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Structure, variations temporelles et interactions biotiques du microbiote souterrain du canola (*B. napus L.*) dans les Prairies Canadiennes

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Structure, variations temporelles et interactions biotiques du microbiote souterrain du canola (*B. napus*) dans les prairies Canadiennes

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Résumé

Les plantes, par leurs racines, offrent une myriade de niches écologiques pour les microorganismes du sol, et ceux-ci la protègent contre les attaques parasitaires et les stress abiotiques, et favorisent son approvisionnement en nutriments et en eau. Cependant, dans le sol, la plante joue aussi un rôle important lorsqu'elle émet depuis ses racines des composés qui influencent la composition des communautés microbiennes dudit sol, ce combiné à un changement du pH du sol par la plante et son apport en matière organique ainsi qu'en oxygène. Ces composés influencent les membres du microbiote souterrain de la plante et donc indirectement la plante elle-même. Plus on a une diversité du couvert végétal, plus la diversité des microorganismes du sol va être élevée et inversement, plus un sol sera divers en matière de microbes plus les plantes qui y poussent tendent à être en bonne santé. Pour une plante en particulier, il n'est pas inhabituel de développer des relations spécifiques avec des microorganismes eux aussi spécifiques qui vont améliorer sa survie. Cependant, une plante peut vivre dans différents environnements et les sols sont divers, donc les plantes doivent s'adapter aux microbes qu'elles trouvent à proximité en sélectionnant les microbes les plus bénéfiques pour elles. Du coup, il est possible que quel que soit l'environnement dans lequel la plante pousse, quelques microbes soit si importants pour sa survie et son développement qu'on les retrouve toujours en association avec ladite plante. Ces microbes toujours en association avec une plante donnée constituent une unité théorique nommée core microbiote dans la littérature scientifique.

La gestion du microbiote des plantes cultivées pourrait améliorer la résistance au stress et la productivité des plantes cultivées et il est donc important d'en comprendre le fonctionnement. A ce jour, le microbiote souterrain des plantes demeure largement une « boîte noire » en raison de son incroyable complexité due à la diversité faramineuse des microorganismes qui le constituent.

Au cours de ma recherche doctorale, j'ai voulu participer à ouvrir encore un peu plus cette « boite noire » pour augmenter la connaissance du fonctionnement et de la structure du microbiote souterrain des plantes. Pour ce faire, j'ai utilisé le canola (*B. napus*) comme plante modèle. J'ai étudié le microbiote racinaire, tel qu'influencé par le niveau de diversification du système cultural, à l'aide d'un dispositif expérimental établi par Agriculture et Agroalimentaire Canada à cinq emplacements dans la prairie canadienne en 2008. Le canola, *B. napus* est une *Brassicaceae* économiquement importante, mais aussi intéressante en tant que plante modèle, car le canola est associé à des communautés microbiennes racinaire moins complexes que bien d'autres plantes, à cause de sa production de composés antimicrobiens. J'ai utilisé le séquençage d'amplicons, des analyses statistiques multivariées et l'analyse de réseau pour approcher cette complexité et: i) vérifier l'impact de la diversification du système de rotation cultural sur les communautés microbiennes souterraines du canola, ii) établir si un core microbiote fongique et bactérien existait bel et bien dans la rhizosphère du canola et le plein sol en culture de canola, iii) identifier de façon claire des espèces clef de voute interagissant intensivement dans les communautés fongiques, bactériennes, et mixtes, et finalement iv) évaluer la persistance des champignons mycorhiziens à arbuscules dans la rhizosphère du canola et le plein sol adjacent cette plante non-hôte, en systèmes culturaux basés sur le canola.

Mes résultats confirment que les communautés fongiques de la rhizosphère du canola et de son sol étaient influencées par la diversification des rotations de cultures, mais démontrent que les communautés bactériennes ne l'étaient pas. La rhizosphère du canola avait un core microbiote fongique variant avec les années, tandis que chez les bactéries, seulement des core espèces ont été identifiées. J'ai aussi relevé des interactions potentielles entre microbiote fongique et microbiote bactérien du canola et identifié des espèces clef de voute. Les fluctuations de l'abondance de ces

espèces pourraient alors faire varier celles de beaucoup d'autres microbes. *Bradyrhizobium* a été l'une de ces espèces. Mes résultats montrent aussi un maintien d'une communauté des champignons mycorhiziens à arbuscules chez le canola même après 10 ans de monoculture.

En résumé, ma recherche apporte une lumière nouvelle dans l'étude du fonctionnement, de la structure et des dynamiques écologiques au sein du microbiote souterrain du canola et sur l'écologie microbienne théorique des plantes notamment en ce qui a trait à ses composantes invariantes telles que le core microbiote et les taxons clef de voûte. Des études en conditions contrôlées sont nécessaires pour vérifier la capacité des microbes clef de voute rapportés ici à influencer les communautés microbiennes du sol et les plantes qui y vivent.

Mots-clés: Champignons, Bactéries, Séquençage d'amplicons, Bio-informatique, Analyse de réseau, Ecologie microbienne, Plante, Canola, Mycorhizes à arbuscules.

Abstract

Plants and soil microbes are closely linked. Plants provides myriads of ecological niches in and on its roots for microbes to thrive. In turn, microbes can protect host plants against pathogen attacks, abiotic stresses, and improve nutrient and water availability. In the distant soil, plant produce volatile compounds shaping microbial communities, with feedback on root-associated communities. The more diversity there is in the plant cover, the higher the diversity of soil microorganisms will be and conversely, the more diverse a soil will be in terms of microbes, the more de plants that grow there trend to be in good health. Certain plants can develop specific relationships with certain microbes improving the fitness of the plant. However, a plant can grow in different environments and soils are diverse, thus plant will have to adapt to the different microbes depending on the environment it is growing in while attracting the ones necessary for its growth. Certain microbes could be so important for a plant's health and development that they are always associated with the plant. Such important microbes form a theoretical group called core microbiota that could be extremely important for plant health and a determinant of the composition of plant-associated microbial communities. The plant subterranean microbiota is often labelled as a "black box" due to the tremendous diversity and interactivity of the microbial communities plants host.

In my thesis research I aimed to "crack the black box" a little further to enhance our understanding of plant subterranean microbial community dynamics and structure. To do so, I used a field experiment established in 2008 by Agriculture and Agri-Food Canada (AAFC) at five different sites in the Canadian Prairies under different crop rotations and canola as model plant. Canola (*B. napus*) is a crop plants of the Brassicaceae family that produces antimicrobial compounds and has "simpler" microbial community in its roots, and rhizosphere. To do so, I used

amplicon sequencing, multivariate analysis, and network analysis. My objectives were i) to verify the impacts of plant cover diversification on canola microbial subterranean community, ii) to verify if a core microbiota of fungi and bacteria could exist in canola rhizosphere and bulk soil and if so, to describe this core, iii) to identify keystone bacteria and fungi, i.e. highly interacting components, in the bacterial and fungal communities associated with canola, and finally, iv) to investigate the persistence of arbuscular mycorrhizal fungi in the rhizosphere and bulk soil of canola, a non-host plant, in canola-based cropping systems.

I found that the diversification of cropping systems influenced the structure of the fungal communities of canola rhizosphere and bulk soil, but diversification had no significant influence on bacterial community structure. A fungal core microbiota varying through years was found in canola rhizosphere, but no bacterial core-microbiota was found. However, we were able to identify a core-specie. Interactions among the fungal and bacterial microbiota in canola rhizosphere and bulk soil were found and *Bradyrhizobium* was among several potentially important keystone taxa. My results also show the maintenance of arbuscular mycorrhizal fungi in canola even after 10 years of monoculture despite this plant is not a host for AMF.

Overall, my PhD research brings a new level of knowledge on the microbial structure and dynamics of canola subterranean microbiota, and also on the theoretical ecology of plant microbiota, particularly regarding its invariable components such as core microbiota and hub-taxa. Further investigations are needed to better understand how keystone species and core species influence the plants and their microbiome.

Keywords: Fungi, Bacteria, Amplicon Sequencing, Bioinformatic, Network analysis, Microbial ecology, plant, canola, Arbuscular Mycorrhiza.

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PGPB: Plant Growth-Promoting Bacteria

AMF: Arbuscular Mycorrhizal Fungi

OTU: Operational Taxonomic Unit

ZOTU: Zero-radius Operational Taxonomic Unit

LSA: Local Similarity Analysis

Lasso: Least Absolute Shrinkage and Selection Operator

gLasso: Graphical Least Absolute Shrinkage and Selection Operator

SPIEC-EASI: Sparse Inverse Covariance and Estimation for Ecological Association Inference

LL: Liberty Link

RR: Roundup Ready

DNA: Desoxyribonucleic Acid

PCR: Polymerase Chain Reaction

RNA: Ribonucleic Acid

PEMANOVA: Permutational Analysis of Variance

ANOVA: Analysis of Variance

MRPP: Multiresponse Permutation Procedure

BMRPP: Blocked Multiresponse Permutation Procedure

NCBI: National Center for Biotechnology Information

DSE: Dark Septate Endophyte

ABIP: Agricultural Bioproducts and Innovation Program

PCARP: Prairie Canola Agronomic Research Program

AAFC: Agriculture and Agrifood Canada

ASV: Amplicon Sequence Variant

FASV: Fungal Amplicon Sequence Variant

BASV: Bacterial Amplicon Sequence Variant

AMF ASV: Arbuscular Mycorrhizal Fungi Amplicon Sequence Variant

NGS: Next Generation Sequencing

BLAST: Basic Local Alignment Research Tool

IndVal: Indicator Values

QC: Québec

SK: Saskatchewan

AL: Alberta

ITS; Internal Transcribed Spacer

Je dédie cette thèse à mes parents et mes camarades qui m'ont soutenu chaque instant malgré les milliers de kilomètres qui nous ont séparés. Enfin je dédie ma thèse à vous lecteur et à tout être humain car le savoir ne saurait être la propriété de quiconque, car l'humanité ne devrait se reconnaitre ni dieu ni maitre et qu'en fin de compte, la propriété c'est le vol.

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Chapitre 1 – Introduction générale

La rhizosphère, son microbiome et la plante

Une plante coexiste avec un très large spectre d'espèces microbiennes dans son environnement naturel (bactéries, champignons, archées, protistes, nématodes, virus). Au sein de ce dernier, la rhizosphère a un rôle tout particulier. En effet, c'est l'endroit où la plante agit sur le sol et sur les communautés microbiennes particulièrement par le biais de ses exsudats racinaires (Bais et al., 2006; Vives-Peris et al., 2020). Cette exsudation dans la rhizosphère permet à la plante de recruter des organismes microbiens, pour son bénéfice (Tkacz et al., 2015a; Pii et al., 2016; Eisenhauer et al., 2017; Zhang et al., 2019). Au sein de la rhizosphère, la plante va pouvoir trouver des organismes mutualistes avec lesquels développer des symbioses, comme avec les bactéries promotrices de croissance (PGPB) ou encore les champignons mycorhiziens. La plante et son microbiome rhizosphérique peuvent être considérés comme un méta-organisme (Berendsen et al., 2012; Hassani et al., 2018; Baedke et al., 2020). Les microbes mutualistes vont permettre à la plante d'accéder plus facilement aux nutriments du sol, mitiger les effets des stress abiotiques comme la sécheresse ou encore prévenir l'infection des racines de leur hôte par des agents pathogènes (St-Arnaud and Vujanovic, 2007; Barriuso et al., 2008; Farina et al., 2012; Fincheira and Quiroz, 2018). Et d'autre part, la plante va subvenir à leur besoin en photosynthétats, par la production d'exsudats racinaires sur lesquels se nourrir ou encore par l'acheminement de sucres et d'acides aminés directement à ses micro-symbiotes (Bonfante, 2003; Trépanier et al., 2005; Begum et al., 2019; Goddard et al., 2021). C'est le cas pour les mycorhizes à arbuscules mais aussi pour d'autres symbioses microbiennes, comme les bactéries endosymbiotiques comme les Rhizobia (Ledermann et al., 2021). Cette dynamique prenant place au sein d'un écosystème extrêmement diversifié, la

rhizosphère est la place d'une intense compétition microbienne pour les ressources du sol et de la plante, ce qui donne alors lieu à de multiples interactions aussi bien symbiotiques que commensales ou parasitaires au sein de la communauté microbienne mais aussi avec la plante (Artursson et al., 2006; Bais et al., 2006; Boby et al., 2008; Handakumbura et al., 2021).

Au sein de cette communauté très dynamique, deux groupes d'organismes particuliers jouent un grand rôle dans la protection et la nutrition de la plante : les bactéries d'une part, et les champignons mycorhiziens d'autre part. Certaines bactéries sont PGPB et sont souvent trouvées à la surface des racines, à l'interface entre la rhizosphère et la plante, elles peuvent aider la plante dans sa nutrition minérale et augmenter sa croissance par le biais de la fixation d'azote ou la solubilisation du phosphate en exsudant des acides organiques (Barriuso et al., 2008; Ghorchiani et al., 2018). Ces bactéries peuvent aussi influencer la production d'hormones de la plante par l'émission d'auxine, de cytokinine, ainsi que d'autres substances volatiles et repousser d'éventuels organismes pathogènes en agissant comme des antagonistes ou en induisant des résistances chez la plante (Palaniappan et al., 2010; Vacheron et al., 2013). Les champignons du sol, et plus particulièrement les champignons mycorhiziens ont aussi une influence très importante sur la plante et sur les communautés des autres microorganismes du sol. On estime que 80% du règne des plantes réalise des symbioses mycorhiziennes (Smith and Read, 2008). Les deux catégories de mycorhizes les plus importante sont les endomycorhizes, notamment représentés par les mycorhizes à arbuscule (AMF) et d'un autre côté, les ectomycorhizes. Les endomycorhizes sont des symbiotes qui rentrent dans l'espace intra-cellulaire de la racine de leur hôte pour créer des structures d'échanges nommées arbuscules ou pelotons (Smith and Read, 2008) qui lui permettent d'accéder aux sucres et aux photosynthétats produits par la plante pour les échanger contre des minéraux tout en augmentant l'approvisionnement de la plante en eau. L'autre catégorie

d'association mycorhizienne la plus répandue est l'ectomycorhize. Cette dernière constitue un manchon autour de la racine de la plante hôte et ne pénètre que dans le milieu intercellulaire de la racine. Les ectomycorhizes ont des fonctions similaires aux endomycorhizes. Cependant ces symbioses sont relativement peu abondantes dans les champs cultivées et se sont préférentiellement développées avec les espèces ligneuses (Smith and Read, 2008). Les mycorhizes sont connus pour leur influence toute particulière sur les communautés bactériennes de la rhizosphère. Elles sont entourées de leur propre microbiome bactérien (Bianciotto et al., 2003; Iffis et al., 2014, 2017) dont des bactéries facilitatrices de symbioses (Fitter and Garbaye, 1994; Iffis et al., 2014; Taktek et al., 2017; Sangwan et al., 2021) et leurs hyphes agissent comme des autoroutes de la rhizosphère en permettant aux bactéries de circuler d'un endroit à l'autre du sol par l'interface labile de leurs hyphes (Nazir et al., 2017). Ils transportent aussi vers la plante hôte les nutriments mobilisés par les champignons ainsi que les bactéries, ces dernières peuvent fixer l'azote de l'air, et comme les champignons solubiliser le phosphore ou minéraliser la matière organique du sol.

La rhizosphère et son microbiome sont intimement liés à la vie et au développement de la plante. Cette dernière est aussi particulièrement influencée par les dynamiques microbiennes qui s'y déroulent. C'est pourquoi comprendre le fonctionnement et les dynamiques de son microbiome est très important. En manipulant le microbiome rhizosphérique, on peut protéger et aider la plante à croître et maintenir ou augmenter sa productivité tout en diminuant l'ajout d'intrants parfois polluants ou difficilement renouvelables.

Le sol agricole

Au cours du 20eme siècle, l'évolution des systèmes et pratiques agricoles a conduit à une mécanisation des moyens de production et à l'utilisation massive d'intrants tels que les fertilisants

et pesticides (Lal 2009). La généralisation de ces moyens à la majorité de la production agricole a permis de multiplier les rendements et de renforcer la sécurité alimentaire (Godfray *et al.*, 2010).

Le fait que la plus grande partie des fertilisants utilisés ne soient pas absorbés par les plantes cultivées conduit à un déséquilibre chimique des sols, ce qui se traduit par des phénomènes de salinisation ou encore de saturation du sol en nitrates et en phosphore (Mosier *et al.*, 1998). Ce déséquilibre se répercute aussi sur d'autres systèmes tels que les nappes phréatiques, ou les systèmes fluviaux entrainant des eutrophisations et menaçant l'équilibre de nombreux écosystèmes (Carpenter *et al.*, 1998). L'injection de grandes quantités d'engrais azoté dans le cycle de l'azote stimule les bactéries dénitrificatrices et crée une production accrue de N_2O, un gaz à effet de serre.

L'agriculture conventionnelle est liée à une baisse significative de la biodiversité dans les agrosystèmes (Phalan *et al.*, 2011, Baudron & Giller, 2014). La manière de gérer le vivant dans les agrosystèmes est souvent focalisé sur un petit nombre d'espèces de plantes (souvent une seule en monoculture) et sur la maitrise de phénomènes abiotiques locaux, cependant, pour que les systèmes aient de la résilience face aux perturbations, il est nécessaire de favoriser une importante biodiversité (Tscharntke *et al.*, 2005). C'est notamment le cas en ce qui concerne la flore microbienne ; le labour et l'épandage de molécules à des fins de contrôle des pathogènes ou encore d'engrais ont une influence sur la diversité en espèces microbiennes des sols cultivés (Lupwayi et al., 2007; Souza et al., 2013; Wang et al., 2020). En modifiant la structure et la composition des communautés de microorganismes, on agit alors sur les interactions qui s'y déroulent.

Or, ce sont ces interactions et cette diversité en microorganismes qui favorisent le développement des plantes. Une diversité fournie en microorganismes dans la rhizosphère induit

de meilleures performances chez les plantes cultivées (van der Heijden *et al.*, 2006, van der Heijden & Hartmann, 2016).

Cette communauté microbienne est aussi très importante en matière de recyclage des nutriments, notamment en ce qui concerne l'azote (N) mais aussi le phosphore (P). Le cycle de l'azote se déroule en partie dans le sol. Cet élément y est intégré par le phénomène de fixation qui converti le diazote sous une forme assimilable par les plantes à savoir NH_4^+ et NO_3^-. Cependant durant ce cycle, la molécule NO_3^- est utilisée par de nombreux microorganismes et est converti en azote N_2 mais aussi protoxyde d'azote N_2O qui est un gaz à effet de serre important (Mosier *et al.*, 1998, Galloway *et al.*, 2008). Ainsi l'importance de l'amendement azoté des agrosystèmes qui est excédentaire perturbe le cycle de l'azote ; par le lessivage de l'azote et une dénitrification accrue, ces pratiques mettent en péril les écosystèmes non culturaux (Carpenter et al. 1998). Il devient alors nécessaire de réduire l'apport en fertilisants des agrosystèmes tout en augmentant la capacité des plantes et des microorganismes à les assimiler, et donc d'acquérir une connaissance plus fine des mécanismes de l'écosystème « sol ».

Le sol est un écosystème complexe comportant un très grand nombre de niches écologiques (Insam, 2001). Cette diversité de niches s'explique notamment par son hétérogénéité en ressource et dans sa structure : le sol est composé de phases aussi bien solides que liquides ou encore gazeuses (Rampazzo *et al.*, 1998). Cette multiplicité de conditions environnementales favorise la diversité biologique qui doit alors s'adapter pour pouvoir exploiter les ressources du milieu.

Le sol est aussi très fortement structuré et modelé par les plantes. Ces dernières, par leurs racines, et leur cycle de vie changent la composition des sols ainsi que leur structure. Cela peut se traduire par un phénomène de succession écologique qui aboutit à l'augmentation progressive de

la matière organique dans le sol et la création d'une couche humus (Thomas & Kevan, 1993, Frouz *et al.*, 2008, Foote & Grogan, 2010), mais aussi par des modifications chimiques induites par les exsudats racinaires destinés à capter des ions pour la plante ou encore à attirer des microorganismes spécifiques par chimiotactisme : la plante va diffuser des composés chimiques à l'extérieur de ses racines tels que des auxines par exemple, ce afin d'attirer des espèces microbiennes qui vont les détecter et se déplacer dans le sol en suivant les gradients de concentration de ces composés.

Le microbiome d'une plante et sa part invariante

Les microorganismes fongiques ou bactériens de la rhizosphère, dont le développement est facilité par la plante, peuvent occuper la même niche écologique que certains pathogènes. Ces microorganismes forment une cohorte dynamique d'organismes. Au fil des saisons, la communauté microbienne de la rhizosphère change (Dunfield et al., 2003; Larkin et al., 2006; Donn et al., 2015; Mendes et al., 2015; Liang et al., 2019), de nouvelles espèces remplaçant les anciennes, tout cela formant une dynamique profondément liée à la vie de la plante. Une partie des fonctions de cette communauté est inféodée au système racinaire et va se retrouver tout au long de la vie de la plante (Vandenkoornhuyse *et al.*, 2015). L'ensemble de ces organismes durablement adaptés à la rhizosphère forme une communauté taxonomique et fonctionnelle que l'on appelle un microbiome (Agler *et al.*, 2016, Berg *et al.*, 2016).

Deux termes sont utilisés afin de définir les ensembles microbiens. Le microbiote, qui désigne l'ensemble des espèces taxonomiques présentes dans un environnement donné et le microbiome qui lui considère l'ensemble des gènes que possèdent les microbes dans l'environnement étudié. Il est toutefois à noter qu'en anglais, le terme microbiome est souvent utilisé pour désigner l'une ou l'autre de ces ensembles. Le terme microbiome apparait dans la

littérature en 2002 dans le cadre de l'étude de la diversité microbienne du système digestif humain (Shanahan, 2002). Cependant, cette notion est applicable à tous les environnements hébergeant des communautés microbiennes, notamment chez la plante. Face à la grande complexité du microbiome des plantes et le fait que ce dernier varie grandement en fonction des environnements, Vandenkoornhuyse et al. (2015) ont divisé la structure d'un microbiote en sous-unités théoriques. L'une d'entre elles est importante en particulier : le core microbiote. Ce dernier est la part invariante des espèces microbiennes inféodées à la plante quelles que soient les conditions environnementales à un temps d'observation *t*. Cet ensemble microbien est invariable et s'il se retrouve systématiquement chez la plante c'est probablement qu'il est favorisé par cette dernière et joue un rôle potentiellement important sur la plante elle-même mais aussi sur l'ensemble de la communauté microbienne (Rout, 2014). Cette définition du core microbiote était appropriée à l'ensemble des études ne comportant qu'un seul échantillonnage à un temps *t* spécifique. Cependant Floc'h et al. (2020) ont souligné l'importance de la subsistance d'une telle structure se maintenant à un temps t+n, ajoutant à la stabilité spatiale, l'importance de la stabilité temporelle dans la définition du core microbiote chez la plante.

Les interactions au sein de la rhizosphère

Au sein du microbiome, les microbes interagissent. En effet, le microbiome des plantes est, comme tout écosystème, un site d'interactions entre les espèces vivantes qui s'y trouvent. Ce consortium d'espèces est particulièrement important pour la plante et agit sur son développement et sa productivité (Smith et al., 1999; Barriuso et al., 2008; Bulgarelli et al., 2013; Bakker et al., 2014). Les interactions microbes-microbes et microbes-plante sont diverses. Si la plante établi des symbioses avec des champignons mycorhiziens ou des bactéries promotrices de croissance (Smith and Read, 2008; Pereira et al., 2019), elle fait aussi face à des organismes pathogènes

(Hajishengallis, Darveau, et Curtis 2012) et à des commensaux. Ce tissu d'interaction est hautement complexe, à l'image de la diversité des espèces qui constituent le microbiote de la plante. On sait par exemple depuis quelques années que les champignons mycorhiziens disposent de leur propre cortège microbien s'organisant parfois en bio-films à la surface des hyphes de manière similaire à ce que l'on peut observer dans la rhizosphère des plantes. En effet, ceux-ci s'entourent de bactéries facilitatrices de symbioses, et sont aussi eux-mêmes en symbiose avec des bactéries qui aident leur nutrition, métabolisme et développement (Garbaye, 1994; Desiro et al., 2014; Iffis et al., 2014; Taktek et al., 2015). De plus, dans le sol, de nombreuses bactéries partagent des micro-habitats où elles interagissent. C'est par exemple le cas de l'hyphosphère des champignons mycorhiziens (Frey-Klett et al., 2011). Le champignon les nourrit par ses exsudats, comme le fait la plante avec ses racines (Agler et al., 2016; Bainard et al., 2016; Hover et al., 2016). Les champignons filamenteux du sol, et notamment les champignons mycorhiziens jouent aussi un rôle dans la mobilité des bactéries dans le sol. Nazir et al. (2017) ont montré que les hyphes pouvaient servir comme un vecteur de transfert bactérien entre deux sources de nutriment différentes. Ces « autoroutes microbiennes » peuvent être empruntées par les bactéries en utilisant l'interface aqueuse existant entre la surface de l'hyphe et le sol. Mais le potentiel d'interaction au niveau du microbiote des plantes ne se résume pas aux interactions décrites précédemment. En effet, la capacité fonctionnelle du microbiome racinaire n'est pas égale à la somme des espèces qui le composent car les microbes interagissent très fréquemment entre eux et forment un réseau complexe de coéquipiers et de compétiteurs. Des myriades d'espèces peuvent intervenir dans le processus de dégradation d'un arbre ou encore dans le cycle de l'azote. Les réseaux d'interactions microbiens sont constitués de milliers d'espèces interdépendantes liées par des relations mutualistes, commensales ou parasitaires. Et ces interactions ont la possibilité d'influencer la capacité de survie de chacun des maillons du réseau, avec des conséquences directes sur la fertilité

du sol et la santé des plantes (Chaffron et al., 2010; Abreu and Taga, 2016; Agler et al., 2016). De fait, l'étude des écosystèmes microbiens fait face à un problème de taille : comment approcher la complexité inhérente aux microbiotes en le rendant compréhensible par un cerveau humain ?

L'accès à l'information microbienne a été augmentée de manière spectaculaire au cours de la dernière décennie par la mise au point des deuxièmes et troisièmes génération de techniques de séquençage (Schadt et al., 2010; Liu et al., 2012; Knief, 2014). Ce bond technologique a permis des analyses plus fines des mécanismes régissant les communautés microbiennes relatives aux plantes, notamment en matière d'expression de gènes (Duffy et al., 2007; Bulgarelli et al., 2013; Mendes et al., 2015) permettant alors l'inférence d'un rôle écologique à des taxons identifiés dans l'écosystème étudié. Nous arrivons maintenant relativement bien à identifier les facteurs influençant la structure des communautés microbiennes et à en étudier les réponses (Kuramae et al., 2011; Chai, 2013; Agler et al., 2016). Cependant les interactions entre microbes sont relativement mal connues. La raison principale est qu'il est difficile d'établir de véritables relations entre les taxons d'un microbiote. En effet, les nouvelles générations de séquençage (NGS) nous donnent une idée de l'abondance de chacun des taxons microbiens dans l'écosystème étudié mais ne renseignent pas sur les relations biochimiques entre eux et encore moins sur leur localisation au sein de l'écosystème. C'est pour cette raison que des approches mathématiques visant à rechercher des liens entre les variations d'abondances de taxons microbiens ont été développées pendant ces dernières années. On nomme ces approches les analyses de réseau (Ings et al., 2009; Deng et al., 2012; van der Heijden and Hartmann, 2016). Tracées à partir des données trophiques pour les écosystèmes macroscopiques, elles s'appuient sur des processus mathématiques dans le cas des écosystèmes microbiens.

Les analyses de réseau, un proxy pour étudier les interactions microbiennes

Afin d'apporter de la compréhension aux interactions microbiennes au sein du microbiote des plantes, des méthodes se basant sur les réseaux corrélatifs ont été mises au point. Ces réseaux corrélatifs relient des nœuds individuels correspondant à une unité taxonomique (OTU) grâce à des liens impliquant une relation biologique, chimique ou encore fonctionnelle. Par exemple, on peut s'attendre à ce que les microbes mutualistes ou mutuellement bénéfiques soient corrélés positivement. En revanche, les microbes ayant des relations antagonistes, telles que la compétition pour le même créneau, peuvent être corrélés négativement. En pratique, les microbes peuvent également être corrélés positivement ou négativement pour des raisons indirectes, en fonction de leurs préférences environnementales. Cette idée est corroborée par l'observation de microbes apparentés phylogénétiquement qui souvent coexistent (Lozupone et al., 2012). Des études de cette dernière décennie montrent d'ailleurs que les co-occurrences microbiennes détectées par corrélations peuvent être utilisées pour déterminer les principaux acteurs microbiens avec un effet structurant sur leur communauté (Steele et al., 2011; Lima-Mendez et al., 2015; Zheng et al., 2021). La corrélation est également un outil puissant pour aider les chercheurs à générer des hypothèses. Par exemple, des espèces dont les abondances sont positivement corrélées pourraient être mutualistes. Ce postulant devant par la suite faire l'objet d'une étude plus approfondie (par exemple, par co-culture ou par séquençage du génome).

Le calcul de réseaux corrélatifs est un défi chez la communauté microbienne du sol. En effet la complexité inhérente aux écosystèmes microbiens, qui hébergent une multitude de fonctions et d'espèces, rend le nombre potentiel d'interaction faramineux. Si l'on prend 45000 comme le nombre d'OTU d'une communauté microbienne standard, pour un ensemble de données ne comportant que des relations bipartites avec n OTU, cela implique (n*(n-1)/2) soit plus d'un

milliard de corrélations bipartites potentielles à tester. Cela en faisant abstraction du fait que les microbes pris en compte vivant en communautés, interagissent vraisemblablement de façon tripartite, quadripartite voire encore plus. De plus, les espèces rares compliquent le traitement statistique en augmentant le nombre de zéros au sein des matrices de calcul (Friedman and Alm, 2012). Enfin, les relations écologiques entre microbes au sein d'un microbiote ne sont probablement pas toute linéaires, exponentielles ou encore périodiques et les tests de corrélations pourraient ne pas détecter un bon nombre de relations bien réelles (Reshef et al., 2011).

Les approches pour calculer les liens corrélatifs entre les espèces d'un microbiome sont diverses. En théorie, toutes les méthodes qui mesurent des relations entre variables peuvent être utilisées. Par exemple il existe des méthodes utilisant des mesures comme la dissimilarité de Bray-Curtis, ou encore les relations linéaires grâce au calcul du coefficient de Pearson ou monotoniques avec celui de Spearman. Certains programmes ont été développés afin de corriger certains biais inhérents à ces précédentes mesures statistiques. C'est par exemple le cas de la méthode d'analyse de réseau la plus connue et répandue : CoNet (Faust and Raes, 2016). Ce logiciel prend en compte les forces et les vicissitudes de chacune des statistiques précédentes en y ajoutant des tests de correction pour, en théorie et de manière optimale, réussir à illustrer les interactions au sein d'un microbiome. L'ensemble des tests de correction prend place dans l'algorithme ReBoot qui combine les informations à partir des à priori particuliers à chacun des tests pour le calcul d'une P-value. Il existe aussi l'analyse locale de similarité (LSA) qui est optimisée pour détecter les relations temporelles non-linéaires lors d'un échantillonnage séquentiel (Ruan et al., 2006). Enfin il y a SparCC développée et basée sur l'analyse log-ratio d'Aitchison (Friedman and Alm, 2012). Cependant toutes ces analyses se basant sur des tests statistiques afin de détecter des relations entre les abondances d'espèces microbiennes comportent tellement de biais qu'il est extrêmement

difficile d'aboutir à quelque chose de clair à l'issue d'une analyse. La plupart de ces méthodes nécessitent une réduction drastique du nombre d'espèces rares dans la matrice d'entrée afin de pouvoir fonctionner. De plus, la quantité de relations faux-positives produite par les méthodes LSA et CoNet rendent difficile la tenue d'une interprétation écologique des résultats (Weiss et al., 2016). Cela fait de la méthode corrélative un candidat médiocre à l'étude des interactions microbiennes.

Cependant, une autre méthode donne des résultats bien plus probants : la méthode des graphes, ou lasso. Cette méthode statistique repose sur une méthode de contraction de la régression dont l'acronyme est « Least Absolute Shrinkage and Selection Operator » (Tibshirani, 2011). Le Lasso était à l'origine destiné à être appliqué à un modèle des moindres carrés. Cependant il a aussi été possible de l'adapter pour les modèles linéaires généralisés. Son intérêt réside dans sa capacité à sélectionner un sous-ensemble de variables dont la variation des coefficients peut s'interpréter de manière géométrique, en statistiques bayésiennes ou en analyse convexe (Friedman et al., 2008; Tibshirani, 2011). La méthode Lasso a été appliquée aux données d'abondance microbiennes très récemment (Kurtz et al., 2015a; Martino et al., 2019). Elle tire son épingle du jeu grâce à trois qualités selon Tibshirani et al. (2011) :

- Le lasso fonctionne dans le cas où le nombre d'échantillons d'un jeu de données est inférieur au total du nombre d'OTU. C'est une des faiblesses des régressions linéaires classiques où le risque d'erreur croît avec l'augmentation de la dimensionalité.
- Le lasso sélectionne aussi de manière parcimonieuse un sous-ensemble restreint de variables grâce au calcul de l'inverse covariance λ.
- Enfin, la méthode est capable de s'affranchir du biais causé par une grande abondance de zéros dans la matrice de données source.

Dans le cas de l'étude des interactions microbiennes à partir de données d'abondance issues des NGS, la méthode Lasso semble donc plus performante que les autres approches décrites précédemment. En effet, nos jeux de données font preuve d'une dimensionnalité extrêmement grande car elle est égale au nombre des espèces de l'écosystème étudié (~ 10000 – 20000). De plus, les matrices de données issues du compte des reads microbiens sont très hétérogènes et comportent un grand nombre d'espèce rares, ce qui entraine souvent des biais statistiques dans les analyses. Enfin la dimensionalité problématique de ces matrices est aussi résolue. En effet, notre nombre d'OTU mesurés p est de l'ordre de centaines voire de milliers alors que le nombre d'échantillons varie généralement d'une dizaine à quelques centaines. Cela implique que tout schéma d'inférence d'interaction significative doit fonctionner dans le régime de données sous-déterminé ($p > n$) qui n'est viable uniquement si d'autres hypothèses sur le réseau d'interaction peuvent être établies comme par exemple des cas de symbioses déjà connus, des consortiums de microbes déjà décrits ou encore une quantification des signaux chimiques particuliers à des types spécifiques d'interactions (parasitisme, mutualisme …), ce qui n'est généralement pas le cas avec les communautés microbiennes d'échantillons environnementaux. N'ayant pas besoin de tout cela, la méthode lasso est prometteuse et produit des résultats plus fiables que les méthodes corrélatives standard (Kurtz et al., 2015a).

Pour l'analyse des interactions microbiennes, une méthode a été publiée par Kurtz et al. (2015), nommée SPIEC-EASI (Sparse Inverse Covariance and estimation for Ecological Association Inference). Comme son nom l'indique, elle repose sur le calcul d'une matrice de covariance $\boldsymbol{\Theta}$. Celle-ci est pondérée par la méthode lasso et on est alors capable de procéder à un regroupement des variables effectivement covariantes par méthode du plus proche voisin en sous-échantillonnant la matrice d'abondance en raisonnant de proche en proche. Cela permet d'éviter

l'apparition de faux-positif en rendant l'OTU sous-échantillonné au sein de la matrice, indépendant des autres voisins avec lesquels il ne covarie pas. Cela étant, la sélections des variables liées par la méthode du plus proche voisin est alors résolue par la méthode lasso, permettant de regrouper de manière non biaisée des OTUs covariant ensemble et ainsi former des hypothèses quant à leurs potentielles interactions.

Objectifs et hypothèses

La rhizosphère des plantes est un écosystème complexe difficile à observer. Dans le cadre de ma thèse je vise à approcher cette complexité grâce à une plante particulière qui présente quelques particularités : le canola. Cette plante est d'importance économique, mais surtout elle produit dans sa rhizosphère des isocyanates antimicrobiens qui sont néfastes pour les microorganismes (Zheng et al., 2014) et donc la complexité du microbiome de sa rhizosphère. En utilisant le canola et grâce aux données de séquençage d'amplicons de sa rhizosphère, de ses racines et de son sol, je compte (1) vérifier l'existence d'un core microbiome bactérien et fongique existant à l'échelle des grandes prairies canadiennes dans la rhizosphère du canola, (2) identifier des points chauds d'interactions microbiennes potentielles entre espèces fongiques et bactériennes au sein de cet écosystème et enfin (3) vérifier si ces structures théoriques, potentiellement en interaction sont stables dans le temps. Et enfin, (4) évaluer la persistance des champignons mycorhiziens à arbuscules dans la rhizosphère du canola et le plein sol adjacent cette plante non-hôte, en systèmes culturaux basés sur le canola.

Dans cette thèse, les premiers et deuxièmes chapitres se penchent sur l'existence possible d'un core microbiome fongique et bactérien chez *B. napus*, tout en identifiant des taxons clef de voûte propres aux communautés fongiques d'une part et aux communautés bactériennes d'autre part. Le troisième chapitre, lui se penche sur les interactions entre champignons et bactéries du

microbiote souterrain du canola et l'identification de taxons clef de voûte au sein de cette communauté unifiée. Enfin, le dernier chapitre, après la détection de champignons mycorhiziens à arbuscules dans la rhizosphère et le sol du canola dans le chapitre trois, se penche sur la composition et le maintien dans le sol et la rhizosphère de cette communauté d'organismes chez cette plante non-hôte.

Mise en contexte du chapitre 2 : Fungal communities of the canola rhizosphere: keystone species and substantial between-year variation of the rhizosphere microbiome

Lors de ma maitrise réalisée entre 2016 et 2018, j'ai articulé mes travaux autour de la compréhension des dynamiques microbiennes fongiques au sein de la rhizosphère du canola. Cependant, ma réflexion et la méthodologie employée alors pour mes analyses de réseau était insatisfaisante car basée sur la méthode CoNet. Cette méthode a le défaut majeur d'être particulièrement sensible à la dimensionalité des données et donc de maximiser au-delà du raisonnable les erreurs de type 2 (faux positifs). La première partie de mon doctorat a alors été de comprendre les analyses de réseaux et leurs faiblesses pour finalement choisir SPIEC-EASI comme méthode fiable permettant de représenter la réalité mathématique des données d'abondance microbiennes en termes de covariances, ce sans n'être biaisé ni par la dimensionalité des données ni par le grand nombre de zéros dans les matrices. Ce chapitre est donc le chapitre de transition entre mes travaux de maitrise et ceux de mon doctorat.

Ce chapitre s'appuie sur les échantillonnages réalisés en 2013 et 2016 avec Agriculture et Agroalimentaire Canada. Les résultats de ce chapitre ont fait l'objet d'une publication dans la revue *Microbial Ecology* en 2020.

Ma contribution dans cet article au doctorat est l'élaboration de l'analyse de réseau, des illustrations et de la rédaction de l'article.

Chapitre 2 – Fungal communities of the canola rhizosphere: keystone species and substantial between-year variation of the rhizosphere microbiome

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Abstract

Rhizosphere microbes influence one another, forming extremely complex webs of interactions that may determine plant success. Identifying the key factors that structure the fungal microbiome of the plant rhizosphere is a necessary step in optimizing plant production. In a long-term field experiment conducted at three locations in the Canadian prairies, we tested the following hypotheses: (1) diversification of cropping systems influences the fungal microbiome of the canola (*Brassica napus*) rhizosphere; (2) the canola rhizosphere has a core fungal microbiome, i.e. a set of fungi always associated with canola; and (3) some taxa within the rhizosphere microbiome of canola are highly interrelated and fit the description of hub taxa. Our results show that crop diversification has a significant effect on the structure of the rhizosphere fungal community but not on the fungal diversity. We also discovered and described a canola core microbiome made up of one ZOTU, *Olpidium brassicae*, and an eco-microbiome found only in 2013 consisting of 47 ZOTUs. Using network analysis, we identified four hub taxa in 2013: ZOTU14 (*Acremonium* sp.), ZOTU28 (*Sordariomycetes* sp.), ZOTU45 (*Mortierella* sp.) and ZOTU179 (*Ganoderma applanatum*), and one hub taxon, ZOTU17 (*Mortierella* gamsii) in 2016, none of these taxa which interacts the most belonged to the core microbiome or eco microbiome for each year of sampling. This temporal variability questions our ideas about plant core microbiome and its stability. Our results provide a basis for the development of ecological engineering strategies for the improvement of canola production systems in Canada.

Keywords: Fungal communities, microbial ecology, agroecosystem, crop rotations, *Brassica napus*

Introduction

The microbiome of the plant rhizosphere constitutes an ecosystem whose interactions and microbial functions are still largely unknown. Throughout their life, plant roots exude chemical compounds creating the rhizosphere environment (Bais et al., 2006). Variations in the composition of rhizodeposits allow plants to shape rhizosphere microbial communities to their benefit (Tkacz et al., 2015a; Pii et al., 2016). The root is an interface allowing exchanges between plants and microorganisms which lead to the formation of symbioses between fungi and bacteria (Garbaye, 1994; van der Heijden et al., 2006).

Plant roots can host mutualistic microbes which help to facilitate nutrient uptake, prevent root infection by pathogens, mitigate the impact of abiotic stress, and modulate plant hormones levels (Doehlemann et al., 2014; Hardoim et al., 2008; Latz et al., 2016; Mozafar et al., 2000). Biological interactions in the rhizosphere exist along a continuum of mutualism to parasitism, with organisms ranging from saprotrophs to obligate biotrophs. Most microbes do not influence plants directly, but they interact with one another and their interactions can affect the plants. Microbial communities may form complex webs of interactions that allow plants to increase their exploitation of the surrounding environment (Bulgarelli et al., 2013). Understanding how the different microbe-microbe interactions are shaped within the microbiome of the plant rhizosphere is a very important topic in ecology.

Next-generation sequencing technologies can increase our understanding of microbiomes by revealing the extreme diversity of the rhizosphere microbiome and its complex microbial functions. In order to gain a better understanding of the structure of microbiomes despite their complexity, a microbiome can be conceptually divided into pools based on the occurrence of species (Ridout and Newcombe, 2016; van der Heijden et al., 2016). Vandenkoornhuyse et al.,

(2015) divided the microbiome into different sub-units since the composition of the microbiome can vary with different environments. They defined a theoretical structure: the core microbiome, which consists of the microbial species that are always part of the plant microbiome regardless of the environmental conditions at time *t*. These taxa are likely to be favored by the plant throughout its existence (Rout, 2014). As most microbiome studies rely on a single sampling time, this definition is appropriate. However, as our study includes two different sampling times, it was necessary to consider the temporal variation in the definition of core microbiome. In this paper, we defined the core microbiome as the set of organisms that are present in the plant microbiome at time *t* and *t+1* in all environments. The microorganisms only present at time *t* or *t+1* were identified as the eco-microbiome, in accordance with the definition of Vandenkoornhuyse et al. (2015).

Some microorganisms forming the microbiome have a greater impact on plants and ecosystem functioning than others because they interact with many partners and antagonists. Agler et al. (Agler et al., 2016) developed the concept of 'hub' taxa to describe the taxa whose abundance is correlated with that of many others within the microbiome. Hub species are often extremely important for plants, and variation in their abundance due to abiotic factors can greatly change the microbiome, influencing plant health (Agler et al., 2016). The microbial networks of rhizosphere microbiomes can be extremely complex. By focusing on hub taxa, information about microbial networks can be organized and the analysis of the microbiome simplified. The taxa in the network can be grouped into communities gravitating around these hub taxa. For example, taxa that recruit microorganisms beneficial to host plants can be identified, such as bacteria closely associated with mycorrhizal networks (Taktek et al., 2017). In the human microbiome, some hub taxa can also be pathogens and articulate tissue infections by consortia of pathogens, threatening the performance of the host (Hajishengallis et al., 2012a). As pathogens can affect microbial communities (Raaijmakers et al., 2009), pathogen hub taxa may occur in the rhizosphere microbiome. The

concept of hub taxa is a useful tool for research aimed at understanding the ecology of the root ecosystem which may lead to applications in agriculture.

Lay et al., (2018) have shown that canola roots and the rhizosphere possess core microbiomes distinct from those of pea and wheat crops grown in the same fields. In the present study, our aim was to investigate the temporal stability of the core microbiome structure and to evaluate its variation with different crop rotations, in order to ascertain whether a persistent canola core microbiome exists. Another aim was to determine if the canola rhizosphere harbors hub taxa, and to visualize the variation in the structure of interactions among the fungi living in the canola rhizosphere microbiome. Using an experiment including three geographic locations and two different years, we sought to identify a universal fungal core microbiome in the rhizosphere of a plant species, specifically canola grown under different climatic conditions and in different biological environments. Canola is one of the most important crops in Canada and a good model plant for studying the rhizosphere microbiome, since it does not form symbiotic relationships with mycorrhizae and it produces antimicrobial isocyanates (Zheng et al., 2014), resulting in simpler rhizosphere communities (Rumberger and Marschner, 2003). To our knowledge, our study is the first to use network analysis to define hub taxa in the canola rhizosphere.

Materials and Methods

Site description and experimental design

The present study used a subset of the plots in a long-term multi-site field experiment established in 2008 to test the influence of canola (*Brassica napus* L.) frequency in crop rotation systems (Harker et al., 2015a). Three sites located in three pedoclimatic zones of the canola-producing area of western Canada were used. Two sites were in Alberta, specifically in Lacombe

(lat. 52.5°N, long. 113.7°W) and Lethbridge (lat. 49.7°N, long. 112.8°W), and the third site was in Scott, Saskatchewan (lat. 52.4°N, long 108.8°W). The Lethbridge site is in a semi-arid region and was irrigated. The climate is cooler in Lacombe than Lethbridge. Scott is in a cool, sub-humid region, with limited growing degree-days. The soil in Lethbridge is a Brown Chernozem with a silty loam texture, while the Dark Brown Chernozems have a loamy texture at the Scott site and a clay loam texture at Lacombe. Annual precipitation (average for 1981-2010) totals 380.2 mm in Lethbridge, 366.2 mm in Scott, and 487.0 mm in Lacombe. Cumulative monthly precipitation in 2013 and 2016 is given for each site in Figure S1.

The experiment included 13 treatments laid out in a randomized complete block design with four blocks at each of the three sites, as detailed in Harker et al., (2015). In 2013, six treatments were sampled, namely, the factorial arrangement of three levels of cropping system diversification: (1) monoculture of canola, (2) wheat-canola, and (3) pea-barley-canola, combined with two canola cultivars: a Roundup Ready® (RR) or a Liberty Link® (LL) (Tableau 1). In 2016, we used an additional treatment: a highly diversified system consisting of lentils-wheat-LL-pea-barley-RR. Thus, there were seven treatments in all. Crops were grown according to best management practices. Information on crop management is described in Harker et al., (2015).

Tableau 1. Selected treatments from a long-term experiment established in 2008 at three different sites in the Canadian prairies. The rotation phases examined in this study in 2013 and 2016 are underlined.

	Cropping systems	
Diversification level	2008–2013	2008–2016
Monoculture	LL-LL-LL-LL-LL-<u>LL</u>[1]	LL-LL-LL-LL-LL-LL-LL-LL-<u>LL</u>
	RR-RR-RR-RR-RR-<u>RR</u>	RR-RR-RR-RR-RR-RR-RR-RR-<u>RR</u>
Low	W-LL-W-LL-W-<u>LL</u>	LL-W-LL-W-LL-W-LL-W-<u>LL</u>
	W-RR-W-RR-W-<u>RR</u>	RR-W-RR-W-RR-W-RR-W-<u>RR</u>
Medium	P-B-LL-P-B-<u>LL</u>	P-B-LL-P-B-LL-P-B-<u>LL</u>
	P-B-RR-P-B-<u>RR</u>	P-B-RR-P-B-RR-P-B-<u>RR</u>
High	-[2]	Len-W-LL-P-B-L-W-P-<u>RR</u>

[1]LL, canola InVigor 5440, a Liberty Link cultivar resistant to glufosinate herbicides; RR, canola 71-45, a Roundup Ready cultivar resistant to glyphosate.

[2] The highly diversified treatment was not used in 2013.

Sampling

Rhizosphere samples were collected during the fourth week of July in 2013 and 2016, which corresponds to the end of canola flowering. Three to four plants randomly selected within each plot were uprooted with a shovel. The shoots were removed, and roots were placed in plastic bags and brought to the laboratory on ice in a cooler. About 5 g of rhizosphere soil per plot was collected by gently brushing the roots. The soil samples were kept at 4 °C before being shipped on ice to Swift Current, Saskatchewan, where they were preserved at -80 °C until DNA extraction.

DNA extraction and amplification

Total microbial DNA was extracted from the 250-mg rhizosphere soil samples using the PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA) in accordance with the manufacturer's recommendations. DNA was eluted in 10 µL tris-HCL buffer and quantified using a Qbit 2.0 spectrophotometer (Invitrogen, Life Technologies, Carlsbad, CA, USA). We constructed amplicon libraries for fungal ITS sequences by using target-specific PCR primers attached to Illumina overhang sequences for Nextera library preparation. The primer pairs were ACACTGACGACATGGTTCTACACTTGGTCATTTAGAGGAAGTAA (ITS1F-Illu) and TACGGTAGCAGAGACTTGGTCTCTGCGTTCTTCATCGAT (5.8A2R-Illu). Each 25-µL PCR reaction consisted of 0.10 µL of forward and reverse primers, with 19.6 µL H_2O, 2.5 µL 25 mM $MgCl_2$, 12.5 µL KAPA HiFi Hotstart ReadyMix (Kapa Biosystem, Cape Town, South Africa) and 1 µL of sample DNA. The reaction conditions were as follows: 95 °C for 5 min, 25 cycles of 94 °C for 45 sec, 52 °C for 60 sec, and 72 °C for 30 sec with a final extension at 72 °C for 7 min. PCR products were verified by electrophoresis on 1% agarose gels. Dual Nextera indices were then attached to PCR products based on the suggested protocol "16S Metagenomic Sequencing Library Preparation" provided by Illumina (part no. 15044223 rev. B). The final purified product was quantified by Qubit Fluorometric Quantitation (Thermofisher Scientific). Libraries were pooled in equimolar amounts before sequencing in rapid paired-ends 250 bp (PE250) mode on an Illumina MiSeq system, using the 500-cycle MiSeq reagent kit v.2 in accordance with the manufacturer's recommendations.

ZOTU formation and bioinformatics pipeline

The 2013 and 2016 sequence files were merged before processing. Paired ends of fungal ITS DNA sequences were joined in USEARCH v.10 (Edgar, 2010) using the function

"fastq_mergepairs". Cutadapt 1.13 was used to remove the primer part of the ITS sequences. Then, we excluded the sequences with more than 300 bp and less than 250 bp to keep only those corresponding to the standard ITS length (Dorn-In et al., 2013). The sequences that did not reach the maxee quality filter (1.0) of USEARCH v10 were removed with the command "fasq_filter" and the singletons were removed using the "fastx_uniques" command. We used the function "unoise3" at a threshold of 1.0 to form the ZOTUs. ZOTUs were then identified using BLAST on the database UNITE, and the identity of ZOTUs of interest was verified manually in the NCBI nt database. As ITS have a low taxonomic resolution, it is possible with the current level of technology used to identify a fungus at the family or genus level, but not at the species level. Thus, all the following identifications at species level in this paper match at 100% similarity the references sequences of NCBI but must be taken with caution as it may not correspond to the real specie identification of the ZOTU.

The MiSeq sequencing data generated as part of this work have been deposited in the NCBI Sequence Read Archive and are available under the project number (will be available before publication).

Data processing and statistical analyses

The dataset was standardized by randomly subsampling the read data from each sample to the lowest number of reads encountered for a sample, using the function "rrarefy" of the vegan package v.2.4.6 in R v. 3.4.3, before calculating the diversity indices. The Chao1, Shannon and Simpson's α-diversity indices were computed using the vegan package v. 2.4.6 in R v. 3.4.3. The effect of crop diversification on the α-diversity indices was tested by analysis of variance (ANOVA) one year at a time, considering 12 blocks, and comparisons between treatment means

were made with Tukey's post-hoc tests using the softwarJMP v. 13.2.1. A random effect was attributed to the block, and fixed effects were attributed to treatments.

The effect of crop diversification on fungal community structure was assessed by permutational multivariate analysis of variance (PERMANOVA) site by site, considering 4 blocks using the function "adonis" of the vegan package v 2.4.6 in R v3.4.3, and the entire (non-subsampled) dataset. The blocked multi-response permutation procedure (blocked MRPP) was performed for the comparison of treatment means using PC-ORD6 (McCune and Mefford, 2011), and Šidák correction for two-way comparisons was applied to p-values. Šidák correction was calculated as follows: $1 - (1 - \alpha)^{1/m}$, where α is 0.05, and m is the number of paired comparisons. Indicator species analysis was performed using the Indicspecies package v 1.7.6 in R 3.4.3 to identify ZOTUs significantly associated with each treatment.

Only the ZOTUs that were found in all plots in both years were assigned to the core microbiome of the canola rhizosphere. The ZOTUs that were present in all plots but only for one year were defined as the eco-microbiome. Potential ecological guilds were created based on the literature reports we found concerning our microbiome taxa by assigning each ZOTU to a guild described as a plant parasite, plant endophyte, or saprotroph.

To assess the interactions among fungal taxa in the microbiome, we created a co-occurrence network using the package Spiec-Easi v 1.0.6 in R 3.4.3 (Kurtz et al., 2015a). The analysis was conducted on the whole rhizosphere microbiome of each year. The input data consisted in the matrix of the raw abundance of ZOTUs of one year of sampling. We first filtered the dataset to remove the ZOTUs with frequency less than 20%. The Spiec-Easi run was done with the algorithm "glasso" with the lambda min ratio set at 10^{-2} and 50 repetitions. We then imported the networks in Cytoscape 3.7.0 for plotting and used the "organic" layout to draw the network. Edges where defined as co-occurrences or mutual exclusion regarding the positives or negatives values of

inverse covariance linking the nodes. Hub-taxa were defined as the nodes possessing a score of betweenness centrality > 0.09. The identification of core microbiome members and hub-taxa aims at targeting the ZOTUs that could be of ecological importance for rhizosphere ecosystem functioning.

Results

Taxonomic affiliation of the fungal microbiome of the canola rhizosphere

After merging the reads from the ITS datasets of summer 2013 and summer 2016, and filtering for quality, we retrieved 7 964 220 reads from the 156 samples, that were assigned to 2 156 ZOTUs. Read number per sample ranged from 8 805 to 112 115. The ZOTUs belong mostly to four fungal phyla: *Ascomycota* (57%), *Chytridiomycota* (21%), *Mortierellomycota* (14%) and *Basidiomycota* (6%) (Figure 1A and 1B). Rarefaction curves indicated that read abundances were close to saturation for all the samples (Figure S2 and S3).

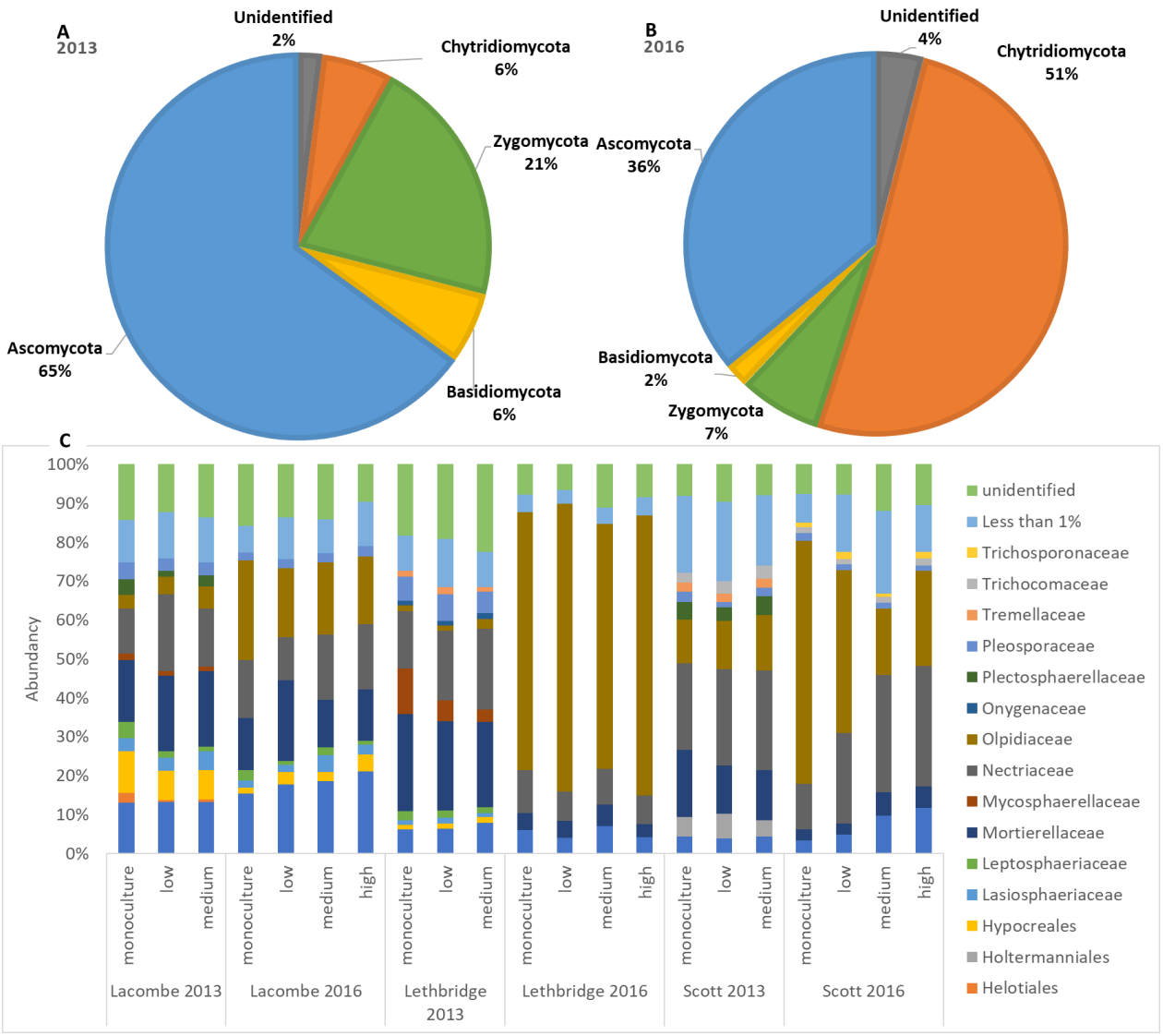

Figure 1. Relative abundance of fungal phyla in the rhizosphere of canola varieties in 2013 (A) and 2016 (B). Variation in taxonomic profiles is characterized by an increase in the abundance of the Olpidiaeae in the phylum Chytridiomycota in 2016. Fungal families also varied with site, crop diversification level and year (C), with an overall increase in the relative abundance of the Olpidiaceae in 2016 in all crop diversification levels.

Effect of treatments on communities

In both years, neither canola cultivar, nor crop diversification had a significant influence on α-diversity indices (Tableau 2). However, crop diversification significantly influenced the structure of the fungal community of canola rhizosphere at all three sites in both years, except at Scott in 2013 and Lethbridge in 2016 (Tableau 3). Environmental conditions modified the effect of crop diversification. Based on PERMANOVA and MRPP *P* values, the crop diversification effect was weaker in 2016 than 2013, except in Scott where the effect of crop diversification was only significant in 2016 (Tableau 3 and 4).

Tableau 2. Mean values of fungal α-diversity indices in the rhizosphere of the canola cultivars under different crop diversification levels, in 2013 and 2016.

Diversification level[2]	Canola cultivar[3]	Richness	Chao1	Shannon	Simpson
		2013[1]			
Monoculture	LL	190.917	285.641	3.963	0.958
	RR	183.917	269.237	3.767	0.932
Low	LL	202.273	290.738	3.911	0.947
	RR	199.000	303.038	3.949	0.949
Medium	LL	192.750	289.399	3.983	0.959
	RR	197.417	296.528	3.943	0.950
		2016[1]			
Monoculture	LL	58.667	68.928	2.166	0.679
	RR	50.667	66.635	2.071	0.662
Low	LL	62.083	53.220	2.235	0.676
	RR	56.000	70.927	2.449	0.756
Medium	LL	60.333	75.649	2.629	0.802
	RR	67.750	75.185	2.609	0.781
High	RR	67.000	81.164	2.485	0.731

[1] For both years, neither canola cultivar nor crop diversification had a significant effect on fungal α-diversity indices by Tukey HSD test (n = 4) (Tableau S1).

[2]Diversification level: Monoculture, canola monoculture; Low, wheat-canola rotation; Medium, pea-barley-canola rotation; High, lentil-wheat-LL-pea-barley-RR. LL, canola InVigor 5440, a Liberty Link cultivar resistant to glufosinate herbicides; RR, canola 71-45, a Roundup Ready cultivar resistant to glyphosate.

Tableau 3. Effects of canola cultivar and crop diversification on the structure of the fungal community in the canola rhizosphere at different sites, in 2013 and 2016, according to PERMANOVA $\alpha = 0.05$, $n = 4$.

		2013		*2016*	
Site	Source	DF [1]	*P-value*	DF	*P-value*
Lacombe	Canola cultivar	1	0.821	1	0.628
	Crop diversification	2	0.001***	3	0.02**
	Canola cultivar × Crop diversification	2	0.925	2	0.098
	Residuals	18		21	
Lethbridge	Canola cultivar	1	0.498	1	0.422
	Crop diversification	2	0.005***	3	0.111
	Canola cultivar × Crop diversification	2	0.017**	2	0.777
	Residuals	18	-	21	-
Scott	Canola cultivar	1	0.695	1	0.639
	Crop diversification	2	0.837	3	0.001***
	Canola cultivar × Crop diversification	2	0.716	2	0.24
	Residuals	18		21	

[1]DF: Degree of freedom

Certain fungal ZOTUs were consistently more abundant in certain cropping systems than in others, as per indicator species analysis (Tableau S3). These indicator species changed between years revealing further the effect of environmental conditions on rhizosphere fungi. Year 2013 was characterized by many indicator species for each crop diversification level as compared to 2016, where the number of indicator species per crop diversification level ranged from 0 to 2 only.

The effect of canola cultivar on the structure of the rhizosphere fungal community was not significant overall. However, at Lethbridge in 2013, there was a significant interaction (P=0.017) between canola cultivar and crop diversification, whereas in 2016 the interaction was not significant (P=0.111). BMRPP comparisons showed that the structure of communities associated with LL and RR differed slightly at the low level of crop diversification in 2013, and in monocultures in 2016 (Tableau 4).

Tableau 4. Comparison of the fungal community structure of the canola rhizosphere as influenced by canola cultivar and crop diversification, at three experimental sites, in 2013 and 2016, based on Blocked Multi-Response Permutation Procedures (BMRPP) and Šidák correction for two-way comparisons ($\alpha = 0.035$, $n = 4$).

Site	Diversification level	Canola cultivar	2013	2016
Lacombe	Monoculture [1]	LL[2]	a [3]	a
	Monoculture	RR	a	a
	Low	LL	b	b
	Low	RR	b	b
	Medium	LL	c	b
	Medium	RR	c	b
	High	RR	- [4]	b
Lethbridge	Monoculture	LL	a	a
	Monoculture	RR	a	b
	Low	LL	c	b
	Low	RR	b	b
	Medium	LL	b	a
	Medium	RR	b	a
	High	RR	-	a
Scott	Monoculture	LL	a	a
	Monoculture	RR	a	a
	Low	LL	a	a
	Low	RR	a	ab
	Medium	LL	a	b
	Medium	RR	a	ab
	High	RR	-	b

[1]Monoculture, canola monoculture; Low, wheat-canola rotation; Medium, pea-barley-canola rotation; High, lentil-wheat-LL-pea-barley-RR.

[2]LL, canola InVigor 5440, a Liberty Link cultivar resistant to glufosinate herbicides; RR, canola 71-45, a Roundup Ready cultivar resistant to glyphosate.

[3] Within each column and site, crop rotations associated with the same letter are not significantly different.

[4] Treatment absent in 2013

The community taxonomic profiles showed that the relative abundance of Chytridiomycota increased from 6% in 2013 to 51% in 2016 whereas the relative abundance of the other main phyla decreased. The relative abundance of *Ascomycota* decreased from 65% in 2013 to 36% in 2016, that of *Basidiomycota,* from 6% to 2%, and that of *Zygomycota,* from 21% to 7% (Figure 1A, Figure 1B). The most abundant families in the community across all sites were the *Olpidiaceae, Nectriaceae, Mortierellaceae* and *Chaetomiaceae* (Figure 1C).

Variation of the canola rhizosphere microbiome

The microbiome in the canola rhizosphere differed between years. From the whole dataset, the set of organisms always present in the microbiome in all treatments in 2013 (i.e. the eco-microbiome of 2013) included 47 ZOTUs (Tableau 5). However, in 2016, the eco-microbiome was composed of only one ZOTU, i.e. ZOTU1, which was identified as *Olpidium brassicae*. As this ZOTU was also present in 2013, in our study we have a canola core microbiome made up of only one ZOTU: ZOTU1. The relative abundance of the ZOTUs in the eco-microbiome of 2013 ranged from 0.02% (ZOTU387) to 6.65% (ZOTU1) of the community (Tableau 5). ZOTU1 was the most abundant at all crop diversification levels: 9.52% in the monocultures, 11.36% at the low level of diversification and 12.42% at the medium level (Tableau S2). Among the eco-microbiome of 2013, 22 ZOTUs corresponded to taxa with potentially pathogenic behavior, 16 were potentially

saprotrophs and three potentially endophytes; the guild of the five remaining taxa was not determined. Crop diversification affected the abundance of three eco-microbiome members: ZOTU27 (*Fusarium solani)*, ZOTU51 (*Dendryphion nanum),* and ZOTU63 (*Thielavia* sp.) (Tableau 5). *Fusarium solani* was more abundant in the wheat-canola system than in the monocultures. In the diversified systems, *Dendryphion nanum* was more abundant in the canola monocultures than in any other system, while *Thielavia* sp. had the highest relative abundance in the monocultures.

Tableau 5. Effect of crop rotation on the relative abundance of ZOTUs forming the eco-microbiome of canola rhizosphere in 2013[1], as determined by ANOVA ($\alpha = 0.05$, $n = 8$) and Spearman's correlation values between the relative abundance of eco-microbiome ZOTUs and canola yield $\alpha = 0.05$, N = 72).

ZOTU	Identity	Guild[2]	Hub taxa[3]	% abundance	Spearman r	Crop-rotation[4]	ZOTU	Identity	Guild	Hub taxa	% abundance	Spearman r	Crop-rotation
ZOTU1	*Olpidium brassicae*	parasite	N	6.6589	0.4165*	0.9654	ZOTU35	*Fusarium solani*	parasite	N	0.5048	0.6799**	0.88
ZOTU10	*Plectosphaerella* sp.	parasite	N	2.7021	0.4756*	0.7643	ZOTU387	*Candida parapsilosis*	saprophyte	N	0.0230	Ns	0.0947
ZOTU102	*Trichoderma koningii*	endophyte	N	0.0948	0.2889*	0.8317	ZOTU39	*Cylindrocarpon* sp.	parasite	N	0.3778	0.5678**	0.9079
ZOTU11	*Fusarium* sp.	parasite	N	1.8167	-0.5063**	0.2103	ZOTU4	*Fusicolla* sp.	parasite	N	3.9051	0.5555**	0.9145
ZOTU123	*Leptosphaeria* sp.	parasite	N	0.1235	-0.2222*	0.1407	ZOTU40	*Cylindrocarpon* sp.	parasite	N	0.4178	0.5951**	0.9892
ZOTU13	*Fusarium avenaceum*	parasite	N	1.4315	0.2659*	0.0924	ZOTU41	*Tetracladium* sp.	saprophyte	N	0.6548	Ns	0.7281
ZOTU15	*Fusarium* sp.	parasite	N	1.3735	-0.5410**	0.9855	ZOTU42	*Alternaria infectoria*	parasite	N	0.5666	-0.4320*	0.6336
ZOTU154	*Mortierella* sp.	saprophyte	Y	0.1863	0.3112*	0.8241	ZOTU49	*Clonostachys rosea*	endophyte	N	0.3511	Ns	0.732
ZOTU16	*Geomyces* sp.	saprophyte	N	1.8497	Ns	0.9466	ZOTU5	*Nectria* sp.	parasite	Y	3.5296	0.4503*	0.9934
ZOTU17	*Mortierella gamsii*	saprophyte	N	1.5999	Ns	0.8354	ZOTU51	*Dendryphion nanum*	parasite	N	0.6841	0.2814*	0.004 **
ZOTU18	*Fusarium* sp.	parasite	N	1.0453	-0.6132**	0.997	ZOTU54	*Cladosporium tenuissimum*	saprophyte	N	0.4334	Ns	0.3295
ZOTU19	*Fusarium redolens*	parasite	N	0.8258	-0.4345*	0.9021	ZOTU56	*Exophiala* sp.	endophyte	N	0.4197	0.5151**	0.9765
ZOTU2	*Humicola grisea*	saprophyte	N	3.3583	0.6595**	0.9382	ZOTU6	*Mortierella* sp.	saprophyte	Y	2.9629	-0.3783*	0.9932

ZOTU21	*Fusarium* sp.	parasite	N	1.0078	-0.2682*	0.9423	ZOTU65	*Coniothyrium cereale*	undefined	N	0.4451	0.2860*	0.3052
ZOTU22	*Mortierella* sp.	saprophyte	N	0.7169	0.3058*	0.9735	ZOTU68	*Vishniacozyma victoriae*	undefined	N	0.3982	-0.2329*	0.9725
ZOTU229	*Aspergillus welwitschiae*	undefined	N	0.0741	Ns	0.8231	ZOTU7	*Mortierella alpina*	saprophyte	N	0.6831	-0.2170*	0.7525
ZOTU24	*Alternaria alternata*	parasite	N	1.0985	-0.4910*	0.7458	ZOTU71	*Cladosporium* sp.	saprophyte	N	3.1653	-0.5052**	0.3778
ZOTU25	*Plectosphaerella* sp.	parasite	N	0.9082	Ns	0.0652	ZOTU78	*Unknown*	undefined	N	0.2468	Ns	0.2826
ZOTU26	*Solicoccozyma aeria*	sapophyte	N	0.9998	Ns	0.9581	ZOTU8	*Phoma sclerotioides*	parasite	N	0.2539	0.4445*	0.9944
ZOTU27	*Fusarium solani*	parasite	N	0.5203	0.2968*	0.0067 **	ZOTU84	*Nectria* sp.	parasite	N	2.2015	Ns	0.9906
ZOTU29	*Ulocladium dauci*	saprophyte	Y	0.8834	-0.5325**	0.8468	ZOTU864	*Trichocladium opacum*	saprophyte	N	0.2117	0.5499**	0.3466
ZOTU3	*Mortierella* sp.	saprophyte	N	5.1457	-0.3121*	0.7355	ZOTU87	*Penicillium* sp.	saprophyte	N	0.0105	Ns	0.6842
ZOTU30	*Fusarium* sp.	parasite	N	0.6605	Ns	0.0704	ZOTU9	*Chaetomium globosum*	saprophyte	N	0.2295	0.2443*	0.9787
-	-	-	-	-	-	-	ZOTU63	*Thielavia* sp.	undefined	N	3.1145	-0.3429*	0.0002 ***

[1] In 2016, the eco-microbiome was composed of one ZOTU only, ZOTU1 *Olpidium brassicae*, which showed a negative correlation with yield (R = -0.76)

[2] possible ecological role.

[3] Taxa with high connectivity in network analysis.

[4] Crop rotations varied from canola monoculture to low (wheat-canola), medium (pea-barley-canola), and high (lentil-wheat-LL-pea-barley-RR) diversification levels; LL, canola InVigor 5440, a Liberty Link cultivar resistant to glufosinate herbicides; RR, canola 71-45, a Roundup Ready cultivar resistant to glyphosate.

Network analysis of the fungal microbiome

In 2013, the network was composed of 85 ZOTUs and 456 edges (Figure 2). Among the ZOTUs forming the network, 22 belonged to the eco-microbiome of 2013: ZOTU2, ZOTU3, ZOTU4, ZOTU5, ZOTU6, ZOTU8, ZOTU9, ZOTU15, ZOTU16, ZOTU18, ZOTU19, ZOTU21, ZOTU22, ZOTU26, ZOTU30, ZOTU35, ZOTU39, ZOTU40, ZOTU42, ZOTU56, ZOTU68 and ZOTU84. The taxonomic affiliations of the ZOTUs are given in Tableau 5. The 2013 network was modular and included 247 co-occurrences and 209 mutual exclusions between fungal taxa. It consisted mainly of an interaction group centering on ZOTU45 (*Mortierella* sp.) and ZOTU28 (*Sordariomycetes* sp.), both of which had the same number of co-occurrences and mutual exclusions, and a smaller group centered on ZOTU14 (*Acremonium* sp.) and ZOTU179 (*Ganoderma applanatum*). One sub-network was disconnected from the main structure; it consisted of a pair of ZOTUs (ZOTU163 and ZOTU207) identified as *Paecilomyces marquandii*. Surprisingly, ZOTU1 (*Olpidium brassicae*), which ranked first in abundance in 2013, had no connection with other ZOTUs, resulting in its absence in the interaction network.

Betweenness centrality, defined as the fraction of the shortest paths between all other nodes in the network containing the given node, highlights central nodes and provides information about network architecture. A score of betweenness centrality greater than the score of 95% of the network taxa could suggest participation in tripartite interactions in the community. The four ZOTUs identified as hub taxa in the 2013 network had a score of betweenness centrality > 0.09. ZOTU14 (*Acremonium* sp.) had a score of 0.15, ZOTU28 (*Sordariomycetes* sp.) a score of 0.11, ZOTU45 (*Mortierella* sp.) a score of 0.095, and ZOTU179 (*Ganoderma applanatum*) a score of 0.090. The ZOTUs with the highest number of mutual exclusions were ZOTU31 (Microascales sp.) with 18 negative interactions and ZOTU45 (*Mortierella* sp.) with 17 negative interactions.

Four ZOTUs accounted for most of the positive interactions in the network: ZOTU45 (*Mortierella* sp.), ZOTU20 (*Tremellomycetes* sp.) and ZOTU58 (*Cryptococcus* sp.) with 18 positive interactions and ZOTU278 (*Penicillium citreonigrum*) with 16 positive interactions. ZOTU45 developed an equal number of mutual exclusions and co-occurrences with other members of the network (18 in each case) and its abundance was negatively correlated with that of the other hub taxa of the same module, ZOTU28, which also had an equal number of mutual exclusions and co-occurrences (7 in each case). This negative relationship between the abundance of these two hub taxa, ZOTU28 and ZOTU45, stands out. The interesting fact is that ZOTU45 was positively linked with 5 of the 6 other ZOTUs with which ZOTU28 was negatively linked (ZOTU33, ZOTU79, ZOTU99, ZOTU8 and ZOTU278) and negatively linked to 3 of the 7 ZOTUs with which ZOTU28 was positively linked (ZOTU135, ZOTU70 and ZOTU76). ZOTU28 was positively linked to ZOTU14 (*Acremonium* sp.), ZOTU135 (*Trichoderma rossicum*), ZOTU22 (*Mortierella hyalina*), ZOTU298 (*Sordariomycetes* sp.), ZOTU168 (*Microascaceae* sp.), ZOTU70 (*Acremonium* sp.) and ZOTU76 (*Mortierella* sp.). ZOTU28 was also negatively correlated with ZOTU33 (*Tremellomycetes* sp.), ZOTU79 (*Penicillium levitum*), ZOTU99 (*Guehomyces pullulans*), ZOTU8 (*Nectria* sp.), ZOTU278 (*Penicillium citreonigrum*), ZOTU45 (*Mortierella* sp.) and ZOTU34 (*Mortierella sarnyensis*). The network of interactions between fungi was simpler in 2016 than in 2013 (Figure 3). It was composed of 12 nodes and 17 interactions with only one hub taxon, ZOTU17 (*Mortierella* sp.), which had a betweenness centrality score of 0.52. The other members of the network were also part of the network in 2013, but ZOTU17 was not. As in 2013, the core microbiome ZOTU1 (*Olpidium brassicae*), was not part of the 2016 network.

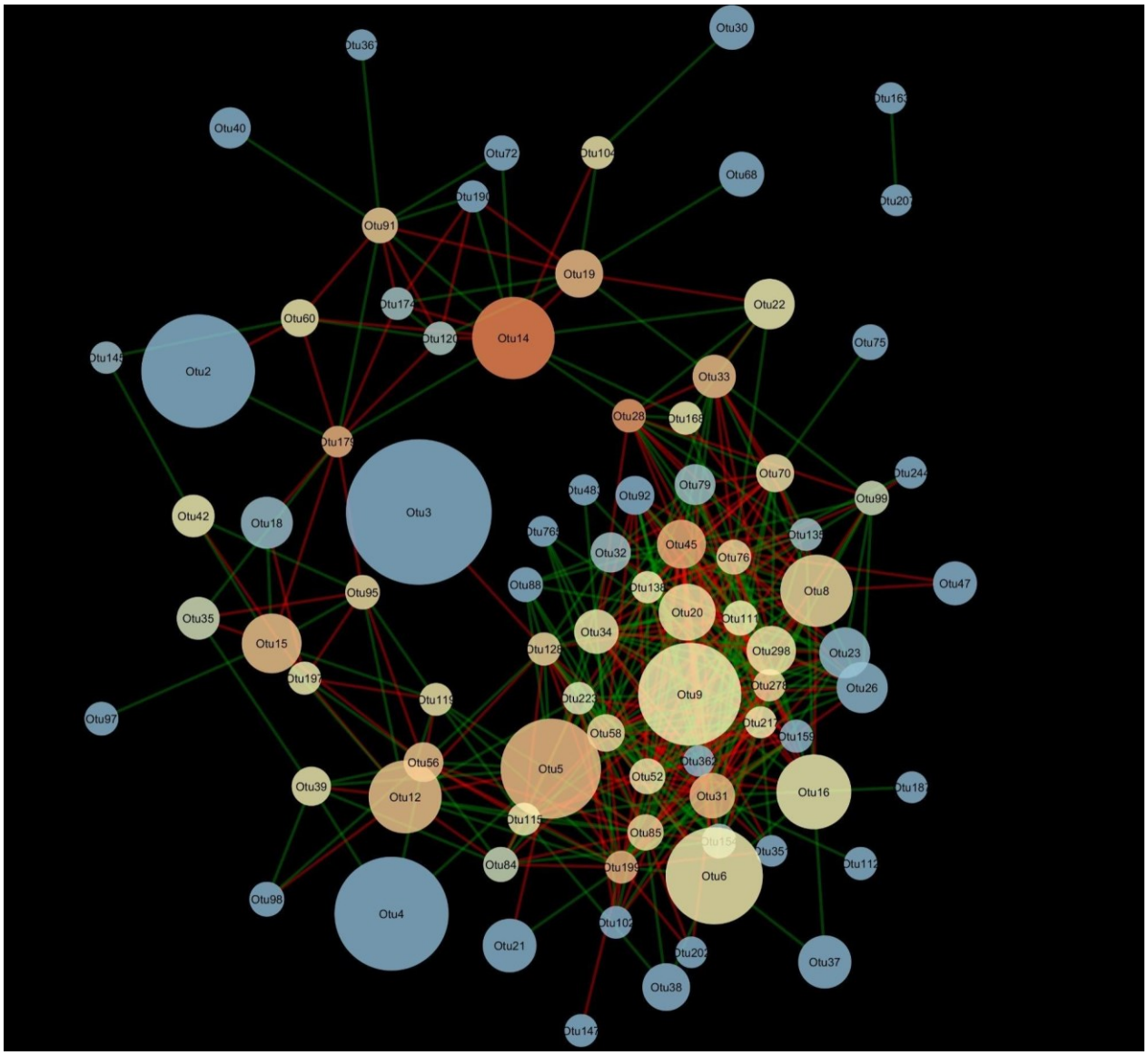

Figure 2. Network of interactions between the fungi forming the microbiome of canola rhizosphere in 2013. ZOTU size is proportional to relative abundance, and ZOTU shades indicate the degree of betweenness centrality: ZOTUs with warm colors are more connected with the other members of the network than the cold colored ones. Green edges indicate positive relationships and red edges, negative relationships.

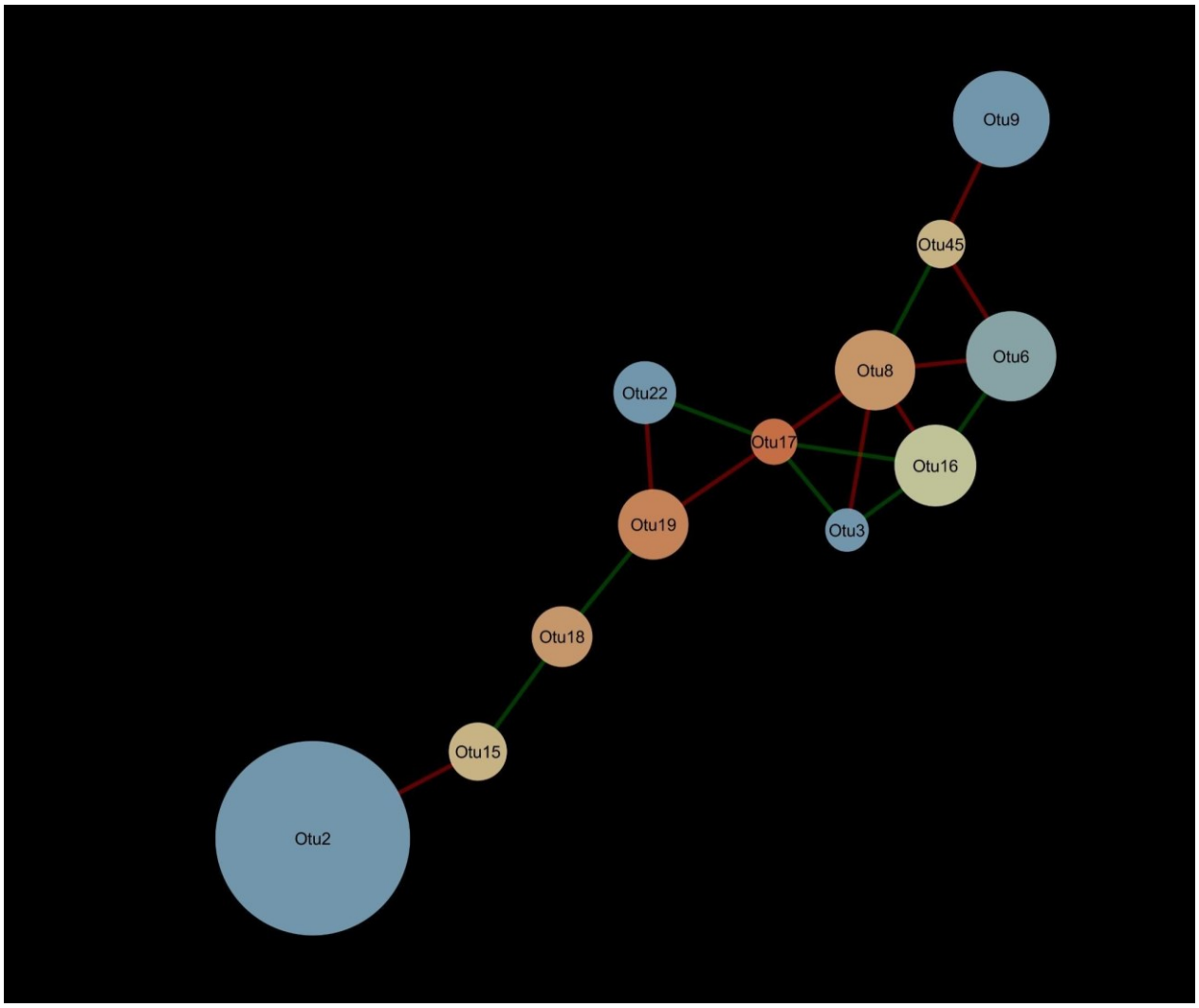

Figure 3. Network of interactions between the fungi forming the microbiome of canola rhizosphere in 2016. ZOTU size is proportional to relative abundance, and ZOTU shades indicate the degree of betweenness centrality: ZOTUs with warm colors are more connected with the other members of the network than the cold colored ones. Green edges indicate positive relationships and red edges, negative relationships.

Correlation between the hub taxa, the core-ZOTUs and canola yield

Spearman's correlations were used to assess the relationship between the eco-microbiome ZOTUs and canola yield for both years (Tableau 5). These correlations do not indicate that there is a causal relationship between the abundance of the fungi and canola yield. They may point to an adaptation to climatic conditions favorable for agricultural production rather than an effect on plant health. We have used these correlation values as a factor for identifying potential fungal species of interest for the enhancement of canola production. We were also able to correlate our hub taxa with yield. In 2013, canola yield was positively correlated with the relative abundance of three hub taxa: ZOTU14 (*Acremonium* sp., R = 0.46), ZOTU28 (*Sordariomycetes* sp., R = 0.28) and ZOTU179 (*Polyporales* sp., R = 0.59). In 2016, canola yield was positively correlated with the abundance of the hub taxon ZOTU17 (*Mortierella* sp., R = 0.47).

There was no consistency in the relationships between canola yield and ZOTU abundance within potential functional guilds of the eco-microbiome (Tableau 5). In 2013, the correlations between potential pathogens and yield were particularly interesting. Surprisingly, the relative abundances of half of the guild of 22 potential pathogens of the eco-microbiome were positively correlated with yield, the abundance of three potential pathogens was not correlated at all, and the abundance of the eight others was negatively correlated with yield. In addition, the relative abundance of two ZOTUs of the genus *Fusarium* (ZOTU13 and ZOTU27), known as a potent crop pathogenic genus, was weakly but positively correlated with yield, and the abundance of another ZOTU, *Fusarium solani* (ZOTU35), showed a strong positive correlation with yield. The relative abundance of two of the three potential endophytes observed (ZOTU56 and ZOTU102) showed a significant positive relationship with yield. Among the 17 potentially saprotrophic ZOTUs of the eco-microbiome, five had abundances that were positively correlated with canola yield, while the other five were negatively correlated. The relative abundance of the only ZOTU forming the core

microbiome, ZOTU1 (*Olpidium brassicae*), was positively correlated with yield in 2013 (R = 0.4135) but showed a strong negatively relationship with yield (*R* = -0.76) in 2016.

Discussion

We validated the existence of a fungal core microbiome in the canola rhizosphere and found that this core microbiome was not only maintained across pedoclimatic zones but also through time. This fungal core microbiome of the canola rhizosphere was composed of only one taxon, ZOTU1 *Olpidium brassicae*, which was present in all samples from the six site-year combinations of this study and across all rotations. We also validated the presence of hub taxa in the canola rhizosphere microbiome for both years of sampling. However, none of these hub taxa belonged to the core microbiome or the eco-microbiome for each year of sampling.

Olpidium brassicae

Olpidium brassicae (ZOTU1) was the only member of the core microbiome of canola rhizosphere in our study. ZOTU1 shared 100% identity with the NCBI reference sequence AB205213 for *O. brassicae*. This fungal species was by far the most abundant in the microbiome of the canola rhizosphere in 2013 and was one of the most abundant fungal species in 2016. It was also the only taxon that met the criteria for retention as a member of the eco-microbiome in 2016. In previous studies, *O. brassicae* was also found to be extremely abundant in the canola rhizosphere (Teakle, 1960; Campbell and Sim, 1994; Campbell et al., 1995; Sekimoto et al., 2011; Lay et al., 2018a). All these authors reported that *O. brassicae* is a plant root pathogen of the *Brassicaceae* family. However, Lay et al., (2018) found no association between *O. brassicae* abundance and canola yield. In the present work, in 2013 when the relative abundance of this fungus was lower, it was positively correlated with yield (Tableau 5) whereas when *O. brassicae* became more

dominant, in 2016, the correlation was strongly negative (R= −0.79). Hilton et al. (Hilton et al., 2013) similarly found canola yield losses in plots where *O. brassicae* was present in large numbers. This finding may point to a shift in the ecological behavior of *O. brassicae* when its population reached a certain threshold. It may also indicate that a minimum amount of rainfall or zoospores is required for the roots to become infected. Lay et al. (Lay et al., 2018a) noted that there was a great deal of taxonomic confusion between *O. brassicae* and *O. virulentus* in the literature, because of the genetic proximity of these two species. It seems also that *O.brassicae* only infects Brassicaceae and that *O. virulentus* is not infecting this family. *O. brassicae* infects plants but does not carry viruses as is the case for *O. virulentus*. However, these correlations with yield appear to indicate that further studies on the abundance of *O. brassicae* are required to determine the influence of this fungus on canola. Thus, as ITS do not have enough taxonomic resolution to consistently identify fungi at species level, isolation work must be considered to validate the presence of *O. brassicae* in canola rhizosphere in our experiment.

Variations in the fungal microbiome

There are previous reports in the literature on geographical and temporal variations in the fungal microbiome of the plant rhizosphere (Gaiero et al., 2013; Edwards et al., 2015; Coleman-Derr et al., 2016). In this study, we expected the canola cultivar and the level of crop diversification to be important factors of variation in the fungal microbiome of canola. Effects of canola cultivar on microbial community structure were previously been reported (Siciliano et al., 1998). However, in our study, canola cultivar had no effect on fungal diversity and little effect on fungal community structure. Canola cultivar influenced community structure only in Lethbridge, and only at the low level of crop diversification in 2013; it also affected community structure in canola monocultures

in 2016. In contrast with the canola cultivar, crop diversification had a strong influence on fungal community structure.

Crop plants modify the soil microbial community from which the following crop will recruit its own fungal associates (Hilton et al., 2013; Smith et al., 2013; Gan et al., 2015). Based on our results and those of other studies (Larkin, 2003; Larkin and Honeycutt, 2006; Lay et al., 2018a), it seems that different crop plants and cropping systems influence the community structure of rhizosphere microorganisms. However, crop diversification had no influence on fungal diversity (Tableau 1 and S1). This means that the number of ZOTUs did not change but their relative abundances varied across the crop rotations. According to the indicator species analysis (Tableau S3), the crop diversification treatments were not correlated with the same fungal taxa in both years of the experiment, even though the influence of the treatments on the fungal microbiome of the rhizosphere was significant. Perhaps climatic conditions influence the adaptation of different taxa to their ecological niche, a situation that could result in a huge variation in community structure and diversity like that found in the two years of the study.

Despite between-year variation in the fungal communities, the fungal microbiome of the canola rhizosphere was mainly composed of *Ascomycota* in both years, which is consistent with previous studies (Bennett et al., 2014; Tkacz et al., 2015b; Gkarmiri et al., 2017; Lay et al., 2018a). Interestingly, in those studies, *Olpidium* was reported to be the most abundant fungal genus in canola roots. In the present study, *Olpidium brassicae* was the most abundant fungus in the rhizosphere microbiome of canola in both years. However, the relative abundance of Olpidiaceae was much higher in 2016 than in 2013 (Figure 1C). Since soil fungi are particularly sensitive to soil moisture levels (Schmitt and Glaser, 2011; Wang et al., 2015; Zhang and Zhang, 2016), this variation may be attributed to the meteorological conditions, which may have caused a greater dominance of *Olpidium brassicae* and hence lower soil fungal diversity in 2016 (Ochoa-Hueso et

al., 2018). It is likely that the wet conditions in July 2016 were favorable for *Olpidium brassicae* zoospore production (Figure S1). The sampling was done at the end of July, a wet period in 2016 which may have favored fungal species like *Olpidium* that have swimming spores (James et al., 2006; Lay et al., 2018a). Furthermore, the drought in June may have reduced fungal diversity, as is common with drought (Toberman et al., 2008). Moisture availability drives biological processes in the prairie biome (Florinsky et al., 2002) and wide fluctuations in available moisture may well be the factor that shapes eco-microbiomes there. This would explain why the eco-microbiome of 2016 consisted solely of *O. brassicae*.

Canola fungal core microbiome and eco-microbiomes

In this study, we included the time variable in the definition of a core microbiome. Using this definition, we found that the fungal core microbiome of the canola rhizosphere was composed of only *O. brassicae*. Lay et al. (Lay et al., 2018a) provide support for this finding since they found *O. brassicae, F. merismoides*, *Fusicolla* sp. and one unidentified soil fungus in the fungal core microbiome of the canola rhizosphere in similar locations in the Canadian prairies in summer 2014. The difference between the relative scarcity of ZOTUs noted in the fungal core microbiome there in 2013 (47), compared to 2016 (1) and the results obtained in 2014 (4) by Lay et al. (2018a) is likely attributed to the difference in precipitation. The drought of June 2016 (Figure S1) most likely reduced fungal diversity while the abundant rainfall in July of the same year promoted zoospore release from *O. brassicae,* hence increasing the relative proportion of this species in our study. The core microbiome also changes with plant phenology (de Campos et al., 2013; Wagner et al., 2015). However, in this study, the same phenological stage was considered in both 2013 and 2016, suggesting that abiotic factors (such as moisture) were a major cause of the variations found in the microbiome. This is consistent with the finding that geographic locations greatly influence fungal

communities (Barnes et al., 2016). We need more sampling points over time to validate the stability of *O. brassicae* as the only core microbiome member of canola rhizosphere. How many more sampling points would be enough to fully characterize the variations in the canola rhizosphere core microbiome? Is the core microbiome that we identified in the Canadian prairies specific to this area or does a similar core microbiome exist in other canola-growing area around the world? These questions warrant further study.

The eco-microbiome of 2013 included many ZOTUs belonging to the genus *Fusarium* (Tableau 5). This is consistent with literature reports (Gkarmiri et al., 2017; Lay et al., 2018a) in which the genus *Fusarium* was found to be dominant in the canola rhizosphere. Colonization of the canola rhizosphere by *Fusarium* can lead to yield losses (Fernandez, 2007; Fernandez et al., 2008), but not always (Hamel et al., 2005; Vujanovic et al., 2006a; Yergeau et al., 2006). In our study, three of the eight *Fusarium* ZOTUs of the eco-microbiome of 2013 were positively correlated with yield (Tableau 5), which raises questions about the function of *Fusarium*. It is likely that *Fusarium* is adapted to soils that are rich in organic matter like the chernozems and acts as a saprophyte without necessarily causing plant diseases (Abdellatif et al., 2010). Non-pathogenic *Fusarium* species and isolates have also been detected in the roots of healthy plants and may even be antagonistic to virulent *Fusarium* species (Vujanovic et al., 2006b). *F. oxysporum*, one of the most commonly found species, has many *formae speciales*, which are usually very host-specific, and other strains which are non-pathogenic (Lievens et al., 2008). The antagonistic abilities of microbial species are particularly marked in endophytes and are potentially beneficial to the plants, protecting them from parasite infection (Clay, 1988; Jumpponen and Trappe, 1998; Xia et al., 2015). This may be the case for ZOTU35, which shared 100% identity with *Fusarium solani*, as it showed a positive correlation with yield in 2013 based on its Spearman's coefficient ($R = 0.70$). *Fusarium solani* is a wide-spectrum pathogenic species, underlying the fact that short sequences

are not well suited to correctly identifying fungi, particularly species of the genus *Fusarium*. It is also possible that *Fusarium solani* and higher canola yield co-occurred because they are both positively correlated with another factor, which was not measured in this study, and that there is no causal relationship.

The eco-microbiome of canola in 2013 included three potentially endophytic taxa: ZOTU102 (*Trichoderma koningii*), ZOTU49 (*Clonostachys rosea*) and ZOTU56 (*Exophiala* sp.). The genus *Trichoderma* is known for its antagonistic and hyperparasitic abilities, which has allowed some strains to be used as biocontrol agents. It has a suppressive effect on *Sclerotinia* spp., a pathogen of canola (Hermosa et al., 2000; Hirpara et al., 2017; Jalali et al., 2017). *Clonostachys rosea* has been reported as a potential pathogen, but it was also found to be nematophagous (Yang et al., 2007) and to have potential for biocontrol (Vega et al., 2008). Lastly, *Exophiala* is a dark septate endophyte (DSE). Certain DSEs are known to form symbiosis relationships increasing the fitness of their host plant (Jumpponen and Trappe, 1998; Jumpponen, 2001). The genus *Exophialia* has also been shown to increase drought resistance in sorghum (Zhang et al., 2017b). This is the first time that the presence of this DSE has been reported in the canola rhizosphere. Future studies should explore the influence of these taxa on canola health and productivity.

Interactions in the microbiome and influence of hub taxa on the community

Among the 85 taxa making up the 2013 co-occurrence network of the canola rhizosphere, only 22 belong to the eco-microbiome. Moreover, among the five ZOTUs classified as hub taxa in 2013 or 2016, none belonged to the eco-microbiome of the same year. This suggests that a significant number of the eco-microbiome members did not interact, or interacted only slightly, with the other members of the fungal microbiome of the canola rhizosphere. Four ZOTUs are identified as hub taxa in 2013—ZOTU14 (*Acremonium* sp.), ZOTU28 (*Sordariomycetes* sp.),

ZOTU45 (*Mortierella* sp.) and ZOTU179 (*Ganoderma applanatum*) —and one ZOTU in 2016, namely ZOTU17 (*Mortierella gamsii*). It has been reported that hub taxa have a very strong influence on the whole microbiome and on the health of the plant (Agler et al., 2016). The influence of the hub taxa on plant performance identified in the present study remains to be tested under controlled conditions, in structured experiments.

The modular structure of the 2013 network suggests two different hotspots of co-occurrences centered on ZOTU45 (*Mortierella* sp.) and ZOTU28 (*Sordariomycetes* sp.), and on ZOTU14 (*Acremonium* sp.) and ZOTU179 (*Ganoderma applanatum*). The negative co-occurrences between the fungal consortia centred on ZOTU45 and on ZOTU28 could indicate a difference between the ecological roles of each consortium if these consortia prefer different ecological niches. The negative co-occurrences between the fungal consortia could also indicate antagonistic relationships between ZOTU45 and ZOTU28. If each of these two hub taxa is surrounded by a consortium of other fungi they could beneficially interact with, the presence or the absence of one of the two hub taxa might have an impact on the abundance of the members of their respective consortium.

As the taxonomic resolution of the identification of ZOTU28 is too low, we cannot make a hypothesis about its potential ecological role in the fungal community. On the other hand, the genus *Mortierella* is a known saprophyte that is well represented in the Canadian prairies (Thormann et al., 2004; Gkarmiri et al., 2017). We noted that the ZOTUs of the genus *Mortierella* were negatively correlated with canola yield (Tableau 5), although the ecological guild of ZOTU45 (*Mortierella* sp.) should be saprophytic. ZOTU28 (*Sordariomycetes* sp.) and ZOTU45 (*Mortierella* sp.) are the only hub taxa that share negative co-occurrences. ZOTU14 (*Acremonium* sp.) and ZOTU28

(*Sordariomycetes* sp.) are positively correlated as is also the case for ZOTU179 (*Ganoderma applanatum*) and ZOTU14 (*Acremonium* sp.).

Acremonium is a genus that is often reported to have a saprophytic lifestyle. A wide range of *Acremonium* species have also been described as phosphorus solubilizers (Gudiño Gomezjurado et al., 2015). ZOTU14 could be a phosphorus solubilizing fungus which enhances soil P fertility, consistent with its positive correlation with canola yield (R = 0.45). *Ganoderma applanatum* is well known in Asian traditional medicine (Peng and Qiu, 2018). *Ganoderma applanatum* is a wood-decay fungus that is commonly found in temperate forest soils (Hood et al., 2018). It is therefore surprising to find this fungus in an agroecosystem characterized by non-ligneous plants; however, the NCBI identification makes ZOTU179 matches with 100% identity and coverage the sequence MF161255.1 labeled as *Ganoderma applanatum.* However, *Ganoderma applanatum* could degrade aromatic organic matter in the soil and thus make nitrogen and nutrients available for its consortium members.

In 2016, the network of potential interactions in the fungal community was less complex. The composition of this network was drastically different from that found in 2013, pointing to a difference in fungal co-occurrence and community structure which might indicate a shift in the functional activity of the fungal community. ZOTU17 (*Mortierella* sp.) was the only ZOTU identified as a hub taxon while it was not part of the 2013 network. ZOTU45 (*Mortierella* sp.) was still part of the network but its very low betweenness centrality value points to a decrease in its importance. Interestingly, ZOTU45 was still positively correlated with ZOTU8 (*Nectria* sp.) and negatively correlated with ZOTU9 (*Penicillium levitum*) in 2016, which is indicative of a persistent pattern of co-occurrence between these taxa.

Conclusion

In this work, we have shown that the core microbiome of the canola rhizosphere should be defined in space and time. We identified a fungal core microbiome which is influenced by rainfall patterns, at least in the Canadian prairies. This core microbiome was composed of a single ZOTU identified as *O. brassicae*. The temporal variability of microbial community structure and composition in the canola rhizosphere raises new questions related to the definition of the plant core microbiome. We identified five hub taxa that could have a significant impact on fungal communities in the canola rhizosphere; however, questions remain about their ecological function. The potentially important role played by *O. brassicae* is highlighted by this research, considering its dominance in the fungal communities and its relationship with canola yield losses when present in abundance in the canola rhizosphere. The diversification level of the cropping system was also found to have a significant effect on fungal community structure. This study provides information about fungal species in the canola rhizosphere that may be important for future enhancement of canola production in Canada through microbiome manipulation or development of new bio-inoculants.

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Mise en contexte du chapitre 3 : Bacterial communities of the canola rhizosphere : network analysis reveal a core microbiome shaping microbial interactions

Après avoir découvert la stabilité d'un core microbiome fongique et aux interactions au sein du microbiote fongique, s'intéresser aux communautés bactériennes avec les mêmes questions a été naturel. L'étude au chapitre 3 est donc basée sur la région 16S bactérienne de l'ADN extrait des échantillons de sol de la rhizosphère qui a servi à l'étude présentée au chapitre 2. L'organisation des composantes du microbiote bactérien de la rhizosphère du canola dans la prairie canadienne et les interactions bactériennes potentielles qui s'y trouvent sont présentées dans ce chapitre.

Ce chapitre s'appuie sur les échantillonnages réalisés en 2013 et 2016 avec Agriculture Canada. Les résultats de ce chapitre ont fait l'objet d'une publication dans la revue *Frontiers in Microbiology* en 2020.

Ma contribution dans cet article a été l'extraction d'ADN, la calibration et mise en place du pipeline bio-informatique, l'analyse statistique des résultats ainsi que l'élaboration de l'analyse de réseau, des illustrations et de la rédaction de l'article.

Chapitre 3 – Bacterial communities of the canola rhizosphere: network analysis reveal a core microbiome shaping microbial interactions

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Abstract

The rhizosphere hosts a complex web of bacteria interacting with one another that may modulate crucial functions related to plant growth and health. Identifying the key factors structuring the bacterial microbiome of the plant rhizosphere is a necessary step towards the enhancement of plant production and crop yield with beneficial associative microorganisms. We used a long-term field experiment conducted at three locations in the Canadian prairies to verify if: (1) the level of cropping system diversity influences the the α- and β-diversity of the bacterial microbiome of the canola (*Brassica napus*) rhizosphere; (2) the canola rhizosphere microbiome has a stable bacterial core; and (3) some highly connected taxa of this microbiome fit the description of hub-taxa. Five and eight years after cropping systems establishment, we sampled the rhizosphere of canola grown in monoculture, in a 2-phase rotation (canola-wheat), in a 3-phase rotation (pea-barley-canola), and in a highly diversified 6-phase rotation. The core microbiome of canola rhizosphere comprised only one Amplicon Sequence Variant, a hub taxon identified as cf. *Pseudoarthrobacter* sp. This ASV was also the only hub taxa found in the networks of interactions in both years at all three sites. We highlight a cohort of bacteria and archaea that were occurring with the core taxa.

Keywords: Bacterial communities, microbial ecology, agroecosystem, crop rotations, *Brassica napus*.

Introduction

A plant in its natural environment coexists with myriads of archaea, bacteria, fungi, as well as with other unicellular eukaryotic microorganisms, which constitute its microbiome. The rhizosphere is a hotspot of microbial interactions between species that have various ecological functions. These microbial communities are particularly important for plant health as they influence its development and its productivity (Barriuso et al., 2008; Bulgarelli et al., 2013; Bakker et al., 2014). Throughout their life, plant roots exude compounds creating the rhizosphere environment (Bais et al., 2006). Spatial and temporal variation in the composition of rhizodeposits allow plants to shape rhizosphere microbial communities to their benefit (Tkacz et al., 2015a; Pii et al., 2016; Eisenhauer et al., 2017).

Plant rhizosphere can host mutualistic microbes such as mycorrhiza or plant growth promoting bacteria (PGPB) that facilitate nutrient uptake, mitigate abiotic stress, and prevent root infection by pathogens (Barriuso et al., 2008; Farina et al., 2012; Fincheira and Quiroz, 2018). Plant-microbe and microbe-microbe interactions are diverse. Plants live in symbiotic and commensal relationships with numerous organisms, but they must also face pathogenic attacks (Hajishengallis et al., 2012). Rhizosphere organisms may impact each other, thus forming a complex web of interactions. For example, we know that mycorrhizal fungi have their own bacterial microbiome (Bianciotto et al., 2003; Iffis et al., 2014, 2017). These bacteria can be endophytic or form biofilm at the surface of the hyphae and can facilitate symbiosis with plants (Fitter and Garbaye, 1994; Iffis et al., 2014; Taktek et al., 2017).

Since the last decade, new generation sequencing (NGS) improved our access to microbial genetic information leading to significant advances in microbial ecology. This technological improvement lead to new ways of analyzing plant microbial communities (Duffy et al., 2007; Bulgarelli et al., 2013; Mendes et al., 2015). Now, we can identify with confidence

the factors shaping the microbial communities of the rhizosphere (Kuramae et al., 2011; Agler et al., 2016). The microbiome of the rhizosphere is extremely large and diverse. To summarize this complexity, we can divide it into pools of microbes based on their functions or occurrence (Ridout and Newcombe, 2016b). In a given community, microbial taxa are likely to be favored by their host, the plant, throughout its existence (Rout, 2014). These taxa are expected to be always part of the plant microbiome regardless the environment at a defined time *t*. According to Vandenkoornhuyse et al. (2015), these taxa always present in association with the plant forms the core microbiome and have preferential interaction with their host. The definition of a pool of micro-organisms always present at t time in the plant microbiome is appropriate for most of ecological study concerning plant microbiota as they mostly rely on a single sampling time. However, as we consider two different times of sampling in this paper, it was necessary to consider temporal variation in our definition of the core microbiome.

The interactions between microbes in the plant rhizosphere remains largely obscure. Next Generation Sequencing techniques provide information on the abundance of the taxa interacting in a microbiome, but it does not reveal the biochemistry happening between the microbes or their spatial organization in the ecosystem. That is why computational approaches aiming at identifying the nature of the links between the variations in the abundance of microbial taxa were developed as a complement to NGS (Ings et al., 2009; Deng et al., 2012; van der Heijden and Hartmann, 2016). Network analysis allows us to identify microbial taxa that are linked to others within the microbiome. Highly connected microorganisms can have a greater impact on plants and ecosystem functioning than others because they interact theoretically with many partners and antagonists; they are named hub taxa (Agler et al., 2016). Interactions occurring in microbial communities are known to be complex and difficult to retrieve with usual statistical methods (Kurtz et al., 2015a). However, the information provided by NGS can be processed through network analysis to identify cohorts represented by hub taxa,

thus simplifying the study of complex microbiome. Taktek et al., (2017) showed taxa that recruit beneficial organisms to host plant, but hub taxa could also be pathogens. Some hub taxa in the human microbiome can articulate infection by consortia of pathogens (Hajishengallis et al., 2012). As pathogens can affect the plant microbiome, this kind of pathogen hub taxa may occur in the rhizosphere. The hub taxa are a useful concept and help the understanding of the ecology of the root and rhizosphere ecosystem, which could lead to application in crop plant root systems.

Floc'h et al. (2020) identified a fungal core microbiome stable across time in canola rhizosphere composed of only one Operational Taxonomic Unit (OTU) identified as cf. *Olpidium brassicae*, but considerable changes in the microbiome across years. In the present study, we seek to test if similar patterns happen in the bacterial microbiome. Our group also recently found the canola microbiome to differ consistently from those of other crops grown in the same fields across the canola-growing zone of western Canada, and to possess a core bacterial microbiome comprising many putative PGPB, including taxa from the *Amycolatopsis*, *Serratia*, *Pedobacter*, *Arthrobacter* and *Stenotrophomonas* genera that were positively correlated with canola yield (Lay et al., 2018a). We also aim to investigate the temporal stability of the bacterial core-microbiome of canola rhizosphere in order to ascertain whether a persistent canola bacterial core microbiome exists. Another aim was to determine if the canola rhizosphere harbors bacterial hub taxa, and to visualize the variation between years in the structure of interactions among the bacteria living in the canola rhizosphere microbiome. We sought to identify a universal bacterial core microbiome in the rhizosphere of a plant species, specifically canola grown over two different years under a range of climatic conditions and biological environments. We thus used a range of crop diversification to have a large biological variation to identify what in the canola bacterial microbiome is not varying: core microbiome and hub

taxa. Canola is a crop of economical importance for the Canada food production and industry. It is also a good model plant to study rhizosphere microbiome as canola produce antimicrobial isocyanates (Zheng et al., 2014) leading to simpler microbial communities in its rhizosphere (Rumberger and Marschner, 2003).

Materials and Methods

Three sites located in three pedoclimatic zones of the canola-producing area of western Canada were used. Two sites were in Alberta, specifically in Lacombe (lat. 52.5°N, long. 113.7°W) and Lethbridge (lat. 49.7°N, long. 112.8°W), and the third site was in Scott, Saskatchewan (lat. 52.4°N, long 108.8°W). The soil in Lethbridge is a Brown Chernozem with a silty loam texture, while the Dark Brown Chernozems have a loamy texture at the Scott site and a clay loam texture at Lacombe. Rhizosphere samples were collected during the fourth week of July in 2013 and 2016, which corresponds to the end of canola flowering. Site description, experimental design and sampling are described in Floc'h et al. (2020). Three to four plants randomly selected within each plot were uprooted with a shovel. The shoots were removed and roots sere placed in plastic bags and brought to the laboratory on ice in a cooler. About 5 g of rhizosphere sol per plot was collected by gently brushing the roots. The samples were kept at 4°C before being shipped on ice to Lethbridge, Alberta where they were preserved at -80°C until DNA extraction. Four treatments were sampled, namely four levels of cropping system diversification organized in a simple randomized block design with four replicates: (1) monoculture of canola, (2) wheat-canola, (3) pea-barley-canola, and (4) lentil-wheat-Liberty Link canola-pea-barley-Roundup Ready canola (Tableau 6). Crops were grown according to best management practices. Information on crop management is described in Harker et al. (2015).

Tableau 6. Selected treatments from a long-term experiment established in 2008 at three different sites in the Canadian prairies (Harker et al., 2015a). The rotation phases examined in this study in 2013 and 2016 are underlined.

Diversification level	Cropping systems	
	2008–2013	2008–2016
Monoculture	RR-RR-RR-RR-RR-<u>RR</u>[1]	RR-RR-RR-RR-RR-RR-RR-RR-<u>RR</u>
Low	W-RR-W-RR-W-<u>RR</u>	RR-W-RR-W-RR-W-RR-W-<u>RR</u>
Medium	P-B-RR-P-B-<u>RR</u>	P-B-RR-P-B-RR-P-B-<u>RR</u>
High	Len-W-LL[2]-P-B-<u>RR</u>	Len-W-LL-P-B-Len-W-P-<u>RR</u>

[1] RR, canola 71-45, a Roundup Ready cultivar resistant to glyphosate.

[2] LL, canola InVigor 5440 Liberty Link, cultivar resistant to gluphosinolate

DNA extraction and amplification

DNA extraction was conducted as described in Floc'h et al. (2020). We constructed amplicon libraries for bacterial 16S RNA gene sequences by using target-specific PCR primers attached to Illumina overhang sequences for Nextera library preparation. The primer pairs were GTGCCAGCMGCCGCGGTAA (515F-Illu) and GGACTACHVGGGTWTCTAAT (806R-Illu), used by the Earth Microbiome Project (http://www.earthmicrobiome.org/emp-standard-protocols/16s/). PCR amplification and sequencing specificities were the same as described in Floc'h et al. (2020).

ASV determination and bioinformatic pipeline

The bioinformatic pipeline used for the processing of our 16S RNA gene sequences from 2013 and 2016 was DADA2 v1.8 (Callahan et al., 2016). We first used Cutadapt 1.13 to remove the primer part of the 16S RNA gene sequences. Then, we excluded the sequences with less than 200 bp as the base quality of the sequences tended to diminish below that threshold in our data with the command "filterAndTrim" with a "maxEE" score of 2, "trunQ" score of 2 and "minLen" argument set to 50. Then we calculated the error rate using the machine learning algorithm implemented in DADA2 with the command "learnErrors". As the error rate was satisfying according to developer's recommendations, we proceeded to merge the forward and reverse sequences using the command "mergepairs". Afterwards, the Amplicon Sequence Variant (ASV) Tableau was calculated and the chimeras eliminated using the command "makeSequenceTableau", resulting in a sequence length ranging from 250 to 253 nucleotides. ASVs were then identified using the naïve Bayesian classifier method on the databases SILVA and RDP, and the identity of ASVs of interest was verified manually in the NCBI using BLAST on the nt database for cross checking. With the taxonomic resolution of the 16S RNA gene, it is generally not possible to identify a bacterium at the species level. Thus, the identifications at species level presented here must be consider with caution despite they perfectly match (100% similarity and coverage) the references sequences of NCBI.

The MiSeq sequencing data generated as part of this work are available on Zenodo (https://zenodo.org/record/3626047#.XisHASZOmV4).

Data processing and statistical analyses

We first wanted to assess the variation occurring in canola rhizosphere caused by the crop diversification systems. The dataset was standardized by randomly subsampling the read data from each sample to the lowest number of reads (13241) encountered for a sample, using the function "rrarefy" of the vegan package v.2.4.6 in R v. 3.4.3, before calculating Chao1,

Shannon and Simpson's α-diversity indices using the same package. The significance of crop diversification effect on α-diversity indices were tested by analysis of variance (ANOVA) one year at a time, combining sites and blocks in one random effect with 12 blocks, and comparisons between treatment means were made with Tukey's post-hoc tests using the R package agricolae v1.3.1.

The core microbiome was determined as the set of organisms that are present in the microbiome at t and t+1 in all our sites and plots.

The effect of crop diversification on bacterial community structure was assessed by permutational multivariate analysis of variance (PERMANOVA), considering 12 blocks, using the function "adonis" of the vegan package v 2.4.6 in R v3.4.3, and the entire (non-subsampled) dataset. The blocked multi-response permutation procedure (BMRPP) was performed for the comparison of crop diversification levels using the R package "RVaidememoire" v0.9 that comprise Šidák correction for pairwise comparison.

Then, after determining the impact of crop diversification on canola rhizosphere, we aimed at identifying its universal bacterial core microbiome and hub taxa. Only the ASVs that were found in all plots in both years were assigned to the core microbiome of the canola rhizosphere.

To assess the interactions among bacterial taxa in the microbiome, we created a co-occurrence network using the package Spiec-Easi v 1.0.6 in R 3.4.3 (Kurtz et al., 2015a). The analysis was conducted over all bacterial rhizosphere microbiome of each year. The input data consisted in the matrix of the raw abundance of ASVs of one year of sampling. We first filtered the dataset to remove the ASVs with a frequency less than 20%. The Spiec-Easi run was done with the algorithm "mb" with the lambda min ratio set at 10^{-2} and 50 repetitions. We then imported the networks in Cytoscape 3.7.1 for plotting and used the "organic" layout to draw the network. Edges where defined as co-occurrences or mutual exclusion regarding the

positives or negatives values of inverse covariance linking the nodes. Betweenness centrality, defined as the fraction of the shortest path between all other nodes in the network containing the given node, and degree score, highlight central nodes and provide information about network architecture. A score of betweenness centrality and degree of connectivity greater than the score of 95% of the network taxa could suggest participation in multipartite interactions in the community and allow us to flag the highly connected taxa as hub-taxa. Hub-taxa were defined as the nodes possessing a score of betweenness centrality > 0.40 and a degree score > 10.

Spearman's correlations between abundance of hub-taxa and of their cohorts with canola yield were computed on R 3.4.3.

Results

Taxonomic affiliation of the bacterial microbiome of the canola rhizosphere

Our bio-informatic pipeline retrieved 2 175 992 reads from the 96 samples, that were assigned to 10 385 ASVs. Read number per sample ranged from 10 938 to 60 896. The ASVs belong mostly to four bacterial phyla that did not vary substantially for both year of study: Proteobacteria (~25%), Actinobacteria (~22.5%), Acidobacteria (~16%) and Chloroflexi (~13%) (Figure 4). Rarefaction curves indicated that read abundances were close to saturation for all the samples (Figure S4).

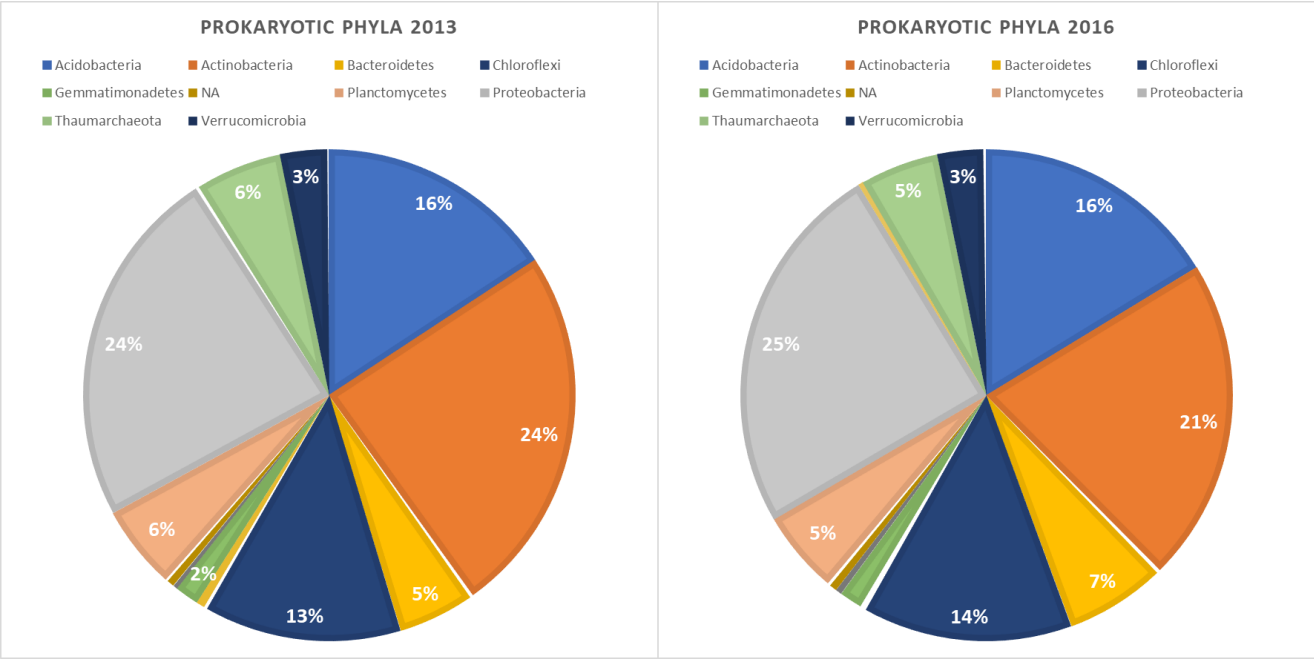

Figure 4. Relative abundance of dominant bacterial phyla in the rhizosphere of canola in 2013 and 2016.

Effect of treatments on communities

Crop diversification had no significant influence on α-diversity indices (Tableau 7) or on the structure of bacterial communities of canola rhizosphere in 2013 (Tableau 8). On the other hand, crop diversification impacted significantly the community structure of canola rhizosphere bacteria in 2016, where the bacterial rhizosphere communities of canola in monoculture and in the highly diversified system were structurally different (Tableau 8). However, this effect of diversification was weak ($P = 0.047$). Indicator species analysis revealed ASVs significantly associated with crop diversifications for both years of sampling. In 2013, the highest level of crop diversification had also the highest number of indicator species (15), whereas monoculture had nine and the diversification treatment with wheat and canola had only one (Tableau 9). No indicator species was found in association with the medium crop diversification level in 2013. In 2016, the monoculture showed the highest number of indicator species with 26 ASVs, the low crop diversification had four and the medium diversification had

one. No indicator species were found in association with the highest level of crop diversification in 2016. ASV108 (cf. *Thermomicrobiales* sp.) was and indicator species of the monoculture in 2013 and 2016; it is also the only indicator species to be found in both years of sampling.

Tableau 7. Mean values of bacterial α-diversity indices in the rhizosphere of canola under different crop diversification levels, in 2013 and 2016.

	2013				2016			
Index	Monoculture[1]	Low	Medium	High	Monoculture	Low	Medium	High
Shannon	5.256	5.297	5.318	5.219	5.397	5.321	5.183	5.227
Simpson	0,990	0,989	0,990[a]	0,988	0,990	0,989	0,985	0,986
Chao1	347,961	385,078	394,167[a]	365,999	441,829	429,286	396,087	428,412
Richness	346,467	382,942	396,768[a]	364,275	436,833	423,442	390,867	420,333

[1]Monoculture, canola monoculture; Low, wheat-canola rotation; Medium, pea-barley-canola rotation; High, lentil-wheat-LL-pea-barley-RR.

No significant differences were detected between the crop rotations by Tukey test $\alpha = 0.05$.

Tableau 8. Effects of crop diversification on the structure of the bacterial community in the canola rhizosphere, in 2013 and 2016, according to PERMANOVA (n = 12) α = 0.05, and significant differences between the structure of bacterial communities per crop diversification level according to Blocked Multi-Response Permutation Procedures (BMRPP) with Šidák correction for two-way comparisons (α = 0.035, n = 12).

	2013		2016	
Source	DF[1]	P-value	DF	P-value
Crop diversification	3	0,202	3	0,047*
residuals	44	NA	44	NA
		MRPP		
Monoculture[2]		a[3]		a
Low		a		ab
Medium		a		ab
High		a		b

[1]DF : Degree of Freedom

[2]Monoculture, canola monoculture; Low, wheat-canola rotation; Medium, pea-barley-canola rotation; High, lentil-wheat-LL-pea-barley-RR.

[3]Within each column, crop rotations associated with the same letter are not significantly different.

Tableau 9. Indicator species analysis of the prokaryotic ASV residing in the rhizosphere of canola in response to cropping diversification treatment in 2013 and 2016. Indicator values (IndVal) were tested for significance by Monte Carlo permutation tests (α = 0.05, 999 permutations). An empty row indicates an absence of indicator specie.

	2013			2016		
Crop diversification[1]	**Indicator species ASV**	**Closest identity**	***P* value**	**Indicator species ASV**	**Closest identity**	***P* value**
	ASV315	*Thermomicrobiales*	0,003**	ASV399	*Acidobacteria* sp.	0,002**
	ASV833	*Paracoccus* sp.	0,001**	ASV202	*Thermomicrobiales*	0,003**
	ASV380	*Chloroflexi* sp.	0,003**	ASV309	*Actinobacteria* sp.	0,002**
	ASV409	*Haliangium* sp.	0,010*	ASV576	*Thermomicrobiales*	0,007**
	ASV16	*Intrasporangiaceae*	0,026*	ASV276	*Chloroflexi* sp.	0,002**
	ASV108[2]	*Thermomicrobiales*	0,031*	ASV848	*Micromonosporaceae*	0,009**
	ASV280	*Chthoniobacter* sp.	0,042*	ASV119	*Rhizobiaceae*	0,013*
	ASV251	*Chthoniobacter* sp.	0,042*	ASV547	*Chloroflexi* sp.	0,003**
	ASV838	*Rhodanobacteraceae*	0,049*	ASV680	*Tepidisphaera* sp.	0,009**
				ASV334	*Rhizobiaceae*	0,015*
				ASV809	*Planctomycetes*	0,020*
Monoculture				ASV315	*Thermomicrobiales*	0,022*
				ASV321	*Thermomicrobiales*	0,034*
				ASV460	*Chloroflexi* sp.	0,032*
				ASV60	*Gaiella* sp.	0,037*
				ASV142	*Chthoniobacter* sp.	0,030*
				ASV181	*Tepidisphaerales*	0,034*
				ASV629	*Solirubrobacter* sp.	0,025*
				ASV552	*Pseudonocardia* sp.	0,036*
				ASV137	*Rubinisphaeraceae*	0,045*
				ASV613	*Chloroflexi* sp.	0,043*
				ASV108	*Thermomicrobiales*	0,039*
				ASV463	*Parafilimonas* sp.	0,041*

				ASV227	*Acidobacteria*	0,045*
				ASV183	*Chitinophagaceae*	0,047*
				ASV1287	*Pirellula* sp.	0,047*
	ASV529	*Rubrobacter* sp.	0,015*	ASV501	*Pyrinomonadaceae*	0,001**
				ASV577	*Streptosporangium* sp.	0,008**
Low				ASV377	*Lysobacter* sp.	0,030*
				ASV697	*Frankiales*	0,036*
Medium				ASV1624	*Acidobacteria*	0,033*
	ASV182	*Pseudomonas* sp.	0,002**			
	ASV214	*Gaiellales*	0,009**			
	ASV34	*Gaiella* sp.	0,012*			
	ASV283	*Haloactinopolyspora* sp.	0,014*			
	ASV498	*Rhizobiales*	0,014*			
	ASV624	*Bacteria*	0,017*			
	ASV93	*Nitrososphaeraceae*	0,016*			
High	ASV751	*Acidobacteria*	0,003**			
	ASV59	*Holophagae* sp.	0,027*			
	ASV24	*Burkholderiaceae*	0,027*			
	ASV53	*Nitrososphaeraceae*	0,025*			
	ASV248	*Iamia* sp.	0,029*			
	ASV127	*Acidobacteria*	0,046*			
	ASV262	*Sphingomonas* sp.	0,040*			
	ASV302	*Acidobacteria*	0,048*			

[1]Monoculture, canola monoculture; Low, wheat-canola rotation; Medium, pea-barley-canola rotation; High, lentil-wheat-LL-pea-barley-RR.

[2]ASV in bold are indicator species found in 2013 and 2016 in the same crop diversification.

Level of significance, "*" = $p < 0.05$, "**" = $p < 0.01$.

Core microbiome of canola rhizosphere

Only one bacterial ASV remained present across all the samples in every crop rotation for both years: ASV1. ASV1 was identified as cf. *Pseudoarthrobacter* sp. according to SILVA and RDP databases and was the most abundant bacterial ASV in the canola rhizosphere in both years of the study. Its relative abundance ranged from 3.4% of the bacterial community in 2013 to 2.6% in 2016 and was not influenced by cropping system diversification.

Network analysis of the bacterial microbiome

A network composed of 47 ASVs and 56 edges was found in 2013 (Figure 5). This network was modular and included 13 mutual exclusions and 43 co-occurrences between bacterial taxa. A module was organized around ASV12 (cf. *Acidobacteria* sp.) which shared 5 co-occurrences and 2 mutual exclusions. Another module was organized around ASV1 (cf. *Pseudoarthrobacter* sp.) which shared 9 co-occurrences and 3 mutual exclusions with other bacterial taxa. In 2016, the interaction network between bacteria was more complex than in 2013, with 51 ASVs and 83 edges (Figure 6). The network showed no modularity but was organized on ASV1 which shared 10 co-occurrences and 3 mutual exclusions with the other members of the network.

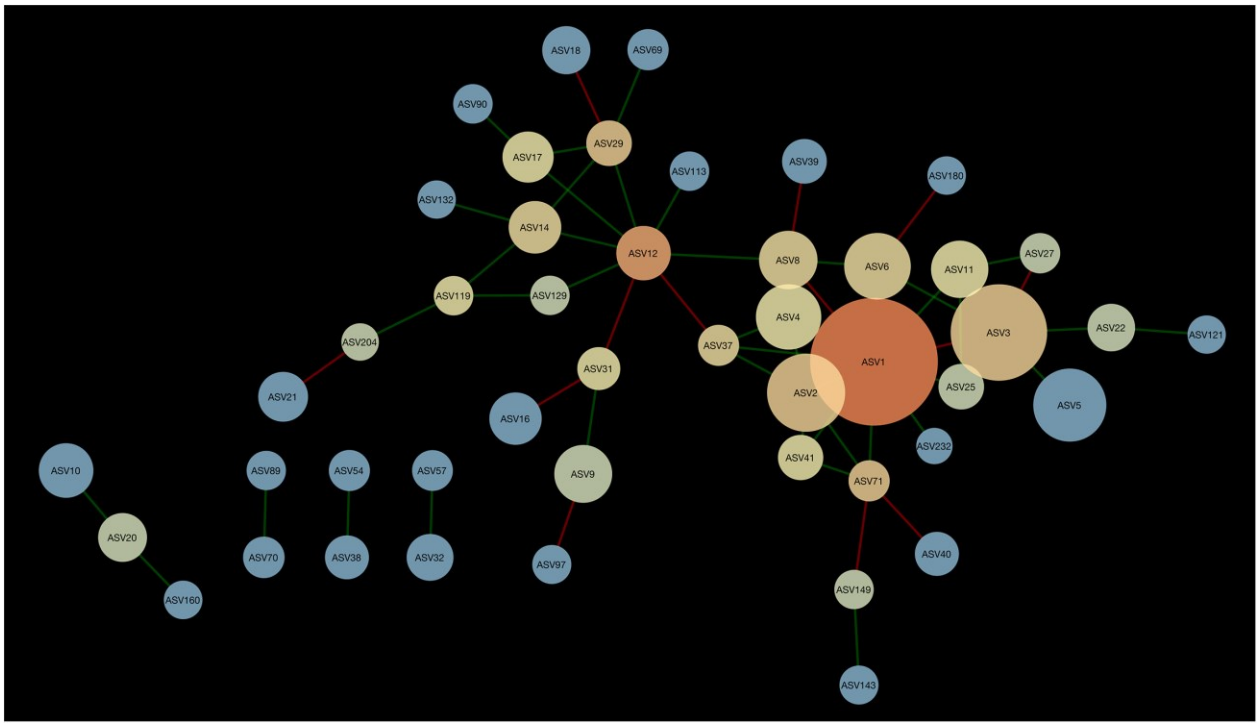

Figure 5. Network of interactions between bacteria forming the microbiome of canola rhizosphere in 2013. Dot size is proportional to the relative abundance of ASV, and shades indicate the degree of betweenness centrality: ASVs with warm colors are more connected with the other members of the network than the cold colored ones. Green edges indicate positive relationships and red edges, negative relationships.

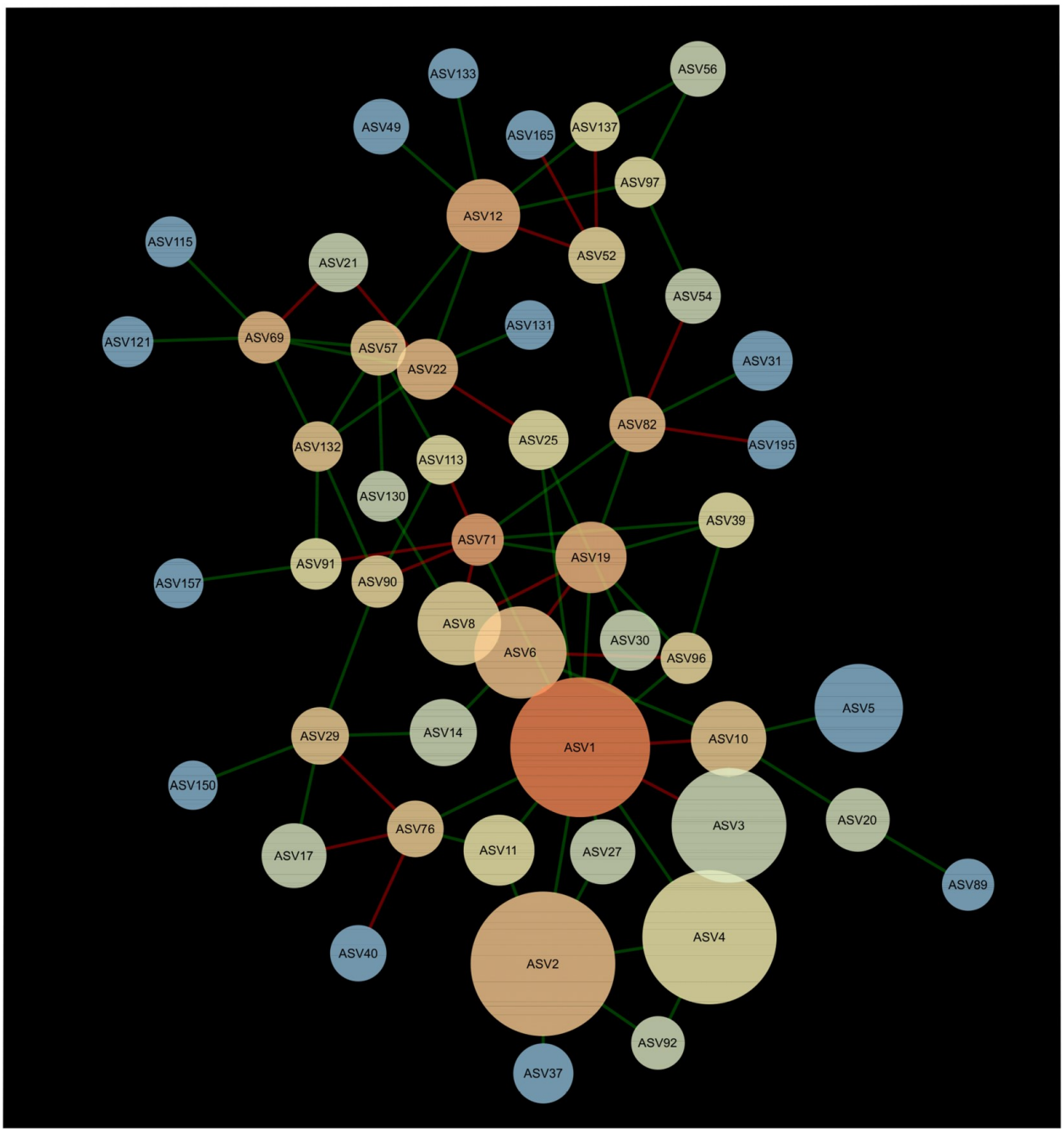

Figure 6. Network of interactions between the bacteria forming the microbiome of canola rhizosphere in 2016. Dot size is proportional to relative abundance of ASV, and shades indicate the degree of betweenness centrality: ASVs with warm colors are more connected with the other members of the network than the cold colored ones. Green edges indicate positive relationships and red edges, negative relationships.

There was one ASV identified as hub-taxa in 2013, and in 2016 with the network analysis, ASV1 (cf. *Pseudoarthrobacter* sp.), that was also the only member of the core-microbiome of canola rhizosphere. In 2013, ASV1 had a score of betweenness centrality of 0.44 and a degree score of 11, and in 2016 a score of betweenness centrality of 0.44 and a degree score of 13. No other ASV of the networks had values of betweenness centrality and degree score that were above the threshold of 95% as it was the case for ASV1. We were able to identify a cohort of bacterial taxa that were always connected with ASV1 in 2013 and in 2016 (Tableau 5). The types of interaction between ASV1 and its cohort members were consistent and remained unchanged between 2013 and 2016: in particular, ASV1 was always positively linked with ASV2 (cf. *Yersinia* sp.), ASV4 (cf. *Stenotrophomonas* sp.), ASV11 (cf. *Stenotrophomonas* sp.), ASV25 (cf. *Candidatus Nitrosocosmicus* sp.) and ASV71 (cf. *Paenarthrobacter* sp), and negatively linked with ASV3 (cf. *Nitrosphaeraceae* sp.) and ASV6 (cf. *Chloroflexi* KD4-96).

Correlation between ASV1 and its cohort members to canola yield

Spearman's correlations were used to assess the relationship between ASV1 and its cohort members with canola yield for both years (Tableau 10). ASV1 and most of its cohort members were not related to canola yield in 2013, only ASV6 showed a strong negative correlation (R = -0.40, $P = 0.0149$). However, in 2016, ASV1 was positively correlated with canola yield (R = 0.46, $P = 0.001$), as it was the case for ASV3 (R = 0.23, $P = 0.05$) and ASV71 (R = 0.45, $P = 0.0012$). ASV6 remained strongly negatively correlated with canola yield (R = -0.41, $P = 0.003$).

Tableau 10. Spearman's correlation between the hub taxa ASV1 and its cohort members with canola yield (N = 48) in 2013 and 2016.

ASV[1]	Identity	Hub taxa[2]	2013		2016	
			% relative abundance	Spearman r	% relative abundance	Spearman r
ASV1	*Pseudoarthrobacter* sp.	Y	3,430	ns	2,692	0,462*
ASV2	*Yersinia* sp	N	1,599	ns	2,829	ns
ASV3	*Nitrosphaeraceae*	N	2,275	ns	1,981	0,286*
ASV4	*Stenotrophomonas* sp.	N	1,129	ns	2,533	ns
ASV6	*Chloroflexi KD4-96*	N	1,172	-0,400***[2]	1,341	-0,412***
ASV11	*Stenotrophomonas* sp.	N	0,831	ns	0,734	ns
ASV25	*Candidatus Nitrosocosmicus* sp.	N	0,397	ns	0,422	ns
ASV71	*Paenarthrobacter* sp.	N	0,234	ns	0,203	0,45***

[1] ASV: Amplicon Sequence Variant

[2] Taxa with high connectivity in network analysis in 2013 and 2016 (see Material and Methods for details).

[2] Level of significance, "*" = $p < 0.05$, "**" = $p < 0.01$ and "***" = $p < 0.001$

Discussion

We validated the presence of a bacterial core microbiome in the canola rhizosphere that was not only stable across pedoclimatic zones but also through years. This core microbiome was composed of only one taxon, ASV1 identified as cf. *Pseudoarthrobacter* sp., which was also identified as a hub taxon and had a cohort of seven bacterial taxa with stable relationships across the two years of the study.

ASV1, cf. *Pseudoarthrobacter* sp.

This taxon was the only member of the bacterial core microbiome detected in the canola rhizosphere and the most abundant ASV for both years of sampling. With our current sequencing technology (Illumina MIseq), it is likely that prokaryotic ASV can be undetected if their abundance is low in a sample. ASV1 was identified as the only member of our core microbiome for both years of sampling. But it is probable that other less abundant prokaryotic members of this core microbiome exist as they were not detected for all our samples. Furthermore, as 16S RNA gene sequences obtained with Illumina MiSeq technology do not have enough taxonomic resolution to to distinguish between closely-related species, certainty about its exact identity remains lacking: ASV1 matches with 100% identity with at least 100 *Arthrobacter* and *Pseudoarthrobacter* sequences in NCBI database. *Arthrobacter* is a genus of gram-positive bacteria from the Micrococcaceae family that was subdivided in several other genera like *Pseudoarthrobacter* by (Busse, 2016). This genus is mainly formed of soil bacterial species (Busse, 2016). *Arthrobacter* is also a genus with many species known as PGPB (Chan and Katznelson, 1961; Manzanera et al., 2015; Ullah and Bano, 2015; Aviles-Garcia et al., 2016; Fincheira and Quiroz, 2018) that colonize the roots and rhizosphere of a large spectrum of agricultural crops, such as rice or tomato. Lay et al. (2018) reported an *Arthrobacter* operational taxonomic unit (OTU) that shared 100% identity

with ASV1 as a member of canola rhizosphere core microbiome for 2014 in similar sites of the Canadian Prairies. They also reported that their *Arthrobacter* OTU was positively correlated with canola yield as it was the case with ASV1 in 2016. Furthermore, an *Arthrobacter* sp. was previously shown to increase canola yield and acts as PGPB (Kloepper, 1988). Additionally, this genus was reported as a highly competitive and fast growing bacteria in canola rhizosphere (Tkacz et al., 2015a). Lay et al. (2018) also reported the presence of *Arthrobacter* sp. in wheat and pea rhizospheres in rotation with canola, but in smaller proportions. That omnipresence and abundance of ASV1 (cf. *Pseudoarthrobacter* sp.) in all our plots suggest a selection by canola and highlight this taxon as a good bacterial candidate as PGPB.

Variations in the bacterial microbiome

Bacterial communities are known to be sensitive to changes in abiotic factors such as pH and humidity, or nutrient availability (Norman and Barrett, 2016; Wan et al., 2020). As plants can actively control their rhizosphere microbiota through root exudates (Bais et al., 2006; Eisenhauer et al., 2017), we expected important changes in the bacterial communities between the crop diversification treatments. That was not the case. In 2013, no effect of crop rotation on bacterial community structure was detected and in 2016, the only two crop rotations that were significantly different were the two extremes: the monoculture of canola and the highest level of crop diversification, and the difference was low ($P = 0.047$). . Indicator species analysis showed those two crop diversification treatments as the ones that had the highest number of indicator species. It is possible that the number of indicator species (26) of the monoculture in 2016 with a dominance of *Chloroflexi* (Tableau 4) could be the source of its significant difference in term of community structure with the highest level of crop diversification, even if no significant differences was found in 2013 between those two crop diversification treatments. Long lasting effect of agricultural

management such as crop rotation are reported in the literature (Buckley and Schmidt, 2001). In the Brazilian Amazon for example, crop management seems to have a significant impact of microbial community structure (Jesus et al., 2009). For temperate environments, our results are consistent with Jesus et al., (2016) who did not find any influence of crop rotation on their soil microbial communities in Michigan.

Therefore, these results need to be tempered. In our study, we accessed the microbiome of canola rhizosphere that is principally buffered by canola root exudates (Rumberger and Marschner, 2003), mitigating the effects of previous crop in the diversification. We do not know if the crop diversification levels influenced the bulk soil bacterial communities. However, our results showed that canola recruited similar bacterial communities between all crop diversification levels in 2013. It is also possible that a part of the canola rhizosphere microbiome can be inherited maternally with the seed microbiome as it is known to be the case for a wide range of plants (Shade et al., 2017). That could explain the similarities of canola rhizosphere community structure between our crop diversification levels. It is also possible that the bacterial communities in our crop diversification were not host-specific but colonize the roots of both canola and the previous crop species used in the crop rotation as it was reported by Lay et al. (2018). They found that the bacterial microbiome of canola rhizosphere was more similar to the one found in pea than the one found in wheat rhizosphere. But here, we did not find significant difference in community structure between the low, medium and high crop diversification in 2013 and only a weak difference in 2016, suggesting that previous crop had no detectable influence on bacterial communities of canola rhizosphere. Thus, we can consider the influence of abiotic variation on bacterial community in our study. A previous study showed that soil type and rain frequency have stronger effect on the microbial community of canola rhizosphere that crop rotations (Schlatter et al., 2019). Floc'h et al. (2020)

also found drastic variation in fungal rhizosphere community structure that could be linked with difference in water availability in canola rhizosphere. In the present study, as the sampling times and place were the same as Floc'h et al., (2020), the differences in the amounts of rainfall prior to sampling between 2013 and 2016 were the same. But this difference between the first year of sampling that was dryer than the second one (Figure S1) is not consistent with the stability of the bacterial community structure observed in 2013 and 2016 as it was the case in Floc'h et al., (2020). This stability is also to be underlined as bacterial microbiome of canola rhizosphere also showed stability in its microbial interactions between 2013 and 2016.

Interactions in the bacterial microbiome

Using the same samples, Floc'h et al. (2020) reported drastic changes between years in the co-occurrence dynamics of fungal communities in the canola rhizosphere. In the present work, if the complexity of the interaction network changed between the two years of sampling, the pool of bacteria forming its nucleus remained the same. The hotspot of interaction was always articulated around ASV1 (*Pseudoarthrobacter* sp.). ASV1 was the only member of the bacterial core-microbiome of canola rhizosphere and the only hub taxa detected with network analysis for both years of the present study. If the fungal hub taxa of canola rhizosphere were subject to change between years of study, it was not the case for bacteria.

For both year of sampling, ASV1 was interacting with seven other taxa: ASV2 (cf. *Yersinia* sp.), ASV3 (cf. *Nitrosphaeraceae* sp.), ASV4 (cf. *Stenotrophomonas* sp.), ASV6 (cf. *Chloroflexi* KD4-96), ASV11 (cf. *Stenotrophomonas* sp.), ASV25 (cf. *Candidatus Nitrosocosmicus* sp.) and ASV71 (cf. *Paenarthrobacter* sp). The persistence of these interactions across time suggests a close interaction of ASV1 with these other members of the community. The fact that ASV6 was negatively linked with ASV1 and strongly negatively correlated with canola yield deserves interest.

This phylum is known to be a common inhabitant of several agricultural plants like potato (İnceoğlu et al., 2011), lettuce (Cardinale et al., 2015) or maize (Peiffer et al., 2013) and was found in a large spectrum of ecosystems as forest soils, grassland, and tundra (Fierer et al., 2012). *Chloroflexi* are also particularly present as indicator species in canola rhizosphere in the monoculture: 3 on 9 ASVs in 2013 and 9 on 26 ASVs in 2016 (Tableau 4). Monoculture of canola was found to have lower yield values across time and favorize accumulation of microbial pathogenic taxa in soil (Hummel et al., 2009; Harker et al., 2015a). *Chloroflexi* have been reported in the canola rhizosphere previously but there was no mention of *Chloroflexi* species being pathogenic to canola (Gkarmiri et al., 2017). Correlations do no indicate that there is a causal relationship between the abundance of the different bacterial ASVs and canola yield. They may point to bacteria that benefit from higher canola growth, or to a condition favorable both for canola productivity and to these bacteria, rather than an effect of the bacteria on plant health. However, we have used the correlation values as an index for identifying potential bacterial ASV of interest for the enhancement of canola production, since the bacteria directly beneficial to canola would be among those showing positive correlation with yield. It is possible that ASV6 could be a commensalistic bacteria occurring with fungal pathogens of canola or other microbes that are favored by monoculture (Floc'h et al., 2020a) or pathogenic itself. Therefore, further studies should be conducted on that ASV, such as test of pathogenicity or cross-kingdom network interactions to verify its occurrence with other pathogenic microbes.

In the cohort of taxa associated with ASV1, two other taxa were positively correlated with canola yield in 2016: ASV3 and ASV71. ASV71 was identified as *Arthrobacter*, so it is phylogenetically closely related to ASV1, and could be a potential PGPB with ASV1 (Manzanera et al., 2015; Ullah and Bano, 2015; Pereira et al., 2019). ASV3 was an archaea identified as a

member of the *Nitrosphaeraceae* family, and was poorly correlated with canola yield. Little information about this family is available. Presence of *Nitrosphaeraceae* was previously reported by Gkarmiri et al. (2017), and Lay et al. (2018) found core microbiome members of canola rhizosphere that were close to *Nitrocosmicus* spp. Another study mention *Nitrosphaeraceae* as microbial taxa retrieved from spacecraft surfaces (La Duc et al., 2012). This family appears to be widely distributed in the environment. As hub taxa can have very strong influence on the whole microbiome and plant performance, ASV1 and its cohort members will need to be isolated and tested under controlled conditions in structured experiments to detail their potential PGPB activity or pathogenic behaviour on canola.

Conclusion

In this work, we have shown that the bacterial core microbiome of canola rhizosphere is stable across time between two years of production that largely differed in their distribution of summer precipitations. We identified a bacterial core microbiome composed of a single ASV identified as cf. *Pseudoarthrobacter* sp. Our previous study on the fungal microbiome of canola rhizosphere showed the presence of a fungal core microbiome strongly influenced by rainfall patterns (Floc'h et al., 2020a), while it appears that it was not the case for the bacterial core-microbiome. We identified the only ASV of the core microbiome to be also a hub taxon in both years of sampling, with a cohort of associated bacteria that did not change their correlation type with it between years. However, questions are raised about the ecological role of this core-hub-taxon and its cohort of related bacteria and archaea. The highest level of diversification of cropping systems was found to have a significant influence on bacterial community structure, but only in 2016, suggesting that the bacterial microbiome of canola rhizosphere could be more stable than its fungal microbiome. The main differences in community structure was between monoculture of

canola rich in *Chloroflexi* indicator species and our highest level of crop diversification. This study provides information about bacterial and archaeal species in canola rhizosphere that could be important for future enhancement of canola production in Canada through microbiome manipulation or development of new cohorts for bio-inoculants.

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Mise en contexte du chapitre 4 : Inter-kingdom networks of canola microbiome reveal *Bradyrhizobium* as keystone species and underline the importance of bulk soil in microbial studies to enhance canola production.

Ayant montré l'existence d'un core microbiote fongique fluctuant au cours du temps et la présence d'une espèce de bactérie comme core, je me suis ensuite intéressé à l'interaction que pouvaient développer les deux règnes fongique et bactérien. Ces deux groupes d'organismes très différents cohabitent dans un même espace où ils sont forcément en interaction. Mais comment s'expriment ces interactions ? C'est la question qui a motivé l'étude du chapitre 4. L'autre raison était aussi la nécessité d'avoir plus de données pour l'établissement de la présence ou de l'absence d'un core microbiote et de hub-taxa stables au cours du temps chez le canola. Cela a été rendu possible grâce à l'échantillonnage de 2018 du même projet que les deux précédent chapitre, à la notable différence que lorsque j'ai modifié le protocole expérimental, j'ai demandé l'échantillonnage des racines et du plein sol du canola ainsi que l'ajout d'un site supplémentaire : Melfort.

Les résultats de ce chapitre ont fait l'objet d'une publication dans la revue *Microbial Ecology* en 2021.

Ma contribution dans cet article a été la mise en place du protocole d'échantillonnage, l'extraction d'ADN, la calibration et mise en place du pipeline bio-informatique, l'analyse statistique des résultats ainsi que l'élaboration de l'analyse de réseau, des illustrations et de la rédaction de l'article.

Chapitre 4 – Inter-kingdom networks of canola microbiome reveal *Bradyrhizobium* as keystone species and underline the importance of bulk soil in microbial studies to enhance canola production

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Abstract

The subterranean microbiota of plants is of great importance for plant growth and health, as root-associated microbes can perform crucial ecological functions. As the microbial environment of roots is extremely diverse, identifying keystone microorganisms in plant roots, rhizosphere and bulk soil is a necessary step towards understanding the network of influence within the microbial community associated with roots and enhancing its beneficial elements. To target these hot spots of microbial interaction, we used inter-kingdom network analysis on the canola growth phase of a long-term cropping system diversification experiment conducted at four locations in the Canadian prairies. Our aims were: to verify whether bacterial and fungal communities of canola roots, rhizosphere and bulk soil are related and influenced by diversification of the crop rotation system; to determine whether there are common or specific core fungi and bacteria in the roots, rhizosphere, and bulk soil under canola grown in different environments and with different levels of cropping system diversification; and to identify hub taxa at the inter-kingdom level that could play an important ecological role in the microbiota of canola. Our results showed that fungi were influenced by crop diversification, which was not the case for bacteria. We found no core microbiota in canola roots but identified three core fungi in the rhizosphere, one core mycobiota in the bulk soil and one core bacteria shared by the rhizosphere and bulk soil. We identified two bacterial and one fungal hub taxa in the inter-kingdom networks of the canola rhizosphere, and one bacterial and two fungal hub taxa in the bulk soil. Among these inter-kingdom hub taxa, *Bradyrhizobium* sp. and *Mortierella* sp. are potentially influential on the microbial community and the plant. To our knowledge, this is the first inter-kingdom network analysis utilized to identify hot spots of interaction in canola microbial communities.

Keywords: sequencing, plant microbiota, soil, rhizosphere, roots

Introduction

Plant subterranean microbiota have often been described as a “black box” (Horton and Bruns, 2001; Cortois and De Deyn, 2012; Pickles and Pither, 2014; Fernandez and Kennedy, 2015) a term referring to an inherent complexity of inner workings that makes a system difficult to grasp in its entirety. We can define plant subterranean microbiota as composed of different biotopes, notably: the root interior, the rhizosphere, and the bulk soil. The root and rhizosphere microbiota are highly influenced by the plant. Rhizodeposits recruit root symbionts and shape the microbial community of the rhizosphere (Rudrappa et al., 2008; Berendsen et al., 2012; Patel et al., 2015). Microbial communities shaped by the plant in the rhizosphere and root interior can protect it against pathogens and enhance its growth and production (Bais et al., 2006; Berendsen et al., 2012; Chaparro et al., 2013; Pageni et al., 2014; Latz et al., 2016). Bulk soil microbiota, while important to plant health, are less influenced by the plant due to their distance from the roots. However, the bulk soil microbiota, along with the seed microbiota, are the inoculum from which the plant recruits its rhizosphere microbiota, and there is a direct link between bulk soil microbial composition and rhizosphere composition, as a substantial portion of the microbiota of these two biotopes interact (Bhattacharyya and Lee, 2016; Schenkel et al., 2018; Raza et al., 2020). In addition, the microbes in the bulk soil can produce volatile compounds that influence plant health and development (Ryu et al., 2003; Cordovez et al., 2018; Schenkel et al., 2018; Garbeva and Weisskopf, 2020).

Due to their proximity, the biotopes of the plant subterranean microbiota should influence each other, and their microbes should interact. Microbial co-occurrence network analysis is a tool increasingly used to apprehend the complexity of microbial dynamics in plant and soil ecosystems (Aderhold et al., 2012; Faust and Raes, 2012; Aires et al., 2015; Kurtz et al., 2015a; Alshawaqfeh et al., 2017; Nahar et al., 2020). Analysis of these networks helps identify microbial taxa of ecological interest, particularly those linked to other members of the

microbial community: the hub taxa (Benedek et al., 2007; Berry and Widder, 2014; Banerjee et al., 2016; Floc'h et al., 2020a, 2020c). Hub taxa can modulate, via their shared interactions, the composition and diversity of the plant microbiota, affecting agronomic production, plant growth and productivity (Berry and Widder, 2014; Agler et al., 2016; van der Heijden and Hartmann, 2016). Network analysis at the inter-kingdom level can reveal different microbial dynamics that are complementary. Fungal and bacterial communities interact with each other, and, considered as a whole, can be used to identify hub taxa at a higher level of ecological complexity than previously reported (Banerjee et al., 2016; Vannini Candida et al., 2016).

In this study, our targeted plant is canola (*B. napus*) from the gluphosinate-tolerant variety L241C, a highly valuable crop grown by producers across the Canadian Prairies (Smith et al., 2013; Rempel et al., 2014). Most studies about canola microbial ecology have targeted the root interior and the rhizosphere (Dunfield and Germida, 2003; Farina et al., 2012; Lay et al., 2018a; Floc'h et al., 2020c, 2020a; Taye et al., 2020). Little is known about the ecology of canola bulk soil microbiota or their overlap with root and rhizosphere microbiota. Within these three biotopes, aside from the hub taxa, other microbes may be ecologically important. Floc'h et al., (2020a, 2020b) reported several fungi and bacteria in the canola rhizosphere that were always present despite variations in crop rotation and environmental conditions. These organisms, also reported in canola roots by Lay et al., (2018a) were identified as core fungi and core bacteria. According to Vandenkoornhuyse et al., (2015), the ever-present taxa associated with a given plant form its core microbiome and have a preferential interaction with their host.

We aimed to identify hub taxa and universal components of the core microbiota in canola roots, rhizosphere, and bulk soil. We used a gradient of crop diversification levels to create variability in these biotopes. Building on Floc'h et al., (2020a, 2020c) who identified hub taxa in the fungal and bacterial fractions of the canola rhizosphere microbiota, respectively, we aimed to consider the canola subterranean microbiome as a whole in order to identify core fungi,

core bacteria, and several inter-kingdom hub taxa that could be of ecological importance for canola health and production.

Material and Methods

Experimental Design and sampling

Our study was conducted in 2018 using a subset of plots from a long-term experiment initiated in 2008. The experiment, which was replicated at four locations in the Canadian Prairies, tested the effects of diversification in canola-based cropping systems, with all rotation phases present each year. Our study used the canola phases of three crop rotation systems. The canola grown was the glufosinate-tolerant variety L241C. The three crop diversification treatments used in this study were: (1) monoculture of canola, (2) wheat-canola, and (3) pea-barley-canola (Tableau 1). These treatments were applied in a randomized complete block design with four blocks at each of four experiment sites.

Tableau 11. Selected crop diversification treatments from a long-term experiment established in 2008 at three different sites in the Canadian prairies (Harker et al., 2015a). The rotation phases examined in this study in 2018 are underlined.

	Cropping systems
Diversification level	2008-2018
Monoculture	C[1]-C-C-C-C-C-C-C-C-C-C
Low	C-W-C-W-C-W-C-W-C-W-C
Medium	P-B-C-P-B-C-P-B-C-P-B-C

[1] C. canola; W. wheat; P. pea; B. barley.

The four experiment sites were located in three pedoclimatic zones of the canola-producing regions of Canada. Two sites were in Alberta in the sub humid Brown soil zone: one in Lacombe (lat. 52.5°N, long. 113.7°W) and the other in Lethbridge (lat. 49.7°N, long. 112.8°W), and two were in Saskatchewan, one in sub-humid Grey soil zone at Melfort (lat. 52.8°N, long. 104.6°W) and another at Swift Current in the semi-arid Brown soil zone (lat. 50.3°N, long. 107.7°W). Crops were grown according to best management practices, as described in Harker et al., (2015). With the exception of the Lethbridge site, the growing season at all sites was characterized by more frequent rain in July just before sampling (Figure S5).

Root, rhizosphere, and bulk soil samples were collected at the end of the canola flowering period, after 50% of flowers on the raceme had opened. This occurred in the fourth week of July 2018. This is the developmental stage of canola that most determine its yield. Three to four plants within each plot were randomly selected and uprooted with a shovel. The shoots were removed, and roots were placed in plastic bags and brought to the laboratory on ice in a cooler. Soil tightly attached on root samples were considered as rhizosphere soil. About 5 g of rhizosphere soil per plot was collected by gently brushing the roots. The brushed roots were then gently washed with sterilized distilled water. The bulk soil was sampled with a 2 cm diameter soil probe at a 7 cm depth, exactly in between two seed rows. The samples were kept at 4°C before being shipped on ice to the laboratory in Québec City, Québec, where they were preserved at -80°C until DNA extraction.

More details on site description, experimental design and sampling methods are provided in Floc'h et al. (2020a).

DNA extraction and amplification

Microbial DNA was extracted from an amount of 250 mg of rhizosphere soil samples using the PowerSoil DNA isolation kit (Qiagen, Toronto, TO, Canada) according to the manufacturer's instructions. DNA was eluted in a volume of 10 µL tris-HCL buffer and quantified using a Qbit 2.0 fluorometer (ThermoFicher, Saint-Laurent, QC, Canada). We constructed amplicon libraries for fungal ITS sequences by using target-specific PCR primers attached to Illumina overhang sequences for Nextera library preparation. The primer pairs were ACACTGACGACATGGTTCTACACTTGGTCATTTAGAGGAAGTAA (ITS1F-Illu) and TACGGTAGCAGAGACTTGGTCTCTGCGTTCTTCATCGAT (5.8A2R-Illu). Each 25-µL PCR reaction consisted of 0.10 µL of forward and reverse primers, with 19.6 µL H_2O, 2.5 µL 25 mM $MgCl_2$, 12.5 µL KAPA HiFi Hotstart ReadyMix (Kapa Biosystem, Cape Town, South Africa) and 1 µL of sample DNA. The reaction conditions were as follows: 95°C for 5 min, 25 cycles of 94°C for 45 sec, 52°C for 60 sec, and 72°C for 30 sec with a final extension at 72°C for 7 min. PCR products were verified by electrophoresis on 1% agarose gels. Dual Nextera indices were then attached to PCR products based on the protocol "16S Metagenomic Sequencing Library Preparation" provided by Illumina (part no. 15044223 rev. B). The final purified product was quantified by Qubit Fluorometric Quantitation (Thermofisher Scientific, Waltham, MA). Libraries were pooled in equimolar amounts before sequencing in rapid paired-ends 250 bp (PE250) mode on an Illumina MiSeq system, using the 500-cycle MiSeq reagent kit v.2 in accordance with the manufacturer's recommendations.

Amplicon libraries for bacterial 16S rRNA gene sequences were constructed by using target-specific PCR primers attached to Illumina overhang sequences for Nextera library preparation. The primer pairs were GTGCCAGCMGCCGCGGTAA (515F-Illu) and

GGACTACHVGGGTWTCTAAT (806R-Illu). This primer set was selected because it is used by the Earth Microbiota Project (http://www.earthmicrobiota.org/emp-standard-protocols/16s/). Two PCR reactions were performed to prepare the amplicon library. In the first PCR reaction, the V4 hypervariable region of prokaryotic 16S RNA genes was amplified using the previously described primers (515F and 806R). The PCR reaction was performed in a 25-µL reaction mixture containing 1 µL of template DNA, 1 × PCR-buffer (Qiagen, Germantown, MD, USA), 1.8 mM $MgCl_2$, 1.25 µL of 5% dimethylsulfoxide (DMSO), 0.2 mM dNTP, 0.5 U Taq DNA polymerase (Roche, Branford, CT, USA), and 0.6 µM of each primer. The 5' ends of the forward and reverse primers were tagged with CS1 (ACACTGACGACATGGTTCTACA) and CS2 (TACGGTAGCAGAGACTTGGTCT), respectively, which were used as anchors for the second PCR reaction. The conditions to amplify the prokaryotic 16S rRNA fragments consisted of an initial denaturation at 94°C for 2 min, 33 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 30 s, followed by a final elongation at 72°C for 7 min.

The second PCR reaction was used to add barcodes to each sample and the Illumina sequencing adapters. This PCR reaction was performed in a 20-µl reaction mixture, containing 1× PCR-buffer (Qiagen, Germantown, MD, USA), 1.8 mM $MgCl_2$, 1 µL of 5% DMSO, 0.2 mM dNTP, 0.5 U Taq DNA polymerase (Roche, Branford, CT, USA), 2 µM of Nextera XT index primers (Illumina Inc., San Diego, CA, USA), and 1 µL of 1/150 dilution of the first PCR products. The PCR conditions were as follows: initial denaturation at 95°C for 10 min, 15 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 1 min followed by a final elongation at 72°C for 3 min. After the second amplification, PCR products were quantified using a Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, Canada) and a Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (D-Mark, Canada). The

amplicon library was purified using calibrated AMPure XP beads (Agencourt Bioscience, Beverly, MA), and the average size and quantity of each library were assessed on the LabChip GX (Perkin Elmer, Waltham, MA) instrument. The library was then sequenced on Illumina MiSeq using the paired-end 250 protocol at the Genome Québec Innovation Centre of McGill University (Montréal, Canada).

ASV determination and bioinformatic pipeline

Bioinformatics were used in QIIME2 environment version 2021.4 (Bolyen et al., 2019). The bioinformatic pipeline used for the processing of our ITS and 16S rRNA gene sequences was DADA2 v1.18.0 (Callahan et al., 2016). First, we used Cutadapt 3.4 to remove the primer part of the ITS and 16S rRNA gene sequences with "minimum-length" at 50 and "p-error-rate" at 0.1. Then, we excluded the sequences with less than 220 bp with the command "—p-trunc-len", as the base quality of the sequences tended to diminish below that threshold in our data. Next, the amplicon sequence variant (ASV) Tableau was calculated, and chimeras eliminated, resulting in a sequence length ranging from 250 to 253 nucleotides. ASVs were then identified using the naïve Bayesian classifier method on the databases SILVA and RDP, and the identities of ASVs of interest were verified manually using BLAST on the NCBI nt database. With the taxonomic resolution of the ITS and 16S RNA gene, it is generally not possible to identify a bacterium or fungus at the species level. Thus, the identifications at species level presented here must be considered with caution despite the fact that they perfectly match (100% similarity and coverage) the NCBI reference sequences.

The MiSeq sequencing data generated as part of this work are publicly available on Zenodo (https://doi.org/10.5281/zenodo.5028181).

Data processing and statistical analyses

To assess the variation induced in the canola rhizosphere by crop diversification systems, the datasets were filtered from their rare ASVs using QIIME2 with "--p-min-frequency" set to 17 and "--p-min-samples" set to 1.

The effect of crop diversification on bacterial and fungal community structure was assessed by permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001), considering 16 random blocks (four blocks per each of the four sites) and our three crop rotation treatments as fixed effects, using the function "adonis" of the vegan package v2.5.7 (Oksanen et al., 2013) in R v4.1.0, and the set of relative abundance data.

We then aimed to identify the universal fungal and bacterial components of the core microbiota and hub taxa in each biotope of the canola subterranean microbiome. We defined the core microbiota of a biotope as the set of microbial ASVs that were present in the microbiota of a particular biotope at all sites and plots. We also looked for ASV core candidates that were present in the samples at 90% and 80% frequency. To assess the interactions among bacterial and fungal taxa in the microbiota, we created a co-occurrence inter-kingdom network using the package Spiec-Easi v1.1.0 in R 4.1.0 (Kurtz et al., 2015a). The analysis incorporated the roots, rhizosphere, and bulk soil fungal and bacterial community. The input data consisted of the raw abundance matrixes of the ITS and 16S ASVs. We first filtered the datasets to remove ASVs with a frequency lower than 20% to avoid rare species. The Spiec-Easi run was conducted with the algorithm "mb" with the lambda min ratio set at 10^{-2} and 50 repetitions. We then imported the networks into Cytoscape 3.8.2 (Smoot et al., 2011) for plotting and used the "organic" layout to draw the networks. Edges were defined as co-occurrences or mutual exclusion based on the positive or negative values of inverse covariance linking the nodes. Betweenness centrality, defined as the fraction of the shortest

path between all other nodes in the network containing the given node, and degree score, highlighted central nodes and provided information about network architecture. A score of betweenness centrality and degree of connectivity greater than 95% of the network taxa suggested participation in multipartite interactions in the community and allowed us to flag the highly connected taxa as hub taxa.

Results

Bioinformatic yield and taxonomic profile of the microbiota of canola roots, rhizosphere, and bulk soil

Sequencing yielded 10,118,613 ITS and 10,273,048 16S reads. Our bio-informatic pipeline retained 6,934,809 (68.03%) non-chimeric ITS-reads and 7,157,985 (68.21%) non-chimeric 16S reads after filtering, trimming, denoising and merging. These reads were respectively assigned to 2140 ITS amplicon sequence variants (ASV) and 9814 16S ASV. Rarefaction curves indicated that read abundances reached saturation for all samples (Figure S5).

Fungal ASV in bulk soil belong mainly to three families: Chaetomiaceae (~16%), Mortierellaceae (~13%) and Nectriaceae (~11%). In the rhizosphere, the most dominant fungal families were Olpidiaceae (~24%), Chaetomiaceae (~12%), Mortierellaceae (~10%) and Nectriaceae (~10%). In the roots, the most dominant fungal families were Olpidiaceae (~54%) and Glomeraceae (11%). Unknown Ascomycota were abundant in the bulk soil (~20%), rhizosphere soil (~13%), and roots (~14%) (Figure 7).

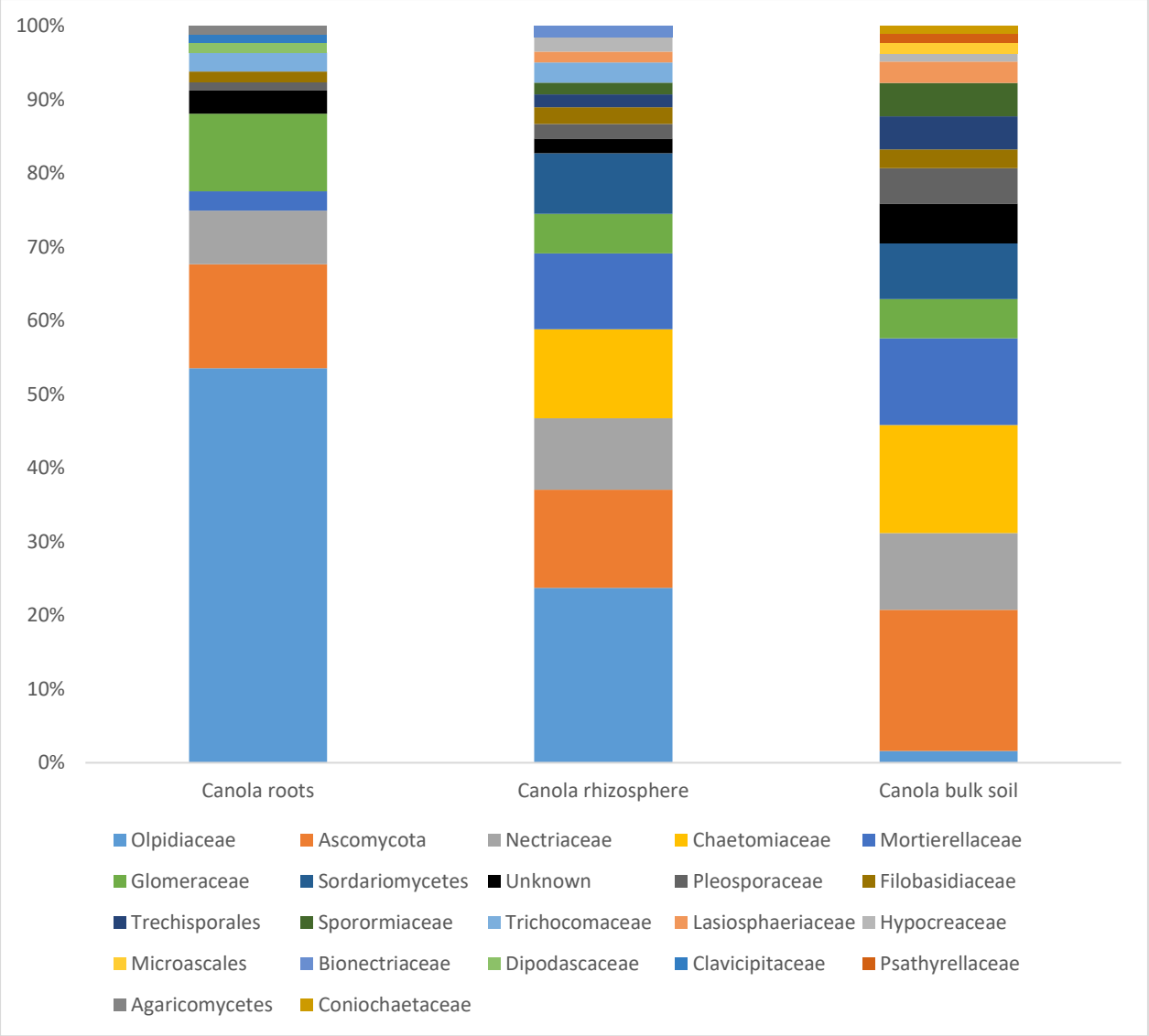

Figure 7. Relative abundance of main fungal families in canola roots, rhizosphere, and bulk soil. The taxa are ordered from the most abundant at bottom to the least abundant to the top of the bars.

The most abundant bacterial higher taxa in the bulk soil were: Actinobacteria (~18%), Thermoleophilia (~15%) and Alphaproteobacteria (11%). In the rhizosphere, Actinobacteria (~24%), Gammaproteobacteria (~16%) and Thermoleophilia (~14%) were the dominant bacterial

higher taxa and in the roots, Gammaproteobacteria (~60%), Bacili (~19%) and Actinobacteria (13%) were the most abundant (Figure 8).

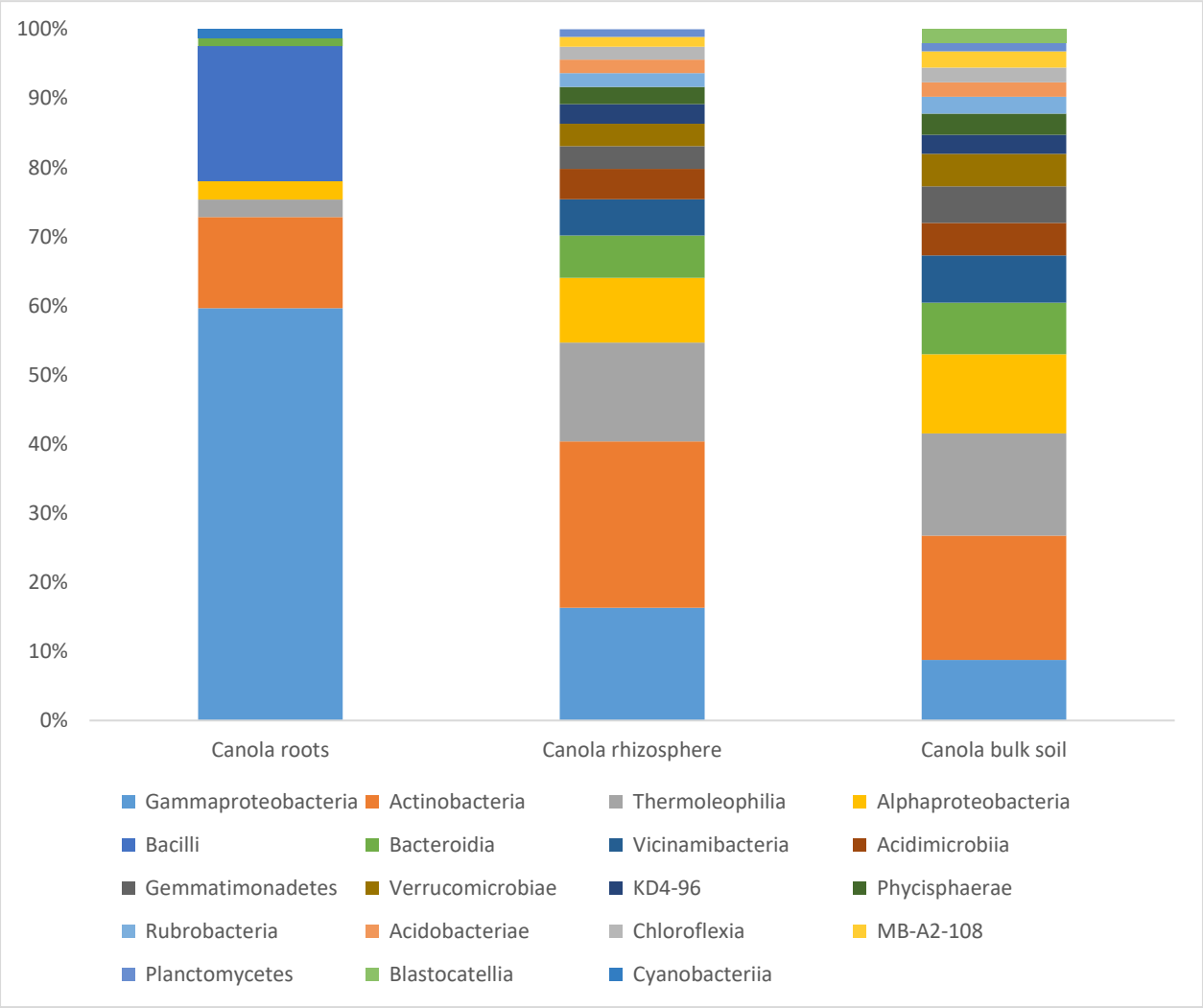

Figure 8. Relative abundance of main bacterial classes in canola roots, rhizosphere, and bulk soil. The taxa are ordered from the most abundant at bottom to the least abundant to the top of the bars.

Principal coordinate analysis (PCoA) showed a site effect on both ITS and 16S communities (Figure S6 and S7). PERMANOVA thus conducted by site reported a significant effect of cropping system diversification on fungal communities in certain sites and microbiota compartments, but no significant effect of diversification on bacterial communities was detected in any compartment of the subterranean microbiota of canola (Tableau 12). Fungal communities were significantly affected by crop diversification in canola roots only in Lethbridge; in the rhizosphere at Melfort and Lacombe; and in the bulk soil at Lacombe and Lethbridge.

Shared microbiota and Core-microbiota of canola roots, rhizosphere, and bulk soil.

The canola roots, rhizosphere, and bulk soil biotopes shared 318 fungal ASV. These ASV represent 59%, 24% and 21% of the fungal communities of the roots, rhizosphere, and bulk soil. Thirty-three fungal ASV representing respectively 5.6% and 2.5% of the root and rhizosphere fungal communities were shared in these ecospheres, but absent in bulk soil. Forty-two fungal ASV found only in the bulk soil and roots made up 7.2% and 2.7% of these fungal communities, respectively. Five hundred and fifty-two fungal ASV representing 42% and 36% of the rhizosphere and bulk soil fungal communities, respectively, were absent in the roots (Figure 9).

The 2405 bacterial ASV that were shared between the three compartments of the canola subterranean microbiota represented 69.4%, 29.9% and 29.2% of the bacterial communities of the roots, rhizosphere, and bulk soil, respectively. The 453 ASV shared exclusively between canola roots and rhizosphere soil represented 13% and 5.6% of the root and rhizosphere bacterial communities. The 268 ASV shared only between bulk soil and roots represented 7.7% and 3.2% of the roots and bulk soil bacterial communities, respectively. There were 4353 ASV shared exclusively between the rhizosphere and bulk soil. These shared ASV represented 54.2% and 53% of the rhizosphere and bulk soil bacterial communities, respectively (Figure 10).

Table 12. Effects of crop diversification on the structure of Bacterial 16S and fungal ITS communities in the canola subterranean microbiome. According to PERMANOVA ($\alpha = 0.05$, $n = 12$) for each site.

ITS communities													
Swift Current													
		Root				Rhizosphere				Bulk soil			
Factor	Df[1]	SumOfSqs	R2	F	P	SumOfSqs	R2	F	P	SumOfSqs	R2	F	P
Cropping system	2	0,548	0,179	0,984	0.48	0,267	0,193	1,082	0.332	0,223	0,194	1,084	0.282
Residual	9	2,506	0,82	NA	NA	1,112	0,806	NA	NA	0,925	0,805	NA	NA
Total	11	3,054	1	NA	NA	1,38	1	NA	NA	1,148	1	NA	NA
Melfort													
Cropping system	2	0,453	0,184	1,02	0.444	0,253	0,222	1,29	0.044*	0,179	0,181	0,996	0.494
Residual	9	1,998	0,815	NA	NA	0,883	0,777	NA	NA	0,808	0,818	NA	NA
Total	11	2,451	1	NA	NA	1,136	1	NA	NA	0,987	1	NA	NA
Lacombe													
Cropping system	2	0,821	0,174	0,948	0.535	0,316	0,267	1,647	0.006**	0,278	0,231	1,352	0.035*
Residual	9	3,895	0,825	NA	NA	0,865	0,732	NA	NA	0,925	0,768	NA	NA
Total	11	4,717	1	NA	NA	1,182	1	NA	NA	1,203	1	NA	NA
Lethbridge													
Cropping system	2	1,071	0,262	1,598	0.002**	0,255	0,211	1,206	0.206	0,241	0,304		0.001***
Residual	9	3,0145	0,737	NA	NA	0,954	0,788	NA	NA	0,553	0,695	NA	NA
Total	11	4,085	1	NA	NA	1,211	1	NA	NA	0,794	1	NA	NA
16S communities													
Swift Current													

		Root				Rhizosphere				Bulk soil			
Factor	Df[1]	SumOfSqs	R2	F	P	SumOfSqs	R2	F	P	SumOfSqs	R2	F	P
Cropping system	2	0,399	0,162	0,874	0.814	0,33	0,176	0,964	0.51	0,422	0,21	1,201	0.138
Residual	9	2,055	0,837	NA	NA	1,54	0,823	NA	NA	1,583	0,789	NA	NA
Total	11	2,454	1	NA	NA	1,871	1	NA	NA	2,005	1	NA	NA
Melfort													
Cropping system	2	0,372	0,218	1,255	0.193	0,309	0,184	1,018	0.418	0,336	0,207	1,18	0.156
Residual	9	1,333	0,781	NA	NA	1,369	0,815	NA	NA	1,283	0,792	NA	NA
Total	11	1,705	1	NA	NA	1,679	1	NA	NA	1,62	1	NA	NA
Lacombe													
Cropping system	2	0,407	0,188	1,043	0.313	0,293	0,161	0,868	0.724	0,303	0,166	0,9	0.647
Residual	9	1,757	0,811	NA	NA	1,518	0,838	NA	NA	1,519	0,833	NA	NA
Total	11	2,165	1	NA	NA	1,811	1	NA	NA	1,822	1	NA	NA
Lethbridge													
Cropping system	2	0,475	0,201	1,132	0.252	0,248	0,187	1,036	0.354	0,176	0,169	0,919	0.586
Residual	9	1,889	0,798	NA	NA	1,078	0,812	NA	NA	0,863	0,83	NA	NA
Total	11	2,364	1	NA	NA	1,326	1	NA	NA	1,04	1	NA	NA

[1]DF, Degree of Freedom

"*" = $p < 0.05$. "**" = $p < 0.01$ and "***" = $p < 0.001$, Level of significance.

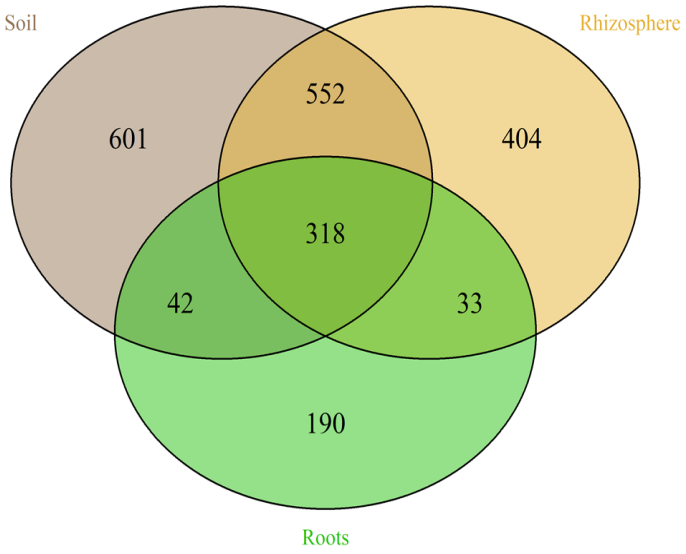

Figure 9. Venn diagram of the ASV of the fungal community shared between root, rhizosphere, and bulk soil, taking all sites in account.

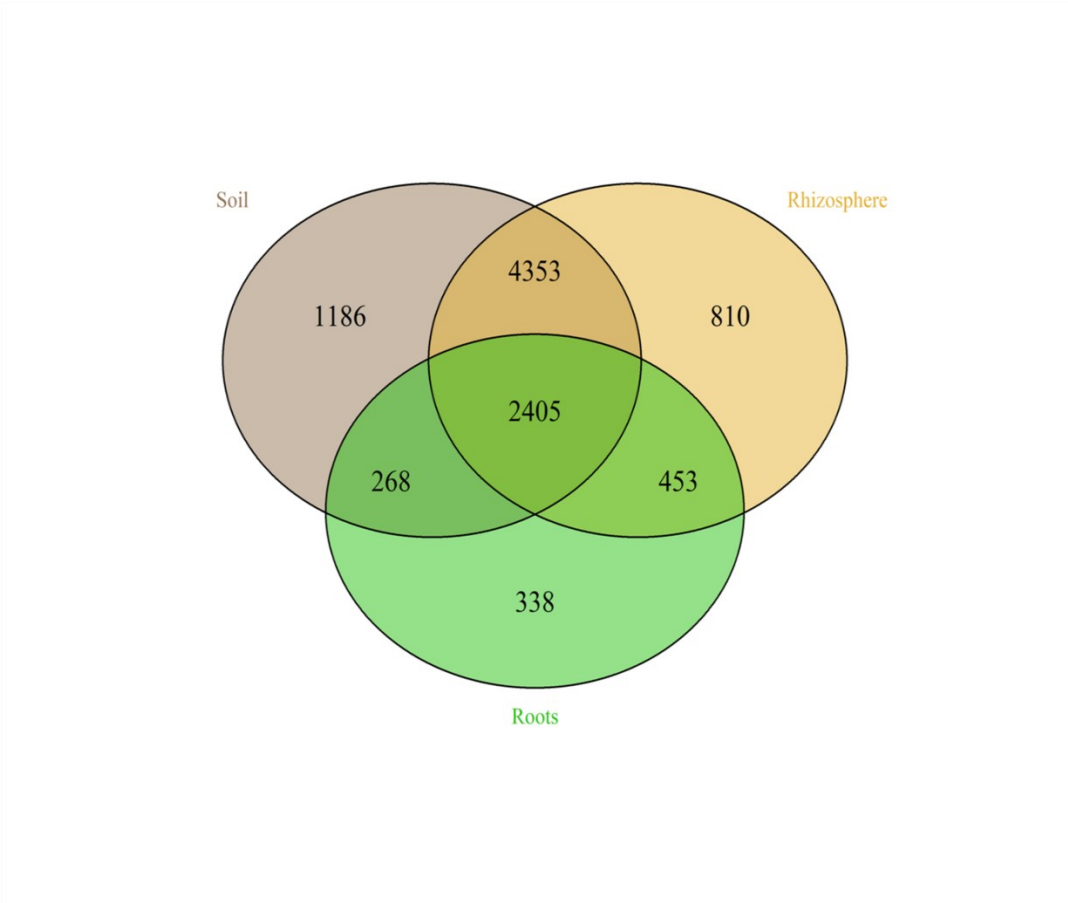

Figure 10. Venn diagram of the ASV of the bacterial community shared between root, rhizosphere and bulk soil, taking all sites in account.

We were unable to detect a core microbiota or ASV core candidates in roots. However, three fungal amplicon sequence variants (FASV) were always present in the rhizosphere of canola, at all sites and regardless of crop rotation specifications. This fungal core microbiota was composed of FASV1 (*Tricocladium* sp.), FASV4 (*Fusarium* sp.) and FASV7 (*Cryptococcus* sp.). Only one fungal ASV, FASV2 (*Fusarium* sp.), was present in the bulk soil sample of all plots. Only one bacterial amplicon sequence variant (BASV) was present in the canola rhizosphere sample of all plots. This BASV, BASV46 (*Marmoricola* sp.), was also the only BASV present in the bulk soil

sample of all plots (Tableau 13). Fungal and bacterial core candidates present at 90% and 80% frequency in our samples are reported in Tableau S4. No BASV core candidates were found at 90% frequency in either soil and rhizosphere.

Table 13. Core fungi and bacterium of canola subterranean microbiome

ASV	Identity	Confidence score
Core fungi in canola rhizosphere		
FASV1	*Trichocladium* sp.	100
FASV4	*Fusarium* sp.	100
FASV7	*Cryptococcus* sp.	100
Core fungi in bulk soil		
FASV2	*Fusarium* sp.	100
Core bacterium in both rhizosphere and bulk soil		
BASV46	*Marmoricola* sp.	99

Inter-kingdom network analysis of the subterranean canola microbiota.

Network analysis detected no potential inter-kingdom interaction in canola roots; thus, no network was drawn.

In the rhizosphere, the inter-kingdom network of putative interactions involved 77 ASV sharing 120 edges and included 33 mutual exclusions and 87 co-occurrences (Figure 11a). This network, formed with the bacterial and fungal abundance datasets, revealed three hub taxa (Tableau S5), namely BASV45 (*Bradyrhizobium sp*.), BASV134 (*Pseudonocardia sp*.) and FASV21

(*Mortierella sp*.). BASV45 (*Bradyrhizobium sp*.) had a betweenness centrality score of 0.25. It was connected to seven BASV and three FASV (Fig. 11B), including the hub taxon FASV21 (*Mortierella sp*.). FASV21 was connected to six BASV and two FASV (Fig. 11D) and had a betweenness centrality score of 0.25. The third inter-kingdom hub taxon, BASV134 (*Pseudonocardia sp*.), was highly connected with FASVs (Figure 11C), with four connections to FASV and 4 connections to BASV. BASV134 had a betweenness centrality score of 0.11.

In the bulk soil, the inter-kingdom co-occurrence network had the highest complexity, with 96 ASV and 149 edges (Figure 12A). All three modules of the network were organized around three inter-kingdom hub taxa (Tableau S5). One of these, FASV8 (*Corynascella inaequalis*), shared seven interactions with FASV and three with BASV (Figure 12C) and had a betweenness centrality score of 0.24. The other two inter-kingdom hub taxa were FASV114 (*Mortierella sp*.) and BASV69 (*Bacterium* sp.). FASV114 shared connections with five FASV and eight BASV (Figure 12D) and had a betweenness centrality score of 0.29. BASV69 shared connections with five FASV and five BASV (Figure 12B) and had a betweenness centrality score of 0.25.

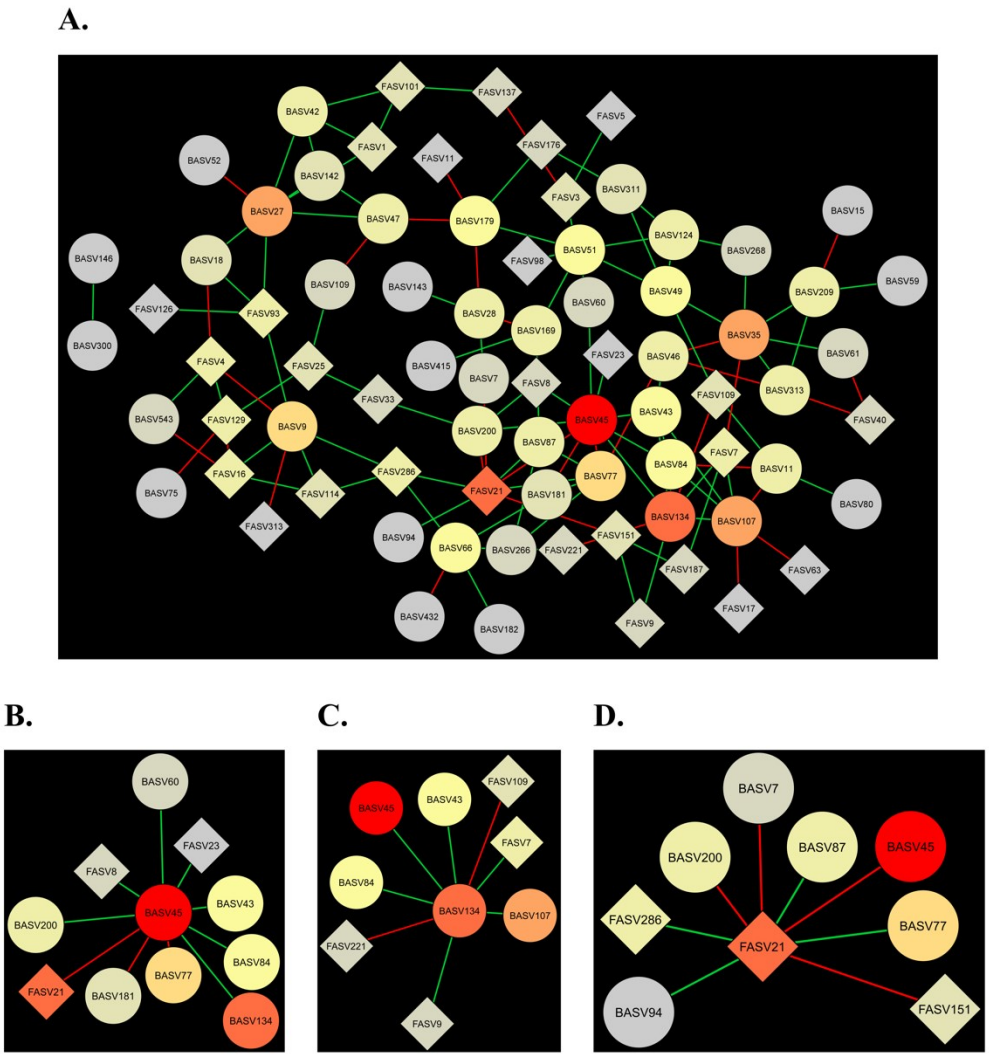

Figure 11. **(A)** Network of inter-kingdom interactions between the bacteria and fungi forming the microbiome of canola rhizosphere. Node shades indicate the degree of connectivity: ASVs with warm colors are more connected with the other members of the network than the cold colored ones. Green edges indicate positive relationships and red edges, negative relationships**. (B)** Sub-network centered around BASV45. **(C)** Sub-network centered around BASV134. **(D)** Sub-network centered around FASV21.

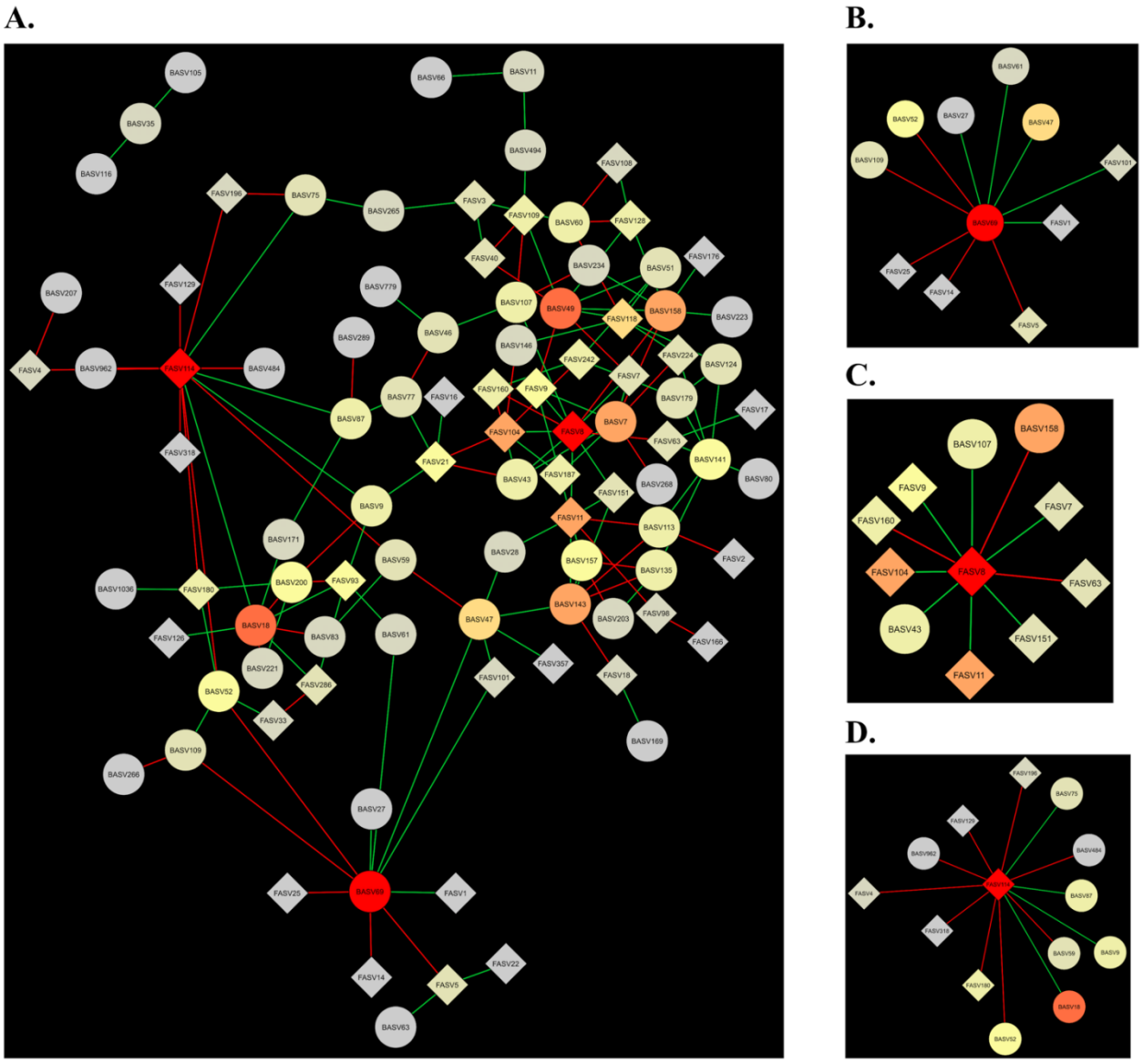

Figure 12. **(A)** Network of inter-kingdom interactions between the bacteria and fungi forming the microbiome of bulk soil in canola field. Node shades indicate the degree of connectivity: ASVs with warm colors are more connected with the other members of the network than the cold colored ones. Green edges indicate positive relationships and red edges, negative relationships. **(B)** Sub-network centered around BASV69. **(C)** Sub-network centered around FASV8. **(D)** Sub-network centered around FASV114.

Discussion

Our results support the existence of stable core bacterial and fungal components of the canola rhizosphere and in the bulk soil of canola-producing fields. Inter-kingdom network analysis revealed hub taxa in rhizosphere and bulk soil, but no significant potential interaction was found in canola roots. Hub taxa BASV45 (*Bradyrhizobium* sp.), BASV134 (*Pseudonocardia* sp.), and FASV21 (*Mortierella* sp.) in the rhizosphere and bulk soil hub taxa FASV8 (*Corynascella* sp.), FASV114 (*Mortierella* sp.) and BASV69 (*Bacterium* sp.) are strongly suspected to act as structuring factors in canola microbial communities and could exclude pathogens or enhance plant health.

Canola subterranean microbiota compartmentation and response to crop diversification

Crop diversification is known to increase canola yield by preventing the accumulation of pests and pathogens in soil (Hilton et al., 2013; Harker et al., 2015a; Floc'h et al., 2020a, 2020c). We have previously shown the insignificance of the crop diversification effect on the bacterial communities of the canola rhizosphere in the Canadian prairies (Floc'h et al., 2020c). In this paper, our results confirm that bacterial communities are insensitive to diversification of cropping systems not only in the canola rhizosphere, but also in its roots and bulk soil (Tableau 12). This could be explained by the fact that, in our case bacteria could be more influenced by soil physical properties and weather conditions than by crop rotations (Leitner et al., 2021).

A contrario, the composition of the fungal communities in canola roots, rhizosphere and bulk soil appeared to be sensitive to cropping systems diversification. The sensitivity of the rhizosphere fungal community was previously reported (Lay et al., 2018a; Floc'h et al., 2020a).

Geographic location and soil physical properties appear to affect fungal community composition as the crop rotation effect was site-dependant (Tableau 12).

Crop rotation systems are known to influence microbial community structure in a wide range of crops (Xuan et al., 2012; Venter et al., 2016; Fan et al., 2020). Crop rotations can be used to modulate biological N-fixation and to modify soil structure, with feedback on microbial communities (Roberts et al., 2011; Suzuki et al., 2012; Soman et al., 2017). Changing the fungal microbiota of canola with crop rotation systems could thus be useful for suppressing pathogens or enhancing canola nutrient uptake. The shaping of canola microbial communities must also take into account the environmental conditions, as fungal communities are subject to substantial geographical variations (Jansa et al., 2014; Barnes et al., 2016; Coleman-Derr et al., 2016).

The dominance of Olpidiomycota in the taxonomic profiles of our fungal communities was similar to that previously reported in canola (Gkarmiri et al., 2017; Lay et al., 2018a; Floc'h et al., 2020a, 2020c). The dominance of Olpidiomycota in the fungal microbiota of the roots and rhizosphere is mainly due to the abundance of *Olpidium brassicae* (Lay et al., 2018a; Floc'h et al., 2020a). Little is known about Olpidiomycota and particularly about *O. brassicae* as it is often misidentified as *O. virulentus* (Lay et al., 2018a). This particularity of a single dominant species spread across the roots and rhizosphere is usually linked to situations of pathogen infestations (Bakker et al., 2013; Shen et al., 2018; Gao et al., 2021) or symbiosis (Andrade et al., 2002) in other crops. The predominance of *O. brassicae* was reported to have little influence on canola yield and health (Lay et al., 2018a; Floc'h et al., 2020a). This high presence of Olpidiomycota in canola roots and rhizosphere could indicate a strong biotic interaction between the plant and these microbes and needs further investigation.

In the soil, compartments like roots, rhizosphere and bulk soil are known to show a concentration gradient of plant chemical compounds that attract microbes (Rudrappa et al., 2008; Patel et al., 2015; van der Heijden and Hartmann, 2016). Thus, the colonization of these three different niches share certain similarities in terms of the composition of their microbial communities. Cordero et al. (2019) reported 77% similarity between bacterial communities in the roots and rhizosphere of canola. In our case, canola subterranean microbiota were similar in terms of the proportion of bacterial ASV shared between the different compartments, with a significant percentage of shared community between the bulk soil and rhizosphere (Figures 9 and 10). The proportion of bacterial species shared between the root interior and rhizosphere of canola (82.4%) was similar to the proportion reported by Cordero et al., (2019). To the best of our knowledge, this is the first report of the percentage of mycobiota shared between canola roots, rhizosphere, and bulk soil communities. We found the same trend in fungi as in bacteria. The fact that a significant part (30~60%) of the microbial communities are shared between these ecological niches could reflect their physical proximity. The fact that between the three biotopes, bacteria are shared more than fungi could be explained by the presence of filamentous fungi that allow bacteria to migrate following their hyphae, thus allowing a wide range of bacteria to navigate between the compartments.

Canola core-microbes in roots, rhizosphere, and bulk soil.

The canola root interior was devoid of any core fungi or bacteria. This lack of detection could be attributed to the fact that canola roots produce glucosinolates that are toxic to microbial life and lead to limited root colonization by fungi and bacteria (Smith et al., 2004, 2013; Lay et al., 2018a). *Olpidium brassicae*, is an obligatory endophyte previously reported as a core fungi in canola rhizosphere in the Canadian prairies (Lay et al., 2018a; Floc'h et al., 2020a). Before Lay et

al. (2018b) resolved the differentiation between *O. virulentus* and *O. brassicae*, *O. brassicae* was labelled as a pathogenic fungus and a plant virus vector. It appeared that *O. brassicae* is not able to carry viruses and its ecology remains largely unknown as the previous study reporting *O.brassicae* behaviour are likely to be victim of the identification confusion between *O. brassicae* and *O. virulentus*. *O. brassicae* was present and dominant in canola roots (Figure 7) but not enough to be flagged as a core fungus according to our threshold of 99%. This difference between our results and results previously published can be explained by the difference in bioinformatics methods used. In this study, DADA2 allowed us to form ASVs (amplicon sequence variants) that are much more discriminating than the 97% identity threshold used in USEARCH to form OTUs. We can thus identify different genetic variants of the same species as different ASVs in DADA2 (Callahan et al., 2016). This permits more precise inference of the structure and ecology of microbial communities. *Olpidium brassicae* shows significant genotypic variation and similarity with other pathogenic species known to affect canola, such as *O. virulentus* (Lay et al., 2018b). Its significant genetic variability and abundance in the roots and rhizosphere of canola suggest that *O. brassicae* should be the target of population genetics studies in the near future.

In the canola rhizosphere in our study, three fungi and one BASV were detected as core microbes: FASV1 (*Trichocladium* sp.), FASV4 (*Fusarium* sp.), FASV7 (*Cryptococcus* sp.) and BASV46 (*Marmoricola* sp.). These three fungi were previously reported by Floc'h et al., (2020a) to be part of the canola rhizosphere fungal core microbiota in 2013. Paired with the observations reported in the previous article, the findings of the present study illustrate the stability over time of the concept of core microbiota in the canola rhizosphere, reinforcing the need for long term studies with recurrent samplings.

In canola bulk soil in our study, a core fungus and core bacterium were found: FASV2 (*Fusarium* sp.) and BASV46 (*Marmoricola* sp.), respectively. The latter was also present as a core bacterium in the canola rhizosphere. Regarding the former, fusaria are well known commensalists and pathogenic fungi, widely abundant in agricultural soils (Ioos et al., 2004; Cha et al., 2016; Klein et al., 2016). As no core microbiota has ever been identified in canola bulk soil, these results should be taken with caution. Core fungi and bacterium are subject to variation in presence and abundance over time and depending on weather conditions (Floc'h et al., 2020a, 2020c).

We were able to identify hub taxa at the intra- and inter-kingdom levels that are known as inter-kingdom hub taxa (Tableau S5). Each of these could be of importance for canola production and manipulation of canola subterranean microbial communities.

BASV45 (*Bradyrhizobium sp*.) is a hub taxa that has been linked to other inter-kingdom hub taxa of the canola rhizosphere. *Bradyrhizobium* is a nitrogen-fixing bacterial genus known to nodulate Fabaceae such as soybeans (*Glycine max*), cowpeas (*Vigna unguiculata*), Bambara groundnuts (*Vigna subterranea*) (Suzuki et al., 2014; Valetti et al., 2016; Grönemeyer and Reinhold-Hurek, 2018). Furthermore, these bacteria demonstrate other ecological functions as plant growth promoting rhizobacteria (PGPR) through hormone secretions and antagonism in non-legume plants (Pageni et al., 2014; Hasegawa et al., 2019). Al-Mallah et al., (1990) and Trinick et al., (1995), reported *Bradyrhizobium*-induced nodular structures on canola roots. Thus, our detection of BASV45 (*Bradyrhizobium sp*.) as an inter-kingdom hub-taxon in the canola rhizosphere highlights this taxon as a potential PGPR for canola production, and as an agent for community manipulation in canola microbial networks. Indeed, high connectivity microbes are potentially beneficial for the plant, particularly in the rhizosphere (Rout, 2014; van der Heijden and

Hartmann, 2016). Given how this taxon interacts with other plant species, ASV45 could be an important actor in the canola microbiome.

In the plant rhizosphere, microbes compete for space. One of the mechanisms used to compete with other microbes is the production of anti-microbial compounds (Whyte, A.C., Gloer, K.B., Koster, B., Malloch, D., 1997; Bandani et al., 2000; de Felício et al., 2015). Such is the case for the second most connected inter-kingdom hub taxon, BASV134 (*Pseudonocardia sp*.). This genus of actinobacteria is known as an important producer of antibiotics associated with leaf cutter ants (Moore et al., 2012; Seipke et al., 2012; Goldstein and Klassen, 2020). This genus can be encountered in a broad range of environments from marine ecosystems to rhizosphere soil (Li et al., 2010; Ye et al., 2016; Song et al., 2019). BASV134 was negatively connected to FASV221 (*Ilyonectria* sp.), a fungus known to cause root rot in a wide spectrum of hosts, including olive trees, panax ginseng, strawberries and avocados (Dann et al., 2012; Úrbez-Torres et al., 2012; Guan et al., 2020; Erper et al., 2021). The fact that our network analysis revealed negative connectivity between these two ASVs could indicate a suppressive effect of BASV134 on FASV221 through antibiosis. It could also indicate that these two species target different exclusive ecological niches and, thus, are not frequently associated. Either way, the fact that BASV45 (*Bradyrhizobium sp*.) is negatively linked to BASV134 (Figure 11C) suggests that *Bradyrhizobium sp*. may have biocontrol abilities in the canola rhizosphere.

The third cross-kingdom hub taxon in the canola rhizosphere was a fungus of the genus *Mortierella* FASV21. *Mortierella* is often reported as a plant growth promoting fungi (PGPF) enhancing plant phosphate nutrition (Zhang et al., 2011; Li et al., 2018, 2020a; Ozimek and Hanaka, 2021). In the canola rhizosphere, *Mortierella* was previously reported as a dominant genus of fungi (Lay et al., 2018a; Monreal et al., 2018; Floc'h et al., 2020a). FASV21, which was

negatively linked to BASV45 (*Bradyrhizobium sp*.), and FASV151 (*Exophiala* sp.), is also a potentially beneficial organism for canola production. FASV151 (*Exophiala* sp.) was reported by (Floc'h et al., 2020a) to be strongly positively linked to canola yield in the Canadian Prairies. *Exophiala* is a genus of fungi belonging to the dark septate endophytes (DSE) group, which hosts a broad range of plant growth promoting fungi (Kauppinen et al., 2013; Zhang et al., 2017c). This mutual exclusion between potentially mutualistic organisms could be explained by these microbes competing for similar ecological niches and may feed on similar rhizodeposits (Hibbing et al., 2010; Foster and Bell, 2012; Ghoul and Mitri, 2016).

Bulk soil is an important part of plant microbiota, but very few studies of canola-related microbiota have taken bulk soil into consideration (Lupwayi et al., 2007, 2009; Hilton et al., 2013; Rathore et al., 2017). Despite the fact that bulk soil is important to plant health and rhizosphere microbiota, most studies of canola-related microbiota have been restricted to root and rhizosphere microbiota. Bulk soil microbial communities emit volatile compounds that can enhance plant growth, protect against pathogens and even influence plant root architecture (Ryu et al., 2003; Cordovez et al., 2018; Schenkel et al., 2018; Garbeva and Weisskopf, 2020). These microbial volatile compounds also have an influence on the structure of rhizosphere microbial communities (Schenkel et al., 2019; Raza et al., 2020). Compared to the rhizosphere microbial network, the bulk soil network showed a higher overall connectivity and a higher number of potential interactions between fungi and bacteria (Figure 6). This greater connectivity is probably due to the higher diversity of organisms found in the bulk soil. This diversity is favorized by the multiples niches bulk soil can provide for microbes, as edaphic conditions in bulk soil aggregates are not homogeneous (Merino-Martín et al., 2021). The bulk soil network had three modules, each centered on a hub taxon. In canola bulk soil, we were able to identify three inter-kingdom hub taxa,

two fungi and one bacterium: FASV8 (*Corynascella* sp.), FASV114 (*Mortierella sp*.) and BASV69 (*Bacterium* sp.). None of these taxa were linked with each other. This finding, coupled with the modularity of the networks, suggests a strong difference in ecological roles between the canola bulk soil hub taxa.

FASV114 (*Mortierella sp*.) was the most connected hub taxon in the network, with a dominance of mutual exclusions. It was negatively linked with *Pseudonocardia* and with FASV129 (*Davidiella* sp.). The latter genus, the teleomorph form of *Cladosporidium*, is known to count among its ranks numerous plant pathogens (Schubert et al., 2007; Bensch et al., 2012). As we discussed previously, *Mortierella* can act as a PGPF. Its negative links with potential pathogens reinforce the need to investigate the impact this genus could have on canola production and health, as it is present as hub taxa in both the bulk soil and rhizosphere soil.

The potential ecological role of the two other hub taxa, FASV8 (*Corynascella sp*.) and BASV69 (unknown bacterium sp.), is rather difficult to attribute, as FASV8 (*Corynascella sp*.) is only reported in donkey dung from Iraq (Guarro et al., 1997) and BASV69 is unknown. FASV8 was positively linked with DSE FASV151 (*Exophiala* sp.). The position of these two hub taxa in bulk soil suggests their potential importance as microbes in canola production and thus the need for subsequent studies defining their ecological functions.

The fungal microbiota in canola roots, rhizosphere and bulk soil respond to cropping system diversification, but show different responses depending on geography and weather conditions. The microbiota in canola roots, rhizosphere and bulk soil demonstrate ecological interconnectivity and recruitment, as a significant part of the microbial communities of these biomes is shared. This first inter-kingdom network analysis of canola rhizosphere and bulk soil microbiota allowed identification of two particular microbes of interest for canola production: *Bradyrhizobium sp*. and

Mortierella sp. The latter is a hub taxon in canola rhizosphere and roots and is linked to *Exophila sp*., a taxon previously described as associated with canola and positively correlated with high canola yield. The hub taxa matching *Bradyrhizobium* and *Mortierella* at the genus level could be of potential interest for bioengineering of canola subterranean microbiota and enhancing canola production.

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Mise en contexte du chapitre 5 : Long-term persistence of arbuscular mycorrhizal fungi in the rhizosphere and bulk soils of non-host *Brassica napus* and their networks of co-occurring microbes

Les résultats du précèdent chapitre ont révélé une relation entre les champignons et les bactéries du sol et de la rhizosphère du canola, ainsi que l'existence de bactéries et champignons clef de voûte à l'échelle interrègne. L'existence de ces taxons a été aussi couplé avec une surprise que je n'ai pas relevé outre mesure dans le chapitre précédent, mais qui a motivé la réalisation du chapitre 5 : la présence notable de clades de champignons mycorhiziens à arbuscule dans la communauté fongique de 2018 dans la rhizosphère et le plein sol du canola. Cette plante étant caractérisée comme non mycorhizienne, l'observation était intéressante. Je me suis alors posé la question de savoir si effectivement l'on était en mesure de retrouver dans la rhizosphère et le sol une communauté de champignons mycorhiziens après 10 ans de monoculture de canola. J'ai aussi voulu savoir si la fréquence du canola dans un système cultural pouvait influencer la composition de la communauté de champignons mycorhiziens associée au canola. Les AMF nécessitant des amorces spécifiques, et j'ai donc procédé à l'amplification de l'ADN des AMF à partir des échantillons de la précédente expérience grâce à Agriculture Canada afin de l'analyser plus en profondeur.

Les résultats de ce chapitre ont fait l'objet d'une publication dans la revue *Frontiers in Plant Science* en 2022.

Ma contribution dans cet article a été la mise en place du protocole d'échantillonnage, la calibration et mise en place du pipeline bio-informatique, l'analyse statistique des résultats ainsi que l'élaboration de l'analyse de réseau, des illustrations et de la rédaction de l'article.

Chapitre 5 – Long-term persistence of arbuscular mycorrhizal fungi in the rhizosphere and bulk soils of non-host *Brassica napus* and their networks of co-occurring microbes

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Abstract

Arbuscular mycorrhizal fungi (AMF) are obligate plant symbionts that improve the nutrition and health of their host. Most, but not all crops form a symbiosis with AMF. It is the case for canola (*Brassica napus*), an important crop in the Canadian prairies that is known to not form this association. From 2008 to 2018, an experiment was replicated at three locations of the Canadian Prairies and it was used to assess the impact of canola on the community of AMF naturally occurring in three cropping systems, canola monoculture, or canola in a two different rotation systems (two-years, canola-wheat and three-years barley-pea-canola). We sampled canola rhizosphere and bulk soils to (i) determine diversity and community structure of AMF, we expected that canola will negatively impact AMF communities in function of its frequency in crop rotations, and (ii) wanted to assess how these AMF communities interact with other fungi and bacteria. We detected 49 AMF Amplicon Sequence Variant (ASV) in canola rhizosphere and bulk soils, confirming the persistence of a diversified AMF community in canola-planted soil, even after 10 years of canola monoculture, which was unexpected considering that canola is among non-mycorrhizal plants. Network analysis revealed a broad range of potential interactions between canola-associated AMF and some fungal and bacterial taxa. We report for the first time that two AMF, *Funneliformis mosseae* and *Rhizophagus iranicus,* shared their bacterial cohort almost entirely in bulk soil. Our results suggest the existence of non species-specific AMF-bacteria or AMF-fungi relationships that could benefit AMF in absence of host plants. The persistence of an AMF community in canola rhizosphere and bulk soils bring a new light on AMF ecology and lead to new perspectives for further studies about AMF and soil microbes interactions and AMF subsistence without mycotrophic host plants.

Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate plant symbionts. Four hundred millions years ago, plants were already in association with arbuscular mycorrhizal fungi (Selosse et al., 2015). The arbuscular mycorrhizal symbiosis has evolved with land plants and, as a result, AMF associate with the roots of 90% of the plant species, including most important crops such as cereals, legumes, and members of Solanaceae (Zhu et al., 2010). The arbuscular mycorrhizal symbiosis brings several benefits for the plant. The AMF facilitate plant nutrition in providing soil nutrients, notably P and N (Smith and Read, 2008), and can mitigate abiotic stresses such as drought (da Silva Folli-Pereira et al., 2013; Begum et al., 2019) and protect plant roots against soilborne pathogen attacks (St-Arnaud and Vujanovic, 2007; Lewandowski et al., 2013; Del Fabbro and Prati, 2014). The association of crop plants with AMF often increases crop yield (Hijri, 2016; Séry et al., 2016; Buczkowska and Sałata, 2020).

In bulk and rhizosphere soils, AMF play an important role in nutrient cycling and in structuring the microbial communities (Smith and Smith, 2011; Iffis et al., 2016; Dagher et al., 2020). AMF interact with the other members of the plant and soil microbiome in various ways. With their hyphae, AMF can be viewed as the bacterial highway of the soil: the labile interface between the hyphae and the soil facilitate bacterial migration (Abeysinghe et al., 2020; de Novais et al., 2020). AMF can also interact with phosphorus solubilizing bacteria (Taktek et al., 2017) or can form a tripartite interaction with other fungi to facilitate symbiosis in plant (Liu et al., 2020). But there is still little knowledge about the interaction of AMF and other microbes in soil and plant rhizosphere.

Certain vascular plants do not host AMF in their roots, and it is the case of canola (*Brassica napus*), an important crop in the Canadian prairies. Canola produces glucosinolates that transform

into iso-cyanates in soil (Smith et al., 2004; Ma et al., 2015). Among others, glucosinolates and iso-cyanates are toxic, which may explain the non-host plant status of canola. But from an analysis of canola-associated soil fungi based on fungal ITS, Floc'h et al., (2021) reported the presence of AMF from the Glomeraceae family in rhizosphere and bulk soil samples from fields that were in canola monoculture for ten years. The AMF depend on a host for their carbon needs (Rich et al., 2017), and the persistence of AMF for such a long period of time in absence of mycotrophic host plants is unexpected. However, axenic AMF growth is known to be stimulated *in vitro* by coculture with plant growth-promoting bacteria (Abdellatif et al., 2019). Thus, the report of Floc'h et al., (2021) motivated the verification of this hypotheses: We expect that canola negatively impacts AMF community in function of its frequency in crop rotations. Furthermore, using network analysis we will explore the potential interactions that AMF develop with other microbes such as fungi and bacteria in canola rhizosphere and bulk soil.

In this study, we used a long-term assay held by Agriculture and Agrifood Canada in three locations of the Canadian Prairies to identify the AMF living in canola rhizosphere and bulk soil, and the samples we used are those of Floc'h et al., (2021) from which we extracted and amplified the 18S region of AMF rDNA to only target AMF. We used Network Analysis to explore the possible interactions of these AMF with the fungi and bacteria also residing in canola rhizosphere and bulk soil.

Material and methods

A subset of plots from a long-term experiment initiated in 2008 and replicated at three locations in the Canadian Prairies was sampled in 2018. The experiment tested the effects of diversification in canola-based crop rotation systems. The cultivar of canola used in this work is the canola gluphosinate resistant L241C. The three crop diversification treatments used in this

study were: (1) monoculture of canola, (2) wheat-canola, and (3) pea-barley-canola (Table 1). These treatments were applied in a randomized complete block design with four blocks. Plot dimension were 3.7 × 15.2 m. All rotation phases were present each year at each of the three locations where the experiment was replicated, but only the canola phases of the rotation was used in this study.

The three experiment sites were located in three pedoclimatic zones of the canola-producing regions of Canada. Two sites were in Alberta in the sub humid Brown soil zone: one in Lacombe (lat. 52.5°N, long. 113.7°W) and the other in Lethbridge (lat. 49.7°N, long. 112.8°W), and a third site was in Swift Current Saskatchewan, in the semi-arid Brown soil zone (lat. 50.3°N, long. 107.7°W). The fourth site (Melfort) used in Floc'h et al., (2021) was discarded due to a lack of material for DNA extraction. Crops were grown according to best management practices, as described in Harker et al., (2015). The growing season at all sites was characterized by more frequent rain events in July just before sampling, Lethbridge was drier (Figure S1).

Rhizosphere and bulk soil samples were collected at the mid bloom stage (50% of flowers opened) of canola development. This occurred in the fourth week of July 2018. Three to four plants within each plot were randomly selected and uprooted with a shovel. The shoots were removed, and roots were placed in plastic bags and brought to the laboratory on ice in a cooler. The soil tightly attached to roots was considered as rhizosphere soil. About 5 g of rhizosphere soil per plot was collected by gently brushing the roots. The brushed roots were then gently washed with sterile distilled water. Bulk soil was taken from the top 0-7cm soil layer using a 2 cm diameter soil probe, exactly in between two plant rows. The samples were kept at 4°C before being shipped on dry ice to the laboratory in Quebec City, Quebec, where they were preserved at -80°C until DNA extraction.

More details on site description, experimental design and sampling methods are provided in (Floc'h et al., 2020b).

DNA extraction and amplification

The bulk and rhizosphere samples DNA was extracted using the PowerSoil™ DNA Isolation Kit (Qiagen, Montreal, QC, Canada) for the characterization of resident AMF. The manufacturer's instructions for both kits were followed, except that soil and rhizosphere DNA was eluted in 50 µl. The DNA extraction of each sample was performed in duplicate, and the duplicates were pooled. The quantity and quality of the DNA extracts were first verified on 1.5% agarose gel stained with Biotium GelRed® diluted at ratio 1:10000 (VWR, Montreal, QC, Canada), run at 70 V for 60 min, and visualized using the Gel-Doc system (Bio-Rad Laboratories, Mississauga, ON, Canada). The quantity and quality of the DNA extracts were also verified using a Qubit Fluorometer 2.0 (Life Technologies, Burlington, ON, Canada) and the Qubit dsDNA HS assay kit. DNA extracts were stored at –20°C until use.

A partial sequence of approximately 800 bp of the nuclear 18S small subunit (SSU) ribosomal RNA gene of AMF was first amplified using the primer pair AML1/AML2 (Lee et al., 2008). The amplification was performed in 20 µl of reaction mixture in triplicate as follows: 1 µl of gDNA, 200 µM of each dNTP, 2 mM of Mg^{2+}, 0.8 µM of each primer, and 2.5 U of Q5 HighFidelity DNA Polymerase NEBNext® Q5 Hot Start HiFi PCR Master Mix (BioLabs, Whitby, ON, Canada). The thermocycling conditions were as follows: initial denaturation at 98°C for 30 s, 20 cycles at 98°C for 10 s, 64°C for 30 s, 65°C for 60 s, and final extension performed at 65°C for 5 min. The DNA was amplified in a Biometra TProfessional thermocycler (Biometra GmbH, Goettingen, Germany). The three amplicon replicates were pooled and purified using the QIAquick

PCR Purification Kit (Qiagen, Montreal. QC, Canada) and eluted in 50 µl of elution buffer. This step is important to prevent interactions between the remaining primers during nested PCR. PCR products were visualized in a GelRed stained 1.5% agarose gel.

In order to comply with the sequencing length capacity of Illumina MiSeq® Reagent Kit v3 (2 × 300 bp), a new primer pair from Dr F. Stefani lab (Ottawa Research and Development Centre, 960 Carling Avenue, Ottawa, ON K1A 06C, Canada) yielding a 490-bp-length amplicon (including primers) was used to target the V3-V4 region of the nuclear 18S rRNA gene: nu-SSU-0450-5′ (5′-CGCAAATTACCCAATCCC-3′) and nu-SSU-0899-3′ (5′-ATAAATCCAAGAATTTCACCTC-3′). Primers were named according to the primer nomenclature system of Gargas and DePriest, (1996). The number in the primer name refers to the 5′ end position of the primer on the 18S sequence standard of *Saccharomyces cerevisiae* (GenBank accession Z75578). Primers were designed based on the guidelines provided by Integrated DNA Technologies (IDT Inc., San Diego, CA, USA). Purified PCR products amplified with AML1/AML2 were used as templates for nested PCR. A one to three bp "heterogeneity spacer" was introduced between the 3′ end of the adapter and the 5′ end of the primer pair nu-SSU-0450-5′/nu-SSU-0899-3′ to dampen the effect of the low sequence diversity issue of the MiSeq platform. The amplification reaction mixture was the same as for the first PCR, except for the primer concentration which was 0.5 µM. The thermocycling conditions were as for the first PCR except for the number of cycles which was reduced to 15 and the annealing temperature which was 59°C. The nested PCR was performed in triplicate. Products were verified by electrophoresis on a GelRed-stained 1.5% agarose gel and replicates were pooled.

Library preparation followed the protocol described in Stefani et al., (2020). Briefly, the PCR products from the nested PCR were purified using Agencourt AMPure® XP beads (Beckman Coulter Inc., Indianapolis, IN, USA), normalized to 1 to 2 ng/µl with the SequalPrep™

Normalization Plate kit (ThermoFisher Scientific) and indexed using the Nextera index kit (Illumina, San Diego, CA, USA). Indexed amplicons were then purified and normalized. Purified indexed amplicons were quantified by qPCR using the LightCycler® 480 system (Roche Molecular Systems Inc., Branchburg, NJ, USA) with the KAPA library quantification kit for Illumina platforms (KAPA Biosystems, MA, USA) to determine the volume of each sample to make up a 1-nM amplicon pool for library preparation. Paired-end sequencing (2 × 300 bp) was carried out using the Illumina MiSeq® sequencer for 500 cycles at the *Centre d'expertise et de service Génome Québec* (Montreal, QC, Canada).

ASV determination and bioinformatic pipeline

Bioinformatics used QIIME2 version 2021.4 (Bolyen et al., 2019). The bioinformatic pipeline used for the processing of nu-SSU-0450-5′ and nu-SSU-0899-3′ 18S small subunit ribosomal RNA gene sequences was DADA2 v1.18.0 (Callahan et al., 2016). First, we used Cutadapt 3.4 to remove the primer part of the nu-SSU-0450-5′ and nu-SSU-0899-3′ RNA gene sequences with "minimum-length" at 50 and "p-error-rate" at 0.1, "—p-times" at 2 and "—p-overlap" at 6. Then, we excluded the sequences with less than 193 bp on the forward sequences and 195 bp on the reverse sequences with the command "—p-trunc-len" with "—p-mas-ee" set to 2, as the base quality of the sequences tended to diminish below that threshold in our data. Next, the amplicon sequence variant (ASV) table was calculated, and chimeras eliminated. 1905 ASVs were identified using the naïve Bayesian classifier method on the NCBI nt database. Only the 62 ASVs belonging to the Mucoromycota and to the Glomeromycota or to an unknown order were kept into a phylogenetic analysis conducted to identify with certainty the ASVs that belong to the group AMF.

The phylogenetic tree was constructed from the alignment of the 62 retained ASVs, 144 sequences from Krüger et al., (2012) and 8 sequences from NCBI/nt database with MAFFT (default settings) with the software UGENE v39.0 (Okonechnikov et al., 2012). A maximum likelihood tree was computed in RAxML v8.2.10 (Stamatakis, 2014) via CIPRES (Miller et al., 2011) with bootsrap resampling set to 1000 and the GTRGAMMA sequence evolutionary model chosen. The ASVs that were not identified as AMF in the tree were removed to produce a tree with 49 AMF ASVs.

The MiSeq sequencing data generated as part of this work are publicly available on Zenodo (https://zenodo.org/record/5639078).

Data processing and statistical analyses

We were not able to perform PERMANOVA to assess the effect of crop diversification on AMF community structure due to data discrepancy, neither we were able to do ANOVA to test for the effect of crop diversification on AMF alpha diversity: AMF were sometimes not found in samples leading to empty row in our matrices. We thus used discriminant analysis with the software JMP v16 (Gonzales, 2021) for community structure and replacement from indicator species analysis, and Kruskal-Wallis test for alpha diversity.

As we wanted to know with what microbes AMF could potentially interact with, we used the exact same samples than Floc'h et al., (2021), we reused the ITS and 16S ASV tables of this study into our analysis. The protocols of DNA extraction and of amplification, sequencing and bioinformatic processing of ITS and 16S sequences are described in Floc'h et al., (2021).

To assess the interactions between AMF and fungi, and between AMF and bacteria, we created a co-occurrence inter-kingdom network using the package Spiec-Easi v1.1.0 in R 4.1.0 (Kurtz et al., 2015b). The analysis considered rhizosphere and bulk soil fungal, bacterial and AMF

communities. The input data consisted of the raw abundance matrices of the AMF, ITS and 16S ASVs. We first filtered the ITS and 16S datasets to remove ASVs with a frequency lower than 20% to avoid rare species. The Spiec-Easi run was conducted with the algorithm "mb" with the lambda min ratio set at 10^{-2} and 50 repetitions. We then imported the networks into Cytoscape 3.8.2 (Smoot et al., 2011) for plotting and used the "organic" layout to draw the networks. Edges were defined as co-occurrences or mutual exclusion based on the positive or negative values of inverse covariance linking the nodes.

Results

Raw sequencing datasets

The 7 513 787 reads obtained from sequencing were inputted in the pipeline yielding 4 253 351 non-chimeric reads with a mean of 50635 reads per sample. Rarefaction curves reached saturation for all samples (Figure S12). These reads were assigned to 1205 Amplicon Sequencing Variants (ASV), which were classified into 49 AMF ASV after phylogenetic filtering and clustering (Figure S10), totalizing 222628 reads distributed heterogeneously across our 72 samples.

Taxonomic profiles and crop rotation effect on the AMF communities of canola rhizosphere and bulk soils

The taxonomic structure of the AMF community differed in canola rhizosphere and bulk soils and varied with crop rotations (Figure 13). However, no significant effect of crop rotations on the alpha diversity of AMF in canola rhizosphere or bulk soil could be detected (Tableau 14).

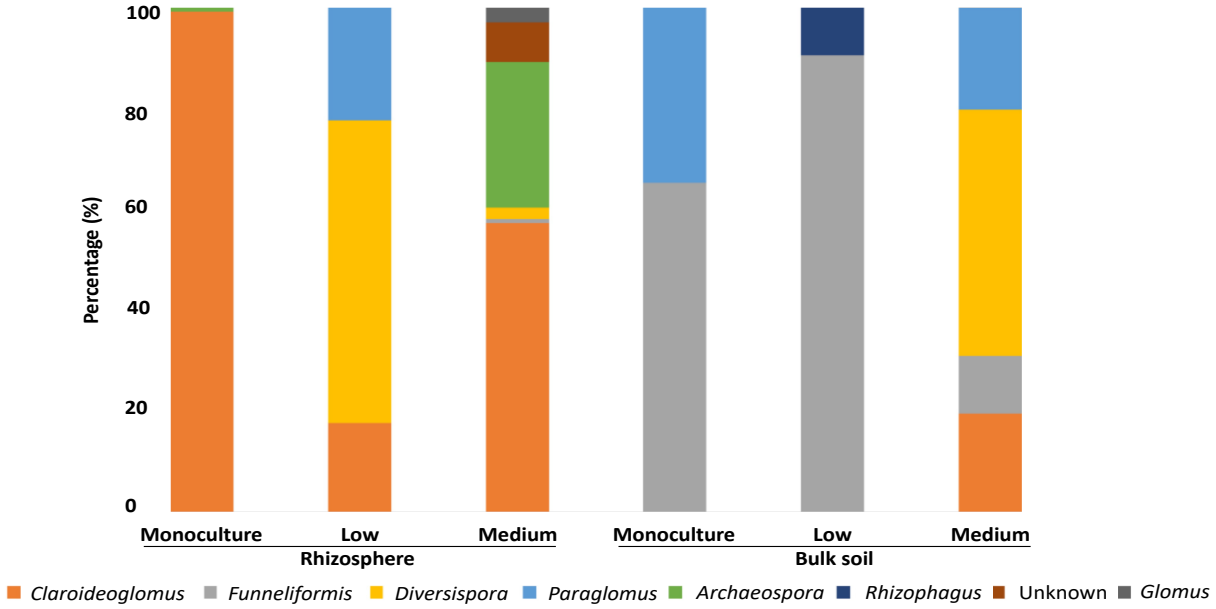

Figure 13. Taxonomic profile of the canola AMF community at genus level in function of the crop rotations and biotope.

Table 14. Results of Kruskal-Wallis tests of the effect of crop rotation diversification on indices of AMF alpha diversity in canola rhizosphere and bulk soil ($\alpha = 0.05$).

Rhizosphere

Index	Crop rotation[1]	Mean	Sd	$\chi 2$	Df[2]	p-value
Shannon	LL	0	0	4.8875	2	0.0868
	W-LL	0.0583	0.0391			
	P-B-LL	0.174	0.1172			
Simpson	LL	0	0	4.8186	2	0.0898
	W-LL	0.0407	0.0273			
	P-B-LL	0.1148	0.0749			
Richness	LL	0.1667	0	4.068	2	0.1308
	W-LL	0.5083	0.0615			
	P-B-LL	0.7917	0.2046			

Bulk soil

Index	Crop rotation	Mean	Sd	$\chi 2$	Df	p-value
Shannon	LL	0.069	0.0503	1.9619	2	0.3749
	W-LL	0.1215	0.0903			
	P-B-LL	0.2271	0.1178			
Simpson	LL	0.0481	0.0351	1.9619	2	0.3749
	W-LL	0.0815	0.0549			
	P-B-LL	0.1519	0.0787			
Richness	LL	0.275	0.0791	3.838	2	0.1468
	W-LL	0.6167	0.152			
	P-B-LL	0.9583	0.1947			

[1]LL : Canola monoculture, W-LL : wheat-canola rotation, P-B-LL : Pea-Barley-Canola rotation

[2]DF : Degree of freedom

In the rhizosphere of monocultured canola, AMF were only represented by two genera: *Claroideoglomus* (99.1%) and *Archaeospora* (0.9%). The 2-crop system (wheat - canola) showed three genera: *Claroideoglomus* (17.7%), *Diversispora* (59.7%) and *Paraglomus* (22.4%). Finally, the 3-crop system (pea, barley and canola) showed six genera: *Claroideoglomus* (57.2%), *Funneliformis* (0.8%), *Diversispora* (2.2%), *Archaeospora* (28.8%), *Glomus* (3.1%), and one unknown AMF genus (7.7%).

In bulk soil, canola monoculture showed again only two genera of Glomeromycota *Funneliformis* (65.1%) and *Paraglomus* (34.8%). The two-crop rotation system showed four AMF genera, but only two were abundant*: Funneliformis* (90.3%) and *Rhizophagus* (9.5%); the other two were an unknown AMF species (0.03%) and *Glomus* (0.03%).

Crop rotations influenced canola AMF community structure both in the rhizosphere and bulk soil as shown by Canonical Analyses (Figures 14 and S11). In the rhizosphere, two AMF ASV (ASV40 *Claroideoglomus* sp. and ASV42 *Archaeospora* sp.) were associated with canola monoculture, five ASVs (ASV86 and ASV1487 belong to *Diversispora*; AS 358 and ASV1667 belong to *Claroideoglomus* ; and ASV711 belongs to *Paraglomus*) were associated with the two-crop rotation system, and six ASVs (two *Claroideoglomus*, two *Glomus*, one *Paraglomus* and an unidentified AMF) were associated with the three-crop rotation system (Figure S11).

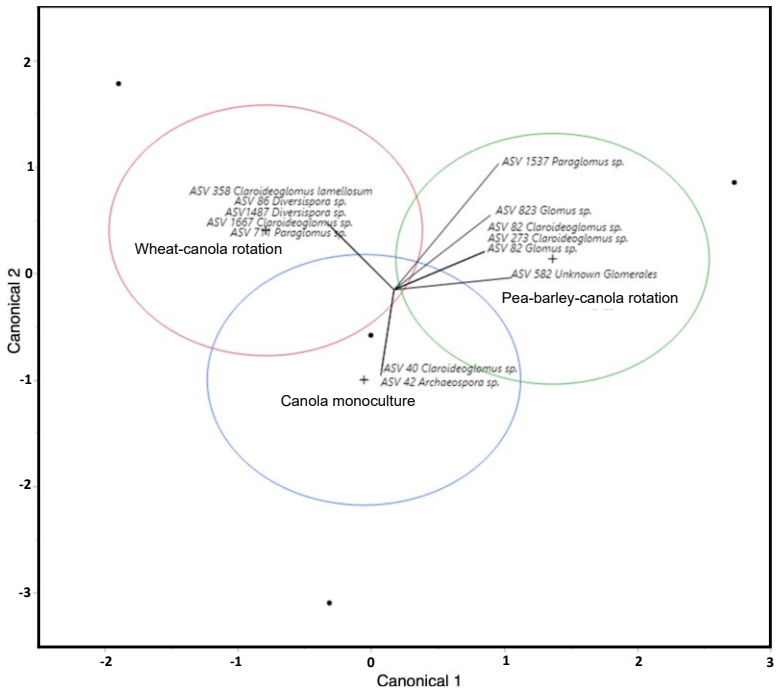

Figure 14. Canonical analysis of AMF community from the canola rhizosphere. Circles in red, blue and green represent the different crop rotations while vectors show different AMF ASV which are likely to be preferentially associated to each of the crop rotations. The more separated the circles are, the more the community structure between the crop rotations are different. The more the arrow is directed to the center of a circle, the more the ASV was associated with a certain crop rotation.

In bulk soil under canola monoculture, we found ASV149 (*Paraglomus occultum*) and ASV18 (*Funneliformis mosseae),* under the two-crop system we found two *Funneliformis*: ASV1254 and ASV10, one *Rhizophagus* ASV1809 and one *Glomus* ASV 1396; and under the three-crop system we found three different *Diversispora*: ASV1774, ASV47 and ASV22, two *Claroideoglomus*: ASV79 and ASV203 and finally, two *Paraglomus*: ASV61 and ASV1462.

Network analysis and the microbial cohorts of canola-associated AMF.

Network analysis revealed that 24 AMF ASVs are putatively interacting in the rhizosphere and 26 in the bulk soil (Figures 15-19). Potential interactions between AMF were always co-occurrences while no mutual exclusions were found.

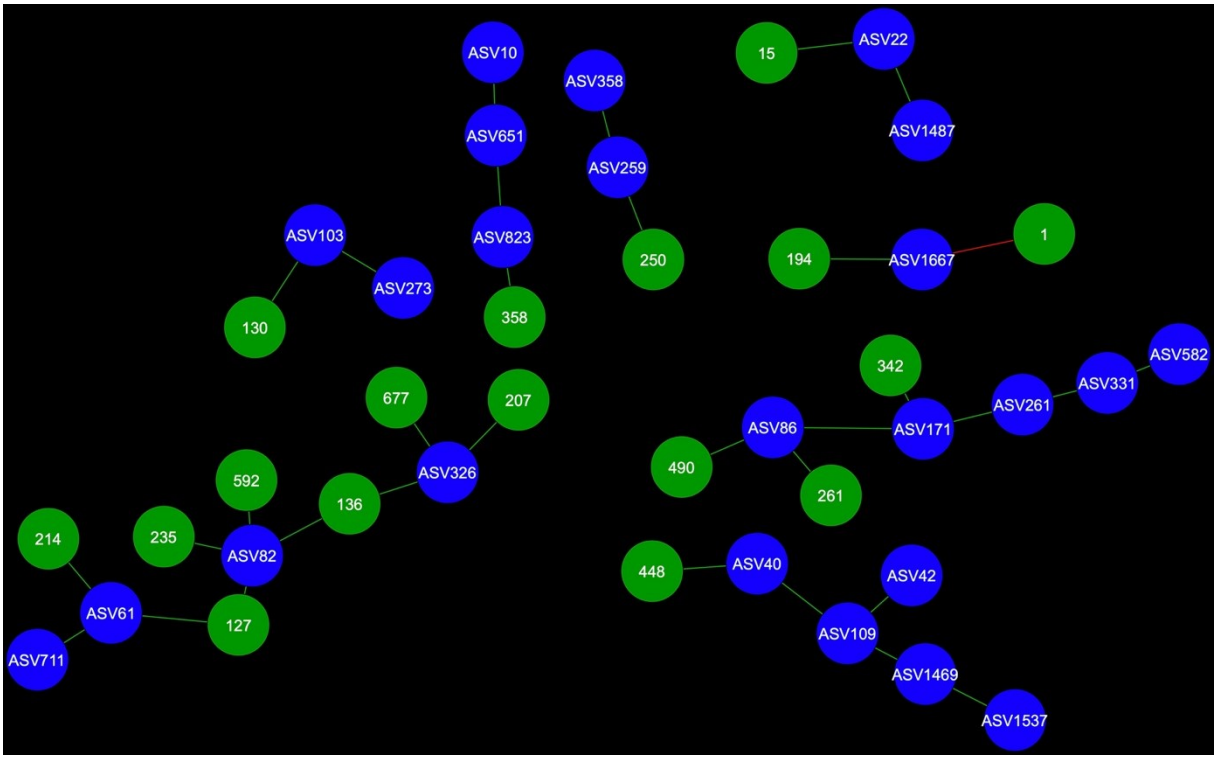

Figure 15. Fungal cohorts of canola AMF in the rhizosphere. The nodes in blue are the AMF ASV and in green the fungal ASV. Red edges signify mutual exclusion whereas green edges signify positive co-occurrence of species.

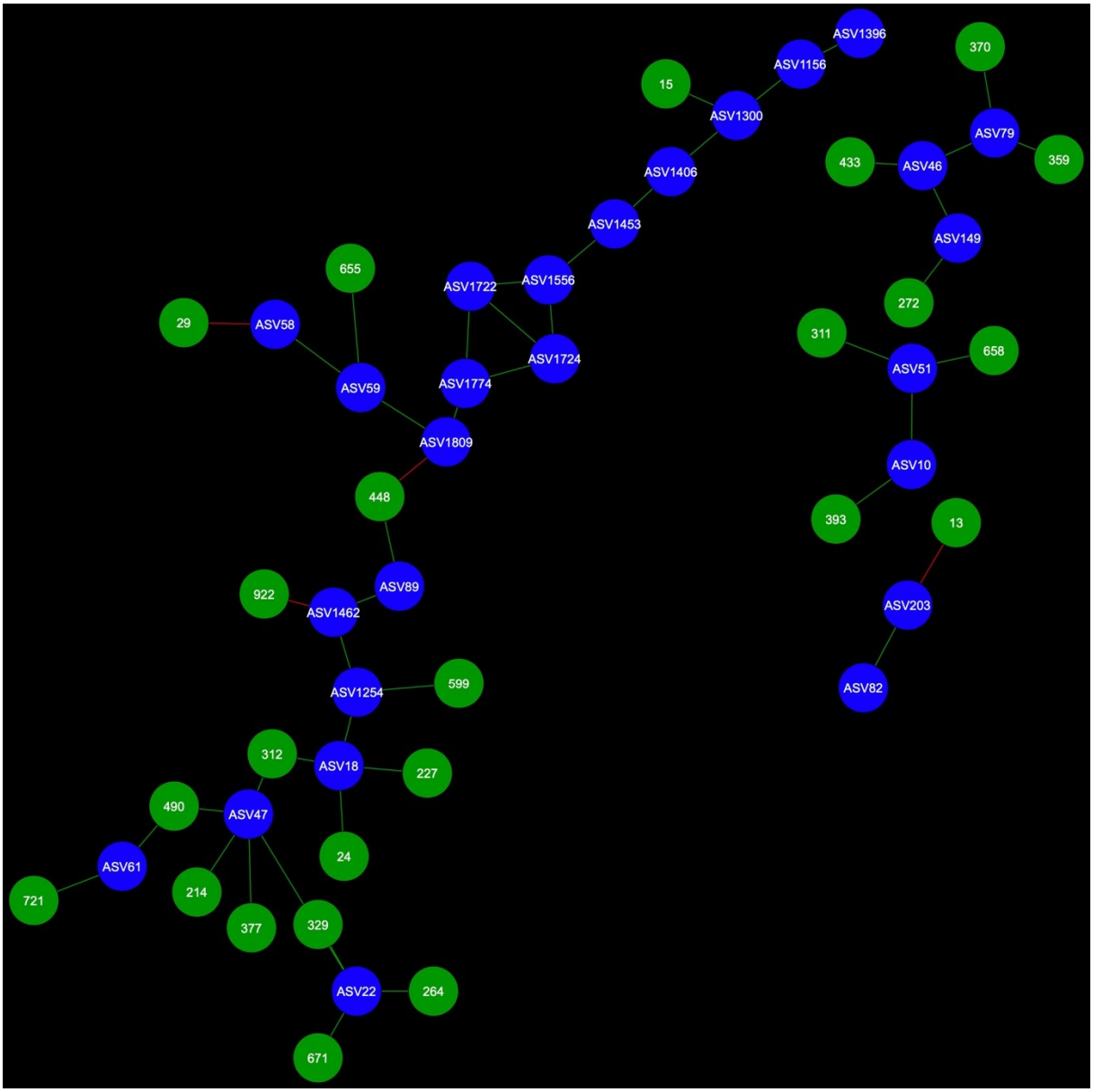

Figure 16. Fungal cohorts of canola AMF in bulk soil. The nodes in blue are the AMF ASV and in green the fungal ASV. Red edges signify mutual exclusion whereas green edges signify positive co-occurrence of species.

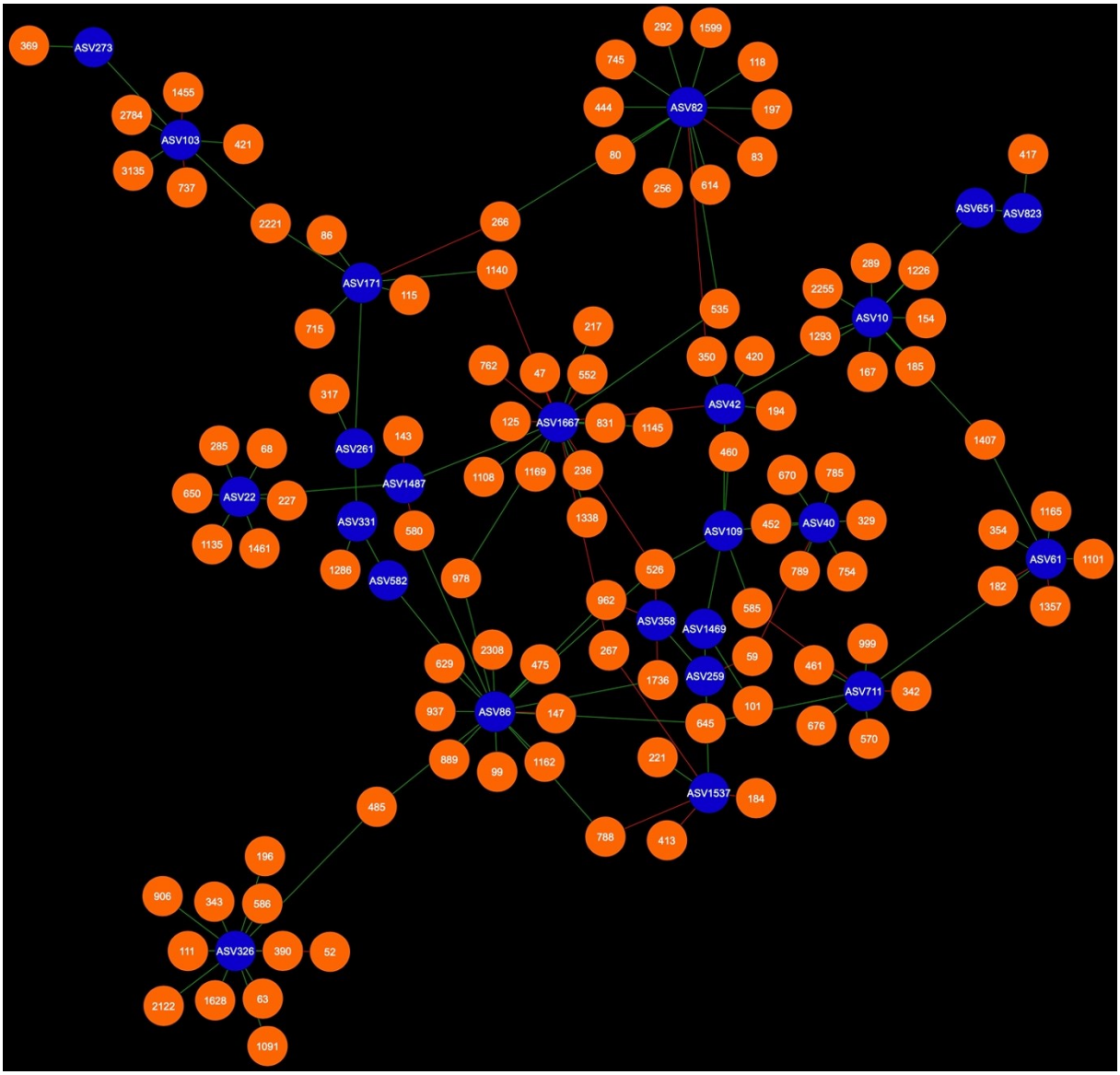

Figure 17. Bacterial cohort of canola AMF in the rhizosphere. The nodes in blue are the AMF ASV and in orange are the bacterial ASV. Red edges signify mutual exclusion whereas green edges signify positive co-occurrence of species.

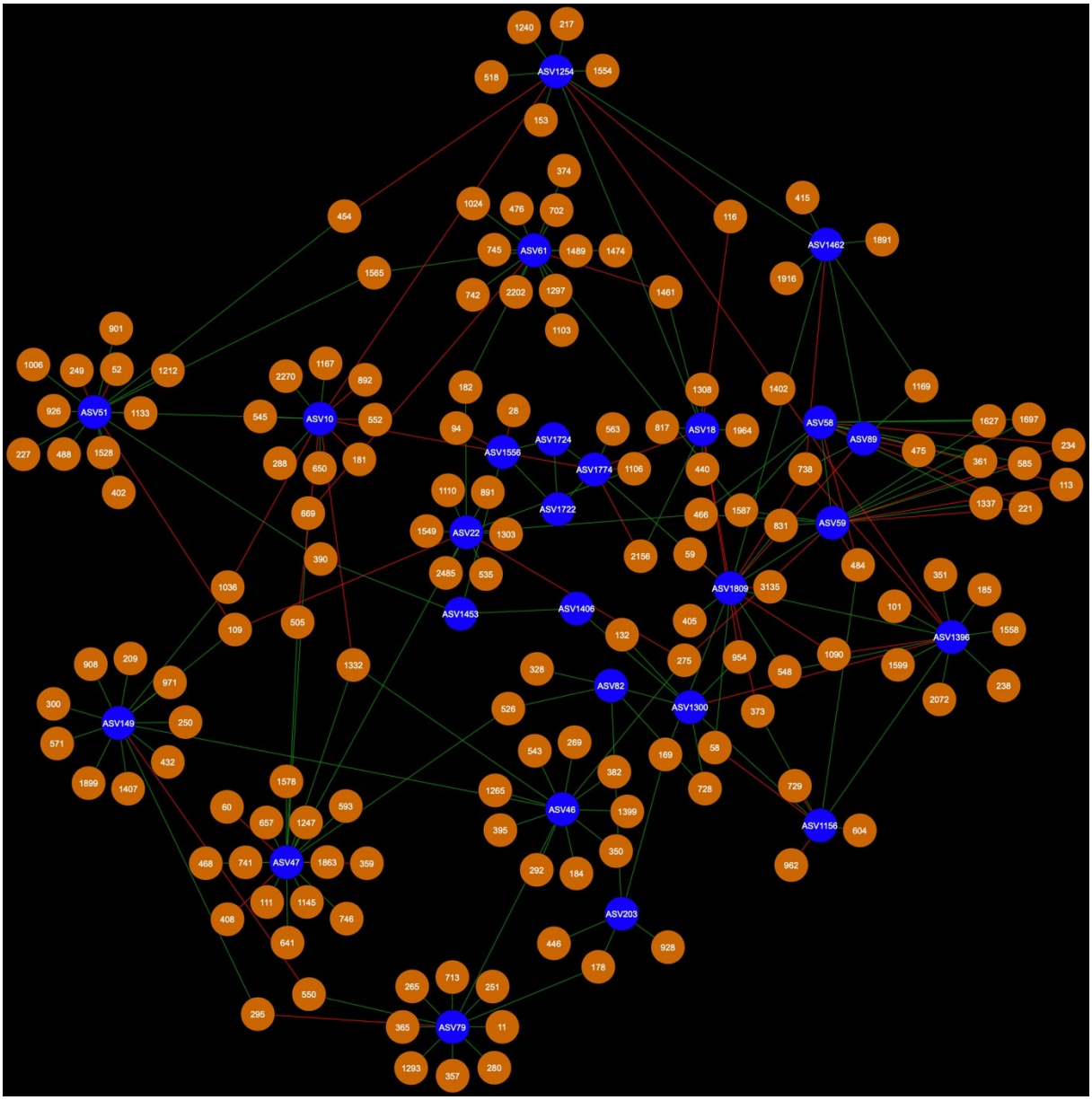

Figure 18. Bacterial cohort of canola AMF in bulk soil. The nodes in blue are the AMF ASV and in orange are the bacterial ASV. Red edges signify mutual exclusion whereas green edges signify positive co-occurrence of species.

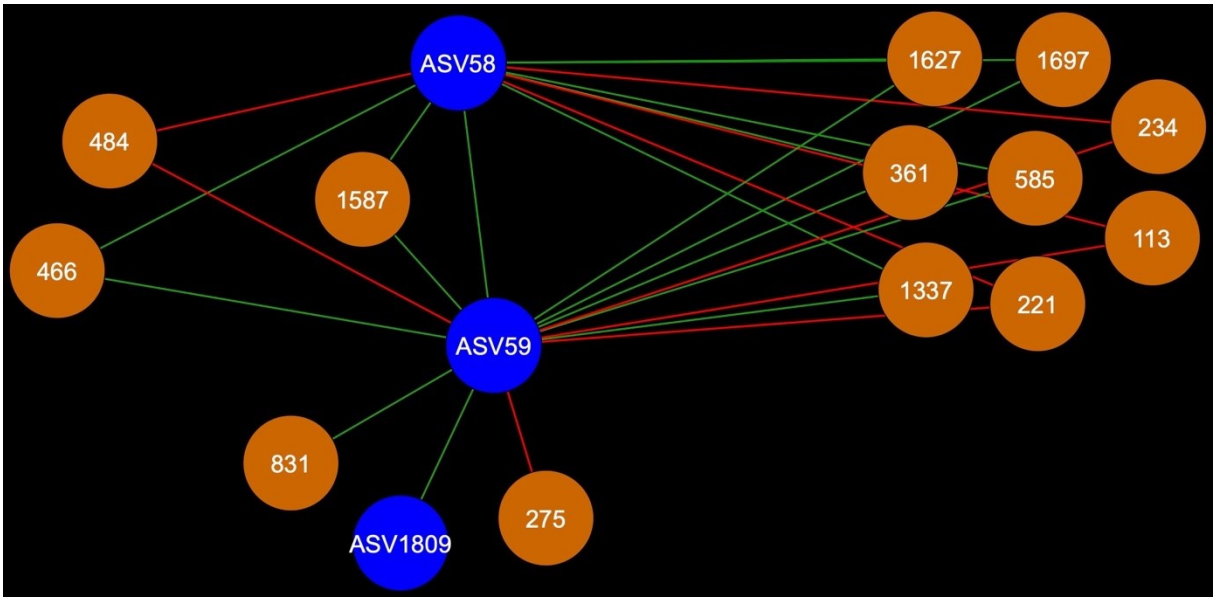

Figure 19. Bacterial cohort shared between AMF ASV 58 (Funneliformis mosseae) and AMF ASV 59 (Rhizophagus iranicus). The nodes in blue are the AMF ASV and in orange are the bacterial ASV. Red edges signify mutual exclusion whereas green edges signify positive co-occurrence of species.

Microbial networks were broadly different in term of connectivity. Fungi-AMF networks showed relatively few potential interactions: 41 nodes and 33 edges in the rhizosphere (Figure 15) and 50 nodes and 48 edges in the bulk soil (Figure 16), whereas potential interactions in the bacteria-AMF network were more abundant with 130 nodes and 142 edges in the rhizosphere (Figure 17) and 175 nodes and 227 edges in the bulk soil (Figure 18). The occurrence of AMF ASVs rarely corresponded with the occurrence of fungal ASV (FASV). In contrast, all AMF ASV except two (AMF ASV1722 and AMF ASV1724, both identified as *Glomus indicum*), have potential relationships with at least one bacterial ASV (BASV), in each of the interkingdom networks (Figures 17 and 18).

In the rhizosphere, AMF cohorts rarely shared common genera with the same type of relationship, but in the bulk soil (Tableau S6), the cohorts were dominated by Vicinamibacteraceae

as this clade was present 21 times across all the AMF cohorts (Tableau S6). In the bulk soil, AMF ASVs rarely shared an important fraction of their bacterial or fungal cohort (Tableaux S6 and S7), but AMF ASV58 (*Funneliformis mosseae*) and AMF ASV59 (*Rhizophagus iranicus*) shared their microbial cohort almost entirely (Figure 19). All the BASV shared between the two AMF ASVs were shared with the same type of relationship.

Discussion

How an AMF community persist after 10-years of canola monoculture?

Arbuscular mycorrhizal fungi (AMF) depend entirely on a host plant for their carbon nutrition as they can't produce palmitic acid by themselves (Trépanier et al., 2005). It is believed that they cannot complete their lifecycle, reproduce, and maintain their community in soil without a living host plant. As our experiment was conducted with a canola cultivar possessing the Liberty® herbicide technology, we can exclude putative effects of weed as a host during canola growth, since no weeds were present in the fields along with canola plants. Harker et al., (2015) reported weeds in this experiment, but their density was only calculated pre-spray before the seeding, during canola growing phase, and particularly in canola monoculture, non-selective herbicide gluphosinate kills the vast majority of weeds reaching 90% mortality on the most common weeds in North America at 70 $g.ha^{-1}$ (Hoss et al., 2003). In this study we used gluphosinate at 900 $g.ha^{-1}$ and thus determined that weed could only have a negligible impact on AMF community in our assay. Canola as other Brassicaceae plants are well documented to be non-mycorrhizal plants and it was assumed that its successive monoculture lead to a poor presence or absence of AMF in its soil. Our study clearly demonstrated the persistence of AMF communities in the soil in which canola was cultivated as a sole crop for a period of 10 years long. Even if the community is very poor (Table S3). More

importantly, we retrieved sequences of several AMF taxa from a narrow zone of influence (rhizosphere) of this presumably non-host crop plant. This could be explained by the following hypotheses:

(i) Arbuscular mycorrhizal fungi may colonize epidermis and external cell layers of canola roots without establishment of functional mycorrhizal symbiosis. Such superficial colonization of canola roots could be sufficient for AMF to undergo limited growth and spore production contributing to their maintenance. Mycorrhizal colonization of the superficial layer of the root cortex of *Arabidopsis thaliana*, another non-host plant of the same family as canola, was previously reported (Veiga et al., 2013; Cosme et al., 2018). AMF can be detrimental to several non-AMF-host plant genera like *Stelaria* and *Pinus* by infecting their root interior (Wagg et al., 2011; Veiga et al., 2012); it is possible that AMF could infect canola in a similar way. Fonseca et al., (2001) reported that when *Brassica rapa* was inoculated with AMF, it reacts and increases concentration of δ15N and δ13C in the shoot, but the influence of AMF in this case is to be relativized as the well-watered regime of the experiment emphasised the dry weight of the plants. Dąbrowska et al., (2012) also reported that canola gene BnMT2 involved with mycorrhizal symbiosis changed its expression patterns when canola was inoculated with *Glomus* spores, leading to longer shoots and a lower fresh biomass on ten-week-old canola seedlings, that suggested a negative effect of the inoculation of AMF spores on canola. As canola roots is not a beneficial environment for AMF, their presence in canola rhizosphere and soil could be from another reason.

(ii) Bacteria are closely or loosly interacting with AMF mycelia forming biofilms (Seneviratne et al., 2008; Lecomte et al., 2011; Iffis et al., 2014; Guennoc et al., 2017). These biofilms can help the AMF acquiring phosphate through phosphate rock solubilization (Taktek et al., 2017) or allowing AMF to resist harsh environmental conditions (Iffis et al., 2016). Root endophyte bacteria

could be actively recruited by AMF after their penetration in the root interior and have beneficial effects on the plant host (Ujvári et al., 2021). They can also increase AMF root colonization (Bourles et al., 2020; Sangwan and Prasanna, 2021; Ujvári et al., 2021). According to Scheublin et al., (2010) and Agnolucci et al., (2015), the most common culturable bacteria ever identified in the biofilms forming on AMF spores are *Pseudomonas*, *Streptomyces*, *Arthrobacter* and *Oxalobacteraceae*. In Taktek et al., (2015) the most frequent bacteria encountered were *Burkholderia*, Lecomte et al., (2011) reported the dominance of *Varivorax*, *Bacillus*, *Kocuria*, *Microbacterium* and *Sphingomonas*, and Iffis et al., (2016) had *Sphingomonas*, *Pseudomonas*, *Massilia* and *Methylobacterium* as their most abundant species associated to AMF vesicles in roots. These taxonomic profiles are very different from what we could identify in AMF cohorts by network analysis, as these genera were not detected in our study. With our more inclusive method, we found a high frequency of *Vicinamibacterales* in the cohorts of AMF living in canola rhizosphere and bulk soil, in the Canadian prairie. *Vicinamibacterales* was associated with tolerance to trace metal contamination of soil, in particular Cu, but its ecological role still remains obscure (Chun et al., 2021). The fact that almost all AMF in canola rhizosphere and bulk soil had a bacterial cohort in the network analysis, comfort the possibility that bacteria could bring advantage to AMF in a hostile environment, facilitating interface with non-host plant. Bacteria acting as host for AMF remains another possibility (Hildebrandt et al., 2006; Horii and Ishii, 2014; Abdellatif et al., 2019)

Arbuscular mycorrhizal fungi may get some carbon from canola through complex interactions with other microbes such as mycorrhiza helper bacteria or *Trichoderma* spp., soil fungi could also be one of the reason AMF were able to maintain their presence in canola rhizosphere and bulk soil through the years. However, the number of interactions of AMF with fungi recorded

here was much lower than the number of interactions with bacteria (Figures 15, 16, 17, 18). It is possible that soil fungus increase yield and overall production of host plants when co-inoculated with AMF and in presence of pathogenic fungi (Martínez-Medina et al., 2009; Nzanza et al., 2012; Commatteo et al., 2019). It can also allow AMF to colonize the rhizosphere of Brassicaceae like *A. thaliana* or canola (*B. napus*) (Poveda et al., 2019; Jatana et al., 2021). For example, *Nectria* and *Leptosphaeria* were found in association with AMF spores (Hijri et al., 2002). However, soil fungi ecology remains largely unknown, and it is difficult to assess their real ecological roles. The fungi associated with AMF in network analysis could be AMF-helpers candidates such as FASV592 (Unknown Nectriaceae) found in the microbial cohort of AMF ASV82 (*Claroideoglomus* sp.) in canola rhizosphere. The fact that AMF ASV47 (*Diversispora* sp.) had FASV377 *Olpidium brassicae* in its cohort is also of interest. *O. brassicae* is the most common fungus found in canola roots and its influence on canola is not yet fully understood (Lay et al., 2018c, 2018b; Floc'h et al., 2021). The fact that *O.brassicae* and *Diversispora* sp. in canola bulk soil could share a relationship of co-occurrence is to be noted and cooperation between AMF and *O. brassicae* remains a possibility.

The presence of AMF in canola rhizosphere and bulk soil could also be explained by a combination of these scenarios. However, we do not rule out that dispersion of AMF propagules by wind, rain and animals may contribute to some extent AMF to persist in canola monoculture overtime (Addy et al., 1998; Smith and Read, 2008; Cornejo et al., 2009; Pepe et al., 2018). Further investigations are needed to shed light of the mechanisms by which AMF persist in soils in the absence of non-host plants. Microbial complexity in soil and plant roots is still poorly understood and microbial interactions in plant microbiomes are just being investigated with mathematical tools since two decades (Friedman et al., 2008; Kurtz et al., 2015b). It is also possible that the AMF

community we were able to retrieve from canola soil and rhizosphere was a remnant of the AMF community of the previous crop from the rotations, but as other studies report presence of AMF taxa in canola rhizosphere (Poveda et al., 2019; Floc'h et al., 2021), and since we found AMF in 10 year old canola monoculture, AMF coexistence with canola is very likely to be reality.

Effects of rotation systems on AMF communities and interactions with their associated microbes

Crop rotation are often used in agriculture to mitigate the accumulation of pathogens that occurs in monoculture and that is the case for canola (Hummel et al., 2009; Harker et al., 2015b). Crop rotation are known to be a tool that affect the subterranean microbiota of plants (Suzuki et al., 2012; Xuan et al., 2012; Souza et al., 2013; Detheridge et al., 2016; Fan et al., 2020). However, crop rotation, depending of their diversification, impact the community structure of canola fungal microbiota but not its bacterial microbiota in this experiment (Floc'h et al., 2020b, 2020d). Crop rotation are also known to have an impact on AMF community in host plants (Higo et al., 2014, 2018; Detheridge et al., 2016; Bakhshandeh et al., 2017; Hontoria et al., 2019). In our case, we were not able to test for the effect of crop rotation on AMF community structure due to data scarcity. However, our ordinations shown a clear differentiation between the AMF community of monoculture, 2-crop and 3-crop systems (Figure 14 and S11). With this, we can hypothesize that AMF community composition, as the composition of fungal community in canola rhizosphere and bulk soil, is influenced by crop rotation diversification.

AMF ASV58 (Funneliformis mosseae) and ASV59 (Rhizophagus iranicus) share their microbial cohort in canola bulk soil

Funneliformis mosseae is a widely spread AMF taxon in natural and disturbed environments. It is able to colonize a large range of hosts (Tanwar et al., 2013; Yang et al., 2017;

Feitosa de Souza et al., 2018; Jie et al., 2019). It was commonly found in highly polluted environments (Hassan et al., 2011; Li et al., 2020b). *Rhizophagus* is also a genus of AMF known for its adaptability and for being an R-strategist (Campagnac and Khasa, 2013; Sędzielewska Toro and Brachmann, 2016; Buysens et al., 2017; Zhang et al., 2018; Cruz-Paredes et al., 2020; Lee et al., 2020). Members of *Rhizophagus* genus have been commercialized and utilized as biofertilizers for more than three decades (Badri et al., 2016; Hijri, 2016; Basiru et al., 2021). *Rhizophagus* is also known for its ability to interact with plant growth-promoting bacteria (Battini et al., 2016; Loján et al., 2017). These two AMF ASV seems to share the same ecological niche and interact with the same bacteria. To the best of our knowledge, it is the first time that an overlap of 90% of bacterial cohort between two AMF species is reported. We know very little about the potential ecology and functions of the members shared in *F. mosseae* (AMF ASV58) and *R. iranicus* (AMF ASV59) cohorts. Among 11 taxa shared between AMF ASV58 and AMF ASV59, seven were identified at genus level and among those, only four genera were the subject of more than two scientific publications: *Sphingomonas*, *Altererythrobacter*, *Luteolibacter*, and *Gemmatimonas* (Table S2). The *Sphingomonas* (BASV234) is the only one with a relation of mutual exclusion with the two AMF ASVs. *Sphingomonas* was isolated from spores of *Rhizophagus irregularis* and showed biofilm-like formation on hyphae (Lecomte et al 2011). It was also found in leek rhizosphere (Nunes da Rocha et al., 2011), and diverse environments including biological soil crusts and fresh water (Ko et al., 2017; Lee et al., 2017; Zhang et al., 2017a). The others three taxa were also found in diverse environments (Jiang et al., 2012; Yuan et al., 2017; Kang et al., 2019; Meng et al., 2019; Dahal et al., 2021), and *Gemmatimonas* was reported as a potential denitrifying bacterium (Chee-Sanford et al., 2019). Potential interactions between *F. mosseae* and *R. iranicus*, and the bacterial taxa represented in their cohorts should deserve further research attention.

Conclusion

A community of AMF can be found in the rhizosphere and bulk soils of canola (*B. napus*), a non-host plant, even after 10-years of canola monoculture. This finding put a new light on the ecology of AMF in rhizosphere and bulk soil in absence of a mycotrophic host plant. AMF forms a broad range of potential interactions with diverse bacterial and fungal species that may be important for AMF, especially in absence of host plants. How AMF interacts with other microbes is unclear, but their associations in networks analysis indicates the possibility of a direct or indirect interaction with other fungi and bacteria, which needs to be clarified.

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Conclusion générale et perspectives

L'objectif principal de ma thèse a été, par l'étude du microbiote souterrain du canola, de vérifier l'existence et le maintien dans le temps de plusieurs concepts d'écologie théorique, à savoir la notion de core microbiote et de taxons clefs de voûte dans le microbiote des plantes en général. L'identification des acteurs microbiens d'importance écologique dans le microbiote souterrain du canola, a permis de mieux comprendre leurs interactions grâce à une approche nouvelle, l'analyse de réseau. Mes travaux ont permis d'identifier les limites de la définition du core microbiote et son instabilité temporelle. En revanche, l'utilisation de l'analyse de réseau pour détecter de potentiels taxons clef de voûte a montré des résultats prometteurs.

L'examen des microbiotes sous étude a montré l'existence d'un core microbiote fongique chez le canola. Ce core fongique peut être constitué d'un trentaine d'espèce à un temps *t*, mais son effectif varie de manière importante entre *t*, *t*+3 ans et *t*+5 ans. La variation annuelle du core fongique observée ici indique qu'il faut considérer la temporalité du microbiote d'une plante sur un temps long pour en saisir ses variations compositionnelles ou la fluctuation de l'abondance des microorganismes toujours présent au sein de ces variations, comme cela a été le cas pour *Olpidium brassicae* dans le cas du canola.

Olpidium brassicae s'est retrouvé en relation étroite avec le canola dans tous les systèmes culturaux et toutes les locations géographiques utilisées dans ma recherche. Sa présence dans les racines et la rhizosphère du canola était soupçonnée de longue date (Campbell et al., 1995) au sein de la famille des Brassicaceae, mais sa confusion avec *O. virulentus* n'a été résolue que récemment par Lay et al., (2018b) qui ont souligné l'incertitude vis-à-vis du rôle écologique de *O. brassicae*

au sein du microbiote de ces plantes. Son aspect omniprésent dans les travaux de cette thèse a mis en évidence une probable importance de ce champignon pour la plante hôte bien qu'à forte concentration il puisse exercer des effets néfastes.

D'autres champignons clef de voûte ont montré une importance probable au sein de la communauté fongique ou interrègne. C'est par exemple le cas pour le genre *Mortierella*, qui est massivement représenté dans les analyses de réseau de cette thèse.

Dans le microbiote bactérien souterrain du canola, les variations de structure des communautés et d'interactions potentielles se sont révélées très différentes de celles observées dans le microbiote fongique. Mon étude sur le canola a permis d'établir des conclusions solides. La première différence majeure entre les deux communautés a été leur organisation théorique : le microbiote fongique fait état d'un core microbiote, mais pas le microbiote bactérien. Si la présence d'un core microbiote bactérien reste à démontrer, la présence d'une bactérie toujours en association avec le canola a en revanche été soulignée avec le cas de *Pseudoarthrobacter* sp. et *Marmoricola* sp., cependant leur présence ne permet pas de vérifier l'hypothèse de l'existence d'un core microbiote. Cette bactérie dont l'écologie au sein du microbiote souterrain du canola, reste à résoudre, fait un bon candidat pour de futures études PGPB étant donné son effet structurant dans les analyses de réseau. Il en va de même pour *Brayrhizobium* qui pourrait être considéré comme un candidat potentiel à l'amélioration par biocontrôle ou par effet PGPB de la production de canola.

Cette thèse de doctorat a aussi permis de mettre en lumière l'utilité des analyses de réseaux par inférence d'inverse covariance pour approcher les interactions microbiennes dans des écosystèmes complexes. Le fait que l'algorithme SPIEC-EASI derrière ces analyse reflète une réalité statistique non biaisée par le grand nombre de zéro dans les matrices d'abondances ou la dimensionalité desdites matrice, permet la mise en place d'études préliminaires à la sélection

d'espèces d'importance écologique pour les agrosystèmes. La mise en lumière des interactions potentielles par analyse de réseau, ici, a permis la formulation d'hypothèses quant au rôle écologique des espèces clef de voûte qui sont cohérentes avec les descriptions précédemment publiées dans la littérature scientifique. Il reste à vérifier dans des études contrôlées si les espèces identifiées dans cette thèse sont d'intérêt agronomique, par exemple en testant leur potentiel de promotion de croissance ou encore d'agent de lutte biologique contre certains pathogènes.

Si la méthode d'analyse et les outils mathématiques que j'ai utilisés pendant ma thèse sont faits de manière à minimiser les biais le plus possible, il me faut néanmoins relever quelques biais inhérents aux méthodes de biologie moléculaire que j'ai utilisées. En effet, pour avoir accès à l'abondance des champignons et bactéries du sol il faut obligatoirement passer par l'utilisation de la PCR (« polymerase chain reaction »). Or en fonction de l'enzyme polymérase utilisée dans la réaction, certains fragments d'ADN du sol sont amplifiés plus que d'autres menant à une vision biaisée de la réelle composition des communautés microbiennes (Hansen et al., 1998; Kanagawa, 2003; Wojdacz et al., 2009). A ce biais méthodologique s'ajoute aussi un problème inhérent au séquençage d'amplicons, qui est la normalisation de la quantité d'ADN pendant les procédures de séquençage. Ce phénomène aboutirait à ce que les données qui en sont issues soient compositionnelles (Gloor et al., 2017) et celles-ci nécessiteraient une correction des abondances relatives issues du séquençage d'amplicons à des abondances absolues, par exemple par le biais de la qPCR (Azarbad et al., 2022). Cela a un impact direct sur les conclusions tirées d'analyses de diversité alpha, particulièrement sensibles à ce genre biais. Dans ma thèse, n'ayant pu utiliser de qPCR pour raisonner en abondances absolues, la majeure partie des analyses sont issues des statistiques multivariées et d'analyse de béta diversité qui sont moins sensibles à la « compositionnalité » des données de séquençage d'amplicons. Cependant il faut garder en

mémoire que nos méthodes pour approcher la réalité biologique du sol vivant ne sont pas aussi optimales que l'on voudrait. L'allégorie de la caverne de Platon illustre parfaitement la façon dont nous approchons la complexité du sol vivant en tant qu'écologues microbiens. Cela ne diminue pas la qualité et l'intérêt des résultats de ma thèse, dont les méthodes d'analyses peuvent être utilisées pour d'autres plantes que le canola de façon tout à fait pertinente (Nahar et al., 2020; Ahmed et al., 2021). Mais il faut toujours garder à l'esprit que notre vision des écosystèmes microbiens du sol est perfectible et que les résultats issus de travaux d'écologie microbienne théoriques doivent être suivi de travaux d'écologie microbienne appliqués de façon à vérifier la réalité des structures microbiennes d'intérêt identifiées par la méthode théorique, par exemple en réalisant des travaux d'isolation des espèces clef de voûte et en testant leur intérêt agronomique.

Enfin, si cette thèse offre des perspectives d'amélioration de production pour le canola et d'établissement d'une méthode d'étude préliminaire du microbiote des plantes en général, elle offre aussi des perspectives en matière d'étude des fonctions microbiennes. Avec le séquençage d'amplicons, nous avons pu avoir un aperçu taxonomique des entités microbiennes présentent dans les différents agroécosystèmes étudiés. Cependant, l'accessibilité à de l'information fonctionnelle vis-à-vis des espèces prises en compte dans mes différentes études a été quasiment impossible de par la nature du séquençage d'amplicon. Or, l'amplification des fonctions microbiennes par métagénomique est un moyen de plus en plus accessible à l'étude du microbiote des plantes et les analyses de réseau y sont tout à fait applicable. Si la métagénomique fonctionnelle n'a pu être utilisée par faute de financement lors de cette thèse, il est tout à fait possible de reprendre mes résultats pour les intégrer à une étude métagénomique du microbiote souterrain du canola ou d'appliquer ma méthodologie à un jeu de données de fonction microbiennes.

C’est principalement en ce fait que ma thèse de doctorat participe à l’avancée de la science : l’optique est de défricher une voie particulièrement complexe en offrant des moyens de synthèse de l’information par le biais d’analyses statistique multivariées et d’analyses de réseau. Si ma thèse offre des possibilités réelle de recherches vis-à-vis de l’amélioration de la production de canola au Canada, elle permet aussi de définir une méthode d’analyse des microbiotes réplicable à l’ensemble des plantes, le tout en offrant la possibilité de pousser plus loin les expérimentations pour identifier de manière subséquente des microbes d’intérêts pour la production végétale en général.

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Annexes

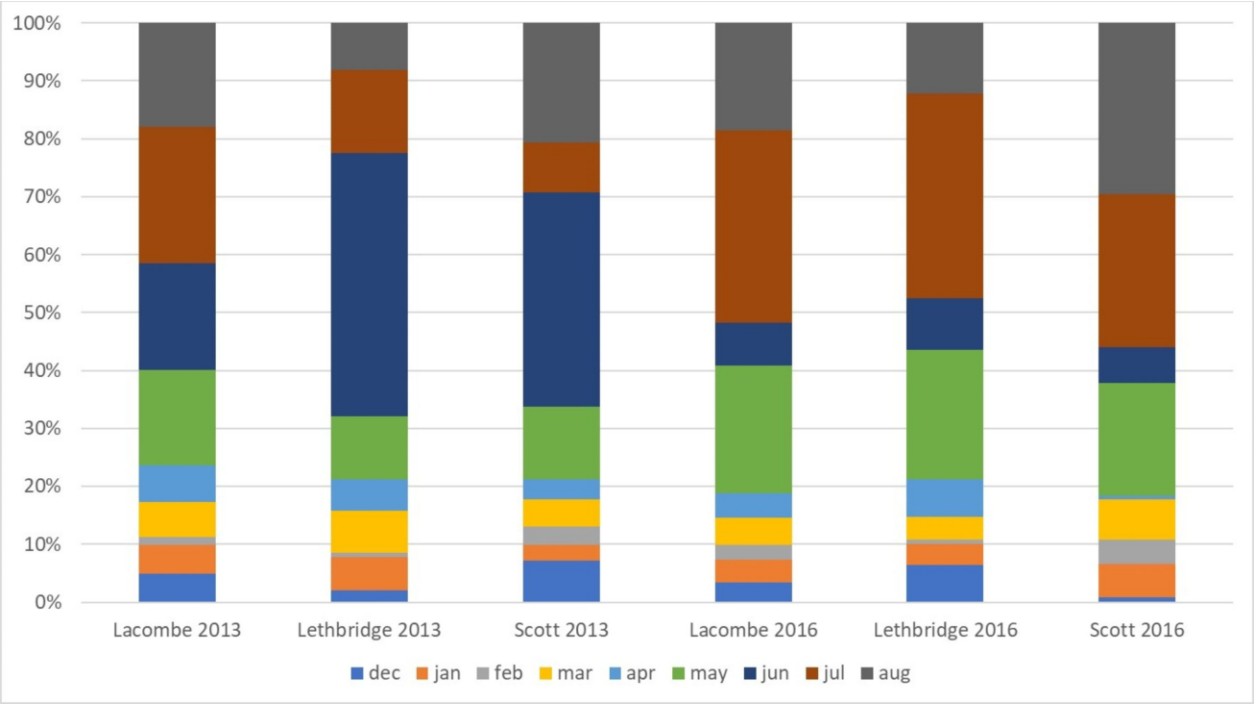


Figure S1. Cumulative precipitation proportion at each site in 2013 and 2016. The fungal communities in the canola rhizosphere may have been influenced by moisture availability in July 2016.

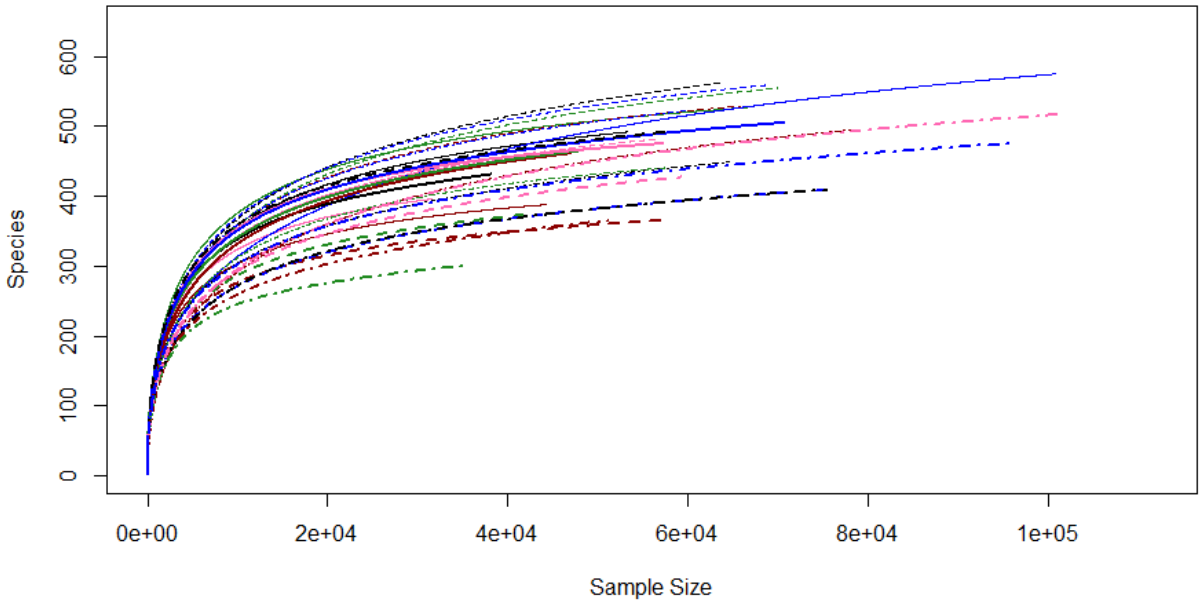


Figure S2: Rarefaction curves for each rhizosphere soil sample, showing the relationship between the number of ZOTUs and the abundance of ITS sequences reads, in the 2013 dataset. The vertical black line indicates the plateau threshold for all curves.

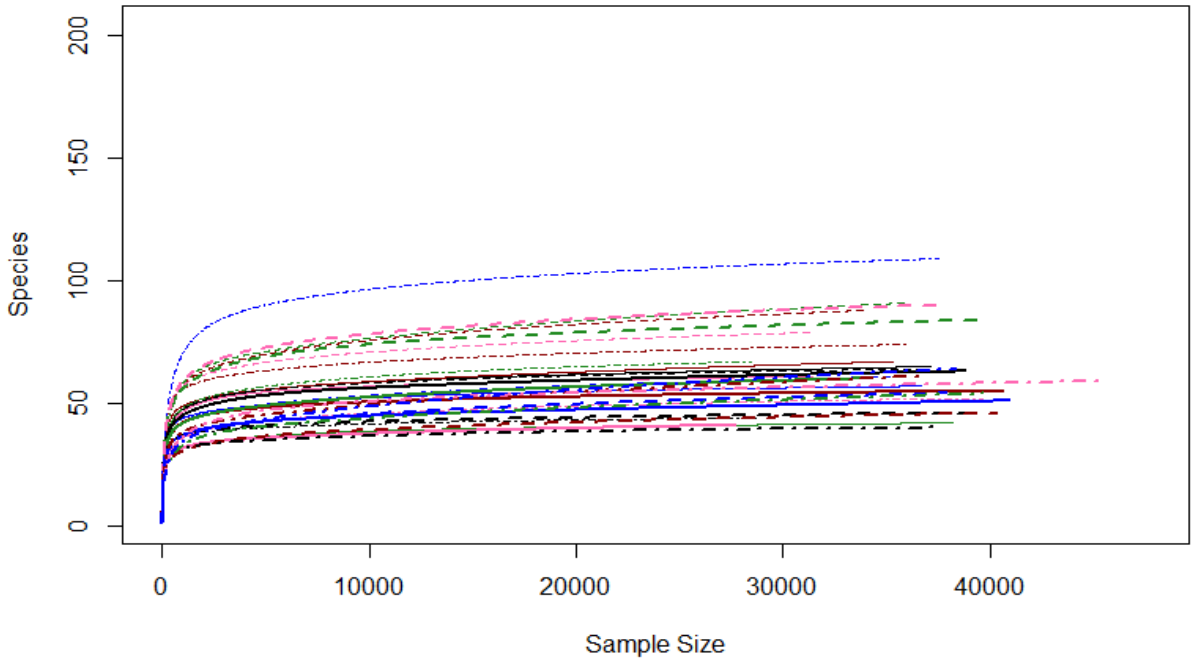


Figure S3: Rarefaction curves for each rhizosphere soil sample, showing the relationship between the number of ZOTUs and the abundance of ITS sequences reads, in the 2016 dataset. The vertical black line indicates the plateau threshold for all curves.

Table S1. Effects of canola cultivar and of crop diversification on the α-diversity of the fungal community of the canola rhizosphere in 2013 and 2016, as determined by ANOVA.

2013						
Index [1]	Canola cultivar [2]		Crop diversification[3]		Cultivar × Crop diversification	
	F [4]	*P*	*F*	*P*	*F*	*P*
Chao 1	0.2634	0.9000	0.8852	0.5358	1.0425	0.4182
Richness	0.0301	0.9982	0.7140	0.6779	0.5908	0.7805
Simpson D	0.7237	0.5801	0.3579	0.9374	0.6337	0.7455
Shannon	0.4604	0.7643	0.3234	0.9531	0.5584	0.8062
2016						
Index	Canola cultivar		Crop diversification		Cultivar × Crop diversification	
	F	*P*	*F*	*P*	*F*	*P*
Chao1	0.0258	0.8728	2.2604	0.1368	0.0417	0.8386
Richness	0.4594	0.5000	2.0936	0.1520	0.9817	0.3249
Simpson D	0.0002	0.9881	3.7672	0.0559	0.4570	0.5010
Shannon	0.0517	0.8207	2.3493	0.1294	0.0945	0.7594

[1] Chao1 = Chao's diversity index, Richness = count of ZOTU, Simpson D = Simpson's diversity index, Shannon = Shannon's diversity index

[2] Canola cultivar: effect on fungal α-diversity of the cultivar InVigor 5440, a Liberty Link cultivar resistant to glufosinate herbicides; and RR, canola 71-45, a Roundup Ready cultivar resistant to glyphosate.

[3] Crop diversification: effect of the crop rotation treatments on fungal α-diversity.

[4] *F*: F-statistic value; *P*: P-value for the ANOVA test.

Table S2: Relative abundance of ZOTUs belonging to the eco-microbiome in 2013 in relation to crop diversification level in the rotation system.

ZOTU	Identity	Monoculture[1]	Low	Medium	ZOTU	Identity	Monoculture	Low	Medium
ZOTU1	*Olpidium brassicae*	0.0952[2]	0.1136	0.1242	ZOTU35	*Fusarium solani*	0.0073	0.0077	0.0098
ZOTU10	*Plectosphaerella* sp.	0.0499	0.0363	0.0483	ZOTU387	*Candida parapsilosis*	0.0004	0.0004	0.0003
ZOTU102	*Trichoderma koningii*	0.0026	0.0014	0.0008	ZOTU39	*Cylindrocarpon* sp.	0.0056	0.0068	0.0063
ZOTU11	*Fusarium* sp.	0.0402	0.0281	0.0219	ZOTU4	*Fusicolla* sp.	0.0518	0.0600	0.0559
ZOTU123	*Leptosphaeria* sp.	0.0023	0.0018	0.0021	ZOTU40	*Cylindrocarpon* sp.	0.0065	0.0084	0.0058
ZOTU13	*Fusarium avenaceum*	0.0165	0.0254	0.0296	ZOTU41	*Tetracladium* sp.	0.0103	0.0124	0.0101
ZOTU15	*Fusarium* sp.	0.0202	0.0196	0.0289	ZOTU42	*Alternaria infectoria*	0.0055	0.0116	0.0115
ZOTU154	*Geomyces* sp.	0.0025	0.0038	0.0031	ZOTU49	*Clonostachys rosea*	0.0038	0.0073	0.0066
ZOTU16	*Mortierella* sp.	0.0289	0.0340	0.0285	ZOTU5	*Nectria* sp.	0.0470	0.0674	0.0589
ZOTU17	*Mortierella gamsii*	0.0293	0.0262	0.0236	ZOTU51	*Dendryphion nanum*	0.0168	0.0093	0.0077
ZOTU18	*Fusarium* sp.	0.0161	0.0165	0.0198	ZOTU54	*Cladosporium tenuissimum*	0.0088	0.0075	0.0054
ZOTU19	*Fusarium redolens*	0.0117	0.0122	0.0175	ZOTU56	*Exophiala* sp.	0.0058	0.0074	0.0078
ZOTU2	*Humicola grisea*	0.0525	0.0554	0.0585	ZOTU6	*Mortierella* sp.	0.0513	0.0541	0.0419
ZOTU21	*Fusarium* sp.	0.0153	0.0168	0.0182	ZOTU63	*Coniothyrium cereale*	0.0023	0.0089	0.0112

ZOTU22	*Mortierella* sp.	0.0096	0.0104	0.0155	ZOTU65	*Vishniacozyma victoriae*	0.0037	0.0092	0.0073
ZOTU229	*Aspergillus welwitschiae*	0.0013	0.0011	0.0013	ZOTU68	*Mortierella alpina*	0.0115	0.0109	0.0117
ZOTU24	*Alternaria alternata*	0.0224	0.0178	0.0145	ZOTU7	*Cladosporium* sp.	0.0789	0.0459	0.0336
ZOTU25	*Plectosphaerella* sp.	0.0143	0.0100	0.0205	ZOTU71	*unknown*	0.0040	0.0058	0.0026
ZOTU26	*Solicoccozyma aeria*	0.0172	0.0171	0.0157	ZOTU78	*Phoma sclerotioides*	0.0054	0.0054	0.0020
ZOTU27	*Fusarium solani*	0.0066	0.0072	0.0120	ZOTU8	*Nectria* sp.	0.0334	0.0431	0.0345
ZOTU29	*Ulocladium dauci*	0.0170	0.0151	0.0120	ZOTU84	*Trichocladium opacum*	0.0035	0.0032	0.0038
ZOTU3	*Mortierella* sp.	0.1033	0.0809	0.0735	ZOTU864	*Penicillium* sp.	0.0002	0.0002	0.0001
ZOTU30	*Fusarium* sp.	0.0028	0.0104	0.0199	ZOTU87	*Chaetomium globosum*	0.0027	0.0027	0.0060
					ZOTU9	*Thielavia* sp.	0.0556	0.0431	0.0494

[1]Crop diversification level: Monoculture, canola monoculture; Low, wheat-canola rotation; Medium, pea-barley-canola rotation; High, lentil-wheat-LL-pea-barley-RR; LL, canola InVigor 5440, a Liberty Link cultivar resistant to glufosinate herbicides; RR, canola 71-45, a Roundup Ready cultivar resistant to glyphosate.

[2]Each relative abundance value is a proportion of the total abundance of fungi in the core microbiome

Table S3: Abundant ZOTUs in the canola rhizosphere in relation to canola cultivar and cropping diversification level, in 2013 and 2016, based on indicator species analysis.

		2013				2016			
Canola[1]	Crop diversification[2]	Indicator species ZOTU	Closest identity	% similarity	*P* value[3]	Indicator species ZOTU	Closest identity	% similarity	*P* value
LL	Monoculture	ZOTU377	*Neurospora crassa*	99%	0.003 **				
		ZOTU1365	*Myrothecium roridum*	99%	0.014 *				
		ZOTU1806	*Zygopleurage zygospora*	95%	0.028 *				
		ZOTU1367	*Humicola* sp.	93%	0.036 *				
RR	Monoculture					ZOTU132	*unknown*	85%	0.032 *
LL	Low	ZOTU1449	unknown	81%	0.030 *	ZOTU147	*Leptosphaeria sclerotioides*	100%	0.034 *
		ZOTU2014	*Kurtzmanomyces* sp.	95%	0.017 *	ZOTU954	*unknown*	98%	0.012 *
		ZOTU2109	*Myrmecridium hiemale*	99%	0.050 *				
		ZOTU531	*Agrocybe pusiola*	99%	0.038 *				
		ZOTU1324	unknown	82%	0.036 *				
		ZOTU1602	*Schizothecium* sp.	94%	0.049 *				
		ZOTU1454	*Schizothecium* sp.	93%	0.049 *				
		ZOTU2195	*Pochonia suchlasporia*	92%	0.049 *				
		ZOTU1495	*Praetumpfia obducens*	90%	0.046 *				
		ZOTU1999	*Dinemasporium morbidum*	100%	0.049 *				
RR	Low	ZOTU1011	*Dactylaria* sp.	90%	0.003 **				
		ZOTU2067	unknown	85%	0.010 **				
		ZOTU1889	*Cystobasidiomycetes* sp.	98%	0.024 *				
		ZOTU1943	*Dioszegia butyracea*	100%	0.048 *				
		ZOTU2143	unknown	88%	0.044 *				

		ZOTU974	*Panaeolus retirugis*	99%	0.045 *				
		ZOTU1168	*Oidiodendron truncatum*	99%	0.049 *				
		ZOTU1786	*Pseudombrophila hepatica*	94%	0.050 *				
		ZOTU2274	mycorrhizal fungus	93%	0.044 *				
		ZOTU904	*Cephalotrichum* sp.	99%	0.044 *				
		ZOTU1883	unknown	83%	0.039 *				
		ZOTU1695	unknown	95%	0.042 *				
LL	Medium	ZOTU1332	unknown	91%	0.010 **				
		ZOTU1440	unknown	81%	0.007 **				
		ZOTU2130	unknown	88%	0.048 *				
RR	Medium	ZOTU473	*Pyrenophora tere*	100%	0.001 ***	ZOTU168	*Kernia* sp.	98%	0.014 *
						ZOTU162	*Myrothecium roridum*	100%	0.038 *
RR	High					ZOTU109	*Hypocreales* sp.	100%	0.019 *

[1]LL, canola InVigor 5440, a Liberty Link cultivar resistant to glufosinate herbicides; RR, canola 71-45, a Roundup Ready cultivar resistant to glyphosate; Low, wheat-canola rotation; Medium, pea-barley-canola rotation; High, lentil-wheat-LL-pea-barley-RR.

[2] Crop diversification level: Monoculture, canola monoculture; Low, wheat-canola rotation; Medium, pea-barley-canola rotation; High, lentil-wheat-LL-pea-barley-RR

[3] P-value from indicator species analysis ($\alpha = 0.05$, 999 permutations in Monte Carlo test).

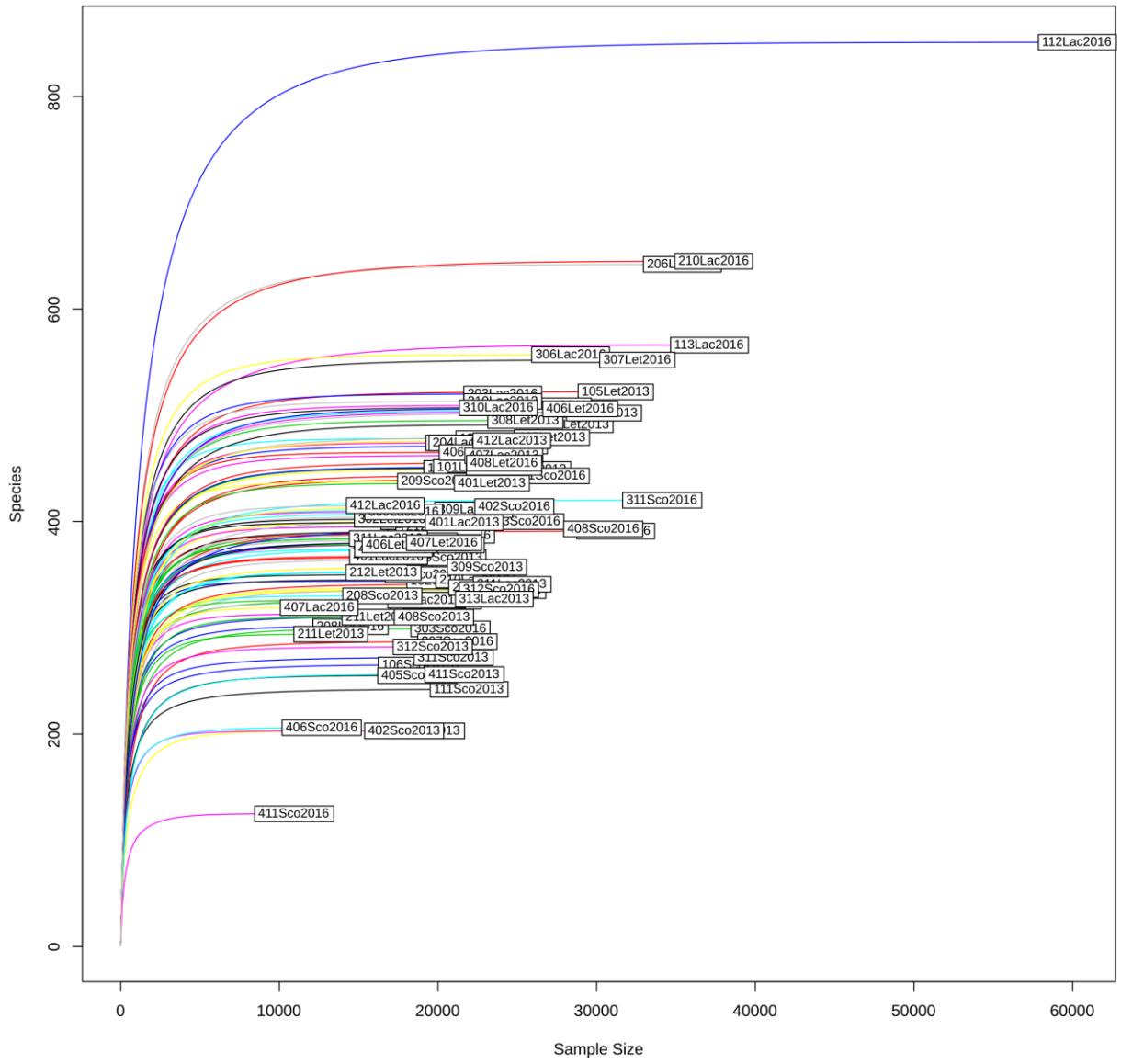


Figure S4: Rarefaction curves for each rhizosphere soil sample, showing the relationship between the number of ASVs and the abundance of 16S RNA gene sequences reads, in the 2013 and 2016 dataset.

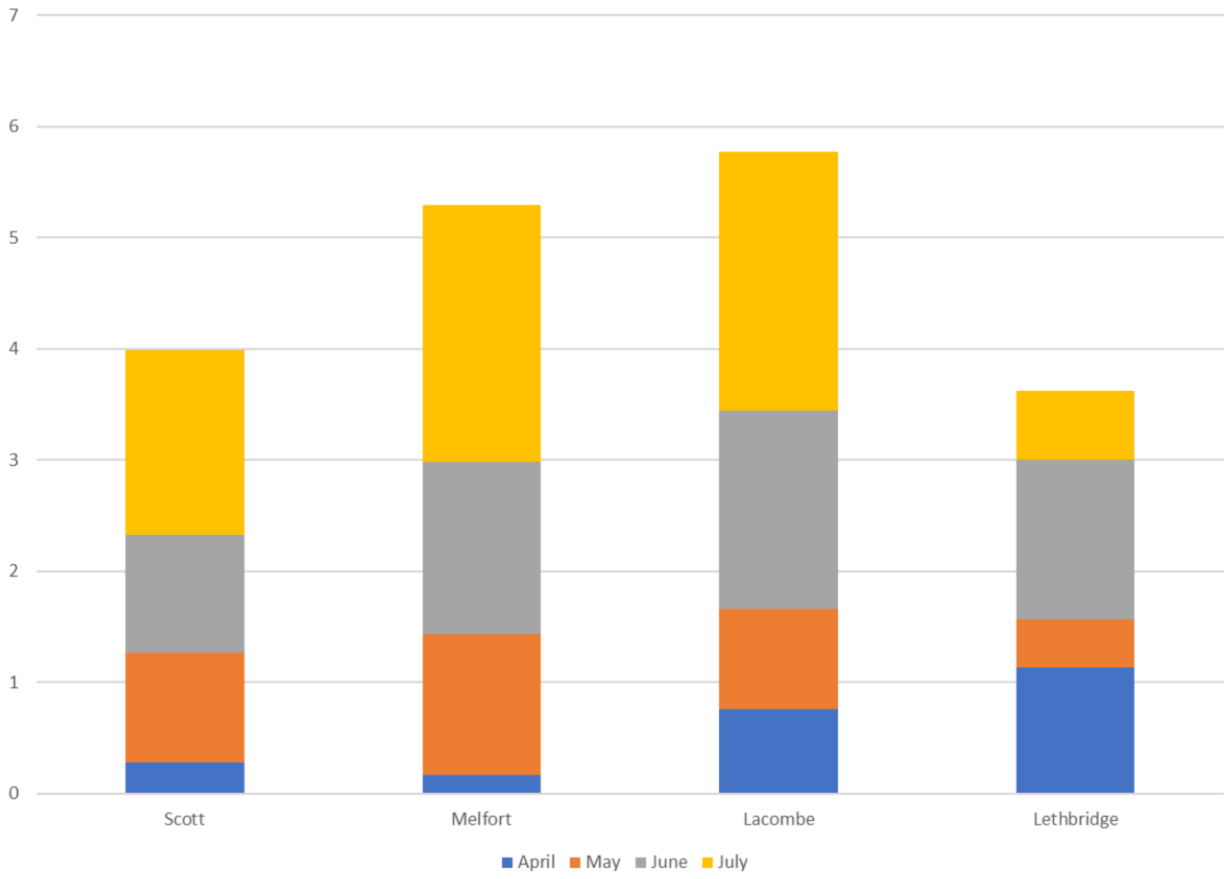


Figure S5. Cumulative precipitations (mm) at each site before sampling in 2018.

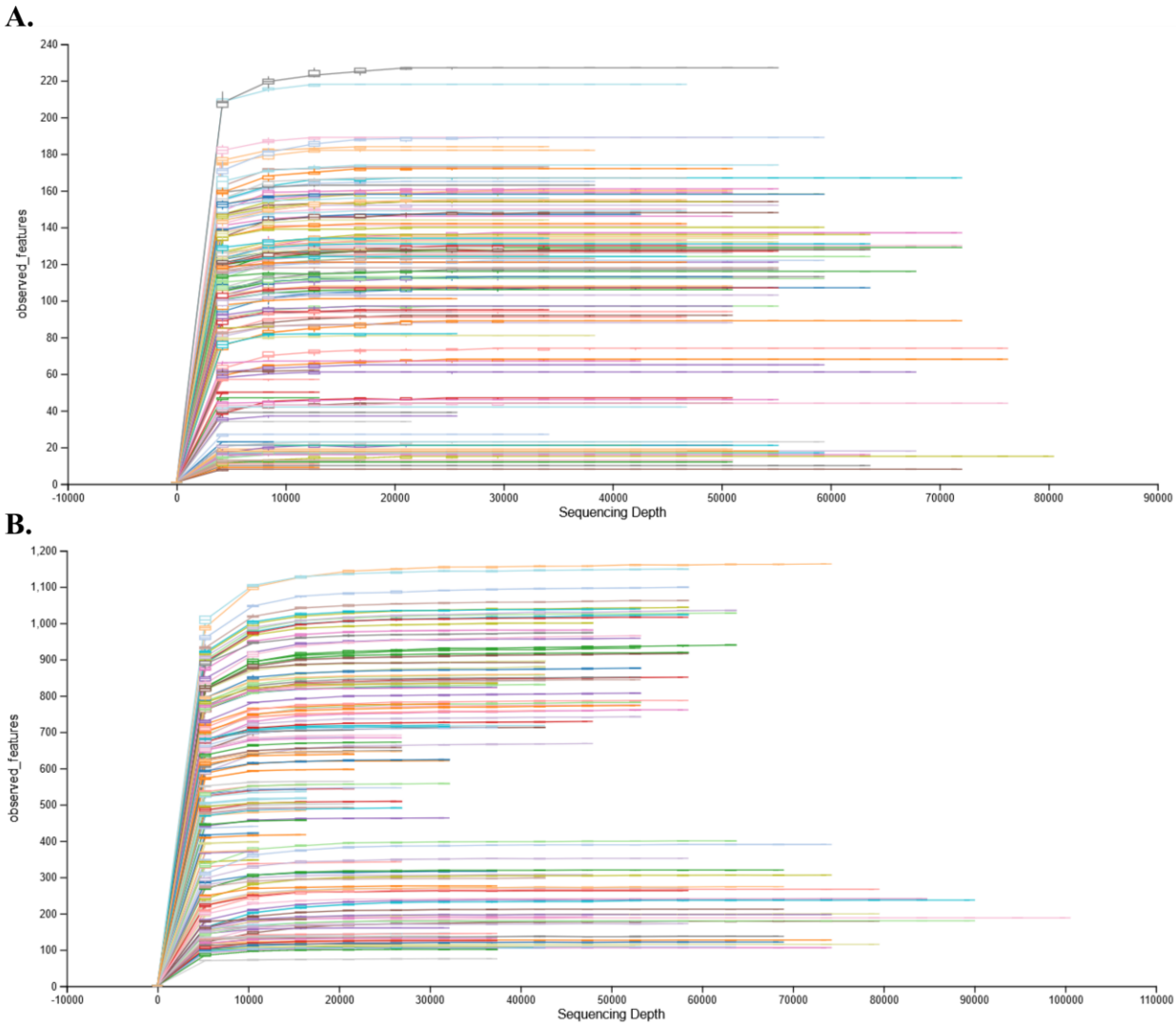


Figure S6. Rarefaction curves for each rhizosphere soil sample, showing the relationship between the number of ASVs and the abundance of (A) ITS DNA gene sequences reads and (B) 16S RNA gene sequences.

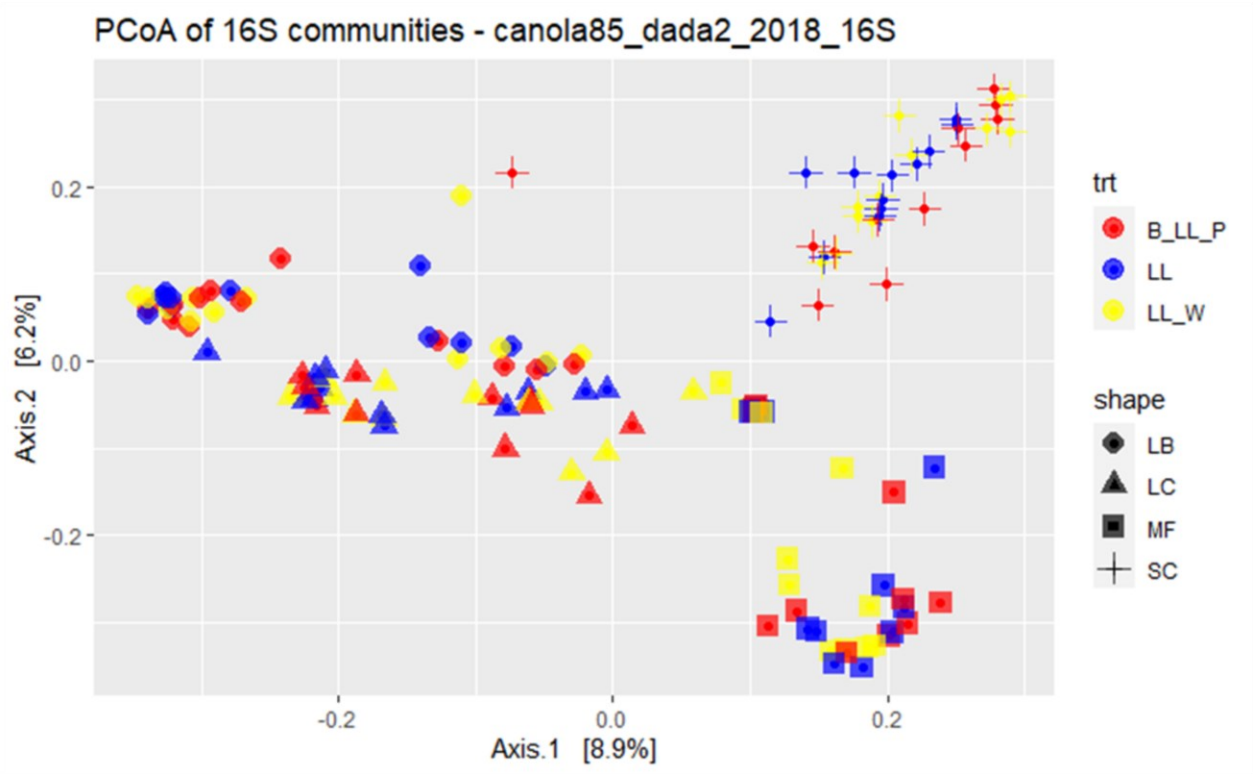


Figure S7. Principal Coordinate Analysis of the bacterial 16S gene community. LL is the cultivar of canola, B is barley, P is pea and W is wheat. LB is the site Lethbridge, LC is Lacombe, MF is Melfort, and SC is Swift Current.

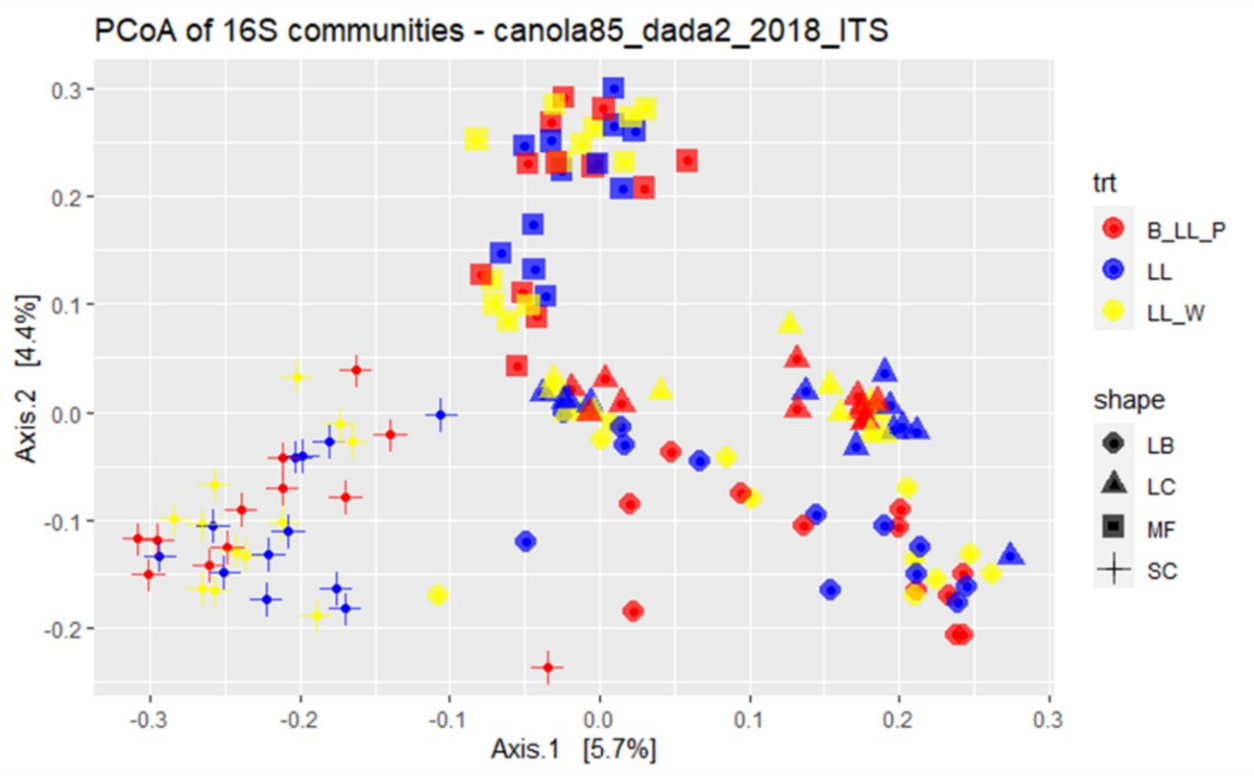


Figure S8. Principal Coordinate Analysis of the fungal ITS gene community. LL is the cultivar of canola, B is barley, P is pea and W is wheat. LB is the site Lethbridge, LC is Lacombe, MF is Melfort, and SC is Swift Current.

Table S4. Bacterial and fungal core candidates found at 90% and 80% frequency in the samples. Confidence score produced by RDP-classifier display the probability of error for the identification reported at clade level.

Core candidates 80% in the rhizosphere

ASV	Identity	Confidence score
	Unknown	
BASV7	*Micrococcaceae*	100
BASV60	*Bacilus* sp.	100
BASV157	*Unknown Chloroflexi*	70
FASV3	*Fusarium* sp.	100
FASV9	*Mortierella* sp.	100
FASV25	*Alternaria* sp.	100
FASV40	*Clonostachys* sp.	100
FASV101	*Thelonectria* sp.	100
FASV129	*Cladosporium* sp.	100

Core candidates 90% in the rhizosphere

ASV	Identity	Confidence score
	Unknown	
FASV2	*Nectriaceae*	100
FASV13	*Fusarium* sp.	100
FASV17	*Fusarium* sp.	94

Core candidates 80% in the bulk soil

ASV	Identity	Confidence score
BASV9	*Pseudonocardia* sp.	99
BASV60	*Bacilus* sp.	100
	Unknown	
BASV200	*Chitinophagaceae*	93
FASV3	*Fusarium* sp.	100

Core candidates 90% in the bulk soil

ASV	Identity	Confidence score
FASV1	*Humicola* sp.	100
FASV7	*Cryptococcus* sp.	100
FASV11	*Nectria* sp.	100
FASV13	*Fusarium* sp.	100

FASV4	*Fusarium* sp.	100
FASV16	*Fusarium* sp.	100
FASV21	*Mortierella* sp.	100
FASV25	*Alternaria* sp.	100
FASV33	*Alternaria* sp.	100
FASV93	*Neonectria* sp.	100
FASV114	*Mortierella* sp.	100
FASV166	*Ophiosphaerella* sp.	100
FASV17	*Fusarium* sp.	94
FASV101	*Thelonectria* sp.	100
FASV129	*Cladosporium* sp.	100

Table S5. Interaction cohorts of inter-kingdom hub-taxa in canola subterranean microbiome. The taxa in bold are inter-kingdom hub-taxa. Confidence score produced by RDP-classifier display the probability of error for the identification reported at clade level.

Cohort of hub-taxa BASV45 inter-kingdom - Rhizosphere

ASV	Identity	Confidence score
FASV8	*Chaetomium mareoticum*	100
FASV21	*Mortierella* sp.	99
FASV23	*Trichoderma pubescens*	99
BASV43	*Sphingomonas* sp.	99.99
BASV60	*Bacillus sp*	99.89
BASV77	*Micromonosporaceae* sp.	72.41
BASV84	***Jatrophihabitans*** sp.	**92.99**
BASV134	*Pseudonocardia* sp.	90.75
BASV181	*Ilumatobacter*	98.24
BASV200	*Chitinophagaceae* sp.	99.99

Cohort of hub-taxa BASV69 inter-kingdom - Soil

ASV	Identity	Confidence score
FASV1	*Humicola nigrescens*	100
FASV5	*Ascomycota* sp.	89
FASV14	*Ascomycota* sp.	75
FASV25	*Alternaria metachromatica*	100
FASV101	*Sordariomycetes* sp.	100
BASV27	*Rubrobacter* sp.	99.99
BASV47	*Chloroflexi KD4-96*	72.03
BASV52	*Skermanella* sp.	99.99
BASV61	*Agromyces* sp.	85.99
BASV109	*Vicinamibacteraceae* sp.	94.91

Cohort of hub-taxa BASV134 inter-kingdom - Rhizosphere

ASV	Identity	Confidence score
FASV7	*Cryptococcus fuscescens*	100
FASV9	*Mortierella hyalina*	98
FASV109	*Microascales* sp.	98
FASV221	*Ilyonectria* sp.	100
BASV43	*Sphingomonas* sp.	99.99
BASV45	***Xanthobacteraceae*** sp.	**99.99**
BASV84	***Jatrophihabitans*** sp.	**92.99**
BASV107	*Gemmatimonadaceae* sp.	72.1

Cohort of hub-taxa FASV8 inter-kingdom - Soil

ASV	Identity	Confidence score
FASV7	*Cryptococcus fuscescens*	100
FASV9	*Mortierella hyalina*	98
FASV11	*Nectriaceae* sp.	99
FASV63	*Dendryphion* sp.	100
FASV104	*Mortierella* sp.	99
FASV151	*Exophiala equina*	100
FASV160	*Tetracladium* sp.	98
BASV43	*Sphingomonas* sp.	99.99

Cohort of hub-taxa FASV21 inter-kingdom - Rhizosphere		
ASV	**Identity**	**Confidence score**
FASV151	*Exophiala* sp.	100
FASV286	*Mortierella* sp.	99
BASV7	*Micrococcaceae* sp.	99.99
BASV45	***Xanthobacteraceae*** sp.	**99.99**
BASV77	*Asanoa* sp.	72.41
BASV87	*Chitinophagaceae* sp.	73.38
BASV94	*Chloroflexi JG30-KF-CM45*	92.33
BASV151	*Gemmatimonas* sp.	79.34
BASV200	*Chitinophagaceae* sp.	99.99
BASV107	*Gemmatimonadaceae* sp.	72.1
BASV158	*Vicinamibacteraceae* sp.	99.99

Cohort of hub-taxa FASV114 inter-kingdom - Soil		
ASV	**Identity**	**Confidence score**
FASV4	*Ascomycota* sp.	100
FASV129	*Davidiella* sp.	100
FASV180	*Leotiomycetes* sp.	100
FASV196	*Lecythophora* sp.	100
FASV318	*Helotiales* sp.	100
BASV9	*Pseudonocardia* sp.	99.99
BASV18	*Blastococcus* sp.	98.89
BASV52	*Skermanella* sp.	99.99
BASV59	*Sphingomonadaceae* sp.	99.99
BASV75	*Pseudonocardia* sp.	99.99
BASV87	*Bacteroidetes* sp.	73.38
BASV484	*Iamia* sp.	74.13
BASV962	*Rhodanobacteraceae* sp.	99.99

Table S6. Bacterial and fungal cohort of AMF ASVs in the canola rhizosphere. Confidence score is given by RDP classifier and correspond to the probability of correct identification.

Cohort of ASV1667 (*Claroideoglomus* sp.)

ASV ID	Identification	Confidence	Interaction Type
BASV47	*Chloroflexi* sp.	100%	Negative
BASV125	*Bacillus* sp.	100%	Negative

BASV217	*Frankiales* sp.	99%	Positive
BASV236	*Vicinamibacteraceae* sp.	99%	Positive
BASV267	*Ilumatobacteraceae* sp.	100%	Negative
BASV526	*Phenylobacterium* sp.	100%	Negative
BASV535	*Sphingomonas* sp.	99%	Positive
BASV552	*Pseudohongiellaceae* sp.	99%	Negative
BASV762	*Steroidobacteraceae* sp.	99%	Negative
BASV831	*Chloroflexi* sp.	92%	Positive
BASV978	*Ilumatobacteraceae* sp.	98%	Positive
BASV1108	*Dinghuibacter* sp.	99%	Positive
BASV1140	*Solirubrobacterales* sp.	98%	Negative
BASV1145	*Gemmatimonas* sp.	98%	Positive
BASV1169	*Vicinamibacteraceae* sp.	99%	Positive
BASV1338	*Tepidisphaerales* sp.	99%	Positive
FASV1	*Humicola* sp.	100%	Negative
FASV194	Unknown *Ascomycota*	100%	Positive

Cohort of ASV86 (*Diversispora* sp.)

ASV ID	Identification	Confidence	Interaction Type
BASV99	*Blastococcus* sp.	100%	Positive
BASV147	*Altererythrobacter* sp.	99%	Negative
BASV475	*Brevundimonas* sp.	94%	Positive

BASV485	Unknown *Latescibacterota*	74%	Positive
BASV526	*Phenylobacterium* sp.	100%	Positive
BASV580	*Solirubrobacter* sp.	100%	Positive
BASV629	*Steroidobacter* sp.	100%	Positive
BASV645	*Acidimicrobiia IMCC26256* sp.	100%	Positive
BASV788	*Devosia* sp.	100%	Positive
BASV889	Unknown *Microbacteriaceae*	82%	Positive
BASV937	Unknown *Gaiellales*	84%	Positive
BASV962	Unknown	72%	positive
BASV978	*Ilumatobacteraceae* sp.	98%	positive
BASV1162	*Microtrichales* sp.	98%	positive
BASV1736	*Vicinamibacterales* sp.	100%	positive
BASV2308	Unknown *Gammaproteobacteria*	73%	positive
FASV261	Unknown *Hypocreales*	100%	positive
FASV490	*Pulchromyces* sp.	92%	positive

Cohort of ASV82 (*Claroideoglomus* sp.)

ASV ID	Identification	confidence	Interaction Type
BASV80	*Nocardioides* sp.	100%	positive
BASV83	Unknown *Chloroflexi*	88%	negative
BASV118	Unknown *Actinobacteria*	75%	positive
BASV197	Unknown *Actinobacteria*	70%	positive

BASV256	*Lapillicoccus* sp.	100%	positive
BASV266	*Reyranella* sp.	98%	positive
BASV292	*Actinobacteria MB-A2-108*	100%	positive
BASV350	Unknown *Gemmatimonadota*	72%	negative
BASV444	Unknown *Thermoleophilia*	89%	positive
BASV535	*Sphingomonas* sp.	100%	positive
BASV614	Unknown *Blastocatellaceae*	100%	positive
BASV745	Unknown *Chloroflexi*	75%	positive
BASV1599	*Sphingobacteriales NS11-12*	100%	positive
FASV127	Unknown *Sordariomycetes*	100%	positive
FASV136	*Mortierella* sp.	100%	positive
FASV235	Unknown *Ascomycota*	100%	positive
FASV592	Unknown *Nectriaceae*	95%	positive

Cohort of ASV326 (*Glomus sp*.)

ASV ID	Identification	confidence	Interaction Type
BASV52	*Skermanella* sp.	100%	negative
BASV63	*Skermanella* sp.	100%	positive
BASV111	Unknown *Nocardioidaceae*	88%	positive
BASV196	*Chitinophagaceae* sp.	99%	positive
BASV343	Unknown *Actinobacteria*	73%	positive
BASV390	*Solirubrobacterales* sp.	99%	positive

BASV485	Unknown *Latescibacterota*	72%	positive
BASV586	*Nocardioidaceae* sp.	100%	positive
BASV906	Unknown *Verrucomicrobiota*	82%	positive
BASV1091	Unknown *Chloroflexi*	70%	positive
BASV1628	*Nocardioides* sp.	90%	positive
BASV2122	Unknown *Acidobacteria*	71%	positive
FASV136	*Mortierella* sp.	100%	positive
FASV207	*Bipolaris* sp.	100%	positive
FASV677	*Cryptococcus* sp.	100%	positive

Cohort of ASV10 (*Funeliformis mosseae*)

ASV ID	Identification	confidence	Interaction Type
BASV154	*Rubrobacter* sp.	100%	positive
BASV167	*Solirubrobacter* sp.	100%	positive
BASV185	*Solirubrobacter* sp.	100%	positive
BASV289	*Sphingomonas* sp.	99%	positive
BASV1226	*Actinocorallia* sp.	100%	positive
BASV1293	*Nitrospira* sp.	100%	positive
BASV1407	*Arenimonas* sp.	100%	positive
BASV2255	*Chthoniobacter* sp.	100%	positive

Cohort of ASV711 (*Paraglomus* sp.)

ASV ID	Identification	confidence	Interaction Type

BASV342	Unknown *Vicinamibacteraceae*	100%	negative
BASV461	*Devosia* sp.	100%	positive
	Unknown		
BASV570	*Gammaproteobacteria*	70%	positive
BASV585	*Galbitalea* sp.	100%	negative
BASV645	*Actinobacteria IMCC26256*	100%	positive
BASV676	*Nocardioides* sp.	100%	positive
BASV999	Unknown *Pedosphaeraceae*	91%	positive

Cohort of ASV61 (*Paraglomus sp*.)

ASV ID	Identification	confidence	Interaction Type
BASV182	*Iamia* sp.	98%	negative
BASV354	*Bryobacter* sp.	100%	positive
BASV1101	Unknown *Gemmatimonadaceae*	99%	positive
BASV1165	*Actinobacteria IMCC26256*	100%	positive
BASV1357	*Haliangium* sp.	100%	negative
BASV1407	*Arenimonas* sp.	99%	positive
FASV127	Unknown *Sordariomycetes*	100%	positive
FASV214	*Podospora* sp.	100%	positive

Cohort of ASV22 (*Diversispora* sp.)

ASV ID	Identification	confidence	Interaction Type

BASV68	*Serratia* sp.	97%	positive
BASV227	*Nocardioides* sp.	100%	positive
BASV285	*Flavisolibacter* sp.	78%	positive
BASV650	*Opitutus* sp.	100%	positive
BASV1135	*Actinobacter MB-A2-108*	100%	positive
BASV1461	Unknown *Vicinamibacteria*	75%	positive
FASV15	*Chaetomium* sp.	100%	positive

Cohort of ASV171 (*Claroideoglomus* sp.)

ASV ID	Identification	confidence	Interaction Type
BASV86	*Acinetobacter* sp.	99%	positive
BASV115	*Gaiella* sp.	96%	positive
BASV266	*Reyranella* sp.	99%	negative
BASV715	Unknown *Chloroflexi*	71%	positive
BASV1140	*Actinobacter 67-14*	100%	positive
BASV2221	*BIrii41* sp.	100%	positive
FASV342	*Stachybotrys* sp.	100%	positive

Cohort of ASV103 (*Claroideoglomus sp*.)

ASV ID	Identification	Confidence	Interaction Type
BASV421	Unknown *Vicinamibacteria*	94%	positive
BASV737	*Hyphomicrobium* sp.	100%	negative
BASV1455	*Labrys* sp.	100%	negative

BASV2221	*BIrii41* sp.	100%	positive
BASV2784	*Bacteroidota AKYH767*	100%	positive
BASV3135	*Xanthomonas* sp.	100%	positive
FASV130	*Sistotrema* sp.	100%	positive

Cohort of ASV1537 (*Archaeospora* sp.)

ASV ID	Identification	confidence	Interaction Type
BASV184	Unknown *Solirubrobacteraceae*	80%	negative
BASV221	Unknown *Acetobacteraceae*	99%	positive
BASV267	Unknown *Ilumatobacteraceae*	100%	negative
BASV413	*Actinobacter 67-14*	100%	negative
BASV788	*Devosia* sp.	99%	negative

Cohort of ASV109 (*Claroideoglomus* sp.)

ASV ID	Identification	confidence	Interaction Type
BASV460	*Chloroflexi KD4-96*	98%	positive
BASV526	*Phenylobacterium sp*	100%	positive
BASV585	*Galbitalea* sp.	100%	positive

Cohort of ASV1469 (*Archaeospora* sp.)

ASV ID	Identification	confidence	Interaction Type
BASV101	Unknown *Intrasporangiaceae*	99%	positive

Cohort of ASV259 (*Claroideoglomus* sp.)

ASV ID	Identification	Confidence	Interaction Type
BASV59	Unknown *Sphingomonadaceae*	100%	negative
FASV250	*Preussia* sp.	100%	positive

Cohort of ASV261 (*Claroideoglomus lamellosum*)

ASV ID	Identification	confidence	Interaction Type
BASV317	*Mycobacterium* sp.	97%	positive

Cohort of ASV273 (*Claroideoglomus sp*.)

ASV ID	Identification	confidence	Interaction Type
BASV369	*Flavisolibacter* sp.	99%	positive

Cohort of ASV331 (*Claroideoglomus* sp.)

ASV ID	Identification	Confidence	Interaction Type
BASV1286	Unknown *Gaiellales*	99%	positive

Cohort of ASV823 (*Glomus* sp.)

ASV ID	Identification	confidence	Interaction Type
BASV417	*Microlunatus* sp.	83%	positive
FASV358	*Sporormiella* sp.	100%	positive

Cohort of ASV40 (*Claroideoglomus sp*.)

ASV ID	Identification	Confidence	Interaction Type
FASV448	*Mortierella* sp.	100%	positive

Table S7. Bacterial and fungal cohort of AMF ASVs in canola bulk soil. Confidence score is given by RDP classifier and correspond to the probability of correct identification.

Cohort of ASV47 (*Diversispora sp*.)

ASV ID	Identification	confidence	Interaction Type
BASV60	*Bacillus* sp.	100%	negative
BASV111	*Kribbella* sp.	88%	positive
BASV359	*Rubrobacter* sp.	99%	negative
BASV408	*Cellulomonas* sp.	98%	negative
BASV468	*Candidatus Alysiosphaera* sp.	99%	positive
BASV505	Unknown *Chitinophagaceae*	99%	positive
BASV526	*Phenylobacterium* sp.	100%	positive
BASV593	Unknown *Vicinamibacterales*	99%	positive
BASV641	*Actinobacteria 67-14*	91%	positive
BASV657	*Actinobacteria 67-15*	99%	positive
BASV669	Unknown *Vicinamibacteraceae*	99%	positive
BASV741	Unknown *Gemmatimonadaceae*	100%	positive
BASV746	*Gemmatimonas* sp.	99%	positive
BASV1145	*Gemmatimonas* sp.	98%	positive
BASV1247	*Actinobacteria IMCC26256*	100%	positive
BASV1332	*Bacteria WS2*	100%	positive
BASV1578	*Gemmatimonadota S0134 terrestrial group*	97%	positive

BASV1863	Unknown *Gemmatimonadaceae*	90%	positive
FASV214	*Podospora* sp.	99%	positive
FASV312	*Podospora* sp.	100%	positive
FASV329	*Mortierella* sp.	100%	positive
FASV377	*Olpidium brassicae*	100%	positive
FASV490	*Pulchromyces* sp.	99%	positive

Cohort of ASV61 (*Paraglomus sp.*)

ASV ID	Identification	confidence	Interaction Type
BASV182	*Iamia* sp.	98%	positive
BASV374	*Cryobacterium* sp.	99%	positive
BASV476	Unknown *Gaiellales*	100%	positive
BASV669	Unknown *Vicinamibacteraceae*	100%	negative
BASV702	Unknown *Ilumatobacteraceae*	100%	positive
BASV742	Unknown *Vicinamibacterales*	72%	positive
BASV745	Unknown *Choloflexi*	76%	positive
BASV1024	*Altererythrobacter* sp.	99%	positive
BASV1103	*Chthoniobacter* sp.	99%	positive
BASV1297	Unknown *Saprospiraceae*	99%	positive
BASV1461	Unknown *Vicinamibacteraceae*	100%	negative
BASV1474	Unknown *Burkholderiales*	70%	positive
BASV1489	Unknown *Pirellulaceae*	97%	positive
BASV1565	Unknown *Vicinamibacteraceae*	100%	positive
BASV1587	*Roseisolibacter* sp.	100%	positive
BASV2202	*Planctomycetota WD2101 soil group*	97%	positive
FASV490	*Pulchromyces* sp.	99%	positive
FASV721	Unknown *Pleosporales*	100%	positive

Cohort of ASV51 (*Funneliformis mosseae*)

ASV ID	Identification	confidence	Interaction Type
BASV52	*Skermanella* sp.	100%	negative
BASV109	Unknown *Vicinamibacteraceae*	99%	negative
BASV227	*Nocardioides* sp.	100%	positive
BASV249	*Gaiella* sp.	100%	negative
BASV390	*Actinobacteriota 67-14*	100%	positive
BASV402	*Sphingomonas* sp.	100%	positive
BASV454	*Chloroflexi OLB14*	100%	positive
BASV488	Unknown *Vicinamibacterales*	99%	positive
BASV901	*Uknown Actinobacteriota*	95%	positive
BASV926	*Nocardioides* sp.	100%	positive
BASV1006	*Rubellimicrobium* sp.	95%	positive
BASV1133	*Chloroflexi KD4-96*	100%	positive
BASV1212	*Nocardia* sp.	99%	positive
BASV1528	*Actinobacteriota 67-14*	98%	positive
BASV1565	Unknown *Vicinamibacteraceae*	100%	positive
FASV311	Unknown *Pleosporales*	100%	positive
FASV658	Unknown *Sporidiobolales*	98%	positive

Cohort of ASV149 (*Paraglomus occultum*)

ASV ID	Identification	confidence	Interaction Type

BASV109	Unknown *Vicinamibacteraceae*	99%	positive
BASV209	*Pseudoxanthomonas* sp.	85%	positive
BASV250	*Blastococcus* sp.	93%	positive
BASV295	Unknown *Xanthobacteraceae*	100%	positive
BASV300	*Solirubrobacter* sp.	98%	positive
BASV432	*Rhodococcus* sp.	94%	positive
BASV550	*Chthoniobacter* sp.	99%	negative
BASV571	*Flavisolibacter* sp.	100%	positive
BASV908	*Altererythrobacter* sp.	84%	positive
BASV971	Unknown *Acidimicrobiia*	100%	positive
BASV1036	Unknown *Gemmatimonadaceae*	99%	positive
BASV1407	*Arenimonas* sp.	100%	positive
BASV1899	*Chthoniobacter* sp.	100%	positive
FASV272	Unknown *Ascomycota*	100%	positive

Cohort of ASV10 (*Funneliformis mosseae*)

ASV ID	Identification	confidence	Interaction Type
BASV181	*Ilumatobacter* sp.	73%	negative
BASV288	*Altererythrobacter* sp.	71%	positive
BASV505	Unknown *Chitinophagaceae*	100%	negative
BASV545	Unknown *Vicinamibacteraceae*	100%	positive
BASV552	*Gammaproteobacteria Blyi10*	100%	negative
BASV650	*Opitutus* sp.	99%	positive

BASV892	*Nocardioides* sp.	100%	negative
BASV1036	Unknown *Gemmatimonadaceae*	99%	negative
BASV1167	*Amaricoccus* sp.	100%	positive
BASV1332	*Bacteria WS2*	100%	negative
BASV2270	Unknown *Chitinophagaceae*	99%	positive
FASV393	Unknown *Phaeosphaeriaceae*	98%	positive

Cohort of ASV1396 (*Glomus sp*.)

ASV ID	Identification	confidence	Interaction Type
BASV101	Unknown *Intrasporangiaceae*	100%	positive
BASV185	*Solirubrobacter* sp.	90%	positive
BASV238	*Candidatus Udaeobacter* sp.	83%	positive
BASV351	*Actinobacteriota IMCC26256*	76%	positive
BASV548	Unknown *Vicinamibacteria*	88%	positive
BASV738	Unknown *Gaiellales*	100%	negative
BASV1090	*Actinobacteriota 67-14*	96%	negative
BASV1402	*Edaphobaculum* sp.	82%	negative
BASV1558	Unknown *Gaiellales*	100%	positive
BASV1599	*Sphingobacteriales NS11-12*	98%	positive
BASV2072	*Flavisolibacter* sp.	100%	positive

Cohort of ASV59 (*Rhizophagus iranicus*)

ASV ID	Identification	confidence	Interaction Type
BASV113	Unknown *Xanthomonadaceae*	100%	negative
BASV221	Unknown *Acetobacteraceae*	100%	negative
BASV234	*Sphingomonas* sp.	99%	negative
BASV275	*Chloroflexi KD4-96*	100%	negative
BASV361	Unknown *Chloroflexi*	80%	positive
BASV466	Unknown *Vicinamibacteraceae*	95%	positive
BASV484	*Iamia* sp.	80%	negative

BASV585	*Galbitalea* sp.	100%	positive
BASV831	*Chloroflexi TK10*	92%	positive
BASV1337	*Altererythrobacter* sp.	100%	positive
BASV1587	*Roseisolibacter* sp.	99%	positive
BASV1627	*Luteolibacter* sp.	98%	positive
BASV1697	*Gemmatimonas* sp.	100%	positive
FASV665	Unknown *Mortierellales*	100%	positive

Cohort of ASV22 (*Diversispora sp*.)

ASV ID	Identification	confidence	Interaction Type
BASV109	Unknown *Vicinamibacteraceae*	99%	negative
BASV182	*Iamia* sp.	98%	positive
BASV275	*Chloroflexi KD4-96*	100%	negative
BASV466	Unknown *Vicinamibacteraceae*	95%	positive
BASV535	*Sphingomonas* sp.	99%	positive
BASV891	*Tepidisphaerales WD2101 soil group*	100%	positive
BASV1106	*Planctomycetota OM190*	100%	positive
BASV1110	*Adhaeribacter* sp.	71%	positive
BASV1303	Unknown *Vicinamibacterales*	98%	positive
BASV1549	Unknown *Gemmatimonadaceae*	90%	positive
BASV2485	*Nocardioides* sp.	95%	positive
FASV264	*Ilyonectria* sp.	100%	positive
FASV329	*Mortierella* sp.	100%	positive
FASV671	*Coniophora* sp.	100%	positive

Cohort of ASV46 (*Paraglomus occultum*)

ASV ID	Identification	confidence	Interaction Type
BASV184	*Conexibacter* sp.	80%	positive
BASV269	Unknown *Micromonosporaceae*	100%	positive

BASV275	*Chloroflexi KD4-96*	100%	positive
BASV292	*Actinobacteriota MB-A2-108*	100%	positive
BASV350	*Gemmatimonas* sp.	78%	positive
BASV382	*Planctomycetota WD2101*	100%	positive
BASV395	*Acidobacteriota RB41*	100%	positive
BASV543	*Streptosporangium* sp.	100%	positive
BASV1265	*Abditibacterium* sp.	100%	positive
BASV1332	*Bacteria WS2*	100%	positive
BASV1399	*Gemmatimonas* sp.	100%	positive
FASV433	*Paecilomyces sp*.	100%	positive

Cohort of ASV58 (*Funneliformis mosseae*)

ASV ID	Identification	confidence	Interaction Type
BASV113	Unknown *Xanthomonadaceae*	100%	negative
BASV221	Unknown *Acetobacteraceae*	100%	negative
BASV234	*Sphingomonas* sp.	99%	negative
BASV361	Unknown *Chloroflexi*	80%	positive
BASV466	Unknown *Vicinamibacteraceae*	95%	positive
BASV484	*Iamia* sp.	80%	negative
BASV585	*Galbitalea* sp.	100%	positive
BASV1337	*Altererythrobacter* sp.	100%	positive
BASV1587	*Roseisolibacter* sp.	99%	positive
BASV1627	*Luteolibacter* sp.	98%	positive
BASV1697	*Gemmatimonas* sp.	100%	positive
FASV29	Unknown Ascomycota	100%	negative

Cohort of ASV79 (*Claroideoglomus sp*.)

ASV ID	Identification	confidence	Interaction Type
BASV11	Unknown *Micrococcaceae*	100%	positive
BASV178	*Actinobacteriota 67-14*	92%	positive
BASV251	*Altererythrobacter* sp.	100%	positive
BASV265	*Bacillus* sp.	100%	positive
BASV280	Unknown *Chloroflexi*	70%	positive
BASV295	Unknown *Xanthobacteraceae*	100%	negative

BASV357	*Actinobacteriota 67-14*	73%	positive
BASV365	*Rubrobacter* sp.	100%	positive
BASV550	*Chthoniobacter* sp.	99%	positive
BASV713	*Luteolibacter* sp.	94%	positive
BASV1293	*Nitrospira* sp.	100%	positive
FASV359	*Mortierella* sp.	100%	positive
FASV370	*Humicola* sp.	100%	positive

Cohort of ASV1809 (*Rhizophagus iranicus*)

ASV ID	Identification	confidence	Interaction Type
BASV58	Unknown *Intrasporangiaceae*	99%	positive
BASV59	Unknown *Sphingomonadaceae*	100%	negative
BASV169	*Iamia* sp.	86%	positive
BASV373	Unknown *Gaiellales*	99%	negative
BASV405	*Sphingomonas* sp.	98%	positive
BASV548	*Acidobacteriota Subgroup 17*	88%	positive
BASV738	Unknown *Gaiellales*	98%	negative
BASV831	*Chloroflexi TK10*	92%	positive
BASV3135	Unknown *Xanthomonadaceae*	100%	negative
FASV448	*Mortierella* sp.	100%	negative

Cohort of ASV1254 (*Funneliformis mosseae*)

ASV ID	Identification	confidence	Interaction Type

BASV116	*Chryseolinea* sp.	99%	negative
BASV153	*Ferruginibacter* sp.	83%	positive
BASV217	*Ferruginibacter* sp.	99%	positive
BASV454	*Chloroflexi OBL14*	100%	negative
BASV518	Unknown *Vicinamibacteraceae*	100%	positive
BASV1240	*Aurantisolimonas* sp.	99%	positive
BASV1402	*Edaphobaculum* sp.	82%	negative
BASV1554	*Roseisolibacter* sp.	100%	positive
FASV599	*Acremonium* sp.	99%	positive

Cohort of ASV18 (*Funneliformis mosseae*)

ASV ID	Identification	confidence	Interaction Type
BASV116	*Chryseolinea* sp.	99%	negative
BASV440	*Flavisolibacter* sp.	72%	positive
BASV817	Unknown *Vicinamibacterales*	100%	positive
BASV954	*Actinobacteriota 67-14*	94%	negative
BASV1308	*Planctomycetota WD2101*	100%	positive
BASV1461	Unknown *Vicinamibacteraceae*	99%	positive
BASV1964	*Edaphobaculum* sp.	89%	positive
BASV2156	Unknown *Kapabacteriales*	100%	positive
FASV24	*Mortierella* sp.	100%	positive
FASV227	*Schizothecium* sp.	99%	positive
FASV312	*Podospora* sp.	100%	positive

Cohort of ASV1156 (*Glomeraceae sp*.)

ASV ID	Identification	confidence	Interaction Type
BASV58	Unknown *Intrasporangiaceae*	99%	negative
BASV373	Unknown *Gaiellales*	99%	positive
BASV484	*Iamia* sp.	80%	positive
BASV604	Unknown *Micropepsaceae*	92%	positive
BASV729	*Qipengyuania* sp.	100%	negative
BASV962	Unknown *Rhodanobacteraceae*	72%	negative

Cohort of ASV1462 (*Paraglomus sp*.)

ASV ID	Identification	confidence	Interaction Type
BASV415	*Opitutus* sp.	99%	positive
BASV738	Unknown *Gaiellales*	100%	negative
BASV1169	Unknown *Vicinamibacteraceae*	100%	positive
BASV1891	Unknown *Gammaproteobacteria*	100%	positive
BASV1916	Unknown *Chitinophagaceae*	83%	positive
FASV922	*Phialocephala* sp.	100%	negative

Cohort of ASV203 (*Claroideoglomus sp*.)

ASV ID	Identification	confidence	Interaction Type
BASV169	*Iamia* sp.	83%	positive

BASV178	*Actinobacteriota 67-14*	92%	positive
BASV446	*Cellvibrio* sp.	100%	positive
BASV928	*Pedobacter* sp.	76%	positive
FASV13	Unknown Nectriaceae	100%	negative

Cohort of ASV1300 (*Claroideoglomus sp*.)

ASV ID	Identification	confidence	Interaction Type
BASV132	Unknown *Gaiellales*	100%	positive
BASV728	*Ferruginibacter* sp.	93%	positive
BASV954	*Actinobacteriota 67-14*	99%	positive
FASV15	*Chaetomium* sp.	99%	positive

Cohort of ASV1774 (*Diversispora* sp.)

ASV ID	Identification	confidence	Interaction Type
BASV563	*Oceanobacillus* sp.	93%	positive
BASV1106	*Planctomycetota OM190*	99%	negative
BASV2156	Unknown *Kapabacteriales*	100%	negative

Cohort of ASV82 (*Claroideoglomus sp*.)

ASV ID	Identification	confidence	Interaction Type
BASV328	*Porphyrobacter* sp.	99%	positive
BASV526	*Phenylobacterium* sp.	99%	positive
BASV728	*Ferruginibacter* sp.	93%	positive

Cohort of ASV89 (*Paraglomus occultum*)

ASV ID	Identification	confidence	Interaction Type
BASV475	*Brevundimonas* sp.	94%	negative
BASV738	Unknown *Gaiellales*	99%	positive
BASV1169	Unknown *Vicinamibacteraceae*	100%	positive
FASV448	*Mortierella* sp.	100%	positive

Cohort of ASV1556 (*Glomus indicum*)

ASV ID	Identification	confidence	Interaction Type
BASV28	*Bradyrhizobium* sp.	100%	negative
BASV94	*Chloroflexi JG30-KF-CM45*	100%	negative

Cohort of ASV1453 (*Diversispora sp*.)

ASV ID	Identification	confidence	Interaction Type
BASV390	*Actinobacteriota 67-14*	100%	positive

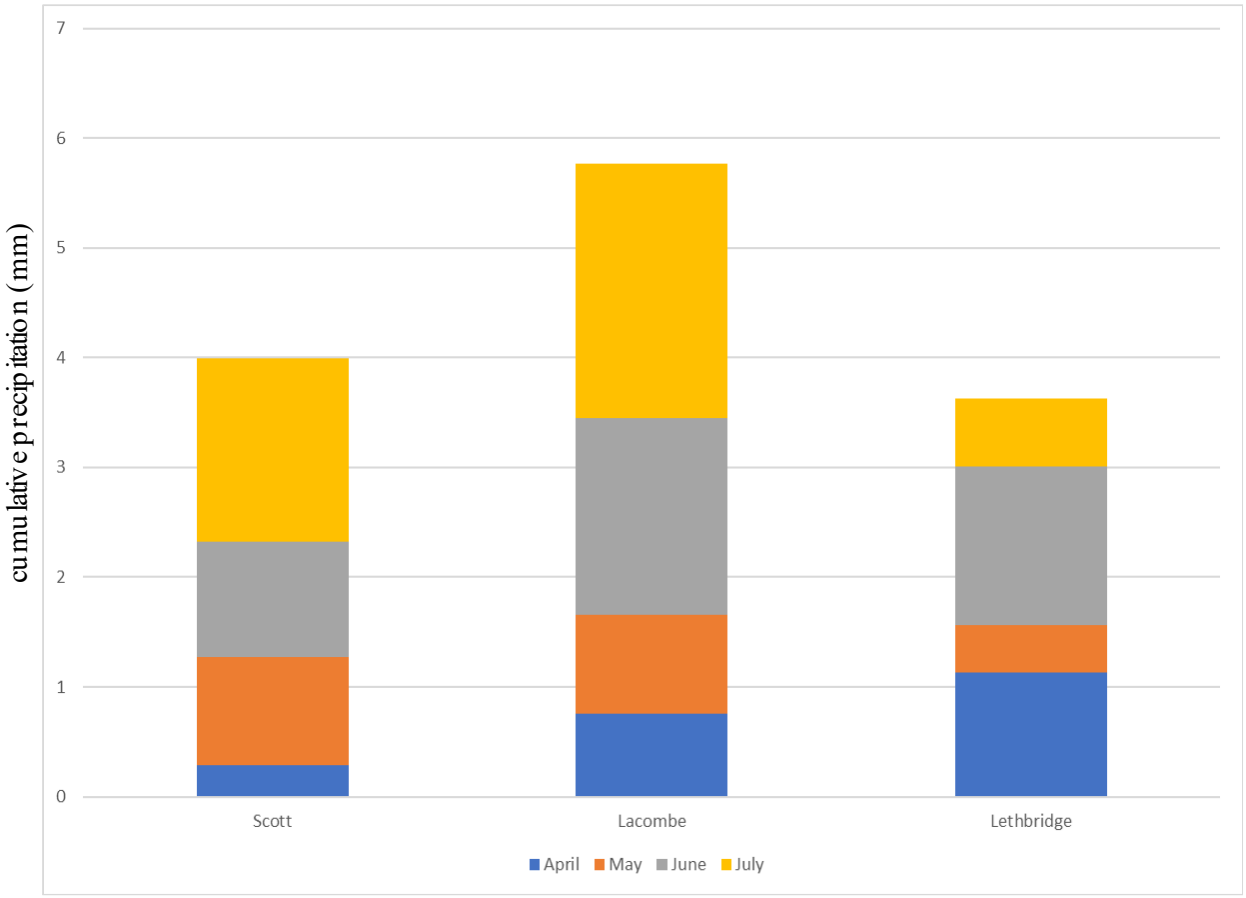
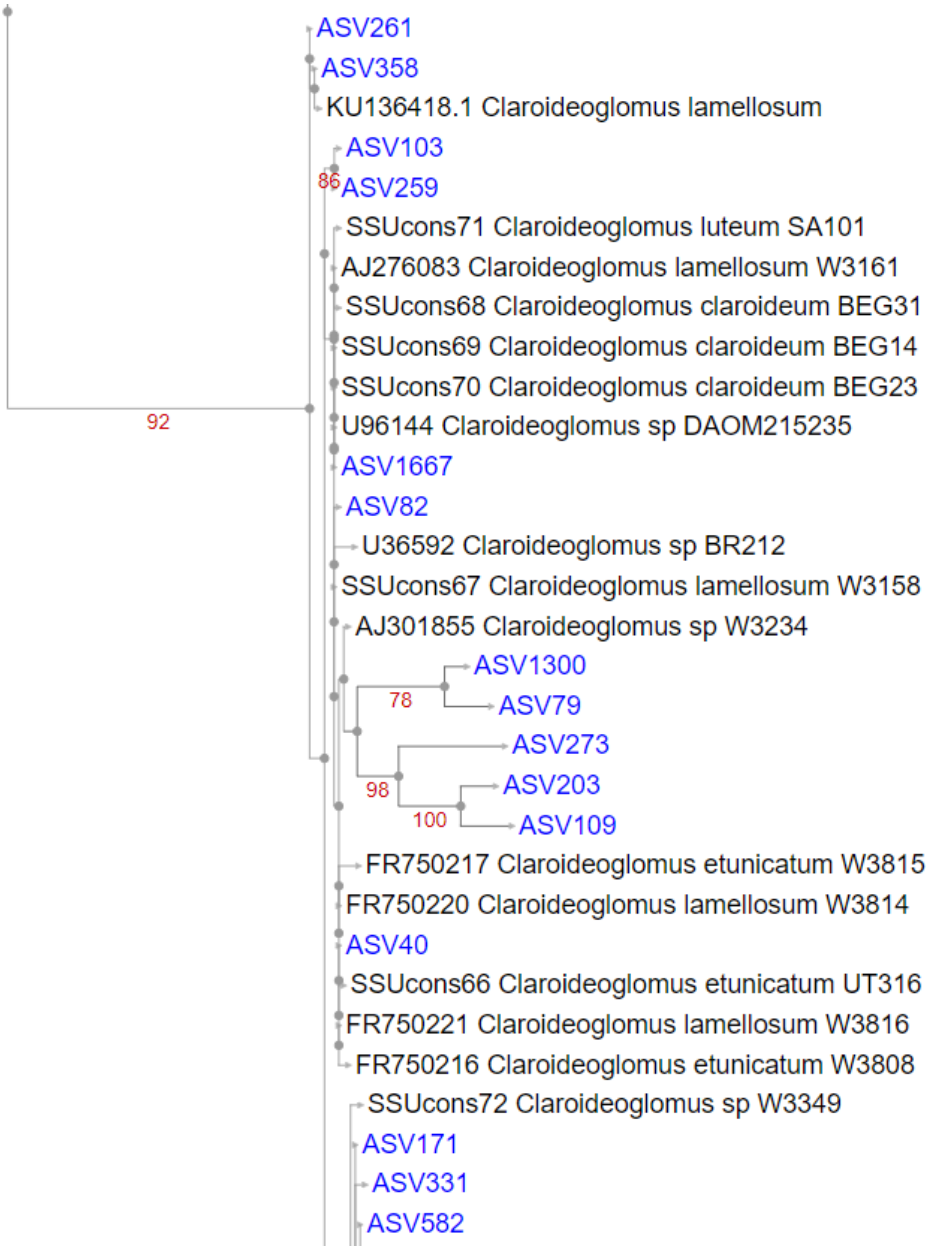


Figure S9. Cumulative precipitations (mm) at each site before sampling in 2018.


Tree scale: 0.1
AFTOL0674 Rhodotorula hordea outgroup
SSUcons86 Paraglomus occultum IA702
98
AJ006799 Paraglomus occultum HA771
AJ301862 Paraglomus brasilianum BR105
MN793990.1 Paraglomus occultum isolate JJ18008
80
ASV89
ASV149
ASV46
ASV1582
100
ASV1462
ASV1583
ASV61
100
ASV711
SSUcons84 Archaeospora trappei W3179
Archeospora ecuadoriana LR596338
100
SSUcons82 Archaeospora trappei NB112
97
SSUcons83 Archaeospora schenckii Att586
ASV42
83
94
ASV1537
91
ASV1469
SSUcons79 Ambispora granatensis JEP2010
SSUcons80 Geosiphon pyriformis GEO1
81
SSUcons78 Ambispora feica W4752
SSUcons76 Ambispora callosa MAFF520057
92
SSUcons77 Ambispora callosa MAFF520058
SSUcons75 Ambispora leptoticha MAFF520055
SSUcons73 Ambispora leptoticha FL130
SSUcons74 Ambispora leptoticha NC176


ASV261
ASV358
KU136418.1 Claroideoglomus lamellosum
ASV103
86
ASV259
SSUcons71 Claroideoglomus luteum SA101
AJ276083 Claroideoglomus lamellosum W3161
SSUcons68 Claroideoglomus claroideum BEG31
SSUcons69 Claroideoglomus claroideum BEG14
SSUcons70 Claroideoglomus claroideum BEG23
92
U96144 Claroideoglomus sp DAOM215235
ASV1667
ASV82
U36592 Claroideoglomus sp BR212
SSUcons67 Claroideoglomus lamellosum W3158
AJ301855 Claroideoglomus sp W3234
ASV1300
78
ASV79
ASV273
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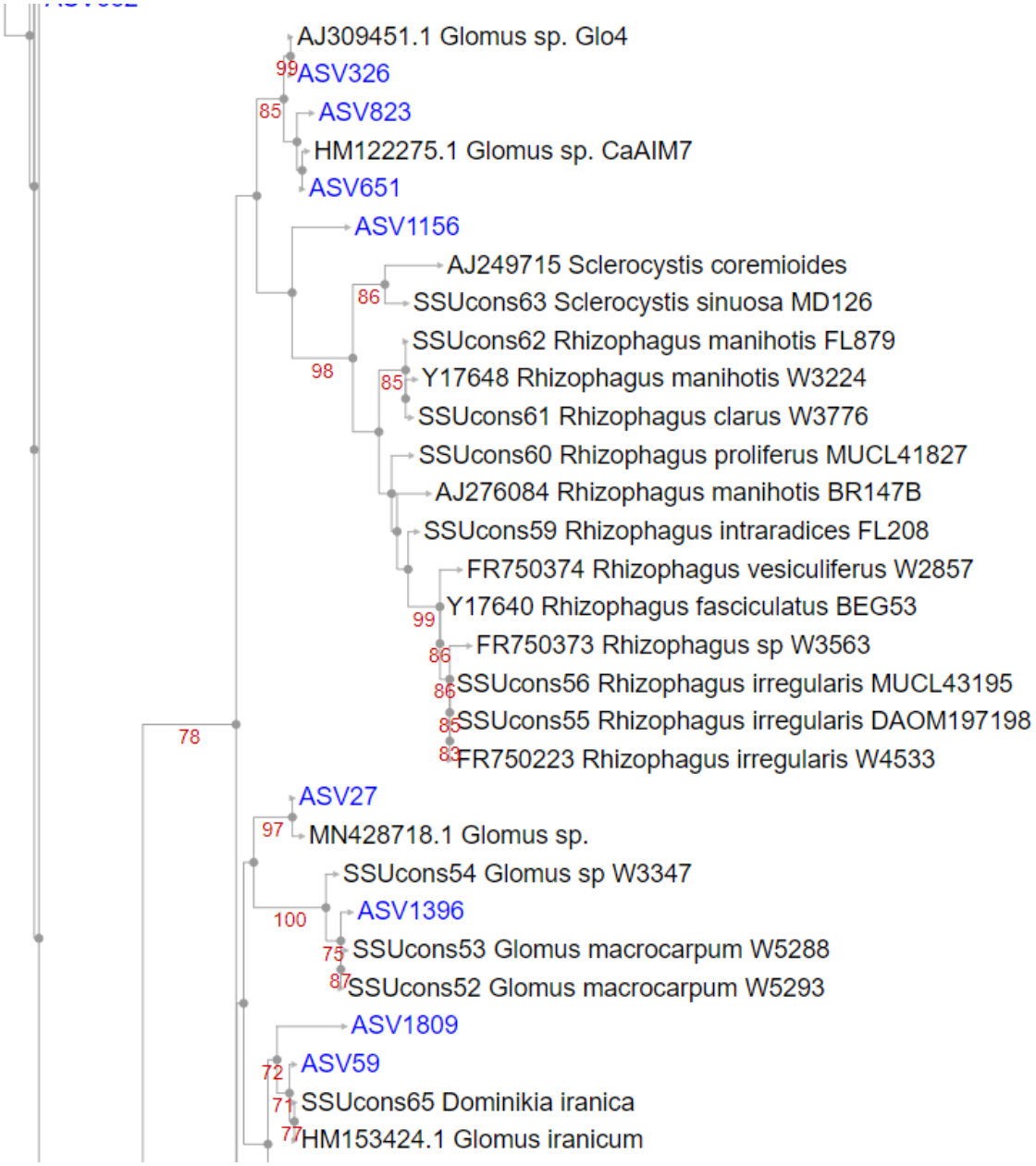
ASV582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FR750374 Rhizophagus vesiculiferus W2857
Y17640 Rhizophagus fasciculatus BEG53
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SSUcons53 Glomus macrocarpum W5288
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SSUcons52 Glomus macrocarpum W5293
ASV1809
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ASV59
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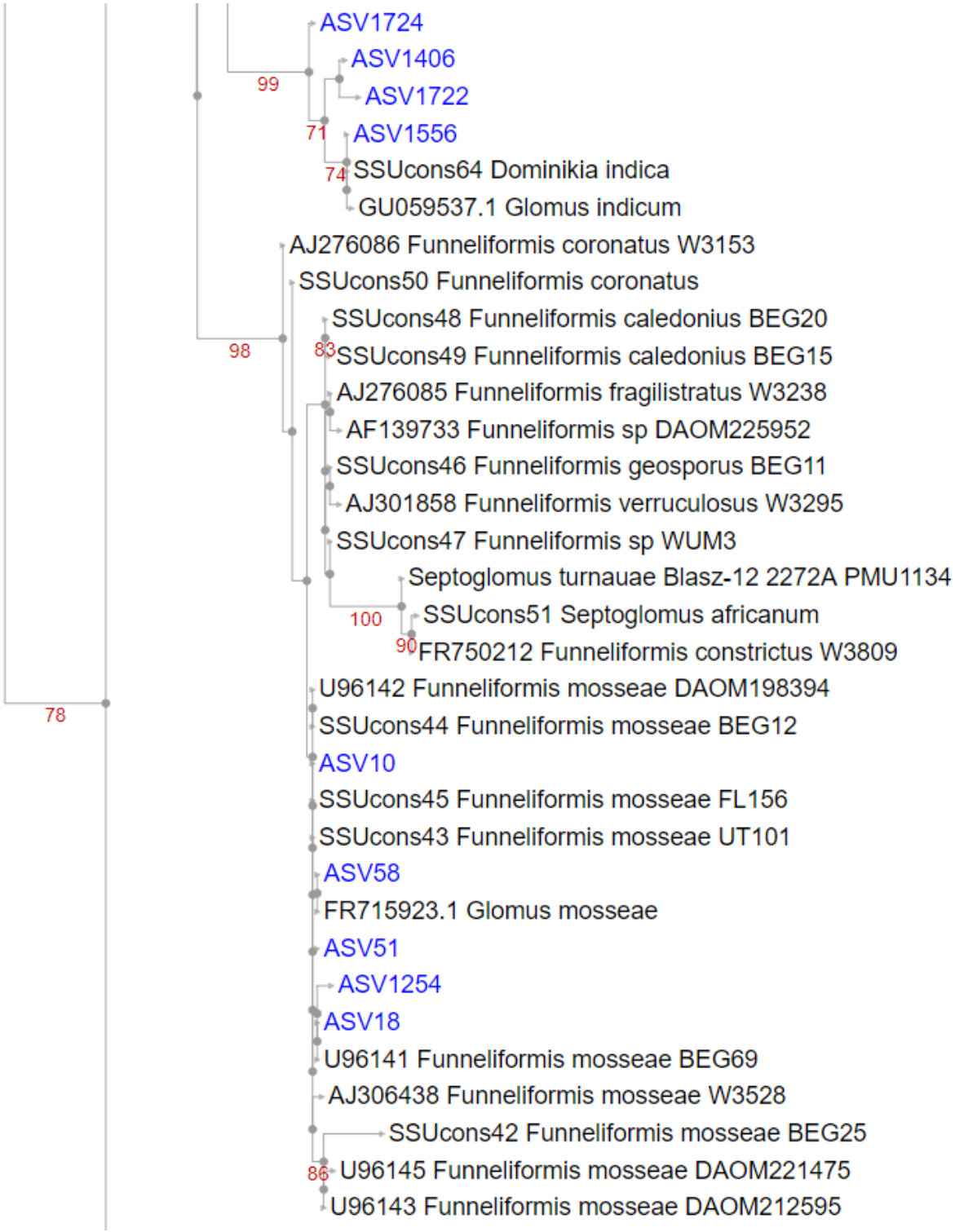
HM153424.1 Glomus iranicum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SSUcons50 Funneliformis coronatus
SSUcons48 Funneliformis caledonius BEG20
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83
SSUcons49 Funneliformis caledonius BEG15
AJ276085 Funneliformis fragilistratus W3238
AF139733 Funneliformis sp DAOM225952
SSUcons46 Funneliformis geosporus BEG11
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SSUcons47 Funneliformis sp WUM3
Septoglomus turnauae Blasz-12 2272A PMU1134
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SSUcons51 Septoglomus africanum
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SSUcons45 Funneliformis mosseae FL156
SSUcons43 Funneliformis mosseae UT101
ASV58
FR715923.1 Glomus mosseae
ASV51
ASV1254
ASV18
U96141 Funneliformis mosseae BEG69
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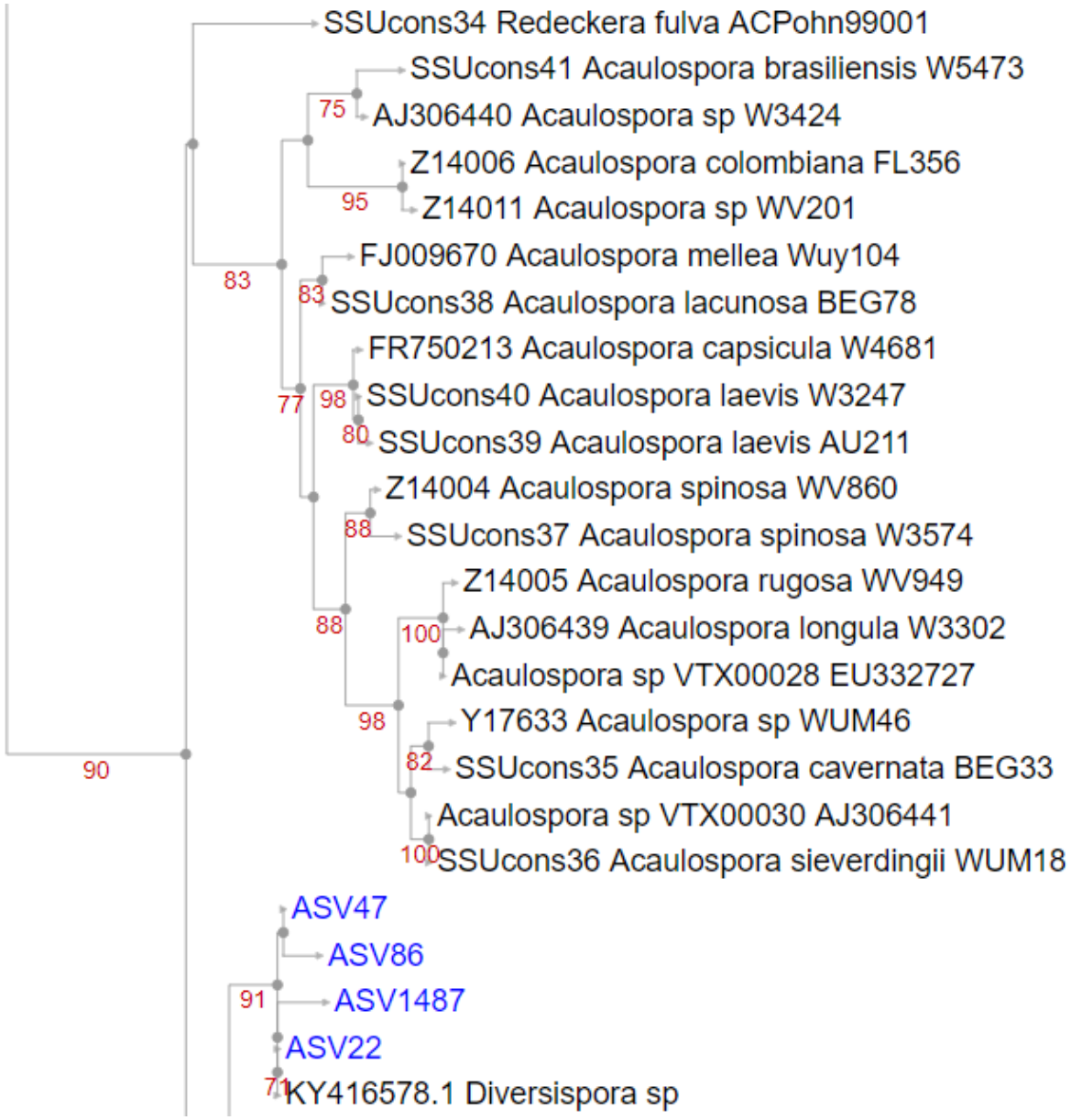
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Z14011 Acaulospora sp WV201
FJ009670 Acaulospora mellea Wuy104
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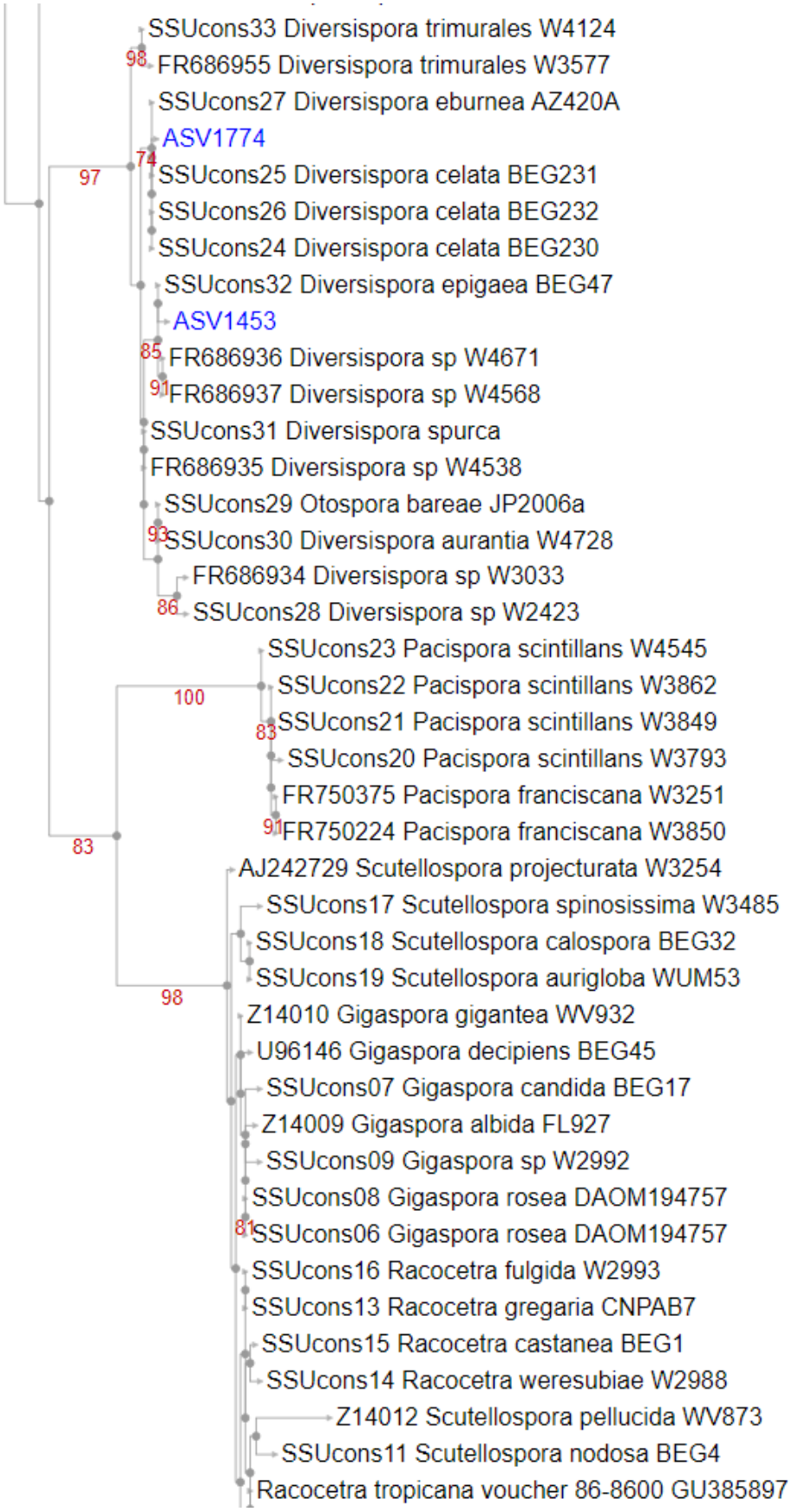
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FR686934 Diversispora sp W3033
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FR750224 Pacispora franciscana W3850
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AJ242729 Scutellospora projecturata W3254
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SSUcons19 Scutellospora aurigloba WUM53
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Z14010 Gigaspora gigantea WV932
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81
SSUcons06 Gigaspora rosea DAOM194757
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SSUcons15 Racocetra castanea BEG1
SSUcons14 Racocetra weresubiae W2988
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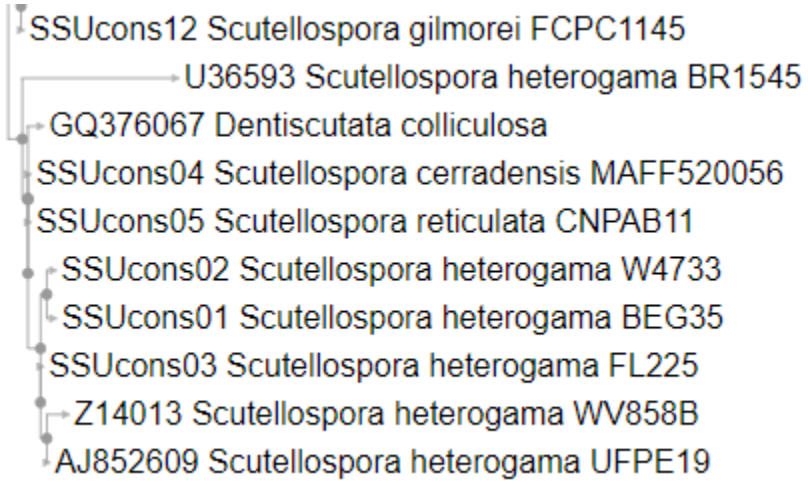

Figure S10. Phylogenetic tree of the 49 ASV identified as AMF.

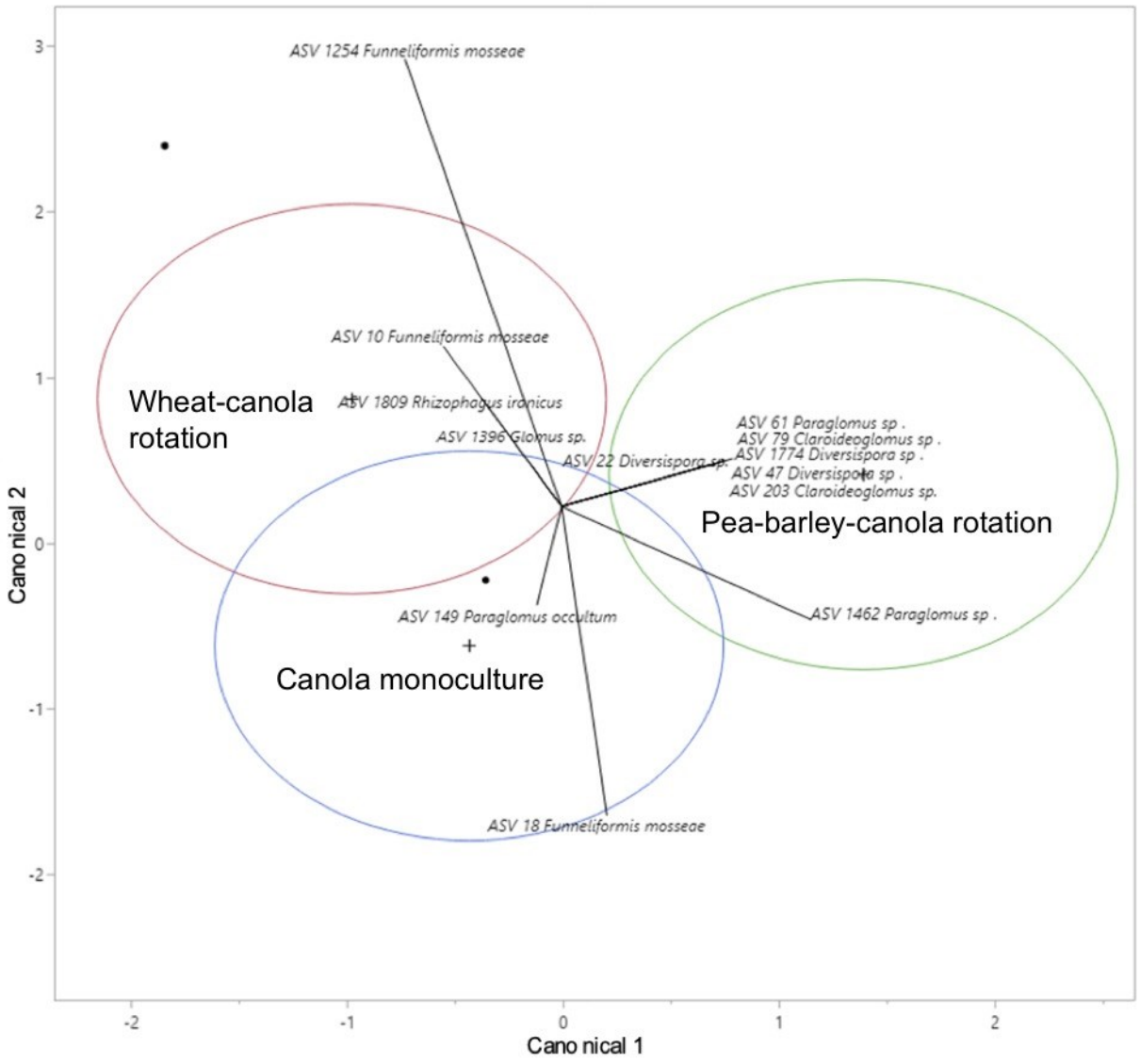

Figure S11. Canonical analysis of AMF community from the canola rhizosphere. Circles in red, blue and green represent different crop rotations while vectors show different glomeromycotan ASV which are likely to be preferentially associated to each of the crop management. The more separated the circles are, the more the community structure between the crop management are different. The more the arrow is directed to the center of a circle, the more the ASV was associated with a certain crop rotation.

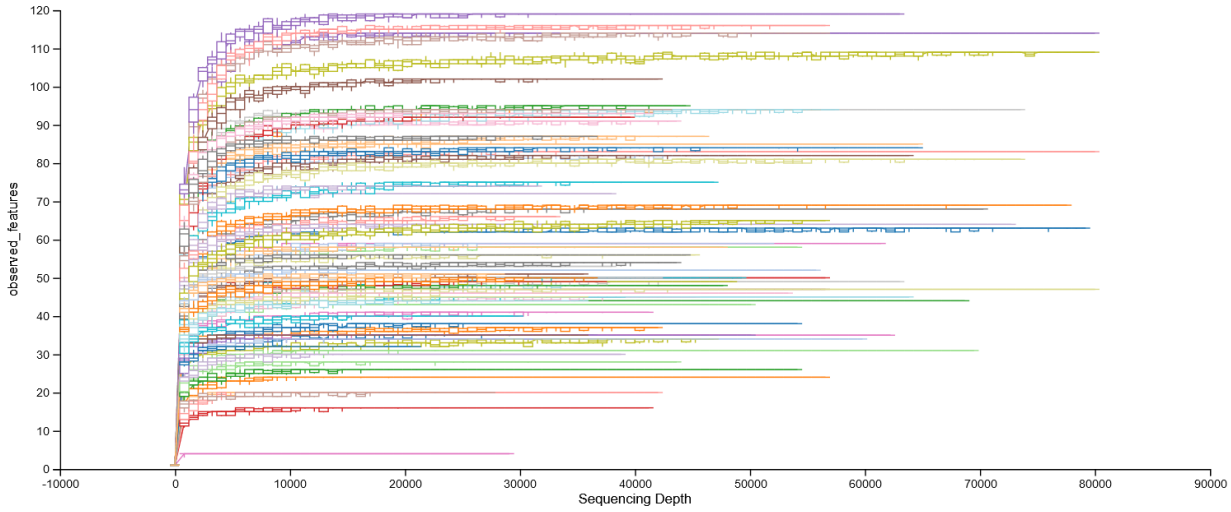

Figure S12. Rarefaction curves for each sample before trimming non-AMF ASV.