Université de Montréal

Arbuscular mycorrhizal fungal communities of 31 durum wheat cultivars (*Triticum turgidum var. durum*) under field conditions in Eastern Canadian province of Quebec

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Résumé

Les champignons mycorhiziens peuvent offrir différents services écosystémiques comme l'amélioration de la nutrition et de la croissance des plantes, la protection contre les maladies et ravageurs, etc. Ainsi, les mycorhizes peuvent présenter des solutions pour réduire l'impact du secteur agricole sur l'environnement en réduisant l'usage des engrais, pesticides, et autres produits. Cependant, les informations disponibles indiquent que le blé peut dans certains cas réagir négativement à la mycorhization : réduction de croissance et de rendement au point de caractériser la relation comme du parasitisme. Nous avons émis l'hypothèse que certains génotypes de blé pourraient engendrer des modifications de la structure des communautés mycorhiziennes associées. Nous avons testé 31 génotypes de blé dur (*Triticum turgidum var.* durum) produits à différentes époques de l'histoire de l'amélioration génétique de cette espèce dans une expérience en champs avec un sol pauvre en phosphore afin de caractériser leur association mycorhizienne dans des conditions de colonisation naturelle. Le séquençage de la sous-unité ribosomique 18S de l'ADN extrait des échantillons indique que la communauté mycorhizienne détectée dans les racines de blé est dominée par *Rhizophagus irregularis* et *R. intraradices* tandis que la communauté dans la rhizosphère et dans le sol est dominée par *Glomus* spp.*, Claroideoglomus* spp. ou *Rhizophagus* spp. Le taux de colonisation variait entre 57.8% (Arnautka) et 84.0% (AC Navigator) mais ces différences ne sont pas statistiquement significatives (*p-value* > 0.05). Bien que les racines de blé dur aient sélectionné un sous ensemble des taxa du sol pour coloniser leur tissues, les résultats suggèrent que tous les cultivars de blé dur sélectionnent des communautés de champignons mycorhiziens similaires. Il semble que la variation génétique du blé dur soit insuffisante pour permettre l'emploi de méthodes d'amélioration génétique traditionnelles pour la production de génotypes s'associant des champignons mycorhiziens arbusculaires performants.

Mots-clés : mycorhizes, champignons mycorhiziens arbusculaires, blé, *Triticum turgidum var. durum,* communauté, microbiome, agriculture

Abstract

Arbuscular mycorrhizal fungi can provide ecosystem services such as growth and nutrition enhancement, protection against pests and diseases, mitigation of abiotic stress, as well as stabilization of the soil. In the agriculture sector, symbiotic relationships between crops and AM fungi are being explored to help reduce agriculture's ecological footprint and enhance its sustainability by decreasing demand for fertilisers, pesticides and other chemicals. However, information available to date indicates that wheat may sometimes show poor response to AM symbiosis (growth depressions and lower yields), and the relationship has in some cases been labeled parasitic. We tested the hypothesis that genetic variation among durum wheat cultivars (*Triticum turgidum var. durum*) might lead to shifts in fungal community structure. We tested 31 durum wheat cultivars released at different times in the history of durum wheat selection in Canada in a field experiment under phosphorus limiting conditions. Based on the 18S ribosomal subunit sequences of DNA extracted from roots, rhizosphere and soil, we found that AM community in durum wheat roots is dominated by *Rhizophagus irregularis* and *R. intraradices*. Meanwhile the community in the rhizosphere and soil have more *Glomus* spp*., Claroideoglomus* spp. or *Rhizophagus* spp. The mycorrhizal community associated with wheat roots is not significantly different between genotypes (*p-value* > 0.05). Mean colonization levels in the roots of the different cultivars varied between 57.8% (Arnautka) and 84.0% (AC Navigator). However, these differences were not statistically significant (*p-value* > 0.05). Despite that durum wheat roots could select a subset of taxa from the soil community to colonize their tissues, results suggest that all cultivars select similar communities. The genetic variation in durum wheat seems insufficient to allow the use of traditional plant breeding methods to produce genotypes associating with highly effective arbuscular mycorrhizal taxa.

Keywords : Mycorrhiza, arbuscular mycorrhizal fungi, wheat, *Triticum turgidum var. durum,* fungal community, microbiome, agriculture

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List of Initials

AM: Arbuscular mycorrhiza

AMF: Arbuscular mycorrhizal fungi

AAFC: Agriculture and Agri-Food Canada

AC: Agricultural Centre (Swift Current Research and Development Centre)

CDC: Crop Development Centre

DNA: Deoxyribonucleic acid

OTU/ZOTU: Operational taxonomic unit/Zero-radius operational taxonomic radius

PCA (PC1/PC2): Principal component analysis (principal component 1/principal component 2)

PCR: Polymerase chain reaction

RLC: Root length colonized

List of Abbreviations

ANOVA: Analysis of variance

Bp: base pairs

Cm: Centimetres

Etc.: Et cætera

Km: Kilometres

M: Million

Min: minute

ML: Millilitres

Mm: Millimetres

µL: microliter

P.: page

W/v: Weight by volume

Mom and Dad, who supported and encouraged me through thick and thin

Lauren and Joaquim, who gave me something to believe in and carry me through

Thank-yous

This project would not have been possible without the help and support of a number of people who I would like to briefly recognize. First of all my supervisors, Dr. Chantal Hamel and Dr. Mohamed Hijri, who provided technical guidance and encouraged me, especially in difficult times. Secondly my committee, Dr. Pierre-Luc Chagnon and Dr. Marc St-Arnaud, who gave helpful insights and avenues to explore.

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Introduction

Arbuscular mycorrhizal (AM) fungi likely evolved around 353-462 million years ago, concurrently with the first land plants. In fact it is hypothesised that AM fungi were instrumental in facilitating the colonization of land by vascular plants (Simon *et al.* 1993). Arbuscular mycorrhiza is the most common type of mycorrhiza found among vascular plants (Wang & Qiu 2006). They are characterized by their ability to colonize a host plant's root cortex cells, forming extensive exchange structures within the root called arbuscules, as well as storage structures called vesicles formed between cortex cells. All AM fungi belong to the phylum Glomeromycota and they form symbioses with more than 80% of all plants on Earth (Smith & Read 2008), providing a range of services from drought and disease protection to nutrient and water uptake (Gianinazzi *et al.* 2010).

The ecology of AM fungi in the environment is complex and governed by a multitude of factors. AM fungi community composition and structure can be influenced by abiotic factors (such as soil type or soil use, phosphorus levels in the soil and other soil physico-chemical properties, climate, time of year), and biotic factors (host plant identity, plant community structure) (Bainard *et al.* 2014, Bainard *et al*. 2015, Dai *et al.* 2012, Degrune *et al.* 2015, Klabi *et al.* 2015). Dai *et al.* (2012) found that the variation in the AM fungal community structure in the Canadian Prairies was best explained by the soil types (Chernozem great groups), whereas Bainard *et al.* (2015) determined that land use (roadsides vs cultivated fields) played a greater role in AM fungal community composition than soil type. Soil chemistry, particularly pH, was shown to be a major driver of AM fungi community structure, and time of year can also play a role in determining community composition, influenced by flux in soil P and climate variables such as soil moisture (Bainard *et al.* 2014). Host plant also affects AM fungal community richness, diversity and composition, but mainly within the plant's roots themselves. As this study showed, the effects of host plants on the fungi in the soil was minimal, and soil chemical factors likely affected them more than the hosts (Bainard *et al.* 2014). In more complex plant assemblage experiments, the introduction of a N-fixing legume was beneficial to AM fungal diversity (Klabi *et al.* 2015).

While plants have an effect on AM fungal communities, the reverse can also be true, with the fungi helping to shape plant communities or impacting the success of different plants. Experiments on prairie grassland communities involving different species of AM fungi, legumes, and grasses (cool-season and warm-season) showed that plant community structure and identity of AM fungi can impact the coexistence of different grasses by favouring some over others, showing that AM fungi are a key factor in understanding plant interactions (Klabi *et al.* 2014).

The agriculture sector is known to be a source of pollution and contamination, especially from the manufacture and application of N and P fertilizers, as well as herbicides and pesticides, and from other agronomic practices. Increasingly, strategies are being explored to reduce the carbon footprint of agriculture and improve its sustainability, such as choosing crop varieties and cultivars specially adapted to the biotic and abiotic conditions of a certain area to reduce the need for inputs, diversifying cropping systems for more effective weed management and improving crop residue management to increase carbon sequestration in soils, among other strategies. The use of AM fungi, either through inoculation or adopting practices that encourage the naturally occurring fungal community, in agroecosystems is one of those strategies (Gan *et al.* 2011). Indeed, AM fungi play key roles in agroecology such as increasing soil stability, improving nutrient use efficiency and thus decreasing the need for fertilization, increasing crop plant tolerance and protection against abiotic and biotic stresses, and increasing the plants' nutritional quality (Gianinazzi *et al.* 2010).

Improving the sustainability of modern agriculture must involve, among other things, a shift toward management and crop breeding practices that favour the exploitation of AM symbioses and take into account the plant microbiome. It has been shown that agricultural practices such as tillage and pesticide application affect microbial community composition for both bacteria (Degrune *et al.* 2015, Yang *et al.* 2012) and fungi (Degrune *et al.* 2015), with some groups increasing or decreasing in abundance depending on use of conventional or reduced tillage (Degrune *et al.* 2015). Adopting tillage and weed control practices specifically tailored to encourage proliferation of healthy and beneficial soil microbes can greatly advantage crops. Brito *et al.* (2013) showed that using chemical as opposed to mechanical control for weeds minimized disturbance of indigenous mycorrhizal communities and existing

extra-radical mycelium, which enhanced early colonization rates in crops, boosting P acquisition and early growth. In addition, encouraging AM colonization may help crops overcome the stress of soil compaction due to the use of heavy agricultural machinery by boosting nutrient uptake in compacted soils (Miransari *et al.* 2009).

Another example of the importance of the plant microbiome in agricultural systems is the effects of crop-microbiome interactions in crop rotations. The crop grown in a field previously can play a role in the success of the subsequent crop, through shaping the microorganism community in the soil. Indeed, durum wheat exhibited higher stand density when planted following the chickpea cultivar CDC Anna compared to other chickpea cultivars, though the effect was absent during particularly dry years (Ellouze *et al.* 2013), and experimentally introducing a root endophytes community to a wheat crop could either worsen or alleviate the allelopathic effect of chickpea cultivar tissues on wheat germination (Ellouze *et al.* 2015). Yang *et al.* (2012) found that terminating a pulse crop early before planting durum wheat lead to favourable conditions for the establishment of a beneficial root endophyte bacterial community and higher grain yield, though the effect was absent during a particularly wet year. Thus it is important for crop management strategies to not only consider the crop itself and its associated microbiome, but also the global management strategy for the whole field.

Wheat has long been recognized as a crop with mixed responses to AM fungi. Some studies found overall positive effects of both inoculated and indigenous AM fungi colonization on wheat. A variety of experiments in different conditions have found increases in grain yield and aboveground and straw biomass as a result of AM colonization, including field experiments under conditions of water stress or organic farming (Pellegrino *et al.* 2015, Al-Karaki *et al.* 2004, Nelson *et al.* 2011), as well as pot/glasshouse experiments in conditions of drought or elevated CO_2 (Karagiannidis & Hadjisavva-Zinoviadi 1998, Zhu *et al.* 2016). Further study by Martín-Robles *et al.* 2018 on a wider range of 27 different crop species including durum wheat, tomato, chickpea, lettuce, cucumber, spinach and others, found that symbiosis with the AM fungus *Rhizophagus irregularis* benefitted plants in all cases in conditions of low P fertility, but that domesticated plants drew no such advantage at high soil fertility. However, in wild progenitors benefits were evident in both low and high fertility

conditions (Martín-Robles *et al.* 2018). Meanwhile other studies found i) negative effects of AM fungi on wheat in the form of growth depression depending on the phosphate fertilization regime, the AM fungal isolate tested, and plant density (Graham & Abbott 2000, Li *et al.* 2008); ii) a mix of positive and negative effects, depending on the wheat cultivar tested and the management strategy used (organic or conventional) (Hetrick *et al.* 1996, Singh *et al.* 2012, Dai *et al.* 2014); or iii) no significant effect on wheat growth at all regardless of added P (with a caveat that, even while no effect was observed, plants were still acquiring important amounts of their phosphorous through fungal pathways) (Li *et al.* 2006).

Hetrick *et al.* (1993) found strong dependence of winter wheat on mycorrhiza in cultivars released prior to 1950, but more variable responses in more recent releases, suggesting an effect of intensive breeding and management practices on mycorrhizal response in wheat cultivars. For durum wheat, there is little evidence that selection under high soil fertility conditions and breeding of modern cultivars impairs their ability to regulate their AM symbiosis compared to landraces (Ellouze *et al.* 2015). Breeding has generally had the consequence of increased AM colonization in durum wheat grown in soil with poor fertility and impacted the wheat's ability to regulate the symbiosis according to soil fertility. However, this impact has been inconsistent: certain cultivars had good but unimproved mycorrhizal regulation (e.g.: Commander, Pathfinder), whereas others had inefficient regulation resulting in significant growth reduction of mycorrhized plants compared to non-mycorrhized controls (e.g.: Hercules, Wascana, Eurostar) (Ellouze *et al.* 2015).

These varied responses suggest that AM fungal effect on wheat is not straight forward and not always strictly beneficial as measured through growth response, but rather is affected by a number of factors including time of year (variation throughout growing season, separation of nutrient flow to plant and fungus over time), growth stage of plants, environmental conditions or stresses, nutrient availability, identity of the plant and fungus, etc. (Jones & Smith 2004). Still, the fact that the relationship between mycorrhizal fungi and plants has persisted and remained stable for millions of years suggests that it confers a distinct selective fitness advantage for both partners (Brundrett 2002), even if some cases would be better classed as parasitism than mutualism (Jones & Smith 2004). During partner selection, there are likely processes at play which favour higher quality partners providing the best return

on the energy investment for the symbiosis, resulting in a selective pressure not to "cheat" in the relationship, thus helping to stabilize the plant-AMF association through evolutionary history (Werner & Kiers 2015).

The objective of this study was to characterize the arbuscular mycorrhizal fungal community associated with the roots of 31 different cultivars released at different times in the history of durum wheat (*Triticum turgidum var. durum*) breeding in order to discern possible differences in AM fungi community composition and colonization levels between genetically diverse durum wheat cultivars. We hypothesised that different durum wheat cultivars may favour the establishment of distinct AM fungal communities (Mao *et al.* 2014). To test this hypothesis, we conducted a field experiment in summer 2016 with a randomized complete block design. Amplicon sequences from 18S rDNA of AM fungi extracted from root, soil, and rhizosphere soil samples were used to characterize fungal communities.

Materials and Methods

The 31 wheat cultivars tested in this project are listed in Appendix 1. A randomized complete block design with four blocks was used; all 31 cultivars were randomized in each block. This gave a total of 124 plots (31 cultivars x 4 blocks). Due to the large number of treatments and the dimension of the field where the experiment was conducted each block was layered in two rows of plots. The final experimental setup is illustrated in Figure 1.

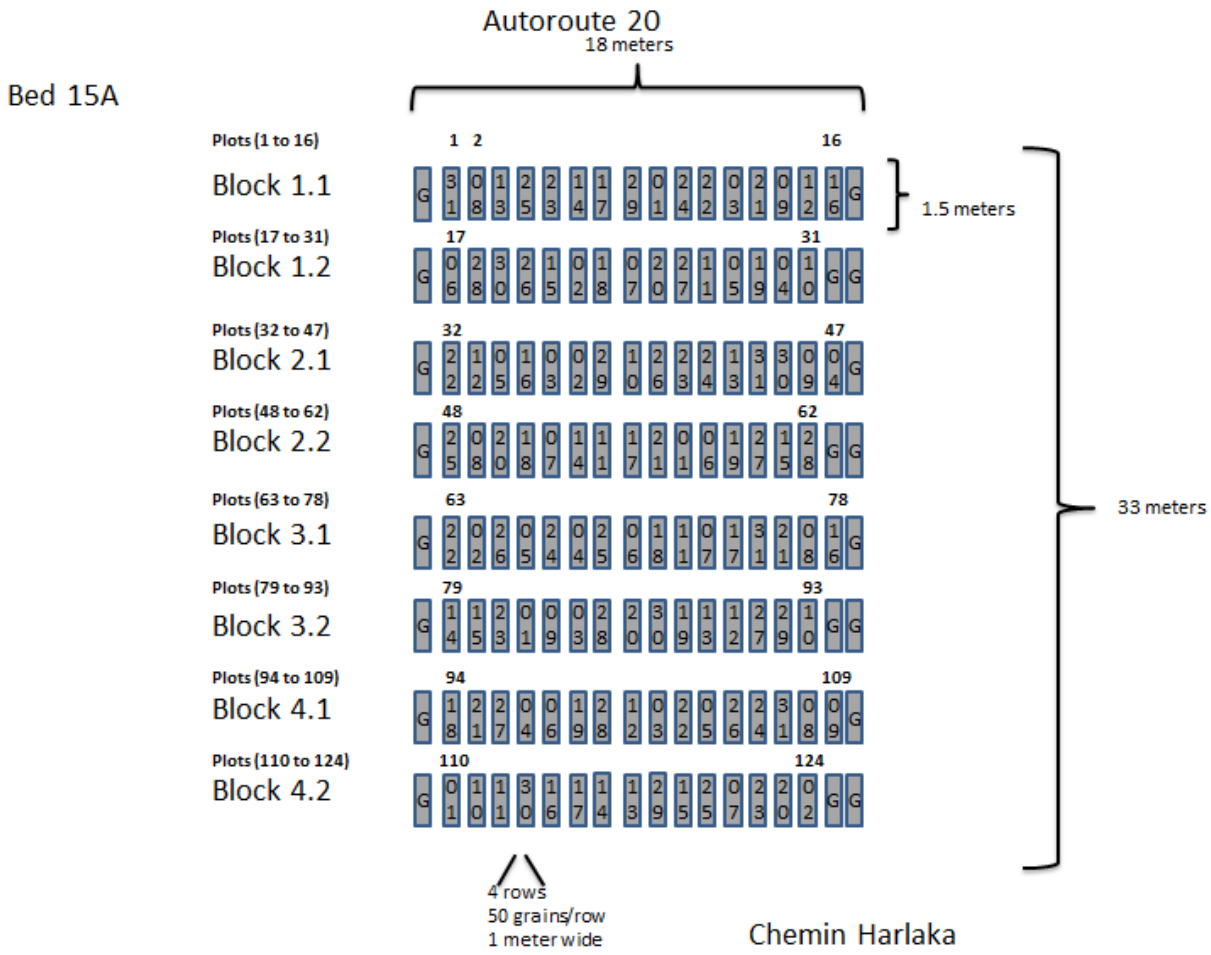

Figure 1. Chart showing the experimental set-up of field trial. A randomized complete block design of four blocks of 36 plots was conducted in summer 2016. The numbers inside each plot represent the cultivar ID numbers (1-31). Numbers above plots represent plot ID numbers (1-124).

Study Area

Field trials were carried out at a site located outside the municipality of Lévis (46°47'40"N 71°08'05"W) in the Canadian province of Quebec. Agriculture and Agri-Food Canada data lists the growing season in this region as lasting between 140 and 150 days (http://www.agr.gc.ca/, retrieved 10/07/2017), and a cool and humid climate is characteristic. The average temperatures for the area in 2016 were 12.5°C, 16.9°C and 19.1°C respectively for May, June, and July according to the Lauzon weather station of Environment and Natural Resources Canada (http://climate.weather.gc.ca/, retrieved 23/08/2017), which was located at 3.5 km from the experimental field. These temperatures are comparable to the 1981-2010 averages for those months (11.0°C, 16.5°C, and 19.3°C).

The soil was a well-drained Saint-André gravelly loam (Fragic Humo-Ferric Podzol or mixed, frigid, Typic Dystrochrept) (Soil Classification Working Group, 1998) with properties listed below (Table I). The field was previously used to grow switchgrass in 2014-2015. Roundup was applied and the field was tilled in fall 2015. Harrowing and fertilization were carried out on 11 May 2016. The plots at time of sowing received 90 kg/ha N as calcium ammonium nitrate (27-0-0) and 45 kg/ha K_2O as potassium chloride (0-0-60). Phosphorous fertilization was not applied in order to make P resources a limiting factor to favour mycorrhizal associations. Plots were seeded using a 4 row cereal seeder on 12 May 2016 as follows: 4 rows of 50 seeds over 1.7 m for each of the 31 varieties of wheat. DyVel herbicide (Dicamba) was applied at 1.25 L/ha on 7 June 2016 for weed control.

Table I. Physical and chemical properties of the field in this experiment. Measurements taken in spring 2015 prior to the beginning of the current project. Mineral nitrogen was extractible with KCl (Maynard & Kalra 1993) and other nutrients were extractible with Mehlich-3 (Mehlich 1984).

Texture	pH	C Total	N Total	C/N		P-PO4	N-NO3	N-NH4
		%	%			mg/Kg	mg/Kg	mg/Kg
Loam	5.30	2.66	0.21	12.73		34.52	52.96	43.32
P	K	Ca	Mg	Al	Fe	Cu	Zn	Mn
mg/Kg	mg/Kg	mg/Kg	mg/Kg	mg/Kg	mg/Kg	mg/Kg	mg/Kg	mg/Kg
133.97	205.11	1632.19	55.44	1346.00	225.39	1.74	1.92	15.92

Sampling

Durum wheat aerial parts, roots and soils were sampled on July 8th and 12th 2016 as follows: six plants were randomly selected from within rows 2 and 3 of each plot (three plants from row 2 and three from row 3). The aboveground portion of each plant was cut and discarded and the root balls were set aside in the shade in plastic bags for transfer to the laboratory. Once there, root balls were kept at 4°C until further processing in the subsequent days. Six samples of soil from between rows were also collected using a 15 cm core sampler, cleaned between every plot by taking a first sample to discard. A single composite sample for the soil between rows of every plot was obtained by combining the six soil cores from each plot, passing them through a 2 mm sieve and transferring them to 15 mL tubes. Tubes of soil were kept in the shade until transfer to the laboratory by car, where they were stored at -20°C. Composite rhizosphere soil samples for each plot were collected in the lab by gently shaking the six root balls in order to dislodge the soil that was attached to them. Soil collected this way was passed through a 0.5 mm sieve for homogenization and to remove debris. All soil samples (rhizosphere and between rows) were stored at -20°C until DNA extraction.

Once the rhizosphere soil was collected, the six root samples from each plot were combined into a composite sample and rinsed, and the fine roots (around 1 mm thickness or less) were cut into 1-2 cm lengths. Root fragments for each plot were separated into 4 parts: 2 parts were put into two plastic Shandon™ tissue cassettes (Thermo Scientific™) for root colonization analysis, temporarily stored in tap water acidified with a few drops of white vinegar at 4°C until all samples were processed, then transferred into a 50% ethanol solution for longer term storage before analysis, still at 4°C. The other 2 parts were stored in 1.5 mL tubes at -80°C for DNA extraction.

Root Colonization Analysis

Root samples prepared for colonization analysis (the cassettes) were treated using the "ink and vinegar" staining method (Vierheilig *et al.* 1998). Cassettes were boiled in 10% w/v KOH solution for 3 min to remove pigmentation and rinsed several times in tap water. Next,

roots and associated fungal structures were coloured by boiling in a 5% ink (Shaeffer black) and white vinegar solution for 3 min. Excess ink was rinsed by soaking roots in tap water acidified with a few drops of vinegar per litre for 20 min. Finally, treated roots were stored in a 50% glycerol solution until they could be analyzed to evaluate their level of colonization.

Root samples were examined under a Zeiss Discovery V20 stereomicroscope coupled with an AxioCam ICc 5 camera. ZEN pro 2012 software was used to visualize the roots on a computer. The level of colonization was evaluated using the gridline intersection method; root samples were spread out in a 90 mm plastic petri dish with a 7x7 gridline drawn on the bottom, and for each intersection of a root with the gridline the presence or absence of mycorrhizal structures was noted (Brundrett 1996, based on Giovannetti & Mosse 1980). In total 39 116 intersects were recorded, giving an average of about 315 intersects per sample. Colonization percentage is represented by the following formula:

$$\left(\frac{\#\ colonized\ intersections}{total\ \#\ intersections}\right) \times 100$$

DNA Extraction

DNA extraction from roots was performed using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions except the elution step, where 75 µL of buffer was used instead of 100 µL, and the flow-through from the first elution was reused for the second elution rather than using fresh buffer. DNA was then stored at -20°C until use. DNA extraction from soil samples was performed using UltraClean Soil DNA Isolation Kit and PowerSoil DNA Isolation Kit (Mo Bio) (see Annex 2). All rhizospheric soil samples were extracted using the PowerSoil kit. The manufacturer's instructions for both kits were used except for the elution step for the PowerSoil kit where 50 µL were used instead of 100 µL. DNA extractions were done in duplicate for each sample then combined in order to increase the amount of extracted DNA. DNA quality was first checked by gel electrophoresis, and then quantified using Qubit Fluorometer (Invitrogen). DNA was stored at -20°C until use.

Polymerase Chain Reaction

PCR was carried out by Genome Quebec according to the following protocol (see Annex 3 for details). PCR was done in one step on the genomic DNA using the Franck primers and CS1/CS2 adapters (Table II), which gives a 400 bp fragment of the 18S gene. Program parameters were as follows: denaturation for 0.5 min at 98°C, then 30 cycles of 10 seconds at 98°C followed by 15 seconds at 60°C followed by 30 seconds at 72°C, then a final extension period of 2 min at 72°C. Then barcoding was done on the amplified DNA products from the first step (diluted 1/200) to incorporate Illumina adapters and index, with the following program parameters: denaturation for 3 min at 98°C, then 10 cycles of 10 seconds at 98°C followed by 30 seconds at 60°C followed by 30 seconds at 72°C, then a final extension period of 2 min at 72°C.

Table II: Primers used in PCR to amplify 400bp section of 18S gene.

Franck_F-CS1	ACACTGACGACATGGTTCTACACGGTAATTCCAGCTCCAATAG
Franck_R-CS2	TACGGTAGCAGAGACTTGGTCTTTGATTAATGAAAACATCCTTGGC

PCR amplification was checked by gel electrophoresis on 2% agarose gel, and DNA concentration was quantified. Equal amounts of DNA were pooled for each sample and the final pool was purified using AMPure XP at a ratio of 0.8 and eluted in water (molecular biology grade). Final quantification on the purified pool was then performed: libraries were quantified with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies) and the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems). Average size fragment was determined using a LabChip GX (PerkinElmer) instrument. Finally, sequencing was performed with the MiSeq Reagent Kit v3 (600 cycles) from Illumina, with all 380 samples pooled for the Miseq run.

Bioinformatics

Amplicon sequence data from Genome Quebec were sorted using sequence barcodes for each sample. Sequence data were analyzed using the UNOISE algorithm in USEARCH in order to identify ZOTUs (zero-radius operational taxonomic units) among the fungal DNA. Forward and reverse reads were first merged with a maximum overlap difference allowance of 10 base pairs (bp). Of 16.9 M pairs, 10.9 M (64.36%) were successfully merged; about 6.0 M pairs (35.64%) were either too different (> 10 bp) or had no alignment and were discarded. Then the primer sequences were removed from either end, giving sequences of median length 353 bp. Any sequences longer than 373 bp or shorter than 333 bp were discarded (142 735 of remaining sequences—or 1.4%—were eliminated this way). Quality filtering discarding sequences with expected errors > 1.00 eliminated another 295 435 (2.9%) of remaining sequences, and dereplication generated the final set of unique sequences (158 057 uniques). The UNOISE algorithm generated a total of 3877 fungal ZOTUs, which were then identified via BLAST using the UNITE and NCBI databases. 102 AMF (Glometomycota) ZOTUs were identified and extracted for further analysis. Samples originating from each of the three categories (roots, rhizosphere soil, and soil between rows) were given unique identifier letters so they could be tracked through further analyses. Samples from root-extracted DNA were given the letter R; samples from rhizosphere soil-extracted DNA the letter S; and samples from between-row soil-extracted DNA the letter E. Read data was turned into percentages so they would be comparable and averaged across replicates.

Statistical Analyses

All statistical analyses were performed in R (v. 3.4.2) except for the PerMANOVA which was done using PC-ORD (v. 6.19). One-way analysis of variance with blocks was done on root colonization % data for each cultivar, as well as species richness and Shannon diversity in the three sample types (roots, rhizosphere, soil). The Bartlett test of homogeneity of variances and the Shapiro-Wilk normality test were applied to the data to ensure compliance with the analysis' requirements. A dendrogram based on average agglomerative clustering (UPGMA) and a heatmap were generated to illustrate the distances between

samples, and principal component analysis was performed to represent the community structure. The AMF communities in each of the three environments were analysed by PerMANOVA and by one-way ANOVA on select fungal OTUs of interest, and the community profiles were represented with bar graphs.

Results

Root Colonization

All root samples for all wheat cultivars showed some degree of colonization by AM fungi, varying between 57.84% (Arnautka) and 84.02% (AC Navigator) (Figure 2). However the differences were not significant (*p-value* from ANOVA = 0.455; *p-value* from Dunnett's Test > 0.05).

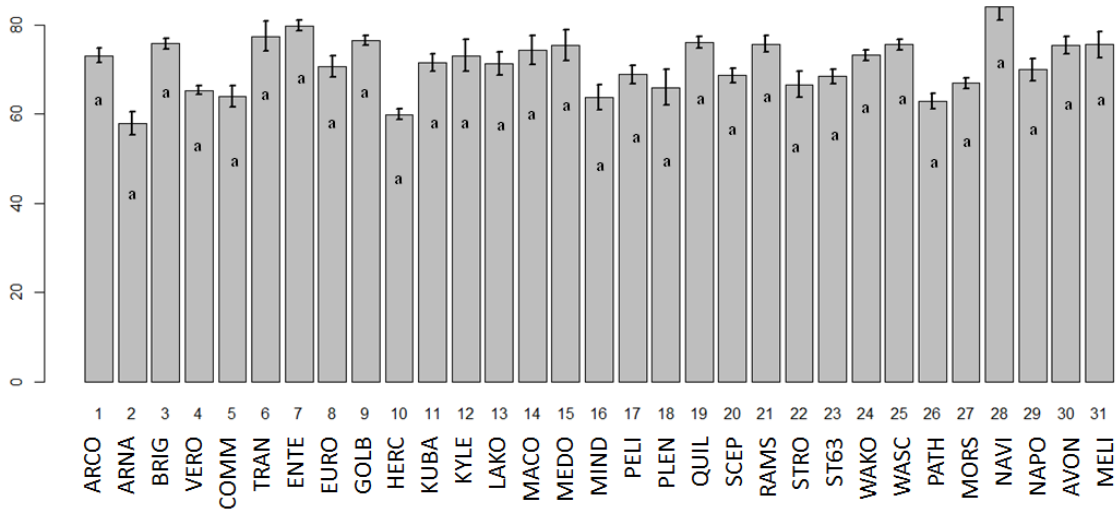

Figure 2. Percentage of AM fungi colonization for each cultivar based on the gridline intersect method (±SE). See Appendix 1 for full cultivar names and other information. Highest observed colonization in AC Navigator (84.02%) and lowest observed colonization in Arnautka (57.84%). *P-value* from ANOVA = 0.455 and Dunnett test > 0.05, no significant difference in means (n = 4)

AMF Community Diversity

The rhizosphere and soil samples showed greater AMF species richness and diversity than the root samples (Figure 3, p.15), which is expected as plants do not indiscriminately form associations with all fungal species in their soil. The Shannon diversity plot shows that there is no significant difference in the diversity of the AMF community between the rhizosphere and the soil, thus theoretically evenness should be higher in the soil samples to balance the lower richness. However the observed difference in evenness is not large enough to be significant, with a *p-value* from Student's t-test of 0.12.

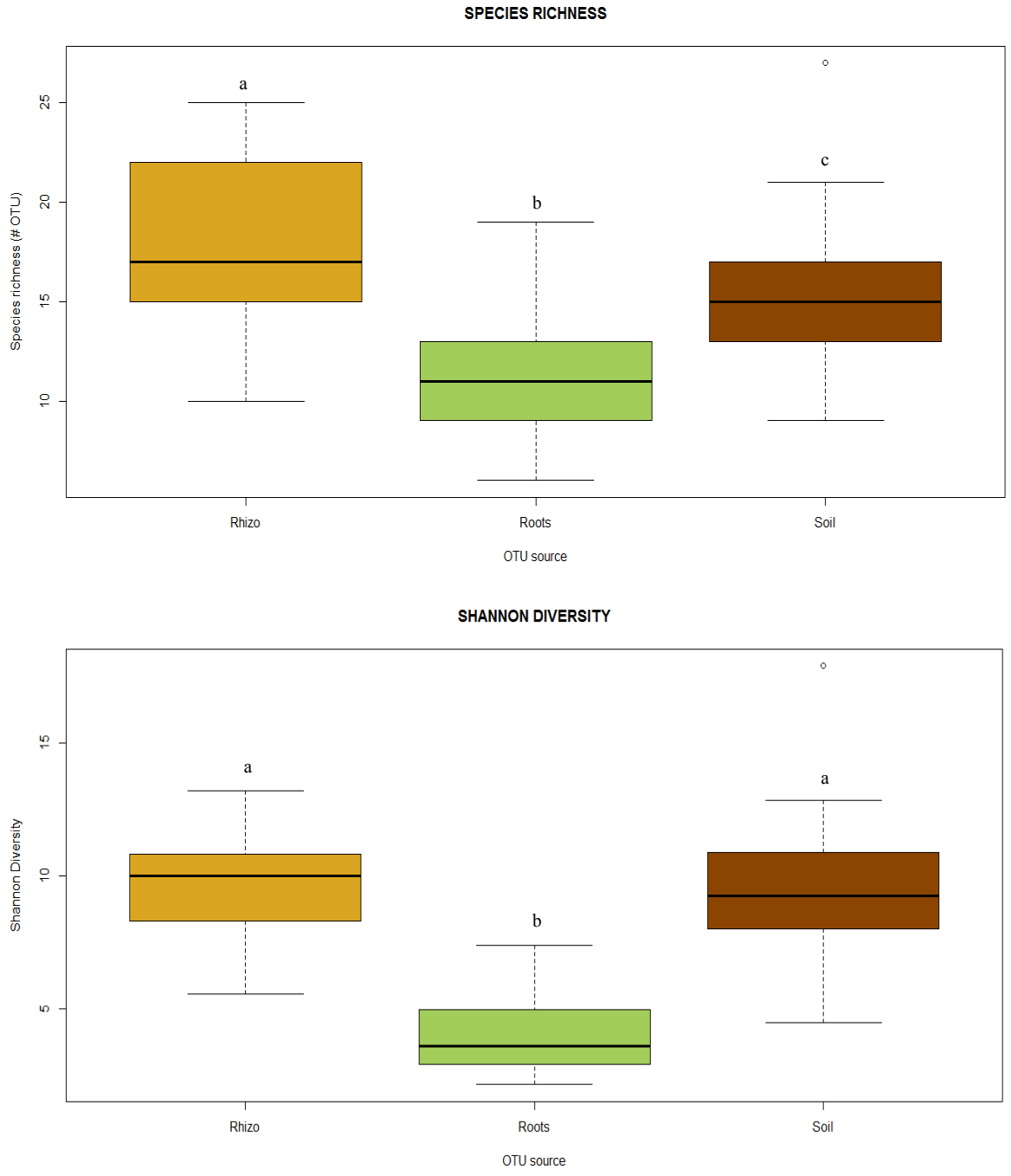

Figure 3. AMF species richness and Shannon diversity coefficient in 31 durum wheat cultivars based on origin of samples (rhizosphere, roots, soil). *P-value* from ANOVA is 5.01e-12 for species richness and <2e-16 for Shannon diversity (n = 31). The horizontal lines represent the median of the datasets and boxes represent the middle 50% of values (25% above the median and 25% below). Whiskers represent maximum and minimum values in each dataset.

AMF Community Structure by Origin

Samples originating from root-extracted DNA form a distinct pattern of AMF colonization compared to soil and rhizosphere samples. Community composition in roots was similar across cultivars, but different from the composition of the rhizosphere and soil communities. This is illustrated by the high degree of clustering between root samples in the dendrogram (Figure 4, p. 17) as well as the distinct pattern in the heat map indicating small distances, and thus high similarity, between these samples (Figure 5, p. 18).

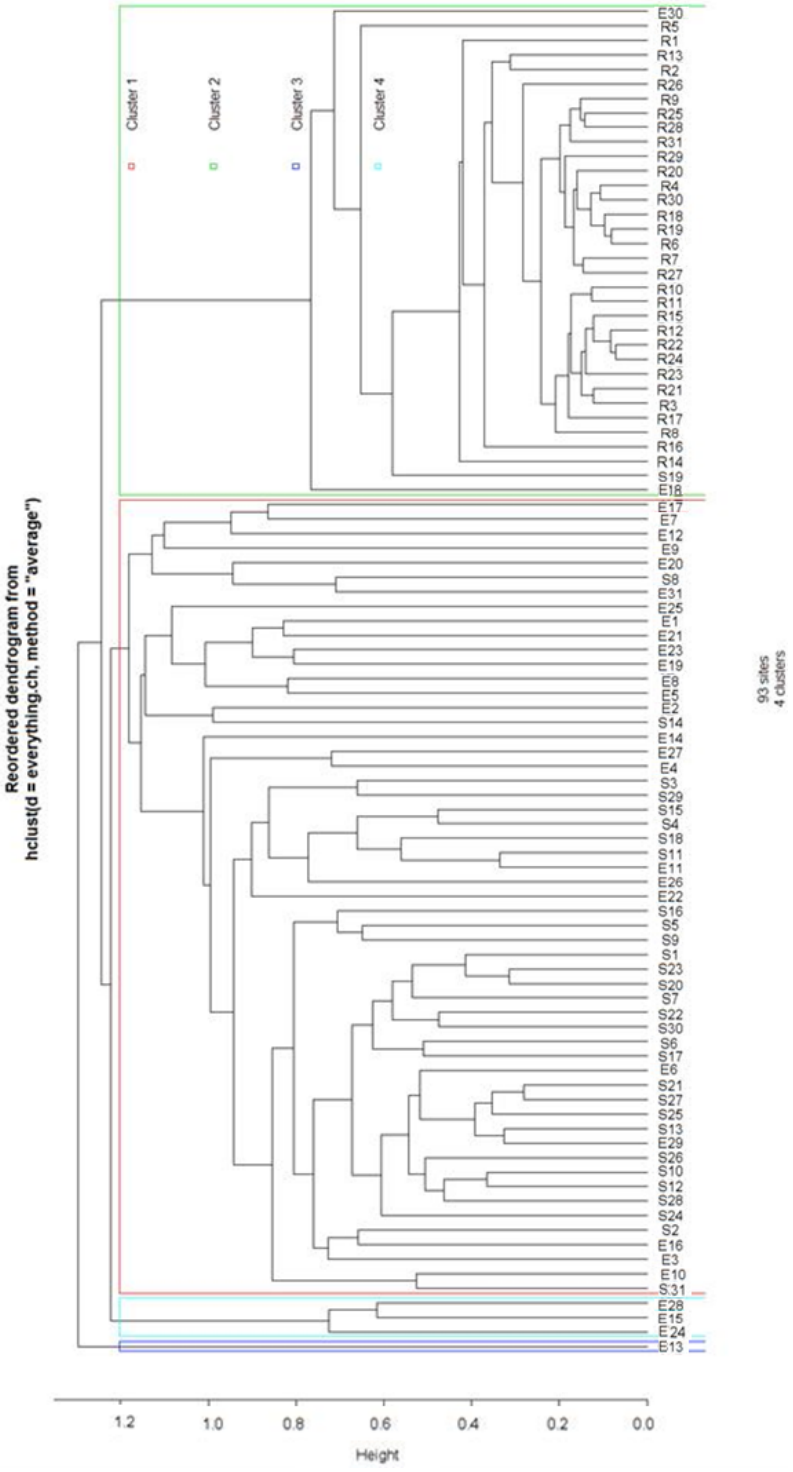

Figure 4. Dendrogram of OTU data based on average agglomerative clustering (UPGMA) of Chord distance, divided into 4 groups. Cluster 2 (green) contains all samples taken from DNA extracted from durum wheat roots (R1-31).

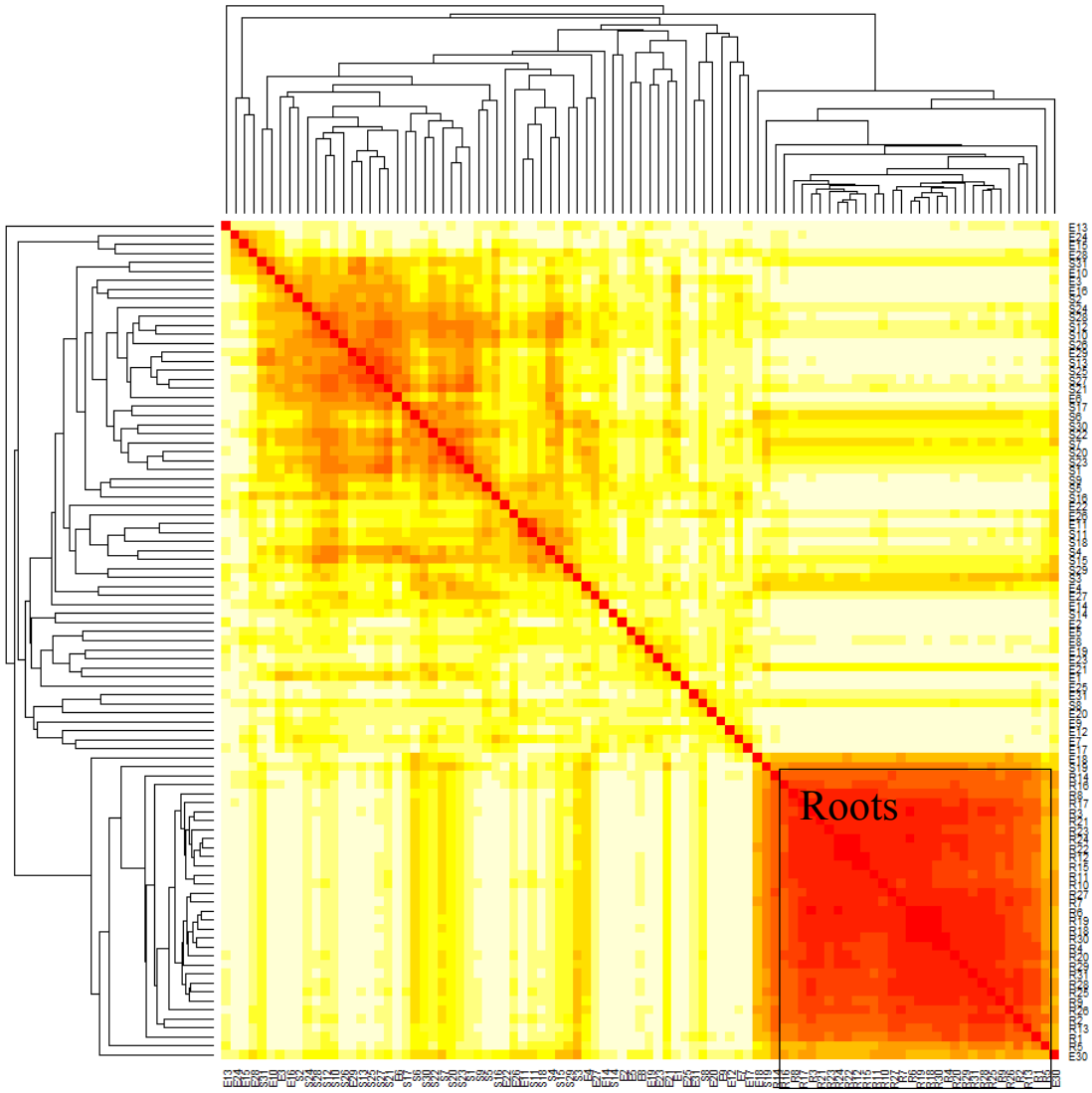

Figure 5. Seriation heatmap showing lower distance objects closer to the diagonal. Note the highly similar root samples (lower right).

The distinct pattern of root sample clustering is most clearly evident in the PCA (Figures 6 & 7, p. 20-21), where the scaling 1 biplot shows samples originating from wheat roots form a tight cluster on the right-hand side of the plot along PC1. It also suggests composite OTU RHIR (made up of the 5 OTUs that were identified as *Rhizophagus irregularis*; 109, 411, 601, 762, 992) and OTU201 (*Rhizophagus intraradices*) dominate the root AMF community. As for the two other groups of samples—rhizosphere and soil—they are not as strongly clustered on the PCA biplot but nevertheless rhizosphere samples are generally grouped in the upper left quadrant of the plot and correlated with OTU133 (*Glomus* sp.) and OTU143 (*Claroideoglomus* sp.), as well as three OTUs which could not be adequately identified (OTU172, OTU389, OTU494). Meanwhile soil samples are generally grouped in the bottom left quadrant of the plot and influenced by OTU340 (*Glomus* sp.), OTU186 (a *Claroideoglomus* sp.*;* either *Claroideoglomus lamellosum* or *Claroideoglomus etunicatum*), and OTU455 (*Glomus* sp. or *Rhizophagus* sp.), as well as one which could not be adequately identified (OTU952).

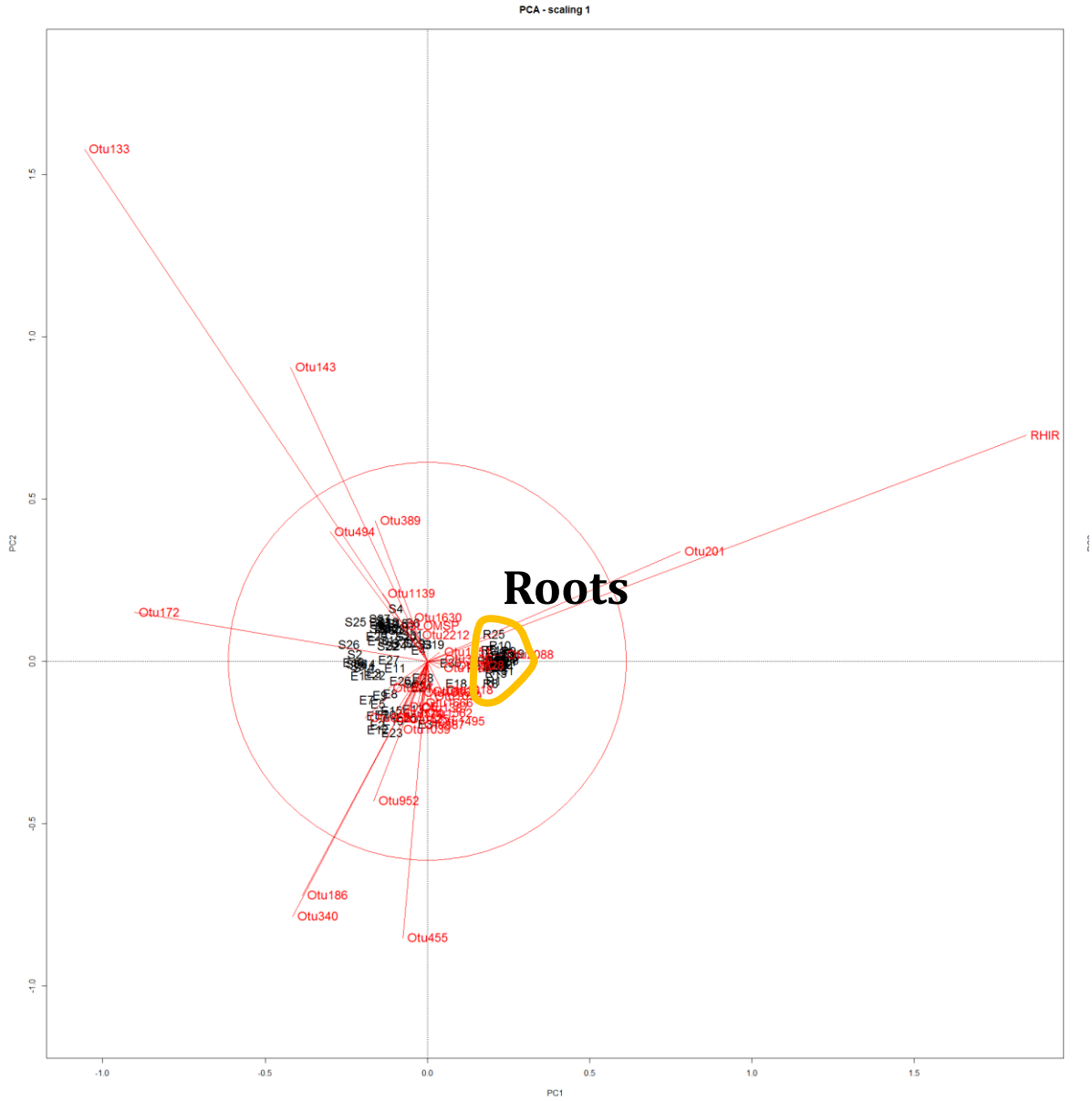

Figure 6. Principal component analysis biplot on Hellinger transformed OTU data, scaling 1. Note the cluster of root originated samples on the right of the plot, exhibiting a unique community of associated AMF.

The scaling 2 biplot (Figure 7) suggests OTUs 133 (*Glomus* sp.), 143 (*Claroideoglomus* sp.), 494, and 389 are correlated together given the small angles between the vectors. Similarly OTUs 340 (*Glomus* sp.), 186 (*Claroideoglomus* sp.) and 952, as well as OTUs RHIR (*Rhizophagus irregularis*) and 201 (*R. intraradices*) are correlated together.

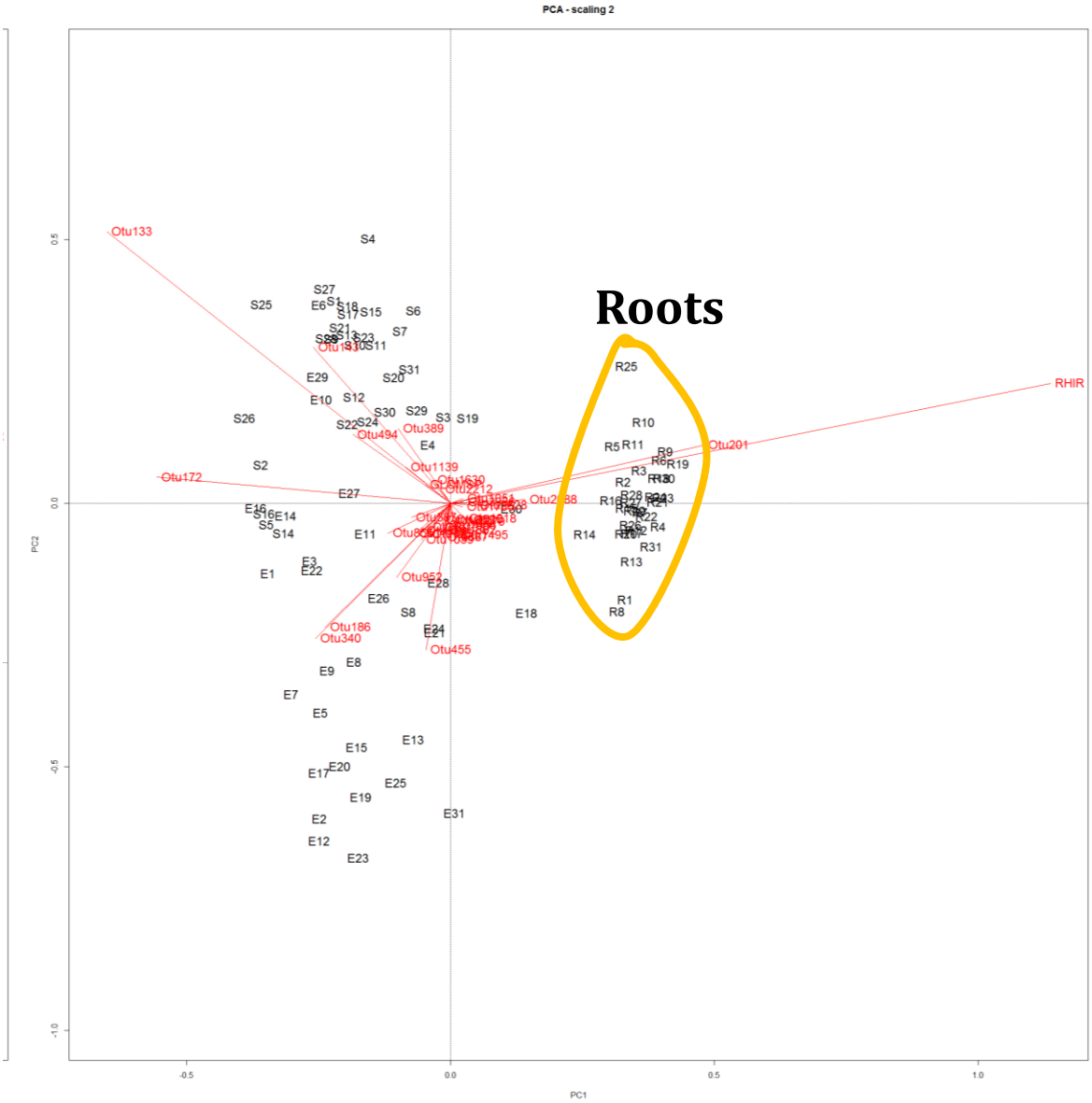

Figure 7. Principal component analysis biplot on Hellinger transformed OTU data, scaling 2. Three types of AMF communities can be distinguished based on the angles between vectors.

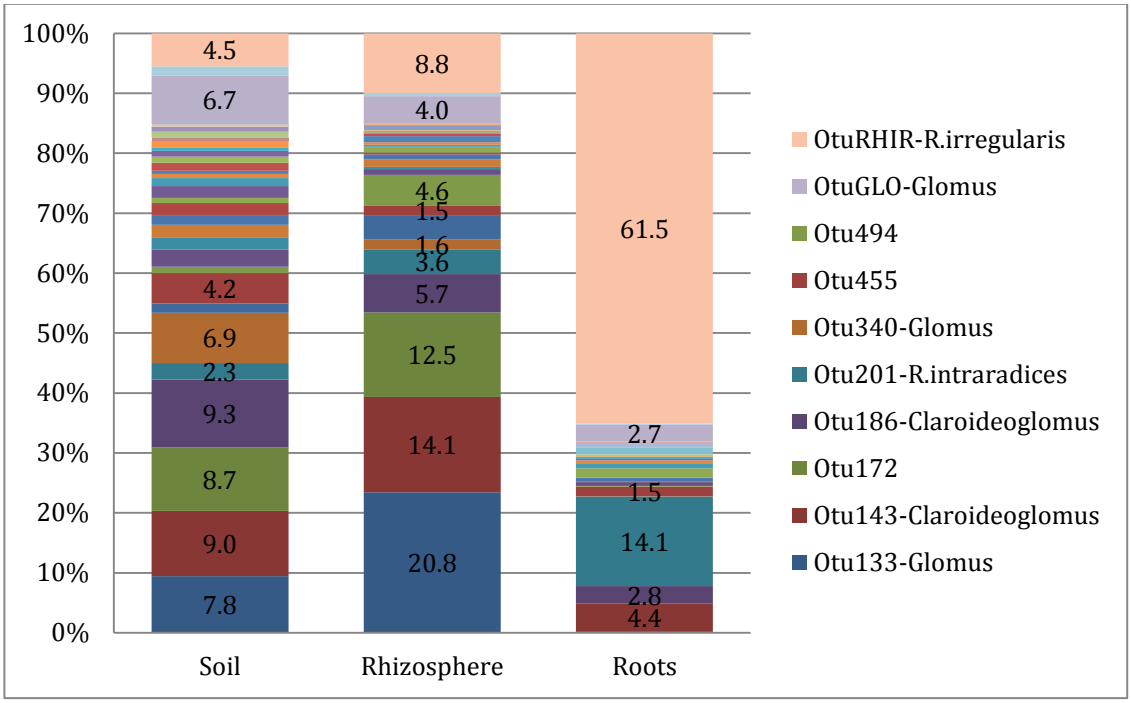

Figure 8. Bar graph illustrating how community structure of wheat-associated AMF changes based on sample origin.

Figure 8 shows the relative abundance of the AMF taxa composing each community found by the PCA above (Figures 6 & 7, p. 20-21). In addition, while both the soil and rhizosphere communities exhibit important proportions of both *Glomus* sp. and *Claroideoglomus* sp. represented by 2 different OTUs each (OTU340/orange and OTU133/dark blue for *Glomus* sp. and OTU186/purple and OTU143/red for *Claroideoglomus* sp.), the relative contribution of each OTU in those two communities reflects the layout of the PCA biplots. Thus the orange band of *Glomus* sp. in Figure 8 is much thicker in the soil community, indicating a greater proportion of OTU340 in those samples. In contrast the dark blue band of *Glomus* sp. in the rhizosphere community in Figure 8 illustrates that OTU133 contributes more. Similarly the purple band of *Claroideoglomus* sp. (OTU186) is thicker in the soil community while the red band of *Claroideoglomus* sp. (OTU143) is thicker in the rhizosphere community.

AMF Community Structure by Wheat Genotype

While the results show a distinct effect of sample origin on community composition, PerMANOVA analysis shows there is no effect of individual wheat genotypes on AMF community (Table III), with a *p-value* > 0.05 in all 3 sample types.

Table III. PerMANOVA results for wheat genotype effect on AMF community. No significant results for all 3 sample types (*p-value* > 0.05)

a) Rhizo

Source	F Observed	F from randomized groups: Mean	Maximum	S.Dev	Number > or = observed F	p *
Bloc	2.36897	1.00605	2.56969	0.27514	2	0.000600
Genotype	1.04039	1.00346	1.41614	0.09410	1686	0.337400

b) Roots

Source	F Observed	F from randomized groups: Mean	Maximum	S.Dev	Number > or = observed F	p *
Bloc	3.07942	1.00467	2.90560	0.31494	0	0.000200
Genotype	0.94852	1.00284	1.47437	0.11020	3339	0.668000

c) Soil

Source	F Observed	F from randomized groups: Mean	Maximum	S.Dev	Number > or = observed F	p *
Bloc	2.53758	0.99993	2.10546	0.19003	0	0.000200
Genotype	1.09089	1.00141	1.27266	0.06755	491	0.098400

* proportion of randomized trials with indicator value
equal to or exceeding the observed indicator value.
p = (1 + number of runs >= observed)/(1 + number of randomized runs)

In addition, ANOVA performed on individual OTUs of interest selected from among the OTUs that stood out in the PCA (Figure 6, p. 20) and the community structure analysis (Figure 8, p. 22) also show that we cannot reject the null hypothesis that there is no difference in means between wheat genotypes (Table IV, p. 24). All but the two RHIR OTUs (all samples combined and roots only) were subjected to square root transformation before analysis to comply with homogeneity of variances and normality requirements. In the case of the two RHIR OTUs the data passed the Bartlett test of homogeneity of variances but failed

the Shapiro-Wilks normality test even after transformation. In all cases for all OTU tested, there were no pairs of genotypes for which Dunnett's test found significant difference.

Table IV: Brief summary table of various ANOVA results for select OTUs with regards to wheat genotype. In no case was a significant result observed (all *p-value* > 0.05) meaning we cannot reject the null hypothesis that there is no difference in the means.

All samples	**Roots**	**Rhizosphere**
OTU RHIR *(R. irregularis)* F-value: 1.315 *p-value*: 0.12953	**OTU RHIR** *(R. irregularis)* F-value: 0.729 *p-value*: 0.8355	**OTU133** *(Glomus* sp.*)* F-value: 1.018 *p-value:* 0.456
	OTU201 *(R. intraradices)* F-value: 1.395 *p-value:* 0.11880	**OTU143** *(Claroideoglomus* sp.*)* F-value: 0.832 *p-value:* 0.71
		OTU172 F-value: 0.644 *p-value:* 0.9134

The community profile graphs below (Figures 9-12, p. 25-28) provide a visual representation of the lack of difference between genotypes for community composition found by the PerMANOVA and ANOVA tests. Figure 9 shows the community as a whole including all samples from the 3 origins (roots, rhizosphere and soil) while Figures 10-12 illustrate in more detail the communities found in each of the 3 areas. The fungal species and OTUs highlighted on the graphs are those which stood out in the PCA tests above.

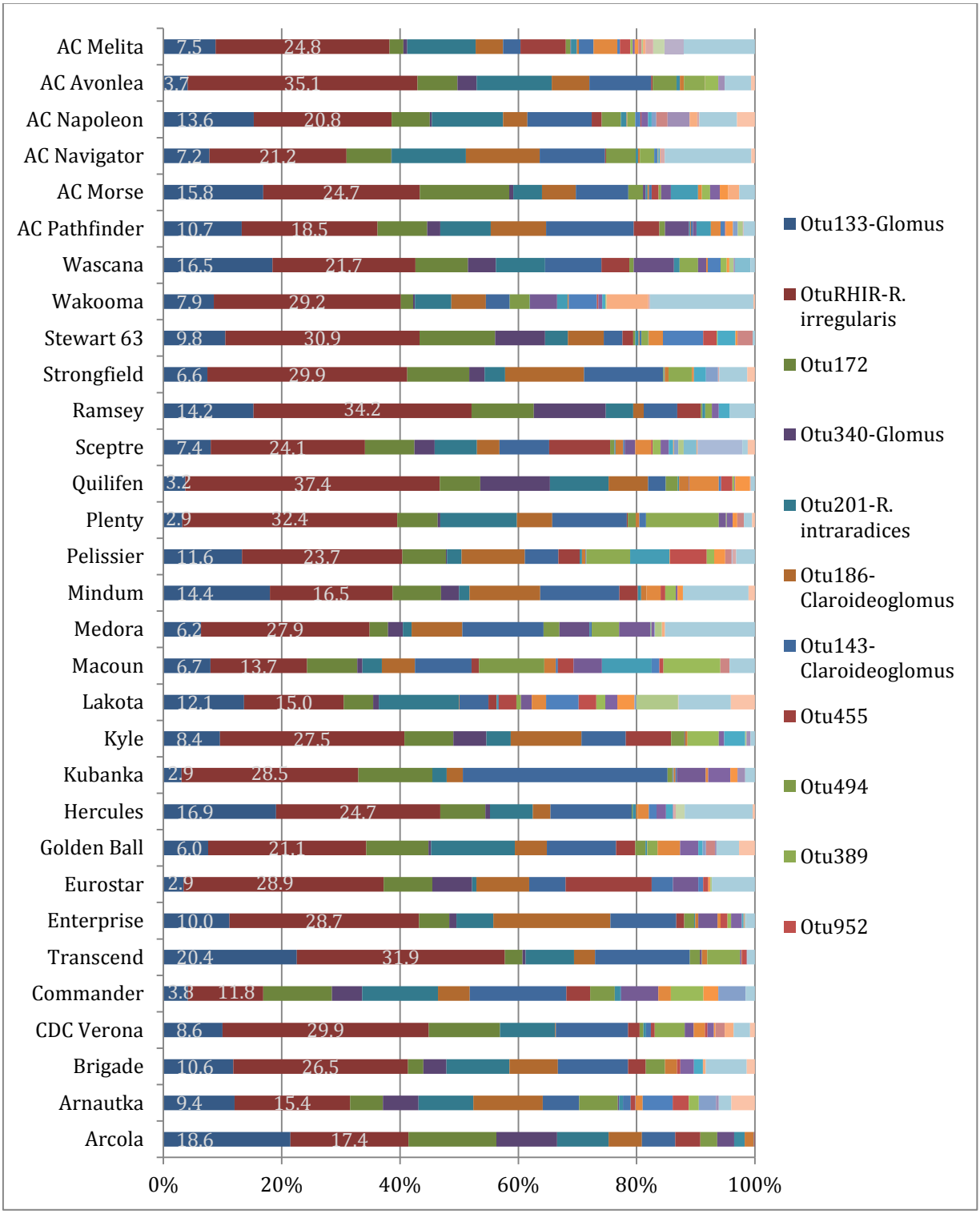

Figure 9. Bar graph illustrating proportions of the most important fungal species in each wheat cultivar's overall community (including roots, rhizosphere and soil) (%). The legend contains the important OTUs identified by the PCA.

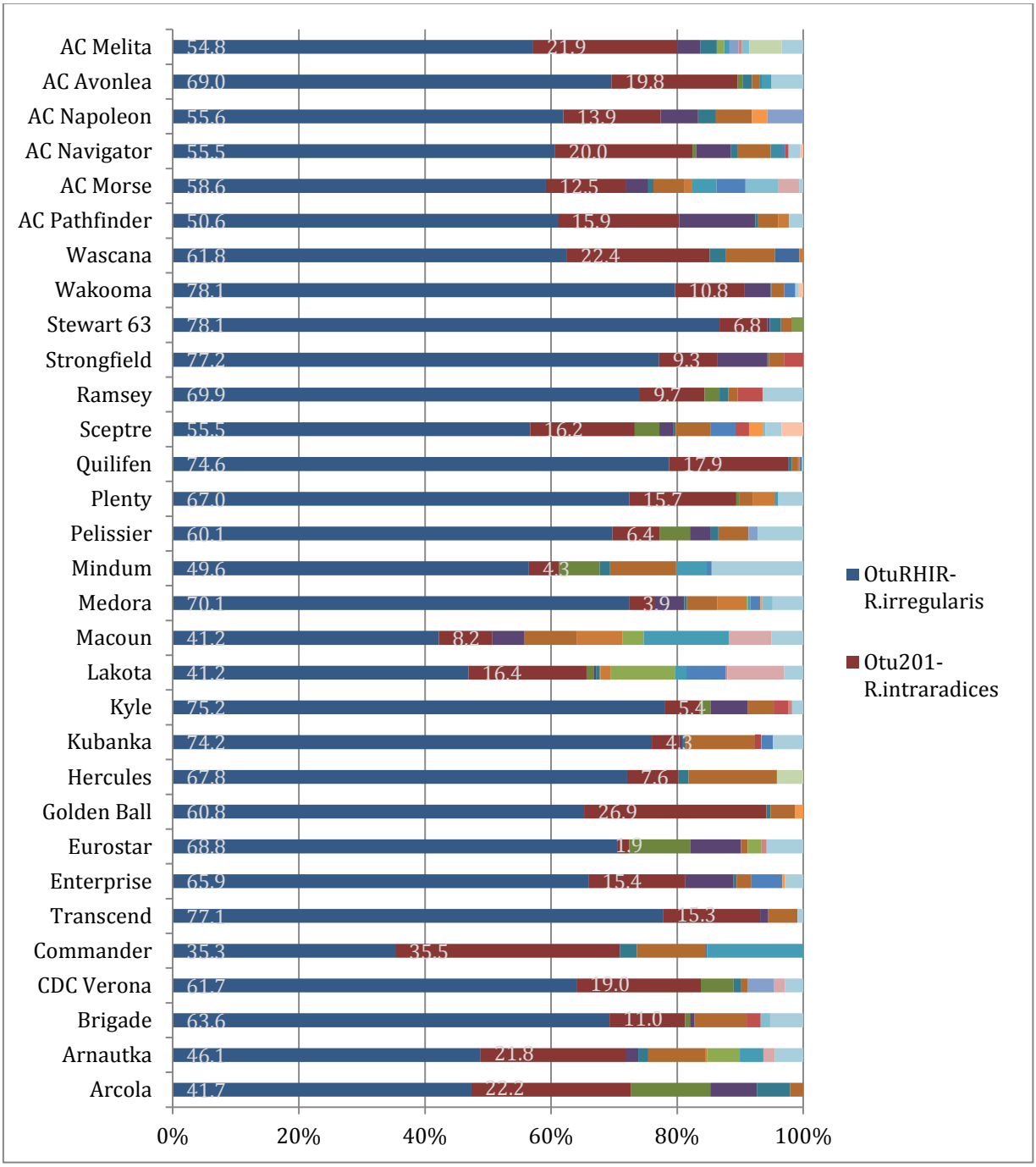

Figure 10. Bar graph illustrating proportions of the most important fungal species in each wheat cultivar's root-associated community (%). The legend contains the important OTUs identified by the PCA which characterize the root-associated community. *P-value* from PerMANOVA = 0.668, n=4.

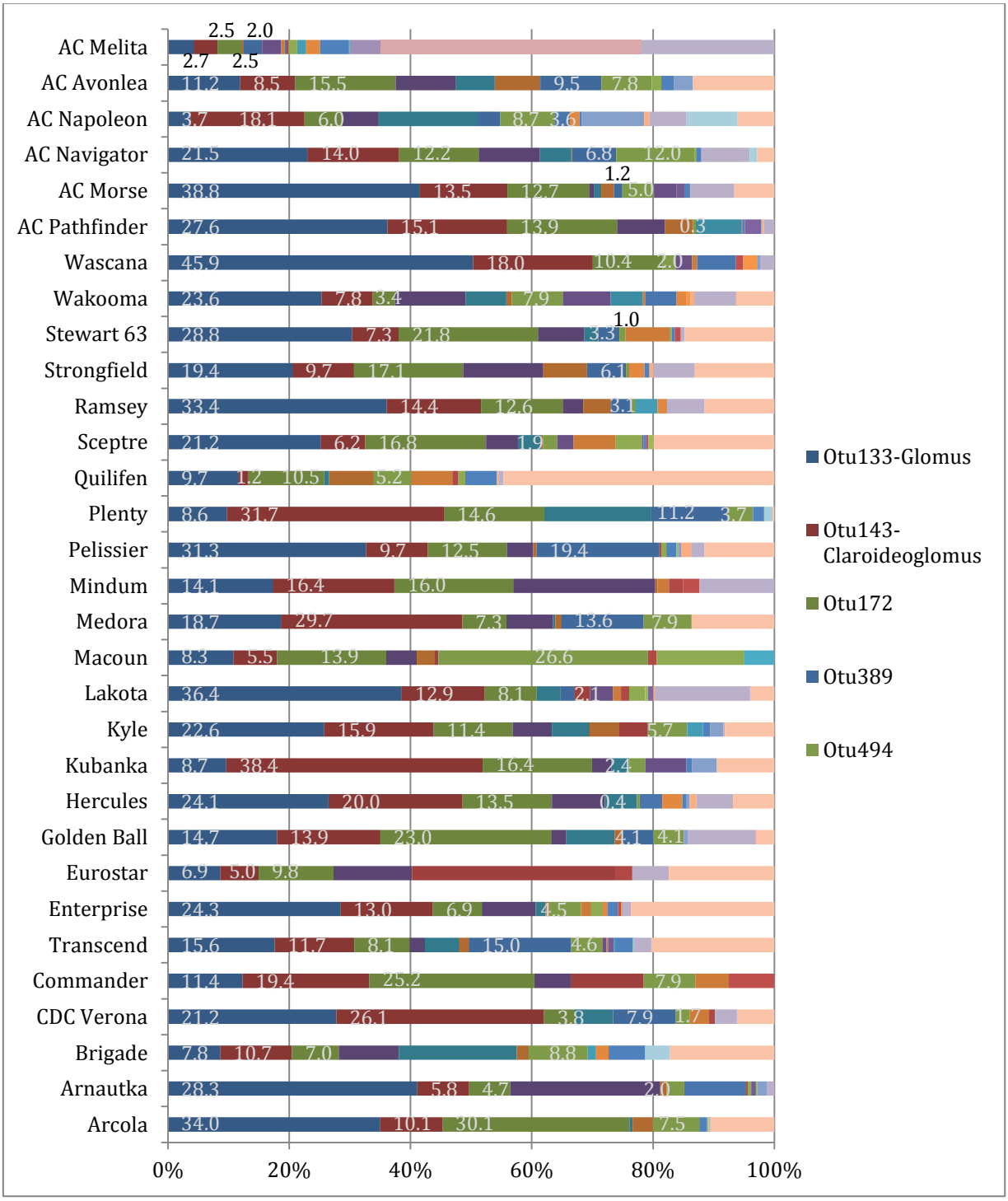

Figure 11. Bar graph illustrating proportions of the most important fungal species in each wheat cultivar's rhizosphere-associated community (%).The legend contains the important OTUs identified by the PCA which characterize the rhizosphere-associated community. *P-value* from PerMANOVA = 0.337, n=4.

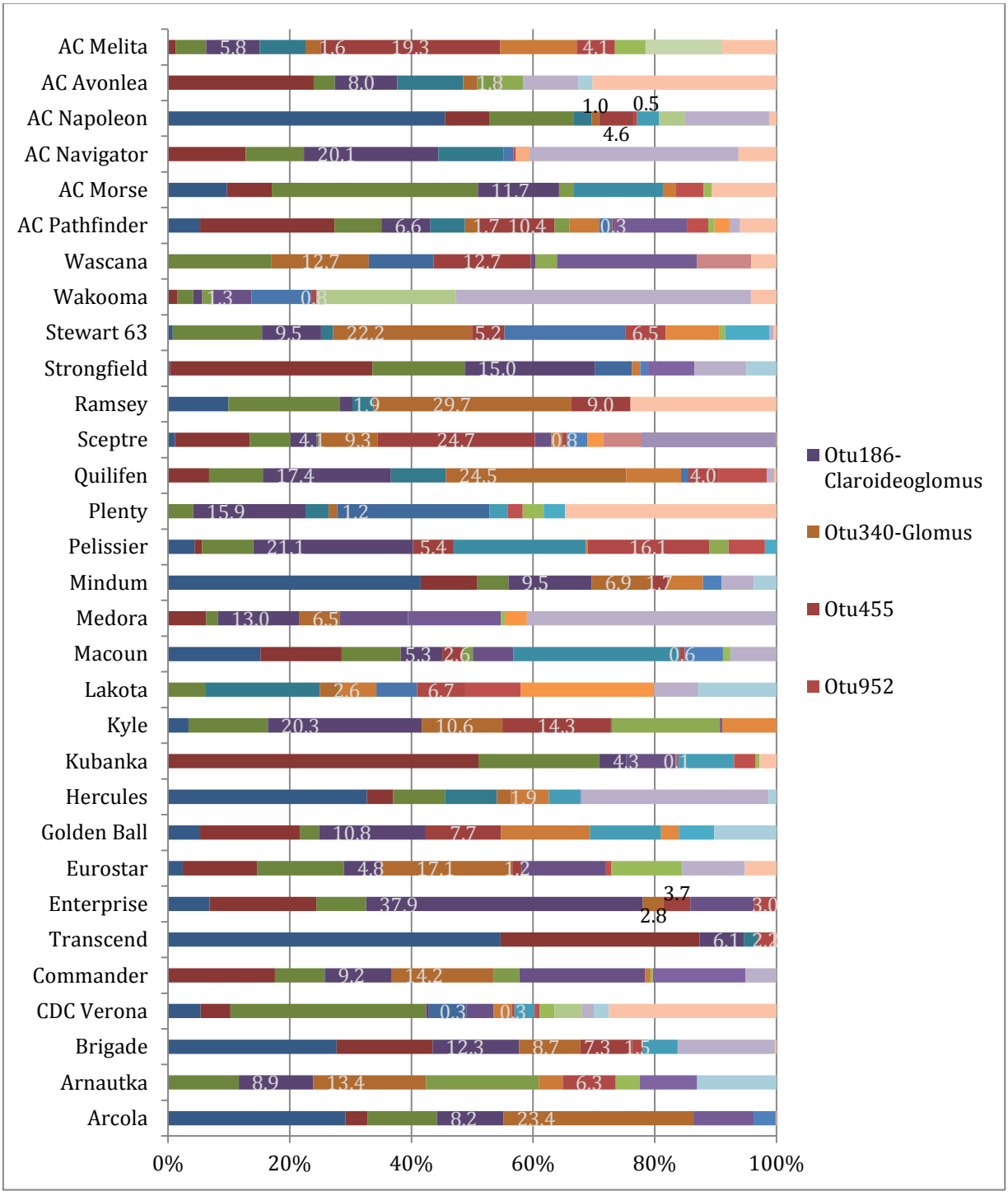

Figure 12. Bar graph illustrating proportions of the most important fungal species in each wheat cultivar's soil-associated community (%).The legend contains the important OTUs identified by the PCA which characterize the soil-associated community. *P-value* from PerMANOVA = 0.098, n=4.

Discussion

Root Length Colonization

The percentages of root lengths colonized (% RLC) (Figure 2) are on the high end compared to previous studies like Ellouze *et al.* 2016 and Singh *et al.* 2012 which found colonization percentages under low fertility conditions in the range of roughly 12-43% and 8-32% respectively. This difference may be due to the fact that those previous experiments were conducted in greenhouses under controlled conditions with a limited number of AMF species tested, whereas the current study was done in natural field conditions where the entire range of resident AMF species could potentially colonize the roots.

The colonization levels reported here are more in line with what Graham & Abbott (2000) observed from "aggressive colonizers" at low P fertility (50-89% RLC compared to 58-84% RLC in this project). In their case the aggressive colonizers included species such as *Scutellospora calospora, Glomus invermaium, Acaulospora laevis* and *Gigaspora decipiens* inoculated onto specimens of Kulin wheat. In my experiment (Figure 10) the most prominent AMF colonizing the roots of durum wheat were two *Rhizophagus* species (*R. irregularis* and *R. intraradices*). These species, especially *R. irregularis,* are common plant colonizers in nature and are widely used as inoculants in both commercial and scientific contexts (Peyret-Guzzon *et al.* 2016), thus finding them strongly represented in this field experiment was not surprising.

In addition to the identity of the AMF colonizing the wheat, another factor which likely contributed to the high % RLC observed was the fertility of the field, specifically the phosphorus fertility. The field where the trial was conducted is naturally poor in phosphorus (Table I), and the decision was made to withhold adding any P fertilization at the time of sowing the wheat in order to favour associations with AMF. In their study on the effect of domestication on AM association at different fertility regimes, Martín-Robles *et al.* (2017) found that at low P fertility levels, both domesticated crops and wild progenitors benefitted similarly from AM association. This is in line with the results presented here, which showed

no significant difference in colonization or AM fungal community composition between durum wheat genotypes (Figure 2; Tables III, IV).

AMF Community Structure

While it is clear that durum wheat genotype did not influence AMF community profile (Figures 9-12), the distinct structure found in the different sample types, specifically roots, suggest there is active selection on the plant's part with regards to AMF symbiosis. Such deliberate partner selection is known to occur in a broad range of plant and AMF species mediated in part by various chemicals which are released into the rhizosphere (Werner & Kiers 2015, Steinkellner *et al.* 2007). Evidence for this selection is seen throughout the results, as root originated samples are consistently more tightly clustered together and more similar to each other than to any other sample. Also, the root community is dominated by 2 species which are comparatively not important in the soil or rhizosphere communities, where they represent less than 10% (compared to 61.5% for *R. irregularis* and 14.1% for *R. intraradices* in the root community) (Figure 8). In addition, the root associated AMF community boasts overall fewer species than the soil or rhizosphere, but interestingly the species richness is higher in the rhizosphere than in the soil, which could be due to fungal species becoming "activated" as they come into contact with the wheat roots and proliferating at high enough levels to be detectable by the analysis (Figure 3). Most likely all the species present in the rhizosphere samples are also present in the soil samples, but in a dormant state and at low enough abundance that they are undetected. All these results together point to a filtering and selection process which favours the proliferation of certain AMF species within wheat roots, and the lack of genotype effect suggests the underlying mechanisms which shape community structure are likely common among durum wheat cultivars, and no single genotype interacts differently with the soil AMF under the trial conditions. The exact factors governing partner selection between AM fungi and durum wheat could be an interesting avenue to explore with further research.

Breeding for AMF Compatibility

Durum wheat breeding and improvement in Canada has been an evolving and ongoing endeavour since the first Canadian cultivar, Stewart 63, was registered in 1963. Since then over 20 new cultivars have been developed and registered, almost all of which were included in this study. Durum wheat breeding has been directed by market demands and quality standards through the years, and as a result more recent registrations exhibit higher yield and grain protein content, higher yellow pigment content and gluten strength as well as lower grain cadmium concentrations (Dexter 2008, Clarke *et al*. 2010). Resistance to diseases and pests such as fusarium head blight, leaf and stem rust, wheat stem sawfly and wheat midge is also an area of interest to breeders, especially as the incidence and severity of pest damage increases as a result of climate change. Canadian durum is already resistant against some diseases, and efforts to maintain and improve resistance in future cultivars are underway (Clarke *et al.* 2010).

There is high genetic diversity available within durum wheat germplasm which allows for such breeding to occur, and breeding for mycorrhizal association is an avenue which could be explored as the push to decrease the need for chemical fertilizers intensifies. Singh *et al.* 2012 demonstrated in a greenhouse experiment using five different durum wheat cultivars that the genetic variation necessary for selection on AM symbiotic formation and function exists within the species. This should serve as a starting point for identifying the genes responsible for AM formation and developing new cultivars with this criterion in mind. Singh *et al.* 2012 also warn against selecting only for AM dependence, but rather breeding efforts should aim to produce cultivars with good AM formation and phenotypic plasticity allowing them to respond effectively to a variety of nutrient conditions.

Building upon the findings of Singh *et al.* 2012 and Ellouze *et al.* 2016 this project sought to investigate the AMF communities of a large set of wheat cultivars (31 as opposed to the 5 tested in Singh *et al.* 2012) in a natural field setting rather than in a greenhouse setting. Similar to Ellouze *et al.* 2016, the current study found no significant effect of wheat genotype on AM root colonization or indeed on AM community composition. The comparison between the two studies is particularly apt because all but one cultivar that was tested in 2016 was also tested in the present study. While wheat genotype was identified as having a significant effect

on root colonization at both low and medium soil fertility in the Singh *et al.* 2012 study, the specific genotypes of interest for the present study (ie. those that were used in the field trial as well as in the 2016 greenhouse trial, namely AC Morse, Commander, and Strongfield) showed no significant differences between them in terms of root colonization by AMF at medium fertility, while at low fertility AC Morse and Strongfield exhibited similar colonization.

This would suggest that in the conditions tested both in the greenhouse experiment of 2016 and 2012, and in the present field trial, wheat genotype in itself does not significantly affect mycorrhizal fungi. A factor which may affect the results could be the fact that all durum wheat cultivars tested are of Canadian origin, which may result in similar reactions to AMF just by virtue of being broadly adapted to similar environmental conditions and developed using similar techniques. It would be interesting to test a broader selection of cultivars from around the world to see whether the observed lack of genotype effect in Canadian cultivars holds true.

Conclusion

This study characterized the AM fungal communities of 31 different durum wheat cultivars in a natural field setting with no artificial inoculation and low soil P fertility. The hypothesis that different cultivars support distinct AM communities was not supported, contrary to what some previous studies have suggested. The AM fungal community found within wheat roots is distinct compared to that in the rhizosphere or surrounding soil, and is dominated by *Rhizophagus irregularis* and *R. intraradices*. When analyzing individual fungal taxa no significant difference was found in their relative abundance when tested by wheat genotype. The results of this research constitute a base which explores a large number of cultivars from the oldest (Arnautka) to the most recent (Transcend) under field conditions which future studies will be able to build on. It would be interesting to see these cultivars subjected to a range of soil fertility conditions which may impact their behaviour in the field.

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Annex 1 – Wheat Cultivar List + Pedigree

#	Cultivar	Pedigree (GRIS*)	Year
1	Arcola	WASCANA/HERCULES	1983
2	Arnautka	LV-RUS; LV-ODESSA	1865
3	Brigade	DT-513/DT-696	2008
4	CDC Verona	D-95253/D-95212	2008
5	Commander	W-9260-BK-03/AC-NAVIGATOR//AC-PATHIFINDER	2005
6	Transcend	DT-707/DT-696	2010
7	Enterprise	DT-716/STRONGFIELD; 9488-C-CK-2/STRONGFIELD	2009
8	Eurostar	G-9575-B-AA-09-C/DT-498//DT-691	2008
9	Golden Ball	(S)LV-SOUTH-AFRICA	1918
10	Hercules	RL-3097/RL-3304//STEWART(TR.DR)/RL-3380	1969
11	Kubanka	LV	1938
12	Kyle	WAKOOMA/DT-322(6962-92-8-5)//(6965-494-1)WAKOOMA/DT-320	1984
13	Lakota	SENTRY,USA//LD-379/LD-357	1960
14	Macoun	RL-3607/DT-182	1974
15	Medora	WARD/MACOUN	1982
16	Mindum	(S)HEDGEROW	1917

17	Pelissier	(S)LV-DZA	1896
18	Plenty	VIC/WASCANA//DT-354	1990
19	Quilifen	-	-
20	Sceptre	D-72110/COULTER	1985
21	Ramsey	CARLETON/(PAL)PI-94701	1955
22	Strongfield	AC-AVONLEA/DT-665	2003
23	Stewart 63	ST-464/8*STEWART,TR.DR; STEWART*8/ST-464	1963
24	Wakooma	LAKOTA*2/PELISSIER; LAKOTAPELISSIER; LAKOTA/2*PELISSIER	1973
25	Wascana	LAKOTA*2/PELISSIER	1971
26	AC Pathfinder	WESTBRED-881/DT-367; DT-367/WESTBRED-881	1999
27	AC Morse	RL-7196/D-84328	1996
28	AC Navigator	KYLE/WESTBRED-881	1999
29	AC Napoleon	VIC/DT-384//DT-471	1999
30	AC Avonlea	SC-8267-AD-2A/DT-612	1997
31	AC Melita	MEDORA/LLOYD	1995

*GRIS = Genetic Resources Information System for Wheat and Triticale (http://www.wheatpedigree.net/, retrieved 20/10/2017)

Annex 2 – Soil DNA Extraction

DNA extraction for the soil between rows was performed with two different kits due to Mo Bio ceasing manufacturing the UltraClean Soil DNA Isolation Kit partway through the extractions and replacing it with the PowerSoil DNA Isolation Kit. Below is the detailed list of which soil samples were treated with which kit. Verification of both kits was done by gel electrophoresis.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
121	122	123	124		UltraClean					PowerSoil				

Annex 3 – Genome Quebec PCR Protocol

1. Targeted PCR

Master Mix Components	1X	419	Final Concentration
Q5 reaction Buffer 5X	1.60	670.4	1 X
DMSO	0.40	167.6	5 %
dNTP mix 10 mM	0.16	67.0	0.2 mM
Q5 HiFi polymerase 2U-ml	0.08	33.5	0.02 U/µl
H2O	4.70	1969.3	
Franck_F-CS1 100µM	0.03	12.6	0.4 µM
Franck_R-CS2 100µM	0.03	12.6	0.4 µM
DNA diluted 1/200	1.00		
Total	8.00	2933.0	

2. Illumina adapters and index incorporation

Master Mix Components	1X	419	Final Concentration
Q5 reaction Buffer 5X	4.00	1676.0	1 X
DMSO	1.00	419.0	5 %

dNTP mix 10 mM	0.40	167.6	0.2 mM
Q5 HiFi polymerase 2U-ml	0.25	104.8	0.025 U/µl
H2O	11.35	4755.7	
Total	17.00	7123.0	

Volume of Master mix to transfer in samples	17
2uM Index volume to add	2
Amplified DNA diluted 1/200 (ul)	1
Final volume	20