PhytoFrontiers™
An Open Access Journal from The American Phytopathological Society

Research

Combining Air Sampling and DNA Metabarcoding to Monitor Plant Pathogens

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Accepted for publication 21 March 2023.

Abstract

Monitoring the air for airborne plant pathogens is an increasingly common method for the management of economically important plant diseases. In Alberta, Canada, several commodity clusters, including dry bean, canola, potato, and wheat, currently support air monitoring research programs for airborne pathogens of interest. In this study, we assessed the feasibility of monitoring for these, and more, plant fungal pathogens simultaneously using two different sampler types (cyclone versus rotation impaction) and by metabarcoding the ITS1 region using the Illumina sequencing platform. We collected air samples from four geographically distant sites across Alberta and monitored four crop types in southern Alberta. Overall, we found weak, but statistically significant, effects of geographic location and crop type on the aeromycobiota community composition. A few common taxa, such as *Ramularia*, *Alternaria*, and *Epicoccum*, constituted the vast majority of reads across all samples. Nevertheless, in each sample, we identified many plant pathogens of interest and organisms that previous research has found antagonistic to those pathogens, highlighting the utility of these approaches in understanding the pathobiome. In assessing the real-world implications of read counts, we discovered that they were only weakly correlated with spore counts quantified by qPCR. The two types of samplers collected different community profiles, reinforcing the importance of carefully considering which sampler type to use in monitoring programs. Taken together, our results show promise for the future of monitoring the air pathobiome, although much more work is required to understand the relationship of airborne communities to their in-field impact on disease development.

Keywords: aerobiology, air microbiome, amplicon sequencing, Illumina, metabarcoding, next-generation sequencing, plant pathogen, qPCR

Funding

Funding was provided by Genome Alberta (A3GP06), the Canadian Agricultural Partnership (CAP) Pulse Cluster Project (ASC-06), the Alberta Pulse Growers Commission, Agriculture and Agri-Food Canada, and the Natural Sciences and Engineering Research Council of Canada (CGSD3-535546-2019).

e-Xtra: Supplementary material is available online.

The author(s) declare no conflict of interest.

Many economically important or novel plant pathogens are dispersed via air currents (Brown 2002; Chen et al. 2018a), and monitoring the air for pathogen inocula has become an important component of many plant disease management programs (Lees et al. 2019; Meyer et al. 2017; Pan et al. 2010; Van der Heyden et al. 2021). Monitoring for airborne inocula can serve several purposes, including identifying novel or emerging plant pathogens, detecting the first seasonal appearance of a pathogen, or informing growers of pathogen levels in a given location or season (Aylor 1999; Carisse et al. 2017; Mahaffee and Stoll 2016; West et al. 2008). In Alberta, Canada, there are currently several commodity organizations independently investigating monitoring programs for airborne plant pathogens including *Phytophthora infestans* (causal agent of late blight in potato), *Puccinia* spp. (rusts in cereal crops; Araujo et al. 2021), and *Sclerotinia sclerotiorum* (white mold in dry bean and sclerotinia stem rot in canola). Airborne spores of the pathogens listed above are identified either through molecular assays, typically quantitative PCR (qPCR), or microscopy.

In aerobiology, as an interdisciplinary and relatively young field of study, many fundamental questions remain unanswered empirically. Complex interactions among fungal biology, vegetation, weather conditions, and air currents (to name only a few) coupled with technological sampling limitations and species identification limitations can make drawing generalizations from air sampling studies difficult (Aylor 1999; Mahaffee and Stoll 2016; Mahaffee et al. 2023). Despite these many limitations, sampling the air for pathogenic spores has become an important part of managing diseases and fungicide use in crops such as onion (Carisse et al. 2009), grape (Thiessen et al. 2017), and strawberry (Van der Heyden et al. 2014).

Advances in DNA sequencing technologies and concomitant reduction of costs have led to an interest in employing high-throughput sequencing (HTS) techniques such as metabarcoding to characterize microbiota from environmental samples, including aerosols (Aguayo et al. 2018, 2021; Banchi et al. 2020; Clare et al. 2022). Although there is still much research remaining to optimize HTS methods and ensure their results are robust for complex environmental samples (Gonzalez et al. 2012; McLaren et al. 2019; O'Sullivan et al. 2021; Porath-Krause et al. 2022; Yeh et al. 2018), these technologies can play an important role in the understanding of plant disease epidemics. In particular, by characterizing a microbial community in a semi-quantitative way, HTS methods provide the potential to identify many plant pathogens simultaneously, along with antagonistic or beneficial plant microbes (Chen et al. 2018a; Vayssier-Taussat et al. 2014).

Early systematic investigations of the air's fungal ecology employing microscopic spore identification found variation in spore release patterns associated with time of day, weather, and location (Gregory and Hirst 1957; Hirst 1953; Lacey 1962). Recent HTS investigations into the aerobiota have largely corroborated earlier findings from Hirst, Gregory, and Lacey (referenced above) that substantial regional differences exist in airborne microbial composition (Banchi et al. 2018; Grantham et al. 2015; Mbareche et al. 2018) and that, on a smaller scale, vegetation may influence airborne microbial composition (Lymperopoulou et al. 2016; Peay and Bruns 2014). These findings have fundamental importance to theories of biogeography and practical importance for the management of economically important plant diseases. Recent calls to focus on the "pathobiome"—the suite of microbes that accompanies disease (or lack of disease)—hold the promise of offering insight into how symbioses among microorganisms could affect disease development in plants (Bass et al. 2019; Vayssier-Taussat et al. 2014).

The air microbiome in Alberta has never been analyzed before, so the hypotheses driving this research project are threefold. The first two hypotheses are based on findings in the fungal aerobiology literature: First, we hypothesize that there are geographical differences in airborne microbial composition. Second, we hypothesize that there are effects of different crop types on airborne microbial composition. The third hypothesis combines ideas of the pathobiome and increasing sampling efficiency: we hypothesize that the use of amplicon sequencing can be useful for the simultaneous monitoring of regionally important agricultural plant pathogens. By monitoring for multiple fungal organisms at the same time, a provincial air monitoring program could help inform all commodity groups of the seasonal risks of their economically important pathogens.

MATERIALS AND METHODS

Survey design and air sampling

Air sampling surveys were designed to test each of the first two objectives. For the first objective, to assess how the air microbiome differs over spatial scales, four sites were selected across Alberta (Table 1). The sampling sites were part of the Sclerotinia stem rot of canola trials conducted at agriculture research centers in, from southernmost to northernmost to southernmost, Lethbridge, Brooks, Lacombe, and Beaverlodge, Alberta (Fig. 1). The distance between the farthest sites in the study, Beaverlodge and Lethbridge, is approximately 750 km; the distance between the closest sites in the study, Lethbridge and Brooks, is approximately 120 km. GRIPST-2009 rotation impaction samplers (hereafter "rotorod samplers"; Aerobiology Research Laboratories, Ottawa, Ontario, Canada) were set up in each of these locations and collected samples from 0800 h to 1600 h on Monday, Wednesday, and Friday. The samplers were set up such that the rods sampled at approximately 1.5 m above the ground, which was also above the canopy of the canola crop. This sampling height was selected to preferentially collect spores being released from nearby and/or within the canopy being sampled (Mahaffee et al. 2023; Van der Heyden et al. 2021) and to collect spores that were most likely coming into contact with the plants. In Lethbridge, samples were also collected on Tuesday and Thursday. To avoid oversaturation of the rods' impaction surfaces by dust and/or spores, the samplers were active for 1 min out of every 10 min. On collection from the field, rods were stored in sterile 1.5-ml Eppendorf

TABLE 1

Summary of locations, crops, and samplers used in the 2019 surveys of this study

		Number of samplers		Number of sites	
Location	Crop	Rotorod	Burkard	Research	Commercial
Geography hypothesis					
Beaverlodge	Canola	1		1	
Lacombe	Canola	1		1	
Brooks	Canola	1		1	
Lethbridge	Canola[z]	1		1	
Crop type hypothesis					
Lethbridge	Canola[z]		1	1	
	Bean		2	1	1
	Potato		2		2
	Wheat		2	1	1

[z] The canola research plot in Lethbridge had one rotorod sampler and one Burkard sampler.

tubes at −20°C until DNA extraction. Cubic meters of air sampled were calculated using the user manual accompanying the rotation impaction samplers, and approximately 2 m^3 of air was sampled each day at each site.

For the second objective, to assess how the air microbiome differs across crop types, air samples were collected by Burkard 7-day volumetric cyclone samplers (Burkard Manufacturing, Hertfordshire, England) in bean, canola, wheat, and potato fields in southern Alberta (Table 1). For all samplers, the intake orifice was approximately 1.2 m above the ground, which was above the canopy of all crops for reasons described previously. For the bean fields, one sampler was placed in the white mold disease nursery at the Lethbridge Research and Development Centre (LeRDC), and the other was placed in a nearby commercial field. For the canola fields, one sampler was placed in a canola research trial at LeRDC directly beside the rotation impaction sampler above. To collect wheat samples, one sampler was placed in a wheat research trial at LeRDC, and another was placed in a nearby commercial wheat field. To collect air samples of the potato fields, the samples were obtained from an existing network of Burkard spore samplers maintained by the Potato Growers of Alberta (PGA). Except for samplers in the potato fields, all Burkard cyclone samplers operated continuously for 24 h a day. Samplers in the potato fields were operated for 3 h per day, from 1300 h to 1600 h, based on the protocol set up by the PGA. Burkard samplers collect air at a rate of 16.5 liters min^{-1} (approximately 1 m^3 h^{-1}), so for samplers in bean, canola, and wheat fields, 24 m^3 of air was sampled each day, and for samplers near potato fields, 3 m^3 of air was sampled each day. The same sampling strategy was carried out in 2021 in one research plot each of beans, canola, and wheat at LeRDC, and in both years, all samplers in the different research plots were within 180 m of each other. For non-potato air samples, seven vials were collected per week (one tube per day for 7 days) by field cooperators and shipped to LeRDC for storage at −20°C until DNA extraction. For potato air samples, samples were collected by the PGA group and sent to the University of Lethbridge for microscopic examination of spores. Samples were stored at −20°C until they were transferred to LeRDC for DNA extraction.

FIGURE 1
Map of Alberta, Canada, showing the sampling locations (large gray circles) in this study.

DNA extraction

The DNA extraction methods were optimized on *Sclerotinia sclerotiorum* ascospores to facilitate future potential for rapid, in-field extraction of DNA for use in in-field, real-time instruments, although these methods were not employed in this project. DNA from the rotorod samplers was extracted as described by Carisse et al. (2009). In brief, each rod was placed in a 2-ml screw cap tube with 100 mg of acid-washed 425- to 600-μm glass beads (Sigma-Aldrich Canada, Oakville, Canada). Eighty microliters of 100% isopropanol were added to each tube, which was then shaken on a FastPrep homogenizer (MP Biomedicals, Irvine, CA) for 20 s at 4.0 m s^{-1}. Isopropanol was evaporated from the tube on a Speed Vac at 60°C, and 300 μl of DNA isolation solution (comprising 10 ng μl^{-1} salmon sperm [Invitrogen, Ottawa, Canada], 5% wt/vol Chelex [Chelex 100, 50-100 mesh, Sigma-Aldrich], 100 mg $liter^{-1}$ bovine serum albumin [New England Biolabs, Whitby, Canada], and nuclease free water) was added to each vial. Vials were vortexed for 5 s and then incubated for 20 min at 105°C on a block heater. Vials were then centrifuged at 13,200 RPM for 5 min at 4°C. Supernatant from each vial was pipetted into new, labeled 1.5-ml tubes, and DNA was stored at −20°C until it was sent for amplicon Illumina sequencing and qPCR.

DNA from the Burkard samplers was extracted using a modified protocol of Ferencova et al. (2017). In brief, DNA was extracted directly from the 1.5-ml Eppendorf tubes once they were retrieved from the samplers as follows: 50 mg of acid-washed 425- to 600-μm glass beads (Sigma-Aldrich) and 100 μl of molecular-grade water with 100 mg $liter^{-1}$ bovine serum albumin were added to each vial. Vials were then placed in a TissueLyserII (Qiagen, Germantown, MD) at 30 Hz for 30 s to dislodge particles from the sides of the vials. Following bead beating, 100 μl of a 10% (wt/vol) Chelex/molecular-grade water mixture was added to each vial. Vials were then vortexed for 10 s and placed in a block heater at 95°C for 20 min. At 5, 10, 15, and 20 min, samples were vortexed for 3 s. Following the final vortex, samples were centrifuged at 13,200 RPM for 1 min. Supernatant from each vial was pipetted into new, labeled 1.5-ml tubes, and DNA was stored at −20°C until it was sent for amplicon Illumina sequencing and qPCR. For the rotorod and Burkard samples, DNA quantification was performed on a Qubit fluorometer (Invitrogen, Burlington, Ontario, Canada).

Mock community and negative controls

A series of positive and negative controls were included to account for biases and contamination in sample collection, DNA extraction, and sequencing protocols. A mock community was created in-house to act as a positive control. The mock community comprised eight *Ascomycota* species (*Blumeria graminis*, *Botrytis cinerea*, *Chaetomium globosum*, *Cladosporium herbarum*, *Fusarium avenaceum*, *Penicillium expansum*, *Sclerotinia sclerotiorum*, and *Sordaria* sp.) and one *Basidiomycota* species (*Puccinia triticina*), each with 10^4 spores (for detailed information, see Supplementary Table S1). Several sets of negative controls were set up as follows: For the rotorod sampler data set, DNA was extracted from rods that were stored but had not been used for sampling. For the Burkard sampler data set, DNA was extracted from Eppendorf tubes that had been placed in the samplers in the fields but had not been actively sampled into. A further set of

negative controls included DNA extracted from the same water used in the DNA extractions, DNA extracted from an empty vial, and water blanks.

Amplicon-based HTS

Following DNA extraction, 10-µl aliquots of DNA were sent to Centre d'expertise et de services Génome Québec (Montreal, Canada) for Illumina amplicon sequencing. Due to low biomass in these samples, the DNA concentrations were typically below 100 ng μl^{-1}. Fungi were targeted by amplifying the internal transcribed spacer (ITS) region using the primer set ITS1-f_KYO1 and ITS2_KYO2 (Toju et al. 2012). Including field and control samples, 542 samples were sequenced in 2019 on Illumina NovaSeq 6000 SP flow cells (generating 250-bp paired-end sequences), and 112 samples from 2021 were sequenced on Illumina MiSeq PE 250.

qPCR

To assess the degree to which Illumina sequencing is quantitative, aliquots of DNA samples that were sequenced on the Illumina platform were also analyzed for DNA of *Sclerotinia sclerotiorum* using a quantitative PCR (qPCR) assay (Reich et al. 2017). The qPCR assay was performed in technical triplicates, and a standard curve was created using DNA extracted from serial dilutions of *S. sclerotiorum* ascospores. Therefore, the qPCR results were summarized as number of ascospores per day.

Bioinformatics and processing of the metabarcodes

Sequencing reads from the rotation impaction samplers and from the Burkard spore samplers were analyzed in parallel for each of the regions amplified. General bioinformatics protocols followed the QIIME2 (v. 2020-6) pipeline for amplicon sequencing analysis, with variations described below (Bolyen et al. 2019). The ITS1 region of the raw paired-end fastq files was extracted using the ITSxpress plugin (Rivers et al. 2018), followed by denoising using DADA2 (Callahan et al. 2016) with default settings, except that –p-min-fold-parent-over-abundance was specified as 2.0. Taxonomic assignment was performed with the RDP classifier (Wang et al. 2007) as implemented in mothur (Schloss et al. 2009) and the classify.seqs() function, specifying the UNITE fungal ITS database as the reference (Nilsson et al. 2019). Reads that were not assigned to the kingdom Fungi were removed prior to statistical analysis.

Statistical analysis

All statistical analyses were performed in R environment (v.4.0.3; R Core Team 2020). The amplicon sequence variant tables generated by the QIIME2 pipeline were imported into R using qiime2R (v.0.99; Bisanz 2018). For alpha diversity analysis, five low read count samples were removed from the data set, and the remaining samples were rarefied to the lowest sample depth of 15,588 for subsequent analysis (Nearing et al. 2022; Weiss et al. 2017). The Shannon diversity index was calculated with the estimate_richness() function in phyloseq (v.1.32.0; McMurdie and Holmes 2013). The differences in the Shannon diversity index were tested using ANOVA and using Location as the main factor for the geography hypothesis and Crop Type as the main factor for the crop type hypothesis. To assess whether there were any seasonal shifts in alpha diversity, a repeated measures ANOVA was performed, and Location or Crop Type was used for replicates, as appropriate. If the ANOVA was significant, pairwise comparison of the means was performed using Tukey's honestly significant difference (HSD) test. ANOVA and Tukey's HSD tests were performed using anova_test() and tukey_hsd() commands in the rstatix package (Kassambara 2020). Generalized linear models using a Gaussian link function were constructed to test the effects of location and environment conditions on Shannon diversity in the rotorod data set. Models from forward, backward, and stepwise were compared based on Akaike's information criterion and residual deviance.

For beta diversity, the Bray-Curtis dissimilarity was calculated on Hellinger-transformed read counts of the non-rarefied data set. Permutational ANOVA was performed with the adonis() command, and dispersion was evaluated with the betadisper() command from the vegan package (v.2.5.6; Oksanen et al. 2019). In situations with a statistically significant ($P < 0.05$) difference, pairwise comparisons were made, and P values were adjusted for multiple comparisons using the Benjamini-Hochberg method and p.adjust() from the stats package. For taxonomic composition analysis, the RAM package (v.1.2.1.7; Chen et al. 2018b) was used to fill in missing taxonomic information with the lowest level available.

Venn diagrams were made with the VennDiagram package (v.1.6.20; Chen 2018), and all other plots were made with ggplot2 (v.3.3.2; Wickham 2016).

Plant pathogens

FUNGuild (Nguyen et al. 2016) was used to assign identified fungal taxa to ecological guilds, and the ones designated as plant pathogens were examined. In particular, causal agents of important grain and pulse diseases in Alberta were examined graphically and statistically (ANOVA) for differential relative abundance of reads based on location or crop type. Fungi of interest included *Alternaria* spp., *Ascochyta* spp., *Blumeria graminis*, *Cochliobolus sativus*, *Fusarium* spp., *Leptosphaeria* spp., *Puccinia* spp., *Ramularia* spp., and *Sclerotinia sclerotiorum*. An exhaustive list is given in Supplementary Table S2.

Disease surveys

For the 2019 rotorod data set, Sclerotinia stem rot disease surveys were performed at the end of the season in each of the canola trial plots where rotorod samplers were sampling. For the 2019 and 2021 Burkard data set, disease surveys were performed at the end of the season in the wheat and bean fields and trial plots but not the potato fields. In all cases, disease incidence and severity were recorded.

RESULTS

Summary of reads

Between 92 and 95% of reads were retained following the DADA2 pipeline for each of the three data sets (rotorod 2019, Burkard 2019, and Burkard 2021; Supplementary Table S3). The total number of unique phyla collected by the rotorod samplers was much greater compared with the Burkard samplers in 2019 or 2021 (10 versus 5 or 4, respectively; Table 2). However, from each sample, the Burkard samplers in 2019 collected 117 ± 93 (mean ± SD) genera, compared with only 86 ± 41 with the rotorod samplers in 2019 and 68 with the Burkard samplers in 2021 ($P < 0.001$). Correspondingly, we recovered more fungal genera in total by Burkard samplers in 2019 (908 genera) compared with both the rotorod sampler in 2019 (769) and the Burkard sampler in 2021 (521).

When plant pathogens as identified by FUNGuild were extracted from each of the data sets, the trends were slightly different (Table 2). Both the rotorod 2019 and Burkard 2019 data contained more plant pathogenic genera per sample (34 ± 23 and 37 ± 22, respectively) than the Burkard data set in 2021 (22 ± 7, $P < 0.001$). In total, the rotorod 2019 and Burkard 2019 data sets had a greater number of unique plant pathogenic genera overall (152 and 138 versus 76, respectively).

Common and core taxa

Out of 1,065 fungal genera identified in this study, 410 (38%) were collected by both types of samplers in both 2019 and 2021 (Fig. 2). Reads from these genera constituted 99% of all reads in this study. In general, the most common genera were similar across data sets. For all data sets, *Ramularia* spp. were most dominant and prevalent (rotorod 2019: 40% in abundance, Burkard 2019: 27%, and Burkard 2021: 57%; Table 3). *Alternaria* was the second most common in most of these data sets at 10, 16, and 19% of reads, respectively. For the 2019 data sets, *Epicoccum* was the third most common taxa collected, with 9% of rotorod 2019 reads and 16% of Burkard 2019 reads being assigned to this genus. In contrast, *Epicoccum* made up a very small proportion (0.14%) of reads in the Burkard 2021 data set. Subsequent ordering of fungal taxa varied by sampler type and amplicon, although all data sets were similar in their top 10 most dominant fungal taxa.

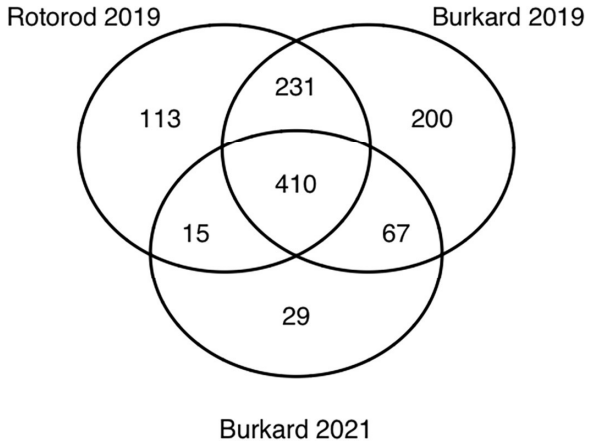

FIGURE 2
Venn diagram summarizing the shared genera among all data sets in this study.

We defined the "core" taxa as the fungal genera shared by locations or crop types (Supplementary Figs. S1 and S2). In 2019, 318 of the 799 (40%) fungal genera were recovered by all Burkard samplers at LeRDC, which constituted over 98% of total abundance. Similarly, in 2021, out of the 521 fungal genera recovered by the same set of Burkard samplers, 226 (43% in diversity, 99% in abundance) were found in all samplers. The remaining fungal genera recovered by these samplers (~60%) accounted for less than 2% in abundance and were collected by individual samplers.

Important plant pathogens

Seasonal dynamics of specific fungi varied by location or crop type. Here, only a handful of regionally important fungi are discussed. For example, *Alternaria* spp. were more abundant in Lethbridge (15.8 ± 7.9% [mean ± SD]) and Brooks (13.5 ± 8.5%) than in Beaverlodge (1.6 ± 1.8%) and Lacombe (4.9 ± 4.5%; pairwise Wilcoxon test, $P < 0.001$). *Blumeria* was, on average, more abundant in Lethbridge (5.9 ± 11.8%) than in any other location (averages ranging from 0.04 to 0.2% of reads; $P < 0.001$; Fig. 3). In contrast, Beaverlodge had a much greater abundance of Sclerotiniaceae spp. (1.1 ± 0.8%) than any of the other locations (averages ranging from 0.06 to 0.32% of reads; $P < 0.001$; Fig. 3).

TABLE 3

Proportion (%) of reads in top 10 taxa recovered by rotorod or Burkard samplers from 2019 and 2021 sampling years through metabarcoding of the ITS1 region[z]

Genus (or lowest rank)	Rotorod 2019	Burkard 2019	Burkard 2021
Ramularia	40.14	26.56	56.67
Alternaria	10.28	16.12	19.48
Epicoccum	9.45	16.37	0.14
Parastagonospora	3.19	1.51	1.41
Blumeria	2.32	2.72	0.66
Vishniacozyma	2.18	2.79	1.53
Stemphylium	2.07	0.05	0.15
Itersonilia	1.95	0.06	0.06
Pleosporaceae sp.	1.57	3.57	2.38
Cladosporium	1.31	0.90	0.96
Drechslera	0.68	2.83	0.03
Sporobolomyces	0.30	2.01	0.80
Aspergillus	0.20	0.94	1.18
Entylomatales sp.	0.12	0.77	1.62
Exobasidium	0.03	1.33	1.26

[z] The genera are arranged in descending order in the rotorod 2019 samples. Note that the top 10 most abundant taxa were not the exact same for each data set, and so this table contains 15 of the taxa that were most common across the whole data set.

TABLE 2

Summary of average and total number of fungal taxa recovered from air samples based on ITS1 metabarcodes[y]

	Amplicon sequence variants			Phyla			Families			Genera		
Sampler and year	Mean[z]	SD	Unique	Mean	SD	Unique	Mean	SD	Unique	Mean	SD	Unique
All reads												
Rotorod 2019	351 a	197	16,956	2	0.7	10	57 b	24	295	86 b	41	769
Burkard 2019	362 a	322	14,903	2	0.7	5	71 a	47	314	117 a	93	908
Burkard 2021	113 b	43	3,760	2	0.6	4	49 b	16	230	69 b	25	521
Plant pathogens												
Rotorod 2019	91 a	32	2,074	2	0.0	2	21 a	6	59	34 a	23	152
Burkard 2019	82 a	57	1,263	2	0.2	2	22 a	12	58	37 a	22	138
Burkard 2021	37 b	13	288	2	0.0	2	15 b	5	40	22 b	7	76

[y] The top panel of the table is a summary of all reads in the data set, whereas the bottom section includes only those reads classified as plant pathogens according to FUNGuild. Rotorod samplers collected samples for the geography hypothesis and Burkard samplers collected samples for the crop type hypothesis.
[z] Rows within the same column and section (all reads vs reads assigned to potential plant pathogens) with different letters are statistically different from each other at alpha = 0.05 following Tukey HSD means separation.

Fusarium spp. were at similar levels across all sites surveyed (0.3 to 1.2% of reads; $P > 0.05$).

Abundance of fungi among crop types in the experimental plots at LeRDC also varied. Here, only results from 2021 are discussed because the data set from 2019 was relatively small, although trends were similar for 2019. *Blumeria*, the causal agent of powdery mildew on wheat, constituted a much greater proportion of reads in the wheat experimental plot (1.7 ± 1.9%) than in the dry bean (0.2 ± 0.3%) or canola (0.4 ± 0.5%) plots ($P < 0.001$; Fig. 3). However, reads of *Fusarium* (0.3 to 0.5% of reads) and Sclerotiniaceae sp. (<0.001% of reads) were similar across all crop types ($P > 0.05$; Fig. 3).

Aeromycobiota diversity

Geography hypothesis (rotorod samplers). The Shannon diversity index was significant for Location effects (ANOVA, $P < 0.0001$), and following Tukey's HSD means separation, Beaverlodge had significantly greater Shannon diversity indices than those at other locations (Fig. 4; Table 4). The alpha diversity remained relatively stable throughout the season at each location, as there was no overall effect of sampling week on Shannon diversity indices (repeated measures ANOVA, $P = 0.96$; Fig. 5). Generalized linear models of Shannon diversity revealed that minimum and mean relative humidity and daily precipitation, in addition

FIGURE 3
The seasonal dynamics of three common plant pathogenic genera revealed by rotorod samplers at each of the four locations used to test whether location influences aerial fungal communities (top panel) and by the Burkard samplers at LeRDC in 2021 to test whether crop type influences aerial fungal communities (bottom panel).

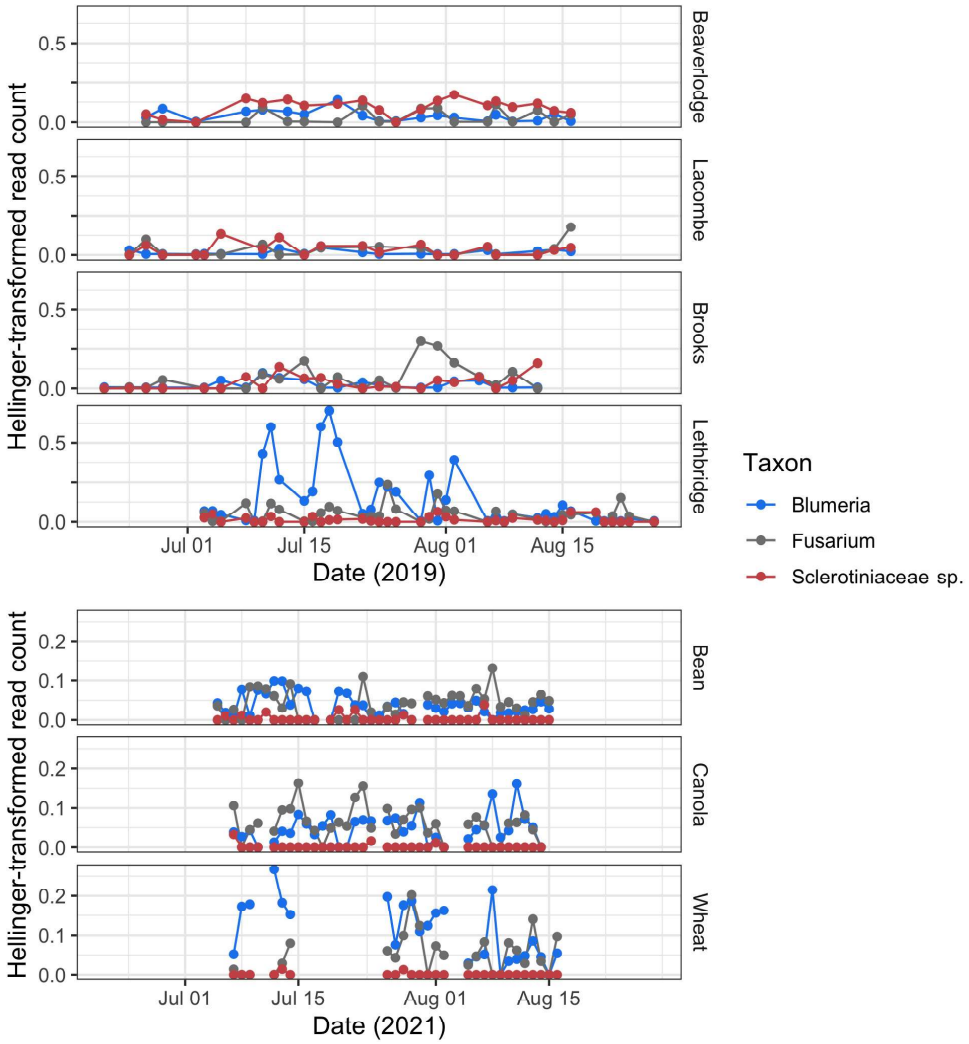

TABLE 4

Means of diversity metrics and environmental variables for the geography hypothesis (rotorod) data set at each of the four locations sampled in Alberta in 2019[y]

	Diversity		Environment		
Location	Shannon	Chao1	Air temperature	Relative humidity	Precipitation
Lethbridge	2.54 a[z]	256 a	18.2 a	60.5 a	97.0
Brooks	2.52 a	215 a	17.1 a	68.0 b	284.8
Lacombe	2.84 a	281 ab	14.3 b	81.2 c	181.9
Beaverlodge	3.45 b	390 b	13.9 b	77.9 c	204.6

[y] Environmental variables are averages of air temperature and relative humidity and seasonal totals of precipitation.
[z] Values in the same column followed by different letters are statistically different following ANOVA and Tukey's honestly significant difference means separation at alpha = 0.05.

to geographic location, had the most important associations with airborne mycobiota diversity (Table 5).

The PermANOVA test showed that community compositional structure based on Bray-Curtis dissimilarity was statistically significant between sampling locations ($P < 0.0001$; Table 6). All pairwise comparisons between locations were statistically different (Benjamini-Hochberg adjusted $P < 0.05$), indicating that each location had its own unique community composition (Supplementary Fig. S3). However, the dispersion tests were also significant ($P < 0.0001$), and so the differences in community composition by location could be due, in part, to unequal dispersions among groups.

Meteorological conditions varied substantially among the locations surveyed: Lethbridge received half as much precipitation (97 mm) as Lacombe (182 mm) and Beaverlodge (205 mm) and one-third as much precipitation as Brooks (285 mm; Table 4). On average, Lacombe and Beaverlodge had similar air temperature, relative humidity, and precipitation over the course of the growing seasons. Both locations had substantially significantly cooler air temperatures (13.9 to 14.3°C) than Lethbridge and Brooks (18.2 and 17.1°C, respectively) and greater relative humidity (78 to 81%) than Lethbridge and Brooks (61 and 68%, respectively). These environmental variables were all statistically significant in the PermANOVA; however, collectively, they explained only ~6% of the variation in community dissimilarity of the samples (Table 6).

Crop type hypothesis (Burkard samplers). ANOVA of the Shannon diversity index revealed that alpha diversity was similar among crop types within both years surveyed (2019: $P = 0.12$; 2021: $P = 0.06$; Fig. 4), but means separation using Tukey's HSD revealed that in 2021, the average Shannon diversity index was statistically greater in canola than in bean ($P = 0.04$). As with the

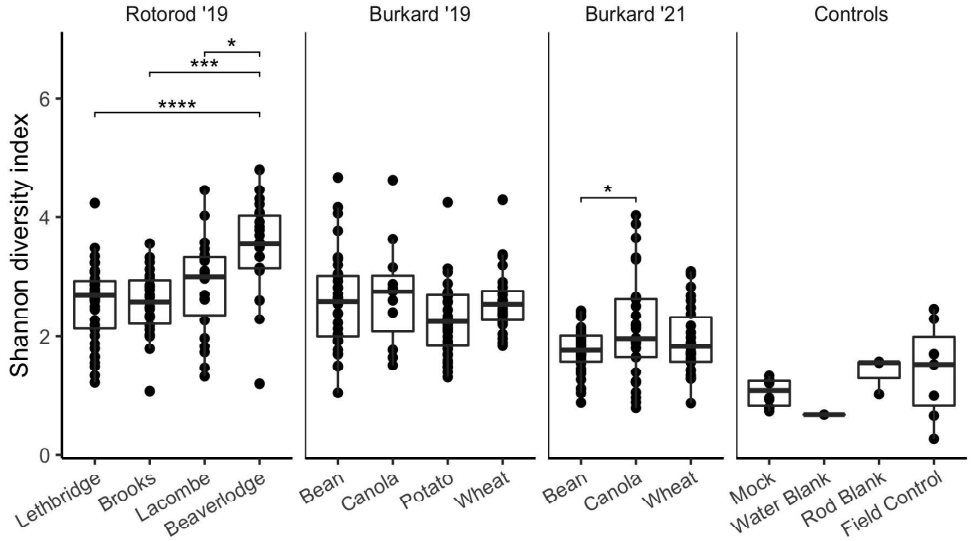

FIGURE 4
Shannon diversity of the ITS1 amplicon sequence variants. Data presented are post quality control; thus, some controls (e.g., water blanks) have been removed entirely from the visualization. Refer to the Results section for a summary of statistical tests performed. Comparisons made by Tukey's honestly significant difference (but not on controls), and only significant differences are shown. Significance levels are denoted at * alpha = 0.05; ** alpha = 0.01; *** alpha = 0.001; **** alpha < 0.0001.

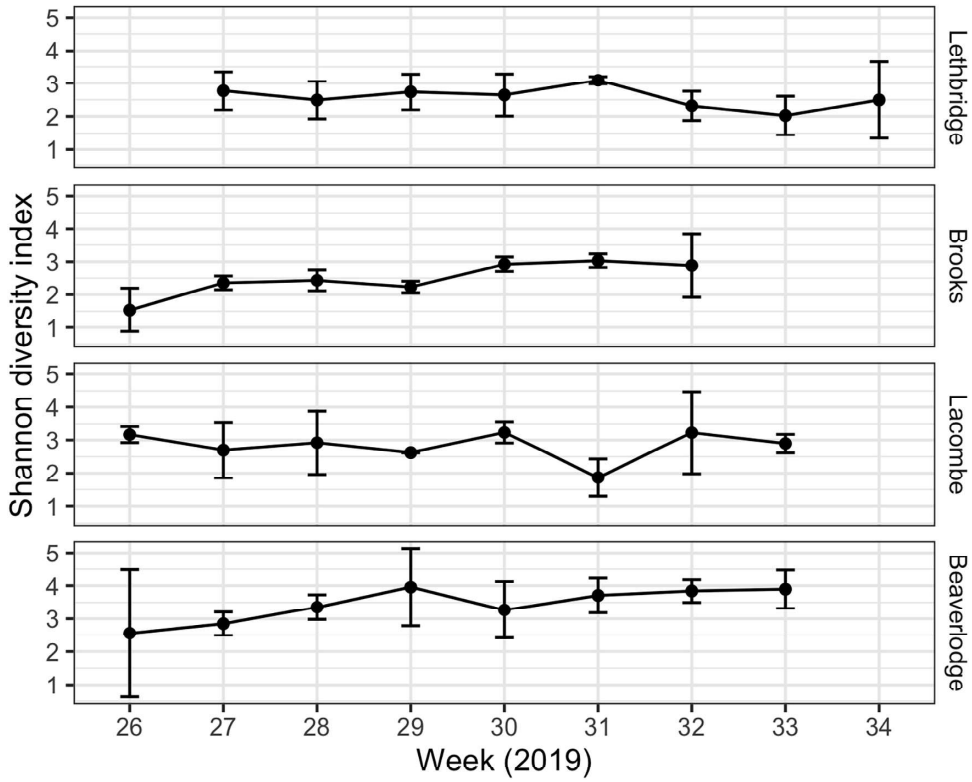

FIGURE 5
Weekly averages of Shannon diversity of the ITS1 amplicon sequence variants (±standard deviation) at each of the locations recovered by rotorod samplers. Week 26 represents the week starting June 24, and Week 34 is the week starting August 19. For all locations combined, Shannon diversity did not vary by week (repeated measures ANOVA, $P = 0.96$), indicating that diversity across the province is stable throughout the growing season.

geography hypothesis, Shannon diversity indices remained relatively stable throughout the season in each of the crops surveyed, with variation within biweekly periods similar to, or greater than, variation among biweekly periods (Fig. 6).

For both years, the PermANOVA test showed a high yearly effect (explained 24% of community heterogeneity, $P < 0.001$; Table 7) and a relatively small effect from crop type (10%, $P < 0.001$) on aeromycobiota compositional shift. Within year, all crops differed in their community composition from one another, except bean and canola plots in 2019 (all other pairwise comparisons significant at $P < 0.01$ following the Benjamini-Hochberg adjustment for multiple comparisons; Supplementary Fig. S4). The tests for dispersion were only significant in 2019 ($P = 0.048$), which indicates that these differences are truly due to crop effects rather than dispersion of the data in 2021. We did not detect interaction effects of sampling year and crop type ($P = 0.07$), which suggests that vegetation-associated air mycobiota remained relatively stable over the two sampling seasons (Table 7).

Comparison of sampler types

There were only four overlapping sampling days available to make comparisons between the rotating impaction sampler and the Burkard sampler in 2019. Nevertheless, from these 4 days of common sampling, it was clear that very different microbial communities were collected by the two sampler types. Even though the samplers were placed beside each other and at the same height, *Ramularia* constituted, on average, 75% of reads from the rotorod samples but only 17% of reads in the Burkard samples. In contrast, *Alternaria* constituted only 8% of reads in the rotorod samples but 19% of reads in the Burkard samples. *Epicoccum*, the third most common genus collected by both samplers, constituted 3% of reads in the rotorod samples but 25% of reads in the Burkard data set. For these 4 days, the Burkard samplers collected a greater diversity of taxa than did the rotating impaction samplers (382 versus 167 genera, respectively). When ordinations of these samples were performed, the samples tended to more closely cluster by sampler type than by day of sampling, indicating the presence of a sampler effect on community composition (Supplementary Fig. S5).

TABLE 5

Summary table of the most parsimonious (lowest Akaike information criterion and residual deviance) generalized linear model for Shannon diversity as determined by forward, backward, and stepwise selection for the rotorod sampler data set[z]

Variable	Estimate	SE	*t*	*P*
Intercept	4.253	0.502	8.47	<0.0001***
Location				
Brooks	−0.067	0.229	−0.29	0.771
Lacombe	0.532	0.219	2.43	0.017*
Beaverlodge	0.898	0.214	4.19	<0.0001***
Precipitation	0.048	0.025	1.94	0.056
Minimum relative humidity	0.034	0.010	3.57	<0.001***
Mean relative humidity	−0.049	0.013	−3.88	<0.001***

[z] SE = standard error; * indicates $P < 0.05$; and *** indicates $P < 0.001$.

TABLE 6

PermANOVA table for the 2019 rotorod data set, testing the effect of location and environmental factors on the microbial community composition for the rotorod sampler data set[z]

Variable	Df	SS	MS	F	R^2	*P*
Location	3	3.58	1.19	4.30	0.118	0.0001
Precipitation	1	0.50	0.50	1.80	0.017	0.0018
Mean temperature	1	0.60	0.60	2.17	0.020	0.0004
Mean relative humidity	1	0.64	0.64	2.29	0.021	0.0005
Residuals	90	24.95	0.28		0.82	
Total	96	30.26			1.00	

[z] Df = degrees of freedom; SS = sum of squares; and MS = mean squares.

TABLE 7

PermANOVA table for the 2019 and 2021 Burkard sampler data sets, testing the effect of crop type and year on the Bray-Curtis dissimilarity index (microbial community composition)[z]

Variable	Df	SS	MS	F	R^2	*P*
Crop type	3	7.38	2.46	10.92	0.104	0.0001
Year	1	17.23	17.23	76.56	0.242	0.0001
Crop type × Year	2	0.67	0.34	1.50	0.009	0.0727
Residuals	204	45.92	0.23		0.645	
Total	210	71.21			1	

[z] Df = degrees of freedom; SS = sum of squares; and MS = mean squares.

Comparison of LeRDC Burkard samplers

Three samplers were placed in experimental plots of dry bean, canola, and wheat at LeRDC in 2019 and 2021, and the distances between samplers ranged from 60 to 180 m. Airborne fungal communities from these samplers were compared to assess how representative a Burkard sampler is over a small region. Ordinations of the daily airborne communities revealed that samples tended to cluster by date, indicating that similar communities were collected by each sampler on each day (Fig. 7). Samples also often clustered by nearby dates, suggesting that community profiles are somewhat stable from day to day. In 2021, ordinations were difficult to interpret due to many more data points, but similar trends were observed as for 2019 (data not shown). When ordinations of samples from 2019 and 2021 were performed, samples clearly separated by year, showing that fungal airborne communities vary dramatically from year to year (PermANOVA: $P < 0.0001$; Table 7; Supplementary Fig. S6).

HTS read counts versus disease levels

2019 was one of the driest years in the last 10 years in southern Alberta, and as a result, only trace levels of disease were observed in the bean, canola, potato, and wheat fields. Similarly, in canola trials in Lethbridge, Brooks, and Lacombe, where the rotorod samplers were sampling, trace levels of Sclerotinia stem rot were observed. In Beaverlodge, however, the mean Sclerotinia stem rot incidence was 32%. Notably, Beaverlodge also had between a 4 and 17 times higher proportion of Sclerotiniaceae spp. reads as compared with the other locations (1.09% of reads compared with 0.06 to 0.20% of reads at the other sites, $P < 0.0001$).

HTS read counts versus qPCR

All sequenced samples were also quantified for *Sclerotinia sclerotiorum* ascospores using a qPCR assay. Most metabarcodes assigned to Sclerotiniaceae were not resolved at the genus level, with some assigned to *Botrytis* and all others assigned to Sclerotiniaceae. For correlations, reads assigned to Sclerotiniaceae were used. Various transformations of Illumina read counts were correlated against the log (no. of ascospores + 1), and the strongest correlations were obtained with Hellinger-transformed read counts ($r = 0.32$, $P < 0.0001$ in 2019; $r = 0.14$, $P = 0.18$ in 2021; Fig. 8). Notably, 2019 had, on average, a much greater proportion of Sclerotiniaceae (0.2% of total reads) than did 2021 (0.005%). In many cases, large amounts of ascospores were quantified with the qPCR assay when there were no read counts of Sclerotini-

aceae in the samples, suggesting that qPCR is a more sensitive detection and quantification method than amplicon sequencing.

Positive and negative controls

Most of the water controls did not form a band on the gel following PCR and were therefore not sequenced. However, one water control and a few of the DNA extraction blanks ("lab blanks") were sequenced (Fig. 9). The dominant taxa in these samples were *Ramularia* and *Blumeria*, also two of the most common fungi in the whole data set. Negative controls intended to assess the background noise or contamination of equipment ("rod blanks" and "field blanks") were similar to the extraction blanks in that they tended to be dominated by *Ramularia*, *Alternaria*, and *Blumeria*, but the patterns of contamination are not clear.

Examining reads from the mock community revealed that DNA extraction methods and Illumina sequencing were highly consistent between samples but that notable biases exist in the workflow (Fig. 9). Across all samples, replicates, and amplicons, six of the nine expected fungal taxa were recovered (*Blumeria*, *Botrytis*, *Chaetomium*, *Cladosporium*, *Fusarium*, and *Penicillium*), and no reads of *Sclerotinia*, *Sordaria*, or *Puccinia* were detected. However, *Cladosporium* and *Penicillium* were absent from four and two replicates, respectively, of the mock community. Most reads for all replicates were assigned to *Botrytis*, and no reads were assigned to *Sclerotinia*, but a small proportion (<1% per sample, typically) was assigned to Sclerotiniaceae. However, when the mock communities were quantified for DNA of *Sclerotinia sclerotiorum* with a qPCR assay, results showed that DNA from the expected number of ascospores (10^4 ascospores) was present in the samples. Furthermore, a sample of pure *Sclerotinia sclerotiorum* ascospores was sequenced and returned 99.7% of reads assigned to Sclerotiniaceae and no reads assigned to *Botrytis*. Taken together, these findings suggest that even though the ITS1 region of the *Botrytis* and *Sclerotinia* species are genetically extremely similar (Amselem et al. 2011), amplicon sequencing of the ITS1 region can still differentiate them.

DISCUSSION

This study was designed to test the hypotheses that airborne fungal communities are influenced by location and vegetation and to assess the utility of HTS for the simultaneous detection of regionally important agricultural plant pathogens. The geography and crop type hypotheses were generally supported by our findings: Airborne fungal communities were statistically different at each location (Lethbridge, Brooks, Lacombe, and Beaverlodge) and in each type of crop field (bean, canola, potato, and wheat), although effect sizes were small ($R^2 = 0.12$ for the geography hypothesis and $R^2 = 0.10$ for the crop type hypothesis). Furthermore, the detection and relative quantification of many fungal plant pathogens of interest—and their potential antagonists—suggest that HTS amplicon sequencing methods have potential for the simultaneous detection of airborne plant pathogens and pathobiome analysis (Hypothesis 3).

The results presented here should be interpreted in light of the controls sequenced. The water controls typically showed little contamination from lab procedures and were removed from analysis during quality control, but the controls designed to assess background contamination in the rods and vials had high

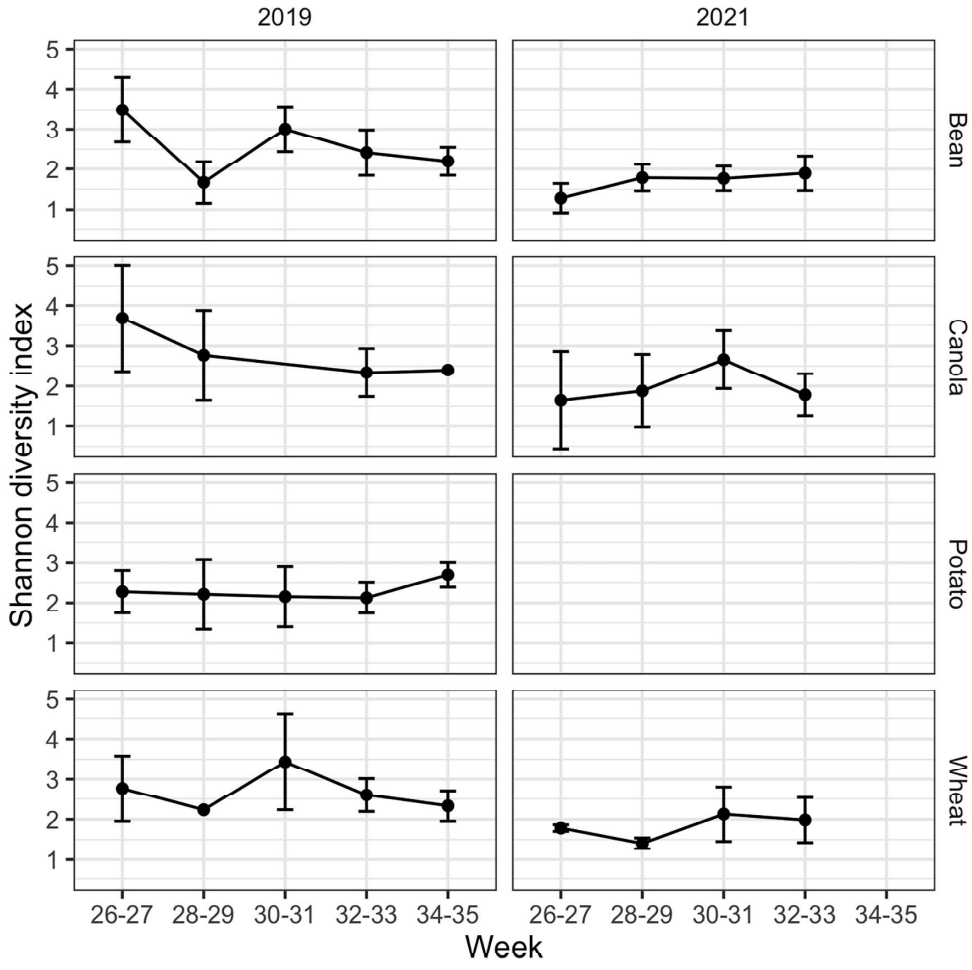

FIGURE 6
Biweekly averages of Shannon diversity of the ITS1 amplicon (±standard deviation) for each of the crop types sampled in 2019 and 2021 in the Burkard data set. Week 26 represents the week starting June 24, and Week 34 is the week starting August 19. For all crop types combined, Shannon diversity indices remained stable over the course of the season for both 2019 (repeated measures ANOVA: $P = 0.23$) and 2021 ($P = 0.15$).

read counts of several common taxa: *Ramularia*, *Stemphylium*, *Botrytis*, *Blumeria*, and others (Fig. 9). The contamination was not systematic across samples and was therefore difficult to account for prior to analysis of the field samples. Additionally, the fact that our samples from different samplers tended to cluster together based on the sampling day (Fig. 7) implies that these were the valid aerobiota because if it were contamination, we would expect to see no, or random, clustering. Contamination, especially in low biomass samples, is a pervasive and important issue in microbiome studies (Eisenhofer et al. 2019), but there is currently no consensus on how best to incorporate this information in analysis. Following PCR, low biomass samples may still fail to make a band on a gel, which is a quality control method typically employed in HTS methods. However, excluding samples on this criterion may result in a 30% false negative rate for sequencing (Minich et al. 2018). One approach to deal with some of the contamination involves simple statistical analysis of the common reads in negative controls (Davis et al. 2018). Some possible issues with this approach for low biomass samples are (i) the DNA quantity being below the detection limit for some assays, at least for some samples, and, (ii) if multiple types of negative controls are used in a study (as for this one), lack of clarity on which one(s) to use as the basis for removing reads (Eisenhofer et al. 2019; Hornung et al. 2019).

Analysis of the mock communities (Fig. 9) revealed a few important trends: First, there was complete loss of some taxa; second, there were taxa that appeared that were not included in the mock community; and third, the relative abundance of read counts was not strongly correlated with starting quantity of fungal biomass, a conclusion that is corroborated by the qPCR results (Fig. 8). Because most microbiome studies do not include positive controls in the form of mock communities (Hornung et al. 2019), it is difficult to assess how surprising these results are. Conversely, studies that do analyze mock communities tend to focus only on the mock communities and do not incorporate analysis of environmental samples (Bakker 2018; Egan et al. 2018; O'Sullivan et al. 2021). Complete loss of some taxa, drastic deviations from the expected read count proportions, and appearance of taxa that should not be in the mock community are all common results from mock community studies (Chandelier et al. 2021; Egan et al. 2018; Gonzalez et al. 2012; Pawluczyk et al. 2015; Yeh et al. 2018). The magnitude of the difference between expected and observed read counts varies from taxon to taxon and by laboratory and computation methods used and can therefore be difficult to generalize across all taxa in a mock community. Therefore, the low correlation between read counts of *Sclerotinia* from Illumina amplicon sequencing and the amount of ascospores quantified by qPCR should not be surprising and align with a recent review of this subject (Lamb et al. 2019).

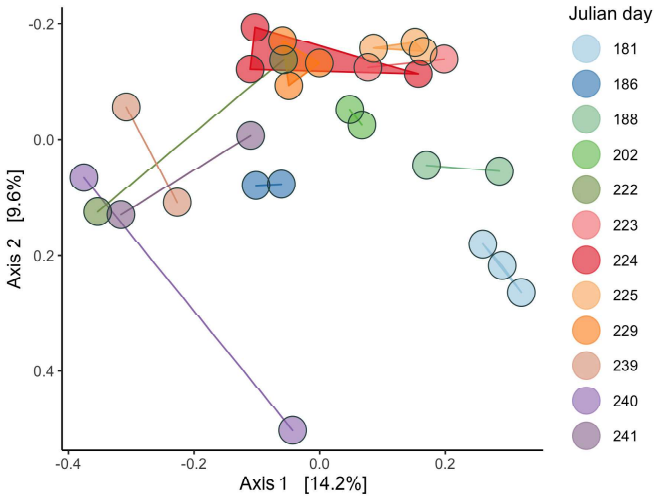

FIGURE 7
Comparison of the ITS1 amplicons from Burkard samplers at Lethbridge Research and Development Centre (LeRDC) in 2019 in each of three experimental plots. All samplers were located within 180 m of each other. Principal coordinate analysis of samples based on the Hellinger-transformed read count and Bray-Curtis dissimilarity of amplicon sequence variants. Lines/polygons connect samples collected on the same day from each of the samplers.

Sources of bias that could contribute to the poor mock community results are discussed in detail by Nguyen et al. (2015) and Hornung et al. (2019), so here, we will focus on specifics of the mock community analyzed in the present research. *Puccinia* spores are notoriously difficult to extract DNA from, so it is likely that our DNA extraction method, which was optimized for *S. sclerotiorum* ascospores, was inadequate to crack the hard *Puccinia* spore wall and could explain why this taxon was absent from the mock community. In addition, primer bias is a well-documented source of bias in amplicon sequencing studies (Schoch et al. 2012; Tedersoo et al. 2015), and the primers used in this study when tested in silico were not able to amplify rust fungi (Chen et al. 2022). Because qPCR of the mock community showed the expected quantity of *S. sclerotiorum* DNA and the taxonomic assignment of DNA from *S. sclerotiorum* was Sclerotiniaceae sp., one explanation for why this taxon was mostly absent from the mock community could be bias in PCR amplification rather than wrong taxonomic assignment to the closely related *Botrytis* genus (Amselem et al. 2011). Taken together, results from our mock community analysis add support to the call for including mock communities in all sequencing runs (Yeh et al. 2018).

The location of the sample collection within the province had an impact on airborne mycobiota diversity. Beaverlodge was the farthest from all other sites in this study and had the highest Shannon and Chao1 diversity of all sites surveyed (Table 4; Fig. 4). Differences in diversity may not be explained by weather conditions alone because Beaverlodge and Lacombe had similar climatological conditions for the 2019 season (Tables 4 and 5). Beaverlodge is an agricultural landscape surrounded by boreal forest, so this higher diversity could be a result of mixing ecosystems and/or land use types (Bowers et al. 2011).

The biggest differences among crop types were seen in the samples from the potato fields, which tended to cluster farther from the samples from dry bean, canola, and wheat. This finding may be an artifact of the different sampling regime employed in the potato samplers. Potato air samples were received from an existing spore monitoring network for late blight in potato fields, which actively sampled for 3 h in the afternoon, compared with the 24 h a day for all other Burkard samplers in this study. Thus, samples from the potato air sampling network samples would not contain fungi that tend to be released in the morning or at night; these spores may rely on active release mechanisms that do not require wind gusts (e.g., *Fusarium graminearum*; Schmale and Bergstrom 2004). Spores deposited at nighttime could also represent fungi from far afield, discussed in more detail below, because nights are generally calmer, and the cooler air with spores from higher in the boundary layer may mix or settle (Aylor 2017a; Betts 2015). Still, ordinations revealed considerable overlap in the community profiles of fungi collected in all these crops (Supplementary Fig. S4). This pattern could indicate that nighttime fungi do not contribute dramatically to different community compositions in this region, in contrast to findings reported by sev-

eral others on the diurnal impact of spore liberation (Hirst 1953; Lagomarsino Oneto et al. 2020).

The most abundant genera recovered in our study were *Alternaria*, *Epicoccum*, and *Ramularia*. These fungi are common across several aerobiology studies (Banchi et al. 2018; Núñez et al. 2017), and their dominance should not be too surprising because they are ubiquitous and fecund fungi causing leaf spots on many plant hosts and involved in soil ecosystems and with plant decomposition (Braga et al. 2018; Lawrence et al. 2015).

Before drawing conclusions from the samplers at LeRDC, it is important to briefly address the complex topics of (i) the origin of the airborne spores collected in the present study and (ii) the utility of using experimental plots, rather than only commercial fields, in the survey. Determining the source of spores in an air sample remains a difficult task because spore dissemination is a result of complex interactions among factors such as fungal biology, plant architecture, turbulence, and other weather events (Aylor 1999; Mahaffee et al. 2023; Schmale and Ross 2015). In general, spores from fungi tend to follow steep dispersal gradients in both the horizontal (Bourdôt et al. 2001; Chandelier et al. 2021; Mahaffee et al. 2023; Rieux et al. 2014; Soubeyrand et al. 2007) and vertical (Cao et al. 2012; Gregory and Hirst 1957; Hirst et al. 1967; Khattab and Levetin 2008; Van der Heyden et al. 2014) directions, but these patterns are largely dependent on air movement. For horizontal movement, spores tend to be deposited within a few meters (<10 m) of a source, and for vertical movement, spores tend to be deposited within a couple meters (<2 m) of the canopy. These trends suggest that placing samplers 1.0 to 1.5 m above ground level (and above the plant canopy), as we did in this study, will predominantly collect spores being released from the near vicinity of the sampler, provided they can escape the crop canopy (Aylor 2017b; Mahaffee et al. 2023; Nicolaisen et al. 2017; Van der Heyden et al. 2021). However, long-distance transport happens frequently, especially in windy areas such as southern Alberta. Indeed, our rare taxa (60% of the taxa recovered representing <2% of read abundance) may reflect long-distance transport. In this context, however, "long-distance" may only mean a distance greater than, perhaps, 10 m. The use of research plots in this survey served two purposes: The first was practical because, given resource constraints, it was easier to monitor our samplers regularly to ensure they were operating smoothly. The second purpose was to help isolate the effect of crop type from location. Even "nearby" commercial fields tend to be at least several kilometers away, and so crop effects could still be confounded by location effects. In our survey design, some of the Burkard samplers were located very close to each other (within 180 m) and immediately surrounded by different vegetation. Nevertheless, research plots may have more complex air biota than commercial agricultural fields, and intermixing of bioaerosols among nearby plots is common but depends on distance among plots, spore biology, and canopy architecture (Aylor 2017c). At LeRDC, the research plots are relatively small (30 × 50 m) and surrounded by buildings and many types of vegetation. In contrast, the commercial sites we surveyed typically were large (6.5×10^5 m^2) and unimpeded by buildings or windbreaks. Therefore, these small plots may still suffer from confounding effects of being too close rather than too far from each other.

With the above caveats in mind, data from the three samplers at LeRDC have some important implications for air monitoring programs. First, these samplers, all within 180 m of each other (representing ~0.14 km^2), collected similar community profiles, as indicated by sampling day clustering together in ordination plots (Fig. 7), suggesting that, at least at a regional scale, aeromycobiota collected by a single sampler may be representative of that area. This finding is reinforced by the results showing that, in both years, 40% of the identified genera were shared between all samplers and constituted over 98% of reads, indicating that a single sampler can collect the vast majority of taxa in that area. Second, in both years of sampling at LeRDC, certain fungi were more prevalent than others in some crops. *Blumeria* spp., the causal agent of powdery mildew on grasses, were much more abundant in the wheat plots than in the canola and bean plots in both years surveyed (Fig. 3). Previous research in *Blumeria* has shown that few spores are collected above the wheat canopy compared with within the canopy, indicating that the spores of this fungus have a steep dispersal gradient (Cao et al. 2012). Similarly, communities of airborne fungi collected within a patch of vegetation were shown to be different from those communi-

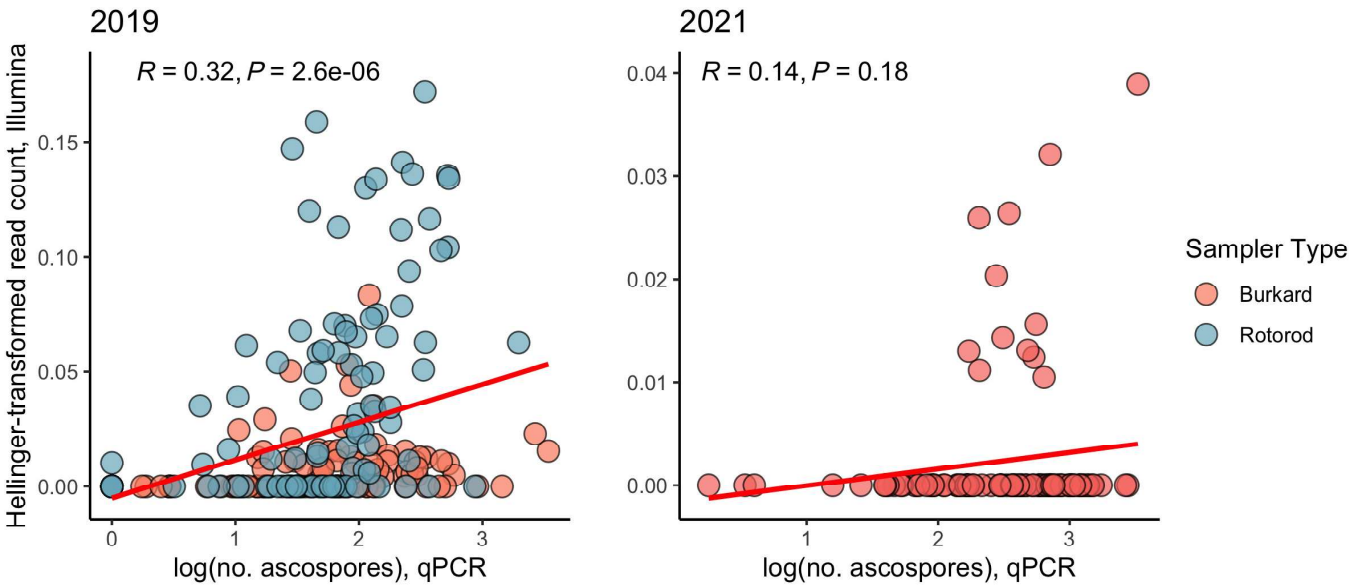

FIGURE 8
Correlations (and linear regression line) of the log (number of ascospores + 1) of *Sclerotinia sclerotiorum* ascospores as quantified by qPCR versus the Hellinger-transformed read count for Sclerotiniaceae sp. as determined in the same samples for samples collected in 2019 (left) and 2021 (right). Note the differences in *y* axis scales between the two plots.

ties collected upwind or downwind from that patch of vegetation (Lymperopoulou et al. 2016). These findings suggest that in cases where specific pathogens are of interest, the samplers should be placed within that host crop.

Third, there were substantial day-to-day changes in the relative abundance of many taxa in each of the three samplers at LeRDC, highlighting the importance of the fine-scale temporal sampling for fungi of interest (Figs. 3 and 7). We are continuing to carry out a nationwide sampling campaign to identify the environmental drivers of these changes. Fourth, there was a clear yearly effect on the community profiles, which shows the importance of sampling every year to address annual changes in airborne fungal communities (Table 7; Supplementary Fig. S6). Annual variation may be a reflection of different meteorological conditions or land use changes (e.g., crop rotations, urban development, deforestation, etc.). Finally, in the plot where both the rotorod and Burkard samplers were placed side by side, fungal communities tended to cluster by sampler type, which points to biases with a chosen sampling method. In the present study, this finding is expected, given the differences in sampling periods (24 versus 8 h for the Burkard and rotorod, respectively), durations (continuous versus 1 out of every 10 min), collection efficiencies of the two samplers (particle size collected: >2 versus >10 to 20 μm), and DNA extraction procedures. Few previous studies have compared these same samplers, but of those that exist, results were similar: little to no correlation of spores collected between the two (Aylor 1993; Bock and Cotty 2006). The effect of sampler types on community profiles has also previously been documented for HTS studies (Chen et al. 2018a; Mbareche et al. 2018).

All samplers collected a wide range of plant pathogens of interest, contributing to the growing body of knowledge that this form of surveillance could be useful in the management of plant disease epidemics (Abrego et al. 2018; Aguayo et al. 2021; Chen et al. 2018a; Nicolaisen et al. 2017). Detection of airborne inoculum can provide earlier warnings than disease scouting that a pathogen has made its first appearance of the season (e.g., in the case of *Phytophthora infestans* of potato; Arocha Rosete et al. 2021) or that a inoculum dispersal has passed a threshold of concern (e.g., in the case of *Botrytis squamosa* in onion; Carisse et al. 2009). In these situations, inoculum detection and quantification could inform the more efficient use of fungicides or aid in the development of more robust predictive models. In addition to plant pathogens, samplers in the present study collected a wide

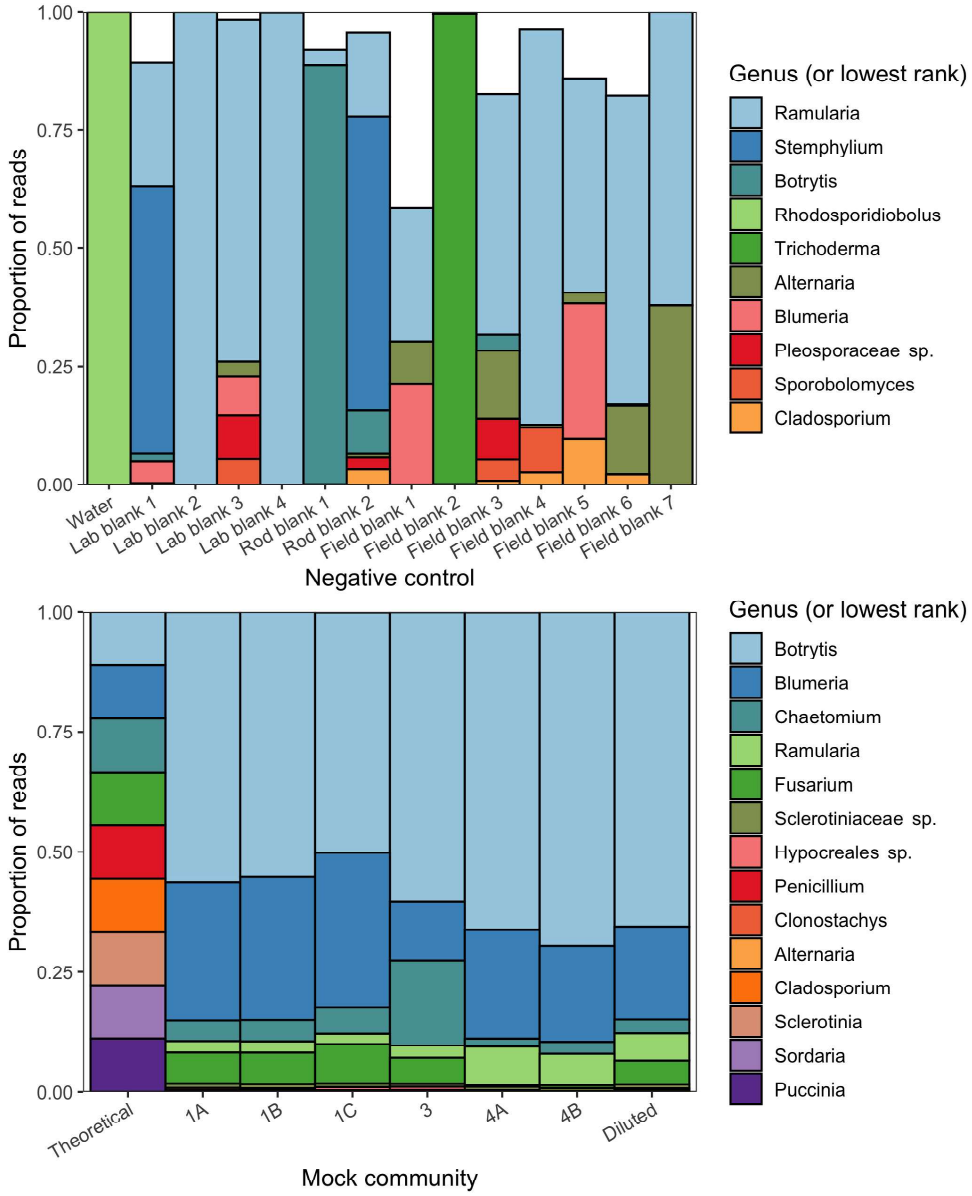

FIGURE 9
Top panel: The top 10 most common taxa found in the negative controls for the ITS1 amplicon. Data represent the proportion of reads in each sample. Bottom panel: The top 10 most common taxa found in the mock community for the ITS1 amplicon. Mock communities with different numbers were communities that were constructed and had DNA extracted independently; communities with different letters following the same number indicate technical replicates of DNA sent for sequencing. Data represent the proportion of reads in each sample. "Theoretical" values are based on the number of spores (and not ITS copy number) included in the mock community. The "Diluted" sample is a 1:10 dilution of the Mock 1 community.

range of antagonistic organisms to plant pathogens. *Epicoccum*, *Fusarium*, and *Alternaria* all exhibited antagonism toward diseases caused by *Sclerotinia sclerotiorum* in field trials (Boland and Inglis 1989; Hannusch and Boland 1996; Hu et al. 2016; Inglis and Boland 1992; Mercier and Reeleder 1987), and these taxa were all commonly recovered by samplers. Therefore, in the spirit of elucidating the pathobiome, future research might determine whether the presence of these taxa could influence disease development in the field. However, as noted above, it remains unclear to what extent relative abundance of read counts correlates with actual organism abundance in the field.

Results from Illumina amplicon sequencing take several weeks or months from sample collection to data interpretation, so the utility of this approach would rely on extensive sampling and surveying to build predictive models. In contrast, whole-genome metagenomic sequencing on the Oxford Nanopore Technologies MinION could provide near real-time results and has already been employed for identifying bacterial, viral (Bronzato Badial et al. 2018), and fungal (Radhakrishnan et al. 2019) plant pathogens. The rapid DNA extraction protocols we describe here might be employed in a field workflow for portable technologies such as whole-genome metagenomic sequencing on the MinION, which could both avoid biases associated with the PCR steps from barcoding methods and sequence longer regions of DNA, which could provide more reliable taxonomic assignments (Mafune et al. 2019). Preliminary trials with the MinION were attempted as part of this research project, but results were inconsistent and so this technology is under consideration for future projects. For an individual sample, MinION sequencing is much more expensive than Illumina sequencing; however, kits that enable multiplexing multiplex samples can greatly reduce per-sample costs for the MinION. Again, more research would be required to interpret the read counts to determine actionable interventions for plant disease management.

Another challenge in practical implementation of some of these microbiome insights is sample size: monitoring enough fields with a range of disease expression over the course of a season. Fortunately, efforts to create national (Chen et al. 2018a) and global (Buters et al. 2018) air monitoring networks could help provide important insights into understanding pathogen dissemination and disease forecasting and management.

ACKNOWLEDGMENTS

We thank the staff at Viterra for their help in collecting samples for this project; the staff at ProMax Agronomy for their help with Burkard spore sampler maintenance; Uriel Karerwa and Natasha Shelstad for their help in collecting and processing field samples; Eric Amundsen, Christine Vucurevich, and Anthony Erickson for their technical expertise; and Jiali Shang and Taifeng Dong for assisting in retrieving weather data and land use data around the sampling sites. We acknowledge the sequencing services provided by Centre d'expertise et de services Génome Québec.

LITERATURE CITED

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