Université de Montréal

Diversité des champignons endophytes mycorhiziens et de classe II chez le pois chiche, et influence du génotype de la plante

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Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de Philosophiæ Doctor (Ph.D.)
en sciences biologiques

avril, 2011

Université de Montréal
Faculté des études supérieures et postdoctorales

Cette thèse intitulée :

Diversité des champignons endophytes mycorhiziens et de classe II chez le pois chiche, et influence du génotype de la plante

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Résumé

Le pois chiche (*Cicer arietinum* L.) a l'avantage de pouvoir assimiler l'azote atmosphérique grâce à son association symbiotique avec des bactéries du genre *Mesorhizobium.* Malgré cet effet bénéfique sur les systèmes culturaux, le pois chiche réduit parfois la productivité du blé qui la suit. Cet effet négatif du pois chiche pourrait provenir d'une réaction allélopathique à ses exsudats racinaires ou résidus, ou de changements inopportuns dans la communauté microbienne du sol induits par la plante. L'amélioration des interactions symbiotiques du pois chiche pourrait améliorer la performance économique et environnementale des systèmes culturaux basés sur le blé.

L'objectif à long terme de ce travail est d'améliorer l'influence du pois chiches sur son environnement biologique et sur la productivité du système cultural. À court terme, nous voulons 1) vérifier l'effet des champignons endophytes sur la performance de cultivars de pois chiche de type desi et kabuli, particulièrement en conditions de stress hydrique, ainsi que sur celle d'une culture subséquente de blé dur, 2) identifier des cultivars de pois chiche capables d'améliorer la qualité biologique de sols cultivés, 3) vérifier que des composés biologiquement actifs sont présents dans les racines des différents cultivars de pois chiches et 4) définir la nature de l'activité (stimulation ou inhibition) des ces composés sur les champignons endomycorhiziens à arbuscules (CMA), qui sont des microorganismes bénéfiques du sol reconnus.

L'inoculation du pois chiche avec des champignons endophytes indigènes en serre a augmenté la tolérance à la sécheresse du cultivar de type kabuli à feuille simple CDC Xena et amélioré la nutrition azotée et phosphatée d'un cultivar de type desi, cv. CDC Nika, cultivé en conditions de stress hydrique. La germination des graines de blé dur fut meilleure lorsque celles-ci étaient semées dans les débris de pois chiche inoculé de type kabuli. Le sol dans lequel le génotype de pois chiche à feuille simple CDC Xena fut cultivé mais duquel tout le matériel végétal de pois chiche fut retiré a fortement inhibé la germination des

semences de blé dur, ce qui suggère un effet des exsudats racinaires sur la communauté microbienne du sol associée à cette variété de pois chiche.

En champ, les cultivars de pois chiche ont influencé différemment la composition des communautés de champignons de la rhizosphère. Les espèces de champignons pathogènes étaient infréquentes et les espèces saprotrophiques et de CMA étaient fréquentes dans la zone des racines du cultivar de type desi CDC Anna. L'effet des composés contenus dans les fractions séparées par HPLC et solubles en solution de méthanol à 25% et 50% de l'extrait racinaire de ce cultivar sur la germination de spores de CMA a été testé *in vitro*. Les deux espèces de CMA utilisées ont répondu différemment à l'exposition aux composés testés, révélant un mécanisme impliqué dans l'association préférentielle entre les plantes hôtes et les CMA qui leurs sont associés.

Nous concluons que le génotype de pois chiche influence la composition de la communauté microbienne qui lui est associée et que cette influence est reliée au moins en partie aux molécules bioactives produites par les racines de la plante. D'autre part, la productivité du pois chiche et de la culture subséquente pourrait être favorisée par la manipulation de leurs champignons endophytes par inoculation.

Mots-clés : Pois chiche, *Cicer arietinum* L., génotypes, biodiversité fongique, symbiose, champignons mycorhiziens à arbuscules, champignons endophytes, sécheresse, allélopathie, composés bioactifs, amélioration des plantes.

Abstract

Chickpea (*Cicer arietinum* L.) has the ability to bring free N into cropping systems, but is only a fair rotation crop, leading to lower yield in following wheat crops, as compared to medic, vetch or lentil. The negative effects of a chickpea plant on the following wheat crops could come from chickpea root exudates, their residues or their influence on the soil microbial community. The identification of chickpea cultivars best able to promote soil biological quality and the growth of a subsequent crop in rotation will help farmers in selecting better crop rotations and, thus, will improve crop management in soil zone growing chickpea.

The global objective of this research is to improve the fitness of chickpea crops to their biological environment and to improve the ability of the plant to enhance soil biological quality. The specific objectives were (1) to verify that the productivity of chickpea and subsequent crops could be promoted through the inoculation by some indigenous endophytic fungi particularly under drought stress conditions (2) to verify the existence of variation in the rhizospheric associations of field-grown chickpea, as it is a necessary condition for the selection of genotypes with improved compatibility with beneficial microorganisms. (3) to identify the biologically active compounds present in the root extracts of chickpea cultivars with contrasting phenotypes, and assess their effect on beneficial and pathogenic soil microorganisms.

The greenhouse experiments show that inoculation with indigenous endophytes increased drought tolerance of the unifoliate Kabuli chickpea CDC Xena and the N and P nutrition of the drought stressed Desi chickpea CDC Nika. Inoculation of both Kabuli chickpea varieties with indigenous endophytes improved wheat seeds germination in tissues amended soil. Residue-free soil previously growing the unifoliate Kabuli chickpea CDC Xena strongly inhibited durum seed germination suggesting an effect of root exudates on the soil microbial community, with this Kabuli chickpea variety.

In a field experiment, the fungal diversity in cultivated Prairie dryland appeared to host a large array of fungal groups known to reduced plant nutrient, water and biotic stresses, and chickpea genotypes influenced differently the composition and biomass of the soil microbial community. The Desi chickpea CDC Anna was associated with high diversity of arbuscular mycorrhizal fungi (AMF) and culturable fungi, favored the proliferation of soil bacteria and fungal genus hosting biocontrol agents, and developed high AM root colonization level, as compared to the three Kabuli genotypes examined. The HPLC fractions of the roots of chickpea cultivar CDC Anna were recovered and the effects of these fractions on AM fungal spore germination were assayed in multi-well plates. Root extract fractions affect in a different ways the percentage of spores' germination of *Glomus etunicatum* and *Gigaspora Rosea*.

We concluded that the genotype of chickpea plants influences the composition of the associated microbial community, and this influence may be related to molecular signals produced by the plants. Furthermore, the productivity of chickpea and subsequent crops could be promoted through the inoculation with indigenous endophytic fungi.

Keywords : Chickpea (*Cicer arietinum* L.), genotypes, fungal diversity, symbiosis, arbuscular mycorrhizae, dark septate endophytes, drought stress, allelopathy, biologically active compounds, plant breeding.

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Remerciements

Au terme de ce travail réalisé au laboratoire de microbiologie de sol à Agriculture et Agroalimentaire Canada, Centre de recherches sur l'agriculture des Prairies semi-arides, Swift Current, Saskatchewan et à l'Institut de Recherche en Biologie Végétale de l'Université de Montréal,

Je dois, en premier lieu, remercier humblement *DIEU* le tout-puissant pour tous sa miséricorde et de m'avoir donné la force pour persévérer jusqu'à la fin de ce travail.

Il m'est agréable de remercier *Docteur Marc St-Arnaud,* mon directeur de recherche à l'Université de Montréal, pour m'avoir accordée sa confiance en dirigeant ce travail. Ses conseils permanents, son soutien, ses longues discussions et ses critiques constructives durant les années de mes études au Doctorat m'ont été d'un grand secours. Je lui exprime mes sincères remerciements et ma profonde considération.

Je voudrais remercier également *Professeur Sadok BOUZID,* mon directeur de recherche à l'Université Tunis El Manar. Il a toujours su m'inciter à donner le meilleur de moi-même lors de mes activités de recherche et a suivi avec beaucoup d'intérêt la progression de mon travail. Je lui exprime mes profonds remerciements pour toute l'attention qu'il a manifestée pour mon parcours.

Je suis particulièrement reconnaissant au *Docteur Chantal Hamel*, ma codirectrice et chef du projet, pour son soutien et sa générosité toujours parfaites. Elle a assuré l'encadrement scientifique de mon travail. J'ai bénéficié de son expérience et de sa compétence pour la conception des protocoles expérimentaux, l'analyse et l'interprétation des résultats ainsi que la structuration et la correction de la thèse. Elle m'a prodigué toujours ses conseils, son savoir-faire et sa disponibilité illimitée. Je lui adresse toute ma gratitude, mon profond respect et ma très haute considération.

Je suis très sensible à l'honneur que me fait *Docteur Stéphanie Pellerin* en acceptant de juger ce travail et de présider le jury de soutenance.

Je suis également redevable aux *Professeurs Hani Antoun* et *Mohamed Hijri* pour avoir accepté de me faire l'honneur de juger ce travail et de faire partie du jury de soutenance.

Je remercie *Professeur Takaaki Ishii* et *Docteur Andre Freire Cruz* pour l'intérêt qu'ils ont toujours porté à ce travail et pour m'avoir accueillie chaleureusement au sein de leur laboratoire à Kyoto au Japon afin d'acquérir les techniques en chimie analytique appliquée aux exsudats racinaires.

Je voudrais remercier également le personnel du laboratoire de microbiologie de sol à Agriculture Canada au près desquels j'ai trouvé compréhension, encouragements et amitié : *Derek Wiebe, Elijah Atuku* et en particulier *Keith Hanson* qui m'a beaucoup aidé lors des expérimentations avec beaucoup de patience, de dévouement et de gentillesse.

Merci également à mes collègues de laboratoire, professeurs et membres de l'Institut de Recherche en Biologie Végétale que j'ai eu la chance de côtoyer.

Je tiens aussi à remercier le gouvernement Tunisien pour m'avoir octroyé en 2005 et 2006 deux bourses d'alternance qui m'ont permis de compléter deux stages de six mois chacun dans les laboratoires d'Agriculture Canada et dont les travaux ont servi de base pour mon projet de Doctorat.

Je voudrais également exprimer mes remerciements à la Banque Islamique de Développement pour m'avoir octroyé une bourse dans le cadre de son «Programme des Bourses de Mérite pour la Haute Technologie» qui m'a donné la chance de poursuivre mes études de Doctorat à l'Université de Montréal.

*M*erci autant à la faculté des études supérieures et postdoctorales de l'Université de Montréal pour la bourse de fin d'études doctorales.

*J*e me dois aussi de remercier ma famille, principalement mes parents, qui m'ont toujours encouragée à aller plus loin et qui m'ont appuyée, moralement et financièrement, durant mes longues années d'études. Je suis aussi très reconnaissant envers mes beaux-parents ainsi que les frères que j'ai rencontré au Canada auprès desquels j'ai trouvé beaucoup de soutient et d'encouragements.

*F*inalement, il sera difficile de trouver les mots pour remercier mon épouse *Mouna*, qui a pu me supporter, a su m'encourager et m'aider, jour après jour et au delà de ses travaux de thèse. Qu'elle trouve ici toute ma gratitude et mon amour.

Avant-propos

Les travaux effectués au cours de ce projet de doctorat ont été présentés sous forme de quatre articles soumis pour publication ou rédigés en vue de l'être :

1) Ellouze Walid, Hamel Chantal, Bouzid Sadok, St-Arnaud Marc (2011) Mycorrhizosphere interactions mediated through rhizodepositions and arbuscular mycorrhizal hyphodeposition and their application in sustainable agriculture, in: S. M. Fulton (Ed.), Mycorrhizal Fungi: Soil, Agriculture and Environmental Implications, Nova Science Publishers, Hauppauge, NY. pp. In press.

2) Ellouze Walid, Hamel Chantal, Bouzid Sadok, St-Arnaud Marc: Endophyte symbiosis benefits chickpea plants and seeds germination of subsequent wheat crops. In preparation for Canadian Journal of Botany.

3) Ellouze Walid, Hamel Chantal, Vujanovic Vladimir, Gan Yantai, Bouzid Sadok, St-Arnaud Marc: Plant genotype modifies the abundance and species composition of the soil microbial community associated with field-grown chickpea. Submitted to PloS One.

4) Ellouze Walid, Hamel Chantal, Cruz Andre Freire, Bouzid Sadok, Ishii Takaaki, St-Arnaud, Marc: Phytochemicals and spore germination: at the root of AMF host preference? In preparation for Journal of Applied Soil Ecology.

Le candidat et ses codirecteurs Dr. Hamel, Prof. Bouzid et Dr. St-Arnaud apparaîtront en tant que co-auteurs sur les quatre articles. Le candidat a été responsable de la conduite de la recherche, l'analyse des données et la préparation des manuscrits. Dr. Hamel et Dr. St-Arnaud ont fourni des révisions, des commentaires et des suggestions tout au long du processus. Dr. Gan, Vujanovic, Cruz et Prof. Ishiii ont fourni de l'expertise scientifique et technique durant la phase expérimentale de ce travail.

Introduction générale

Le pois chiche (*Cicer arietinum* L.) joue un rôle important dans les systèmes agricoles des pays de la Méditerranée et dans les prairies de l'Amérique du nord, où cette légumineuse annuelle a récemment été introduite (Gan and Noble, 2000). Elle y est employée comme alternative aux céréales traditionnelles en réponse à un besoin de diversification des cultures (Miller et al., 2002). C'est une culture de grand intérêt économique et social. Elle a la capacité de fixer l'azote atmosphérique grâce à son association symbiotique avec des bactéries du genre *Mesorhizobium.* Malgré l'apport d'azote biologiquement fixé par le pois chiche, dans certaines circonstances, l'introduction de cette plante dans la rotation des cultures entraîne une diminution du rendement d'une culture subséquente de blé, en comparaison avec la luzerne (*Medicago sativa*), la vesce commune (*Vicia sativa*) ou la lentille (*Lens culinaris*) (Ryan et al., 2010). Cet effet négatif du pois chiche pourrait provenir de ses exsudats racinaires, de ses résidus ou de son influence sur la communauté microbienne du sol et particulièrement, sur les champignons mycorrhiziens arbusculaires (CMA). Par ailleurs, il a été montré que les effets inhibiteurs des racines de pois chiches sur les cultures subséquentes dépendent de la variété de pois chiches ainsi que des caractéristiques génétiques de la culture subséquente en rotation (Chaichi and Edalati-Fard, 2005).

Dans les écosystèmes terrestres, la plupart des plantes vivent en symbiose avec des champignons endophytes et des CMA. Ces symbioses assurent la tolérance à plusieurs stress qui pourraient limiter la croissance des plantes. Les endophytes sont d'importants colonisateurs fongiques des racines des plantes dans l'écozone des prairies semi-arides caractérisées par le déficit hydrique (Khidir et al., 2010). Les interactions au sein des associations entre plantes et endophytes ont été largement considérées comme mutualistes, car les champignons confèrent des bénéfices à leurs hôtes à travers l'amélioration de l'absorption des nutriments, une tolérance accrue contre les agents pathogènes des racines, et une meilleure capacité de résister aux conditions environnementales défavorables (Mandyam and Jumpponen, 2005). Les CMA jouent un rôle crucial dans les écosystèmes naturels et agricoles en agissant comme biofertilisants, épurateurs, éléments structurant la

matrice du sol, bioprotecteurs et agents de lutte biologique. Ils ont un grand potentiel d'application en agriculture, en horticulture, en sylviculture, pour la production de produits alimentaires et de biomasse. Les effets bénéfiques des CMA en agriculture écologique ont été récemment revus par Ellouze et al. (2008) et Fraser et al. (2009).

La structure des communautés microbiennes dans la rhizosphère est influencée par les plantes à travers la libération de produits bioactifs par leurs racines (Estabrook and Yoder, 1998). Des différences génotypiques dans la composition et la quantité des exsudats racinaires des espèces végétales cultivées et indigènes ont été signalées chez les plantes possédant différents niveaux de tolérance au stress nutritif, à la toxicité ionique, et aux maladies (Rengel, 2002).

Dans cette thèse, nous avons vérifié les hypothèses suivantes:

1) Les champignons endophytes indigènes améliorent la nutrition et la tolérance des plantes de pois chiche au stress hydrique et réduit leur effet négatif sur la productivité d'une culture subséquente de blé dur.

2) Certains cultivars de pois chiches augmentent sélectivement les microorganismes bénéfiques et réduisent la prolifération des agents pathogènes d'origine tellurique.

3) Les racines des différents génotypes de pois chiche produisent différentes molécules bioactives qui influencent la germination des spores des CMA.

Pour une meilleure compréhension des interactions entre plantes et microorganismes du sol, nous avons revu dans le premier chapitre les connaissances actuelles sur les mécanismes par lesquels l'association symbiotique avec les CMA, considérés comme la pierre angulaire de la durabilité des agro-écosystèmes, influence les communautés microbiennes de la mycorhizosphère et par la suite, nous avons discuté des applications possibles de ces mécanismes en agriculture durable.

Pour développer la compréhension de la fonction écologique des champignons endophytes indigènes sur les cultures de pois chiche et les cultures subséquentes de blé dur dans les zones arides, nous avons étudié dans le deuxième chapitre l'effet de l'inoculation avec des endophytes indigènes sur la performance des plantes de pois chiches en absence et en présence de stress hydrique, en serre. Nous avons également testé l'effet allélopathique potentiel des tissus et des exsudats racinaires du pois chiche en présence et en absence d'endophytes indigènes sur l'émergence de blé dur. Nous avons vérifié l'hypothèse selon laquelle les champignons endophytes indigènes améliorent la nutrition et la tolérance des plantes de pois chiche au stress hydrique et réduit l'effet allélopathique d'une culture de pois chiche antérieure sur le blé dur.

Pour améliorer la connaissance des microorganismes du sol naturellement associés aux plantes de pois chiche cultivées dans les zones arides des Prairies canadiennes, nous avons vérifié dans le troisième chapitre l'hypothèse selon laquelle certains cultivars de pois chiches augmentent sélectivement les microorganismes bénéfiques et réduisent la prolifération des agents pathogènes d'origine tellurique. Nous avons déterminé dans ce chapitre comment la variation génétique chez le pois chiche pouvait influencer l'abondance et la diversité des champignons du sol et identifier des cultivars de pois chiche capables de stimuler et améliorer la qualité biologique des sols cultivés.

Pour comprendre la relation entre la composition des extraits racinaires et l'augmentation sélective des microorganismes bénéfiques, nous avons identifié dans le quatrième chapitre les composés biologiquement actifs présents dans les extraits racinaires des différents cultivars de pois chiches et défini la nature de leur activité (stimulation ou inhibition) sur les microorganismes bénéfiques du sol. Nous avons vérifié l'hypothèse selon laquelle les racines des différents génotypes de pois chiche produisent différentes molécules bioactives qui influencent la germination des spores des CMA.

La conclusion générale vise à donner les résultats principaux de chacun des chapitres et leurs applications futures.

Objectifs

L'objectif global de cette thèse était de développer la connaissance sur les interactions entre le pois chiche et son environnement biologique en vue de renforcer la capacité de cette plante à améliorer la qualité biologique du sol et la productivité des systèmes culturaux basés sur la production de blé dur.

Les objectifs spécifiques sont :

1) Définir l'effet des champignons endophytes sur la performance de cultivars de pois chiches de type desi et kabuli, particulièrement sous conditions de stress hydrique, ainsi que sur la performance d'une culture subséquente de blé dur.

2) Identifier des cultivars de pois chiche capables de stimuler et d'améliorer la qualité biologique des sols cultivés.

3) Identifier les composés biologiquement actifs présents dans les extraits racinaires de différents cultivars de pois chiches et définir la nature de leur activité (stimulation ou inhibition) sur des microorganismes bénéfiques du sol, les CMA.

Chapitre 1 : Mycorrhizosphere interactions mediated through rhizodepositions and arbuscular mycorrhizal hyphodeposition and their application in sustainable agriculture

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1.1 Abstract

Arbuscular mycorrhizal (AM) fungi are ubiquitous in soils around the world where they form symbiotic associations with the majority of plant species. The beneficial effects of AM fungi are well known. They include improved plant nutrient uptake, enhanced N_2-fixation in legumes, increased plant tolerance to biotic and abiotic stress and improved soil structural quality, and are seen as the cornerstone of sustainability in agro-ecosystems. In soil, AM fungi form extensive mycelial networks connected to plant roots. These AM mycelial networks, which increase the surface for nutrient and water absorption, also offer large surfaces for interactions with other microorganisms. These fungi often interact positively with biofertilizers and biocontrol agents. The presence of AM fungi in plant roots and soils was repeatedly shown to reduce the incidence of diseases caused by pathogenic nematodes and fungi and to improve plant health. AM fungi exert profound effects on soil microorganisms through competition for nutrients, and modification of root exudation, plant physiology and signaling. Better understanding of the interactions between AM fungi and other microorganisms is necessary for the development of sustainable management of soil fertility and crop production. The purpose of this review is to outline the current knowledge on the mechanisms by which the symbiotic association with mycorrhizal fungi alters the existing mycorrhizosphere microbial community and discusses the possible applications these mechanisms may have in sustainable agriculture.

1.2 Introduction

The arbuscular mycorrhizal symbiosis is a widespread mutualistic association between plants and fungi from the phylum Glomeromycota (Redecker and Raab, 2006). The origin of AM associations corresponds with the appearance of land plants, 450 million years ago (Pirozynski and Dalpé, 1989; Simon et al., 1993). This association is fundamental in the plant kingdom; strictly speaking, most plants do not have roots, they have mycorrhizae (Wilhelm, 1966). AM fungi play a crucial role in agro- and natural ecosystems

by acting as biofertilizers, bioremediator, soil conditioner, bioprotectors and biocontrol agents and have wide applications in agriculture, horticulture, forestry, and biomass production.

The beneficial effects of AM fungi in ecological agriculture have been recently reviewed by Ellouze et al., (2008) and Fraser et al., (2009). The mycorrhizal symbiosis occupies a central position in rhizosphere development and many types of interactions involving this symbiosis and important microbial groups have been reported (Barea et al., 2004). The functional and morphological complexity of the rhizosphere is increased by mycorrhizal fungi, which in turn support a mycorrhizosphere (Linderman, 1988; Nehl and Knox, 2006).

Various microorganisms have been tested in interaction studies with AM fungi, including saprophytes (McAllister et al., 1995), pathogens (Lioussanne et al., 2008b; Meyer and Linderman, 1986), biocontrol agents (Wyss et al., 1992) and other plant symbionts (Barea et al., 1987). The purpose of this review is to outline the current knowledge on the mechanisms by which the symbiotic association with mycorrhizal fungi alters the existing mycorrhizosphere microbial community and their possible application in sustainable agriculture.

1.3 The mycorrhizosphere

The rhizosphere is colonized by bacteria, fungi and other microorganisms and thus constitutes a living interface between roots and the soil. It is a dynamic environment determined by interactions between the soil, plants and roots-associated microflora. The rhizosphere is a unique habitat where only a subset of the soil microflora is able to survive and multiply (Foster, 1986; Manoharachary and Mukerji, 2006; Marilley and Aragno, 1999; Marschner and Timonen, 2005). In this environment, plant beneficial microorganisms are of peculiar importance: they interact and determine plant success (Aragno, 2003).

The mycorrhizosphere refers to the soil zone near and influenced by mycorrhizal roots, hyphae, spores, and fruit bodies. Arbuscular mycorrhizal (AM) fungi are typically abundant in soil, and may account for some 25% of the soil microbial biomass (Hamel, 2007; Hamel et al., 1991; Olsson et al., 1999) and up to 80% of the fungal biomass (Bååth et al., 2004; Kabir et al., 1997) in certain agricultural soils. A gram of soil can contain up to 30 m of AM fungal extraradical hyphae (Smith and Read, 1997). Most crop plant species form mycorrhizae, thus the concept of mycorrhizosphere applies to most crops (Timonen and Marschner, 2006).

Arbuscular mycorrhizal fungi have a large influence on soil microorganisms as they increase the size of C and energy input to soil by stimulating plant productivity and modifying the physical and chemical environment of the soil (Johansson et al., 2004). They influence mycorrhizosphere communities quantitatively and qualitatively with impact on plant health (Azcón et al., 1991; Barea, 2000; Linderman, 1988; Meyer and Linderman, 1986; St-Arnaud and Elsen, 2005; St-Arnaud et al., 1995; St-Arnaud and Vujanovic, 2007). Plants provide reduced carbon compounds to their mycorrhizal fungal partners to grow mycorrhizal mycelial networks and improve their capacity for soil nutrient and water extraction. In turn, better plant nutrition reduces root exudation and, thus, the food supply for rhizospheric microorganisms. The supply of photosynthates, as substrates to soil microbiota, is a key factor in mycorrhizosphere formation (Barea, 2000). Mycorrhizal fungi induced shifts in soil microbial communities were also attributed to the regulation of the symbiosis. For example, mycorrhizal colonization of roots triggers plant defense mechanisms against potential pathogens attack (Cordier et al., 1998; Pozo et al., 2002; Pozo et al., 2009; Vigo et al., 2000).

Arbuscular mycorrhizal fungi exert profound effects on other mycorrhizosphere microorganisms either directly or indirectly via their impact on host, but also through direct effects. Arbuscular mycorrhizal fungi are competitive soil microorganisms because they tap directly on the ultimate source of carbon and energy for soil organisms, plant photosynthesis, and have first access to carbon of plant origin. Direct effects include

provision of energy-rich carbon compounds derived from host assimilates, which are transported to the mycorrhizosphere via fungal hyphae, changes in soil pH, competition for nutrients, and exudation of inhibitory or stimulatory compounds.

1.4 Mycorrhizosphere interactions through rhizodepositions

Rhizosphere soil is strongly influenced by the root, through rhizodeposition (Marx et al., 2007) and respiration, as well as a result of ions and minerals uptake (Aragno, 2003). Roots release 1 to 25% of plants' net photosynthesis as soluble and insoluble compounds into the rhizosphere (Marschner and Timonen, 2006; Merbach et al., 1999). Rhizodeposition products are composed of exudates, lysates, mucilage, secretions and dead cell material, and include gaseous compounds (Lynch and Whipps, 1990). Root exudates constitute the major part of the rhizodeposition and are mainly composed of soluble low-molecular weight substances such as carbohydrate monomers, amino acids, organic acids, phytosiderophores, flavonoids, plant hormones and vitamins (Farrar et al., 2003; Lynch and Whipps, 1990; Marschner, 1995). Mycorrhiza establishment is known to change mineral nutrient composition, hormonal balance, C allocation patterns, and other aspects of plant physiology (Barea, 2000; Marschner and Timonen, 2006) including root exudates composition (Bansal and Mukerji, 1994; Marschner et al., 1997), which influences the rhizosphere environment. Mycorrhizal fungi may reduce the amount of root exudates in the rhizosphere by taking up carbohydrates directly from root cells before they reach the surrounding soil (Timonen and Marschner, 2006). Mycorrhizal plants transfer more assimilates to their roots than non-mycorrhizal plants (Eissenstat et al., 1993). Larger C investments in AM roots are explained by the need for fungal growth, respiration and synthesis of the wide range of compounds that may stimulate beneficial microorganisms or antagonize phytopathogenes (Finlay and Söderström, 1992; Kope and Fortin, 1989; Kucey and Paul, 1982). The variation in the amount and composition of rhizodeposition induced by AM symbiosis formation can result in dramatic changes in the activity and composition of soil microbial communities, as microbial species differ in metabolism and have different ability to use different carbon sources (Marschner and Timonen, 2006; Neumann, 2005)

There are conflicting reports in the literature regarding the impact of AM fungi on rhizodeposition. While some researchers have reported reduced amounts of carbohydrates (Bansal and Mukerji, 1994; Mada and Bagyaraj, 1993; Ocampo and Azcon, 1985), altered composition of amino acids (Laheurte et al., 1990; Mada and Bagyaraj, 1993) and stimulated release of phenolics, gibberellins and nitrogen (Mada and Bagyaraj, 1993) in roots colonized by AM fungi (Bansal and Mukerji, 1994; Marschner et al., 1997), others found no evidence of AM fungal influence on rhizodeposition (Azaizeh et al., 1995). Lioussanne et al. (2008b) reported higher concentrations of proline and isocitrate in root exudates in presence of *G. intraradices* after 24 weeks of growth, while after 16 weeks, proline concentration in exudate was not affected and the isocitrate concentration was reduced. Furthermore, the presence of *G. intraradices* had no influence on the amounts of amino acids, organic acids and measured sugars released into the mycorrhizosphere in this study. Other studies hypothesize that mycorrhizal fungi regulate rhizosphere events such as secondary AM infection and *Striga* germination in reducing the production of strigolactones, which is a group of sesquiterpenes lactones known so far as rhizospheric signaling molecules (Akiyama and Hayashi, 2006; Lendzemo et al., 2009; Lendzemo et al., 2007; Matusova et al., 2005). A variety of factors could explain these contrasting results, including differences in the plant and fungus species involved, the experimental system used, and environmental conditions (Bending et al., 2006).

1.4.1 Interaction of AM fungi with rhizobacteria

Interactions between functional groups of the soil microflora affect both plant and soil development (Andrade et al., 1997; Schreiner et al., 1997). Bacteria and fungi influence each other (Garbaye, 1994; Paulitz and Linderman, 1991) and, thus, their influence on the plant-soil system depends on the outcome of their interaction (Bethlenfalvay et al., 1997; Meyer and Linderman, 1986). In particular, mycorrhizal infection influences the composition of the rhizosphere bacterial community (Bansal and Mukerji, 1994; Buwalda and Goh, 1982; Christensen and Jakobsen, 1993; Dixon et al., 1989; Graham et al., 1981; Jones et al., 2004; Mansfeld-Giese et al., 2002; Marschner et al., 1997; Shachar-Hill et al.,

1995) and the density of bacterial population within the mycorrhizosphere (Bending et al., 2006). Negative as well as positive effects of AM fungi on bacteria have been reported.

Beneficial fungi and bacteria are a major focus in rhizosphere research. The large group of plant growth-promoting rhizobacteria (PGPRs) includes bacteria with different lifestyles (Perotto and Bonfante, 1997). PGPRs are either symbiotic, for example the *Rhizobium* spp., or free-living (Glick, 1995; Kloepper et al., 1980). They are often associated with mycorrhizal fungal spores and hyphae and may even be found within these structures (Bonfante and Anca, 2009; St-Arnaud and Elsen, 2005).

1.4.1.1 Interactions of AM fungi with free-living PGPRs

Several studies have confirmed synergism between AM fungi and free-living PGPRs such as *Burkholderia cepacia* Palleroni & Holmes (Ravnskov et al., 2002) and *Pseudomonas fluorescens* Migula (Edwards et al., 1998). Root exudates collected from tomato roots colonized by *Glomus fasciculatum* were more attractive to *Azotobacter chrococcum* and *Pseudomonas fluorescens* than exudates collected from non-colonized roots (Sood, 2003). Meyer and Linderman (1986) saw more facultative anaerobic bacteria and less fluorescent pseudomonas in the rhizosphere of mycorrhizal plants, but no effect of AM fungi on the total number of bacteria. Secilia and Bagyaraj (1987) reported an increase in total bacteria, nitrogen fixers and Gram negative bacteria in the rhizosphere of mycorrhizal plants. Later studies reported an increase in the total number of plant associated bacteria in presence of the AM fungus *G. intraradices* (Ravnskov and Jakobsen, 1999). More recent studies show that the AM fungi might affect plant and soil microbial activity by stimulating the production of root exudates, phytoalexins, and phenolic compounds (Dalpé and Monreal, 2004; Norman and Hooker, 2000).

In a study with split-root pepper plants (Marschner et al., 1997), ^{14}C exudation was decreased on the root side colonized by *Glomus intraradices* but not on the non-mycorrhizal side. However, the population density of the introduced *P. fluorescens* 2-79RL in the rhizosphere was decreased on both sides of the root system. This result was

confirmed by a subsequent study on the effect of *G. mosseae* colonization on bacterial community structure in a split-root maize (Marschner and Baumann, 2003). Both studies showed that the effect of AM fungi colonization on rhizospheric bacterial community structure is systemic and involves more than mere changes in the abundance of root exudation.

The mycorrhizosphere may be favorable or unfavorable to microbial proliferation. Free-living N_2-fixing bacteria were more numerous in the mycorrhizosphere of *Panicum maximum* (Secilia and Bagyaraj, 1987). Similarly, the number of autotrophic NH_4^+-oxidizing bacteria were higher in the mycorrhizosphere of *G. mosseae* and *G. fasciculatum* growing with *Zea mays* than in non-mycorrhizosphere soil, with the reverse situation for the numbers of denitrifying and NH_4^+-producing organisms (Amora-Lazcano et al., 1998; Bending et al., 2006).

1.4.1.2 Interactions of AM fungi with symbiotic N_2-fixing bacteria

AM fungi and symbiotic N_2-fixing bacteria are particularly important. The N_2-fixing bacteria belong to the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Azorhizobium*, collectively termed rhizobia. It has been shown that dual inoculation with AM fungi and rhizobia increases plant growth and N_2-fixation to a greater extent than inoculation with rhizobia alone (Ibijbijen et al., 1992; Vejsadova et al., 1993; Vejsadova et al., 1992). Measurements of the $^{15}N/^{14}N$ ratio in plant shoots indicated enhancement of the N_2-fixation rates in nodulated mycorrhizal plants, relative to that achieved by the same rhizobium strain in non-mycorrhizal plants (Jeffries et al., 2003; Toro et al., 1998). Mycorrhizal fungi may also enhance rhizobial cell densities in root nodules, which tend to decrease under drought stress (Tate, 1995). The AM symbiosis can influence nodule function and rhizobia population by changing the rates of water movement into, through and out of host plants in addition to reducing oxidative damage. The interactions between mycorrhizal fungi and rhizobium are complex and may change during the development of the nodules (Marschner and Timonen, 2006).

Establishment of an AM fungus within a root can alter rhizosphere populations (Ames et al., 1984; Meyer and Linderman, 1986) and this may affect the distribution or development of nodules throughout the root system (Ames and Bethlenfalvay, 1987). Bethlenfalvay et al. (1985) demonstrated a competitive interaction between an AM fungus and *Bradyrhizobium japonicum,* where prior establishment of the fungus inhibited subsequent nodule development in soybean. Several studies, indicate that rhizobia and AM fungi can compete for colonization sites on legume roots and thus reduce symbiotic efficiency (Chalk et al., 2006). Moreover, AM symbiosis formation initially reduces the rate of nodule formation, presumably as a result of competition for plant carbon, but at later stages normally enhances plant nodulation and N_2-fixation by enhancing water uptake and more importantly, plant P nutrition (Barea and Azcón-Aguilar, 1983). De Varennes and Goss (2007) suggests that the positive interaction of rhizobia, AM fungi and legumes is modulated by the rate of early AM colonization, and soil disturbance created by tillage impairs this interaction by delaying the colonization of roots by the fungal partner in a field situation.

1.4.2 Interactions of AM fungi with mycorrhizosphere fungal community

1.4.2.1 Interactions of AM fungi with saprotrophic microorganisms community

Arbuscular mycorrhizal fungi have a competitive advantage over saprophytic soil microorganisms because they have first access to carbon of plant origin. Olsson et al. (1998) reported that AM fungal mycelium can reduce the size of saprophytic fungal communities in calcareous dune sand. Green et al. (1999) observed that the presence of external mycelium of *G. intraradices* suppressed *T. harzianum* population development and β-glucuronidase (GUS) activity, and suggest that nutrient competition is a likely means of interaction.

Interactions with saprophytic populations could influence decomposition processes (Finlay and Söderström, 1992). Hodge et al. (2001) reported that *Glomus hoi*, an AM

fungus, enhances organic matter decomposition. In absence of AM effect on the abundance of microbial PLFA biomarkers, they concluded to the direct involvement of *G. hoi* in N mineralization from organic residues, although hyphospheric effects could have been masked by a large soil volume in the organic residue compartment used. Arbuscular mycorrhizae could stimulate organic matter decomposition through their effect on soil microorganisms. Qualitative as well as quantitative changes in microbial community structure may also be responsible for faster organic matter decomposition in presence of arbuscular mycorrhizae in a process that links mineralization to plant nutrient demand (Atul-Nayyar et al., 2009).

The AM fungi can modify the composition the soil microbial community through different mechanisms and, thus, change soil functionality. Extraradical hyphae of AM fungi may also bring available C to microorganisms of the hyphosphere allowing them to mineralize recalcitrant soil organic matter, as described in the model of Schimel and Weintraub (2003). More research is needed to clarify the impact of AM fungi on saprotrophic microorganisms.

1.4.2.2. Interactions of AM fungi with the pathogenic fungal community

Root colonization with AM fungi changes root exudate composition which, in turn, exhibits a different bioactive effect on pathogens within the soil (Jones et al., 2004; Lioussanne et al., 2008b; Meyer and Linderman, 1986; Norman and Hooker, 2000; Sharma et al., 1992). The antagonistic effects of mycorrhizal fungi against plant pathogenic fungi can be due to the inability of pathogens to sense the presence of root when exudation levels is reduced by AM fungi (Timonen and Marschner, 2006), but there might be more to this AM effect, as exudates composition are modified by AM fungi. Exudates released from strawberry roots colonized by *Glomus etunicatum* and *Glomus monosporum* reduced sporulation and zoospore production by the pathogen *Phytophthora fragariae*, in vitro (Norman and Hooker, 2000). Recently, it was shown that proline concentration in exudates liberated by AM tomato roots was higher than in exudates from nonmycorrhizal roots

(Lioussanne et al., 2008b) suggesting that AM colonization can modify the composition of tomato root exudates. Increased proline concentration in tomato plants infected by *Phytophthora nicotianae*, was thought to prevent pathogen-root encounter by reducing the accumulation of zoospores in the vicinity of roots (Lioussanne et al., 2008b). These results support earlier observations of reduced *Fusarium* populations in the soil surrounding mycorrhizal tomato roots and suggest the involvement of AM fungi in the control of soil-borne diseases (Caron, 1989)

A positive effect of tomato root exudates from plants colonized by the AM fungus *Glomus mosseae* on microconidia germination of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) was reported by Scheffknecht et al. (2006). The higher the level of AM colonization, the higher the rate of microconidial germination, indicating that alterations of the exudation pattern depended on the degree of AM root colonization. These quantitative and/or qualitative alterations in root exudation of the *Fol* host tomato through mycorrhization are not specific to the *Fol*-tomato interaction. Similar changes in the bioactivity of root exudates of 12 non-host species from eight plant families showed that mycorrhizae-induced changes in root exudates were unrelated to plant susceptibility to *Fol* (Scheffknecht et al., 2007).

Information on the effect of AM root colonization on pathogens comes from greenhouse experiments using treated soil or *in vitro* systems using transformed roots. Artificial conditions allow the isolation of the organisms under study from other biotic and abiotic effects present in field situation but may be misleading. Field studies should always be used to validate results obtained under artificial conditions (Filion et al., 1999; St-Arnaud and Elsen, 2005). Observations from field surveys (e.g. Hamel et al., 2005), interpreted under the light of controlled conditions study results, would greatly help us understand the AM-pathogenic fungi interactions that are actually occurring in real situation.

1.5 Mycorrhizosphere interactions mediated through arbuscular mycorrhizal hypho-deposition

Research has been focused on the biology of AM symbiotic plant root systems. However, attention has begun to shift to the biology of AM extraradical mycelia and their contribution to the spatial and temporal heterogeneity of microbial diversity and function in the soil. This mycelium may be an important site for microbial interactions. In fact, recent evidence suggest that rhizosphere microbial community could be much more sensitive to the presence of AM mycelia than to the modification in root exudation mediated by the AM symbiosis (Lioussanne et al., 2010).

The extraradical mycelium of AM fungi constitutes a large surface area interacting with the surrounding soil environment and representing an interesting source of C for the soil microflora (George et al., 1995). The AM extraradical mycelium exudes various compounds into the soil (Wright et al., 1996; Wright and Upadhyaya, 1996) influencing the chemical composition and pH of the soil environment (Bago and Azcón-Aguilar, 1997). The analysis of exudates from a *Glomus* revealed the presence of low-molecular-weight sugars and organic acids, and also unidentified high-molecular-weight compounds (Toljander et al., 2007).

The hyphosphere effect is created by the release of carbonaceous exudates by AM hyphae (Toljander et al., 2007), but also by the rapid (5 to 6 days) turnover of these hyphae (Staddon et al., 2003). The C compounds released have different bioactivity on different soil microorganisms of the hyphosphere and as a result, mycelial exudates can not only increase microbial growth and vitality, but can also change the composition of the microbial community (Duponnois et al., 2008; Toljander et al., 2007). For example, Filion et al. (1999) found a stimulation of *Pseudomonas chlororaphis* growth and *Trichoderma harzianum* germination in the presence of *G. intraradices* mycelia. In contrast, conidial germination of *F. oxysporum* f. sp. *chrysanthemi* was reduced, while the growth rate of *Clavibacter michiganensis* subsp. *michiganensis* was not affected. Scheublin et al. (2010)

found specificity in bacterial attachment to AM hyphae and suggested that hyphal exudates shape hyphosphere bacterial communities through specific signaling that makes AM hyphae attractive to certain types of bacteria and not to others.

The hyphosphere effects can be nutritional or not. For example, Toljander et al. (2006) reported preferential attachment of some soil bacterial isolates to living AM fungal hyphae, but also to dead hyphae. Preferential attachment to dead hyphae suggests the existence of physical mechanisms for bacterial attachment to AM hyphae.

The bi-compartment *in vitro* system, where the AM fungi is grown on genetically transformed carrot (*Daucus carota*) roots in one compartment and where only AM mycelium is allowed to grow in the other (St-Arnaud et al., 1996), has been an important tool to study the direct effects of AM fungi on soil microorganisms. It revealed the AM mycelium as an important microbial community structuring agent in soil.

1.6 Mycorrhizospheric bioactive molecules and their application in sustainable agriculture

Public concerns about climate change, food safety, and water quality, raise the need for a profound change in the way we produce crops. Biotechnologies harnessing biodiversity to enhance the contribution of natural plant symbiotic relationships to crop nutrition appear an option to improve the efficiency of nutrient use by crop plants and reduce the environmental impacts of fertilizers. Recent advances in research on chemical signaling in microorganisms open new promising avenues in biotechnology development.

The soil rooting zone is a place where communication between plants and soil microorganisms through signaling molecules is a key determinant of the soil microbial community composition, with impact on the success of the plant involved. The development and function of the AM symbiosis is governed by the cross-talk between

plants and AM fungi (Brachmann, 2006). The identification of plant signal molecules promoting AM fungal infectivity and fungal signal molecules stimulating root receptivity could lead to the development of biotechnology tools for the management of the AM symbiosis in crop production (Gianinazzi and Vosátkatka, 2004).

Knowledge on the regulation of the AM symbiosis is accumulating and the stimulating activity of some compounds naturally produced by plants such as flavonoids, sesquiterpenes, ethylene and polyamines has been reported (Brachmann, 2006; Horii et al., 2009). Alginate oligosaccharide (Ishii et al., 2000) and nucleoside derivative (Kuwada et al., 2006) were also shown to enhance AM fungi growth. Stimulation of AM hyphal growth (Ishii et al., 1997) and root colonization (Cruz et al., 2004) were found in response to eupalitin, a flavonoid of Bahiagrass roots, and a bioactive peptide was recently discovered in the root extract of this plant (Horii et al., 2009). Tryptophan dimer promotes the growth and induces a chemotaxic response in germinating AM fungal spores, attracting growing hyphae. Short molecular peptides such as tryptophan dimer (Trp–Trp) and Leu–Pro remarkably stimulated spore formation of *G. clarum*, *G. etunicatum* and *Gigaspora albida* in absence of roots or root exudates (Ishii and Horii, 2009). When applied to *Citrus iyo*, a 25% MeOH eluate of an extract of brown alga, *Laminaria japonica*, raised AM colonization level and spore production (Kuwada et al., 2000).

Research on bioactive molecules may lead to improved AM inoculants production and may lower their price. Alternatively, cultivars with improved signaling capacity could be developed through marker-assisted selection by plant breeding programs. Care should be taken, however, with the manipulation of plant signals, as some signal molecules may result in the attraction of undesirable organisms (Steinkellner et al., 2007) and reduce crops' ability to manage their rhizosphere.

The AM fungi also release diffusible signal molecules offering potential for the development of green biotechnologies. These AM fungal signals could be formulated and applied to crops to enhance the contribution of indigenous AM fungi to crop production. They could be introduced into the formulation of AM inoculants to promote their

infectivity (Gianinazzi and Vosátkatka, 2004; Hamel and Strullu, 2006; Mabood et al., 2006).

1.7 Mycorrhizosphere inoculation with multiple beneficial microorganisms in sustainable agriculture

Considering the beneficial effects of many microorganisms in promoting plant growth and health, co-inoculation of crop plants with beneficial microorganisms appears as an attractive complement or alternative to conventional agrochemicals (Avis et al., 2008). However, the interaction between AM fungi and other beneficial microorganisms may vary from mutualistic to antagonistic. A good understanding of the microbe–microbe and plant-microbe interactions taking place in the mycorrhizosphere is the first step toward the development of effective co-inoculation technologies. Several research works have compared the effects of plant inoculation with more than one beneficial microorganism to single inoculation (Table 1.1). The effects of co-inoculation on plants vary from additive or synergistic, to negative or neutral according to the microbial combination used (Avis et al., 2008; Gianinazzi and Vosátkatka, 2004). The development of technologies for plant inoculation with multiple microorganisms is currently attracting much interest in the inoculant industry.

Table 1-1 Reports of effect of inoculation of plants with multiple beneficial microorganisms.

Host plant	AM fungus	Other microorganisms	Outcome	Reference
Tomato (*Solanum lycopersicon* L.) in pot and field grown	*G. intraradices*	*Pseudomonas fluorescens, Trichoderma harzianum*	All the biocontrol agents were effective at controlling Fusarium wilt and stimulating yield; their combination was most effective.	Srivastava et al., (2010)
Tomato (*Solanum lycopersicon* L.) in pot	*G. intraradices*	*Clonostachys rosea*	Synergistic microbial effect in dual inoculation despite a mutual inhibition of isolates.	Ravnskov et al., (2006)
Chickpea (*Cicer arietinum* L.) in pot	*G. mosseae*	*P. fluorescens, Azotoacter chroococcum, Azospirillum brasilense, Rhizobium sp.*	*P. fluorescens* and *G. mosseae* improved growth better than any combination treatment.	Siddiqui and Mahmood, (2001)
Chickpea (*Cicer arietinum* L.) in pot	*G. intraradices*	*Pseudomonas striata, Rhizobium sp.*	Combined inoculation best reduced galling caused by the root-rot disease complex (*Meloidogyne incognita* and *Macrophomina phaseolina*) and improved	Sayeed Akhtar and Siddiqui, (2008)

			plant growth and nutrition.	
Chickpea (*Cicer arietinum* L.) in pot	*G. fasciculatum*	*Rhizobium sp., Pseudomonas striata, Penicillium variable*	The AM fungus in combination with the *Rhizobium* and the *Pseudomonas* improved nutrition and yield, but decreased nutrition and yield resulted from co-inoculation with the AM fungi, the *Rhizobium* and P. variable.	Zaidi et al., (2003)
Clover (*Trofolium repens* L.) in pot	*G. mosseae*	*actinomycetes*	Synergistic effect of the microorganisms on growth and nutrition	Franco-Correa et al., (2010)
Cucumber (*Cucumis sativus* L.) in pot	*G. intraradices*	*Paenibacillus macerans* and *Paenibacillus polymixa*	The bacteria had suppressive effects on the AM fungus, with co-inoculation causing growth depression.	Larsen et al., (2009)

The potential value of inoculation with microbial consortia in crop production was demonstrated mostly in controlled condition studies, but it is important to test the effect of co-inoculated microorganisms in field trial. One possible advantage of inoculating beneficial microorganisms with the AM fungi is that these fungi may offer an opportunity to improve the ability of beneficial microorganisms to establish and persist in soil after inoculation. AM fungi are very competitive in soil as they have first access to plant resources. They may favor the persistence of microorganisms introduced through inoculation if these ones are adapted to life in the hyphosphere. Therefore, it is important to

better understand the interactions occurring in the mycorrhizosphere and the role of roots and AM extraradical mycelia exudates in the dynamics of microbial populations.

1.8 Conclusion

The mycorrhizosphere is a complex environment where AM fungi play a central role in increasing the nutrition and stress tolerance of host plants by modifying their metabolism and improving their reaction against pathogens (Smith and Read, 1997). The influence of AM extraradical mycelia on soil microbial growth and community composition has large ecological significance.

More efforts should be made to study the mycorrhizosphere under field conditions, because observations made in situ have much more relevance than those made in controlled condition studies. The new molecular and biotechnological methods to measure the abundance of AM fungi in soil, which are now brewing in specialized laboratories, will certainly open new possibilities in mycorrhizosphere ecology and contribute solutions for a more sustainable world. Important and rapid progress in the field of AM-soil microbial interaction is expected.

1.9 Acknowledgement

This study was supported by a scholarship to Mr. Walid Ellouze from the Islamic Development Bank Merit Scholarship Programme for High Technology.

Chapitre 2: Endophyte symbiosis benefits chickpea plants and seeds germination of subsequent wheat crops

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2.1 Abstract

Chickpea has the ability to bring free N_2 into cropping systems, but is only a fair rotation crop, leading to lower yield in following wheat crops, as compared to medic, vetch or lentil. A greenhouse experiment was conducted. It had three factors: chickpea cultivars (2 Kabuli and 2 Desi), levels of irrigation (sufficient and insufficient water) and inoculation (presence and absence of endophytes naturally occurring in a typical cultivated field of the Canadian Prairie). To test that indigenous endophytic fungi improve nutrition and water stress tolerance in chickpea plants, one set of plants was harvested at the time of symbioses development and another set was harvested at seed maturation. Inoculation with indigenous endophytes increased drought tolerance of the unifoliate Kabuli chickpea CDC Xena and the N and P nutrition of the drought stressed Desi chickpea CDC Nika. To test that indigenous endophytic fungi reduce the allelopathic effect of Kabuli chickpea on durum wheat, the dry tissues (shoots and roots) of the two Kabuli varieties (from the first harvest time) were mixed with their respective growing soil. All plant debris were sieved out of part of the growing soil to assess the effect of root exudates alone. Inoculation of both Kabuli chickpea varieties with indigenous endophytes improved wheat seeds germination in tissues amended soil. Residue-free soil previously growing the unifoliate Kabuli chickpea CDC Xena strongly inhibited durum seed germination suggesting an effect of root exudates on the soil microbial community, with this Kabuli chickpea variety.

Key Words: Chickpea (*Cicer arietinum* L.), garbanzo bean, plant genotype, soil microbial community structure, drought stress, plant symbioses, dark septate endophytes, dryland agriculture, soil resource use efficiency, allelopathy.

2.2 Introduction

Chickpea (*Cicer arietinum* L.) is an important rotation crop in cereal-based production systems. It has been grown in semiarid regions of the world for hundreds of years, primarily in India, Pakistan, and the countries in the Middle East and parts of Africa

(Kumar and Abbo, 2001). This crop was recently introduced to the semiarid regions of the northern Great Plains of North America where it is being used as an alternative to traditional cereals for crop diversification (Miller et al., 2002).

As other legumes, chickpea brings atmospheric nitrogen to soil increasing the fertility of agricultural soils in addition to being a very economically and socially important crop. However, chickpea is only a fair rotation crop, leading to lower yield in following wheat crops, as compared to medic, vetch or lentil (Ryan et al., 2010). The negative effects of a chickpea plant on the following wheat crops could come from chickpea root exudates, their residues or their influence on the soil microbial community.

Endophytic fungi are important fungal colonizers of plant roots in the semiarid prairie ecozone characterized by water deficit (Khidir et al., 2010). The interactions in plant-endophyte associations have been widely regarded as mutualistic because the fungi have been demonstrated to confer benefits to their hosts through improved nutrient uptake, increased tolerance against root pathogens and improved ability to withstand adverse environmental conditions (Mandyam and Jumpponen, 2005).

Semiarid lands are influenced by a variety of abiotic and biotic factors. Seed germination and seedling emergence in these ecosystems are limited by drought, nutrient deficiency, extreme high or low temperatures, salinity and herbivore predation (Maun, 1994). Early seedling emergence and establishment of plants are important phases controlling the abundance and distribution of mature plants (Wäli et al., 2009) and contribute more to crop yield than later emerging plants (Gan et al., 1992). Endophytic fungi have been demonstrated to increases seed germination of agronomically important forage and turf grasses such as *Lolium perenne* (Clay, 1987), *Festuca arundinacea* (Pinkerton et al., 1990), *Bromus setifolius* (Novas et al., 2003) and *Achnatherum inebrians* (Zhang et al., 2010). However, endophyte-plant interactions in natural ecosystems does not always benefit the plant as they can vary from mutualistic to parasitic with environmental conditions and the genotypes of interacting species (Saari et al., 2009; Wäli et al., 2009).

To expand understanding of the ecological function of endophytic fungi in dryland, we investigate the effect of inoculation with indigenous endophytes on the performance of chickpea plants under two levels of water availability in the greenhouse. We also tested the potential allelopathic properties of un-inoculated and inoculated chickpea tissues and root exudates on the emergence of durum wheat. We hypothesized that indigenous endophytic fungi improve nutrition and water stress tolerance in chickpea plants and reduce the allelopathic effect of chickpea on durum wheat.

2.3 Materials and methods

2.3.1 Previous crops plant materials, experimental design and conditions

The experiment was conducted in the greenhouse of the Semiarid Prairie Agricultural Research Centre, in Swift Current. Temperature was 24/15°C day/night (±2°C) and photoperiod was 16 hours. It had a split-plot design with two water levels, 30% of field capacity (deficient water) and 70% of field capacity (sufficient water) randomized in main plots. Two inoculation treatments, *Mesorhizobium ciceri* + endophytes and *M. ciceri* only, were applied to two classes of chickpea varieties bred at the Crop Development Centre of the University of Saskatchewan, Canada (CDC Frontier, a large-seeded kabuli with fern leaves; CDC Xena, a large-seeded kabuli with unifoliate leaves; CDC Anna and CDC Nika, two desi type). The factorial combinations of inoculation and cultivar were randomized in the subplots. There were four repetitions and two sets of plants. One set was harvested at the time of symbioses development (7 weeks after plants transplantation) and another set was harvested at seed maturation (16 weeks after transplantation of pre-germinated seeds).

Chickpea seeds were germinated in the greenhouse in seed trays using calcined clay (Professional Gardener co. Ltd., Calgary, AB) as a growth substrate and two pre-germinated seeds were transplanted in each pot. Pots were later thin to one plant per pot. Pots contained 6 kg of an air-dry pasteurized (80°C for 3 hours) mixture of light loam and sand (87.3% sand, 7.2% clay and 5.5% silt) with a pH of 6.5 and an EC of 0.48 mS. The soil mix contained 19.7 mg kg^{-1} NH_4-N and 14.1 mg kg^{-1} NO_3-N as per KCl extraction (Maynard and Karla,

1993), and 21.3 mg kg^{-1} P and 324.5 mg kg^{-1} K as per sodium bicarbonate extraction (Olsen et al., 1954), 0.57% of organic C and 0.08% of total N after pasteurization. Half of the plants were inoculated at the time of transplantation by spreading 3 g of surface sterilized roots of crested wheatgrass (*Agropyron cristatum* (L) Gaertn) colonized by fungal endophytes on a circular plane 10 cm below the soil surface. The crested wheatgrass roots and the soil were taken from the same location, at the interface between a cultivated soil and a native pasture near Swift Current SK, in the semiarid zone of the Canadian Prairie. Crested wheatgrass roots were washed and sanitized by agitation in 10% Chloramine-T for 10 min, and rinsed in distilled water. Roots were chopped into 1-cm fragments. Control inoculant was similarly applied in control pots. It was prepared by autoclaving the surface sterilized roots of crested wheatgrass during 20 min. The granular *Rhizobium* inoculant Nitragin GC® (LiphaTech Inc., Milwaukee, WI) was applied on the roots of chickpea plants at the time of transplantation.

At the first harvest, early morning plant water potential was determined in a pressure chamber (Plant Water Status Console Model 3005, Soil Moisture Equipment Corp). The youngest part of the shoot tip cut just before the 5th leaf from the apex was used for this determination. Plant shoots water potential was measured immediately after cutting the shoot. The N_2-fixing efficiency of the nodules was determined by the acetylene reduction assay in which the entire nodulated roots were placed in jars with 10% (v/v) acetylene according to the procedure described by Turner and Gibson (1980). Gas samples were analyzed for ethylene concentration using a Varian Star 3600 CX gas chromatograph fitted with a Porapak N column. Fresh roots, shoots and nodules were separated and weighted. Nodule number per plant was determined. Root volume was determined by the water displacement technique, i.e., by measuring, with a graduated cylinder, the volume of water displaced by the roots (Böhm, 1979). Root length and surface area were measured using the root analysis system WinRhizo, after staining the roots (Costa et al., 2001). Roots, shoots and nodules were dried separately for 3 days in a convection oven at 55°C to constant weight and dry weights were recorded.

At the second harvest, plant shoots were dried and ground before N and P analysis. Tissue digestion (Thomas et al., 1967) was completed and N and P concentrations were measured on the autoanalyzer. One soil sample per pot was taken and placed at -12°C until fatty acid methyl ester (FAME) analysis of AMF abundance and soil microbial community profiling.

2.3.2 Subsequent crops plant materials and experimental design and conditions

After the first harvest, dried plant materials were mixed with the soil in designated pots to assess and elucidate the source of negative effects of previous chickpea crop on the emergence of subsequent durum wheat (*Triticum turgidum* L.) crop. In addition, *Brassica nigra*, an allelopathic plant (Tawaha and Turk, 2003), was used as a positive control and pea (*Pisum sativum* L.), which is the best previous crop for durum wheat in our region, was used as a negative control. The soil of half of the pots that had grown Kabuli chickpea varieties was sieved to remove plant debris with the aim of assessing the effect of root exudates. Twenty durum wheat seeds were placed in each pot. Plant emergence was monitored by counting the number of emerged seedlings in each pot daily for 17 days.

2.3.3 Fatty Acid Methyl Esters (FAME) Analysis

Phospholipid fatty acids (PLFAs) were purified from soil lipid extracts and analyzed as a measure of active soil microbial biomass, using the method described in Hamel et al. (2006). Briefly, total soil lipids were extracted from fresh soil (4 g dry weight equivalent) in dichloromethane (DCM) : methanol (MeOH) : citrate buffer (1:2:0.8 v/v). Lipid-class separation was conducted in silica gel columns. The neutral, glyco- and phospholipids fractions were eluted by sequential leaching with DCM, acetone and MeOH, respectively. The glycolipid fraction was discarded. The neutral and phospholipid fractions were dried under a flow of N_2 at 37°C in the fume hood, dissolved in 2 mL of MeOH for PLFA and stored at -20°C. Fatty acid methyl esters were created through mild acid methanolysis. Ten microliters of methyl nonadecanoate fatty acid (19:0 Sigma-Aldrich) was added to serve as

internal standard and samples were dried under a flow of N_2 at 37°C in the fume hood. Samples dissolved in 50 µL of hexane were analyzed using a Varian 3900 gas chromatograph (GC) equipped with a CP-8400 auto sampler and a flame ionization detector (FID). Helium was the carrier gas (30 mL min^{-1}) and the column was a 50-m Varian Capillary Select FAME # cp7420. Sample injection (2 µL) was in 5:1 split mode. The injector was held at 250°C and the FID at 300°C. The initial oven temperature, 140°C, was held for 5 min, raised to 210°C at a rate of 2°C min^{-1}, and then raised from 210 to 250°C at a rate of 5°C min^{-1}, and held for 12 min.

Identification of peaks was based on comparison of retention times to known standards (Supelco Bacterial Acid Methyl Esters #47080-U, plus MJS Biolynx#MT1208 for 16:1ω5). The abundance of individual PLFAs was expressed as µg PLFA g^{-1} dry soil. Amounts were derived from the relative area under specific peaks, as compared to the internal standard (19:0) peak value, which was calibrated according to a standard curve made from a range of concentrations of the 19:0 FAME standard dissolved in hexane. Fatty acids were named according to the ω -designation described as follows: total number of carbons followed by a colon; the number of double bonds; the symbol ω; the position of the first double bond from the methyl end of the molecule. Cis- and trans-isomers are indicated with c or t, respectively. Methyl (meth) and hydroxy (OH) groups are labeled at the beginning, where applicable. Iso and anteiso forms are indicated by i- and a-, respectively.

Individual fatty acids have been used as signatures for various groups of micro-organisms (Hamel et al., 2006; Pankhurst et al., 2002). The FAME 18:2ω6c and 18:1c were used as indicators of fungal biomass (Frostegård and Bååth, 1996; Petersen and Klug, 1994) and FAME 16:1ω5, as indicator of arbuscular mycorrhizal fungi (Balser et al., 2005; Spring et al., 2000). FAMEs 3OH-12:0, a-12meth-15:0, i-13meth-15:0, 15:0, 14:0, 2OH-14:0, i-14meth-16:0, 16:1ω7c, i-15meth-17:0, 17:0, 2OH-16:0 and 18:1t were chosen to represent bacterial PLFAs based on the bacterial standards used.

2.3.4 Isolation of fungi, DNA sequencing and ITS sequence analysis

The sterilized crested wheatgrass roots, used as inoculum, were plated on Potato dextrose agar (PDA) medium supplemented with neomycin sulfate (12 mg l^{-1}) and streptomycin sulfate (100 mg l^{-1}) (Vujanovic et al., 2002), and incubated in the dark at 21°C. After incubation for 3 to 15 days, pure cultures were obtained by transferring agar plug containing young hyphae emerging from the root fragments to fresh new agar plates. Cultures were identified based on the internal transcribed spacer (ITS) region of the rRNA gene. Fungal DNA extraction was carried out using an UltraClean microbial DNA isolation kit (MoBio Laboratories) following the manufacturer's instructions. The ITS region of each fungus was amplified by polymerase chain reaction (PCR) in 25 µl reaction volumes, each containing 11 µl sterile distilled H_2O, 12.5 µl Taq PCR Master Mix Kit (Qiagen Laboratories), 0.25 µl of each fungal specific primer ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990), and 1 µl of extracted genomic DNA. The amplifications were performed in an Eppendorf's Mastercycler eP S gradient thermocycler using the following conditions: initial denaturation step at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final extension step at 72°C for 7 min. Reactions were performed with negative controls containing no DNA. The resulting PCR products were electrophoresed in 1% (w/v) agarose gels, stained with ethidium bromide, and visualised under UV light.

Sequencing reactions were performed in a commercial laboratory (Genome Quebec Innovation Centre). ITS sequences were analysed with the Basic Local Alignment Search Tool (BLAST) through GenBank (http://www.ncbi.nlm.nih.gov/). Sequences were deposited in GenBank under the accession numbers: JF690986; JF690987; JF690988; JF690989 and JF690990.

2.3.5 Statistical analysis

The time at which the point of inflection occurs also called the median emergence time ($T_{0.5}$) and the maximum emergence rate (MER) were calculated as described by (Nasr and Selles, 1995):

$$T_{0.5} = a / b$$

$$\text{MER} = (M \times b) / 4$$

In the equation, a is the constant of integration and b is the emergence rate constant and M is a parameter describing the maximum number of seedlings that eventually emerged (France and Thornley, 1984).

The data sets were analysed by ANOVA using JMP 6 (SAS Institute, Cary, USA). A P value of 0.05 was used as threshold to accept the significance of effects. The weight of plant fragments added to pots was used as co-variable. Treatment means were compared based on least significant differences (LSD), where significant treatment effects were found. The data was tested for normality using Shapiro-Wilk's test and non-normal data was transformed prior to analysis, as required by the tests.

Phylogenetic distance analysis was conducted by *MEGA* version 4.0.2 (Tamura et al., 2007) using DNA sequences selected for their similarity to the reference data in Genebank. Branch support was assessed by bootstrapping (maximum parsimony, 1000 replicates).

2.4 Results

2.4.1 Plant morphology and influence of indigenous endophytes on plant performance

After 7 weeks of growth under greenhouse conditions, the root system of Kabuli chickpea (CDC Frontier and CDC Xena) was significantly larger than that of the Desi

varieties (CDC Nika and CDC Anna) (Fig. 2.1). Based on plant dry mass, nodule number and nitrogenase activity, it appears that the differences between chickpeas are more related to cultivars than to classes (Desi and Kabuli). CDC Frontier had much more dry mass, root nodules and nitrogenase activity than any other cultivars (Fig. 2.1). At symbiosis development, endophytes inoculation induced a negative effect on all measured parameters except for nodule number at 30% of field capacity, where nodule number was very low and no difference was observed between inoculated and control plants (Fig. 2.1).

Inoculation with indigenous endophytes did not influence plant water potential, except for an inoculation-induced increase (Fig. 2.2) in the drought susceptible CDC Xena grown under condition of soil water sufficiency.

At seed maturation (16 weeks after transplantation of pre-germinated seeds), inoculation of chickpea plants with indigenous endophytes improved the N and P nutrition of water stressed CDC Nika (Fig. 2.3).

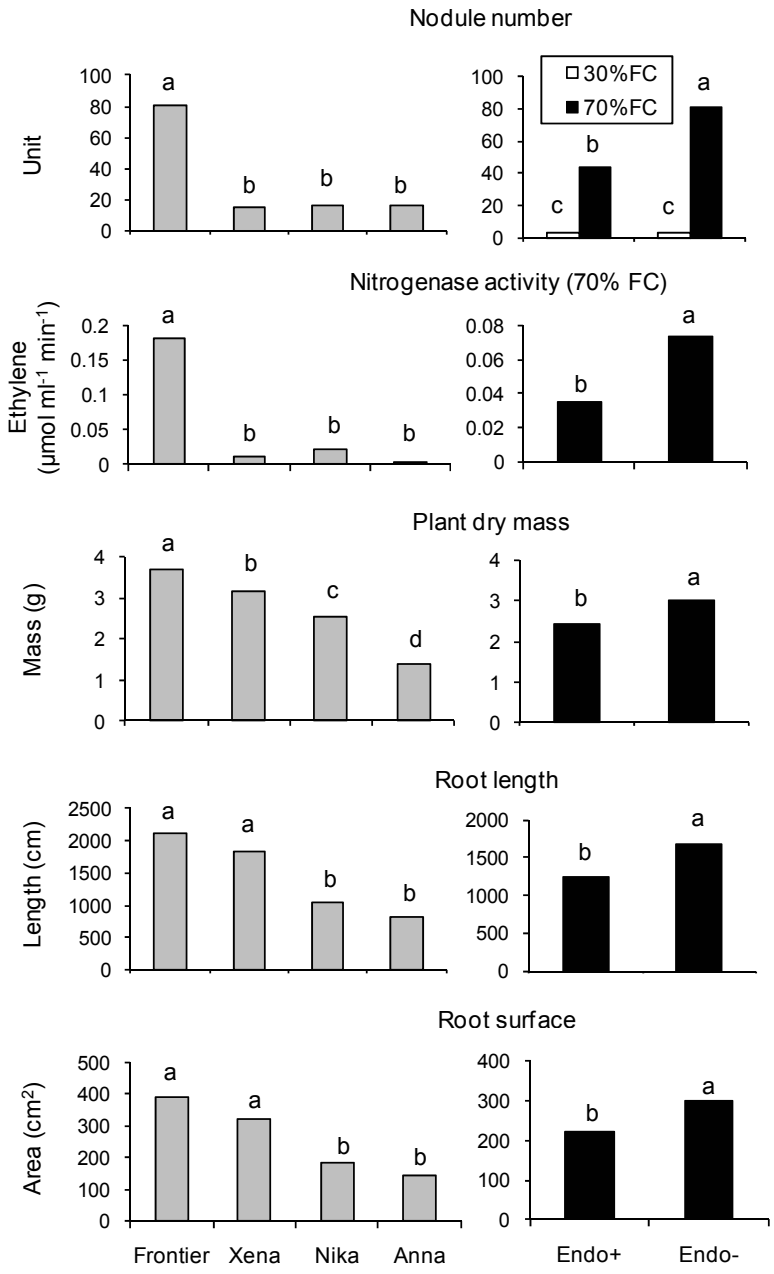

Figure 2-1 Nodule number, nitrogenase activity, plant dry mass, root length and root surface area of chickpea, as influenced by cultivar (*n*=16) and endophyte inoculation (*n*=32; *n*=16 for nodule number) at symbioses development (7 weeks after plants transplantation). Endo+, endophytes inoculated; Endo-, control; 30% FC, soil moisture maintained at 30% of field capacity; 70%, soil moisture maintained at 70% of field capacity. Bars with the same letter are not significantly different according to LSD (*P=0.05*).

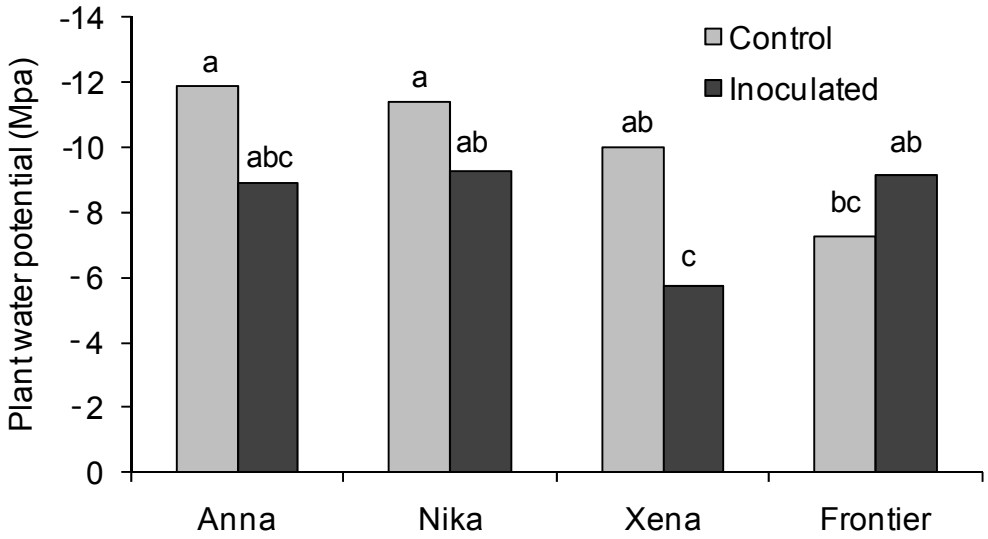

Figure 2-2 Plant water potential of different chickpea genotypes under condition of soil water sufficiency measured early morning 7 weeks after transplantation of pre-germinated seeds. Means followed by the same letter are not significantly different according to LSD *P=0.05* (*n*=4).

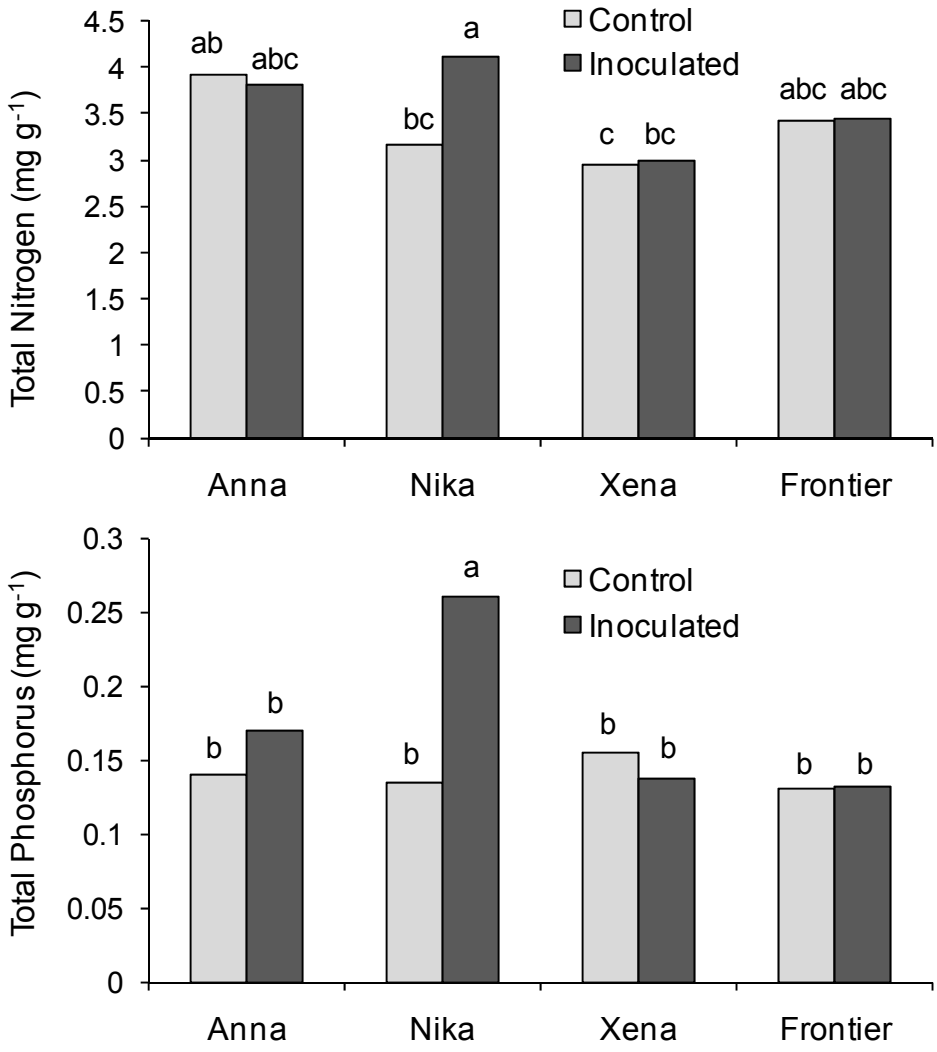

Figure 2-3 Effect of inoculation on total N and P in water stressed chickpea shoots tissue at seed maturation (16 weeks after transplantation of pre-germinated seeds). Means followed by the same letter are not significantly different according to LSD *P = 0.05* (*n*=4).

2.4.2 Whole soil microbial diversity analysis based on fatty acid methyl esters (FAME)

Frontier increased the abundance of three soil microbial PLFA markers under condition of soil water sufficiency, only (Fig. 2.4). The effect of CDC Frontier on the soil microbial community was limited to these three markers and there was no significant effect

on total microbial PLFA markers and overall, the soil microbial marker profiles associated with the chickpea cultivars were not different.

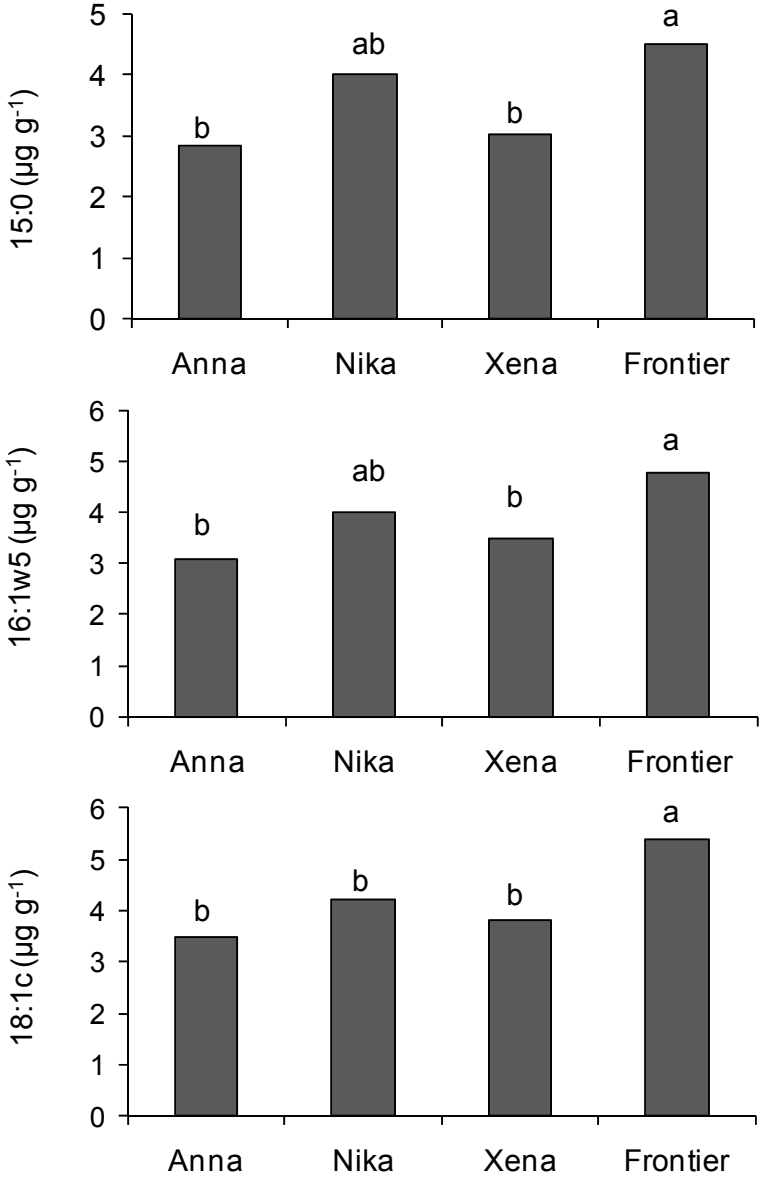

Figure 2-4 Variation in the abundance of the bacterial PLFA fingerprints 15:0 and 16:1w5 and the fungal PLFA fingerprint 18:1c in the rooting soil associated with different chickpea genotypes under condition of soil water sufficiency. Means are not significantly different when labelled with the same letter according to LSD $P = 0.05$ (n=8).

2.4.3 Diversity of culturable endophytes in crested wheatgrass root inoculum

Sixteen fungal cultures were obtained from the roots of crested wheatgrass. ITS sequence analysis revealed that they were of five different species, all Ascomycetes. Only the isolate CWG-F2-E13 could be identified to the species level as *Pseudogymnooscus roseus* Raillo by blasting our sequence in GenBank in addition to morphological observations performed at Agriculture and Agri-Food Canada National Mycological Herbarium, Ottawa. The cultures obtained from the other isolates were sterile and yet unreported in public databases. The ITS sequence of the isolate CWG-F3-E6 is consistent with a phylogenetic placement within the order Pleosporales, in the family Leptosphaeriaceae, probably near the genus Phaeosphaeria. The ITS sequences of isolates CWG-F1-E3 and CWG-F5-E16 fell within the order Helotiales and isolate CWG-F4-E15, within the order Sordariales (Fig. 2.5).

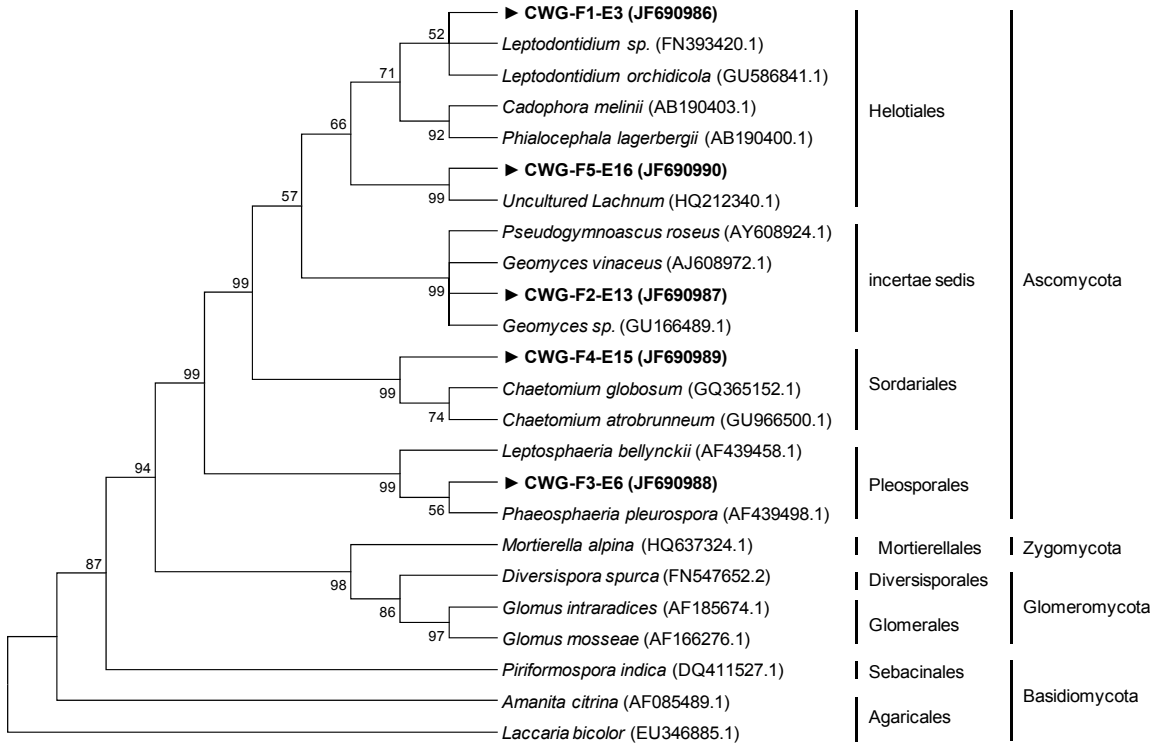

Figure 2-5 Phylogenetic analysis of the ITS sequences of the endophytic isolates contained in the root inoculums. Bootstrapping values greater than 50% calculated from 1,000 replicates are given above the branches. Names and Genbank codes in bold and preceded by a triangle (►) represent the sequences of the five isolates under study. In italics are the names of rDNA sequences of fungi downloaded from Genbank.

2.4.4 Effects of plant tissues on durum germination and emergence

Maximum emergence rate under the influence of chickpea and Brassica residues were largely similar, supporting the hypothesis of an allelopathic effect of chickpea on a subsequent crop of wheat (Fig. 2.6a). The median emergence time did not differ significantly after chickpea cultivars in the same class, but was longer in Desi chickpea (CDC Nika and CDC Anna) exudates+residue treated soil than in Kabuli or pea exudates+residue treated soil (Fig. 2.6b). The shortest median emergence time was found

under the influence of Kabuli (CDC Frontier and CDC Xena) chickpea and pea (Fig. 2.6b). Brassica, known to have allelopathic properties (Tawaha and Turk, 2003), induced the longest median emergence time. Under the influence of Desi chickpea, an intermediate median emergence time was recorded (Fig. 2.6b). Drought stress triggered the development of allelopathic properties in pea, which importantly reduced wheat maximum emergence rate, but this effect of drought was not seen in chickpea (Fig. 2.6a).

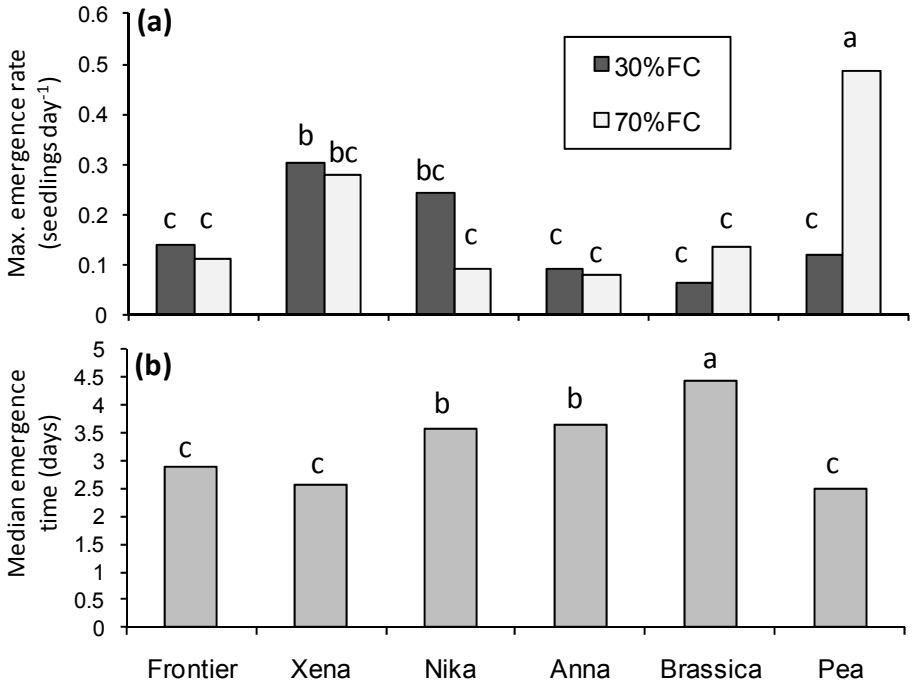

Figure 2-6 Influence of chickpea cultivars residues on maximum emergence rate (a) and median emergence time of AC Avonlea durum wheat seeds (b), as compared to black mustard, an allelopathic species, and to CDC Handel pea, a crop plant that stimulated the yield of a following crop of durum. Means followed by the same letter are not significantly different according to LSD (*P=0.05*; *n*=4 for (a) and *n*=8 for (b)).

2.4.5 Inoculation effect on subsequent wheat crop

The tissues of both inoculated Kabuli chickpea cultivars increased durum wheat maximum emergence rate as compared to noninoculated tissues (Fig. 2.7a), but only

endophytes-inoculated CDC Frontier tissues decreased median emergence time significantly (Fig. 2.7b). Tissues of CDC Frontier were more allelopathic than its exudates and effectively reduced durum wheat maximum emergence rate (Fig. 2.7c). The influence of tissues and exudates of CDC Xena did not different. Endophytes inoculation reduced emergence time of durum wheat grown with chickpea tissues (Fig. 2.7d).

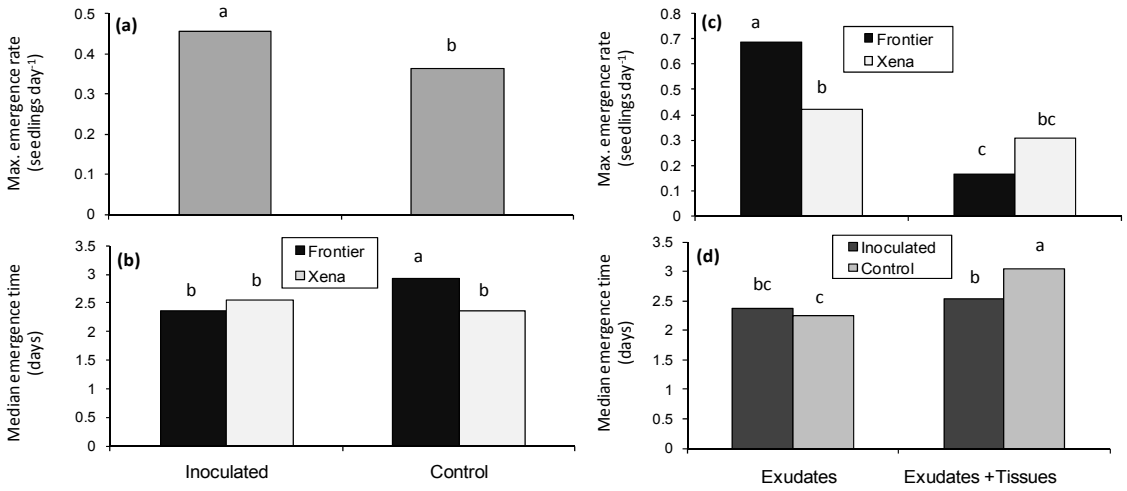

Figure 2-7 Influence of inoculation on the combined influence of the residues and exudates of chickpea CDC Frontier and CDC Xena on maximum emergence rate (a) and median emergence time of durum (b), and effect of the root exudates and tissues of these chickpea genotypes on maximum emergence rate (c) and median emergence time (d). Means followed by the same letter are not significantly different according to LSD ($P = 0.05$; n = 8 for (a) and 4 for (b), (c), and (d).

2.5 Discussion

2.5.1 Influence of indigenous endophytes on plant performance

The effects of inoculation on chickpea growth and nodulation varied with time. They were negative at early plant development stage, but positive or neutral later on. Similar results were obtained in previous work with perennial ryegrass (*Lolium perenne* L.) genotypes (Hesse et al., 2003). In the present study, reduction in growth at symbiosis

development due to inoculation may occur when the endophytic fungi draw on plant photosynthesis without providing nutritional benefits either because their hyphal networks are not yet developed or because soil nutrient level is below a minimum threshold. The first scenario is the likely explanation for chickpea growth reduction after 7 weeks of growth under greenhouse conditions, since the difference between inoculated and non-inoculated plants had disappeared at seed maturation and positive effects of indigenous endophytes were identified on shoot nitrogen and phosphorus concentration of the drought stressed Desi chickpea CDC Nika, with increases of 30% and 93%, respectively, in these parameters relative to un-inoculated controls. This situation is unlikely to be encountered in the field where the extraradical hyphal networks of endophytic fungi is conserved from year to year, in no-tilled soil or in soil submitted to shallow tillage. Inoculation also reduced nodule development. This was repeatedly observed in pot experiment inoculated by arbuscular mycorrhizal fungi (Catford et al., 2003; Zhang et al., 1995), but never reported from field experiment. The simultaneous development of the rhizobial and endophytic symbioses is a large carbon drain on a plant. The amount of photosynthetically active radiation is typically low in greenhouse where risk of overheating in summer dictates the practice of shading and short day length in winter cannot be completely compensated by supplemental lightings.

The chickpea plants varied in their response to endophyte inoculation with environmental conditions and the genotypes of interacting cultivar. These results are consistent with the evidence reported in several previous studies where positive (Rodriguez et al., 2008), neutral (Jumpponen and Trappe, 1998) or negative (Stoyke and Currah, 1993) effects of endophyte infection on different plant genotypes performance have been detected. In the present study, whereas presence of endophyte fungi led to better plant water potential for the cultivar CDC Xena, this was only true under condition of soil water sufficiency, and there was no effect under condition of soil water deficit. Inoculation resulted in better N and P nutrition of CDC Nika, only in condition of water stress. No effect of inoculation was detected for the other chickpea cultivars used, which does not negate the possibility of important functions for these endophytes in semiarid ecosystems.

2.5.2 Diversity of culturable endophytes in crested wheatgrass roots

The endophytes recovered from crested wheatgrass roots were Ascomyceteous of the Helotiales, Pleosporales, Sordariales and undefined taxonomic order "Incertae sedis". The dominance of Ascomyceteous endophytes in the present study corroborates the findings of Porras-Alfaro et al. (2011; 2008), who found that soils and plant roots of semiarid grasslands are colonized predominantly by Ascomycota.

The widespread occurrence of endophytes in extreme and stressful environments means that they are well adapted to these ecosystems and suggests an ecological function for these fungi in drylands. The positive effect of inoculation showed here could be a result of an individual, additive or a synergic interaction between fungal isolates and chickpea plants.

Four isolates from our study could not be identified to the species level by blasting sequences in GenBank or by morphological observations by experts of the National Mycological Herbarium, Ottawa, Canada. The matching ITS reference sequences for these isolates were lacking in public databases and cultures were sterile. This may be evidence that we have isolated new endophytes species from crested wheatgrass roots. However, they could be isolated and described before but not yet sequenced.

2.5.3 Potential source of chickpea plant allelopathic effect

In this study, chickpea tissues demonstrated harmful allelopathic effects on durum germination and emergence, including reduced maximum emergence rate and increased median emergence time as compared to pea which is the best previous crop for durum wheat in our region. Chickpea was also reported as a fair rotation crop, leading to lower yield in following wheat crops, as compared to medic, vetch or lentil in the Mediterranean region (Ryan et al., 2010). The negative effects of a chickpea plant on the following wheat crops could come from products of secondary metabolism (allelochemicals) such as phenolic compounds released into the environment through root exudation and/or through decomposition and leaching of plant residues in soil (Rice, 1984).

For pea plants, the exudates + tissues produced under drought stress reduced significantly durum wheat maximum emergence rate, but this was not so for pea plants grown under condition of soil water sufficiency. This response of pea plants to drought could be attributable to a greater production and release of allelochemicals under stress conditions (Einhellig, 1987). Release of allelochemicals in chickpea plants did not appear to be influenced by abiotic stress. No effect of drought on allelopathic properties was detected in chickpea cultivars. This concurs with Li and Copeland (2000) results showing the absence of abiotic stress effect on malonate, a toxic organic acid playing the role of a defensive chemical in chickpea plants.

Modification of the soil microbial communities does not seem to be involved in chickpea allelopathic effects. The soil microbial community associated with CDC Frontier was different from that associated with other chickpea genotypes, but this difference was not expressed at the level of allelopathic effects.

2.5.4 Effects of endophyte inoculation of chickpea plant on durum seeds germination and emergence

Results of this study indicate that endophytes inoculation reduced emergence time of durum wheat grown with chickpea tissues. Positive effects of endophyte infection have been detected in past studies focusing on the performance of grass populations (Clay, 1987; Novas et al., 2003; Pinkerton et al., 1990; Zhang et al., 2010). Furthermore, same tendency was shown in the case of the endophyte-infected red fescue seedlings where germination strategy and growth may be beneficial to surviving in the harsh conditions (Wäli et al., 2009).

2.6 Conclusion

With the growing interest in reducing chemical inputs, the use of beneficial microbial endophytes in the improvement of biomass production and stress tolerance may be an environment friendly way to increase food production. In the present study we found

that indigenous endophytes can improve drought tolerance, and N and P nutrition in chickpea. Moreover, chickpea's allelopathic effect on a subsequent durum wheat crop is reduced by endophytes inoculation. Genetic variation in the response of chickpea to endophytic infection indicates the possibility to produce chickpea genotypes better adapted to the microflora of Canadian Prairie soils through plant breeding.

2.7 Acknowledgements

The authors would like to gratefully acknowledge the excellent technical assistance of Keith Hanson. We also thank Dr. Tharcisse Barasubiye, Dr. Sarah Hambleton and Dr. Keith A. Seifert for their guidance in fungal identification. This project was supported by a grant from the Alberta Pulse Growers and AAFC to Hamel and a scholarship from the Islamic Development Bank Merit Scholarship Programme for High Technology to Ellouze.

Chapitre 3: Plant genotype modifies soil fungal diversity and arbuscular mycorrhizal colonization of chickpea crops in the Canadian Prairie

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3.1 Abstract

Many soil microorganisms can enhace plant growth, which leads to a more efficient use of nutrients by crop plants. Genetic variation in plant promotion of beneficial soil microbial resources would allow the selection of crop varieties with reduced dependence on agro-chemicals. This study was conducted to document the fungal resources in dryland agriculture of the Canadian Prairie, and determine how genetic variations in chickpea may influence soil fungi and fungal diversity. Four chickpea genotypes with contrasting phenotypes were evaluated under field conditions. Species of the Ascomycota accounted for 93% of the culturable fungi detected, most belonging to the Hypocreales and some to the Pleosporales, which are orders hosting several dark septate endophytic species. The detection of 15 Glomeromycotan ribotypes indicated that the diversity of arbuscular mycorrhizal fungi (AMF) can be high in intensively managed agricultural soils. Five of these ribotypes belong to the Glomerales, five to the Diversisporales, four to the Paraglomerales and one to the Archaeosporales. The Desi chickpea CDC Anna was associated with high diversity of AMF and culturable fungi, favored the proliferation of soil bacteria and fungal genus hosting biocontrol agents, and developped high AM root colonization level, as compared to the three Kabuli genotypes examined. The fungal diversity in cultivated Prairie dryland appeared to host a large array of fungal groups known to reduced plant nutrient, water and biotic stresses, and chickpea genotypes influenced differently the composition and biomass of the soil microbial community. Therefore, it seems possible to enhance the soil microbial community and the services it provides to food production, through the selection of chickpea genotypes.

Key Words: Chickpea (*Cicer arietinum* L.), garbanzo bean, plant genotype, soil microbial community structure, soil fungal diversity, arbuscular mycorrhizae, plant symbioses, dark septate endophytes, dryland agriculture, soil resource use efficiency, plant breeding.

3.2 Introduction

Several soil fungal species can increase plant tolerance to environmental stresses associated with a changing climate, whereas other fungal species can improve the efficiency of nutrient and water use by plants, in a world with diminishing resources. The arbuscular mycorrhizal fungi (AMF) are recognized for their beneficial influence in the whole plant-soil ecosystem (Smith and Read, 2008). The AMF are widespread biotrophic fungi with host-preference rather than specificity (Vandenkoornhuyse et al., 2003). The large networks of hyphae formed in the soil by AMF are multipurpose (Leake et al., 2004). They mobilize soil nutrients, transport nutrients across the depletion zone around roots (Liu et al., 2007), and transfer them to the plant symplast via a symbiotic interface formed at the level of root cells' plasma membrane (Govindarajulu et al., 2005; Parniske, 2008). The AMF associated to roots can improve their host plant productivity by stimulating decomposers activity and linking mineralization to plant nutrient demand (Atul-Nayyar et al., 2009; Hodge and Fitter, 2010). This symbiotic association can protect plants against soil-borne diseases (St-Arnaud and Vujanovic, 2007) through improved nutrition, modulation of plant defense pathways and soil sanitization (Lioussanne et al., 2008a), and mitigate the impact of cold (Paradis et al., 1995) and of drought stress in plants including wheat (Al-Karaki et al., 2004; Aroca et al., 2008) and pulses (Porcel et al., 2006; Porcel et al., 2003; Ruiz-Lozano et al., 2001) by enhancing plant physiological response. The AMF improve plant growth and also enhance soil structural quality. Their extensive hyphal networks act as sticky strings in soil, holding and packaging soil particles and microaggregates into macroaggregates, and leading to better soil aeration and water relation (Augé, 2004; Six et al., 2004). The AMF contribute importantly to the soil carbon pool (Rillig, 2004) by enhancing photosynthesis and diverting belowground between 4% and 20% of the carbon fixed by a host plant (Smith and Read, 2008).

The origin of AMF dates back to the Early Devonian, over 400 million years ago, when they helped plants' ancestors to acquire nutrients from the soil, allowing them to colonize the continents of Earth (Strullu-Derrien and Strullu, 2007). Today, they

collectively form the Phylum Glomeromycota and continue to improve plant fitness (Schüßler et al., 2001).

Fungi of other phyla also colonize asymptomatically plant roots. Endophytic fungi with melanized hyphae, which are sometimes called dark septate endophytes (DSE), are found in stressful (Rodriguez et al., 2008; Sonjak et al., 2009) or cold environments (Narisawa et al., 2007) such as the high Artic (Fujimura et al., 2008; Piercey et al., 2004) and Antarctica (Mandyam and Jumpponen, 2005) where AMF are sporadic or absent (Bledsoe et al., 1990). The DSE are important fungal colonizers of plant roots in the Prairie (Khidir et al., 2010; Mandyam and Jumpponen, 2008), a biome characterized by water deficit, where the DSE abundance in plant roots often exceeds that of AMF (Khidir et al., 2010; Mandyam and Jumpponen, 2008; Medina-Roldan et al., 2008). The abundance of DSE in the roots of grassland plants suggests an ecological function for these fungi in dryland. Some DSE may be important in reducing plant disease incidence (Narisawa et al., 2002), giving plants access to organic nutrient pools (Upson et al., 2009), and in reducing drought and heat stresses (Rodriguez et al., 2008). DSE fungi may also interact with AMF (Mandyam and Jumpponen, 2008; Scervino et al., 2009) and influence plant community dynamics (Mandyam and Jumpponen, 2005).

The whole range of positive to negative interactions between DSE and plants has been documented in the literature; this is not unexpected because a range of different fungi are referred to as DSE, and different DSE may have different influences on plant growth. Some DSE fungi can improve plant nutrition, produce growth hormones, improve plant health, and others can reduce plant growth (Kageyama et al., 2008). Whereas DSE in the Canadian boreal forest were examined, little is known on the distribution of DSE in the Canadian grassland other than the rare occurrence of *P. fortinii* in this environment (Piercey et al., 2004).

Fungal pathogens cause plant diseases when they meet with a susceptible host in a favorable environment (Susilo et al., 2004). They have considerable negative impact on human wellbeing through major yield losses in agriculture, and also have consequences for

biodiversity conservation (Anderson et al., 2004). In natural vegetation, pathogenic microbes can have important effects on the composition and functioning of plant communities (Van der Putten, 2003). Soil-borne pathogens have been shown to affect plant competition by reducing the competitive ability of their host (Van der Putten and Peters, 1997). Diseased plants are often replaced by other genotypes or species (Van der Putten et al., 2007). Plants use several strategies to defend themselves against damage caused by pathogens. The strategies may include tolerance and resistance to pathogen attack, the use of constitutive or induce defense mechanisms, and escaping attacks with seasonal growth patterns incompatible with the life cycle of pathogens. Reducing plant compatibility with pathogenic microorganisms has been an important target for crop genetic improvement programs throughout the world.

Environment models predict changes in climate that will occur as an unprecedented rate (Lane and Jarvis, 2007; Redden et al., 2010) and the impacts of climate change on agricultural ecosystems may be devastating in regions where the climate is already stressful, such as in the drylands of the Northern Great Plains of America. The impacts of climate change on the biosphere, however, are difficult to predict, and thus, improving the general tolerance of crop plants to environmental stress appears as a good strategy to mitigate the impact of a changing climate and improve the stability of food production. Improving the compatibility of crop plants with beneficial fungal endophytes naturally occurring in cultivated soils may be a way to increase stress tolerance in crops and improve crop efficiency of nutrient and water use in these times of diminishing natural resources (Fixen, 2009), which would help reduce greenhouse gas emissions from cultivated soils (Buczko and Kuchenbuch, 2007; Snyder et al., 2009).

The objective of this study was to verify the existence of variation in the rhizospheric associations of field-grown chickpea, as it is a necessary condition for the selection of genotypes with improved compatibility with beneficial microorganisms. Through this research, we also wanted to improve knowledge of the soil microorganisms naturally associated with an important crop plant used in dryland agriculture, in the Canadian Prairie.

3.3 Materials and methods

3.3.1 Site description and treatments

Chickpea with contrasting phenotypes and lineages were used. They include two classes of chickpea, Desi and Kabuli, with three varieties bred at the Crop Development Centre of the University of Saskatchewan, Canada, and one variety introduced from Europe. They were: (1) CDC Xena, a large-seeded Kabuli with unifoliate leaves; (2) CDC Frontier, a large-seeded Kabuli with fern leaves; (3) Amit, a small-seeded Kabuli with fern leaves; (4) CDC Anna, a Desi type. These chickpea genotypes were grown in 2 m x 10 m field plots at the South Farm of the Semiarid Prairie Agricultural Research Centre, in Swift Current SK (50° 15'N, 107° 44'W), in 2005 and 2006. The soil, a Brown Chenozem (Aridic Haploboroll in US soil classification) with silt loam texture, an organic carbon content of 20 g kg^{-1}, and a pH ($CaCI_2$) of 6.8, was planted with wheat the previous year (Gan et al., 2009b). Growing season precipitations were 219 mm in 2005 and 194 mm in 2006. The four genotype treatments were repeated four times in complete blocks.

Plots were seeded on 10 May 2005 and 6 May 2006, when noon soil temperature at 10-cm depth was about 10°C and 13°C, respectively. Seeding rates were determined based on pre-seeding germination tests, targeting a plant density of 40 plants m^{-2}. Seeds were treated with carbathiin, thiabendazole, and metalaxyl, as per manufacturer recommendations, to minimize soil- and seed-borne diseases. Seeds were placed 4 cm deep using a hoe press drill equipped with C shanks, side band openers, fertilizer boxes, granular *Rhizobium* inoculant box, and a seed splitter. Row spacing was 25.4 cm. All plots received a blanket application of 0-45-0 fertilizer with the seeds to supply phosphorus. Plots received 5.5 kg ha^{-1} of granular inoculant Nitragin GC® (LiphaTech Inc., Milwaukee, WI) containing a minimum of 100 million viable cells of *Mesorhizobium ciceri* per gram. Nitrogen and potassium fertilizers were not required on these crops and soils, and were not applied. Weed control was achieved using a previous fall broadcast application of ethalfluralin, a pre-seeding burn-off treatment with glyphosate and in-crop application of sethoxydim at mid-seedling stage, all as recommended by manufacturers. Ascochyta blight

was noticed on the chickpea crops at the mid- to late-seedling stage, and controlled by two foliar applications of chlorothalonil (Syngenta Crop Protection Canada, Inc.) at 0.5 - 0.65 L product ha^{-1} and pyraclostrobin (Bayer Crop Science, USA) at 0.6 - 1.0 L product ha^{-1}.

3.3.2 Soil and root sampling

Root and rooting soil were taken directly on the plant row at physiological maturity, using a hand corer (2.5 cm diameter x 7.5 cm length). Four cores were taken and pooled to produce one sample per plot. Roots were freed from the soil, which was further sieved through 2 mm. The soil was subsampled into four parts; one part of the soil was used for the determination of soil moisture, and the second part was placed at -12°C until fatty acid methyl ester (FAME) analysis and DNA extraction, as described below. A third part was placed at 4°C until fungal culture isolation, and the fourth part of the soil was air dried and used for soil pH measurement in water (Hendershot et al., 1993) using a pH meter.

Roots were thoroughly washed on a 2-mm sieve after sampling to minimize fine root losses. The chickpea roots were then cut into 1 cm fragments, mixed, and 2 replicates from each plot were taken and placed in plastic cassettes. Roots were cleared and stained using an ink and vinegar solution as described by Vierheilig et al. (1998). The percentage of chickpea root colonization was determined by the gridline-intersect method (Giovannetti and Mosse, 1980).

3.3.3 Fatty Acid Methyl Esters (FAME) Analysis

The abundance of active soil microbial biomass in soil samples was determined by quantification of phospholipid fatty acids (PLFAs), using the method described in Hamel et al. (2006). Four grams of soil (dry weight equivalent) were extracted in 9.5 ml mixture of dichloromethane (DCM) : methanol (MeOH) : citrate buffer (1:2:0.8 v/v) as described in Chapter 2, Section 2.3.3. Lipid-class separation was conducted in silica gel columns. The transmethylation and quantification of PLFAs by gas chromatography was determined following the method of described in Chapter 2, Section 2.3.3. Individual fatty acids have

been used as signatures for various groups of micro-organisms (Hamel et al., 2006). The FAME 18:2ω6c and 18:1c were used as indicators of fungal biomass (Frostegård and Bååth, 1996) and FAME 16:1ω5, as indicator of arbuscular mycorrhizal fungi (Balser et al., 2005). FAMEs 3OH-12:0, a-12meth-15:0, i-13meth-15:0, 15:0, 14:0, 2OH-14:0, i-14meth-16:0, 16:1ω7c, i-15meth-17:0, 17:0, 2OH-16:0 and 18:1t were chosen to represent bacterial PLFAs based on the bacterial standards used.

3.3.4 Isolation of fungi

Dilution series were prepared from the 2005 soil samples. One milliliter from a 10^{-4} dilution was spread on PDA culture media supplemented with neomycin sulfate (12 mg l^{-1}) and streptomycin sulfate (100 mg l^{-1}) (Vujanovic et al., 2002). Five plates per plot were prepared. Plates were incubated at room temperature in the dark, and observed after 3 days. Total fungal colony-forming units (CFU) were counted for each plate. The three plates with the largest biodiversity where selected. All the colonies developing in these plates were transferred onto new Petri plates. Fungal cultures were stored in sterile distilled water, in 2 mL containers for further references.

3.3.5 DNA sequencing and ITS sequence analysis of culturable fungal diversity

The fungal isolates were grown in Petri dishes containing Potato dextrose agar (PDA) medium supplemented with neomycin sulfate (12 mg l^{-1}) and streptomycin sulfate (100 mg l^{-1}) (Vujanovic et al., 2002). Cultures were classified into 140 morphotypes (St-Germain and Summerbell, 1996) and identified based on the internal transcribed spacer (ITS) region of the rRNA gene. Fungal DNA extraction was carried out using an UltraClean microbial DNA isolation kit (MoBio Laboratories) following the manufacturer's instructions. The ITS region of each fungus was amplified by polymerase chain reaction (PCR) in 25 µl reaction volumes, each containing 11 µl sterile distilled H_2O, 12.5 µl Taq PCR Master Mix Kit (Qiagen Laboratories), 0.25 µl of each fungal specific primer ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990), and 1 µl of

extracted genomic DNA. The amplifications were performed in an Eppendorf's Mastercycler eP S gradient thermocycler using the following conditions: initial denaturation step at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final extension step at 72°C for 7 min. Reactions were performed with negative controls containing no DNA. The resulting PCR products were electrophoresed in 1% (w/v) agarose gels, stained with ethidium bromide, and visualised under UV light.

Sequencing reactions were performed in a commercial laboratory (Genome Quebec Innovation Centre). ITS sequences were analysed with the Basic Local Alignment Search Tool (BLAST) through GenBank (http://www.ncbi.nlm.nih.gov/). Sequences were submitted to GenBank under the accession numbers shown in the phylogenetic trees (Fig. 3.4).

3.3.6 DNA sequencing and SSU sequence analysis of AMF diversity

DNA extraction from chickpea rooting soil was carried out using an UltraClean soil DNA isolation kit (MoBio Laboratories) following the manufacturer's instructions.

A nested-PCR approach was employed to amplify AMF DNA. The first PCR round, using the primers NS1 (White et al., 1990) and NS41 (Simon et al., 1992), amplified a 1.5-kb fragment of the 18S rRNA gene. PCR was conducted with an Eppendorf's Mastercycler eP S gradient thermocycler in 25 µL volume made of: 2.5 µL 10×PCR buffer, 0.5 µL 25 mM MgCl2, 0.25 µL 2.5 units/µL Taq DNA polymerase, 0.5 µL 2.5 mM dNTP, 2.5 µL 5 µM NS1, 2.5 µL 5 µM NS41, 0.5 µL Tween 1%, 1 µL DMSO, 0.125 BSA, 13.625 µL dd H_2O and 1 µL of extracted DNA (diluted 1:100). The PCR conditions were as follows: 95 °C for 3 min; 35×(94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min); 72 °C, 10 min. The PCR products were analyzed by agarose gel electrophoresis (1.0% (w/v) agarose), stained with ethidium bromide, and visualized using a Gel Imaging System (GelDoc, Bio-Rad Laboratories).

The product of the first PCR round with a visible band was diluted 1:10 and used as template in subsequent nested PCR round. The primers for this second stage was AML1 and AML2 (Lee et al., 2008). PCR was conducted in 50 µL volume in the following reaction system: 5 µL 10×PCR buffer, 0.25 µL 2.5 units μL^{-1} Taq DNA polymerase, 1 µL 2.5 mM dNTP, 5 µL AML1 (5 µM), 5 µL AML2 (5 µM), 32.75 µL dd H_2O and 1 µL product of the first PCR. The conditions for the second PCR round were: 95 °C for 15 min; 30×(94 °C, 30 s; 58 °C, 40 s; 72 °C, 55 s); 72 °C, 10 min.

Equal amounts of the PCR products from 2005 and 2006 samples were combined to obtain a pooled DNA sample from each experimental treatment. Each of these four pooled DNA samples were then cloned using One Shot® TOP10 Chemically Competent *E. coli* and the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Approximately, 50 positive clones were isolated from each cloning reaction, sequenced, and compared to reference sequences in Genbank (http://www.ncbi.nlm.nih.gov/). Plasmid DNA extraction and sequencing reactions were performed in a commercial laboratory (Genome Quebec Innovation Centre). SSU rRNA sequences were submitted to GenBank under the accession numbers shown in the phylogenetic trees (Fig. 3.1).

3.3.7 Statistical analysis

The Simpson's Index of Diversity (1 – *D*) (Pielou, 1969) (Equation 1) and Shannon's diversity index (*H'*) (Margelef, 1958) (Equation 2) were calculated as:

$$1 - D = 1 - \frac{\sum_{i=1}^{S} n_i(n_i-1)}{N(N-1)} \quad [1] \qquad H' = -\sum_{i=1}^{S} \frac{n_i}{N} \ln \frac{n_i}{N} \quad [2]$$

Where n_i is the number of individuals in species *i, S* is the number of species and *N* is the total number of individuals in the community. A higher index reflects higher diversity of the fungal community associated with the different field-grown chickpea genotypes.

The significance of chickpea genotype effects on fungal species richness and diversity and on PLFA fingerprints variables were assessed by analysis of variance

(ANOVA) using JMP 6 (SAS Institute, Cary, USA). A P value of 0.05 was used as threshold to accept the significance of effects. The significance of difference between treatment means was assessed by the least significant difference (LSD), when significant treatment effects were found. The data was tested for normality using Shapiro-Wilk's test and non-normal data was transformed prior to analysis, as required by the tests. The cosines transformation was applied to the PLFA fingerprint (i-methyl-17:0 + 17:0) to meet the requirement of normality before statistical analysis.

Principal component analysis (PCA) and canonical redundancy analysis (RDA) were carried out to determine the relationship between the distribution of the most abundant fungal species and chickpea genotypes. The rare species, i.e. those associated with less than two chickpea genotypes were not included in the analyses, and the species abundances data were Hellinger-transformed prior to analysis (Legendre and Gallagher, 2001; Legendre and Legendre, 1998). These PCA and RDA were completed with the R Project for Statistical Computing version 2.11.0 (R Development Core Team, 2011). The rdaTest application was used to compute the RDA (Legendre and Durand, 2008).

Phylogenetic distance analysis was conducted by *MEGA* version 4.0.2 (Tamura et al., 2007) using DNA sequences selected for their similarity to the reference data in Genebank. Branch support was assessed by bootstrapping (maximum parsimony, 1000 replicates).

3.4 Results

3.4.1 Diversity of AMF communities in chickpea rooting soil

A total of 15 different arbuscular mycorrhizal (AM) fungal ribotypes were found in this study. Of these, five belong to the orders Glomerales (three Group A, two Group B (Schwarzott et al., 2001)), five to the Diversisporales, four to the Paraglomerales and one to the Archaeosporales (Fig. 3.1). Eleven AM fungal ribotypes were found in the rooting soil of CDC Anna, a Desi type of chickpea, and fewer ribotypes were found in the rooting soil

of the Kabuli chickpea genotypes Amit, CDC Frontier and CDC Xena (Fig. 3.1). Ribotypes of the Diversisporales were more frequently associated with the Desi chickpea than with the Kabuli genotypes (Fig. 3.1 and 3.2). The *Archaeospora* specie was only found with the Desi type - CDC Anna (Fig. 3.1). Paraglomerales were more frequently found with CDC Anna, Amit and CDC Frontier, while *Glomus* group A was the most frequent ribotypes found with CDC Xena (Fig. 3.1 and 3.2).

Over the 2005 growing season, the genotypes CDC Anna and Amit had greater (*P=0.0001*) root colonization than genotypes CDC Xena and CDC Frontier, but not in 2006 (*P = 0.873*) (Fig. 3.3).

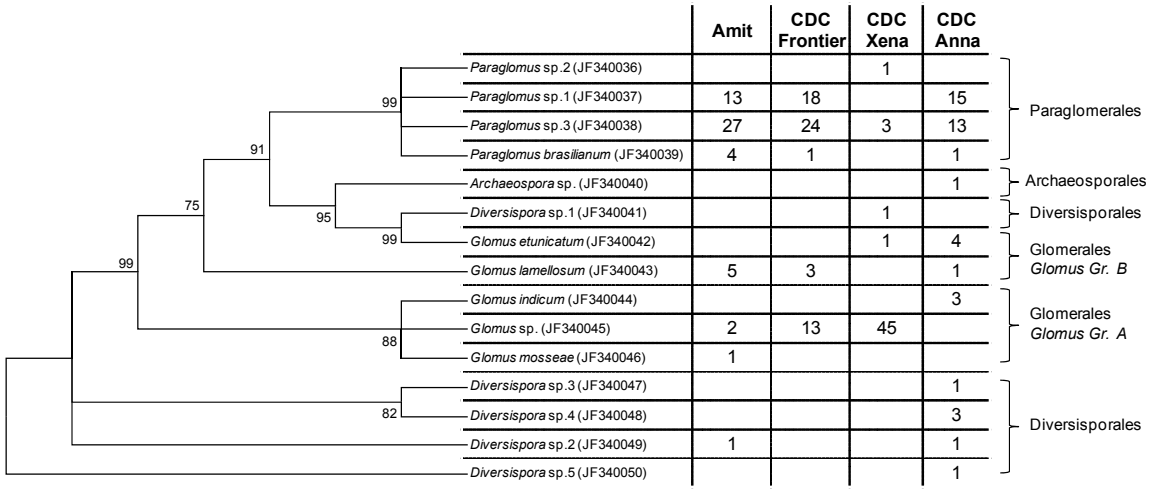

Figure 3-1 Identity of the AMF (*Glomeromycota*) associated to the field-grown chickpea crops. Identity was inferred from phylogenetic analysis of SSU rRNA sequences. Bootstrapping values greater than 75% calculated from 1,000 replicates are given above the branches. The number of clones corresponding to each AMF taxa are listed in the table.

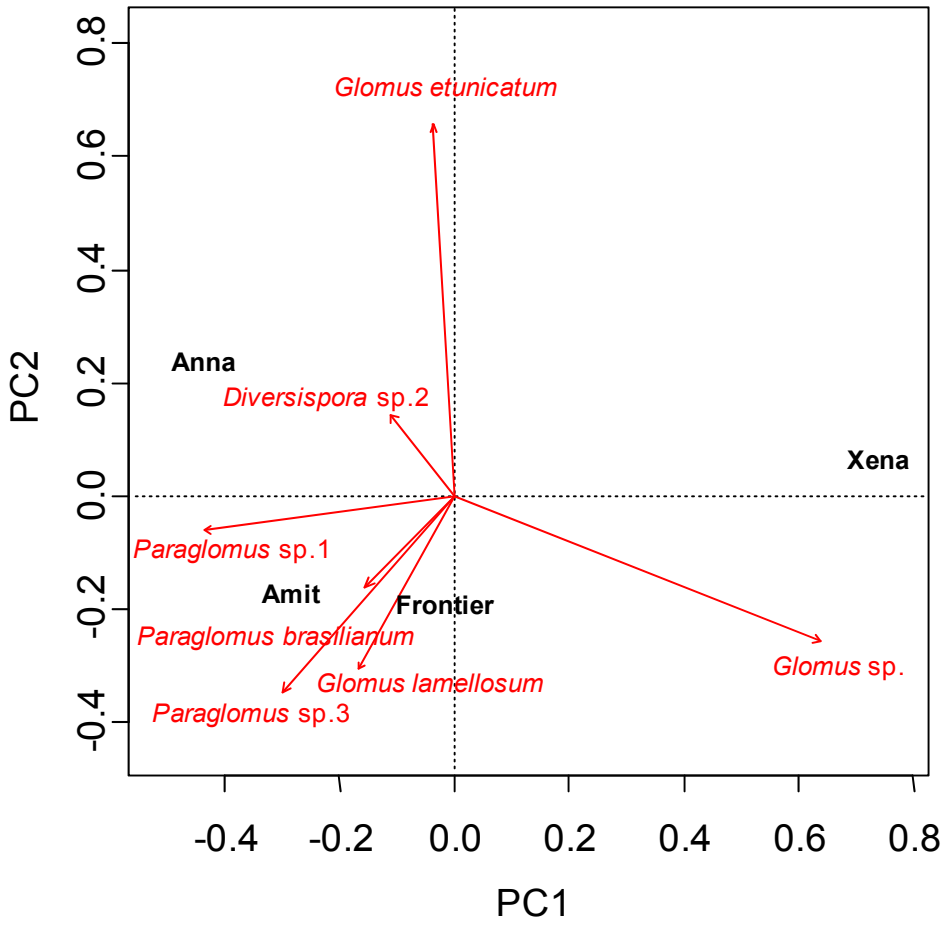

Figure 3-2 Relationship between AMF taxa and chickpea genotypes. Rare taxa, i.e. those detected in only one chickpea treatment, were not included in the Principal Component Analysis (*N*=32).

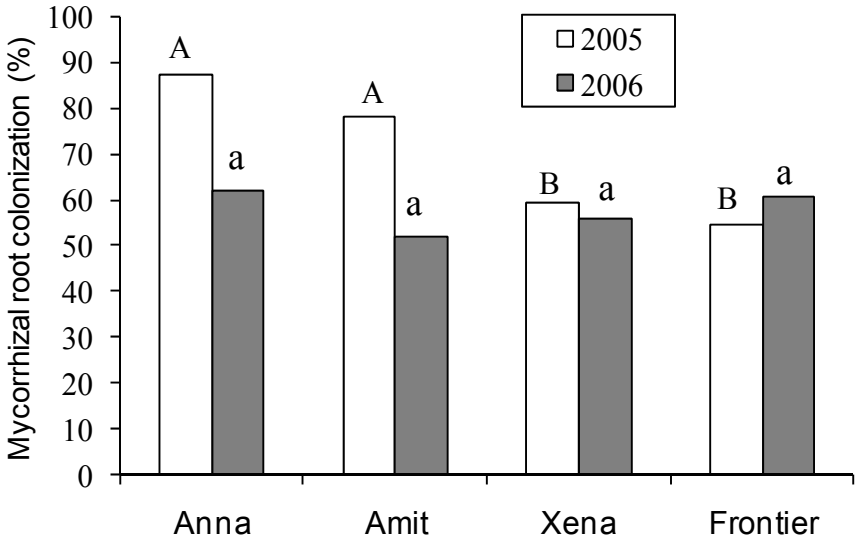

Figure 3-3 Level of arbuscular mycorrhizal root colonization of field-grown chickpea genotypes at harvest, in 2005 and 2006. Means are not significantly different when labelled with the same letter within a year (*n*=4).

3.4.2 Diversity of culturable fungal communities in chickpea rooting soil

The 1360 colony forming units (CFUs) isolated from field soil growing different chickpea genotypes were separated into 68 operational taxonomical units (OTUs), based on rRNA genes ITS sequence analysis (Fig. 4). Most fungi Ascomyceteous (92.6%), others belong to the Zygomycota (5.9%) and Basidiomycota (1.5%). Ascomycota species belong to the orders Hypocreales (50.8%), Eurotiales (25.4%), Pleosporales (7.9%), Onygenales (4.8%), Sordariales (4.8%), Microascales (3.2%), Capnodiales (1.6%) and undefined taxonomic order "Incertae sedis" which belong to the family Myxotrichaceae (1.6%).

The Simpson's Index of Diversity (*1 – D*) and Shannon's Diversity index (*H'*) of the culturable fungal community were largest in the CDC Anna rooting zone (Table 3.1). The fungal diversity associated with the roots of Kabuli genotype Amit had low indices.

Table 3-1 Diversity index of the culturable fungal community in the rooting soil of different chickpea genotypes.

Chickpea genotypes	**CDC Anna**	**CDC Frontier**	**CDC Xena**	**Amit**
Simpson's Index of Diversity (1 – D)	0.891 a	0.881 ab	0.869 ab	0.828 b
Shannon's Diversity index (*H'*)	2.284 a	2.223 ab	2.192 ab	1.998 b

Means followed by the same letter are not significantly different according to LSD ($P < 0.05$; n=9).

The distribution of the most abundant culturable fungal species was related to chickpea genotypes, as revealed by RDA (*P=0.016, N=36*). Desi genotype CDC Anna was associated with two species of fungi, *Bionectria ochroleuca* (F3 and F5) and *Trichoderma sp.* (F36) (Fig. 3.5), whereas Kabuli genotypes were principally associated with *Penicillium* species. CDC Xena was associated with *Penicillium sp.* (F28, F32 and 34), *P. kurssanovii* (F29), and *P. canescens* (F25). Amit was associated with *P. canescens* (F24) and CDC Frontier with *P. ochrochloron* (F30), *P. canescens* (F26), and *P. aurantiogriseum* (F23), in addition to *B. Ochroleuca* (F4) and *Fusarium sp.* (F11) (Fig. 3.5).

The distribution of *Penicillium* species F25: *P. canescens*, F29: *P. kurssanovii* and F32: *Penicillium sp.* was negatively related with that of *Fusarium redolens* (F7), *Fusarium solani* (F8) *Gibberella avenacea* (F10), *Fusarium sp.* (F11), and *Hypocrea lixii* (F15 and F17). *Penicillium canescens* (F24) was negatively related to *Bionectria ochroleuca* (F3, F5 and F6) (Fig. 3.5).

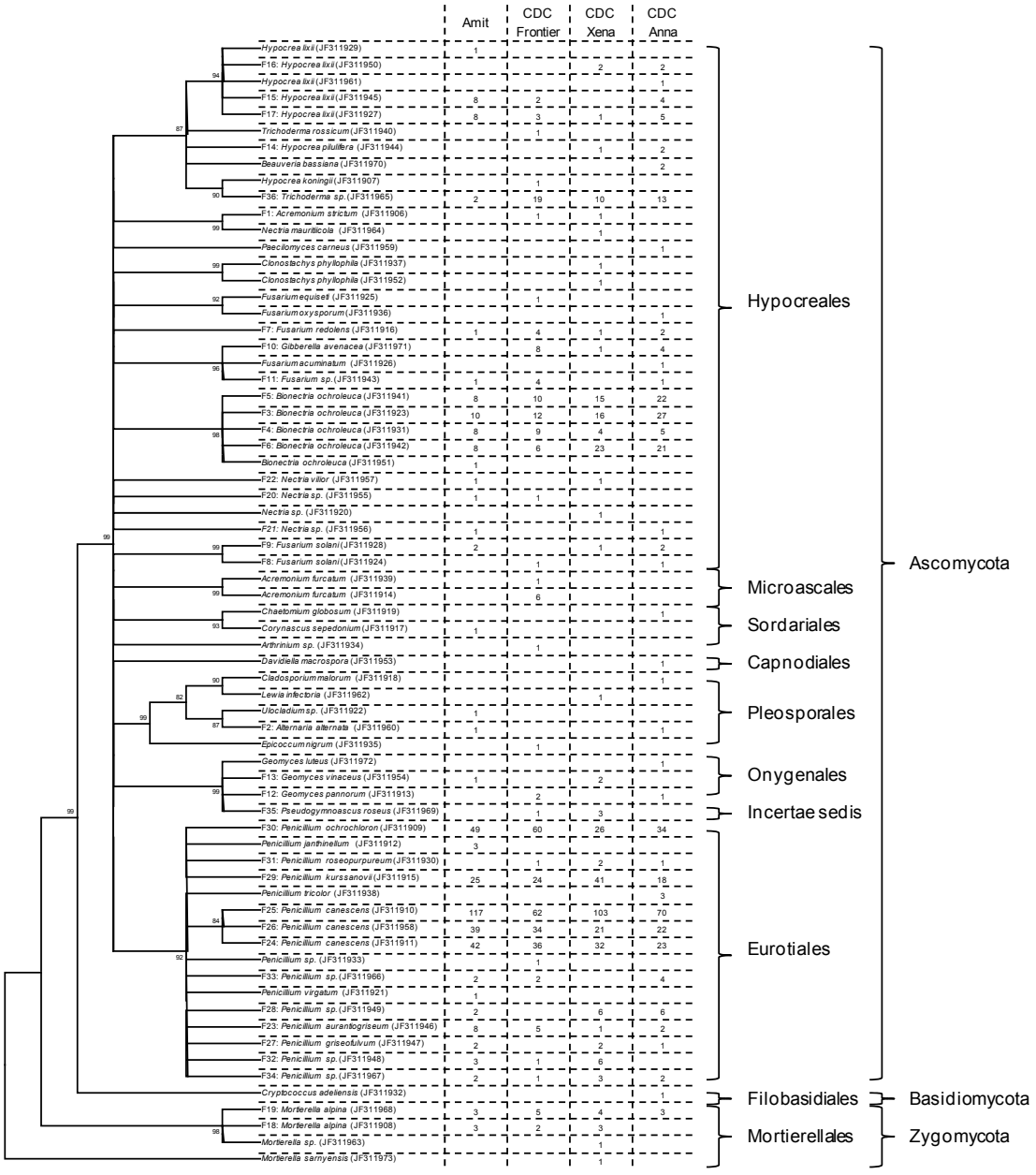

Figure 3-4 Identity of the culturable fungi associated to the field-grown chickpea crops. The phylogenetic analysis used ITS rRNA sequences of the fungal mycelia isolated from the rooting soil of different chickpea genotypes. Bootstrapping values greater than 75% calculated from 1,000 replicates are given above the branches. The number of colony forming units of each fungal species, as influenced by chickpea genotype, is presented in the table.

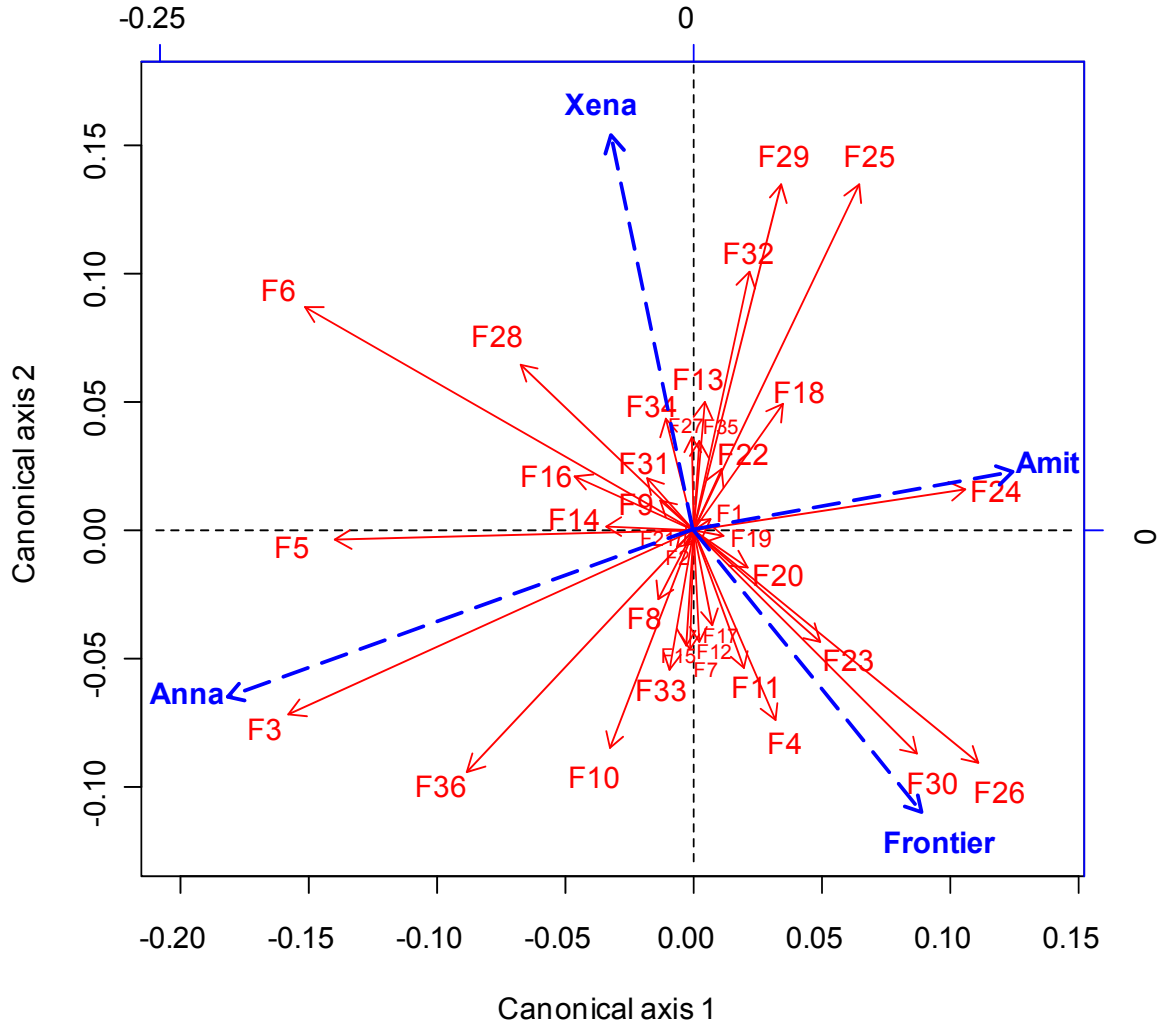

Figure 3-5 Relationship between culturable fungal species and chickpea genotypes. Rare taxa, i.e. those detected in only one chickpea treatment, were not included in the canonical redundancy analysis (RDA) (P=0.016, N=32). The numbers associated with fungal species are listed in Figure 3.4.

3.4.3 Whole soil microbial diversity analysis based on fatty acid methyl esters (FAME)

Chickpea genotypes had significant effects on the phospholipids fatty acids (PLFA) microbial markers only in 2005 (Table 3.2). The Kabuli genotype Amit was associated with the highest abundance of four bacterial PLFA markers (14:0, i-15:0, 2OH-14:0, i-17:0+17:0) and the PLFA marker for AMF (16:1ω5), followed by the Desi genotype CDC Anna, and the two Kabuli genotype CDC Xena and CDC Frontier, which were associated with low abundance of microbial biomass markers (Fig. 3.6).

Table 3-2 ANOVA on the significance of chickpea genotypes effects on microbial phospholipid fatty acid (PLFA) markers and other soil-related variables measured in 2005 and 2006.

Soil-related variable	Growing season	df	Sum of Squares	F Ratio	*p*
14:0	**2005**	**3**	**72.551**	**8.647**	**0.005****
	2006	3	711.430	1.205	0.363^{ns}
i-13meth-15:0	**2005**	**3**	**74.681**	**4.644**	**0.032***
	2006	3	413.182	1.295	0.334^{ns}
2OH-14:0	**2005**	**3**	**240.660**	**3.890**	**0.049***
	2006	3	3364.526	2.325	0.143^{ns}
16:1ω5	**2005**	**3**	**15.205**	**4.092**	**0.044***
	2006	3	16.779	1.100	0.398^{ns}
i-15meth-17:0+	**2005**	**3**	**3.266**	**7.530**	**0.008****
17:0	2006	3	1.561	1.113	0.394^{ns}
Soil pH	2005	3	0.201	0.666	0.594^{ns}
	2006	**3**	**0.349**	**5.630**	**0.019***
Soil Moisture	**2005**	**3**	**65.242**	**7.603**	**0.008****
	2006	3	0.157	0.251	0.859^{ns}

NS: nonsignificant; * Significant at the 5% level; **Significant at the 1% level; ***Significant at the 0.1% level.

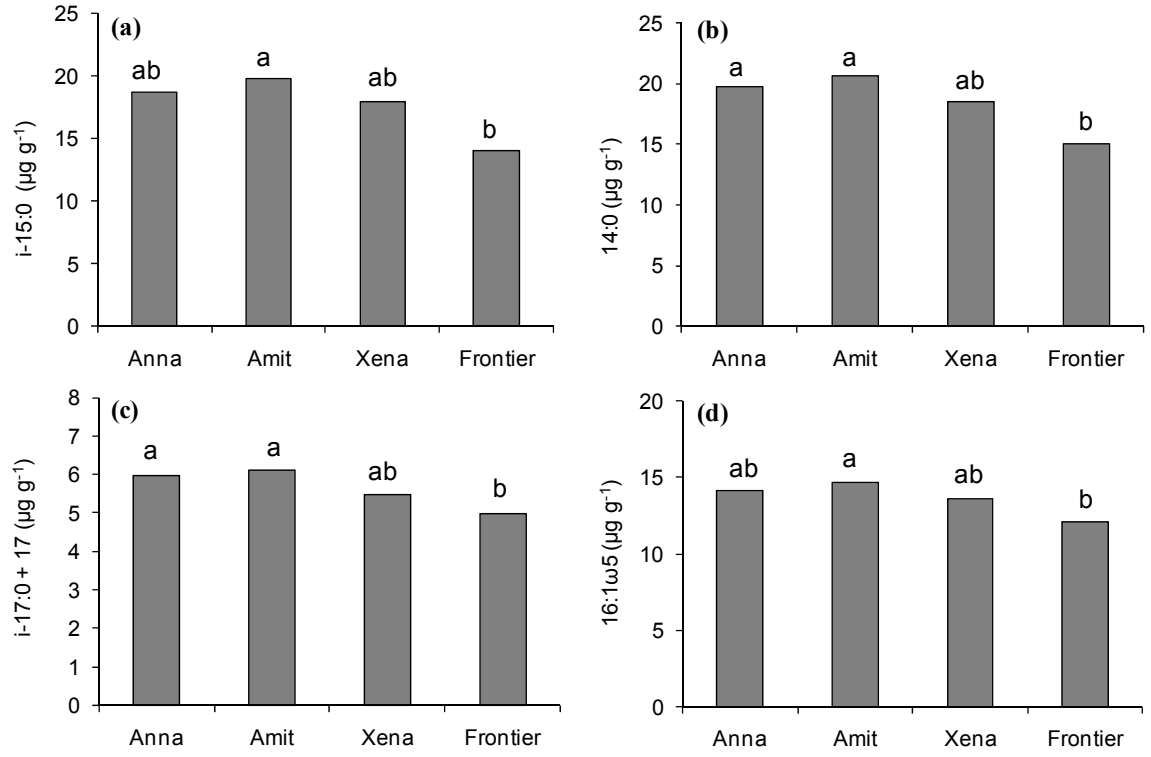

Figure 3-6 Chickpea genotype-induced variation in the biomass of soil microbial groups. Abundance of bacterial (A, B and C) and AMF phospholipid fatty acid (PLFA) markers (D) in the rooting soil associated with different chickpea genotypes, in 2005. Means are not significantly different when labelled with the same letter (*n*=4).

3.4.4 Soil pH and moisture percentage

The effects of chickpea genotypes on soil pH and moisture were measured at plant physiological maturity (Table 1). Genotypes CDC Anna and CDC Frontier reduced soil pH in 2006 (*P = 0.019*), but not in 2005 (*P = 0.594*) (Fig. 3.7a). Soil planted with genotype CDC Frontier had more moisture than other soils in 2005 (*P = 0.008*), but not in 2006 (*P = 0.859*) (Fig. 3.7b).

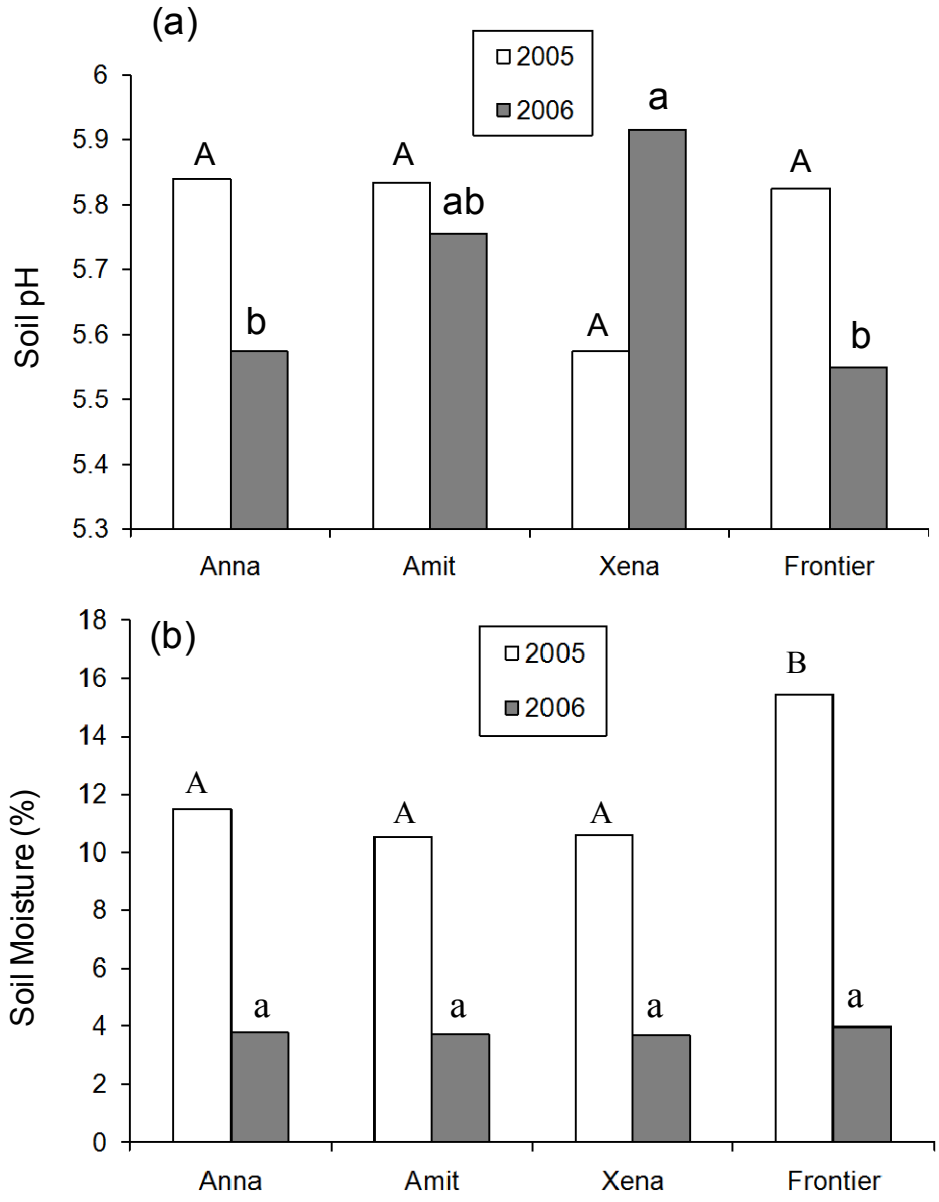

Figure 3-7 Chickpea genotype-induced variation in soil properties in 2005 and 2006. Soil pH (A) and soil moisture content (B) in field plots at plant physiological maturity. Means are not significantly different when labelled with the same letter within a year (n=4).

3.5 Discussion

3.5.1 Effect of chickpea genotype on AMF diversity

Chickpea genotypes had a significant effect on the AMF diversity in the rooting soil. The highest level of AMF diversity was found in the rooting soil of CDC Anna, a Desi type chickpea. AMF community in field associated with the roots of Kabuli genotypes was less diverse. As the large-seeded domestic Kabuli chickpeas originated from the small-seeded Desi chickpeas by selection and mutation (Moreno and Cubero, 1978; Singh, 1997), it is possible that the Kabuli type has lost genes regulating the formation of the AM symbiosis during evolutionary selection and became less dependent on mycorrhizal associations (Plenchette and Fortin, 2009). The examination of more Desi and Kabuli chickpea genotypes would be required to confirm this hypothesis.

3.5.2 Effect of chickpea genotype on mycorrhizal root colonization

Among Kabuli genotypes, the European variety Amit had the highest level of AMF diversity in this study. The genotype Amit has also had the best nodulation and N-fixation among chickpea genotypes (Gan and Liang, 2010) and the high percentage of mycorrhizal colonization (Fig. 3.3). The relationship between the super-nodulating character and improved ability to develop mycorrhiza was previously reported in leguminous species such as *Glycine max* (L.) Mirr., *Medicago truncatula, Pisum sativum* and *Lotus japonicus* (Morandi et al., 2000; Shrihari et al., 2000; Zakaria Solaiman et al., 2000). These observations suggest the presence of common factors influencing both the mycorrhizal and rhizobium symbioses in chickpea.

The root systems of CDC Frontier and CDC Xena are significantly larger than that of CDC Anna and Amit (Ellouze et al., 2006; Gan et al., 2010), which had higher percentage of mycorrhizal colonization in 2005. These observations support that genotypes with shorter root lengths are more dependent on a mycorrhiza than genotypes with longer root lengths (Baylis, 1970; Baylis, 1975; John, 1980; Koide and Li, 1991), and concur with

findings made on other plant species, such as Welsh onion *(Allium fistulosum* L) (Tawaraya et al., 1999), rye (*Secale cereale* L.) (Baon et al., 1994), white clover (*Trifolium repens* L) (Crush and Caradus, 1980) and pasture species (Schweiger et al., 1995). Root properties such as total length, branching, and rate of growth may affect the degree of mycorrhizal colonization. Plants with large root systems may invest more carbon in root development than in mycorrhiza formation (Apple et al., 2005; Brundrett, 2002; Gan et al., 2009a). Variation in mycorrhizal development and dependency was reported in other plant species (An et al., 2010).

The significant difference in the percentage of mycorrhizal colonization of chickpea roots between genotypes found in 2005 may be related to several factors: variation in precipitation, temperature, winter snow cover, pesticide use, etc. Clark et al. (2009) found a positive relationship between root colonization of two shrub species and cumulative precipitation. The variation in precipitation had no effect on the percentage of root colonization for the genotypes less dependent on mycorrhizal infection. The influence of environment in determining plant-microbial interaction is well recognized, but complex and hardly predictable.

Results do not support that genotype influence on soil pH is a determinant of AMF diversity. The highest and lowest species richness levels, found with CDC Anna and CDC Frontier respectively (Fig. 3.1) were both found at low soil pH, indicating that pH is not the main driver of AMF diversity, at least in the pH range found in this study. Soil pH had no effect on colonisation levels, which is in agreement with previous studies (Porter et al., 1987; Uhlmann et al., 2004; Wang et al., 1993) reporting the absence of soil pH effect on root colonisation despite marked effects of soil pH on AMF species composition.

3.5.3 Effect of chickpea genotype on soil microbial resources

Kabuli genotype CDC Frontier was associated with low AMF diversity, low biomass of bacteria and extraradical AMF, and was poorly colonized by AMF. The same tendency was shown in the case of Gram-negative and Gram-positive bacteria where CDC

Frontier was associated with a poor abundance of active bacterial biomass in soil as compared with Amit. These observations suggest that CDC Frontier has a poor ability to influence the AM fungal and bacterial communities of the soil. They may have poor root exudates composition and amount (Marschner et al., 2007) or they may support fungal communities antagonizing AMF and bacteria.

The influence of year on genotype effects on the soil pH, AM root colonization and microbial biomasses underlines the importance of the environmental influence on plant-microorganisms interactions under field conditions. In a previous experiment at the same location, Hamel et al. (2006) suggest that sudden events such as heavy rainfall on a dry soil may play an important role in the seasonal variation of the active soil microbial biomass. Since a high number of species may translate into the maintenance of a high number of ecological functions, crop genotypes maximizing soil microbial diversity should contribute to yield stability in agro-ecosystems.

3.5.4 Effect of chickpea genotype on culturable fungi

Chickpea genotype differentially influenced the culturable fungi. Highest biodiversity of culturable fungi was associated with Desi genotype CDC Anna, which concurs with the high AMF diversity found in the rooting soil of this genotype. Interestingly, the two most abundant culturable fungal isolates in CDC Anna rooting soil were *Bionectria ochroleuca* (anamorph: *Clonostachys rosea*, syn.: *Gliocladium roseum*) and *Trichoderma sp.*, taxa well known for their biocontrol activity (Cota et al., 2008; Wijesinghe et al., 2011). CDC Anna seems to favour the growth of beneficial microbial populations over that of pathogenic organisms. The low diversity index for culturable fungi observed with Kabuli chickpea Amit, was associated with good levels of AM fungal diversity, which suggests that this genotype may better associate with symbiotic fungi than free-living fungi.

3.5.5 Soil microbial diversity in dryland agriculture

The most abundant AM fungal ribotypes encountered belong to the orders Glomerales and Diversisporales, an information that confirms and extends previous finding (Jansa et al., 2002; Sjoberg et al., 2004). The genus *Diversispora* could be a dominant AM fungal taxon in cultivated soil of the Canadian Prairie. The order Paraglomerales was also well represented. All of the AMF found belong to genera morphologically very close to the genus *Glomus*. The dominance of the rapidly sporulating species of the genus *Glomus* and of genera morphologically similar to *Glomus*, in cultivated soils, could be explained by the selection pressure exerted by cropping practices. Hamel (1996) hypothesized that soil tillage would most likely favour the proliferation of more vigorous AM fungal species that can colonize new plants through spores, fragmented hyphal networks and infected root pieces and that have the ability to re-establish networks after mechanical disruption. This could explain the absence of slowly sporulating species that mainly use spores or intact mycelium to infect new plants as found in the Gigasporaceae family (Castillo et al., 2006; Daniell et al., 2001). In the present experiment, soil samples were taken at physiological maturity of chickpea plants (late summer) and the species that are active early in the growing season such as the genus *Acaulospora* (Hijri et al., 2006), might have been missed.

Despite intensive agricultural management practices such as repeated although very shallow (7.5 cm) tillage, fertilization and agrochemicals used in this dryland soil, our study revealed a high AM fungal diversity (15 different AM fungal ribotypes). This result is in agreement with previous work of Oehl et al. (2003), Franke-Snyder et al. (2001) and Hijri et al. (2006) who have shown that the diversity of AMF is not always low in arable soils as reported by Daniell et al. (2001) and Helgason et al. (1998). In the present study, we used the set of PCR primers designed by Lee et al. (2008) as they have an excellent coverage of all known AMF groups including the ill documented families *Paraglomaceae* and *Ambisporaceae* whose sequences were not amplifyed by previous primers. The region amplified using this set of primers is also relatively long (795 bp), which offers the possibility of a more reliable phylogenetic placement of some environmental AMF sequences.

Fungal isolation and molecular analysis revealed Ascomycetes as the most abundant culturable fungi (92%) in a typical Canadian chickpea-growing soil, which reflects the abundance of ascomycetes in Canadian dryland agriculture. Ascomycota is the largest fungal phylum. Its species occur in numerous ecological niches and virtually all terrestrial and aquatic ecosystems including some of the most extreme environments on earth. They may decompose organic substrates or act as mutualists, parasites, or pathogens (Schoch et al., 2009). Dark septate endophytes are Ascomycetes, mostly of the orders Hypocreales and Pleosporales. Several taxa identified in this study belong to the genera *Acremonium*, *Alternaria*, *Cladosporium*, *Fusarium*, *Lewia*, *Penicillium* and *Trichoderma* which are known to host root endophytes (Maciá-Vicente et al., 2008). This is consistent with previous works reporting that plant roots in semi-arid grassland were most abundantly colonised by DSE than by AMF (Barrow and Aaltonen, 2001). The diversity of dark septate endophytic fungal species appears to be important in Canadian Prairie dryland.

The analysis of fungal diversity in cultivated dryland reported here, shows that *Penicillium* and *Trichoderma*/*Hypocrea* were among most frequent saprophytic fungi, by contrast, *Penicillium* and *Aspergillus* are dominant in tropical forests soils (Saravanakumar and Kaviyarasan, 2010). *Fusarium*, was the pathogenic genus most frequenlty seen. Most *Fusarium* species are pathogenic to plants, and the diseases they cause are often more severe in dry than wet climate (Agrios, 2005).

3.6 Conclusion

Crop genotypes serve as an important factor in shaping soil fungal communities in chickpea fields. Selection of genotypes with favourable compatibility with AMF and other beneficial fungal endophytes is possible, as shown by the variation in the microbial assemblages associated with the different chickpea genotypes evaluated in this study. Growing chickpea with improved compatibility with beneficial fungi can be a good strategy to enhance soil nutrient use in the Canadian Prairie where the soil fungal resources appears to be rich. The quality of soil fungal resource is evidenced by the high dominance of Ascomyceteous and the large diversity of Glomeromycotan species, the two phyla well-

known to host several species able to increase the efficiency of nutrient use by plants and to improve plant tolerance to biotic and abiotic stresses.

3.7 Acknowledgements

The authors would like to gratefully acknowledge the excellent technical assistance of Keith Hanson, Cal McDonald, and Lee Poppy. We also thank Stéphane Daigle for his guidance in the statistical analyses. This project is supported by a grant from the Alberta Pulse Growers and AAFC to Hamel and Gan, a NSERC discovery grant to St-Arnaud and a scholarship to Ellouze from the Islamic Development Bank Merit Scholarship Programme for High Technology.

Chapitre 4: Comparative study of biologically active compounds present in the root extracts of different chickpea genotypes and their effect on arbuscular mycorrhizal fungal spores germination

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4.1 Abstract

Plant symbioses are regulated by the production of phytochemicals that are selectively perceived and trigger responses in symbiotic partners. We hypothesize that the roots of different chickpea genotypes produce different arrays of phytochemicals and that some of these phytochemicals influence the germination of arbuscular mycorrhizal (AM) fungal spores. The root extracts of five chickpea genotypes were first fractionated based on solubility in methanol and further fractionated by HPLC. The phytochemical profiles of root extracts varied with the chickpea genotype. These profiles shared similarity within a chickpea type, but the phytochemical profiles of Desi and Kabuli chickpea were very different. The HPLC fractions of the roots of chickpea cultivar CDC Anna were recovered and the effects of these fractions on AM fungal spore germination were assayed in multi-well plates. Root extract fractions either had no influence or repressed germination. Whereas, *Glomus etunicatum* spore germination was very sensitive to HPLC fractions soluble in 25% methanol, the spores of *Gigaspora rosea* were quite insensitive to these fractions. *Gigaspora rosea* spore germination was slightly more sensitive to HPLC fractions soluble in 50% methanol than *G. etunicatum* spores. The genetic variation observed in the production of bioactive phytochemicals by chickpea roots suggests the possibility to select chickpea genotypes for better compatibility with AM fungi. But the differential response of AM fungal species to chickpea phytochemicals suggests that the development of chickpea varieties with better AM symbioses would have to consider the AM fungal species residing in cultivated soils of a target region.

Key Words: Chickpea (*Cicer arietinum* L.), garbanzo bean, root extracts, biologically active compounds, plant genotype, arbuscular mycorrhizae, plant symbioses, plant breeding.

4.2 Introduction

The structure of rhizosphere communities is affected by plants through the release of attractants and repellents from their roots (Estabrook and Yoder, 1998). Plants modify their biological environment using a wide range of phytochemicals. The production of volatile phytochemicals by plants was well studied (Arimura et al., 2009) and led to practical applications in the field of insect pests monitoring and control. Rhizospheric interactions are shorter range than phyllospheric interactions and phytochemicals with restricted mobility are also effective in the regulation of the biological environment of a plant. For example, roots produce strigolactones that spontaneously hydrolyze in soil water; nevertheless, strigolactones are important signals molecules triggering branching in arbuscular mycorrhizal (AM) fungi and initiating symbiosis development (Parniske, 2008).

AM fungi form symbiotic association with the vast majority of land plants. They can provide their host plants with improved plant nutrient use efficiency, enhanced N_2-fixation in legumes, reduced plant disease incidence, increased plant tolerance to stress and improved soil physico-chemical quality (Jeffries et al., 2003).

Knowledge on the regulation of the AM symbiosis is accumulating. The initiation and the maintenance of the AM symbiosis appear to involve different regulatory mechanisms. Whereas the control of the AM fungi by the host plants in an established association involves hormonal regulation in processes similar to those involved in plant defence against pathogens (Vierheilig et al., 2008), the initiation of the symbiosis involves an exchange of signals between the plants and the fungi, and the recognition of these signals by the partners (López-Ráez et al., 2011; Parniske, 2008). In natural ecosystems, the fine balance between various plant and fungal metabolic pathways that is necessary for the creation of an effective symbiosis was achieved by a long history of natural selection, in natural ecosystems. Crop plants, which are often very different from their wild ancestor, are well nurtured and protected in agroecosystems, and depend little on the AM symbiosis. The imperfect regulation of the AM symbiosis by crop plants may have very little

consequences on crop yield, and crop plants may have a reduced ability to develop successful AM symbioses, in contrast to their wild relatives.

As other legumes, chickpea imports atmospheric N into cropping systems through its symbiosis with *Mesorhizobium ciceri*. Chickpea is an important component of wheat-based rotation but is only a fair rotation crop, leading to lower yield in following wheat crops, as compared to medic, vetch or lentil (Ryan et al., 2010). The negative effects of a chickpea plant on the following wheat crops could come from chickpea root exudates, their residues or their influence on the soil microbial community and, in particular, on the AM fungi. Furthermore, it was shown that the inhibitory effects of chickpea roots on a subsequent crops was dependent on the chickpea cultivar as well as on the genetic characteristics of the crop following in rotation (Chaichi and Edalati-Fard, 2005). In this study, we hypothesize that different chickpea cultivars produce root exudates differing in composition and that certain biologically active compounds present in these exudates influence AM fungal spores germination.

4.3 Materials and methods

4.3.1 Experimental design

Compounds with bioactivity on AM fungal spore germination were sought within the root extract of different chickpea genotypes. Five chickpea cultivars with contrasting phenotypes were selected for this study: Kabuli types CDC Frontier, CDC Xena and Amit, and Desi types CDC Anna and CDC Nika. Their roots were extracted, extracts were parsed based on solubility in methanol (MeOH), and materials soluble in 25% and 50% MeOH were further separated by HPLC. HPLC fractions from CDC Anna were recovered and assayed on *Glomus etunicatum* and *Gigaspora rosea*, for their effects on spore germination.

4.3.2 Plant materials and conditions

Approximately 400 g of chickpea roots of each cultivar were produced in large flats (122 cm x 122 cm x 15 cm in depth). Plants were grown in a sand: calcined clay (Professional Gardener co. Ltd., Calgary, AB) (1:1, v:v) mix containing a root and soil inoculum of the AM fungus *Glomus intraradices* Schenck & Smith (DAOM 181602). *G. intraradices* was multiplied on maize (*Zea mays* L.) in calcined clay. Maize stems were discarded, root were chopped in segment of about 1 cm and returned to the calcined clay. Approximately 10 L of this AM fungal inoculant was mixed in the growth substrate of each flat, using a cement mixer. There was about 250 chickpea plants m^{-2}.

Plants were grown in the greenhouse under a 24/15°C (±2°C) day/night temperature regime. Natural day light was supplemented with high intensity discharge lamp (Alto 400 watt low pressure sodium, Philips, Somerset, NJ) providing photosynthetically active radiation for 16 h day^{-1}. Plants were examined daily and watered as needed. They received weekly a modified Long Ashton nutrient solution (Hewitt, 1966) 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 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 32.9 NaFe-EDTA.

Plant roots were harvested six weeks after emergence, washed in water, spin dry, and frozen at -24°C.

4.3.3 Root extraction and extracts fractionation

The scheme of the root extraction/fractionation procedure is summarized in Figure 4.1. Approximately 400 g of roots from each cultivar were soaked three times in 80% MeOH solution for 24 h at room temperature. The extracts were concentrated in a rotary evaporator at 40°C and filtered before fractionation by flash chromatography on an octadecyl-silica 45 x 400 mm column through successive elution with 0%, 10%, 25%, 50%, 75% and 100% MeOH solutions. Fractions were concentrated in a rotary evaporator at 40°C. The 25% and 50% MeOH fractions were purified by autofocusing using a Rotofor (Bio-Rad) before separation by HPLC with a UV detector at 220 nm, and a C18 column.

The mobile phase consisted of a 10% ethanol solution with a flow rate of 1.2 mL min^{-1}. Ten peaks were produced by the 25% MeOH-soluble materials, and eleven by the 50% MeOH-soluble materials, on the HPLC (Fig. 4.2). These 21 HPLC fractions were recovered. The 25% MeOH-solubles HPLC fractions were evaporated and dissolved in 5 mL of 25% MeOH, and the 50% MeOH-soluble fractions were evaporated and dissolved in 10 mL of 50% MeOH.

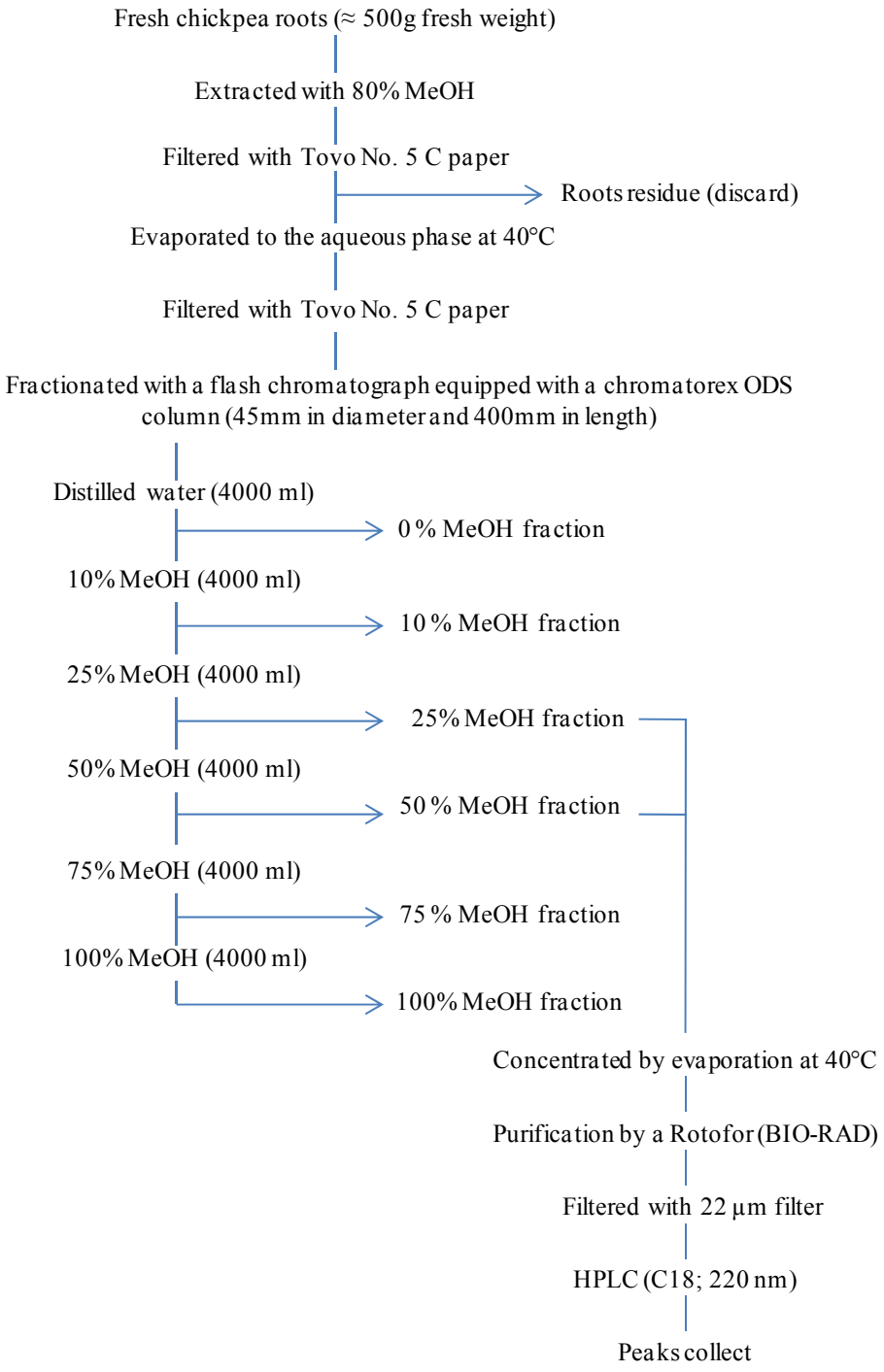

Figure 4-1 Flow chart for separation of compounds present in the root extracts of chickpea cultivars (Modified from Ishii et al., 1997).

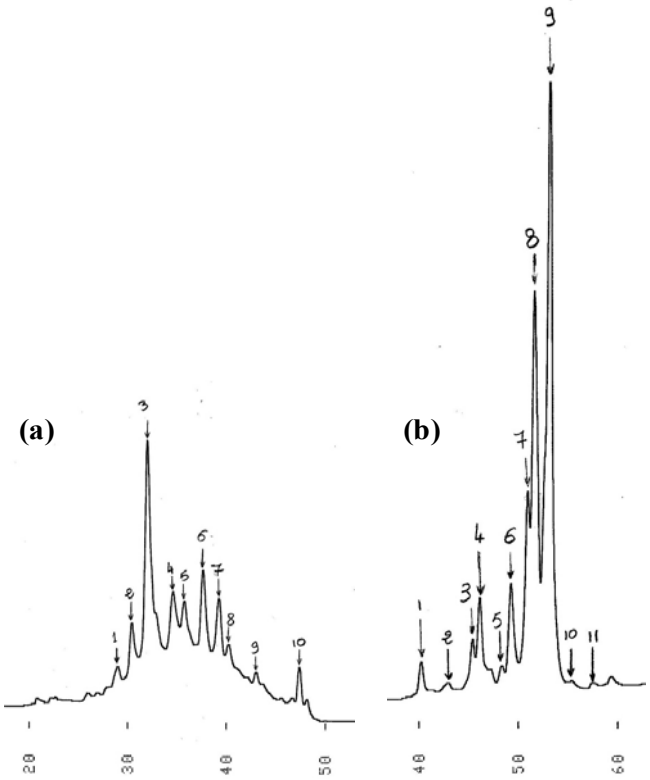

Figure 4-2 The arrows show the HPLC fractions collected (peaks) from the root extracts of Desi chickpea cultivar Anna that are soluble in (a) 25% methanol and (b) 50% methanol.

4.3.4 AM fungal spores germination bioassay

One hundred spores of AM fungi were exposed to three concentrations of each HPLC fraction (10, 50, or 100 mg fresh root equivalent mL^{-1}), of the 25% and 50% MeOH-soluble materials from CDC Anna. Treatments were replicated four times.

The germination assays were performed in sterile 8-well chambered coverglass (Lab-Tek) for *G. etunicatum* spores and in sterile 24-well culture plates for *Gigaspora rosea* spores. AM fungal spores were extracted from leek pot cultures by wet sieving (Gerdemann and Nicolson, 1963) followed by centrifugation in 60% sucrose (w:v) (Daniels and Skipper, 1982). Spores of *G. etunicatum* and *Gi. rosea* were surface sterilized for 12 and 15 min, respectively, using a solution made of 0.7 g chloramines T, 5.6 mg streptomycin and 2.1 mg chloramphenicol in 100 mL distilled water, with a few drops of

Tween 80 (Horii and Ishii, 2006; Yu et al., 2009). After sterilization, spores were rinsed seven times, re-suspended in sterilized distilled water and cold treated for four to six weeks at 4°C prior to use to improve germination (Juge et al., 2002).

Solutions were prepared by diluting volumes of the HPLC fractions to obtain the target concentrations of 10, 50, and 100 mg fresh root equivalent mL^{-1} in distilled water. Control stock solutions were prepared using identical amounts of 25% or 50% MeOH solutions. One hundred of surface sterilized AM fungal spores were placed in each well with 300 µL of a potentially bioactive or control solution, at the prescribed concentration. Spores of *G. etunicatum* were incubated in the dark at room temperature for 20 days, and spores of *Gi. rosea*, for 5 days. Spores were examined directly in the wells, using an inverted microscope (100 to 200X) and spores were considered to be germinated when germ tubes length was as long as the diameter of the spore or longer.

HPLC fractions assessment was made through a series of 12 essays, each testing the effects of one of the three rates of the HPLC fractions soluble in either 25% or 50% MeOH, on either *G. etunicatum* or *Gi. rosea.*

4.3.5 Statistical analysis

The significance of treatment effects on AM fungi spores germination compared to the controls were assessed by analysis of variance (ANOVA) using JMP 3.2.6 (SAS Institute, Cary, USA). A *P* value of 0.05 was used as threshold to accept the significance of effects, except when indicated otherwise. The significance of difference between treatment means was assessed by the least significant difference (LSD), where significant treatment effects were found. The data was tested for normality using Shapiro-Wilk's test and non-normal data was transformed prior to analysis, as required by the tests. A square root transformation was applied to the counts of *G. etunicatum* at the two higher rates of 25% MeOH-soluble HPLC fractions, and cosines transformation was applied to the counts of *Gi. rosea* at the lowest rate of the 50% MeOH-soluble fractions, to meet the requirement of normality before statistical analysis.

Principal component analysis (PCA) was carried out to compare the biochemical composition of the different chickpea root extracts, as described by their profile of HPLC peak areas. The areas under the peaks were measured using the image-processing software 'ImageJ' version 1.42 (Abramoff et al., 2004) and the percentage of every peak in each extract was determined. The data were Hellinger-transformed prior to PCA analysis (Legendre and Gallagher, 2001; Legendre and Legendre, 1998) using the R Project for Statistical Computing version 2.12.2 (R Development Core Team, 2011).

4.4 Results

4.4.1 HPLC analysis of root extracts

HPLC analyses of the 25% and 50% MeOH-soluble fractions revealed several differences between the chickpea cultivars root extract compositions (Fig. 4.3). Up to 14 and 13 different HPLC fractions respectively soluble in 25% and 50% MeOH were detected in the different chickpea genotypes (Fig. 4.4). The peaks identified as 5a, 5b, 10a and 10b in the 25% MeOH-soluble HPLC fractions (Fig. 4.4a) and those identified as 1a and 4a in the 50% MeOH-soluble HPLC fractions (Fig. 4.4b) were absent from the root extracts of the cultivar CDC Anna but present among root extracts of at least one of the other cultivars. The differences in the HPLC profiles of chickpea cultivars root extract (Fig. 4.3), suggest difference in bioactivity. Similarity in the composition of root extracts within each of the two chickpea classes, i.e. Desi and Kabuli, was revealed by the PCA analyses (Fig. 4.4). The Desi-types CDC Anna and CDC Nika were clustered separately from the three Kabuli cultivars Amit, CDC Xena and CDC Frontier (Fig. 4.4), suggesting a relationship between chickpea root phytochemicals production and genotype.

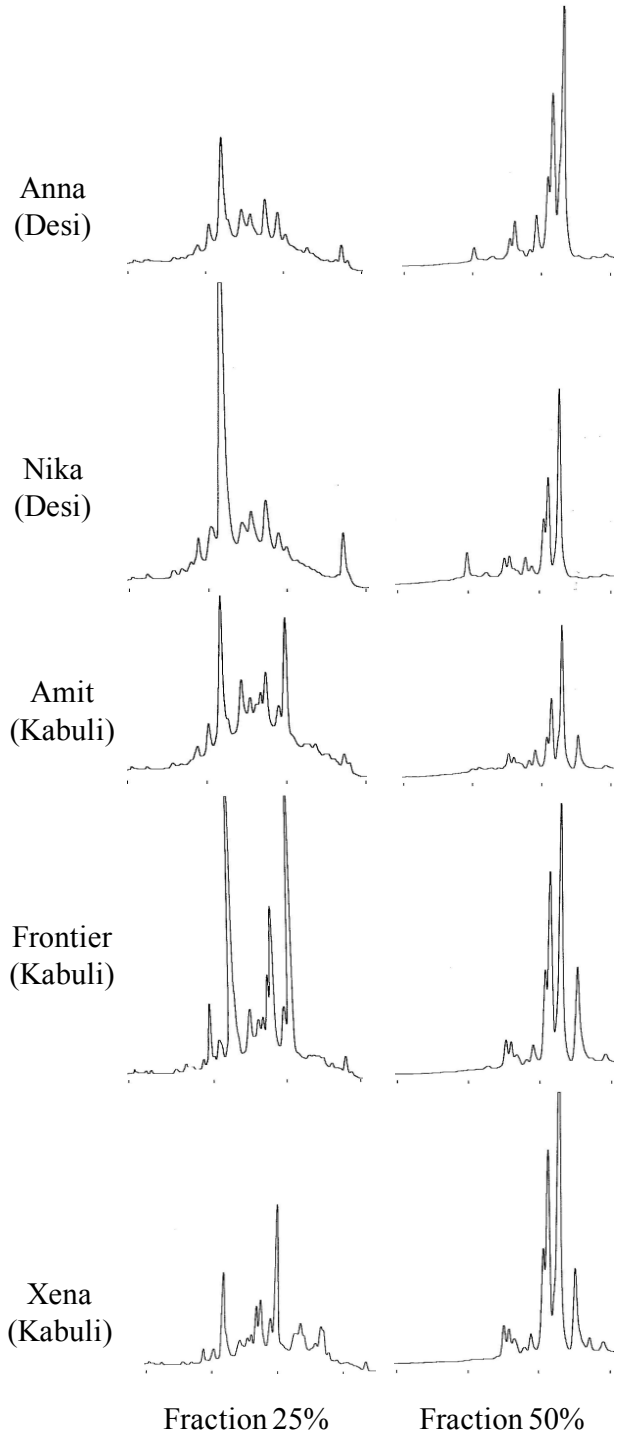

Figure 4-3 The HPLC chromatograms of the fractions of the root extracts of chickpea cultivars that are soluble in 25% and 50% methanol.

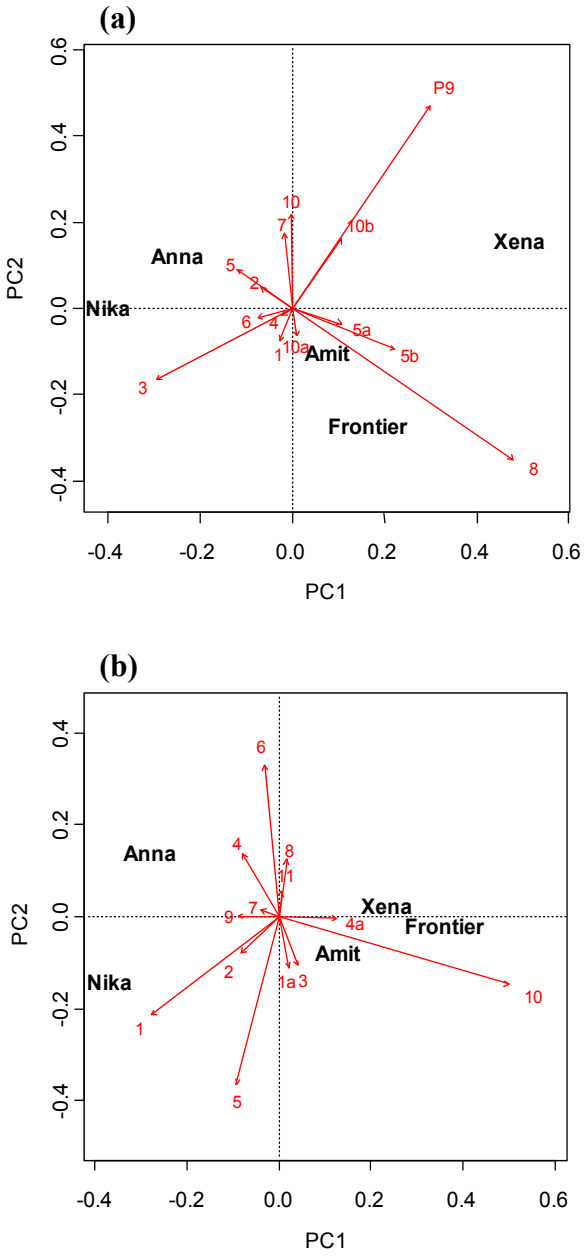

Figure 4-4 PCA biplots of the relationship between the composition of root extracts fractions soluble in (a) 25% and (b) 50% methanol, and chickpea cultivars. Numbers represent the peaks produced by HPLC analysis of the chickpea root extract, as shown in Fig.2.

4.4.2 AM fungal spores germination bioassay

The HPLC fractions assessed in the present study affected spore germination differently in *G. etunicatum* and *Gi. rosea*. The 25% MeOH-soluble HPLC fractions of chickpea root extracts corresponding to peaks 3, 6, 7, 9 and 10 (Fig. 4.2) significantly inhibited *G. etunicatum* spores germination at the low concentration (Fig. 4.5a), and those corresponding to the peaks 1, 2, 4, 5 and 8 significantly inhibit *G. etunicatum* spores germination at the medium rate of application. By contrast, the 25% MeOH-soluble HPLC fractions had little effect on the spore germination of *Gi. rosea* (Fig. 4.5b); only a few fractions inhibited the germination of its spores, when used at the highest concentration. Compounds contained in the 50% MeOH-soluble HPLC fractions of chickpea root extracts were more effective in repressing *Gi. rosea* spore germination. These fractions had a more similar effect on the germination of *G. etunicatum* and *Gi. rosea* spore than the fractions soluble in 25% MeOH, although three of them had a significant effect only on the spores of one species (Fig. 4.6).

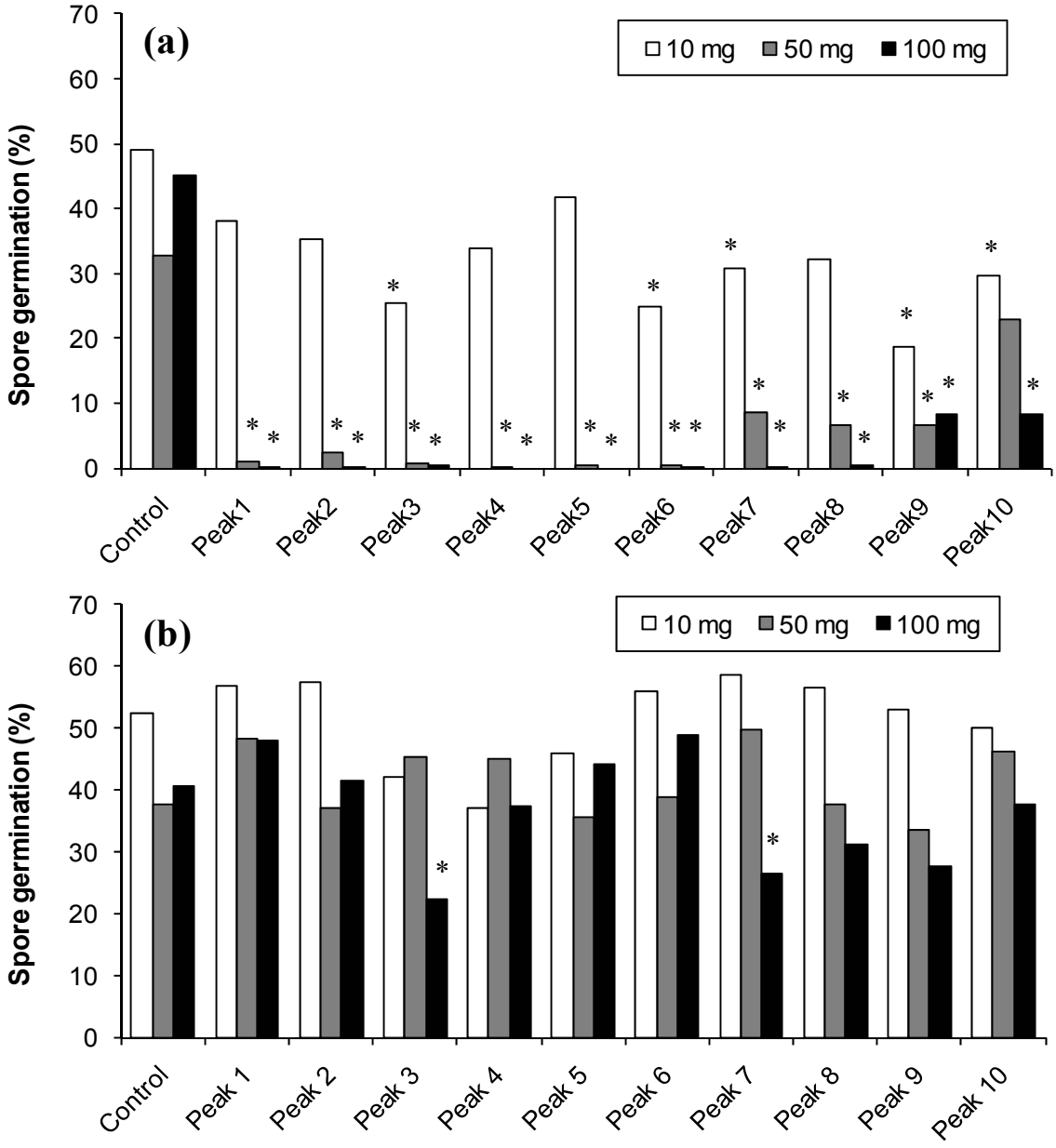

Figure 4-5 Percentage of inhibition of spore germination caused by compounds contained in the HPLC fractions of chickpea root extracts that are soluble in 25% MeOH, in two isolates of AM fungi (a) *G. etunicatum* and (b) *Gi. rosea*. Stars indicate a statistically significant difference from the control (Student's t test, $P < 0.05$).

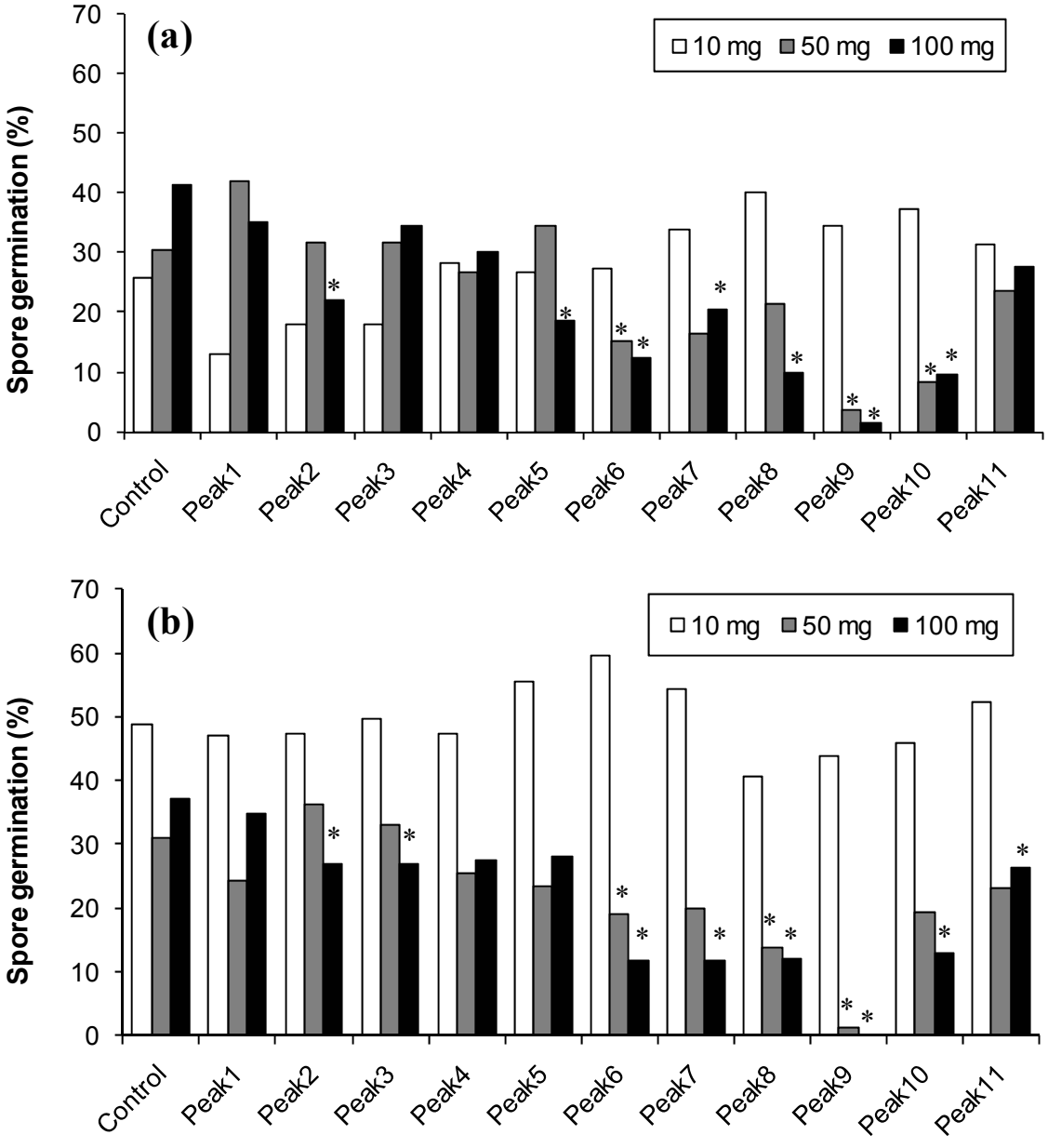

Figure 4-6 Percentage of inhibition of spore germination caused by compounds contained in the HPLC fractions of chickpea root extracts that are soluble in 50% MeOH, in two isolates of AM fungi (a) *G. etunicatum* and (b) *Gi. rosea*. Stars indicate a statistically significant difference from the control (Student's *t* test, $P < 0.05$).

4.5 Discussion

Genotypic differences in qualitative and quantitative composition of root exudates in crop and native plant species were reported for genotypes that differ in tolerance to nutrient deficiencies, ion toxicities, and pathogen attack (Rengel, 2002). The results presented here also show qualitative and quantitative variations in the HPLC profiles of chickpea cultivars root extracts soluble in either 25% or 50% MeOH, suggesting difference in the influence of these cultivars on rhizosphere organisms. A number of fractions were found in the roots extract of certain genotypes but were absent in the root extracts of others. The largest difference in root extracts was found between the two types of chickpea cultivars, Desi and Kabuli, indicating the existence of a wide genetic variation in phytochemical production in chickpea, and the possibility to select genotypes for the production of certain phytochemicals.

HPLC fractions repressed the germination of AM fungal spores or had no effect, in bioassays conducted in multi-well plates with extracts from the variety CDC Anna. This repression is the expression of the control of the plant on the AM fungal symbionts. These results are consistent with the evidence reported in previous study by Stevenson et al. (1995) where the amounts and composition of compounds exuded into the rhizosphere by *Fusarium* wilt-resistant and *Fusarium* wilt-susceptible chickpea genotypes were different. They found that wilt-resistant chickpea genotypes exuded an unidentified apolar ethyl-acetate-extractable compound, but the latter was absent in the root exudates of the wilt-susceptible chickpea genotypes. The apolar compound had antifungal properties and inhibited germination of *Fusarium oxysporum* f.sp. *ciceri* spores, thus conferring disease resistance to wilt-resistant chickpea genotypes. Similarities between mechanisms operating in the control of the plant on the AM fungal symbionts and those operating in the plant interactions with pathogens do point towards two effects of the same mechanism (Pozo et al., 2010; Vierheilig et al., 2008).

All HPLC fractions soluble in 25% MeOH repressed the germination of *G. etunicatum* spores, in bioassays conducted in multi-well plates with extracts from the

variety CDC Anna. The inhibitory effect of these fractions was shown to be concentration dependent. This result is consistent with an earlier study where Stevenson et al. (1994) found that chickpea resistant genotypes released the pterocarpans medicarpin and maackiain in their exudate at more than 10 times the concentration of that in the Fusarium wilt sensitive genotypes.

In contrast to the high susceptibility of *G. etunicatum* to root fractions soluble in 25% MeOH, only a few 25% MeOH soluble fractions had an effect on spore germination in *Gi. rosea,* when used at the highest concentration. Differential response of *G. etunicatum* and *Gi. rosea* to the 25% MeOH-soluble fractions suggests a genus specific effect of these compounds on spore germination, which is in agreement with previous results suggesting some degree of fungal host preferences (Gollotte et al., 2004; Scervino et al., 2005; Vandenkoornhuyse et al., 2002). A higher similarity of effects on *G. etunicatum* and *Gi. rosea* spore germination was observed with the compounds contained in the 50% MeOH-soluble HPLC fractions of chickpea root extracts, suggesting the non-specificity of this group of compounds.

The inhibition of AM fungal spore germination observed in the present work was previously reported by Chabot et al. (1992), who studied the effect of the flavonoid compounds biochanin A, genistein and hesperetin on spore germination of *Gigaspora margarita*. Tsai and Phillips (1991) also observed an inhibition of germination of *G. etunicatum* and *Glomus macrocarpum* spores in the presence of the isoflavone formononetin. Despite the repressive effect of some compounds on the germination of AM fungal spores found in the present and in earlier experiments, different effects may possibly be observed with the same compounds when tested on different AM fungal species or other fungal growth parameters such as hyphal length, hyphal branching and the formation of auxiliary cells and secondary spores (Scervino et al., 2005).

4.6 Conclusion

The differential influence of chickpea root extracts on the two AM fungal species used in this experiment suggests the presence of a high level of complexity in the regulation of the chickpea-AM fungi symbiosis. This differential influence also predicts difficulties in the selection of chickpea genotypes for improved compatibility with AM fungi, as this compatibility is also influenced by the identity of the AM fungal species making up the AM fungal community in any given soil.

4.7 Acknowledgements

The authors would like to gratefully acknowledge the excellent technical assistance of Keith Hanson and Mouna Fakhfakh. This project was supported by a grant from the Alberta Pulse Growers to Dr. Chantal Hamel and a scholarship to Mr. Walid Ellouze from the Islamic Development Bank Merit Scholarship Programme for High Technology.

Conclusion générale

Cette thèse avait pour objectif de développer les connaissances sur les interactions entre le pois chiche et son environnement biologique en vue de renforcer la capacité de cette plante à améliorer la qualité biologique du sol et la productivité des systèmes culturaux basés sur la production de blé dur.

Les résultats obtenus dans les expériences en serre nous ont permis de mieux comprendre la fonction écologique des champignons endophytes indigènes sur le pois chiche et la culture subséquente de blé dur, en zones arides. Ils montrent que l'inoculation par des champignons endophytes indigènes ascomycètes peut augmenter la tolérance à la sécheresse du cultivar de pois chiche de type kabuli à feuille simple CDC Xena, en condition de suffisance hydrique, et améliorer la nutrition azotée et phosphatée du pois chiche de type desi CDC Nika en conditions de stress hydrique. La réponse du pois chiche à l'inoculation par des champignons endophytes dépend des conditions environnementales, selon les résultats obtenus ici et avec ceux signalés dans plusieurs études antérieures qui montraient des effets positifs (Rodriguez et al., 2008), neutres (Jumpponen and Trappe, 1998) ou negatifs (Stoyke and Currah, 1993) de l'inoculation sur la performance des génotypes de différentes plantes. D'autre part, l'inoculation par des champignons endophytes a réduit l'effet allélopatique des résidus de culture de pois chiche de type kabuli sur la germination et l'émergence d'une culture subséquente de blé dur. La même tendance fut observée dans le cas de plants de fétuque rouge infectés par des champignons endophytes de classe II dont la stratégie de germination et de croissance pourrait être bénéfique pour survivre dans les conditions difficiles (Wäli et al., 2009). L'utilisation de champignons endophytes bénéfiques pour améliorer la production végétale et la tolérance des cultures à divers stress pourrait être un moyen de réduire la dépendance des cultures envers les intrants chimiques, d'accroître la production alimentaire et d'améliorer la durabilité des systèmes culturaux.

Au champ, les résultats obtenus nous ont permis d'améliorer nos connaissances sur les microorganismes du sol naturellement associés aux plantes de pois chiche cultivées dans la zone aride des Prairies canadiennes. Il montre que les cultivars de pois chiche ont influencé différemment la composition des communautés de champignons de la rhizosphère. Le cultivar de type desi CDC Anna fut associé à la diversité fungique contenant le moins d'espèces pathogènes et le plus d'espèces saprophytiques et mycorhiziennes arbusculaires. La communauté des CMA associée aux racines des génotypes de type kabuli en champ était moins diversifiée. Vu que le pois chiche kabuli a été sélectionné génétiquement à partir du desi (Moreno and Cubero, 1978; Singh, 1997), il est possible que le type kabuli ait perdu des gènes régulant la formation de la symbiose avec les CMA lors de la sélection évolutive et soit devenu moins dépendant des champignons mycorhiziens (Plenchette and Fortin, 2009).

Parmi les génotypes de kabuli étudiés, la variété européenne Amit a été associée à la diversité de CMA la plus importante. Le génotype Amit a également exhibé les niveaux de nodulation et de fixation d'azote atmosphérique les plus importants (Gan and Liang, 2010), et obtenu le pourcentage de colonisation mycorhizienne le plus élevé. La relation entre la capacité de former des nodules et la capacité de développer des mycorhizes a été précédemment rapportée dans le cas d'autres légumineuses telles que *Glycine max* (L.) Mirr., *Medicago truncatula, Pisum sativum* et *Lotus japonicus* (Morandi et al., 2000; Shrihari et al., 2000; Zakaria Solaiman et al., 2000). Ces observations suggèrent la présence de facteurs communs qui influencent la symbiose mycorhizienne et rhizobienne chez le pois chiche.

Malgré les pratiques agricoles intensives utilisées dans les zones arides des prairies canadiennes, notre étude a révélé une grande diversité de CMA dans ce sol. Ce résultat est en accord avec des travaux antérieurs de Oehl et al. (2003), Franke-Snyder et al. (2001) et Hijri et al. (2006), qui ont montré que la diversité des CMA n'est pas toujours faible dans les sols arables tel que rapporté par Daniell et al. (2001) and Helgason et al. (1998). D'autre part, les résultats montrent que les champignons cultivables les plus abondants dans les zones agricoles arides des prairies canadiennes sont des ascomycètes appartenant aux

genres *Acremonium*, *Alternaria*, *Cladosporium*, *Fusarium*, *Lewia***,** *Penicillium* et *Trichoderma* qui contiennent des champignons endophytes foncés à septations (Maciá-Vicente et al., 2008). Ceci est cohérent avec des travaux antérieurs indiquant que les racines des plantes dans les prairies semi-arides ont été plus souvent colonisées par des champignons endophytes foncés à septations que par des CMA (Barrow and Aaltonen, 2001). La diversité des champignons endophytes foncés à septations semble aussi être importante dans des zones arides des prairies canadiennes.

Dans le but de comprendre la relation entre la composition des extraits racinaires des différents cultivars de pois chiches et l'augmentation sélective des microorganismes bénéfiques, les extraits de racines de cinq génotypes de pois chiche ont été fractionnés en fonction de leur solubilité dans le méthanol et par HPLC. Les résultats montrent que les profils phytochimiques des extraits de racines varient avec le génotype de pois chiche. Cette variation génétique couplée à la variation dans la réponse à l'infection du pois chiche par les champignons endophytes d'une part et la variation des assemblages microbiens et les profils phytochimiques des extraits de racines associés aux différents génotypes de pois chiche d'autre part, suggère la possibilité de sélectionner des génotypes de pois chiche mieux adaptés à l'environnement biologique des sols. De tels génotypes possèderaient une meilleure compatibilité avec les champignons mycorrhiziens arbusculaires (CMA) et avec d'autres champignons endophytes bénéfiques. Les tests conduits avec des composés présents dans les deux fractions (25% et 50% MeOH) des extraits racinaires du cultivar de pois chiches CDC Anna, qui ont été isolés à l'aide du HPLC, ont montré que les espèces de CMA réagissent différemment à l'exposition à différents composés. Ceci suggère la présence d'un niveau élevé de complexité dans la régulation de la symbiose pois chiches-CMA et prédit également des difficultés dans la sélection de génotypes de pois chiche pour une meilleure compatibilité avec les CMA, car cette compatibilité est également influencée par l'identité des espèces constituant la communauté des CMA d'un sol donné.

Cultiver du pois chiches compatibles avec les champignons bénéfiques peut être une bonne stratégie pour améliorer l'utilisation des nutriments du sol dans les Prairies Canadiennes où les ressources fongiques du sol semblent être riches. La qualité des

ressources fongiques du sol est mise en évidence par la forte dominance des Ascomycètes et la grande diversité des espèces de Glomeromycètes, deux phyla reconnus pour leur abondance d'espèces bénéfiques qui accroîssent l'efficacité de l'utilisation des nutriments du sol par les plantes et améliorent leur tolérance aux stress biotiques et abiotiques.

Suite à ces travaux, les perspectives d'études de l'amélioration des interactions symbiotiques du pois chiche, qui nous permettront de renforcer les performances économiques et environnementales des systèmes culturaux basés sur le blé, sont largement ouvertes. L'obtention de nouveaux cultivars capables d'améliorer la qualité du sol ainsi que des bioproduits stimulant les micro-organismes bénéfiques du sol sont des exemples de nouvelles biotechnologies qui pourraient être développées à partir des connaissances acquises dans ce projet.

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