Investigations of low input turfgrasses

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Dedication

This thesis is dedicated to my Aunt Michelle LaBeau, Mother Lynnette LaBeau, Uncle Art LaBeau and Grandmother Jane LaBeau. As well as to those friends such as Nick Weiss, Dane Verret, Jeanetta Langhorne, Audrey Merriweather, Lucas Plowman, Nick Hayes and Selam Yoseif who have supported me throughout this research project.

Abstract

The development of low-input turfgrasses has been challenging for plant breeders. Turfgrasses typically lack resources for crop improvement that are common in other crops, such as a fully sequenced genome. New tools are needed to study turfgrass and decrease its environmental impact. Two approaches were developed and utilized in this project: investigating the rhizosphere associated with low-input turfgrasses and quantifying silica body deposition in various turfgrass species.

Understanding how microorganisms associate with different species and cultivars of turfgrass may in the future provide researchers the ability to breed for turfgrass plants that form better mutualistic and beneficial relationships with soil microorganisms. In some plants, plant genotype seems to have a significant influence on shaping the microbial community structures. Previous research in Poaceae has shown that a plant genotype has the ability to influence the rhizosphere microbial community structure. The objectives of this study were to (1) determine if turfgrass species affects soil microbial community structure and (2) identify differences in soil microbial community structure of turfgrasses based on nitrogen fertility regime. Rhizosphere and rhizoplane soil samples were taken from six species of turfgrass, prairie junegrass (*Koeleria macrantha* (Ledeb.) Schult.), Kentucky bluegrass (*Poa pratensis* L.), hard fescue (Festuca trachyphylla (Hackel) Krajina), colonial bentgrass (Agrostis capillaris L.), as well as tufted hairgrass (Deschampsia caespitosa (L.) P. Beauv.) and DNA isolated from each sample was then amplicons were pair end sequenced using Illumina HiSeq 2000 at a read length of 2 x 150 base pairs. Data was analyzed using WinSCP, Putty and Mothur. Our results show that the rhizosphere community structure is influenced by location, fertility, and genotype.

We also found that *Thermodesulfobacteria* and *Thaumarchaeota* are associated with fertilized turfgrass soils. Weed incidence, a measure of turf performance, was found to associate to the *Actinobacteria, Verrumicrobia, and Bacteroidetes.* Results from this research will inform turfgrass researchers about the impacts of turfgrass species and nitrogen fertility on soil microbial communities.

The deposition of silicon into epidermic cells of grass species is thought to be an important mechanism that plants use to defend against pests and environmental stresses. Turfgrass cultivars within the same species exhibit different tolerances under traffic stress and some of these differences may be attributed to silica bodies. There are a number of techniques available to study the size, density and distribution pattern of silica bodies in grass leaves. None of those techniques, however, can provide a high-throughput and accurate analysis, especially for a great number of samples. The purpose of this study was to develop a high-throughput method that uses the fluorescence-emitting nature of silica bodies along with fluorescence microscopy and image software to investigate the size, density, and distribution patterns of these bodies in the perennial grass *Koeleria macrantha*. Dry ashing, followed by fluorescent microscopy, was utilized to image silica bodies in prairie junegrass. ImageJ was used to analyze the images for size, number and orientation. We found that dry-ashing and fluorescent microscopy can be combined with ImageJ to create a high throughput method to study silica bodies in turfgrass. Our results indicated that abaxial and adaxial deposition in turfgrasses are significantly different and that turfgrass genotypes showed significant differences in number of carbon inclusions and silica body deposition.

Table of Contents

Acknowledgments i

Dedication ii

Abstract iii

List of tables vi

List of figures vii

Literature Review 1

Chapter 1. Rhizosphere community structure of common cool season turfgrasses 10

Introduction 11

Materials and Methods 17

Results & Discussion 21

Chapter 2. A novel method to characterize silica bodies in grasses 34

Introduction 34

Materials and Methods 37

Results & Discussion 40

Literature Cited 52

Appendix 1. Assessment of microbial communities in greenhouse-grown *Koeleria macrantha* accessions 69

Appendix 2. Assessment soil microbial communities of nineteen cool-season turfgrass species grown in Minnesota 74

List of Tables

Chapter 1

Table 1.1. Spearman correlation of rhizosphere microbial phyla, turfgrass quality, and weed intercept in St. Paul**.** 33

Table A.1 Cool season turfgrasses used in the species demonstration study. 82

List of Figures

Chapter 1

Figure 1.1 Principal component analysis of cool season turfgrass samples at the St. Paul location. 26

Figure 1.2 Distribution of abundant orders found in rhizoplane samples of cool season turfgrass. 27

Figure 1.3 Principal component analysis of cool season turfgrass samples. 28

Figure 1.4 Principal component analysis of cool season turfgrass samples. 29

Figure 1.5 Distribution of taxonomic genus of OTUs found to differ significantly by phyla at St. Paul and Chaska, Minnesota, USA. The OTUs were found to vary significantly by using the Kruskal–Wallis test. 30

Figure 1.6. Redundancy analysis showing variation in phyla explained by species at St. Paul in October 2013. 31

Figure 1.7. Linear discriminant analysis of effect sizes of species, cultivar, species x cultivar, fertilized, and fertilized x species. 32

Chapter 2

Figure 2.1 Size and distribution pattern analysis of silica bodies in *K. macrantha* 'Ireland' using Adobe Photoshop CS5. **a** A randomly selected microscopy image of ashed leaf sample. **b** The selection of silica bodies using "Magic Wand Tool" (background selection) and "Select->Inverse" tool. **c** Pixel reads of entire image. **d** Pixel reads of the selected silica bodies. **e** A list of statistical results. 46

Figure 2.2 *Box plot* illustrates the size variation of silica bodies in 9 junegrass accessions. *Small circles* demonstrate samples with statistically loud noises. 47

Figure 2.3 Property differentiation of silica bodies examined by brightfield and fluorescence microscopy. **a** Brightfield and **b** fluorescence microscopic examination of *K. macrantha* 'Ireland', showing two types of mineral structures developed in silica cells: Type I structures emitted green autofluorescence; Type II structures did not emit green autofluorescence (*red circled*). **c** Brightfield and **d** fluorescence microscopic examination of *K. macrantha* 'Russian Federation', showing that Type I structures developed in silica cells (short cells, *arrows*) emitted autofluorescence. There was also emission of green fluorescence from long cells (*red circle*) defined as type III silica bodies. Images **a–d** share the same magnification rate. 48

Figure 2.4 Silica body distribution differences between abaxial and adaxial leaf epidermis of KM-MN. **a** The abaxial epidermis under brightfield microscopy. **b** The abaxial epidermis under fluorescence microscopy. **c** The adaxial epidermis under brightfield microscopy. **d** The adaxial epidermis under fluorescence microscopy. **e** *Box plot* illustrates the distribution differences of silica bodies between abaxial and adaxial leaf epidermis. Images **a–d** share the same magnification rate. 49

Figure 2.5 Characterization of carbon inclusions in the silica bodies. **a** Carbon inclusions within silica bodies demonstrated brown to black colored granules under brightfield microscopy. **b** Carbon inclusions within silica bodies did not emit green

fluorescence under fluorescence microscopy. **c** Box plot illustrates the distribution differences of silica bodies among 10 accessions (silica body number per 10,000 µm^2, white circles demonstrate loud noises). **d** Box plot exhibits the number differences of carbon inclusions per silica body from the selected four accessions (white circles indicate statistically loud noises). IR ‘Ireland’, CN ‘China’, CO ‘Colorado’, MO ‘Mongolia’, BA ‘Barkoel’, BL ‘Barleria’, MN ‘Minnesota’, RU ‘Russian Federation’, NE ‘Nebraska’, SP ‘Spain’, CI carbon inclusion, SB silica body. Images a and b share the same magnification rate. 50

Figure 2.6 Illustration of sample preparation using dry ash method. **a** Dry ashing was performed by heating leaf-blade samples on a Corning Hot Plate at >608.2 °F/320 °C. **b** Unburned leaf tissues were placed between two micro-slides. **c** Incompletely burned tissue demonstrated brown to black color. **d** Completely dry ashed tissue showed gray to white color. 51

Appendices

Figure A.1 Principal component analysis of *Koeleria macrantha* samples. 76

Figure A.2 Principal component analysis of *Koeleria macrantha* samples. 77

Figure A.3. Distribution of taxonomic genus of OTUs found to differ significantly by origin of *Koeleria macrantha*. 78

Figure A.4. Distribution of taxonomic order of OTUs found to differ significantly by origin of *Koeleria macrantha*. 79

Figure A.5 Distribution of taxonomic genus of OTUs found to differ significantly by species and cultivar. 80

Figure A.6. Redundancy Analysis showing variation in phyla explained by species at St. Paul for the species demonstration study in October 2013. 81

Literature Review

Turfgrass production often requires significant inputs for cultivation

As of 2002, turfgrass acreage was estimated to be 60 million acres in the United States (Lubowski et al., 2006). Turfgrass covers a significant land area of 1.9% of the continental United States, which is an area three times larger than any irrigated crop (Milesi et al., 2005). The turfgrass industry has a significant impact on the economy: in 2005, the annual turfgrass market was around $62.2 billion and the turfgrass industry consists of 822,849 jobs in the United States (Haydu et al., 2006). Management of this turfgrass acreage is intensive and utilizes a large amount of inputs, particularly fertilizer (Jenkins, 1994).

Conventional agriculture relies on fertilizer inputs that may degrade the environment. Of all the fertilizer used in agriculture only 30-50% is used by plants while approximately 45% of phosphorus is taken by plants (Cassman et al., 1999; Smil 1999 and 2000). Fertilizer used in crop production not taken up by organisms is leached through the soil and can run off to bodies of water, where it is associated with being deleterious to terrestrial and aquatic ecosystems and water quality and this fertilizer used in agriculture is contributing to the shift in atmospheric composition (Tilman et al., 2001; Vitousek et al., 1997; Carpenter et al., 1998; Matson, 2002).

Large amounts of pesticides are also applied to turfgrasses worldwide, especially in highly managed landscapes. Cohen et al. (1999) looked at water surrounding 36 golf courses and 3.6% of 16,587 water samples exceeded maximum contamination levels with turfgrass pesticides. As the human population continues to increase, pressures on delicate

ecosystems will likely increase. Since the industrial revolution, this period of time has seen one of the highest periods of extinction of earth history and unlike past abiotic influenced extinction we are now experiencing human influenced extinction.

New avenues need to be explored to reduce our impact on the environment. Breeding plants to more positively affect the soil rhizosphere and better utilizing new knowledge about silica bodies are new avenues for exploration and discovery. The rhizosphere is associated with decreased disease pressure, which means less pesticides and fungicides. The rhizosphere can decrease the need for fertilizers by utilizing the microbial communities to produce the fertilizer. Silica bodies have shown an ability to reduce the impact of abiotic and biotic stressors in various grass species; decreases in basal temperature, insect herbivory and fungal diseases has been recorded in the literature (Rémus-Borel et al., 2005; Ma & Takahashi, 2002; Wang et al., 2005).

Methods to study turfgrass to improve its cultivation are needed to decrease turfgrasses impact on the environment. We are undergoing a human associated mass extinction and modern extinction rates are significantly high and are increasing (Ceballos et al., 2015). Nitrogen fertilizers cause increased emission of nitrous and nitrogen oxides from turfgrass areas (Maggiotto et al., 2000). Pesticides that are associated with turfgrass production and the turfgrass industry have been detected in the surface waters of urban watersheds (Cohen et al., 1999). Nutrient runoff has been associated with eutrophication of local waterways (Sharpley, 1994). Mowing turfgrass often requires significant amounts of fuel as well as greenhouse gasses associated with the combustion of that fuel necessary to run the mowers. Grass plants that are mowed emit four times the CO_2 than

lawns that are mowed less frequently (Allaire, 2008). It is prudent for turfgrass breeders to explore new approaches to improving grasses for low-input systems and thereby decrease the impact of turfgrass cultivation. In this thesis, I will report on two possible approaches: (1) selecting turfgrasses that result in more favorable soil microbial communities, and (2) selecting grasses with high levels of silica bodies.

Turfgrasses and Soil Microbial Communities

How does the soil microbial community benefit a plant?

The soil microbial community is diverse and these microbes can have various functions. Plant root exudates play a role in how these microbial communities perform; sometimes this interaction is positive and sometime it is negative. An example of a positive interaction is exudates causing other plants to produce leafy volatiles to attract predators to the herbivore thus decreasing herbivory (Kessler, 2001). A negative interaction example would include allelopathy in tall fescue (Smith & Martin, 1994) where the exudate is reducing the growth of neighboring plants. Weir (2004) found that some plants produce allelopathic compounds that can diffuse through the rhizosphere that inhibit the growth of other plants.

Plant root exudates are often the communication molecules between plants and microorgansisms. These exudates can play a role in communication between plants and microbes. Simons et al. (1997) found that that organic acids produced by bacteria can cause chemotaxis responses; they also found that amino acids such as leucine, arginine and histidine can allow colonization of root tips by *Pseudomonas fluorescens*.

Pseudomonas flurescens 2-79 can synthesize phenazine-1-carboxylic acid, which inhibits the growth of *Gaeumannomyces graminis* and other fungal root pathogens; this bacterium confers disease resistance to plants by intricate mutualistic relationships (Thomashow et al., 1990). Hyphal growth and duration of growth are increased in the presence of plant root secreted flavonoids (Bécard, 1992).

Rhizosphere microbes can also aid in decreasing herbivory. Herbivory of the plant *Elymus repens* L. (Gould) (commonly known as couch grass) will cause the plant to produce volatiles that decrease the herbivory by attracting parasitoid wasps; these volatiles can be excreted into the roots and recognized by other plants in the surrounding rhizosphere and these plants will then produce these volatiles decreasing further herbivory by aphids (Glinwood et al., 2003).

These complex relationships between organisms appear to be controlled by root exudates. The exudates can produce plant hormones that affect the growth and development of the plant. In addition, organisms can produce antimicrobial compounds that can decrease the colonization of the rhizosphere by deleterious plant infecting microbes. The more that is understood about these complex systems, the better chance there is that landscape management pressures upon delicate ecosystems can be reduced and new ways of managing landscapes.

Plant and microorganism symbiosis is important for the survival of some plants and microorganisms Plants have coevolved with many microorganisms this coevolution has selected for some of these microbes to have mutual relationships with plants (Chisholm et al., 2006). These mutual relationships may reduce disease pressure, affect

the growth and development of the plant as well as decrease herbivory. The microbial community structure in the rhizosphere is critical for plant performance as it aids the plant in nutrient uptake and helps protect against pathogen infection (Berendsen et al., 2012). Certain exudates have larger effects on the microbial community structure of the rhizosphere than others; for instance, organic acids have a greater effect on changing the microbial community structure than do sugars in both richness of the community as well as the shifts of dominant taxa (Shi al., 2011).

Exudates can affect gene regulation in both plants and microorganisms. These exudates can cause gene activation in fungi; Tamasloukht et al. (2003) found that gene activation caused by plant root exudates boosted fungal respiratory activity and increased hyphal branching. This study showed that plant exudates have an effect on fungi but it has also been shown that fungal exudates can effect plant gene activation; the pMtENOD11-gusA gene was expressed in the roots of *Medicago truncatula* (Gaertn.) by a fungal diffusible factor. These results give some insight into the communication between plants and microbes.

Plant diseases can often be inhibited by certain rhizosphere bacteria. The rhizosphere has been shown to improve plant cultivation and result in decreased plant disease incidence (Garbeva et al., 2004; Kloepper et al., 1980). Studies have also shown a beneficial rhizosphere produces a bacterial environment that increases plant usable nutrients and that plants will promote certain microbial communities for increased access to limiting nutrients (Hamilton & Frank, 2001; Hinsinger, 1998).

How does a plant affect the soil microbial community structure?

In the past several years, there has been clear evidence that plants affect the structure of soil microbial communities. Significant differences in microbial community structure observed between *Zea mays* L. (maize) and *Beta vulgaris* L. (sugar beet) as well as among maize cultivars; different species as well as cultivars show different distributions of bacterial phyla (Philippot, et al., 2013). Limited breeding research has been carried out related to the rhizosphere. Ismail et al. (2007) found many genes in *Oryza sativa* associated with superior performance in nutrient limited soils (Ismail et al., 2007). Oger et al. (1997) used a transgenic approach to study how *Lotus corniculatus* affects soil microbes; they transformed plants to produce the opines mannopine and nopaline and found that 12 different species of bacteria were associated with transformed plants. Peiffer et al. (2013) found a statistically significant association of cultivar and microbial community structure by comparing the sequenced the genomes of 28 different maize cultivars with rhizosphere community structure. Maize genotype has been shown to affect the microbial community structure, biomass, activity and growth as well as when maize is fertilized in different amounts the microbial community structure will differ based on fertilizer amount (Aira et al., 2010). These results point to the potential of improving plants that have a more positive influence on soil microbial communities.

Different species of grasses have been found to differ in the bacteria and fungi found in their rhizospheres. The grasses *Stipa, Hilaria and Bromus* show different microbial composition of the rhizosphere; these three grass genera with very different root architecture (Kuske, 2002). Maize genotype has been shown to affect the microbial

community structure, biomass, activity and growth as well as when maize is fertilized in different amounts the microbial community structure will differ based on fertilizer amount (Aira et al., 2010). Another study in maize found that there is a significant effect of maize line on microbial community structure but so does genetic group (tropical, corn belt dent, stiff stalk, northern flint and European flint). This result seems to indicate that there is some genic association between plant genotype and the rhizosphere microbial community structure (Bouffaud et al., 2012). A similar association has been found in Arabidopsis where host genotype affected ribotype profiles of soil bacteria (Bulgarelli, 2015) (ribotyping is a molecular technique used to differentiate strains of bacteria). Lundberg et al. (2012) established that Arabidopsis genotype can create either differential recruitment of beneficial microbes and/or differential exclusion. This suggests that genotype could be selected for the recruitment of beneficial microbes and the exclusion of deleterious or benign.

Plant rhizosphere community structure appears to have limited plasticity influenced by plant genotype. The plant trait of having a beneficial rhizosphere microbial community structure has been found to improve plant nutrient acquisition, useful to exploit for bioremediation (Anderson et al., 1993), increase plant vigor (Jones and Darrah, 1994), improve drought resistance (Yang et al., 2009), increase the build up of humus, improve carbon sequestration (Clemmenson, 2013), and affect disease resistance (Zamioudis, 2012).

Silica bodies and plant stress

Grasses have specialized silica cells that produce bodies that are distinguishable as grass phytoliths/silica bodies. Little is currently know about the importance of these silica bodies in turfgrass growth and development, and even more importantly, there is a lack of information about how to effectively and efficiently image and quantify these bodies, which is an important first step in further research. Silica bodies have not been studied in turfgrass plants and are worthy of further investigation due to their likely role in stress tolerance. Selecting turfgrasses based on silica body deposition may improve grass performance and decrease inputs needed to grow these turfgrasses.

In *Oryza sativa* L. increased silica bodies and silica fertilizer increases yield, decreases disease and pests in rice (Ranganathan et al., 2006). Silica seems to be a very important for the growth and development of some grass species. In the rice shoot several fold more Si can be found than the amount of N, P or K (Savant, 1996). This amount of silica may signify that silica has a very important role in plant growth and development. Applications of silica based fertilizer to rice paddies significantly increases grain yield (Takahashi, 1974). Silica fertilizer has been shown to improve rice resistance to fungi (Mengel & Kirby, 2001).

Plant silica has shown to be very effective in mitigating stressors such as fungal colonization, herbivory, wear, and drought. Silica bodies are found to deter feeding and increase the abrasiveness of the blades of grass (Vicari & Bazely, 1993). Soluble silica fertilizer when compared to a no additional silica control has the ability to decrease fungal disease severity of brown patch and dollar spot by up to 30% (Uriarte et al., 2004). Trenholm (2001) applied potassium silicate at two foliar rates (1.1 and 2.2 kg Si ha^{-1}) and

as a soil drench of 22.4 kg Si $ha^{-1}$and found that application of K + Si reduced wear tolerance in *Paspalum vaginatum* Sw. (seashore paspalam).

Silica bodies have an influence on the effects of drought on grasses. Drought tolerance research in St. Augustinegrass shows that when this grass is fertilized with silica, it shows a significant difference in color, density, and quality when compared to the controls; color improved 13.6%, density by 8.5% and quality by 19% (Trenholm et al., 2004). Bentgrass that received silica fertilizer showed a 1-degree Celsius decrease in leaf temperature when compared to control at room temperature; when the plants were placed in a growth chamber and the temperature was between 35-40 °C the plants with silica fertilizer were on average about 3 °C cooler but up to 4.14 °C temperature was measured using thermal infrared imaging (Wang et al., 2005).

In summary, these two approaches can help plant breeders better understand low-input turfgrasses and enhance the development of new cultivars. Rhizosphere studies in turfgrass could help plant breeders select for plant genotypes that increase beneficial relationships with microorganisms to decrease the necessary inputs needed to grow superior turfgrass. Dry-ashing combined with fluorescent microscopy can be used to select grass genotypes that improve plant silica and possibly decrease fungal disease, wear, drought and herbivory stressors.

Chapter 1: Rhizosphere microbial community structure of common cool season turfgrasses

Summary

Professional turfgrass managers, as well as other consumers, have shown increased interest in low-input turfgrasses as a way to lessen the environmental and economic impacts of turfgrass management. An overlooked, but important, component of a turfgrass system is the rhizosphere. Recent advances in the area of metagenomics allow us to better understand this critical area surrounding turfgrass roots. Previous research in Poaceae has shown that a plant's genotype has the ability to influence the rhizosphere microbial community structure. Understanding how microorganisms associate with different species and cultivars of turfgrass may in the future provide researchers the ability to breed for turfgrass plants that form better mutualistic and beneficial relationships with soil microorganisms. The objectives of this study were to (1) determine whether a turfgrass species affects the structure of the soil microbial community and (2) characterize the effect of nitrogen fertilization on turfgrass-associated soil microbial community using a nitrogen fertility regime as an indicator. Turfgrass species plots were maintained at a 7.6 cm mowing height for three years at two nitrogen fertility levels (no fertility and 98 kg ha^{-1} yr^{-1}). Three separate rhizosplane soil samples were taken from each plot during ideal growing conditions in the fall of 2013, first soil was sampled then plots were fertilized and a week later soil samples were again collected from six species of turfgrass (*Koeleria macrantha, Poa pratensis, Lolium perenne*,

Agrostis capillaris, Festuca trachyphylla, and *Deschampsia cespitosa)*. DNA was isolated from turfgrass rhizosphere soil and the V4 hypervariable region of the bacterial 16S rRNA gene was sequenced using HiSeq Illumina. Data was then analyzed using Mothur. We found that grass species, soil type/location and fertility regime can have a significant effect on the rhizosphere community structure. Turfgrass plots that receive no fertilizer treatment had more diverse rhizosphere community structures; averaged among all samples *Proteobacteria* makes up 28.45% *Proteobacteria*; 1648 genus were observed in these samples. Principle component analyses showed that the samples cluster by location, species and cultivar. These data indicate that microbial community structure varied by location, species and cultivar. Results from this research will be able to inform turfgrass researchers about the impacts of both grass species and nitrogen fertility on soil microbial communities.

Introduction

There are about 50 million acres of managed turfgrass in the United States, which is more than the acreage of irrigated corn or wheat (Lindsey, 2005) and the turfgrass industry has an estimated value of $40 billion annually (National Turfgrass Federation, 2009). Significant land use devoted to managed turfgrass practices can adversely impact the environment due to applications of pesticides and fertilizers, along with greenhouse gasses that are associated with mowers and other types of lawn care equipment (Lindsey, 2005). Excessive use of certain fertilizers and the subsequent fertilizer runoff can be deleterious to terrestrial and aquatic ecosystems and water quality; additionally,

volatilization of nitrogen fertilizer can cause shifts in atmospheric composition (Tilman et al., 2001; Vitousek et al., 1997; Carpenter et al., 1998).

An understudied, but important component of a turfgrass system is the microbial community found surrounding the dense root systems that support turf stands. Research into the rhizosphere community structure of turfgrasses may provide a new means to improve turfgrasses and lead to the creation of cultivars for the market that utilize less pesticides, water, and fertilizer, which is imperative to addressing the overall impact of turfgrass cultivation and maintenance.

The rhizosphere is the soil-root-interface area surrounding a plant root system, which is colonized by distinct populations of microbes that are impacted by compounds released by plant roots (exudates). The rhizosphere includes organisms such as bacteria, fungi, viruses, protozoa, nematodes, and arthropods. In one gram of soil there are about $1x10^9$ microorganisms but this amount can be as high as $2x10^{10}$ billion near the soil surface adjacent to a plant (Clark, 1967); molecular study suggests that this same amount of soil can have 10,000 microbial species (Øvreås, 2000) or up to 8.3 million microbial different species in non-contaminated soil (Gans et al., 2005).

The function and structure of the rhizosphere community are important components of plant production. Many micro and macro nutrients essential for plant growth and development are mediated by microbes in the soil; the microbes play a significant role in availability and uptake of the nutrients (Miransari, 2013). The microbial community structure can be associated with plant disease suppression or prevalence (Bezemer and van Dam, 2005; Hayat et al., 2010). The microbes that make up

the rhizosphere community structure are also integral in soil aggregation, creation of soil organic matter, and the breakdown of crop residues (Bronick and Lal, 2005; Lützow et al., 2006).

This diverse microbial community structure is affected by different abiotic and biotic factors such as pH, temperature, soil type, plant age, plant species, and soil amendments (Marschner et al., 2004). The rhizosphere is very plastic: alterations in the soil type or plant species will have a large effect on what species of microorganism can exist in that rhizosphere (Marschner et al., 2004).

Plants release exudates can influence these microorganisms in a way that both the plant and the microorganisms benefit. The exudates are a means to communicate with microorganism as well as can provide the symbiotic organism with a means to sustain life, which can be a metabolic source or environment (Bouwmeester et al, 2007). The plant root releases exudates of photosynthetically-fixed carbon in both organic and inorganic forms. The organic forms of carbon tend to be very diverse and have a tendency to have the greatest effect on chemical, physical, and biological processes that occur in the rhizosphere. Research shows that roots release ~ 10-250 mg C/g^{-1} of root produced which ranges from 10-40% of a plants overall photosynthetically-fixed carbon (Newman and Bowen, 1974). This large investment in exudate production and secretion are likely evolutionarily significant and associated with the formation of relationships with bacteria and fungi in the surrounding soil.

Interestingly, it has been shown that plants have means to alter the microbial community structures, and that this occur in significantly different ways depending on plant species (Grayston, 1997). For instance, Lemanceau (1995) showed that different species of plant alter the species and biovars of fluorescent pseudomonads found associating with root tissue in different ways; *Linum* usitatissimum L. (flax) had more *P. putida* bv while *Solanum lycopersicum* L. (tomato) root had more *P. fluorescens bv. II* associated with it roots. In *Triticum spp.*, cultivar has also been found to significantly influence the microbial community structures of the rhizosphere (Germida & Siciliano, 2001). Further exploration of the rhizosphere is needed to determine how turf breeders and managers can optimize the rhizosphere microbial community that exploits these mutual microbial-plant relationships.

Soil microbial community structures can differ based on plant genotype differences within a species. Marshner et al. (2004) found that both soil type and plant species can have a significant influence on altering the makeup of the rhizosphere community structure. In *Zea mays,* at any field environment studied, genotypes affect OTU richness but the way the genotype effects microbial community structure varies between the field environments (Peiffer et al., 2013). Ultimately, selection of plant genotypes may be important for the plant to manipulate the rhizosphere microbiome toward the maximization of symbiotic mutualism (Miller et al., 1989). Likely, specific plant genotypes create unique environments in the rhizosphere that attract or benefit specific microorganisms; this trait could be exploited by breeders to aid plants by using

plant genotype to select unique microbial residents whose populations are influenced by the plant genotype.

Until recently, means to investigate microbial populations and community structure were lacking, mainly due to the fact that only <1% of microbes can be cultured in the lab (Frostegård & Bååth, 1996); thus, a realistic characterization of what is occurring in the soil has in the past evaded researchers (Kirk et al, 2004). Current high-throughput sequencing technology gives the opportunity for the characterization of bacterial communities within soil samples (Jones et. al, 2009).

High-throughput DNA sequencing technology allows for the study of bacterial communities within soil samples (Jones et. al, 2009). Recent advances in sequencing technologies have permitted a more comprehensive approach to investigate structures and function of soil bacterial community through analysis of DNA samples isolated from plant rhizosphere (Huse et al., 2008). Mothur, which is a bioinformatics tool for analyzing 16S sequences, is often used to determine the distribution of bacterial taxa in a sample (Schloss et al. 2009). These data can be utilized by turfgrass scientists to have a better understanding of the dynamics of rhizosphere community structure under different variables such as fertility regime, plant cultivar or soil type.

The rhizosphere has been observed to alter minerals from inaccessible forms into accessible forms (Bais et al., 2006); this is useful to the plant because these microorganisms increase nutrient availability to forms the plant can utilize. The bacteria and fungi in the rhizosphere can be useful for bioremediation (Anderson et al., 1993), can increase plant vigor (Jones and Darrah, 1994), improve drought resistance (Yang et al.,

2009), increase humus build up and carbon sequestration (Clemmenson, 2013); and increase disease resistance (Zamioudis, 2012).

In addition to the genetics of the plant, how a plant system is managed can also affect soil microbial community structure. Donnison et al. (2000) showed that different nitrogen hay meadow management can significantly alters the soil microbial community structure and reduced the fungal-to-bacterial phospholipid fatty acid ratios. Similar findings of the difference in microbial community structure and fungal biomass were observed in unimproved grasslands and improved grasslands (Federle, 1986). Microbial community structure differed in high and low fertility grasslands (Innes et al., 2004). The turfgrass *Poa annua* shows a significantly altered microbial community structure when under different fertility regimes when maintained as a golf green (Beirn et al., 2016).

Investigation into the effects of fertilization on the rhizosphere community structure are needed in turfgrass models. This data could provide turfgrass breeders information helpful in determining which turfgrass cultivars would be utilized for certain environments and guide turfgrass managers in more sustainable management practices. Thus there is a possibility that the effect on the rhizosphere community structure by plant genotypes as well as cultural practices could be exploited to improve turfgrass quality and production. Research into the understanding of the rhizosphere may reduce the human environmental impact that is associated with turfgrass management and production. Plant breeders will be able to use data on which bacteria and fungi are surrounding plant roots as a phenotype for selection of improved cultivars.

The goal of our research was to determine if these interesting relationships between microbial communities and plants are important in turfgrass systems. The research objectives of this study were to 1) determine how nitrogen fertilization affects soil microbial community structure in a mature turfgrass stand; and 2) determine how turfgrass species affects soil microbial community structure in a mature turfgrass stand.

Materials and methods

Field Experiment

Samples for this study were taken from mature turfgrass research plots at both the Turfgrass, Research, Outreach, and Education Center at the University of Minnesota in St. Paul, MN and the University of Minnesota Landscape Arboretum in Chaska, MN. Trial design and establishment is described in detail by Hugie and Watkins (2016). Briefly, 18 entries representing seven turfgrass species were established in late summer 2009 in a split-split plot design; there were three fertilizer and three mowing heights. The present study commenced in spring 2012. For our study, beginning in spring 2012, we sampled plots in one of the main plots (8.3 cm mowing height) that were subjected to one of two fertilizer treatments (1) 0 g N m^{-2}, 0 g P_2O_5 m^{-2}, 0 g K_2O_5 m^{-2}; 2) 9.8 g N m^{-2}, 0 g P_2O_5 m^{-2}, 9.8 K_2O_5 g m^{-2} (applied as a split application with 4.9 g N m^{-2} applied in late May and 4.9 g N m^{-2} applied in early September). From establishment in 2009 through 2013 Renaissance ® All Natural 9-0-9 fertilizer was used; from 2014 on Natural Origins 10-0-8 was used. Soil samples were collected by taking 3 soil samples from each individual

sub-sub plot using a 3.81cm plug stacker (Miltona, MN) with 4 replicated plots of the following turfgrasses in St. Paul and 3 replicated plots of turfgrasses in Chaska: one entry of perennial ryegrass (*Lolium perenne* 'Arctic Green')*;* two entries of hard fescue (*Festuca trachyphylla* 'SR3150', Reliant IV*; Agrostis capillaris* 'Glory' and 'Allister'*; Koeleria macrantha* 'Barkoel'; *Deschampsia cespitosa* 'DCS Bulk', 'DCM'. Soil samples were collected pre and post fertilization in October 2nd and October 9th of 2013. Rhizosphere soil is those soil macroparticles attached to root tissue and rhizoplane soil consists of microparticles attached to the root tissue. To collect rhizosphere soil, the plug was shaken and the resulting soil attached to the roots was collected and placed in a glass vial. Soil samples were collected for each individual sub-sub plot, a total of three soil samples were collected and analyzed separately; each sub-sub plot has three replicates included in the study.

Visual ratings of turf quality were taken on a 1-9 scale (1 = poor, 5 = minimally acceptable, 9 = excellent) prior to sampling. Turf quality ratings were based on color, uniformity, density, and texture. Quantitative data were collected on percent live turfgrass cover and percent weed cover using the grid intersect method by placing a 0.3×0.9 m grid with 99 intersects on each plot. Live turfgrass cover and weed cover were scored at each intersect and converted into a percentage.

Sample Processing

In the present study we characterized the rhizosphere bacterial community structures of six species of cool season turfgrass: colonial bentgrass, deschampsia, hard fescue, Kentucky bluegrass, junegrass and perennial ryegrass at two locations: St. Paul and Chaska, Minnesota.

Rhizosphere soil samples were taken from turfgrasses documented in the tables below (Table 1.1, Table 1.2, Table 1.3). Each plug of turfgrass had its aboveground plant organs aseptically removed by using a scissors sprayed with 70% ethanol and wiped clean with autoclaved paper towels. Loose soil was manually removed from the roots by kneading and shaking with sterile gloves (sprayed with 70% ethanol). The roots were transferred from the empty tube to a new sterile 50 mL tube with 25 mL sterile phosphate buffer (0.1 % Na-pyrophosphate, pH 7.0, and 0.1 % Tween 20), cleaned for remaining debris with sterile tweezers and transferred to new sterile buffer tubes until the buffer was clear after vortexing (without major sediment on the tube bottom). The roots were then agitated at 4,000 rpm for 20 minutes. The agitation further disrupted tiny soil aggregates and attached microbes, cleaning the root exterior. After agitation, 25 ml of supernatant was collected and centrifuged and the resulting supernatant was decanted off, and then stored at -80°C until processing.

The plug was shaken by hand (using sterile gloves sprayed in 70% ethanol) until the bulk soil was removed. Soil that remained attached to the roots was placed in a glass vial. The soil was promptly stored in sterilized glass vials in a freezer at a -80°C. Each plug of turfgrass had its above-ground plant organs are aseptically removed.

16S rDNA Sequencing

DNA from the soil samples was quantified using the Qubit dsDNA HS kit (Thermo Fisher Scientific, Waltham, MA, USA). Amplicons preparation and sequencing were performed by the University of Minnesota Genomics Center. The V5-V6 hypervariable regions of the 16S rDNA were PRC amplified using the BSF784/1046R primer set (Claesson et al. 2010) and barcode-indexed (Staley et al., 2015). Replicate sequence data was generated by paired-end sequencing of purified amplicon pools on the Illumina MiSeq (2 × 150 bp read length). Sequence data were then analyzed using Mothur 1.36.0. Illumina sequencing combined using PCR and with barcoded primers, yielding amplified products that are separated and categorized following sequencing is used in this study based on Mamanova et al. 2010. Illumina sequence reads were processed using Mothur version 1.36.0 (Schloss et al. 2009). Sequence reads were trimmed of primer sequences and multiplexing barcodes specific to each site. Sequences that differed by greater than one nucleotide from primer sequences, had at least one ambiguous base, had had greater than 8 nucleotide homopolymers, and which had a quality score smaller than thirty-five nucleotides in a window of fifty nucleotides were removed. Sequence chimeras were identified and removed using UCHIME (Edgar et al. 2011). Sequences were then aligned against the SILVA database (Pruesse et al. 2007), and classification of OTUs at 97% identity using furthest neighbor clustering was done using Mothur and the Ribosomal Database Project (RDP) taxonomic database (release 9) (Cole et al. 2013). Sample quantities were normalized in libraries created at the

University of Minnesota Genomics Center (UMGC) and libraries were paired end sequenced at a read length of 100 bp and average insert size between 260 and 320 bp over two lanes on the HiSeq 2000 platform by the UMGC (St. Paul, MN).

Data Analysis

Replicated soil samples were pooled. Diversity indices [number of OTUs (Sobs) and Shannon index, sequencing coverage estimation, UniFrac analysis (Lozupone and Knight, 2005), ANOSIM analysis (Clarke, 1993), principal component analysis (PCoA), Mantel tests (Sokal and Martin, 1995), Kruskal–Wallis analysis (Acar and Sun, 2013), and analysis of molecular variance (AMOVA) (Excoffier et al., 1992)] were performed using Mothur ver. 1.36.0 (Schloss et al., 2009). For statistical tests in Mothur, replicates were grouped via use of .design files, but were maintained as separate groups. Bray–Curtis distance matrices were for community comparisons (Bray and Curtis, 1957). All statistical analyses were conducted at $\alpha = 0.05$.

Results & Discussion

Our experimental design allowed us to observe the effects of fertility, turfgrass genotype and location/soil type on the rhizosphere community structures under turfgrass field conditions. We found that fertility regime, location and turfgrass genotype significantly influenced α- and β-diversity rhizosphere microbiota.

Redundancy analysis indicated that *Actinobacteria* were positively correlated with turf performance quantified by weed intercept (r = 0.263, p = 0.002), while *Verrucomicrobia* and *Bacteroidetes* was negatively correlated with WI (r = -0.187, -0.288, p=0.031, 0.001). The five most common bacterial OTUs found in the turfgrass rhizosphere included *Actinobacteria*, *Proteobacteria* and *Firmicutes* which have been associated with disease suppression in other studies (Mendes et al., 2011; Pankhurst et al., 2002; & Peng et al., 1999). In our study, we judged low levels of weed pressure as a measure of better turfgrass performance and function (Hugie and Watkins, 2016); We found a significant correlation between *Actinobacteria* and WI, which suggests that *Actinobacteria* contribute to healthy turfgrass; however, this is not clear evidence of a direct effect. Further studies into the effectiveness of *Actinobacteria* in predicting superior low-input turf performance are needed in addition to studies investigating *Verrucomicrobia*, *Bacteroidetes* and turfgrass performance. Future turfgrass trials should more closely investigate the relationship between turf performance and bacterial community structure. These bacterial communities may aid in the prediction of superior turf and may possibly inform turf scientists what communities are associated with turf traits such as turf quality and weed intercept data.

The OTU composition of the rhizoplane datasets were investigated and utilizing Kruskal-Wallis P<0.05 by species showing the OTUs whose abundance differed significantly. The OTUs that differed by phyla over an average of 1% of sequence reads included: *Proteobacteria*, *Actinobacteria*, *Bacteriodetes*, *Firmicutes*, *Acidobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Planctomycetes*, *Chlamydiae*, and *Chloroflexi*. Weinert

et al (2011) showed that in *Solanum tuberosum* (potato) that about 4% of OTUs were controlled by genotype while 40% was due to indigenous soil microbes. Bulgarelli (2015) also observed these differences in rhizosphere community structure based on plant genotype was in barley (*Hordeum vulgare* L.).

Significant differences in microbial community structure seems to be due to soil type and other abiotic factors (Figure 1.3). Turfgrass species at the Chaska site associated together and turfgrass species at the St. Paul site associated together (Figure 1.4) suggesting that location and soil type is very important, a finding that is similar to that of Schreiter et al. (2014) in lettuce. Also, there is genetic diversity of how these traits are expressed which could be exploited by breeders. Future, multi-location testing will be integral to find cultivars with wide adaptation and ability to create favorable microbial communities in diverse soils. This may make breeding for a turfgrass that works well under many conditions as well as forms increased mutual associations with microorganisms expensive and time consuming. Future research will need to investigate genotype by environment interaction and identify those elite cultivars that show no rank changes at different locations and maintain a positive interaction.

We found that turfgrass species tend to be associated with more diverse soil bacterial communities when grown with fewer fertilizer inputs (Figure 1.5). Nitrogen amendments have also been shown to suppress soil respiration and microbial biomass, promoting copiotrophs such as *Actinobacteria* and *Firmicutes* while reducing the abundance of oligotrophs such as *Acidobacteria* and *Verrucomicrobia* (Ramirez et al. 2012). In our study, the only significant differences in phyla abundance were between fertilized and

not-fertilized plots. The LDA score is the magnitude of difference in abundance between groups and it showed that *Armatimonadetes* are more abundant in not-fertilized plots while *Thermodesulfobacteria* and *Thaumarchaeota* are more abundant with fertilizer. Observing a greater abundance of *Thermodesulfobacteria* could likely be due to the increased amount of sulfur that is in the fertilizer applied to those plots (Natural Origins 10-0-8 contains 3.3% sulfur in the form ammonium sulphate and sulphate of potash). Another study has also found impacts of fertilizer in OTU richness, diversity and relative abundance of functionally significant taxa in both bulk and rhizosphere soils (Fernandez et al., 2016). *Thaumarcheota* is a group of chemolithoautotrophic ammonia-oxidizers that play a role in nitrogen and carbon cycling; which oxidizes ammonia using ammonia monooxygenase enzyme (Leininger et al., 2006; Zhalnina, 2012). *Desulfobacteria* show an increase in abundance in enriched-N marshes when compared to reference marshes (Kearns et al., 2016). Increases in *Desulfobacteria* may be environmentally problematic since they are a marine sulfate reducer oxidizing organic carbon completely to carbon dioxide (Strittmatter et al., 2009). Since *Desulfobacteria* tend to increase in abundance under increasing fertility this could be inferred as fertilizers often contain sulfur in the form calcium sulfate.

Conclusion

The present study shows that the rhizosphere community structures in perennial turfgrass systems are very diverse and there appears to be significant differences at the order and genus taxonomic level. Future research needs to be done at more time points as well as looking only at one model turfgrass species with a diverse germplasm. This

research informs turfgrass breeders that further research may find how much plasticity in rhizosphere community structure can be accomplished by genotype and the genotypic influenced plasticity on the rhizosphere community structure and its associated effect of various soil types. Fertility appeared to have an influence on rhizosphere community structure and further study may help turfgrass managers find a balance between fertility regime and effect on the microbes in the rhizosphere.

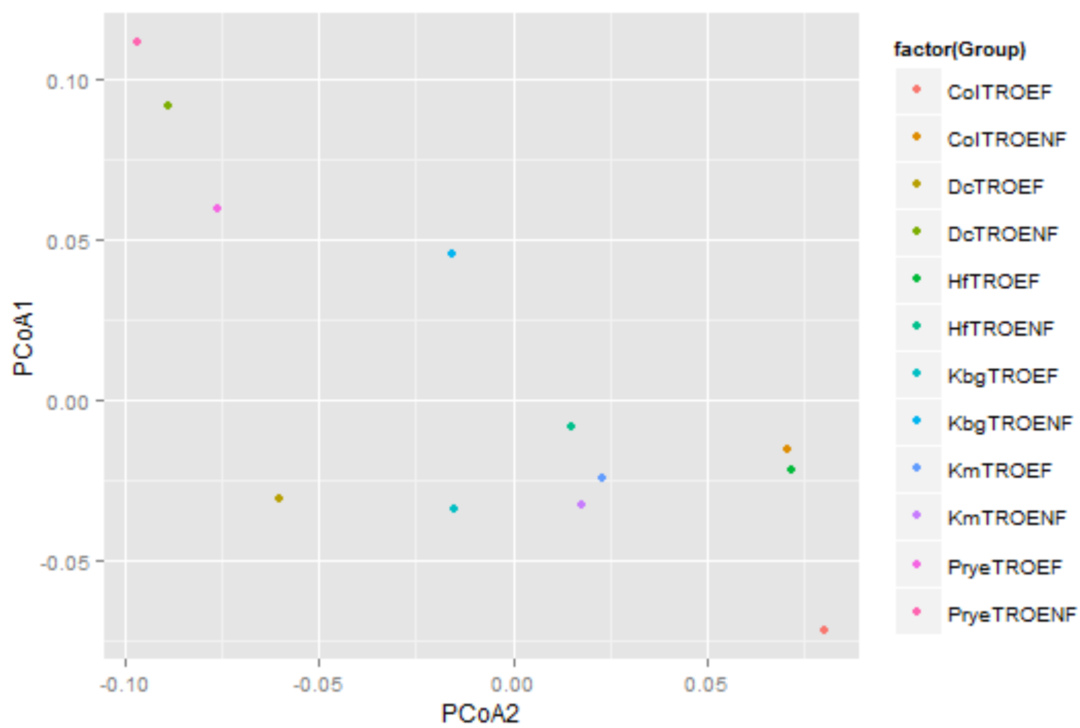


Figure 1.1. Principal component analysis of cool season turfgrass samples at the St. Paul location (all replicates merged for ordination).

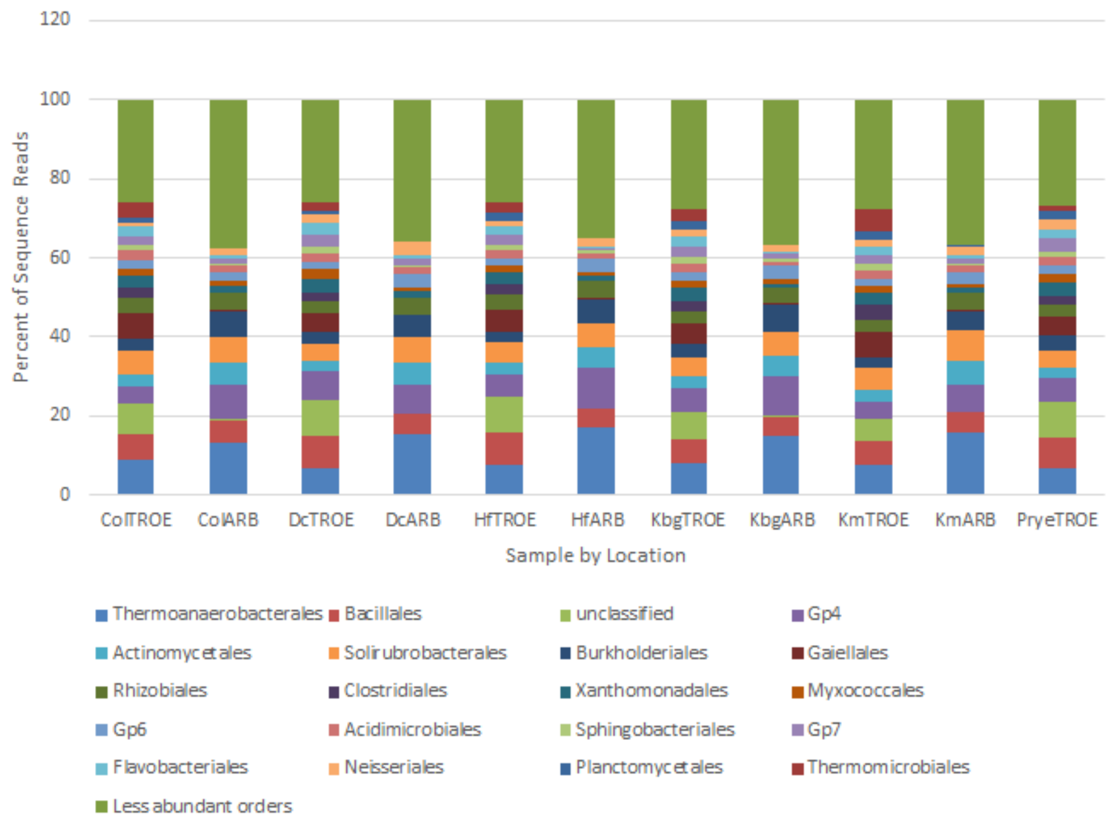


Fig 1.2. Distribution of abundant orders found in rhizoplane samples of cool season turfgrass.

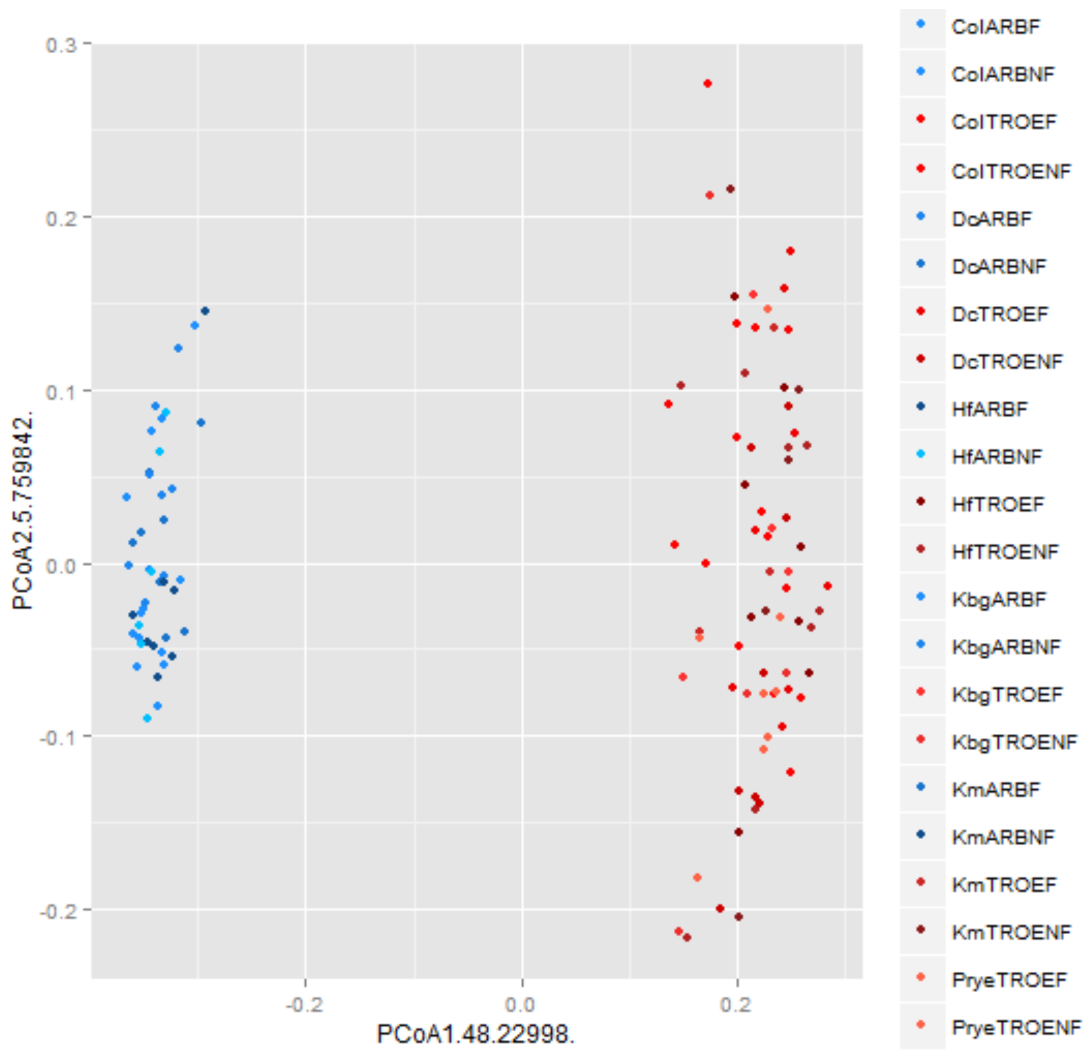


Figure 1.3. Principal component analysis of cool season turfgrass samples.

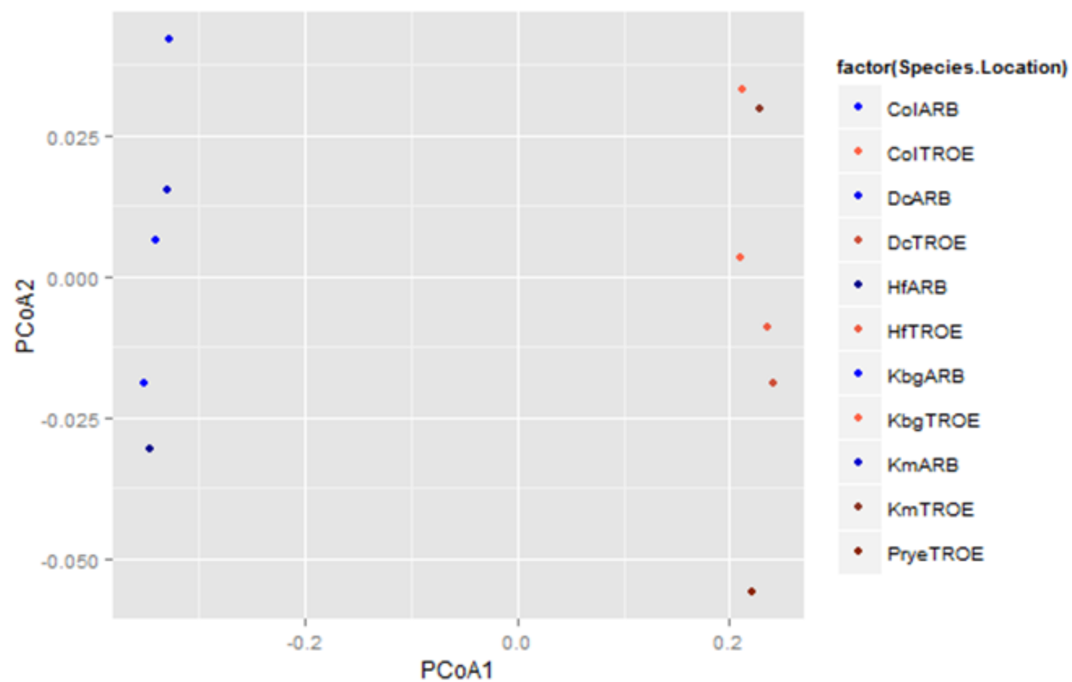


Figure 1.4. Principal component analysis of cool season turfgrass samples (all replicates merged for ordination).

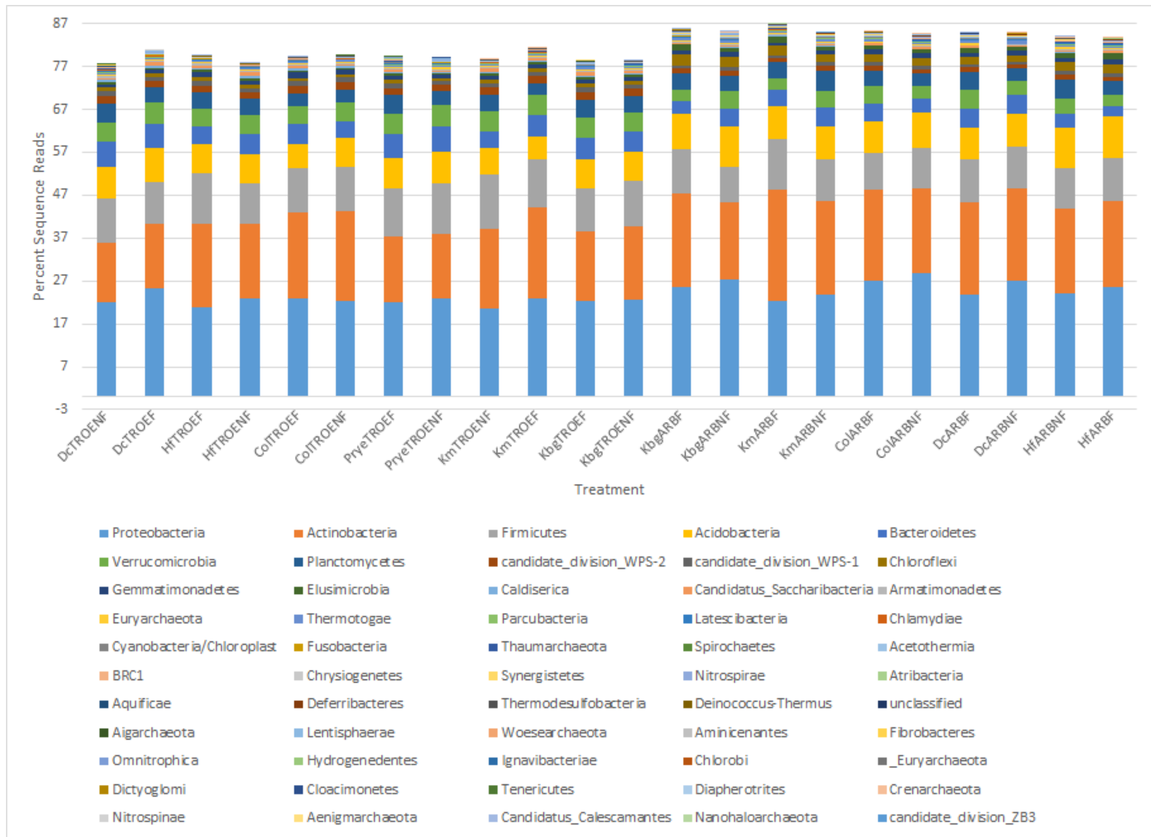


Figure 1.5. Distribution of taxonomic genus of OTUs found to differ significantly by phyla at St. Paul and Chaska. The OTUs were found to vary significantly by using the Kruskal–Wallis test.

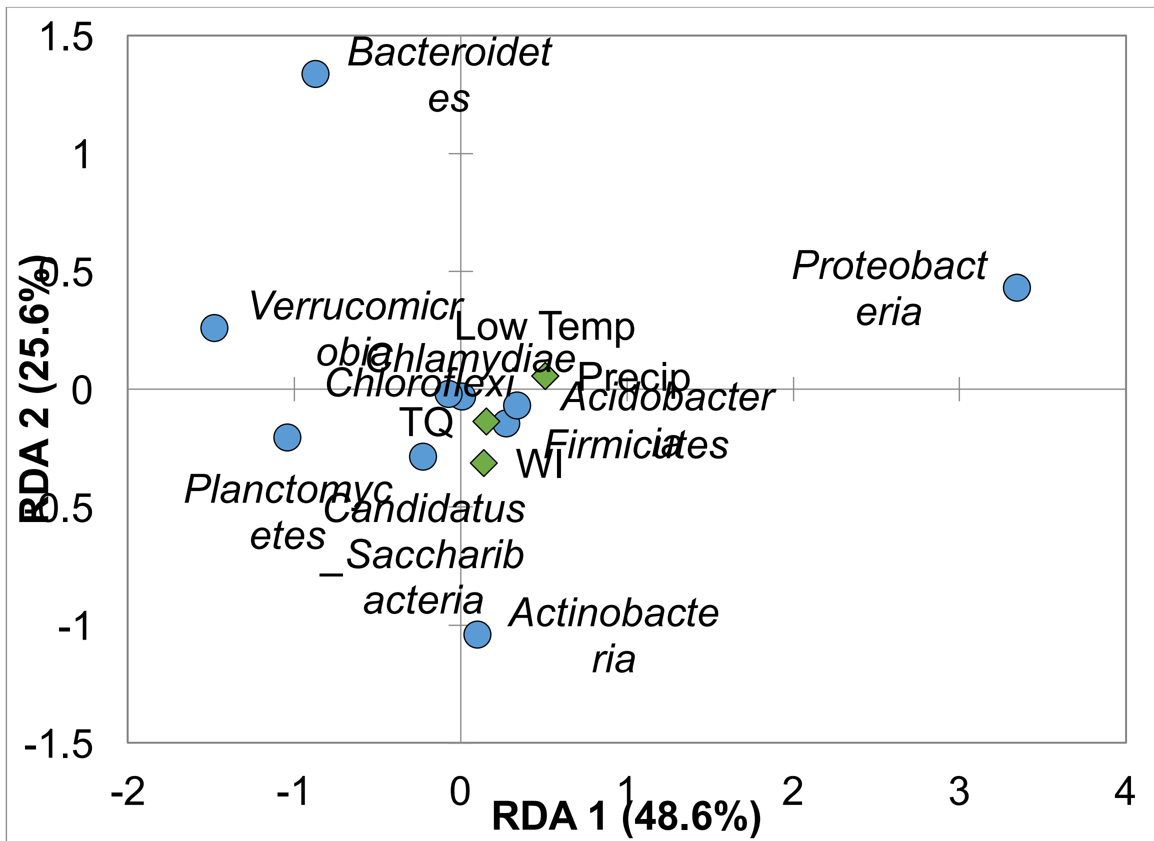


Figure 1.6. Redundancy analysis showing variation in phyla explained by species at St. Paul in October 2013.

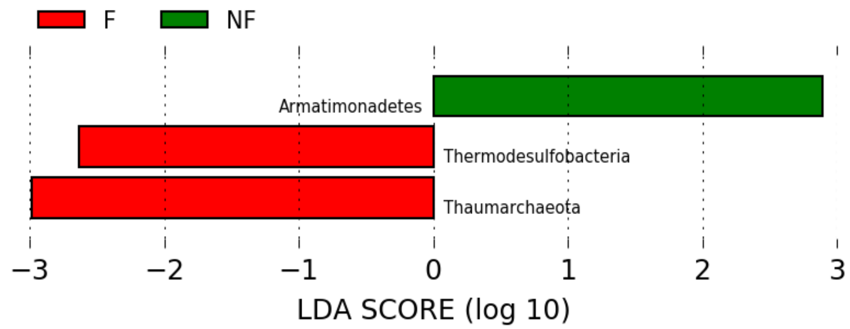


Figure 1.7. Linear discriminant analysis of effect sizes of species, cultivar, species x cultivar, fertilized, and fertilized x species. The LDA score is the magnitude of difference in abundance between groups. This is showing that *Armatimonadetes* are more abundant in not-fertilized plots while *Thermodesulfobacteria* and *Thaumarcheota* are more abundant with fertilizer. No effect of species or cultivar on abundances of phyla.

Variables	TQ	Weed Intercept	*Proteobacteria*	*Actinobacteria*	*Planctomycetes*	*Verrucomicrobia*	*Bacteroidetes*
TQ	**1**	**0.512**	0.050	0.142	-0.065	**-0.214***	-0.132
WI	**0.512***	**1**	0.014	**0.263***	-0.007	**-0.187***	**-0.288***
Proteobacteria	0.050	0.014	**1**	-0.166	**-0.624**	**-0.515**	-0.101
Actinobacteria	0.142	**0.263**	-0.166	**1**	-0.017	**-0.244**	**-0.485**
Planctomycetes	-0.065	-0.007	**-0.624**	-0.017	**1**	**0.434**	**-0.191**
Verrucomicrobia	**-0.214**	**-0.187***	**-0.515**	**-0.244**	**0.434**	**1**	**0.210**
Bacteroidetes	-0.132	**-0.288***	-0.101	**-0.485**	**-0.191**	**0.210**	**1**
Firmicutes	0.031	0.114	**-0.419**	**0.428**	**0.216**	-0.076	**-0.306**
Acidobacteria	0.012	0.125	-0.043	**-0.391**	0.013	0.004	0.091
Chlamydiae	-0.035	0.033	**-0.260**	**0.362**	0.017	-0.019	**-0.205**
Candidatus_Saccharibacteria	0.019	0.085	**-0.239**	0.019	**0.298**	0.144	**-0.239**
Chloroflexi	-0.136	-0.030	**-0.350**	**0.240**	**0.530**	**0.220**	**-0.428**

Table 1.1. Spearman correlation of rhizosphere microbial phyla, turfgrass quality, weed intercept in St. Paul. The highlighted data are significantly different.

Chapter 2: A novel method to characterize silica bodies in grasses

Introduction

The epidermal cells of grasses (Poaceae) are arranged in parallel rows with combinations of diverse cell types (Evert, 2006). Some of these cells are specific for silicon (Si) deposition and are called silica cells. The Silicon accumulated in the silica cells develops into the mineral structures of amorphous hydrated silica ($SiO_2 \cdot nH_2O$) having various shapes and properties called silica bodies, silica phytoliths, or plant opal (Chen et al., 1996; Piperno and Sues, 2005).

Silica bodies are one of the most durable structures in grass tissues that remain as particles in the soil even after all other organic parts of plant have naturally decayed or degraded. These particles in the soil and ash can be very important research tools for systematic botanists (Chen et al., 1996; Prychild et al., 2003), environmental biologists (Sangster and Hodson, 1986), archeologists (Rapp and Mulholland, 1992; Piperno and Flannery, 2001), paleontologists/paleobotanists (Wilding, 1967; Pearsall, 1978; Thomasson et al., 1986; Ciochon et al., 1990; Prasad et al., 2011; Lu et al., 2009), and geologists (Folger et al., 1967; Zhang et al., 2012; Zhang et al., 2011).

The amounts of silica in plant tissues suggest that silicon has a very important role in growth and development. For example, in rice (*Oryza sativa* L.), several fold more Si can be detected in shoots compared with the amounts of nitrogen, phosphorus, or

potassium (Savant et al., 1996), reaching up to ten percent of its dry mass (Epstein, 1994; Ma et al., 2002).

Functional analyses of plant silica have shown that silicon is critical for mitigating stressors such as fungal infection (Chen et al., 2012; Park et al., 2006), herbivory (Hunt et al., 2008; Garbuzov et al., 2011), wear (Peters, 1982; Massey and Hartley, 2009), and drought (Schaller et al., 2013; Ma, 2010; Chen et al., 2011; Richmond and Sussman, 2003). Mature silica bodies have been found to deter herbivory and increase the abrasiveness of grass leaf blades (Vicari and Bazely, 1993; Massey et al., 2007a; Massey et al., 2007b). In addition, ample silica bodies have been associated with photosynthetic activities (Chen et al., 2011; Detmann et al., 2012; Agarie et al., 1996), although the mechanism for this response remains unclear (Agarie et al., 1996).

Because we are interested in improving stress tolerance response in turf grasses, we wanted to develop a method to efficiently identify and quantify silica bodies in perennial grasses. Such a method could also be extended to other grass species, such as important forage grasses and cereals. In searching for an easy, economical, and fast method to study the morphology and distributional patterns of silica bodies in turf grasses and other plants, we found a number of available techniques. These include dry ash method, wet oxidation method, scanning electron microscopy (SEM) method, and X-ray image analysis. Among which, dry ash-imaging is one of the most commonly used methods for studying silica bodies in modern plants. To study grass leaves, ash imaging has been a method-of-choice to many researchers; however, this method is extremely labor intensive when analyzing the size, density, and distribution patterns using brightfield light microscopy and

researchers have to manually measure a great number of silica bodies in order to perform a statistically meaningful analysis (Chen et al., 1996; Chen et al., 2002).

This method can be accomplished by placing samples in porcelain crucibles and into a muffle furnace, or an oven, for 1–2 h at 500 °C, but some morphological changes might occur to certain, lightly silicified phytoliths when the temperature exceeds 600 °C (Chen et al., 1996; Chen et al., 2002; Wu et al., 2012). The wet oxidation method was developed to examine the isolated silica bodies and is suitable for measuring the abundance of silica bodies in plant tissues, but does not work well for analyzing the distribution patterns of silica bodies (Chen et al., 1996; Gallego and Distel, 2004). In comparison to the dry ash method, the wet oxidation method results in less damaged silica bodies, especially when the samples are exposed in an environment of 600 °C or higher (Sun et al., 2012). Due to the limitation of applying light microscopy to examine surface morphology at extra high magnification, scanning electron microscopy (SEM) can also be used to study silica bodies (Sangster, 1968; Theunissen, 1994). The SEM method can be combined with X-ray analysis to provide information on surface structure and composition of silica bodies (Kaufmian et al., 1969; Takeoka et al., 1983; Lichtenberger and Neumann, 1997). Here we report a method to study silica bodies using fluorescence microscopy to visualize green autofluorescence in combination with the dry ash-imaging technique. This method was developed using a perennial grass species, *Koeleria macrantha*, commonly known as junegrass, and has potential to be used in all fields of paleobotany and modern plant sciences on silica body research.

Materials & Methods

Plant material and sample collection

To examine the structure and properties of silica bodies, mature *Koeleria macrantha* leaf blades were collected from plants grown in the greenhouse in a 2:1 mixture of Sunshine MVP (Sungro Horticulture) and MVP:Turface (PROFILE Products LLC) substrates with no additional fertilizer. Plant material was derived from: (a) populations from the University of Minnesota turfgrass breeding program derived from material collected in either Colorado (KM-CO), Nebraska (KM-NE) or Minnesota (KM-MN) (Currie and Perry, 2007; Rajendiran et al., 2012; Tang et al., 2012); (b) the cultivar 'Barkoel'; (c) several accessions from the United States Department of Agriculture National Plant Germplasm System including PI 430287 (Ireland), PI 387927 (Canada), W6 33040 (Russia Federation), PI 207489 (Afghanistan), W6 13043 (China), and PI 302912 (Spain).

The middle section of each leaf blade was cut along the transverse plane and used for ash-imaging sample preparation with removal of the tip and leaf base (Figure 2.6b). Each leaf blade was cut into two pieces, one of which was placed on the adaxial and the other on the abaxial side of the leaf onto a glass microslide (VWR Micro Slides were used in this study). On the same microslide, one other leaf from a different plant of the same accession was sampled as a biological replicate (Figure 2.6b). A total of 24 plants (thus 12 slides) per accession were analyzed.

Dry ash-imaging sample preparation

A microslide with the leaf samples on was then covered with another glass microslide in an effort to not disturb the placement of the leaves and to add appropriate weight to keep the ash sample intact (Figure 2.6b). The slides were then heated on either a Corning Hot Plate Stirrer PC-351 (Figure 2.6a) or a Tek-Pro Heat-Stir 36 H2397-1 (not shown) placed in a fume hood. The hot plate temperature was gradually increased every 5 min up to 320 °C. The temperature was approximated using an infrared thermometer Ryobi IR001 (CW0938) read at >608.2 °F/320 °C. The ash process usually took 2–3 h depending on the accession. Grass leaf samples first turned dark brown or black (Figure 2.6c), and then gray (Figure 2.6d) when the ashing process completed. To end the heating process, we turned off the hot plate and kept the slides on the plate for 1 h or longer to slowly cool them down. (*Note: The hot plate stirrer and glass slides are extremely hot while preparing the ash samples; do not move the slides directly to a cooler place while they are still hot, which often result in broken slides*.) Slow heating and cooling down prevents the microslides from cracking. For the dry ash samples preparation, we recommend to use a hot plate instead of a coiled electric stove, where the slides often break due to its uneven heating surface.

Microscopy imaging

After the slides were cooled down for at least 1 h, the top microslide was then carefully removed and discarded. A 1 ml plastic transfer pipette (Fisher Scientific, Pittsburgh, PA, USA) was cut at the 0.5 ml measurement to make an ease cedar wood oil

application, and a single drop of cedar wood oil (Electron Microscopy Sciences, Hatfield, PA, USA) was applied. A cover slip was then placed on the microscopy slide without disrupting the sample. Cedar wood oil was allowed to diffuse fully under the cover slip with slightly warming the slide on an alcohol burner. The slides were then imaged using an Ernst Leitz Wetzlar 307143.004 microscope (Wetzlar, Germany) and photographed with a SPOT Insight 4 Camera (Diagnostic Instruments, USA). The auto-fluorescence was detected using a Green Fluorescence Protein filter cube (SN: 31001, excitation at 480 nm, beamsplitter at 505 nm, and emission at 535 nm) that was manufactured by Chroma (Chroma Technology Corp, Bellows Falls, VT, USA). The camera interference program used to take the images was SPOT Basic v4.6. Up to 5 sets of brightfield and fluorescent images per object on the slide were taken at 200× and 800× magnification rate. Duplicate fluorescent images were analyzed using Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA, USA) and/or Image J (imagej.net) for silica body occupancy rate of leaf surface (percentage of surface area), the size of silica bodies, and the pattern of silica body distribution (number of silica bodies per unit) on epidermis. Granule-like structures in silica bodies are carbon inclusions, which were counted and recorded using ImageJ, Analyzing Particles tool. The data collected from 20 silica bodies per accession, each with the number of carbon inclusions and their spatial distribution patterns, such as tightly clustered, loosely clustered, randomly distributed, or single granule.

Image and data analysis

First, a random selected fluorescence image was imported into Adobe Photoshop CS5 (Figure 2.1a). Second, we used the Photoshop Magic Wand Tool to select the dark

image background without silica bodies. Third, the Select-Inverse tool was utilized to select all the silica bodies (Figure 2.1b). Fourth, we used the Window-Histogram function to read the pixel counts of the entire image (Figure 2.1c) and the counts of selected silica bodies (Figure 2.1d). To count the number of silica bodies in an image, we used the Window->Tools->Count Tool in the Photoshop CS5, the number of silica bodies was automatically shown. For those who do not have a licensed Adobe Photoshop CS5 or advanced version, the freeware ImageJ is available (http://imagej.nih.gov/ij/download.html) with tutorials videos on YouTube (youtube.com). Using the Analyzing Particles tool in imageJ we could count the number of silica bodies automatically as well. All statistically significant differences were tested at $P < 0.05$ level. The results were analyzed by ANOVA using R 3.1.2. (R, 2011). The Tukey multiple comparison test was used to test the significant differences of silica bodies among all accessions studied (Shaffer, 1995).

Results and Discussion

Combination of fluorescence microscopy and image software provides an opportunity to study plant silica bodies with high efficiency

Silicon-containing structures in plants often display auto- or inducible fluorescence emission that can be examined by fluorescence microscopy (Soukup et al., 2014). Comparing to brightfield microscopy, the fluorescence emission created ideal conditions for image analysis in high throughput studies, because noisy ash background was eliminated and samples demonstrated clear shape, number, and the distribution pattern of

silica bodies (Figure 2.1a). Autofluorescence micrographs of an ash-image sample can be easily processed using image software such as Adobe Photoshop and Image J for high-throughput analysis, which include occupancy rate of the leaf epidermis, size and number of silica bodies per unit of leaf surface (Figure 2.1a–e). Results can be obtained from a single image or from a random combination of sample images. For example, by analyzing a single image of *K. macrantha* 'Ireland', we observed 11 silica bodies in an area of 4909 μm^2 (Figure 2.1), which converted to 2240 silica bodies in an area of 1 square millimeter (2240 sb/mm^2) abaxial leaf epidermis; the silica bodies occupied 8.1 % of the leaf surface (abaxial). The average size of each silica body was 36 μm^2 with a standard deviation of 7.94.

With the same approach we analyzed other accessions using 10 randomly selected images per accession and found that KM-MN and KM-CO exhibited averages of 13,676 sb/mm^2 and 13,568 sb/mm^2, respectively, which is approximately 6.1 times more silica bodies per square millimeter comparing to the Ireland accession. The sizes of the silica bodies also differed significantly among accessions; for example, we observed the largest silica bodies with an average size of 52.8 μm^2 in 'Barkoel', whereas the smallest silica bodies with an average size of 26.7 μm^2 in 'Canada' (Figure 2.2) (Dixon, 2000; Piperno and Pearsall, 1998).

Three types of silica bodies or silica body related mineral structures were detected by brightfield microscopy (BM) and fluorescence microscopy (FM). Under brightfield microscopy, structures remaining in the ash-imaging process are considered silica bodies (Chen et al., 1996; Chen et al., 2002). In this study, we compared images from the same

ash sample under brightfield and fluorescence microscopy and observed three different types of silica bodies or silica body related structures: Type I silica bodies were developed in the short silica cells and detected under both brightfield and fluorescence microscopy (Figure 2.3). Type II silica bodies were also developed in the short silica cells, but only seen under the BM (Figure 2.3a), and could not be detected under FM (Figure 2.3b). Type III silica bodies were only detected under FM, which were not developed in silica cells (short cells), instead, Type III silica bodies were likely developed in the silicon-enriched long-cells and trichomes (Figure 2.3c, d). We frequently observed a number of structures that appeared to look like silica bodies under BM (Figure 2.3a), which likely contained less silica and more other chemicals, such as carbon seen in the type I silica bodies as carbon inclusions. However, we were not able to utilize correlative fluorescence microscopy (Jahn et al., 2007) with X-ray analysis in combination with the ash-imaging method to confirm that the intensity of autofluorescence corresponded to the level of silica deposition. Evidence from previous studies using scanning electron microscopy and X-ray analysis suggested that structures developed in silica cells could be classified into two different types based on the silica profile (Kaufman et al., 1979; Kaufman et al., 1981). The majority of silica cells demonstrated significant silica deposition; only a small portion of the short silica cells (~4 %) exhibited silica body-like structures with barely detected silica signal (Kaufman et al., 1981).

Silica bodies presented in both abaxial and adaxial epidermis of *K. macrantha*

Most of ash-imaging analysis demonstrated a single layer of silica bodies in grass leaves (Chen et al., 1996; Chen et al., 2002). By carefully examining the ash-image samples of *K. macrantha*, we found that silica bodies were presented in both abaxial and adaxial leaf epidermis. We observed two layers of silica bodies in some accessions of junegrass at different focus depths of the dry ash slides. Results from the samples that have only one side of epidermal cell layers, either abaxial or adaxial with removal of the opposite epidermis have confirmed that both the abaxial and adaxial epidermis developed silica bodies (Figure 2.4). The density, shape, and size of silica bodies in the adaxial epidermis (Figure 2.4a), however, differed from those of the abaxial epidermis (Figure 2.4b).

No autofluorescence was detected from carbon inclusions. Carbon inclusions are the residues of organic matter entrapped in silica bodies during the process of silicification (Gallagher et al., 2015; Rowlett and Pearsall, 1993; Krull et al, 2003) which can occupy approximately 0.85 % of the total volume of silica bodies (Gallagher et al., 2015; Rowlett and Pearsall, 1993; Krull et al, 2003). Recent studies suggested that the carbon inclusions in plant silica bodies contributed to the enhancement of long-term soil carbon sequestration in agro-ecosystem (Jones and Beavers, 1964; Currie and Perry, 2007; Rajendiran et al., 2012). Nevertheless, we did not observe autofluorescence emitted from the carbon inclusions inside silica bodies (Figure 2.5b). The number and distribution pattern of carbon inclusions in silica bodies varied in different *K. macrantha* accessions and cultivars. There was a statistically significant difference in the number of carbon

inclusions within silica bodies among accessions: the lowest average was found in 'Barkoel' with 2.08 carbon inclusions per silica body and the highest average was found in KM-CO with 5.56 carbon inclusions per silica body (Figure 2.5d). Since the number of carbon inclusions in each accession is stable, and the difference between accessions can be used to distinguish the uniqueness in each accession. In addition, the number and size of carbon inclusions can be used to measure the process of cell silicification and the correlation between silica deposition and carbon accumulation (Chen et al., 1996; Chen et al., 2002).

Silica bodies could be a means to improve turfgrasses, especially for the reduction of certain stresses such as plant herbivory. Increasing silica body content is shown to decrease folivore herbivory (Massey et al., 2006). Sorghum lines with different silica body deposition show different drought stress responses; the sorghum genotype with higher silica body deposition in the root and leaf tissue showed increased drought tolerance (Lux et al., 2002). Additions of porous hydrate calcium silicate improved wear resistance, insect resistance and decreased the disease severity caused by *Rhizoctonia solani* in zoysiagrass (Saigusa, 2000). It appears that silica bodies may be a means to improve a grasses ability to mitigate fungal disease severity, improve wear and insect resistance as well as decrease herbivory and drought stress. These structures require more study in relation to breeding low input turfgrasses.

Conclusion

We propose a method of combining fluorescence microscopy and image processing software for the quantification of silica bodies in *Koeleria macrantha* leaf

tissue, which can be applied to biological, ecological and geological studies of grass species. We observed differences between junegrass accessions for both size and density of silica bodies in leaf epidermis. In addition, we identified differences between accessions for carbon inclusions. This study outlines a means to investigate silica bodies in grass models utilizing a novel high throughput method.

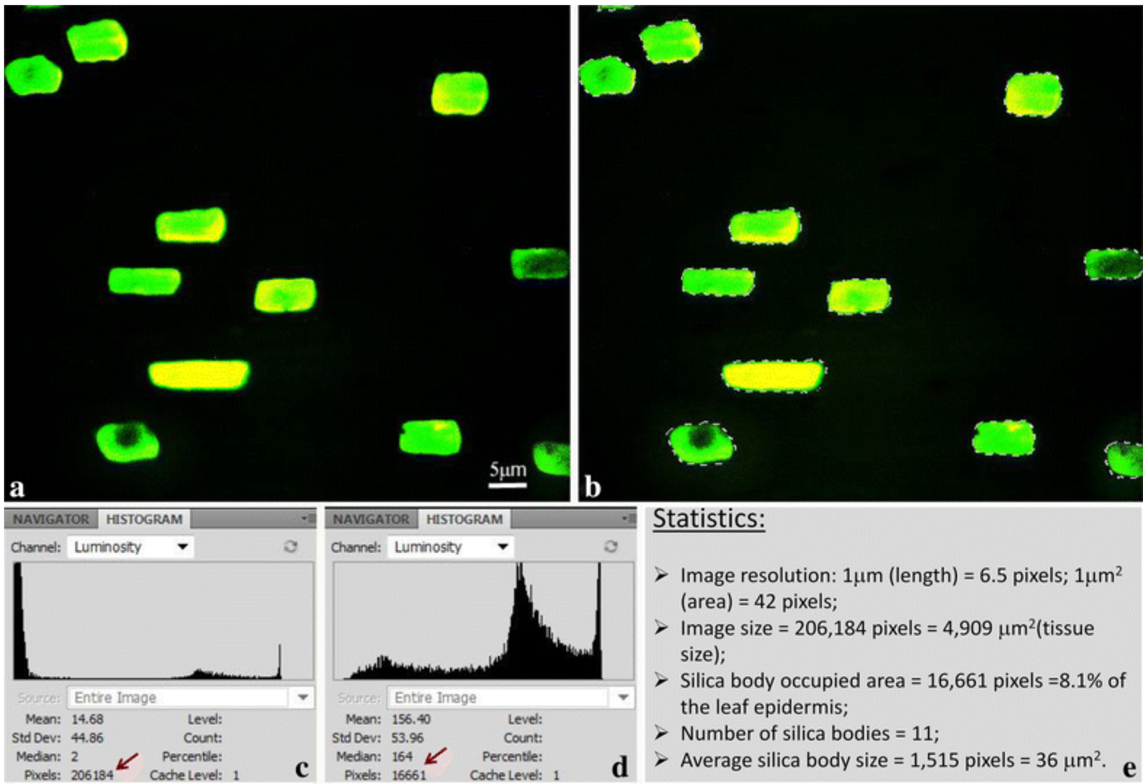


Figure 2.1
Size and distribution pattern analysis of silica bodies in *K. macrantha* 'Ireland' using Adobe Photoshop CS5. **a**A randomly selected microscopy image of ashed leaf sample. **b** The selection of silica bodies using "Magic Wand Tool" (background selection) and "Select->Inverse" tool. **c** Pixel reads of entire image. **d** Pixel reads of the selected silica bodies. **e** A list of statistical results

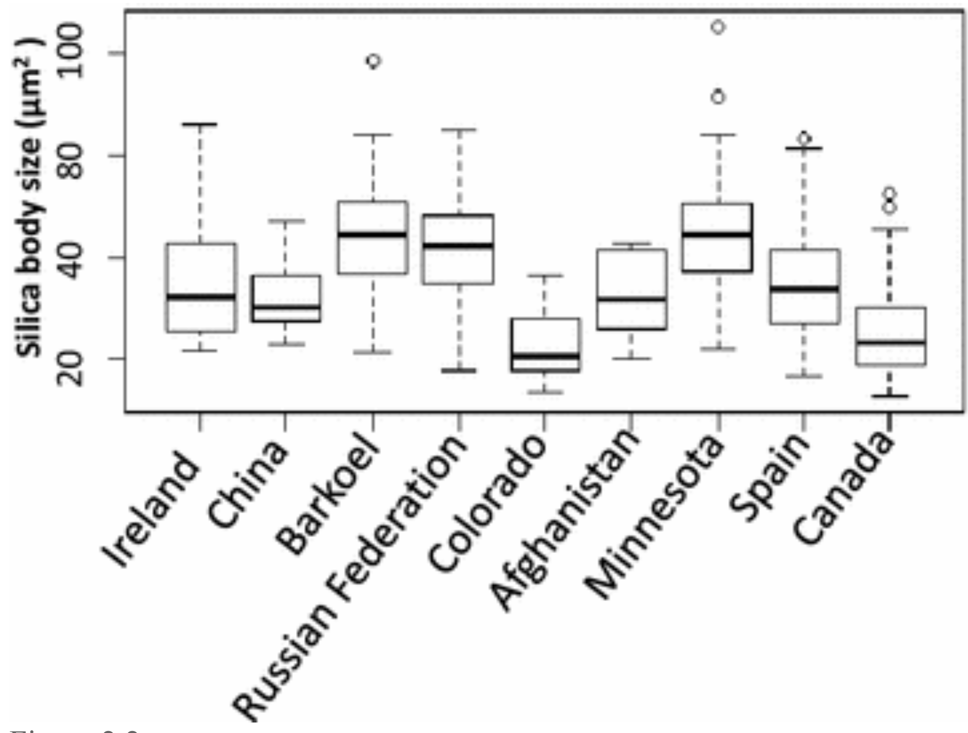


Figure 2.2
Box plot illustrates the size variation of silica bodies in 9 junegrass accessions. *Small circles* demonstrate samples with statistically loud noises

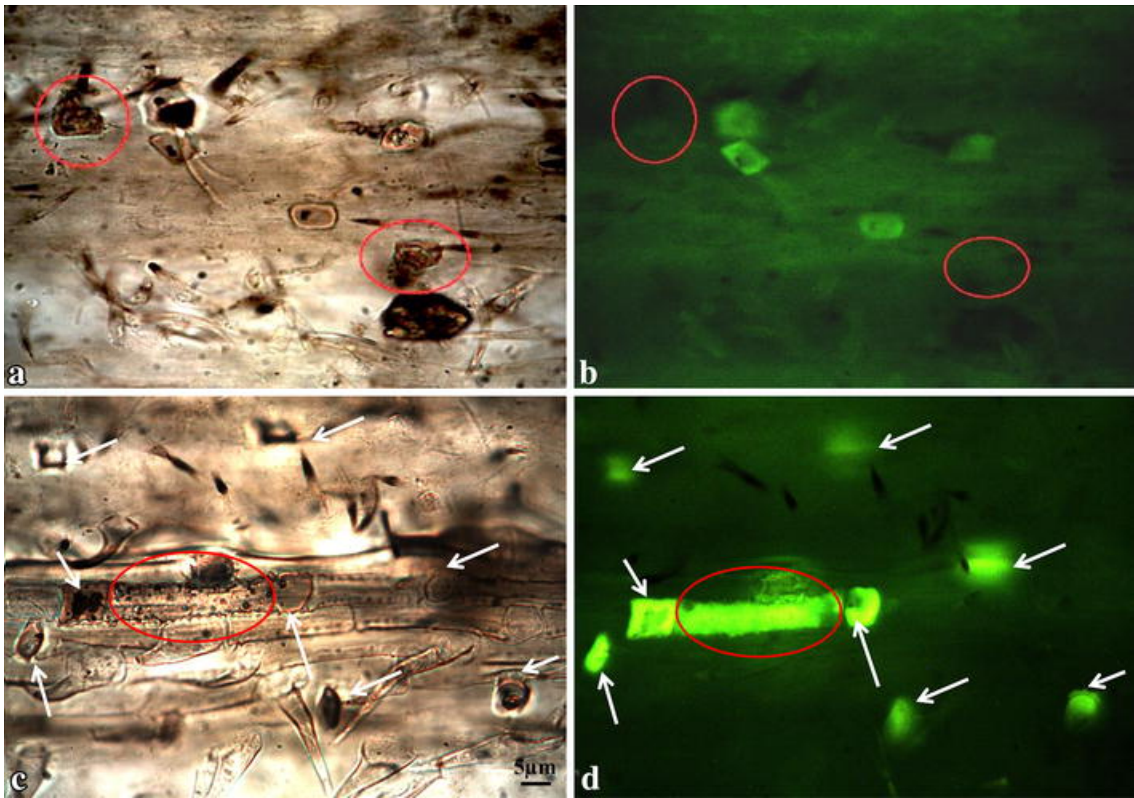


Figure 2.3
Property differentiation of silica bodies examined by brightfield and fluorescence microscopy. **a** Brightfield and **b** fluorescence microscopic examination of *K. macrantha* ‘Ireland’, showing two types of mineral structures developed in silica cells: Type I structures emitted green autofluorescence; Type II structures did not emit green autofluorescence (*red circled*). **c** Brightfield and **d** fluorescence microscopic examination of *K. macrantha* ‘Russian Federation’, showing that Type I structures developed in silica cells (short cells, *arrows*) emitted autofluorescence. There were also emission of green fluorescence from long cells (*red circle*) defined as type III silica bodies. Images **a**–**d** share the same magnification rate

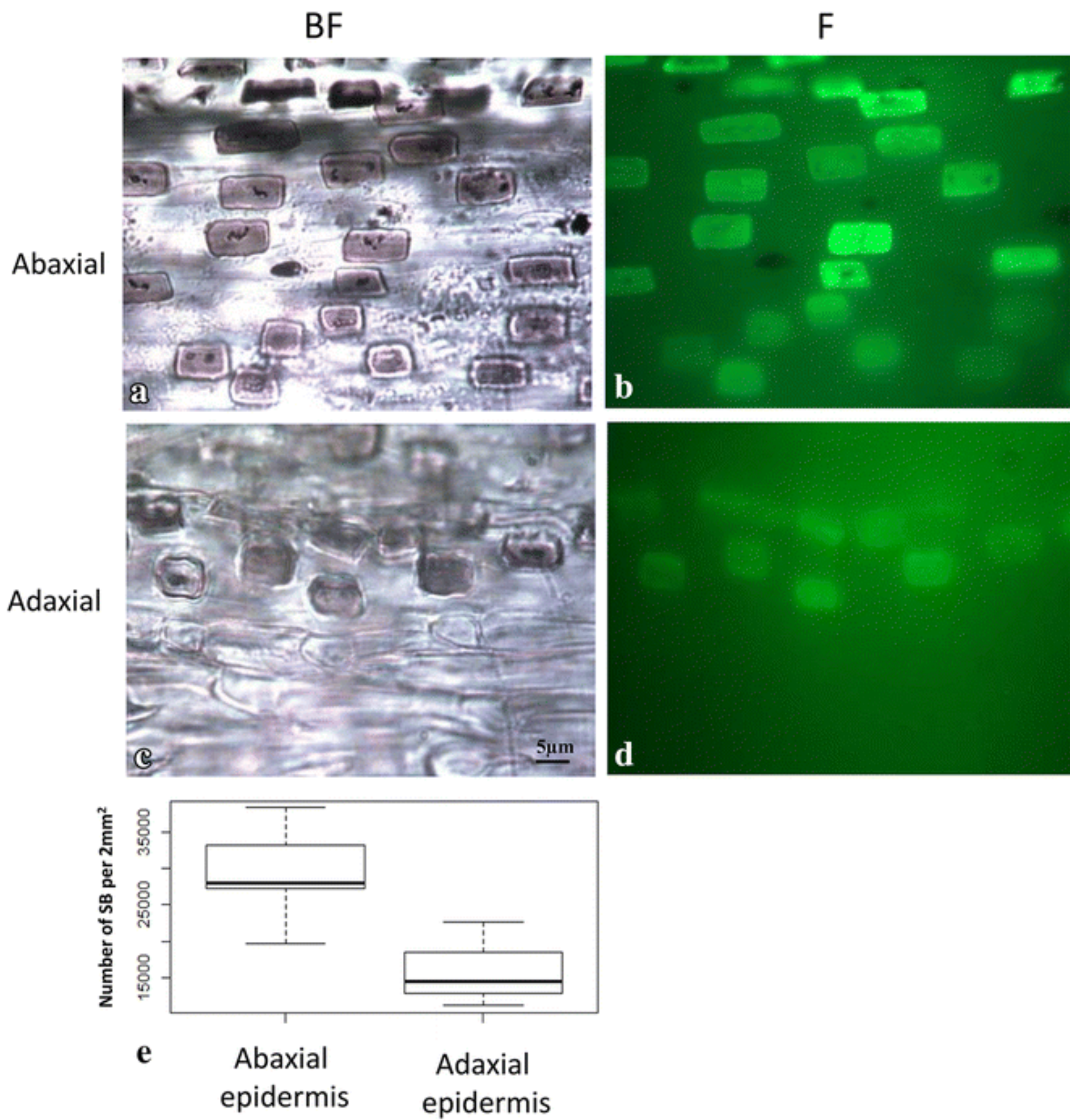
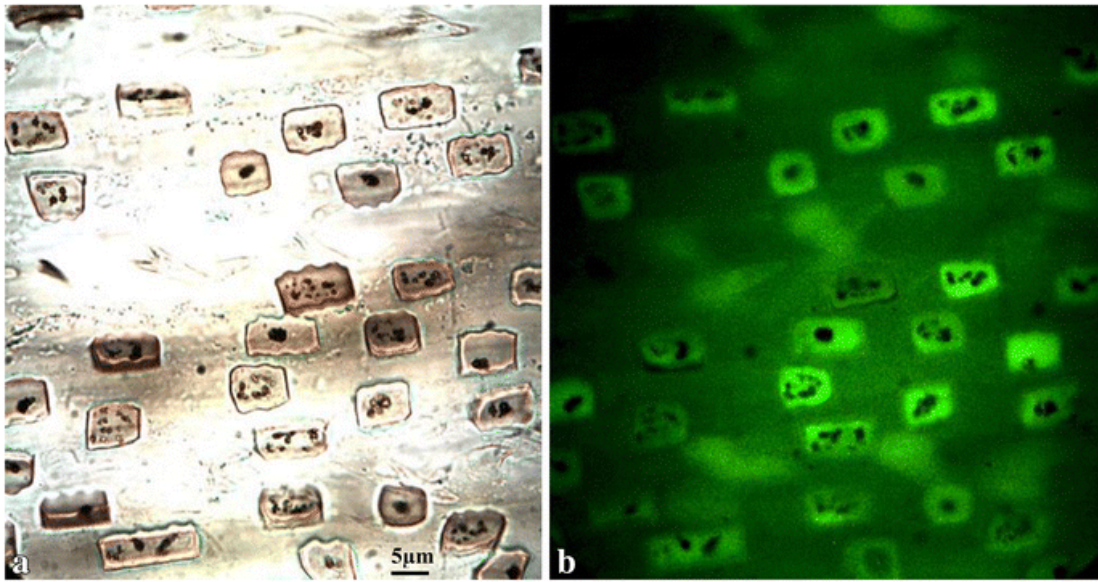


Figure 2.4
Silica body distribution differences between abaxial and adaxial leaf epidermis of KM-MN. **a** The abaxial epidermis under brightfield microscopy. **b** The abaxial epidermis under fluorescence microscopy. **c** The adaxial epidermis under brightfield microscopy. **d** The adaxial epidermis under fluorescence microscopy. **e***Box plot* illustrates the distribution differences of silica bodies between abaxial and adaxial leaf epidermis. Images **a**–**d** share the same magnification rate



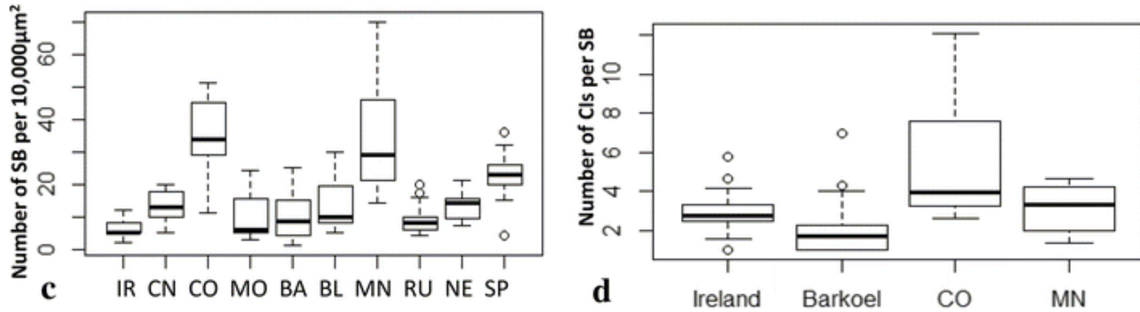


Figure 2.5
Characterization of carbon inclusions in the silica bodies. **a** Carbon inclusions within silica bodies demonstrated *brown* to *black colored* granules under brightfield microscopy. **b** Carbon inclusions within silica bodies did not emit green fluorescence under fluorescence microscopy. **c** *Box plot* illustrates the distribution differences of silica bodies among 10 accessions (silica body number per 10,000 μm^2, *white circles* demonstrate loud noises). **d** *Box plot* exhibits the number differences of carbon inclusions per silica body from the selected four accessions (*white circles* indicate statistically loud noises). *IR* 'Ireland', *CN* 'China', *CO*'Colorado', *MO* 'Mongolia', *BA* 'Barkoel', *BL* 'Bar leria', *MN* 'Minnesota', *RU* 'Russian Federation', *NE* 'Nebraska', *SP* 'Spain', *CI* carbon inclusion, *SB* silica body. Images **a** and **b** share the same magnification rate

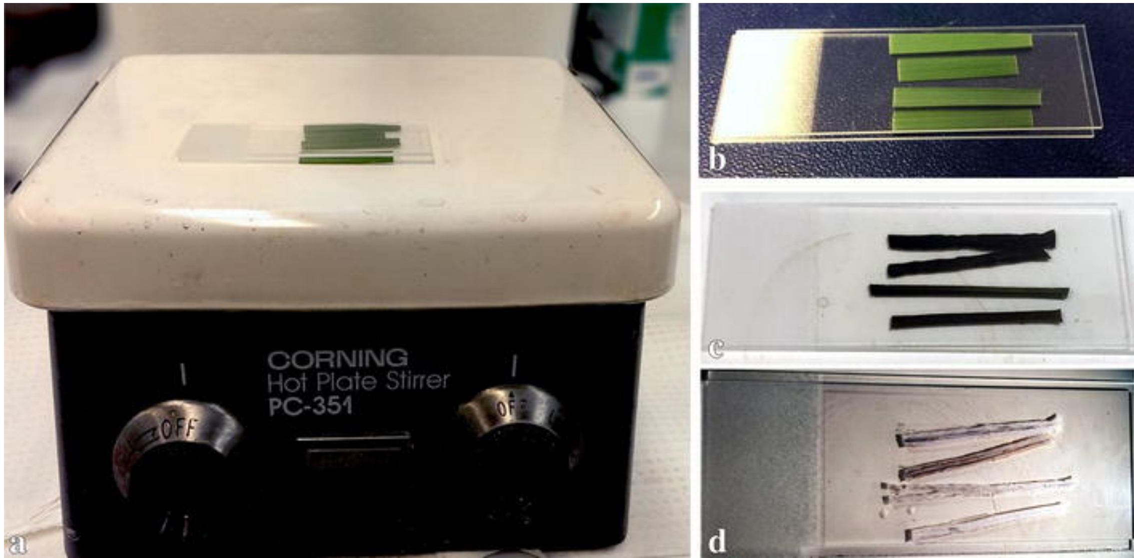


Figure 2.6
Illustration of sample preparation using dry ash method. **a** Dry ashing was performed by heating leaf-blade samples on a Corning Hot Plate at >608.2 °F/320 °C. **b** Unburned leaf tissues were placed between two microslides. **c** Incompletely burned tissue demonstrated *brown* to *black color*. **d** Completely dry *ashed* tissue showed *gray* to *white color.*

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Appendix 1

Assessment of microbial communities in greenhouse-grown *Koeleria macrantha* accessions

Plant breeders ultimately might be interested in whether or not there is sufficient variability in plant populations in how they affect soil microbial communities. Studies show that there appears to be a plant genetic influence on microbial community structures of the rhizosphere (Philippot et al., 2013; Peiffer et al., 2013; & Bulgarelli, 2015). The research objectives of were to see if the same plant species created significant differences in microbial community structure based on plant genotype. This following study utilized the aforesaid methods outlined in chapter 1; the following method outlines the differences used to study microbial communities in greenhouse-grown *Koeleria macrantha,* Seeds were obtained from University of Minnesota collections as well as the United States Department of Agriculture National Plant Germplasm System. One hundred seeds were sown into a flat of 2:1 ration of Sunshine MVP (Sun Gro Horticulture, Agawam, Washington): Turface (Profile Products LLC, Buffalo Grove, Illinois). Thirty-two plants were randomly selected from each accession or collection and transplanted when at the three-leaf stage. The greenhouse temperature during the day was between 20°C and 23 during the day and between 18.9°C and 21.1°C at night. The photoperiod was 16 hours supplemented with high pressure sodium lamps when incident light below 250 watts/m^2 for 30 minutes. The plants were irrigated daily, and fertilized weekly with a nutrient mix that contains 200 ppm nitrogen, 22 ppm phosphorous, 83 ppm potassium, 114 ppm sulfur, 2.5 ppm iron, 750 ppb magnesium, 100 PPb boron, 50 ppb copper, 280 ppb manganese, 500 ppb molybdenum, and 81 ppb zinc.

Between population: **differences between individuals in a population**

For the study investigating differences between populations of prairie junegrass, each of the randomly selected plants was vegetatively propagated into 16 single tiller clones which were potted into 4-inch pots into a 2 part Sunshine MVP (Sun Gro Horticulture, Agawam, Washington) to 1 part Turface (Profile Products LLC, Buffalo Grove, Illinois) mix. Additionally, for the *between* population study five different individual genotypes were randomly selected from the initial group of 32 plants for each population, and were then vegetatively cloned so that each genotype could be replicated three times (three clonal plants for each genotype within a population).

Within population**:** **differences between populations within the same species**

For the comparison of genotypes within a population, vegetative cloning occurred by taking 16 single tillers of junegrass from a single mother plants and they were potted in 4-inch pots into a 2 part Sunshine MVP (Sun Gro Horticulture, Agawam, Washington) to 1 part Turface (Profile Products LLC, Buffalo Grove, Illinois) mix. For the *within* population study, several genotypes were randomly selected and clonally propagated to produce five clones for each genotype. After the plants had grown for 90 days, rhizoplane soil was collected. Three subsamples of root tissue were collected per individual clone. Root tissue was collected and frozen in a -70C freezer on April 16th, 2015.

Results & Discussion

Figure A.1 and Figure A.2 shows that the clonal populations of junegrass associate closely to one another, thus there are plant genetic influences on the rhizosphere community structure. This finding is very similar to research by Bulgarelli et al. (2015) who found that in barley (*Hordeum vulgare L.*) host genotype accounts for approximately 5.7% of the variance in the rhizosphere community structure (Bulgarelli et al., 2015). Figure A.3 and Figure A.4 show that there are differences within populations and thus each individual in a population can produce a significantly different rhizosphere community structure but still be more closely associated with its own population and that each population produces a significantly different rhizosphere community structure. This result parallels the finding of Weinert et al. (2011) who showed that in three different potato cultivated varieties that a significant amount of OTUs detected were cultivar specific and *Streptomycetaceae* reacted in a cultivar-dependent fashion. Thus different genotypes of the same species differ in their rhizosphere community structure. This finding was also observed again in young potato plants where there was genotype specific changes in the rhizosphere community structure (İnceoğlu et al., 2011).

Figure A.1 shows there are differences that is genetic diversity within a population and that different populations still tend to be closely related in what microbial community structure is produced by the plant in the rhizosphere. Increases in phylogenetic distance between plant species also differences in rhizosphere community structural composition also increase (Wieland et al. 2001; Pongsilp et al. 2012; Bouffaud et al. 2014).

This plant host genotype having an influence on the microbes in the soil is also observed in *Agave spp.* where rhizosphere community structures show significant differences between cultivated and native agave (Coleman-Derr et al., 2016); this is similar to our findings (Figure A.3 and A.4). Similar results were found *Beta vulgaris;* the ancestor of beets, which showed significant difference in rhizosphere composition when compared to modern sugar beets (Zachow et al., 2014).

We identified 30001 and 35963 OTUs in the between clonal populations and within clonal populations. Individual soil sample had 2430±293 and 2400±246 in the between and within populations.

Between Population

Classified OTUs belonged to 55 phyla and several dominant phyla were observed which included *Proteobacteria*, *Actinobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Bacteroidetes*. Only 5 OTUs could not be assigned at the phyla level. The five most abundant phyla averaged across all samples is 87.42 ± 0.95% (Max=89.03% (Afghanistan); Min=85.65 (Ireland)).

Rare OTU belonged to 48 phyla and several dominant phyla were observed which included *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia*. The five most abundant phyla averaged across all samples is 75.74 ± 2.97% (Max=78.98% (South Africa); Min=66.78% (Ireland)).

Within Population

Classified OTUs belonged to 56 phyla and several dominant phyla were observed which included *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia*. Only 4 OTUs could not be assigned to a phyla. The five most abundant phyla averaged across all samples is 88.91 ± 1.34% (Max=90.47% (South Africa 4); Min=86.72% (Ireland 2)).

Rare OTU belonged to 48 phyla and several dominant phyla were observed which included *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia*. The five most abundant phyla averaged across all samples is 83.03 ± 1.71% (Max=86.00% (South Africa 5); Min=80.19% (Minnesota 5)).

Appendix 2

Assessment soil microbial communities of nineteen cool-season turfgrass species grown in Minnesota

Introduction

Different species of plant is shown to produce very different rhizoplane community structures surrounding turfgrass roots (Peiffer et al., 2013). This study looks at a nineteen different species of turfgrass. There appears to be significantly different rhizoplane community structures based on turfgrass species.

Methods

This study was established by seeding on September 3rd 2013 at the University of Minnesota Turfgrass Research Outreach and Education Center in St. Paul, MN. The turfgrass species and cultivars utilized in this study can be found in Table A.1. Outside of plots and turfgrass plants used the methods aforementioned in chapter 1 were used in this study.

Results and Discussion

Turf performance as measured by weed intercept (WI) was positively correlated with *Proteobacteria* and *Bacteroidetes* ($r = 0.254, 0.269$ and $p = 0.019, 0.013$) negatively correlated with *Planctomycetes* and *Firmicutes* ($r = -0.278, -0.224$, $p = 0.010, 0.040$). A curious finding that as aforementioned *Firmicutes* and *Proteobacteria* are associated with suppressive soils; in the species demo study there was a significant negative correlation between WI and *Firmicutes*. Investigation into the disease suppression that may be afforded by *Firmicutes* is prudent. *Actinobacteria*, *Proteobacteria* and *Firmicutes* have

been associated with disease suppressiveness (Mendes et al., 2011; Pankhurst et al., 2002; & Peng et al., 1999).

The OTU composition of the rhizoplane datasets were investigated and utilizing Kruskal-Wallis $P<0.05$ by species showing the OTUs whose abundance differed significantly. The OTUs that differed by phyla over 1% of sequence reads included: *Proteobacteria*, *Firmicutes*, *Verucomicrobia*, *Actinobacteria*, *Bacteriodetes*, *Planctomycetes*, candidate division WPS, and *Acidobacteria*. The species demonstration study shows that there is significant difference between different species of turfgrass and that the rhizosphere community structures can be incredibly varied that is appears to be genetically based and this may be exploited. More investigations into the rhizosphere of common cool season turfgrasses can elucidate the impact of genotype on the various microbial community structures. As can be seen in Figure A.5 there is a correlation between species and phyla.

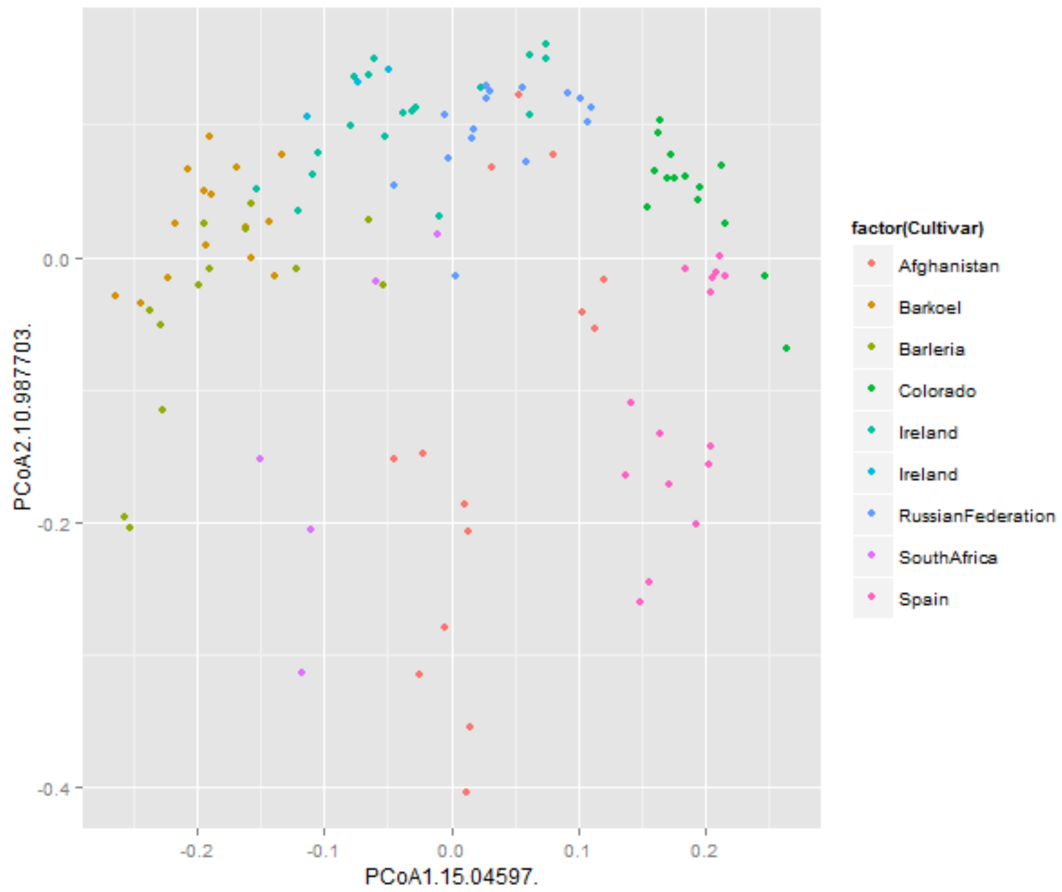

Figure A.1. Principal component analysis of *Koeleria macrantha* samples.

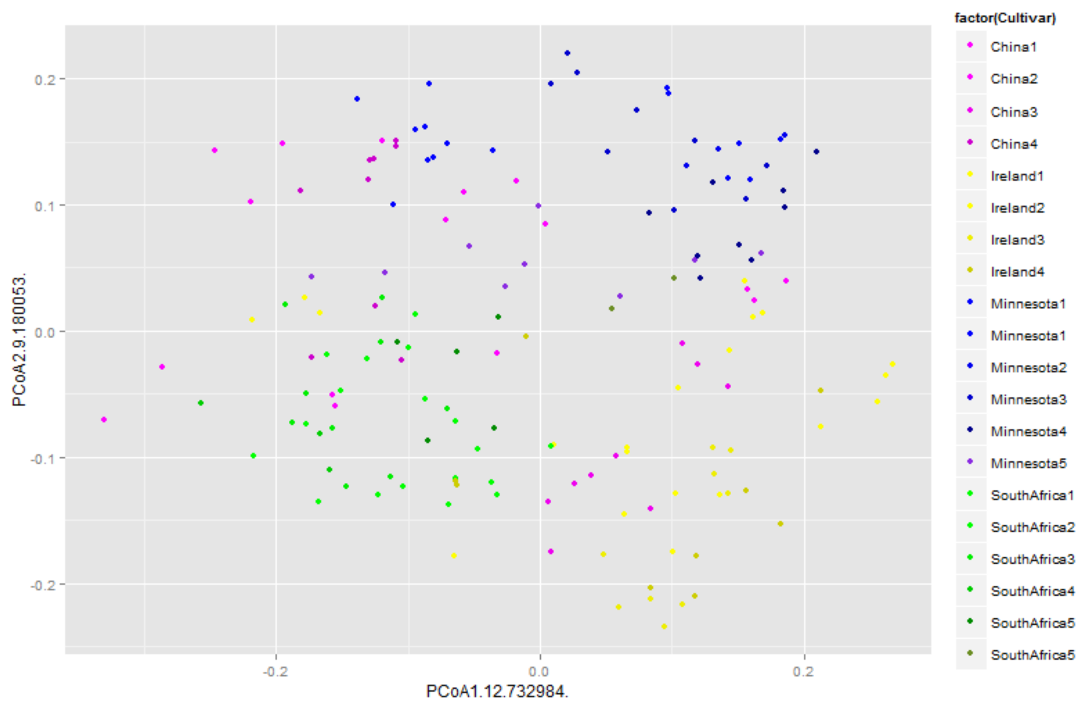

Figure A.2. Principal component analysis of *Koeleria macrantha* samples.

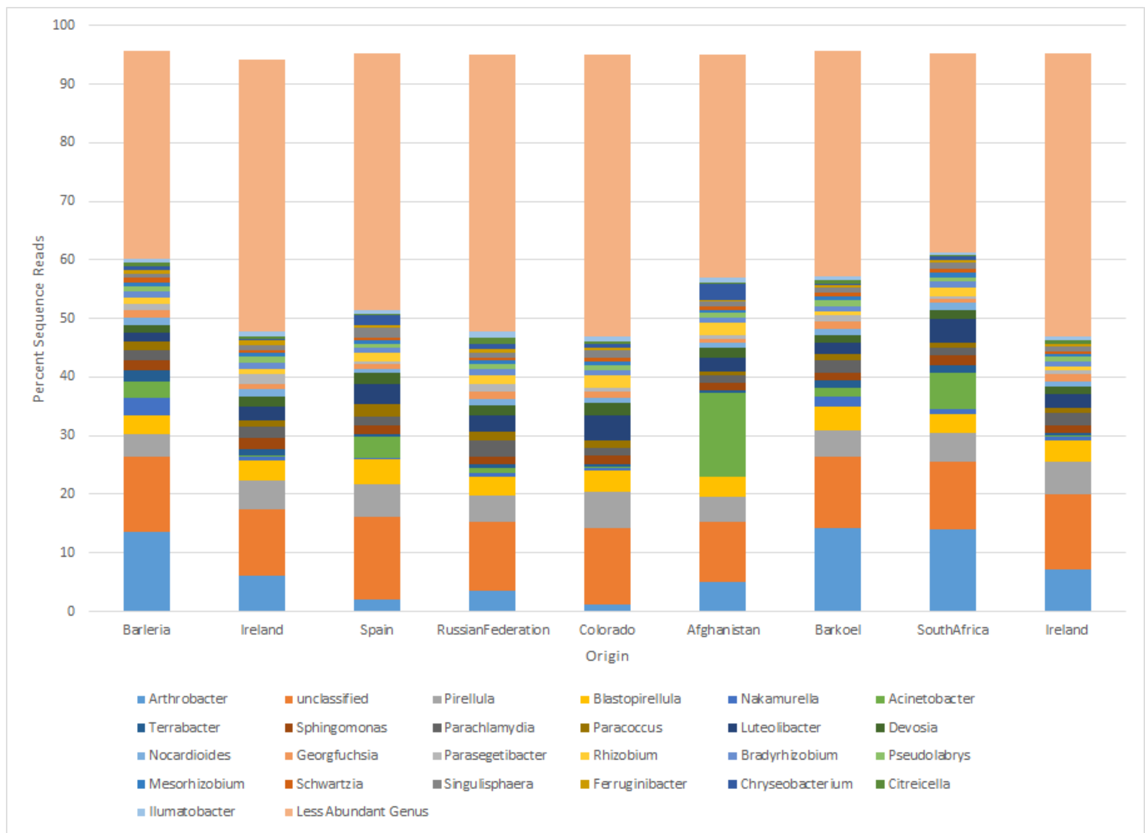

Figure A.3. Distribution of taxonomic genus of OTUs found to differ significantly by origin of *Koeleria macrantha*. The OTUs were found to vary significantly by using the Kruskal–Wallis test.

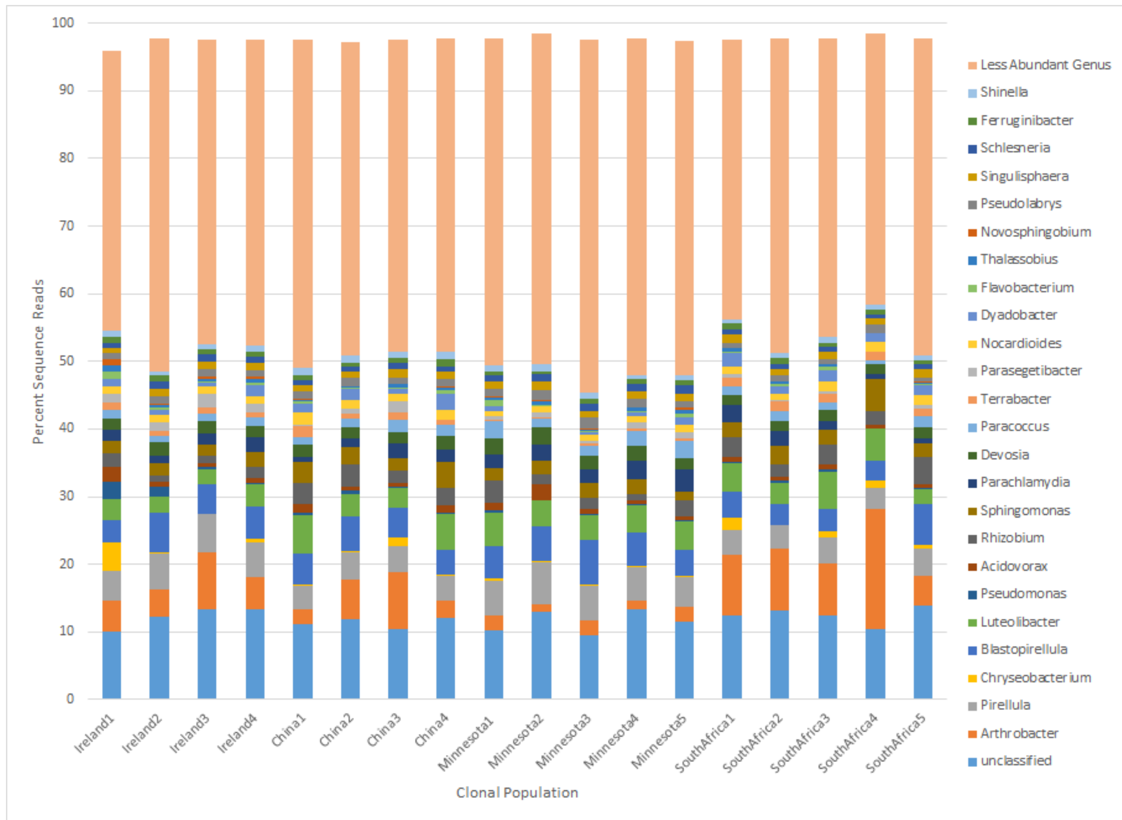

Figure A.4. Distribution of taxonomic order of OTUs found to differ significantly by origin of *Koeleria macrantha*. The OTUs were found to vary significantly by using the Kruskal–Wallis test.

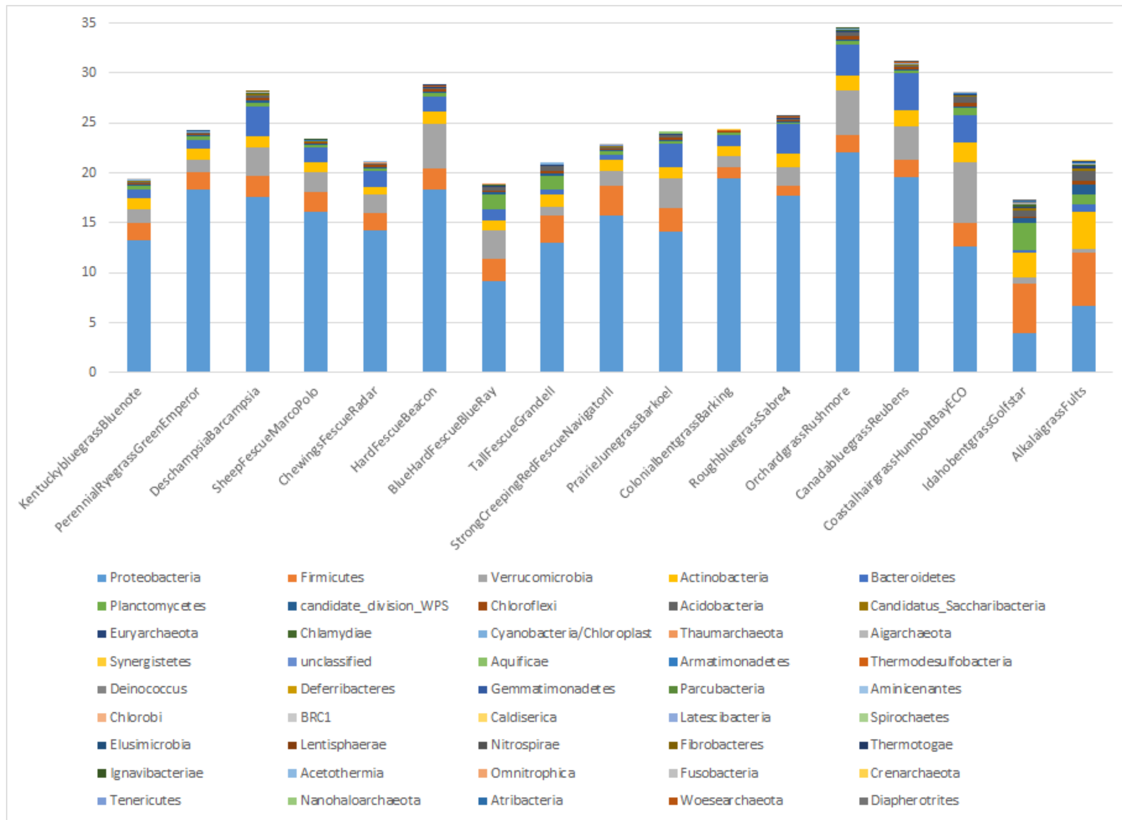

Figure A.5. Distribution of taxonomic phyla of OTUs found to differ significantly by species and cultivar. The OTUs were found to vary significantly by using the Kruskal–Wallis test.

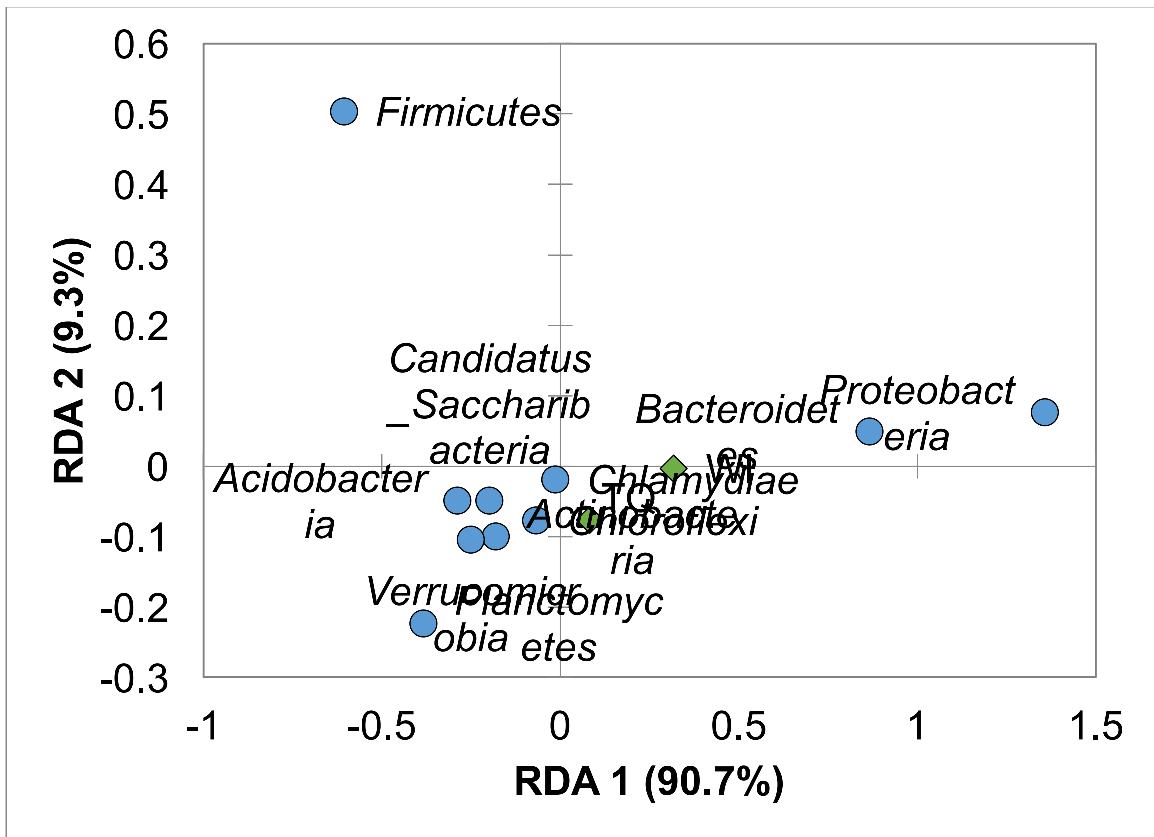

Figure A.6. Redundancy Analysis showing variation in phyla explained by turfgrass species at St. Paul for the species demonstration study in October 2013.

Scientific name	Common name	Cultivar
Agrostis capillaris	Colonial bentgrass	Barking
Dactylis glomerata	Orchardgrass	Rushmore
	Coastal hairgrass	Humbolt Bay ECO
Agrostis idahoensis	Idaho bentgrass	Golfstar
Puccinellia distans	Alkalaigrass	Fults
Poa pratensis	Kentucky bluegrass	Bluenote
Deschampsia cespitosa	Deschampsia	Barcampsia
Koeleria macrantha	Prairie junegrass	Barkoel
Festuca rubra subsp. rubra	Strong creeping red fescue	Navigator II
Puccinellia maritima	Alkalaigrass	Maritima Oceana
Festuca ovina	Blue hard fescue	BlueRay
Festuca arundinacea	Tall Fescue	Grande II
Lolium perenne	Perennial ryegrass	Green Emperor
Poa trivialis	Rough bluegrass	Sabre 4
Poa compressa	Canada bluegrass	Reubens
Festuca longifolia	Hard fescue	Beacon
Festuca rubra subsp. litoralis	Slender creeping red fescue	Shoreline
Festuca rubra subsp. commutata	Chewings fescue	Radar
Festuca ovina	Sheep Fescue	Marco Polo

Table A.1. Cool season turfgrass species used to investigate species differences in rhizoplane soil samples.