silencing components of root exudation machinery (e.g., ABC transporters [Brechenmacher et al. 2009; Sugiyama et al. 2007]) might not result in efficient depletion of isoflavonoids in rhizodeposits. Similarly, the use of adsorbents might disrupt quorum signals between bacteria and cause nonspecific modifications in root-surface microbial communities and exert unpredicted, indirect impacts on nontarget organisms, including the plant producing the flavonoids (Hassan and Mathesius 2012). Subsequent identification of specific bacterial phylotypes that are different between VC and IFSi roots through culture-dependent and sequence-based culture-independent methods (e.g., pyrosequencing) will reveal the specific influence of rhizodeposit isoflavonoids on bacterial communities in the soil. Identifying bacteria impacted by isoflavonoids will allow us to better understand how these rhizodeposits influence the rhizosphere and what benefits, if any, soybean derives from rhizodeposit isoflavonoids. This knowledge could be applied to agricultural pursuits to promote plant growth and increase food production in a sustainable and environmentally friendly manner by altering relevant rhizosphere bacterial communities.

5. Conclusion

Results from our experiments clearly demonstrate that root isoflavonoids significantly influence rhizosphere bacterial community composition. Identifying

bacterial communities influenced by isoflavonoids in the soybean rhizosphere through pyrosequencing and/or culture-based experiments would reveal information that would i) improve our scientific understanding of communication between plant and rhizosphere microbes, and ii) ultimately aid better rhizosphere management and sustainable agriculture.

6. Materials and Methods

6.1. Plant materials, soil, and growth conditions

Soybean (*Glycine max* cv. Williams 82) seeds were surface-sterilized via submersion and agitation in a 10% bleach solution for 4 min, followed by rinsing with distilled water six to seven times and submersion and agitation in a 70% ethanol solution for 2 min and again rinsing with distilled water six to seven times. The seeds were then sown in 4-in pots filled with an autoclaved vermiculite/perlite (1:3) mixture and were watered with Hoagland solution (Hoagland and Arnon 1950; Table 1.1).

Table 2.1. Chemical components of Hoagland, nitrogen-free plant nutrient solution (N⁻PNS), and micronutrient solutions.

Hoagland Solution			
<i>Solution</i>	<i>Reagents</i>	<i>Stock Volume (g Chemical)</i>	<i>mL Stock/L Hoagland</i>
<i>Hoagland I</i>	Ca(NO ₃) ₂ • 4H ₂ O	1,078mL (227.27g)	5.6mL
<i>Hoagland II</i>	MgSO ₄ • 7H ₂ O	1,014mL (125g)	4mL
<i>Hoagland III</i>	KNO ₃	989mL (125g)	4mL
	KH ₂ PO ₄	(33.65g)	-
<i>Hoagland IV</i>	NaFeEDTA	952mL (4.0857g)	8mL
<i>Micronutrients (10,000x Conc.)</i>	See below	See below	0.1mL
<i>dH₂O</i>	N/A	N/A	978.3mL

N⁻PNS Solution		
<i>Reagents</i>	<i>Stock Volume (g Chemical)</i>	<i>mL Stock/L PNS</i>
MgSO ₄ • 7H ₂ O	200mL (12.3g)	2mL
CaCl ₂ • 2H ₂ O	400mL (29.4g)	4 mL
KH ₂ PO ₄ • 3H ₂ O	100mL (3.4g)	1 mL
K ₂ SO ₄	400mL (22.0g)	4 mL
FeCl ₃ • 6H ₂ O	250mL (0.62g)	2.5 mL
Micronutrients (10,000x Conc.)	See below	0.5 mL
dH ₂ O	N/A	986mL

Micronutrient Solution (10,000x Concentration)		
<i>Reagents</i>		<i>g/L dH₂O</i>
H ₃ BO ₃	Boric Acid	1.42g
MnSO ₄ • H ₂ O	Manganese Sulfate	0.77g
ZnSO ₄ • 7H ₂ O	Zinc Sulfate	1.73g
CuSO ₄ • 5H ₂ O	Cupric Sulfate	0.37g
NaMoO ₄ • 2H ₂ O	Sodium Molybdate	0.24g
CoCl ₂ • 6H ₂ O	Cobalt Chloride	0.025g
NiSO ₄	Nickel Sulfate	0.01g

Growth conditions were as follows: 50% relative humidity, 16 h of light, 8 h of dark, approximately 25°C day and 20°C night temperatures.

The soil used to isolate rhizosphere bacteria was obtained by pooling approximately 200 samples from agricultural fields with a history of soybean cultivation from South Dakota and western Minnesota submitted to the South

Dakota State University (SDSU) soil-testing laboratory. The samples were cleared of plant materials, were mixed well, and were stored at 4°C until further use (“soybean field soil”). Physical and chemical properties of the soil samples are listed in Table 1.2.

Table 1.5. Physical and chemical properties of “Soybean Field Soil.”
Soil Composition

Sample Number	% Sand	% Silt	% Clay
1	28	39.5	32.5
2	32	35.5	32.5
3	32	42.5	25
4	27	47.5	25
5	28	42	30
Mean	29.4	41.4	29
Standard Deviation	2.41	4.39	3.79

Sample Number	Parts per Million (PPM)							
	Sulfur	Zinc	Manganese	Boron	Iron	Copper	Sodium	NO ₃ N
1	11.45	1.65	27.5	0.41	16.6	1.425	20.95	6.8
2	10.95	1.75	27.85	0.375	14.25	2.7	16.75	6.6
3	11.5	1.35	28.2	0.38	13.15	0.715	16.45	5.7
4	11.5	1.55	27.5	0.44	15.6	0.705	24	7.6
5	10.1	1.35	26.35	0.41	15.8	0.7	18.15	7.6
Mean	11.1	1.53	27.48	0.403	15.08	1.249	19.26	6.86
Standard Deviation	0.61	0.18	0.7	0.03	1.37	0.87	3.19	0.79

Sample Number	Water pH	Buffer pH	Parts per Million (PPM)				Percent (%)
			Phosphorus	Potassium	Calcium	Magnesium	Organic Matter
1	7.1	7.7	36.5	191.5	3727	529.5	3.4
2	7.1	7.7	25	190.5	4128	517.5	3.3
3	7.1	7.7	29	250	4298	556	3.3
4	7.1	7.7	38	231.5	4256	609	3.3
5	7	7.6	36	232.5	3944	555	3.4
Mean	7.08	7.68	32.9	219.2	4070.6	553.4	3.34
Standard Deviation	0.04	0.04	5.62	26.78	236.36	35.22	0.05

6.2. DNA vectors and plant transformation

The DNA vectors (control and IFSi constructs) have been previously described (Subramanian et al. 2005). Fourteen-day-old seedlings (possessing their first trifoliolate leaves) were used for composite plant generation as described previously (Collier et al. 2005), except that *Agrobacterium rhizogenes* cultures (VC or IFSi) used for transformation were cultured for 16 to 20 h in Luria Bertani broth supplemented with 50 mg of kanamycin per liter at 30°C, and then, were centrifuged at $3,500 \times g$ for 8 min at 4°C and resuspended in nitrogen-free plant nutrient solution. The plants were grown under previously described conditions (Subramanian et al. 2006). After 3 weeks, successfully transformed roots were identified by green fluorescent protein epifluorescence via a fluorescein isothiocyanate filter, using an Olympus SZX16 Epi-Fluorescence Stereo Microscope, marked with “Tough-Tags” (Diversified Biotech), and were subsequently planted in soybean field soil.

6.3. Isolation of rhizosphere soil

To obtain rhizosphere samples, plants were removed from soil after 1 and 3 weeks and roots were subjected to three consecutive sonications. A Fisher Scientific FS20 model sonicator (input: 117 V⁻ 50 to 60 Hz 1 ϕ , output: 70 W 42 kHz \pm 6%) was used for this experiment. The harvested roots were first shaken gently in a still pool of distilled (d)H₂O to remove larger soil particles. Next, they were severed from the plant and were placed in separate 15 ml tubes with 10 ml of phosphate buffered saline Tween20 (PBST). These tubes were subjected to a 60 s sonication to collect DS from the roots. The roots were then moved to new

15 ml centrifuge tubes with 10 ml of fresh PBST and were subjected to another 60 s sonication to collect MS from the plant roots. After that the roots were relocated to new 15 ml tubes containing 10 ml of fresh PBST and were subjected to a 10 min sonication to collect PS from the roots. The material released from roots was harvested by centrifugation ($5,000 \times g$ for 10 min or $4,500 \times g$ for 15 min).

To address concerns that sonication might disrupt bacterial cells and result in DNA contamination across the PS, MS, and DS zones we subjected *Escherichia coli* K12, a strain well-known for its relatively weak cell-wall properties, to our successive sonication method and evaluated differences in CFU and found no significant difference between control and cell suspensions subjected to 10 min of sonication, suggesting that there was no significant disruption of bacterial cells (Fig. 1.16).

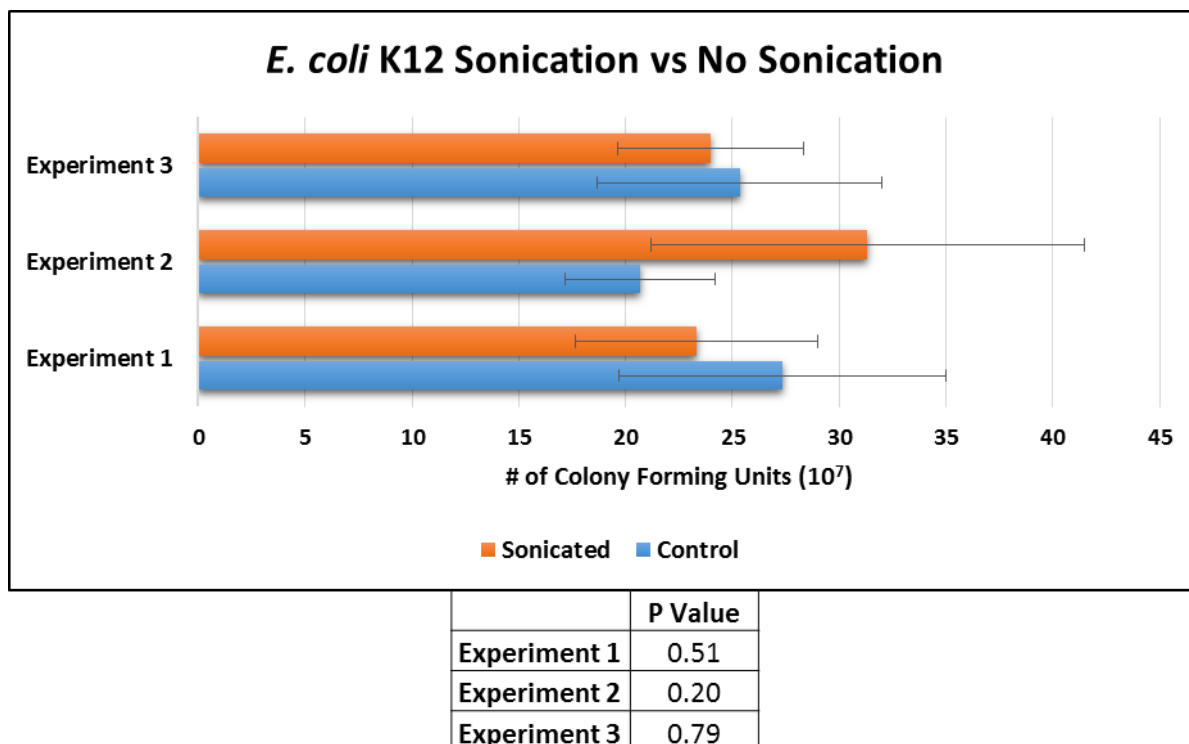


Figure 1.16. Bar graph comparing number of CFUs from *E. coli* K12 liquid cultures subjected to either a 10 min sonication or no sonication.

Bar graph comparing the number of colony forming units (CFUs) from *Escherichia coli* K12 liquid cultures either subjected to a 10 min sonication (Sonicated) or not subjected to sonication (Control). Three replicate plates with a 10^{-7} dilution were used for each treatment in each experiment. Error bars were derived from standard deviations. *P* values derived from two-tailed *t*-tests for each experiment are depicted in the table below the bar graph.

6.4. DNA isolation, PCR and DGGE

DNA was extracted from 0.09 to 0.47 g of harvested rhizosphere materials using the PowerSoil DNA isolation kit (MO BIO Laboratories, Inc.), following the manufacturer's protocol.

The 16S rRNA variable regions V3 to V5 were amplified using a Gene Amp PCR System 9700 model thermocycler machine in a 30- μ l reaction mixture (0.2 μ l *Taq* DNA polymerase (Promega/Invitrogen), 6 μ l of PCR buffer, 0.15 μ l of dNTP (10 mM, Promega), 1.8 μ l of $MgCl_2$ (25 mM), 1.2 μ l of forward primer

(0.01 mM), 1.2 µl of reverse primer (0.01 mM), 18.95 µl of Nanopure H₂O, 0.5 µl (approximately 100 ng) of template DNA. PCR parameters were as follows: preliminary denaturation at 94°C for 5 min, (94°C for 30 s, 56°C for 45 s, 72°C for 1 min) for 30 cycles, final elongation at 72°C for 7 min, and 10°C indefinitely for storage. Forward primer F357 containing a 5' 40-base GC-clamp (Brons and van Ems 2008; Muyzer et al. 1993) and reverse primer R907 (Teske et al. 1996) were used for DNA amplification in this experiment.

PCR products were subjected to denaturing gradient gel electrophoresis as described previously (Muyzer et al. 1993). PCR product (40 µl) was resolved using a 35 to 70% denaturant gradient gel in 1.25× Tris-acetate-EDTA buffer in a DCode System (BioRAD). Electrophoresis was executed at 60°C at 20 V until DNA moved through the cap gel, and then, at 70 V for 16 h. Gels were stained for 20 min in SYBR gold (30 µl in 300 ml of dH₂O [Invitrogen]), and images were captured with UV transillumination (BioRAD Chemidoc XRS).

6.5. DGGE gel image analysis

Quantity One (BioRAD) software was used to capture the intensity of the data and ascertain and subtract the amount of background noise within each DGGE image as previously described (Rettedal 2011). The resulting quantitative data were then rounded to the nearest whole number in Microsoft Excel and were subsequently analyzed further using the *vegan* package (Oksanen et al. 2013) of R software (R Core Team 2013) (version 3.0.2). General diversity indices (Shannon, Simpson, and Inverse-Simpson) were obtained by executing the respective commands and then plotting the indices against one another. Unconstrained

ordination analyses were accomplished by first implementing the detrended correspondence analysis commands (using “*iweigh=-1*,” “*iresc=4*,” and “*ira=0*” to downweigh rare species, execute four rescaling cycles, and perform a detrended analysis using the *decorana* method) and, then, plotting the data (only displaying the “sites”). Nonparametric multivariate analysis of variance for partitioning distance matrices among sources of variation was performed using *adonis*. Linear models were tested for each variable and their statistical significance determined. Due to the non-normal distribution of the community data, it was standardized in R (via the *center_scale* command with *scale = FALSE* to only subtract the mean), and all resulting values were increased by 1,000 and were subject to a \log_{10} transformation prior to hierarchical clustering. Cluster analyses were performed on the normalized, logarithmically transformed data by calculating Euclidean dissimilarity matrices (using the *vegdist* and *hclust* commands) and plotting the data.

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Chapter 2: Isolation of Rhizosphere Bacterial Communities from Soil

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1. Abstract

Rhizosphere bacterial communities have become a major focal point of research in recent years, especially regarding how they affect plants and vice versa (Philippot *et al.*, 2013). Changes in microbial density and diversity within the rhizosphere occur in a spatial temporal manner. The soil zone closest to the plant roots has the most density and diversity of microbes (Clark, 1940). The lack of methods to consistently isolate rhizosphere samples in a spatially defined manner is a major bottleneck in rhizosphere microbiology. We hypothesized that microbes with increasing affinities to and distance from the plant root can be isolated using increasing strengths of physical disruption. Sonication is an excellent choice due to the ability to gently remove rhizosphere soil and bacterial biofilms without damaging plant roots (Doi T *et al.*, 2007; Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012). In addition, simply increasing the time of sonication can increase the amount of physical force. We used such an approach to consistently isolate microbial communities with different affinities to the soybean roots (White *et al.*, 2014). This article describes the use of successive sonication to isolate distal, middle, and proximal soil from the rhizosphere of soybean roots.

2. Materials and Reagents

1. Soybean seedlings (*Glycine max*) in the vegetative stage (~ V3 to V5 period)
2. Soil with a history of soybean cultivation
3. dH₂O
4. K₂HPO₄ (VWR International, catalog number: BDH0266-500 g)

5. KH_2PO_4 (VWR International, catalog number: BDH0268-500 g)
6. NaCl (Sigma-Aldrich, catalog number: S7653-1 kg)
7. Tween-20 (Sigma-Aldrich, catalog number: P9416-100 ml)
8. Phosphate buffered saline Tween 20 (PBST) (see Recipes)

3. Equipment

1. Razor blade
2. Tweezers
3. 15 ml conical-bottom polypropylene centrifuge tubes (3 per sample) (VWR International, catalog number: 89039-670)
4. 50 ml conical-bottom polypropylene centrifuge tubes (3 per sample) (VWR International, catalog number: 21008-940)

Note: Needed if plant roots are too large for 15 ml centrifuge tubes.
5. Styrofoam raft to suspend centrifuge tubes in sonicator (homemade)
6. Sonicator (Input: 117 V-50-60HZ 1 ϕ , Output: 70 W 42KHZ +/-6%) (Thermo Fisher Scientific, model: FS20)
7. Centrifuge with a fixed angle rotor for 15 and 50 ml conical bottom tubes at 4 °C capable of at least 5,000 \times g relative centrifugal force (120 V 12 A 60 Hz 1,300 W) (Example: Eppendorf, model: 5804R 15 amp version)

4. Procedure

1. Either directly sow plant seeds or plant seedlings into soil of interest and allow seeds/seedlings to grow for desired amount of time (minimum of 1 week suggested for soybean plants).

Notes:

- *Although larger roots (ex. mature tree roots) are not recommended for this procedure, representative samples of the root system can be used depending on the research question.*
- *Amount of growth time depends on the research focus, for example the impact of a particular root exudate or the plant growth stage on the soil microbial community.*

2. Carefully remove plant seedlings by saturating the soil with dH₂O or gently loosening the soil by hand to avoid damage to the roots.

Notes:

- *Using an excessive amount of dH₂O during saturation (i.e. resulting in a soil consistency thinner than mud) risks a loss of sample size and rhizosphere bacteria.*

3. Submerge the roots in a still pool of dH₂O and gently shake the roots (as if painting a picture or dunking a teabag) to remove the larger soil particles. Skip this step if plant seedlings were removed by soil saturation in the

previous step. See Figure 2.1 for example of soybean roots before and after the removal of large soil particles.



Figure 2.1. Thirty-six day old soybean roots (A) before and (B) after submersion in a still pool of dH₂O to remove large soil particles.

The amount of soil clinging to the plant roots can vary depending on soil properties, the root architecture, and the size(s) of the plant roots.

4. Use a razor blade to sever the plant roots (cutting near the plant stem).
5. Place the severed roots into separate, labeled 15 ml centrifuge tubes filled with 10 ml of PBST, ensuring they are completely submerged (may use tweezers to gently push roots deeper into the tube).

Notes:

- *Roots should be placed into the centrifuge tube vertically.*
- *Ensure the centrifuge tube is not packed with the root sample. The number of roots placed into one tube depends on root size and/or the desire to keep root samples separate (ex. pooling all roots from one plant together, pooling multiple roots from several*

plants together, or keeping each root from one plant separate).

Overly large roots, or too many roots in one tube, will lead to poor sample isolation whereas tiny roots, or too few roots in one tube, will yield a miniscule sample size.

- *For seedlings with larger root systems, use a 50 ml centrifuge tube filled with 45 ml of PBST in this step and all subsequent steps. See Figure 2.2 for demonstrative sample of an acceptable amount of roots in a single tube.*



Figure 2.2. Soybean roots submerged in 10 ml of PBST within a 15 ml centrifuge tube.

6. Firmly secure the centrifuge tube lids, then place the tubes in a floating raft within a sonicator filled with dH₂O.

Notes:

- *Ensure the centrifuge tubes do not touch the bottom or sides of the sonicator (see Figure 2.3 for demonstrative diagram).*

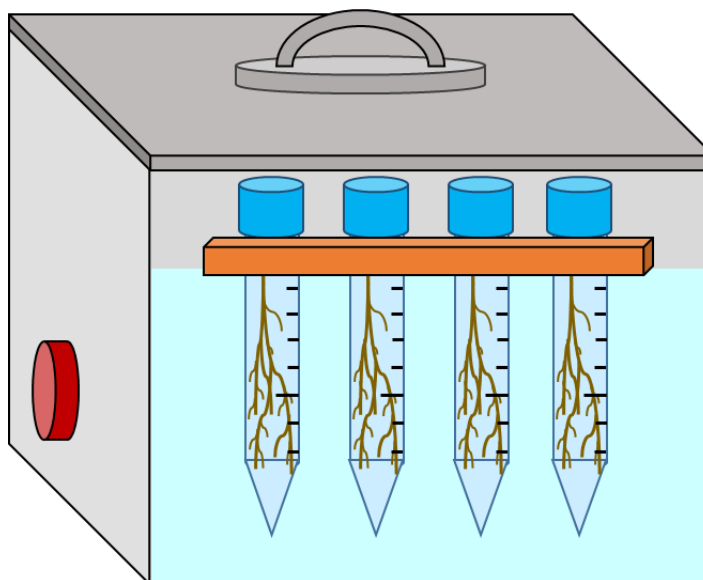


Figure 2.3. Diagram demonstrating how to properly load samples and floating raft into the sonicator filled with dH₂O.

Centrifuge tubes should be submerged up to the 10 or 45 ml line (dependent on if a 15 or 50 ml centrifuge tube was used). Tubes should not touch the bottom or edges of the sonicator.

7. Subject the centrifuge tubes to sonication for 60 s, then turn off the sonicator (see Figure 2.4 for sonication summary).

Notes:

- *This sonication yields the rhizosphere soil furthest from the plant root or soil with least affinity to the plant root, noted as the “distal soil” sample.*
- *Use the same sonication time for both the 15 and 50 ml centrifuge tubes.*

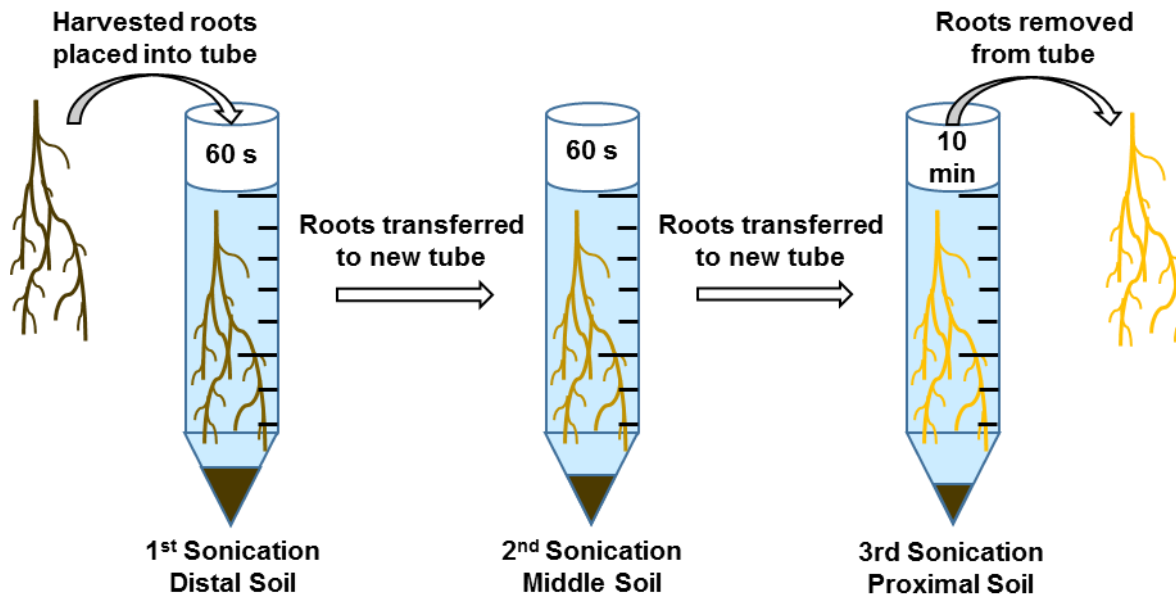


Figure 2.4. Diagram of successive sonication procedure for isolation of distal, middle, and proximal soil samples from plant roots.

Distal soil samples consist of the rhizosphere soil furthest from and with least affinity to the plant root. Middle soil samples consist of the rhizosphere soil that is closer to and with relatively less affinity the plant root. Proximal soil samples consist of the rhizosphere soil closest to and with highest affinity to the plant root. Image adapted from a previous article (White et al., 2014).

8. Using tweezers, gently remove the root(s) from the current centrifuge tube(s) and transfer into a new, labeled centrifuge tube (or tubes) containing 10 ml of fresh PBST.

Notes:

- *Keep roots/samples separated in the same manner used for the first sonication.*
 - *Do not pool roots/samples from different centrifuge tubes together.*
9. Firmly secure the centrifuge tube lids, place the tubes in the floating raft within the sonicator, and subject the tubes to sonication for 60 s. Then turn off the sonicator.

- *Note: This sonication yields the rhizosphere soil that is closer to the plant root, noted as the “middle soil” sample.*

10. Using tweezers, gently remove the root(s) from the current centrifuge tube(s) and transfer into a new, labeled 15 ml centrifuge tube (or tubes) containing 10 ml of fresh PBST.

Notes:

- *Again, keep roots/samples separated in the same manner used for the first sonication. Do not pool roots/samples from different centrifuge tubes together.*

11. Firmly secure the centrifuge tube lids, place the tubes in the floating raft within the sonicator, and subject the tubes to sonication for 10 min. Then turn off the sonicator.

Notes:

- *This sonication yields the rhizosphere soil closest to the plant root including any biofilms, noted as the “proximal soil” sample. At this point, soil should not be visible on the plant root.*

12. Using tweezers, gently remove the root(s) from the current centrifuge tube(s) and either discard the roots or place them into a new, labeled centrifuge tube (or tubes) filled with fresh PBST, then store the tubes at 4 °C until needed. Harvested samples may then be immediately used for bacterial cultivation or further processed for DNA or RNA isolation. If seeking to isolate DNA or RNA, complete the next 2 steps of the protocol. For bacterial cultivation, promptly subject the samples to a series of 6 to 10 fold dilutions using sterile dH₂O and

select several of these dilutions for plating (dilutions $>10^{-3}$ recommended). When plating the chosen dilutions, ensure the appropriate nutrient medium (or media) is chosen. One hundred microliters of the chosen dilution should be dispensed onto the center of the petri dish and spread across the media using a flame-sterilized glass spreader. The petri dish should then be inverted and incubated under the ideal cultivating conditions (*i.e.* time and temperature). See Figure 2.5 for an example of bacterial cultivation via petri dish.

Notes:

- *Distal, middle, and proximal soil samples are all useful for bacterial cultivation. However, proximal soil samples are preferable as they contain the bacteria that most likely affect the plant directly and vice versa.*
- *Possible media for bacterial cultivation include a soil extract medium such as SESOM, DR2A + supplements, and R2A solidified with agar or gellan (Tamaki et al., 2005; Vilain et al., 2006).*

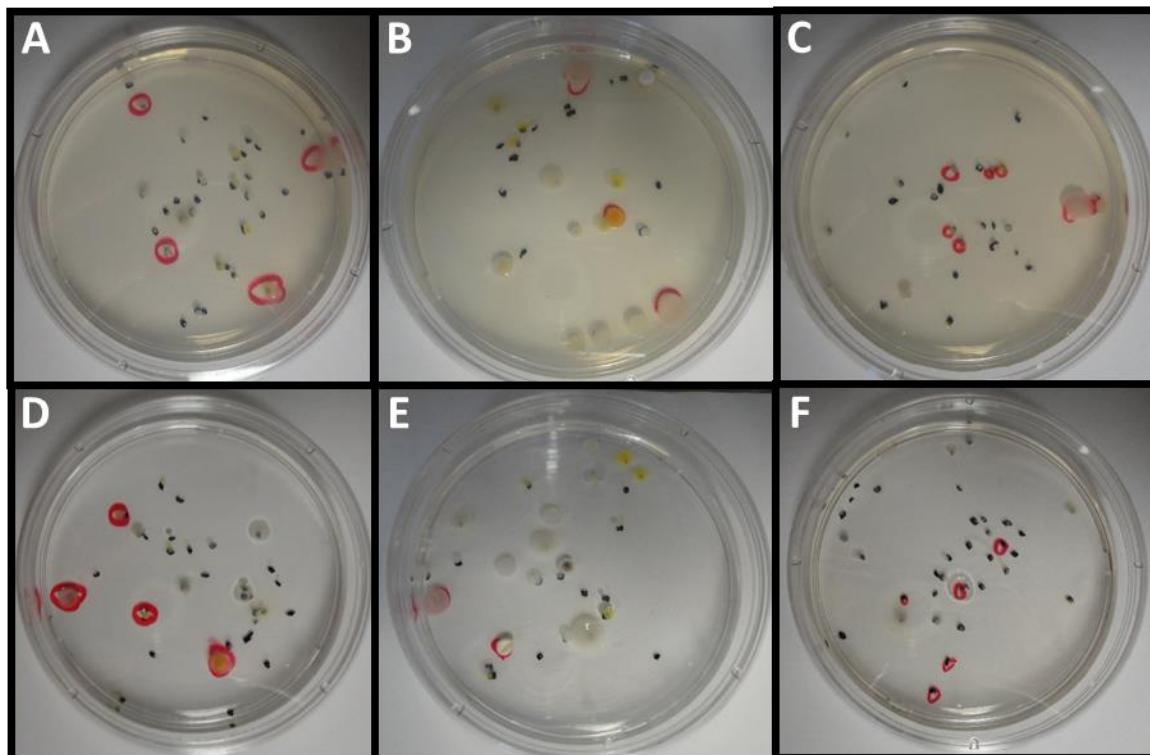


Figure 2.5. Bacterial cultivation of proximal soil samples from untransformed soybean roots on nutrient media solidified with (A-C) agar or (D-F) gellan.

Nutrient media consisted of (A,D), R2A (B,E) DR2A+, and (C,F) SESOM. Bacterial samples acquired from a 10^{-5} dilution. Black dots and red circles indicate the presence of individual bacterial colonies.

13. After securing the lids on all the centrifuge tubes, place them into a 4 °C centrifuge and subject them to centrifugation at 5,000 $x g$ for 10 min or 4,500 $x g$ for 15 min (depending on the limits of the centrifuge).
14. Once centrifugation is complete, discard supernatant and either immediately use the pellets for DNA or RNA isolation or store them at -80 °C until needed.

5. Limitations of the Method

1. Sonication times may vary depending on the types of plant roots used as well as the properties of the soil in which they were grown.
2. It is uncertain how useful this procedure is for soil fungi.

3. Sample sizes will be small (likely < 0.3 g when using 15 ml centrifuge tubes) and decrease from sonication to sonication, with proximal soil samples being the smallest. This might be an issue for methods such as proteomics and metabolomics that generally require a larger sample size.
4. Age of the plant makes a difference (root system is very large at later stages). This procedure is better suited for smaller root sizes. For perennial plants or older plants with large root systems, one can use a golf cup cutter (4" to 8" diameter) to obtain a soil core (6" to 12" deep) and obtain root segments from that by placing it in water and allowing the soil to separate from the roots. Obviously, this would depend on whether the representative samples of the root system would suffice to answer the research question.

5. Recipes

1. Phosphate buffered saline Tween 20 (PBST) (500 ml, pH of 7.2)
 - a. Add 0.605 g of K_2HPO_4 to 300 ml of dH_2O , stir until K_2HPO_4 is completely dissolved
 - b. Add 0.17 g of KH_2PO_4 to mixture, stir until KH_2PO_4 is completely dissolved
 - c. Add 4.1 g of $NaCl$ to mixture, stir until $NaCl$ is completely dissolved.
 - d. Adjust pH with $NaOH$ or HCl until final pH is 7.2
 - e. Add dH_2O to mixture until the final volume is 500 ml, stir to ensure even distribution

- f. Sterilize solution via autoclaving (liquid cycle, 121 °C for 30 min)
- g. Add 250 µl of Tween20 to mixture, gently swirl to ensure even distribution

Notes:

- *Adding Tween20 before autoclaving will result in frothing overflow due to bubble formation*

- h. Store at room temperature (~20 °C)

2. Acknowledgments

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Chapter 3: Root Isoflavonoids and Hairy Root Transformation

Influence Key Bacterial Taxa in the Soybean Rhizosphere

Original Document: White, L.J., Ge, X., Brözel, V.S., and Subramanian, S. (2017) Root isoflavonoids and hairy root transformation influence key bacterial taxa in the soybean rhizosphere. *Environmental Microbiology*.

1. Abstract

Rhizodeposits play a key role in shaping rhizosphere microbial communities. In soybean, isoflavonoids are a key rhizodeposit component that aid in plant defense and enable symbiotic associations with rhizobia. However, it is uncertain if and how they influence rhizosphere microbial communities. Isoflavonoid biosynthesis was silenced via RNA interference of isoflavone synthase in soybean hairy root composite plants. Rhizosphere soil fractions tightly associated with roots were isolated, and PCR amplicons from 16S rRNA gene variable regions V1–V3 and V3–V5 from these fractions were sequenced using 454. The resulting data was resolved using *MOTHUR* and *vegan* to identify bacterial taxa and evaluate changes in rhizosphere bacterial communities. The soybean rhizosphere was enriched in Proteobacteria and Bacteroidetes, and had relatively lower levels of Actinobacteria and Acidobacteria compared with bulk soil. Isoflavonoids had a small effect on bacterial community structure, and in particular on the abundance of Xanthomonads and Comamonads. The effect of hairy root transformation on rhizosphere bacterial communities was largely similar to untransformed plant roots with approximately 74% of the bacterial families displaying similar colonization underscoring the suitability of this technique to evaluate the influence of plant roots on rhizosphere bacterial communities. However, hairy root transformation had notable influence on Sphingomonads and Acidobacteria.

2. Introduction

Plants play a prominent role in shaping soil microbial community structure, particularly within the rhizosphere. Multiple studies have demonstrated the effects of

plant roots on soil microbial community size and diversity (Kaiser et al., 2001; Gottel et al., 2011; Lundberg et al., 2012; Peiffer et al., 2013). The impact of plant roots on soil microbes has been attributed to multiple factors such as plant community diversity, species, genotype and developmental stage as well as root morphology and exudation (Philippot et al., 2013; Sugiyama et al., 2014). Rhizodeposition in particular exerts a more direct effect on soil microbes as the composition of rhizodeposits differs depending on the plant's interactions with insects, soil microbes, and other plants as well as its species, genotype and developmental stage (Walker et al., 2003). The root exudates are composed of multiple organic compounds such as amino acids, nucleotides, sugars, vitamins, organic acids and plant growth regulators. These compounds serve various functions such as mediating chemical interference between plants, altering soil chemistry to help regulate soil nutrient availability, or providing a carbon source for soil microbes (Bais et al., 2006; Lugtenberg and Kamilova, 2009). Plants oftentimes use root exudates to attract beneficial microbes and dissuade pathogenic microbes. For example, flavonoids can help facilitate a symbiotic relationship with nodule-forming *Rhizobia*, or help stave off infection by *Fusarium oxysporum* (Zhang et al., 2009; Banasiak et al., 2013). Beneficial microbes are recruited to aid in nitrogen fixation, increase stress tolerance and promote plant growth as well as defend against harmful microbes using protective biofilms or antibiotics produced by the beneficial microbes. Both beneficial and pathogenic microbes use root exudates as nutrient sources and/or chemoattractants (Bais et al., 2006; Lugtenberg and Kamilova, 2009). The sheer variety and functions of root exudates as well as the complexity of plant-microbe interactions provide a challenge

for rhizosphere studies. Focusing on root exudates of specific plant species that play more active roles in plant-microbe interactions allows researchers to better analyze the exudates' effects on the rhizosphere microbiome. The plant species of interest in our study was *Glycine max* (soybean) due to the crop's increasingly important role as livestock feed, biodiesel fuel and biocomposite building material as well as its production of the root exudates known as isoflavonoids, which are more likely to play a role in signaling bacterial communities rather than acting as an energy source.

Isoflavonoids are a particularly useful group of root exudates for studying plant-microbe interactions due to their ability to regulate nodulation factors, aid plant defense against pathogenic microbes and, as previously mentioned, because they are solely detected in plants belonging to the legume family (Hassan and Mathesius, 2012). Daidzein and genistein are two particular isoflavonoids produced by soybean that induce *Bradyrhizobium japonicum* nod genes and suppress *Sinorhizobium meliloti* nod genes as well as aid against the pathogenic microbe *Phytophthora sojae* (Subramanian et al., 2005; Bais et al., 2006; Subramanian et al., 2006). Isoflavonoids are also secreted by soybean roots into the surrounding environment (D'Arcy-Lameta, 1986; Graham et al., 2007). Given the apparent, active role these isoflavonoids play in plant-microbe interactions, one may reasonably surmise they help shape the rhizosphere microbial community structure. In a previous study, we sought to determine the magnitude of the isoflavonoids' impact on the rhizosphere bacterial community diversity of soybean. As root isoflavonoid levels directly influence root exudate isoflavonoid levels (D'ArcyLameta, 1986), we expected that silencing of isoflavone synthase (IFS), a key enzyme necessary for isoflavone biosynthesis, would

result in a significant reduction of isoflavones secreted by the roots. Secreted isoflavonoids amount to approximately 2%–20% of the root isoflavonoids (D'Arcy-Lameta, 1986; Graham et al., 2007). We demonstrated that IFS-RNAi led to a > 95% reduction in root isoflavonoids and a 50%–85% reduction in secreted isoflavonoids. For example, we observed an approximately 75% reduction in secreted daidzein, and an approximately 50% reduction in secreted genistein in IFS-RNA interference (IFS-RNAi) soybean roots challenged with *Phytophthora sojae* (Graham et al., 2007). Similarly, we observed a 75%–85% reduction in daidzein and a 60%–70% reduction in genistein in root exudates of uninoculated IFS-RNAi soybean roots (Table 3.1).

Table 8.1. Quantification of daidzein and genistein in root secretions of control and IFS-RNAi roots.

Plant Genotype	Experiment I			Experiment II		
	Root Weight (mg)	Daidzein in Exudate (nmoles)	Genistein in Exudate (nmoles)	Root Weight (mg)	Daidzein in Exudate (nmoles)	Genistein in Exudate (nmoles)
Control	4429	45.92	26.77	1345	30.26	8.25
IFS-RNAi	4538	11.52	8.42	1380	4.4	3.1
% Reduction		74.91	68.53		85.48	62.5

Composite plants with transgenic roots were placed in glass tubes containing 10 ml sterile deionized water and incubated in a plant growth chamber (16 h light, 25°C day and 20°C night temperature) with gentle shaking. After 48 h, plants were removed, and the roots were harvested and weighed. Root secretions from approximately equal amount of roots (by weight) were pooled for each genotype, and isoflavonoids were extracted using equal volumes of ethyl acetate in a separating funnel. The extraction was repeated two additional times and all extractions were pooled. Ethyl acetate was allowed to evaporate and the residue was dissolved in a final volume of 100 µl of 80% methanol. Total daidzein and genistein were assayed by HPLC as described by [Subramanian et al. 2006. Plant J. 48 (2): 261-273].

We previously examined the bacterial diversity of root soil samples from three regions in the rhizosphere – noted as distal, middle and proximal – for 3 root types – untransformed, vector control and IFS-RNAi – at 1 and 3 weeks post planting. Our

results showed there was a significant difference in the rhizosphere bacterial community diversity of roots with normal isoflavonoid levels compared with roots with reduced isoflavonoid levels. Additionally, they appeared to have a temporal gradient effect on the rhizosphere, with the isoflavonoids exerting greater influence as more time passed (White et al., 2015). Although the study showed the basic impact of isoflavonoids on the soybean bacterial community diversity it did not clarify how the community was affected, such as whether specific bacterial groups were suppressed or enhanced. Such knowledge is crucial when attempting to define the impact of root exudates on rhizosphere microbes and subsequently using that knowledge for rhizosphere engineering. In order to better define how isoflavonoids impacted the soybean rhizosphere bacterial community, we silenced isoflavonoid biosynthesis in hairy root composite plants through IFS-RNAi, isolated root proximal soil samples through successive sonication, identified bacterial phyla, families, genera and OTUs from 16S rRNA using pyrosequencing, and examined the resulting data through various statistical analyses.

3. Results

3.1. Bacterial community structure of the soybean rhizosphere

We previously isolated proximal soil samples from unaltered soybean roots, transgenic vector control roots and IFS-RNAi roots (White et al., 2015). Transgenic roots were verified by the use of GFP as a selectable marker (Fig. 3.1) and consistent silencing of IFS genes and significant reduction in root isoflavonoids were confirmed by qPCR and HPLC analyses respectively (White et al., 2015).

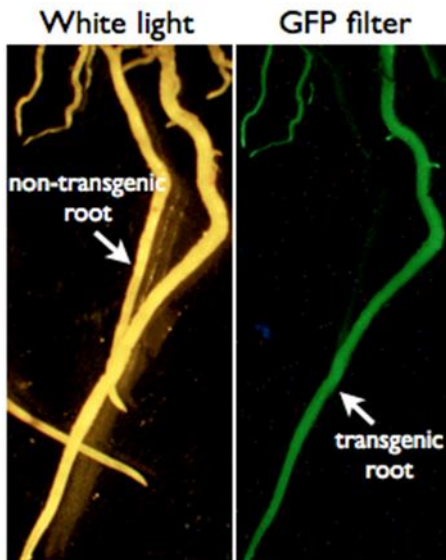


Figure 3.1. Transgenic and non-transgenic soybean roots imaged under a white light (left) and through a GFP filter (right).

Roots exhibiting epifluorescence under a GFP filter indicate successful stable transformation (i.e. transgenic roots).

Here, we amplified and sequenced 16S variable regions V1–V3 and V3–V5 from (i) bulk soybean field soil (SFS; 2 replicates) without soybean roots, (ii) proximal soil (White et al., 2015) from unaltered soybean roots (UNR; 3 replicates), (iii) proximal soil from vector control roots (VC; 5 replicates) and (iv) proximal soil from IFS-RNAi roots (IFSi; 5 replicates). High quality sequences of 16S amplicons (V1–V3 and V3–V5) were processed through an analysis pipeline (Table 3.2 and Fig. 3.2) involving *MOTHUR* to obtain operational taxonomic units (OTUs).

Table 3.2. Sequence tallies for the individual samples and sample types for variable regions V1-V3 and V3-V5 before data analysis.

Variable Regions V1-V3					
Replicates	Number of Roots*	Number of Sequences	Genotypes	Number of Sequences	Total
VC Rep1	8	15295	VC	69043	226746
VC Rep2	3	13556			
VC Rep3	3	9917			
VC Rep4	3	10191			
VC Rep5	9	20084			
IFSi Rep1	5	10579	IFSi	86047	
IFSi Rep2	5	11532			
IFSi Rep3	4	16060			
IFSi Rep4	8	37972			
IFSi Rep5	8	9904			
SFS Rep1	--	10245	SFS	22231	
SFS Rep2	--	11986			
UNR Rep1	2	12813	UNR	49425	
UNR Rep2	2	21251			
UNR Rep3	2	15361			
Variable Regions V3-V5					
Replicates	Number of Roots*	Number of Sequences	Genotypes	Number of Sequences	Total
VC Rep1	8	14951	VC	57227	177707
VC Rep2	3	7272			
VC Rep3	3	10440			
VC Rep4	3	10341			
VC Rep5	9	14223			
IFSi Rep1	5	12469	IFSi	61025	
IFSi Rep2	5	8611			
IFSi Rep3	4	9411			
IFSi Rep4	8	10791			
IFSi Rep5	8	19743			
SFS Rep1	--	8051	SFS	13735	
SFS Rep2	--	5684			
UNR Rep1	2	13347	UNR	45720	
UNR Rep2	2	13326			
UNR Rep3	2	19047			

* Each replicate was composed of rhizosphere fractions isolated from at least this many roots

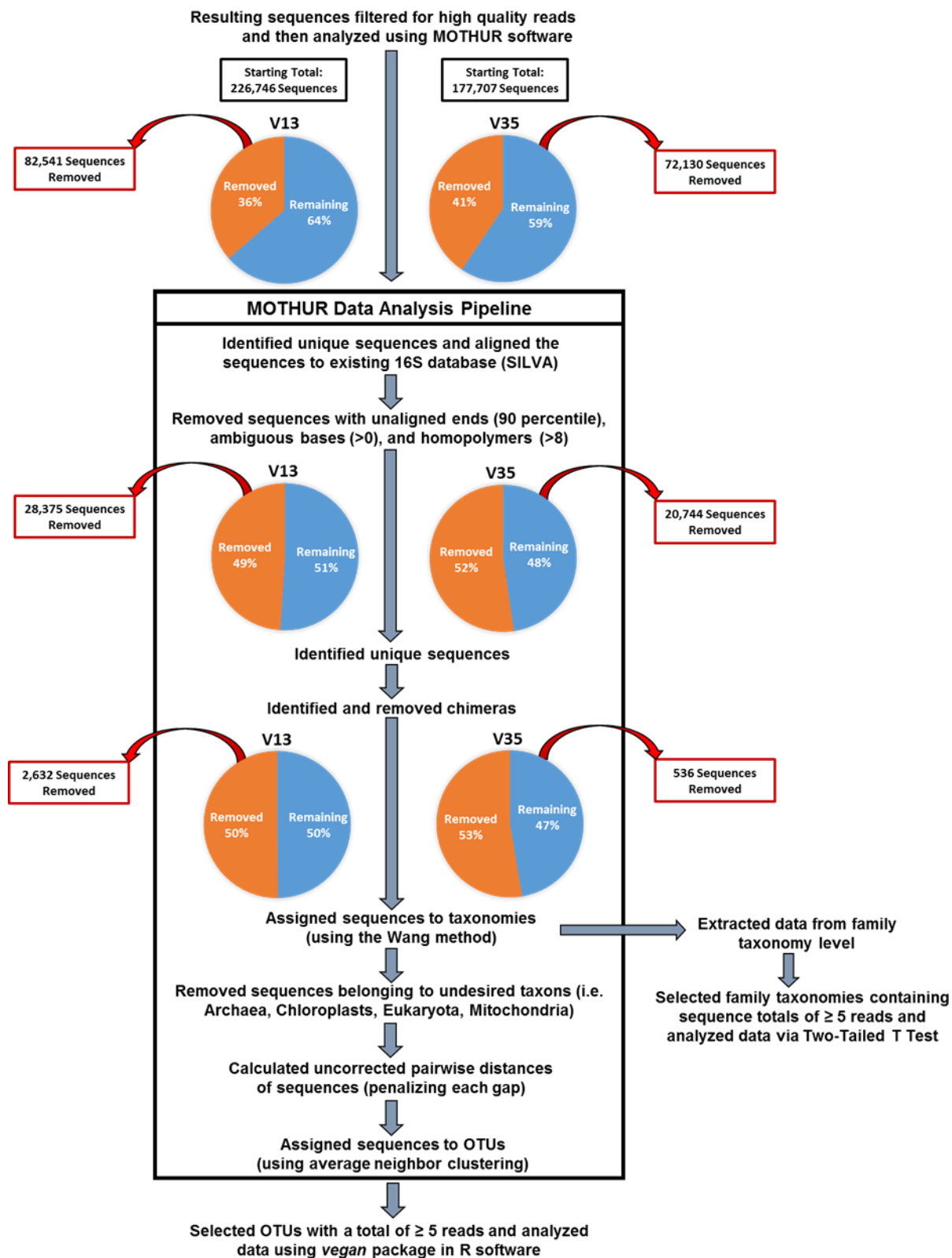


Figure 3.2. Pyrosequencing data analysis pipeline.

Data analysis pipeline used to process pyrosequencing data to identify bacterial taxa and evaluate differences in abundance between samples.

We eliminated very low abundance OTUs by removing those that had < 5 reads in all 15 samples combined. The abundance data of each OTU in different samples were used to calculate Shannon, Simpson and Inverse-Simpson general diversity indices. The results clearly showed that SFS samples had the lowest diversity compared with UNR, VC and IFSi samples (Fig. 3.3) in agreement with previous reports of enriched diversity in the rhizosphere compared with bulk soil (Peiffer et al., 2013; Sugiyama et al., 2014).

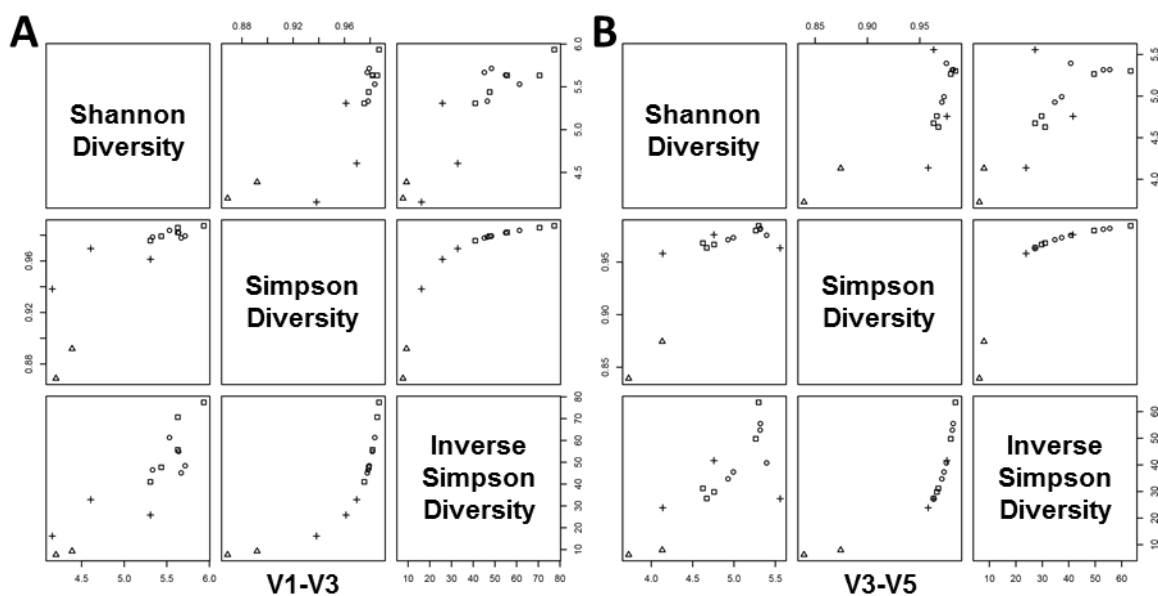


Figure 3.3. Comparison of diversity indices for SFS and UNR, VC and IFSi PS samples 3 wpp.

Comparison of Shannon, Simpson, and inverse-Simpson Diversity index plots for soybean field soil (SFS) and proximal soil samples from untransformed (UNR), vector control (VC), and IFS-RNAi (IFSi) roots at 3 weeks after planting (\triangle = SFS, $+$ = UNR, \square = VC, \circ = IFSi). Diversity indices calculated from both (A) V1-V3 and (B) V3-V5 libraries indicated that the SFS samples had the lowest diversity followed by the UNR samples, and that the VC and IFSi samples exhibited similar, but highest diversity.

Next we compared the community structures in the different samples using detrended correspondence analysis (DCA) and hierarchical cluster analysis (Figs.

3.4-3.5) with the ultimate goal of determining the influence of isoflavonoids on the rhizosphere bacterial community (Hill and Gauch, 1980).

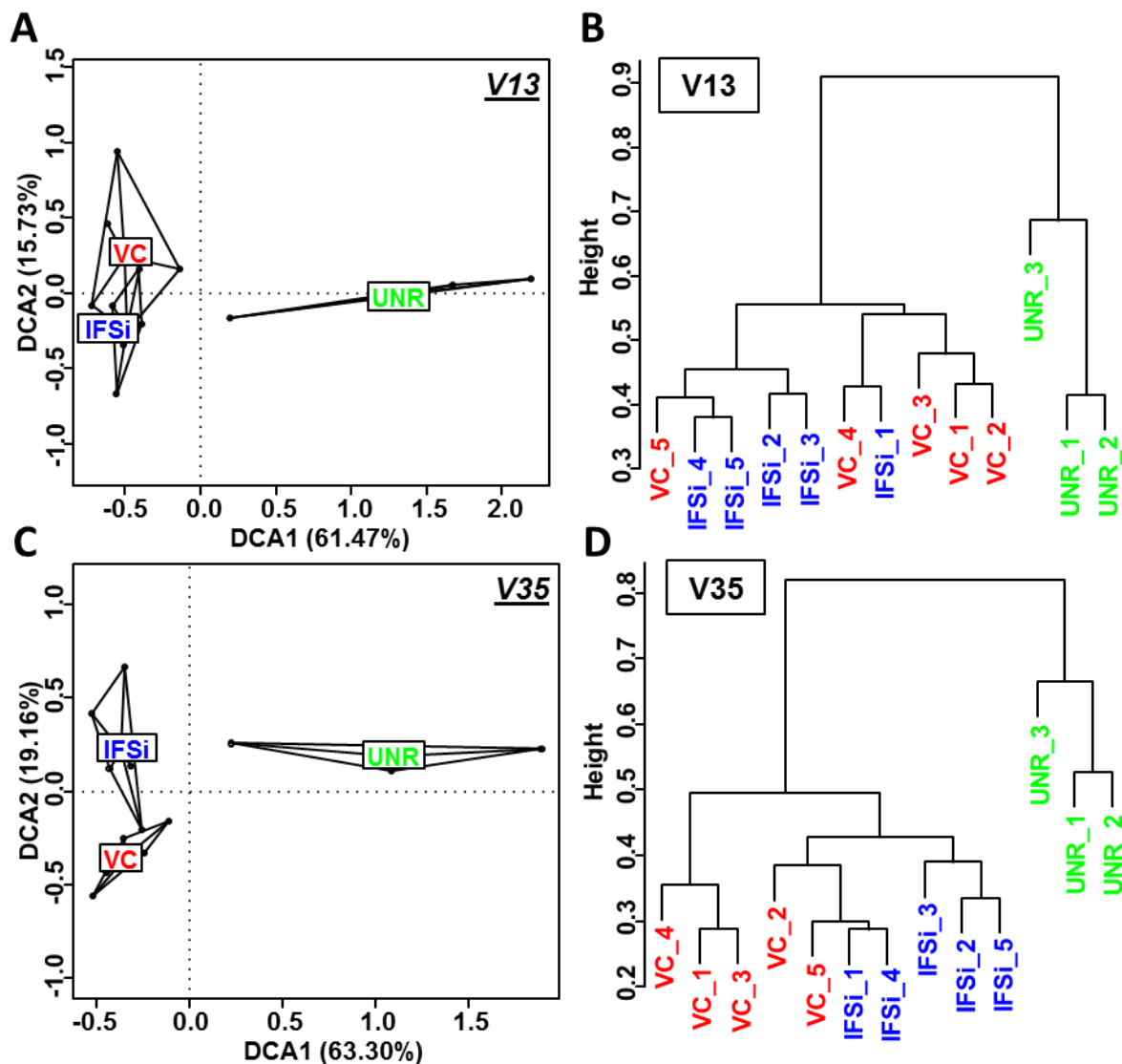


Figure 3.4. DCA and hierarchical clustering analyses indicating the extent of dissimilarities among UNR, VC and IFSi soil samples.

(A, C) DCA plots displaying the separation of proximal soil samples from untransformed (UNR), control (VC), and IFS-RNAi (IFSi) roots 3 weeks post planting. DCA1 and DCA1 represent the major axes of dissimilarity. Data points of the same sample type from different experiments are depicted connected by a line. V13 and V35 indicate if the plots were obtained using sequences of PCR amplicons from V1-V3 or V3-V5 variable regions of the 16S rRNA gene. (B, D) Dendrograms displaying the hierarchical clustering of proximal soil samples from UNR, VC and IFSi roots 3 weeks post planting. Numbers listed after the sample labels specify their experiment of origin. V13 and V35 indicate if the plots were obtained using sequences of PCR amplicons from V1-V3 or V3-V5 variable regions of the 16S rRNA gene.

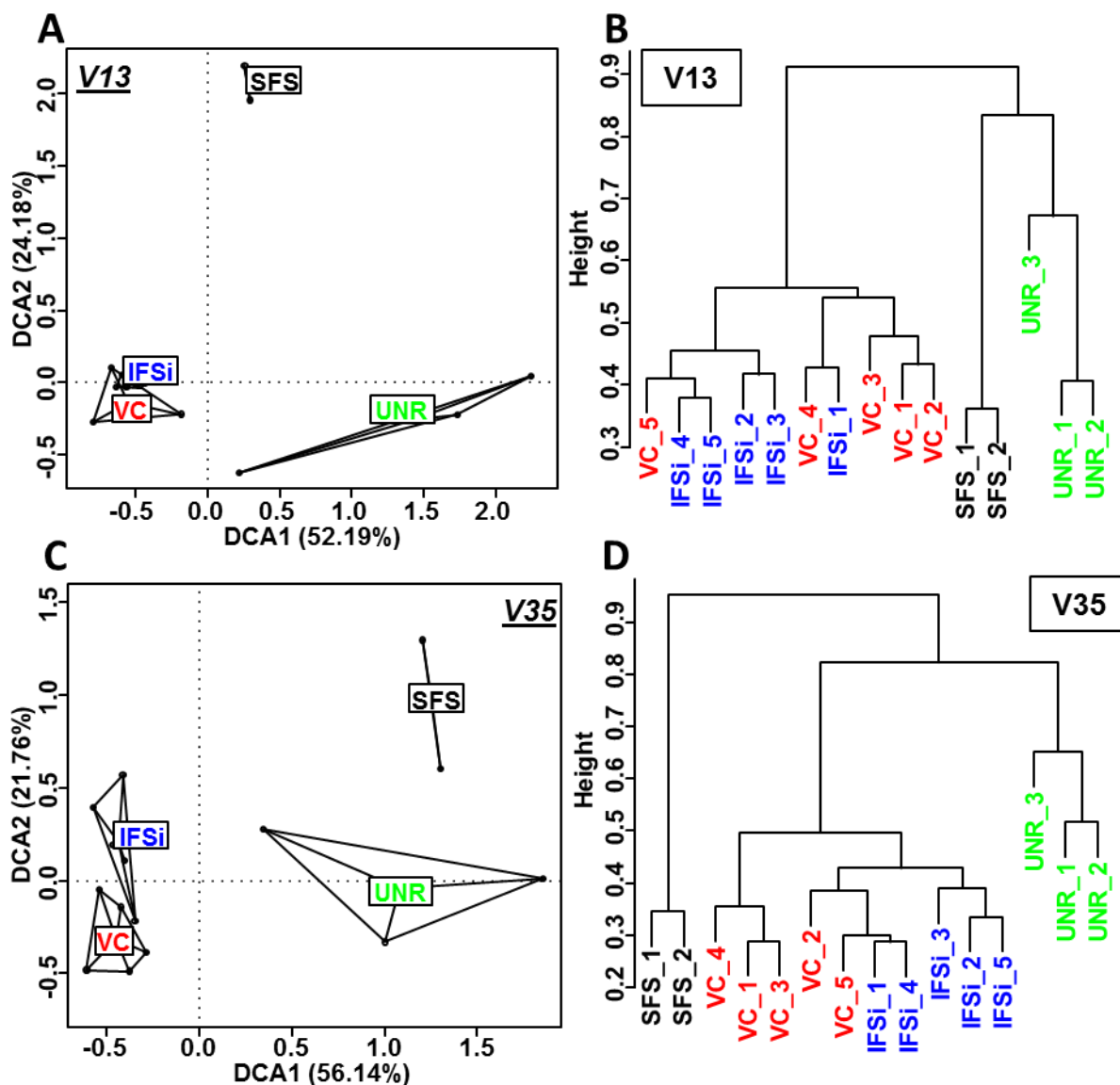


Figure 3.5. DCA and hierarchical clustering analyses indicating the extent of dissimilarities among SFS, UNR, VC and IFSi soil samples.

(A, C) DCA plots displaying the separation of soybean field soil (SFS) samples and PS samples from untransformed (UNR), control (VC), and IFS-RNAi (IFSi) roots 3 weeks post planting. DCA1 and DCA2 represent the major axes of dissimilarity. Data points of the same sample type from different experiments are depicted connected by a line. V13 and V35 indicate if the V1-V3 or V3-V5 variable regions of the 16S rRNA were amplified. (B, D) Dendrograms displaying the hierarchical clustering of SFS samples and proximal soil samples from SFS and UNR, VC, and IFSi roots 3 weeks post planting. Numbers listed after the sample labels specify their experiment of origin. V13 and V35 indicate if the V1-V3 or V3-V5 variable regions of the 16S rRNA were amplified.

Our first objective was to ascertain differences in bacterial community structure between the bulk soil (SFS) and soil proximal to untransformed soybean roots (UNR). Both DCA and hierarchical clustering analyses indicated there were large differences in bacterial community structure between the SFS and UNR samples (Fig. 3.5; Compare SFS vs. UNR). The first two axes for the DCA plots accounted for approximately 75%–78% of the variance. The differences between the SFS and UNR samples were noted as statistically significant based on *adonis*, a nonparametric multivariate analysis of variance tool ($P < 0.01$; Bray–Curtis distance matrices).

These observations were further verified via capscale and constrained ordination analysis (Supporting Information Figs. 3.6 A-B and 3.7 A-B). Results from analysis of V1–V3 and V3–V5 amplicons were in agreement with each other further strengthening our conclusions.

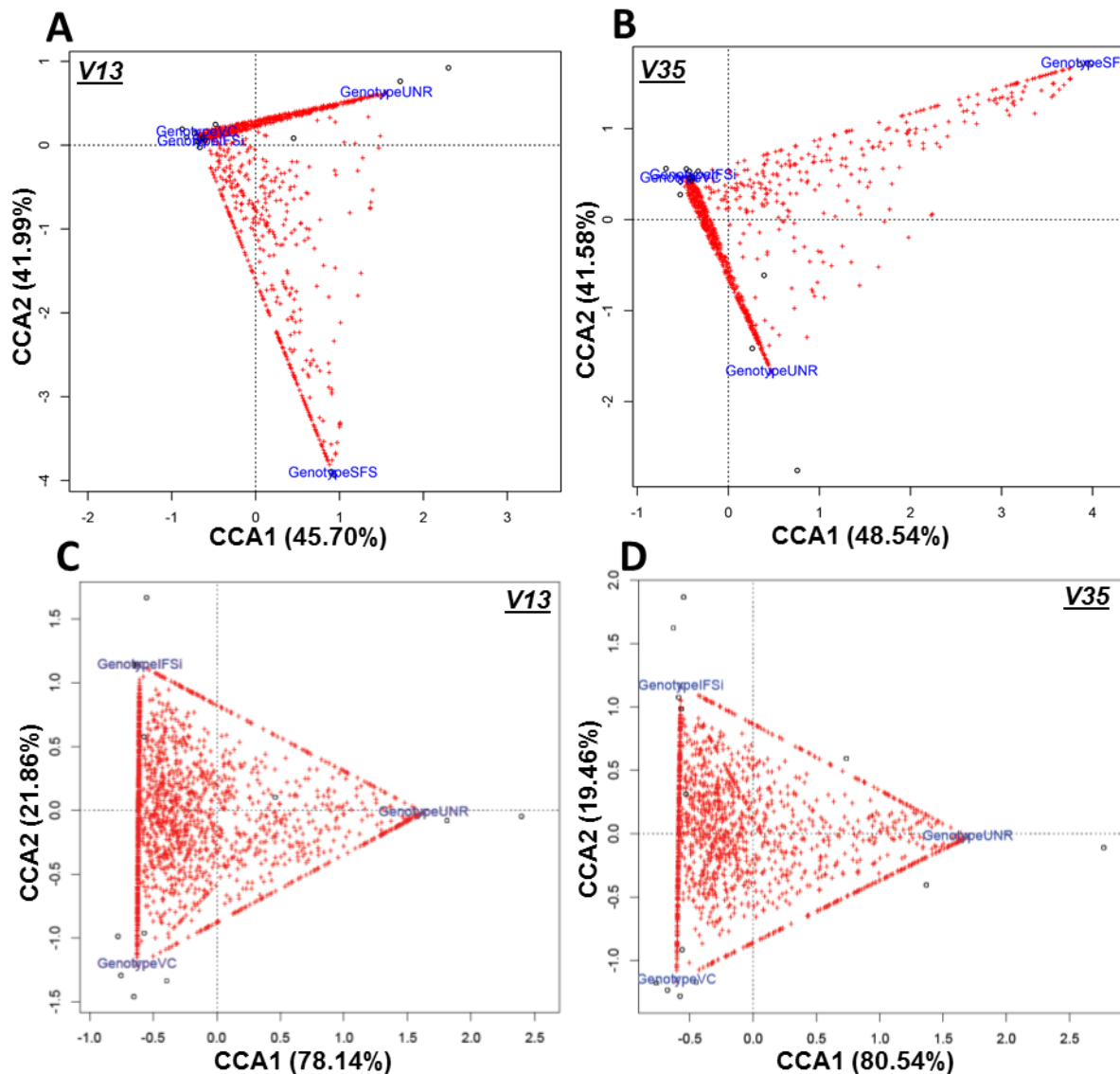


Figure 3.6. CCA of OTU profiles for SFS samples and UNR, VC and IFSi root soil samples 3 wpp.

(A, B) Constrained correspondence analysis of OTU profiles for samples from soybean field soil (SFS) and the proximal soil of untransformed (UNR), vector control (VC), and IFS-RNAi (IFSi) roots 3 weeks after planting. (C, D) Constrained correspondence analysis of OTU profiles for samples from the proximal soil of untransformed (UNR), vector control (VC), and IFS-RNAi (IFSi) roots 3 weeks after planting. In agreement with the results shown in Figure 3.4, SFS and UNR samples showed definitive separation compared to VC and IFSi samples. Although VC and IFSi samples exhibited overlapping (see A, B), they still showed a separation from one another that was better seen when SFS samples were excluded from the graph (see C, D). V13 and V35 indicate if the V1-V3 or V3-V5 variable regions of the 16S rRNA were amplified.

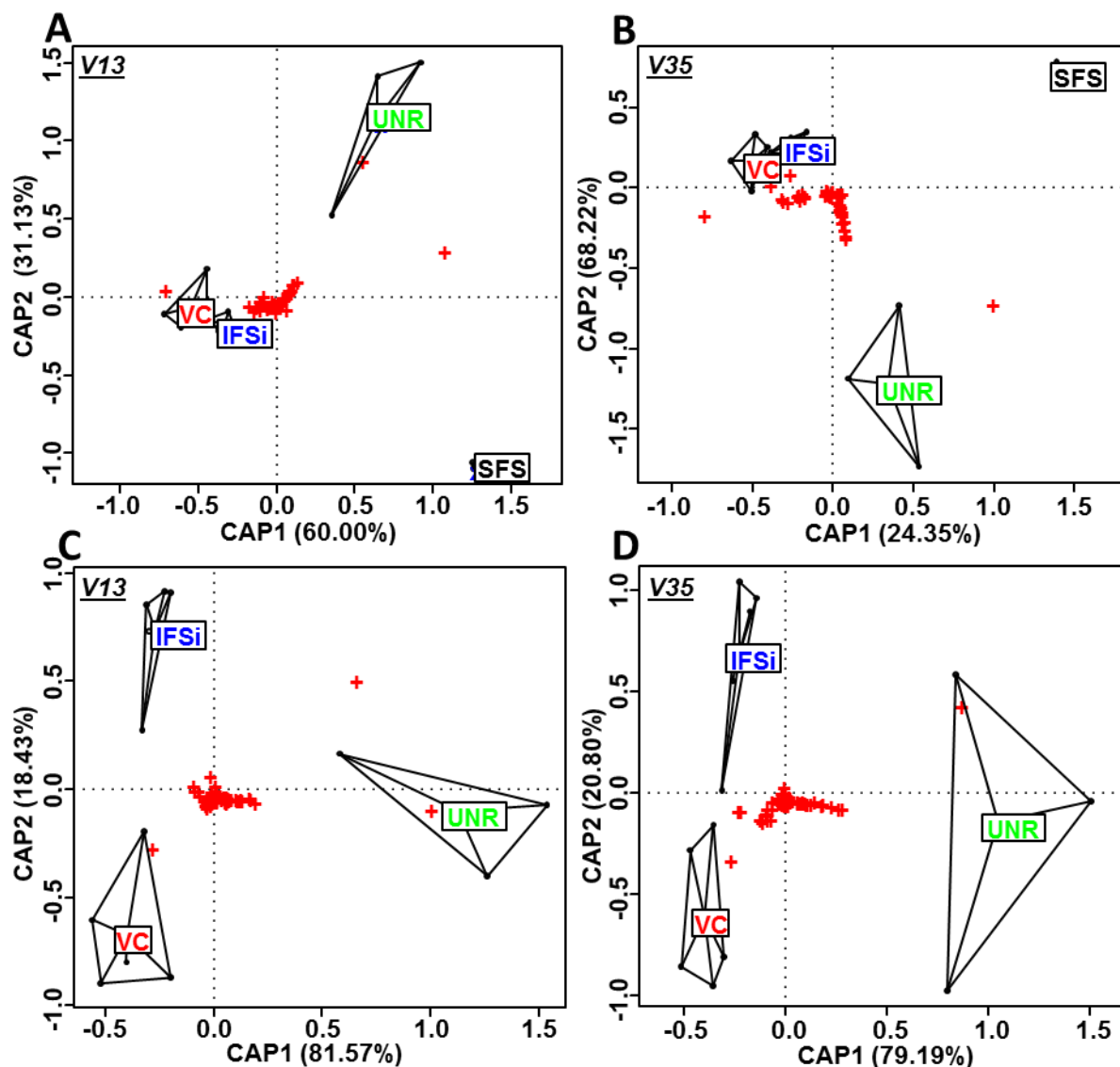


Figure 3.7. Capscale of OTU profiles for SFS samples and UNR, VC and IFSi root soil samples 3 wpp.

(A, B) Capscale of OTU profiles for samples from soybean field soil (SFS) and the proximal soil of untransformed (UNR), vector control (VC), and IFS-RNAi (IFSi) roots 3 weeks after planting. (C, D) Capscale of OTU profiles for samples from the proximal soil of untransformed (UNR), vector control (VC), and IFS-RNAi (IFSi) roots 3 weeks after planting. In agreement with the results shown in Figure 3.4, SFS and UNR samples showed definitive separation compared to VC and IFSi samples. Also, VC and IFSi samples displayed separation from one another with limited overlapping. Significance values were $P < 0.01$ for the SFS, UNR, VC and IFSi samples. V13 and V35 indicate if the V1-V3 or V3-V5 variable regions of the 16S rRNA were amplified.

Our second objective was to determine the impact of the hairy root

transformation procedure on the bacterial community structure by comparing the

UNR and VC samples. As we had previously reported using DGGE (White et al., 2015), the samples acquired from the VC roots differed largely from those from UNR roots (Fig. 3.4; Compare UNR vs. VC). The first two axes of the DCA plots accounted for approximately 77%–83% of the variance. Hierarchical clustering showed completely separate branches for the UNR samples compared with VC and IFSi samples.

The impact of the hairy root transformation procedure was also verified as statistically significant (*adonis* $P < 0.01$; Bray–Curtis distance matrices) and supported by additional constrained ordination analyses (Figs. 3.6 C-D and 3.7 C-D).

Our third and most important objective was to discover the influence of isoflavonoids on the bacterial community structure by comparing the VC and IFSi samples. Although the samples gathered from the isoflavonoid-deficient IFSi roots did not exhibit drastic differences compared with the VC roots, we still detected changes in the bacterial community (Fig. 3.4; Compare VC vs. IFSi). For example, while there was some conservative overlap between VC and IFSi samples in both the DCA plots and hierarchical clustering, they were clearly distinguishable from each other. The separation was more prominent in the V3–V5 library compared with the V1–V3 library. These differences were also supported by other constrained ordination analyses (Figs. 3.6 C-D and 3.7 C-D). However, statistical analysis deemed the differences to be not significant (*adonis* $P < 0.13$ and $P < 0.21$ for V1–V3, $P < 0.11$ and $P < 0.08$ for V3–V5; Bray–Curtis

distance matrices). This suggested that only a small proportion of OTUs were influenced by isoflavonoids in proximal soils.

In summary, our results indicate that (i) the bacterial community structures are significantly influenced by soybean roots in proximal soils, (ii) transformed hairy roots had a clear effect on the bacterial community structure compared with untransformed roots and (iii) soybean root isoflavonoids did not have a significant effect on the bacterial community structure of proximal soils.

3.2. Bacterial taxa in the soybean rhizosphere

After detecting variations in bacterial communities amongst the various sample types, we sought to find changes at specific taxonomic levels within said communities. As before, we evaluated differences between bulk soil and soil proximal to soybean roots, and differences due to hairy root transformation, or isoflavonoids. Our first objective was to determine which bacterial taxa within our samples were enriched or reduced by untransformed soybean roots in proximal soils compared with the soybean field soil samples. Given that hairy root transformation itself influenced the bacterial community structure, we anticipated this comparison would help identify which bacterial taxa colonize soybean in the ‘natural’ environment. Our analysis pipeline included a step to compare each OTU to known sequences (SILVA database version 102) and obtain potential taxonomies. In SFS samples, Proteobacteria (30%), Actinobacteria (28%–34%) and Acidobacteria (10%–13%) were the three most abundant phyla. In contrast, the most abundant phyla in untransformed root soil samples were Proteobacteria (79%) and Bacteroidetes (8%–11%). This indicated that unaltered soybean roots

promoted members of Proteobacteria and Bacteroidetes and reduced Actinobacteria and Acidobacteria (Fig. 3.8; Compare SFS vs. UNR). Proximal soils of VC and IFSi roots also had similar profiles but, compared with UNR samples, the abundance of Proteobacteria was lower (56%–60%) whereas that of Bacteroidetes was higher (16%–22%). This indicated that the hairy root transformation influenced rhizosphere bacterial communities even at the phylum level (Fig. 3.8; Compare SFS vs. VC and IFSi).

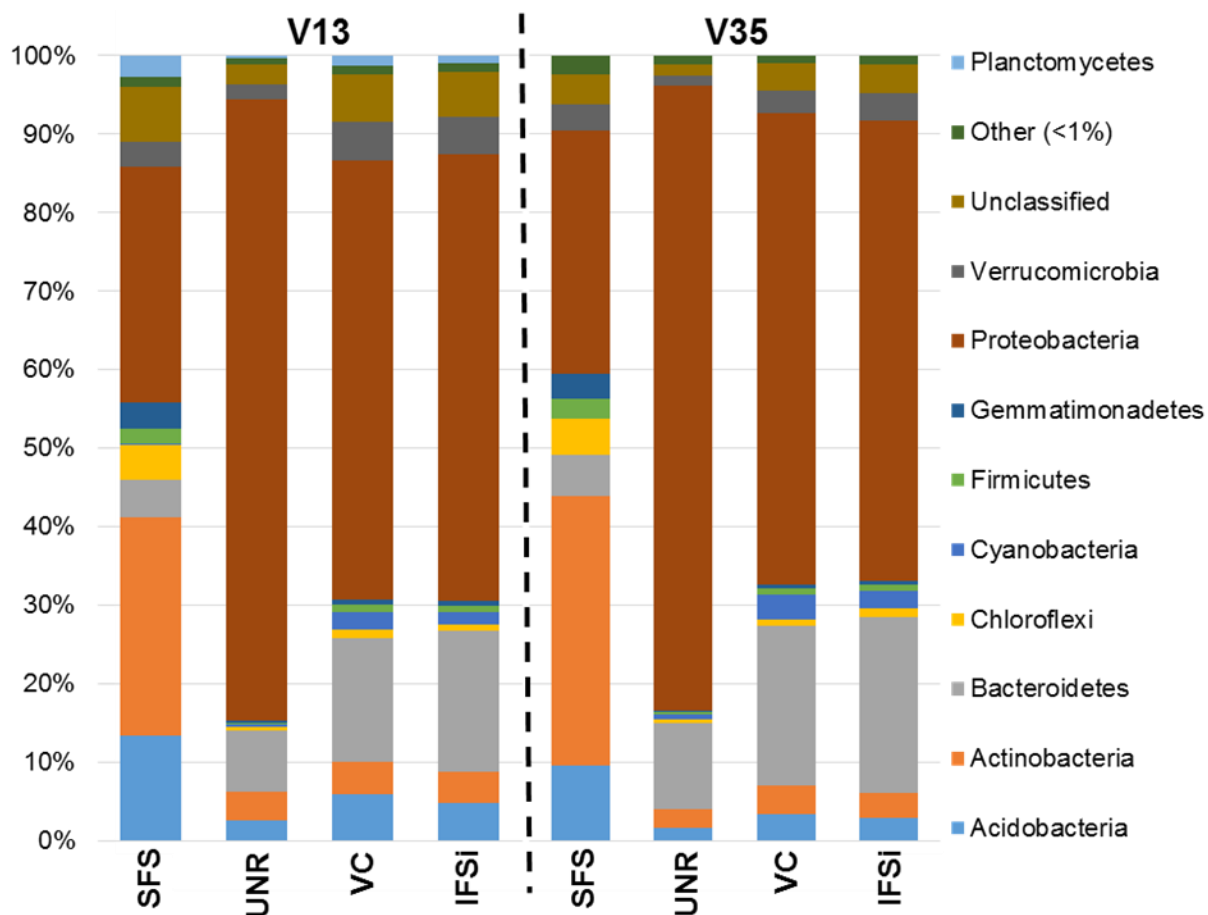


Figure 3.8. Stacked bar graphs comparing bacteria phyla proportions from SFS, UNR, VC and IFSi root soil samples.

Stacked bar graphs comparing proportions of bacteria phyla from soybean field soil (SFS) samples to untransformed (UNR), vector control (VC) and IFS-RNAi (IFSi) root samples. V13 and V35 indicate if the graphs were obtained using sequences of PCR amplicons from V1-V3 or V3-V5 variable regions of the 16S rRNA gene. ‘Other (<1%)’ includes the phyla whose proportions account for < 1% of the bacterial community in each of the 4 sample types. The ‘Other < 1%’ includes Candidate division OD1, Candidate division TG-1 (only for V35), Candidate division TM6, Candidate division TM7, Candidate division WS3, Chlorobi, Chlamydiae (only for V35), Fibrobacteres (only for V13), Nitrospirae, Planctomycetes (only for V35) and WCHB1-60.

In agreement with results from DCA and hierarchical cluster analyses,

there was little if any difference between VC and IFSi roots at the phylum level.

Both V1–V3 and V3–V5 libraries yielded near identical results indicating that our analysis pipeline provided reliable taxonomic classifications at this level.

The differences between samples were more prominent at the family level. A total of 194 families were detected in the V1–V3 library and 206 families were detected in the V3–V5 library. Eighty-five of the V1–V3 library families and ninety-one of the V3–V5 library families were listed as ‘unclassified’ or ‘uncultured’. Of the remaining named families, 101 were detected by both libraries, 8 were identified only in the V1–V3 library, and 14 were only identified in the V3–V5 library. Approximately 77% of those families detected in both libraries and possessing P -values ≤ 0.05 – calculated using two-tailed t -tests – exhibited the same enrichment or reduction trends between the V1–V3 and V3–V5 libraries (Table 3.3) in different comparisons.

Table 3.3. Average bacterial family abundancies in SFS, UNR, VC and IFSi samples. Results of Student’s t -tests to compare proportions of bacterial families between different samples. SFS vs. UNR to evaluate enrichment in the rhizosphere of untransformed roots, SFS vs. VC to evaluate enrichment in rhizosphere of hairy root composite plants and VC vs. IFSi to evaluate changes due to reduction in root isoflavonoids. Average proportions in each sample type and t -test p -values are shown.

Bacterial Family	Variable Regions V1-V3						
	Average Proportion				P-Value		
	SFS	UNR	VC	IFSI	SFS vs UNR	SFS vs VC	VC vs IFSI
Acidimicrobiales	0.0294	0.0040	0.0041	0.0067	0.0076	0.0507	0.0454
Acidobacteriaceae	0.1272	0.0257	0.0570	0.0472	0.0189	0.0830	0.1646
Actinomycetales	0.1140	0.0262	0.0297	0.0273	0.0072	0.0051	0.6642
AKIW543	0.0646	0.0018	0.0044	0.0035	0.0698	0.0750	0.4185
Bdellovibrionaceae	0.0009	0.0019	0.0084	0.0109	0.5862	0.0004	0.0900
Beijerinckiaceae	0.0011	0.0010	0.0007	0.0011	0.8760	0.4199	0.4197
Bradyrhizobiaceae	0.0071	0.0128	0.0114	0.0130	0.1230	0.0250	0.4149
Burkholderiaceae	0.0012	0.0151	0.0002	0.0004	0.1350	0.0024	0.5355
Caulobacteraceae	0.0080	0.0167	0.0247	0.0237	0.3011	0.0068	0.8301
Chitinophagaceae	0.0332	0.0224	0.0612	0.0687	0.5722	0.0001	0.0409
Comamonadaceae	0.0090	0.0671	0.0685	0.0916	0.0487	0.0008	0.0257
Cryomorphaeae	0.0000	0.0007	0.0038	0.0066	0.1891	0.0042	0.1041
Cytophagaceae	0.0030	0.0044	0.0131	0.0194	0.7386	0.0078	0.0675
Flavobacteriaceae	0.0024	0.0346	0.0565	0.0618	0.0076	0.0033	0.6889
Gemmatimonadaceae	0.0327	0.0023	0.0057	0.0062	0.0036	0.0000	0.7614
Hyphomicrobiaceae	0.0037	0.0085	0.0217	0.0207	0.4213	0.0002	0.7442
Nannocystineae	0.0049	0.0047	0.0143	0.0125	0.9494	0.0071	0.4486
Nitrosomonadaceae	0.0145	0.0007	0.0030	0.0022	0.0177	0.0586	0.3182
Nitrospiraceae	0.0022	0.0000	0.0007	0.0004	0.0053	0.0008	0.2092
Opitutaceae	0.0026	0.0035	0.0113	0.0129	0.7419	0.0335	0.6504
Oxalobacteraceae	0.0088	0.0608	0.0394	0.0375	0.0012	0.0086	0.8138
Planctomycetaceae	0.0164	0.0022	0.0081	0.0060	0.0065	0.0100	0.3559
Pseudomonadaceae	0.0029	0.1627	0.0187	0.0108	0.1892	0.0288	0.1821
Rhizobiaceae	0.0001	0.0247	0.0126	0.0123	0.1155	0.0000	0.8718
Rhodospirillaceae	0.0123	0.0040	0.0047	0.0041	0.0566	0.0067	0.7364
Shinella_genera_incertae_sedis	0.0000	0.0050	0.0105	0.0108	0.1471	0.0020	0.8440
Sinobacteraceae	0.0114	0.0031	0.0088	0.0095	0.0641	0.0334	0.6571
Solirubrobacterales	0.0324	0.0012	0.0010	0.0009	0.0743	0.0808	0.9160
Sorangineae	0.0096	0.0111	0.0299	0.0282	0.7929	0.0004	0.6330
Sphingobacteriaceae	0.0021	0.0100	0.0053	0.0051	0.0016	0.0349	0.7957
Sphingomonadaceae	0.0425	0.1198	0.0858	0.0944	0.0437	0.1932	0.1812
Verrucomicrobiaceae	0.0021	0.0100	0.0226	0.0211	0.3921	0.0000	0.5024
Xanthobacteraceae	0.0194	0.0122	0.0023	0.0046	0.1179	0.0190	0.0592
Xanthomonadaceae	0.0242	0.0711	0.0631	0.0429	0.0518	0.0061	0.0489
0.1.2.1.3.1unclassified	0.0115	0.0002	0.0011	0.0005	0.0095	0.0015	0.1964
0.1.2.1.5.1unclassified	0.0207	0.0020	0.0016	0.0007	0.0281	0.0216	0.1579
0.1.15.4.1.1unclassified	0.0276	0.0008	0.0022	0.0017	0.0333	0.0292	0.4546
0.1.16.1.1.1unclassified	0.0007	0.0022	0.0052	0.0062	0.1313	0.0038	0.5242
0.1.24.1.6.7unclassified	0.0116	0.0007	0.0020	0.0020	0.0362	0.0612	0.9893
0.1.24.1.8.6unclassified	0.0035	0.0153	0.0069	0.0081	0.0205	0.0895	0.4349
0.1.24.1.9.1unclassified	0.0045	0.0066	0.0133	0.0167	0.5554	0.0253	0.2805
0.1.24.6.1.1unclassified	0.0034	0.0053	0.0074	0.0079	0.5379	0.0080	0.7106

Bacterial Family	Variable Regions V3-V5						
	Average Proportion				P-Value		
	SFS	UNR	VC	IFSI	SFS vs UNR	SFS vs VC	VC vs IFSI
Acidimicrobiales	0.0333	0.0022	0.0044	0.0039	0.0422	0.0832	0.6767
Acidobacteriaceae	0.0887	0.0145	0.0313	0.0263	0.0568	0.1373	0.2321
Actinomycetales	0.1015	0.0182	0.0209	0.0179	0.0050	0.0367	0.3991
AKIW543	0.1265	0.0021	0.0068	0.0063	0.0002	0.0000	0.8177
Bdellovibrionaceae	0.0002	0.0024	0.0133	0.0154	0.3317	0.0050	0.5088
Beijerinckiaceae	0.0033	0.0005	0.0003	0.0011	0.2141	0.2289	0.0103
Bradyrhizobiaceae	0.0137	0.0257	0.0198	0.0191	0.1517	0.0194	0.7943
Burkholderiaceae	0.0006	0.0085	0.0000	0.0002	0.1154	0.5000	0.1778
Caulobacteraceae	0.0127	0.0226	0.0347	0.0363	0.2763	0.0105	0.8125
Chitinophagaceae	0.0278	0.0294	0.0660	0.0780	0.9542	0.0017	0.1273
Comamonadaceae	0.0075	0.0450	0.0632	0.0853	0.0771	0.0005	0.0238
Cryomorpaceae	0.0000	0.0019	0.0143	0.0143	0.4226	0.0010	0.9999
Cytophagaceae	0.0089	0.0134	0.0319	0.0391	0.6477	0.0036	0.3446
Flavobacteriaceae	0.0034	0.0367	0.0607	0.0642	0.0355	0.0019	0.8261
Gemmatimonadaceae	0.0291	0.0015	0.0050	0.0042	0.0008	0.0057	0.4881
Hyphomicrobiaceae	0.0118	0.0194	0.0513	0.0562	0.4826	0.0038	0.4125
Nannocystineae	0.0095	0.0036	0.0089	0.0131	0.1057	0.8035	0.1087
Nitrosomonadaceae	0.0063	0.0005	0.0004	0.0015	0.2010	0.2100	0.0040
Nitrospiraceae	0.0027	0.0002	0.0008	0.0001	0.0014	0.0011	0.0292
Opitutaceae	0.0027	0.0027	0.0065	0.0067	0.9803	0.2516	0.9065
Oxalobacteraceae	0.0010	0.0237	0.0087	0.0068	0.0697	0.0251	0.4854
Planctomycetaceae	0.0032	0.0032	0.0008	0.0011	0.9925	0.4918	0.6246
Pseudomonadaceae	0.0039	0.2030	0.0384	0.0302	0.1924	0.0040	0.2916
Rhizobiaceae	0.0038	0.0812	0.0432	0.0364	0.1230	0.0007	0.3161
Rhodospirillaceae	0.0090	0.0034	0.0039	0.0039	0.0554	0.0515	0.9935
Shinella_genera_incertae_sedis	0.0000	0.0067	0.0202	0.0160	0.0663	0.0005	0.1091
Sinobacteraceae	0.0131	0.0030	0.0077	0.0090	0.1445	0.3700	0.3156
Solirubrobacterales	0.0185	0.0000	0.0010	0.0004	0.0186	0.0011	0.2372
Sorangiiineae	0.0070	0.0075	0.0216	0.0272	0.9450	0.0005	0.2489
Sphingobacteriaceae	0.0056	0.0239	0.0179	0.0167	0.0073	0.0005	0.6679
Sphingomonadaceae	0.0611	0.1231	0.1108	0.0997	0.0874	0.1073	0.2347
Verrucomicrobiaceae	0.0002	0.0042	0.0077	0.0093	0.4426	0.0002	0.3479
Xanthobacteraceae	0.0279	0.0133	0.0060	0.0078	0.0040	0.0002	0.5260
Xanthomonadaceae	0.0103	0.0486	0.0373	0.0230	0.0177	0.0013	0.0127
0.1.4.3.1.12unclassified	0.0049	0.0035	0.0096	0.0082	0.6902	0.0417	0.4949
0.1.14.4.1.1unclassified	0.0161	0.0003	0.0005	0.0010	0.0130	0.0174	0.2320
0.1.14.10.1.1unclassified	0.0096	0.0020	0.0029	0.0027	0.0187	0.0112	0.7996
0.1.15.1.1.1unclassified	0.0004	0.0017	0.0077	0.0078	0.3589	0.0044	0.9704
0.1.21.1.6.7unclassified	0.0124	0.0011	0.0037	0.0020	0.0006	0.0004	0.0699
0.1.21.1.9.1unclassified	0.0011	0.0085	0.0101	0.0087	0.2529	0.0051	0.6157
0.1.22.1.1.1unclassified	0.0063	0.0005	0.0020	0.0043	0.0022	0.0017	0.0271

The remaining families also showed similar trends in enrichment or reduction, but the difference was not statistically significant (i.e., no family showed enrichment in one library but reduction in the other library for the same comparison). Given the comparable number of families identified by either variable region, we conclude that either variable region could be used for future rhizosphere bacterial community analyses in soybean.

To obtain an overall view of abundance differences of specific bacterial families in our dataset, we calculated deviation from the mean abundance in each sample type (Fig. 3.9).

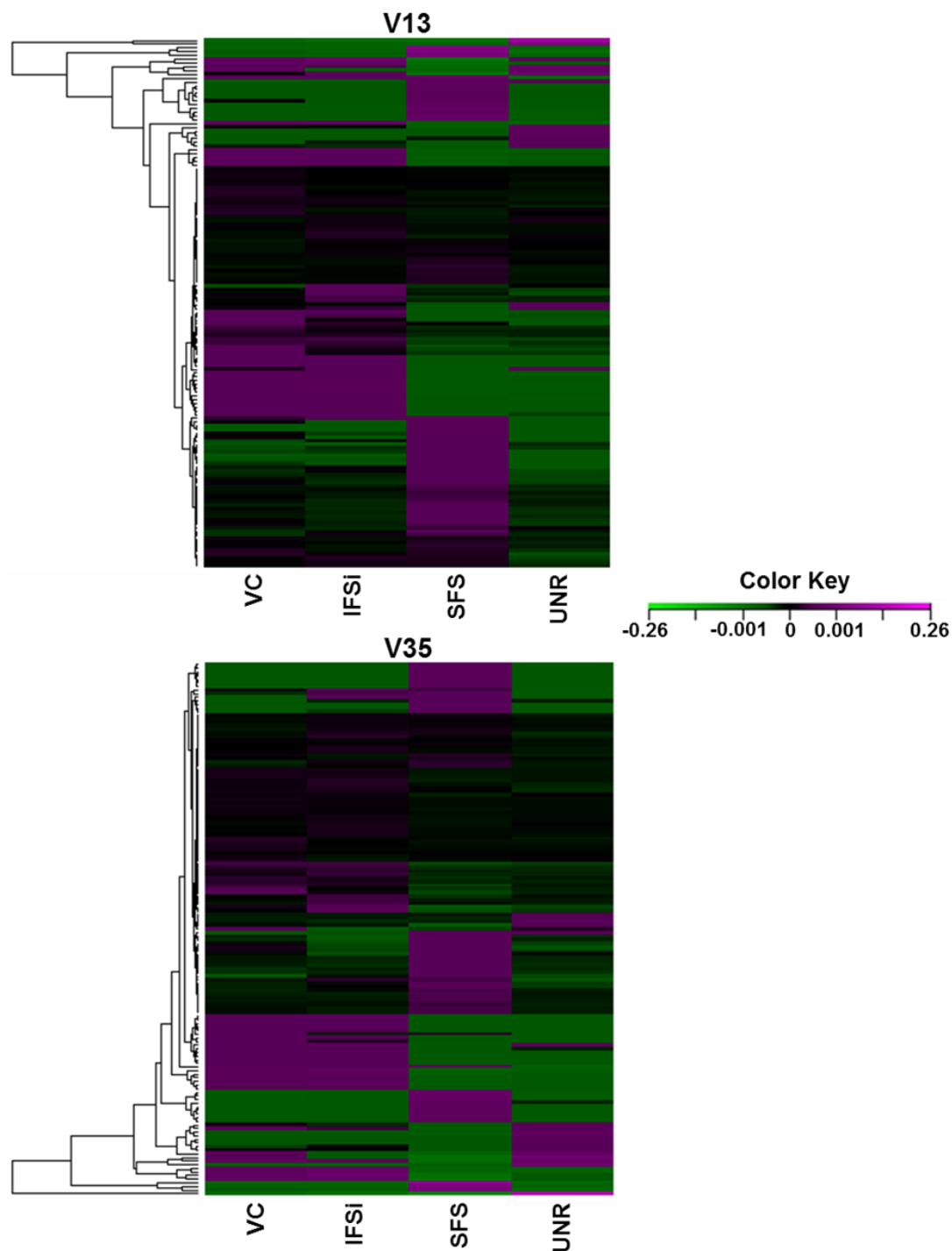


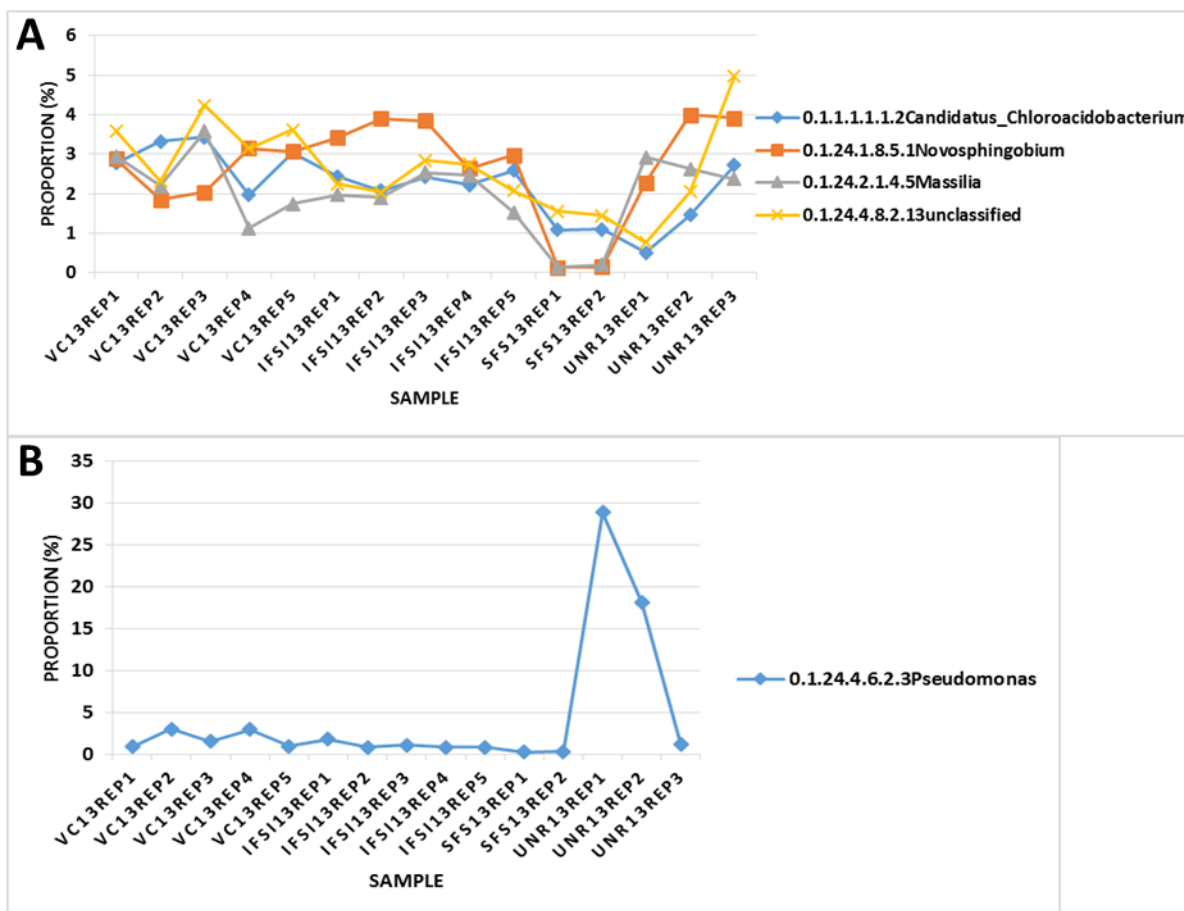
Figure 3.9. Heat maps showing bacterial family enrichment or reduction in SFS, UNR, VC and IFSi soil samples.

Heat maps displaying enrichment (purple) or reduction (green) from average abundance (black) for each bacterial family in each sample type: soybean field soil samples (SFS), untransformed (UNR), vector control (VC) and IFS-RNAi (IFSi) root soil samples. V13 and V35 indicate if the heat maps were obtained using sequences of PCR amplicons from V1-V3 or V3-V5 variable regions of the 16S rRNA gene. V13 heat map consists of 140 families and V35 heat map consists of 147 families.

About 30% of the bacterial families showed little or no difference in abundance across the samples in both libraries. Another 30% of the families had the highest abundance in SFS samples, and were at or below average levels in all three proximal soil samples. We observed different patterns among the remaining families. A good proportion of the families (20%) had lower than average abundance in SFS and UNR samples, but were higher in VC and IFSi samples suggesting that these families are enriched only in hairy roots and might not colonize untransformed roots. We also observed groups of families (8%) that were enriched only in the UNR samples, but not VC samples. These bacterial families probably only colonized untransformed roots and not hairy roots. It may not be possible to use hairy root transformation to study the association of these families with soybean roots. However, we observed a group of families (3%) enriched in both UNR and VC samples compared with SFS samples. Since these families appear to similarly colonize both untransformed and hairy roots, their association with soybean roots can be effectively studied using hairy root transformation methods. Finally, we observed a small number of families that appeared to be differentially abundant between VC and IFSi suggesting that their colonization of soybean roots might be influenced by isoflavonoids.

We also evaluated similarities among different bacterial genera in their relative abundance in the different samples using hierarchical cluster analysis. Bacterial genera with similar relative abundances were clustered together displaying interesting patterns. We identified clusters with specific discernible patterns such as genera with similar increased or reduced abundance in

rhizosphere versus bulk soil (Figs. 3.10 and 3.11) as well as genera with similar increased or reduced abundance in rhizospheres of untransformed versus hairy root composite plants (Figs. 3.12 and 3.13).



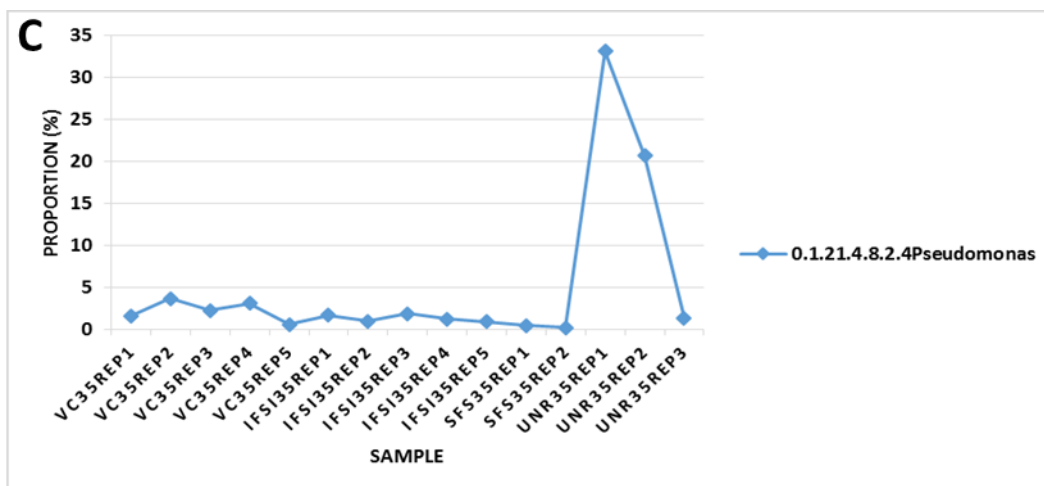
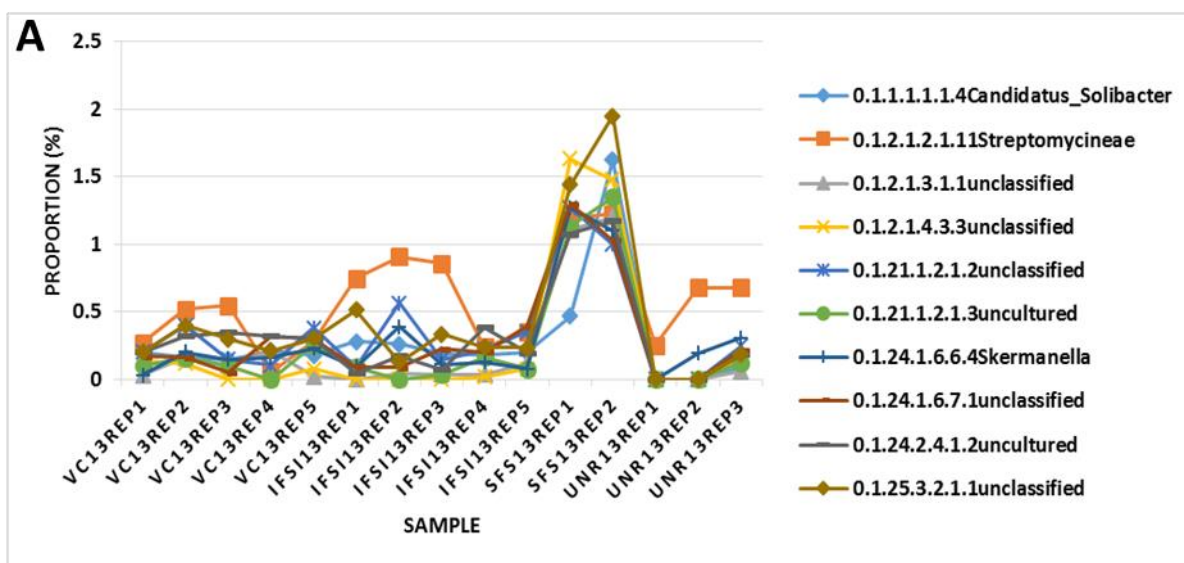
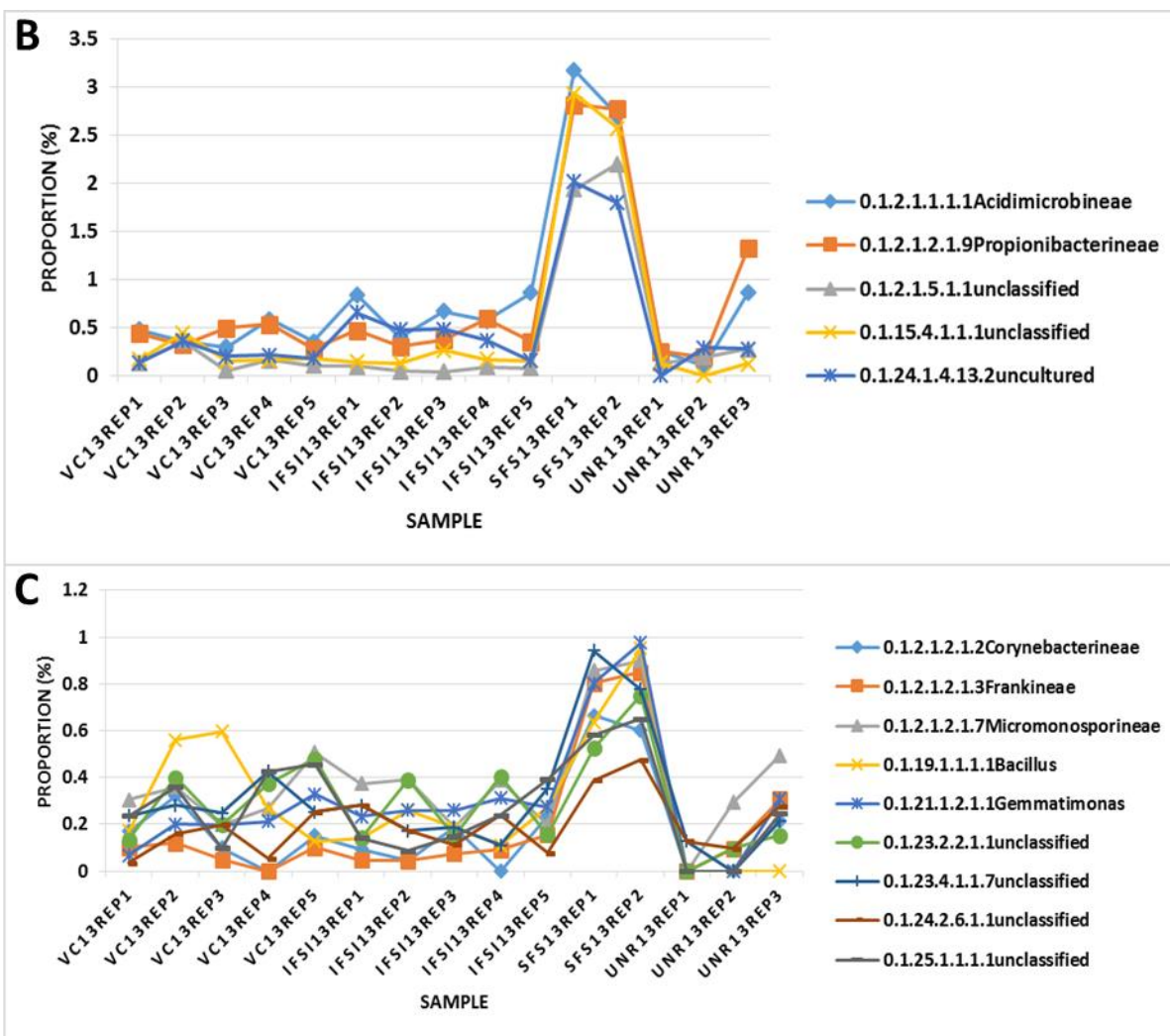
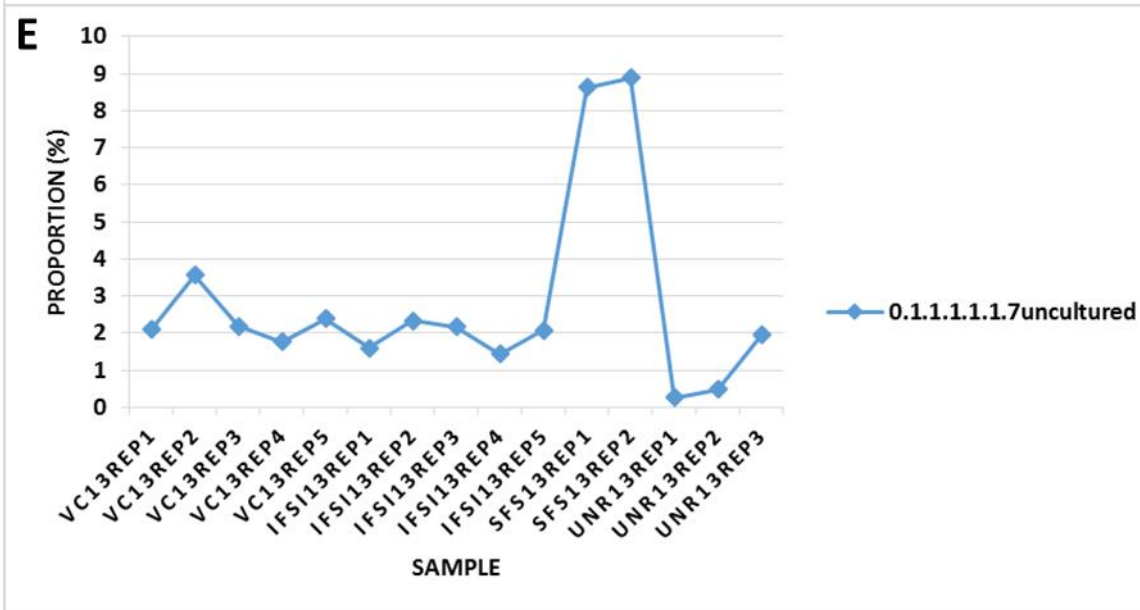
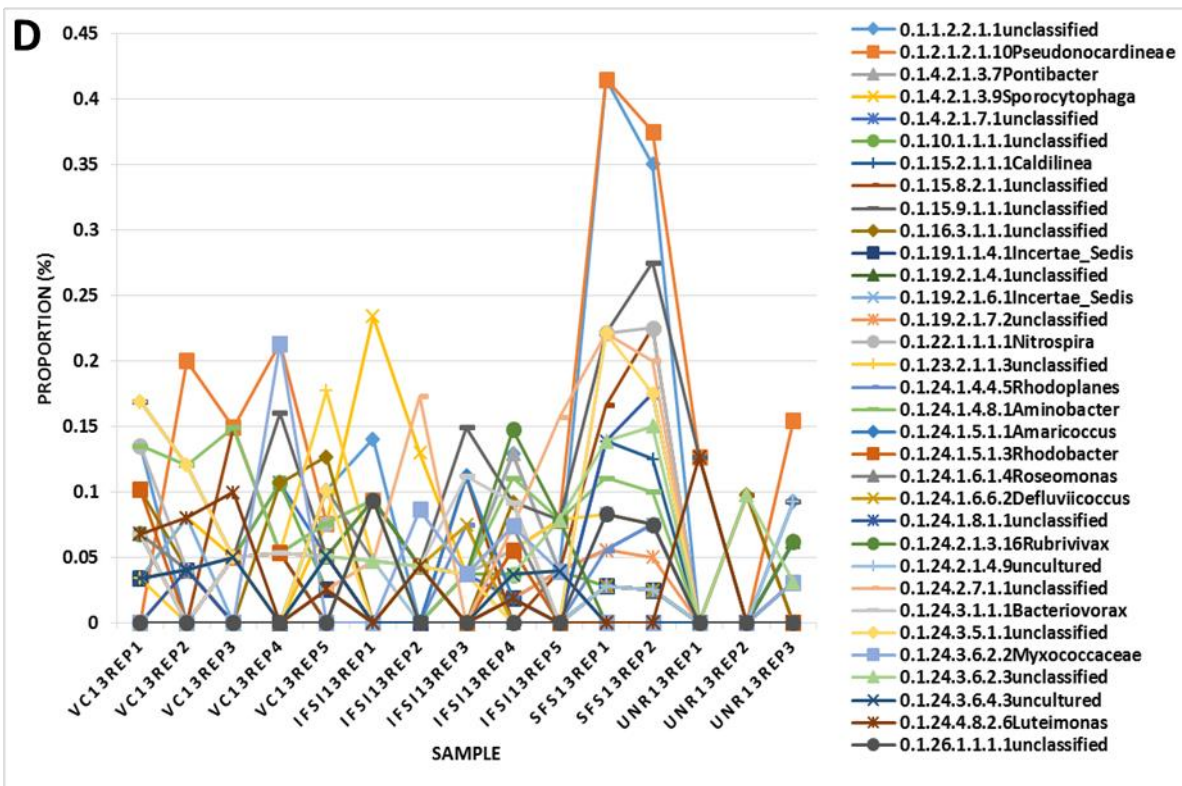


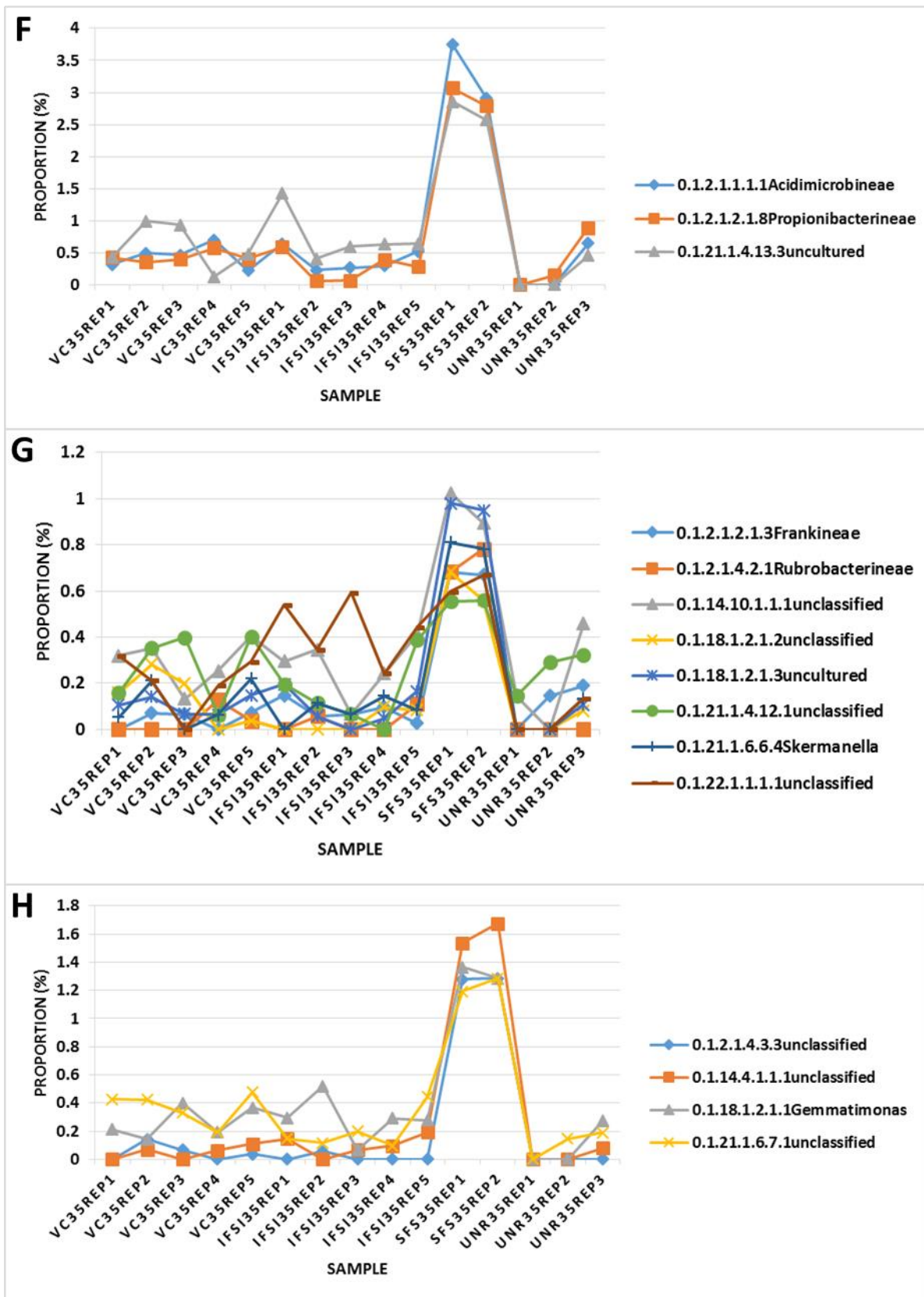
Figure 3.10. Bacterial genera clustered with a relatively increased abundance in rhizosphere soil vs. bulk soil.

Clusters **A-B** were obtained based on abundance identified using read counts of variable regions V1-V3. Cluster **C** was obtained based on abundance identified using read counts of variable regions V3-V5. Sample labels (x-axis) indicate if the sample was from soybean field soil (SFS) or untransformed soybean (UNR), vector control (VC), or IFS-RNAi (IFSi) rhizosphere soil samples. Proportion values (y-axis) were calculated by dividing the total number of sequences for each bacterial genus by the total number of sequences within each sample.









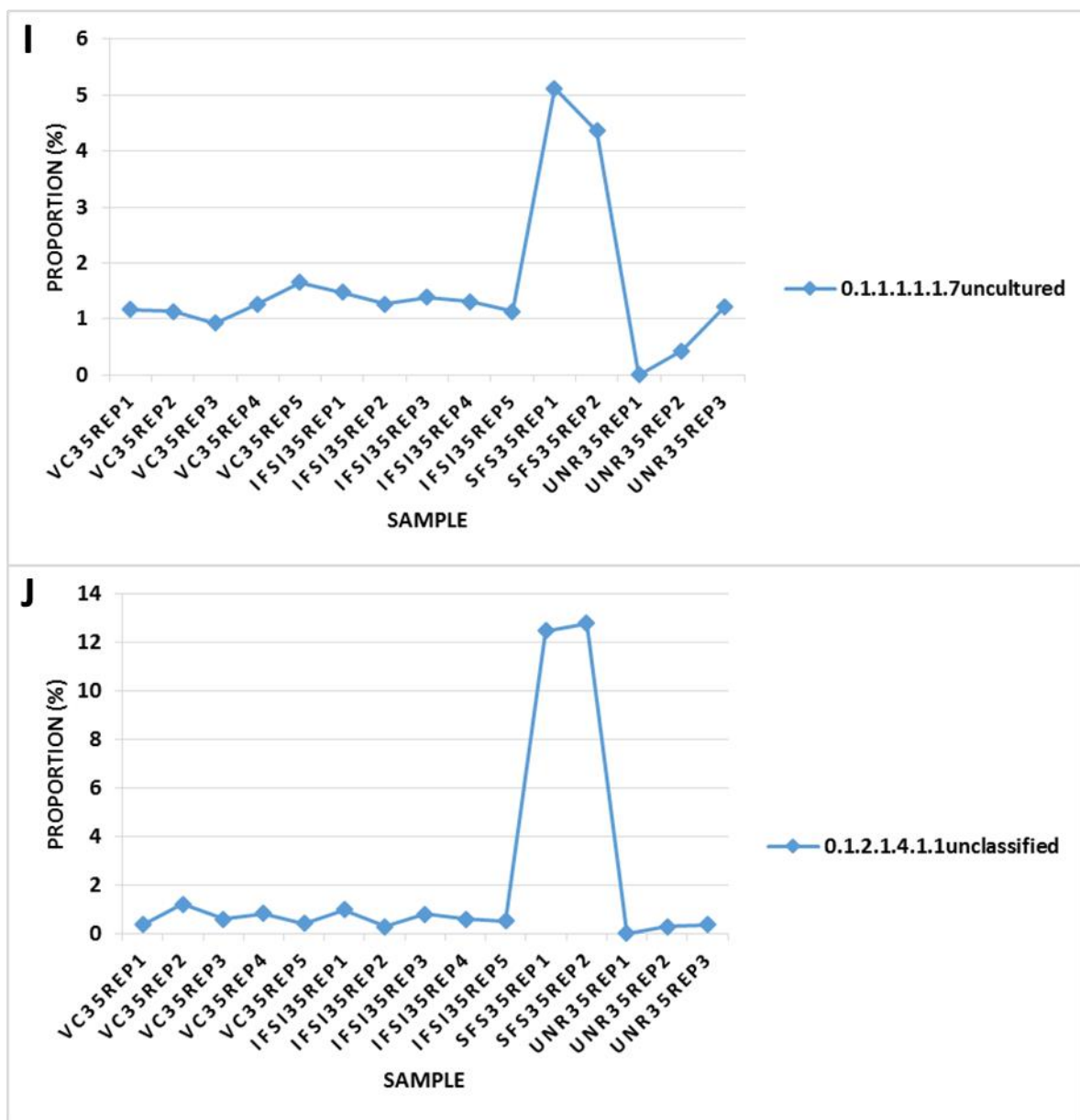
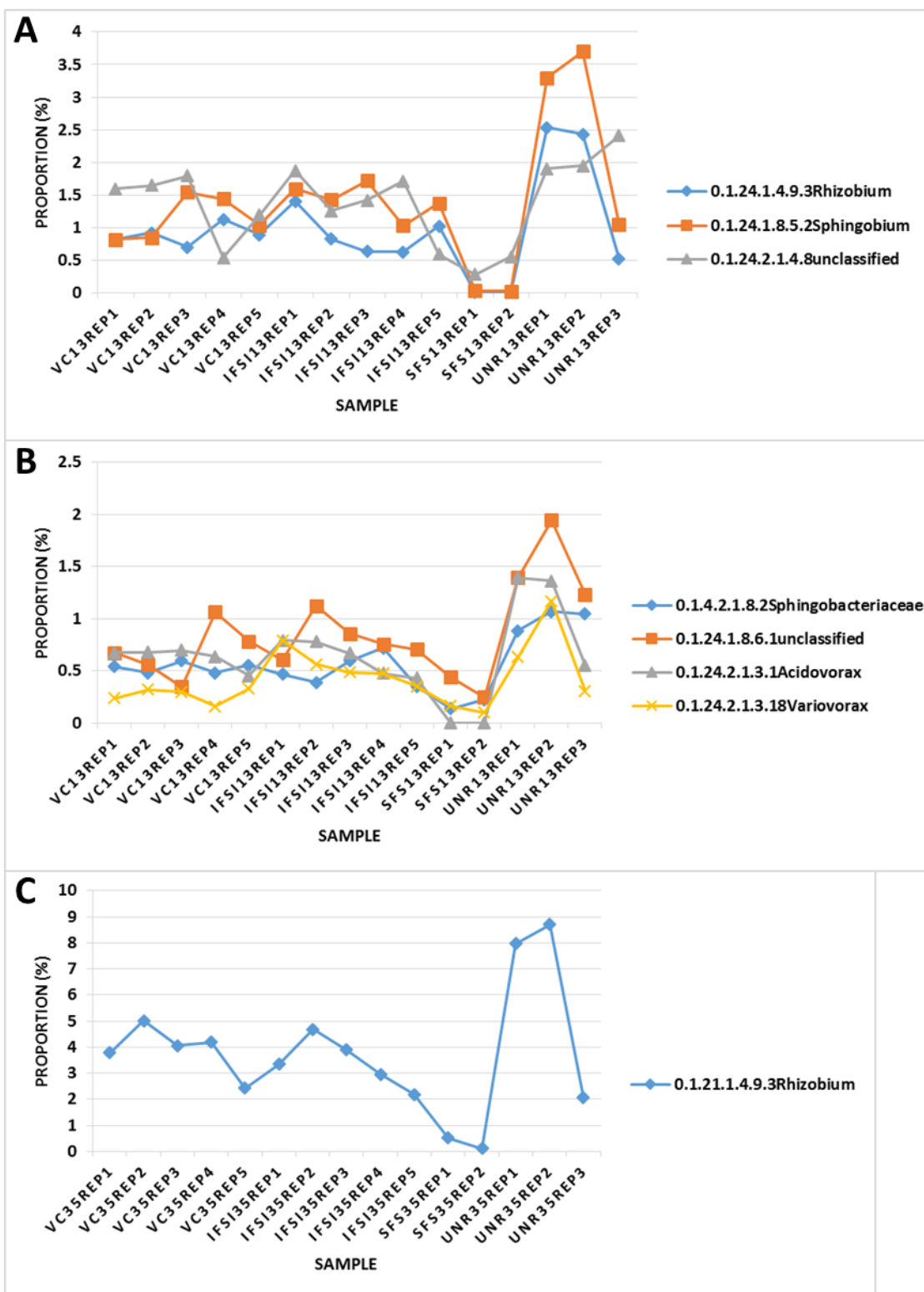


Figure 3.11. Clusters of bacterial genera with a relatively reduced abundance in rhizosphere soil vs. bulk soil.

Clusters A-E were obtained based on abundance identified using read counts of variable regions V1-V3. Clusters F-J were obtained based on abundance identified using read counts of variable regions V3-V5. Sample labels (x-axis) indicate if the sample was from soybean field soil (SFS) or untransformed soybean (UNR), vector control (VC), or IFS-RNAi (IFSi) rhizosphere soil samples. Proportion values (y-axis) were calculated by dividing the total number of sequences for each bacterial genus by the total number of sequences within each sample.



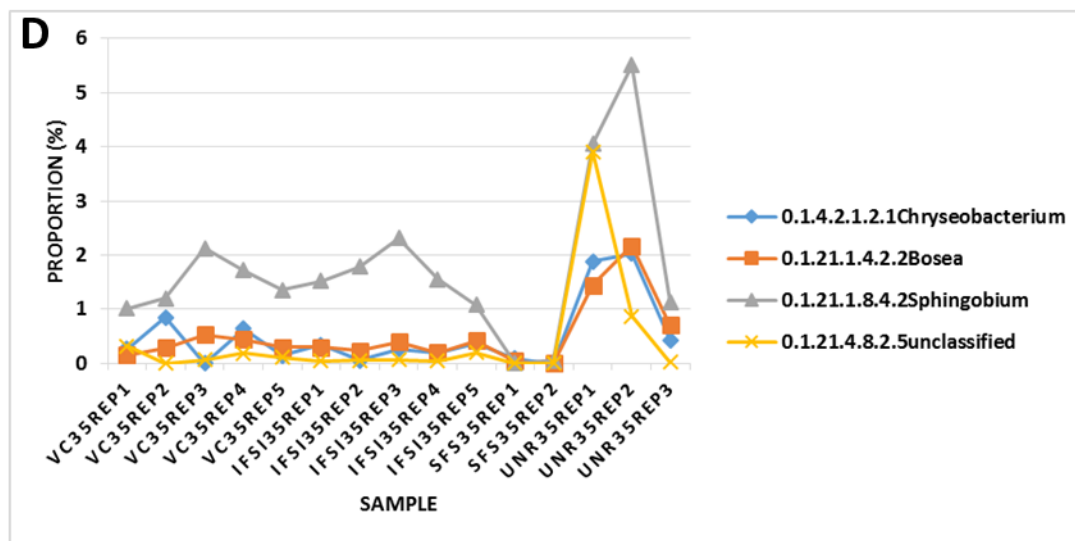
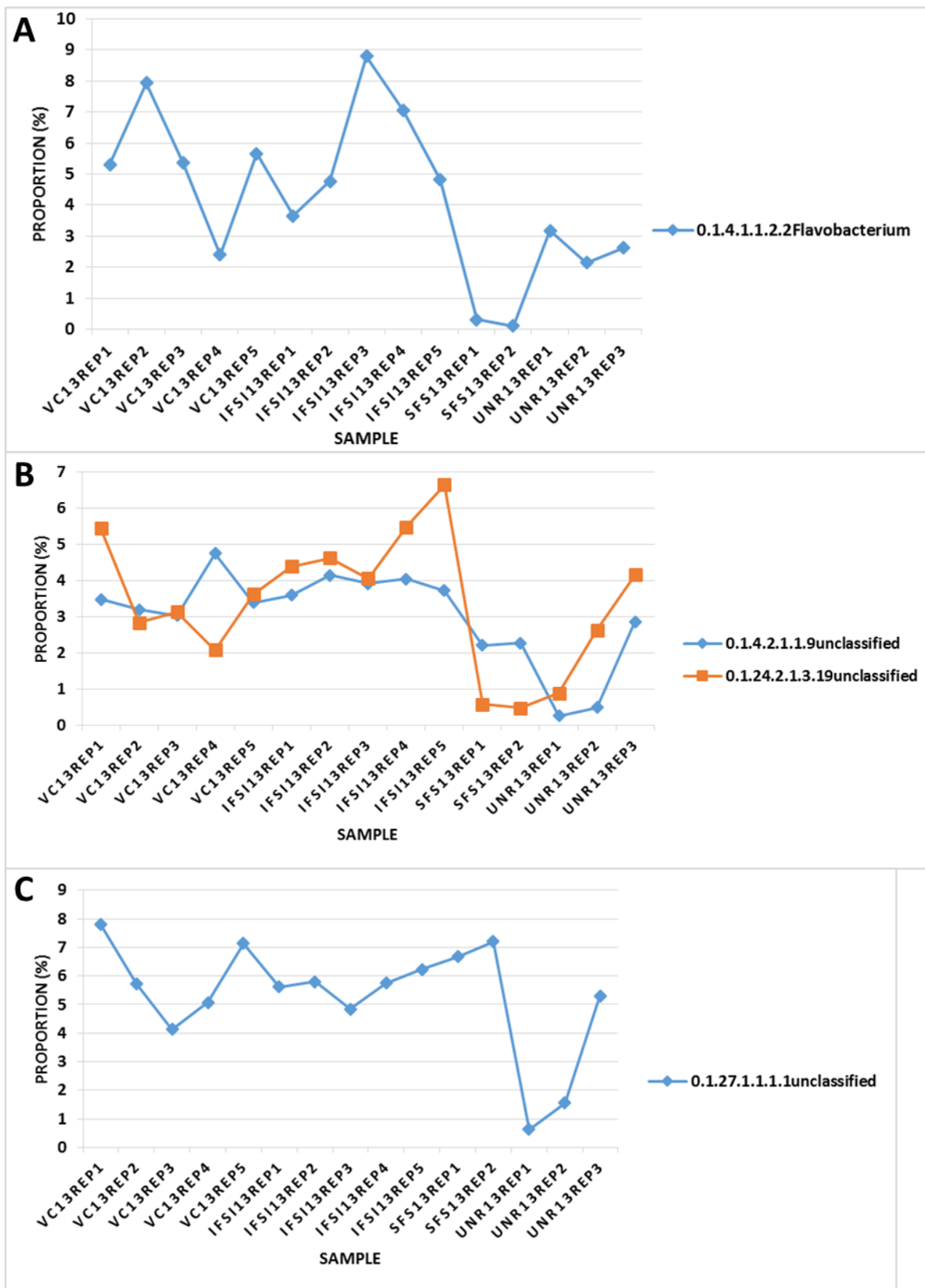
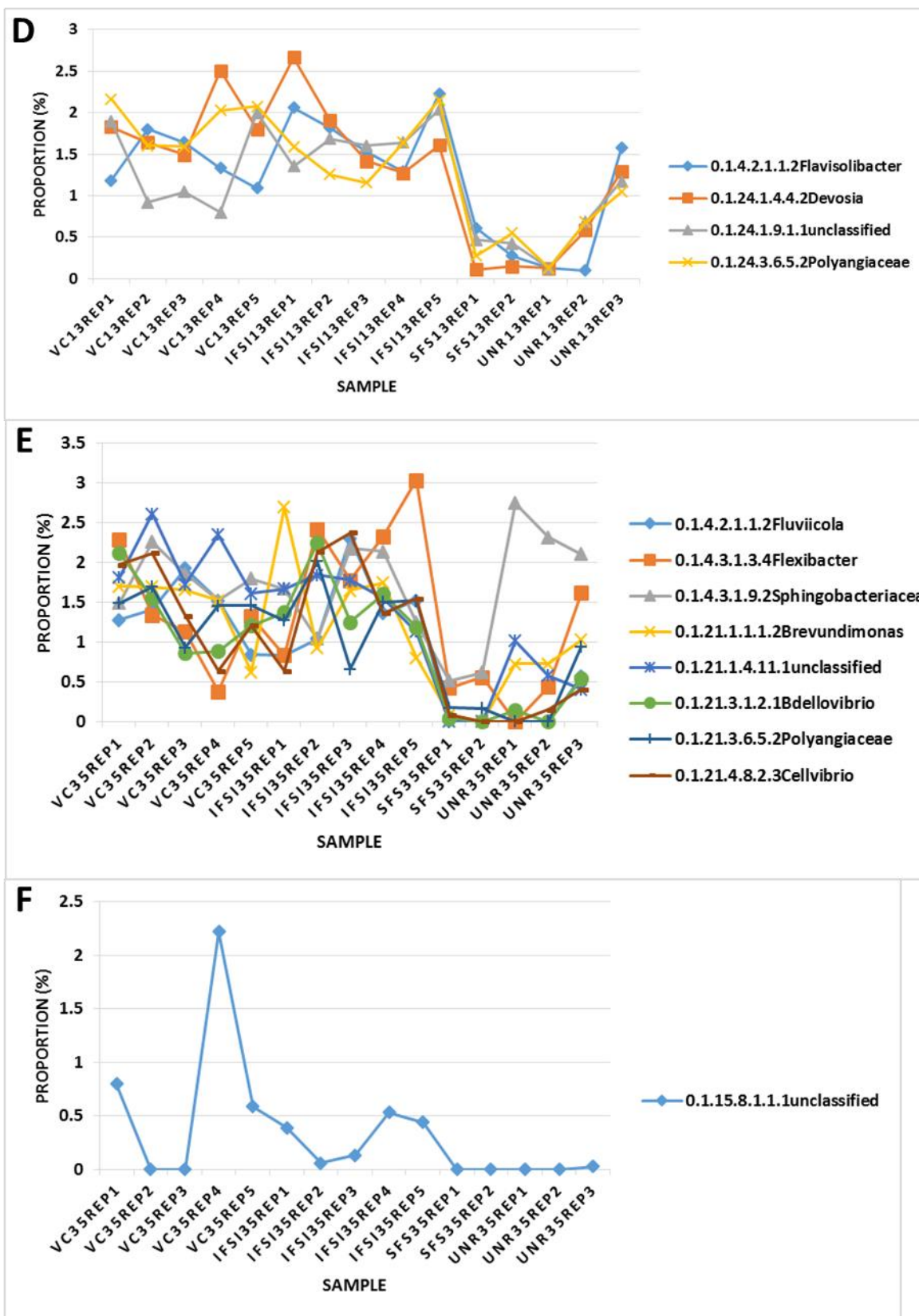


Figure 3.12. Clusters of bacterial genera with a relatively reduced abundance in rhizospheres of hairy root composite plants vs. untransformed plants.

Clusters A-B were obtained based on abundance identified using read counts of variable regions V1-V3. Clusters C-D were obtained based on abundance identified using read counts of variable regions V3-V5. Sample labels (x-axis) indicate if the sample was from soybean field soil (SFS) or untransformed soybean (UNR), vector control (VC), or IFS-RNAi (IFSi) rhizosphere soil samples. Proportion values (y-axis) were calculated by dividing the total number of sequences for each bacterial genus by the total number of sequences within each sample.





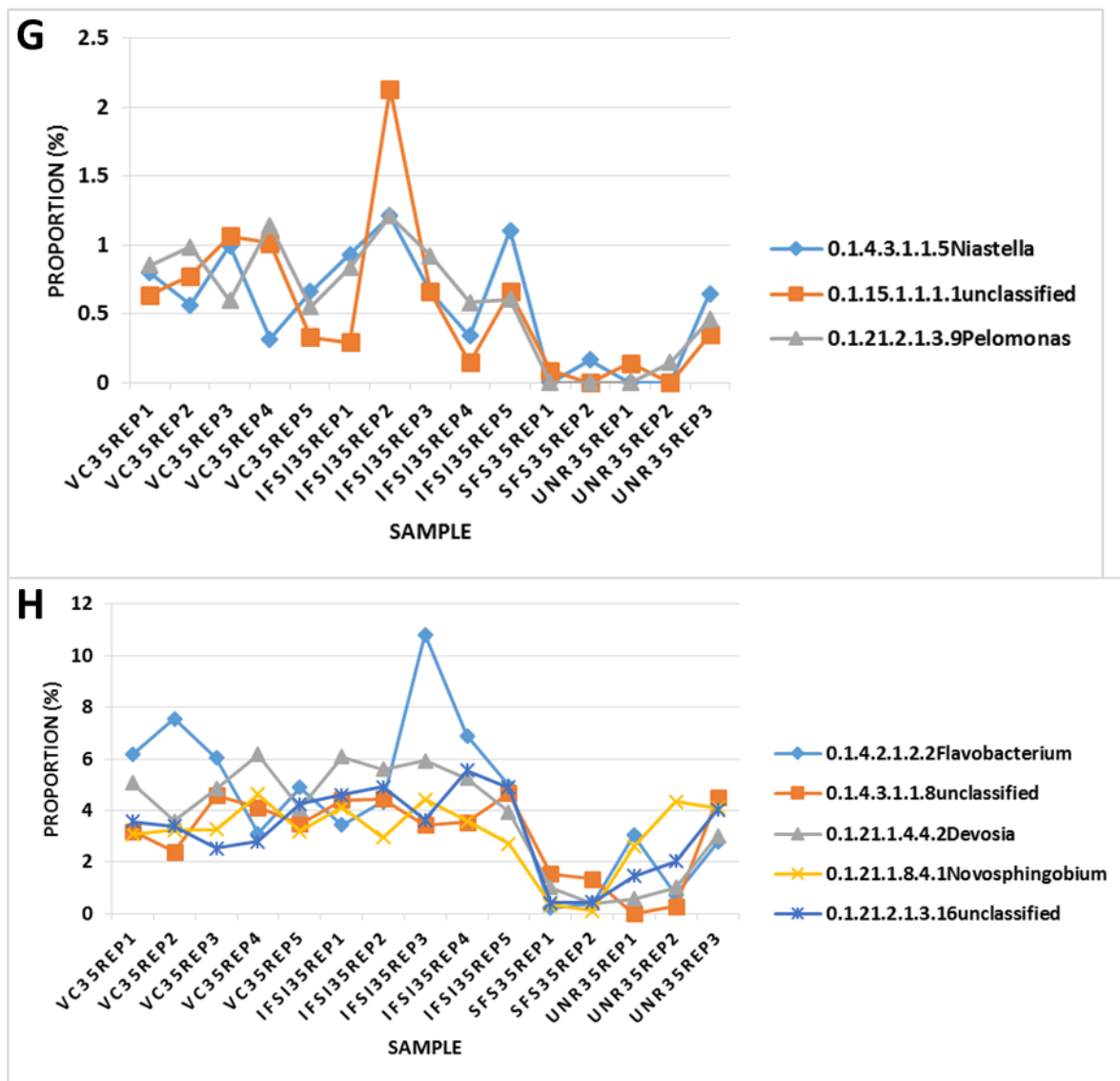
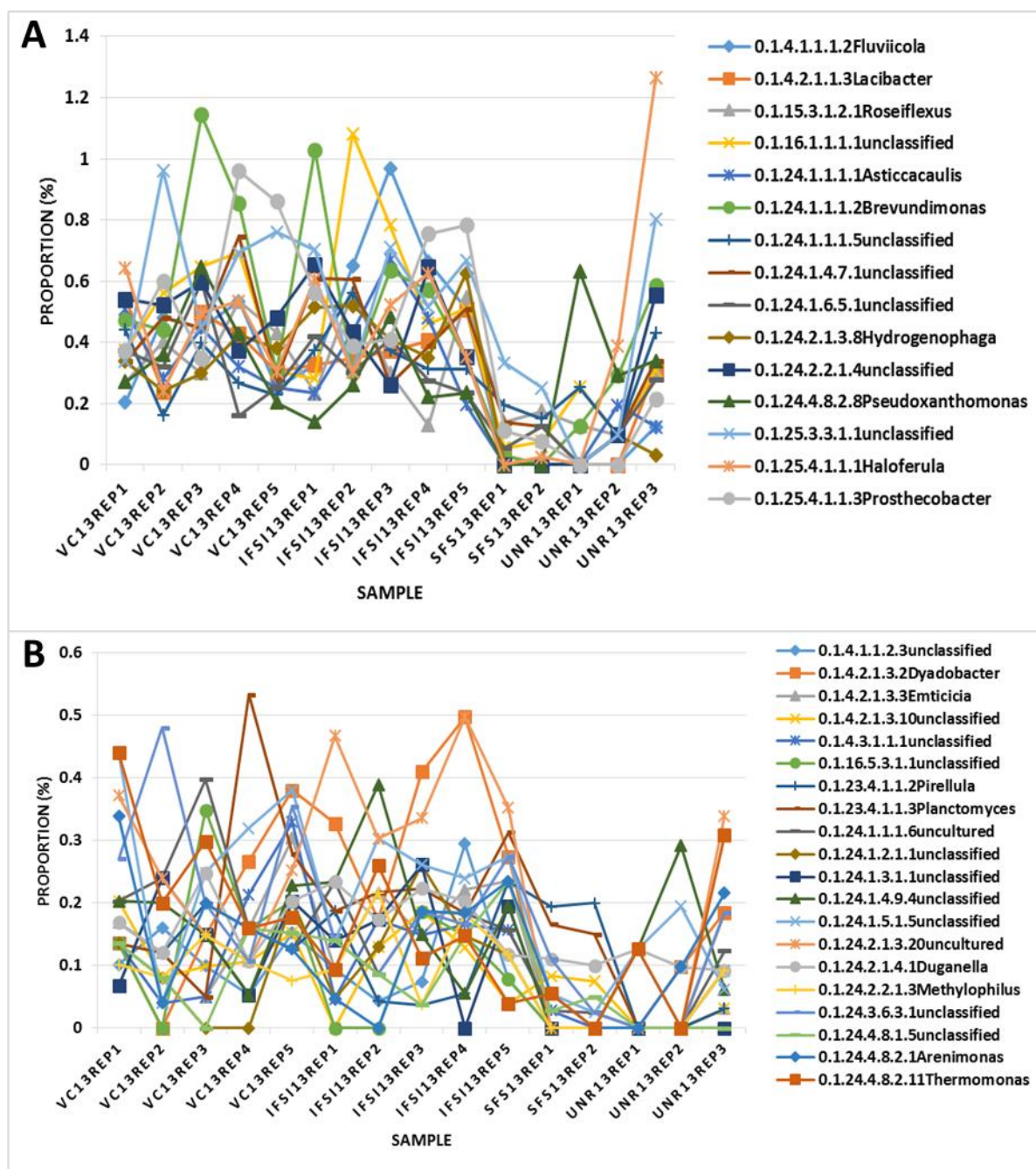


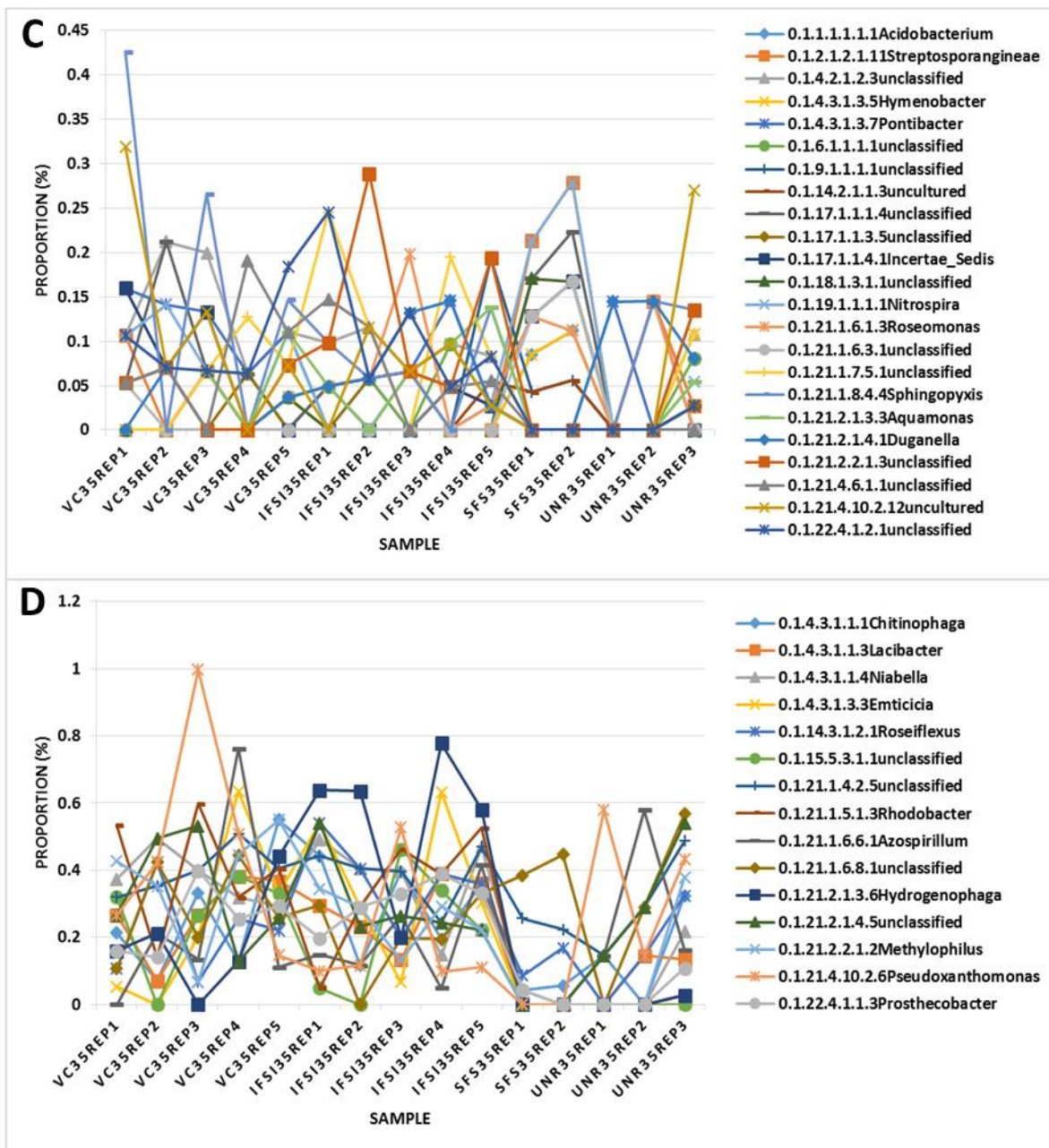
Figure 3.13. Clusters of bacterial genera with a relatively higher abundance in rhizospheres of hairy root composite plants vs. untransformed plants.

Clusters A-D were obtained based on abundance identified using read counts of variable regions V1-V3. Clusters E-H were obtained based on abundance identified using read counts of variable regions V3-V5. Sample labels (x-axis) indicate if the sample was from soybean field soil (SFS) or untransformed soybean (UNR), vector control (VC), or IFS-RNAi (IFSi) rhizosphere soil samples. Proportion values (y-axis) were calculated by dividing the total number of sequences for each bacterial genus by the total number of sequences within each sample.

Multiple clusters with similar patterns, but differences in relative proportions were identified (Figs. 3.10–3.14). However, none of the clusters displayed a strong change in genera proportions due to the absence of

isoflavonoids in agreement with the observation that only a small number of bacterial families displayed any change in abundance. It is likely there were too few genera with a consistent pattern of change in response to the lack of isoflavonoids, resulting in said genera being sorted into other clusters.





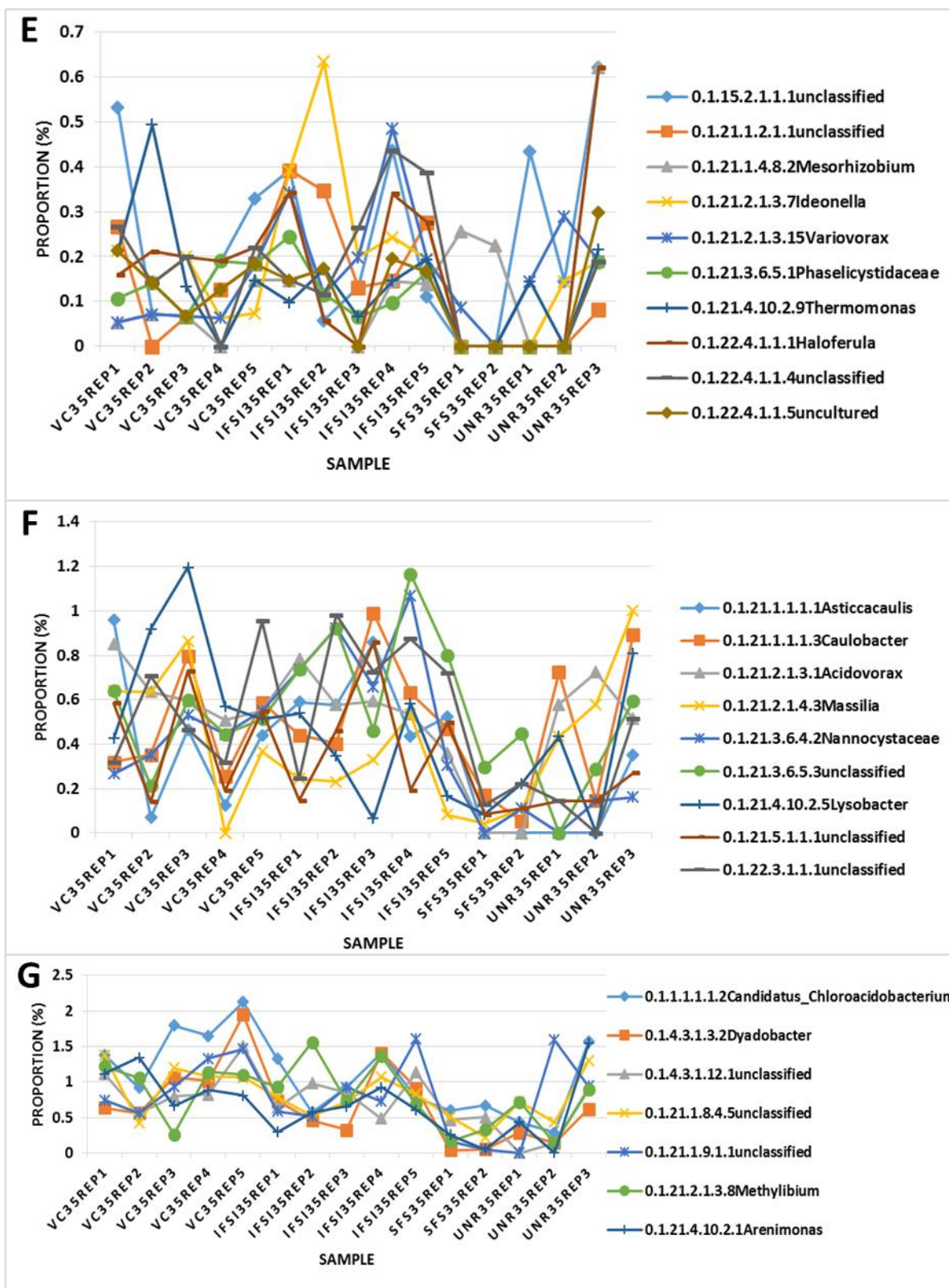


Figure 3.14. Clusters of bacterial genera with similar relative abundances in different samples with no discernible pattern among the different samples.

Clusters A-B were obtained based on abundance identified using read counts of variable regions V1-V3. Clusters C-G were obtained based on abundance identified using read counts of variable regions V3-V5. Sample labels (x-axis) indicate if the sample was from soybean field soil (SFS) or untransformed soybean (UNR), vector control (VC), or IFS-RNAi (IFSi) rhizosphere soil samples. Proportion values (y-axis) were calculated by dividing the total number of sequences for each bacterial genus by the total number of sequences within each sample.

In addition to patterns based on relative abundance in different samples, we also observed clusters of genera with similar functional attributes. For example, genera containing associative N fixers *Ensifer*, *Azospirillum*, *Bosea* and *Burkholderia* clustered together displaying a higher relative abundance in rhizosphere versus bulk soil (Fig. 3.15).

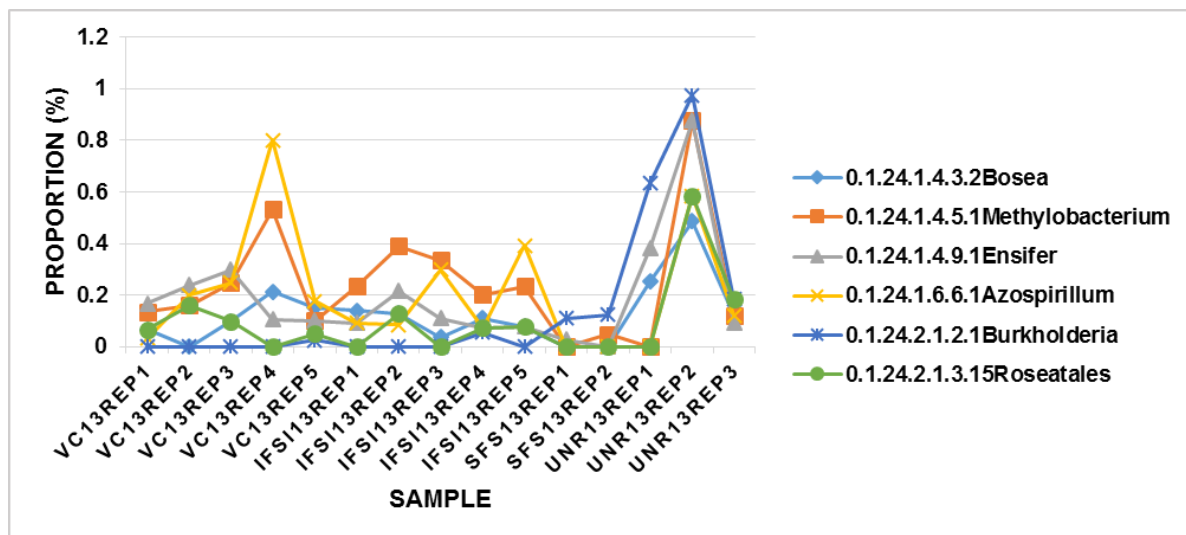


Figure 3.15. Cluster plot of nitrogen fixing bacterial genera with comparable abundance in SFS, UNR, VC and IFSi soil samples.

Cluster plot displaying genera with comparable abundance in soybean field soil (SFS), untransformed soybean (UNR), vector control (VC) and IFS-RNAi (IFSi) samples. Genera showed relatively high enrichment in the rhizosphere of untransformed soybean roots and were primarily composed of associative nitrogen fixers. Plot was obtained using sequences of PCR amplicons from the V1-V3 variable region of 16S rRNA gene.

Similarly, *Bdellovibrio* – considered to be a good indicator of the presence of gram negative bacteria – clustered well with a group of gram negative genera such as *Flexibacter*, *Methylibium*, *Pelomonas* and *Optitutus* (Fig. 3.16).

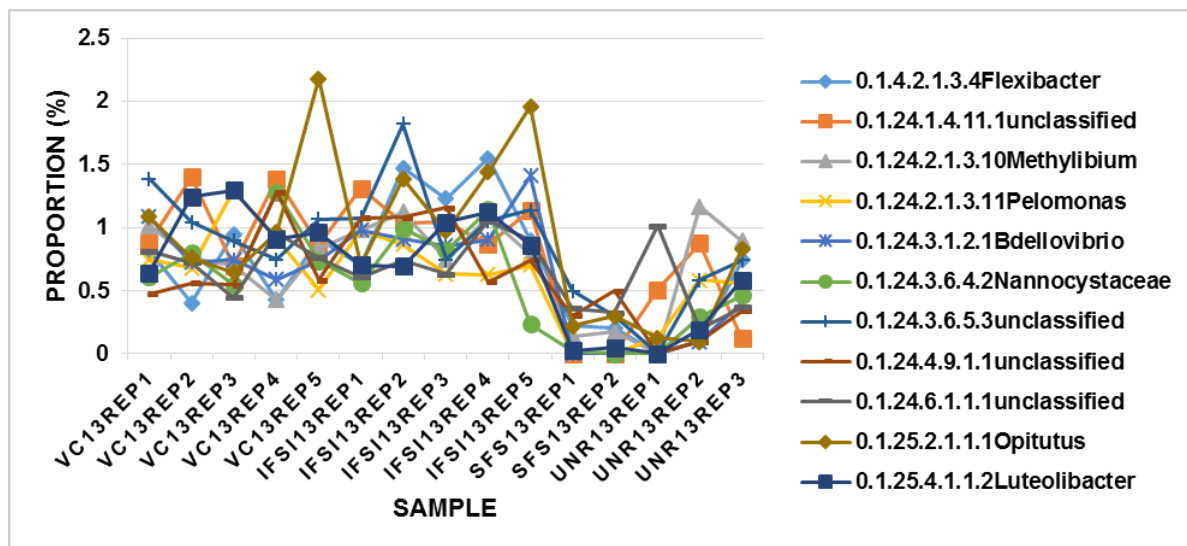


Figure 3.16. Cluster plot of gram negative bacterial genera with comparable abundance in SFS, UNR, VC and IFSi soil samples.

Cluster plot displaying genera with comparable abundance in soybean field soil (SFS), untransformed soybean (UNR), vector control (VC) and IFS-RNAi (IFSi) samples. Genera showed relatively high enrichment in the rhizosphere of hairy root composite plants and were composed of *Bdellovibrio* and gram negative bacteria. Plot was obtained using sequences of PCR amplicons from the V1-V3 variable region of 16S rRNA gene.

These patterns suggested that evaluating clusters of uncultured and previously uncharacterized OTUs with genera of known significance or functions might help hypothesize dependencies and/or functional similarities between them.

3.3. Bacterial families influenced by root exudate isoflavonoids

We compared the proportional abundance of each family in different samples to evaluate their enrichment in specific samples. In V1–V3 libraries from UNR samples, 16 families had a statistically significant differential abundance

compared with SFS samples (6 enriched, 10 reduced; Student's t -test $P < 0.05$;

Fig. 3.17).

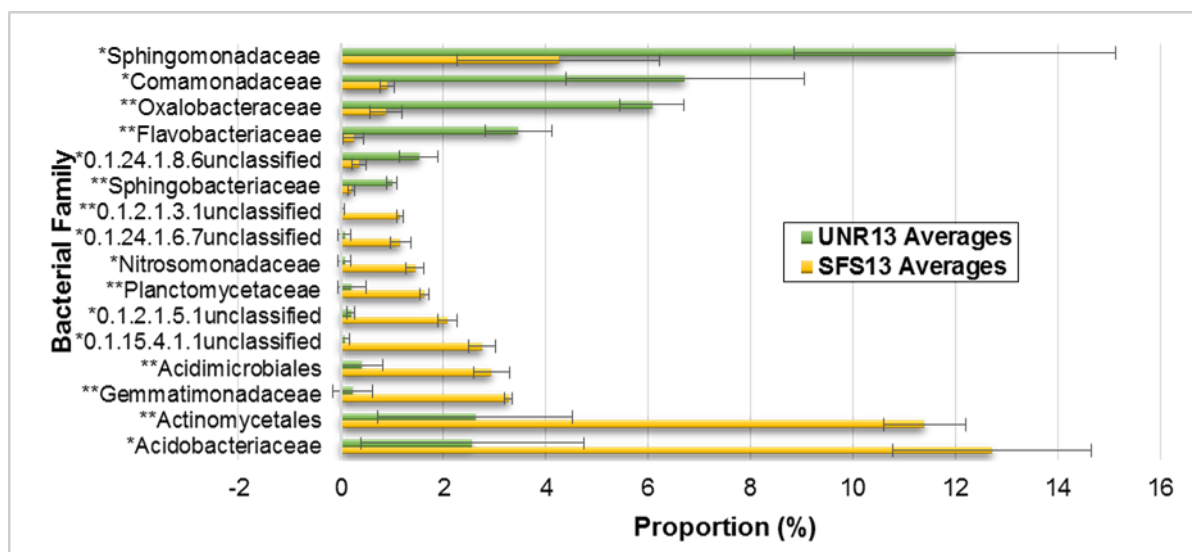


Figure 3.17. Bar graph comparing bacterial family relative abundances from SFS and UNR soil samples for V13 region.

Bar graph comparing relative abundance of selected bacteria families from soybean field soil (SFS) samples to untransformed (UNR) root soil samples. “13” indicates the graph was obtained using sequences of PCR amplicons from V1-V3 variable region of the 16S rRNA gene. Asterisks indicate the level of statistical significant difference, if any, between the samples (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Error bars indicate standard deviation values.

In V3–V5 libraries 12 families were significantly differentially abundant

(3 enriched, 9 reduced; Student's t -test $P < 0.05$; Fig. 3.18) between these

samples.

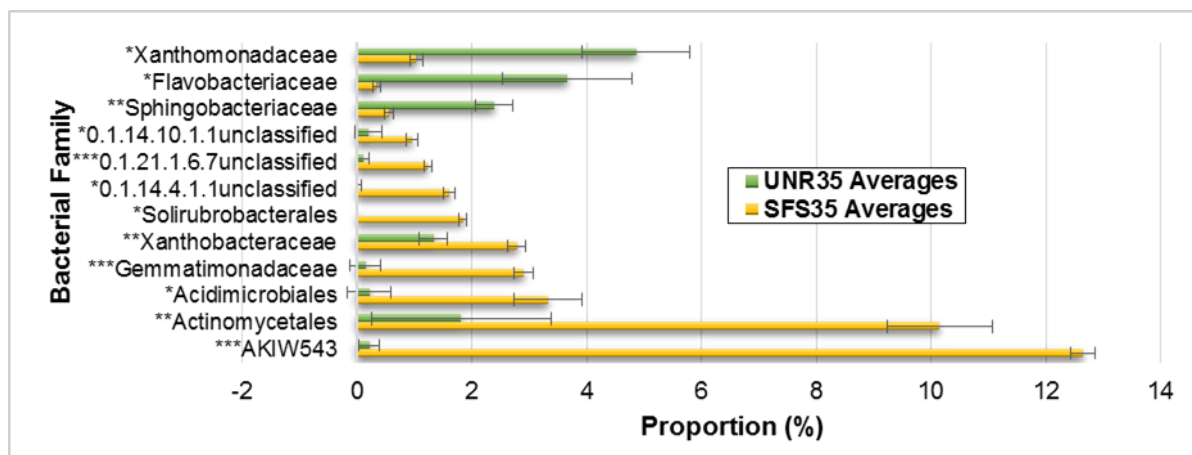


Figure 3.18. Bar graph comparing bacterial family relative abundances from SFS and UNR soil samples for V35 region.

Bar graph comparing relative abundance of selected bacteria families from soybean field soil (SFS) samples to untransformed (UNR) root soil samples. “35” indicates the graph was obtained using sequences of PCR amplicons from the V3-V5 variable region of the 16S rRNA gene. Asterisks indicate the level of statistical significant difference, if any, between the samples (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Error bars indicate standard deviation values.

Five of these families were detected by both libraries, therefore a total of 23 bacterial families were differentially abundant (7 enriched and 16 reduced) in proximal soils of untransformed soybean roots relative to the bulk field soil. Such changes amongst bacterial families were unsurprising since many plants are renowned for manipulating their environment, and the bacteria within, to suit their needs (Marschner et al., 2002; Micallef et al., 2009; Gottel et al., 2011).

Compared with the phylum level analysis, only a small number of families were detected by both V1–V3 and V3–V5 libraries. However, the ones that were detected by both libraries showed similar trends of enrichment or reduction.

Our second objective was to determine which families were affected by the hairy root transformation by comparing VC samples to the SFS samples. In V1–V3 libraries from VC samples, 32 families were significantly differentially

abundant (22 enriched, 10 reduced; Fig. 3.19 A) while in V3–V5 libraries 28 families were differentially abundant (20 enriched, 8 reduced; Fig. 3.19 B) compared with the bulk field soil.

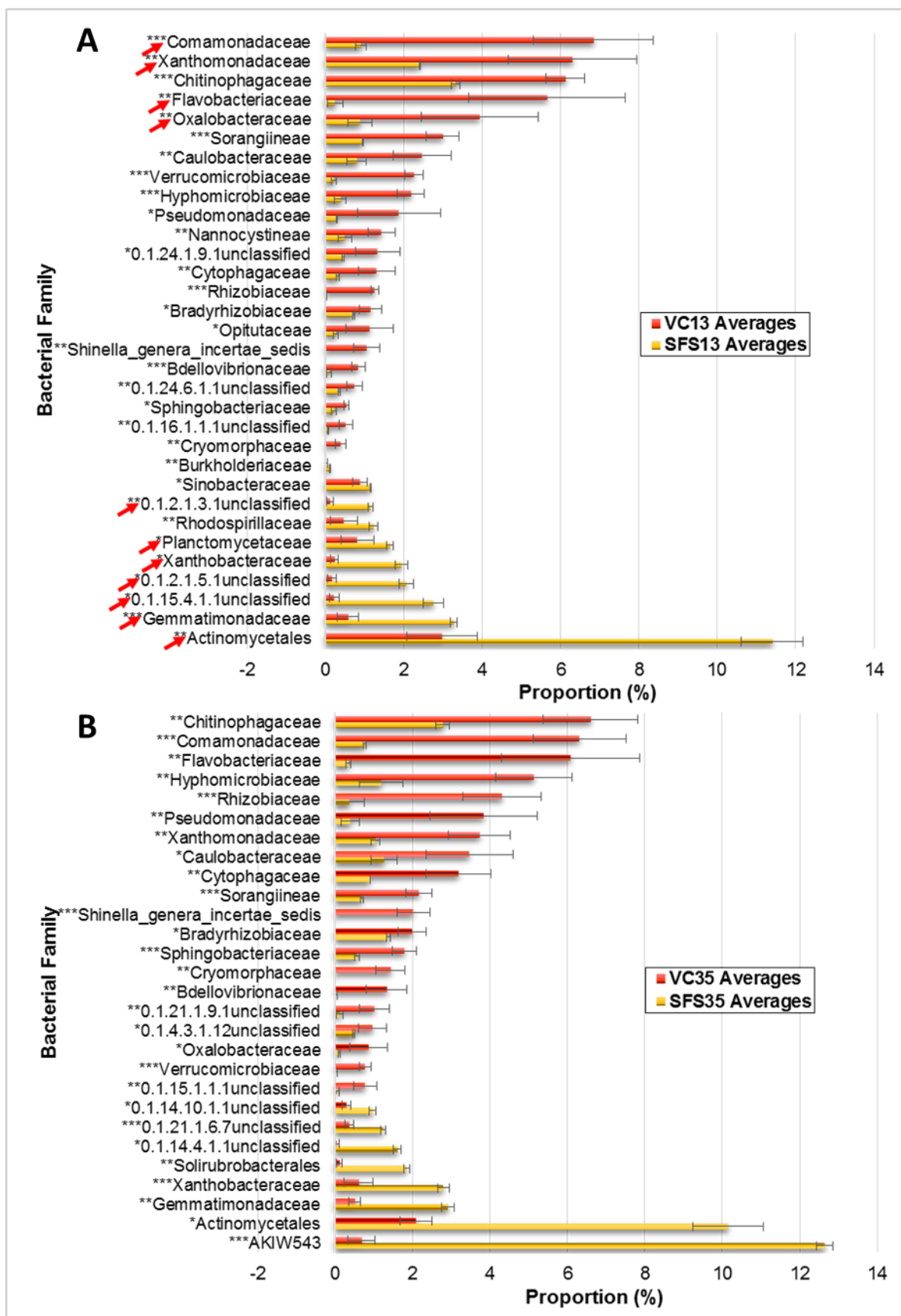


Figure 3.19. Bar graphs comparing bacterial family abundancies of SFS and VC soil sample.

Bar graphs comparing relative abundance of selected bacteria families from soybean field soil (SFS) samples to vector control (VC) root soil samples. (A) “13” and (B) “35” indicate if the graphs were obtained using sequences of PCR amplicons from the V1-V3 or V3-V5 variable regions of the 16S rRNA gene. Red arrows indicate families that were also detected in untransformed root (UNR) soil samples. Asterisks indicate the level of statistical significant difference, if any, between the samples (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Error bars indicate standard deviation values.

Of these, 20 families were detected by both libraries, thus a total of 40 families were differentially abundant (25 enriched and 15 reduced) in proximal soils of hairy roots. Seventeen of the 23 bacterial families that were differentially abundant in untransformed roots showed a similar pattern of colonization in hairy roots as well (5 of them were enriched and 12 reduced; Fig. 3.19 A-B – families marked with red arrows). Therefore, hairy root transformation impacted numerous bacterial families that were otherwise unaffected in proximal soils of untransformed soybean roots. However, the majority of the families (74%) that were differentially abundant in UNR samples showed similar trends of differential abundance in VC samples making them amenable for studies using hairy root transformation (Table 3.3). Notable exceptions were Sphingomonadaceae (enriched in UNR, $P = 0.04$; unaltered in VC, $P = 0.19$) and Acidobacteriaceae (reduced in UNR, $P = 0.02$; unaltered in VC, $P = 0.08$).

Our third objective was to identify which bacteria families were affected by isoflavonoids by comparing the abundance of bacterial families between VC and IFSi samples. The V1–V3 library detected 4 families that were differentially abundant in IFSi samples (3 increased, 1 reduced; Student’s t -test $P < 0.05$; Fig. 3.20) relative to the vector control samples.

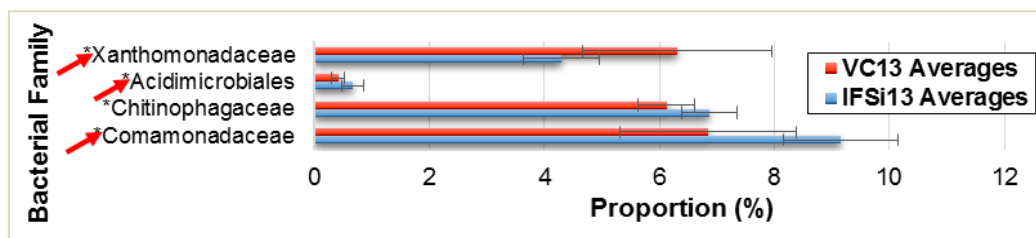


Figure 3.20. Bar graph comparing bacterial family relative abundances from VC and IFSi soil samples for V13 region.

Bar graph comparing relative abundance of selected bacteria families from vector control (VC) samples to IFS-RNAi (IFSi) root soil samples. “13” indicates the graph was obtained using sequences of PCR amplicons from the V1-V3 variable region of the 16S rRNA gene. Red arrows indicate families that were also detected in untransformed root (UNR) soil samples. Asterisks indicate the level of statistical significant difference, if any, between the samples (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Error bars indicate standard deviation values.

The V3–V5 library detected 6 families that were differentially abundant (4 increased, 2 reduced; Student’s t -test $P < 0.05$; Fig. 3.21).

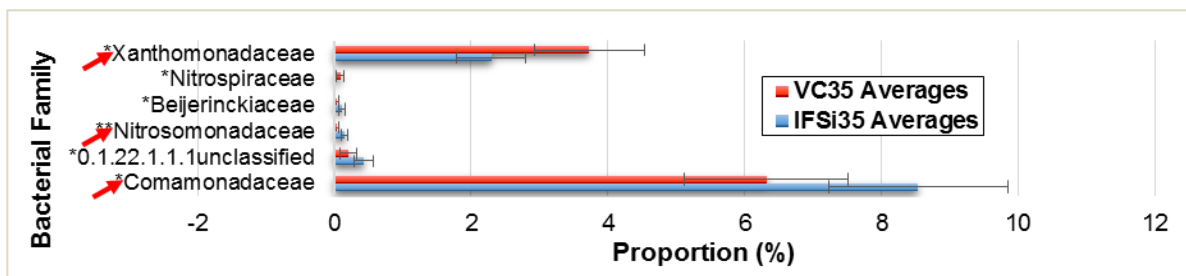


Figure 3.21. Bar graph comparing bacterial family relative abundances from VC and IFSi soil samples for V35 region.

Bar graph comparing relative abundance of selected bacteria families from vector control (VC) samples to IFS-RNAi (IFSi) root soil samples. “35” indicates the graph was obtained using sequences of PCR amplicons from the V3-V5 variable region of the 16S rRNA gene. Red arrows indicate families that were also detected in untransformed root (UNR) soil samples. Asterisks indicate the level of statistical significant difference, if any, between the samples (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Error bars indicate standard deviation values.

Two families were detected by both libraries, and therefore the abundances of 6 families were increased and 2 families were reduced in proximal soil in response to a reduction in the levels of root isoflavonoids. Bacteria of

Xanthomonadaceae and Comamonadaceae were enriched in proximal soils of untransformed and vector control roots. Reduction of root isoflavonoids resulted in a 25% decrease in the abundance of Xanthomonads in proximal soils suggesting that isoflavonoids might promote their presence in the proximal soils of soybean roots. On the other hand, the abundance of Comamonads increased approximately 35% suggesting that isoflavonoids might inhibit their presence in proximal soils. Bacteria of Acidimicrobiales and Nitrosomonadaceae were reduced in proximal soils of untransformed and vector control roots. In the absence of isoflavonoids, there was a small but significant increase in their abundance suggesting that isoflavonoids might suppress their presence in proximal soils.

4. Discussion

Interactions between plants and soil microbes are subject to increasing interest as the need for sustainable agriculture and environmental preservation rises. Discovering changes in soil microbial communities due to plant roots is one step closer to such goals. Our study focused on soybean rhizosphere bacterial communities at the phylum, family, genus and OTU levels. Initial analysis of the phyla showed Proteobacteria dominated the soybean rhizosphere, followed by Bacteroidetes. Actinobacteria and Acidobacteria were the third and fourth most prominent phyla, but were greatly reduced by soybean roots. A previous soybean rhizosphere study corroborated the dominance of these four, known bacterial phyla during the vegetative, flowering and mature stages of soybean growth, with the exception of Firmicutes acting as yet another dominant phylum during the vegetative and

flowering stages. Although the study also listed Proteobacteria as the most dominant phylum at all soybean growth stages, Actinobacteria was the second most dominant phylum rather than Bacteroidetes, which acted as the third or even fifth most dominant phylum. During the vegetative stage – which was used in our study – Bacteroidetes was preceded by Acidobacteria and nearly tied with Firmicutes in relative abundance within the rhizosphere. However, the previous study used a later vegetative stage – at the beginning of flowering – compared with our study, which used 8-week-old plants with no signs of flowering. The difference in vegetative stages may partially account for the differences in bacteria phyla dominance (Sugiyama et al., 2014). Alternatively, the differences in dominance may be due to different phylum abundance levels in the initial bulk soil, soil type or available nutrients (Xu et al., 2009; Mendes et al., 2014; Wang et al., 2014). Despite minor discrepancies, this trend of predominant phyla was also depicted in the rhizospheres of other plant species. The maize rhizosphere was also dominated by Proteobacteria, followed by Bacteroidetes and Actinobacteria (Peiffer et al., 2013). This was the case in *Arabidopsis thaliana* as well, although Acidobacteria showed an abundance comparable to Actinobacteria (Lundberg et al., 2012). The rhizosphere of *Populus deltoids* deviates from this pattern with Bacteroidetes failing to register as a dominant phylum and Verrucomicrobia being the third most prominent phylum. However, Proteobacteria and Acidobacteria were still among the most prominent phyla (Gottel et al., 2011). Despite minor discrepancies, Proteobacteria was the indisputably dominant phylum across all four different plant species. This may, in part, be attributable to its initially large presence in soil lacking plant roots. However Actinobacteria, an originally

prominent phyla in the soybean field bulk soil, was drastically reduced in the soybean rhizosphere. Interestingly, the *A. thaliana* rhizosphere showed a slight increase in Actinobacteria in the rhizosphere (Lundberg et al., 2012). This indicates plant roots can actively influence bacteria, likely by altering the environment within the rhizosphere. Indeed, even the initially dominant Proteobacteria shows an increased presence in soybean rhizosphere samples. Whether these shifts in abundancies are due to the presence of one or multiple compounds produced by the plant roots is uncertain. To that end, we focused on the effect of isoflavonoids on the bacterial community structure as well as specific families within the soybean rhizosphere.

Isoflavonoids are mainly renowned for aiding in plant defenses against harmful microbes as well as inducing rhizobial nod factors (Hassan and Mathesius, 2012). Indeed, isoflavonoids have been shown to induce *Bradyrhizobium japonicum* nod genes and inhibit *Sinorhizobium meliloti* nod genes in leguminous plants (Peck et al., 2006; Subramanian et al., 2006). As for plant defense, pterocarpan – derivatives of isoflavonoids – are known to act as antifungal agents for legumes. For example, pisatin production has been noted to reduce damage in *Pisum sativum* L. (pea) caused by the *Nectria haematococca* fungus (Naoumkina et al., 2010). However, other studies have implied that isoflavonoids can also act as metal chelators in *Medicago sativa* (alfalfa), stimulate symbiotic mycorrhizal infection in a *Medicago truncatula* mutant, modulate auxin transportation in soybean, and break down auxin in white clover (Hassan and Mathesius, 2012). Although isoflavonoids are depicted serving various functions, it is not known if and how they influence rhizosphere bacterial communities. Our study focused on their impact on soybean rhizosphere bacterial

community structure as well as specific bacterial families. To that end, we examined samples acquired from bulk soybean field soil (SFS) as well as proximal soil from unaltered soybean roots (UNR), vector control roots (VC) and isoflavone synthase interference roots (IFSi). Statistical analyses of OTU bacterial community structures of these samples revealed a conservative difference between the IFSi and VC samples. This limited difference was also depicted in the subsequent comparisons of bacteria family proportions and supported by the denaturing gradient gel electrophoresis analysis in our previous study (White et al., 2015). Of the 194–206 families detected by the V1–V3 and V3–V5 libraries, only 8 were notably affected by reduced isoflavonoid levels (6 increased, 2 reduced). Intriguingly, few or no genera within these families showed a statistically significant difference in proportions attributable to low isoflavonoid levels. This discrepancy is likely because the sum of smaller changes at the genus level yield a larger, notable change at the family level. Four of the affected families belonged to the Proteobacteria phylum, although they did not necessarily share the same abundance trends (e.g., Xanthomonadaceae was reduced whereas Comamonadaceae was increased by low isoflavonoid levels). The remaining families belonged to the Actinobacteria, Bacteroidetes, Nitrospirae and Verrucomicrobia phyla. These families serve important functions within the rhizosphere, either for the plant or other bacterial families. Chitinophagaceae contains species capable of degrading chitin or hydrolyzing cellulose to generate nutrient sources, such as glucose, which other bacteria may be able to use (Rosenberg, 2014). Beijerinckiaceae, Nitrospiraceae and Nitrosomonadaceae families contain nitrogen fixers as well as nitrite and ammonia-oxidizers capable of providing essential sources

of nitrogen, such as nitrate, for soybean (Daims, 2014; Marin and Arahal, 2014; Prosser et al., 2014). Closer inspection of genera within the affected families may help clarify why they were positively or negatively impacted by the absence of isoflavonoids. For example, Comamonadaceae contains the phytopathogenic genus *Acidovorax*, which is capable of inducing bacterial leaf blight, bud rot and leaf spot (Willems, 2014). The increase of Comamonadaceae in the absence of isoflavonoids may indicate this plant-pathogenic genus is normally suppressed by isoflavonoids. On the other hand, we detected the *Lysobacter* and *Stenotrophomonas* genera within the Xanthomonadaceae family. The *Lysobacter* genus consists of bacterium that lyse other bacterium (both gram-negative and gram-positive) as well as filamentous fungi whereas the *Stenotrophomonas* genus has a narrow nutritional spectrum limited to maltose, lactose, cellobiose, trehalose and salicin (Christensen and Cook, 1978; Palleroni and Bradbury, 1993). The decrease of Xanthomonadaceae is possibly due to a lack of nutritional sources for such genera, possibly because isoflavonoid-deficient roots fail to attract the microbes that contain or produce the necessary nutrients. Ultimately, further studies are necessary to definitively determine why the aforementioned families were impacted by the absence of isoflavonoids.

Most of the previously mentioned phyla accounted for large portions of the bulk soybean field soil bacterial community, indicating isoflavonoids can potentially impact key, influential soil bacteria. However, several families listed as significantly, differentially abundant in VC and IFSi proximal soil samples were not noted as such in UNR proximal soil samples. This differential effect was also detected in the overall bacterial community structure at the OTU level, indicating hairy root transformation

exerted an additional influence on the rhizosphere bacterial community. The large proportion of Rhizobiaceae in VC roots is to be expected as this family contains *Agrobacterium rhizogenes*, which was used to induce hairy root transformation and generate the VC and IFSi roots in the first place (Carareto Alves et al., 2014). On the other hand, the reduced proportion of the Rhodospirillaceae family is curious since our samples contained the *Azospirillum* genus, which is known to contain plant-growth-promoting bacteria that predominantly colonize the plant root surface (Baldani et al., 2014). The apparent impact of the hairy root transformation is not necessarily unexpected since hairy root cultures have been noted to steadily produce high quantities of secondary metabolites in multiple plant species. Plants increase the production of these metabolites in response to damage by pathogens, such as members in the *Agrobacterium* genus (Bulgakov, 2008; Chandra, 2012). This increase in secondary metabolite production likely impacted the soil bacterial community by preventing the establishment of normally competitive bacterial strains. Alternatively, other bacterial strains were possibly attracted by the secondary metabolites and simply outcompeted other strains. Collectively, the differentially affected families accounted for approximately 1%–7% of the VC and IFSi proximal soil bacterial communities. However, the remaining families accounted for twice the proportion of these same communities (12%–16%). Also, the families depicted as differentially abundant in VC as well as UNR samples displayed similar differential abundancy trends. Another potential concern with the use of composite hairy root plants is the presence of a mixture of transformed and untransformed roots in these plants. However, since root exudate influence the rhizosphere in very close proximity

to the root surface, exudation from untransformed roots is unlikely to influence microbial diversity of neighboring roots. This indicated that hairy root transformation is still a useful tool for evaluating the impact of plant roots on rhizosphere microbial communities.

Overall, our results revealed the composition of bacterial communities closely associated with soybean roots in the rhizosphere – especially from soils with a history of soybean cultivation – and identified specific bacterial taxa that are influenced by hairy root transformation and root isoflavonoids in the soybean rhizosphere.

5. Materials and Methods

5.1. Plant materials, DNA vectors, plant transformation and rhizosphere soil isolation

The DNA vectors (vector control and IFS-RNAi constructs) used in this study have been previously described (Subramanian et al., 2005). For composite plant transformation, soybean (*Glycine max* cv. Williams 82) seeds were surface sterilized and grown as previously described (White et al., 2015). Fourteen-day-old seedlings containing their first trifoliolate leaves were used for composite hairy root plant generation as previously described (Collier et al., 2005) with slight modifications (described in White et al., 2015). After 3 weeks, roots that were successfully and stably transformed were identified through GFP epifluorescence using the FITC filter in an Olympus SZX16 Epi-Fluorescence Stereo Microscope, marked with ‘Tough-Tags’, (Diversified Biotech) and then planted in soybean field soil (described in (White et al., 2015)). Rhizosphere soil samples were

isolated as previously described (White et al., 2015), but only proximal soil samples from the 3 week time period were used for this experiment. This study ultimately focused on four sample types, noted as soybean field soil (SFS) and untransformed soybean (UNR), vector control (VC) and IFS-RNAi (IFSi) rhizosphere soil samples.

5.2. DNA isolation, PCR and pyrosequencing

DNA was acquired from 0.09 to 0.47 g of soil sample via a PowerSoil® DNA isolation kit (MO BIO Laboratories, Inc. Carlsbad, CA) in accordance with the manufacturer's protocol. The 16S rRNA variable regions V1–V3 and V3–V5 were amplified using a Gene Amp® PCR System 9700 model thermocycler machine (100/120/220/230/240 VAC 50/60 Hz, Max Power 725VA) and a 30 µL reaction mixture containing 0.2 µL Taq DNA polymerase, 6 µL PCR buffer, 0.15 µL dNTP, 1.8 µL MgCl₂, 1.2 µL forward primer, 1.2 µL reverse primer, 18.95 µL nanopure H₂O, 0.5 µL (100 ng) template DNA. PCR parameters were as follows: preliminary denaturation at 94°C for 5 min, (94°C for 30 s, 56°C for 45 s, 72°C for 1 min) 22 cycles, final elongation at 72°C 7 min, 10°C indefinitely for storage. PCR was limited to 22 cycles to ensure sequence amplification remained in the logarithmic phase to avoid generating artificial proportions of sequences detected within the bacterial community of each sample. Forward primer 27F and reverse primer 533R (Weisburg et al., 1991; Huse et al., 2008) were used for V1–V3 amplification while forward primer F357 without the GC clamp (Muyzer et al., 1993; Brons and van Ems, 2008) and reverse primer R907 (Teske et al., 1996) were used for V3–V5 amplification in this experiment. The aforementioned

primers were outfitted with distinct sequence tags ('barcodes') for each sample to enable pooling the amplicons prior to library construction and pyrosequencing (Table 3.4). Equal amounts of PCR products from each sample were mixed together and sent to the Beckman Coulter Genomics Inc. for pyrosequencing (Roche 454).

Table 3.4. Sequences of barcode tags and primers used in the study.

Sample	V1-V3 Barcode	V1-V3 Forward Primer	V1-V3 Reverse Primer
SFS Rep1	TCTACGTAGC	5'-TCTACGTAGCAGAGTTTGATCCTGGCTCAG-3'	5'-TCTACGTAGCTTACCGCGGCTGCTGGCAC-3'
SFS Rep2	TGTACTIONACTC	5'-TGTACTIONACTCAGAGTTTGATCCTGGCTCAG-3'	5'-TGTACTIONACTCTTACCGCGGCTGCTGGCAC-3'
UNR Rep1	ACGACTACAG	5'-ACGACTACAGAGAGTTTGATCCTGGCTCAG-3'	5'-ACGACTACAGTTACCGCGGCTGCTGGCAC-3'
UNR Rep2	CGTAGACTAG	5'-CGTAGACTAGAGAGTTTGATCCTGGCTCAG-3'	5'-CGTAGACTAGTTACCGCGGCTGCTGGCAC-3'
UNR Rep3	TACGAGTATG	5'-TACGAGTATGAGAGTTTGATCCTGGCTCAG-3'	5'-TACGAGTATGTTACCGCGGCTGCTGGCAC-3'
VC Rep1	TACTCTCGTG	5'-TACTCTCGTGAGAGTTTGATCCTGGCTCAG-3'	5'-TACTCTCGTGTTACCGCGGCTGCTGGCAC-3'
VC Rep2	TAGAGACGAG	5'-TAGAGACGAGAGAGTTTGATCCTGGCTCAG-3'	5'-TAGAGACGAGTTACCGCGGCTGCTGGCAC-3'
VC Rep3	TCGTCGCTCG	5'-TCGTCGCTCGAGAGTTTGATCCTGGCTCAG-3'	5'-TCGTCGCTCGTTACCGCGGCTGCTGGCAC-3'
VC Rep4	ACATACGCGT	5'-ACATACGCGTAGAGTTTGATCCTGGCTCAG-3'	5'-ACATACGCGTTTACCGCGGCTGCTGGCAC-3'
VC Rep5	ACGCGAGTAT	5'-ACGCGAGTATAGAGTTTGATCCTGGCTCAG-3'	5'-ACGCGAGTATTTACCGCGGCTGCTGGCAC-3'
IFSi Rep1	ACTACTATGT	5'-ACTACTATGTAGAGTTTGATCCTGGCTCAG-3'	5'-ACTACTATGTTTACCGCGGCTGCTGGCAC-3'
IFSi Rep1*	CACGCTACGT	5'-CACGCTACGTAGAGTTTGATCCTGGCTCAG-3'	5'-CACGCTACGTTTACCGCGGCTGCTGGCAC-3'
IFSi Rep2	ACTGTACAGT	5'-ACTGTACAGTAGAGTTTGATCCTGGCTCAG-3'	5'-ACTGTACAGTTTACCGCGGCTGCTGGCAC-3'
IFSi Rep2*	CAGTAGACGT	5'-CAGTAGACGTAGAGTTTGATCCTGGCTCAG-3'	5'-CAGTAGAGTTTACCGCGGCTGCTGGCAC-3'
IFSi Rep3	AGACTATACT	5'-AGACTATACTAGAGTTTGATCCTGGCTCAG-3'	5'-AGACTATACTTTACCGCGGCTGCTGGCAC-3'
IFSi Rep3*	CGACGTGACT	5'-CGACGTGACTAGAGTTTGATCCTGGCTCAG-3'	5'-CGACGTGACTTTACCGCGGCTGCTGGCAC-3'
IFSi Rep4	AGCGTCGTCT	5'-AGCGTCGTCTAGAGTTTGATCCTGGCTCAG-3'	5'-AGCGTCGTCTTTACCGCGGCTGCTGGCAC-3'
IFSi Rep5	AGTACGCTAT	5'-AGTACGCTATAGAGTTTGATCCTGGCTCAG-3'	5'-AGTACGCTATTTACCGCGGCTGCTGGCAC-3'
Sample	V3-V5 Barcode	V3-V5 Forward Primer	V3-V5 Reverse Primer
SFS Rep1	ACGAGTGCGT	5'-ACGAGTGCGTTACGGGAGGCAGCAG-3'	5'-ACGAGTGCGTCCCCGTCAATTCCTTTGAGTTT-3'
SFS Rep2	ACGCTCGACA	5'-ACGCTCGACATACGGGAGGCAGCAG-3'	5'-ACGCTCGACACCCCGTCAATTCCTTTGAGTTT-3'
UNR Rep1	AGACGCACTC	5'-AGACGCACTCTACGGGAGGCAGCAG-3'	5'-AGACGCACTCCCCGTCAATTCCTTTGAGTTT-3'
UNR Rep2	AGCACTGTAG	5'-AGCACTGTAGTACGGGAGGCAGCAG-3'	5'-AGCACTGTAGCCCCGTCAATTCCTTTGAGTTT-3'
UNR Rep3	ATCAGACACG	5'-ATCAGACACGTACGGGAGGCAGCAG-3'	5'-ATCAGACACGCCCCGTCAATTCCTTTGAGTTT-3'
VC Rep1	ATATCGCGAG	5'-ATATCGCGAGTACGGGAGGCAGCAG-3'	5'-ATATCGCGAGCCCCGTCAATTCCTTTGAGTTT-3'
VC Rep2	CGTGTCTCTA	5'-CGTGTCTCTATACGGGAGGCAGCAG-3'	5'-CGTGTCTCTACCCCGTCAATTCCTTTGAGTTT-3'
VC Rep3	CTCGCGTGTC	5'-CTCGCGTGCTACGGGAGGCAGCAG-3'	5'-CTCGCGTGCTCCCCGTCAATTCCTTTGAGTTT-3'
VC Rep4	TCTCTATGCG	5'-TCTCTATGCGTACGGGAGGCAGCAG-3'	5'-TCTCTATGCGCCCCGTCAATTCCTTTGAGTTT-3'
VC Rep5	TGATACGTCT	5'-TGATACGTCTACGGGAGGCAGCAG-3'	5'-TGATACGTCTCCCCGTCAATTCCTTTGAGTTT-3'
IFSi Rep1	CATAGTAGTG	5'-CATAGTAGTGTACGGGAGGCAGCAG-3'	5'-CATAGTAGTGCCCCGTCAATTCCTTTGAGTTT-3'
IFSi Rep1*	N/A	N/A	N/A
IFSi Rep2	CGAGAGATAC	5'-CGAGAGATACTACGGGAGGCAGCAG-3'	5'-CGAGAGATACCCCGTCAATTCCTTTGAGTTT-3'
IFSi Rep2*	N/A	N/A	N/A
IFSi Rep3	ATACGACGTA	5'-ATACGACGTATACGGGAGGCAGCAG-3'	5'-ATACGACGTACCCCGTCAATTCCTTTGAGTTT-3'
IFSi Rep3*	ATAGAGTACT	5'-ATAGAGTACTTACGGGAGGCAGCAG-3'	5'-ATAGAGTACTCCCCGTCAATTCCTTTGAGTTT-3'
IFSi Rep4	TCACGTACTA	5'-TCACGTACTATACGGGAGGCAGCAG-3'	5'-TCACGTACTACCCCGTCAATTCCTTTGAGTTT-3'
IFSi Rep5	CGTCTAGTAC	5'-CGTCTAGTACTACGGGAGGCAGCAG-3'	5'-CGTCTAGTACCCCGTCAATTCCTTTGAGTTT-3'

* = sample used >2 barcodes for a primer set

5.3. DNA sequence data preparation

The libraries of the 30 samples obtained from pyrosequencing were initially processed using btrim software (Kong, 2011) to remove all sequences < 300 nucleotides and ensure their average quality scores were 20 (the window size was 3; Supporting Information Table S2). The remaining sequences were then

reoriented so they all read from forward primer to reverse primer using a custom-made Perl script. Afterward, the 15 libraries containing variable regions V1–V3 and the 15 libraries containing variable regions V3–V5 were merged into two separate files identified as V1_V3 and V3_V5. These files were then processed using a data analysis pipeline developed in *MOTHUR* (Schloss et al., 2009) (version 1.29.2; Fig. 3.2). The first step of this pipeline was to identify unique sequences within the files. Next, the sequences were aligned using the 16S SILVA database (Pruesse et al., 2007; Quast et al., 2013). The resulting files were then screened to remove sequences starting before or ending after 90% of the other sequences, containing ambiguous bases, or possessing > 8 homopolymers. Afterward, the files were filtered to remove columns solely containing gaps in all sequences. Unique sequences were then identified within the resulting files. After that, sequences likely resulting from pyrosequencing errors as well as potential chimeric sequences were identified and subsequently removed. The remaining sequences were then assigned to a taxonomy outline from the SILVA database using the Wang method and a cutoff bootstrap value of 80 (Wang et al., 2007). The resulting taxonomy files were then used to assign the sequences to operational taxonomic units (OTUs). Then any sequences belonging to undesired taxons (Archaea, Eukaryota, Chloroplast and Mitochondria) were removed and the resulting files were again filtered to remove columns only containing gap characters. Afterward, distance matrices were created for the files where each gap within a sequence was penalized and only OTUs with distances ≤ 0.10 were generated. The resulting distance matrices were then used to cluster the sequences

together into OTUs using the average neighbor clustering algorithm, and consensus taxonomies were generated for the resulting OTUs. The files acquired from the *MOTHUR* data analysis pipeline were used to examine the sequence totals from a taxonomic perspective and an OTU perspective.

5.4. *Taxonomic data analysis*

The taxonomic perspective examined the proportions of the resulting sequence totals from an OTU distance of 0.02 at each taxonomic level for each bacteria. These proportions were calculated for each sample by dividing the total number of sequences for a specific bacterial group at a specific taxonomic level by the total number of sequences for that particular sample. The resulting values were then used to calculate the average proportion of a particular bacterial group at a specific taxonomic level for each sample type (SFS, UNR, VC and IFSi). These averages were then used to compare the various sample types using two-tailed t-tests. Also, standard deviations across all replicates of a particular sample type (e.g., SFS13REP1 and SFS13REP2) were calculated. The calculated averages were used to generate bar graphs at the family taxonomic level. Bacterial groups containing a total of < 5 reads, or possessing *P* values > 0.05 were excluded. The calculated averages were also used to create stacked bar charts that examined the bacterial community structure for each sample type at the phylum level. Any bacterial phylum containing < 5 reads across all 15 samples was excluded. The calculated averages were further processed to generate heat maps of the bacterial families for the sample types (SFS, UNR, VC and IFSi) of the V1–V3 and V3–V5 variable regions. The overall mean for each family was first

calculated from the averages of the sample types. These values were then subtracted from the previously calculated averages of each sample type. The resulting data was then used to generate heat maps using the *gplots* package (Warnes et al., 2015) (version 2.12.1) for R software (R Core Team, 2013) (versions 3.0.2–3.0.3). The heatmaps were generated using the *heatmap.2* command (using ‘*distfun5 dist*’ to calculate the dissimilarity between the rows and columns, ‘*hclustfun5 hclust*’ to determine the hierarchical clustering and ‘*dendrogram – “row”*’ to draw the dendrogram for the rows).

The taxonomic sequence proportion data at the genus level was used for additional cluster analysis through R software. As before, bacterial groups consisting of < 5 reads, or possessing *P* values > 0.05 were excluded. These groups were initially placed into hypothetical clusters via K-means clustering to determine the smallest number of clusters that displayed both trends among sample types as well as differences or similarities among individual samples (V1–V3 genus: 17 clusters V3–V5 genus: 17 clusters). Once the ideal number of clusters was determined, the *K*-means clustering command was again executed with the proper parameters. The resulting, clustered data was then used to generate line graphs to display trends amongst individual samples and sample types.

5.5. *OTU data analysis*

The OTU perspective examined the proportions of the resulting sequence totals for the various OTUs. Ultimately, only OTUs with a total of ≥ 5 reads were included in subsequent analyses. The proportions for each sample were calculated

by dividing the total number of sequences for a particular OTU in a particular sample by the total number of OTUs for said sample. The resulting values were then multiplied by 100. These values were analyzed using the *vegan* package (Oksanen et al., 2016) (version 2.0-9) for R software following the previously described pipeline (White et al., 2015) except the OTU data was not standardized, increased in value, or subjected to a log₁₀ transformation. Also, cluster analyses were executed by calculating Bray–Curtis rather than Euclidean dissimilarity matrices before plotting the data.

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APPENDIX

1. Appendix 1: R Software *Vegan* Package Data Analysis Pipeline

1.1. Command Summaries

diversity – Examines rarefaction species richness and ecological diversity indices. Shannon, Simpson, and Inverse-Simpson diversity indices examine the number of species in each sample (i.e. richness) as well as their relative abundance (i.e. evenness) to determine the community diversity.

cca – Conducts correspondence analysis (cca), canonical correspondence analysis (i.e. constrained correspondence analysis), or partial constrained correspondence analysis. Cca focuses on data variation explained by the chosen constraints rather than focusing on all variation within the data. Partial cca is useful for removing the impact of some conditioning (ex. random) variables before conducting the cca. This pipeline used sample types and treatments as the constraints and conditions.

anova/permutest – Conducts an analysis of variance (ANOVA) type of permutation test for redundancy analysis (rda), distance-based rda (dbrda, capscale) or cca to determine the significance of constraints. The function can assess significance for each term (i.e. constraining variable), marginal term, or constrained axis. This pipeline executed ANOVA according to constrained axis, term, and marginal term on the data acquired from cca. A total of 99 permutations were completed using the full model.

envfit – Fits environmental factors or vectors onto an ordination diagram. Vectorfit locates directions within the ordination space that focus on which environmental vectors exhibit the fastest change and which show maximal correlations with the ordination configuration. Factorfit determines ordination score averages for factor levels and treats unordered and ordered factors in similar ways. This pipeline used data from a previous cca, detrended correspondence analysis (decorana), and nonmetric multidimensional scaling and specified the sample types/treatments as the environmental variables. Also, a *P* value of 0.05

and data acquired from a previous cca were used as the chosen weights when analyzing the cca data.

rda – Conducts a redundancy analysis or a principal component analysis to examine the significance of constraints. Unlike cca, it uses unweighted rather than weighted singular value decomposition and ordinary, unweighted linear regression when analyzing data. This pipeline used sample types and treatments as the constraints and conditions.

capscale – Executes a constrained analysis of principal coordinates in a linear and metric manner. Acts similarly to rda, but it can use non-Euclidean dissimilarity indices such as Bray-Curtis distance. This pipeline used the Bray-Curtis distance matrices and focused on the sample types/treatments for the variables of interest.

decorana – Conducts orthogonal correspondence analysis or detrended correspondence analysis and basic reciprocal averaging. It removes unwanted curvature by using detrending to replace the orthogonalization of the axes and rescales the axes after extraction. The rescaling helps equalize the weighted variance of species scores upon the axis segments rather than utilizing the widths of species responses. This pipeline directed the function to downweigh rare species, execute 4 rescaling cycles, and conduct detrended correspondence analysis.

metaMDS – Conducts an unconstrained ordination method known as nonmetric multidimensional scaling (NMDS) and determines a stable

solution utilizing several random starts. It also standardizes scaling in the results to allow for easier configuration interpretation and adds species scores to the site ordination. Once the final results are acquired, the function attempts to fix indeterminacy of scaling and orientation of the axes within the NMDS process. This pipeline executed the command three times, two of which implemented the “previous.best” parameter.

vegdist – Computes a number of dissimilarity indices of the input data, depending on which indices the user specifies. Some indices are useful for detecting underlying ecological gradients (i.e. Bray-Curtis, Gower, Jaccard and Kulczynski), others for handling varying sample sizes (i.e. Binomial, Morisita, Horn-Morisita, Cao and Chao), and still others for handling unknown and variable sample sizes (i.e. Raup-Crick and Mountford). This pipeline computed the Euclidean, Bray-Curtis, and Cao dissimilarity indices.

meandist – Detects the mean between and within block dissimilarities. It calculates a mean of between-cluster and within-cluster dissimilarities as well as an attribute n of grouping counts. This pipeline used data computed using the Bray-Curtis dissimilarity index and a weight.type parameter value of 1. The aforementioned weight helped provide the correct test for the mean within cluster dissimilarity as well as an acceptable approximation for the classification strength.

hclust – When coupled with the “*plot*” function, draws a dendrogram of the input data matrix based in between-group and within-group dissimilarities. This pipeline generated dendrograms using the data acquired after using the Euclidean or Bray-Curtis dissimilarity index either directly after processing or after further processing the data using the “*mean.dist*” function.

mrpp – Determines if there is a significant difference between two or more sampling units or groups using a multiple response permutation procedure. The user may choose the distance metric used to measure the dissimilarity between two observations. It operates similarly to analysis of variance as it compares dissimilarities among and within groups. This pipeline examined Euclidean, Bray-Curtis, and Cao distances.

adonis – Implements ANOVA via distance matrices by partitioning distance matrices among variation sources and fitting linear models (ex. factors) to metric and semimetric distance matrices. Additionally, it implements a permutation test using pseudo-F ratios. When working with a multivariate data set it is comparable to the multivariate ANOVA. It also acts similar to redundancy analysis. The user may specify the number of permutation to be used during analysis. This pipeline implemented the Bray-Curtis, Euclidean, and Cao methods to calculate pairwise distances within the input data when executing the command. Also, the pipeline directed “*adonis*” to complete 99 permutations and to take the sample types and treatments into consideration whilst analyzing the data.

betadisper/TukeyHSD – Analyze multivariate homogeneity of variances (i.e. group dispersions). The “*betadisper*” function handles non-euclidean distances between group centroids and objects by reducing their original distances to principal coordinates to help assess beta diversity. The “*TukeyHSD*” function generates a set of confidence intervals based on differences between the mean distance-to-centroids from levels of grouping factors possessing the specified family-wise probability of coverage. These functions can measure the variance (i.e. multivariate dispersion) of a group of samples by calculating the average distances of the group members to either the spatial median or the group centroid in multivariate space. They can also test if the variances of one or more groups are different by subjecting the distances of the group centroid to the group members to ANOVA. This pipeline used the functions on data generated after executing the “*vegdist*” function using the Bray-Curtis dissimilarity index.

1.2. Example Pipeline

Input:

```
library(vegan)
```

Output:

```
Loading required package: permute
```

```
Loading required package: lattice
```

```
This is vegan 2.0-9
```

Input:

```
dggefig8data = read.csv("/Volumes/SSlab_RAID/Laura/DGGE/dggefig8data.csv",
  header=TRUE)
```

```
dggefig8geno = read.csv("/Volumes/SSlab_RAID/Laura/DGGE/dggefig8geno.csv",
  header=TRUE)
```

```
dggefig8shdiv <- diversity(dggefig8data)
```

```
dggefig8shdiv
```

Output:

```
[1] 5.803384 5.742236 5.770404 5.765056 5.821366 5.753768 5.945040
5.877472 5.698171 5.732413 5.861938 5.973640 5.683820 5.581633
5.490877
```

Input:

```
dggefig8sim <- diversity(dggefig8data,"simpson")
dggefig8sim
```

Output:

```
[1] 0.9959221 0.9955815 0.9955997 0.9953774 0.9955534 0.9955425
     0.9965353 0.9961673 0.9950882 0.9947533 0.9957854 0.9966014
     0.9952611 0.9943126 0.9936526
```

Input:

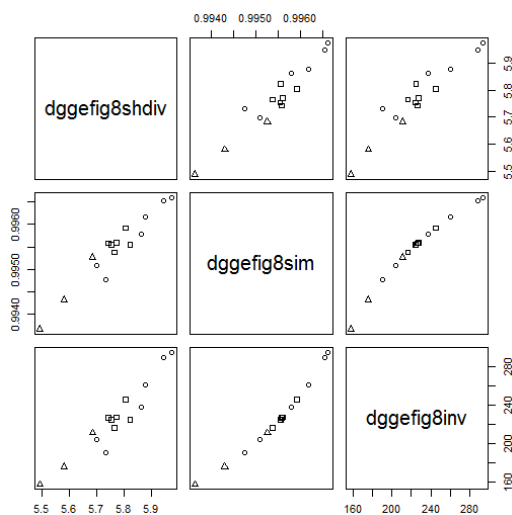
```
dggefig8inv <- diversity(dggefig8data,index="invsimpson")
dggefig8inv
```

Output:

```
[1] 245.2219 226.3230 227.2569 216.3282 224.8901 224.3420 288.6289
     260.9157 203.5924 190.5963 237.2681 294.2357 211.0176 175.8258
     157.5444
```

Input:

```
pairs(cbind(dggefig8shdiv,dggefig8sim,dggefig8inv),pch=c(rep(0,6),
  rep(1,6),rep(2,3)))
```

Output:**Input:**

```
dggefig8cca <- cca(dggefig8data~Genotype,data=dggefig8geno)
dggefig8cca
```

Output:

```
Call: cca(formula = dggefig8data ~ Genotype, data = dggefig8geno)
```

	Inertia	Proportion	Rank
Total	0.4754	1.0000	
Constrained	0.2581	0.5429	2
Unconstrained	0.2173	0.4571	12

Inertia is mean squared contingency coefficient

Eigenvalues for constrained axes:

CCA1	CCA2
0.18807	0.07002

Eigenvalues for unconstrained axes:

CA1	CA2	CA3	CA4	CA5	CA6	CA7
0.049123	0.043211	0.035812	0.018453	0.015214	0.013607	0.011015
CA8	CA9	CA10	CA11	CA12		
0.009671	0.008397	0.006226	0.003598	0.002982		

Input:

```
anova(dggefig8cca)
```

Output:

```
Permutation test for cca under reduced model
```

```
Model: cca(formula = dggefig8data ~ Genotype,
data = dggefig8geno)
```

	Df	Chisq	F	N.Perm	Pr(>F)
Model	2	0.2581	7.1258	199	0.005 **
Residual	12	0.2173			

```
---
```

```
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05
'.' 0.1 '.' 1
```

Input:

```
anova(dggefig8cca,by="axis")
```

Output:

```
Model: cca(formula = dggefig8data ~ Genotype, data = dggefig8geno)
```

	Df	Chisq	F	N.Perm	Pr(>F)
CCA1	1	0.1881	10.3853	199	0.005 **
CCA2	1	0.0700	3.8664	199	0.005 **
Residual	12	0.2173			

```
---
```

```
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05
'.' 0.1 '.' 1
```

Input:

```
anova(dggefig8cca,by="terms")
```

Output:

```
Permutation test for cca under reduced model
```

```
Terms added sequentially (first to last)
```

```
Model: cca(formula = dggefig8data ~ Genotype,
data = dggefig8geno)
```

	Df	Chisq	F	N.Perm	Pr(>F)
Genotype	2	0.2581	7.1258	99	0.01 **
Residual	12	0.2173			

```
---
```

```
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05
'.' 0.1 '.' 1
```

Input:

```
anova(dggefig8cca,by="margin")
```

Output:

```
Permutation test for cca under reduced model
```

```
Marginal effects of terms
```

```
Model: cca(formula = dggefig8data ~ Genotype,
data = dggefig8geno)
```

	Df	Chisq	F	N.Perm	Pr(>F)
Genotype	2	0.2581	7.1258	199	0.005 **
Residual	12	0.2173			

```
---
```

```
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05
'.' 0.1 '.' 1
```

Input:

```
permutest(dggefig8cca,permutations=99,model="full")
```

Output:

```
Permutation test for cca
```

```
Call: cca(formula = dggefig8data ~ Genotype,
data = dggefig8geno)
```

```
Permutation test for all constrained eigenvalues
```

```
Pseudo-F: 7.125839 (with 2, 12 Degrees of Freedom)
```

```
Significance: 0.01
```

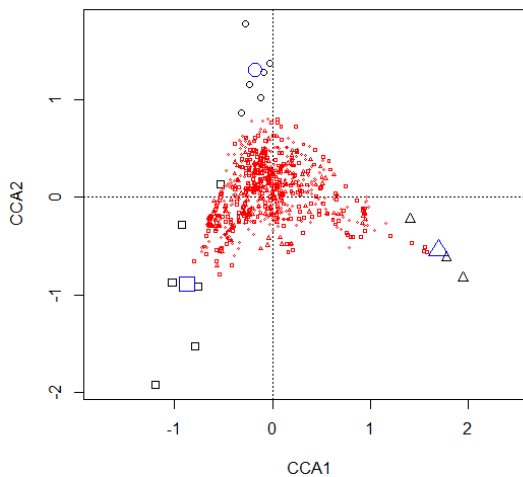
```
Based on 99 permutations under full model.
```

Input:

```

plot(dggefig8cca)
plot(dggefig8cca, display="sites", type="n")
with(dggefig8geno, points(dggefig8cca, display="sites", pch=c(rep(0, 6),
  rep(1, 6), rep(2, 3))))
with(dggefig8geno, points(dggefig8cca, display="species", pch=c(rep(0, 6),
  rep(1, 6), rep(2, 3)), col="red", cex=0.4))
with(dggefig8geno, points(dggefig8cca, display="lc", pch=c(rep(0, 6), rep(1, 6),
  rep(2, 3)), col="blue", cex=2))

```

Output:**Input:**

```

dggefig8ccaefit <- envfit(dggefig8cca~Genotype, dggefig8geno, perm=99,
  w=weights(dggefig8cca))
dggefig8ccaefit

```

Output:

```
***FACTORS:
```

```
Centroids:
```

	CCA1	CCA2
GenotypeIFSi_MS	-0.1730	1.2969
GenotypeUNR_MS	1.6972	-0.5447
GenotypeVC_MS	-0.8741	-0.8876

```
Goodness of fit:
```

	r2	Pr(>r)
Genotype	0.8857	0.01 **

```
---
```

```
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05
'.' 0.1 '.' 1
```

```
P values based on 99 permutations.
```

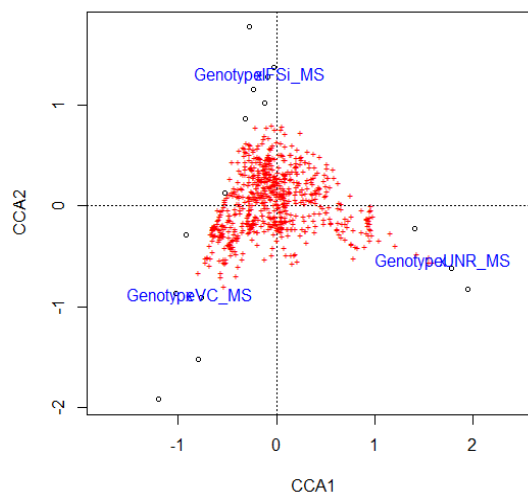
Input:

```

plot(dggefig8cca, type="p")
plot(dggefig8ccaefit, p.max=0.05, col="blue")

```

Output:



Input:

```
dggefig8rda <- rda(dggefig8data~Genotype,data=dggefig8geno)
dggefig8rda
```

Output:

```
Call: rda(formula = dggefig8data ~ Genotype, data = dggefig8geno)
```

	Inertia	Proportion	Rank
Total	7.055e+06	1.000e+00	
Constrained	4.332e+06	6.140e-01	2
Unconstrained	2.723e+06	3.860e-01	12

Inertia is variance

Eigenvalues for constrained axes:

RDA1	RDA2
3326589	1005258

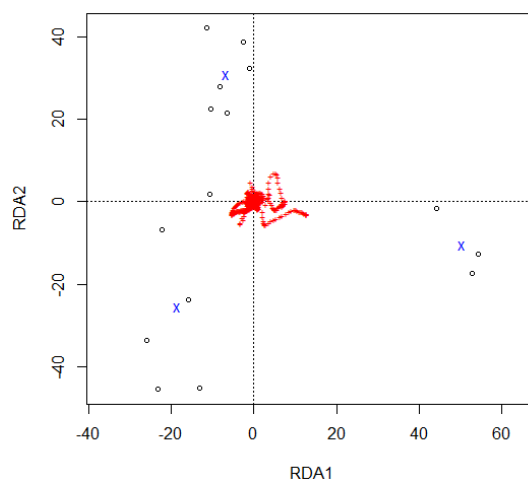
Eigenvalues for unconstrained axes:

PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
764777	663089	349097	274675	185444	128439	101474	80991	67572
PC10	PC11	PC12						
56197	32936	18015						

Input:

```
plot(dggefig8rda)
```

Output:



Input:

```
dggefig8caps <- capscale(dggefig8data~Genotype,dggefig8geno,dist="bray")
dggefig8caps
```

Output:

```
Call: capscale(formula = dggefig8data ~ Genotype,
data = dggefig8geno, distance = "bray")
```

	Inertia	Proportion	Rank
Total	1.034235		
Real Total	1.037232	1.000000	
Constrained	0.579827	0.559014	2
Unconstrained	0.457405	0.440986	12
Imaginary	-0.002997		1

Inertia is squared Bray distance

Eigenvalues for constrained axes:

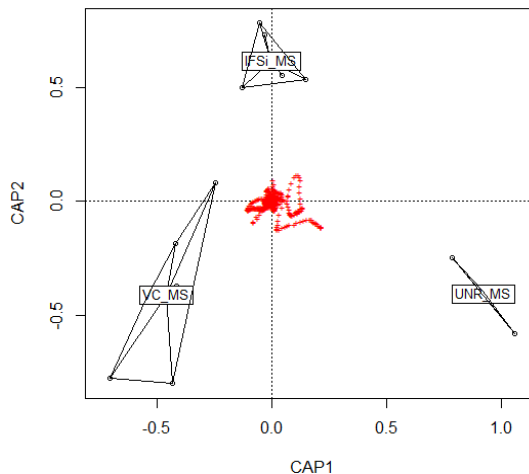
CAP1	CAP2
0.4028	0.1770

Eigenvalues for unconstrained axes:

MDS1	MDS2	MDS3	MDS4	MDS5	MDS6	MDS7
0.121930	0.092695	0.079436	0.048431	0.032392	0.029344	0.016506
MDS8	MDS9	MDS10	MDS11	MDS12		
0.013229	0.009210	0.008637	0.004559	0.001037		

Input:

```
plot(dggefig8caps)
with(dggefig8geno,ordispider(dggefig8caps,Genotype,label=TRUE))
with(dggefig8geno,ordihull(dggefig8caps,Genotype,label=FALSE))
```

Output:**Input:**

```
dggefig8dca <- decorana(dggefig8data,iweigh=1,iresc=4,ira=0)
dggefig8dca
```

Output:

```
Call:
decorana(veg = dggefig8data, iweigh = 1, iresc = 4, ira = 0)
```

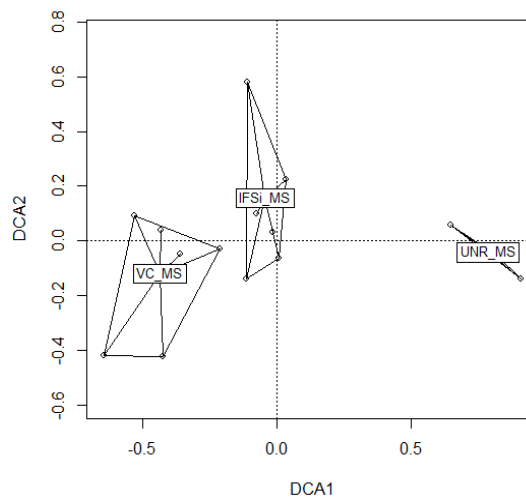
Detrended correspondence analysis with 26 segments.
Rescaling of axes with 4 iterations.
Downweighting of rare species from fraction 1/5.

	DCA1	DCA2	DCA3	DCA4
Eigenvalues	0.1916	0.05590	0.04123	0.035535
Decorana values	0.1950	0.03778	0.02146	0.007043
Axis lengths	1.5529	1.00258	0.75454	0.668909

Input:

```
plot(dggefig8dca,display="sites")
with(dggefig8geno,ordihull(dggefig8dca,Genotype,label=FALSE,col="black"))
with(dggefig8geno,ordispider(dggefig8dca,Genotype,label=TRUE,col="black"))
```

Output:



Input:

```
dggefig8mds <- metaMDS(dggefig8data)
```

Output:

```

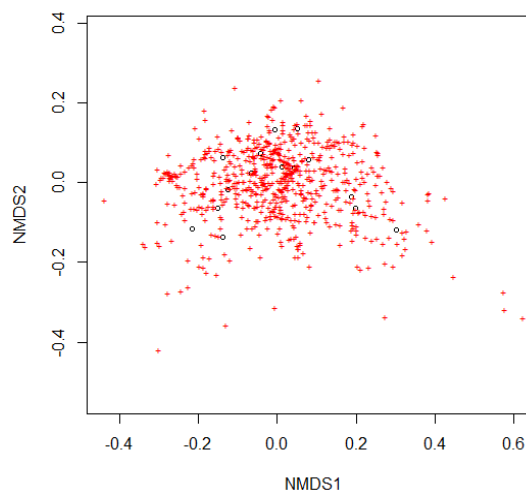
Square root transformation
Wisconsin double standardization
Run 0 stress 0.07805188
Run 1 stress 0.09602721
Run 2 stress 0.09581756
Run 3 stress 0.07805023
... New best solution
... procrustes: rmse 0.001170683  max resid 0.00308235
*** Solution reached

```

Input:

```
plot(dggefig8mds)
```

Output:



Input:

```
dggefig8mds <- metaMDS(dggefig8data,previous.best=dggefig8data)
```

Output:

```

Square root transformation
Wisconsin double standardization
Run 0 stress 0.5572142
Run 1 stress 0.09741051
... New best solution
... procrustes: rmse 0.1694327  max resid 0.2853891
Run 2 stress 0.07805309
... New best solution
... procrustes: rmse 0.09208478  max resid 0.2624023
Run 3 stress 0.07805212
... New best solution
... procrustes: rmse 0.002173156  max resid 0.005550453
*** Solution reached

```

Input:

```
dggefig8mds2 <- metaMDS(dggefig8data,previous.best=dggefig8data)
```

Output:

```

Square root transformation
Wisconsin double standardization
Run 0 stress 0.5572142
Run 1 stress 0.07805023
... New best solution
... procrustes: rmse 0.1770708  max resid 0.2961972
Run 2 stress 0.09184945
Run 3 stress 0.09581706
Run 4 stress 0.07805319
... procrustes: rmse 0.001262811  max resid 0.003085313
*** Solution reached

```

Input:

```
dggefig8dcaefit <- envfit(dggefig8dca~Genotype,dggefig8geno,perm=99)
dggefig8dcaefit
```

Output:

```

***FACTORS:

Centroids:
                DCA1   DCA2
GenotypeIFSi_MS -0.0451  0.1566
GenotypeUNR_MS  0.7848 -0.0424
GenotypeVC_MS   -0.4370 -0.1214

Goodness of fit:
                r2   Pr(>r)
Genotype 0.8192   0.01 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05
'.' 0.1 '.' 1
P values based on 99 permutations.

```

Input:

```
dggefig8mds2efit <- envfit(dggefig8mds2~Genotype,dggefig8geno,perm=99)
dggefig8mds2efit
```

Output:

```

***FACTORS:

Centroids:
                NMDS1  NMDS2
GenotypeIFSi_MS  0.0235  0.0791
GenotypeUNR_MS  0.2301 -0.0738
GenotypeVC_MS   -0.1386 -0.0422

Goodness of fit:
                r2   Pr(>r)
Genotype 0.8241   0.01 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05
'.' 0.1 '.' 1
P values based on 99 permutations.

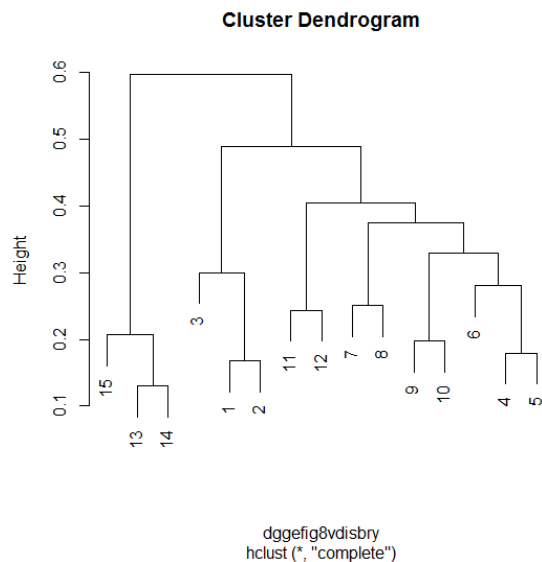
```

Input:

```

dggefig8vdiseuc <- vegdist(dggefig8data,method="euclidean")
dggefig8vdisbry <- vegdist(dggefig8data,method="bray")
dggefig8vdiscao <- vegdist(dggefig8data,method="cao")
dggefig8vdisbrydend <- hclust(dggefig8vdisbry)
plot(dggefig8vdisbrydend)
groupsfig8 <- factor(c(rep(0,6),rep(1,6),rep(2,3)),labels=c("VC_MS",
"IFSi_MS","UNR_MS"))

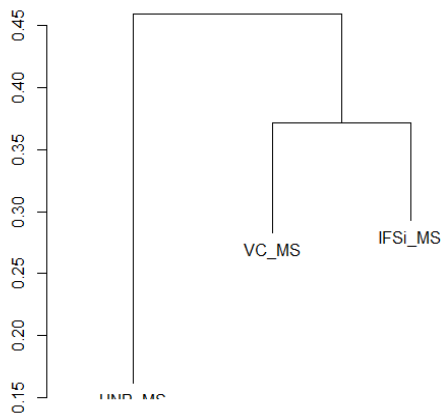
```

Output:**Input:**

```

dggefig8.mdist.bry <- meandist(dggefig8vdisbry,groupsfig8,weight.type=1)
plot(dggefig8.mdist.bry,"dendrogram")

```

Output:**Input:**

```

dggefig8mrpp.euc <- mrpp(dggefig8data,groupsfig8,distance="euclidean")
dggefig8mrpp.euc

```

Output:

```
Call:
mrpp(dat = dggefig8data, grouping = groupsfig8,
      distance = "euclidean")

Dissimilarity index: euclidean
Weights for groups: n

Class means and counts:

      VC_MS IFSi_MS UNR_MS
delta 2778  2409   1701
n       6     6     3

Chance corrected within-group agreement A: 0.3245
Based on observed delta 2415 and expected delta 3575

Significance of delta: 0.001
Based on 999 permutations
```

Input:

```
dggefig8mrpp.bry <- mrpp(dggefig8data, groupsfig8, distance="bray")
dggefig8mrpp.bry
```

Output:

```
Call:
mrpp(dat = dggefig8data, grouping = groupsfig8,
      distance = "bray")

Dissimilarity index: bray
Weights for groups: n

Class means and counts:

      VC_MS IFSi_MS UNR_MS
delta 0.2826 0.2927 0.1612
n       6     6     3

Chance corrected within-group agreement A: 0.2941
Based on observed delta 0.2624 and expected delta 0.3717

Significance of delta: 0.001
Based on 999 permutations
```

Input:

```
dggefig8mrpp.cao <- mrpp(dggefig8data, groupsfig8, distance="cao")
dggefig8mrpp.cao
```

Output:

```
Call:
mrpp(dat = dggefig8data, grouping = groupsfig8,
      distance = "cao")

Dissimilarity index: cao
Weights for groups: n

Class means and counts:

      VC_MS IFSi_MS UNR_MS
delta 0.8249 0.7122 0.7366
n       6     6     3

Chance corrected within-group agreement A: 0.1975
Based on observed delta 0.7621 and expected delta 0.9497

Significance of delta: 0.001
Based on 999 permutations
```

Input:

```
dggefig8ado.bry <- adonis(dggefig8data ~ Genotype, data=dggefig8geno,
                          method="bray", permutations=99)
dggefig8ado.bry
```


Output:

Call:

```
adonis(formula = dggefig8data ~ Genotype, data = dggefig8geno,
permutations = 99, method = "bray")
```

Terms added sequentially (first to last)

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Genotype	2	0.57981	0.289903	7.6554	0.56061	0.01 **
Residuals	12	0.45443	0.037869		0.43939	
Total	14	1.03424			1.00000	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05
'.' 0.1 '.' 1

Input:

```
dggefig8ado.cao <- adonis(dggefig8data ~ Genotype, data=dggefig8geno,
method="cao",permutations=99)
```

dggefig8ado.cao

Output:

Call:

```
adonis(formula = dggefig8data ~ Genotype, data = dggefig8geno,
permutations = 99, method = "cao")
```

Terms added sequentially (first to last)

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Genotype	2	3.0733	1.53663	5.1222	0.46054	0.01 **
Residuals	12	3.5999	0.29999		0.53946	
Total	14	6.6732			1.00000	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05
'.' 0.1 '.' 1

Input:

```
contmat <- cbind(c(0,1,-1),c(1,0,-1),c(1,-1,0))
```

contmat

Output:

	[,1]	[,2]	[,3]
[1,]	0	1	1
[2,]	1	0	-1
[3,]	-1	-1	0

Input:

```
Genotypes <- dggefig8geno$Genotype
```

Genotypes

Output:

```
[1] VC_MS VC_MS VC_MS VC_MS VC_MS VC_MS IFSi_MS
IFSi_MS IFSi_MS IFSi_MS IFSi_MS IFSi_MS UNR_MS UNR_MS UNR_MS
Levels: IFSi_MS UNR_MS VC_MS
```

Input:

```
contrasts(Genotypes) <- contmat[,1:3]
```

contrasts(Genotypes)

Output:

	[,1]	[,2]
IFSi_MS	0	1
UNR_MS	1	0
VC_MS	-1	-1

Input:

```
trt2v3 <- model.matrix(~Genotypes)[,2]
```

trt2v3

Output:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
-1	-1	-1	-1	-1	-1	0	0	0	0	0	0	1	1	1

Input:

```
trt1v3 <- model.matrix(~Genotypes)[,3]
```

Output:

```
trtlv3
  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15
-1 -1 -1 -1 -1 -1  1  1  1  1  1  1  0  0  0
```

Input:

```
dggefig8.ado <- adonis(dggefig8data~Genotype,data=dggefig8geno,
  method="bray",permutations=99)
```

```
dggefig8.ado
```

Output:

```
Call:
```

```
adonis(formula = dggefig8data ~ Genotype, data = dggefig8geno,
  permutations = 99, method = "bray")
```

```
Terms added sequentially (first to last)
```

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Genotype	2	0.57981	0.289903	7.6554	0.56061	0.01 **
Residuals	12	0.45443	0.037869		0.43939	
Total	14	1.03424			1.00000	

```
---
```

```
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05
'.' 0.1 '.' 1
```

Input:

```
dggefig8.ado.con <- adonis(dggefig8data~trt2v3+trtlv3,method="bray",
  permutations=99)
```

```
dggefig8.ado.con
```

Output:

```
Call:
```

```
adonis(formula = dggefig8data ~ trt2v3 + trtlv3, permutations =
  99, method = "bray")
```

```
Terms added sequentially (first to last)
```

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
trt2v3	1	0.39173	0.39173	10.3444	0.37877	0.01 **
trtlv3	1	0.18807	0.18807	4.9664	0.18185	0.01 **
Residuals	12	0.45443	0.03787		0.43939	
Total	14	1.03424			1.00000	

```
---
```

```
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05
'.' 0.1 '.' 1
```

Input:

```
groupsfig8 <- factor(c(rep(0,6),rep(1,6),rep(2,3)),labels=c("VC_MS",
  "IFSi_MS","UNR_MS"))
```

```
dggefig8vdisbry <- vegdist(dggefig8data,method="bray")
```

```
dggefig8bdispbry <- betadisper(dggefig8vdisbry,groupsfig8)
```

```
anova(dggefig8bdispbry)
```

Output:

```
Analysis of Variance Table
```

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Groups	2	0.022408	0.0112040	8.0579	0.006045 **
Residuals	12	0.016685	0.0013904		

```
---
```

```
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05
'.' 0.1 '.' 1
```

Input:

```
(dggefig8bdispbry.HSD <- TukeyHSD(dggefig8bdispbry))
```

Output:

```
Tukey multiple comparisons of means
 95% family-wise confidence level

Fit: aov(formula = distances ~ group, data = df)

$group
      diff      lwr      upr    p adj
IFSi_MS-VC_MS  0.005750997 -0.05168421  0.06318620 0.9615509
UNR_MS-VC_MS  -0.093536887 -0.16388036 -0.02319342 0.0103745
UNR_MS-IFSi_MS -0.099287883 -0.16963135 -0.02894441 0.0070241
```

1.3. References

1. Oksanen, J., Blanchet, G.F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D. et al. (2016) *vegan: Community Ecology Package*. In.
2. R Core Team (2015) *R: A Language and Environment for Statistical Computing*. In: R Foundation for Statistical Computing.

2. Appendix 2: MOTHUR Software Data Analysis Pipeline**2.1. Command Summaries**

unique.seqs – Identifies unique sequences within the input file and groups identical sequences together to better visualize the bacterial diversity amongst the samples.

summary.seqs – Summarizes the sequences to track the total percentage of sequences removed later in the pipeline.

align.seqs – Aligns sequences to a 16S rRNA SILVA database – uploaded by the user – to ensure they belong to actual organisms rather than random sequences resulting from pyrosequencing errors or from the interactions of forward and reverse primers during PCR.

screen.seqs – Removes sequences with unaligned ends, possessing 1 or more ambiguous bases, and a chain of homopolymers > 8 to remove sequences of poor or questionable quality.

filter.seqs – Removes any empty data columns generated in previous commands. Does not remove sequences.

pre.cluster – Removes sequences likely generated by pyrosequencing errors.

chimera.uchime – Identifies sequences that are likely chimeras (i.e. sequences created from a combination of two different “parents”).

remove.seqs – Removes sequences flagged as chimeras.

classify.seqs – Assigns sequences to their respective taxonomies ranging from the kingdom level down to the species level.

phylo.type – Assigns sequences to OTUs based on their taxonomies.

summary.tax – Summarizes taxonomic information of the sequences (including the assigned names and groups of the sequences).

remove.lineage – Removes sequences belonging to undesired taxons (Archaea, Chloroplasts, Eukaryota, and Mitochondria).

dist.seqs – Calculates uncorrected pairwise distances between aligned sequences.

cluster – Groups related sequences together and assigns them to OTUs.

classify.otu – Generates consensus taxonomies for OTUs. Resulting output files possessing taxonomic and OTU data summaries may be used

for additional analyses in *MOTHUR* or other bioinformatics software (ex. R software).

make.shared – Generates a file specifying the number of times each OTU is found in each sample. Can also produce files containing data useful for plotting as rank abundance plots.

2.2. Example Pipeline

INPUT:

```
unique.seqs(fasta="Name".merge.fasta)
```

OUTPUT:

```
"Name".merge.names  
"Name".merge.unique.fasta
```

INPUT:

```
summary.seqs(fasta="Name".fasta, name="Name".names)
```

OUTPUT:

```
"Name".merge.unique.summary
```

INPUT:

```
align.seqs(fasta="Name".merge.unique.fasta, reference=silva.bacteria.fasta, processors=8, flip=t)
```

OUTPUT:

```
"Name".merge.unique.align  
"Name".merge.unique.align.report  
"Name".merge.unique.flip.accnos
```

INPUT:

```
screen.seqs(fasta="Name".merge.unique.align, name="Name".merge.names, optimize=start-end,  
criteria=90, maxambig=0, maxhomop=8, group="Name".groups)
```

OUTPUT:

```
"Name".merge.unique.good.align  
"Name".merge.unique.bad.accnos  
"Name".merge.good.names  
"Name".good.groups
```

INPUT:

```
summary.seqs(fasta="Name".merge.unique.good.align, name="Name".merge.good.names)
```

OUTPUT:

```
"Name".merge.unique.good.summary
```

INPUT:

```
filter.seqs(fasta="Name".merge.unique.good.align, vertical=T, processors=8)
```

OUTPUT:

```
"Name".filter  
"Name".merge.unique.good.filter.fasta
```

INPUT:

```
unique.seqs(fasta="Name".merge.unique.good.filter.fasta, name="Name".merge.good.names)
```

OUTPUT:

```
"Name".merge.unique.good.filter.names  
"Name".merge.unique.good.filter.unique.fasta
```

INPUT:

```
summary.seqs(fasta="Name".merge.unique.good.filter.unique.fasta,  
name="Name".merge.unique.good.filter.names)
```

OUTPUT:

```
"Name".merge.unique.good.filter.unique.summary
```

INPUT:

```
pre.cluster(fasta="Name".merge.unique.good.filter.unique.fasta,
name="Name".merge.unique.good.filter.names, group="Name".good.groups)
```

OUTPUT:

```
"Name".merge.unique.good.filter.unique.precluster.fasta
"Name".merge.unique.good.filter.unique.precluster.names
"Name".merge.unique.good.filter.unique.precluster."Sample1".map
"Name".merge.unique.good.filter.unique.precluster."Sample2".map
"Name".merge.unique.good.filter.unique.precluster."Sample3".map
"Name".merge.unique.good.filter.unique.precluster."Sample4".map
```

INPUT:

```
summary.seqs(fasta="Name".merge.unique.good.filter.unique.precluster.fasta,
name="Name".merge.unique.good.filter.unique.precluster.names)
```

OUTPUT:

```
"Name".merge.unique.good.filter.unique.precluster.summary
```

INPUT:

```
chimera.uchime(fasta="Name".merge.unique.good.filter.unique.precluster.fasta,
name="Name".merge.unique.good.filter.unique.precluster.names, group="Name".good.groups,
processors=8)
```

OUTPUT:

```
"Name".merge.unique.good.filter.unique.precluster.uchime.chimeras
"Name".merge.unique.good.filter.unique.precluster.uchime.accnos
```

INPUT:

```
remove.seqs(accnos="Name".merge.unique.good.filter.unique.precluster.uchime.accnos,
fasta="Name".merge.unique.good.filter.unique.precluster.fasta,
name="Name".merge.unique.good.filter.unique.precluster.names, group="Name".good.groups)
```

OUTPUT:

```
"Name".merge.unique.good.filter.unique.precluster.pick.names
"Name".merge.unique.good.filter.unique.precluster.pick.fasta
"Name".good.pick.groups
```

INPUT:

```
summary.seqs(fasta="Name".merge.unique.good.filter.unique.precluster.pick.fasta,
name="Name".merge.unique.good.filter.unique.precluster.pick.names)
```

OUTPUT:

```
"Name".merge.unique.good.filter.unique.precluster.pick.summary
```

INPUT:

```
classify.seqs(fasta="Name".merge.unique.good.filter.unique.precluster.pick.fasta,
name="Name".merge.unique.good.filter.unique.precluster.pick.names,
group="Name".good.pick.groups, template=silva.bacteria.fasta, taxonomy=silva.bacteria.silva.tax,
cutoff=80, processors=8)
```

OUTPUT:

```
"Name".merge.unique.good.filter.unique.precluster.pick.silva.wang.taxonomy
"Name".merge.unique.good.filter.unique.precluster.pick.silva.wang.tax.summary
```

INPUT:

```
phylotype(taxonomy="Name".merge.unique.good.filter.unique.precluster.pick.silva.wang.taxonomy,
name="Name".merge.unique.good.filter.unique.precluster.pick.names)
```

OUTPUT:

```
"Name".merge.unique.good.filter.unique.precluster.pick.silva.wang.tx.list
"Name".merge.unique.good.filter.unique.precluster.pick.silva.wang.tx.sabund
"Name".merge.unique.good.filter.unique.precluster.pick.silva.wang.tx.rabund
```

INPUT:

```
summary.tax(taxonomy="Name".merge.unique.good.filter.unique.precluster.pick.silva.wang.taxonomy,
name="Name".merge.unique.good.filter.unique.precluster.pick.names,
group="Name".good.pick.groups)
```

OUTPUT:

```
"Name".merge.unique.good.filter.unique.precluster.pick.silva.wang.tax.summary
```

INPUT:

```
remove.lineage(fasta="Name".merge.unique.good.filter.unique.precluster.pick.fasta,
name="Name".merge.unique.good.filter.unique.precluster.pick.names,
group="Name".good.pick.groups, taxonomy=silva.bacteria.silva.tax, taxon=Archaea-Eukaryota-
Chloroplast-Mitochondria)
```

OUTPUT:

```

silva.bacteria.silva.pick.tax
"Name".merge.unique.good.filter.unique.precluster.pick.pick.names
"Name".merge.unique.good.filter.unique.precluster.pick.pick.fasta
"Name".good.pick.pick.groups

```

INPUT:

```

summary.seqs(fasta="Name".merge.unique.good.filter.unique.precluster.pick.pick.fasta,
name="Name".merge.unique.good.filter.unique.precluster.pick.pick.names)

```

OUTPUT:

```
"Name".merge.unique.good.filter.unique.precluster.pick.pick.summary
```

INPUT:

```

filter.seqs(fasta="Name".merge.unique.good.filter.unique.precluster.pick.pick.fasta, vertical=T,
processors=8)

```

OUTPUT:

```

"Name".filter
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.fasta

```

INPUT:

```

summary.seqs(fasta="Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.fasta,
name="Name".merge.unique.good.filter.unique.precluster.pick.pick.names)

```

OUTPUT:

```
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.summary
```

INPUT:

```

dist.seqs(fasta="Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.fasta, cutoff=0.10,
calc=eachgap, processors=8)

```

OUTPUT:

```
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.dist
```

INPUT:

```

cluster(column="Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.dist,
name="Name".merge.unique.good.filter.unique.precluster.pick.pick.names)

```

OUTPUT:

```

"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.sabund
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.rabund
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.list

```

INPUT:

```

classify.otu(taxonomy=
"Name".merge.unique.good.filter.unique.precluster.pick.silva.wang.equalized.taxonomy,
list="Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.list,
name="Name".merge.unique.good.filter.unique.precluster.pick.pick.names,
group="Name".good.pick.pick.groups)

```

OUTPUT:

```

"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.unique.cons.taxonomy
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.unique.cons.tax.summary
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.0.01.cons.taxonomy
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.0.01.cons.tax.summary
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.0.02.cons.taxonomy
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.0.02.cons.tax.summary
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.0.03.cons.taxonomy
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.0.03.cons.tax.summary

```

INPUT:

```

make.shared(list="Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.list,
group="Name".good.pick.pick.groups)

```

OUTPUT:

```

"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an.shared
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an."Sample1".rabund
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an."Sample2".rabund
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an."Sample3".rabund
"Name".merge.unique.good.filter.unique.precluster.pick.pick.filter.an."Sample4".rabund

```

2.3. References

1. Schloss, P.D., Gevers, D., and Westcott, S.L. (2011) Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PloS one* **6**: e27310.
2. Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B. et al. (2009) Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology* **75**: 7537-7541.