Wheat rhizosphere microbiome: response to application of the Plant Growth Promoting Rhizobacterium *Bacillus subtilis* T29 and *Fusarium pseudograminearum.*

By

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DECLARATION

I, Magalane Pheladi Gqozo, declare that the thesis, which I hereby submit for the degree Doctor of Philosophy (Plant Pathology) at the University of Pretoria, is my own work and has not been previously submitted by me for a degree at this or any other tertiary institute.

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SUMMARY

Soil microbial communities have demonstrated enormous potential for promotion of plant health and productivity. In particular, the diversity of the soil community may play an important role in promoting plant growth and health. However, previous research has focused on profiling both fungal and bacterial communities and neglected the role that pathogen presence and plant growth promoting rhizobacteria (PGPR) inoculation play in influencing plant growth. The objectives of this study were i) to determine the structure and composition of wheat root-associated microbial communities in different wheat growing regions of South Africa (SA); ii) to determine the variations in colonization and survival potential of *Bacillus subtilis* T29 in the rhizosphere of different wheat cultivars following artificial inoculation for growth promotion; iii) to determine the effect of *B. subtilis* T29 and the wheat pathogen, *Fusarium pseudograminearum*'s presence and on the structure and composition of the resident microbiome. To achieve this, metagenomic sequencing targeting the 16S rRNA was employed to decipher soil microbial diversity while droplet digital PCR (ddPCR) was used to identify and quantify *B. subtilis* T29 in wheat rhizosphere soils. Microbial community analysis revealed structural and compositional differences between rhizosphere and non-rhizosphere soils. Findings of the study indicated that non-rhizosphere soil had a higher diversity compared to cultivated rhizosphere soils. Cultivar and soil properties also had an effect on composition and distribution of microbial populations. For the first time, this study revealed that *B. subtilis* T29 can successfully colonize the rhizosphere of different wheat cultivars grown in South Africa and increase wheat growth. Most importantly, correlation analysis of colonization and plant growth revealed that higher levels of *B. subtilis* T29 colonization do not always

result in the highest plant mass increase. Comparison of taxonomic community profiles revealed major changes in fungal populations after introduction of *B. subtilis* T29 and/or *F. pseudograminearum* to the wheat rhizosphere soil. Marginal changes were however observed on bacterial populations after introduction of *B. subtilis* T29 and/or *F. pseudograminearum*. The above data provide an important insight into the diversity and composition of bacterial and fungal wheat microbiomes of different wheat cultivars from different agroecological conditions. The data also provide more information on the response of native fungi and bacteria after bioinoculation with a rhizobacteria and a wheat pathogen. The application of *B*. *subtilis* T29 provides a potential alternative strategy for increasing wheat growth, while minimising damage to the natural microbiomes. Overall, the results highlight the importance of understanding how microorganisms antagonize or benefit each other, which will contribute greatly to improving plant biomass production when manipulating agricultural soil microbial communities. Data gathered in this study therefore can be useful in future crop management strategies specific to the target production region to obtain a microbial community structure favoring improved plant health and productivity.

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Chapter 1

1.1. General introduction

Wheat (*Triticum sativum* L.) is one of the most important staple foods cultivated worldwide. Wheat, along with maize and rice, provides 50% of daily caloric intake in humans and is a critical food source in regions with rapid population growth such as Asia, Africa and the Middle East (Donn et al., 2015). The crop is ranked second after rice in cereal production worldwide (Fao, 2017; Verma and Suman, 2018). Approximately 731.5 million metric tonnes of wheat were produced in the 2018/19 season worldwide, which saw a 4.1% declined from 2017/18 season (USDA, 2020).

In South Africa (SA), wheat is considered an important cereal crop and it ranks second after maize in terms of the cultivation area with production ranging from 1.3 to 1.5 million tons per year (Grain SA, 2018). The major wheat production areas in SA are the Free State, Western Cape and the Northern Cape Provinces. Other provinces such as Gauteng and North-West are also ranked as minor producers of wheat (Nhemachena and Kirsten, 2017). As observed with the global downward trends with regards to crop production, the Free State Province saw a drop (up to 50%) in annual wheat production in the 2015/2016 season (Daff, 2016). This has resulted in a need for strategic interventions to increase the annual wheat production to at least 2.7 million tonnes in order to feed the country's growing population and avoid food price increases (DAFF, 2016).

One of the main factors contributing to the decline in wheat production is diseases and wheat is very susceptible to a number of them in the field with yield losses of up to 90% (Verma and Suma, 2018). Diseases caused by soilborne plant pathogens reported to be the most problematic, difficult to predict, detect, diagnose and ultimately control. Yield losses worldwide as a result of soilborne pathogens in wheat and other crops such as cotton and maize are estimated to be as high as 50% (Panth et al., 2020). On the other hand, only a limited number of fungicides and fumigants are effective in the control of soilborne diseases. Moreso, most of these fungicides are either banned, or in the process of being phased out worldwide due to health concerns and environmental pollution (Carlson, 2017). Moreover, the negative impact of chemicals on the environment and human health, has become the driving force for an urgent need for alternatives that are both environmentally and consumer friendly (Stewart, 2001; Schisler et al., 2004; Welbaum et al., 2004; Finkel et al., 2017; Singh et al., 2017; Carlson, 2017).

Application of microorganisms naturally occurring to the rhizosphere is generally seen as one of the optimistic ways to alleviating plant diseases and increasing crop yields. This environmentally friendly biotechnological approach offers an alternative to chemical control of plant diseases and pests, and furthermore reduces the need for application of chemical fertilizers (Dobbelaere et al., 2003). Among these alternatives, the development of plant growth-promoting rhizobacteria (PGPR) inoculants is promising and their use has seen a constant increase over the past few years (Backer et al., 2018). Plant growth promoting rhizobacteria have been successfully used in agriculture to promote plant growth and also to control diseases resulting in significant increases in yields (Zahir et al., 2004; Lugtenberg and Kamilova, 2009). In recent years, the use of PGPR as

biocontrol agents has acquired relevance, but most of these PGPR products have mainly been adopted commercially in countries like the United States of America (USA), United Kingdom (UK) and Australia (Qiao et al., 2017).

Bacillus subtilis, is one of the biocontrol agents widely recognised for its antagonistic properties to wheat diseases (Cao et al., 2011; Zhao et al., 2013) and plant growth promotion ability (Mercado-Flores et al., 2014). *Bacillus subtilis* is a Gram-positive, spore-forming bacterium that is widely distributed in the soil environment (Wang et al., 2018). The disease suppression activity by *B. subtilis* is the net result of multiple mechanisms, including plant growth promotion (PGP), antibiosis, competition for space and nutrients, lysis of pathogen hyphae, and induced systemic resistance (ISR) (Wang et al., 2018). Research has documented the suppression of several important plant pathogens following *B. subtilis* application and these include *Fusarium sp*. (Cao et al., 2011; Zhao et al., 2013), *Rhizoctonia solani* (Kuhn) (Kumar et al., 2012), *Sclerotium rolfsii* (Sacc) (De Curtis et al., 2010), *Sporisorium reilianum* (Kuhn) (Mercado-Flores et al., 2014), and *Verticillium dahliae* (Kleb) (Li et al., 2013). Liu et al. (2009) reported a significant reduction of take-all diseases in wheat of as much as 55.3% as a result of *B. subtilis* E1R-J application, in comparison to untreated control plants.

However, the introduction of an exogenous bacteria as an inoculant has the potential to affect resident microbiome, and vice versa (Sowinski et al., 2007). Such interferences may result in increased, decreased or no effect on PGPR effectiveness. Due to these potential effects, the need to study the microbial ecology of the rhizosphere following PGPR applications has become imperative (Sowinski et al., 2007). Several studies (Sowinski et al., 2007; Trabelsi and Mhamdi, 2013; You et al., 2016) investigating the

effects of microbial inoculants on soil ecosystems indicated that inoculation could significantly affect soil microbial communities. Such changes, in some cases, might affect the abundance of soil pathogens, thereby promoting plant health (Sang and Kim, 2012). In a study by You et al. (2016), the inoculation of *B. subtilis* Tpb55 increased the diversity and species richness of the bacterial community in the tobacco rhizosphere. Another study, focussing on the effects of *B. subtilis* Tpb55 on tobacco rhizosphere microbial community, confirmed the previous results and speculated that improving the diversity of the soil bacterial community and ecosystem stability might explain the strain's biocontrol efficacy on tobacco black shank (Han et al., 2016).

Similarly, Zhang et al. (2014) reported that the suppression ability of bioorganic fertilizers containing *B. subtilis* N11 against banana *Fusarium* wilt was associated with soil microbial community regulation, with increased densities of bacteria and actinomycetes but decreased numbers of fungi in the rhizosphere soil. Generally, susceptibility of the rhizosphere to invasion by soil pathogens is inversely related to the diversity of the rhizosphere microbiome (Matos et al., 2005), whereby increased diversity can result in decreased pathogen virulence. Moreover, promoted microbes can compete with pathogens for resources, produce compounds that are inhibitory to pathogens, or parasitize pathogens. Indeed, most rhizosphere bacteria and fungi are prolific producers of metabolites that inhibit the growth or activity of competing microorganisms.

On the other hand, Li et al. (2016) found that *B. subtilis* B068150 did not significantly change the diversity of microbial communities in a cucumber rhizosphere, indicating that the effects of different strains on the soil microbial community are variable. Natural variation is the major challenge of using *B. subtilis* in the field (Le et al., 2016; Wang et

al., 2018). The colonization of *Bacillus* isolates is the critical factor for successful biocontrol under natural conditions (Wang et al., 2018). Biocontrol strains are mostly screened under controllable conditions such as the laboratory or greenhouse. However, many factors including pesticide application or pesticide residue are unfavorable for colonization under field conditions (Compant et al., 2010; Lee et al., 2014). The application of biocontrol *B. subtilis* is also affected by temperature, humidity, soil type, pH amongst other factors (Lee et al., 2014). Therefore, it is difficult to predict how an organism may respond when released to the natural environment compared to the controlled environment under laboratory or greenhouse conditions. Studies on the success of PGPR application in wheat under field conditions in SA are to date not available. Therefore, identifying resident microbiome and studying its shift upon PGPR inoculation could benefit sustainable wheat production systems.

This study is proposed to unravel aspects of soil microbial diversity in the wheat growing regions of SA. Also, to investigate the dynamics of the rhizosphere microbial communities in the presence of the PGPR *B*. *subtilis* T29 and the pathogen *F*. *pseudograminearum*. This research will focus on the nature and composition of the wheat rhizosphere microbiome from three different provinces i.e.: Free State, Western Cape and Gauteng Provinces, to ensure more sustainable production systems for the future. Detailed aims of this research are listed below.

1.2. Aim and objectives of the research.

The main aim of this study was to investigate wheat performance and the rhizosphere microbiome of wheat as affected by the plant beneficial bacterium *B. subtilis* and the pathogenic fungus *F. pseudograminearum*. To further describe the dynamics of bacterial and fungal communities and diversity associated with the wheat rhizosphere, furthermore, study the colonization efficacy of *B. subtilis* T29 and its effect on shaping the wheat rhizosphere microbiome during growth promoting and control of *F. pseudograminearum.* This was investigated through the following objectives:

- To profile bacterial and fungal communities and diversity on wheat rhizosphere from three wheat growing regions in SA.
- To investigate how variations in field soil physico-chemical properties affect associated microbiomes, particularly pathogenic and beneficial populations on wheat rhizosphere in three different agro-ecological sites.
- To investigate *B*. *subtilis* T29 in terms of colonization efficacy and growth promotion on wheat on different cultivars and soils.
- To evaluate microbial population, shift on wheat rhizosphere caused by *B. subtilis* T29 and *F*. *pseudograminearum* application in soil.

The research hypothesis is thus:

Ho: The application of biocontrol agents in the soil affects the diversity and composition of resident microbial communities.

H1: The application of biocontrol agents in the soil does not affects the diversity and composition of resident microbial communities.

These objectives will be addressed in the following way:

Chapter 2: A review of control of plant pathogens on wheat and its impact on soil microbial diversity.

Chapter 3: Bacterial microbiomes in the wheat seedling rhizosphere

Chapter 4: Fungal diversity and community composition of wheat rhizosphere and non-rhizosphere soils from three different agricultural production regions of South Africa

Chapter 5: Root colonization and plant growth promotion by *Bacillus subtilis* T29 on different wheat cultivars.

Chapter 6: Effects of *Bacillus subtilis* T29 on wheat growth and health under *Fusarium pseudograminearum* pressure and its impact on the rhizosphere microbiome.

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Chapter 2: Literature review

Control of wheat pathogens and dynamics of soil microbial communities

2.1. Introduction

Wheat (*Triticum aestivum L.)* is an edible cereal grain (Belderok et al., 2000). It is grown on more acreage than any other commercial crop and continues to be the most important food grain source for humans worldwide (FAO, 2017). Wheat is an important basic food in many regions across the world accounting for nearly 55% of the carbohydrates and 20% of the food calories consumed annually (Breiman and Graur, 1995). Globally, wheat is the most important source of vegetable protein in the human diet and has a higher protein content than the other major cereals including maize (*Zea mays L.)* and rice (*Oryza sativa L*.) (Nutrient data, 2013). Wheat is amongst the top five produced crops, including rice, maize, potatoes and sugarcane worldwide (FAO, 2017).

In South Africa (SA), during the 2018/19 season, wheat production contributed approximately 11% to the gross value of field crops (DAFF, 2019). However, there are constraints to the high production of wheat. This includes both abiotic and biotic factors and amongst them are unpredictable weather conditions and drought, low soil fertility and the devastating impact of pests and diseases (Scott, 1990; Pannar, 2009). Globally, productivity increases in wheat have slowed to 0.9% per year, yet there is a dire need to increase production to at least 1.5% per year by 2050 to avoid food price increases (Fischer and Edmeades, 2010). Additionally, yield declines have been experienced in most cereal systems which follow wheat rotations, and these dominate many global food

production systems. These declines are often as a result of undiagnosed soil biological causes (Lobell et al., 2009). Although intensive agriculture has contributed to increases in crop yields in previous years, detrimental effects on the physical and biological properties of soils have often been reported to be associated with such production systems (Pimentel et al., 1995; Bouwman et al., 2009). Furthermore, to maximize crop yields, chemical fertilizers are used to compensate for a loss in soil fertility in intensively managed agricultural systems, while tillage is practiced. However, these practices further disrupt microbial communities (Johnson et al., 1997).

Soil microbial communities and their associated functions largely determine the productivity of agroecosystems (van der Heijden et al., 2008). Soil microbes, therefore, play a key role in the cycling of nutrients such as nitrogen or phosphorus as well as providing plant protection against biotic and abiotic stresses, thus increase plant production (Bender et al., 2016; Lladó et al., 2017). The composition of the soil microbiome presents the major driver in shaping the bacterial and fungal communities associated with plant roots (Bulgarelli et al., 2013; Vandenkoornhuyse, 2015). Several recent microbial community studies have described the root microbiomes of *Arabidopsis thaliana* (Bulgarelli et al., 2012; Lundberg et al., 2012), clover (Hartman et al., 2017), maize (Peiffer et al., 2013), rice (Edwards et al., 2015), sugarcane (Yeoh et al., 2015), and grapevine (Zarraonaindia et al., 2015) and reported significant effects of soil type on root microbiome community composition. Soil and root microbial communities are intricately linked. Therefore, root microbial communities are affected by agronomic practices (Seghers et al., 2004). Moreover, maintaining a diverse and well-balanced

microbiome at the plant-soil interface is vital in crop production (Syed Ab Rahman et al., 2018).

Microorganisms that can be both beneficial and pathogenic to the crop are present in the wheat soil microbiome (Mendes et al., 2013). Wheat is affected by many organisms, and these include bacteria, viruses, nematodes, and fungi (Crop Disease Management Bulletin, 2013). In Ethiopia, where it is thought wheat originated, 30 fungal, three bacterial, one viral and four nematodal diseases have been identified (Eshetu, 1985). However, only few are important constraints for wheat production specifically the rusts, *Septoria* sp., *Fusarium* spp. and the smuts (Singh et al., 2002).

In South Africa, several fungal diseases affect wheat. *Rhizoctonia* and *Fusarium* species cause root and crown rot, which severely reduce yields in wheat production (Scott, 1990). Crown rot of wheat is considered the most important wheat disease in the Free State Province (Van Wyk et al., 1988) and the Western Cape Province (Lamprecht et al., 2006) of South Africa. All South African wheat cultivars are susceptible to these diseases although different levels of severities have been recorded. Chemical control methods have been reported mostly ineffective in controlling these diseases (Lamprecht et al., 2006). With effective control of crown rot being challenging, management of the disease in the Western Cape Province is routinely done by either implementing reduced tillage or no-tillage practices during conservation farming (Lamprecht et al., 2006; Conservartion Farming Work Group of the Western Cape Province, 2011).

Even when optimal doses of chemicals are used, environmental and public concerns have been raised over their harmful effects on human health and the environment

(Remoundou and Koundouri, 2009). Alternatively, methods that reduce soilborne pathogen inoculum levels have been used; these include crop rotation and management practices that reduce the loss of soil moisture and plant stress (Draper, 2000; Pannar, 2009; DAFF, 2010). There is thus a need for innovative disease control strategies that are more effective in order to improve crop yields and minimize reliance on the use of chemicals. One such possible option is the manipulation of the rhizosphere microbiome to encourage the proliferation of microorganisms that promote plant growth and control disease. Therefore, future studies are required to establish the effect of the plant growth promoting microorganisms on natural microflora, while increasing plant productivity and improving plant health.

2.1. The host- wheat

2.1.1. Origin and history of wheat

Wheat (*Triticum aestivum L.*) is a nutritious and concentrated cereal grain that has the capacity to be easily stored, transported, and processed into different types of food (Belderok et al., 2000). It is assumed to have originated from wild grasses since its exact place of origin is unknown (DAFF, 2010). Wheat was first seen in the Levant region of the Near East and Ethiopian Highlands; thus, it is assumed to be the place of its origin (Feldman and Kislev, 2007). The development of agriculture and cultivation of wheat occurred approximately 9 000 to10 000 years ago (Katz, 2003).

‘Wild einkorn’, the most common variety of wheat prior to the Neolithic revolution, led to the evolution of wild emmer by crossing with wild goat weed. Emmer is the oldest cultivated variety of wheat, grown as early as 8700 B.C.E. in ancient Turkey, and then introduced to Mesopotamia, Egypt, Rome, and Greece (Kiple and Kriemhild, 2000). During these prehistoric times, the cultivation of wheat increased rapidly extending to North Africa and the Indus valley of Northern India by 4000 B.C.E, Northern China by 3000 B.C.E. and Western Europe by 2000 B.C.E. Wheat was introduced to the New World by the Spanish, and eventually reached America by the early 17th century through English settlers in the North American colonies (Smith, 2004).

Wheat was therefore considered as a key factor that enabled the emergence of city-based societies at the start of civilization because it was one of the first crops that could be easily cultivated on a large scale. It also had an advantage of yielding a harvest that provides long-term storage (Rapsomanikis et al., 2003).

2.2. Wheat production in South Africa

Commercial wheat production in SA started in the early 1910s with varieties brought by the Dutch traders to Cape Town (then the Cape of Good Hope). In SA, the main uses of wheat are human consumption (especially for making flour for the bread industry), industrial (important sources of grain for alcoholic beverages, starch, and straw), and animal feed (bran from flour milling as an important source of livestock feed, grain as animal feed) (DAFF, 2010). There are two basic types of commercially cultivated wheat in SA which differ in genetic complexity, adaptation and use: (1) bread wheat (*Triticum*

aestivum) and (2) durum wheat (*Triticum turgidum*) (Nhemachena and Kirsten, 2016). Wheat is produced in 32 of SA's 36 crop production regions and occurs in both summer and winter rainfall regions. The main wheat-producing provinces are the Western Cape (winter rainfall), Free State (summer rainfall) and Northern Cape (irrigation), Mpumalanga (irrigation) and North West (mainly irrigation) (SAGL, 2012). In recent years, SA's wheat area production has stagnated at around 500,000 hectares, and this is approximately a million hectares less than two decades ago (USDA, 2019). The Western Cape produces almost 50% of SA's total wheat production. The other major wheat producing areas are the irrigation fields in the Northern Cape and the Free State. Together these three provinces produced almost 85% of SA's total wheat crop in the 2018/19 season (USDA, 2019). Wheat is planted between mid-April and mid-June in the winter rainfall areas and between mid-May and end of July in the summer rainfall areas. Production under irrigation has been reported to potentially have a higher yield than dry land wheat production (PANNAR, 2009). On the other hand, dry land productivity in SA has been shown to be exceptionally low compared to that of the major wheat-producing countries in the world including Australia and Pacific North West (Nhemachena and Kirsten, 2016).

2.3. Economic importance of wheat in South Africa

South Africa mainly produces bread wheat, while durum wheat represents an exceedingly small percentage of the annual wheat production in the country. The wheat industry contributes about R 4 billion to the gross value of agricultural production in SA and currently provides about 28 000 job opportunities (Van der Vyfer and Nordier, 2013;

DAFF, 2019). The annual wheat production in SA for 2018/2019 production cycle was 1.8 million tonnes which represented an increase of 20 percent from the previous year's drought crop of 1.5 million tonnes (USDA, 2019). However, the overall trend for the past 10 years show that wheat production has been decreasing (Nhemachena and Kirsten, 2017). The Crop Estimates Committee (CEC) forecasts SA's wheat and wheat products imports for the 2019/20 cycle, at 1.9 million tonnes (USDA, 2019).

2.4. Commercial wheat varieties/cultivars grown in South Africa.

Cultivar choice is an important production decision; and if planned correctly, could contribute greatly to reduce diseases and optimise yields (ARC, 2019). A huge variety of cultivars are available in SA, and these are divided into three categories, namely cultivars for dry land production in the North, winter production in the South and irrigation mainly in the North (Wheat production guideline, 2017). The suitable cultivar is chosen based on factors such as cultivar adaptability, yield potential, grading and quality, diseases and pest's resistance, seed price, hectoliter mass, straw strength, aluminium tolerance, photoperiod and vernalization, shatterproof and preharvest sprouting tolerance and agronomic characteristics (ARC, 2019). An innovation investment targeting wheat variety improvements has been established in SA, targeting the need to improve yield potential, resistance/tolerance to biotic and abiotic stresses and nutritional and processing quality and adaptability to the production area (Lantican et al., 2005; Atack et al., 2009).

An analysis of the sources of wheat varietal improvements during the different growth periods indicates that wheat breeding was driven initially by individual breeders and

agricultural colleges. The current main sources of wheat varietal improvements in SA are Sensako, the Agricultural Research Council's Small Grain Institute (ARC–SGI) and Pannar seeds (Nhemachena and Kirsten, 2016). The most popular varieties identified for further analysis of cost attribution and the benefits of wheat varietal improvements were Gariep, Elands and Duzi. However, there are many wheat cultivars available in South Africa. No one cultivar can be resistant to all the fungal diseases, therefore, fungicide application remains of importance in the sustainable production of small grains in SA (ARC, 2019). Tables 2.1, 2.2 and 2.3 below outlines the different cultivars suited for the different growing regions and their disease resistance or susceptibility to the different diseases.

Table 2.1. Wheat cultivars recommended for dry land regions and their disease resistance/susceptibility to different wheat diseases in South Africa (ARC, 2019)

Cultivar	**Stem rust**	**Leaf rust**	**Stripe rust**
Eland (PBR)	MR	S	MS
Gariep	R	S	S
Koonap (PBR)	R	S	R
Matlabas (PBR)	S	S	S
PAN 3111 (PBR)	R	S	MR
PAN 3118 (PBR)	R	S	S
PAN 3120 (PBR)	R	MS	MS
PAN 3161 (PBR)	R	MS	MR
PAN 3195 (PBR)	R	S	MR
PAN 3198 (PBR)	R	R	MR

PAN 3368	MR	MS	MR
PAN 3379 (PBR)	MS	MS	MS
Senqu (PBR)	R	MS	R
SST 3149 (PBR)	MR	R	R
SST 316 (PBR)	MR	S	R
SST 317 (PBR)	MR	S	R
SST 347 (PBR)	MR	MS	MS
SST 356 (PBR)	MS	S	R
SST 374 (PBR)	MS	S	MS
SST 387 (PBR)	R	S	R

*****PBR – Cultivars protected by plant breeder's rights. MR = Moderately Resistant. S= Susceptible. MS= Moderately Susceptible. R= Resistant.

Table 2.2. Wheat cultivars recommended for irrigation regions and their disease resistance/susceptibility to different wheat diseases in South Africa (ARC, 2019)

Cultivar	**Stem Rust**	**Leaf Rust**	**Stripe Rust**
Baviaans (PBR)	S	MS	R
Duzi (PBR)	S	S	R
Kariega	S	MS	R
Koedoes (PBR)	MS	S	S
Krokodil (PBR)	MS	S	S
PAN 3400 (PBR)	MS	S	R
PAN 3471 (PBR)	S	MS	R
PAN 3497 (PBR)	S	S	R

PAN 3514 (PBR)	MS	R	R
PAN 3623 (PBR)	S	S	R
Renoster (PBR)	S	S	MS
Sabie (PBR)	S	MS	R
SST 806 (PBR)	S	MS	R
SST 8135 (PBR)	MS	MS	R
SST 8154 (PBR)	MS	S	R
SST 8156 (PBR)	MS	MS	R
SST 822 (PBR)	MS	MS	R
SST 835 (PBR)	MS	MS	MR
SST 843 (PBR)	MS	MS	R
SST 866 (PBR)	S	MS	R/MS
SST 867 (PBR)	S	MS	MR

*****PBR – Cultivars protected by plant breeder's rights. MR = Moderately Resistant. S= Susceptible. MS= Moderately Susceptible. R= Resistant.

Table 2.3. Wheat cultivars recommended for winter rainfall regions and their disease resistance/susceptibility to different wheat diseases in South Africa (ARC, 2019).

Cultivar	**Stem rust**	**Leaf rust**	**Stripe rust**
Baviaans (PBR)	S	MS	R
PAN 3408 (PBR)	MS	MS	R
PAN 3471 (PBR)	S	MS	R
Ratel (PBR)	MR	MS	R

SST 0117 (PBR)	S	MR	MR
SST 0127 (PBR)	MR	MR	R
SST 0147 (PBR)	R	MR	MR
SST 0166 (PBR)	MS	MR	R
SST 015 (PBR)	S	S	R
SST 027 (PBR)	MR	MS	R
SST 056 (PBR)	MS	MS	MR
SST 087 (PBR)	S	S	R
SST 096 (PBR)	S	MS	MR
SST 88 (PBR)	S	S	MR
Tankwa (PBR)	MS	MS	R

*****PBR – Cultivars protected by plant breeder's rights. MR = Moderately Resistant. S= Susceptible. MS= Moderately Susceptible. R= Resistant.

2.5. *Fusarium* crown rot of wheat: it's effect on wheat production in South Africa.

There are many factors affecting wheat production in SA and these are variable climatic conditions (including dry, warm winters), low soil fertility, pests and diseases, cultivar choice and cultivation methods (Dube et al., 2020). With regards to pests and diseases, increasing incidences of root diseases such as eyespot, take-all and crown rot have become a major threat to wheat yields and the profitability of the business in recent years (ARC, 2019). Soilborne diseases such as crown rot are very difficult to control because of the heterogenous incidence and lack of knowledge on the epidemiological aspects of the pathogen (Veena et al., 2014). Therefore, for the purpose and interest of this study,

a detailed description of the causal agent of crown rot and disease development in wheat will be given. Other soilborne pathogens causing crown/root diseases will not be discussed in this literature review as they were not investigated in detail in this thesis.

The filamentous fungal genus *Fusarium* has a worldwide distribution and contains at least 300 phylogenetically distinct species/species complexes (O'Donnell et al., 2015; Fang et al., 2020). In most cases, the *Fusarium sp* infects crops as a complex. In wheat, crown rot is caused by the predominant pathogens which are members of the *Fusarium graminearum* species complex (FGSC), namely *Fusarium culmorum*, *Fusarium pseudograminearum*, and *Fusarium graminearum* (Fang et al., 2020). Most species within the FGSC appear to be restricted to specific geographic regions (Shen et al., 2012). Crown rot caused by *Fusarium pseudograminearum*, is a chronic disease of wheat and barley in many arid and semi-arid cropping regions of the world (Kazan and Gardiner, 2018). In SA has particularly become more prevalent in the Western Cape, but it is also present in other small grain producing areas in the country. The disease is especially important in areas where wheat is cultivated under dry land conditions, and it is difficult to control. Crop rotation with leguminous crops is recommended to combat this disease (ARC, 2019). The most effective control strategy of crown rot to date is a well-planned and managed crop rotation system, which aims at eliminating annual grasses and volunteer wheat which may serve as a source of inoculum at least 12 months prior to crop establishment (ARC, 2017).

2.5.1. Classification of *Fusarium pseudograminearum*

Fusarium pseudograminearum (O'Donnell and Aoki) is a member of the *Nectriaceae* family. It is also known as *Gibberella coronicola* (O'Donnell and T. Aoki), which is the teleomorph stage. The fungus is usually referred to by its anamorph (asexual form) name because the sexual phase is usually not present for crown rot of wheat (Backhouse et al., 2004). It is a monoecious fungus with a narrow host range (Ferreira et al., 2015). *F. pseudograminearum* often co-exist with other FCR-causing *Fusarium* species, including *Fusarium culmorum*, *Fusarium avenaceum*, *Fusarium poae* and *Fusarium graminearum*, as well as other pathogens including *Bipolaris sorokiniana*, *Gaeumannomyces graminis* and *Phythium* spp. (Akinsanmi et al., 2004, 2006; Smiley et al., 2005; Chakraborty et al., 2006; Gebremariam et al., 2017).

2.5.2. Symptoms of *Fusarium pseudograminearum* on wheat

Seedlings infected with *F. pseudograminearum* can die before or after emergence. If infected seedlings survive, typical disease symptoms are browning of the coleoptile, sub crown internode, lower leaf sheaths and adjacent stems and nodal tissues; this browning can become evident within a few weeks after planting or throughout plant development. Infected plants may develop white heads with no or shrivelled grains as shown in Figure 2.1. Disease symptoms are exacerbated under water limitation (Kazan and Gardiner, 2018).

Figure 2.1: Symptoms of *Fusarium* crown rot of wheat, (a) crown browning and necrosis and (b) whitehead formation (Alahmad et al., 2018).

2.5.3. Epidemiology of *Fusarium pseudograminearum*

The fungus, *F*. *pseudograminearum* overwinters as chlamydospores or mycelia on plant debris (Kazan and Gardiner, 2018). The stubble of the previous year's crop and chlamydospores acts as the source of the inoculum, and at this stage the pathogen is in its saprophytic stage and survives as spores and mycelia. Once dry conditions persist, disease development is triggered. The pathogen forms conidia which enter the plant through the coleoptile, progressing into the sub crown internode and leaf sheaths and, subsequently, into the stem epidermal tissues, frequently penetrating tissues via stomatal openings. The pathogen then moves into the hypodermis to induce typical browning of the stem as shown in Figure 2.2 and, subsequently, into vascular tissues (Knight and Sutherland, 2013, 2016).

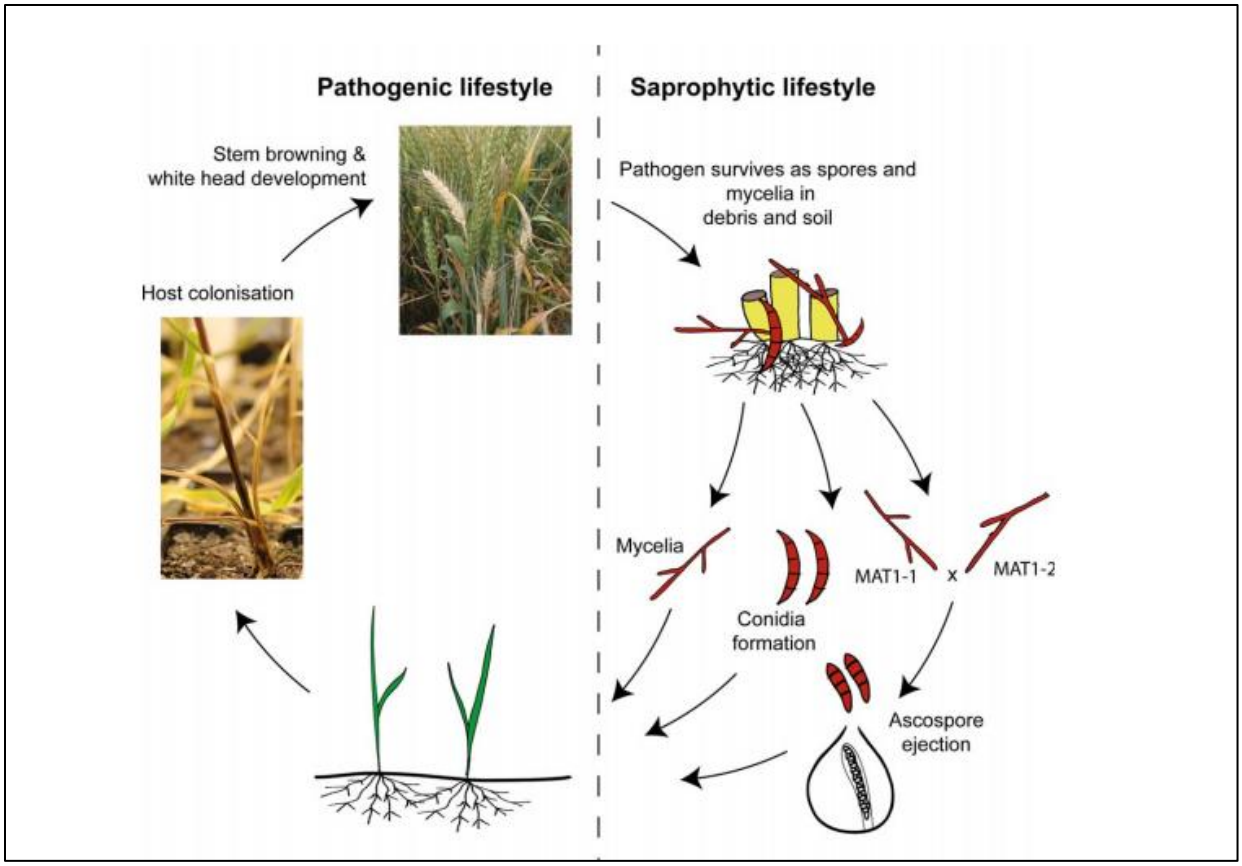

Figure 2.2: Lifecycle of *Fusarium pseudograminearum* on wheat (Kazan and Gardiner, 2018).

2.5.4. Management strategies of *Fusarium pseudograminearum*

The management of soilborne plant pathogens that live in or near the rhizosphere can be difficult due to the formation of resistant survival structures that help in long-term survival of the pathogens (El-Mougy et al., 1996). The successful control of crown rot depends largely on the manipulation of management practices and the use of tolerant cultivars (Kazan and Gardiner, 2018). Various control strategies are therefore employed which include cultural, chemical, and biological practices.

2.5.1.1. Cultural control of *Fusarium pseudograminearum*

The successful growth of wheat crops in areas where crown rot occurs depends on effective management of subsoil moisture during the fallow period, a process in which stubble retention and no-tillage or minimum tillage are essential components (Moya-Elizondo, 2013). Stubble retention is generally the preferred management practice for crown rot even though it was previously regarded as a reservoir for inoculum. *Fusarium pseudograminearum* is a necrotrophic pathogen and does not need a living host to survive. Therefore, stubble removal or break crops that are grass free are imperitive to lower inoculum in field. It is, therefore, important that in depth research be done to ensure that growers have confidence in the alternative crops that are resistant to the crown rot fungus and the crops can be grown profitably on a regular basis (Kazan and Gardiner, 2018). Other key cultural practices which are currently being used to minimize the incidence and severity of crown rot are crop rotation with leguminous crops, management of nitrogen applications and conservation tillage to enhance subsoil moisture in fallow and reduced risk of late season moisture stress (Burgess et al., 2001).

Fusarium crown rot on wheat is also controlled using tolerant wheat varieties. Although there are no commercial wheat varieties that are completely resistant to FCR, resistance in the form of tolerance to the disease can be used (Chakraborty et al., 2006). Amongst the FCR tolerant varieties used in Australia are 2-49, Sunco, Kukri, Brundage, Gene, Weatherford, Madson, Temple, and Tubbs (Smiley et al., 2005; Chakraborty et al., 2006). Infection of the host by the pathogen can still take place in the tolerant varieties, but the host will have the ability to endure infection and produce an acceptable yield (Moya-Elizondo, 2013). It is also important to mention that no single management strategy has

been shown to eliminate root and crown rots, but combined practices have proven to be useful (Moya-Elizondo, 2013).

2.5.1.2. Chemical control of *Fusarium pseudograminearum*

In the absence of other effective control strategies, chemicals are considered as an option. Seed treatments are helpful against soil-borne pathogens, and this is because after treatment the emerging plant can withstand early infection (Cook, 1980). Over the past 50 years, pesticide use in agriculture has gradually increased and is still common practice nowadays. The ideal pesticide should only be toxic to the target organisms, biodegradable and should not leach into ground water (Cook, 1980). Unfortunately, this is rarely the case with most pesticides and the widespread use of pesticides in modern agriculture has become an increasing concern. Generally, chemical pesticides usage has recently raised agricultural and public concerns. The agricultural concerns are focused on the increase in disease resistance to the available chemical controls whilst public concerns are focused on the negative effects that the chemicals have on the environment as well as on human health (Horrigan et al., 2002).

Although fungicides are not completely effective in controlling crown and root diseases, they are still used to a large extent (El-Mougy et al., 1996). Application of fungicides containing Difenoconazole and metalaxyl-M, Imazalil and Prothioconazole among others is most widely recognized as the control method of choice of *Fusarium* crown and root rots (Watkins et al., 2013). The use of fungicides as seed treatments is limited to the early stages of wheat growth and their effectiveness is generally not maintained beyond two to

four weeks of application (Moya-Elizondo and Jacobsen, 2016). Chemical seed treatments applied early in the season are recommended for the control of *Fusarium* crown and root rot (Balmas et al., 2006) in winter wheat, but infections can still be observed later in the season on mature plants (Stein, 2010). Difecoconazole-mefenoxam seed treatment fungicide reduced *Fusarium* crown rot severity on wheat by 29-50% in greenhouse trials (Moya-Elizondo and Jacobsen, 2016). The challenge in using fungicides to control FCR is because *F. pseudograminearum* attacks the plant at different developmental stages when favorable conditions permit (Johansson et al., 2003), therefore a seed-treatment that has an extended effect would be preferred (Johansson, 2003).

2.6. Biological control of *Fusarium pseudograminearum*

As a response to the search for safe and environmentally friendly disease control methods, biological control method was developed (Campbell, 1989). Biological control is defined by Baker (1987) as, 'the decrease of inoculum or the disease-producing activity of a pathogen accomplished through one or more organisms, including the host plant'. Biological control, therefore, supplements the use of chemical pesticides.

The use of biological control has become a preferred strategy for disease control because other methods such as the development of resistant plant cultivars, has often been too slow, and economic pressure on land use has limited some of the traditional cultural techniques of control (Burger, 1988; Mbili, 2011). Attempts have been made previously to find biocontrol agents (BCAs) that are effective against *Fusarium* diseases in wheat

(Johansson, 2003). *Trichoderma* spp. has previously shown strong inhibitory effects on *F. pseudograminearum* when sprayed onto straw colonized by this pathogen (Wong et al., 2002), suggesting that this treatment might reduce FCR inoculum levels in the field. Other biocontrol agents such as *Bacillus* spp. and *Trichoderma harzianum* have also shown significant protection against FCR in glasshouse trails (Moya-Elizondo et al., 2016). Furthermore, the use of plant growth promoting rhizobacteria (PGPR) is slowly gaining momentum across the world. This is due to their ability to promote plant growth and control plant disease as well. Various mechanisms such as induced systemic resistance (ISR), competition for space and nutrients, production of nutrients is used by PGPRs to aid in plant growth promotion and control of plant diseases (Compant et al., 2005). It is in implementing all these control strategies when the microbial community surrounding a plant rhizosphere change and the implication of these changes in root microbial communities on crop production need to be understood.

Microorganisms that colonize the rhizosphere can be classified according to their effects on plants and their interaction with roots. Many microorganisms such as bacteria, fungi, protozoa, and algae coexist in the rhizosphere with a good percentage being either pathogenic or beneficial to plant growth (Saharan and Nehra, 2011). Amongst these are the most important and abundant group of bacterial microbes inhabiting the rhizosphere that can be beneficial to the plants and they are referred to as plant growth promoting organisms (Kloepper at el., 1980).

Plant growth promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize the plant rhizosphere and enhance its growth and protect the plant against diseases (Kloepper at el., 1980). The rhizosphere represents a thin layer of soil

surrounding plant roots that is of importance for root activity and metabolism (Lynch, 1990). Direct and indirect mechanisms are implemented by the PGPRs to accomplish this. The direct mechanisms of plant growth promotion may involve the synthesis of substances by the bacterium or facilitation of the uptake of nutrients from the environment (Glick et al., 1999). The direct growth promoting mechanisms are as follows: (i) nitrogen fixation (Calvo et al., 2017); (ii) solubilization of phosphorus; (iii) sequestering of iron by production of siderophores (Yasmin, 2007); (iv) production of phytohormones (Bhattacharyya et al., 2015; Joseph et al., 2007); and (v) lowering of ethylene concentration (Kloepper et al., 1989; Glick et al., 1999).

The indirect promotion of plant growth occurs when PGPR lessens or prevents the deleterious effects of plant pathogens by production of inhibitory substances or by increasing the natural resistance of the host (Cartieaux et al., 2003). The indirect mechanisms of plant growth promotion by PGPR include (i) antibiotic production (Santoyo et al., 2012); (ii) depletion of iron from the rhizosphere (Martínez-Absalón et al., 2014); (iii) synthesis of antifungal metabolites (Leclère et al., 2005); (iv) production of fungal cell wall lysing enzymes; (v) competition for sites on roots and (vi) induced systemic resistance (Glick et al., 1999).

Inoculation of crop plants with certain strains of PGPR at an early stage of plant development can improve biomass production through increased root and shoot growth (Rangarajan et al., 2003). Presently, most research involving bacterial biocontrol agents is centered on species of *Bacillus*, *Pseudomonas*, *Streptomyces* and *Burkholderia*. However, a wide diversity of other biocontrol agents also contributes towards increased production in agriculture (Kumar et al., 2011).

Several studies clearly demonstrated the beneficial effects of plant growth promoting rhizobacteria on growth and yield of different crops at different climates and soils (Glick et al., 2007). Wheat yield increased up to 30% with *Azotobacter* inoculation and up to 43% with *Bacillus* inoculation (Kloepper et al., 1991). In a series of laboratory experiments with Rhizobial inoculation on wheat conducted by Zahir et al. (2004), an increase in root elongation of up to 20% was recorded while root dry weight, shoot elongation and shoot dry weight increased by up to 13%, 38% and 36%, respectively.

Seed inoculation of barley with different PGPR increased root weight by 8.9-16.7% and shoot weight by 28.6-34.7% compared to the untreated control plants (Canbolat et al., 2006). Furthermore, Shaharoona et al. (2008) conducted an experiment on the effect of combined use of PGPR and nitrogen fertilizer on wheat and reported that the *Pseudomonas fluorescens* strain significantly increased the root weight by 19-43%, number of tillers per plant by 10-21%, grain yield by 15-43% and straw yield by 22-39% in comparison to un-inoculated control plants.

Other studies including the inoculation of maize with bacteria strains of *Pseudomonas alcaligenes*, *Bacillus polymyxa*, and *Mycobacterium phlei* significantly increased the shoot dry weight (17-30%), root dry weight (19-52%) and total dry matter of maize increased up to 38% (Egamberdiyeva, 2007). The use of PGPR offers a great potential to sustainable agriculture. However, additional studies are necessary to understand the consequences that PGPR could exert on the core microbiome (internal or external) of the plant species to be inoculated and of the surrounding soil microbiota (Orozco-Mosqueda et al., 2018).

2.8. The root microbiome

The microbiome represents regions or zones where the microbes can interact with the plant, either outside (rhizosphere), inside (endosphere) or on (phyllosphere) the plant (Dong et al., 2019). The rhizosphere is the soil portion influenced by plant roots (Hartmann et al., 2008). It serves as a hot spot for numerous organisms and is considered as one of the most complex ecosystems on earth (Hinsinger and Marschner, 2006; Pierret et al., 2007; Jones and Hinsinger, 2008; Hinsinger et al., 2009; Raaijmakers et al., 2009). The microbiome is composed of several different types of organisms, including bacteria, fungi, protozoa, archaea, and viruses (Muller and Sacs, 2015). The microbiome can play a beneficial role in protecting the plant from potential pathogens while at the same time improving growth, health, and production, thereby conferring an adaptive advantage to plants (Haney et al., 2015; Berg et al., 2016). In particular, the bacterial microbiome carrying out beneficial interactions is important for sustainable agriculture and has attracted more attention, compared to other groups of microrganisms (Morales and Holben, 2011).

Root-associated microorganisms are mainly recruited from the surrounding soil. The root microbiome is strongly influenced by the composition of the soil microbial species pool that is present in the vicinity of the roots (Morales and Holben, 2011). Several studies, using high-throughput amplicon sequencing, have demonstrated the strong connection between the soil bacterial communities and root-associated bacteria in *Arabidopsis thaliana* (Bulgarelli et al., 2012; Lundberg et al., 2012; Schlaeppi et al., 2014). Soil microbial community structure and the associated environmental parameters have also shown to be drivers of root-associated bacterial community structure than plant genotype

or species (Bulgarelli et al., 2012; Lundberg et al., 2012; Shakya et al., 2013; Schlaeppi et al., 2014). This further outlines the importance of local environmental parameters, notably soil properties, as determinants of soil microbial community composition and root microbiota (Shakya et al., 2013; Schreiter et al., 2014). Therefore, further research is required to investigate and highlight factors that affect the root.

2.9. The role of the root microbiome on plant health

Pathogens can have a severe impact on plant health. The influence of the root microbiome on plant health is evidenced most clearly in disease-suppressive soils (Berendsen et al., 2012). Disease suppressive soils are defined by Cook and Baker (1983) as soils in which the pathogen does not establish or persist, if established it causes no damage. The microflora of most soils is starved. As a result, there is a fierce battle in the rhizosphere between the microorganisms that compete for plant-derived nutrients (Raaijmakers et al., 2009). The success of a pathogen is influenced by the microbial community of the soil in which the infection takes place. Every natural soil could suppress a pathogen to a certain extent (Berendsen et al., 2012). Moreover, the rhizosphere microbiome uses certain mechanisms for pathogen suppression. These include production of antibiotic compounds, consumption of pathogen stimulatory compounds, competition for (micro) nutrients and production of lytic enzymes (Lugtenberg and Kamilova, 2009; Doornbos et al., 2012).

In addition to direct effects on deleterious microbes in the rhizosphere, many beneficial soil-borne microorganisms have been found to boost the defensive capacity of the plant

such as induction of systemic resistance (ISR) (Berendsen et al., 2012). Induction of ISR in Arabidopsis (*Arabidopsis thaliana*) by the plant growth-promoting rhizobacterium *Pseudomonas fluorescens* WCS417 is well studied. Although specific microorganisms are able to protect the plant either directly or indirectly against pathogens, their efficacy is largely influenced by the rest of the microbial community. First, the pathogen-suppressing microorganisms should be presenting sufficiently high numbers to have a significant effect (Raaijmakers et al., 1995; Berendsen et al., 2012). Second, microorganisms that are regarded as commensals, because they neither harm nor benefit the plant directly, can compete effectively with the pathogen-suppressing biocontrol bacteria. It has also been documented that upon pathogen attack, plants recruit their own microbiome (Dudenhoffer et al., 2016). Changes in rhizosphere composition upon infection might be the result of the induced excretion of antimicrobial compounds by infected roots. Bais et al. (2002) observed that infection of sweet potato by *Pythium* elicited the secretion of rosmarinic acid, containing antimicrobial activity. *Fluorescent pseudomonads* produce the antibiotic 2,4-Diacetylphloroglucinol (DAPG), which has been extensively studied as a protectant against soil-borne diseases and has been directly linked to disease suppression (Raaijmakers and Weller,1998). *Bacillus subtilis* also releases the antibiotics surfactin and iturin into the rhizosphere, which plays a major role in plant disease suppression while also conferring increased plant growth promotion (Kinsella et al., 2009). Thus, studying the microbial composition of crop plants aid in creating a sustainable approach to disease control and plant productivity and to date, there is an increase in studies on the beneficial effects of soil microbes on plant health and the associated mechanisms.

2.10. Factors affecting the rhizosphere microbiome.

Several factors such as biotic and abiotic parameters may affect microbial populations prevailing in the plant rhizosphere. The level to which microbial communities are influenced by biotic and abiotic factors is not totally understood. Such factors include cultivation practices (Abdelfattah et al., 2016), geographic location (Igiehon and Babalola, 2018), prevailing environment, cultivar type (Corneo et al., 2016), plant age (Abdelfattah et al., 2016), growth stage (De-la-Pena et al., 2010) and seasonality (Figure 2.3). Specific climatic features have been found to correlate with the microbial assemblages (Bokulich et al., 2013), which implies that a link between the field prevailing condition and a consortium of microbial inhabitants exists. Considered in combination, the factors that influence the rhizosphere of wheat impose a non-random “microbial terror” (Igiehon and Babalola, 2018), which becomes a dominant factor for regional differences among rhizosphere populations in wheat. These factors will be discussed in further detail in the section below.

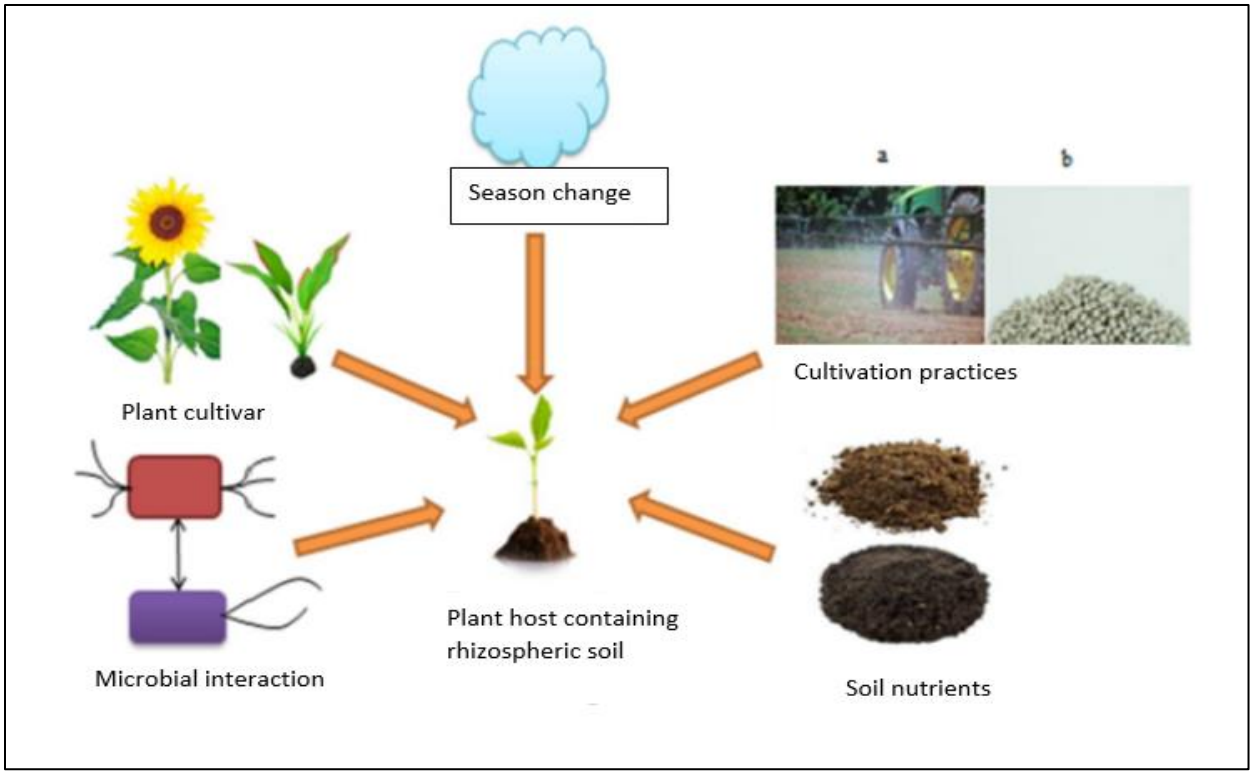

Figure 2.3: Factors influencing the rhizosphere microbiome (Adapted from Igiehon and Babalola, 2018).

2.10.1. Crop management practices

Field cultivation practices may also influence the microbial profiles associated with the rhizosphere. Several studies have pointed out the differences in microbial communities of organically and conventionally produced crops (Carmichael, 2018). Recent work (Li et al., 2019) has shown that the roots of organically grown pepper inhabit larger unique taxa of rhizosphere microbiota than the roots of pepper (*Capsicum annuum*) under a conventional pest management practice. Similarly, Edwards et al. (2019) also detected

several unique fungal or bacterial taxa exclusive to organically grown rice roots sampled at vegetative stage and this was attributed to the management practices.

Microbial population dynamics are also influenced by pesticide spray programmes that also modify the community profile of different plant organs (Abdelfattah et al., 2016). For instance, fungal communities of strawberry fruit reacted differently to fungicide sprays. A one-month holding period was sufficient to alter the fungal profiles on the strawberry plants (Abdelfattah et al., 2016). An abundance of *Ascomycota*, mainly *Leotiomycetes* (88.2%) and *Dothideomycetes* (99%) was reported in untreated (unsprayed) leaves and fruit, respectively, while in the treated (sprayed) samples, *Ascomycota* abundance remained similar in all organs. *Basidiomycota,* particularly, *Tremellomycetes,* were found in slightly lower quantities (8.2%) in non-sprayed leaves of strawberries than those sprayed, and much less abundant in immature and mature fruit (Abdelfattah et al., 2016). Intensive cultivation and the use of fungicides was associated with high prevalence of postharvest pathogens, particularly, *Botrytis* and *Cladosporium spp*. (Abdelfattah et al., 2016). This agreed with previous work (Pinto et al., 2014; Singh et al., 2015), which confirmed that the use of chemicals modulate the overall genetic diversity in natural environments. With respect to the wheat field microbiome, Rascovan et al. (2016) revealed a significant variation between wheatfield locations, but no major modifications in community structure due to chemical or biological sprays. The authors attributed this to a strong adaptation of native rhizospheric communities to abiotic and biotic factors of the production areas.

2.10.2. Soil physicochemical properties

The soil is a source of a large variety of carbon, which provide energy for survival of microorganisms and makes it a conducive environment for microbial diversity and richness (Igiehon and Babalola, 2018). Soil type greatly affect microbial assemblages associated with plant's rhizosphere (Andrew et al., 2012; Abbott and Robsson, 1985; Inceoglu et al., 2012). This further influence root exudates released by roots and finally plant health (Lundberg et al, 2012; Igiehon and Babalola, 2018). Soil can differ in structure, organic matter, pH, texture, and nutrient status (Dumbrell et al., 2010; Igiehon and Babalola, 2018). In particular, soil pH and availability of nutrient such as carbon have been observed to affect the diversity of crop pathogenic nematodes, bacteria, fungi and other beneficial microorganisms (Toljander et al., 2008; Rotenberg et al., 2005). Latour et al. (1996) found that soil type affected the abundance and composition of *Pseudomonas* species in flax and tomato rhizospheres. Sequence analysis of the rhizosphere bacterial community of different *Arabidopsis thaliana* cultivars revealed that soil type greatly affects rhizosphere microbial diversity (Gelsomino et al., 1999; Lundberg et al., 2012). This was further confirmed by Gelsomino, Keijzer-Wolters (Bulgarelli et al., 2012), who demonstrated that bacterial community structures were alike in soils of the same type.

2.10.3. Cultivar type and developmental stage

The type of cultivar and crop developmental stage also shapes microbial community patterns. Several studies have documented an effect of plant cultivar on indigenous

microbiota present in the rhizosphere (Bouffaud et al., 2012; Inceoglu et al., 2012; Corneo et al., 2016).

Plants can determine the composition of the root microbiome by active secretion of compounds that specifically stimulate or suppress members of the microbial community (Doornbos et al., 2012). Recent evidence suggests that differences between plant genotypes in a single gene can have a significant impact on the rhizosphere microbiome (Berendsen et al., 2012). Chemical signals emitted by soil microorganisms are received and recognized by plants and then addressed through the release of chemical compounds in the form of root exudates. Secretion of these compounds varies between different plant species (Rovira, 1969), ecotypes (Micallef et al., 2009), and even distinct roots within a plant (Uren, 2007). The diverse compounds released by plants as root exudates create a unique environment in the rhizosphere and include sugars, amino acids, flavonoids, aliphatic acids, proteins, and fatty acids (Badri et al., 2009). All these different compounds can attract and initiate both symbiotic and pathogenic interactions within the rhizosphere (Bais et al., 2006). Root exudate composition and concentration change according to the signals received from the environment and the rhizosphere, age of the plant (De-la-Pena et al., 2010), soil type (Rovira, 1969), and biotic and abiotic factors (Flores et al., 1999, Tang et al., 1995).

Apart from the cultivar type, developmental stage also plays a crucial role, with regards to microbial community and diversity in most horticultural crops. Amongst widely grown crops, effect of developmental stage was reported on tomato (Ottesen et al., 2013), grape (Sternad Lemut et al., 2015), olive (Abdelfattah et al., 2015), strawberry (Abdelfattah et al., 2016) and wheat and rice (Wang et al., 2016). In all these highlighted crops, early

stages of development (immature fruit stages) had lower incidence of decay causing fungal groups, which later increased in abundance as the fruit matured. In a study by Houlden et al. (2008), it was revealed that bacterial communities on pea and sugar beet seedlings had low relative activity when compared with established plants, where activity stabilized as communities became established.

2.10.4. Seasonal changes

Climate change has different effects, ranging from local cooling to global warming, shifting vegetation zone and augmented extreme weather events and all these effects have indirect impacts on the rhizosphere microbiome (Berendsen et al, 2012). Increases in carbon dioxide levels, a component that is alleged to be the key driver of climate change, could also directly affect rhizosphere interactions by changing root exudation patterns and the soil food web (Igiehon and Babalola, 2018). Previous studies have demonstrated a seasonal variation in microbial decomposition processes in temperate forest soils which was related to a seasonal shift in availability of substrates and a seasonal variation in soil temperature and moisture (Kaiser et al., 2011, 2010). Koranda et al. (2013) further demonstrated that microbial community changes due to alterations in resource availability, which results in functional differences between microbial communities, implies that the distinct microbial communities differ in their physiological capacities. Summer and winter seasons affect microbial community composition. In a study by Koranda et al. (2013), there were functional differences between summer and winter soil communities in response to addition of C substrates, reflecting microbial

adaptation to availability of different types of C sources during different seasons. Furthermore, the winter community responded to the addition of complex C substrates with significantly enhanced actual cellulase/amylase activity and reduced mineralization of soil organic matter. Both suggest adaptation of the winter community to degradation of complex C substrates (Koranda et al., 2013).

2.11. The influence of PGPR inoculation on the rhizosphere microbiome

A strategy to improve plant health and development includes the selection and modification of the rhizosphere microbiome (Chaparro et al., 2012; Mendes et al., 2013; Swenson et al., 2000). In recent years, the understanding regarding soil microbial diversity, drivers of microbial diversity in agriculture systems and relationship between biodiversity and ecosystems functions has improved (Singh and Trivedi, 2017). Key knowledge on the critical role played by the microbial community in the rhizosphere, particularly nutrient acquisition, and disease resistance, has also improved. However, the ability to manipulate the microbial diversity for improved production is either limited to altering management practices or through the addition of microbial inoculates. The use of microbial inoculants has so far, had limited success in field conditions, mainly due to competition with the indigenous microflora of soils. However, there is strong evidence to suggest that plants and their associated microbiota (particularly of rhizosphere) constantly communicate with each other for resource requirements and defense against pathogen and parasite attack. *In situ* microbiome engineering (Mueller and Sachs, 2015) can be the choice of tool for harnessing the microbiome for beneficial outcomes in agriculture.

Adding beneficial microorganisms to those already present in the soil can maximize plant nutrient uptake (Kirankumar and Jagadeesh, 2010), increase plant growth (Cummings, 2009; Guiñazú et al., 2010; Hayat et al., 2010), confer resistance to abiotic stress (Selvakumar and Panneerselvam, 2012), and suppress disease (Chithrashree et al., 2011; Okubara and Bonsall, 2008). These benefits can be particularly pronounced with microorganisms called plant growth promoting rhizobacteria (PGPR) that effectively colonize the root soil interface, or rhizosphere (Hayat et al., 2010). Plants interact with a variety of PGPRs that can increase photosynthetic capacity (Zhang et al., 2008; Xie et al., 2009), conferring drought and salt tolerance (Xie et al., 2009; Zhang et al., 2008; Dimkpa and Weinand, 2009), and improving the effectiveness of the plant's own iron acquisition mechanisms (Xie et al., 2009).

The development of plant growth-promoting rhizobacteria (PGPR) as inoculants is constantly increasing. For example, there are now close to 25 million hectares of soybeans inoculated with *Bradyrhizobium japonicum* in South America and close to 500 000 hectares of wheat and maize inoculated with commercial *Azospirillum* inoculants in Argentina and Mexico (Fuentes-Ramirez and Caballero-Mellado, 2005; Izaguirre-Mayoral et al., 2007). Most PGPR are active when in the rhizosphere, thus most rhizobacterial communities are drawn from the soil and fewer originate from seed-associated microorganisms (Castro-Sowinski et al., 2007). The introduction of many exogenous bacteria as an inoculant has the potential to affect the resident microorganisms, and similarly, an inoculant may be affected by them. Such interferences may result in increased, decreased or no effect on PGPR effectiveness. Due to this reason, there is a need to study the microbial ecology of the rhizosphere following PGPR applications

(Castro-Sowinski et al., 2007). Certain microbiota groups may be enhanced, while others may be inhibited, or the introduced PGPR may not affect the population structure at all (Nacamulli et al., 1997; Schwieger and Tebbe, 2000; Bacilio-Jimenez et al., 2001; Dobbelaere et al., 2003).

The application of PGPR was observed to result in more roots in the soil volume, as was demonsatrated in maize trials following *Azospirillum brasilense* inoculation (Dobbelaere et al., 2003). Chihaoui et al. (2015) further analyzed the effect of inoculation of *Agrobacterium* sp. 10C2, a nodule-inhabiting endophyte on nodulation, plant growth, and the rhizosphere microbiome of *Phaseolus vulgaris*, and noticed that this strain does not form nodules and is non-pathogenic. Inoculation with the 10C2 strain promoted a significant increase in the number of nodules and plant biomass. The beans that were produced showed a significant increase in the contents of phosphorus, polyphenols, and flavonoids, and total antioxidant capacity. To evaluate the effect of the 10C2 strain on the microbiome, the terminal restriction fragment length polymorphism (TRF) technique of PCR-amplified 16S rRNA genes was used. Fifteen days after the inoculation of the 10C2 strain, the TRF richness was unchanged, but the bacterial community structure did change. However, when bean plants were cultivated in these soils for a much longer period, i.e., 75 days, both the TRF richness and structure were affected by the 10C2 strain. Interestingly, the TRF richness increased in the rhizosphere soil, but not in the soil without the plant (Chihaoui et al., 2015). In another study by Chen et al., 2021, Co-inoculation of Trichoderma compound agent and *B*. *subtilis* on wheat roots resulted in increased wheat growth, available nitrogen and changed in bacterial diversity were

observed. This shows that PGPR inoclulation influences the resident microbiome, however more research is needed in this area.

2.12. Conclusion

Wheat diseases incited by *Fusarium* spp. has major economic importance particularly in developing countries where wheat constitutes a major part of the diet. Minimising crop yield loses is mainly through the use of chemical pesticides and cultural practices, however, due to the increased concern on human and environmental health new sustainable methods on increasing crop yield and controlling diseases are being researched. One path toward sustainability involves a greater reliance on the beneficial functions afforded by the use of biocontrol agents, particularly plant growth promoting rhizobacteria. Research on the use of PGPR for plant growth and disease control has been rigorously done and proved to be successful when used mostly in a controlled environment. There is however, lack of technical knowledge regarding the handling and use of biocontrol agents which is a major obstacle in the successful adoption of the technology. Additionally, poor performance and inconsistencies under commercial conditions have been the major limitation to the adoption of biocontrol agents for the control of soilborne disease in wheat. Meanwhile, though still relatively new, there are discoveries in exploiting the plant-associated microbiome in agricultural crop production. The plant-associated microbiome play an important role towards plant health, productivity, and shaping the ecosystem. Knowledge on the microbiome of specific functional plant tissues is already established for most horticultural products. Therefore,

the primary focus and future perspectives should be on behaviours and changes of the soil microbiome upon manipulation with biocontrol agents. This will ultimately translate to reduced crop losses and less chemical applications, hence improved food security and economic returns in agriculture.

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Chapter 3

Bacterial diversity and community composition of wheat rhizosphere and non-rhizosphere soils from three different agricultural production regions of South Africa

Plant productivity and protection is to a greater extent shaped by rhizosphere microbial communities which play a significant role in co-regulating soil-microbial-plant interactions, nutrient uptake, and other host physiological processes. Knowledge of the drivers and extent of variation in bacterial communities, whose presence and activities are partly a function of plant genotype and age, remains limited. This study investigated the composition and diversity of bacterial communities (using high-throughput sequencing targeting the 16S rRNA gene) associated with wheat rhizosphere soils, as well as physicochemical properties during seedling growth, from three geographically distinct wheat-growing regions of South Africa. A decline in bacterial diversity was observed from the non-rhizosphere to the rhizosphere. Rhizosphere soils shared features with non-rhizosphere soils such as predominance of Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Actinobacteria. Wheat cultivars varied in their “microbial enrichment” effect especially if grown under different geographical environments. Redundancy analysis showed that soil chemical properties (mainly pH) were the main drivers of variation in bacterial communities. Overall, early in the season, rhizosphere bacterial community diversity in wheat is low with recruitment being mainly influenced by the environment and is to a lesser extent cultivar dependent. This information can be very

useful in developing natural disease management strategies or to improve wheat production, yield and quality.

Keywords: bacterial communities, 16S rDNA gene, soil chemical properties, redundancy analysis

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3.1. Introduction

Soil microbial communities, in particular the rhizospheric microorganisms which exist at densities of approximately 10^{11} cells per gram of soil within a narrow zone around the root of a plant, drive a number of globally important processes (Fuhrman, 2009) including elemental cycling and energy flows (Wu, 2018; Egamberdieva et al., 2008). Generally considered the plant's second genome (Berendsen et al, 2012; Wu et al, 2018), these microbial communities enhance agricultural production (Lupwayi et al., 1998) by promoting nutrient absorption and inhibiting potential pathogens (Wu et al., 2018; Lugtenberg, 2014; Raaijmakers and Lugtenberg, 2013; Whipps, 2001; Thakore, 2006). The rhizosphere microbial community structure is influenced by a number of factors including, soil characteristics (Singh et al., 2007; Kuramae et al., 2012), land use history (Debenport et al., 2015) and plant species (Burns et al., 2015; Lima et al., 2015), genotype and plant developmental stages (Inceoglu et al., 2010; Marques et al., 2014).

Data on root-associated bacterial populations have largely been done under laboratory conditions, with field studies mostly limited to non-rhizosphere microbial communities which are poorly correlated to crop growth and yield (Singh et al., 2007; Marques et al., 2014; Peiffer et al., 2013). Microbial studies on a number of different plant species including potato (Marques et al., 2014), sweet potato (Peiffer et al., 2013), maize (Donn et al., 2015) and rice (Breidenbach et al., 2016) have provided insight into the impact of plant host and field management strategies on observed bacterial communities (Donn et al., 2015; Edwards et al., 2015; Corneo et al., 2016). Work done on wheat has been limited to the study of rhizosphere associated bacterial communities and how these communities evolved over time as mediated with respect to plant growth stages and cultivar (Edwards et al., 2015; Corneo et al., 2016). However, not much information has been reported on microbial community diversity of rhizosphere and nearby non-rhizosphere soils in intensive wheat cropping systems, from different production regions at the vegetative growth stage (critical stage in wheat production).

Understanding the bacterial population dynamics of the wheat rhizosphere is the first step towards successful manipulation of microbial communities to reduce plant pathogen pressure and increase yeid (Bever, 2012). The invasion of cereals crown and root tissues by soilborne pathogens at the vegetative stage, has significant consequences on the plant's capacity for efficient nutrient and water uptake, and survival in winter (Smalla et al., 2001; Cook and Veseth, 1991; Weise, 1987). The current study is to our knowledge, the first to investigate the dynamics of the diversity in soil bacterial communities associated with the vegetative (four to five leaf) stage of wheat growth and development

from three major and climatically different production regions. This study, therefore, investigated fungal composition and diversity of wheat rhizosphere in three wheat growing regions of SA and to further understand the dynamics in the benficial bacterial populations in these production areas.

3.2. Materials and methods

3.2.1. Site description and soil collection

A total of 256 whole plants with soil and 64 nearby field soil samples were collected from three different agro-ecological regions which included Napier, Western Cape Province (Site A), Pretoria, Gauteng Province and Bethlehem, Free State Province (Site C) in South Africa. The wheat cultivars grown included ‘Kariega’ and ‘SST88’ (Site A), ‘Kariega’ (Site B) and ‘Eland’ (Site C). Detailed site data is shown in Table 1 including annual rainfall, average temperature, and soil chemical properties (management practices). Eight replicated plots were selected for sampling in each location. Each replicate consisted of 10 samples collected and pooled, with one sample consisting of three plants.

Non-rhizosphere soil was collected in the field between rows approximately 40 cm away from the sampled plants with a clean auger (washed and disinfected with 70% ethanol between sampling) from a soil depth of approximately 10 cm. Non-rhizosphere soil is characterized as the non-cultivated soil often seen in between rows or along the edge of the plot. Plants (selected at the 4-5 leaf stage with crown root system fully developed)

were collected by harvesting the whole plant with the roots that was gently removed (with an auger) from a soil depth of approximately 10 cm.

Plant samples were processed by gently shaking off excess soil and collecting rhizosphere soil as that portion that remained attached to the roots. Samples were placed inside zipper bags and transported to the laboratory in cooler boxes for storage at 4°C and processing within two days.

3.2.2. Analysis of soil chemical properties

The soil chemical properties were analyzed according to Mehlich, (1972) and Bray et al. (1945) with minor modifications. Briefly, the soil pH was determined in a soil suspension with deionized water (1:10 w/v), while soil organic carbon was analyzed by titration of wet oxidation in concentrated H_2SO_4 and 2 M $K_2Cr_2O_7$. Available soil phosphorus (AP) was determined using a flow analyzer and finally, potassium (K), calcium (Ca), magnesium (Mg) and sodium (Na) were determined by flame emission and atomic absorption spectroscopy (Bray et al., 1945 and Mehlich, 1972).

3.2.3. DNA extraction and sequencing

Total community DNA was extracted from 0.25g fresh soil using the MoBio PowerSoil™ DNA Isolation Kits (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA concentration in each sample was determined

using Nanodrop ND-2000 UV–VIS Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) prior to further analysis.

Sequencing was done using the following primers; 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 909R (5'-CCCCGYCAATTCMTTTRAGT-3') targeting the bacterial V4 -V5 region in the 16S rRNA gene region (Tamika et al., 2011). Sequencing was performed at Molecular Research DNA (MR DNA) (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines.

3.2.4. Data analysis and taxa classification

Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, USA). The sequences from the Illumina were joined and depleted of bar codes. Sequences with ambiguous base calls and those <150 bp (Gu et al., 2017) were discarded. Noises, singletons and chimeras were removed from sequences. Generated operational taxonomy units (OTUs) were defined by clustering at a similarity threshold of 97%.

Diversity indices (Shannon, Chao1 and Observed richness) (Abdelfattah et al., 2016) for each sample were calculated in 'Visualization and Analysis of Microbial Populations Structure (VAMPS)' (http://vamps.mbl.edu). Normalization of the OTU table was done with rarefaction to an even depth of 1 000 sequences in order to maintain homogeneity of samples. The indices were then calculated from the rarefied OTU table (Ondov et al., 2011). To determine uniqueness of detected OTUs between phenological stages and

sites, Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html) was used. Alpha diversity was calculated using Chao1 and Shannon-Weaver indices. Principle coordinates analysis (PCoA), based on distance matrix of Bray-Curtis estimation, was used to evaluate similarities or differences between samples. The alpha diversity indexes relative to the samples were represented by box plots and statistically compared using a standard t-test. Redundancy analysis (RDA) was performed with Canoco (version 4.5 for Windows; Ithaca, NY, United States) to determine which environmental variables best explained the assemblage's variability. The ordination in the x- and y-axes and the length of the corresponding arrows indicated the importance of each physicochemical factor in explaining the taxon distribution across communities. We further tested for significant differences in the relative abundance of taxonomic groups using one-way ANOVA and Tukey's multiple comparison test. Test results with $P < 0.05$ were considered statistically significant. All statistical analyses were executed in R version 3.4.3 (R Core Team, 2017).

3.3. Results

3.3.1. Core bacteria OTU composition

Rhizosphere and non-rhizosphere bacteria communities of three wheat cultivars, SST88, Kariega and Eland grown in three different production regions were compared based on V1–V3 Sequence region of the 16S rDNA gene. A total of 1 158 673 valid reads were generated from eight rhizosphere and six non-rhizosphere soil samples at 3% distance after paired-end alignments, quality filtering, and deletion of chimeric sequences and singletons. All Sequences were assigned to 10 455 OTUs and ranged from 3287 to 5856

per sample. The core microbiome was characterised by 665 OTUs for rhizosphere and 1041 OTUs (665/1041) for non-rhizosphere soil samples and were present in all samples, and included the phyla Proteobacteria (271/400), Actinobacteria (134/83), Bacteroidetes (86/128), Acidobacteria (38/64), Chloroflexi (31/59), Firmicutes (28/53), Planctomycetes (24/46), Verrucomicrobia (24/49)) Gemmatimonadetes (7/23), Nitrospirae (7/13), Cyanobacteria (6/8) and fusobacteria (3/3) (Figure 3.1).

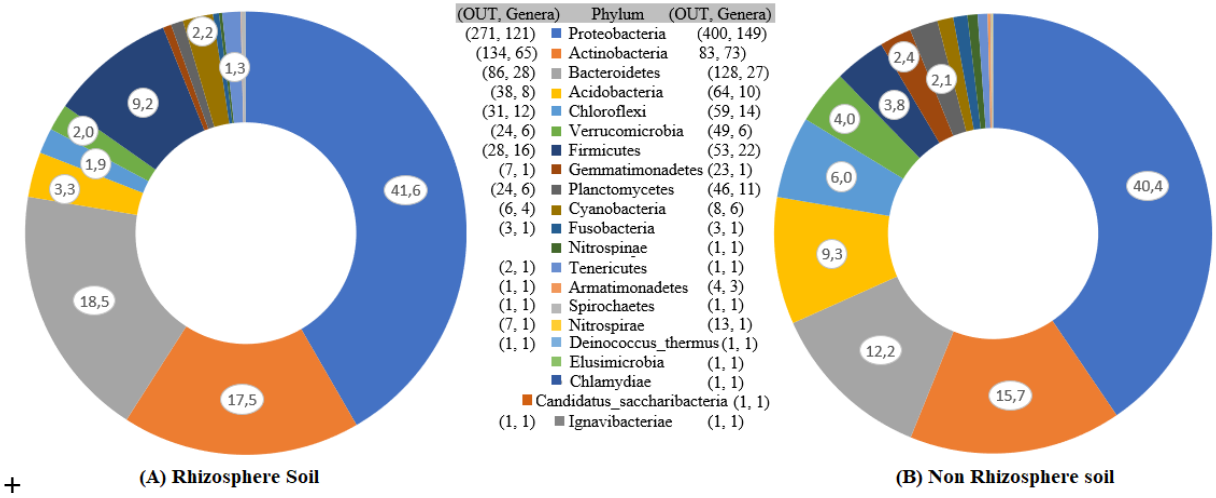

Figure 3.1: Phylogenetic distributions of bacterial operational taxonomic unit (OTUs) that were present in all rhizosphere (A) and field soil samples (B) across all cultivars and their locations. Phyla are listed with number of OTUs and the number of classifiable taxa associated with each phylum.

3.3.2. Comparative analysis of wheat cultivars

The rhizosphere microbial community of the three cultivars was characterised by 26 phyla including *Proteobacteria* (40%), *Bacteroidetes* (19%) and *Actinobacteria* (16%) which dominated the data set. Comparison of the three cultivars showed significant differences ($P < 0.01$) in relative abundances of a number of phyla including *Chlamydiae, Acidobacteria* and *Ignavibacteriae* which had higher relative abundance in the Eland cv at Site C only. *Fibrobacteres*, *Cyanobacteria* and *Gemmatimonadetes* had higher relative abundance in the Kariega cultivar (cv) grown at Site A only. The phyla *Armatimonadetes* and *firmicutes* had higher relative abundances in the Eland cv at Site C and Kariega cv at Site B. Kariega cultivar which was grown at both Site A and B had similar higher relative abundance of phyla *Verrucomicrobia* and *Bacteroidetes* as the SST88 cv grown at Site A. Unique to cultivars grown at Site A, Kariega cv and SST88 cv was the higher relative abundance of phyla *Spirochaetes, Elisimimocrobia and Planctomycetes.*

The average relative abundances (RA) and the classified sequences were associated with 24 phyla, but only six (Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Acidobacteria and Verrucomicrobia) had a frequency greater than 1% (Figure 1). These phyla accounted for more than 90% of bacterial sequences observed in both non-rhizosphere and rhizosphere soils from the three growing regions. Overall, the dominant phyla in the non-rhizosphere soil were Proteobacteria (34% - 44%), Actinobacteria (21% - 34%), Bacteroidetes (2% - 7%), Acidobacteria (4% - 11%) and Firmicutes (2% - 10%) while rhizosphere soil were composed of Proteobacteria (31% - 39%), Actinobacteria (10% - 42%), Bacteroidetes (2% - 8%), Acidobacteria (1% - 10%) and Firmicutes (2% - 16%) (Table 3). Minor differences on the extent to which different cultivars on the same

site/region could modify the rhizosphere communities was observed. The percentage increase in RA (enrichment in rhizosphere soils) between ‘SST88 cv’ and ‘Kariega cv’ at Site A for most the phyla was similar except for Cyanobacteria, Firmicutes and Deltaproteobacteria, (149% vs 198%, 79% vs 124% and 56% vs 10%, respectively). On the other hand, differences in percentage increase in RA were observed between Site A and Site B for ‘Kariega cv’ in most of the bacterial groups including Acidobacteria (59% vs 160%), Actinobacteria (33% vs 18%), Spingobacteria (-22% vs 6%), Cyanobacteria (198% vs -87%), Firmicutes (124% vs -57%), Gemmatimonadetes (-20% vs 187%), Alphaproteobacteria (64% vs 99%), Planctomycetes (3% vs 89%), Deltaproteobacteria, Verrucmicrobia (10% vs 128%), Firmicutes (35% vs 159%). At Site C, the bacterial enrichment in the rhizosphere soil of Eland cv was observed only in four phyla including Actinobacteria (35%), Firmucutes (190%), Betaproteobacteria (80%) and Gammaproteobacteria (31%).

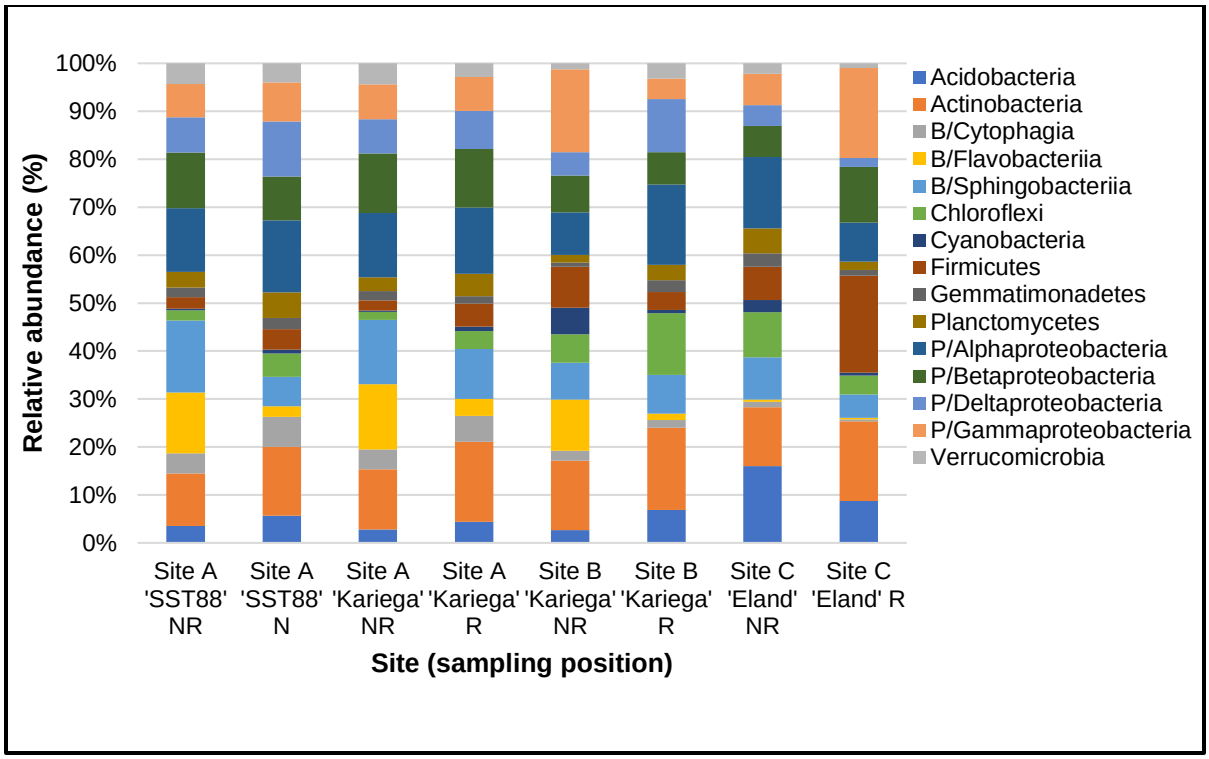

Figure 3.2: Bacterial community composition of different phyla (and classes of Proteobacteria and Bacteroidetes) on non-rhizosphere and rhizosphere of wheat varieties grown in agro-ecologically different regions.

The effect of wheat cultivar and site on relative abundances of observed OTUs showed significant differences with a PERMANOVA test statistic for cultivar abundances of the OTUs. A significant effect ($P < 0.001$) was found for both cultivar and site, although no significant interaction could be determined for the two (Table 1). A principal coordinate analysis plot based on Bray-Curtis similarity of relative abundances was used to visualise the differences. About 60.8% of observed variation could be explained by the first two principal coordinates. The three cultivars were generally well separated with significant relative abundance variation of OTUs, observed between the Kariega cv grown on different sites (A and B) and minimal separation between Kariega cv and SST88 cv grown

at the same site. The first axis which appeared to correspond to host cultivar differences explained the majority (41,5%) of the observed variation (Figure 3).

Table 3.1: Permutational multivariate analysis of variance (PERMANOVA) of main factors tested and their interactions for wheat rhizosphere and non-rhizosphere soil from the same field sites

Sources of variation	Df	F-value	R-squared	P-value
Non-Rhizosphere				
Site	2	11.81	0.887	0.06
Rhizosphere Samples				
Site x Cultivar	3	17.26	0.928	0.006
Cultivar	2	5.166	0.674	0.012
Pairwise Comparison				
Site (Kariega cv: Site A x Site B)	1	12.67	0.864	0.333
Cultivar (Kariega cv X SST88 cv at Site A)	1	18.49	0.902	0.333

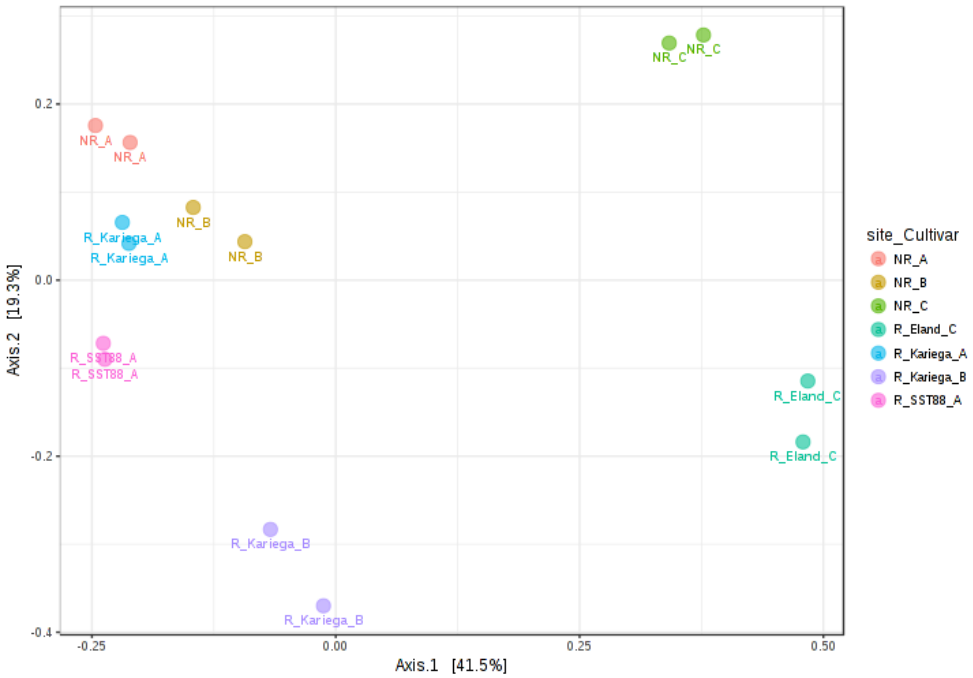

Figure 3.3: Principal coordinate analysis (PCoA) based on non-rhizosphere (NR_) and rhizosphere (R_) soil, OTUs abundances for three wheat cultivars (Kariega, Eland and SST88) which were sampled in three production regions (Western Cape, Site A, Gauteng, Site B and Free state, Site C) in South Africa.

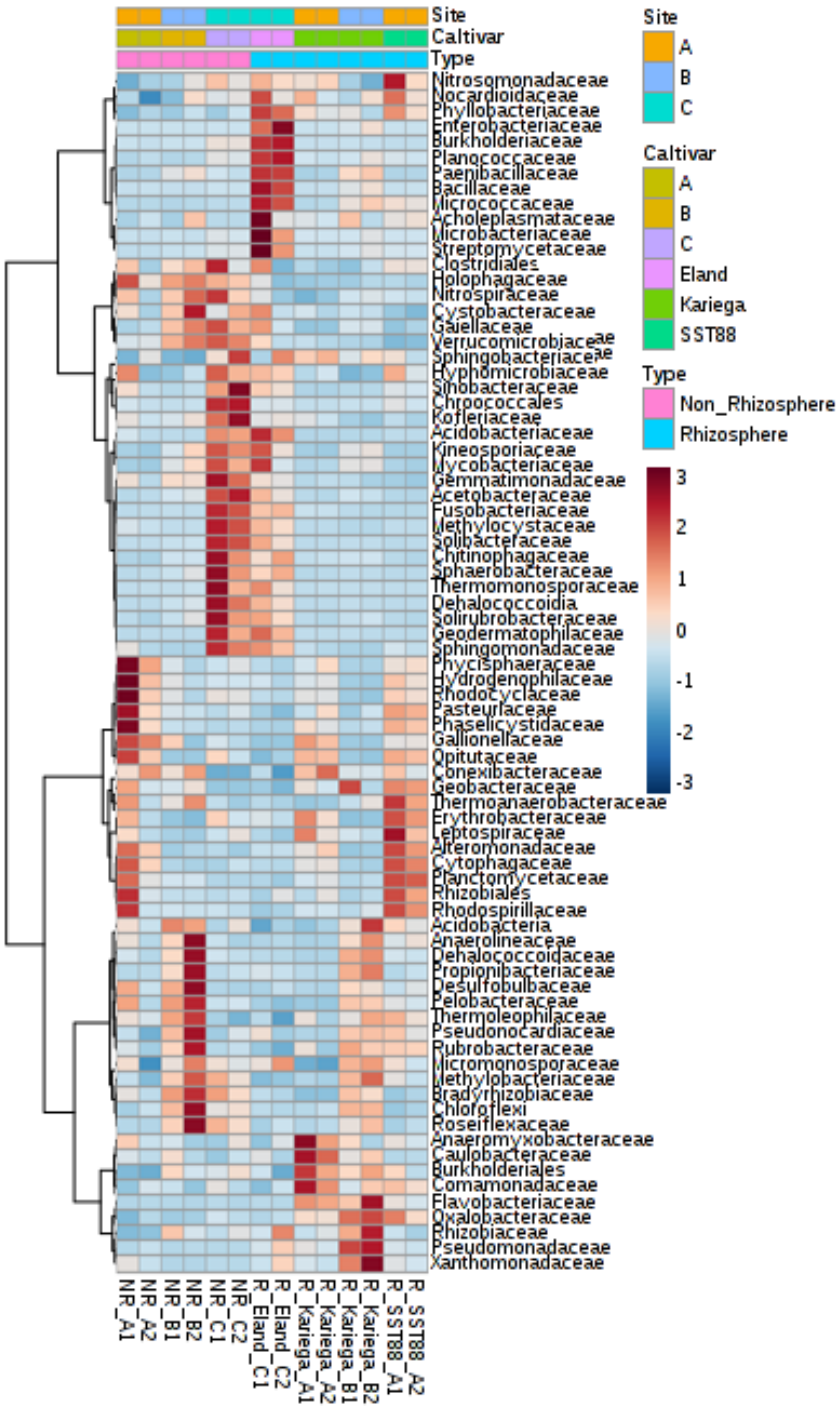

Figure 3.4: A heat map displaying the frequencies of OTUs with abundance > 0.01% at order level of classification for rhizosphere and non-rhizosphere soils of three different cultivars grown in three different production regions in South Africa.

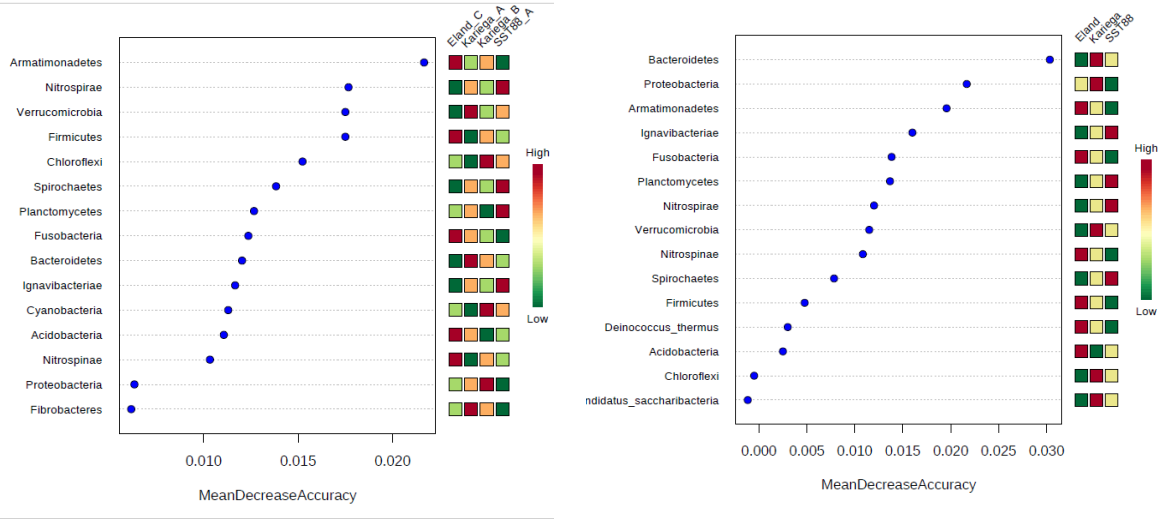

Figure 3.5: Random forest displaying the frequencies of OTUs with abundance >0.01% at phyla level of classification present in all the samples.

Host-associated OTUs whose abundance differences between the three cultivars, were determined after removing low abundance sequences and could be used for reproducible predictive characterisation of the three cultivars with random forest machine learning (figure 3.5). Removal of low abundance sequences (0.01%) from the analysis reduced the number of OTUs from 7558 to 2457.

Bacterial diversity as measured by Shannon and Simpson indices were significantly higher in all non-rhizosphere soil samples than rhizosphere samples except for samples from Site A, irrespective of being different cultivars. Considering diversity in Kariega samples from different sites had no significant diversity differences.

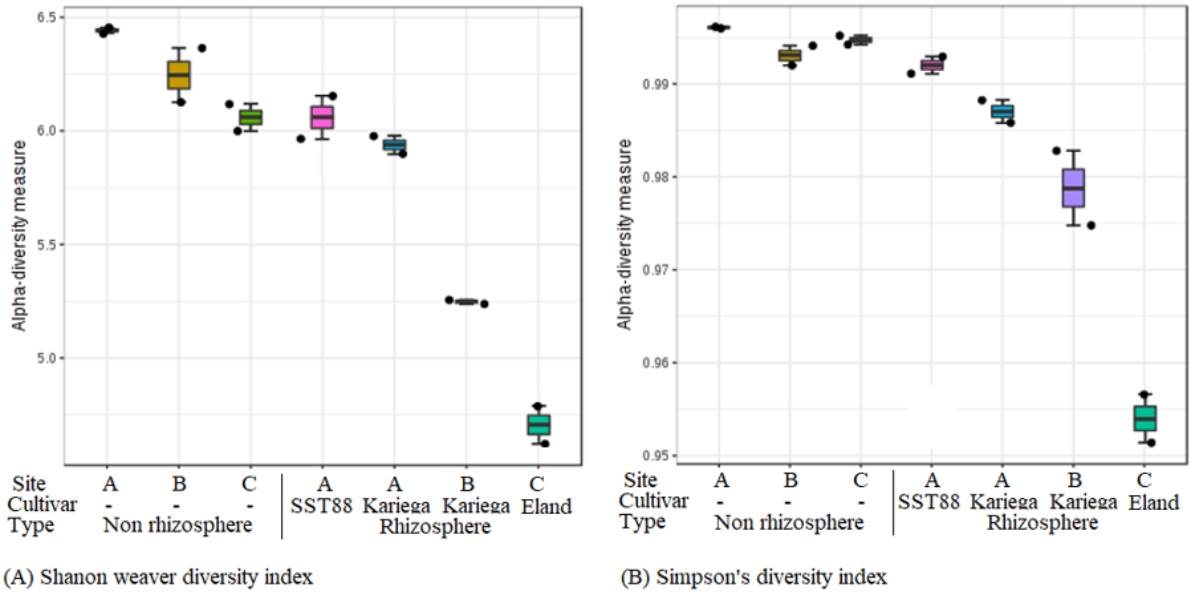

Figure 3.6: Diversity indices based on Shannon weaver (A) and Simpson's (B) diversity index of non-rhizosphere (NR_) and rhizosphere (R_) soil samples, OTUs abundances for three wheat cultivars which were sampled on three sites in different production regions in South Africa.

Bacterial diversity as measured by Shannon weaver and Simpson's diversity index and the observed richness was consistently higher in non-rhizosphere soil compared to rhizosphere samples across the three regions (sites) (Table 2). The observed richness of total OTU in non-rhizosphere soil ranged from 496 to 587 as compared to rhizosphere soil with, 329 to 513 (Table 2). A general decline in diversity was observed in bacterial communities for all the three indices i.e. Shannon-Weaver (10.1%), CHAO (23.4%) and ACE (23.2%) when non-rhizosphere soils was compared rhizosphere soils (Table 2). Different cultivars showed varied diversity changes even when grown on the same site (production region). The wheat cultivar 'SST88' had a 4.6% difference in community diversity (from non-rhizosphere to rhizosphere soil) while 'Kariega cv' had a 7% change

in diversity at Site A. However, considering ‘Kariega cv’, Site B had almost double the diversity change (11.5%) compared to Site A (7%) which was associated with higher Mg (36.4%) (Table 1). Minor differences in community change (2.4%) were observed between ‘Kariega cv’ and ‘SST88 cv’ both grown at Site A. However, the extent of modification for ‘Kariega cv’ grown at Site B was ~5% higher compared to that observed for Site A. The influence of different environmental settings at different sites was also observed at Site C, ‘Eland cv’ which had a 19.4% change (decline from non-rhizosphere to rhizosphere soils) in diversity and this was associated with highly acidic (pH value: 4.42) soils with higher AP (35.19 mg/kg) and lower Mg (74.14 mg/kg) levels (Table 1).

The ANOVA results show that there were significant ($P < 0.05$) differences in soil pH, sodium (Na), calcium (Ca), magnesium (Mg), available potassium (AK), available phosphorus (AP), electrical conductivity (EC) and organic carbon (OC) (Table 1). Site A had significantly higher soil pH, EC, Ca, Na and OC compared to the other two sites. At Site A, The EC, was two times that of Site B with the Ca and OC two times that of Sites B and C while the Na level was ten times that of Site B. Site C had the lowest pH (4.42) of all the sites which was associated with high levels of AP.

3.3.3. Bacterial richness and diversity

Bacterial diversity as measured by ACE, CHAO and Shannon indices and the observed richness was consistently higher in non-rhizosphere soil compared to rhizosphere samples across the three regions (sites) (Table 2). The observed richness of total OTU in non-rhizosphere soil ranged from 496 to 587 as compared to rhizosphere soil with, 329

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3.3.4. Bacterial structure and composition

3.3.4.1. Interactions of bacterial abundance with soil chemical properties

Redundancy analysis was performed to study the relationship between the soil chemical properties and the abundance of bacterial phylum. Redundancy analysis axis 1 explained 59.0% of the total variation and RDA axis 2 explains 31.9% of the total variation in non-rhizosphere soil, while RDA axis 1 explaining 75.6% of the total variation and RDA axis 2

explains 14.9% of the total variation in rhizosphere soil, respectively. Redundancy analysis showed relative abundance of bacteria was affected by soil chemical properties in both the rhizosphere and non-rhizosphere soil (Figure 3.7a and b). The abundance of bacteria at Site A in the non-rhizosphere soil correlated with pH, OC, AK, Ca and Na while the abundance of bacteria at Sites B and C correlated with AP and Mg respectively (Figure 3.7a). A similar trend was observed in bacterial abundances in rhizosphere soils at the three sites (Figure 3.7b). Strong associations were found among Mg and EC with the abundance of Gammaproteobacteria and Betaproteobacteria clustering along axis-1 in the rhizosphere soil at Site A (Figure 3.7a). A strong positive association found in most of the bacterial classes i.e., Deltaproteobacteria, Planctomycetes, Verrucomicrobia, Gemmatimonadetes, Deltaproteobacteria Flavobacteria, Sphingobacteria and Alphaproteobacteria. Alphaproteobacteria, with soil pH, clustering along axis-2 in the rhizosphere soil at Site A. The abundances of Acidobacteria and Firmicutes were found to have a strong negative association with Mg and EC at Site C. However, Cytophagia and Cynobacteria showed a strong association with OC, AK, Ca and Na, but were negatively associated with AP in the rhizosphere soil at Site A. A strong positive association was noticed among Mg and the abundance of Acidobacteria, Planctomycetes, Gemmatimonadetes and Alphaproteobacteria in the rhizosphere soils at Site C on the first RDA axis (Figure 3.7b). On the other hand, pH was clustered along the RDA axis-2 and was strongly associated with Gammaproteobacteria, Flavobacteria, Cytophagia, Betaproteobacteria, Sphingobacteria and Verrucomicrobia AT Site A. At Site B, a strong association between AP and Actinobacteria and Gammaproteobacteria was noticed. These observations clearly indicate that soil chemical properties mainly soil pH

play a significant role in shaping the bacterial community composition in wheat rhizosphere.

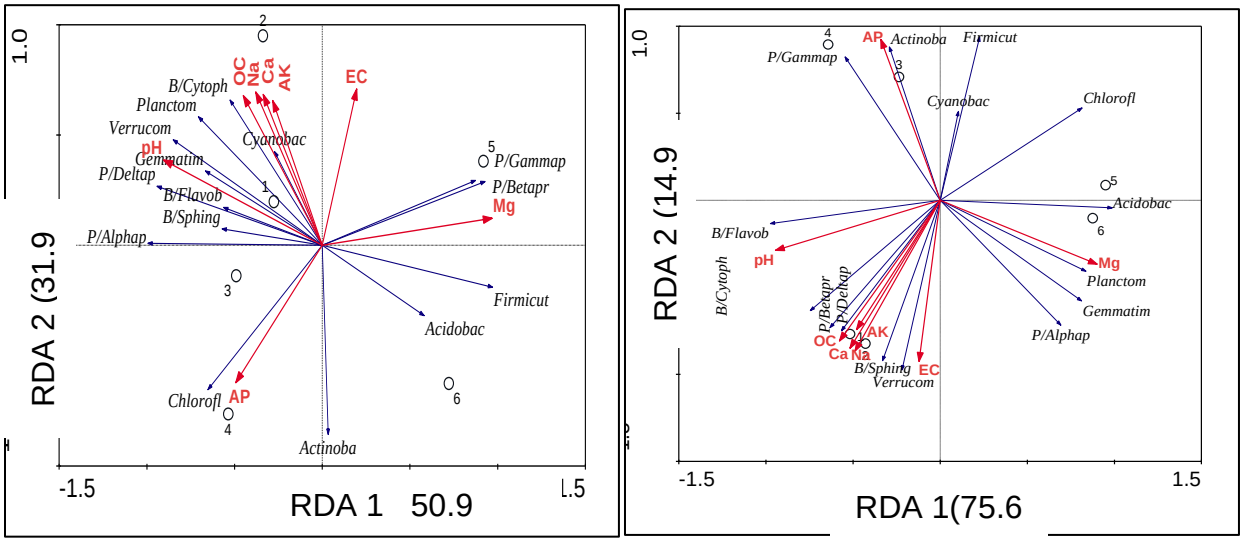

Figure 3.7: Redundancy analysis (RDA) of the correlations between the bacteria taxa and soil physiochemical properties in non-rhozosphere soil (a) and rhizosphere soil (b) in three main wheat growing regions (sites) in South Africa. The arrow length and direction correspond to the variance that can be explained by the environmental and response variables. The direction of an arrow reflects the extent to which the given factor is influenced by each RDA variable. The perpendicular distance between the abundance of bacterial taxa and environmental variable axes in the plot indicates their correlations. The smaller the distance, the stronger the correlation. EC, electrical conductivity; soil organic carbon % (OC); AP, available phosphorus; AK, available potassium; Na, sodium; Mg, magnesium; Ca, calcium; pH.

3.4. Discussion

Understanding the drivers and extent of variation in wheat soil-associated bacterial communities is a crucial step towards developing management strategies to obtain a microbial community structure favoring crop health and productivity. In the current study we found that the rhizosphere soil favoured a lower bacterial diversity dominated by some groups that was not prevalent in the non-rhizosphere soils. The current study is to our knowledge the first to investigate the extent and drivers of variation in soil bacterial communities associated with wheat from different wheat production regions at the vegetative stage.

In this study, the rhizosphere and non-rhizosphere soil bacterial communities of wheat grown in three production regions at the four to five leaf stage when the crown root system was fully developed was profiled. A developed crown zone during wheat growth helps with the acquisition of nutrients by the plant and plant survival during winter is increased (Cook and Veseth, 1991). Soilborne pathogens target and invade the crown and root tissues of cereals at the seedling stage thereby decreasing the plant's capacity for efficient nutrient and water uptake (Weise, 1987; Zillinsky, 1983). The study showed lower bacterial diversity that was associated with dominant communities in rhizosphere compared to non-rhizosphere soils and this was consistent with previous reports (Kowalchuk et al., 2002; Inceoglu et al., 2011; Yun et al., 2016). Although rhizosphere communities, including those that are loosely or tightly associated with the roots are simplest early in the season (sampling stage of the current study) and increase with plant age and senescence (Edwards et al., 2015), minor differences in diversity changes in rhizosphere associated bacterial communities were noticed between cultivars 'SST88'

and ‘Kariega’ at Site A while a more pronounced difference in diversity change was noticed in ‘Kariega cv’ between Site A and Site B. Differences in soil pH were also evident in these two sites. Soil from Site A has a high pH whereas soil from Site B had as intermediate pH. In a stusy by Yun et al. (2016) soil structure and characteristics was reported to have a stronger effect on potato rhizosphere bacterial community diversity than genotype and this is likely the case in the present study. More so, the highest decrease in diversity (19.4%) was observed in soil characterized by low soil pH and high AP levels (Site C) and this agrees with the findings of Yun et al. (2016).

Similar to the findings of Don et al. (2015), the current study demonstrated the ability of wheat roots at the vegetative stage to enrich and promote the dominance of specific bacterial groups including Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Acidobacteria within the rhizosphere. Proteobacteria and Acidobacteria are dominant phyla and are widely distributed across a range of ecosystems (terrestrial to marine ecosystems) (Chaudhry et al., 2012). The Proteobacteria group plays a key role in organic matter decomposition (Su et al., 2010) and nitrogen fixation in the soil (Zimmermann et al., 2006) while Acidobacteria play an important ecological role through synergism with other microorganisms in degrading polysaccharides of plant and fungal origin (Wei et al., 2017) hence their predominance in the wheat rhizosphere. Wheat cultivars seemed to vary more in the bacterial enrichment abilities especially in different environmental settings characterized by different soil chemical properties. For instance, the phyla Acidobacteria, and Planctomycetes did not differ significantly between the rhizospheres of ‘SST88 cv’ and ‘Kariega cv’ at Site A while the same phyla significantly increased in RA in the rhizosphere of ‘Kariega cv’ at Site B compared to Site A. To date, work done in

wheat revealed cultivar/genotype effect as the main cause of variation in bacterial communities (Corneo et al., 2016). However, the current study further suggests that differences noticed in the enrichment abilities in wheat cultivars could be driven mainly by soil chemical properties (Sclemper et al., 2017). Furthermore, we noticed that modification in specific bacteria diversity was to a lesser extent cultivar dependent, similar to the findings of Sclemper et al. (2017).

Gammaproteobacteria phylum has been reported to increase at low pH which was consistent with our findings, however the enrichment of Betaproteobacteria and Firmicutes under low pH contradicts observations of Wei et al. (2017) and Kuramae et al. (2017), Kuramae et al. (2012) who reported that the phyla normally increase with pH. This may therefore suggest an indirect influence by the plants. Some bacteria belonging to the Firmicutes pylum are known to play an important role on wheat growth (Kumar and Verma, 2017) and previous studies have shown that different strains of *Bacillus*, *Paenibacillus* and *Lysinibacillus* through direct or indirect mechanisms inhibit or control potential pathogens such as *Fusarium oxysporum* (Leclere et al., 2005) and *Botrytis cinerea* (Mandal et al., 2018; Timmusk et al., 2005; Chen et al., 2009; Lyngwi et al., 2016). Mechanisms to inhibit potential pathogens include production of antibiotics such as lipopeptides (Koumoutsi et al., 2004; Ongena and Jacques, 2008). Although a number of common phyla were shared between rhizosphere and non-rhizosphere soil samples, the non-rhizosphere soil had more unique OTUs, hence the observation of such phylum as Thermotogae only in non-rhizosphere soil across the three sampled regions. With this in mind, we further investigated the extent to which soil chemical properties contributed in shaping the bacterial communities in different wheat production regions.

Our results showed significant differences in the soil chemical properties between different sites. High levels of available potassium (AK), calcium (Ca), magnesium (Mg) and sodium (Na) at Site A which possibly resulted in high soil pH were depicted. In the RDA study, the bacterial community composition was shown to be influenced by a number of parameters, although pH was the main significant contributor. We demonstrated through the multivariate analysis that apart from AK, Ca, Na, soil organic carbon (OC) and electrical conductivity, soil pH greatly influenced abundance of Deltaproteobacteria, Alphaproteobacteria, Planctomycetes, Verrucomicrobia, Gemmatimonadetes, Flavobacteria and Sphingobacteria in rhizosphere and non-rhizoshere soils at Site A. The phylum Gemmatimonadetes was reported to be vulnerable to soil acidity (Shi et al., 2015) hence was most abundant in alkaline soils at Site A. Furthermore, at Site B, Mg influenced the abundance of Gammaproteobacteria, Actinobacteria, Firmicutes and Cynobacteria in the non-rhizosphere soil while in the rhizosphere soil it influenced the abundance of Chloroflexi and Actinobacteria, Plactomycetes, Gemmatine and Alphaproteobacteria. Members of the Acidobacteria produce a wide range of enzymes and thrive under low pH levels (Shi et al., 2015) and narrow carbon sources (Lee et al., 2015) which is characteristic of Site C. The current study showed that more diverse bacterial communities in the wheat rhizosphere were associated with high soil pH levels which in turn were strongly associated with high levels of Ca, Mg, AK and OC.

In conclusion, we analyzed wheat bacterial communities in the rhizosphere and non-rhizosphere soil sampled early in the season from three wheat growing regions. Overall, rhizosphere soils were characterized by a lower bacterial diversity coupled with

predominance of Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Acidobacteria in all three sites. However, cultivars varied in their microbial enrichment capabilities especially at different sites. Soil chemical properties (mainly soil pH) was the main driver of recruitment in the rhizosphere and to a lesser extent cultivar for specific bacterial groups.

This study provides better understanding of the critical seedling growth stages of wheat and how production region and cultivar may influence bacterial populations on the wheat rhizosphere. The study further outlines that when wheat is planted in different environmental systems, the initial phase of microbial population construct differs and is mainly driven by soil pH. Future biocontrol systems should take regional impact factors into account. This study provides baseline information for future adaptive research to ensure successful establishment of PGPR or biocontrol systems as it gets affected by soil structure and microbial stability diversity and dominance of competitive or enhancing microbes. Moreover, it is important to also outline fungal changes at seedling growth stages of wheat and also study the effects that may be caused by cultivar and soil properties.

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Chapter 4

Fungal diversity and community composition of wheat rhizosphere and non-rhizosphere soils from three different agricultural production regions of South Africa

Understanding complex interactions among plant genotypes, environmental conditions and microbiome structure provides crucial information for sustainable farming practices towards disease control in agriculture. In this study, fungal diversity and composition in wheat rhizosphere and non-rhizosphere soils were investigated. Special emphasis was placed on pathogenic and beneficial genera. Wheat rhizosphere and non-rhizosphere soil from three different wheat growing regions were analyzed using Illumina high-throughput sequencing. The analysis showed a significant decline in the fungal diversity and richness from non-rhizosphere to rhizosphere soils. Ascomycota and Basidiomycota were the dominant fungal phyla detected in both rhizosphere and non-rhizosphere soils across the three test sites. Genera known to include wheat pathogens detected included *Fusarium*, *Phoma* and *Colletotrichum* genera while, beneficial groups included *Trichoderma*, *Aureobasidium* and *Acaulospora*. The presence of *Fusarium* was observed to be inversely proportional to that of *Aureobasidium,* a well-known antagonist of the *Fusarium* spp. This information could provide new opportunities to explore the potential of manipulating natural fungal antagonistic microorganisms for use in controlling soilborne pathogenic fungi in wheat.

Keywords: Fungal communities, *Triticum sativum L.*, High-throughput ITS sequencing, Microbiome, Soil microbiology

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4.1. Introduction

In South Africa (SA), wheat (*Triticum sativum* L.) is considered an important cereal crop and it ranks second after maize in terms of the area under cultivation (533 000 ha) with production ranging from 1.3 to 2 million tons per year (DAFF, 2016). Major wheat production areas in SA are the Free State, the Western Cape and Northern Cape Provinces. Other provinces such as Gauteng and North-West are ranked as minor producers of wheat (Nhemachena and Kirsten, 2017). As with global downward trends, the Free State Province has shown a drop (up to 50%) in annual wheat production (Daff, 2016). Production in other irrigation and winter wheat areas also declined, but not as drastically. This calls for strategic intervention to increase production to at least 2.7 million tons per year in order to feed the country's growing population and avoid food price increases (Fischer and Edmeades, 2017).

Several factors have been attributed to the decline in SA wheat production including unpredictable weather conditions and drought, poor soil fertility and occurrence of pests and diseases (Scot, 1990). Due to poor soil quality most of the local wheat producers resort to fertilizer use (FAO, 2005). This together with lime can increase the total variable input costs to as much as 17 to 30% in wheat production (DAFF, 2010).

Additionally, root and crown diseases in wheat also negatively impact on wheat germination and yield losses in SA (Lamprecht et al., 2006). Economically important diseases of wheat include take-all caused by *Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *graminis*, crown rot caused by *Fusarium pseudograminearum* O'Donnell & Aoki, common root rot caused by *Rhizoctonia solani (*Burgess et al., 2001; Kuzdralinsk et al., 2014) etc. The prevalence of these soilborne pathogenic fungi in wheat differs from one geographic region to another (Kuzdralinsk et al., 2014). Disease control of these pathogens is reasonably successful through different cultivation practices such as the use of disease resistant cultivars, crop rotation, use of pathogen-free seeds, appropriate planting date and plant density as well as by chemical and biological control methods.

Cultivation practices have been reported to influence the plant and soil microbiome (Sergaki et al., 2018) and therefore it is imperative to understand how microbial communities differ from one production area to the other. This will help in the development of a more effective holistic plant protection strategy. In many cases, diseases are associated with plant microbiome imbalances or shifts which makes exploitation of the entire microbiome a desirable strategy. More so, the plant microbiome has been known to be one of the key determinants of plant health and productivity (Berg et al., 2016).

The study of root-associated microbial communities has been important in understanding their ecological role in natural environments (Cavaglieri et al., 2009). Soil microorganisms that establish positive interactions with plant roots play a key role in agricultural environments and are promising for their potential use in sustainable agriculture (Di Cello et al., 1997). The rhizosphere community contains multiple

species of microbes that exert beneficial effects on plant growth and health (Zhao et al., 2018). These include nitrogen-fixing bacteria, mycorrhizal fungi, plant growth promoting rhizobacteria (PGPR), biocontrol microbes and protozoa. On the other hand, soilborne pathogens colonize the rhizosphere causing plant diseases through breaking the protective microbial “shield” and overcoming the plant’s innate defense mechanisms (Mendes et al., 2013). The complexity and diversity of microbes in the rhizosphere are essential for maintaining homeostasis in the soil ecosystem (Garbeva et al., 2004; Raaijmakers et al., 2009). Therefore, understanding both bacterial and fungal population dynamics of the wheat rhizosphere is the first step towards successful manipulation of microbial communities to reduce plant pathogen pressure and increase yield (Bever, 2012). The previous chapter focused only on bacterial community composition between the wheat rhizosphere and non-rhizosphere and the differences between the wheat growing regions and the current chapter studied the difference in rhizosphere fungal communities between the three growing regions. To our knowledge, studies on soil fungi in wheat focused on fungal diversity across different farming systems (Hagn et al., 2003; Sommermann et al., 2018; Abdelfattah et al., 2016). However, no studies have been conducted to investigate fungal diversity with special emphasis on plant pathogenic and beneficial communities in the wheat rhizosphere at the seedling stage in SA. Therefore, the aim of our study was to assess the fungal composition and diversity of wheat rhizosphere in three wheat growing regions of SA and to further understand the dynamics in the pathogenic and beneficial fungal populations in these production areas.

4.2. Materials and methods

4.2.1. Site description and soil collection

A total of 240 rhizosphere and 60 non-rhizosphere soil samples were collected from three different wheat growing agro-ecological regions in SA i.e., Napier, Western Cape Province (Site A), Pretoria, Gauteng Province (Site B) and Bethlehem, Free State Province (Site C). Rhizosphere soil was sampled from four different cultivars and the cultivars were specific for each region. The wheat cultivars grown included 'Kariega' and 'SST88' (Site A), 'Kariega' (Site B) and 'Eland' (Site C). From the 240 rhizosphere soil samples, 80 were collected at 20 m spacing in each site, from each cv. in a statistical designed classical W-shaped pattern. Eight plots were selected for sampling within each location. From each plot, we collected 30 rhizosphere samples that were evenly distributed across the sampling site. The non-rhizosphere soil samples comprised of 20 samples per site. Soil was collected in the field between rows approximately 40 cm away from the sampled plants with a clean auger (washed and disinfected with 70% ethanol between sampling) from a soil depth of approximately 10 cm. Approximately 10 g of rhizosphere soil were collected (from each plantlet at the 4-5 leaf stage with the crown root system fully developed) by uprooting the whole plant and gently shaking off all the access soil from the roots. Only the soil in direct surrounding the roots was gently shaken and brushed off to represent the root zone soil.

Soil samples were placed inside marked zipper bags and transported to the laboratory in cooler boxes for storage at 4 °C to minimize the development of commensals. Soil samples were processed within two days. Each prepared soil sample was stored in an ultralow temperature freezer at −80 °C for DNA extraction.

4.2.2. DNA extraction and sequencing

Total community DNA was extracted from 0.25g soil using the MoBio PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA concentration in each sample was determined using Nanodrop ND-2000 UV–VIS Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) prior to further analysis.

The fungal ITS region was amplified with illumine Miseq using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS2 (GCTGCGTTCTTCATCGATGC) (White et al., 1990). Sequencing was performed at Molecular Research DNA (MR DNA) (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines.

4.2.3. Data processing and bioinformatic analysis

Sequence data were processed using MR DNA analysis pipeline (www.mrdnalab.com, MR DNA, Shallowater). The sequences from the Illumina were joined and depleted of bar codes. Sequences with ambiguous base calls and those <150 bp (Abdelfattah et al., 2016) were discarded. Noises, singletons and chimeras were removed from sequences. Generated operational taxonomy units (OTUs) were defined by clustering at a similarity threshold of 97%.

Normalization of the OTU table was done with rarefaction to an even depth of 1 000 sequences in order to maintain homogeneity of samples. The indices were then calculated from the rarefied OTU table (Smalla et al., 2001). Alpha diversity was calculated using Chao1 and Shannon-Weaver indices. Principle coordinates analysis

(PCoA), based on distance matrix of Bray-Curtis estimation, was used to evaluate similarities or differences between samples. The alpha diversity indexes relative to the samples were represented by box plots and statistically compared using a standard t-test. We further tested for significant differences in the relative abundance of taxonomic groups using one-way ANOVA and Tukey's multiple comparison test. Test results with $P < 0.05$ were considered statistically significant. All statistical analyses were executed in R version 3.4.3 (R Core Team, 2017).

4.3. Results

4.3.1. Fungal richness and diversity

Fungal communities were characterized by next-generation sequencing of nuclear ribosomal internal transcribed spacer-1. A total of 1 598 148 valid reads were generated from 14 soil samples at 3% distance after paired-end alignments, quality filtering, and deletion of chimeric and singletons. Sequences ranged from 34 767 to 432 036 per sample, with a median read count of 114 153 per sample. The high-quality reads were clustered into 1 268 microbial OTUs at 97% similarity after the removal of OTUs that were unassigned or not assigned to the target species.

The species richness and diversity of the soils from the three sites is presented in Figure 4.1. The t-test revealed a significant difference ($P < 0.05$) on the species richness (Chao) and diversity (Shannon-Weaver) between rhizosphere and non-rhizosphere soil samples (supplementary Table 4.1). The OTU richness was significantly lower ($P < 0.05$) in the rhizosphere soil (SST88 cv at Site A- 774.1 ± 19.01, Kariega cv at Site A- 847.4 ± 41.32 and Kariega cv at Site B- 929.9 ± 27.22 and Eland

cv at Site C- 753.9 ± 28.14,) soil compared to non-rhizosphere (Site A- 800.2 ± 23.65 and Site B- 946.2 ± 21.97, Site C- 736.9 ± 28.98) as shown in Figures 4.1 A and B, respectively. Similarly, the fungal diversity was lower in rhizosphere soil samples (Kariega cv at Site A- 3.085 ± 0.10, Eland cv at Site C- 3.93 ± 0.43, SST88 cv at Site A- 4.41 ± 0.38 and Kariega cv at Site B- 4.49 ± 0.02) compared to non-rhizosphere (Site A- 3.58 ± 0.18, Site B- 4.03 ± 0.31 and Site C- 4.65 ± 0.27) soil samples across the three sites. Rhizosphere soil from Kariega cv at Site B had a significantly ($P < 0.05$) higher fungal diversity of 4.49 ± 0.02 while rhizosphere from Kariega cv at Site A had the lowest fungal diversity of 3.085 ± 0.10. In addition, significant differences ($P < 0.05$) in fungal diversity and species richness were observed between the rhizosphere soils of Kariega cv and SST88 cv grown at Site A. The Kariega cv rhizosphere soil showed significantly ($P < 0.05$) lower species richness and diversity compared to SST88 cv rhizosphere soil. Meanwhile, significant differences ($P < 0.05$) were also observed in species richness and diversity between Kariega cv grown in Site A and Site B. Similar to the observations in the rhizosphere soil samples, the highest alpha diversity in non-rhizosphere soils was observed at Site B (4.65 ± 0.27) while Site C (3.58 ± 0.18) had the lowest alpha diversity.

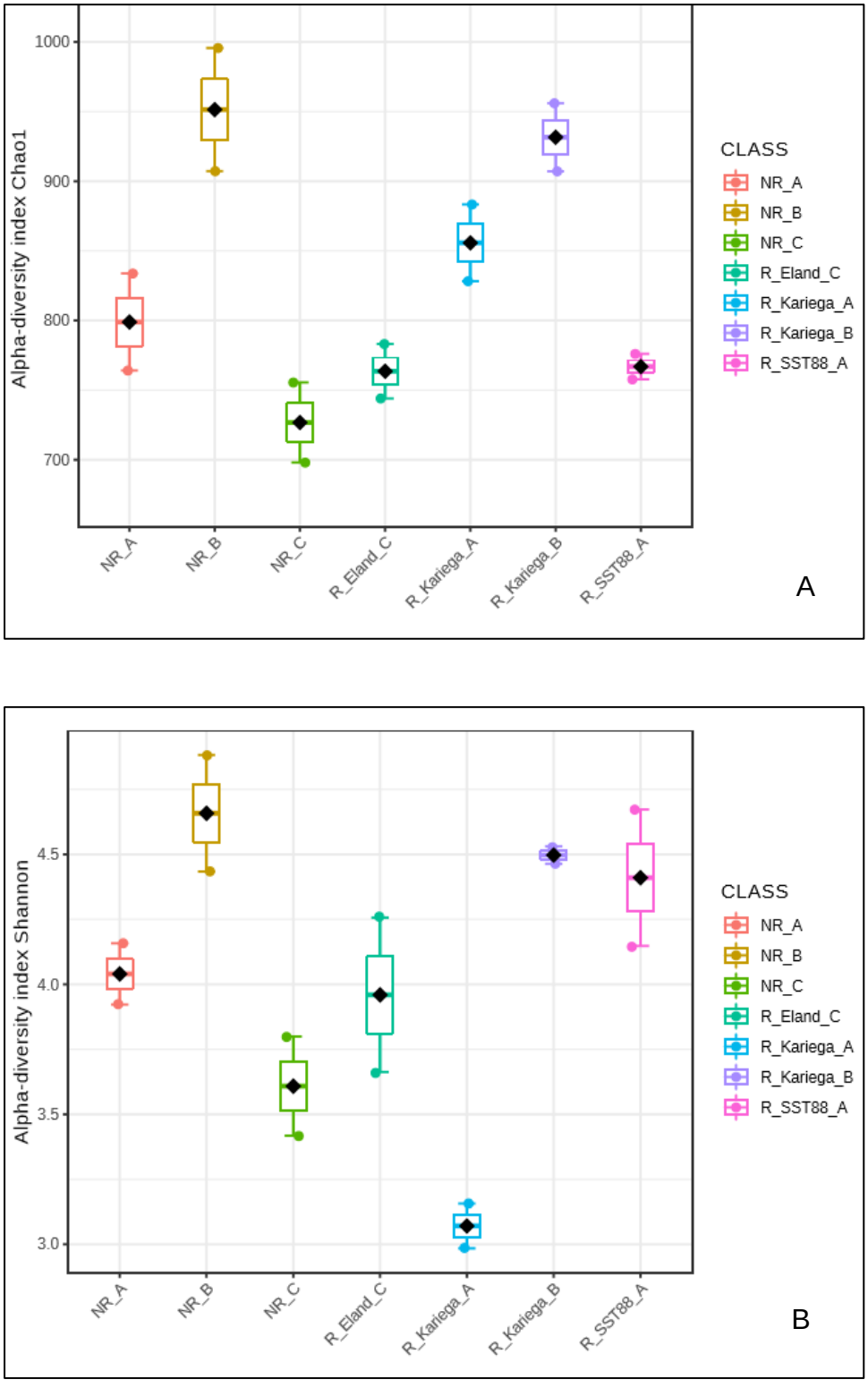

Figure 4.1: Alpha diversity of the estimated OTU richness (Chao1 (A)) and diversity (Shannon (B)) indexes of the 16S rRNA gene libraries for clustering at 97% identity for wheat rhizosphere and non-rhizosphere soil. NR_A, non-rhizosphere soil at Site A;

NR_B, non-rhizosphere soil at Site B; NR_C, non-rhizosphere soil at Site C; R_Eland_C, Eland rhizosphere soil at Site C; R_Kariega_A, Kariega rhizosphere soil at Site A; R_Kariega_B, Kariega rhizosphere soil at Site B; R_SST88_A, SST88 rhizosphere soil at Site A.

The effect of wheat cultivar and site on relative abundances of observed OTUs showed significant differences with a PERMANOVA test statistic for cultivar abundances of the OTUs. A significant effect ($P < 0.05$) was found for both cultivar and site, although no significant interaction could be determined for the two (supplementary Table 4.2). Based on Bray-Curtis dissimilarity analysis, the beta diversity of the fungal communities between three sites were significantly different ($P < 0.01$). About 41.5% of observed variation could be explained by the first two principle coordinates (Figure 4.2). The rhizosphere and non-rhizosphere OTUs clustered together according to sampling site in principle component analysis. In addition, the separation of the different sampling sites was clearly visible. The three cultivars (SST88 cv, Kariega cv and Eland cv) were generally well separated although, minimal separation was observed in fungal communities in the rhizosphere of SST88 cv and Kariega cv at Site A.

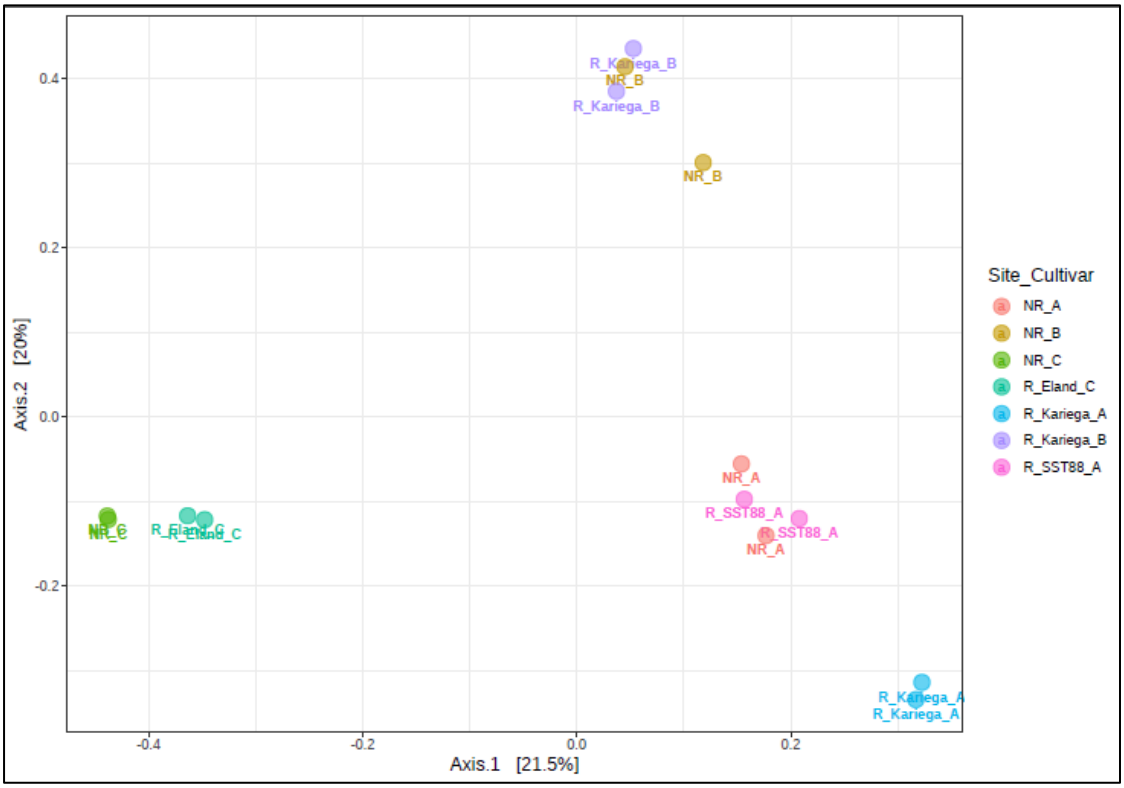

Figure 4.2: Principal component analysis of the fungal community composition on wheat rhizosphere and non-rhizosphere soil collected from different regional sites in South Africa, according to illumina sequencing data. NR_A, non-rhizosphere soil at Site A; NR_B, non-rhizosphere soil at Site B; NR_C, non-rhizosphere soil at Site C; R_Eland_C, Eland rhizosphere soil at Site C; R_Kariega_A, Kariega rhizosphere soil at Site A; R_Kariega_B, Kariega rhizosphere soil at Site B; R_SST88_A, SST88 rhizosphere soil at Site A.

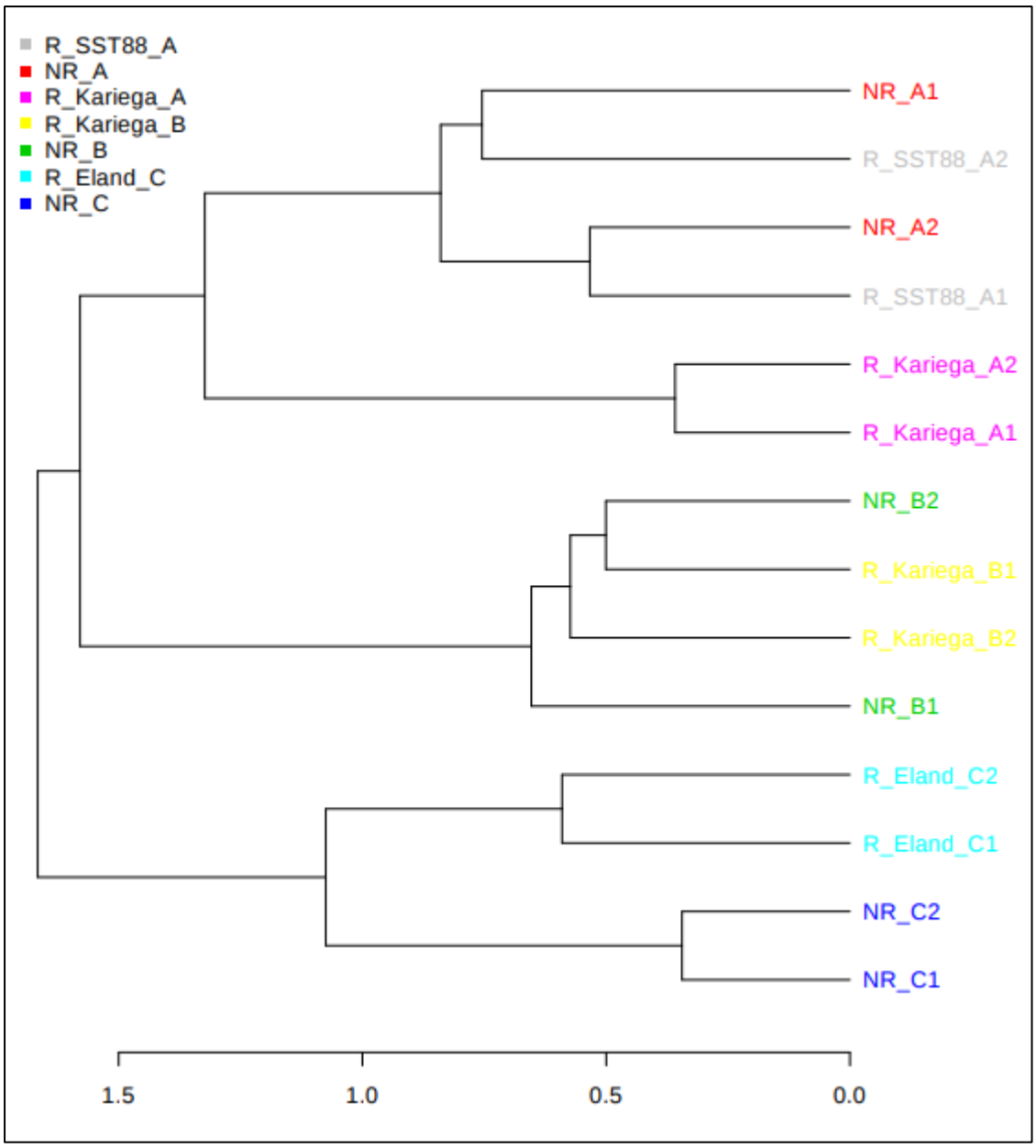

Figure 4.3: Dendrogram from hierarchical cluster analysis (using Bray- Curtis Index) of fungal composition at OTU level for rhizosphere and non-rhizosphere soil of wheat. NR_A, non-rhizosphere soil at Site A; NR_B, non-rhizosphere soil at Site B; NR_C, non-rhizosphere soil at Site C; R_Eland_C, Eland rhizosphere soil at Site C; R_Kariega_A, Kariega rhizosphere soil at Site A; R_Kariega_B, Kariega rhizosphere soil at Site B; R_SST88_A, SST88 rhizosphere soil at Site A.

4.3.2. Relative abundance

In the rhizosphere soils, the phylum Ascomycota (41.6- 60.4%) was the most abundant irrespective of sampling site. The second most dominant phylum was Basidiomycota (10.7-25.4%). Although the fungal composition was similar between the different cultivars, their relative abundance was different. The abundance of the Ascomycota phylum in the rhizosphere soils in different sites were as follows, Kariega cv at Site A- 41.6%), SST88 cv at Site A- 60.4%, Kariega cv at Site B- 54.5% and Eland cv at Site C- 47.1% while that of the Basidiomycota phylum were Kariega cv Site A- 25.4%, SST88 cv at Site A- 21.7% and Eland cv at Site C- 25.5%.

Across all sites the rhizosphere soils, the Ascomycota phylum was generally dominated by fungal member classes of Sordariomycestes, Dothideomycetes, Eurotiomycetes and Orbiliomycetes with *Fusarium*, *Aureobasidium* and *Colletotrichum* being the most dominant genera. Basidiomycota were dominated by member classes Agaricomycetes and Tremellomycetes. Similar to the rhizosphere, fungi belonging to the phyla Ascomycota (Site A- 25.5%, Site B- 44.5% and Site C- 5.3%) and Basidiomycota (Site A- 22.3%, Site B- 45.1% and Site C- 0.7% were observed in higher relative abundances in the non-rhizosphere soil. Meanwhile, Monoblepharidomycota was more dominant in the non-rhizosphere soil of Site A (45.6%). And the unclassified fungi, mainly from the Mucoromycotina class dominated Site C (93.7%) soil.

Significant ($P < 0.05$) differences in the relative abundance of specific fungal classes were observed between the rhizosphere of Kariega cv and that of SST88 cv at Site A (supplementary Figure 4.1). For example, Pezizomycetes, Sordariomycetes, Tremellomycetes, Mucoromycotina and Monoblepharidomycetes were 4.8, 3, 2.4, 3.9

and 22 times higher in SST88 cv than in Kariega cv at Site A, respectively. While in Glomeromycetes, Mortierellomycotina and Leotiomycetes the relative abundance differences were 19, 9.11 and 3 times higher in Kariega cv than in SST88 cv at Site. A more pronounced variation of the two cultivars was observed at family level. The pair wise comparisons showed that, the Acaulosporaceae family was significantly ($P < 0.05$) higher in the rhizosphere of Kariega cv (23%) compared to that of SST88 cv (0.35%) at Site A. However, the abundance of the Nectriaceae family was significantly ($P < 0.05$) higher in the SST88 cv rhizosphere (22.5%) compared to the Kariega cv (3.5%) rhizosphere.

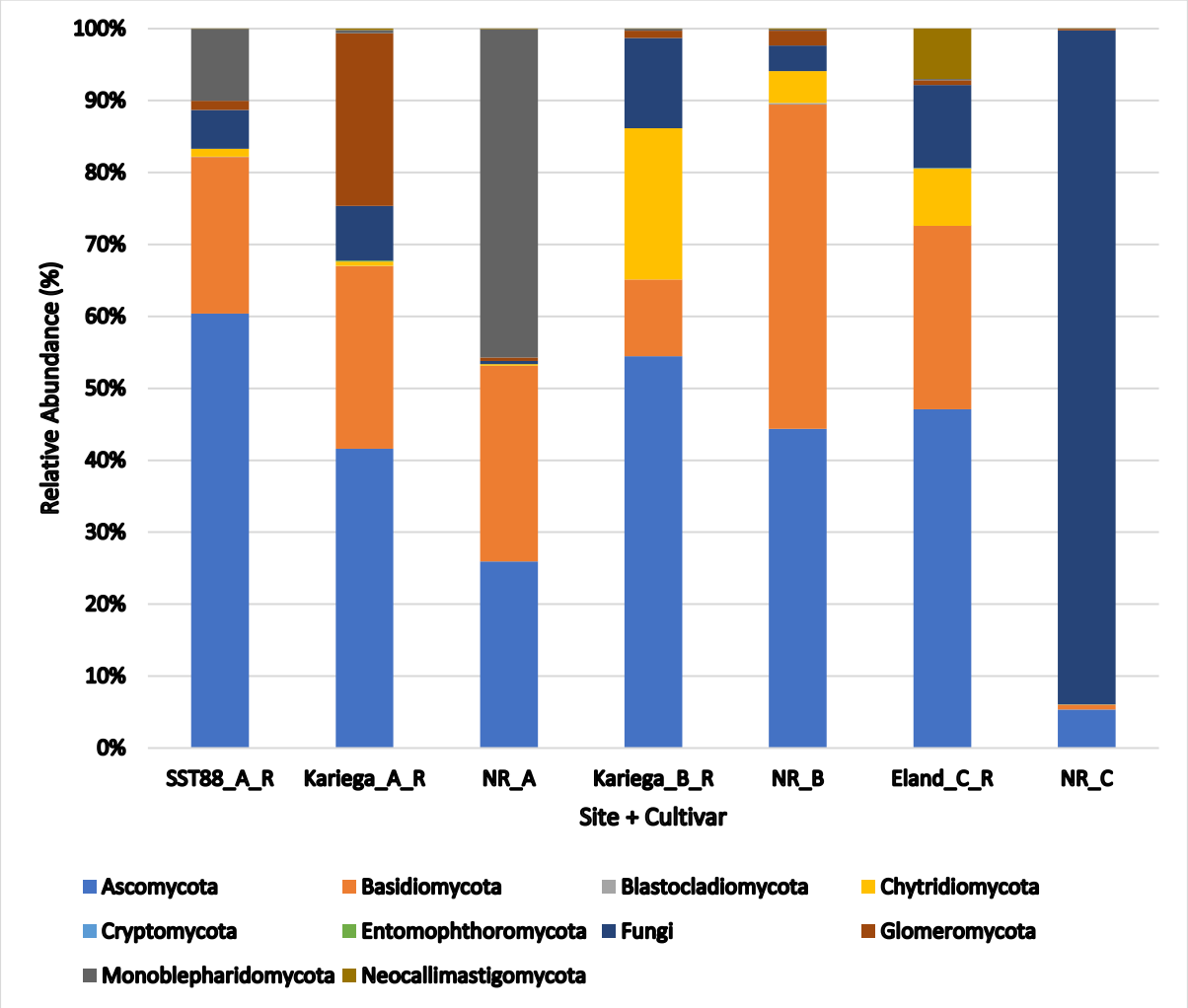

Figure 4.4: Overall relative abundance of fungal phyla of wheat on SST88 and Kariega

rhizosphere (R), and non-rhizosphere (NR) soil at Site A, Kariega rhizosphere (R) and non-rhizosphere soil at Site B and Eland rhizosphere and non-rhizosphere soil at Site C. NR_A, non-rhizosphere soil at Site A; NR_B, non-rhizosphere soil at Site B; NR_C, non-rhizosphere soil at Site C; Eland_C_R, Eland rhizosphere soil at Site C; Kariega_A_R, Kariega rhizosphere soil at Site A; Kariega_B_R, Kariega rhizosphere soil at Site B; SST88_A_R, SST88 rhizosphere soil at Site A.

4.3.3. Pathogenic and beneficial fungal general

We further assessed the abundance of fungal genera pathogenic (*Colletotrichum, Cladosporium, Fusarium, Aspergillus*, *Alternaria*, *Cateractispora*, *Penicillium*, *Sclerotinia*, and *Phoma*) and beneficial (*Aureobasidium, Trichoderma and Acaulospora)* to wheat at the three sites. Amongst the pathogenic fungi, the *Fusarium* genus (0.92% - 21.1%) was the most abundant across all the sites. Overall, no significant ($P > 0.05$) changes were observed in the abundance of *Fusarium*, *Colletotrichum*, *Cladosporium*, *Aspergillus* and *Cateractispora* from the non-rhizosphere to the rhizosphere soils (Table 4.1). Meanwhile, a significant ($P < 0.05$) increase was observed in the abundance of *Aureobasidium* from the non-rhizosphere to the rhizosphere soil. The pairwise comparisons showed significantly ($P < 0.05$) higher abundances of *Fusarium* (21.1%) in the rhizosphere of SST88 cv compared to non-rhizosphere soil at Site A. In contrast, the abundances of *Fusarium* decreased significantly ($P < 0.05$) from the non-rhizosphere soil to the rhizosphere of Kariega cv at the same site. As a general observation, the abundance of *Fusarium* was inversely proportional to that of *Aureobasidium*. The *Colletotrichum* genus was present at abundances above 3% in both the rhizosphere of Kariega cv and non-rhizosphere

soils at Site B while, in the other two sites, the presence of *Colletotrichum* was almost negligible (<0.5%). The *Cateractispora*, *Penicillium*, *Sclerotinia*, and *Phoma* genera were significantly ($P < 0.05$) enriched in the rhizosphere of Kariega cv at Site A when using the pairwise comparisons. The opposite was noticed in the rhizosphere of SST88 cv at the same site. *Acaulospora* was noticed to be enriched in the rhizosphere of most of the sampled soils except for the rhizosphere of Eland cv at Site C, where it decreased substantially.

Table 4.1: Pathogenic and beneficial fungal genera that were affected by wheat rhizosphere at three sites in South Africa.

		Site A						Site B			Site C		
		SST88			Kariega			Kariega			Eland		
		NR	R		NR	R		NR	R		NR	R	
Pathogenic fungi	*Phoma*	0.125	0.06	-	0.125	0.14	+	0.61	0.655	+	0.06	0.345	+
	Alternaria	0.035	0.055	+	0.035	0.065	+	0.075	0.28	+	0.005	0.005	x
	Colletotrichum	0.05	0.13	+	0.05	0.04	-	3.29	3.61	+	0.015	0.185	+
	Alternaria	0.035	0.055	+	0.035	0.065	+	0.075	0.28	+	0.005	0.005	x
	Bipolaris	0	0	x	0	0	x	0.48	0.985	+	0	0.1	+
	Cladosporium	0.405	0.98	+	0.405	0.98	+	4.735	4.01	-	0.135	2.24	+
	Fusarium	10.41	21.085	+	10.41	3.28	-	1.97	2.785	+	0.915	1.545	+
	Ramulispora	0	0	x	0	0.01	+	0.015	0.645	+	0	0	x
	Paraphaeosphaeria	0.02	0.415	+	0.02	0	-	0.015	0.01	+	0	0.02	+
	Leptosphaeria	0.025	0.03	+	0.025	0	-	0	0.02	+	0	0.04	+
	Sclerotinia	0.03	0.01	-	0.03	0.04	+	0.035	0.045	+	0	0.09	+
	Paraphaeosphaeria	0.02	0.415	+	0.02	0	-	0.015	0.01	-	0	0.02	+
	Aspergillus	1.225	1.435	+	1.225	0.15	-	0.55	0.2	-	0.005	0.365	+
	Penicillium	0.75	0.79	-	0.75	2.24	+	0.505	0.685	+	0.19	3.525	+

	Cateractispora	0.79	0.135	-	0.79	1.525	+	0	0.035	+	0.01	0.005	-
Beneficial fungi	*Aureobasidium*	3.455	8.175	+	3.455	12.25	+	9.155	16.51	+	1.945	7.365	+
	Acaulospora	0.015	0.365	+	0.015	23.35	+	0.08	0.335	+	0.015	0.135	+
	Trichoderma	0.105	0.6	+	0.105	0.76	+	0.46	0.165	-	0.535	3.66	+

“+” denotes fungi with high relative abundance in rhizosphere soil than in non-rhizosphere soil. “-“ denotes fungi with lower relative abundance in rhizosphere than in non-rhizosphere soil. “x” denotes no changes in relative abundance between rhizosphere and non-rhizosphere soil. Wheat cultivars (SST88, Kariega and Eland); NR, Non-rhizosphere; R, Rhizosphere.

4.4. Discussion

The present study assessed microbial diversity and composition in wheat rhizosphere and non-rhizosphere soils from three different wheat growing sites (regions) through Illumina sequencing of the ITS1 and ITS2 regions. Special emphasis was placed on understanding the dynamics of pathogenic and beneficial fungal populations at the vegetative growth stage. In this study, diverse and significantly different fungal populations were detected in the rhizosphere of the different cultivars tested. Similar observations were made with non-rhizosphere soils in the three different growing sites. Microbial diversity and richness play a key role in the sustainable development of soil health, ecosystem function and crop production (Kennedy and Smith, 1995). Fungal genera comprising of major wheat root and crown rot pathogens as well as beneficial (disease control and facilitate nutrient availability) microbes were also detected. This information is valuable in the development of sustainable disease management strategies such as those that employ natural or artificially applied biological control agents (Chandrashekara et al., 2012).

Fungal diversity along with species richness (OTU numbers) declined from the non-rhizosphere to the rhizosphere soils across the three sites. Similar trends were previously observed in other plants such as Arabidopsis (Shakya et al., 2013), rice (Lundberg et al., 2012; cotton Qiao et al., 2018) and Populus (Edwards et al., 2015). This observation was attributed to differences in the microbial community composition between rhizosphere soils and the surrounding non-rhizosphere soils. Wang et al. (2017) also reported higher fungal diversity in non-rhizosphere compared to rhizosphere soil in wheat during the flowering stage under conservative tillage farming systems. The narrowing of the fungal populations in the rhizosphere was mainly

attributed to the selection pressure exerted by plant roots on the surrounding soil microbial community. The current study demonstrated the ability of wheat roots at the vegetative stage to enrich and promote the dominance of specific fungal groups including Ascomycota, Basidiomycota and unclassified fungi under the subdivision Mucoromycotina. Similar trends were also observed in previous studies by Lauber et al. (2008) and Nemergut et al. (2008). They reported that Ascomycota and Basidiomycota were the dominant fungi in vegetated soils. Fungi within the Ascomycota phylum grow quickly and become dominant during the early stages of plant growth because of their ability to use the carbon resources immediately released by roots into the soil (Hannula et al., 2012). Most of the plant pathogens are members of the Ascomycota phylum (Carris et al., 2012). On the other hand, Basidiomycota consists mainly of saprotrophic yeasts which are the primary agents of plant litter decomposition, and their hyphal networks grow throughout the soil interface. They represent highly dynamic channels through which nutrients are readily distributed (Crowther et al., 2012). This trend was observed in all cultivars and in non-rhizosphere soil across all sites except for non-rhizosphere soil of Site C, where Mucoromycotina was mostly abundant.

Wheat cultivars differed in recruiting other fungal communities especially in different geographical settings which were characterised by different environmental conditions. For instance, the phyla Chytridiomycota did not differ significantly between the rhizospheres of SST88 cv and Kariega cv at Site A. However, the same phyla significantly increased in abundance in the rhizosphere of Kariega cv at Site B compared to Site A. Additionally, different cultivars also showed enrichment of different fungal groups at the same site. For instance, SST88 cv and Kariega cv at Site A enriched different fungal groups namely Monoblepharidomycota and Glomeromycota,

respectively. In the meantime, Kariega cv at Site B enriched Chytridiomycota while Neocallimastigomycota and Chytridiomycota were enriched by Eland cv at Site C. A study done by Mommer et al. (2016) indicated that plants often select their rhizobiomes *via* root exudation. Furthermore, Wardle (2002); Wagg et al. (2014); Hannula et al. (2017) suggested that fungal populations are strongly influenced by plant genotype as they affect nutrient availability and cycling processes. Moreover, geographic locations were observed to be another factor affecting microbial populations (Igiehon and Babalola, 2018; Philippot et al., 2013). Previous studies have reported that geographical location as well as crop management practices can affect soil microbial community composition and consequently the composition of rhizosphere microbial communities (Göre and Bucak, 2007; Sapkota et al., 2017; Soman et al., 2017).

In this study, differences in fungal communities between two wheat cultivars (Kariega and SST88) grown on the same site were noticed. Three fungal orders i.e., Hypocreales, Agaricales and Mortierellales were abundant in the rhizosphere of SST88 cv while Lobulomycetales, Rhizophlyctidales and Agaricales were abundant in the rhizosphere of Kariega cv at Site A. This was not surprising since host genotype is a major factor in determining the composition of root communities (Sapkota et al., 2015). This observation highlights the importance of investigating microbial diversity and community composition of different cultivars during the process of developing disease control strategies. Notably, fungal diversity in the rhizosphere was strongly influenced by cultivar in all the three sites. For instance, differences in rhizosphere fungal diversity were observed between cultivars SST88 and Kariega at Site A. It has been reported that different genotypes of the same plant species when grown in the same soil are often associated with distinct microbial communities (Berendsen et al.,

2012; Berg and Smalla, 2009). Studies on chickpea showed that different cultivars produced different arrays of root phytochemicals (Cruz et al., 2012; Ellouze et al., 2012) resulting in different soil fungal communities (Yang et al., 2012; Ellouze et al., 2013).

Further variations in fungal diversity were observed between rhizosphere and non-rhizosphere soils in the three sites. Essel et al. (2019) demonstrated diversity indices where fungal OTUs were less in the wheat rhizosphere compared to non-rhizosphere soil under different tillage and crop rotation systems. These diversity variations were well defined at Site C where the diversity of non-rhizosphere soil was 0.72 times higher that of the rhizosphere soil. The current study noted that lower fungal diversity in non-rhizosphere soils was associated with the enrichment of certain fungi groups. For example, Mucorales, classified under the Mucoromycotina subphylum was more abundant (93.7%) in non-rhizosphere soil compared to rhizosphere soil (11.52%) at Site C. As a result, Site C had the lowest fungal diversity on non-rhizosphere soil.

Certain pathogenic fungi belonging to the Ascomycota phylum, known to cause major disease in wheat were observed in this study. Plant pathogenic fungal genera such as *Phoma*, *Colletotrichum* and *Fusarium* were observed in high abundance across all the three sites. Soil-borne pathogens identified included *Fusarium solani, F. oxysporum, F. equiseti, F. pseudograminearum, F. avenaceum, Fusarium sp, Phoma tropica, Phoma sp, Giberella zeae*. Pathogenic fungi from the *Fusarium spp* are reported to cause economically important diseases on wheat in South Africa (SA) namely *Fusarium* crown rot, *Fusarium* Head blight, *Fusarium* wilt and *Fusarium* root rot (Lamprecht et al., 2006). *Fusarium* was most abundance in SST88 cv Site A compared to the other cultivars. Furthermore, *Fusarium* was the most abundant pathogenic

genus observed in all the rhizosphere and non-rhizosphere samples. This observation can be attributed to the production of wheat under dry land farming systems in SA (DAFF, 2016) where the occurrence of *Fusarium* was previously reported to be the main cause of major diseases in wheat. Diseases caused by fungi in the genera *Bipolaris and Fusarium* are favored by dry soil conditions (Wegulo et al., 2013).

Fungal species known to have biological control of *Fusarium* pathogens were observed in conjunction with pathogens in this study. Fungal genera known to have a beneficial effect namely, *Aureobasidium*, *Acaulospora* and *Trichoderma* were observed in the current study. Han et al. (2016) and Berendsen et al. (2012) reported that *Aureobasidium* and *Trichoderma* represent genera that contain antagonistic species which could potentially be used in the suppression of plant pathogens and ultimately the control of soilborne diseases in food crops. Previous studies on the efficacy of fungal biological control agents focused more on *Trichoderma,* a genus with species well known for their biological control properties (Gajera et al., 2013; Lecomte et al., 2016; Mukherjee et al., 2012). Although the *Trichoderma* genus was observed in this study in both rhizosphere and non-rhizosphere soils across the three sites, it was however, present in lower abundances of <1%. Interestingly, *Aureobasidium* another potential biological control group (Wachowska and Głowacka, 2014) was observed in higher abundances of between 1% and 16%.

Fungi belonging to the *Aureobasidium* genus are mostly saprotrophic and polymorphic fungi (Gniewosz and Duszkiewicz-Reinhard, 2008) and some of their species can be used as biological control agents to protect wheat plants from pathogenic *Fusarium species* (Wachowska et al., 2013). *Aureobasidium pullulans* was reported to be commonly found on the surface or inside the tissue of cereal kernels where they

suppress the growth of phytopathogens (Wachowska and Głowacka, 2014). In the present study, *A. pullulans* was the only species detected under the Aureobasidium genus and its abundance was observed to be inversely proportional to that of the *Fusarium* species in both the rhizosphere and non-rhizosphere soils. The relative abundance of *A. pullulans* was also observed to be increasing from the non-rhizosphere to the rhizosphere soils across all the three sites.

Wachowska and Głowacka (2014) also observed an antagonistic effect of *A. pullulans* against *Fusarium culmorum* known to cause winter wheat spikes. Furthermore, Wachowska et al. (2015) demonstrated that *A. pullulans* could be used in an integrated approach to protect wheat grain from being colonized by pathogenic *Fusarium* species. The antagonistic effect of *A. pullulans* was reported to be related to production of extracellular enzymes as well as grain phytochemicals (Castoria et al., 2001). Root pathogens such as *Fusarium* species are highly efficient colonizers of the host plant's rhizosphere and rhizoplane thus, having the potential to significantly influence populations of other rhizosphere colonizers including microorganisms that can help in the biological control of other root pathogens.

Other beneficial groups observed in this study included fungi belonging to the Glomeromycota genus. *Acaulospora*, a member of the Glomeromycota genus was detected in high abundances (24.0%) in the rhizosphere of Kariega cv at Site A. On the other note, the same fungi were detected in low frequencies (>2%) in SST88 cv at the same site as well as in other cultivars from other sites. The low abundance of *Acaulospora* has also been reported previously (Cui et al., 2018). A study by Singh and Adholeya (2013) revealed lower abundance of arbuscular mycorrhizas (AM) fungi in agricultural fields. The Glomeromycota family comprises of arbuscular mycorrhizas

which are characterized by the formation of unique structures, arbuscules and vesicles (Brundrett, 2002). Fungi of the *Glomeromycota* family have been previously reported to be associated with wheat roots (Cui et al., 2018). Khaitov and Teshaev (2015) suggested that soil properties are an important factor influencing the AM root colonization and they further reported the colonization of the rhizosphere by *Glomeromycota* in the two cotton varieties tested. The AM fungi is of agricultural importance as it helps plants to capture soil nutrients such as phosphorus, sulfur and nitrogen as well as other important micronutrients including magnesium, iron and manganese (Brundrett, 2002). The AM fungal species distribution was reported to be strongly dependent on the location (Castillo et al., 2016) and these findings agree with the findings of the current study.

In conclusion, the current study indicated that the soil fungal diversity and composition of wheat rhizosphere and non-rhizosphere soil differs from one production region to the other. Wheat cultivar also affect fungal composition in the rhizosphere. The rhizosphere soils were abundant of pathogenic populations compared to the non-rhizosphere soil. Important wheat known fungal pathogenic genera detected in both the rhizosphere and non-rhizosphere soils included *Fusarium*, *Phoma*, *Colletotrichum* and *Paraphaeosphaeria* and their presence was inversely proportional to *Aureobasidium*, *Acaulospora* and *Trichoderma* which are deemed as beneficial fungi in wheat production. The findings of the current study can serve as a baseline study for future development of antagonistics on soil health, plant growth and biocontrol research and gives an outline on the pathogenic fungal populations occurring in the major wheat production areas of SA. Furthermore, developing a good antagonist is usually based on its ability to colonize the rhizosphere, hence further research must be conducted on colonization potential of PGPR strains.

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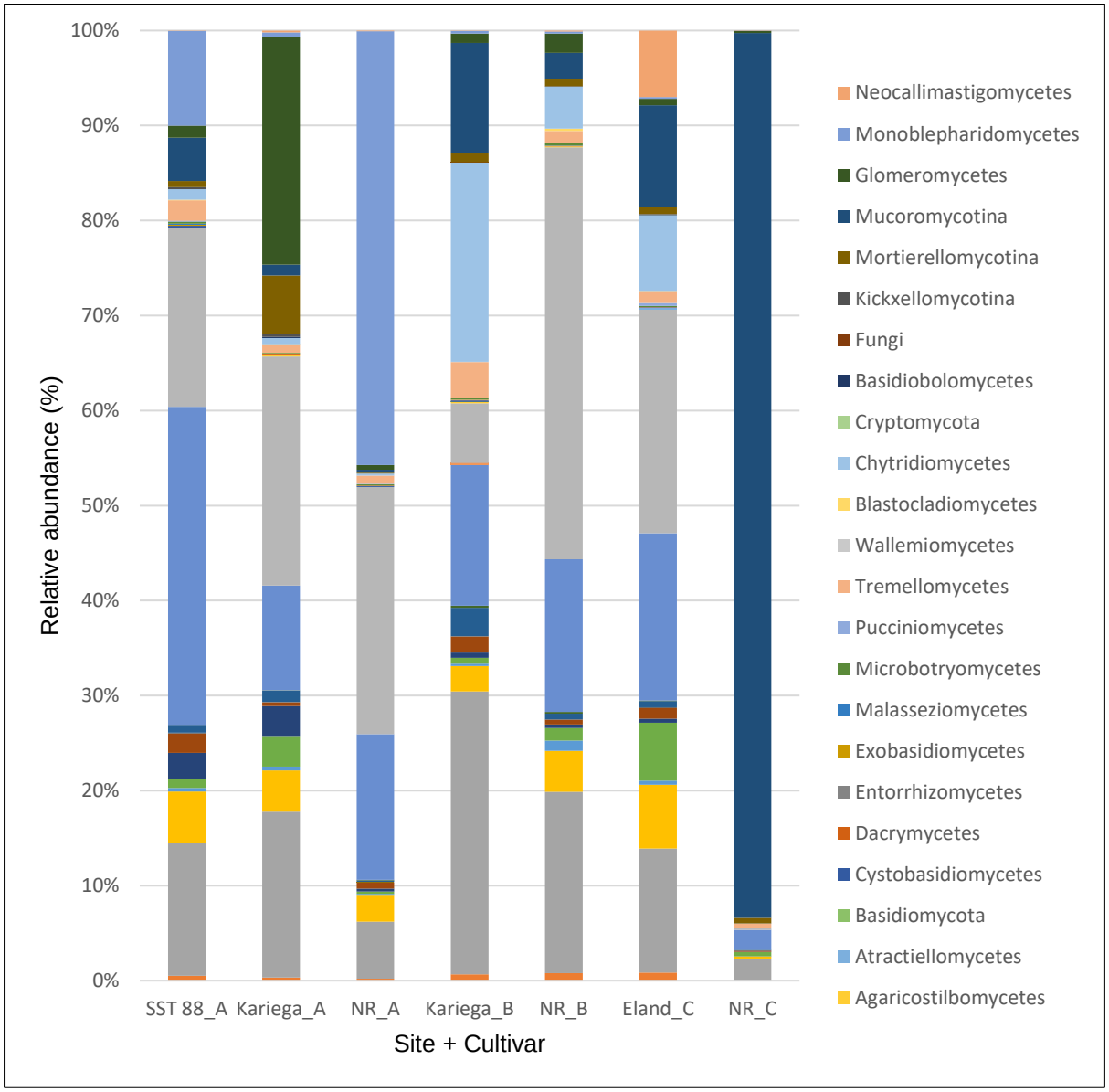
Supplementary data

Supplementary Table 4.1: Permutational multivariate analysis of variance (PERMANOVA) of main factors tested and their interactions for wheat rhizosphere and non-rhizosphere soil from the same field sites.

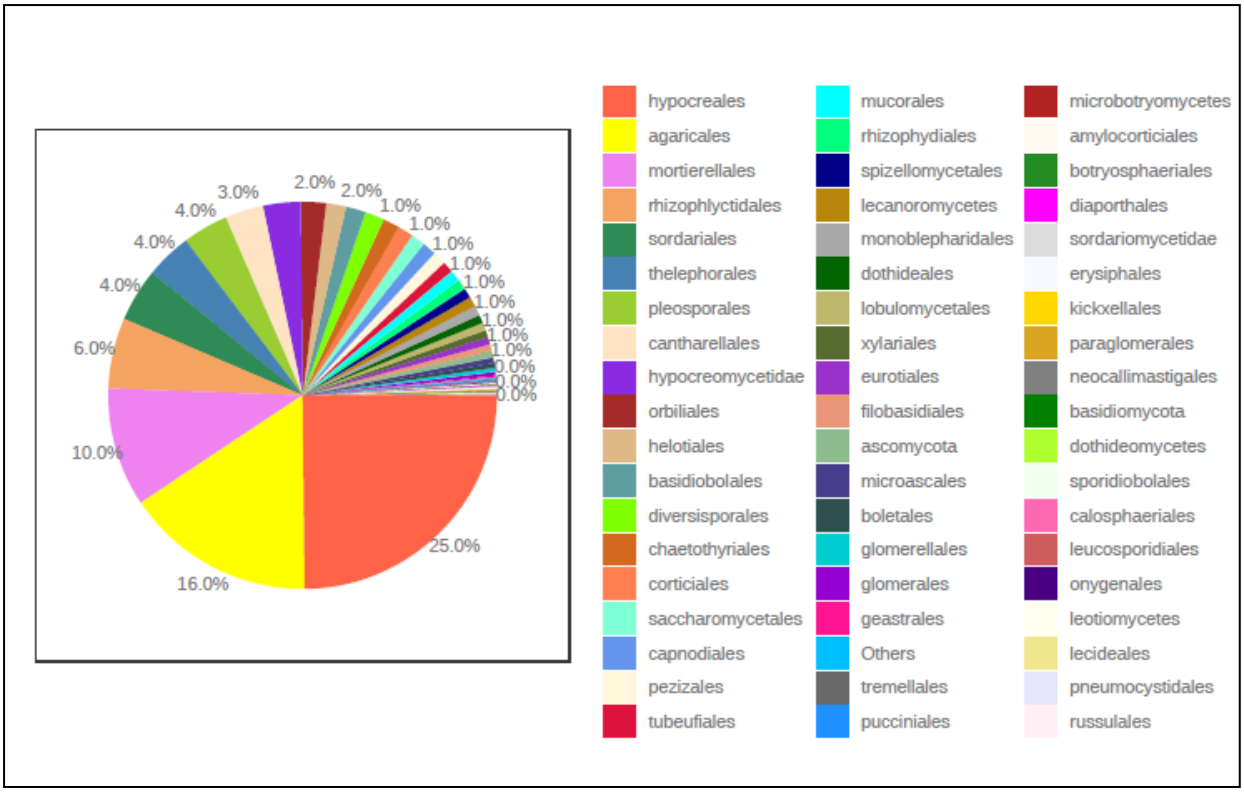
	Chao index		Shannon index	
	F-value	**P-value**	**F-value**	**P-value**
Rhizosphere (R)	36.44	0.002	24.73	0.017
Non-rhizosphere (NR)	20.10	0.001	15.87	0.025
R vs NR	14.39	0.014	8.31	0.034

Supplementary Table 4.2: Descriptive statistics on OTU richness (Chao 1) and species diversity (Shannon-Weaver).

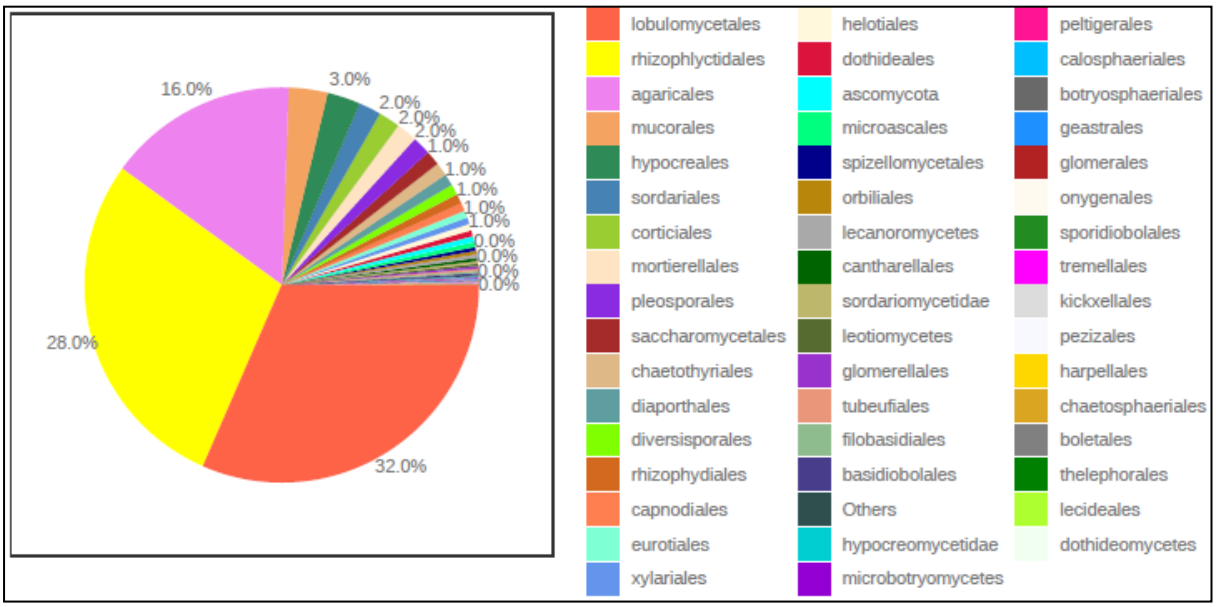
Sources of variation	Df	F-value	R-squared	P-value
Non-Rhizosphere				
Site	2	4.49	0.749	0.009
Rhizosphere Samples				
Site x Cultivar	3	2.79	0.528	0.060
Cultivar	2	3.62	0.731	0.012
Pairwise Comparison				
Site (Kariega: Site A x Site B)	1	6.48	0.764	0.033
Cultivar (Kariega x SST88 at Site A)	1	2.88	0.590	0.043



Supplementary Figure 4.1: Alpha diversity of the estimated OTU richness (Chao1 (A)) and diversity (Shannon (B)) indexes of the 16S rRNA gene libraries for clustering at 97% identity for wheat rhizosphere and non-rhizosphere soil. NR_A, non-rhizosphere soil at Site A; NR_B, non-rhizosphere soil at Site B; NR_C, non-rhizosphere soil at Site C; R_Eland_C, Eland rhizosphere soil at Site C; R_Kariega_A, Kariega rhizosphere soil at Site A; R_Kariega_B, Kariega rhizosphere soil at Site B; R_SST88_A, SST88 rhizosphere soil at Site A.



Supplementary Figure 4.2: Overall relative abundance of fungal order on SST88 wheat rhizosphere (R) at Site A.



Supplementary Figure 4.3: Overall relative abundance of fungal order on Kariega wheat rhizosphere (R) at Site A.

Chapter 5

Root colonization and plant growth promotion by *Bacillus subtilis* T29 on different wheat cultivars

Apart from exerting a plant growth promoting effect, rhizobacterial inoculants should be able to compete successfully against the native microflora to colonize and establish in the rhizosphere. The *Bacilus subtilis* T29 strain was tested for its ability to promote growth and colonize the rhizosphere of different wheat cultivars. Rhizosphere colonization was assessed using droplet digital PCR technique. Seed treatment with *B. subtilis* T29 resulted in increased plant height, shoot and root volume and total dry mass. Results showed that *B. subtilis* T29 was able to colonize the rhizosphere of different wheat cultivars and that colonization efficiency was influenced by the type of soil used. The average concentration of *B. subtilis* T29 on Free State rhizosphere soil samples (17.44 copies μL^{-1} of DNA) were comparatively higher to those from Western Cape Province (16.7 copies μL^{-1} of DNA). The plant growth-stimulating ability of *B. subtilis* T29 was also affected by cultivar. *Bacillus subtilis* T29 significantly stimulated the shoot and root mass of Eland cv, whereas Duzi cv was less affected by *B. subtilis* T29 inoculation. The results of this study suggest that inoculation of wheat with *B. subtilis* T29 can improve plant growth in agricultural field soil. Prior to a selection of good bacterial inoculants, it is recommended to select cultivars that benefit from association with these bacteria. In addition, this study provides a strain-specific ddPCR approach to monitor this rhizobacterial inoculant in soil.

Keywords: *Bacillus subtilis*, rhizosphere, droplet digital PCR, wheat cultivars, root colonization

5.1. Introduction

Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that colonize plant roots and have beneficial effects on plant growth and development (Kloepper and Beauchamp, 1992; Mendis et al., 2018). Diverse bacterial genera such as *Alcaligenes*, *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Klebsiella*, *Pseudomonas* and *Serratia* include specific strains which are reported as PGPR (Hurek and Reinhold-Hurek, 2003; Podile and Kishore, 2007; Kumar et al., 2011; Saharan, 2011). Inoculation of crop plants with certain strains of PGPR at an early stage of development improves biomass production through direct and indirect effects on root and shoot growth (Saharan, 2011). Direct effects of PGPR on plants include providing fixed nitrogen and phytohormones, increasing the availability of nitrogen, soluble phosphate and minerals in the soil and control plant pathogens through competition for space and nutrients (Mendis et al., 2018). Some PGPR are also responsible for promoting plant growth indirectly by eliciting the Induced Systemic Resistance (ISR) response (Abdelrahman et al., 2016; Jogaiah et al., 2018; Mendis et al., 2018).

Due to an increased concern on the repeated use of pesticides in agriculture (Özkara et al., 2016), there has been a growing demand for development of PGPR as an alternative to pesticides usage (Joshi and McSpadden, 2006; Arrebola et al., 2010). Members of the genus *Bacillus* have received much attention in research due to their ability to produce heat resistant spores which makes *Bacillus* more amenable to commercial formulation. Therefore, most of the commercially available PGPR products contain *Bacillus* strains (Kloepper et al., 2004). Many of these *Bacillus* spp.

have been found to be effective biocontrol agents of plant pathogens (Mendis et al., 2018). Furthermore, several *Bacillus*-based products developed from *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus pumilus* are now commercially available (Parvathi et al., 2009). Some examples of biological products available commercially would include Serenade and VOTiVO developed from *B. amyloliquefaciens* QST713 and *Bacillus firmus* respectively (Castillo et al., 2013).

One critical requisite of an effective biological agent is that the PGPR strain must be a good root colonizer (Mendis et al., 2018). The term “root colonization” of PGPR describes the active growth of introduced bacteria on or around roots in the presence of native microflora (Kloepper and Beauchamp, 1992; Mendis et al., 2018). Quantification of root colonization by PGPR is thus an essential part of discovering and evaluating promising PGPR strains. Dilution-plate counting is a common method used in quantification of root colonizing bacteria (Simons et al., 1996). However, dilution-plate counting could underestimate the number of bacteria associated with roots due to some of the cells failing to grow into colonies under the plating conditions (Mendis et al., 2018). The droplet digital Polymerase Chain Reaction (ddPCR) is a new an overly sensitive detection tool that has been used recently in quantifying microbes. This recent technology provides precise quantification and detection of the target nucleic acid in a sample (Carmichael, 2018). Application of ddPCR have mainly focused on clinical research areas such as cancer (Albano et al., 2015; Beltrame et al., 2015; Combaret et al., 2015) and HIV (Myerski et al., 2019). On plants, applications of the ddPCR include quantification of wheat fungal pathogens (Liu et al., 2020), *Botrytis cinerea* on table grapes (Carmichael, 2018), potato bacterial pathogens (Dreo et al., 2014) and it has also been used for analysis of genetically modified organisms

(Koppel and Bucher, 2015). However, to our knowledge its application in plant pathology is still new, specifically in quantification of beneficial bacteria in wheat rhizosphere (Mendis et al., 2018.

The previous two chapters outlined both the fungal and bacterial community composition in the wheat rhizosphere of different cultivars grown in the main wheat growing regions of South Africa. This chapter will further determine the survival and colonization efficacy of a potential antagonist (*B. subtilis* T29) in the presence of the the outlined microbiome using absolute quantification.

5.2. Materials and methods

5.2.1. Bacterial cultures

Rhizobacterial cultures were obtained from the University of Pretoria's Plant growth promoting rhizobacteria (UP-PGPR) culture collection. The rhizobacterial strain was previously identified as part of two studies by Hassen (2007) and Pretorius (2012), respectively. This strain is being used as a model system on studies focusing on both biological control of diseases and growth promotion on cereals. The rhizobacterial strain was selected based on its performance in previous greenhouse trials on wheat (*Triticum aestivum L*.) (Makgolane, 2016) and maize (*Zea mays L*.) (Rudolph, 2012).

5.2.2. Bacterial inoculum preparation and enumeration

The bacterial culture was revived on nutrient agar (NA) after storage at -80˚C in a biofeezer. A single colony was selected and sub-cultured onto NA and incubated for

24 hours at a temperature of 25 °C to verify purity. Subsequently, a single bacterial colony was again selected and inoculated into sterile nutrient broth (100ml) (Biolab, Wadeville). The inoculated broth was incubated at 25 °C on a rotary shaker (152rpm) for 24h. A Petroff hausser counting chamber (Marienfeld, Germany) was used to determine the concentration of bacterial cells in the suspensions. The culture was then transferred to 50ml centrifuge tubes and centrifuged at 3000rpm for 10 min. Thereafter the pellet was re-suspended in sterile Ringer's solution (Merck) and adjusted to a concentration of approximately $2x10^8$cfu/ml.

5.2.3. Soil sampling

Soil sampling was conducted as described in section 3.2.1.

5.2.4. Greenhouse trial

Wheat seeds were surface sterilized with 70% ethanol for 5 min, 1% sodium hypochlorite for 1 min and rinsed five times with sterile distilled water, whilst control seeds were wetted with sterile distilled water. The seeds were left to dry in an incubator for 2 hours and further inoculated with 1 ml rhizobacterial suspension ($1x10^9$ cells/ml), sterile water was used for control seeds. Seeds were planted into 10 cm diameter plastic pots containing soil sampled from agricultural wheat fields in the Western Cape, Napier and Free State, Bethlehem. Three wheat cultivars (cv.) were selected based on popularity and disease susceptibility for each region and planted in soil sampled from the corresponding region. Free State; Eland cv, Duzi cv and Kariega cv, Western Cape; SST056 cv, SST88 cv and SST0117 cv. Pots were watered every second day

with municipal tap water. Three months after planting, the plants were harvested by carefully separating the roots from the soil. Rhizosphere soil of the lower upper and middle part of the root and bulk soil was collected and stored. Excess soil was rinsed off using tap water and plant length was recorded. The roots were excised from the foliage, and their mass was determined separately. The plants were then dried in an oven at 50 °C oven for 48 hours and the dry mass was recorded.

Bacillus subtilis isolation from rhizosphere soil

Pot soil from treated rhizosphere was collected in an eppendorf tube and exposed to 80 °C on a heatblock to kill non-spore forming bacteria. The soil wash from the heated soil was prepared and plated on nutrient agar. The plates were incubated for 48 hours at 25 °C. Pure colonies were sent to Inqaba Biotec for further identification (figure 5.1).

5.2.5. DNA extraction

The rhizosphere soil retrieved from above was used. Total community DNA was extracted from 0.25g fresh soil using the MoBio PowerSoil™ DNA Isolation Kits (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA concentration in each sample was determined using Nanodrop ND-2000 UV–VIS Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) prior to further analysis.

5.2.6. Primer design

Specific primer sets were designed using the Primer3 software (http://biotools.umassmededu/bioapps/primer3_www.cgi) from the specific sequence of *Persea americana* deposited in the NCBI GenBank (Table 4.1). The primer pairs were chosen and validated in silico using primer BLAST specific analysis (http://www.ncbi.nlm.nih.gov/Blast.cgi) and then according to the melting profiles obtained from the quantitative real time PCR conditions (qPCR), as described later.

5.2.6.1. Primer specificity and efficiency (Real-Time PCR)

The qPCR reactions were performed using iTaq universal Sybr® Green supermix mix (Bio Rad) on a qPCR (Biorad) under the following conditions: an initial holding cycle (10 min. at 95 °C), followed by 40 cycles of two steps of denaturation and annealing/extention (15 sec at 95 °C and 60 sec at 60 °C respectively). The PCR amplification was carried out in a total volume of 20 µL, containing 1 µL of DNA (duplicates), 0.25 µM of each primer, and 10 µL of iTaq universal Sybr® Green supermix mix. All the assays included no-template controls, to determine the non-specific amplification. To determine the specificity of the amplicons, melting curve analysis was performed over the range of 60 to 95 °C. The qPCR efficiency (E) of each primer pair was determined using standard curves generated according to the equation E = 10−1/slope of five triplicate cDNA pool dilutions (undiluted, 0.25, 0.0625, 0.015, and 0.003).

5.2.6.1 Controls and method optimization (ddPCR)

To test the sensitivity of the primers, DNA from *Pseudomonas* T19 University of Pretoria culture collection) isolated from Sorghum rhizosphere was used as a negative template control. Template DNA of *B. subtilis* T29 isolated from sorghum (from UP-PGPR culture collection) was used as a positive control and identified by inqaba biotec. In order to obtain a clear separation between the negative and positive droplets from the ddPCR output, optimization of the positive control to the best amplitude was necessary. Three dilutions, (1:10, 1:100 and 1:1000) of the positive control with sterile PCR water were tested. The diluted 1:100 diluted template had the best amplitude and was used for the ddPCR analysis.

Gene	NCBI accession number	Primer sequence (forward/reverse)	Amplicon size	PCR efficiency (%)	R^2 of standard curve
Bacillus subtilis	MN128547.1	TTGCCACCTACGTATTACCG TAACTGCTTGCACCTTGACG	161	106	0.985

5.2.6.2. Droplet digital PCR assays for *Bacillus subtilis* quantification

The DNA from the rhizosphere soil was used to detect and quantify *B. subtilis* using the QX100™ Droplet Digital™ PCR system (Bio-Rad, Pleasanton, CA, USA) (detection limit, 1 DNA copy μL^{-1}). The reaction mix for ddPCR consisted of 1µL DNA, 11.05µL Supermix (2Xqx200 EvaGreen Mix) (Bio-Rad), 9.51µL RNase-DNase Free Water and

0.22µL of each primer. Primers specific to *B. subtilis* (designed above) (F 5'-TTGCCACCTACGTATTACCG-3'; R 5'TAACTGCTTGCACCTTGACG-3') were used. All samples were analysed in duplicates. Droplet generation was performed, thereafter, the PCR reaction was performed in a T100™ thermal cycler (Bio-Rad). The amplification conditions were 5 min. at 95 °C DNA polymerase activation, followed by 39 cycles of 30 sec at 95 °C for denaturing and 60 sec at 54.5 °C for annealing and extension, followed by a final hold of 5min at 90 °C for droplet stabilization and cooling to 4 °C. After thermal cycling, the plate was transferred to a droplet reader (Bio-Rad), and the data was generated using QuantaSoft version 1.7.40917 provided with the ddPCR system.

5.2.7. Data and statistical analysis

The ddPCR data was first analysed in QuantaSoft™ software (Bio-Rad) version 1.7.40917 following manufacturer's guidelines. Wells with less than 10,000 droplets were not included in the analysis. QuantaSoft automatically did a calculation of the target concentrations in copy number per µl. The data were then statistically analysed using Analysis of Variance (ANOVA) to determine differences between cultivars and sites. Pearson's correlations were used also to correlate plant mass and concentration of *B. subtilis*.

5.3. Results

Bacillus subtilis isolation from rhizosphere soil: *Bacillus subtilis* was identified using 16s rDNA sequencing on the wheat rhizosphere. Other bacteria identified were *Bacillus cereus*, *Bacillus mobilis* and *Lysinibacillus fusiformis*.

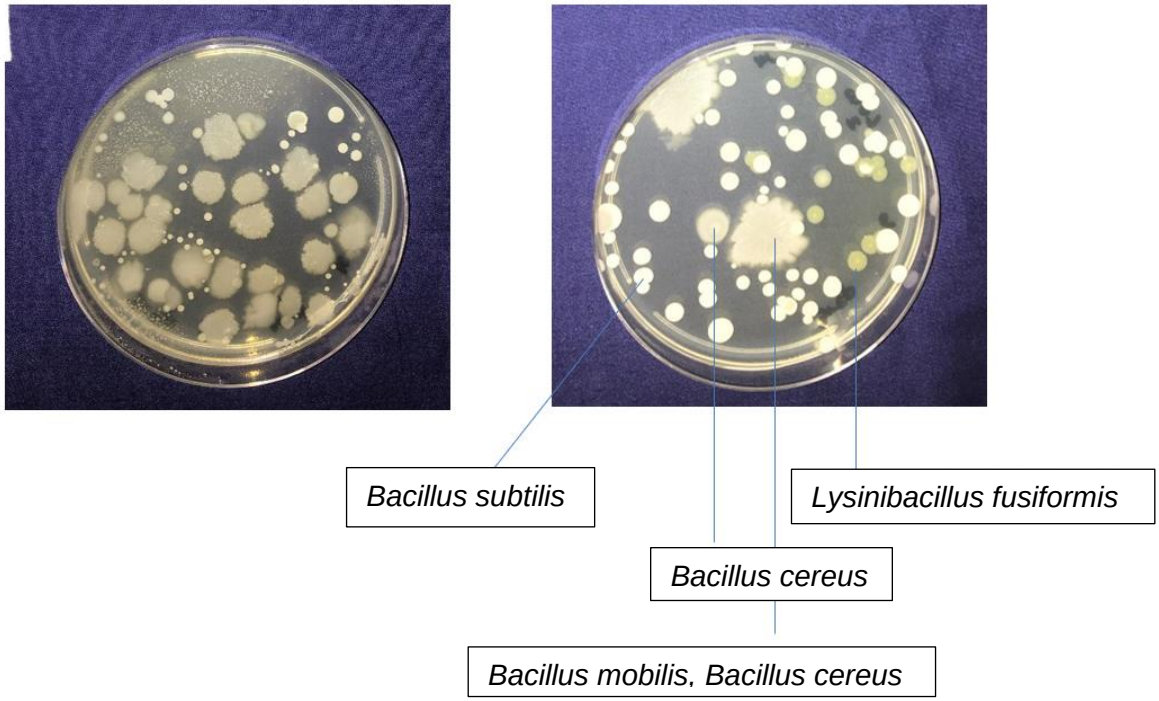
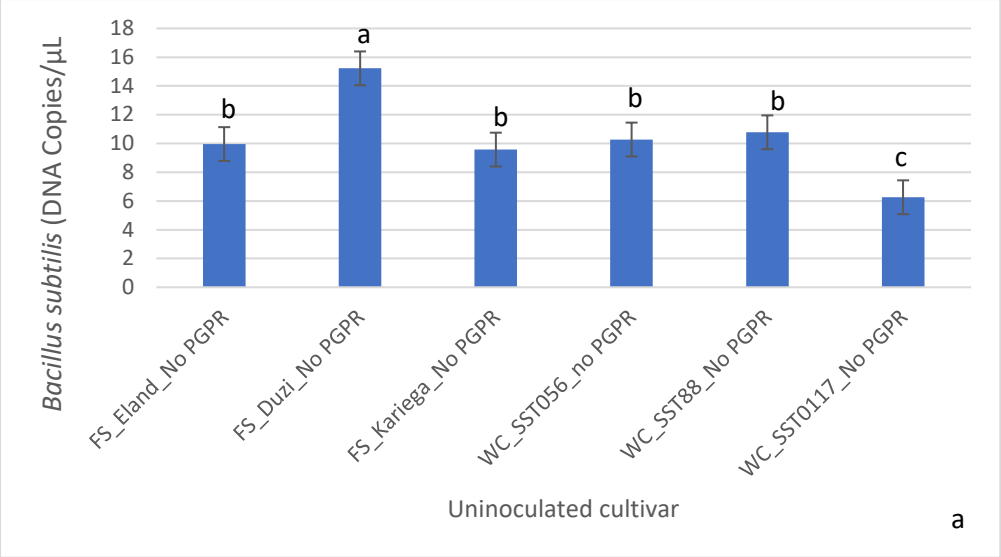


Figure 5.1: Bacteria identified growing on the wheat rhizosphere.

Bacillus subtilis T29 was detected in all rhizosphere soil samples. On average, the concentration of *B*. *subtilis* T29 on inoculated samples was higher (17.1 copies μL^{-1} of DNA) than uninoculated samples (10.3 copies μL^{-1} of DNA) (Figure 5.2, a and b). In the inoculated samples, the average concentration of *B. subtilis* T29 in rhizosphere soil samples from the Free State Province cultivars (17.44 copies μL^{-1} of DNA) were comparatively higher to those results from the Western Cape Province cultivars (16.7 copies μL^{-1} of DNA). Concerning the results of cultivars from the Free State Province, rhizosphere soil from Kariega cultivar (cv) had the highest *B. subtilis* T29 concentration

(21.1 copies μL^{-1} of DNA), while the lowest concentration (15.1 copies μL^{-1} of DNA) was observed from Duzi cv. While the SST056 cv from the Western Cape Province had the highest *B. subtilis* T29 concentration (19.8 copies μL^{-1} of DNA) in the rhizosphere and SST88 cv had the lowest concentration (13.7 copies μL^{-1} of DNA). On the uninoculated samples, the Free State (11.0 copies μL^{-1} of DNA) had comparatively higher *B. subtilis* T29 concentration to those from the Western Cape (9.1 copies μL^{-1} of DNA) (Figure 5.1).

When comparing the highest colonization of *B. subtilis* T29 on the different cultivars (inoculated versus uninoculated), SST0117 cv had the highest percentage increase (160%) against the unicoculated SST0117 cv. Followed by Kariega cv showing a percentage increase of 120,4%, SST056 cv with a percentage increase of 92, 2% and Eland with a percentage increase of 61,9%. The lowest percentage increase in *B. subtilis* colonization was detected on SST88 cv, while Duzi cv had no colonization increase of *B. subtilis* (-0,91%) (Figure 5.3).



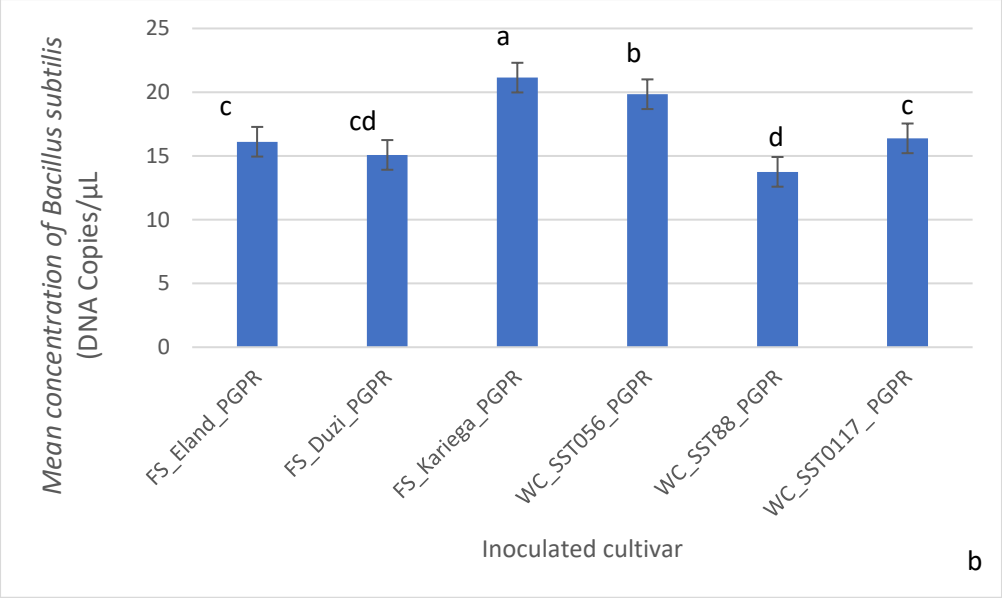
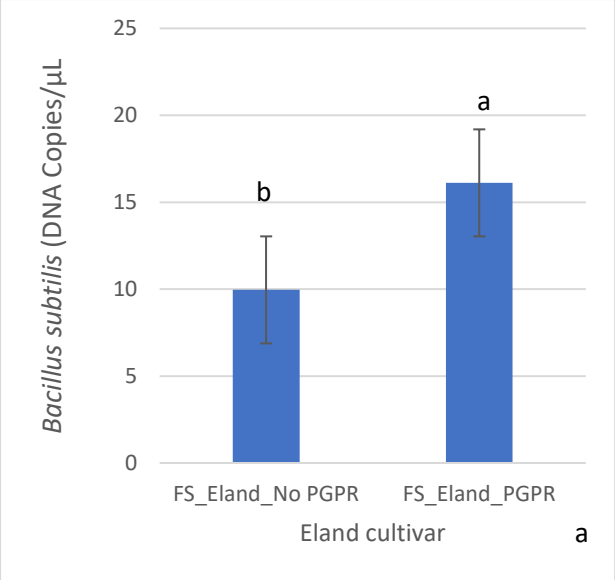
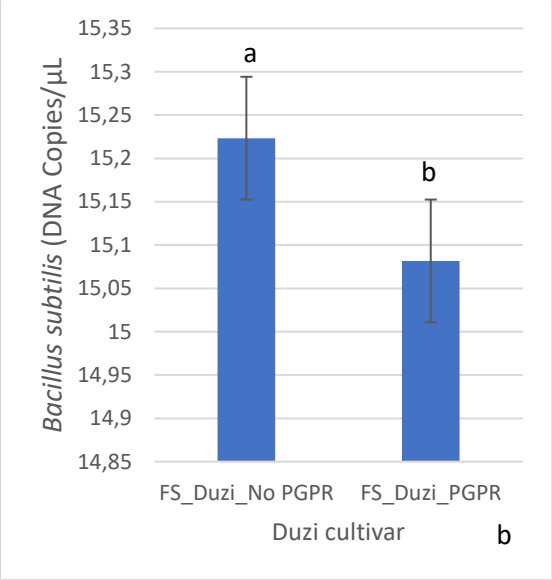
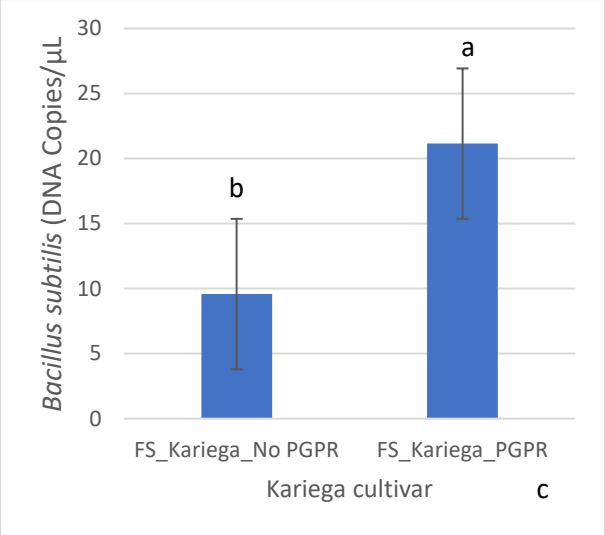
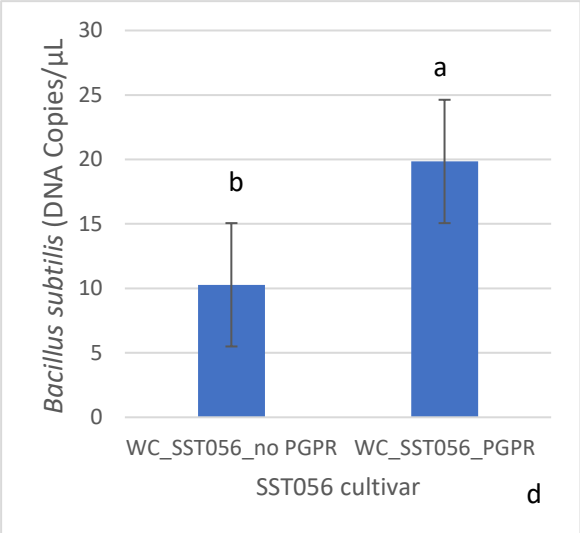


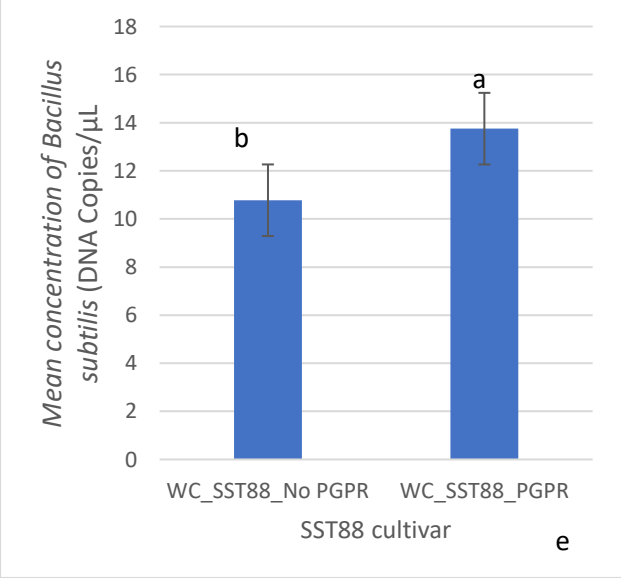
Figure 5.2: Mean concentration of *Bacillus subtilis* T29 on the roots of different wheat cultivars, a – *Bacillus subtilis* T29 inoculated cultivars and, b – uninoculated cultivars. Error bars show standard error.


Bacillus subtilis (DNA Copies/µL
25
20
15
10
5
0
b
a
FS_Eland_No PGPR
FS_Eland_PGPR
Eland cultivar
a


Bacillus subtilis (DNA Copies/µL
15,35
15,3
15,25
15,2
15,15
15,1
15,05
15
14,95
14,9
14,85
a
b
FS_Duzi_No PGPR
FS_Duzi_PGPR
Duzi cultivar
b


Bacillus subtilis (DNA Copies/µL
30
25
20
15
10
5
0
b
a
FS_Kariega_No PGPR
FS_Kariega_PGPR
Kariega cultivar
c


Bacillus subtilis (DNA Copies/µL
30
25
20
15
10
5
0
b
a
WC_SST056_no PGPR
WC_SST056_PGPR
SST056 cultivar
d


Mean concentration of Bacillus subtilis (DNA Copies/µL
18
16
14
12
10
8
6
4
2
0
b
a
WC_SST88_No PGPR
WC_SST88_PGPR
SST88 cultivar
e

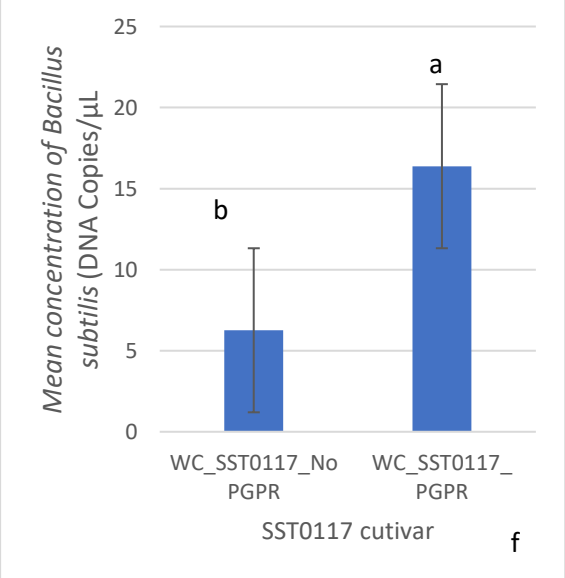

Mean concentration of Bacillus subtilis (DNA Copies/µL
25
20
15
10
5
0
b
a
WC_SST0117_No PGPR
WC_SST0117_ PGPR
SST0117 cutivar
f

Figure 5.3: Mean concentration of *Bacillus subtilis* T29 on the roots of different wheat cultivars, a – Eland, b – Duzi, c – Kariega, d – SST056, e- SST88 and f – SST0117. Error bars show standard error.

To determine whether *B. subtilis* T29 is able to promote plant growth, seeds of different wheat cultivars were treated and planted in field soil. Based on the phenotypes of the wheat crop, *B. subtilis* T29 treatment notably improved growth. Furthermore, dry mass of roots, shoots and whole plants treated with *B. subtilis* T29 were higher than these parameter for the untreated cultivars. Eland cultivar (cv) had a significant percentage increase (50%) in total dry mass when comparing treated versus untreated Eland cv compared to the other cultivars. On Duzi cv, the application of *B. subtilis* T29 had no significant effect on the dry mass of the plant, in fact, the treated plants showed a decrease in total dry mass as compared to the untreated plants. SST056 cv, SST88 cv, SST88 cv and Kariega cv showed a slight increase of 14,5%, 14,6%, 11,1% and 10% respectively as compared to their untreated controls (Table 5.1). *Bacillus subtlis* T29 concentrations was negatively correlated with the total dry weight and root weight and positively correlated with shoot weight for the aforementioned cultivars (Table 5.2).

Table 5.1: Effect of *Bacillus subtilis* T29 on the dry mass of wheat.

	Seedling mass in grams			**% change in mass**		
				Mean dry mass		
Treatment	**Dry_Root**	**Dry_Shoot**	**Dry_Total**	**Root**	**Shoot**	**Total**
FS_Eland_PGPR	0.25a	2.16a	2.41a	92	46.9	50.6
FS_Eland_No PGPR	0.13b	1.47b	1.6b			

FS_Duzi_PGPR	0.05a	1.07ab	1.12ab	-44.4	-10.1	-12.5
FS_Duzi_No PGPR	0.09a	1.19a	1.28a			
FS_Kariega_PGPR	0.07a	1.43a	1.5a	-22.2	13.49	10
FS_Kariega_No PGPR	0.09a	1.26b	1.35b			
WC_SST056_PGPR	0.21a	1.84a	2.05a	50	11.5	14.5
WC_SST056_no PGPR	0.14ab	1.65b	1.79b			
WC_SST88_PGPR	0.71a	1.59b	2.3a	491	-18.4	11.1
WC_SST88_No PGPR	0.12b	1.95a	2.07ab			
WC_SST0117_ PGPR	0.11a	1.85a	1.96a	37.5	13.5	14.6
WC_SST0117_No PGPR	0.08ab	1.63b	1.71b			

Different letters above error bars indicate significant differences in mean values ($p<0.05$) within columns.

Table 5.2: Correlation analysis between *Bacillus subtilis* T29 concentration in rhizosphere soil and wheat growth.

Factor	Correlation coefficient	Probability value
Total dry weight	-0.37*****	0.47
Shoot weight	0.07	0.9
Root weight	-0.53*****	0.27

*****correlation coefficients for factors that had a negative correlation with *Bacillus subtilis* T29 concentration in rhizospheric soil.

5.4. Discussion

Quantification of root colonization by plant growth promoting rhizobacteria (PGPR) is an important part of tracking strains in the field and provides valuable information concerning the persistence of these microbes in the soil. Results from such studies can lead to the discovery of potential plant growth promoting rhizobacteria. The survival and colonization of PGPR in the plant rhizosphere is the basis for plant growth promotion (Compant et al., 2010). However, in many cases, PGPRs do not achieve the desired effect because of insufficient numbers in the rhizosphere or failure of rhizosphere or plant colonization (Lugtenberg et al., 2001).

The choice of cultivar used in the current study for each region soil was influenced by what commercial farmers plant in each region. *Bacillus subtilis* was detected in both inoculated and uninoculated rhizosphere samples. Additionally, a trend of increased concentration of *B. subtilis* in the rhizosphere soil after inoculation with *B. subtilis* T29 was observed, indicating that *B. subtilis* T29 successfully colonized the plant root system. The presence of *B. subtilis* T29 in all samples confirms the ubiquity of the bacteria. Saxena et al. (2020) reported that *Bacillus* is one of the predominant bacterial genera found in soil. This explains why *B. subtilis* was also detected in uninoculated rhizosphere samples. Furthermore, it was reported that spore-producing bacteria of *Bacillus* genus successfully colonized the wheat rhizosphere, when applied at 10^4-10^6 CFU per seed (Kuzmina and Melentiev, 2003). Upon successful colonization, the colonization of *B. subtilis* T29 on each cultivar's roots differed per sample after a period of three months in the current study, with the inoculated samples showing a significantly higher colonization level of the bacterium, with an exception of Duzi cv.

This study further revealed that colonization efficacy of *B. subtilis* was influenced by wheat cultivar. For example, the Kariega cultivar (cv) had the highest *B. subtilis* T29 colonization levels, while the lowest colonization levels were observed for SST88 cv. The influence of cultivar on microbial colonization of plant roots has been documented in previous studies (Canarini et al., 2019; Habibi et al., 2019). Many biotic and abiotic factors influence the colonization of plants by different or specific PGPR, with cultivar being one of the major factors (Habibi et al., 2019). In a study by Canarini et al. (2019), microbial colonization of plant roots was influenced by root exudates. This is because the root exudates play a key role in the selective stimulation of surrounding microorganisms (Canarini et al., 2019). The metabolic activity of the microorganisms is maintained at a high-level owing to the continuous release of root exudates by the plant root (Chiarini et al., 1998). Host specificity may be another factor influencing the colonization of PGPR to diverse crops (Canarini et al., 2019). Elbeltagy et al. (2001) demonstrated that an endophytic bacterium (*Herbaspirillum* sp. strain B501) isolated from the stems of wild rice did not colonize cultivated rice after inoculation under aseptic conditions. This may be the case in our current study, where *B. subtilis* T29 was grown and inoculated under aseptic conditions. Additionally, its growth promotion efficacy was lower when compared to our previous studies (Makgolane, 2016).

Moreover, the success in plant growth promotion of crops has been found to greatly vary between plant species with growth promotion depending on the growth system (*in vitro* culture, greenhouse, or field), substrates, plant health and soil pH, type, and moisture content (Luisa et al., 2018). In addition, growth promotion is related to the ability of these bacteria to germinate and colonize plant roots (De Souzaet et al., 2015; Kloepper et al., 1980; Ugoji et al., 2006; Luisa et al., 2018). In the current study, the

growth promotion efficacy of *B. subtilis* T29 varied with wheat cultivar. The highest percentage increase in total dry mass was observed from Eland cv treated with *B. subtilis* T29. SST88 cv, SST056 cv, SST0117 cv and Kariega showed an increased percentage in total dry mass, although the increase was not significant. However, no increase in total dry mass was observed from Duzi cv treated with *B. subtilis* T29. These results are consistent with those reported by Luisa et al. (2018), who indicated that total dry weight and shoot length of tomato plants were increased by 10.7% and 69.3% respectively after *B. subtilis* was applied to the soil. A similar pattern of improved plant growth following *Bacillus* inoculation was also reported by Cabra et al. (2017) and Islam et al. (2019). Our findings also agreed with the observation of Alam et al. (2003), who reported of cultivar variation in the stimulation of rice growth inoculated with plant growth-promoting bacteria. Islam et al. (2019) also reported differences in growth promotion of tomato plants between two tomato cultivars in a greenhouse study. Different species or cultivars may produce different types of root exudates, which may support the activity of the inoculums or serve as substrate for the formation of biologically active substances by the inoculums (Khalid et al., 2004). In general, the *B. subtilis* T29 isolate significantly increased root length, shoot length and also exhibited biomass increment in the treated wheat cultivars. This observation is in concordant with the results obtained in earlier studies conducted with the *Bacillus* strains (Almoneafy et al., 2014; Sana et al., 2014), where *Bacillus* inoculation did not result in plant growth promotion. The increase in shoot and root mass in response to *B. subtilis* T29 may also be attributed to the enhanced ability of the rooting system to synthesis phytohormones, increase uptake and availability of nutrients (Figueiredo et al., 2016). Application of *Bacillus* species to seeds or roots has been shown to cause

alteration in the composition of rhizosphere microflora leading to an increase in growth of plants (Vessey and Buss 2002).

The relationship between plant growth promotion and bacterial rhizospheric colonization is frequently reported in the soil science literature (Beauregard, 2015; Gao et al., 2016), but to our knowledge, no study to date, has verified this relationship particularly in wheat production. In this study, a non-correlation of concentration and plant growth was observed. Although Kariega cv had the highest density of *B. subtilis* T29, it's percentage increase in dry plant mass was not significant compared to Eland cv. Eland cv had the highest increase in total dry mass. This might be due to different cultivars and soils being used in the study. This study revealed that higher PGPR populations do not always translate to plant growth. On the other hand, some studies have determined the importance of maintaining a high population of PGPR on roots to obtain good plant growth (Chin-a-Woeng et al., 2003; Gao et al., 2013; Kloepper et al., 1980). Nevertheless, it is possible that inoculated populations of beneficial bacteria activate their mechanisms for growth promotion over a short period, generating changes in plant growth without colonizing the plant.

Colonization studies of PGPR, specifically focussing on with *B. subtilis*, are limited (Luisa et al., 2018). This study was the first to show the capacity of *B. subtilis* T29 to colonize different wheat cultivars in SA and further persist on the roots for a sufficiently long periods of time to enable the effects on plant growth and soil processes. Several factors significantly influenced the quantity of *B*. *subtilis* T29 on wheat roots and its growth promotion efficacy. Significant behaviours between *B. subtilis* T29 DNA yield and wheat cultivar were shown in this study. Kariega cv, SST056 cv, Eland cv SST0117 cv and SST88 cv were associated with a significantly higher average DNA

levels of *B. subtilis* T29 at maturity of wheat compared to the untreated samples. However, the colonization efficacy was not correlated to an increase in plant growth. Proper selection of cultivar can be critical when aiming at obtaining higher levels of *B. subtilis* T29 colonization. Wheat growth promotion by *B. subtilis* T29 was also observed to be influenced by the type of cultivar used. Eland cv had the highest increase in plant mass following treatment with *B. subtilis* T29 compared to the untreated Eland cv, while Duzi cv had no change in plant mass upon *B. subtilis* T29 treatment.

Although some agro-ecological factors were considered in this study, there was no sufficient results to conclude on because not all metrological and ecological factors were accounted for and these could further explain some of the variations observed. Future studies should incorporate factors such as weather conditions (temperature), soil physico-chemical composition, track the changes in microbial diversity on the roots upon PGPR inoculation, study different growth stages and the same cultivars should be considered per region. It is also recommended to conduct the studies in the field. Agro-ecological factors play an important role on the epidemiology of soil microbes, however, the presence of other microbes (beneficial and pathogenic) on the root systems of plants present a challenge in understanding the behaviour of bioinoculants in the field. This calls for urgent attention and require long-term research to ensure more sustainable increase in wheat yields for future food security. This study provides some insight into the colonization of *B. subtilis* T29 in different wheat cultivars and further effect on growth promotion response. In the next chapter, we further explore the dynamics of the rhizosphere soil microbial communities after the intruductions of bio-inoculants of the above PGPR *B. subtilis* T29 and a fungal wheat pathogen, *F. pseudograminearum*.

5.5. References

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Chapter 6

Bacillus subtilis T29 effects on wheat productivity and health under Fusarium pseudograminearum pressure.

Fusarium crown rot (FCR) caused by *Fusarium pseudograminearum* significantly affects the productivity of wheat grain crops. The use of biocontrol agents is an alternative to agrochemicals. However, little is known about the impact of potentially occurring pathogens and the beneficial inoculants on indigenous microorganisms associated with wheat roots. This study aimed at understanding the fungal and bacterial population on wheat rhizosphere after inoculation with *Bacillus subtilis* T29 and *F. pseudograminearum*. Furthermore, the effect of *B. subtilis* T29 on wheat growth and biological control against *F. pseudograminearum* was investigated. The bacterial strain *B. subtilis* T29 displayed a high level of antagonism and reduced the incidence of *Fusarium* crown rot by 39%. The fungal phyla Ascomycota (36.9%), Chytridiomycota (38.1%) and Basidiomycota (9.7%) and bacterial phyla Proteobacteria (34.4%), Acidobacteria (23.6%), Bacteriodetes (7.4%), Acidobacteria (6.9%), Planctomycetes (6.7%) and Firmicutes (5.74%), were the most abundant in all samples. Interestingly, the microbial diversity was enhanced by treatment with both the pathogen, and the co-inoculated biological control agent. The highest diversity was observed in *B. subtilis* T29 + *F. pseudograminearum* inoculation treatment. Certain potential biocontrol species (*Burkholderia cepacian, Azospirillum oryzae and Paenibacillus alvei)* were unique to the *B. subtilis* T29 alone or in combination with *F. pseudograminearum* treatments. This study suggests shift in both bacterial and fungal communities of the rhizosphere at seedling stage upon introduction of *B. subtilis* T29

alone or in combination with *F. pseudograminearum* treatments. Therefore, understanding microbe–microbe dynamics and their effects on the composition of microbial communities is essential to identify microbial determinants that shape microbial communities, with the ultimate goal of achieving good plant health.

Keywords: *Bacillus subtilis*, *Fusarium pseudograminearum*, microbial communities, biocontrol

6.1. Introduction

Crop losses due to plant diseases pose a major threat to food security worldwide. The impact ranges from a modest reduction of plant growth to more serious damages leading to plant death and reduction in yields (Cao et al., 2011; Mazzola and Freilich, 2017). Diseases such as *Fusarium* head blight, root rot and crown rot caused by *Fusarium graminearum*, *Fusarium* culmorum and *Fusarium pseudograminearum* respectively, are some of the diseases that pose significant threats to wheat production and other economically important cereal crops (Powell et al., 2017). Many approaches have been taken to control infestations by these pathogenic organisms. These approaches include the use of resistant varieties, the production of genetically modified resistant plants, and the use of synthetic chemical fungicides (Chakraborty et al., 2006; Moya-Elizondo and Jacobsen, 2016). One major drawback with the use of resistant varieties is that their development takes a long time. On the other hand, fungicides are only effective during the early growth stages of cereals i.e., between the first two to four weeks for wheat (Balmas et al., 2006), the control of *Fusarium* crown rot relies heavily on integrated pest management strategies (Moya-Elizondo and Jacobsen, 2016). Not only that, a limited number of fungicides are effective in

controlling soilborne diseases; most of these are either banned, or in the process of being phased out (Carlson, 2019). In addition, the use of the agrochemicals has been proved to be harmful to beneficial microbiomes inhabiting the soil environment (Pal and Gardener, 2006), and also pose a threat to human health (Nicolopoulou-Stamati et al., 2016). Hence, it is imperative vital to search for alternative plant disease control strategies that are environmentally friendly yet effective.

There is a growing interest in the use of plant growth promoting rhizobacteria (PGPR) as supplements or alternatives to the use of chemical pesticides. Many bacterial genera have been utilized as PGPR, including *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas,* and *Serratia (*Bhattacharyya and Jha, 2012). Among these, members of the rod-shaped, endospore-forming Gram-positive *Bacillus* group are the most commonly commercialized for plant growth and disease control (Borriss, 2011). *Bacillus sp*. are globally known as biological control agents (BCAs) and are being used to control plant pathogens, increase plant immunity and growth under field and greenhouse conditions (Shafi et al., 2017). This is mainly due to their ability to produce volatile compounds such auxins and gibberellins (Tahir, 2017) which aid in plant growth promotion and activation of plant defence mechanisms by triggering the induced systemic resistance (ISR) (Compant et al., 2005). Moreover, species belonging to the *Bacillus* genus, for example, *Bacillus subtilis* have a unique ability to replicate rapidly, resistant to adverse environmental conditions and have a broad biocontrol spectrum (Shafi et al., 2017).

There are several studies that described the use of *Bacillus sp*. as growth promoters and biocontrol agents against *Fusarium* infections in wheat (Grosu et al., 2015, Khan

et al., 2017). Studies carried out under greenhouse conditions showed improved root and shoot growth as well as very low pathogen frequencies in *Bacillus sp*. inoculated plants compared to untreated control plants. Zhao et al. (2014) found that *B. subtilis* strain SG6 exhibited strong antagonism against *F. graminearum* in dual culture plate assays and inhibited sporulation in the pathogen. While the benefits of *Azospirillum*, *Bacillus* and other biocontrol agents inoculations on plants are well supported in literature, further research is required to fully understand how they may persist in the soil post inoculation, which is an important aspect in large scale and commercial crop production contexts.

The interaction between the plant and below ground microbiome is crucial for plant health as plants communicate with their rhizosphere microbiome to control the pathogens (Berendesen et al., 2012). Upon pathogen attack, plants are able to recruit protective microorganisms, and enhance microbial activity to suppress plant pathogens in the rhizosphere (Berendesen et al., 2012). Upon pathogen attack, plants are able to recruit protective microorganisms, and enhance microbial activity to suppress pathogens in the rhizosphere (Berendesen et al., 2012). Most previous studies have focused on the effects of PGPR applications on individual or specific beneficial microbes but neglected their global effect on microbial communities (Almario et al., 2013). Risk assessment and colonization studies for specific biocontrol agents showed minor and only transient effects on the rhizosphere community (Scherwinski et al., 2007; Adesina et al., 2009; Chowdhury et al., 2013; Schmidt et al., 2014). In addition, the impact of pathogen inoculations on the crop's indigenous microbiome is severely underexplored (Berendesen et al., 2012). More so, there is limited studies on the effects of these growth promoting bacteria on agricultural field soils. Therefore, the effect of rhizobacteria treatments and pathogen inoculation on the microbial

community of the wheat rhizosphere is to date poorly understood. Further studies should aim at understanding their interactions with resident microbial communities and how this can be used to further improve disease control. Most previous studies have focused on the effects of PGPR applications on individual or specific beneficial microbes but neglected their global effect on microbial communities (Almario et al., 2013).

The current study aimed at investigating the biocontrol efficacy of *B. subtilis* T29 against *F. pseudograminearum* and its growth promoting ability on wheat. Additionally, the effect of *B. subtilis* T29 on the rhizospheres fungal and bacterial community was further studied by sequencing the 16s rDNA and ITS gene regions using Illumina sequencing. Knowledge on the effects of BCA application on the wheat rhizosphere microbial community composition and disease occurrence could inform future studies on inoculum recommendations and particular rhizobacteria affected upon pathogen infection during wheat cultivation. This will ultimately, better our understanding of the ecological consequences of the use of microbial inoculants and how it impacts the rhizosphere microbiota.

6.2. Materials and Methods

6.2.1. Rhizobacterial inoculum source

The rhizobacterial (*Bacillus subtilis* T29) culture was obtained from the University of Pretoria's plant growth promoting rhizobacteria (UP-PGPR) culture collection. Further details regarding the isolate selection, maintenance and inoculum preparation are shown in Appendix 1.

6.2.2. Rhizobacterial inoculum preparation and seed inoculation

Wheat seeds (Eland cv.) obtained from a commercial wheat farm from the Free State Province, South Africa were used for this trial. The seeds were surface sanitized with 70% ethanol for 5 min followed by immersion in 1% sodium hypochlorite for 1 min and rinsed five times with sterile distilled water. Inoculation of the wheat seeds with rhizobacterial isolate (*Bacillus subtilis* T29) was done using a method adapted from Idris et al. (2007). Rhizobacterial suspensions were added to the seeds placed in sterile petri dishes. The seeds were incubated in the laminar flow at room temperature for a period of 1 hour to allow attachment of the rhizobacteria to the seed coat. Seeds treated with sterile distilled water served as the untreated control treatment.

6.2.3. Fungal inoculum preparation

Fusarium pseudograminearum M7816N obtained from the Agriculture Research Council (ARC), Stellenbosch, South Africa (Dr S. Lamprecht (Private Bag X 5017, Stellenbosch, 7599) was used in this study. The fungal cultures were received in sterile McCartney bottles on potato dextrose agar (PDA) (Biolab, Wadeville, South Africa) slants. The inoculum was prepared by cutting a 5mm x 5mm block from the actively growing fungal culture on the agar slant and placing it at the centre of a PDA plate containing half strength PDA (½ PDA). The culture was incubated for five days at 25 °C after which sub-cultures were prepared by cutting a 5mm x 5mm blocks from the edge of the fungal culture and placing them on half PDA plates.

Conidial suspensions were produced in a mung bean liquor medium according to Bai and Shaner (1996). To prepare this medium, 40 g of mung beans was placed in a 1 L

Erlenmeyer flask containing 1 L of distilled water and autoclaved at 121 °C for 15 min. The mung bean suspension was thereafter filtered through a sterile cheese cloth. The mung bean filtrate was autoclaved twice, inoculated with a 2 mm^2 disk from a pure *F. pseudograminearum* culture, and placed on a shake incubator at 25 °C for 5 days. After the incubation period, the liquid cultures were stored in a refrigerator (4°C). For inoculation, the spore suspension was prepared by carefully pipetting and avoiding clumps, to a sterile flask and adding sterile distilled water to adjust the inoculum concentration. Conidia were counted under a microscope using a haemocytometer to 1 x 10^5.

6.2.4. Soil collection

Field soil was collected from Richmond farm in Bethlehem in the Free State Province. Soil was collected in the wheat field between rows, approximately 40 cm away from seedlings in a statistical designed classical W-shaped pattern. A clean spade was used, the soil collected was at least 10 cm deep from the top. Soil was placed inside marked zipper bags and transported inside boxes to the greenhouse at the University of Pretoria. Soil was homogeneously mixed before the trial.

6.2.5. Greenhouse trial design

Treated and untreated (sterile distilled water) seeds were planted in soil collected from a commercial wheat farm in SA. Sterilized plastic pots (10 cm in diameter) were used, and two seeds were planted per pot. The pots were watered with municipal tap water every second day. Ten days after planting, seedlings were inoculated with *F.*

pseudograminearum spore suspension by pipetting 1 ml of the inoculum and introducing it to the root base of each plant. Treatments included: 1) Uninoculated control plants; 2) Plants inoculated with *F. pseudograminearum* suspension; 3) Plants inoculated with *B. subtilis* T29 suspension; 4) Plants inoculated with both *B. subtilis* T29 and *F. pseudograminearum*. The treatments were arranged in a complete randomized block design and the pots were kept on benche in the greenhouse. Each treatment was comprised of 10 replicate pots and each replicate included two seedlings per pot. The experiment was repeated twice. Four weeks after *F. pseudograminearum* application, plants were harvested. Excess soil was shaken off the roots whereas soil attached to the roots was stored in Eppendorf tubes for further microbial population studies. Roots were rinsed with tap water and their symptoms recorded. The roots were excised from the foliage and their mass was determined. Thereafter, the plants were dried in a 50 °C oven for 48 hours and the dry mass was recorded.

6.2.6. Disease symptom assessment

Li-rating: Disease severity was assessed at harvest according to the Wildermuth and McNamara '0-5 scale' as modified by Li et al. (2008), where 0 = no obvious symptoms; 1 = visible necrotic lesion on coleoptile or 1st leaf sheath; 2 = the 1st leaf sheath and below sub-crown internode partially necrotic; 3 = the 2nd leaf sheath and the below sub-crown internode completely necrotic with up to 50% reduction in seedling height; 4 = the 3rd leaf or leaf sheath and the below sub-crown internode partially or completely necrotic with more than 50% reduction of seedling height; 5 = whole plant severely to completely necrotic.

6.2.7. Analysis of soil chemical properties

The soil chemical properties were analyzed according to Mehlich (1972) and Bray et al. (1945) with minor modifications. Briefly, the soil pH was determined in a soil suspension with deionized water (1:10 w/v), while soil organic carbon was analyzed by titration of wet oxidation in concentrated H_2SO_4 and 2 M $K_2Cr_2O_7$. Available soil phosphorus (AP) was determined using a flow analyzer and finally, potassium (K), calcium (Ca), magnesium (Mg) and sodium (Na) were determined by flame emission and atomic absorption spectroscopy (Bray and Kurtz, 1945; and Mehlich, 1972).

6.2.8. DNA extraction and sequencing

The rhizosphere soil retrieved from above was used. Total community DNA was extracted from 0.25 g fresh soil using the MoBio PowerSoil™ DNA Isolation Kits (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA concentration in each sample was determined using Nanodrop ND-2000 UV–VIS Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) prior to further analysis. (Pooling DNA).

Sequencing was done using the following primers: 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 909R (5'-CCCCGYCAATTCMTTTRAGT-3') targeting the bacterial V4-V5 region in the 16S rRNA gene region (Gu et al., 2017) and ITS1 (TCCGTAGGTGAACCTGCGG) and ITS2 (GCTGCGTTCTTCATCGATGC) (White et al., 1990) targeting the fungal ITS region. Sequencing was performed at Molecular Research DNA (MR DNA) (www.mrdnalab.com, Shallowater, TX, USA) on

a MiSeq following the manufacturer's guidelines. Raw sequence data are available in fastq format on NCBI-SRA under the BioProject accession number: MN128547.1.

6.2.9. Data analysis and taxa classification

Sequence data were pre-processed using MR DNA analysis pipeline (MR DNA, Shallowater). The sequences from the Illumina were joined and depleted of bar codes. Sequences with ambiguous base calls and those <150 bp (Abdelfattah et al., 2016) were discarded. Noises, singletons and chimeras were removed from sequences. Generated operational taxonomy units (OTUs) were defined by clustering at a similarity threshold of 97% using the Usearch algorithm (Diskin et al., 2019). Final OTUs were taxonomically classified using BLASTn against a curated database derived from RDPII to NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu) and compiled into each taxonomic level. Non-rarefied sequences were used for α-diversity and compositional analysis, while rarefied sequences (at a depth of 1000 sequences) were used to determine β-diversity. The α-diveristy of the fungal and bacterial communities were calculated using the MicrobiomeAnalyst online pipeline (http://microbiomeanalyst.ca/) (Govender et al., 2006; Galsurker et al., 2020).

To determine uniqueness of detected OTUs between cultivars and sites (non-rhizosphere and rhizosphere soil), Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html) was used.

6.2.11. Statistical analysis

General Linear Models procedure of Statistical Analysis Systems (SAS) version 9.4 (Institute Inc., Carry NC, USA) was used to determine differences in plant growth and soil chemical parameters. The means were compared using Tukey's LSD test at a significance level of $P < 0.05$. Redundancy analysis (RDA) was performed with Canoco (version 4.5 for Windows; Ithaca, NY, United States) to determine which environmental variables best explained the assemblage's variability. The ordination in the x- and y-axes and the length of the corresponding arrows indicated the importance of each physicochemical factor in explaining the taxon distribution across communities. Species abundance of selected fungi and bacteria were analysed for significant difference. The normality of the data was checked by means of Shapiro-Wilk's test before comparing species abundance in soil treated with *B. subtilis* T29 and *F. pseudograminearum*. When data met the assumption of normality, ANOVA followed by Tukey's HSD post-hoc for pairwise comparison of the different treatments was conducted. If data was not normally distributed, then Kruskal-Wallis test was applied for the individual treatments followed by pairwise comparisons using Wilcoxon rank sum test. Test results with $P < 0.05$ were considered statically significant.

6.3. Results

6.3.1. Wheat growth promotion and biocontrol of *Fusarium pseudograminearum* by *Bacillus subtilis* T29

The effect of *Bacillus subtilis* T29 on wheat growth and biocontrol of *Fusarium pseudograminearum* indicated that the untreated control plants were healthy and

showed no signs of *F. pseudograminearum* infection (Figure 6.1a and 6.2). *Fusarium pseudograminearum* had a significant ($P < 0.05$) negative effect on the total mass of wheat seedlings and it reduced the plant fresh and dry mass by 29 and 25% respectively, compared to the untreated control (Table 1). The sub-crown area of *F. pseudograminearum* treated plants were completely necrotic and a reduction in plant height was noticed in some wheat seedlings. No significant differences were observed on root masses between both the *B. subtilis* T29 and *F. pseudograminearum* inoculation treatments when compared to the untreated control plants. The *B. subtilis* T29 + *F. pseudograminearum* treatment improved dry plant mass significantly by 38% when compared to *F. pseudograminearum* treatment. When *F. pseudograminearum* inoculation treatment was compared to the *F. pseudograminearum* + *B. subtilis* T29 treatment, it is evident that the presence of *B. subtilis* T29 in the combination treatment reduced the negative effect of *F. pseudograminearum*, based on the plant mass.

Figure 6.1: a) Biocontrol activity of *Bacillus subtilis* T29 against *Fusarium pseudograminearum* M7816N (1- Plants inoculated with *Bacillus subtilis* T29 + *Fusarium pseudograminearum*, 2- Plants inoculated with *Bacillus subtilis* T29, 3-

Uninoculated control plants and 4- Plants inoculated with *Fusarium pseudograminearum*, b) Wheat growth promotion by *Bacillus subtilis* in a wheat greenhouse trial (1- Plants inoculated with *Bacillus subtilis* T29 and 2- Uninoculated control plants.

Table 6.1: Biocontrol of *Fusarium pseudograminearum* by *Bacillus subtilis* T29 on wheat.

	Seedling mass in grams							% Change in mass					
		Mean fresh mass			Mean dry mass			Mean fresh mass			Mean dry mass		
Treatment	**Severity**	**Root**	**Shoot**	**Total**	**Root**	**Shoot**	**Total**	**Root**	**Shoot**	**Total**	**Root**	**Shoot**	**Total**
Untreated control	0.22c	1.01a	1.19b	2.20a	0.22a	0.33b	0.56ab	50.60	15.53	29.33	48.34	14.38	25.96
Fusarium	3.67a	0.67c	1.03c	1.70d	0.15b	0.29bc	0.44b	0	0	0	0	0	0
T29 + *Fusarium*	2.22b	0.67c	1.45a	2.12c	0.20ab	0.41a	0.61a	0.75	40.39	24.79	35.10	39.73	38.15
T29	0.19d	0.91b	1.48a	2.39a	0.20ab	0.37ab	0.58ab	35.8	43.6	40.5	33.3	27.5	31.8

In each column, means followed by the same letter are not significantly different according to Fisher"s LSD test at P = 0.05. Percentage change in mass= [(treatment-disease control)/disease control x 100]. Values are means of 10 replicate pots containing 2 plants each. Therefore, negative values are treatments that are less than the disease control and positive values are treatments with a higher mass than the disease control.

Figure 6.2: *Fusarium* crown rot symptoms in wheat seedlings (crown idicated by red arrows). A- Plants inoculated with *Fusarium pseudograminearum*, B - Plants inoculated with *Bacillus subtilis* T29, C- Plants inoculated with *Bacillus subtilis* T29 and *Fusarium psuedograminearum* and D- Uninoculated control plants.

6.3.2. Exogenous application of *B. subtilis* T 29 and *F. pseudograminearum* on the wheat rhizosphere microbiomes

Microbial diversity

A total of 1 256 437 ITS and 1 529 573 16S high quality sequences were recovered from 12 wheat rhizosphere soil samples, after paired-end alignments, quality filtering, and deletion of chimeric sequences and singletons. The sequences ranged from 42 235 to 155 863 per samples and all were assigned to 3 161 fungal OTUs at 97% similarity. A total of 3 161 fungal OTUs were subjected to the classification analysis. The fungal OTUs varied between the treatments. The highest (1 195) fungal OTUs were recorded in the *B. subtilis* T29 + *F. pseudograminearum* treated rhizosphere soils, while the lowest (1 247) being from the *B. subtilis* T 29 treatment. Bacterial sequences ranged from 43 948 to 103 304 per sample and were assigned to 11 048 OTUs at 97% similarity (Figure 6.3). A total of 11048 bacterial OTUs were subjected to the classification analysis and these OTUs varied between the different treatments. The highest (5 579) number of bacterial OTUs was recorded in the *F. pseudograminearum* treated rhizosphere soils with the lowest (4 388) being recorded in the *B. subtilis* T29 treated rhizosphere soils.

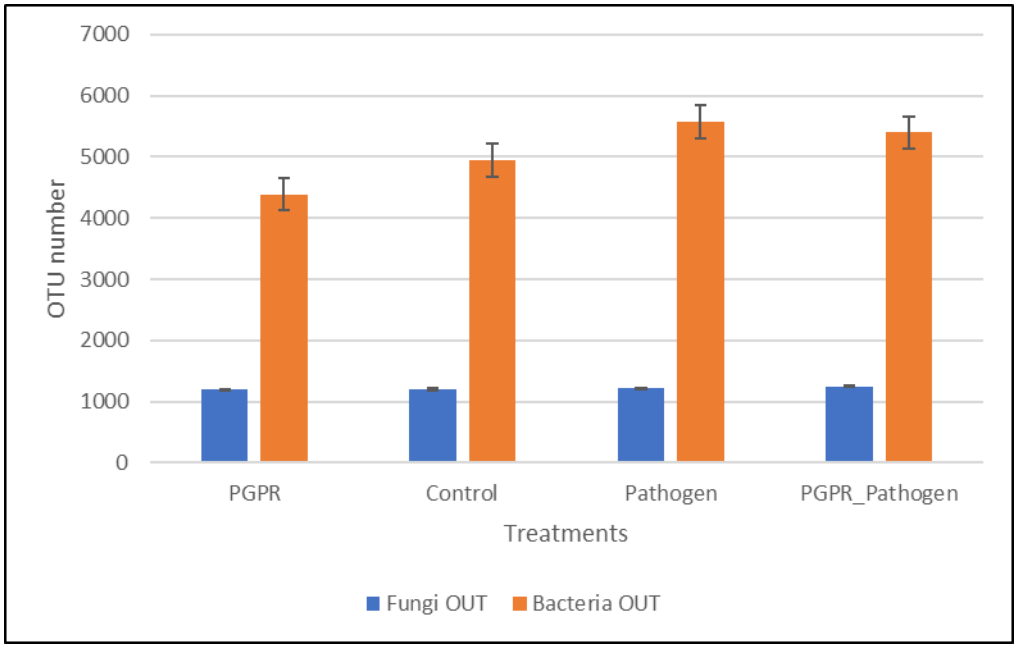


Figure 6.3: The distribution of operational taxonomical units (OTUs) found in wheat rhizosphere soil with different treatment of *Bacillus subtilis* T29 and *Fusarium pseudograminearum*.

The wheat rhizosphere microbiome analysis between the inoculation treatments using the best-hit revealed the distribution and sharing of the same OTUs of fungi and bacteria in rhizosphere soil (Figure 6.3). Several unique taxa were detected exclusively in each treatment, as indicated by the Venn diagrams (Figure 6.4a). The *B*. *subtilis* T29 treated rhizosphere soil recorded the highest (8, 2%) number of unique fungal OTUs compared to the untreated control, *F. pseudograminearum* treated and *B. subtilis* T29 + *F. pseudograminearum* treated rhizosphere soils. Meanwhile, the *F. pseudograminearum* treated rhizosphere soil showed the least (5.1%) number of unique fungal OTUs. A total of 363 (43.7%) fungal OTUs were shared between all the treatments. The *F. pseudograminearum* soils shared about 4% OTUs with both the untreated control and *B. subtilis* T29 + *F. pseudograminearum* treated rhizosphere

soils. The least (1.8%) number of shared OTUs were observed between the untreated control and *B. subtilis* T29 treated rhizosphere soils (Figure 6.4a), showed that the fungal communities in these two treatments are not as similar as in other treatments. Meanwhile, fungal communities between *B. subtilis* T29 and *B. subtilis* T29 + *F. pseudograminearum* treated rhizosphere soils were more similar compared to the other treatments with a total of 40 shared OTUs. The fungal communities of the untreated control and *F. pseudograminearum* treated soil were the second most similar with a total of 33 (4%) shared OTUs, followed by *B. subtilis* T29 + *F. pseudograminearum* and *F. pseudograminearum* treated rhizosphere soil with 32 (3.9%) shared OTUs. At family level, it was interesting to see that there were no common fungal families shared between *B. subtilis* T29 and untreated control, while five families (Amylocorticiaceae, Geastraceae, Pleurotaceae, Erythrobasidiaceae and Pacisporaceae) were shared between untreated control and the *F. pseudograminearum* treatment. Furthermore, four fungal families (Cyphellaceae, Pluteaceae, Venturiaceae, Hysteriaceae) were shared between *B. subtilis* T29 and *F. pseudograminearum* treated samples. There were six (Geoglossaceae, Uropyxidaceae, Aureobasidiaceae, Backusellaceae, Erysiphaceae, Discinaceae) shared fungal families between *B. subtilis* T29 and *B. subtilis* T29 + *F. pseudograminearum* treatments.

In contrast to the observations made in fungal populations, the *F. pseudograminearum* treated rhizosphere soils had the highest (7%) number of unique bacterial OTUs, while the *B. subtilis* T 29 treated rhizosphere soils had the lowest (3%) number of unique OTUs. Fifty-five percent of the bacterial OTUs were shared among all the treatments. The untreated control and *B. subtilis* T29 treated rhizosphere soils shared the least (0.7%) number of bacterial OTUs, while the *B. subtilis* T29 treated and *B. subtilis* T29

+ *F. pseudograminearum* treated rhizosphere soil had the most similar OTUs compared to all the other treatments (Figure 6.4b). At family level, there were no common bacterial families shared between *B. subtilis* T29 and untreated control, and *B. subtilis* T29 and *F. pseudograminearum*. Five bacterial families (Clostridialesfamilyxiii.incertaesedis, Vulgatibacteraceae, Acidithiobacillaceae, Desulfohalobiaceae and Bogoriellaceae) were shared between *B. subtilis* T29 and *B. subtilis* T29 + *F. pseudograminearum*. We also observed some bacterial species of beneficial interest to wheat production being unique to certain treatments i.e. *Burkholderia cepacian* and *Azospirillum oryzae* were only identified in untreated control samples while *Paenibacillus alvei* and *Paenibacillus telluris*, were unique to the *F. pseudograminearum* treated rhizosphere soils.

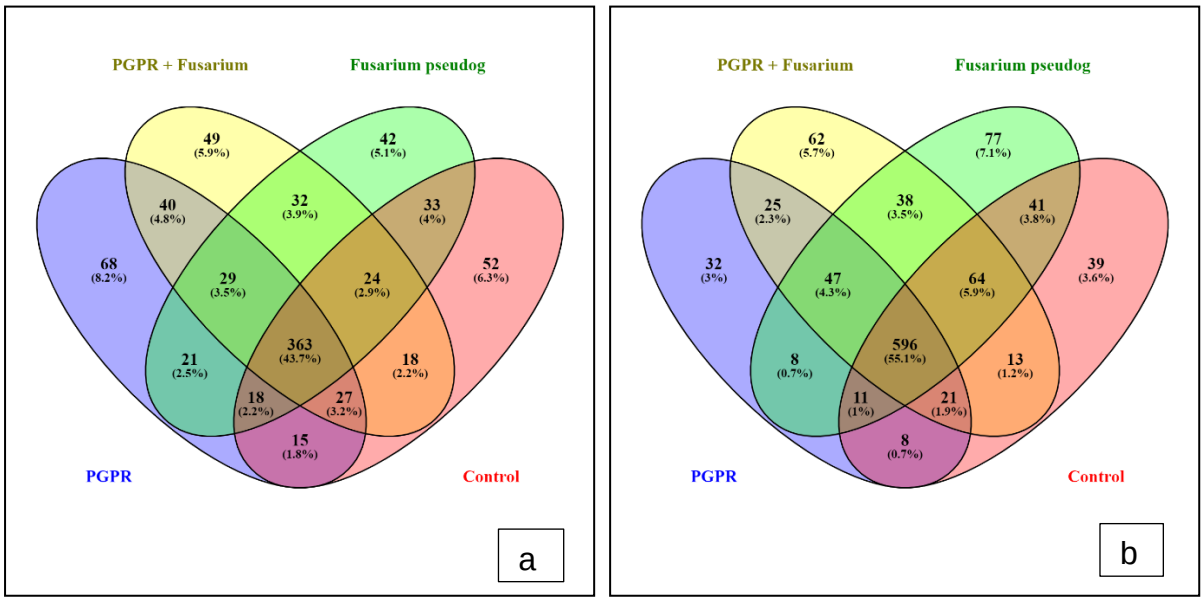


Figure 6.4: Venn diagram displaying the distribution of unique and shared, fungal (a) and bacterial (b) OTUs on wheat rhizosphere soil treated with PGPR (*Bacillus subtilis T29*) and/or *Fusarium pseudograminearum*.

The species diversity and richness of the untreated and treated rhizosphere soils is shown in Figure 6.5 and 6.6 The t-test revealed significant differences (P = 0.012; [ANOVA] F = 7.05) on the fungal diversity (Shannon index) between the untreated and treated rhizosphere soils. Similar observations were made with bacterial diversity with both the *B. subtilis* T29 and *F. pseudograminearum* treatments increasing the fungal diversity by 79% and 77%, respectively when compared to the untreated control. The *B. subtilis* T29 + *F. pseudograminearum* treated rhizosphere soils recorded the highest fungal diversity (5.617, Shannon index), which was about 85% higher compared to the untreated control soil. The untreated wheat rhizosphere soils had the lowest fungal diversity and richness (3.036, Shannon index and 626.86, Chao index; respectively). The observed fungal species richness was highest (484.33, Chao index) in *B. subtilis* T29 + *F. pseudograminearum* treated rhizosphere soils and was approximately 2.4% more than that of the untreated control rhizosphere soils (Figures 6.5).

Similar observations were made in the bacterial community diversity and richness. The t-test revealed significant differences (P = 0.036; [ANOVA] F = 4.63) on the bacterial diversity and richness (Shanon and Chao indices, respectively) in the rhizosphere between treatments. The species diversity (6.917, Shannon index) and richness (2122.21, Chao index) were higher in *B. subtilis* T29 + *F. pseudograminearum* treated soils while the untreated control rhizosphere had the lowest bacterial diversity (6.674, Shannon index) and richness (2081.99, Chao index). Similar to the fungal communities, the observed bacterial species richness was highest (2122.21, Chao index) in *B. subtilis* T29 + *F. pseudograminearum* treated rhizosphere soils while the *B. subtilis* T29 treated rhizosphere soils had the lowest species richness (2064.54) (Figure 6.6).

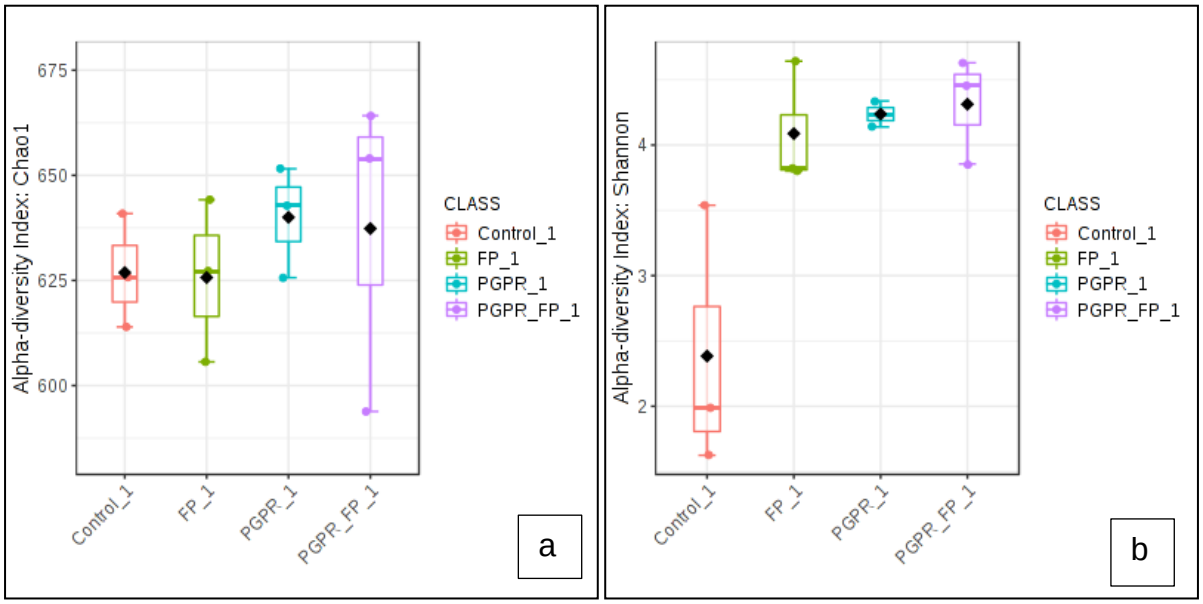


Figure 6.5: Alpha diversity of the estimated OTU richness (Chao1 (a)) and diversity (Shannon (b)) indexes of the ITS region clustering at 97% identity for wheat rhizosphere soil treated with PGPR (*Bacillus subtilis* T29) and/or *Fusarium pseudograminearum* (FP).

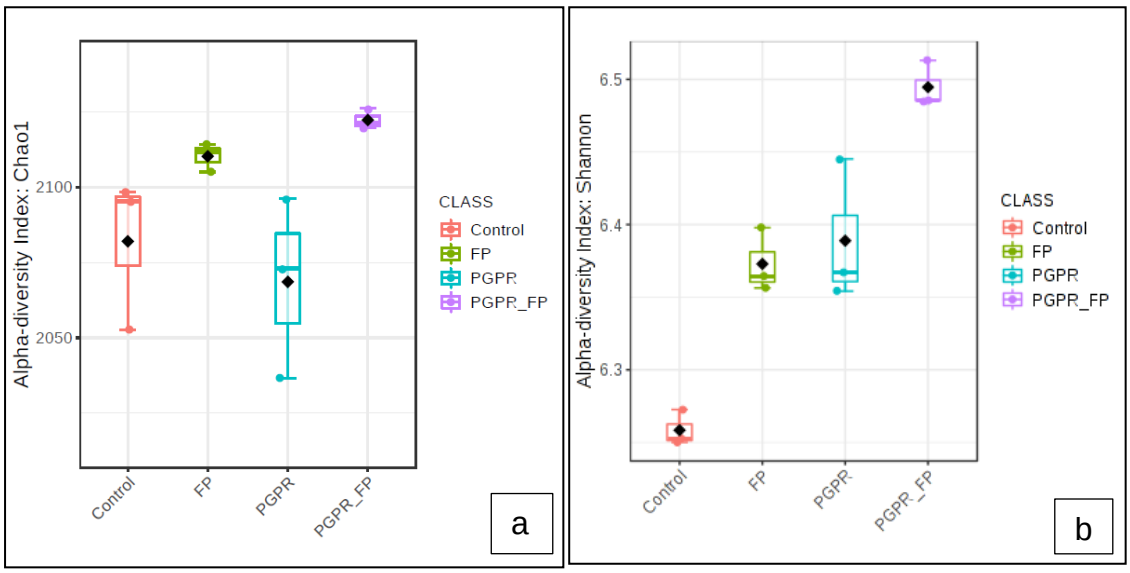
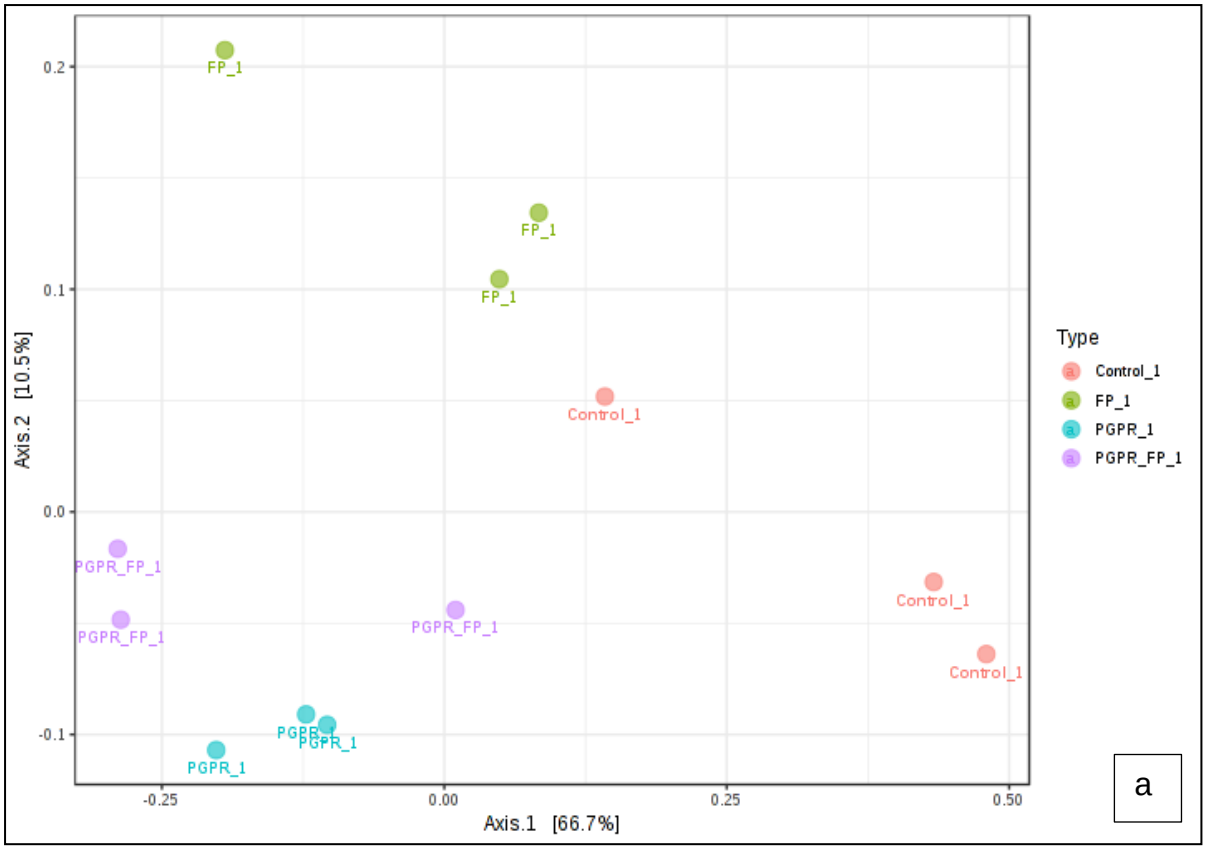


Figure 6.6: Alpha diversity of the estimated OTU richness (Chao1 (a)) and diversity (Shannon (b)) indexes of the 16S rRNA gene libraries for clustering at 97% identity for

wheat rhizosphere soil treated with PGPR (*Bacillus subtilis* T29) and/or *Fusarium pseudograminearum* (FP).

6.3.3. Microbial community structure

The effect of *B*. *subtilis* T29 and *F*. *pseudograminearum* inoculation treatments on relative abundances of observed OTUs showed significant differences with a PERMANOVA test statistic for the different treatments' abundances of the OTUs. Based on Bray-Curtis dissimilarity analysis, the beta diversity of the fungal communities between three treatments showed significant difference ($P < 0.001$). About 77.2% of the observed variation could be explained by the first two principal coordinates (Axis 1 = 66.7% and Axis 2 = 10.5%) (Figure 6.7a). The fungal OTUs clustered together according to their different treatments in principal component analysis. As expected, fungal communities from the *B. subtilis* T29 treated rhizosphere soils were more similar to those of the *B. subtilis* T29 + *F. pseudograminearum*. The beta diversity of bacterial communities between three inoculation treatments was also significantly different ($P < 0 .001$), with 69.9% (Axis 1= 56.6% and Axis 2 = 13.3%) of the sequences explaining the observed variation. Similar to fungal communities, the bacterial community in the *B. subtilis* T29 treated rhizosphere soils was more similar to that of the *B. subtilis* T29 + *F. pseudograminearum* treated rhizosphere soils when compared to other treatments. On the other hand, *F. pseudograminearum* treated rhizosphere soils showed more variation compared to the other treatments.



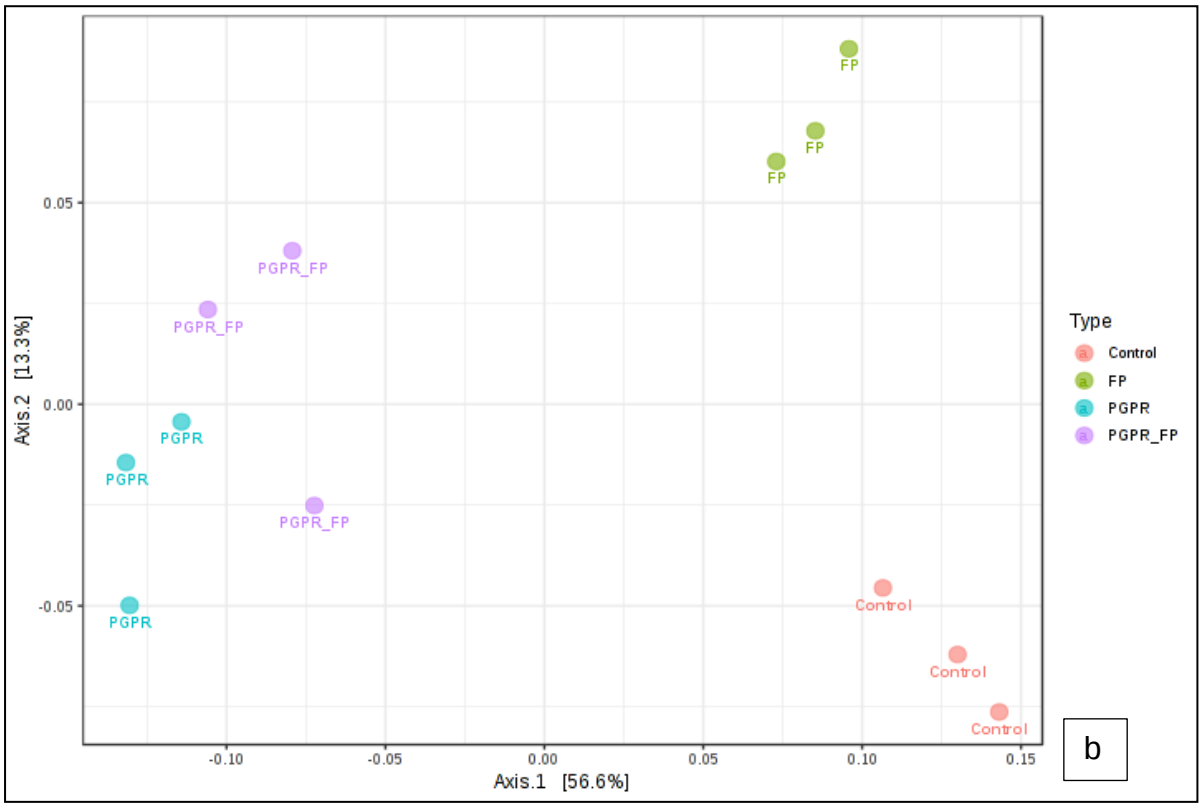
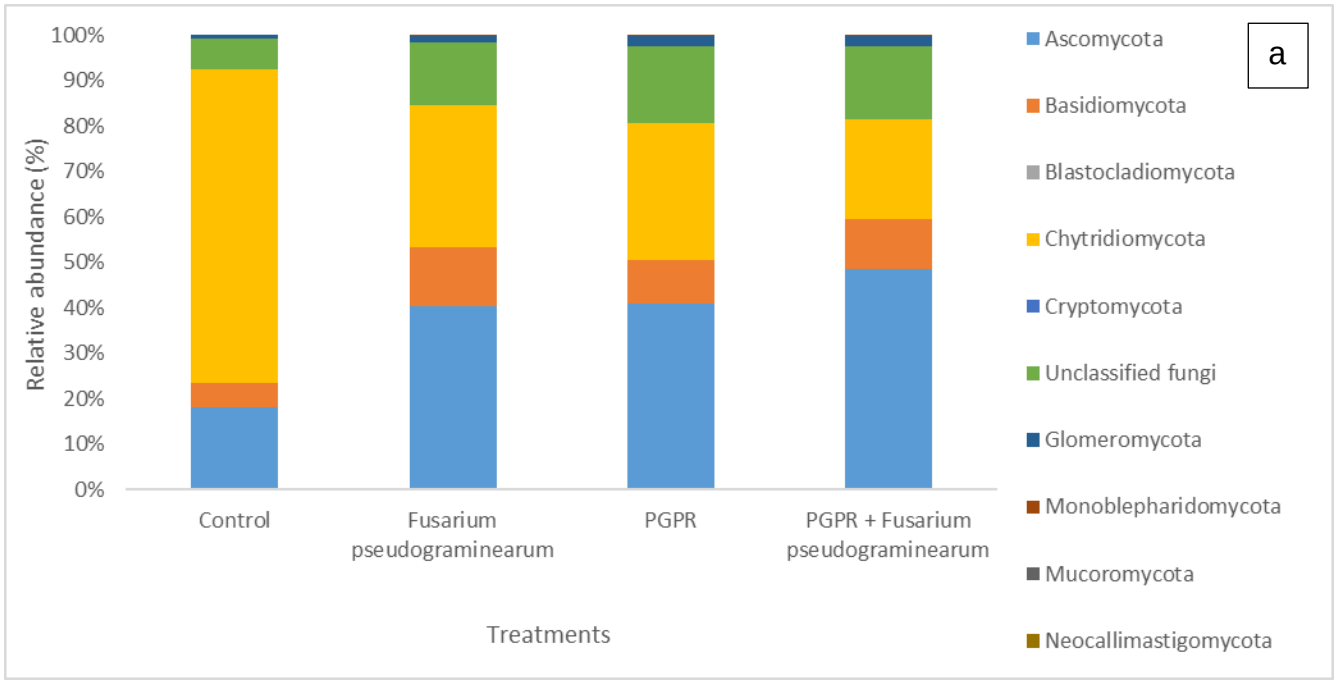


Figure 6.7. Principal component analysis of fungal (a) and bacterial (b) community

composition on wheat rhizosphere soil treated with *Bacillus subtilis* T29 and/or *Fusarium pseudograminearum* (FP).

The assessment of fungal and bacterial community compositions revealed that the treatment of the wheat rhizosphere with either *B. subtilis* T29 alone or in combination with *F. pseudograminearum* had an impact on certain fungal taxa (Figure 6.8a and 6.8b). The five most abundant fungal phyla identified across all treatments were Ascomycota (36.9%), Basidiomycota (9.7%), Chytridiomycota (38.1%) and Glomeromycota (1.7%). Neocallimastigomycota, Mucoromycota, Monoblepharidomycota and Blastocladiomycota were detected at a very low frequency (<1%) across all the treatments (Figure 6.8a). The main classes within the Ascomycota phylum were Sordariomycetes (9%), Dothideomycetes (4%) and Eurotiomycetes (2%), whereas Basidiomycota were represented by the Agaricomycetes (4%) and Tremellomycetes (0.9%) classes. The Chytridiomycota phylum was dominated by members of the Chytridiomycetes class (73%).

The nine most dominant bacterial phyla across the treatments included, Proteobacteria (34.4%), Acidobacteria (23.6%), Bacteriodetes (7.4%), Actinobacteria (6.9%), Planctomycetes (6.7%), Firmicutes (5.74%), Chloroflexi (4.3%), Gemmatimonadetes (3.7%) and Verrucomicrobia (2.4%). Armatimonadetes, Cyanobacteria, Fusobacteria and Nitrospirae were detected at frequencies lower than 1% across all treatments (Figure 6.8b). Proteobacteria was dominated by members of the classes Alphaproteobacteria (13.1%), Betaproteobacteria (8.8%), Deltaproteobacteria (7.9%) and Gammaproteobacteria (4.3%). Acidobacteria were represented by the Acidobacteria (17.1%), Solibacteres (4%) and Holophagae (2.4%) classes. Firmicutes were dominated by the Clostridia (4%) and Bacilli (1%) classes.



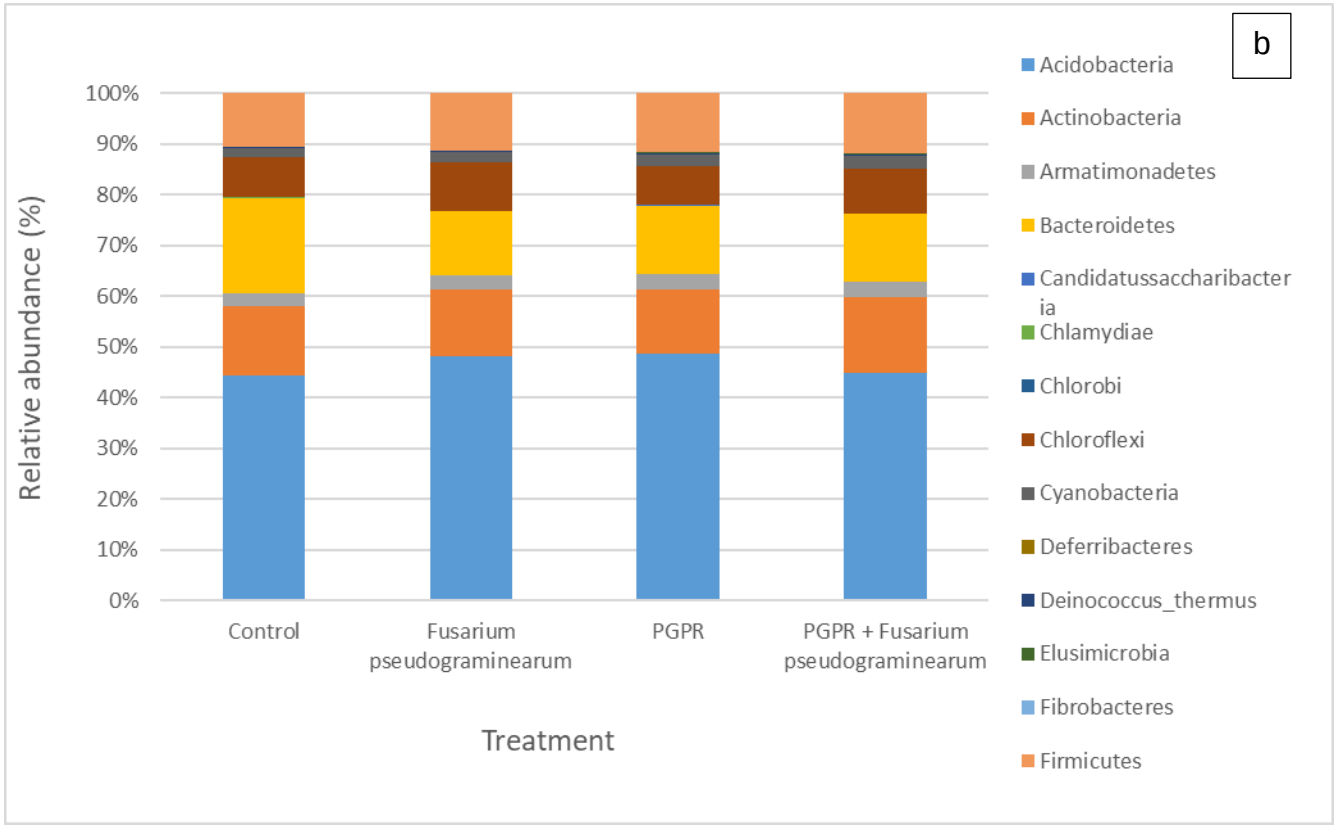


Figure 6.8: Fungal (a) and bacterial (b) community composition of different phyla on wheat rhizosphere soil treated with PGPR (*Bacillus subtilis* T29) and/or *Fusarium pseudograminearum*.

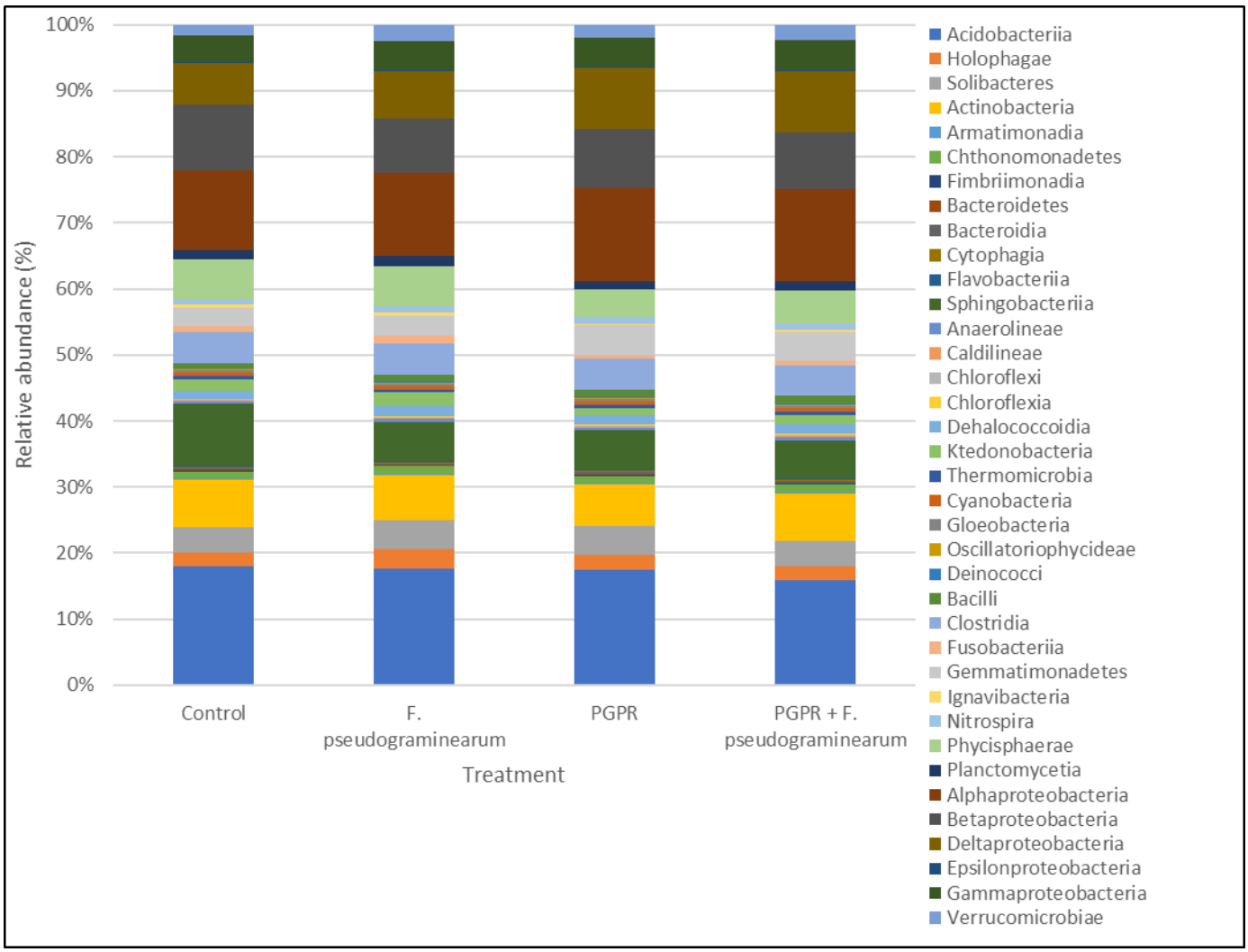


Figure 6.9: Fungal (a) and bacterial (b) community composition of different classes on wheat rhizosphere soil treated with PGPR (*Bacillus subtilis*) and/or *Fusarium pseudograminearum*.

6.3.4. Core microbiome genera

The dominating fungal populations across all treatments are shown in Figure 6.10. *Lobulomyces*, *Mortierella*, *Fusarium*, *Chaetomium* and *Trichoderma* were the most

prevalent fungal populations in all treatments. The least detected populations were fungi belonging to the genera *Coniochaeta* and *Chaetosphaeria*.

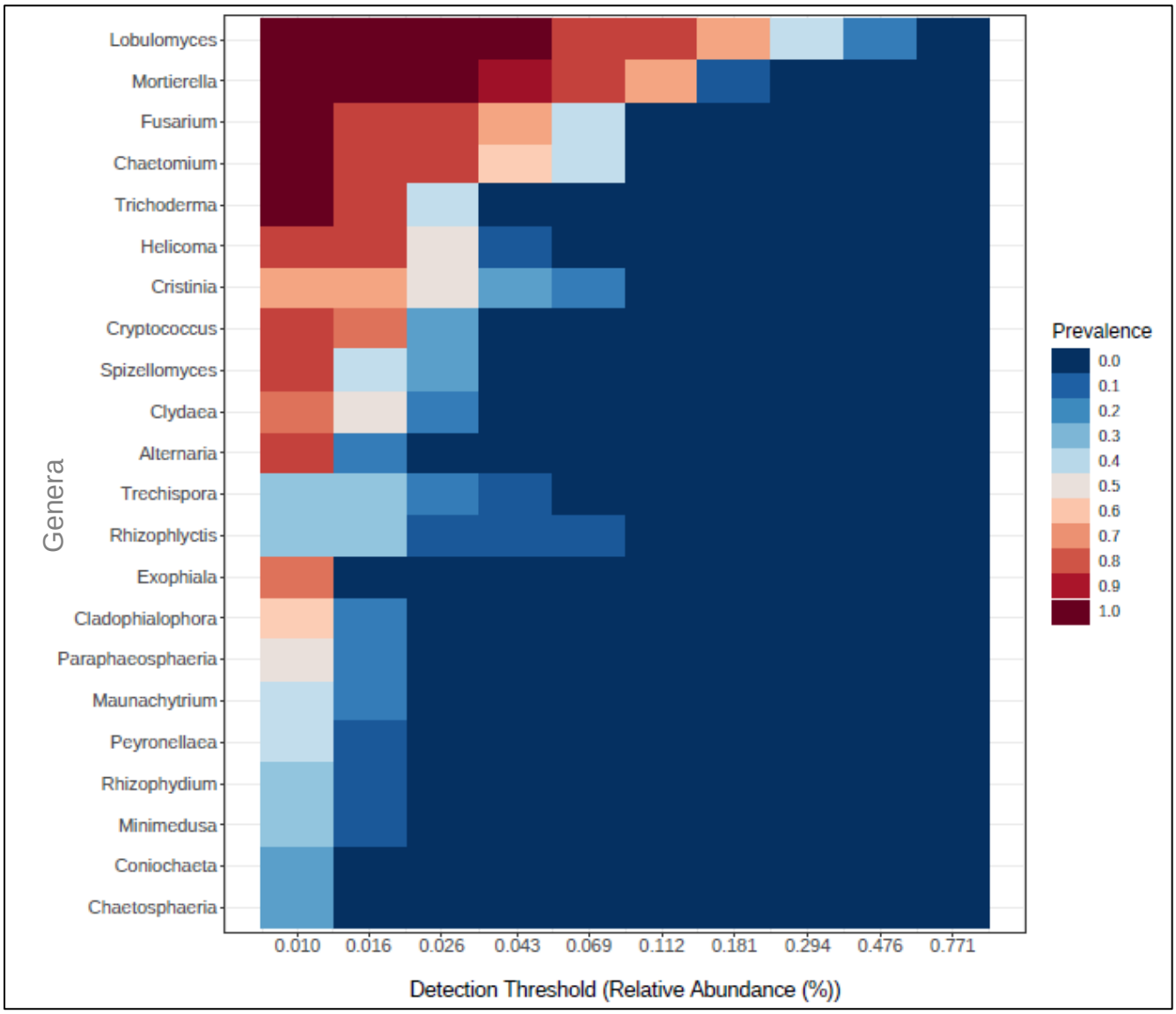


Figure 6.10: Core fungal genera detected on all wheat rhizosphere soils (untreated/ treated with PGPR (*Bacillus subtilis*) and/or *Fusarium pseudograminearum*).

6.3.5. Variations of potential pathogenic and beneficial microbial species

We further investigated the treatment effect on the prevalence of known pathogenic and beneficial fungal species to wheat (Table 4). Fungal species previously reported to be associated with crown root rot during development in wheat namely, *Fusarium equiseti, Fusarium sp*, *F. pseudograminearum, Fusarium oxysporum, Fusarium ciliatum* and *Rhizoctonia solani,* all members of the Ascomycota phylum were detected in the current study. Other pathogenic fungal species detected included *Fusicolla acetilerea, Fusicolla aquaeductuum, Fusicolla violacea and Fusidium griseum.* The relative abundances of most fungal species including *Fusicolla violacea* (Kruskal-Wallis test: $\chi^2 = 5.95$, P = 0.11), *Fusicolla acetilerea* (Kruskal-Wallis test: $\chi^2 = 6.72$, P = 0.08), *F. pseudograminearum* (Kruskal-Wallis test: $\chi^2 = 2.66$, P = 0.44) and *Rhizoctonia solani* (Kruskal-Wallis test: $\chi^2 = 3.31$, P = 0.34) were not significantly different between treatments.

Significant differences were observed in the relative abundances of *Fusarium sp.* (P < 0. 001; [ANOVA] F = 46.3), *Fusarium oxysporum* (P = 0.007; [ANOVA] F = 48.56) and *Fusarium equiseti* (P = 0.005; [ANOVA] F = 9.80) across all the treatments. Furthermore, pairwise comparisions showed that the relative abundance of *Fusarium equiseti* was significantly different between *B. subtilis* T29 vs the untreated control (P = 0.02), *B subtilis* T29 and *F. pseudograminearum* vs the untreated control (P = 0.004) and *B subtilis* T29 and *F. pseudograminearum* vs *F pseudograminearum* (P = 0.029). Pairwise comparisons also indicated that there were significant differences in the relative abundances of *F. oxysporum* between *B. subtilis* T29 and control (P = 0.02) as well as between *B subtilis* T29 + *F. pseudograminearum* and control (P = 0.005). For *Fusarium* sp, pairwise comparison indicated between treatments showed

significant differences between *B. subtilis* T29 and control (P = 0.002), *B. subtilis* T29 + *F. pseudograminearum* and control (P = 0.00), *B. subtilis* T29 and *F. pseudograminearum* (P = 0.02), *B subtilis* T29 + *F. pseudograminearum* and *F. pseudograminearum* (P = 0.009), *B subtilis* T29 + *F. pseudograminearum* and *B subtilis* T29 (P = 0.003).

No significant differences were observed between the treatments in certain beneficial fungal species i.e., *Trichoderma rossicum* (Kruskal-Wallis test: $\chi^2 = 5.31$, P = 0.15). Significant differences were however observed in the relative abundances of *Trichoderma hamatum* (P = 0.018; [ANOVA] F = 6.19), *Sarocladium strictum* (P = 0.014; [ANOVA] F = 6.78). For *Sarocladium strictum* relative abundances, pairwise comparisons of treatments showed significant differences between *B subtilis* T29 + *F. pseudograminearum* and control (P = 0.02) as well as between *B subtilis* T29 and *F. pseudograminearum* and *F. pseudograminearum* (P = 0.04). Lastly, significant differences were observed between *F. pseudograminearum* and control (P = 0.01) as well as between *B subtilis* T29 + *F. pseudograminearum* and *F. pseudograminearum* (P = 0.04) in the relative abundance of *Trichoderma hamatum*.

Table 6.2: Relative abundance of pathogenic and beneficial fungal species detected in wheat rhizosphere treated with *Bacillus subtilis* T29 and/or *Fusarium pseudograminearum*

Species	*Bacillus subtilis* T29	Untreated control	*Fusarium pseudograminearum*	*Bacillus subtilis* T29 + *Fusarium pseudograminearum*
Pathogenic fungi				
Fusarium_ciliatum	0,0006	0,0002	0,0007	0,0008
Fusarium_equiseti	0,0031	0,0007	0,0016	0,0039
Fusarium_oxysporum	0,0367	0,0152	0,0324	0,0441
Fusarium_pseudograminearum	0,0001	0,0001	0,0005	0,0002
Fusarium_sp_	0,0250	0,0047	0,0110	0,0440
Fusarium_solani	0,0031	0,0013	0,0023	0,0032
Fusicolla_acetilerea	0,0019	0,0004	0,0060	0,0023
Fusicolla_aquaeductuum	0,0003	0,0001	0,0004	0,0003
Fusicolla_violacea	0,0034	0,0017	0,0044	0,0177
Fusidium_griseum	0,0006	0,0003	0,0005	0,0007
Rhizoctonia_solani	0,0045	0,0022	0,0036	0,0024
Beneficial fungi				

Sarocladium_strictum	0,0008	0,0001	0,0002	0,0010
Trichoderma_spirale	0,0003	0,0003	0,0005	0,0005
Trichoderma_hamatum	0,0091	0,0075	0,0173	0,0125
Trichoderma_rossicum	0,0018	0,0004	0,0008	0,0011
Acaulospora_cavernata	0,0001	0,0001	0,0001	0,0002

Rhizospheric soil obtained from untreated control, *Bacillus subtilis* T29 and *Fusarium pseudograminearum* treated soils.

6.3.6. Interactions of beneficial and pathogenic populations with soil chemical properties

Redundancy analysis was performed to study the relationship between the soil chemical properties and the abundance of fungal and bacterial genus, either pathogenic or beneficial to wheat. Redundancy analysis axis 1 explained 15,5% and axis 2 showed 14.6% of the total variation in fungi, while on bacteria RDA axis 1 explains 37, 5% and 15.9% explained by axis 2 of the total variation, respectively. Redundancy analysis showed that relative abundance of fungi was affected by soil chemical properties in *Bacillus subtilis* T29 and *Fusarium pseudograminearum* treated soil. The soil PH and Na content was strongly associated with the relative abundance of *Macrophomina* and *Trichoderma* fungal genera in the untreated control treatment clustering along axis 1. Other pathogenic genera like *Fusarium*, *Phoma* showed a strong correlation with Na and Mg in *B*. *subtilis* T29 and *F*. *pseudograminearum* inoculation treatments. Bacterial populations on all treated and untreated soil were affected by the soil chemical properties. A similar trend as above was observed. The relative abundance of *Streptomyces* was strongly associated with Ca, S and K in untreated soils, while *Bacillus* was associated with both *B*. *subtilis* and B. subtilis + *F*. *pseudograminearum* inoculation treatments and *Agrobacterium* was associated with Mg in *F. pseudograminearum* inoculation treatment.

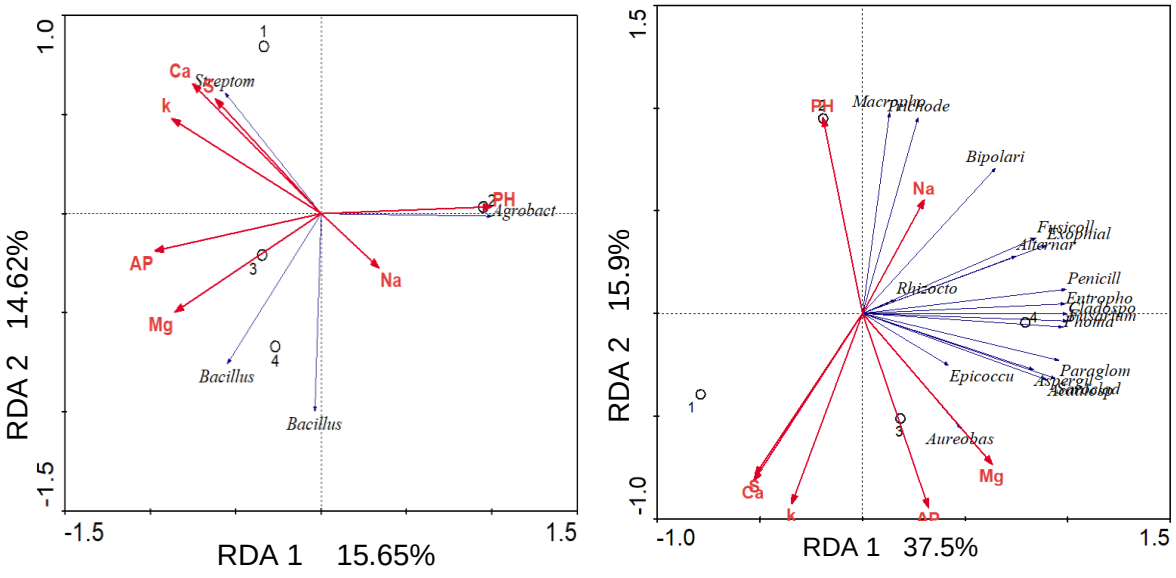


Figure 6.11: Redundancy analysis (RDA) of the correlations between the bacteria (a) and fungal (b) taxa and soil physiochemical properties in the different treatments; 1) untreated, 2) *Fusarium pseudograminearum* 3) *Bacillus subtilis* T29, and 4) *Bacillus subtilis* T29 + *Fusarium pseudograminearum* treated wheat rhizosphere. The arrow length and direction correspond to the variance that can be explained by the environmental and response variables. The direction of an arrow reflects the extent to which the given factor is influenced by each RDA variable. The perpendicular distance between the abundance of bacterial taxa and environmental variable axes in the plot indicates their correlations. The smaller the distance, the stronger the correlation. AP, available phosphorus; Na, sodium; Mg, magnesium; Ca, calcium; pH.

6.4. Discussion

Plant growth promotion and biocontrol activities are important features of commercial biocontrol agents used in sustainable agriculture. To date, members of the genus *Bacillus* are preferred for preparing bioformulations with beneficial impact on plant growth and health because of their spore forming ability (Borris, 2011; Qiao et al., 2017). In the current study, it was observed that *Bacillus subtilis* T29 was able to promote wheat growth (shoot and root) and showed biocontrol activity against *Fusarium pseudograminearum* in a pot trial at the vegetative stage. The deleterious effects of *F. pseudograminearum* was reduced by 24.7% and 38.1% of the fresh and dry total mass, respectively. The reduction of the negative effects of *F. pseudograminearum* on the plant mass by *B. subtilis* T29 indirectly indicates that *Fusarium* crown rot, caused by *F. pseudograminearum* was suppressed. Similar results were reported by Ugoji and Laing (2007), who also observed a similar effect of the *B. subtilis* bacterium on controlling *Rhizoctonia solani* on the roots of maize seedlings. Pathogen suppression specifically on the roots is mostly attributed to the mechanisms of biocontrol (competition for space and nutrients) deployed by the bacteria functioning in the root area (Ugoji and Laing, 2007).

Bacillus subtilis T29 significantly reduced the crown rot (*F. pseudograminearum*) severity by 65% in a pot experiment using field soil in the current study. These results suggest that *B. subtilis* T29 is a promising candidate as an efficient biocontrol agent for the control of *F. pseudograminearum* in wheat at the seedling stage. This observation agrees with the findings of Ugoji and Laing (2007); Makgolane (2016) and Carlson (2019). In this context, it is important to understand the effect of *B. subtilis* T29 applications on the

rhizosphere microbial community compositions so as to fully understand how it affects resident populations upon introduction.

The findings of the current study demonstrated the biocontrol activity and plant growth promotion of *B. subtilis* T29 and this agrees to findings of, Pretorius (2012), Rudolph (2014) and Makgolane (2016). These studies indicated that *B*. *subtilis* T29 strain has biocontrol abilities against *Fusarium graminearum* (Pretorius, 2012) and *F*. *pseudograminearum* (Makgolane, 2016) of wheat, as well as *Rhizoctonia solani* of maize (Rudolph, 2014). The effect of *B. subtilis* T29 on plant growth was, however, not significant as there were no substantial increases of both the total fresh and dry mass in the current study. This observation may be due to the fact that, the *B. subtilis* T29 treatment was applied on wheat seeds before planting and no subsequent applications were made after the plants had germinated and during the course of seedling growth. Past trials of wheat treated with *R. solani* and *Fusarium oxysporum* (Pretorius, 2012; Rudolph, 2014), seedlings received a *B. subtilis* T29 application before transplanting to pathogen infested soil, and four additional *B. subtilis* T29 applications thereafter. It has been reported that repetition of inoculation greatly influences the efficacy of rhizobacteria since the numbers of bacteria introduced into the soil usually decline rapidly over time (Bull *et al.,* 1991; Milus and Rothrock, 1997; Georgakopoulos, 2002). Therefore, one time application of *B. subtilis* T29 to the plants could have affected both biocontrol and plant growth promoting efficacy in this study.

Understanding the microbial community structure of plants is important because rhizosphere associated communities are key regulators of plant performance (Schmidt et al., 2019). This study further demonstrated that both the tested microorganisms (*B.*

subtilis T29 and *F. pseudograminearum*) greatly influenced the structure of root associated microbiome. The *B. subtilis* T29 and *F. pseudograminearum* treatment increased the both the fungal and bacterial diversity by 85% and 4%, respectively when compared to the untreated control rhizosphere soil. In agreement to our findings, Bae and Knudsen, (2005) reported an increase in fungal and bacteria populations after the application of *Trichoderma harzianum* ThzID1-M3 in natural soils. However, this was subsequently followed by reduced biocontrol efficacy due to competition for space and nutrients, and this may explain the observed reduction in biocontrol and growth promotion efficacy of *B. subtilis* T29 in the current study compared to that previously recorded in studies by Makgolane (2016) and Calrson (2019).

The use of microorganisms to control plant diseases has been a common practice during the last few decades (Glick et al., 2007; Calvo et al., 2017). The application of biocontrol agent directly into the soil to control plant diseases was previously shown to have a minor or transitory impact on the resident organisms or on biogeochemical cycles (Winding et al., 2004; Cavaglieri et al., 2005; Principe et al., 2007). The present study gave insights to the general structure of the wheat microbiome at the seedling stage and further showed the impact of the common wheat pathogen *F. pseudograminearum*, and its antagonistic *B. subtilis* T29 on fungal and bacterial communities in the rhizosphere. The study revealed a more diverse fungal and bacterial community following the *B. subtilis* and *F. pseudograminearum* co-inoculation treatment compared to the untreated control. Furthermore, the change in the bacterial populations was not as distinct as that in fungal populations. This observation agrees with that of Erlacher et al. (2014) who indicated that co-inoculation of the biocontrol agent *B. amyloliquefaciens* FZB42 and pathogenic *R.*

solani enhanced the microbial diversity of the lettuce rhizosphere (Erlacher et al., 2014). In the current study, the *B. subtilis* T29 treatment application to the wheat rhizosphere increased both fungal and bacterial diversity compared to a stand-alone *F. pseudograminearum* treatment. Moreover, co-inoculations with both *B. subtilis* T29 and *F. pseudograminearum*, resulted in more diverse fungal and bacterial (5.617 and 6.917, respectively) communities compared to either the /4/+*B. subtilis* T29 (5.435 and 6.821, respectively) or *F. pseudograminearum* (5.382 and 6.708) stand-alone treatments. These results indicate that the indigenous microbiome shifts as a consequence of pathogen attack but *B. subtilis* has the ability to compensate for the negative effects (Erlacher et al., 2014).

The fungal and bacterial community composition was also affected by the application of either *B. subtilis* T29 and/or *F. pseudograminearum* as compared to untreated control plants. Similar observations were made by Krober et al. (2014), where the application of *B. amyloliquefaciens* FZB42 resulted in a shift in the composition of the indigenous microbial community in the lettuce rhizosphere. However, these changes were transient. Other studies showed that *Pseudomonas spp*. (Erlacher et al., 2014; Blouin et al., 2004), *Enterobacter cowanii* (Götz et al., 2006), and *Sinorhizobium meliloti* (Miethling et al., 2000) altered the composition of the root bacterial community to the same extent as that reported for fungal plant pathogens such as *R*. *solani* (Chowdhury et al., 2013). A great variation was observed in fungal community composition compared to bacterial communities in our study. Qiao et al. (2017) reported that *B. subtilis* PTS-394 application influenced Eukaryota in the rhizosphere microbial community and the effect lasted up to 14 days after inoculation while that of the bacterial community lasted up to three days

after inoculation. However, in the present study, assessments were only focused on a single time point i.e., three weeks after *F. pseudograminearum* inoculation. Future studies should focus on several important stages of wheat growth and development.

Data of the current study also demonstrated that the introduction of *B. subtilis* T29 had a significant influence on the microbial diversity in the wheat rhizosphere. Moreover, the introduction of the disease-causing pathogen, *F. pseudograminearum* also resulted in shifts of both the fungal and bacterial communities. Contrary to our findings, Li et al. (2016) reported no significant differences in microbial diversity between the untreated and *B. subtilis* B068150 inoculated cucumber rhizosphere. The effects of B068150 on native microbial communities were also reported to be temporary (Domsch et al., 1983). Similarly, the application of a commercially available *B. amyloliquefaciens* FZB42 had a temporary shift on the microbial composition of the indigenous lettuce rhizosphere (Krober et al., 2014). However, the majority of the reported non-target studies of BCAs pointed to no substantial effects on bacterial abundances (Winding et al., 2004).

Our comparison of the taxonomic profiles of the wheat rhizosphere agreed to the findings of Don et al. (2016). In contrast to the untreated control rhizosphere, *B. subtilis* T29 and *F. pseudograminearum* treated rhizosphere soils were associated with an increase in the relative abundances of the Ascomycota, Basidiomycota and Glomeromycota phyla with a reduction in that of the Chytridiomycota phylum. Furthermore, Chytridiomycota was the most abundant phylum in the untreated control rhizosphere soils. The occurrence of Chytridiomycota in soil fungal populations has been documented with relative abundances reaching up to 63% in the bulk soils of disturbed Australian soils (James et al., 2006). The *Lobulomyces poculatus* species belonging to the Chytridiomycota phylum

was significantly enriched in the untreated control rhizosphere soils (67%) compared to all the other treatments. There are no previous reports on *L. poculatus* dominating wheat rhizosphere or other soil, and the application of *B. subtilis* and *F. pseudograminearum* treatments in the current study, resulted in a decrease of *L. poculatus* by 19% and 26%, respectively, compared to the untreated control. However, this species still remained the most abundant 76% in the Chytridiomycota phylum.

Several studies of the plant rhizosphere habitat revealed that Acidobacteria, Bacteroidetes, Actinobacteria, Firmicutes and Proteobacteria were detected as the most abundant phyla (Don et al., 2016; Lui et al., 2017). The current study showed that Proteobacteria and Acidobacteria were the most prominent bacterial taxa in the wheat rhizosphere at the seedling stage.

Bacillus sp. has been found to be a significant contributor to the rhizosphere within many other crops including rice, maize, potato, grapevine, coffee, and coconut (Rosenblueth and Martínez-Romero, 2006). In the current study, the relative abundance of the genus *Bacillus* (Firmicutes), however, was very low in comparison to the total bacteria population in all treatments, despite its external addition (*B. subtilis* and *B*. *subtilis* + *F*. *pseudograminearum*) to seeds. The low relative abundances of *Bacillus sp*. in rhizosphere have been documented previously and it has been linked to the nutrient content in soil (Persson et al., 2003). *Bacillus sp*. relative abundances were the lowest in the untreated control rhizosphere, followed by the *F. pseudograminearum* and *B. subtilis* T29 treatments with the highest relative abundance being observed in *B. subtilis* T29 + *F. pseudograminearum* inoculated rhizosphere soil. However, *Bacillus* is reported to be one of the most abundant bacteria in the soil (Köberl et al, 2011) and in the rhizosphere

of other crops such as lettuce (Chowdhury et al., 2013). The observed reductions *in B. subtilis* OTUs in the current study may be due to the selection or enrichment of specific fast-growing taxa in the wheat rhizosphere. For instance, bacteria that belongs to the Proteobacteria and Actinobacteria phyla, have generally been characterized as fast-growing populations (Goldfarb et al., 2011).

Enrichments of fungal genera that may contain pathogens including *Rhizoctonia and Fusarium sp*. were noticed following the application of *B. subtilis* T29, *F. pseudograminearum* and *B. subtilis* T29 + *F. pseudograminearum*. Upon pathogen attack, plants recruit beneficial populations to their roots to stimulate the plants' immune system that results in increased resistance to the pathogens (Brendenson et al., 2018).

The application of biocontrol agents to the soil can cause changes to both the beneficial and pathogenic microbial populations present on the wheat rhizosphere, and so does the increase in pathogen pressure, as observed in this study. This current study demonstrated a shift in both the beneficial and pathogenic populations following the applicationof either *B. subtilis* T29 or *F. pseudograminearum*. The co-inoculation of *B. subtilis* T29 and *F. pseudograminearum* increased the relative abundance of *Fusarium oxysporum* by 2.9 times in the wheat rhizosphere when compared to the untreated control. Despite this observation, the application of biocontrol agents in the soil has been proven to be more beneficial than detrimental towards plant health by conferring resistance to abiotic stress (Selvakumar et al., 2012), and suppress plant diseases (De Vleesschauwer and Höfte, 2009). These living microorganisms are dynamic and potentially self-sustaining, thus, reducing the need for repeated applications, and can

avoid the problem of pests and pathogens evolving resistance to the treatments (Lucas, 2011).

It was notable in this study that, the soil physico-chemical properties also had an effect on the distribution of both pathogenic fungal and beneficial bacterial populations. Previous research has shown that soil physico-chemical properties may influence the biological balance considerably and Li et al. (2016), concluded that the survival of a PGPR, *B. subtilis* B068150 at low population densities in sand was related to a lower level of organic matter and lower water potential (Acea et al., 1998; Bergelson et al., 2008; Li et al., 2016) compared to other soil types. The current study showed that the population of *Fusarium*, a major wheat pathogen was associated with high Na and Mg levels in the soil. Therefore, the occurrence of *Fusarium* in the soil can possibly be controlled by manipulating the Na and Mg levels in the soil. The *Bacillus* genus on the other hand, was associated with low Na and this finding has not been reported previously. Lareen et al. (2016) reported that the nutrient content in soil can determine the composition of microbial communities associated with roots although their quantitative contribution in influencing rhizosphere communities remains unclear. These physico-chemical properties of soils can directly select for specific microbes by creating niche environments that can benefit certain types of microbes (Lareen et al., 2016) and this agrees with the findings of the current study. Soil physico-chemical properties can also influence the availability of plant root exudates affecting microbial recruitment by the plant (Lareen et al., 2016). For instance, soil pH and nutrient availability (e.g., carbon, nitrogen, phosphate) have been found to affect the abundances of crop pathogenic bacteria, fungi and nematodes as well as beneficial microbes (Höper et al., 1995; Duffy et al., 1997; Lacey and Wilson, 2001; Rasmussen et

al., 2002; Rimé et al., 2003; Hamel et al., 2005; Rotenberg et al., 2005; Toljander et al., 2008; Dumbrell et al., 2010).

The current study outlines the changes that take place in the wheat rhizosphere microbiome upon the introduction of *B. subtilis* and *F. pseudograminearum*. A change in the number of OTUs, diversity and the abundance of the fungal and bacterial population was observed. In the absence of either *B. subtilis* or *F. pseudograminearum*, a shift on the fungal and bacterial composition was further seen. The findings of the current study corroborate the exploitation of potential beneficial root microbiome can provide sustainable solutions in wheat production (Philippot et al., 2013). Creating a rhizosphere ambiance between microbial populations and soil physico-chemical properties might allow plants to establish robust beneficial microbial communities to obtain a variety of benefits and enhance their ecological competitiveness. However, the main motivation should still be to recruit a high diversity of beneficial microbes to sustain nutrient supply and receive protection against pathogens and opportunists. Therefore, understanding microbe–microbe dynamics and their effects on the composition of microbial communities is essential to identify microbial determinants that shape microbial communities. This knowledge can provide solutions to create beneficial microbiomes as already present in some suppressive soils. It has been suggested that stable populations of beneficial microbes that are selectively recruited and maintained in the rhizosphere by the plant, subdue pathogens through secretion of secondary metabolites (Doornbos and Van Loon, 2012). This ultimately leads to disease suppression and perhaps some level of exclusion of the pathogen from the soil.

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Chapter 7

General discussion and conclusions

The soil microbiome is critical for the conservation of soil health, which is key to agricultural sustainability (Chaparro et al., 2012; Lehman et al., 2015). Soil health has significant implications on the productivity of agricultural soils (Dubey et al., 2019). Thus, the degradation of soils due to long-term inadequate agricultural practices in wheat producing areas of South Africa (SA), have led to an urgent need for research and development of more sustainable agricultural practices in order to delay, and if possible revert, the consequences of soil impoverishment. This thesis contributes with an environmental-friendly alternative approach that could be exploited to improve the wheat growth in SA. Plant associated microbial communities, specifically those in the rhizosphere, are increasingly important in helping overcome biotic (Mendes et al., 2011; Berendsen et al., 2012) and abiotic stresses and increasing productivity. This research was conducted to profile the wheat rhizosphere microbiome and further understand how to exploit soil microbial communities and provide plants with healthy soils to increase plant health and productivity. Moreover, results from these studies may be extended to other crops of agricultural importance.

Understanding how wheat plants and the rhizosphere microbiome interact naturally in different environments crucial to our understanding of the key microbial communities that are essential for plant health and growth. It has been stated that plant productivity and protection is shaped by the rhizosphere microbial communities (Wu et al., 2018), therefore, chapter 3 of this study focused on understanding the typical bacterial

communities present on the rhizosphere of different wheat cultivars in three regions of SA. The results provided insight into the implications of land-use change from its natural state to agricultural land, on bacterial diversity and composition. Our results further highlighted the effect of cultivar and soil chemical properties on the diversity and distribution of bacterial communities. The study showed lower bacterial diversity that was associated with dominant communities in rhizosphere compared to non-rhizosphere soils and this was consistent with previous reports (Kowalchuk et al., 2002; Inceoglu et al., 2011; Yun et al., 2016). Interestingly, minor differences in diversity changes in rhizosphere associated bacterial communities were noticed between different cultivars grown on the same region. However, a more pronounced difference in diversity change was noticed on the same cultivar, grown in different regions. Findings from this study further revealed that soil characteristics also influenced diversity and distribution of microbial communities. Differences in soil pH were also evident in these different regions. Soil structure and characteristics was reported to have a stronger effect on rhizosphere bacterial community diversity than genotype in past studies (Yun et al., 2016) and this is likely the case in the present study. Additionally, it is important to note that soil pH influenced recruitment in the rhizosphere and to a lesser extent cultivar for specific bacterial groups. This study highlights certain factors to consider during bacteria microbiome manipulation for increased wheat production in agriculture. The study further outlines that when wheat is planted in different environmental systems, the initial phase of microbial population construct differs and is mainly driven by soil pH. Future biocontrol systems should take regional impact factors into account. This study therefore provides

baseline information for future adaptive research to ensure successful establishment of biocontrol systems as it gets affected by soil structure and plant cultivar.

Knowledge of the structure and diversity of the fungal community in the rhizosphere will lead to a better understanding of pathogen-antagonist interactions. Especially during bio-inoculation studies with potential biocontrol and growth promotion studies. Additionally, fungi are important components of every terrestrial ecosystem, as decomposers, food sources, pathogens or mutualists (Wood, 2017). As yield losses caused by fungal pathogens represent a major threat to global food production (Sebold et al., 2020), and in SA, most of the wheat diseases are caused by fungi (ARC, 2019). Therefore, in chapter 4 of this research, included a fungal microbiome assessment, which gave for the first time, an insight into the factors contributing to fungal community composition and diversity in wheat. A diverse and different fungal population was detected in the rhizosphere of different cultivars and the geographic locations also influence fungal diversity and composition. This is an important finding since different cultivars are planted in preferred regions in SA. Therefore, recruitment of desired microbial communities on certain cultivars could be done strategically.

Besides exerting a plant growth promotion or protection effect, a plant growth promoting rhizobacteria (PGPR) strain should be able to successfully colonize the rhizosphere (Mendis et al., 2018). We confirmed that *Bacillus subtilis* T29 inoculation is contributing to increase the biomass yields of seedlings and thus optimistic with the idea that this positive effect on plant growth can be reproduced in field experiments. Chapter 5 further highlighted that factors such as cultivar significantly influenced *B. subtilis* T29 colonization on the root system of wheat. Expectedly, *B. subtilis* T29 treated wheat plant showed

higher concentration of *B. subtilis* T29 on the root system. Interestingly abundance of *B. subtilis* T29 on different cultivars was not positively correlated with increased wheat growth on the same cultivars. This result suggests that higher levels of PGPR colonization will not always result in maximum plant growth, especially in the presence of native microflora. It has been reported that the success of plant growth promotion depends on plant genotype (Luisa et al., 2018). This is because different species or cultivars may produce different types of root exudates, which may support microbial activity (Khalid et al., 2004). This chapter thus highlights the importance of cultivar choice to achieve increased yield. It was therfore important to determine the microbial shift upon *B. subtilis* T29 introduction to resident microbiome. This study allowed to attribute the plant growth promoting effect observed in wheat seedlings with the presence of the introduced rhizobacterial inoculant *B. subtilis* T29 within the rhizosphere. In addition, it provides a strain-specific ddPCR approach to monitor this rhizobacterial inoculant in natural soil.

In chapter 6, *B. subtilis* T29 and *Fusarium pseudograminearum* were added to the soil microbial community. The addition of these microbes to the soil caused distinct changes in the soil microbial community. The knowledge gained from this application of antagonist and pathogen can be used to model these interactions and complexities in agricultural field soil. This approach was able to identify pathogenic species of concern and beneficial microbes after exposure to *B. subtilis* T29 and/or *F. pseudograminearum*. Therefore, it can be used to predict soil microbial community dynamics upon exposure to PGPR and pathogen and further identify microbes that could be used to enhance and create healthy soils for increased wheat production.

While this study sought to understand the soil microbiome in Frees State, Western Cape and Gauteng province, the findings from this study can contribute to the global request to map Africa's soil microbiome. Ultimately, understanding the behaviour of resident microbiome upon pathogen attack and PGPR inoculation. The results of this study have broader implications for improving our ability to manipulate the soil microbial communities for improved plant health and growth. Finally, more research could develop specific microbial partners that work synergistically to enhance plant production, increasing overall health of the agroecosystem. Understanding how microorganisms antagonize or benefit each other will contribute greatly to improving plant biomass production when manipulating agricultural soil microbial communities. Results from this study are based on data collected from samples of both field and greenhouse conditions. It may be valuable to conduct studies in the field especially on the influence of *B. subtilis* and *F. pseudograminearum* on resident microbiome. It is important to study naturally assembled soil communities because they are more complex and contain thousands of microbial species (van Elsas et al., 2012).

Future research should combine metagenomics with other high throughput methods such as proteomics, metabolomics and transcriptomics to fully understand the ecological functions of soil microbiomes. Further experiments can also be conducted on representatives of the genera that showed significant effect with added PGPR to confirm enhanced PGPR performance.

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Appendix A

Bacterial culture origin and maintenance

The *Bacillus subtilis* T29 isolate used in the current study was obtained from the University of Pretoria"s PGPR culture collection and was previously identified during studies by Hassen (2007), Pretorius (2012) and Makgolane (2016), respectively. The isolate was selected based on its performance in previous trials with sorghum and wheat. It was obtained from the culture collection in lyophilised form, revived on nutrient agar (Bioloab, Wadeville) and checked for purity. Pure cultures were preserved in eppendorf tubes containing 70% glycerol and stored at -70°C. When needed the culture was streaked onto Nutrient agar (NA) (Biolab).

Appendix B

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Fungal diversity and community composition of wheat rhizosphere and non-rhizosphere soils from three different agricultural production regions of South Africa

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ABSTRACT

Understanding complex interactions among plant genotypes, environmental conditions and microbiome structure provides crucial information for sustainable farming practices towards disease control in agriculture. In this study, fungal diversity and composition in wheat rhizosphere and non-rhizosphere soils were investigated. Special emphasis was placed on pathogenic and beneficial genera. Wheat rhizosphere and non-rhizosphere soil from three different wheat growing regions were analyzed using Illumina high-throughput sequencing. The analysis showed a significant decline in the fungal diversity and richness from non-rhizosphere to rhizosphere soils. Ascomycota and Basidiomycota were the dominant fungal phyla detected in both rhizosphere and non-rhizosphere soils across the three test sites. Genera known to include wheat pathogens detected included *Fusarium*, *Phoma* and *Colletotrichum* genera while, beneficial groups included *Trichoderma*, *Aureobasidium* and *Acaulospora*. The presence of *Fusarium* was observed to be inversely proportional to that of *Aureobasidium*, a well-known antagonist of the *Fusarium* spp. This information could provide new opportunities to explore the potential of manipulating natural fungal antagonistic microorganisms for use in controlling soil-borne pathogenic fungi in wheat.

1. Introduction

Current models predict that the world's population will reach 9 billion by 2050 and that crop yields will need to increase by 70–100% to sustain the population growth (Reid and Greene, 2012). Wheat, along with maize and rice provides 50% of human calories and are critical food sources in regions with rapid population growth such as Asia, Africa and the Middle East (Donn et al., 2015). However, global wheat production has slowed to 0.9% per year. At this level the 2050 targets will not be met and an increase to 1.5% per year should be the target (Fisher and Edemeades, 2010).

In South Africa (SA), wheat (*Triticum sativum* L.) is considered an important cereal crop and it ranks second after maize in terms of the area under cultivation (533,000 ha) with production ranging from 1.3 to 2 million tons per year (DAFF, 2016). Major wheat production areas in SA are the Free State, the Western Cape and Northern Cape Provinces. Other provinces such as Gauteng and North-West are ranked as minor producers of wheat (Nhemachena and Kirsten, 2017). As with global downward trends, the Free State Province has shown a drop (up to 50%) in annual wheat production (Daff, 2016). Production in other irrigation and winter wheat areas also declined, but not as drastically. This calls for strategic intervention to increase production to at least 2.7 million tons per year in order to feed the country's growing population and avoid food price increases (DAFF, 2016).

Several factors have been attributed to the decline in SA wheat production including unpredictable weather conditions and drought, poor soil fertility and occurrence of pests and diseases (Scott, 1990). Due to poor soil quality most of the local wheat producers resort to fertilizer use (FAO, 2005). This together with lime can increase the total variable input costs to as much as 17 to 30% in wheat production (DAFF, 2010). Additionally, root and crown diseases in wheat also negatively impact on wheat germination and yield losses in South Africa (Lamprecht et al., 2006). Economically important diseases of wheat include take-all caused by *Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *graminis*, crown rot caused by *Fusarium pseudograminearum* O'Donnell & Aoki, common root rot caused by *Rhizoctonia solani* (Burgress et al., 2001; Kuzdraliński et al., 2014) etc. The prevalence of these soil-borne pathogenic fungi in wheat differs from one geographic region to another (Kuzdraliński et al., 2014). Disease control of these pathogens is reasonably successful through different

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Appendix C

Table 3.2: Soil chemical composition of three wheat production sites in South Africa selected for the soil microbiome study.

Region	Cultivar	pH (kcl)	EC (µs/cm)	Available P (mg/L)	Available K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	Na (mg/kg)	Organic carbon %
Western Cape (Site A)	'Kariega' 'SST88'	6.14a	245.6a	3.25c	219.2b	1001.3a	110.0b	45.99a	1.61a
Gauteng (Site B)	'Kariega'	5.64b	106.5c	6.95b	200.9a	455.0c	172.6a	5.44c	0.75c
Free State (Site C)	'Eland'	4.42c	170b	35.19a	222.1c	466.3b	74.1c	7.72b	0.81b

Table 3.3: Comparison of the estimated OTU richness and diversity indexes of the 16S rRNA gene libraries for clustering at 97% identity**.**

Site	Wheat Variety – Origin	Sampling Depth	Observed Richness (s)	ACE	CHAO	Shannon-Weaver	Inverse Simpson
Site A	'SST88' – NR	88162	562.0	982.8	1394.1	6.0	262.9
	'SST88' – R	59931	490.0	780.1	1060.4	5.7	150.3
	% difference	**32.02173**	**12.8**	**20.6**	**23.9**	**4.6**	**42.8**
	'Kariega' – NR	89789	587.0	1132.0	1489.9	6.1	293.9
	'Kariega' – R	75283.5	489.5	808.4	1136.3	5.7	105.8
	% difference	**16.1551**	**16.6**	**28.6**	**23.7**	**7.0**	**64.0**
Site B	'Kariega' – NR	80924.5	521.5	913.1	1275.8	5.8	166.8
	'Kariega' – R	80161	393.0	732.7	1019.2	5.2	66.9
	% difference	**0.943472**	**24.6**	**19.8**	**20.1**	**11.5**	**59.9**
Site C	'Eland' – NR	74417	518.0	835.5	1200.3	5.9	233.5

	‘Eland’ – R	54828.5	339.0	552.0	785.0	4.7	31.4
	% difference	**26.32261**	**34.6**	**33.9**	**34.6**	**19.4**	**86.6**
Overall	NR	81439	538.0	935.3	1306.0	5.9	226.2
	R	67551	427.9	718.3	1000.2	5.3	88.6
	% difference	**17.05325**	**20.5**	**23.2**	**23.4**	**10.1**	**60.8**

NR, Non-rhizosphere soil and R, Rhizosphere soil.

Table 3.4: Overall relative abundance of bacterial phyla in Site A 'SST88' non-rhizosphere (NR) and rhizosphere (R), Site A 'Kariega' NR and R, Site B 'Kariega' NR and R and Site C 'Eland' NR and R

Phylum		Relative abundance (%)								Increase in relative abundance (%)			
		Site A 'SST88'		Site A 'Kariega'		Site B 'Kariega'		Site C 'Eland'		Site A 'SST88'	Site A 'Kariega'	Site B 'Kariega'	Site C 'Eland'
		NR	R	NR	R	NR	R	NR	R	R	R	R	R
Proteobacteria	Alphaproteobacteria	----	15.04	13.42	13.86	8.86	16.76	14.90	8.14	13.38	3.24	89.22	-45.39
	Betaproteobacteria	11.63	9.13	12.39	12.19	7.66	6.70	6.47	11.65	-21.49	-1.59	-12.52	-79.96
	Deltaproteobacteria	7.33	11.48	7.19	7.93	4.87	11.10	4.32	1.86	56.64	10.30	127.91	-57.01
	Gammaproteobacteria	6.96	8.18	7.27	8.56	17.31	8.58	6.52	8.58	-17.47	-2.38	-75.23	31.60

Bacteroidetes	Cytophagia	4.25	6.26	4.16	5.38	2.04	1.56	1.15	0.41	47.34	29.47	-23.24	-64.58
	Flavobacteria	12.62	2.18	13.65	3.56	10.70	1.31	0.42	0.38	-82.69	-73.94	-87.73	-10.60
	Sphingobacteria	15.03	6.16	13.38	10.40	7.63	8.10	8.83	4.82	-59.03	-22.30	6.15	-45.39
Chloroflexi		2.17	4.86	1.65	3.77	5.97	12.87	9.43	3.95	123.82	127.88	115.44	-58.11
Acidobacteria		3.51	5.68	2.79	6.11	2.65	6.14	16.02	6.14	61.79	56.86	158.95	45.41
Actinobacteria		10.95	14.36	12.53	16.72	14.52	17.18	12.27	16.59	31.10	33.42	18.33	35.23
Cyanobacteria		0.32	0.80	0.30	0.88	5.52	0.70	2.52	0.63	149.00	198.19	-87.38	-74.95
Firmicutes		2.39	4.27	2.14	4.81	8.57	3.70	6.96	20.18	78.55	124.55	-56.78	190.15
Gemmatimonadetes		1.97	2.32	1.94	1.55	0.87	2.48	2.78	1.18	17.79	-20.06	186.63	-57.64
Planctomycetes		3.28	5.33	2.83	4.66	1.61	3.21	5.20	1.75	62.49	64.50	98.86	-66.41
Verrucomicrobia		4.32	3.95	4.36	2.83	1.23	3.18	2.22	0.96	-8.51	-35.15	158.66	-56.72

Table 3.5: Soil chemical composition from the different treatments.

Treatment	pH(kcl)	AP(mg L^{-1})	Ca(mg kg^{-1})	Na(mg kg^{-1})	S(mg kg^{-1})	Organic Carbon%	K	Mg(mg kg^{-1})
Untreated control	5.48	32.81	573.56	50.73	29.35	0.8	226.22	94.27
***Bacillus subtilis* T29**	5.45	35.26	562.26	49.24	28.51	0.83	223.32	94.56
Fusarium pseudograminearum	5.53	26.53	548.36	49.24	27.72	0.87	215.22	91.69
Bacillus subtilis* T29 + *Fusarium pseudograminearum	5.39	34.29	555.86	53.19	28.02	0.84	220.32	95.53

AP - Available phosphorus, Ca - Calcium, Na - Sodium, S - Sulfur, K - Potasium, Mg - Magnesium.