ASSOCIATION OF CHICKPEA WITH SOIL FUNGI: A COMPARISON OF CULTIVARS

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By

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ABSTRACT

Certain crop plants are susceptible to pathogens or unable to develop efficient microbial symbioses. These crops adversely impact soil biological quality with consequences on plant health and productivity of cropping systems. Chickpea is a rotational pulse crop with two types: kabuli and desi, and several cultivars. Cultivation of chickpea has inconsistent effects on soil microbial communities and subsequent wheat crops. I conducted field studies and used high throughput molecular analyses to explore the variations among chickpeas to identify cultivars developing fungal communities that are conducive to plant health and productivity. I also carried out greenhouse studies and used biochemical analyses to investigate the response of chickpea cultivars to arbuscular mycorrhizal (AM) fungi and non-AM fungal endophytes and identify the influence of root and root metabolites on the endophytic and pathogenic fungi. Cultivars and types of chickpeas and environmental conditions promoted different fungal communities in the root endosphere. *Funneliformis* and *Claroideoglomus* were the dominant AM fungal genera and *Fusarium* and *Alternaria* were the dominant non-AM fungal genera in the roots of chickpea. The roots of cultivars CDC Corrine, CDC Cory and CDC Anna hosted the most diverse fungal communities in contrast to CDC Alma and CDC Xena roots which hosted the least diverse communities. Plant response to AM and non-AM fungal endophytes varied with genotype and type of chickpea. The root symbiosis effectively promoted plant growth in CDC Cory, CDC Anna and CDC Frontier and stimulated nitrogen fixation in CDC Corrine. Cultivars of chickpea responded differently to dual inoculation of the AM and non-AM fungal endophytes. Co-inoculation with AM and non-AM fungal endophytes had additive effects on CDC Corrine, CDC Anna and CDC Cory but non-AM fungal endophytes reduced the positive effect of AM fungi in

Amit and CDC Vanguard. Desi chickpea appeared to form more efficient symbioses with soil fungal resources than kabuli chickpea. Protein(s) up-regulated in the mycorrhizal roots of the desi chickpea CDC Anna suppressed the growth of the fungal endophytes *Trichoderma harzianum* and *Geomyces vinaceus* and of the pathogens *Fusarium oxysporum* and *Rhizoctonia* sp. The formation of AM symbiosis decreased the production of root bioactive metabolites soluble in 25% methanol. Some of the root metabolites stimulated the growth of *Trichoderma harzianum* and *Geomyces vinaceus*, and a few inhibited *Rhizoctonia* sp. and *Fusarium oxysporum*. A few metabolites with contrasting effects on the different fungal species were detected. The non-protein phytochemicals had selective effects on the endophytes and pathogens whereas the antifungal proteins of mycorrhizal roots were non-selective. Overall the study reveals a "genotype effect" of chickpea on the soil microbiota suggesting the possibility to improve the performance of this crop through the selection of genotypes improving the communities of root associated fungi, by associating and responding to beneficial fungi and repressing the pathogens.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AM: Arbuscular mycorrhizal

ANOVA: analysis of variance

DCA: Detrended correspondence analysis

DNA: Deoxyribonucleic acid

Per-MANOVA: Permutational multivariate analysis of variance

MRPP: Multi-response permutation procedures

NMDS: Non-metric multidimensional scaling

PCA: Principle component analysis

PCR: Polymerase chain reaction

PDA: Potato dextrose agar

rDNA: Ribosomal DNA

SPARC: Semiarid Prairie Agricultural Research Centre

HPLC: High-performance liquid chromatography

MPLC: Medium pressure liquid chromatography

EDTA: Ethylenediaminetetraacetic acid

MeOH: Methanol

ODS: Octa decyl silane

SDS: Sodium dodecyl sulfate

CDC: Crop Development Centre

N: Nitrogen

OUT: Operational taxonomic unit

VOC: Volatile organic compounds

NPAA: non-protein amino acid

ITS: Internal transcribed spacer

SA: Salicylic acid

JA: Jasmonic acid

1. GENERAL INTRODUCTION

The roots of plants are associated with soil microbes throughout their life. The influence of the root microbiome on plant fitness is crucial so that it is "referred to as the second genome of the plant" (Berendsen et al., 2012). Soil fungi represent a diverse group of microorganisms including saprophytes, symbionts and pathogens that are involved in processes such as decomposition of organic substances, promotion of plant growth, and development or prevention of disease (Bridge and Spooner, 2001). The majority of crop plants belong to families that form root symbioses with arbuscular mycorrhizal (AM) fungi and septate fungal endophytes. Arbuscular mycorrhizal symbiosis is important for serving the host with nutritional benefits (Jakobsen et al., 2003), whereas endophytes increase plant tolerance to stressful conditions such as drought and disease (Mandyam and Jumpponen, 2005). Arbuscular mycorrhizal fungi is a group of soil fungi that represent similar lifestyles and functionalities forming the phylum Glomeromycota (Schüβler et al., 2001). The term fungal endophyte refers to a miscellaneous group of allied fungi that asymptomatically reside in the tissues of plants (Saikkonen et al., 1998) and could be classified as Ascomycete or Basidiomycete (Kageyama et al., 2008).

Despite the fact that AM fungi and fungal endophytes commonly co-occur in terrestrial ecosystems, there is a gap in knowledge on the influence of such multipartite symbioses on plant performance. Current knowledge on the effects of the symbiotic associations of plants with AM fungi and fungal endophytes is inadequate and unbalanced. Despite the wealth of reports that describe the functions of AM symbiosis, little is known about the influence of fungal endophytes on plant performance.

Fungal endophytes colonize the intercellular space of the root cortex and are capable of forming mutualistic associations similar to mycorrhiza, however the interfaces formed by endophytes differ from the ones observed in mycorrhizal symbioses (Barrow and Aaltonen, 2001). While both of these fungal symbionts compete for organic carbon from the host plant, their contribution to plant fitness depends on the environmental conditions in which the multipartite symbioses occur. The function of the AM symbiosis could be optimal when P is limiting (Koide, 1991), whereas the fungal endophytes are important in extreme environmental conditions (Mandyam and Jumpponen, 2005).

Root symbioses with AM fungi and fungal endophytes are usually examined separately. However, recent reports indicate that fungal endophytes interact with and modify the development of AM fungi (Scervino et al., 2009), as well as the outcome of the AM symbiosis (Chandanie et al., 2009; Muller, 2003), suggesting that the association of root with AM fungi and fungal endophytes must be studied as a multipartite association. However the study of this co-symbiosis could be difficult, as these fungal symbionts are taxonomically distant and show different levels of host and environmental preferences. It appears that fungal endophytes are generalists and can be associated with nearly all plant species around the globe (Saikkonen et al., 1998) whereas the formation of the AM symbiosis involves a certain level of specificity (Sanders, 2003).

A symbiosis involving AM fungi and fungal endophytes is the outcome of the complex interaction between the AM fungi, fungal endophytes and plant genetics. For instance, simultaneous colonization of roots by the fungal endophyte *Trichoderma harzianum* and AM fungi *Glomus mosseae* enhanced plant growth in cucumber, but had no effect on growth of

melon (Chandanie et al., 2009; Martínez-Medina et al., 2011).

A comprehensive understanding of the mechanisms involved in the formation and function of multipartite symbioses could be used to promote the formation of efficient associations. In addition to fungal symbionts that associate roots, soil-borne fungal pathogens might colonize plant roots and cause disease in crop plants leading to massive yield reductions (Horbach et al., 2011).

A wealth of studies show that specific plant species host specific microbial communities (Berendsen et al., 2012), suggesting that the structure and function of the plant microbiome could be regulated by the genome of the plant. Plants use various mechanical or biochemical mechanisms to interact with soil microorganisms (Sarkar et al., 2009). The roots of plants have the ability to release a wide range of metabolites that modify the structure and function of their microbiome (Bakker et al., 2012; Hartmann et al., 2009).

Primary metabolites such as sugars and amino acids develop nutritional niches for soil microbes and secondary metabolites act as tools to stimulate or inhibit microbial populations (Badri et al., 2013; Collemare and Lebrun, 2011; Jones et al., 2004). The pattern of plant's secondary metabolite production can be altered by soil microbes (Badri et al., 2010; Nelson, 2004). Recent reports indicate that colonization of roots by fungal endophytes could influence the biochemistry of the roots and influence the establishment and outcome of the AM symbiosis (Peipp et al., 1997; Shoresh et al., 2005).

On the other hand, colonization of roots by AM fungi might influence the interactions between roots and fungal endophytes. The formation of the AM symbiosis involves hormonal activities similar to those used for defense against pathogens, hence, it might affect the

secondary metabolism and the mechanisms through which plants interact with other fungi, including fungal endophytes (Balestrini and Lanfranco, 2006; Cruz et al., 2008; Vierheilig et al., 2008). Altogether, the outcome of a multipartite symbiosis involving AM fungi and fungal endophytes in roots could range along a continuum from mutualism to parasitism depending on the combination of host plant and fungal species involved and the environmental conditions to which they are exposed (Jumpponen, 2001; Mandyam et al., 2012; Monzon and Azcón, 1996; Sousa et al., 2012).

Chickpea is a rotational legume and moderately well adapted to the semiarid climate of southern Saskatchewan where several cultivars of both kabuli and desi chickpea are cultivated in rotation with wheat. Lower yield of wheat is usually obtained after it follows chickpea compared with other pulse crops, such as pea and lentil. The poor rotational effect of certain chickpea crops could be, in part, attributed to the influence of its roots on the soil microbiome, in particular on the endophytic and pathogenic fungal species that potentially impact the biomass and yield of the next wheat crop. Moreover, several root diseases caused by fungal pathogens such as *Fusarium oxysporum* and *Rhizoctonia* can infect chickpea, reducing yield and negatively impacting the subsequent crop. By contrast, fungal endophytes such as *Trichoderma* sp. can improve the biomass and yield of plants (Chaeichi and Edalati-Fard, 2006; Nene et al., 1991; Rudresh et al., 2005). Cultivars of chickpea were recently reported to produce different arrays of bioactive phytochemicals within their roots and selectively promote microbial communities in the rhizosphere (Ellouze et al., 2013; Yang et al., 2012).

The selection of cultivars that promote the proliferation of beneficial AM fungi and

endophytes while inhibiting fungal pathogens in the root endosphere could improve soil health and productivity of the cropping systems.

In this thesis research, field and greenhouse experiments as well as biochemical and molecular methods were combined to generate knowledge on the association of chickpea root and soil fungal communities including:

1 - The influence of cultivars and types (desi & kabuli) of chickpea on the structure of the fungal community residing in the root endosphere of this crop plant.

2 - The role of chickpea cultivar and type on the response of this plant to colonization by AM fungi and fungal endophytes.

3 - Information on the production of phytochemicals in chickpea roots and their influence on soil-borne fungi.

4 - The influence of colonization of roots by AM fungi on the profile of roots phytochemicals and its subsequent effects on fungal endophytes and pathogens.

In chapter 2, the fungal communities associated with the roots of thirteen cultivars of chickpea were characterized. In addition, the plant growth promoting ability of important fungal endophytes and pathogens was assessed on chickpea. Chapter 3 verifies the existence of a genotypic variation in the response of chickpea to associations with AM fungi and non-AM fungal endophytes. Furthermore, chapter 3 investigates the influence of fungal endophytes on the formation and function of the AM symbiosis and verifies that the fungal symbiosis can be modified by the genotype of chickpea. The influence of root phytochemicals and the formation of AM symbiosis in chickpea on fungal endophytes and pathogens were explored in chapter 4. Chapter 5 discusses the overall results of this study.

2.0 CULTIVARS OF CHICKPEA SHAPE THE STRUCTURE OF FUNGAL COMMUNITIES IN THE ROOT ENDOSPHERE

2.1 Preface

This chapter reports the characterization of the fungal communities associated with the roots of thirteen cultivars of chickpea and reveals the existence of genotypic variation in the structure of the AM and non-AM fungal communities hosted in the root endosphere. This observation and other finding showing genotypic variations in the response of chickpea to AM fungal and non-AM fungal endophytes (Chapter 3), suggest the possibility for the selection of chickpea varieties that can develop more efficient associations with soil fungal resources.

2.2 Abstract

Crop plants regulate their microbiome impacting the health and productivity of the entire cropping system. Genetic variations among cultivars of crop species could result in the establishment of different structures and functions of the microbial communities associated with the roots. As a N_2-fixing crop, chickpea generally improves soil fertility; however, its cultivars have different impacts on subsequent crop in rotation. I conducted a 2-year field experiment with 13 cultivars of chickpea, testing the effect of cultivar on the root mycota using 454 amplicon pyrosequencing. *Funneliformis* and *Claroideoglomus* were the dominant AM fungal genera and *Fusarium* and *Alternaria* were the dominant non-AM fungal genera colonizing the endosphere of chickpea. The cultivars CDC Corrine, CDC Cory and CDC Anna had the highest fungal diversity, and in contrast CDC Alma and CDC Xena had the

lowest fungal diversity in roots. Chickpea cultivar and environmental conditions had significant effects on the structure of the root fungal community, suggesting the possibility of selecting cultivars that promote beneficial microbial environments promoting plant health and the productivity of the cropping system.

2.3 Introduction

Plant microbiome also known as “the second genome of the plant” has a crucial impact on plant health and crop productivity (Berendsen et al., 2012). In particular, the roots of plants are associated with a wide variety of soil microbial communities throughout their life. Fungi represent a diverse group of soil biota that is involved in decomposition of organic materials, promotion of plant growth, and development or suppression of disease (Bridge and Spooner, 2001). The integration of symbiotic species such as AM fungi into cropping systems could increase crop yield through improved plant health, nutrition and tolerance to abiotic stresses (Finlay, 2008). In contrast, soil-borne fungal pathogens cause a variety of diseases in crop plants leading to massive economic losses through the reduction of yield and contamination of food by various mycotoxins (Horbach et al., 2011).

The use of fungicides has long been a common practice to control soil-borne pathogens. However, agrochemicals may have non-target effects on beneficial soil microorganisms, and exacerbate the incidence of disease by reducing the diversity of the soil microbiome (Yang et al., 2012). Moreover, fungicides are often applied when symptoms are visible, which is too late to effectively prevent the negative impact of disease on crop production (Ghorbani et al., 2010). An effective approach to control soil-borne diseases is to create environmental conditions unsuitable for the plant pathogens that cause the infection (Weller, 1988). Crop

rotation is a pivotal practice preventing the buildup of pathogenic communities in soil. However, the choices of rotational crop species are usually narrow making it difficult to adopt the long rotations that effectively control the proliferation of pathogens (Bennett et al., 2012). A complementary strategy is the use of resistant plants or certain cultivars to develop healthy microbial environments.

Research on plant-microbe interactions has shown that susceptible crop varieties do not show disease symptoms when they are exposed to pathogens in specific soils, known as "disease-suppressive soils" (Weller et al., 2002). Interaction between root and soil microbes is a dynamic process in which plants employ fine-tuned mechanisms to shape the structure and function of their microbiome (Berg and Smalla, 2009; Sarkar et al., 2009). Thus, the genotypes of a plant species growing in the same soil could be associated with distinct microbial communities (Berendsen et al., 2012).

Plant roots release various bioactive substances that are able to attract or inhibit specific microbial groups and initiate symbiosis or pathogenesis (Badri et al., 2013; Bais et al., 2006). Certain plants are able to select protective microbial species or increase microbial activity to develop a disease-suppressive soil (Berendsen et al., 2012). Research has revealed that the genetic variations within a plant species can also influence the composition and function of root and soil microbiomes (Ellouze et al., 2013; Schweitzer et al., 2008). This suggests the feasibility of breeding genotypes that promote the establishment of the protective and symbiotic microbial species in the rhizosphere.

Chickpea is a high-value pulse crop that is well adapted to the semiarid climate of southern Saskatchewan where several cultivars of chickpea of both kabuli and desi types are

cultivated in rotation with wheat. Cultivars of chickpea were recently reported to produce different arrays of root phytochemicals and establish different rhizobacterial communities (Yang et al., 2012). Selection of cultivars that promote colonization of beneficial AM fungi and endophytes while inhibiting fungal pathogens in the root endosphere could improve soil health and productivity of cropping systems. Considering the intraspecific variations among the cultivars of chickpea, I hypothesized that the chickpea cultivar influences the composition of the fungal communities in the root endosphere. To test this hypothesis I conducted a 2-year field experiment and used 454 pyrosequencing of amplicon to examine the influence of chickpea cultivars on AM fungal and non-AM fungal communities of the root endosphere. Thirteen cultivars of chickpea with different phenotypic features and agronomic characteristics representing the chickpea mapping population were examined and their influence on the structure of the fungal root endophyte community was described in the field experiment reported in this chapter. In addition, a greenhouse experiment was conducted to assess the effects of important beneficial and pathogenic fungal species on chickpea growth.

2.4 Material and Methods

2.4.1 Experimental design and site description

The influence of 13 cultivars of chickpea on the fungal communities of roots was described in a field experiment. These cultivars were selected from kabuli and desi types of chickpea and had different phenotypes (Table 2.1) and genotypes (Fig. A.1) (Diapari et al., 2014) representing the mapping population. The field experiment was set up with four replicates as a randomized complete block design in 2010 and repeated in 2011 on a different location in the same field (Figs. B.1 and B.2).

Table 2.1. Main features of the cultivars of chickpea used in the field study.

Cultivar	Class	Leaf Type	Ascochyta Blight Resistance	Height (cm)	Days to Flower	Maturity[†]	Seed Weight g/1000	Seed Shape	Reference
Amit	Kabuli	Fern/Compound	Fair	46	56	L	259	Round	(Miller et al., 2002)
CDC Alma	Kabuli	Fern/Compound	Poor	41	54	L	368	Ram-head	(Thompson and Tar'an, 2014)
CDC Frontier	Kabuli	Fern/Compound	Fair	45	56	L	350	Ram-head	(Warkentin et al., 2005)
CDC Leader	Kabuli	Fern/Compound	Fair	41	55	M	389	Ram-head	(Taran et al., 2013)
CDC Luna	Kabuli	Fern/Compound	Fair	39	53	ML	369	Ram-head	(Taran et al., 2009)
CDC Orion	Kabuli	Fern/Compound	Fair	45	51	L	438	Ram-head	(Taran et al., 2011)
CDC Xena	Kabuli	Unifoliate	Very Poor	44	54	L	464	Ram-head	(Liu et al., 2003)
CDC Cabri	Desi	Fern/Compound	Fair	48	51	M	304	Plump	(Warkentin et al., 2005)
CDC Corinne	Desi	Fern/Compound	Fair	44	55	M	245	Angular	(Taran et al., 2009)
CDC Cory	Desi	Fern/Compound	Fair	48	57	M	273	Angular	(Thompson and Tar'an, 2014)
CDC Vanguard	Desi	Fern/Compound	Fair	42	53	ML	221	Plump	(Warkentin et al., 2009)
CDC Anna	Desi	Fern/Compound	Fair	40	52	L	210	Plump	(Vandenberg et al., 2003)
CDC Nika	Desi	Fern/Compound	Fair	40	50	L	320	Plump	(Vandenberg et al., 2003)

[†]Maturity: M = medium; ML = medium-late; L = late

The plots were established in wheat stubble on an Orthic Brown Chernozem soil located at the South Farm of the Semiarid Prairie Agricultural Research Centre near Swift Current, SK, Canada (latitude 50° 18' N; longitude 107° 41' W) (Table 2.2). The experimental area received more precipitation in 2010 than 2011 (Fig. 2.1). The mean temperature was below the normal in 2010, but it was approximately normal in 2011 (Fig. 2.2). Monoammonium phosphate was applied with the seed at a rate of 33 kg ha^{-1} in both years. Seeds were coated with *Mesorhizobium ciceri* Nitragin Nitrastick GC peat powder inoculant as per the instruction of the manufacturer (110 g of inoculant per 25 kg seeds) (Nitragin Inc. Brookfield, WI). Plants were seeded on 2 × 6 m plots pre-treated with the herbicides Roundup Weathermax (Monsanto Company, St. Louis, MO) and Pursuit (American Cyanamid Company, Prince-ton, NJ) at rates of 133 mL ha^{-1}, and 4.8 mL ha^{-1} respectively. The fungicides Bravo® (Syngenta Crop Protection Canada Inc., Guelph, ON, a.i. chlorothalonil) and Headline® Duo (BASF Canada Inc., Mississauga, ON, a.i. pyraclostrobin and boscalid) were sprayed. Chickpea is a susceptible crop to Ascochyta blight, a disease caused by the fungal pathogen *Ascochyta rabiei.* Ascochyta blight can dramatically affect chickpea seed quality and result in up to 96% yield loss in susceptible cultivars (Chongo et. al., 2000). Therefore, the use of fungicide is a necessary practice to control the disease in chickpea production.

2.4.2 Root sampling and processing

Ten normally growing plants were randomly selected from each plot at the mid-flowering stage. Roots were separated from the shoots using scissors and transported to the lab using a cooler, where the roots were washed and cut into 4-cm fragments.

Table 2.2. Physical and chemical characteristics of soil[†] in the experimental sites[‡]

Soil Property	2010	2011
Texture	Silt loam to loam	Silt loam to loam
pH[§]	6.5	6.5
Mineral N (kg ha^{-1})	9	8
Available K (kg ha^{-1})	326	491
Available S (kg ha^{-1})	35	15
$NaHCO_3$ extractable P (kg ha^{-1})	36	39

[†]Depth: 0-15 cm
[‡]Located at the South Farm of the Semiarid Prairie Agricultural Research Centre near Swift Current, SK Canada.
[§]1:1 soil: water

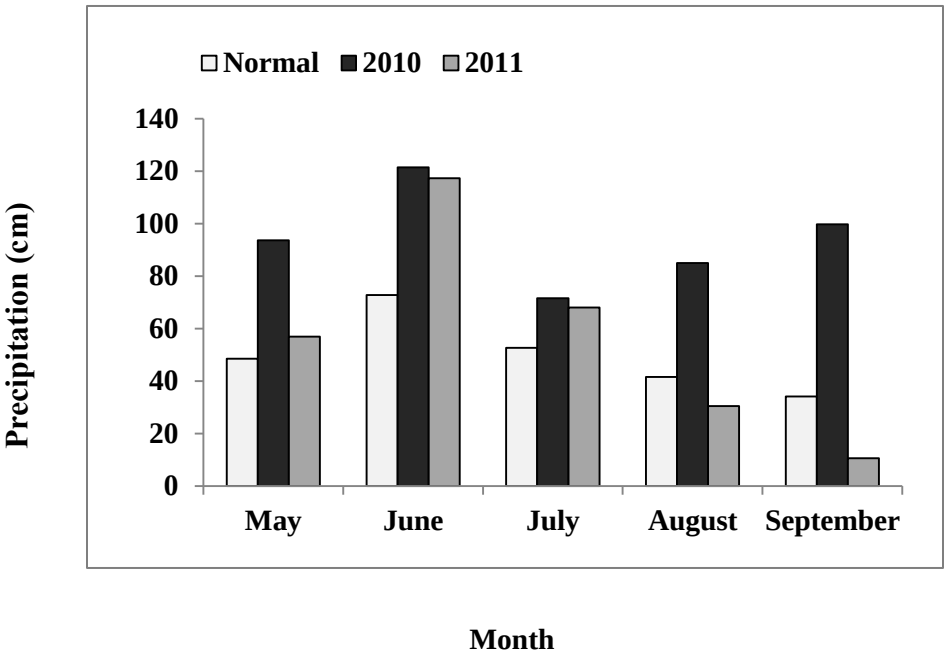


Fig. 2.1. Total precipitation during the growing season in 2010, 2011 and normal conditions (average for 1981–2010), Swift Current, SK– Data received from Environment Canada.

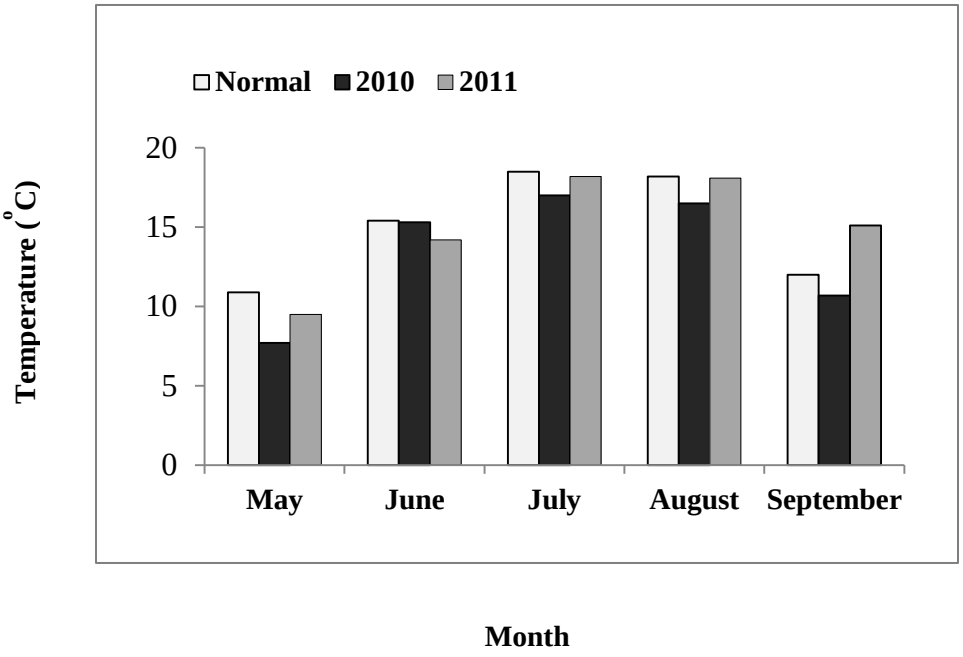


Fig. 2.2. Mean temperature during the growing season in 2010, 2011 and normal conditions (average for 1981–2010), Swift Current, SK– Data received from Environment Canada.

A subsample of the roots was stored at -20°C until molecular analysis of the fungal communities of the root endosphere. Another subsample was cleared and stained to visually assess the level of AM fungal colonization of plant roots.

2.4.3 Assessment of root colonization by AM fungi

Root samples were cleared by boiling in KOH solution (100 g L^{-1}) for 12 min and staining them in a boiling solution of Schaeffer black ink and vinegar (50 g L^{-1}) for 3 min (Vierheilig et al., 1998). The extent of root length colonized by AM hyphae, arbuscules and vesicles was determined (McGonigle et al., 1990). One hundred intersects per sample were examined at 400X magnification under a compound microscope.

2.4.4 DNA extraction, PCR amplification, purification and pyrosequencing

Fresh root samples of 50 mg were freeze dried and milled with a tungsten bead in a micro-centrifuge tube by vigorously shaking for 3 min. Total DNA was extracted from each root sample using Qiagen Plant DNeasy kits (QIAGEN, Mississauga, ON) according to the manufacturer's instructions. The 18S ribosomal DNA sequences were amplified using NS1/NS4 and AMV4.5-NF/AMDGR primer sets (Table 2.3) to analyze the structure of the AM fungal community of the root endosphere (Lumini et al., 2010). Nested PCR was to generate sufficient numbers of amplicons using AMV4.5-NFand AMDGR primer set. In the first polymerase chain reaction (PCR), each template was amplified in 20 μL reactions containing 16 μL of Platinum PCR Supermix (Invitrogen, Mississauga, ON), 0.2 μL NS1, 0.2 μL NS4 (20 mM solutions), 2.6 μL of ultrapure water, and 1.0 μL of template DNA. The final concentration of the reagent mix was 0.0165 U/mL Taq DNA Polymerase, 1.24 mM $MgCl_2$, 16.5 mM Tris–HCl (pH 8.4), 41.25 mM KCl, 165 mM (each) dNTP, and 0.2 mM

(each) primer per 10 mL volume. All the amplifications were conducted in a Veriti 96-well fast Thermal Cycler (Applied Biosystems, Foster City, CA). The PCR was initiated with a denaturation step of 94 ^{o}C for 3 min followed by 35 cycles at 94^{o}C for 45 s of denaturation, 51^{o}C for 45 s of annealing, 72 s for 1 min of elongation and a final elongation at 72^{o}C for 7 min. The PCR products were diluted (1:20) and used for the second PCR. The DNA templates were amplified in 20 µL reactions containing 16 µL of Platinum PCR Supermix (Invitrogen Inc.), 0.2 µL AMV4.5-NF, 0.2 µL AMDGR (20 mM solutions), 2.6 µL of ultrapure water and 1.0 µL of diluted PCR product. The PCR conditions were 10 min for denaturation at 95°C, 35 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 1 min of elongation at 72°C, followed by 9 min of final elongation at 72°C. The internal transcribed spacer (ITS) region of the template DNA samples was also amplified using ITS1F/ITS2 primer sets (Table 2.3) to analyze the structure of the non-AM fungal community of the root endosphere (Buée et al., 2009). The 30 µL PCR reactions contained 22.5 µL of Platinum PCR Supermix (Invitrogen, Mississauga, ON) 0.3 µL ITS1F, 0.3 µL ITS2 (20 mM solutions) and 3.9 µL of ultrapure water and 3.0 µL of template DNA. The PCR conditions were 94^{o}C for 4 min, 35 cycles of 30 s at 94^{o}C (denaturation), 50^{o}C for 1 min (annealing) and 72^{o}C for 90 s (elongation), followed by 10 min at 72^{o}C. The amplicons were barcoded with one of 32 Roche's Multiplex Identifiers. The PCR products were purified using the AMPure PCR Purification Kit (Agencourt Bioscience, Beverly, MA), analyzed by Agilent Bioanalyzer (Agilent Technologies, Edmonton, AB) for quality control and sequenced using 454 GS FLX amplicon pyrosequencing at the Laboratory for Advanced Genome Analysis at the Vancouver Prostate Centre, Vancouver, BC.

Table 2.3. Primer, adaptors and key sequences used for PCR amplification of fungal DNA used for molecular analyses of the roots-associated fungal communities in 13 cultivars of chickpea

Primer	Sequence (5´ to 3´)	Amplicon size (bp)	Reference
ITS-1F	CTTGGTCATTTAGAGGAAGTAA	400	(Buée et al., 2009)
ITS2	GCTGCGTTCTTCATCGATGC		
NS1	GTAGTCATATGCTTGTCTC	1100	(Lee et al., 2008)
NS4	CTTCCGTCAATTCCTTTAAG		
AMV4.5NF	AAGCTCGTAGTTGAATTTCG	350	(Lumini et al., 2010)
AMDGR	CCCAACTATCCCTATTAATCAT		
Adaptor / Key (Forward)	CCATCTCATCCCTGCGTGTCTCCGAC / TCAG	-	-
Adaptor / Key (Reverse)	CCTATCCCCTGTGTGCCTTGGCAGTC / TCAG		

2.4.5 Bioinformatics analysis

In total, 502,383 raw sequence reads were obtained. The average lengths of the ITS and 18S rDNA sequence reads were 255 and 278 bp respectively. Unfortunately, one replicate of raw sequences from each year was lost due to a technical issue during sequencing. The raw sequences were processed using Mothur V.1.28.0. The command 'trim.seqs' was used to screen the short and low quality sequences. Any ITS sequences shorter than 150 bp in length were removed from the dataset. The 18S rDNA sequence reads between 230 and 250 bp in length were considered for further analysis (Lumini et al., 2010). All sequences containing ambiguous base pairs, excessively long homopolymers and chimeras, and low quality reads (average score ≥ 25) were excluded from downstream analysis. The command 'unique.seqs' was used to reduce the reads to unique sequences. The chimeric sequences were detected with the command "chimera.uchime" and excluded with command "remove.seqs". The sequences of each sample were subsampled using the command "sub.sample" to normalize the number of sequences in the OTU dataset (Table D.1). The clean ITS and 18S rDNA sequences were aligned against SILVA (http://www.arb-silva.de) and ITS sequences against UNITE (http://unite.ut.ee/repository.php) databases, respectively (Kõljalg et al., 2013) using the command "align.seqs". The poorly aligned sequences were detected using the command "screen.seqs" and removed from the dataset with the commands "filter.seqs" (vertical=T, trump=.) and "remove.seqs". The sequence errors were excluded with the command "pre.cluster". The sequences were classified using the command "classify.seqs". Any sequences that belong to phyla other than Glomeromycota (arbuscular mycorrhizal fungi) were excluded from the 18S rDNA dataset with the command "get.lineage". A distance

matrix was generated with the command “dist.seqs”. The sequences were clustered into operational taxonomic units (OTU) based on 97% similarity using a furthest neighbor clustering algorithm with the command “cluster.seqs”. The command “split.abund” was used to remove the OTU clusters containing only one or two sequences (singletons and doubletons). The representative sequences for each OTU were identified with the command “get.oturep”. The largest sequence of each OTU was used for BLAST search against the NCBI nucleotide collection database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Matches with more than 94% similarity and 90% query coverage were considered. Hits with BLAST scores < 200 were considered to represent unknown or unclassified fungi. The number of reads for each OTU was obtained for each sample using command “classify.otu” and used to form the database of the relative abundance of the OTUs. This data was used to analyze the structure of the communities of AM fungi and non-AM fungi. Chao richness and Shannon diversity indices were estimated for each plot (In subsampled and non-subsampled data) using the command “collect.single”.

2.4.6 Collection and identification of fungal isolates

Forty-nine fungal isolates were received from the fungal collection of Semiarid Prairie Agricultural Research Centre in Swift Current, SK. The fungal isolates had been recovered from the roots of chickpea, pea, lentil and wheat grown at the same research farm. These isolates were used as references to assess the potential influence of the non-AM fungal root colonizing species on chickpea growth found in the field study. Isolates were cultured on the half-strength PDA (Potato Dextrose Agar) medium (Chet and Baker, 1980). The actively growing hypha were collected from the edge of the plates after 72 h and used for molecular

identification of the fungal species. Fungal hyphae were transferred into 2 mL sterile micro-centrifuge tubes, freeze dried by liquid nitrogen and ground. Genomic DNA was extracted from the fungal hyphae using Qiagen DNeasy Plant Mini Kit according to manufacturer's instructions and stored at - 20ºC. The DNA was PCR-amplified using universal fungal primer set ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS-4 (TCCTCCGCTTATTGATATGC). PCR reactions consisted of 17 µL of Platinum Supermix (Invitrogen, Mississauga, ON), 3 µL of distilled water, 1 µL of each primers and 2 µL of DNA template. PCR conditions were set up as 30 s at 95°C, 40 s at 60°C, 40 s at 72°C, 5-min extension at 72°C, 4°C. Amplified ITS region (about 600bp) PCR products were purified using AMPure DNA purification kit (Agencourt Bioscience, Beverly, MA) and sequenced at Plant Biotechnology Institute after quality control. Sequences were blasted against NCBI Gene Bank database for the identification of fungal species.

2.4.7 Greenhouse experiment

Seeds of the chickpea cultivar CDC Frontier were surface sterilized by successive immersion in 95% ethanol for 30 s, sterile distilled water for 30 s, 2.5% Javex solution (sodium hypochlorite) for 2 min, and sterile distilled water for 2 min. The seeds were kept on a sterile moist filter paper in Petri dishes for 72 h at 25^{o}C in the dark to germinate. Pots were planted with seven germinated seeds. Seed roots and the underneath soils were treated with 1.5 g of a peat-based *Mesorhizobium ciceri* inoculant (Nitragin Nitrastick GC®, Nitragin Inc., Brookfield, WI). Designated plants were inoculated with four plugs of actively growing fungal mycelium cut from PDA medium (Chet and Baker, 1980) and planted in 4 L pots containing pasteurized calcined clay (90^{o}C, 1 h). A control was inoculated with sterile agar

plugs and rhizobial inoculant. The pots were arranged in a randomized complete block design and replicated four times in the greenhouse. Plants were kept under 16/8 hours at 24/16^{o}C temperatures day/night in the greenhouse. Supplemental lighting was applied during the daytime using high intensity discharge lamps (Alto 400 watt low pressure sodium, Philips, Somerset, NJ). Plants were watered with distilled water and fertilized with modified Long Ashton nutrient solution containing (in mg L^{-1}) 554 KCl, 200 $NaH_2PO_4 \cdot H_2O$, 244 $MgSO_4$, 520 $CaCl_2 \cdot H_2O$, 1.7 $MnSO_4$, 0.25 $CuSO_4 \cdot 5H_2O$, 0.30 $ZnSO_4 \cdot 7H_2O$, 3.0 H_3O_3, 5.0 NaCl, 0.09 $(NH_4)_6$ $Mo_7O_{24} \cdot {}_4H_2O$ and 32.9 NaFe-EDTA (Hewitt, 1966). Plants were kept under water limiting conditions and the soil moisture was adjusted weekly by weighing the pots. Plants were harvested at maturity and the dried shoot biomass was measured.

2.4.8 Statistical Analysis

All the data collected in this study was tested for normality using the Shapiro-Wilk's test and non-normal data was square root transformed before analysis.

2.4.8.1 Field experiment and fungal community analyses

Two-way ANOVA was conducted to test the significance of the effect of cultivar, year and their interaction on the level of root colonization. Tukey-Kramer HSD post hoc test was used to assess the significance of difference between means in this analysis (R package V.2.15.2). The analyses related to the pyrosequencing work were completed in three replicates for both 2010 and 2011 due to missing data. The two years of the relative abundance data were combined and the community analyses were completed in six replicates (n = 6). A two-way factorial permutation based multivariate analysis of variance (Per-MANOVA) was performed on the relative abundance of 5 dominant AM and 14 dominant

non-AM fungal OTUs (both as collective and separate communities), using SØrensen distances to test the effect of cultivar, year and their interaction on the structure of fungal communities of the root endosphere. Detrended correspondence analysis (DCA) was performed on the same data to determine fungal communities associated with different cultivars of chickpea. Non-metric multidimensional scaling (NMDS) was performed to find a relationship between the cultivars of chickpea based on agronomic performances (PC-ORD V. 4.34). One-way analysis of variance (ANOVA) was performed to test the significance of the effect of cultivar on richness and diversity indices. Tukey-Kramer HSD post hoc test was used to assess the significance of difference between means (R package V.2.15.2). The significance of the effects of chickpea type (desi and kabuli) on the richness and diversity of the fungal communities was tested by orthogonal contrasts, using JMP V.6 (SAS Institute Inc. Cary, NC USA). Spearman correlation test was performed to detect relationships between the fungal genera, and between fungal genera and plant performance (R package V.2.15.2).

2.4.8.2 Greenhouse study

One-way ANOVA was performed to test the significance of non-AM fungal endophytes and pathogens on plant biomass. The biomass of plants inoculated with different non-AM fungi were compared to control plant biomass using Dunnett's test in R package multcomp. A *p*-value of less than 0.05 was indicative of statistical significance.

2.5 Results

2.5.1 Sequence analysis of field samples

A total of 139,88218S rDNA and 109,163 ITS sequence reads were obtained from AM and non-AM fungi respectively after the cleaning and removal of short, ambiguous, low-quality and chimeric sequences. The average length of ITS read sequences was 255 bp and that of 18S rDNA reads was 278 bp. The reads were further clustered into 44 AM and 105 non-AM fungal OTUs (Figs. C.1 and C.2). *Glomus, Claroideoglomus, Funneliformis, Paraglomus, Rhizophagus* and *Diversispora* were the AM fungal genera present in the roots of chickpea. *Funneliformis* and *Claroideoglomus* were the most abundant genera, accounting for more than 50% of the total sequence reads obtained in 2010 and 2011. The relative abundance of *Diversispora, Glomus* and *Paraglomus* were significantly different ($p < 0.05$) between the two years (Fig. 2.3 a). The non-AM fungal community was dominated by the genera *Fusarium, Alternaria, Cryptococcus, Acremonium, Cladosporium, Bionectria, Mortierella, Rhodotorula, Chaetomium, Penicillium, Pyrenophora, Microdochium, Trichoderma* and *Paecilomyces. Fusarium* and *Alternaria* were the dominant non-AM fungal genera in chickpea roots encompassing the most deleterious soil-borne fungal pathogens. The relative abundance of *Alternaria, Paecilomyces, Microdochium, Cladosporium* and *Acremonium* significantly varied ($p < 0.05$) between two years (Fig. 2.3 b). *Fusarium* was more abundant in 2010. *Trichoderma* was more abundant in 2011. The genus *Fusarium* includes important pathogens that associate with various crop plants. The composition of root fungi varied for AM ($p < 0.02$) and non-AM ($p < 0.04$) fungal genera in different cultivars of chickpea (Fig. 2.4 and 2.5). The relative abundance of *Claroideoglomus, Diversispora* and unclassified genera varied in different cultivars. *Claroideoglomus* was abundant in CDC Anna, but was scarce in CDC Nika. *Diversispora* was abundant in CDC Corrine, CDC Frontier and CDC Nika (Fig. 2.4). *Fusarium* was abundant in CDC Nika, but it was scarce in CDC Corrine.

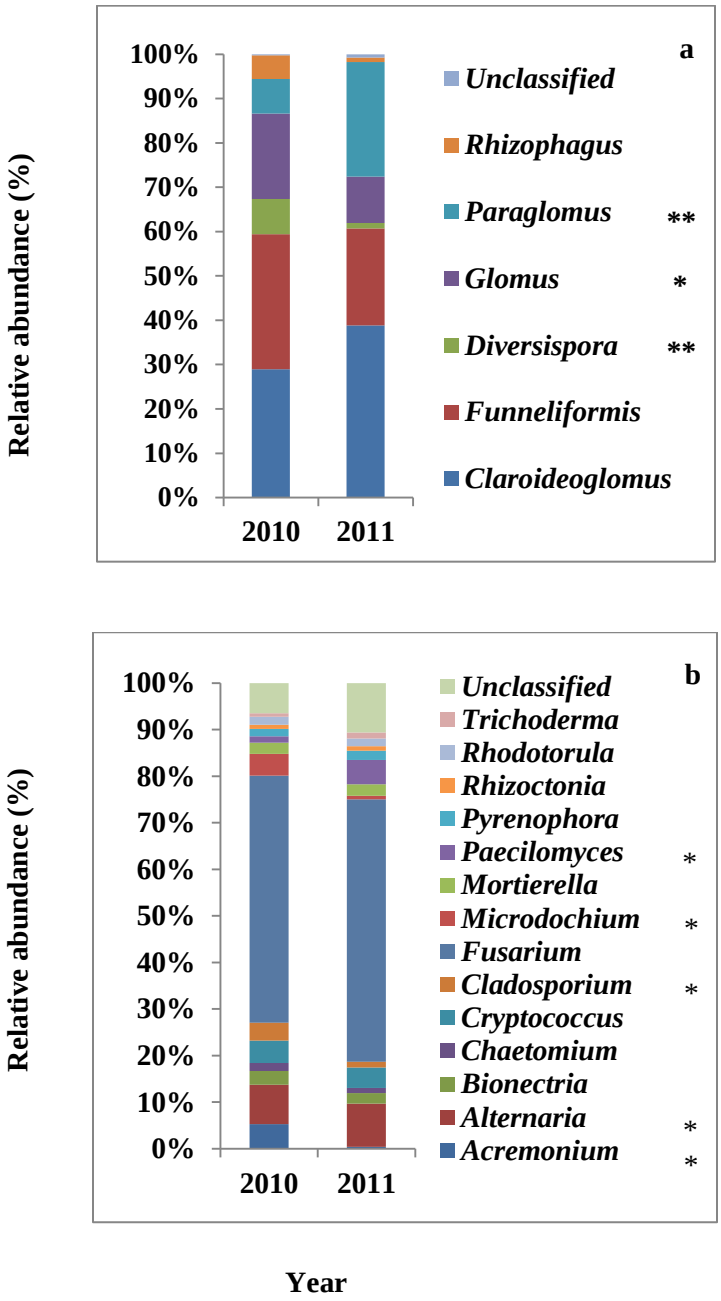


Fig. 2.3. Relative abundance of arbuscular mycorrhizal (AM) (a) and non-AM (b) fungal genera in the roots of chickpea, detected by pyrosequencing (n = 3). Data is some of the relative abundance of fungal genera in 13 chickpea cultivars including Amit, CDC Anna, CDC Alma, CDC Frontier, CDC Luna, CDC Leader, CDC Vanguard, CDC Xena, CDC Corrine, CDC Cory, CDC Orion, CDC Cabri and CDC Nika. Significant differences between years are indicated by * ($p \leq 0.05$) and ** ($p \leq 0.01$). Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK. NS1/NS4 and AMV4.5-NF/AMDGR primer sets were used for PCR amplification of fungal DNA (Nested PCR).

2.5.2. Fungal Community Structure

Cultivars of chickpea and the year they were grown significantly influenced the structure of root fungal communities. The interaction between cultivar and year was not significant indicating that the cultivars have similar effects in different environmental conditions (Table 2.4). The fungal community of CDC Anna was different from that of nine cultivars and that of CDC Orion was different from that of eight cultivars. CDC Luna and CDC Nika were associated with different fungal communities in the root endosphere (Table 2.5) (Fig. 2.6).

2.5.3. Richness and diversity indices

Chickpea cultivar had a significant influence on the richness and diversity indices of AM and non-AM root fungal communities (Tables 2.6 and D.2). CDC Corrine had the highest level of richness and diversity of both AM fungal and non-AM fungal communities. In contrast, CDC Alma and CDC Xena had the lowest richness and diversity of non-AM fungal and AM fungal communities respectively. Overall fungal richness and diversity were higher in the roots of desi than kabuli chickpeas (Table 2.7).

2.5.4. Relationships between root fungal communities and plant performance

The abundance of *Fusarium* and *Glomus* in roots was negatively correlated with the yield of cultivars of chickpea (Fig. 2.7). *Fusarium* is a fungal genus containing several pathogenic species causing disease and reducing the biomass of many plant species. The relative abundance of *Paraglomus* and *Trichoderma* were correlated with high yield, harvest index

and seed weight. In contrast *Fusarium, Glomus* and *Alternaria* were negatively correlated with yield, harvest index and seed weight (Table 2.8 and Fig. 2.7).

2.5.5. Root colonization

Colonization of root by AM fungi significantly varied in the cultivars of chickpea ($p < 0.0001$) and root colonization was different in 2010 and 2011. However the interaction of chickpea cultivars and year was not significant (Table 2.9). CDC Cory and CDC Leader had higher levels of colonization than other cultivars except CDC Frontier and CDC Frontier had a higher level of root colonization than CDC Nika (Fig. 2.8).

2.5.6 Effects of non-AM fungi on chickpea growth in the greenhouse assay

The non-AM fungal isolates indigenous to the South Farm had a significant effect on the biomass of chickpea (Table 2.10). The non-AM fungal isolates influenced chickpea biomass ranging from positive to neutral and negative (Table 2.11 and Fig. 2.9). Three fungal isolates including *Trichoderma harzianum, Geomyces vinaceus* and *Mortierella alpina* significantly increased the biomass of chickpea. However, several *Fusarium* isolates with ITS regions similar to that of *Fusarium redolence, Fusarium solani* and *Fusarium oxysporum* decreased the biomass of chickpea.

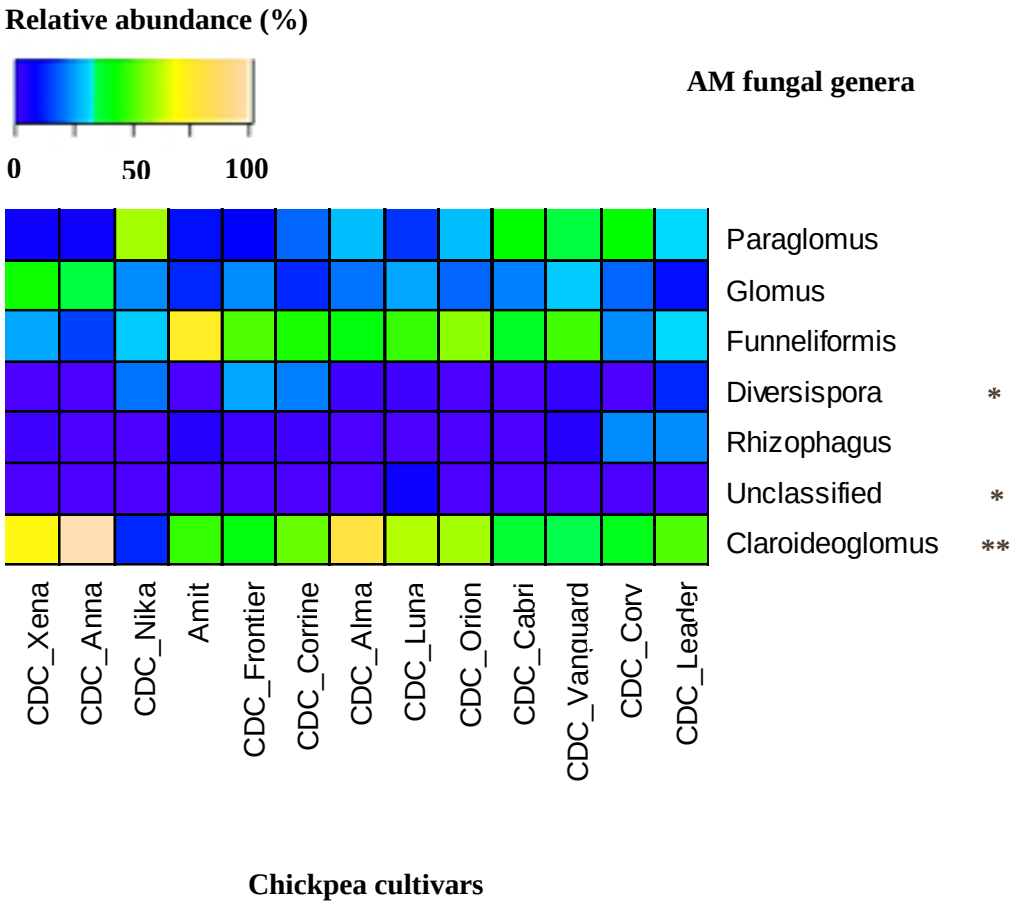


Fig. 2.4. Relative abundance of arbuscular mycorrhizal (AM) fungal genera associated with the roots of 13 cultivars of field-grown chickpea, detected by pyrosequencing (n = 6). Data is the average of the relative abundance of AM fungal genera over two years. Significant differences between cultivars are indicated by * ($p \leq 0.05$) and ** ($p \leq 0.01$). Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011. NS1/NS4 and AMV4.5-NF/AMDGR primer sets were used for PCR amplification of fungal DNA (Nested PCR).

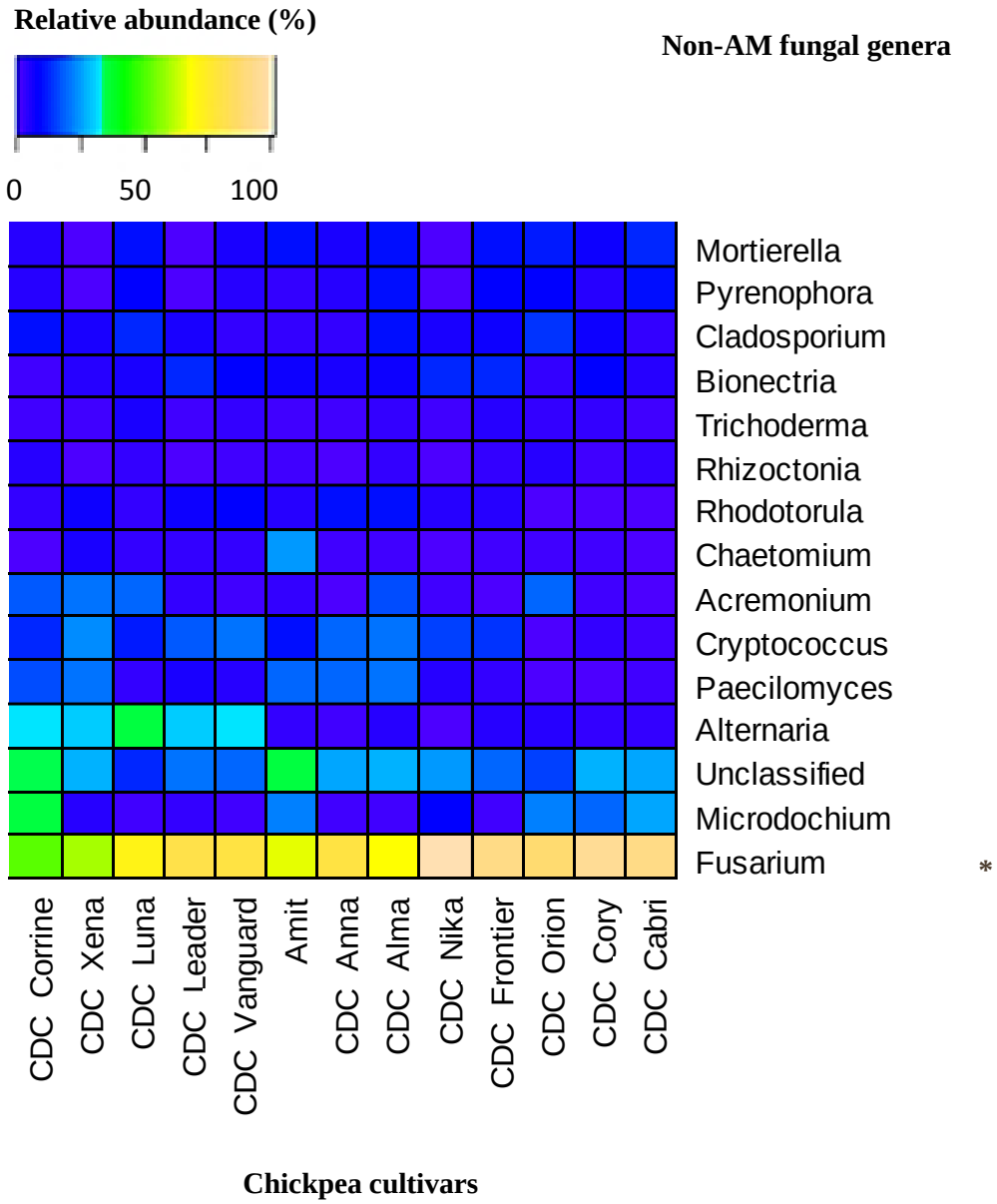


Fig. 2.5. Relative abundance of non-arbuscular mycorrhizal (AM) fungal genera associated with the roots of 13 cultivars of field-grown chickpea, detected by pyrosequencing (n = 6). Data is the average of the relative abundance of non-AM fungal genera over two years. Significant differences between cultivars are indicated by * ($p \leq 0.05$). Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011. ITS1F/ITS2 primer set was used for PCR amplification of fungal DNA.

Table 2.4. Effects of cultivar[†] and year on the structure of root-associated fungal communities in field-grown chickpea. The fungal communities consisted of the relative abundance of AM[‡] and non-AM[§] genera detected by pyrosequencing[¶].

Source	d.f.	SS	MS	F value	*p* value
Cultivar	12	9.6995	0.80829	1.279	0.0442*
Year	1	1.8227	1.8227	2.8842	0.0034**
Cultivar*Year	12	6.8568	0.5714	0.90419	0.7453
Residual	52	32.861	0.63195		
Total	77	51.24			

Significant effects are indicated by * ($p \leq 0.05$) and ** ($p \leq 0.01$) according to PerMANOVA ($n = 6$).

[†]Chickpea cultivars include Amit, CDC Anna, CDC Alma, CDC Frontier, CDC Luna, CDC Leader, CDC Vanguard, CDC Xena, CDC Corrine, CDC Cory, CDC Orion, CDC Cabri and CDC Nika.

[‡]Arbuscular mycorrhizal: Consisting of genera *Glomus, Claroideoglomus, Funneliformis, Paraglomus, Rhizophagus* and *Diversispora.*

[§]Consisting of genera *Fusarium, Alternaria, Cryptococcus, Acremonium, Bionectria, Microdochium, Trichoderma Paecilomyces, Mortierella, Rhodotorula, Chaetomium, Cladosporium, Penicillium,* and *Pyrenophora.*

[¶]Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011. Primer sets used for PCR amplification of fungal DNA were ITS1F/ITS2 (Non-AM genera), and NS1/NS4 and AMV4.5-NF/AMDGR (AM genera).

Table 2.5. Pairwise comparison of the structure of root-associated fungal communities in field-grown chickpea cultivars. The fungal communities consisted of the relative abundance of AM[†] and non-AM[‡] genera over two years, according to pyrosequencing[§].

Cultivar	Amit	CDC Alma	CDC Anna	CDC Cabri	CDC Corinne	CDC Cory	CDC Frontier	CDC Leader	CDC Luna	CDC Nika	CDC Orion	CDC Vanguard	CDC Xena
Amit	-	0.28	0.12	0.21	0.53	0.59	0.15	0.61	0.47	0.2	0.02	0.09	0.62
CDC Alma	0.28	-	0.03*	0.46	0.35	0.29	0.16	0.7	0.19	0.62	0.01*	0.46	0.65
CDC Anna	0.12	0.03*	-	0.01*	0.01*	0.01*	0.009**	0.02*	0.16	0.009**	0.01*	0.01*	0.06
CDC Cabri	0.21	0.46	0.01*	-	0.42	0.39	0.37	0.5	0.06	0.8	0.02*	0.77	0.43
CDC Corinne	0.53	0.35	0.01*	0.42	-	0.69	0.91	0.94	0.16	0.44	0.17	0.44	0.96
CDC Cory	0.59	0.29	0.01*	0.39	0.69	-	0.44	0.69	0.22	0.13	0.04*	0.32	0.65
CDC Frontier	0.15	0.16	0.009**	0.37	0.91	0.44	-	0.84	0.14	0.16	0.09	0.36	0.86
CDC Leader	0.61	0.7	0.02*	0.5	0.94	0.69	0.84	-	0.4	0.19	0.05	0.65	0.96
CDC Luna	0.47	0.19	0.16	0.06	0.16	0.22	0.14	0.4	-	0.04*	0.01*	0.18	0.48
CDC Nika	0.2	0.62	0.009**	0.8	0.44	0.13	0.16	0.19	0.04*	-	0.02	0.54	0.26
CDC Orion	0.02*	0.01	0.01*	0.02*	0.17	0.04*	0.09	0.05	0.01*	0.02	-	0.01*	0.03*
CDC Vanguard	0.09	0.46	0.01*	0.77	0.44	0.32	0.36	0.65	0.18	0.54	0.01*	-	0.56
CDC Xena	0.62	0.65	0.06	0.43	0.96	0.65	0.86	0.96	0.48	0.26	0.03*	0.56	-

[†]Arbuscular mycorrhizal: Consisting of genera *Glomus, Claroideoglomus, Funneliformis, Paraglomus, Rhizophagus* and *Diversispora*.

[‡]Consisting of genera *Fusarium, Alternaria, Cryptococcus, Acremonium, Bionectria, Microdochium, Trichoderma Paecilomyces, Mortierella, Rhodotorula, Chaetomium, Cladosporium, Penicillium,* and *Pyrenophora.*

Significant differences are indicated by * ($p \leq 0.05$) and ** ($p \leq 0.01$) according to PerMANOVA ($n = 6$).

[§]Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011. Primer sets used for PCR amplification of fungal DNA were ITS1F/ITS2 (Non-AM genera), and NS1/NS4 and AMV4.5-NF/AMDGR (AM genera).

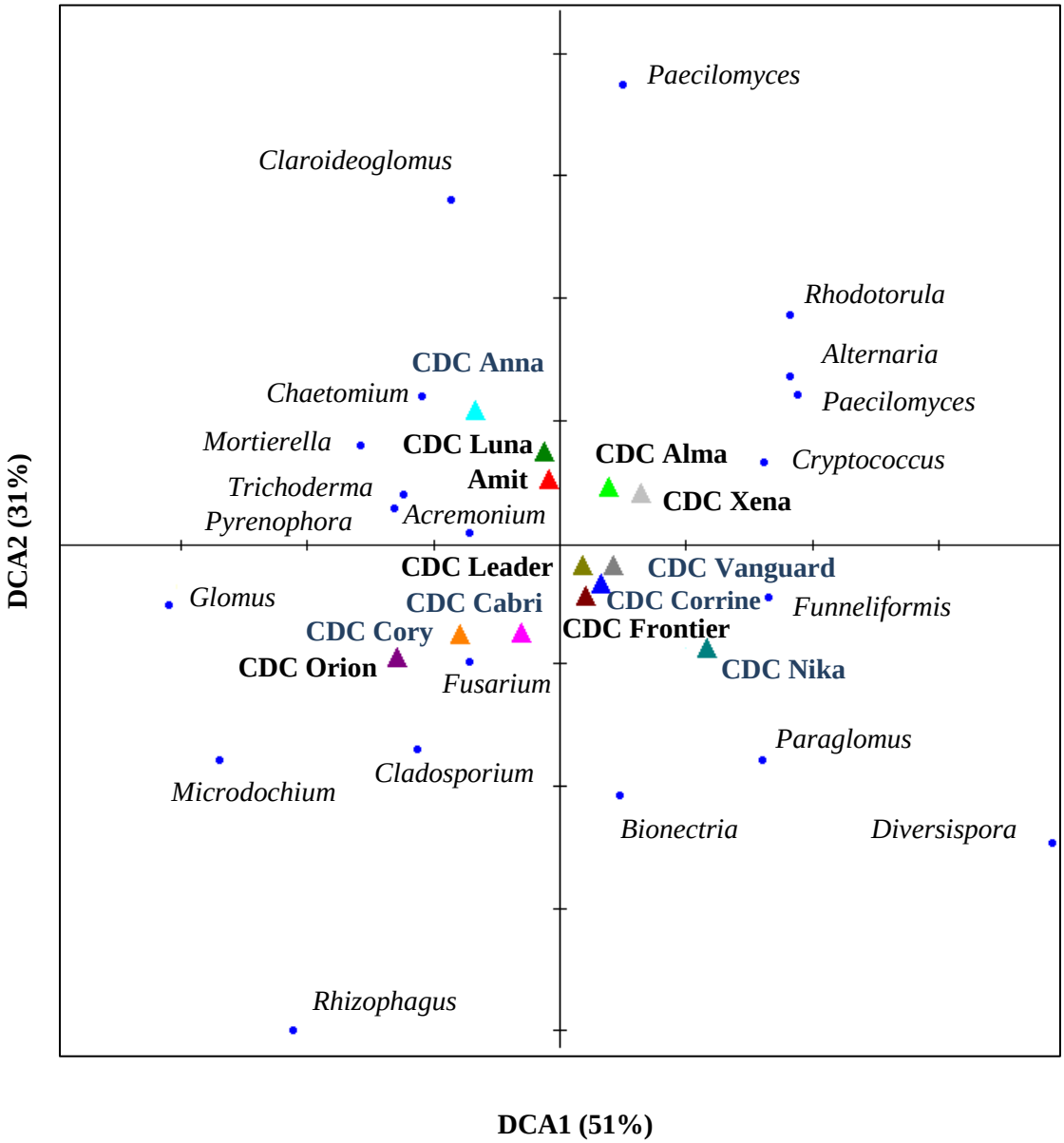


Fig. 2.6. Relationship between the root-associated fungal communities and field-grown chickpea cultivars, according to detrended correspondence analysis (DCA). Data points are means (n = 6) of the relative abundance of arbuscular mycorrhizal (AM) and non-AM fungal genera detected by pyrosequencing, over two years. Kabuli type cultivars are indicated in black and desi type cultivars are indicated in gray. Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011. Primer sets used for PCR amplification of fungal DNA were ITS1F/ITS2 (Non-AM genera), and NS1/NS4 and AMV4.5-NF/AMDGR (AM genera).

Table 2.6. Chao richness and Shannon diversity indices[†] of AM[‡] and non-AM[§] fungal communities associated with the roots of field-grown chickpea cultivars over two years, detected by pyrosequencing.

Chickpea		**Chao**	**Shannon**	**Chao**	**Shannon**
Genotype	*Type*	*AM community*		*Non-AM community*	
CDC Corrine	Desi	736 a	4.48 a	3365 a	6.69 a
CDC Cory	Desi	686 ab	4.33 ab	3018 b	6.63 a
CDC Anna	Desi	615 cd	4.29 ab	2300 d	6.52 ab
CDC Cabri	Desi	587 d	4.21 ab	2622 c	6.55 ab
CDC Vanguard	Desi	644 bc	4.37 a	3084 ab	6.58 a
CDC Nika	Desi	430 f	4.15 ab	1705 f	6.01 bc
Amit	Kabuli	515 e	4.12 ab	2189 de	6.23 abc
CDC Leader	Kabuli	513 e	4.21 ab	2852 bc	6.33 abc
CDC Orion	Kabuli	660 bc	4.27 ab	2031 de	6.01 bc
CDC Frontier	Kabuli	649 bc	4.40 a	2314 d	6.31 abc
CDC Alma	Kabuli	396 f	3.92 b	2086 c	6.50 ab
CDC Luna	Kabuli	664 bc	4.43 a	1934 ef	6.01 bc
CDC Xena	Kabuli	615 cd	4.40 a	1966 f	5.88 c

Data are presented as means (n = 6). Within a column, Means followed by different letters are significantly different at $p \leq 0.05$.

[†]Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011. Primer sets used for PCR amplification of fungal DNA were ITS1F/ITS2 (Non-AM community), and NS1/NS4 and AMV4.5-NF/AMDGR (AM community).

[‡]Arbuscular mycorrhizal: Consisting of genera *Glomus, Claroideoglomus, Funneliformis, Paraglomus, Rhizophagus* and *Diversispora.*

[§]Consisting of genera *Fusarium, Alternaria, Cryptococcus, Acremonium, Bionectria, Microdochium, Trichoderma Paecilomyces, Mortierella, Rhodotorula, Chaetomium, Cladosporium, Penicillium,* and *Pyrenophora.*

Table 2.7. Chao richness and Shannon diversity indices[†] of AM[‡] and non-AM[§] fungal communities associated with the roots of two types of field-grown chickpea over two years, detected by pyrosequencing.

Chickpea type	Chao	Shannon	Chao	Shannon
	AM community		*Non-AM community*	
Desi	616 a	4.29 a	2676 a	6.50 a
Kabuli	532 b	4.26 b	2242 b	6.16 b

Data are presented as means (n = 39). Within a column, Means followed by different letters are significantly different at $p \leq 0.05$.

[†]Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011. Primer sets used for PCR amplification of fungal DNA were ITS1F/ITS2 (Non-AM community), and NS1/NS4 and AMV4.5-NF/AMDGR (AM community).

[‡]Arbuscular mycorrhizal: Consisting of genera *Glomus, Claroideoglomus, Funneliformis, Paraglomus, Rhizophagus* and *Diversispora*.

[§]Consisting of genera *Fusarium, Alternaria, Cryptococcus, Acremonium, Bionectria, Microdochium, Trichoderma Paecilomyces, Mortierella, Rhodotorula, Chaetomium, Cladosporium, Penicillium,* and *Pyrenophora.*

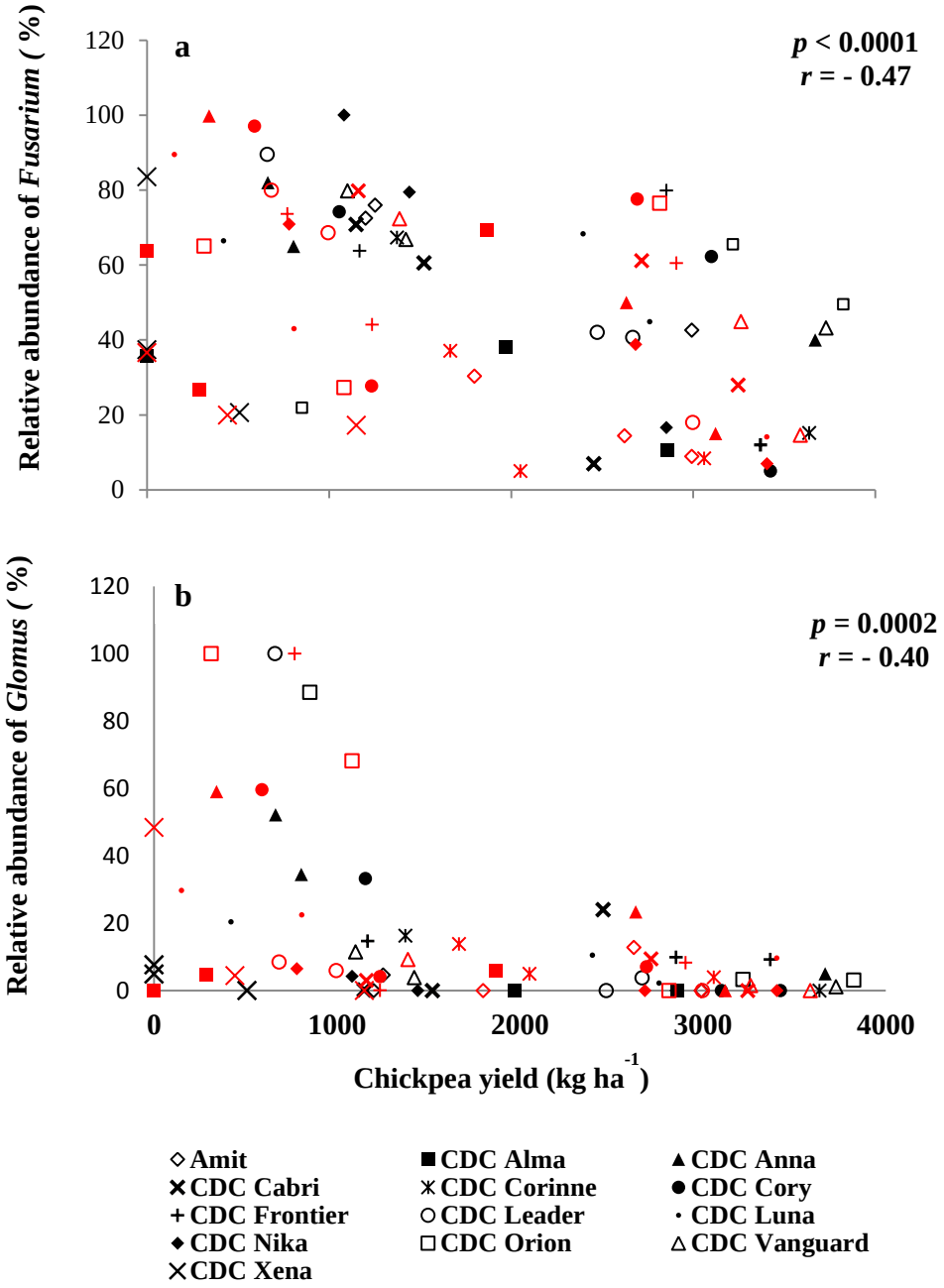


Fig. 2.7. Spearman correlations between yield and the relative abundance of *Fusarium* (a) and *Glomus* (b) associated with the roots of 13 cultivars of field-grown chickpea (*N* = 78). Data is the relative abundance of the fungal general over two years detected by pyrosequencing. Primer sets used for PCR amplification of fungal DNA were ITS1F/ITS2 (*Fusarium*), and NS1/NS4 and AMV4.5-NF/AMDGR (*Glomus*). Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011. Data in 2010 is in red and in 2010 is in black.

Table 2.8. Significant relationships[†] between the agronomic performance of field-grown chickpea cultivars[‡] and the relative abundance of root-associated fungal genera, over two years, detected by pyrosequencing[§].

Genus	Agronomic performance	*p*-value	r
Glomus	Mature biomass	0.01**	0.28
Glomus	1000 Seed weight	0.008**	- 0.29
Glomus	Harvest Index	0.0003***	- 0.39
Paraglomus	1000 Seed weight	0.007**	0.29
Paraglomus	Harvest Index	0.01**	0.28
Paraglomus	Yield	0.014*	0.27
Rhizophagus	Mature biomass	0.009**	0.29
Fusarium	1000 Seed weight	0.0003***	- 0.39
Fusarium	Harvest Index	0.0001***	- 0.43
Alternaria	1000 Seed weight	0.04*	- 0.26
Alternaria	Harvest Index	0.008**	- 0.29
Alternaria	Yield	0.008**	- 0.29
Trichoderma	1000 Seed weight	0.004**	0.31
Trichoderma	Harvest Index	0.002**	0.34
Trichoderma	Yield	0.0001***	0.41

*Significant at $p \leq 0.05$
**Significant at $p \leq 0.01$
***Significant at $p \leq 0.001$
[†]Data presented as Spearman correlations across 13 cultivars of chickpea ($N = 78$). Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011.
[‡]Consisting of Amit, CDC Anna, CDC Alma, CDC Frontier, CDC Luna, CDC Leader, CDC Vanguard, CDC Xena, CDC Corrine, CDC Cory, CDC Orion, CDC Cabri and CDC Nika.
[§]Primer sets used for PCR amplification of fungal DNA were ITS1F/ITS2 (*Fusarium, Alternaria* and *Trichoderma*), and NS1/NS4 and AMV4.5-NF/AMDGR (*Glomus* and *Paraglomus*).

Table 2.9. Effects of cultivar[†] and year on the level of root colonization by AM[‡] fungi in field-grown[§] chickpea (n = 4).

Source	d.f.	SS	MS	F value	*p* value
Cultivar	12	3595	299.6	8.754	< 0.0001***
Year	1	601	601	17.559	< 0.0001***
Cultivar *Year	12	624	52	1.519	0.135
Residuals	78	2670	34.2		

***Significant at $p \leq 0.001$

[†]Consisting of Amit, CDC Anna, CDC Alma, CDC Frontier, CDC Luna, CDC Leader, CDC Vanguard, CDC Xena, CDC Corrine, CDC Cory, CDC Orion, CDC Cabri and CDC Nika

[‡]Arbuscular mycorrhizal

[§]Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011.

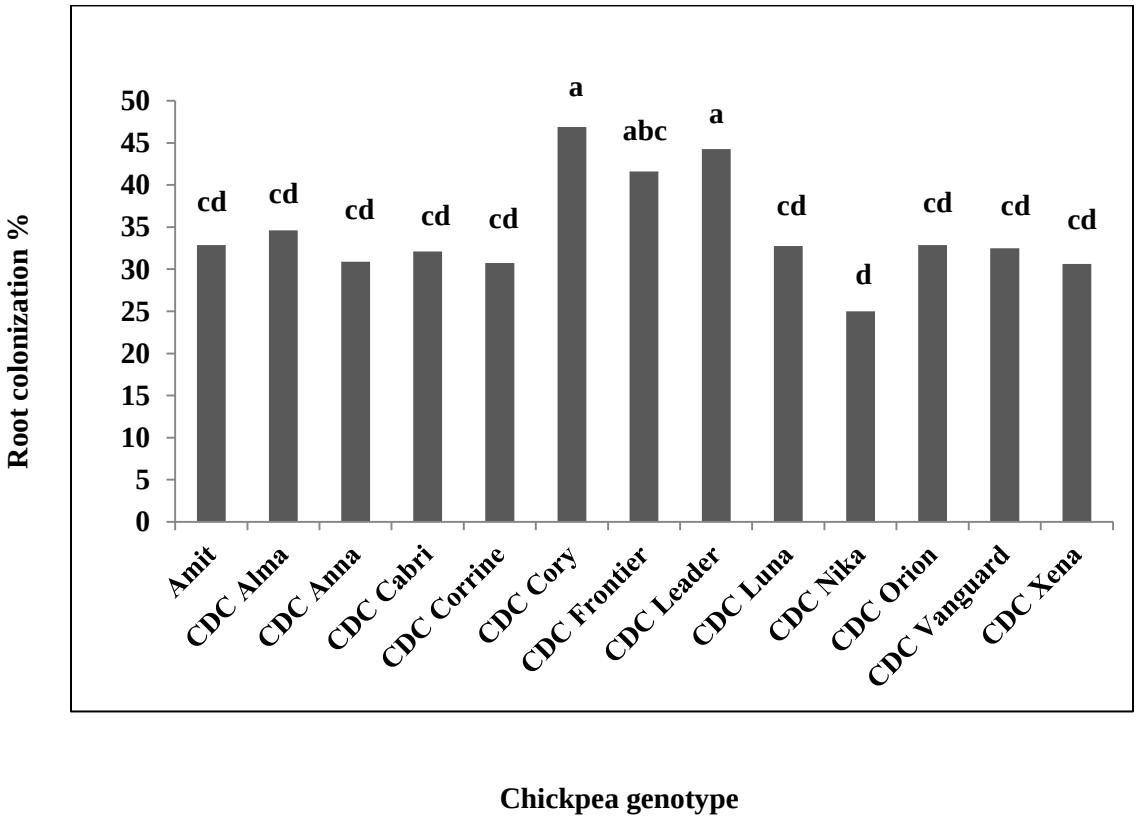


Fig. 2.8. Level of root colonization by arbuscular mycorrhizal (AM) fungi in field-grown cultivars of chickpea. Data presented as means (n = 8) of root colonization by AM fungi over two years. Significant differences between cultivars ($p \leq 0.05$) are indicated by different letters. Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011.

Table 2.10. Effect of non-AM[†] fungal isolates[‡] on the biomass of chickpea cultivar CDC Frontier, in the greenhouse (n = 4).

Source	**d.f.**	**SS**	**MS**	**F value**	***p* value**
Isolates	49	31.26	0.6380	3.618	< 0.0001 ***
Residuals	150	26.45	0.1763		

***Significant at $p \leq 0.001$

[†]Arbuscular mycorrhizal

[‡]Consisting of isolates of *Trichoderma harzianum, Geomyces vinaceus, Mortierella alpina, Acremonium furcatum, Penicillium commune, Penicillium canescens, Bionectria ochroleuca Penicillium kurssanovii, Fusarium solani, Fusarium oxysporum* and *Fusarium redolens*, indigenous to the research farm of the Semiarid Prairie Agricultural Research Centre, near swift current, SK.

Table 2.11. Effects of 49 isolates[†] of non-AM[‡] fungi on the biomass of chickpea cultivar CDC Frontier, in the greenhouse.

Isolate ID	Fungal species	Plant biomass (g)
IS_1	*Trichoderma harzianum*	3.69 *****
IS_10	*Geomyces vinaceus*	3.18 *****
IS_5	*Mortierella alpina*	3.19 *****
IS_19	*Trichoderma harzianum*	2.59
IS_18	*Mortierella alpina*	2.52
IS_45	*Trichoderma harzianum*	2.49
IS_13	*Trichoderma harzianum*	2.48
IS_46	*Mortierella alpina*	2.48
IS_14	*Geomyces pannorum*	2.47
IS_22	*Trichoderma harzianum*	2.46
IS_11	*Trichoderma harzianum*	2.45
IS_24	*Trichoderma harzianum*	2.44
IS_23	*Trichoderma harzianum*	2.42
IS_3	*Trichoderma harzianum*	2.4
IS_6	*Acremonium furcatum*	2.38
IS_16	*Mortierella alpina*	2.38
IS_7	*Mortierella alpina*	2.34
IS_42	*Trichoderma harzianum*	2.33
IS_2	*Trichoderma harzianum*	2.32
IS_15	*Penicillium commune*	2.28
IS_9	*Mortierella alpina*	2.27
IS_21	*Trichoderma harzianum*	2.26
IS_40	*Penicillium canescens*	2.25
-	*Control*	2.23
IS_12	*Acremonium furcatum*	2.19
IS_4	*Mortierella alpina*	2.17
IS_8	*Acremonium sp*	2.17
IS_35	*Bionectria ochroleuca*	2.16
IS_38	*Bionectria ochroleuca*	2.16
IS_17	*Penicillium kurssanovii*	2.14
IS_30	*Fusarium solani*	2.13
IS_32	*Bionectria ochroleuca*	2.13
IS_49	*Fusarium redolens*	2.12
IS_44	*Bionectria ochroleuca*	2.09
IS_48	*Penicillium canescens*	2.07
IS_37	*Bionectria ochroleuca*	2.04
IS_47	*Fusarium solani*	2.04
IS_27	*Fusarium oxysporum*	1.95
IS_26	*Fusarium redolens*	1.91
IS_43	*Fusarium redolens*	1.91
IS_28	*Fusarium solani*	1.86
IS_20	*Fusarium oxysporum*	1.82
IS_41	*Fusarium solani*	1.75
IS_34	*Fusarium solani*	1.65

Table 2.11 (continued)

Isolate ID	Fungal species	Plant biomass (g)
IS_39	*Fusarium solani*	1.63
IS_33	*Fusarium redolens*	1.61
IS_36	*Fusarium redolens*	1.59
IS_25	*Fusarium redolens*	1.53
IS_29	*Fusarium solani*	1.53
IS_31	*Fusarium redolens*	1.52

[†]Indigenous to the research farm of the Semiarid Prairie Agricultural Research Centre, near swift current, SK.

[‡]Arbuscular mycorrhizal

Data presented as means (n = 4). Significance effects of fungal isolates on chickpea biomass as compared to non-inoculated control are indicated by * ($p \leq 0.05$) according to Dunnett's test.

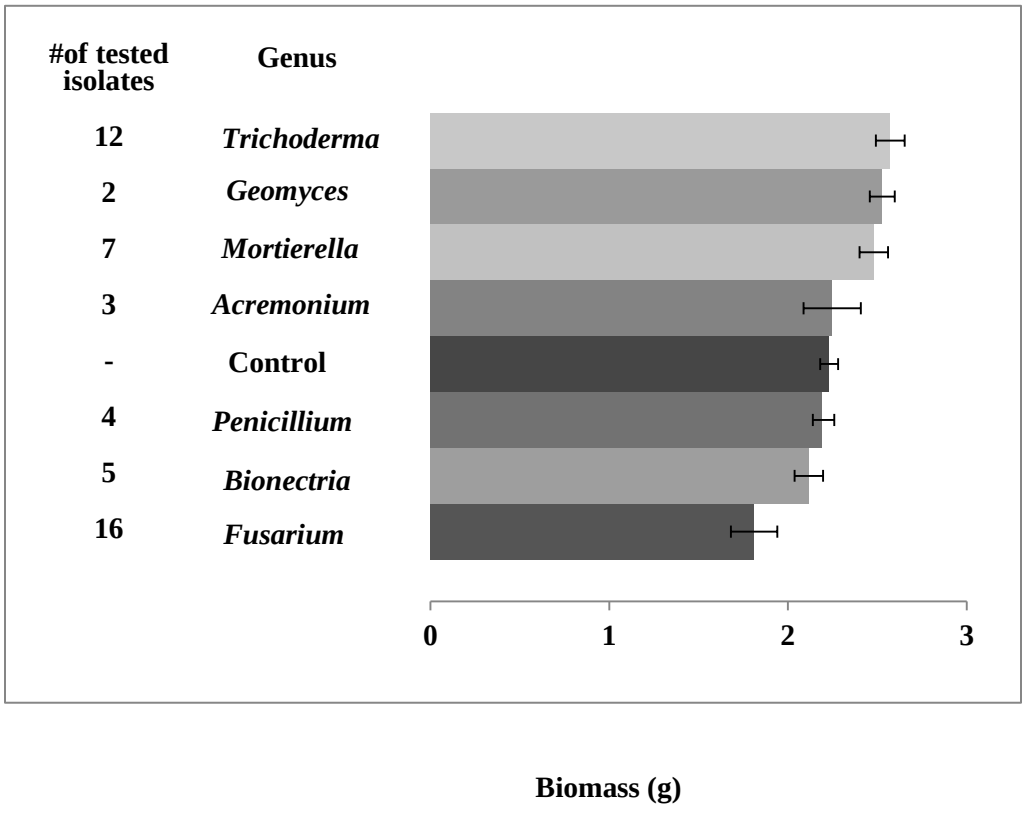


Fig. 2.9. Effect of different groups of non-arbuscular mycorrhizal (AM) fungal isolates on the biomass of chickpea cultivar CDC Frontier, in the greenhouse. The fungal isolates were indigenous to the research farm of the Semiarid Prairie Agricultural Research Centre, near swift current, SK. Data are presented as means shoot biomass (n = 4) with standard errors.

2.6. Discussion

Soil fungal communities, particularly those colonizing roots are central to plant growth and health (Shakya et al., 2013). The roots of plants mediate their biological environment through the release of a variety of metabolites that shape the structure and function of a safe and growth promoting microbiome. The composition of those metabolites varies in different plant species providing selective driving forces maintaining microbial diversity in mixed plant communities (Bakker et al., 2012; Berg, 2009; Hartmann et al., 2009).

Agricultural plants grow where they are seeded by humans rather than in the environments to which they are adapted which may explain in part the extent of the environmental pressures affecting them. The selection of crop plant varieties able to improve soil biology is considered as a way to reduce our dependence on agrochemicals while improving crop productivity. This study shows that there is genetic variation in the influence of chickpea on the fungal community of the root endosphere. This genetic variation could be used to select genotypes using conventional breeding techniques; however a first step is to understand soil-borne microbial diversity and the capabilities of chickpea to modify the soil microbiome.

I found that the chickpea cultivars influence the richness, diversity and structure of AM and non-AM fungal communities of the root endosphere (Tables 2.6 and D.2). The observation that different plant genotypes can differentially alter the structure of the root microbiome, suggests the possibility of selecting genotypes that promote specific microbial environments leading to improved plant health and productivity of the cropping system. Interactions between plant roots and symbiotic and pathogenic microbes simultaneously influence the composition of root exudates and the structure of the root microbiome. These

complex interactions impact plant performance, nutrient cycling and ecosystem functioning (Singh et al., 2004). Some of the interactions are general and take place through all species, whereas others are more specific, and some are even genotype specific (Badri et al., 2010; Nelson, 2004; Roberts and Ellis, 1989). A comprehensive understanding of the mechanisms that drive the root associated microbial community is still obscure and even an optimal microbiome is yet to be identified. The formation of symbioses with specific microbial groups, the production of stimulating or antimicrobial compounds, the induction of plant defense responses, and the competition for soil nutrient resources are the most important mechanisms influencing soil microbial communities (Azcón-Aguilar and Barea, 1997; Badri et al., 2013; Hu et al., 2010; Sarkar et al., 2009; Vierheilig et al., 2008).

Previous studies showed that the root microbiome affects the structure of soil microbial communities, and genetic variations naturally occurring in crop plants or certain crop genotypes could be used to promote beneficial soil microorganisms and control soil-borne phytopathogens (Aira et al., 2010; Krupinsky et al., 2002; Lioussanne et al., 2010; Maiti et al., 2011; Plenchette et al., 2005). Cultivars of chickpea were recently reported to have different profiles of root bioactive phytochemicals (Cruz et al., 2012; Ellouze et al., 2012) and are associated with different rhizobacterial communities (Yang et al., 2012).

This study showed that desi chickpeas support a higher richness and diversity of fungal communities in the root endosphere. The variation in the association of desi and kabuli chickpea with soil fungi could be attributed to the process of selection of cultivars overtime. There is evidence that desi cultivars originated from kabuli chickpeas (Moreno and Cubero, 1978; Singh, 1997). The process of the selection of new crop plant genotypes usually takes

place on fertile soils to generate varieties for enhanced yield production or for resistance to pathogens. This process may lead to the loss of genes and phytochemicals of kabuli cultivars needed for successful development of associations with symbiotic fungi. The involvement of these specific genes and phytochemicals in initiation and regulation of symbiosis with soil fungi can enhance the diversity of fungal communities of the root endosphere. In addition the disease resistant varieties might also be less capable of forming associations with a diversity of soil-borne fungi.

The fungal community associated with CDC Anna was different from the other cultivars. CDC Anna has a positive impact on the subsequent wheat crop indicating that the high diversity and specific community of root endosphere can improve the productivity of the cropping system beyond the chickpea phase (Ellouze et al., 2013). CDC Alma and CDC Xena roots hosted the lowest fungal richness and diversity. Interestingly, only these two cultivars are highly susceptible to the leaf disease Ascochyta blight (Table 2.1) and their agronomic performance was different to the other cultivars (Fig. 2.10). The weak association of CDC Alma and CDC Xena with soil fungi could be attributed to the induction of the immune systems of these susceptible cultivars by the fungal pathogen *Ascochyta rabiei*. Changes in the plant immune system and production of antifungal compounds or other mechanisms involved in the reaction of plant to *Ascochyta rabiei* might indirectly supress the formation of the associations between root and soil-borne fungi. This information suggests that there could be a relationship between the fungal diversity in the root endosphere and chickpea response to infecting phytopathogens.

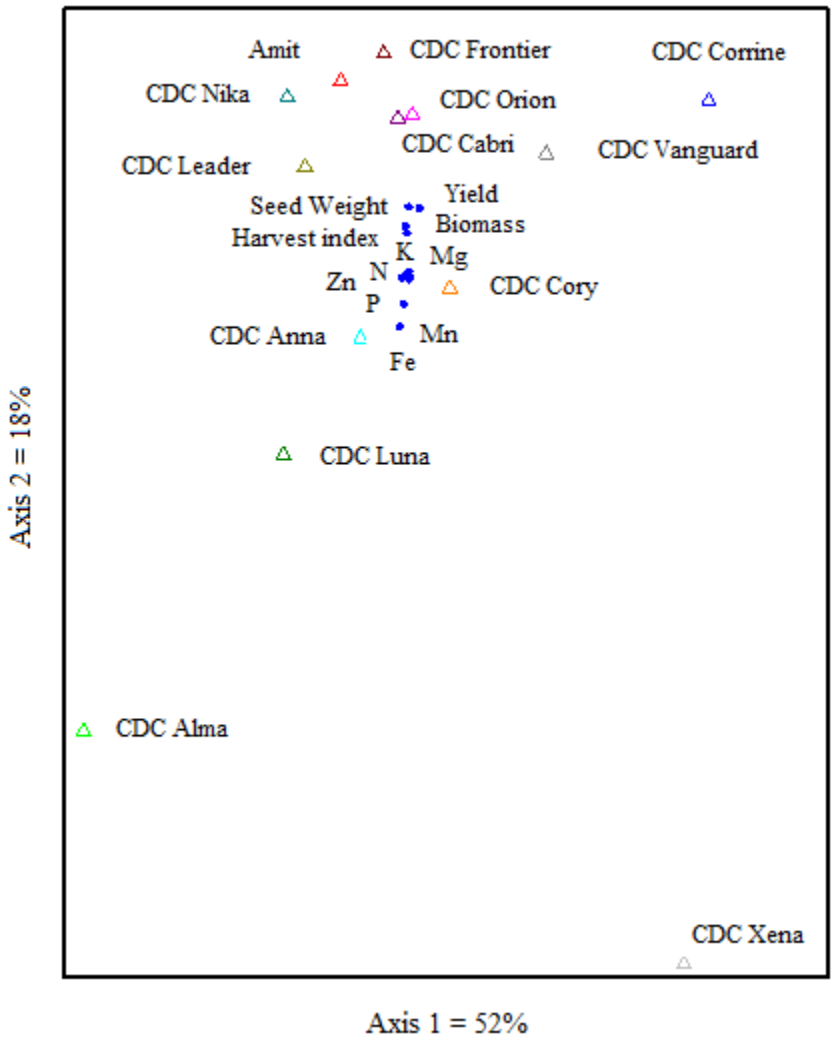


Fig. 2.10. Ordination plot relating the 13 field-grown cultivars of chickpea based on their shoot biomass, seed weight, harvest index, yield and concentration of N, P, K, Fe, Mn, Mg and Zn over two years according to non-metric multidimensional scaling. Data presented as means (n = 8).

Arbuscular mycorrhizal fungi are a ubiquitous group that generally establish symbioses with the majority of crop plants and can improve nutrient uptake, control phytopathogens and increase drought tolerance and yield in a variety of crop plants (Borowicz, 2001; Jeffries et al., 2003). The results of this study suggest that AM fungi are not all the same functionally. I observed that the abundance of the AM fungi *Paraglomus* and *Rhizophagus* was correlated with chickpea productivity (Table 2.8) but that of *Glomus* was negatively related with chickpea performance (Fig. 2.7 b). The AM fungi may act as parasites reducing crop performance (Johnson et al., 1997). The *Glomus* OTUs being mainly classified under *G. iranicum, G. indicum*. A similar observation in wheat leads to the proposal that these AM fungal species might be a sink and hence drain carbon from the host plant than other AM fungal species (Dai et al., 2014). In this study chickpea cultivars CDC Xena and CDC Anna were associated with a higher abundance of genus *Glomus* in the root endosphere (Fig. 2.4).

Fusarium and *Alternaria* were abundant members of the non-AM fungal community of the chickpea root endosphere. *Fusarium* and *Alternaria* are the main microorganisms responsible for common root rot disease and yield reduction in a variety of agricultural crops (Nene et al., 1991; Tsuge et al., 2013). The genus *Fusarium* also includes some species of fungal endophytes such as *F. equiseti* that have beneficial effects on the host plant (Maciá-Vicente et al., 2009). The greenhouse experiment showed that the *Fusarium* species are the most important root pathogenic species reducing chickpea biomass (Fig. 2.9). The relative abundance of *Fusarium* varied in chickpea cultivars and was the lowest in the root endosphere of CDC Corrine. This cultivar exhibited a high level of richness and diversity of AM and non-AM fungal communities in roots suggesting a negative relationship between

diversity of root microbial community and abundance of the dominant genus *Fusarium*. Pathogenic *Fusarium* species are possibly more abundant in chickpea roots than the beneficial species. Recent research support that *Fusarium* infection and disease severity could be mediated by changes in the structure of the root microbiome (Klein et al., 2012).

In contrast to this, cultivars CDC Xena and CDC Alma were associated with a relatively low abundance of *Fusarium* although they hosted an overall low diversity of fungi in their root endosphere. The low abundance of *Fusarium* in CDC Xena and CDC Alma could be attributed to the lower richness and diversity of these cultivars to soil-borne fungi.

Fungal endophytes are free-living species colonizing plant roots. They are mycoparasites of some other fungi and express different levels of host preference rather than specificity. *Trichoderma* species are opportunistic, avirulent plant symbionts that can improve plant nutrient uptake and provide resistance to biotic and abiotic stresses (Hanhong, 2011; Harman et al., 2004). I observed a correlation between the abundance of root *Trichoderma* and chickpea growth. This could take place through providing the plant with nutrients or antagonizing pathogenic fungi such as *Fusarium* species that are very abundant in roots. The results of the greenhouse experiment indicated that chickpeas inoculated with the fungal endophytes *Trichoderma harzianum* and *Mortierella* produced the highest biomass (Table. 2.11).

In addition to the specific suppression of soil-borne pathogens by certain microorganisms, microbial diversity is a key component of general disease suppression acting against a wide range of phytopathogens (Alabouvette, 1999; Altieri, 1999; Naeem et al., 1994). In agricultural ecosystems soil biodiversity can provide several other ecological services such as

nutrient cycling, detoxification of chemical contaminants, regulation of hydrological processes and local microclimate (Altieri, 1999). Cropping systems with chickpea cultivars that promote soil microbial diversity or select for particular rhizospheric microorganisms that suppress some phytopathogens can improve plant and soil health and productivity of the overall cropping system.

Environmental conditions influenced the structure of the root fungal communities of chickpea. It is well known that abiotic factors can modify microbial communities (Andrew et al., 2012; Castro et al., 2010; Yang et al., 2012). I conducted the field trials in two years, including different soil conditions, temperature and moisture regimes, (Fig. 2.1 and 2.2) and found that the environment affects the structure of the fungal communities of roots. Ellouze et al. (2013) reported that the effect of chickpea on soil microbial community disappears under stress.

This study suggests that intraspecific variations in the association of crop plants with soil microbial communities can be used to select varieties that are conducive to plant health and productivity.

3.0 GENOTYPIC VARIATION IN THE RESPONSE OF CHICKPEA TO ARBUSCULAR MYCORRHIZA AND FUNGAL ENDOPHYTES

3.1 Preface

In chapter 2, field observations revealed that chickpea genotypes recruit different fungal root endosphere communities. This chapter provides evidence of genotypic variation in the response of chickpea to AM and non-AM fungal endophytes. Furthermore, this chapter demonstrates the non-AM fungal endophytes on the formation and function of the AM symbiosis and shows that the outcome of a multipartite symbiosis depends on the genotype of chickpea.

3.2 Abstract

Plant roots form symbioses with AM and non-AM fungal endophytes that influence plant growth and crop productivity. The genotype of the host plant influences the outcome of a root symbiosis. This suggests that intraspecific variations could exist in the functionality of root symbioses in chickpea. Here, I tested the effect of 13 cultivars of chickpea on the function of root symbioses formed by the AM, non-AM fungi, and both types of fungal endophytes, in the greenhouse. Intraspecific variation in chickpea and the identity of the fungi colonizing the roots of the plants influenced the function of the symbioses. The AM symbiosis increased the biomass and nitrogen fixation activity of most cultivars of chickpea, whereas the influence of non-AM fungal endophytes on these varieties was neutral to positive. The root symbioses

effectively promoted plant growth in CDC Cory, CDC Anna and CDC Frontier, and stimulated nitrogen fixation in CDC Corrine. Cultivars of chickpea responded differently to co-inoculation with AM and non-AM fungal endophytes. The co-inoculation had additive effects on CDC Corrine, CDC Anna and CDC Cory but non-AM fungal endophytes reduced the positive effect of AM fungi on Amit and CDC Vanguard. The genetic variation found in chickpea could be used to select varieties that form efficient symbioses with the AM and non-AM fungal endophytes using conventional breeding techniques.

3.3 Introduction

Plant roots form mutualistic associations with soil microorganisms to alleviate biotic and abiotic environmental stresses (Gianinazzi-Pearson, 1996). Arbuscular mycorrhizal fungi form symbioses with more than 80% of land plant species. They provide nutritional benefits to their hosts, particularly under P-limiting conditions, and protect them against pathogens in exchange for carbohydrates (Harrier, 2001; Jeffries et al., 2003). Certain non-AM fungal endophytes are also capable of developing mutualistic associations that promote plant growth, especially under stressful conditions such as drought (Mandyam and Jumpponen, 2005). Fungal endophytes are a diverse group of soil fungi that asymptotically colonize plant roots (Jumpponen, 2001). Fungal endophytes release secondary metabolites that may influence plant physiology and interact with other fungal species including phytopathogens (Schulz et al., 2002; Sumarah et al., 2011). Effects of mycorrhizal fungi and fungal endophytes on plants can be beneficial or harmful depending on the host plant, the fungal species involved, and the conditions of the environment in which they interact. Any component of the association influences the formation and efficiency of symbiosis (Andrade-

Linares et al., 2011; Jumpponen, 2001; Mandyam et al., 2012; Monzon and Azcón, 1996; Sousa et al., 2012). Different root colonizing fungi can have a variety of effects on a single host plant. It was shown that AM fungal species, and even isolates of the same species, can differently influence plant growth and development, and may increase, reduce or have no effect on the biomass of a host plant (Abbott and Robson, 1982; Van der Heijden and Kuyper, 2001). Only certain fungi are able to enhance plant growth and development (Kleczewski et al., 2012; Mandyam et al., 2012).

Plant species and genotypes of the same species form symbioses that vary in form and effectiveness (Krishna et al., 1985; Singh et al., 2012; Weishampel and Bedford, 2006). The formation and function of plant symbiosis is initiated and regulated by a range of phytochemicals produced by roots (Badri et al., 2009; Harrison, 2005). Recent studies reported that the profile of bioactive compounds produced in wheat and chickpea roots vary with cultivar (Cruz et al., 2012; Ellouze et al., 2012). Some investigators proposed that the effect of plant genotype can even override the effect of the fungal endophytes in influencing the outcome of the symbiosis (Cheplick, 2008). The cultivars of crop plants may differ in architectural features such as length and abundance of roots which can influence the uptake of soil nutrients and the formation and function of root symbioses (Baon et al., 1994; Römer et al., 1988). Comparison of the responsiveness of tomato cultivars to mycorrhiza formation revealed that the cultivars with good ability to extract soil P are less responsive to the AM symbiosis (Bryla and Koide, 1998). The formation and function of the symbiosis is a dynamic process that can be influenced by the microbial communities associated with roots. Fungal endophytes were reported to interact with AM fungi and modify the level of AM

fungal colonization of roots and the efficacy of the symbiosis (Muller, 2003; Novas et al., 2009). The outcome of a symbiosis between roots and soil fungi is the result of the interaction of the host plant with all the fungal species colonizing its roots (Mandyam et al., 2012). An effect of plant genetics on the interaction between endophytic and mycorrhizal species in a symbiosis is expected, but has yet to be explored.

Chickpea is a high value crop used in wheat-based cropping systems of the semiarid prairie of Canada. Genotypes of chickpea were recently reported to produce different arrays of bioactive phytochemicals within roots and selectively promote microbial communities in the rhizosphere (Ellouze et al., 2013; Yang et al., 2012). The selection of genotypes of chickpea that form efficient symbiosis with native AM fungi and other fungal endophytes could improve the fitness of chickpea crops in a given environment and enhance the performance of a cropping system. Genotypic variation is a necessary condition for effective genetic selection.

This study was conducted to test the hypothesis that there is genotypic variation in the response of chickpea to mycorrhiza and fungal endophytes. I also hypothesized that the colonization of roots by non-AM fungal endophytes influences the formation and function of the AM symbiosis differently in different genotypes of chickpea. These hypotheses were tested in a greenhouse experiment.

3.4 Material and Methods

3.4.1 Experimental design

A greenhouse experiment was conducted to detect possible variation in the response of

cultivars of chickpea to inoculation with AM fungi and non-AM fungal endophytes. Thirteen cultivars of chickpea representing the mapping populations of chickpea were selected (Table 2.1 and Fig. A.1). The chickpea cultivars were subjected to one of four inoculation treatments: inoculation with (1) AM fungi (2) non-AM fungal endophytes (3) mixture of AM and non-AM fungal endophytes and (4) a mock inoculation control treatment. Pots were arranged in a randomized complete block design with four blocks in the greenhouse, and biomass production, N-fixation activity and nutrient concentrations in plant tissues measured.

3.4.2 Source of AM fungi and non-AM fungal endophytes

Spores of *Diversispora eburnea* (3244B), *Claroideoglomus etunicatum* (2639A), and *Glomus* sp (4350D) isolated from cultivated soils of the province of Saskatchewan, Canada were used in this study. Isolates of the non-AM fungal endophytes *Trichoderma harzianum* (P134 D1 11) and *Mortierella alpina* (P156 D2 50), also from Saskatchewan, were selected because of their positive effect on the growth of chickpea (Chapter 2). All these isolates belong to the collection of the Soil Microbiology Laboratory of Semiarid Prairie Agricultural Research Centre, Swift Current, SK.

3.4.3 Preparation of AM fungal inoculants

The AM fungal isolates were propagated on maize (*Zea mays* L.) in 16-L pots containing calcined clay (Montmorillonite, Pro'schoice Sports Field Products, Chicago, Illinois) for 90 days. Maize seeds were surface sterilized by successive immersion in 95% ethanol for 30 s, sterile distilled water for 30 s, 2.5% Javex solution (sodium hypochlorite) for 2 min, and sterile distilled water for 2 min and germinated on moist filter paper in Petri dishes prior to use. The seedlings were inoculated with 100 spores of *D. eburnea*, *C. etunicatum*, and

Glomus sp. Each species was propagated in three pots and in total nine pots were kept under 16/8 h day/night conditions at 24/16^{o}C in the greenhouse. Supplemental lighting was provided during daytime using high intensity discharge lamps (Alto 400 watt low pressure sodium, Philips, Somerset, NJ). Plants were watered with distilled water as needed and fertilized with a modified Long Ashton nutrient solution containing (in mg L^{-1}) 554 KCl, 200 $NaH_2PO_4 \cdot H_2O$, 244 $MgSO_4$, 520 $CaC_{l2} \cdot H_2O$, 1.7 $MnSO_4$, 0.25 $CuSO_4 \cdot 5H_2O$, 0.30 $ZnSO_4 \cdot 7H_2O$, 3.0 H_3O_3, 5.0 NaCl, 0.09 $(NH_4)_6$ $Mo_7O_{24} \cdot 4H_2O$ and 32.9 NaFe-EDTA. Plants were harvested 12 wk after emergence and their roots were collected and cut into 1-cm fragments. The root fragments and growth medium of each AM fungal culture was pooled and hand mixed. The AM fungal spores were extracted from three representative samples of each AM fungal culture by the sucrose centrifugation and flotation method (Walker et al., 1982), collected on a 125 µm sieve, and counted using a compound microscope. Specific amounts of each mixture that contained approximately 50 spores were used as AM fungal inoculants. Thus, the AM fungal inoculant used in this study consisted of a mixture of root fragments and growth substrates containing approximately 150 spores of three AM fungal species.

3.4.4 Preparation of non-AM fungal inoculants

Trichoderma harzianum and *M. alpina* were propagated in half-strength potato dextrose broth. The cultures were grown for 36 h on a Thermolyne Big Bill orbital shaker at 80 rpm. The liquid culture of each fungal endophyte was filtered under sterile conditions and 2 g of each species were mixed with 1L of sterile distilled water on a magnetic stirring plate and immediately used to inoculate the designated chickpea plants.

3.4.5 Pot establishment and growing conditions of experimental plants

Cultivars of chickpea were grown in 4-L pots containing pasteurized soil (90^{o}C, 1 h) (Table 3.1). Seeds were surface sterilized as described in section 3.4.3. The seeds were germinated on a moist sterile filter paper in Petri dishes at 25^{o}C for 72 h in the dark. Pots were planted with seven germinated seeds. The germinated seeds and planting holes were treated with 1.5 g of a peat-based *Mesorhizobium ciceri* inoculant (Nitragin Nitrastick GC®, Nitragin Inc., Brookfield, WI). Designated plants were inoculated with the AM fungal inoculant which was mixed with soil in the rooting zone, or with 2 mL of the liquid culture of the non-AM fungal endophyte using a pipette. Dual-inoculated plants were treated with both AM and non-AM fungi, as described above. The control (mock inoculated) plants were treated with autoclaved inoculants of both AM and non-AM fungi. Plants were kept under 16/8 h day/night photoperiod at 24/16^{o}C in the greenhouse. Supplemental light was applied during the daytime using high intensity discharge lamps (Alto 400 watt low pressure sodium, Philips, Somerset, NJ). Plants were similarly watered with distilled water, and fertilized with a modified Long Ashton nutrient solution (100 mL per week), as described in section 3.4.3.

3.4.6 Data collection

Plant shoots were cut at ground level after 90 days. Roots were collected, washed, cut in 4-cm fragments and mixed. A sub-sample of roots was cleared in 10% KOH and stained in 5% Schaeffer black ink in vinegar (Vierheilig et al., 1998) for the assessment of fungal colonization using the gridline intercept method (Giovannetti and Mosse, 1980). Assessment of root colonization level was conducted at 400X magnification under a compound microscope. AM and non-AM fungal hyphae were distinguished based on their

morphological features. AM fungal hypha were non-septate and contained vesicles or arbuscules whereas the non-AM fungal hypha were septate and not associated with vesicular and arbuscular structures. Plant shoots were dried at 65^{o}C, weighed and ground. Subsamples of ground tissues were digested in H_2SO_4/Se/Na_2SO_4 (Varley, 1966). The digests were analyzed for N (Noel and Hambleton, 1976) and P (Milbury et al., 1970) concentrations on a segmented flow auto-analyzer (Technicon, AAII System, Tarrytown, NY) and for K concentration by atomic absorption spectrometry (Anonymous, 1987). Another series of subsamples were digested with $HClO_4$/HNO_3 (Jones, 1991) and analyzed for Fe, Mg, Zn and Mn content, using atomic absorption spectrometry (Anonymous, 1987) at the Analytical Chemistry Laboratory of the Semiarid Prairie Agricultural Research Centre in Swift Current, SK. To evaluate the biological nitrogen fixation activity, a third series of ground plant subsamples was further pulverized using a bead-miller (Retsch, MM301) and analyzed for the abundance of isotopic N (δ^{15}N) on a mass spectrometer (V.G. Isotech, Aston Way, Middlewich, Cheshire, CW10 OHT, United Kingdom) at the Isotope Laboratory of the Lethbridge Research Centre, in Lethbridge, AB.

3.4.7 Statistical analysis

The data collected in this study was tested for normality using the Shapiro-Wilk's test and non-normal data was transformed before analysis. Two-way analysis of variance (ANOVA) was conducted to test the significance of the effects of cultivar, inoculation and the interaction of these factors on plant biomass, nutrient concentrations in plant tissues, N-fixation and level of root colonization, at α = 0.05. The Tukey-Kramer HSD post hoc test was used for comparison of treatment means.

Table 3.1 Physical and chemical characteristics of the soil[†] used in the greenhouse experiment.

Texture	pH[‡]	EC	NH_4-N	NO3-N	PO4-P	K	O.C.[§]	Total N
		(mS)	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(g kg^{-1})	(g kg^{-1})
Loamy Sand	6.48	0.48	19.72	14.13	21.92	357	0.57	0.08

[†] Soil was collected from Bulin farm, located at 25 km North West of Swift current, SK (Range 15, Township 16, and Southwest quarter of section 17).
[‡]1:1 soil: water
[§] Organic carbon

Correlation analysis was made between plant performance indicators. These analyses were performed using the package agricolae (de Mendiburu, 2010) in R V.2.15.2. The patterns of response to the different inoculation treatments of the chickpea cultivars were compared using multi response permutation procedure (MRPP) with pairwise comparisons in PC-ORD 4.3.4. A principle component analysis (PCA) plot was computed with PC-ORD (V. 4.34) to illustrate the similarities and differences in the response patterns of the cultivars.

3.5 Results

3.5.1 Effects of cultivar and inoculation on the performance indicators of chickpea

Cultivar of chickpea and the fungal treatments applied significantly influenced plant biomass, $\delta^{15}N$, root colonization level, and N, P and Mg concentrations in plant tissues (Table 3.2). Only the concentrations of K, Fe, Zn and Mn in plant shoots were unaffected by the treatments. Significant interactions between cultivar and fungi for all of the variables that were influenced by the treatments applied clearly demonstrated that chickpea cultivars respond differently to the AM and non-AM fungal associations.

The roots of inoculated plants were colonized, as expected. Inoculation with AM fungi increased the biomass and nutrient content of all cultivars of chickpea (Table 3.3 and 3.4). The non-AM fungal endophytes only influenced the biomass of mycorrhizal Amit, CDC Anna and CDC Vanguard. The non-AM fungal endophytes increased plant biomass in mycorrhizal CDC Anna, but reduced that of mycorrhizal Amit and CDC Vanguard compared with control (Fig. 3.1). This reduction of plant biomass by the endophyte in mycorrhizal Amit and CDC Vanguard was correlated with reduced N-fixation ($p < 0.001$).

Table 3.2. The effects of chickpea cultivar[†], fungal inoculation[‡], and the interaction of these factors on plant biomass, $\delta^{15}N$, root colonization level and concentrations of N, P, K, Mg, Fe, Mn and Zn in plant tissues (n = 4).

Source	Biomass	$\delta^{15}N$	Colonization	P	N	K	Mg	Fe	Mn	Zn
Fungi	< 0.001***	< 0.001***	< 0.001***	< 0.001***	0.001*	0.85	0.01*	0.22	0.10	0.57
Cultivar	< 0.001***	< 0.001***	< 0.001***	< 0.001***	< 0.009**	0.18	< 0.001***	0.51	0.13	0.44
Fungi*Cultivar	< 0.001***	0.003**	< 0.001***	0.02*	0.002**	0.45	< 0.001***	0.56	0.15	0.33

*Significant at $p \leq 0.05$, **Significant at $p \leq 0.01$, ***Significant at $p \leq 0.001$

[†]Cultivars tested are Amit, CDC Alma, CDC Anna, CDC Cabri, CDC Corrine, CDC Cory, CDC Frontier, CDC Leader, CDC Luna, CDC Nika, CDC Orion, CDC Vanguard and CDC Xena

[‡]Inoculation treatments: (1) Arbuscular mycorrhizal (AM) fungi *(Diversispora eburnea, Claroideoglomus etunicatum* and *Glomus* sp) (2) Non-AM fungal endophytes (*Trichoderma harzianum* and *Mortierella alpina*) and (3) a mixture of AM fungi and Non-AM fungal endophytes and mock inoculant as control.

Table 3.3. Biomass production and $\delta^{15}N$ in chickpea cultivars[†] inoculated with mock inoculant (C), AM fungi[‡] (A), non-AM fungal endophytes[§] (E), and a mixture of AM and non-AM fungal endophytes (AE) in the greenhouse.

	Inoculation	**Cultivar**												
		Am[†]	Al	An	Ca	Cr	Co	Fr	Le	Lu	Ni	Or	Va	Xe
Biomass (g)	C	3.0b	2.7b	3.1c	2.8b	2.7c	2.2b	3.8b	3.9c	2.7b	4.7b	3.1b	2.8b	3.1b
	E	3.7b	3.2b	3.5c	3.0b	3.0c	2.8b	4.4b	4.5b	3.2b	5.1b	3.2b	3.2b	3.2b
	A	4.7a	3.9a	4.4b	3.8a	3.6b	3.3ab	5.7a	5.2a	3.9a	5.9a	4.0a	4.8a	3.9a
	AE	3.4b	3.9a	5.6a	3.8a	4.7a	4.3a	5.6a	5.1a	3.9a	5.8a	3.9a	3.3b	4.1a
$\delta^{15}N$	C	1361c	1542a	1587a	1649b	1418c	1745a	1696ab	1520b	1383a	1849a	1357c	1624ab	1456a
	E	1596ab	1623a	1372a	1773a	1649b	1544a	1656b	1564b	1639a	1532b	1634b	1424b	1476a
	A	1851a	1712a	1655a	1965a	1937a	1827a	1790a	1525b	1661a	1573b	1672ab	1650a	1570a
	AE	1660ab	1770a	1593a	1652b	1875ab	1835a	1793a	1845a	1550a	1903a	1821a	1812a	1606a

Data is presented as means (n = 4). Means within a single sub-column followed by a different letter are significantly different at $p \leq 0.05$.

[†]Cultivars tested are Am = Amit, Al = CDC Alma, An = CDC Anna, Ca = CDC Cabri, Cr = CDC Corrine, Co = CDC Cory, Fr = CDC Frontier, Le = CDC Leader, Lu = CDC Luna, Ni = CDC Nika, Or = CDC Orion, Va = CDC Vanguard, Xe = CDC Xena.

[‡]Arbuscular mycorrhizal: *Diversispora eburnea, Claroideoglomus etunicatum* and *Glomus* sp

[§]*Trichoderma harzianum* and *Mortierella alpina*

Table 3.4. Level of root colonization and concentrations of P, N and Mg in the shoots of chickpea cultivars[†] inoculated with mock inoculant (C), AM[‡] fungi (A), non-AM fungal endophytes[§] (E), and both the AM and non-AM fungal endophytes (AE) in the greenhouse.

	Inoculation	**Cultivar**												
		Am[†]	Al	An	Ca	Cr	Co	Fr	Le	Lu	Ni	Or	Va	Xe
P (g kg^{-1})	C	2.6ab	2.4b	2.4b	2.4a	2.0b	2.4a	2.9a	2.6a	2.4b	2.1ab	2.2ab	2.4a	2.0a
	E	2.3b	2.4b	2.6ab	2.4a	2.6a	1.9b	2.3ab	2.5ab	2.5ab	2.0b	2.1b	2.2ab	2.0a
	A	2.6ab	3.1a	3.0a	2.1b	2.6a	2.0ab	2.2b	2.2b	2.8a	2.4a	2.4a	2.1a	2.1a
	AE	3.0a	2.7ab	2.3b	2.2ab	2.4a	2.4a	2.4ab	2.3ab	2.4b	2.1ab	2.2ab	2.3ab	2.2a
N (g kg^{-1})	C	47.1a	44.6a	48.3ab	41.1a	43a	46.7a	52.3a	45.7a	41.1b	42.2a	41a	44.2a	44.4a
	E	41.7a	45.7a	44.5b	41.0a	43.1a	39.3b	44.1b	42.9a	41.9b	44.2a	41a	44.8a	45.0a
	A	47.2a	47.9a	56.1a	43.1a	53.5a	41.5ab	46.3ab	41.3a	51.6a	46.7a	42a	45.1a	45.4a
	AE	44.8a	46.9a	46.6ab	43.3a	45.2a	43.2ab	47.3ab	45.5a	43.2ab	46.6a	44.7a	42.9a	44.1a
Mg (g kg^{-1})	C	5.3b	4.9b	5.1b	4.4b	4.56c	4.5b	4.8a	5.69b	4.8b	4.4b	4.8a	5.1ab	4.8a
	E	5.4ab	5.3ab	5.3b	5.2ab	5.0bc	4.6b	5.0a	5.2b	5.4ab	4.5ab	5.0a	5.2ab	5.2a
	A	5.8ab	5.8a	6.3a	5.4a	5.5ab	5.9a	5.2a	6.3a	6.0a	5.3a	5.1a	5.6a	5.3a
	AE	6.4a	5.7a	5.1b	5.5a	5.8a	6.0a	5.1a	6.0a	5.5ab	5.2a	5.1a	4.7b	5.9a
Colonization (%)	C	0c	0b	0d	0c	0d	0d	0c	0c	0c	0c	0c	0c	0c
	E	8b	9a	8c	6b	7c	8c	13b	11b	8b	6b	8b	7b	7b
	A	13a	11a	14b	11a	14b	14b	15ab	12b	13a	12a	14a	14a	11a
	AE	14a	12a	23a	15a	24a	22a	19a	15a	15a	14a	17a	13ab	12a

Data is presented as means (n = 4). Means within a single sub-column followed by different letter are significantly different at $p \leq 0.05$.
[†]Cultivars tested are Am = Amit, Al = CDC Alma, An = CDC Anna, Ca = CDC Cabri, Cr = CDC Corrine, Co = CDC Cory, Fr = CDC Frontier, Le = CDC Leader, Lu = CDC Luna, Ni = CDC Nika, Or = CDC Orion, Va = CDC Vanguard, Xe = CDC Xena.
[‡]Arbuscular mycorrhizal: *Diversispora eburnea, Claroideoglomus etunicatum* and *Glomus* sp.
[§]*Trichoderma harzianum* and *Mortierella alpina*

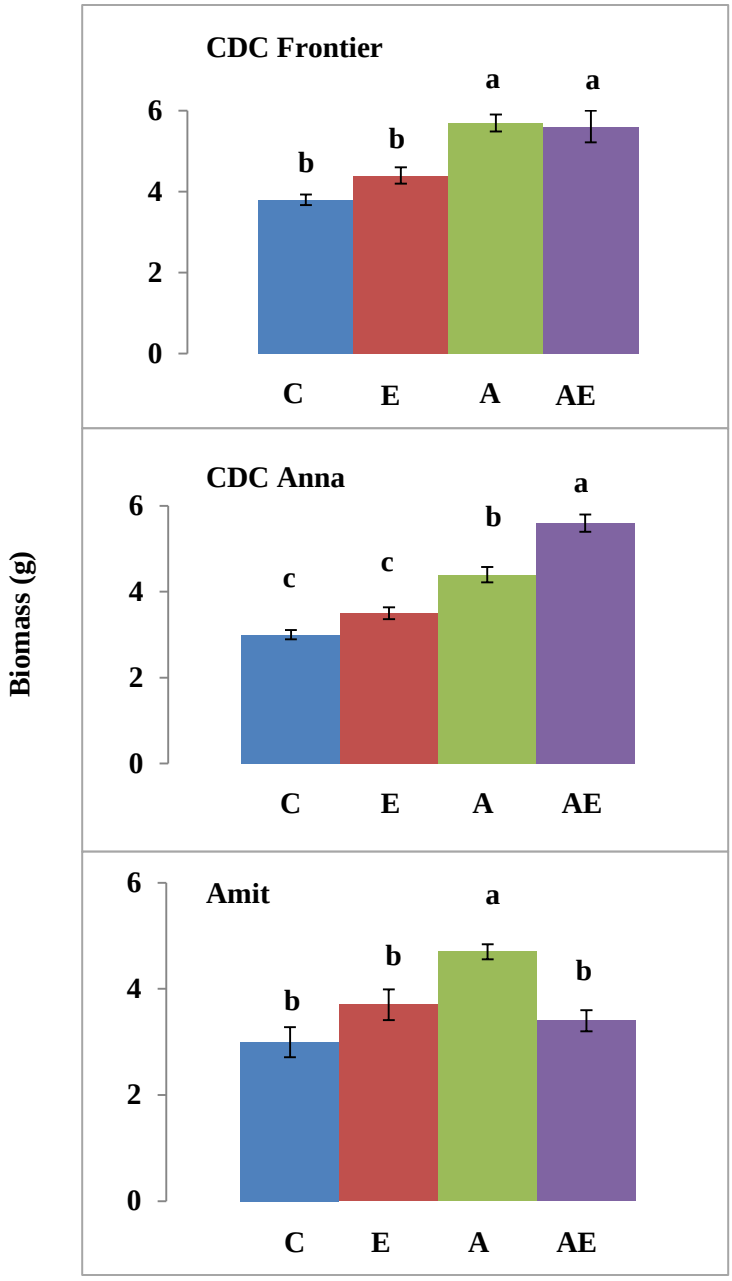


Fig. 3.1. Typical patterns of the biomass productions of chickpea cultivars inoculated with mock inoculant (C), non-arbuscular mycorrhizal (AM) fungal endophytes (E), AM fungi (A) and a mixture of AM and non-AM fungal endophytes (AE). Data is presented as means (n = 4). Bars with different letters are significantly different (α = 0.05). Non-AM fungal endophytes used for inoculation were *Trichoderma harzianum and Mortierella alpina* and AM fungi were *Diversispora eburnea*, *Claroideoglomus etunicatum* and *Glomus sp.*

The non-AM fungal endophytes reduced the concentration of P in mycorrhizal CDC Anna and CDC Luna (Table 3.4). The non-AM fungal endophytes reduced the concentration of Mg in mycorrhizal CDC Anna and CDC Vanguard compared with control but had no effect on the concentration of N in plant tissue (Table 3.4). The simultaneous reduction of P and Mg concentration and increase in plant biomass in these chickpea cultivars are consistent with a model of nutrient dilution in greater biomasses. The level of root colonization was higher in dual inoculated plants than in AM inoculated plants in CDC Anna, CDC Corrine, CDC Cory and CDC Leader (Table 3.4).

3.5.2 Response patterns of chickpea cultivars representing the mapping population

Multi response permutation procedure (MRPP) revealed that there is a significant difference in the pattern of response of cultivars of chickpea to inoculation treatments ($p < 0.0001$). Pairwise comparisons demonstrated the differences in the overall response of each cultivar to inoculation with AM fungi, non-AM fungal endophytes and dual inoculation (Table 3.5). Furthermore, Principle component analysis (PCA) illustrates that some chickpea cultivars have different patterns of responsiveness to the root-associated community fungal symbionts. For instance, CDC Cory and CDC Vanguard were more responsive to single inoculation of AM fungi (Fig. 3.2), whereas CDC Frontier was more responsive to single inoculation of fungal endophyte (Fig. 3.3) and, CDC Cory and CDC Anna were more responsive to dual inoculation of AM fungi and non-AM fungal endophytes (Fig. 3.4).

Table 3.5. Pairwise comparisons of the response† patterns of chickpea cultivars to inoculations with AM‡ fungi, non-AM fungal endophytes§, and AM and non-AM fungal endophytes.

Cultivar		**Cultivar**	***p* value**
CDC Frontier	vs	CDC Anna	0.04*
CDC Frontier	vs	CDC Nika	0.03*
CDC Frontier	vs	CDC Xena	0.02*
CDC Frontier	vs	CDC Vanguard	0.03*
CDC Frontier	vs	CDC Cabri	0.03*
CDC Frontier	vs	CDC Corrine	0.04*
CDC Frontier	vs	CDC Cory	0.30
CDC Frontier	vs	Amit	0.03*
CDC Frontier	vs	CDC Alma	0.05*
CDC Frontier	vs	CDC Luna	0.03*
CDC Frontier	vs	CDC Orion	0.04*
CDC Frontier	vs	CDC Leader	0.03*
CDC Anna	vs	CDC Nika	0.03*
CDC Anna	vs	CDC Xena	0.04*
CDC Anna	vs	CDC Vanguard	0.05*
CDC Anna	vs	CDC Cabri	0.04*
CDC Anna	vs	CDC Corrine	0.03*
CDC Anna	vs	CDC Cory	0.07
CDC Anna	vs	Amit	0.08
CDC Anna	vs	CDC Alma	0.07
CDC Anna	vs	CDC Luna	0.03*
CDC Anna	vs	CDC Orion	0.03*
CDC Anna	vs	CDC Leader	0.03*
CDC Nika	vs	CDC Xena	0.14
CDC Nika	vs	CDC Vanguard	0.06
CDC Nika	vs	CDC Cabri	0.83
CDC Nika	vs	CDC Corrine	0.02*
CDC Nika	vs	CDC Cory	0.11
CDC Nika	vs	Amit	0.05*
CDC Nika	vs	CDC Alma	0.46
CDC Nika	vs	CDC Luna	0.25
CDC Nika	vs	CDC Orion	0.10
CDC Nika	vs	CDC Leader	0.19
CDC Xena	vs	CDC Vanguard	0.03*
CDC Xena	vs	CDC Cabri	0.40
CDC Xena	vs	CDC Corrine	0.03*
CDC Xena	vs	CDC Cory	0.03*
CDC Xena	vs	Amit	0.10
CDC Xena	vs	CDC Alma	0.05*
CDC Xena	vs	CDC Luna	0.02*
CDC Xena	vs	CDC Orion	0.04*

Table 3.5 (continued)

Cultivar		Cultivar	*p* - value
CDC Xena	vs	CDC Leader	0.04*
CDC Vanguard	vs	CDC Cabri	0.05 *
CDC Vanguard	vs	CDC Corrine	0.03*
CDC Vanguard	vs	CDC Cory	0.04*
CDC Vanguard	vs	Amit	0.07
CDC Vanguard	vs	CDC Alma	0.06
CDC Vanguard	vs	CDC Luna	0.02*
CDC Vanguard	vs	CDC Orion	0.10
CDC Vanguard	vs	CDC Leader	0.03*
CDC Cabri	vs	CDC Corrine	0.03*
CDC Cabri	vs	CDC Cory	0.03*
CDC Cabri	vs	Amit	0.12
CDC Cabri	vs	CDC Alma	0.24
CDC Cabri	vs	CDC Luna	0.03*
CDC Cabri	vs	CDC Orion	0.38
CDC Cabri	vs	CDC Leader	0.24
CDC Corrine	vs	CDC Cory	0.17
CDC Corrine	vs	Amit	0.05*
CDC Corrine	vs	CDC Alma	0.04*
CDC Corrine	vs	CDC Luna	0.04*
CDC Corrine	vs	CDC Orion	0.03*
CDC Corrine	vs	CDC Leader	0.03*
CDC Cory	vs	Amit	0.16
CDC Cory	vs	CDC Alma	0.09
CDC Cory	vs	CDC Luna	0.08
CDC Cory	vs	CDC Orion	0.03*
CDC Cory	vs	CDC Leader	0.04*
Amit	vs	CDC Alma	0.19
Amit	vs	CDC Luna	0.15
Amit	vs	CDC Orion	0.02*
Amit	vs	CDC Leader	0.07
CDC Alma	vs	CDC Luna	0.54
CDC Alma	vs	CDC Orion	0.07
CDC Alma	vs	CDC Leader	0.28
CDC Luna	vs	CDC Orion	0.07
CDC Luna	vs	CDC Leader	0.03*
CDC Orion	vs	CDC Leader	0.04*

*Significant at $p \leq 0.05$ according to MuIti-response Permutation Procedures (MRPP)

[†]Response variables include biomass, N-fixation, root colonization level and concentrations of N, P and Mg in the shoot tissues.

[‡]Arbuscular mycorrhizal: *Diversispora eburnea, Claroideoglomus etunicatum & Glomus* sp.

[§]*Trichoderma harzianum* and *Mortierella alpina*

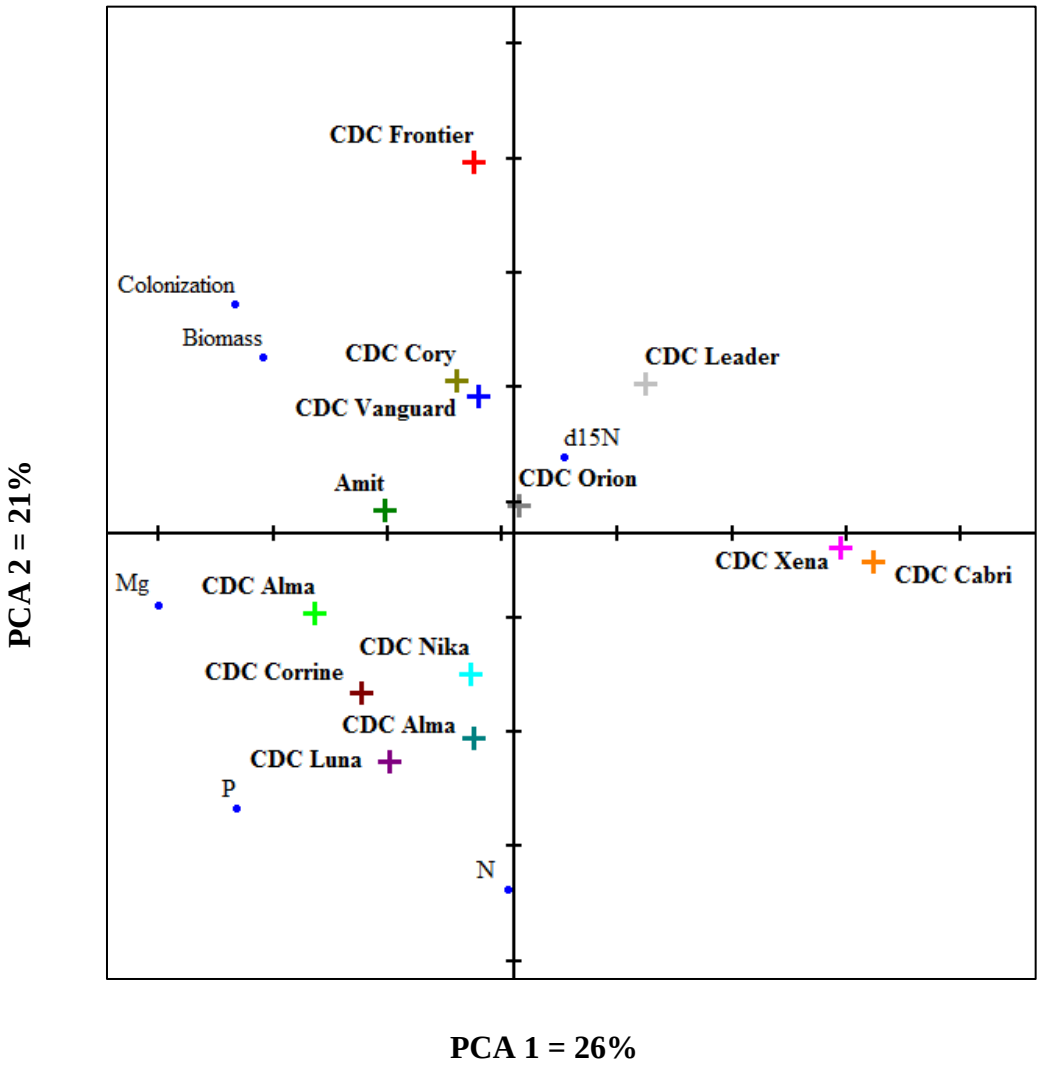


Fig. 3.2. Biplot of the principal component analysis of the response of chickpea cultivars to inoculation with arbuscular mycorrhizal (AM) fungi. The pattern of response considers the response of biomass, $\delta^{15}N$, root colonization and concentrations of N, P and Mg in plant tissues (n = 4). AM fungal species used, include *Diversispora eburnea*, *Claroideoglomus etunicatum* and *Glomus sp.*

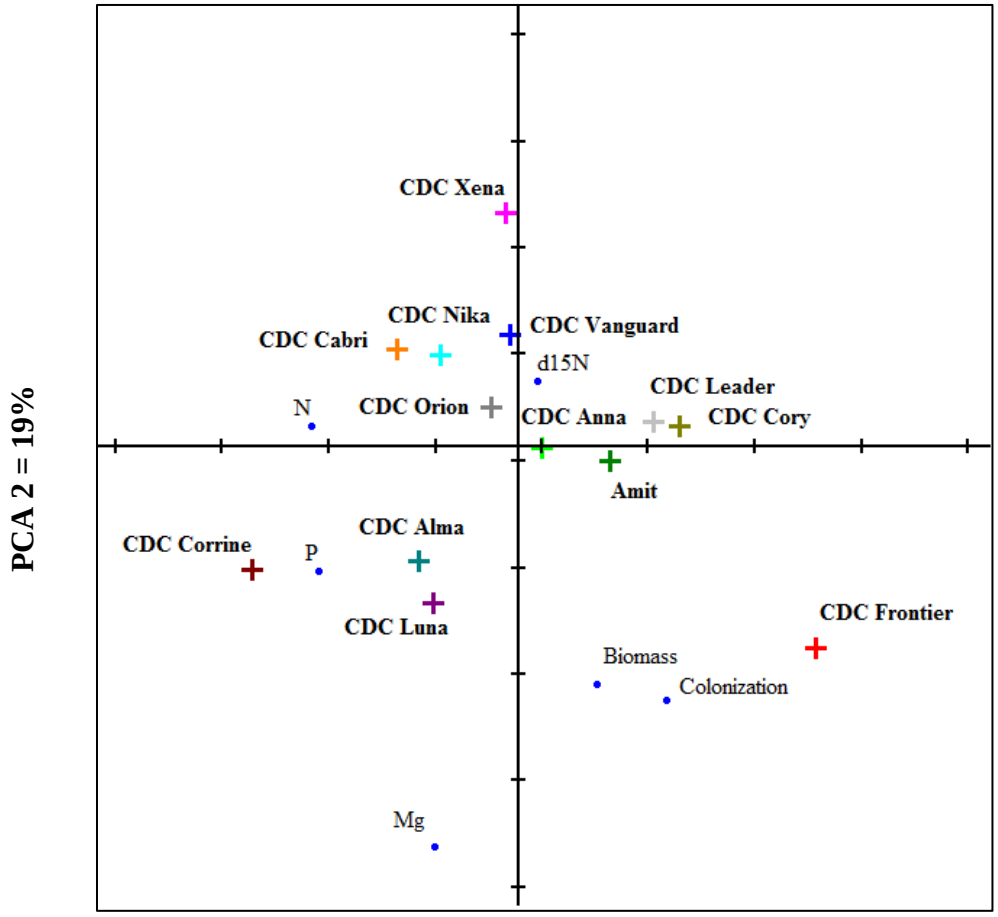


Fig. 3.3. Biplot of a principal component analysis of the response of chickpea cultivars to inoculation with non-arbuscular mycorrhizal (non-AM) fungal endophytes. The response considers the response of biomass, $\delta^{15}N$, root colonization and concentrations of N, P and Mg in plant tissues (n = 4). Non-AM fungal species used include *Trichoderma harzianum* and *Mortierella alpina.*

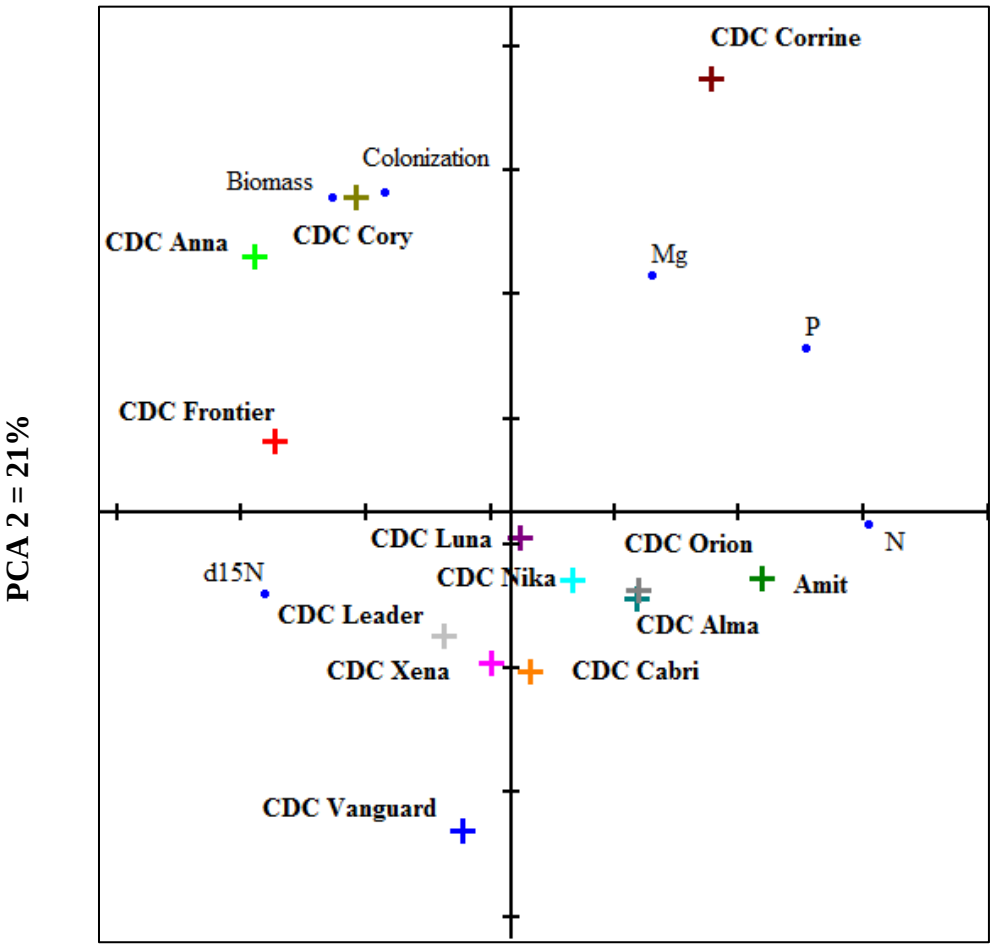


Fig. 3.4. Biplot of a principal component analysis of the response of chickpea cultivars to inoculation with arbuscular mycorrhizal (AM) and non-AM fungal endophytes. The response considers the response of biomass, $\delta^{15}N$, root colonization and concentrations of N, P and Mg in plant tissues (n = 4). AM fungal species used, include *Diversispora eburnea*, *Claroideoglomus etunicatum* and *Glomus sp.* Non-AM fungal species used, include *Trichoderma harzianum* and *Mortierella alpina.*

3.6 Discussion

3.6.1 Genetic variation in plant response to symbioses

Numerous reports indicate that the effectiveness of the AM symbiosis depends on the genotype of the host plant (Krishna et al., 1985; Linderman and Davis, 2004). Our findings, like those of others, show that there is significant variation in responsiveness of genotypes of chickpea to AM and non-AM fungal endophytes, supporting the possibility to select genotypes that form more efficient associations with naturally occurring soil fungi. A conclusive explanation for the basis of this variation is beyond the scope of this work. However, intraspecific variations in symbiosis-specific genes and genes regulating the physiology and morphology of the host plant could interact and result in a certain level of response (Balestrini and Lanfranco, 2006; Estaun et al., 1987; Linderman and Davis, 2004).

I found that, at least in terms of biomass, desi chickpeas in particular CDC Corrine, CDC Cory and CDC Anna, were more responsive than other cultivars to fungal symbioses suggesting that a type-specific feature of chickpea is involved in or linked to the formation of efficient fungal associations. Genetic variations in the responsiveness of chickpea could result from variations in the ratio of the costs to benefits derived from the symbiosis. The fungi living in the root endosphere are thought to rely on photosynthate to fulfill their needs for C and energy. The cost of maintaining the mycorrhizal association sometimes offsets the nutritional benefits obtained by the host plant. Genetic selections conducted in fertile soils might have inadvertently selected varieties with less capacity to positively respond to mycorrhizal symbiosis (Koide and Elliott, 1989; Olsson et al., 2010). It is notable that kabuli chickpeas were derived from desi chickpeas through selections (Moreno and Cubero, 1978;

Singh, 1997). The selection for high-yielding varieties, which takes place on fertile substrates, might lead to the loss of genes and phytochemicals or other features that are necessary for the formation of efficient symbioses. Moreover, selections for disease resistance could also select for resistance against symbioses due to common pathways in regulation of symbiosis and disease resistance (Toth et al., 1990). Hence, breeding programs targeting high yield and disease resistance could have selected kabuli chickpeas responding poorly to fungal symbiosis.

The inherent ability of chickpea genotypes to acquire nutrients, particularly P, could be related to responsiveness to the AM symbiosis. Bryla and Koide (1998) showed that the ability of plants to acquire and efficiently use P determines the level of their responsiveness to mycorrhizal symbiosis, so that only cultivars with low P uptake capability are likely to respond positively to AM fungi. It was observed P fertilization had no effect on the seed yield of kabuli chickpea, but improved the seed yield of desi chickpea (Walley et al., 2005). The lack of response of kabuli chickpea to P fertilization suggests that kabuli chickpea plants are more capable of taking up P at normal P levels than desi chickpeas. Thus, kabuli chickpeas may respond poorly to mycorrhizal symbiosis. This study indicates that among kabuli cultivars CDC Frontier was more responsive than others. In fact CDC Frontier could benefit from mycorrhizae, but also it might have lost the ability to effectively regulate the co-occurrence of AM and non-AM fungal endophytes. In general, cultivars of chickpea responded positively to AM and non-AM fungal colonization. The increase of biomass production ranged from 1 to 89%. This result contrasts with previous reports of response to AM symbiosis ranging from positive to negative in different genotypes of marigold

(Linderman and Davis, 2004). Kaeppler et al. (2000) who worked on maize varieties observed positive responses to mycorrhizae ranging from 66 to 653% (Kaeppler et al., 2000). The present study clearly demonstrates that in addition to mycorrhizae, fungal endophytes that associate with roots can impact the outcome of the symbiosis. As reported by other studies, the single inoculation of chickpea with fungal endophytes generated an effect on growth that varied between neutral and positive (Mayerhofer et al., 2013). I conclude that non-AM fungi can interact with mycorrhiza and influence plant biomass production.

3.6.2 Response of chickpea to co-inoculation with AM and non-AM fungal endophytes

I found that non-AM fungal endophytes can interact with the formation and function of mycorrhizal symbiosis in chickpea while on the other hand, the interaction could be modified by the genotype of the plant. Recent studies show that fungal endophytes influence mycorrhizae along a continuum from synergism to antagonism depending on the host plant. *Trichoderma harzianum*, a common soil dweller that was also used in this study, increased the colonization of roots by *Glomus mosseae* (syn: *Funneliformis mosseae*) and plant growth in cucumber (Chandanie et al., 2009), but had no effect on melon (Martínez-Medina et al., 2011). *Trichoderma koningii* reduced the level of root colonization by *G. mosseae* and the biomass of maize and lettuce (McAllister et al., 1994).

Interactions between non-AM fungal and AM fungal endophytes occur through a combination of mechanisms including competition between extraradical mycelia for nutrients and colonization sites (Green et al., 1999; Pozo and Azcón-Aguilar, 2007), morphological and phytochemical changes in roots (Malinowski et al., 1999; Shoresh and Harman, 2008), and activation of plant defence systems (Yedidia et al., 2003). Fungal endophytes may

directly influence other fungal species by releasing various secondary metabolites (Miller et al., 2012; Sumarah et al., 2011; Vinale et al., 2008), altering the level of secondary metabolites inside the roots (Peipp et al., 1997) and by interacting with plant hormonal signalling and modifying the proteome and metabolism of the plant (Gravel et al., 2007; Harman et al., 2004; Shoresh et al., 2005; Vassilev et al., 2006). The co-inoculation of AM and non-AM endophytes has resulted in specific plant hormonal profiles that were different from those produced by single inoculation (Martínez-Medina et al., 2011). *Trichoderma harzianum* induces the systemic production of specific metabolites and antibiotics (Yedidia et al., 2003) including salicylic acid (SA) and jasmonic acid (JA) that are involved in reactions against pathogens (Pieterse et al., 2003; Sticher et al., 1997). It was noted that the level of SA is lower in mycorrhizal responsive plants than in non-responsive plants (Blilou et al., 1999). In addition, the levels of SA and JA are lower in plants co-inoculated with *T. harzianum* and AM fungi than in plants only inoculated with *T. harzianum* (Martínez-Medina et al., 2011). As a requirement of the formation of mycorrhizal symbiosis, the reduction in plant SA and JA could be triggered by the AM fungi. The genetic capacity for the suppression of these defensive hormones by AM fungi, or for their stimulation by *T. harzianum* in particular genotypes of chickpea may be involved in the successful establishment and function of mycorrhizal symbiosis. On the other hand, changes in the level of phytohormones occurring by the interactions between AM fungi and fungal endophytes could influence plant physiology and biomass production.

Tucci et al. (2011) reported that the effects of *T. harzianum* on plant growth and response to pathogens depend on the genotype of tomato. In their study, *T. harzianum* triggered the SA

pathway only in responsive genotypes and further inoculation of a plant with the pathogen *Botrytis cinerea* triggered the JA pathway in a genotype dependent level. Since the production of JA and SA could control the colonization of roots by the AM fungi, genotypic variations in the inductions of these compounds by *T. harzianum* could influence root colonization and the outcome of the AM symbiosis (Tucci et al., 2011).

Here, for the first time I report that cultivars of chickpea respond differently to co-inoculation with AM and non-AM fungal endophytes. The cultivars CDC Vanguard and Amit responded positively to AM fungi in the absence of non-AM fungal endophytes, but did not respond when they were also exposed to the non-AM fungal endophytes. In contrast fungal endophytes caused an additive positive response in CDC Corrine, CDC Cory and CDC Anna. This result shows a potential influence of ubiquitous endophytic fungi that are common residents of Saskatchewan chickpea-growing soils, on the formation and function of the AM symbiosis of chickpea. It is noteworthy that the differences in the response of chickpea cultivars to non-AM fungal endophytes were observed using only two fungal species. Since the roots of plants are exposed to a wide diversity of fungal speices in the field, more variations in the response of chickpea cultivars to AM symbiosis could be expected.

This study is a first step towards the elucidation of plant genetic factors that control the multipartite symbiosis formed in chickpea by AM and non-AM fungal endophytes that may lead to the development of chickpea cultivars forming beneficial associations with indigenous fungal resources. Resilient genotypes that benefit from mycorrhizae, but at the same time that are not mycorrhiza dependent, would effectively utilize soil resources for the production of high and stable yield.

4.0 MYCORRHIZA-INDUCED PHYTOCHEMICALS TO SUPPRESS FUNGAL ENDOPHYTES AND PATHOGENS

4.1 Preface

This chapter investigates the potential influence of the bioactive phytochemicals produced in mycorrhizal and non-mycorrhizal roots of chickpea on soil-borne fungal endophytes and pathogens.

4.2 Abstract

Plant roots shape the soil microbiome by releasing a wide array of phytochemicals. Root metabolite production is a dynamic process modified by various symbiotic and pathogenic microorganisms. Chemical regulation of the soil microbial community by chickpea was explored. Proteins and low-molecular-mass phytochemicals were extracted from chickpea (*Cicer arietinum* L.) roots colonized or not by the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis*, fractionated by flash chromatography and high pressure liquid chromatography (HPLC). The bioactivity of the fractions obtained was tested on the soil-borne fungal endophytes *Trichoderma harzianum* and *Geomyces vinaceus* and on the pathogens *Fusarium oxysporum* and *Rhizoctonia* sp. in multiwell plates. One protein fraction from the AM roots which seemingly contained a 34 KDa chitinase/chitin-binding domain and 24 KDa non-specific lipid transfer protein non-selectively repressed the endophytes and pathogens. Several bioactive fractions of low-molecular-mass phytochemicals were obtained.

By contrast to the protein fraction, the low-molecular-mass fractions were often selective. A few compounds stimulated specific fungal species but inhibited others. The different response of fungal species to the phytochemicals could be involved in the so called host 'preference' of fungal endophytes or 'resistance' to pathogens. Overall, the phytochemicals in AM root extracts were more suppressive than non-AM root extracts. These results support that the AM symbiosis stimulates the production of protein and low molecular weight compounds that suppress fungal endophytes and pathogens.

4.3. Introduction

Plant roots release phytochemicals modifying the structure and function of the soil microbiome (Bakker et al., 2012; Hartmann et al., 2009). Root phytochemicals are a wide range of organic and inorganic substances that mediate the physical and chemical properties of the soil and influence microbial growth and development (Dakora and Phillips, 2002). Primary metabolites such as sugars and amino acids create nutritional niches for soil microorganisms and secondary metabolites act as tools to stimulate or inhibit the microorganisms (Badri et al., 2013; Collemare and Lebrun, 2011; Jones et al., 2004).

Numerous phytochemicals influence soil microbes. Certain flavonoids turn on the nod genes in rhizobia or trigger the germination of AM fungal spores, initiating symbioses (Fries et al., 1997; Gianinazzi-Pearson et al., 1989; Rengel, 2002). Strigolactones inhibit the fungal pathogens *Fusarium oxysporum* and *F. solani* and the volatile organic compounds (VOC) trans-2-hexenal and 1-hexanol suppress *F. graminearum* and *F. avenaceum* (Birkett et al., 2004; Cruz et al., 2012; Dor et al., 2011; Kretzschmar et al., 2012; Steinkellner et al., 2007). Tryptophan dimers stimulate the growth of AM fungal hyphae (Horii et al., 2009) whereas

hydrogen cyanide inhibits cellular respiration of herbivores and pathogens (Wittstock and Gershenzon, 2002).

Chemotaxonomic analysis provided evidence that taxonomically related plant groups often produce similar types of secondary metabolites (Wink et al., 2010). For instance, species of the genus Solanaceae mainly use sesquiterpenes, whereas legumes depend on isoflavonoids to resist pathogens (Bonanomi et al., 2009). However, several plant families produce various secondary metabolites and a number of phytochemicals can be found within plant groups that are unrelated in a taxonomic context (Hammerschmidt, 1999; Smith, 1996; Wink et al., 2010). Very similar compounds could exhibit inconsistent functions against microbial communities and even a particular substance may have variable impacts on related microbial groups (Badri et al., 2013; Bonanomi et al., 2009). Legumes roots select beneficial N_2-fixing rhizobia through the release of canavanine, a non-protein amino acid (NPAA) that inhibits soil bacteria except rhizobia (Cai et al., 2009; Wink et al., 2010).

The pattern of plant secondary metabolite production can be altered by soil microorganisms (Badri et al., 2010; Nelson, 2004). Arbuscular mycorrhizal fungi are ubiquitous soil fungi that develop a mutualistic symbiosis with the majority of plant species. The AM symbiosis provides the host plants with improved nutrition, water use efficiency, health status, and tolerance to abiotic stresses such as drought and metal toxicity. Arbuscular mycorrhizal fungi play a central role in many ecological processes such as nutrient cycling, organic matter decomposition and weathering of minerals (Finlay, 2008). The AM symbiosis is initiated by a series of signaling events, followed by physiological and morphological changes in the root system, and further regulated to maintain the symbiotic nature of the

association. The regulation of AM symbiosis by host plants involves hormonal activities similar to those used for defense against phytopathogens. These interactions could influence secondary metabolism and the mechanisms through which plants influence the soil microbiome (Balestrini and Lanfranco, 2006; Cruz et al., 2008; Vierheilig et al., 2008).

Chickpea contributes soil N to cropping systems through biological N_2-fixation. Although, it produces a high value yield, chickpea often leads to lower yield in the following wheat crop compared to pea and lentil (Miller et al., 2003). The poor rotation effect of chickpea could be partly attributed to its influence on the soil microbiome. In particular, chickpea roots could stimulate the endophytic and pathogenic fungal species that potentially impact biomass and yield in succeeding wheat crop. Several root rot pathogens such as *Fusarium oxysporum* and *Rhizoctonia* can infect chickpea, reducing yield and promoting the proliferation of these pathogens, but endophytes such as *Trichoderma* sp. can improve the biomass and yield of chickpea and other crops (Chaeichi and Edalati-Fard, 2006; Nene et al., 1991; Rudresh et al., 2005).

In this study, the mechanisms involved in the biochemical regulation of soil-borne fungal endophytes and pathogens by chickpea roots were explored. I hypothesized that the roots of chickpea contain biologically active proteins and low-molecular-mass phytochemicals that influence the growth of fungal endophytes and pathogens. Because the AM symbiosis may influence the production of bioactive phytochemicals, I examined phytochemical production in both AM and non-AM chickpea roots.

4.4 Material and Methods

Low-molecular-mass and protein compounds were isolated from mycorrhizal and non-

mycorrhizal roots of CDC Anna chickpea and their effects on model soil-borne fungal endophytes and pathogens were tested *in vitro*. Some of the bioactive compounds were subsequently identified.

4.4.1 Plant growth conditions and collection of roots

Mycorrhizal and non-mycorrhizal CDC Anna chickpea were grown in large flat containers (236 cm × 94 cm × 5 cm) containing a mixture of pasteurized sand and calcined clay (Pro's Choice Sports Field Products, Chicago, IL) (1:1, v:v). Two flats of mycorrhizal and two flats of non-mycorrhizal plants including 70 plants / flat were grown in the greenhouse. Seeds were inoculated with *Mesorhizobium ciceri* (Nitragin Nitrastick GC®, Nitragin Inc. Brookfield, WI). Mycorrhizal plants were inoculated with the AM fungus *Rhizophagus intraradices* syn. *Glomus irregularis,* which was added to the planting hole prior to seeding (Myke®, Premier Tech Biotechnologies, Rivière-du-Loup, QC). Plants were kept in the greenhouse under a photoperiod of 16 h d^{-1} at 24/16°C day/night temperatures. Natural daylight was supplemented with high intensity discharge lamps (Alto 400 watt high pressure sodium, Philips, Somerset, NJ) during daytime. Plants were watered with distilled water as needed and fertilized with half strength Long Ashton nutrient solution containing (in mg L^{-1}) 554 KCl, 200 $NaH_2PO_4 \cdot H_2O$, 244 $MgSO_4$, 520 $CaCl_2 \cdot H_2O$, 1.7 $MnSO_4$, 0.25 $CuSO_4 \cdot 5H_2O$, 0.30 $ZnSO_4 \cdot 7H_2O$, 3.0 H_3O_3, 5.0 NaCl, 0.09 $(NH_4)_6\ Mo_7O_{24} \cdot 4H_2O$ and 32.9 NaFe-EDTA (Hewitt, 1966). Plants were harvested 8 wk after emergence and the roots were collected, washed and stored at -20°C for a few days until extraction.

4.4.2 Preparation and fractionation of root homogenate

Four hundred grams of roots from mycorrhizal and non-mycorrhizal plants were

separately immersed in 0.05M Tris-HCl-NaCl buffer, ground and filtered (5B filter paper Advantec, Tokyo). The residues were used for methanol (MeOH) extraction. The filtrate was subjected to flash chromatography with a diol column (50×200 mm, Chromatorex Diol-Fuji Silysia Chemical Ltd. Kasugai) to separate the high and low molecular weight compounds. The high-molecular-weight compounds were separated by elution of the column using 2 L of 0.05M Tris-HCl-NaCl buffer. The low-molecular-weight compounds were subsequently collected by elution of the column using 2 L of MeOH.

4.4.3 Extraction of root proteins

Root proteins were extracted from the high-molecular-weight fraction of the root homogenate using ammonium sulfate. The fraction was saturated with 35% ammonium sulfate and kept for 24 h in the refrigerator at 5^{o}C. The ammonium sulfate concentration was increased to 95% and the solution kept at 5^{o}C for another 24 h. The solution was centrifuged at 8000 g for 30 min at 4°C. The supernatant was discarded and the pellet collected and re-suspended. Proteins were purified from the suspension using medium pressure liquid chromatograph (MPLC) equipped with a diol column (30×300 mm) (Chromatorex Diol-Fuji Silysia Chemical Ltd. Kasugai).

4.4.4 Methanol extraction of roots

The root residues were immersed in 1 L of 80% MeOH for 72 h at room temperature (Ishii et al., 1997), replacing the MeOH every 24 h. The MeOH extracts and low molecular weight fraction of the root homogenate were mixed. The mixture was concentrated using a rotary evaporator at 40°C and fractionated on an octa decyl silane (ODS) 45 x 400 mm column through successive elution with 0%, 10%, 25%, 50%, 75%, and 100% (v/v) MeOH solutions.

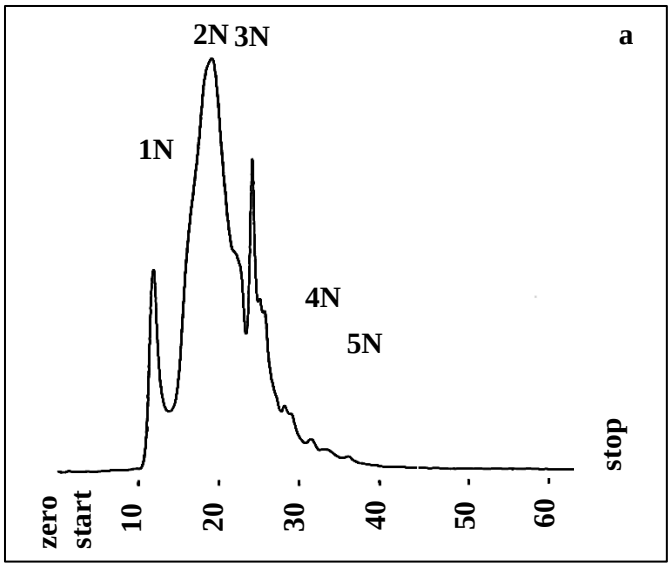
The eluted fractions were collected and separately concentrated using a rotary evaporator at 40^{o}C and stored at 4°C.

4.4.5 HPLC fractionation of root proteins

Protein extracts were fractionated on a diol column (Develosil 300 diol-5, 8×300 mm, Phenomenex, Torrence, CA) at 280 nm, first using a constant pH and then using a gradient pH. The mobile phase consisted of 0.2 M Tris-HCl and 0.2 M NaCl solution with a flow rate of 0.5 mL min^{-1}. The protein extracts of the AM and non-AM roots were each separated into five fractions at the constant pH of 7.5 (Fig. 4.1), including the non-AM fractions corresponding to peaks of 1N, 2N, 3N, 4N, 5N, and the AM fractions corresponding to peaks of 1M, 2M, 3M, 4M and 5M. The fractions corresponding to peaks 2N, 3N, 2M and 3M were re-fractionated at a cycle of gradient pH (Figs. 4.2 and 4.3) and recovered. One cycle of the gradient pH dropped from 7.5 to 6 in 30 min and back to pH 7.5 in 30 min. The protein fractions were freeze-dried and stored at -20^{o}C until use.

4.4.6 HPLC fractionation of MeOH extracts

The 25% methanol extracts were fractionated on an octa decyl silane (ODS) column at a flow rate of 2.5 mL min^{-1} and absorbance of 254 nm and the fractions were recovered. The low-molecular-mass extract of mycorrhizal roots yielded 24 HPLC fractions and the extract of non-mycorrhizal roots yielded 22 fractions (Table 4.1).



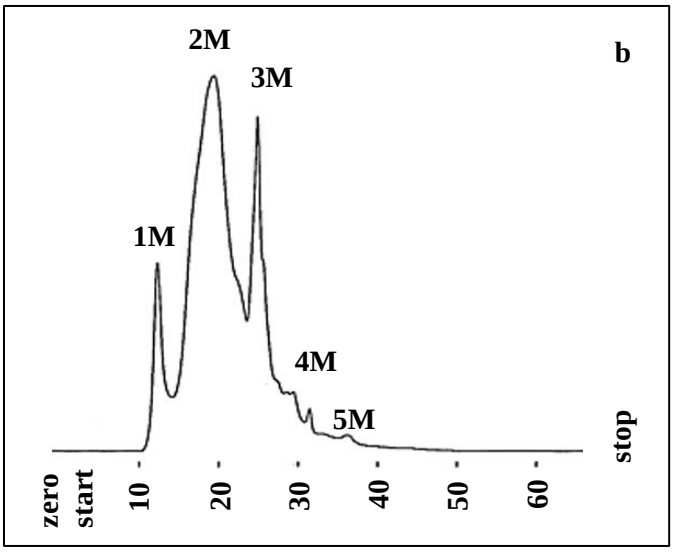
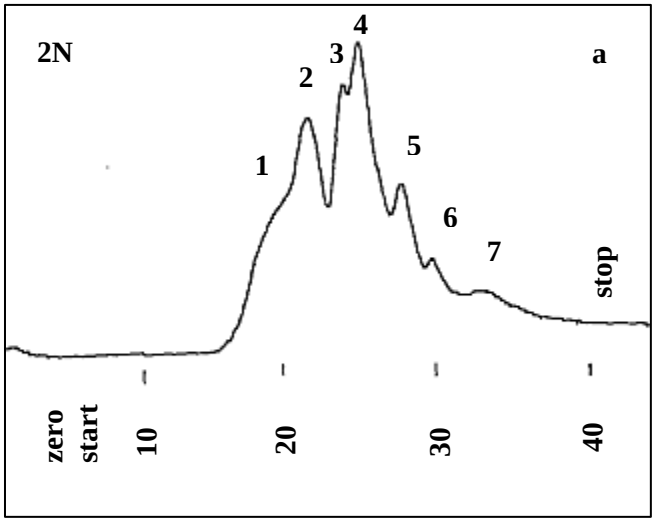


Fig. 4.1. HPLC chromatograms of the fractionation of total proteins content of non-mycorrhizal (a) and mycorrhizal (b) roots of CDC Anna chickpea on a diol column (8×300 mm) at 280 nm and the constant pH of 7.5. Mycorrhizal roots were colonized by arbuscular mycorrhizal fungus *Glomus intraradices*.



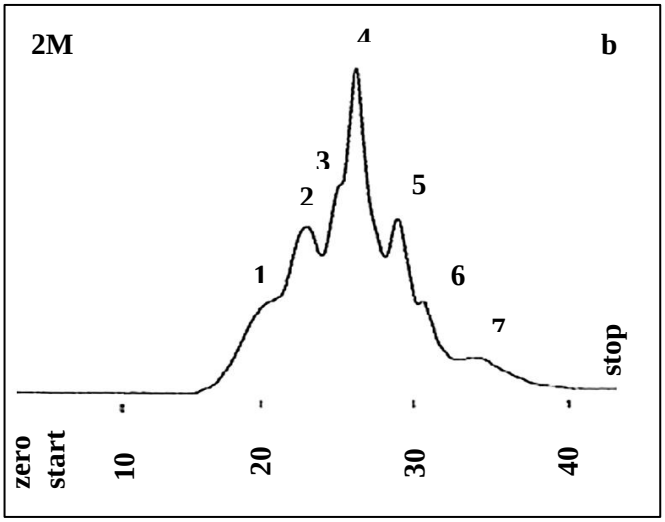


Fig. 4.2. HPLC chromatograms of the fractionation of 2N and 2M protein fractions from non-mycorrhizal (a) and mycorrhizal (b) roots of CDC Anna chickpea on a diol column (8×300 mm) at 280 nm and the gradient pH of 6 to 7.5. Mycorrhizal roots were colonized by arbuscular mycorrhizal fungus *Glomus intraradices*.

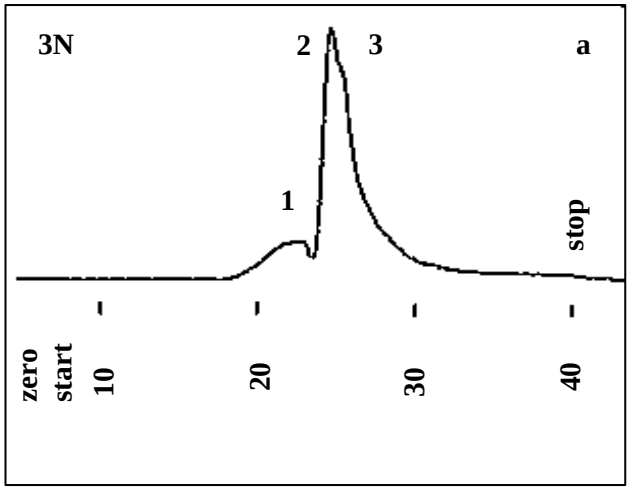


Fig. 4.3. HPLC chromatogram of the fractionation of 3N and 3M protein fractions from non-mycorrhizal (a) and mycorrhizal (b) roots of CDC Anna chickpea on a diol column (8×300 mm) at 280 nm and the gradient pH of 6 to 7.5. Mycorrhizal roots were colonized by arbuscular mycorrhizal fungus *Glomus intraradices*.

Table 4.1. Overview of HPLC fractions of low-molecular-mass[†] and proteins compounds[‡] extracted from AM[§] and non-AM roots of chickpea cultivar CDC Anna.

Phytochemical	Extract	Separation pH	AM treatment	Number of fractions
Low molecular mass	25% MeOH	7.5	Non-AM	22
Low molecular mass	25% MeOH	7.5	AM	24
Protein	Total	7.5	Non-AM	5
Protein	Total	7.5	AM	5
Protein	2NC	6 – 7.5	Non-AM	7
Protein	2MC	6 – 7.5	AM	7
Protein	3NC	6 – 7.5	Non-AM	3
Protein	3MC	6 – 7.5	AM	6

[†]Fractionated on a octa decyl silane (ODS) column at 254 nm
[‡]Fractionated on a diol column (8×300 mm) at 280 nm
[§]Arbuscular mycorrhizal (root) was colonized by *Glomus intraradices*

4.4.7 Fungal growth bioassay

The influence of proteins and low-molecular-mass metabolites of chickpea roots on fungal endophytes were tested using *Trichoderma harzianum* and *Geomyces vinaceus* and on pathogens using *Rhizoctonia* sp. and *Fusarium oxysporum* as model fungi. The fractions were tested at three concentrations in 96-well multiwell plates. The experiment was arranged in a randomized complete block design with four replicates. The concentration of proteins in fractions was quantified using the Qubit® Protein Assay Kit in a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA). A 100 µg mL^{-1} stock solution of each of the lyophilized protein fractions was prepared in 0.05M Tris-HCl buffer with a pH of 7.5. Dilutions of 5, 25 and 50 µg mL^{-1} were prepared and filter sterilized (MCE 0.2 µM, Fisher Scientific, Pittsburgh, PA). Pure cultures of the four model fungi were grown on potato dextrose agar (PDA) medium for 4 d (Chet and Baker, 1980). Six plugs (5 mm diameter) of actively growing mycelia were transferred into sterile Erlenmeyer flasks containing 30 mL of sterile distilled water and mixed for 2 min using a sterile magnetic stirbar at the minimum speed. Each well of the microtitre plate was set up with 170 µL of potato dextrose broth (PDB), 20 µL of the mixture of mycelia and 10 µL of a protein fraction. The control treatment received sterilized fungal mycelia. The plates were shaken for 5 min and absorbance was read on a Bio-Tek microplate reader at 630 nm prior to incubation at 25^{o}C in dark. Each plate represented an experimental block. The plates were randomized daily during the incubation time. Absorbance was measured at 630 nm daily after shaking the plate for 5 min until no change in absorbance was observed.

4.4.8 Gel electrophoresis

The protein fraction that was found bioactive through bioassays was further analyzed on SDS-PAGE gel electrophoresis using a modified protocol described by Vujanovic et al. (2009). The freeze-dried proteins were re-suspended in the sample buffer (0.062 M Tris pH 6.8, 5% sodium dodecyl sulfate [SDS]), 10% glycerol, 0.05% bromophenol blue, and 10% β-mercaptoethanol) and separated on a 12% polyacrylamide gel on Bio-Rad Mini-Protean II apparatus at 150 V along with a standard protein marker (BIO-RAD Ltd. Mississauga, ON). The gels were silver-stained according to Shevchenko et al. (1996) and scanned using a 1200C Epson scanner (Epson, Tokyo). The gel image was digitalized using software TotalLab Quant V. 1.0.0.1 (TotalLab Limited, Newcastle, UK).

4.4.9 Mass spectrometry and identification of proteins

Protein bands were excised from the polyacrylamide gel, and digested with trypsin at the Advanced Analysis Centre (AAC) of the University of Guelph. Matrix assisted laser desorption ionization-time of flight-tandem mass spectrometry (MALDI-TOF-TOF-MS/MS) acquisition was performed (Bienvenut et al., 2002). The raw mass spectrometry data were processed and converted into theoretical calculated masses and corresponding scored peptide sequences using the software PEAKS (Ma et al., 2003). The sequences were searched at NCBI, UniProtKB, and antimicrobial plant peptides PhytAMP databases to identify the proteins (Hammami et al., 2009).

4.4.10 Statistical analysis

A one-way analysis of variance (ANOVA) with Dunnett's post hoc multiple-comparison test was performed in R version 2.14.1 (package multcomp) to assess the significance of the

effect of protein and low-molecular-mass fractions on the growth of fungal mycelia, separately. A *p*-value of less than 0.05 was used as the threshold to reject the null hypothesis (Foit et al., 2010).

4.5 Results

4.5.1 Bioassay testing protein fractions

Only one protein fraction expressed bioactivity in the conditions of the bioassay (Table 4.2). This HPLC protein fraction 3 from mycorrhizal root extract (3M), inhibited the growth of all fungi tested at all three concentrations used (Fig. 4.4). In contrast, the HPLC protein fraction 3 from the non-mycorrhizal root extract 3N had no influence on fungal growth.

4.5.2 Gel electrophoresis and identification of proteins

Protein fraction 3 from mycorrhizal root extract 3M separated into four protein bands on the SDS-PAGE gel and only two bands were obtained from the same fraction of the non-mycorrhizal root extract 3N. Two up-regulated protein bands P1 and P2 were only detected in the mycorrhizal fraction (Fig. 4.5). These peptides were associated with chitinase/chitin-binding domain and non-specific lipid transfer proteins (Table 4.3).

Table 4.2. Time course of the effects of a bioactive protein fraction from mycorrhizal chickpea roots[†] on the growth of fungal endophytes[‡] and pathogens[§], in 96-well plat assay

Fungi	concentration (μg mL^{-1})	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
G. vinaceus	0	0.09	0.10	0.13	0.32	0.53	0.64	0.68	0.71	0.74	0.77
G. vinaceus	5	0.08	0.09	0.09	0.11	0.43	0.56**	0.65	0.71	0.72	0.74
G. vinaceus	25	0.08	0.09	0.09	0.09	0.32*	0.50**	0.54**	0.59***	0.61***	0.64***
G. vinaceus	50	0.09	0.09	0.09	0.09	0.09**	0.17***	0.27**	0.47***	0.56***	0.59***
F. oxysporum	0	0.096	0.10	0.13	0.27	0.56	0.77	0.93	1.05	1.09	
F. oxysporum	5	0.09	0.10	0.08	0.10	0.21	0.46**	0.65	0.79	0.89	
F. oxysporum	25	0.08	0.08	0.08	0.09	0.17*	0.24**	0.42**	0.68***	0.76***	
F. oxysporum	50	0.10	0.09	0.10	0.10	0.10**	0.10***	0.15***	0.38***	0.48***	
T. harzianum	0	0.08	0.08	0.13	0.29	0.56	0.98	1.32	1.41		
T. harzianum	5	0.08	0.08	0.09	0.25	0.53	0.76**	0.93	1.00		
T. harzianum	25	0.08	0.08	0.09	0.23	0.44*	0.70**	0.82**	0.89***		
T. harzianum	50	0.08	0.08	0.10	0.13	0.30**	0.49***	0.59***	0.61***		
Rhizoctonia sp.	0	0.08	0.08	0.08	0.08	0.21	0.62	0.76	0.80		
Rhizoctonia sp.	5	0.08	0.08	0.09	0.10	0.19	0.50**	0.69	0.73		
Rhizoctonia sp.	25	0.08	0.09	0.09	0.10	0.12*	0.48**	0.52**	0.54***		
Rhizoctonia sp.	50	0.08	0.08	0.09	0.09	0.10**	0.13***	0.29***	0.33***		

Data is presented as means (n = 4) of optical density at 630 nm. Significant differences between treatments and control (25% MeOH) were indicated by * ($p \leq 0.05$), ** ($p \leq 0.01$) and *** ($p \leq 0.001$).

[†]The roots of cultivar CDC Anna was colonized by *Glomus intraradices*

[‡]*Trichoderma harzianum* and *Geomyces vinaceus*

[§]*Fusarium oxysporum* and *Rhizoctonia* sp

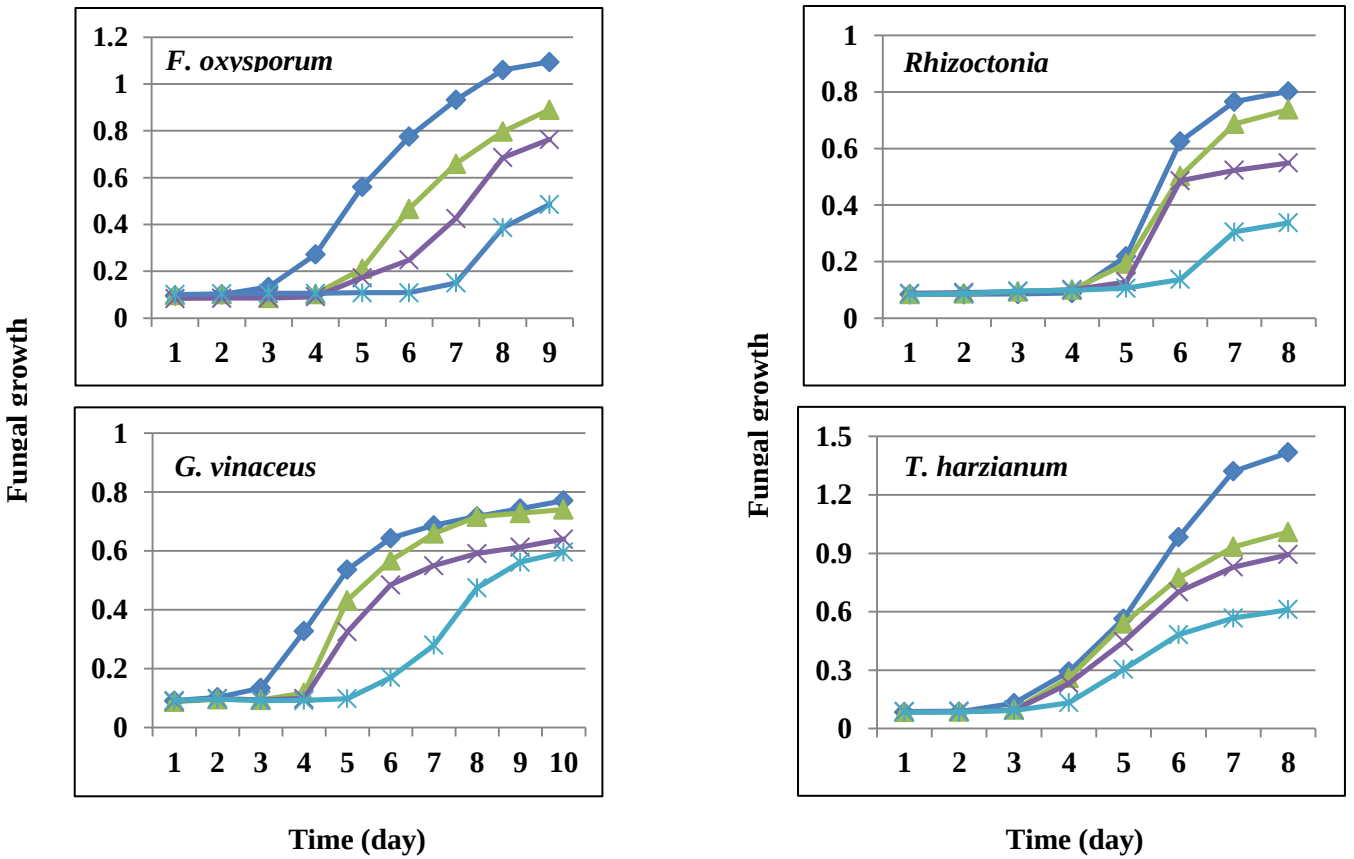


Fig. 4.4. The effect of bioactive protein fraction extracted from chickpea (Cultivar CDC Anna) roots colonized by *Glomus intraradices*, on the growth of fungal endophytes (*T. harzianum* and *G. vinaceus*) and pathogens (*F. oxysporum* and *Rhizoctonia* sp.) at different concentrations, *in vitro*. ■ 0 µg mL^{-1} ▲ 5 µg mL^{-1} × 25 µg mL^{-1} ✳ 50 µg mL^{-1}. Fungal growth was measured by optical density at 630 nm. The protein fraction was isolated from total protein content of roots using HPLC equipped with diol column (8×300 mm) at 280 nm, at two stages first using a constant pH = 6 and then using a gradient pH between 6 and 7.5.

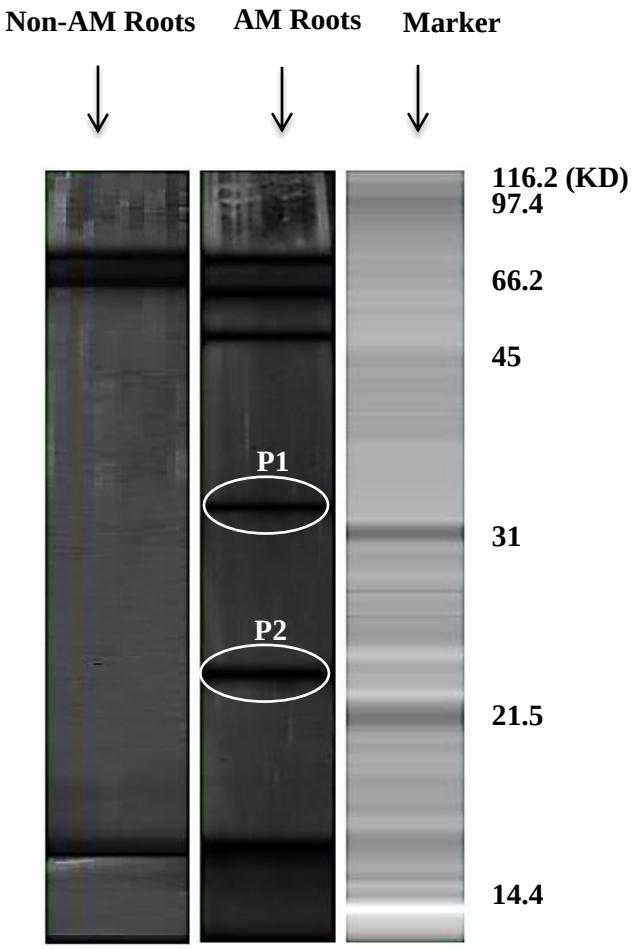


Fig. 4.5. SDS PAGE gel image of a protein fraction (3) extracted from arbuscular mycorrhizal (AM) and non-AM roots of chickpea cultivar CDC Anna. The P1 and P2 protein bands were up-regulated in AM roots. The gel image was digitalized using software TotalLab Quant V. 1.0.0.1 (TotalLab Limited, Newcastle, UK). The AM roots were colonized by AM fungus *Glomus intraradices*. The protein fractions were isolated from total protein content of chickpea roots using HPLC equipped with diol column (8×300 mm) at 280 nm, at two stages first using a constant pH = 6 and then using a gradient pH between 6 and 7.5.

Table 4.3. Identity of the differentially expressed bands[†] in the bioactive[‡] proteins fraction (3M) extracted from AM[‡] roots of CDC Anna Chickpea

Protein Band	Identification	gi no.	Score (%)	Sequence
P1	Non-specific lipid transfer protein	122249428	69.79	YLTGGA
P1	Chitin-binding domain	117956268	68.8	GMCCSQFGY
P1	Chitinase	357454531	68.8	GMCCSQFGY
P2	Non-specific lipid transfer protein	67937775	71.79	GVSNLNSMAK

[†] Protein bands were sequenced using MALDI-TOF mass spectrometry
[‡] inhibited fungal endophytes and pathogens
[‡]Arbuscular mycorrhizal roots, colonized by *Glomus intraradices*

4.5.3 Bioassay testing methanol fractions

Seven of the 25% MeOH-soluble HPLC fractions from mycorrhizal roots were bioactive, whereas 14 fractions of non-mycorrhizal roots extracts were bioactive (Table 4.4 and 4.5). The effect of these fractions varied with the concentration applied and the fungal species tested. Nineteen fractions stimulated the growth of the endophytes *T. harzianum* and *G. vinaceus*, and six fractions inhibited the pathogens *Rhizoctonia* sp. and *F. oxysporum*. One fraction also stimulated *F. oxysporum*. Thirty six percent of the MeOH-soluble HPLC fractions from the mycorrhizal root extract had growth-promoting effects when applied at a conducive concentration, while 13% were suppressive (Table 4.4). Fifty nine percent of the non-mycorrhizal fractions had promoting effects when applied at a conducive concentration whereas 14% were suppressive (Table 4.5). The MeOH soluble fractions had selective effects on the endophytes and pathogens. Among the fractions from the mycorrhizal root extract, four fractions had only growth-promoting effects, one fraction had suppressing effect, and two fractions were sometimes growth-promoting and sometimes growth-suppressing, depending on the fungal species. Among the fractions from non-mycorrhizal root extract, ten were growth-promoting, one was growth-supressing, and the effects of four fractions varied with fungal species *T. harzianum* and *Rhizoctonia* sp. were respectively promoted and suppressed by several fractions. The MeOH fractions of the AM roots had less growth promoting effects on fungal endophytes than the non-AM roots (Table 4.6).

Table 4.4. Time course of the effects of low molecular mass phytochemicals (25% MeOH fractions) from mycorrhizal chickpea roots[†] on the growth of fungal endophytes[‡] and pathogens[§], in 96-well plat assay

Fungi	Fr.[¶]	Con.[#]	Effect	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
G. vinaceus	17	100%	Stim.[††]	0.18	0.47	0.56	0.56*	0.64**	0.74**	0.77**	0.77**	0.79**	0.81**
Rhizoctonia sp.	8	100%	Sup.[‡‡]	0.08	0.08	0.10	0.17	0.47	0.57*	0.69*	-	-	-
Rhizoctonia sp.	10	100%	Sup.	0.08	0.09	0.15	0.24	0.46	0.55*	0.72*	-	-	-
Rhizoctonia sp.	13	100%	Sup.	0.08	0.08	0.11	0.24	0.52	0.63*	0.70*	-	-	-
T. harzianum	9	100%	Stim.	0.09	0.14	0.30	0.62*	0.96*	1.25*	1.36*	-	-	-
T. harzianum	10	66%	Stim.	0.10	0.15	0.30	0.66*	1.11*	1.26*	1.35*	-	-	-
T. harzianum	10	100%	Stim.	0.08	0.24	0.38	0.76*	1.06*	1.24*	1.38**	-	-	-
T. harzianum	13	100%	Stim.	0.08	0.15	0.34	0.73*	1.04*	1.22*	1.33*	-	-	-
T. harzianum	15	100%	Stim.	0.08	0.26	0.45	0.77*	1.11*	1.34*	1.36*	-	-	-
T. harzianum	21	100%	Stim.	0.08	0.17	0.34	0.78*	1.08*	1.23*	1.33*	-	-	-
F. oxysporum	13	100%	Sup.	0.08	0.11	0.71**	0.86***	0.89***	-	-	-	-	-

Data is presented as means ($n = 4$) of optical density at 630 nm. Significant differences between treatments and control (25% MeOH) were indicated by * ($p \leq 0.05$), ** ($p \leq 0.01$) and *** ($p \leq 0.001$). Fractions with non-significant effect at * ($p \leq 0.05$) were omitted from the table.

[†]The roots of cultivar CDC Anna was colonized by *Glomus intraradices*

[‡]*Trichoderma harzianum* and *Geomyces vinaceus*

[§]*Fusarium oxysporum* and *Rhizoctonia* sp

[¶]Fraction

[#] Concentration

[††] Stimulation

[‡‡]Suppression

Table 4.5. Time course of the effects of low molecular mass phytochemicals (25% MeOH fractions) from non-mycorrhizal chickpea roots[†] on the growth of fungal endophytes[‡] and pathogens[§], in 96-well plat assay

Fungi	Fr.[¶]	Con.[#]	Effect	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
G. vinaceus	9	33%	Stim.[††]	0.08	0.09	0.10	0.14	0.46	0.56	0.62	0.67	0.70*	0.72*
G. vinaceus	13	66%	Stim.	0.08	0.08	0.09	0.13	0.47	0.60*	0.65*	0.68***	0.70***	0.71***
G. vinaceus	14	100%	Stim.	0.08	0.09	0.09	0.13	0.52*	0.69***	0.76***	0.80***	0.82***	0.83***
G. vinaceus	21	100%	Stim.	0.08	0.08	0.09	0.15	0.41	0.57	0.65*	0.71**	0.75**	0.77**
Rhizoctonia	9	100%	Sup.[‡‡]	0.08	0.08	0.09	0.09	0.20	0.43***	0.52***	0.54***	0.55***	-
Rhizoctonia	11	66%	Sup.	0.08	0.08	0.08	0.09	0.19	0.43***	0.54***	0.58***	0.58***	-
Rhizoctonia	21	66%	Sup.	0.08	0.08	0.08	0.09	0.19	0.41***	0.49***	0.51***	0.52***	-
T. harzianum	2	33%	Stim.	0.09	0.18	0.73	0.93	1.08	1.14***	1.18***	-	-	-
T. harzianum	4	100%	Stim.	0.08	0.18	0.84	0.96*	1.08**	1.16***	1.21***	-	-	-
T. harzianum	7	33%	Stim.	0.08	0.18	0.73	0.96	1.15	1.26***	1.28***	-	-	-
T. harzianum	7	100%	Stim.	0.08	0.15	0.64	0.76	0.88	0.97***	1.00***	-	-	-
T. harzianum	8	66%	Stim.	0.08	0.18	0.70	0.95	1.06	1.15***	1.19***	-	-	-
T. harzianum	9	33%	Stim.	0.08	0.19	0.75	1.00	1.17*	1.27***	1.31***	-	-	-
T. harzianum	9	66%	Stim.	0.08	0.18	0.72	0.93	1.05	1.14	1.17***	-	-	-
T. harzianum	10	66%	Stim.	0.08	0.17	0.66	0.91	1.04	1.14**	1.21***	-	-	-
T. harzianum	13	100%	Stim.	0.088	0.18	0.784	0.95	1.10*	1.21***	1.27***	-	-	-
T. harzianum	15	100%	Stim.	0.09	0.18	0.78	0.95	1.06	1.17***	1.22***	-	-	-
T. harzianum	17	66%	Stim.	0.08	0.18	0.64*	0.81*	0.93***	0.98***	1.00***	-	-	-
T. harzianum	18	33%	Stim.	0.08	0.17	0.74	0.94	1.04	1.12**	1.17***	-	-	-
T. harzianum	18	66%	Stim.	0.08	0.18	0.72	0.95	1.05	1.13**	1.17***	-	-	-
T. harzianum	18	100%	Stim.	0.08	0.18	0.75	0.99**	1.12***	1.21***	1.26***	-	-	-
T. harzianum	22	66%	Stim.	0.08	0.18	0.71	0.90	1.03	1.13***	1.20***	-	-	-
T. harzianum	22	100%	Stim.	0.08	0.18	0.73	0.92	1.09***	1.19***	1.26***	-	-	-
F. oxysporum	14	66%	Stim.	0.09	0.18	0.96*	1.39***	1.43***	-	-	-	-	-
F. oxysporum	17	100%	Sup.	0.08	0.12	0.58*	0.70***	0.83***	-	-	-	-	-

Data is presented as means (n = 4) of optical density at 630 nm. Significant differences between treatments and control (25% MeOH) were indicated by * ($p \leq 0.05$), ** ($p \leq 0.01$) and *** ($p \leq 0.001$). Fractions with non-significant effect at * ($p \leq 0.05$) were omitted from the table.

[†]The roots of cultivar CDC Anna was colonized by *Glomus intraradices*

[‡]*Trichoderma harzianum* and *Geomyces vinaceus*

[§]*Fusarium oxysporum* and *Rhizoctonia* sp

[¶]Fraction

[#] Concentration

[††] Stimulation

[‡‡]Suppression

Table 4.6. Synthesis of the effects of low-molecular-mass phytochemicals[†] from AM[‡] and non-AM roots of chickpea cultivar CDC Anna on the growth[§] of fungal endophytes[¶] and pathogens[#]

Fungi	***T. harzianum***						***G. vinaceus***						***Rhizoctonia* sp.**						***F. oxysporum***					
	Concentration (Equivalent to root biomass)																							
Fraction	**5mg**		**10 mg**		**15 mg**		**5 mg**		**10 mg**		**15 mg**		**5 mg**		**10 mg**		**15 mg**		**5 mg**		**10 mg**		**15 mg**	
	N[††]	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M
1																								
2	+[‡‡]																							
3																								
4					+																			
5																								
6																								
7	+				+																			
8			+															-						
9	+		+			+	+										-							
10			+	+		+												-						
11													-		-									
12																								
13					+	+			+									-						-
14											+										+			
15					+	+																		
16																								
17			+								+												-	
18	+		+		+							+												
19																								
20			+		+	+																		
21											+		-		-									
22																								
23																								
24																								

[†]The phytochemicals were extracted by 25% methanol solution, and re-suspended into 25% ethanol prior to bioassay
[‡]Arbuscular mycorrhizal roots, colonized by *Glomus intraradices*
[§]Fungal growth was measured by optical density at 630 nm (n = 4) in 96-well plates
[¶]*Trichoderma harzianum* and *Geomyces vinaceus*
[#]*Fusarium oxysporum* and *Rhizoctonia* sp
[††]N: Non-mycorrhizal root, M: Mycorrhizal root
[‡‡]: growth promotion, -: growth suppression, empty cells: no effect

4.6 Discussion

The AM and non-AM chickpea roots produced bioactive proteins and low-molecular-mass phytochemicals that impact the growth of soil-borne fungal endophytes and pathogens. I observed that the low-molecular-mass phytochemicals selectively influenced the growth of fungal species. Several low-molecular-mass phytochemicals stimulated the growth of *T. harzianum. Trichoderma* species are well-known endophytic symbionts that colonize a wide range of host plants. They are able to improve seed germination and plant nutrient uptake, and trigger systemic resistance to disease in their host plant (Bailey et al., 2009; Brotman et al., 2008). Colonization of roots by endophytic fungi involves some level of host specificity (Maciá-Vicente et al., 2009; Mandyam et al., 2013). The different responses of *T. harzianum* and *G. vinaceus* to particular phytochemicals suggest that specific roots compounds are responsible for the 'host preference' of fungal endophytes.

By contrast to the endophytes, few compounds were found to suppress *Fusarium oxysporum* and *Rhizoctonia* species, which are major fungal pathogens causing root rot diseases in a wide variety of plants (Nene et al., 1991; Zhang et al., 2013). The presence of various antimicrobial compounds in plant tissues was repeatedly reported (Bonanomi et al., 2009; Osbourn, 1996; Paul Schreiner and Koide, 1993). *Arabidopsis thaliana* released p-hydroxybenzamide and vanillic acid that inhibit *Fusarium oxysporum* and *Rhizoctonia* (Walker et al., 2009). Plant species express different degrees of resistance against different pathogens. The variations in the response of *F. oxysporum* and *Rhizoctonia* to the root phytochemicals suggest that specific root substances could be involved in 'host resistance' to soil-borne fungal pathogens.

The simultaneous presence of functionally similar bioactive compounds within roots suggests that plants produce and integrate multiple overlapping metabolites to modify their microbial

environment. I found that a specific compound can act as a stimulator for a specific species of fungi while also acting as an inhibitor or neural on other species. In a recent study by Badri et al. (2013) natural blends of phytochemicals derived from the root exudates of *Arabidopsis thaliana* was repeatedly applied to soil at various relative concentrations. Further, high throughput DNA sequencing analysis revealed that one particular compound can influence various soil microbes, but the same compound can stimulate or suppress different groups of microbes.

This study identified antifungal proteins up-regulated in the mycorrhizal roots of chickpea (Table 4.3). The protein fraction contained chitinase, chitin-binding domain and a non-specific lipid transfer protein that non-selectively suppressed the growth of the endophytic and pathogenic fungi. Chitinases are potent enzymes that catalyze the degradation of chitin, the primary constituent of fungal cell walls (Salzer et al., 2000; Selitrennikoff, 2001). Several isoforms of chitinases are involved in induced resistances to pathogen infections, the development of microbial symbioses and the enhancement of tolerance to abiotic stresses (Boller and Métraux, 1988; Collinge et al., 1993; Liu et al., 2004; Salzer et al., 2004). The inhibitory activity of plant chitinase against soil-borne fungi has been confirmed *in vitro*, however several species of mycorrhizal and pathogenic fungi were not suppressed by some isomorphs of chitinases (Dumas-Gaudot et al., 1996). Class I chitinases enhance plant resistance to *Rhizoctonia* solani (Broglie et al., 1991). Chitinases are able to degrade the residues of chitin released from the cell walls of mycorrhizal fungi that would otherwise induce the plant defense response and suppress the symbiosis (Salzer et al., 1997). These observations suggest different roles of plant chitinases in the interaction of plants with symbiotic and pathogenic fungi. I did not detect an active chitinase in the non-AM roots, which could be due to the low concentration of chitinase in the roots of chickpea. Previous reports indicate that the activity of chitinases is

low in plant tissues unless it is triggered by microbial infections or other abiotic stresses (Lawton et al., 1992).

The protective effect of colonization by mycorrhizal fungi against soil-borne pathogens of various genera including *Fusarium*, *Rhizoctonia*, *Macrophomina*, and *Verticillium* co-occurs with over-expression of chitinase in a variety of plants. The AM symbiosis could reduce the abundance of pathogens in the root tissues (Pozo et al., 1998; Salzer et al., 2004; Whipps, 2004). Chitin binding domains are structural components of chitinases playing essential roles in substrate affinity and antifungal activity of chitinases (Iseli et al., 1993). Some chitin binding domains have direct antifungal properties (Broekaert et al., 1992).

Nonspecific lipid transfer proteins are small cysteine-rich proteins that are ubiquitous and abundant in the plant tissues of agriculturally important crops (Elmorjani et al., 2004). They are involved in plant development and responses to environmental stresses. Nonspecific lipid transfer proteins can be induced by AM fungi to selectively suppress spore germination and growth of fungal species (Blilou et al., 2000; Sun et al., 2008). This study concurs with those of others suggesting that mycorrhization influences the profile of metabolites produced in chickpea roots with potential impacts on the fungal communities associated with roots.

Plants produce antifungal protein and non-protein phytochemicals as defense reactions against microbial colonizers. The production of these antifungal compounds could be triggered by mycorrhizal symbiosis through the so called “autoregulation mechanisms” (Vierheilig et al., 2008). The AM symbiosis promotes the systemic induction of plant defense mechanisms, which enhance plant resistance to infection by microbial pathogens (Campos-Soriano et al., 2012).

Roots produce phytochemicals with antifungal properties against a wide range of pathogenic fungi. It was reported that *Fusarium oxysporum* f. sp. *ciceri* was only inhibited by the root

exudates of a wilt-resistant chickpea genotype, indicating the genetic basis of the variation in the bioactivity of chickpea roots on soil fungi (Stevenson et al., 1995). This suggests that conversely, the selection of chickpea genotypes with improved mutualistic associations, can be based on the screening of plants possessing a certain phytochemical profile.

5.0 DISCUSSION AND SYNTHESIS

The increasing emphasis on sustainable, efficient agriculture has raised interest in the management of soil microbial communities using their natural capacities to maintain soil health and fertility. Improving the contribution of beneficial microbes to soil quality requires a better understanding of the factors influencing the dynamics and functionalities of the microbial communities (Grayston et al., 1998; Johansson et al., 2004). In this research, I examined the natural effect of chickpea roots on soil fungi as well as associations with soil fungal communities. This research is a first step towards the identification of chickpea varieties that improve the biological quality of soils by associating with, and responding to beneficial fungal species while repressing pathogens. The results described in Chapter 4 indicate the capacity of chickpea root metabolites to control specific fungal species. Chapter 2 reveals the significance of cultivars of chickpea in structuring root-associated fungal communities. Chapter 3 demonstrates that chickpea cultivars have different responses to beneficial soil fungi. These findings contribute to knowledge of the fitness of chickpea to its microbial environment and provide a basis for the development of the next-generation of varieties adapted to naturally occurring soil fungi in the Canadian Prairie.

5.1 Chickpea roots in the soil biological environment

Plants have a natural ability to release a spectrum of metabolites that selectively attract and repel soil microorganisms. Ultimately, these metabolites shape the structure and function of soil microbial communities (Badri et al., 2013). Based on previous research (Bednarek and Osbourn, 2009; Cruz et al., 2012; Scalbert and Williamson, 2000) the composition of plant metabolites

differs with species, genotype, tissue and physiological stage. Thus, the structure of soil microbial communities is expected to be constantly shifting in agricultural settings. In this research, the observation that the metabolites of chickpea roots selectively influence fungal species, suggests that root metabolites could have important roles in structuring the fungal communities of soil and root endosphere. Manipulating root metabolite production, therefore, could create microbial niches promoting the proliferation of beneficial fungi while inhibiting pathogens. This research found several root metabolite fractions with general or specific bioactivities on important fungal species that inhabit cultivated soils of the chickpea growing region of Canada. A recent study showed that the profile of root metabolites differ among cultivars of chickpea (Ellouze et al., 2012). The simultaneous variations in the profile of root metabolites and the structure of root fungal communities of cultivars of chickpea supports the conclusion that the root metabolites could be selecting root-associated fungi. The selective effects of the bioactive metabolites from chickpea roots on fungal endophytes and pathogens could be used to generate varieties that enhance the sustainability of agriculture by improving the contribution of soil fungi to crop production.

5.2 Cultivar dependent interactions of chickpea with soil fungi

Cultivars of chickpea determined the diversity of root-associated fungal communities. Cultivars also determined the response of chickpea to symbioses with AM and non-AM fungal endophytes. For example, the cultivar CDC Corrine, which hosted a high diversity of root fungal communities, also responded more positively to inoculation with selected native AM and non-AM fungal endophytes (Table 5.1). There are reports indicating that soil microbial diversity is often associated with plant health (Garbeva et al., 2004).

Table 5.1. Synthesis of the influence of chickpea cultivars on the root-associated fungal communities[†], and the response[‡] of cultivars to AM[§] fungi and non-AM fungal endophytes

Cultivar	Type	Richness[¶] (AM)	Diversity[#] (AM)	Richness (non-AM)	Diversity (non-AM)	Dominance of *Fusarium* in roots	Response to AM	Response to non-AM
Amit	Kabuli	Low	Medium	Low	Medium	Medium	Good	Negative
CDC Alma	Kabuli	Low	Low	Low	Medium	Medium	Fair	Neutral
CDC Frontier	Kabuli	Medium	High	Medium	Medium	High	Good	Neutral
CDC Leader	Kabuli	Low	Medium	Low	Medium	High	Fair	Neutral
CDC Luna	Kabuli	Medium	High	Low	Medium	Medium	Fair	Neutral
CDC Orion	Kabuli	Medium	Medium	Low	Medium	High	Fair	Neutral
CDC Xena	Kabuli	Medium	High	Low	Low	Low	Fair	Neutral
CDC Cabri	Desi	Medium	Medium	Medium	Medium	High	Fair	Neutral
CDC Corinne	Desi	High	High	High	High	Low	Fair	Positive
CDC Cory	Desi	High	Medium	High	High	High	Fair	Positive
CDC Vanguard	Desi	Medium	High	High	High	High	Good	Negative
CDC Anna	Desi	Medium	Medium	Medium	Medium	High	Good	Positive
CDC Nika	Desi	Low	Medium	Low	Medium	High	Good	Neutral

[†]Assessed in field-grown chickpeas, using 454 pyrosequencing (n = 6). Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011. Primer sets used for PCR amplification of fungal DNA were ITS1F/ITS2 (Non-AM fungi), and NS1/NS4 and AMV4.5-NF/AMDGR (AM fungi).

[‡]Tested in the greenhouse. AM fungal species used for the study include *Diversispora eburnea, Claroideoglomus etunicatum* and *Glomus* sp and non-AM fungal endophytes used include *Trichoderma harzianum* and *Mortierella alpina*. All the species were native to Saskatchewan soils.

[§]Arbuscular mycorrhizal

[¶]Chao richness index

[#]Shannon diversity index

It also is notable that plants are more responsive to AM symbiosis when inoculated with total soil inoculum and multiple AM fungal species compared with inoculation with single AM fungal species (Hoeksema et al., 2010). However, to date, there is no indication for a relationship between the diversity of root-associated fungal communities and plant response to AM symbiosis. The co-occurrence of the high diversity of root associated fungal communities and negative response of CDC Vanguard to non-AM fungal endophytes (Table 5.1), suggests that there could also be other factors involved in controlling the response of plants to fungal symbionts. The higher responsiveness of plants to multiple AM symbioses could be due to a functional complementarity among AM fungi in providing benefits to the host (Hart and Reader, 2002; Maherali and Klironomos, 2007) or due to the higher chance for the presence of compatible AM fungal species in the mixed inoculum (Vogelsang et al., 2006). The higher response of some cultivars to AM symbiosis observed in this study could be attributed to the natural capability of these cultivars to form an association with the fungal species that were used. My findings suggest that the chickpea varieties that associate with a high diversity of soil microbial communities could have a better chance to form efficient microbial symbioses. Although, other factors including plant genetics appear to interact in the outcome of an association between roots and symbiotic soil microorganism.

5.3 Differences in microbial relations of desi and kabuli chickpea

The low diversity of root-dwelling fungal communities associated with kabuli chickpea and the relative weakness of this type of chickpea in forming efficient fungal symbioses could have developed through breeding practice reducing the ability of the plant to use soil fungal resources. Kabuli chickpeas were derived from desi chickpea varieties through long-

term selections (Moreno and Cubero, 1978; Singh, 1997). The differences in fungal associations between the types of chickpea could be due to the loss of genes coding for certain metabolites originally present in the ancestors. It could also be the side effect of resistance against fungal pathogens acquired through years of selective breeding, as similar mechanisms are thought to be involved in regulation of AM symbiosis and control of root-infecting fungal pathogens (Vierheilig et al., 2008).

5.4 Dynamics of *Fusarium* in chickpea roots

The observation that *Fusarium* was the dominant non-AM fungal species in the roots of field grown chickpea (Fig. 2.5) and its effect in reducing plant biomass and yield in the field and greenhouse studies (Figs. 2.7 and 2.9) suggests that *Fusarium* could negatively impact the yield of chickpea in southern Saskatchewan. The genus *Fusarium* contains various fungal species that cause root and crown rot diseases and reduce the biomass of chickpea (Doohan et al., 2003; Navas-Cortés et al., 2000; Nene et al., 1991). However, the genetic variation found in the colonization of chickpea roots by *Fusarium* indicates the potential ability of chickpea to control *Fusarium*. The roots of CDC Corrine and CDC Xena contained the lowest relative abundance of *Fusarium* among all cultivars. The lower abundance of *Fusarium* in CDC Corinne could be attributed to the highest level of diversity of root associated fungi as suppression of soil-borne pathogens is often attributed to high soil microbial diversity (Altieri, 1999; Brussaard et al., 2007). In contrast, the diversity of root associated fungal communities was the lowest in CDC Xena, suggesting the possibility of the involvement of other mechanisms that inhibit the association of roots with soil-borne fungi including *Fusarium* species. It is notable that CDC Xena is the most susceptible cultivar to the fungal

pathogen *Ascochyta rabiei*. Cho and Muehlbauer (2004) found significant differences in the expression patterns of defence-related genes in blight resistant and susceptible genotypes of chickpea inoculated with *A. rabiei*. Although the differences in the gene expressions, were not related to blight resistance in the recombinant lines, such variations in gene expressions could have non-target effects on other fungi that inhibit the association of roots with other fungal pathogens including *Fusarium* species through induced systemic resistance.

5.5 Future research

This research shows that the metabolites derived from the roots of CDC Anna have selective influence on some soil microorganisms. More research in required to identify the bioactive metabolites produced in the roots of chickpea. CDC Corrine, CDC Cory and CDC Anna would be among the best plants to investigate as their roots are associated with the highest level of fungal diversity and they respond best to fungal symbionts. CDC Xena is also an interesting model due to its low level of colonization by *Fusarium*. Key root metabolites could be used to generate markers for the selection of varieties that form efficient fungal symbioses and resist *Fusarium* invasion.

Colonization of roots by AM fungi appears to influence the composition of root metabolites through the stimulation or direct release of secondary metabolites (Larose et al., 2002). These metabolites might influence non-target organisms such as *Ascochyta rabiei*, a fungal pathogen causing the leaf disease Ascochyta blight. Considering the importance of Ascochyta blight in chickpea, the possibility of a systemic influence of AM fungal colonization of roots is worth investigating. Metabolites systemically induced or produced by AM fungi might reduce the impact of Ascochyta in the leaves. Metabolites released from *T.*

harzianum were shown to suppress the growth of *Ascochyta rabiei* in vitro (KüÇük et al., 2007).

This study indicates that the outcome a multipartite symbioses with the AM and non-AM endophytes in chickpea is cultivar-dependent and varies from negative to positive. It opens the possibility for the development of cultivars of chickpea that improve soil microbial quality and best respond to the soil microbial community feedback, resulting in the improvement of soil microbiology.

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APPENDIX A

Diversity /phylogenetic analysis of chickpea genotypes

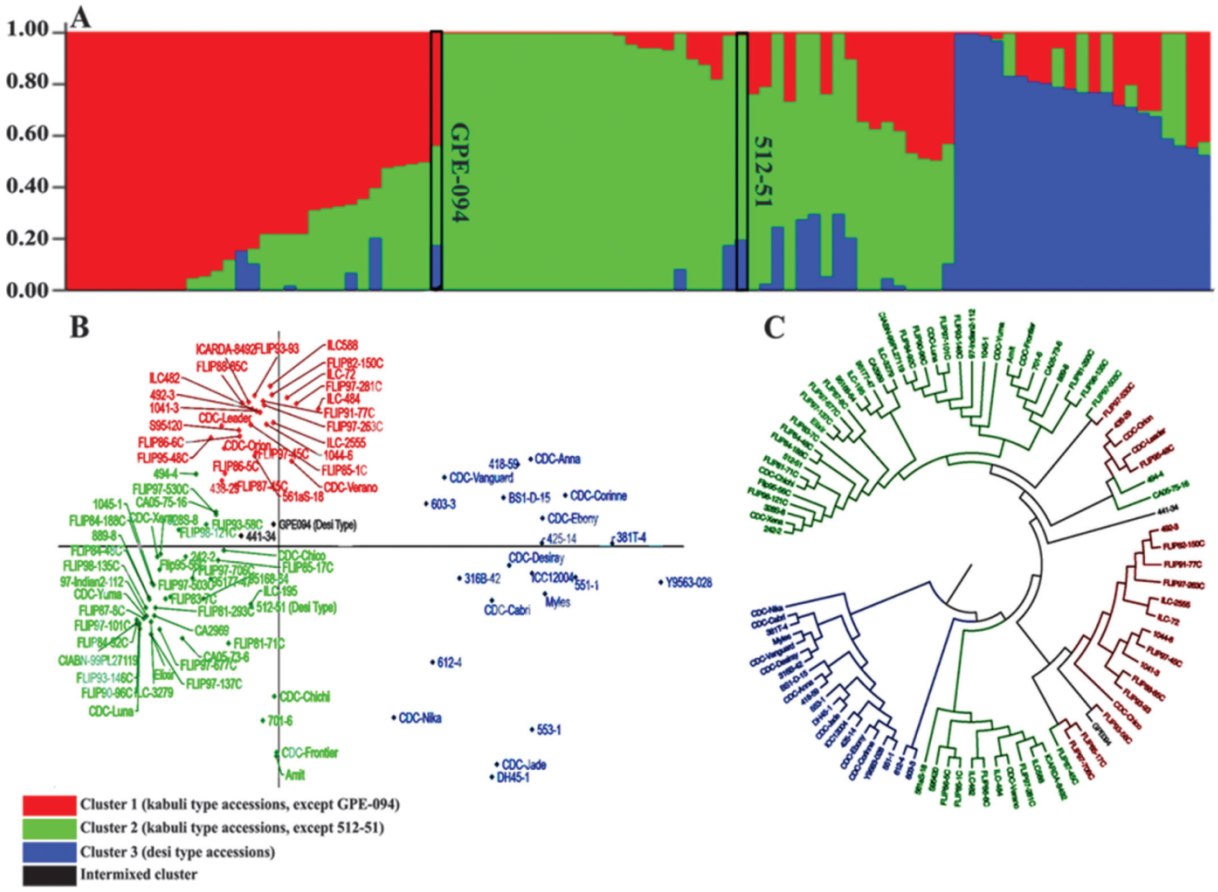

Fig. A.1. Structure plot of the chickpea association mapping population with K = 3 clusters based on all polymorphic SNP markers. Each individual is represented by a thin vertical line and estimated membership fractions in clusters. Two accessions, GPE094 and 512-51, are morphologically desi type, but they clustered with the kabuli type due to a larger portion of kabuli ancestors. (B) Principal coordinates analysis (PCoA), the x axis represents the eigenvalue for principal component 1 (PC1) and the y axis for PC2. (C) The neighbor-joining phylogenetic tree (NJTree), based on Nei (1972) standard genetic distance with 10 000 individual bootstraps (With permission from Genome, Courtesy of *Diapari et al., 2014).
The figure was reproduced from "Genetic diversity and association mapping of iron and zinc concentrations in chickpea (*Cicer arietinum* L.) by: Marwan Diapari, Anoop Sindhu, Kirstin Bett, Amit Deokar, Thomas D. Warkentin, and Bunyamin Tar'an. Genome 57: 1–10 (2014).

APPENDIX B

Research Plot Layouts

Treatment	Plot#	Treatment	Plot#
BORDER		**BORDER**	
CDC Alma	**1**	Amit	**27**
CDC Vanguard	**2**	CDC Corrine	**28**
CDC Cabri	**3**	CDC Vanguard	**29**
CDC Leader	**4**	CDC Xena	**30**
CDC Luna	**5**	CDC Nika	**31**
CDC Anna	**6**	CDC Leader	**32**
CDC Cory	**7**	CDC Cabri	**33**
CDC Frontier	**8**	CDC Orion	**34**
Amit	**9**	CDC Anna	**35**
CDC Nika	**10**	CDC Luna	**36**
CDC Corrine	**11**	CDC Alma	**37**
CDC Orion	**12**	CDC Frontier	**38**
CDC Xena	**13**	CDC Cory	**39**
BORDER		**BORDER**	
CDC Cory	**14**	CDC Luna	**40**
CDC Anna	**15**	CDC Nika	**41**
Amit	**16**	CDC Frontier	**42**
CDC Corrine	**17**	CDC Cabri	**43**
CDC Alma	**18**	CDC Orion	**44**
CDC Xena	**19**	CDC Cory	**45**
CDC Vanguard	**20**	CDC Xena	**46**
CDC Frontier	**21**	CDC Leader	**47**
CDC Cabri	**22**	CDC Alma	**48**
CDC Orion	**23**	Amit	**49**
CDC Leader	**24**	CDC Anna	**50**
CDC Nika	**25**	CDC Vanguard	**51**
CDC Luna	**26**	CDC Corrine	**52**
BORDER		**BORDER**	

67m

15m | 6m | 6m | 6m | 15m

18m

Fig. B.1. Research Plot Layout 2010

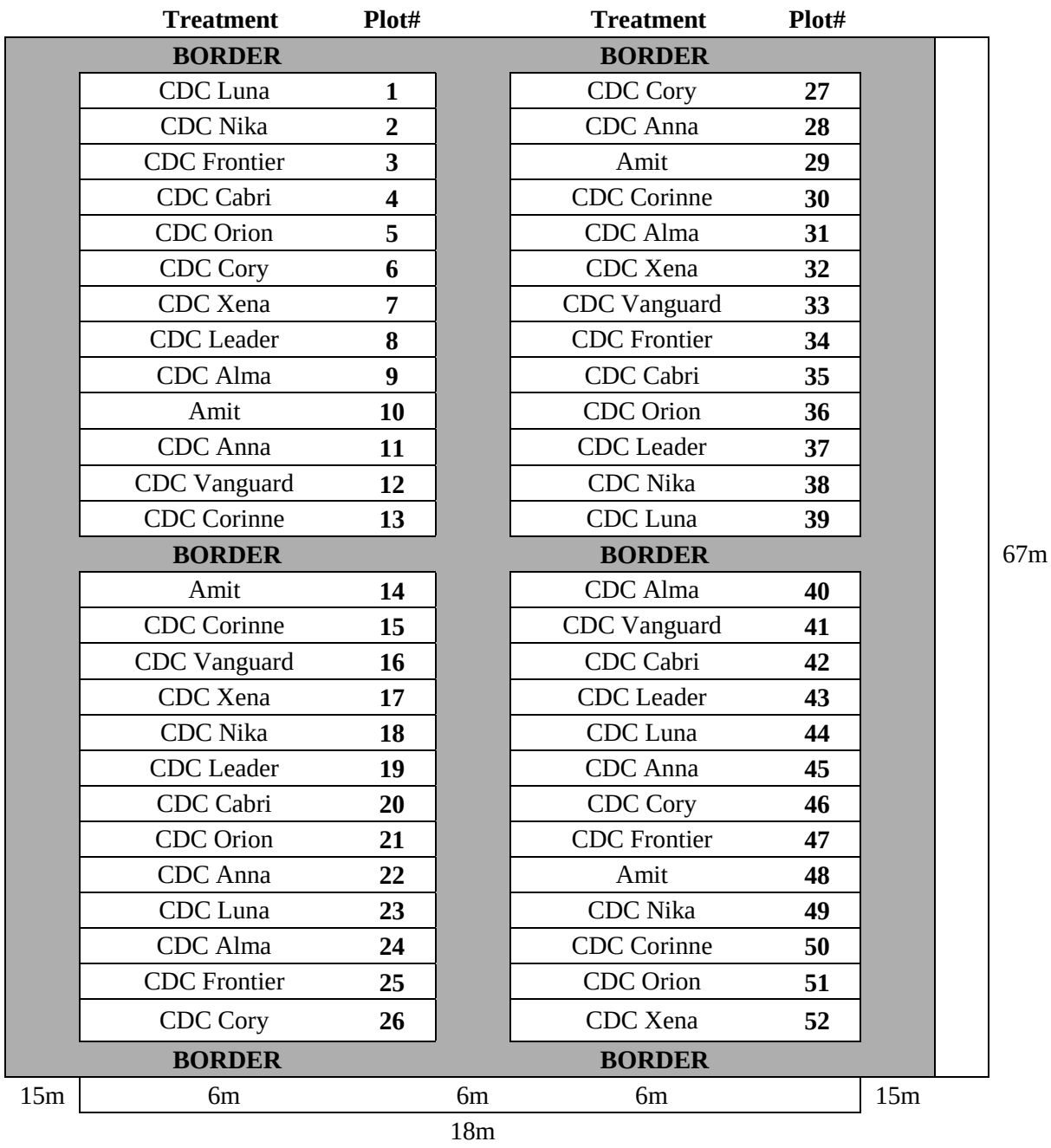

Fig. B.2. Research Plot Layout 2011

APPENDIX C

The Identity and distribution of fungal OTU reads in the root endosphere community of 13 cultivars of chickpea

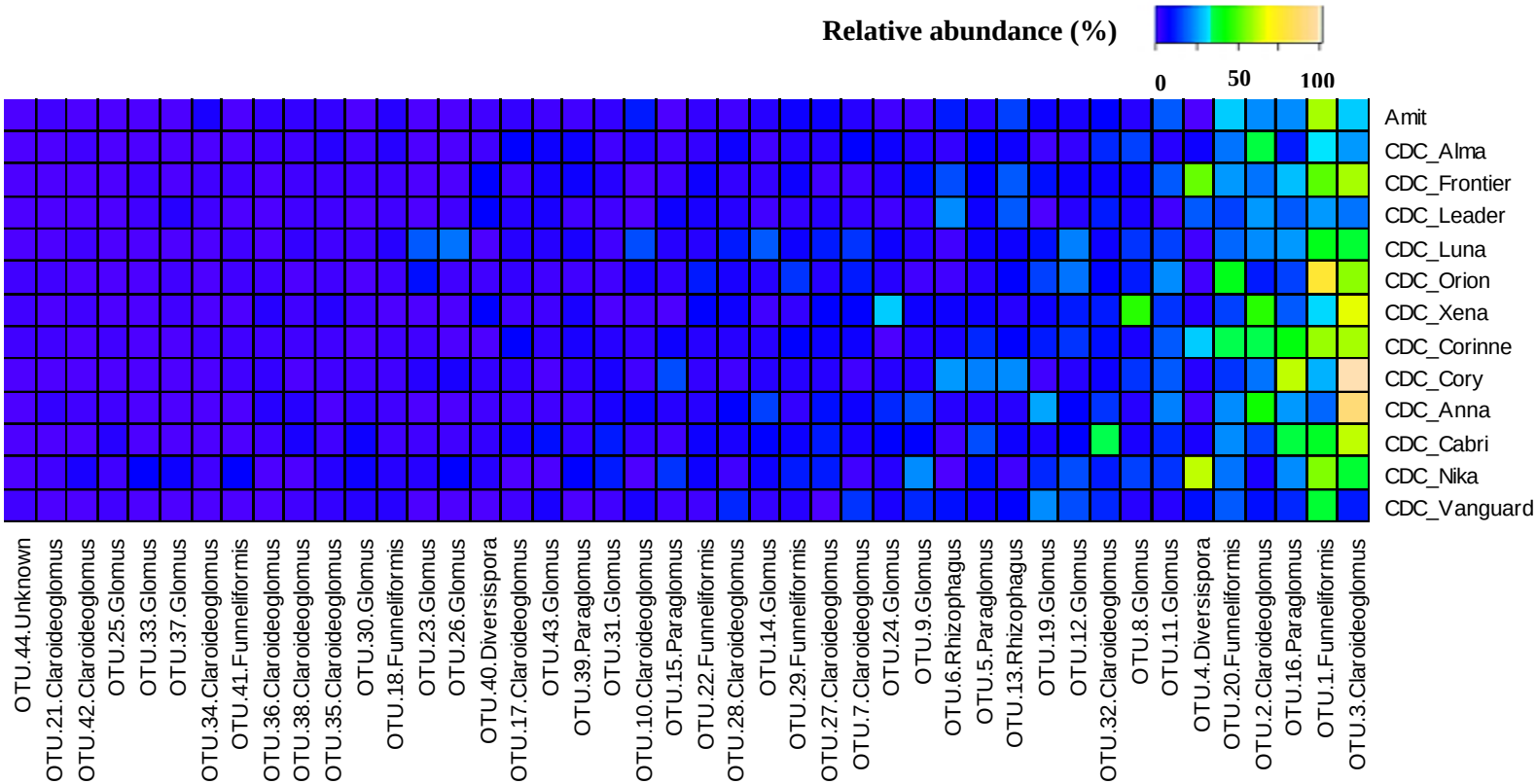

Fig. C.1. Distribution of AM fungal OTU reads in the root endosphere community of 13 cultivars of field-grown chickpea (n = 6). Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011. NS1/NS4 and AMV4.5-NF/AMDGR primer sets were used for PCR amplification of fungal DNA (nested PCR). Significant difference in the structure of AM fungal community among the cultivars was detected by Per-MANOVA (p = 0.0002).

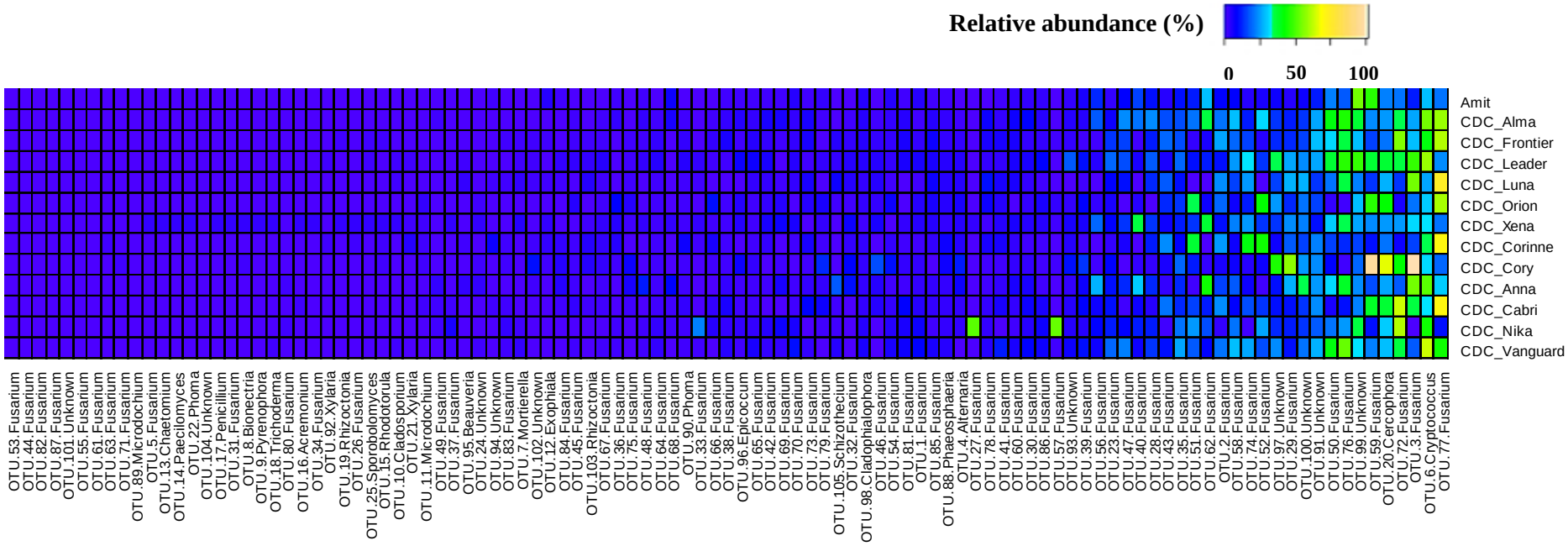

Fig. C.2. Distribution of non-AM fungal OTU reads in the root endosphere community of 13 cultivars of field-grown chickpea detected by pyrosequencing (n = 6). Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011. ITS1F/ITS2 primer set was used for PCR amplification of fungal DNA. Significant difference in the structure of non-AM fungal community was detected among the cultivars according to Per-MANOVA (p = 0.0004).

APPENDIX D

Sequence processing/Analysis information

Table D.1. Pre and post-cleaning sequence numbers[†] of AM[‡] and non-AM[§] fungal communities

Cultivar	Pre-cleaning AM Seq. number	Post-cleaning AM Seq. number	Post-subsampling AM Seq. number	Pre-cleaning Non-AM Seq. number	Post-cleaning Non-AM Seq. number	Post-subsampling Non-AM Seq. number
CDC Corrine	29784	11037	3000	14726	7726	3000
CDC Cory	27644	10897	3000	10389	8389	3000
CDC Anna	29487	11072	3000	15937	9937	3000
CDC Cabri	24202	9838	3000	18166	8166	3000
CDC Vanguard	29317	11818	3000	11829	7829	3000
CDC Nika	23001	11756	3000	12675	9675	3000
Amit	20092	9179	3000	10249	9249	3000
CDC Leader	27432	10972	3000	12167	8067	3000
CDC Orion	26837	11884	3000	11093	8053	3000
CDC Frontier	26713	10424	3000	10485	8985	3000
CDC Alma	27253	10449	3000	10378	7378	3000
CDC Luna	20851	9146	3000	12486	8486	3000
CDC Xena	29121	11410	3000	10069	7223	3000

[†]Assessed in field-grown chickpeas, using 454 pyrosequencing (n = 6). Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011. Primer sets used for PCR amplification of fungal DNA were ITS1F/ITS2 (Non-AM fungi), and NS1/NS4 and AMV4.5-NF/AMDGR (AM fungi).

[‡]Arbuscular mycorrhizal: Consisting of genera *Glomus, Claroideoglomus, Funneliformis, Paraglomus, Rhizophagus* and *Diversispora.*

[§]Consisting of genera *Fusarium, Alternaria, Cryptococcus, Acremonium, Bionectria, Microdochium, Trichoderma Paecilomyces, Mortierella, Rhodotorula, Chaetomium, Cladosporium, Penicillium,* and *Pyrenophora.*

Table D.2. Non-subsampled and subsampled Chao richness and Shannon diversity indices[†] of AM[‡] and non-AM[§] fungal communities associated with the roots of field-grown chickpea cultivars over two years, detected by pyrosequencing.

Cultivar	Non-subsampled AM richness	Subsampled AM richness	Non-subsampled AM diversity	Subsampled AM diversity	Non-subsampled non-AM richness	Subsampled non-AM richness	Non-subsampled non-AM diversity	Subsampled non-AM diversity
CDC Corrine	736 a	313 a	4.48 a	3.05 a	3365 a	1068 a	6.69 a	4.85 ab
CDC Cory	686 ab	249 ab	4.33 ab	3.01 ab	3018 b	1001 ab	6.63 a	5.07 ab
CDC Anna	615 cd	178 bc	4.29 ab	3.15 a	2300 d	825 ab	6.52 ab	5.21 a
CDC Cabri	587 d	195 bc	4.21 ab	2.91 ab	2622 c	641 ab	6.55 ab	4.81 ab
CDC Vanguard	644 bc	176 bc	4.37 a	2.71 ab	3084 ab	651 bc	6.58 a	4.88 ab
CDC Nika	430 f	197 bc	4.15 ab	2.55 ab	1705 f	842 ab	6.01 bc	4.95 ab
Amit	515 e	211 abc	4.12 ab	2.67 ab	2189 de	409 d	6.23 abc	4.66 ab
CDC Leader	513 e	144 bc	4.21 ab	2.62 ab	2852 bc	809 ab	6.33 abc	5.02 ab
CDC Orion	660 bc	149 bc	4.27 ab	2.61 ab	2031 de	661 bc	6.01 bc	4.74 ab
CDC Frontier	649 bc	171 bc	4.40 a	2.74 ab	2314 d	719 abc	6.31 abc	4.81 ab
CDC Alma	396 f	143 bc	3.92 b	2.61 ab	2086 c	655 bc	6.50 ab	4.69 ab
CDC Luna	664 bc	191 bc	4.43 a	2.78 ab	1934 ef	692 bc	6.01 bc	4.72 ab
CDC Xena	615 cd	130 c	4.40 a	2.24 b	1966 f	606 c	5.88 c	4.44 b

Data are presented as means ($n = 6$). Within a column, Means followed by different letters are significantly different at $p \leq 0.05$.

[†]Chickpea cultivars were grown in the South Farm of Semiarid Prairie Agricultural research Centre near swift Current, SK in 2010 and 2011. Primer sets used for PCR amplification of fungal DNA were ITS1F/ITS2 (Non-AM fungi), and NS1/NS4 and AMV4.5-NF/AMDGR (AM fungi).

[‡]Arbuscular mycorrhizal: Consisting of genera *Glomus, Claroideoglomus, Funneliformis, Paraglomus, Rhizophagus* and *Diversispora.*

[§]Consisting of genera *Fusarium, Alternaria, Cryptococcus, Acremonium, Bionectria, Microdochium, Trichoderma Paecilomyces, Mortierella, Rhodotorula, Chaetomium, Cladosporium, Penicillium,* and *Pyrenophora.*