A Field-Based Study of Phyllosphere Mycobiomes in Apple Orchards Under Varying Agricultural Practices

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Abstract

Microbial communities in the phyllosphere are key players in plant health and disease resistance, yet their response to agricultural practices remains poorly understood under field conditions. Here, we compare fungal community composition and diversity across conventional and organic apple orchards using ITS amplicon sequencing. Leaf samples were collected from six sites at three distinct time points during the 2023 growing season: once in May, July, and August, corresponding approximately to monthly intervals throughout the summer, while flower samples were collected from the same trees in May. Our analyses reveal that agricultural practices are significantly associated with fungal community structure, with effects intensifying from May to July. While conventional practices are associated with increased absolute abundance of commensal and potentially beneficial taxa, higher abundance of several genera which include known pathogens is also observed at organic sites. Alpha diversity declined over time at conventional orchards but remained stable at organic sites. These patterns suggest that shared interventions and distinct inputs impose contrasting selective pressures on the phyllosphere mycobiome, thus affecting not only community composition but also diversity. Our findings underscore the ecological relevance of field-based microbiome research and offer insights to support the development of sustainable orchard management strategies informed by fungal community dynamics.

Introduction

In 2024, the world's total population reached 8.2 billion humans, with an expected increase of 1.5 billion by 2050 [1]. As the world population keeps growing, agriculture faces increasing pressure not only to meet rising food demands, but also to ensure efficient, stable, and sustainable production systems that can support long-term productivity under variable environmental conditions. In parallel, pressures on ecosystems biodiversity and functions have never been so strong, and global change is deeply impacting biomes' temperature and precipitation regimes [2]. Now more than ever, crop producers face the heightened challenges of optimizing land use and limiting the spread of plant bacterial and fungal diseases, while navigating social expectations to minimize local environment disruptions. On the one hand, conventional agricultural practices most often rely on the use of chemicals such as antibiotics, fungicides, and insecticides to maintain crop productivity. On the other hand, these practices can be damageable to the environment in the long-term as they can lead to water pollution [3] and to the emergence of resistances in microbial pathogens [4, 5]. While the use of strong chemicals in agriculture provided crucial benefits to human society in terms of food security during the Green Revolution of the 1950s, the book "Silent Spring" by Rachel Carson [6] revealed how these practices, when used without restrictions, are detrimental to ecosystems [7]. Thus, sustainable agricultural practices (e.g., organic), even if they are less effective in terms of productivity per land area in the short term, are considered to be an important alternative to preserve ecosystem biodiversity and functions [8]. Yet, organic practices also include some treatments (e.g., biocontrol products such as "Blossom Protect" [9], "Bt" [10]), which can impact the microbial communities associated with crops. In this context, research on the effects of agricultural practices (conventional and organic) on plant-microbe interactions is crucial to advance our knowledge of these ecosystems and support crop producers.

Plant flowers, leaves, and roots host diverse microbial communities (i.e., assemblies of microorganisms including bacteria and fungi) that can impact plant host growth and general health [11, 12]. For instance, members of the *Arabidopsis* microbiota can antagonize the widely distributed foliar pathogen *Pseudomonas syringae* [13], and plant growth promoting rhizobacteria (PGPR) are foreseen to be key players in plant growth [14, 15]. As the impact of agricultural practices on rhizosphere (i.e., roots)

microbial communities has been extensively researched [16, 17], the current study focuses on the aerial parts of plants (i.e., flowers and leaves), commonly named the phyllosphere [11], an environment that has gained scientific interest in the last two decades due to its known impacts on general plant health.

Plant-microbe studies are often performed in controlled settings, such as tissue culture plates filled with agar [18], growth chambers on a variety of substrates such as calcined clay [19], or greenhouses with controlled lighting, temperature, and humidity [20]. Although these controlled growth experiments provide instrumental understanding of molecular mechanisms and simplified interactions, they offer little extrapolation potential to *in situ* dynamics, which is what crop producers truly experiment and deal with. Scientific initiatives are often criticized to be too far from the real world or to not make the effort to connect with users [21]. In view of this, initiatives from our group [22, 23] but also from others (see **Table S1**) have addressed plant-microbe systems directly in the field. This comes with various challenges linked to access to the sites, access to information on practices, and study design constraints [24]. Nonetheless, a few studies have investigated the influence of crop management practices on either phyllosphere bacterial or fungal alpha and beta diversity (**Table S1**). However, because of the difficulties mentioned above, these studies often yield different and sometimes contradictory results, thus reinforcing the need to investigate how agricultural practices impact the structure of microbial phyllosphere communities in the field to reach scientific consensus.

Fungi, although expected to be less abundant than bacteria [11] in the phyllosphere, are known to affect host plant and ecosystems through drought tolerance improvement [25], pathogen regulation [9], and leaf litter decomposition [26]. In a previous study, we identified *Aureobasidium*, *Sporobolomyces*, and *Mycosphaerella* as the three main fungal genera observed in the apple tree (*Malus domestica*, Borkh.) phyllosphere [22]. Our work also showed that site and time are strong drivers of microbial (both bacteria and fungi) community composition and diversity in field conditions [22, 23]. Yet, in Boutin *et al.* (2024) [22], when stratifying our data by agricultural practices, the organic site showed no significant difference in alpha diversity across the three time points, in sharp contrast with the observable trend at the two conventional sites. Therefore, to broaden the impact of our

research and investigate this discrepancy, we extended our sampling design of the apple tree phyllosphere in orchards to include more sites of varying agricultural practices.

Here, we aim to compare fungal community composition and diversity across conventional and organic agricultural practices on both apple tree flowers and leaves. More precisely, our main objectives are to (1) assess the associations between agricultural practices and fungal community composition, (2) characterize how alpha diversity varies across time, sites, and agricultural practices, and (3) identify differentially abundant taxa within conventional and organic sites. These analyses are performed on a large dataset including four to seven cultivars at each site, as well as a smaller subset of two cultivars present at every sites. Building on previous results from our group [22, 23] and others (**Table S1**), we expect that fungal community assemblies will diverge between agricultural practices, and that these differences will be reflected in the differential abundance of specific taxa. Yet, alpha diversity should be affected to a lesser extent. We expect selective pressure from agricultural chemicals usage to be higher at conventional sites, leading to stronger alterations in the community composition of dominant fungal taxa, including apple leaf pathogens such as *Venturia inequalis* (the causal agent of apple scab). Despite this selective pressure, we anticipate that alpha diversity (Shannon index) will be reduced at conventional sites. Finally, we expect to observe spatial (site specific) and temporal variation within fungal community composition and diversity, linked to fungal taxa responding to climatic cues and treatments, as well as dispersing from local reservoirs (e.g., soil, land use).

To achieve our objectives, we sampled apple flowers and leaves at six orchards (three conventional and three organic) at three time points during the summer of 2023 Specifically, flowers were sampled in May, while leaves were sampled in May, July, and August. Targeting the Internal Transcribed Spacer (ITS) region of fungal ribosomal DNA, we sequenced fungal DNA extracted from phyllosphere samples to assess community composition and diversity. Our results highlight a strong association between agricultural practices and phyllosphere (leaves and flowers) fungal community composition and diversity, explaining part of the variation that was attributed to site identity in previous works.

133 **Materials and methods**

134 *Sampling*

135 Six orchards (A-B1-B2-C-D1-D2) from the Eastern Townships region of Québec (Canada)
136 were sampled in 2023. These orchards (sites) were selected based on available cultivars,
137 agricultural practices (conventional or organic), and spatial proximity. Some orchards were
138 selected as pairs of contrasting practices with spatial proximity. For example, B1 and B2
139 are respectively conventional and organic orchards situated less than a kilometer apart.
140 Sites D1 and D2 are conventional and organic plots within the same orchard. These two
141 pairs of sites were chosen to test for differences in agricultural practices while controlling
142 for the distance. More details about the sites and the sampled cultivars are available in
143 **Table S2**. At each site, three trees from each cultivar were randomly selected. In May, leaf
144 and flower samples were collected. In July and August, after the end of the blooming
145 period, only leaf samples were collected. In the same day of leaf and flower collection,
146 samples were stored at -20ºC in the laboratory until cell collection and DNA extraction.

147 *Fungal cells collection and DNA extraction*

148 Using the same method as previously described by Boutin *et al.* [22], samples were thawed
149 at room temperature approximatively one hour before proceeding to cell collection.
150 Then, 100 ml of 1X Redford Buffer [27] was added to each sampling bag. The bags were
151 shaken during five minutes, then the buffer was decanted equally into two 50 ml tubes.
152 These tubes were centrifuged at 4000 g for 20 minutes and the supernatants were discarded.
153 The pellet of the first tube was resuspended in 800 µl of solution CD1 from the DNeasy®
154 PowerSoil® Pro Kit (QIAGEN). The content of the first tube was transferred to the second
155 tube to resuspend its pellet. Up to 1 ml was transferred to a bead beating tube, which was
156 kept at -20ºC until DNA extraction. Before DNA extraction, bead beating tubes containing
157 the microbial pellets and CD1 were thawed at room temperature. Tubes were placed in a
158 Bead Mill Homogenizer (Fisher) set to the following parameters (speed: 3.25 m/s; time:
159 2 minutes; cycles: 5; breaks: 30 seconds). The rest of the extraction followed the quick start
160 protocol and DNA was eluted in 75 µl of solution C6. Extracted DNA was stored at -20ºC
161 until it was sent to the sequencing platform.

162

Sequencing

The ITS1 region was amplified using the ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) primers. Samples were sequenced at the CERMO genomic platform (Université du Québec à Montréal) on the Illumina MiSeq platform at 2x300 bp.

Amplicon sequence variants processing

A total of 9,333,318 sequences from 364 samples were processed using the DADA2 pipeline v1.34 [28] in R v4.4.0 [29] using default parameters unless otherwise stated. Briefly, low complexity sequences and those containing ambiguous nucleotides were discarded using the *filterAndTrim* function with *rm.lowcomplex = TRUE, maxN = 0*. Primers were removed using cutadapt v2.10 [30] with parameters *-n 2 -m 21 -M 300*, then sequences were quality-filtered using *filterAndTrim*, allowing a maximum expected error *maxEE = c(4, 4)* and a minimum length *minLen = 100*. After learning the error model, sample compositions were inferred using *learnErrors(pool = "pseudo").* Forward and reverse sequences were merged, then bimeras removed using *method = "consensus"*. The taxonomy of the resulting amplicon sequence variants (ASVs) was assigned using the UNITE ITS database v10.0, 04-04-2024 [31]. ASVs with fewer than a total of 10 sequences across all samples or with no taxonomic classification at the Phylum level were removed. Then, samples with fewer than a total of 2,000 sequences were discarded, resulting in a total of 329 samples kept for analysis (the 35 discarded samples are listed in **Table S3**. This resulted in a total of 5,022,964 total sequences (mean 15,267 ± 8,189 per sample) and 2,812 ASVs (mean 216 ± 144 per sample). On average, each ASV was found in 25 ± 39 samples. Sample metadata, sequence and taxonomy tables were built into a phyloseq object (phyloseq v1.46.0 [32]) for further analyses. For alpha and beta diversity analyses (see below), samples were rarefied to 2,500 sequences to control for uneven sequencing depth using the *rarefy_even_depth* function from the vegan package v2.7-1 [33]. This resulted in a total 822,500 sequences and 2,795 ASVs (mean 146 ± 112 per sample). On average, each ASV was found in 17 ± 31 samples after rarefaction (**Table S4**).

Statistical analyses

Statistical analyses were performed using base R functions and the rstatix v0.7.2 and vegan packages. Plots were generated using the ggplot2 v3.5.2, ggpubr v0.6.1, ggrain v0.0.4, ggh4x v0.3.1, see v0.11.0, and patchwork v1.3.1 packages.

For each dataset, we analyzed alpha diversity based on the Shannon index and beta diversity based on the Bray-Curtis index. Shannon diversity indices were calculated using the *estimate_richness* function from phyloseq. Beta diversity was estimated by first variance-stabilizing the rarefied count tables using the *varianceStabilizingTransformation* function from DESeq2 v1.48.1, then computing the Bray-Curtis dissimilarity index using the *vegdist* function from vegan. Permutational multivariate analyses of variance (PERMANOVA) were applied to the resulting dissimilarity matrices using the *adonis2* function from vegan with 999 permutations. Homogeneity of group dispersions was verified using the *betadisper* function (**Table S5**). The dissimilarity matrices were visualized using principal coordinates analysis (PCoA). Aggregate sample composition bar charts are based on the mean relative abundance of each taxon, showing the top 20 taxa that were most relatively abundant on average.

Flower fungal beta and alpha diversity

To characterize the fungal communities associated with apple tree flowers, we analyzed a subset of 54 flower samples (35 from conventional and 19 from organic orchards), collected across four sites (A, B1, B2, and C). Sites D1 and D2 were excluded due to the absence of flowers at the time of sampling.

Beta diversity dynamics were tested using the following model: [*Community* ~ *(Practice/Site)* × *Cultivar*] with 'Site' nested in 'Practice' to reflect the sampling design. A Kruskal-Wallis test revealed that alpha diversity was not normally distributed; therefore, the non-parametric Wilcoxon–Mann–Whitney test was used to confirm whether organic and conventional sample diversity originated from the same distribution.

Leaf fungal beta diversity

To assess the influence of agricultural practices on the fungal communities associated with apple tree leaves, we analyzed all 275 leaf samples, encompassing all sites and cultivars.

222 Beta diversity dynamics were tested using the following model: [*Community ~*
223 *(Practice/Site) × Time + (Practice/Site) × Cultivar*] with 'Site' nested in 'Practice' to
224 reflect the sampling design. Permutations were constrained within individual trees
225 (TreeID) to account for repeated measures. Separate analyses were also conducted for the
226 Honeycrisp (n = 51) and Spartan (n = 53) cultivars to confirm that our results were robust
227 despite uneven cultivar representation across sites. To control for temporal effects, we
228 repeated the PERMANOVA and PCoA independently for each sampling time point (May,
229 July, August), using the model: [*Community ~ (Practice/Site) × Cultivar*], allowing to
230 estimate the variance explained by agricultural practices at each time point. To further
231 highlight site-specific patterns, ordinations were visualized to highlight pairs of sites that
232 were spatially closed to one another: (1) for sites B1 and B2 as well as (2) for sites D1 and
233 D2. To visualize the variation in fungal class composition across the PCoA ordination, we
234 performed an *envfit* analysis from the vegan package to test for correlations between the
235 relative abundance of fungal classes and the ordination axes. Significance was assessed
236 using permutation tests, and a Bonferroni correction was applied to account for multiple
237 comparisons.

238 *Leaf fungal alpha diversity*

239 Diversity was compared using the Kruskal-Wallis non-parametric test, followed by post
240 hoc pairwise Dunn's tests with Bonferroni correction. These analyses were performed to
241 assess variation in diversity across sampling time points (May, July, August) within each
242 site, across time points within each agricultural practice, and across agricultural practices
243 at each time point.

244 All analyses (for composition and alpha diversity) were also performed separately
245 for the Honeycrisp ($n_{Flowers} = 8$; $n_{Leaves} = 51$) and Spartan ($n_{Flowers} = 9$; $n_{Leaves} = 53$) cultivars
246 to confirm that our results were robust to uneven cultivar representation across sites.

247 *Differential absolute abundance analyses*

248 Differences in absolute abundance between organic and conventional sites were
249 estimated using the log-linear model implemented in ANCOM-BC2 [34] and are expressed
250 as natural log fold differences (LFD) in absolute abundances. The following procedure was

done separately for flower and leaf samples. First, non-rarefied sequence counts were aggregated at the genus level, discarding any ASV with no taxonomic assignment at that level. Genera found fewer than 10% of samples (6 flower and 28 leaf) were not considered. The model controlled for cultivar, as well as sampling month in the case of the leaf samples. *P*-values were manually corrected using the Holm procedure, because ANCOM-BC2's *p*-value correction only accounts for the number of tests done in a single run, while the command was executed once for leaf and once for flower samples. Genera with an adjusted p-value < 0.01 and having passed the pseudo-count sensitivity check were considered as differentially abundant (enriched) in either group. Only genera with an absolute LFD > 1 were reported.

Results

Flower samples across agricultural practices

We first explored the impact of agricultural practices on flower fungal community composition and diversity. From the principal coordinates analysis (PCoA), we observed that samples from conventional and organic practices clustered separately (**Figure 1A**). The permutational variance analysis (PERMANOVA) revealed that agricultural practices explained up to R^2 = 20% (P = 0.001) of the variation observed within those samples (**Table 1**). Site identity was also a driver of fungal community structure, explaining R^2 = 13% of the variation (**Table 1**). In terms of alpha diversity, flower samples from organic sites had a lower mean Shannon index than those from conventional sites (conventional = 4.3, organic = 3.7; $P < 0.0001$) (**Figure 1B**). The three main fungal genera found in samples from conventional sites were *Cladosporium*, *Aureobasidium*, and *Fomes* which respectively represented 8%, 8%, and 7% of the total number of sequences. In organic sites, they were *Ramularia, Didymella, Gyoerffyella*, representing 17%, 17%, and 9% of sequences (**Figure 1C**).

We then sought to identify genera enriched in samples from either organic or conventional sites. To that end, we estimated fold differences in absolute abundances for each genus using a log-linear model (ANCOM-BC2), controlling for cultivar type (**Figure 2**). In flower samples, eight and twenty-six genera were found to be differentially enriched (adjusted $p < 0.01$) in conventional and organic sites, respectively.

281 Lecanoromycetes (n = 2 genera) and Eurotiomycetes (n = 1 genus) were only enriched in
282 conventional sites, while Agaricomycetes (n = 11 genera), Phaeococcomyces and
283 Cystobasidiomycetes (n = 1 genus each) were only enriched in organic sites. The most
284 differentially enriched genus, *Exidia*, was estimated to be almost 37 times more abundant
285 (3.6 ± 0.2 LFD) in organic sites compared to conventional sites.

286 Overall, we obtained similar results when looking at flower samples from
287 Honeycrisp and Spartan cultivars only. Indeed, samples clustered separately by practice
288 type (**Figure S1A**). Of note, practices explained an even greater amount of the variation
289 observed within samples (**Table S6**), and the difference in alpha diversity was not
290 statistically significant (conventional = 4.4, organic = 3.9; $P = 0.07$; **Figure S1B**). The two
291 most relatively abundant genera (*Ramularia* and *Aureobasidium*) (**Figure S1C**) were the
292 same as for the whole dataset.

293 *Leaf samples across agricultural practices*

294 The impact of agricultural practices was then investigated for leaf fungal communities.
295 In the PCoAs, samples from conventional and organic sites did cluster independently
296 across all sampling time-points (**Figure 3**). When all leaf samples were analyzed together,
297 agricultural practices explained 13% of the variation among samples (**Table 2A**). When the
298 three time-points were evaluated independently, a greater proportion of the variation was
299 explained by the agricultural practices in July (32%) and August (27%) than in May (13%)
300 (**Table 2B-D**). Site identity explained from 15 to 31% of the variation across sampling
301 time-points (**Table 2**). With samples from Honeycrisp and Spartan only, fungal
302 communities from different agricultural practices also clustered separately (**Figure S2**), as
303 agricultural practices explained 14% of the variation (**Table S7**).

304 For leaves, fungal community composition was also explored across sampling time-
305 points (May, July, and August) for both conventional and organic sites. The most relatively
306 abundant fungal genera were *Aureobasidium*, *Sporobolomyces*, and *Cladosporium*
307 (**Figure 4**). Similar community profiles were observed when analyzing the data subset
308 including only Honeycrisp and Spartan cultivars. (**Figure S3**).

For both practices, our results show that there is a shift in fungal communities' composition between May and July (**Figure 4**), which is confirmed by the change in the absolute abundance of certain taxa (**Figure 5**) as well as by general changes in the relative abundance of specific fungal classes (**Figure S5**). In leaf samples, 10 and 28 genera were found to be differentially enriched in conventional and organic sites, respectively (**Figure 5**). Lecanoromycetes genera (n = 2) identified as significantly enriched were again all associated with conventional sites, and both Tremellomycetes (n = 5) and Cystobasidiomycetes (n = 3) genera were only found to be enriched in organic sites. The most differentially enriched genus was an unnamed Dothidotthiaceae, estimated to be 75 times more abundant (4.32 ± 0.13 LFD) in organic sites compared to conventional.

In terms of alpha diversity, a general decrease in Shannon index through time is observed at conventional sites (**Figure 6A-B**). Oppositely, alpha diversity remains stable from May to August at organic sites (**Figure 6A-B**). When comparing both practices through sampling month, alpha diversity was higher at conventional sites in May compared to organic sites, while the opposite was observed in July and August (**Figure 6C**).

Discussion

Understanding the dynamics of microbial communities in agroecosystems is essential for developing sustainable agricultural practices. While many observational microbiome studies have focused on spatiotemporal variation in microbial assemblages [35, 36], few have examined these dynamics under *in situ* conditions with direct relevance to producers. In this study, we extended the design of our previous works to assess how fungal communities in the apple phyllosphere respond to agricultural practices (conventional vs. organic) across multiple sites (six orchards) and time points (three sampling months). Our results demonstrate that agricultural practices are associated with alterations in the composition and diversity of fungal communities of the apple tree flowers and leaves, with effects intensifying from May to July before stabilizing in August (**Tables 2-3**; **Figures 1-3**). Our work also confirms that both site and month of sampling are strongly correlated with changes in fungal community structure (**Table 1; Table 2A**), reinforcing the idea that orchard-specific conditions, such as microclimate and management practices, contribute to shaping the local phyllosphere mycobiome.

339 Although practices were broadly categorized as conventional or organic due to
340 limited access to detailed treatment records, this binary classification still revealed clear
341 and consistent differences in fungal assemblages between orchard types. Working *in-field*
342 inevitably introduces constraints, such as incomplete knowledge of specific inputs or
343 treatment schedules, yet it also ensures ecological relevance and direct applicability of
344 findings for growers. Despite these limitations, we observed distinct fungal community
345 profiles between conventional and organic orchards (**Figures 1-4**), suggesting that the use
346 of synthetic versus organic inputs could exert differential selective pressures on the
347 phyllosphere mycobiome, for example resulting in shift in community composition.

348 *Flower mycobiome of organic orchards host more relatively abundant plant pathogens*

349 In flower samples, fungal communities from conventional and organic orchards formed
350 distinct clusters in PCoA ordinations (**Figure 1A**), a pattern supported by PERMANOVA
351 results showing that agricultural practice explains a significant proportion of the variation
352 in fungal community dissimilarities. Notably, genera such as *Alternaria*, *Didymella*, and
353 *Ramularia*, all of which contain potential plant pathogens species (e.g., *Alternaria mali*,
354 *Ramularia collo-cygni*, *Didymella pomorum*) [37–39], were more relatively abundant in
355 organic orchards (**Figure 1C**). Differential abundance analysis confirmed that *Didymella*
356 and *Ramularia* were significantly enriched at organic sites (**Figure 2**). This dominance of
357 a few potentially pathogenic taxa may contribute to the lower alpha diversity observed in
358 organic flower samples (**Figure 1C**), consistent with previous findings that pathogen
359 proliferation can reduce overall community evenness and richness [40, 41]. Interestingly,
360 Agaricomycetes such as the *Exidia* genus (known as saprotroph jelly fungi) were only
361 enriched at organic sites. This suggests either that products used in conventional orchards
362 may reduce the abundance of members of this fungal genus, or that organic cultivation
363 methods favor them.

364 *The effect of agricultural practices on leaf mycobiome beta diversity increases with time*

365 Similar patterns were observed in leaf samples, where agricultural practices were linked to
366 fungal composition and diversity. Importantly, the proportion of variance in fungal
367 community composition explained by practice increased over the growing season, from
368 13% of community variation in May to 32% in July and 27% in August (**Table 2**).

369 This temporal trend likely reflects the timing of orchard treatments, which may have been
370 applied shortly before or after the May sampling, thus exerting stronger effects later in the
371 season. In conventional orchards, the mid-season shift in community structure was
372 characterized by an increased relative abundance of *Cladosporium*, *Sporobolomyces*, and
373 *Aureobasidium*, genera commonly associated with beneficial or commensal roles in the
374 phyllosphere [42–44]. Notably, the red pigmented *Sporobolomyces* produce carotenoids,
375 thus have antimicrobial properties and the potential to control for pathogens [45, 46].
376 Different species of *Cladosporium* also show biocontrol potential against plant pathogens
377 [42, 47]. Moreover, *Aureobasidium* is the active ingredient in the biocontrol product
378 Blossom Protect [9], which is widely applied in both organic and conventional orchards to
379 suppress fire blight, potentially explaining its consistent presence across all sites
380 (**Figure 4**). In contrast, organic sites showed a higher abundance of potential pathogens
381 such as members of the *Dothidotthiaceae*, known to cause cankers and leaf spots [48].

382 These findings highlight how overlapping and distinct management interventions
383 across organic and conventional regimes impose unique selection pressures on
384 phyllosphere fungal communities, leading to both shared dominant taxa, such as
385 *Aureobasidium*, and divergent overall community structures. Conventional practices
386 appear be associated with dominance by potentially beneficial or commensal taxa, while
387 organic practices showed potentially pathogenic genera to be enriched in leaf mycobiomes.
388 Yet, it is important to note that these interpretations are based on relative abundance data
389 derived from metagenomic sequencing, which reflects DNA presence rather than active
390 fungal biomass or viability. In terms of diversity, alpha diversity declined over time in
391 conventional orchards but remained stable in organic sites, a pattern consistent with our
392 previous observations [22]. Interestingly, alpha diversity was higher at conventional sites
393 in May, while organic sites showed greater diversity in July and August. This contrasts with
394 patterns observed in bacterial communities, where diversity was lower in organic sites in
395 May but showed no significant differences between practices in July and August [23].
396 These divergent trends suggest that agricultural practices may have distinct and taxon-
397 specific impacts on fungal versus bacterial communities. Taken together, our results
398 provide *in-field* information about flower and leaf mycobiome dynamics highlighting that
399 agricultural practices are associated with shifts in fungal community assembly and

diversity across the season. By linking fungal community dynamics to orchard practices, we provide actionable insights for growers seeking to optimize plant health and disease resistance through microbiome-informed strategies.

Apple tree cultivar is a weak but significant driver of fungal phyllosphere communities

Consistent with earlier observations by Boutin et al. (2024) [22], cultivar identity explained a small but statistically significant portion of the variation in leaf-associated fungal community structure (**Table 2**). This modest effect aligns with previous studies showing that host species identity tends to exert a stronger influence on microbial communities than cultivar-level differences [35, 49, 50]. Nonetheless, the reproducibility of this pattern across independent datasets underscores that, although this effect is weaker than month of sampling, site identity or agricultural practices, accounting for cultivar identity is relevant to understand the dynamics of the apple phyllosphere mycobiome.

Limitations

While our study provides ecologically relevant insights into the influence of agricultural practices on apple phyllosphere fungal communities, several limitations should be acknowledged. First, working under field conditions meant that we could not fully control environmental variables or access detailed treatment records for each orchard (e.g., absence of flowers at sites D1 and D2, not all cultivars present at all sites). As a result, agricultural practices were broadly categorized as conventional or organic, which may mask important nuances in management strategies and input types. Second, our sequencing approach targeted the ITS1 region, which, while widely used for fungal community profiling, has known biases in taxonomic resolution and amplification efficiency across fungal taxa. These methodological constraints may have influenced the detection and relative abundance of certain groups, which should be held into account when interpreting or comparing the results with other studies. Finally, although our sampling design captured temporal and spatial variation, it remains observational in nature and cannot establish causal relationships between specific treatments and microbial outcomes. Future studies incorporating controlled experiments, metagenomic approaches, and detailed agronomic

429 metadata will be essential to refine our understanding of how orchard management shapes
430 fungal community structure and function.

431 *Conclusion*

432 This study provides novel information about how agricultural practices are associated with
433 fungal community composition and diversity of the apple tree phyllosphere in field
434 conditions. By leveraging a robust sampling design across multiple sites, cultivars, and
435 months of sampling, we were able to quantify the relative influence of agricultural
436 practices, time, and site on fungal assemblages, track alpha diversity dynamics throughout
437 the growing season, and identify differentially abundant taxa associated with each practice.
438 Our findings offer new insights into how agricultural practices used for pathogen control
439 may differentially affect the structure of fungal communities, including shifts in relative
440 abundance of both pathogenic and beneficial taxa. Beyond documenting these patterns, our
441 work contributes to a growing body of research emphasizing the ecological relevance of
442 phyllosphere fungi, which play key roles in plant health, disease resistance, and microbial
443 interactions. Studying these communities *in situ* allows us to generate actionable
444 knowledge for growers, highlighting how site-level decisions can influence microbial
445 composition and, ultimately, crop resilience and productivity.

446 While our results reveal clear differences between conventional and organic
447 systems, determining which practice is more sustainable remains an open question. Future
448 studies are required to develop integrative sustainability indices and link microbial
449 community structure to disease incidence, yield, and broader environmental and socio-
450 economic outcomes. We therefore advocate for future research that bridges microbial
451 ecology with on-farms agronomic performance and sustainability science to guide the
452 development of microbiome-informed orchard management strategies.

453 **TABLES**

454 **Table 1.** Factors explaining the variation within all flower samples (PERMANOVA on
455 Bray-Curtis dissimilarities)

456

	R^2 (%)	F	Pr(>F)	
practice	20.2	16.9	0.001	***
cultivar	12.4	1.5	0.008	**
practice:site[*1]	12.7	5.3	0.001	***
practice:cultivar	8.9	1.2	0.058	ns
practice:site:cultivar	7.7	1.3	0.050	*
Residual	38.1			
Total explained	61.9			

457 [*1] Site is nested within practice.

Table 2. Factors explaining the variation within all leaf samples (PERMANOVA on Bray-Curtis dissimilarities). All leaf samples from all time-points (**A**) and separated for May (**B**), July (**C**) and August (**D**).

A

	R^2(%)	F	Pr(>F)	
practice	13.8	117.4	0.001	***
time	19.1	81.1	0.001	***
cultivar	4.4	5.3	0.001	***
practice:site[*1]	15.4	32.7	0.001	***
practice:time	6.0	25.7	0.001	***
practice:cultivar	2.1	2.9	0.001	***
practice:site:time	7.6	8.0	0.001	***
practice:site:cultivar	4.5	2.8	0.001	***
Residual	27.1			
Total explained	72.9			

B

	R^2(%)	F	Pr(>F)	
practice	13.2	20.3	0.001	***
cultivar	10.2	2.2	0.001	***
practice:site[*1]	24.6	9.5	0.001	***
practice:cultivar	5.6	1.4	0.011	*
practice:site:cultivar	12.2	1.4	0.001	***
Residual	34.3			
Total explained	65.7			

C

	R^2(%)	F	Pr(>F)	
practice	32.6	108.4	0.001	***
cultivar	8.3	3.9	0.001	***
practice:site[*1]	26.6	22.1	0.001	***
practice:cultivar	4.3	2.4	0.001	***
practice:site:cultivar	10.4	2.5	0.001	***
Residual	17.8			
Total explained	82.24			

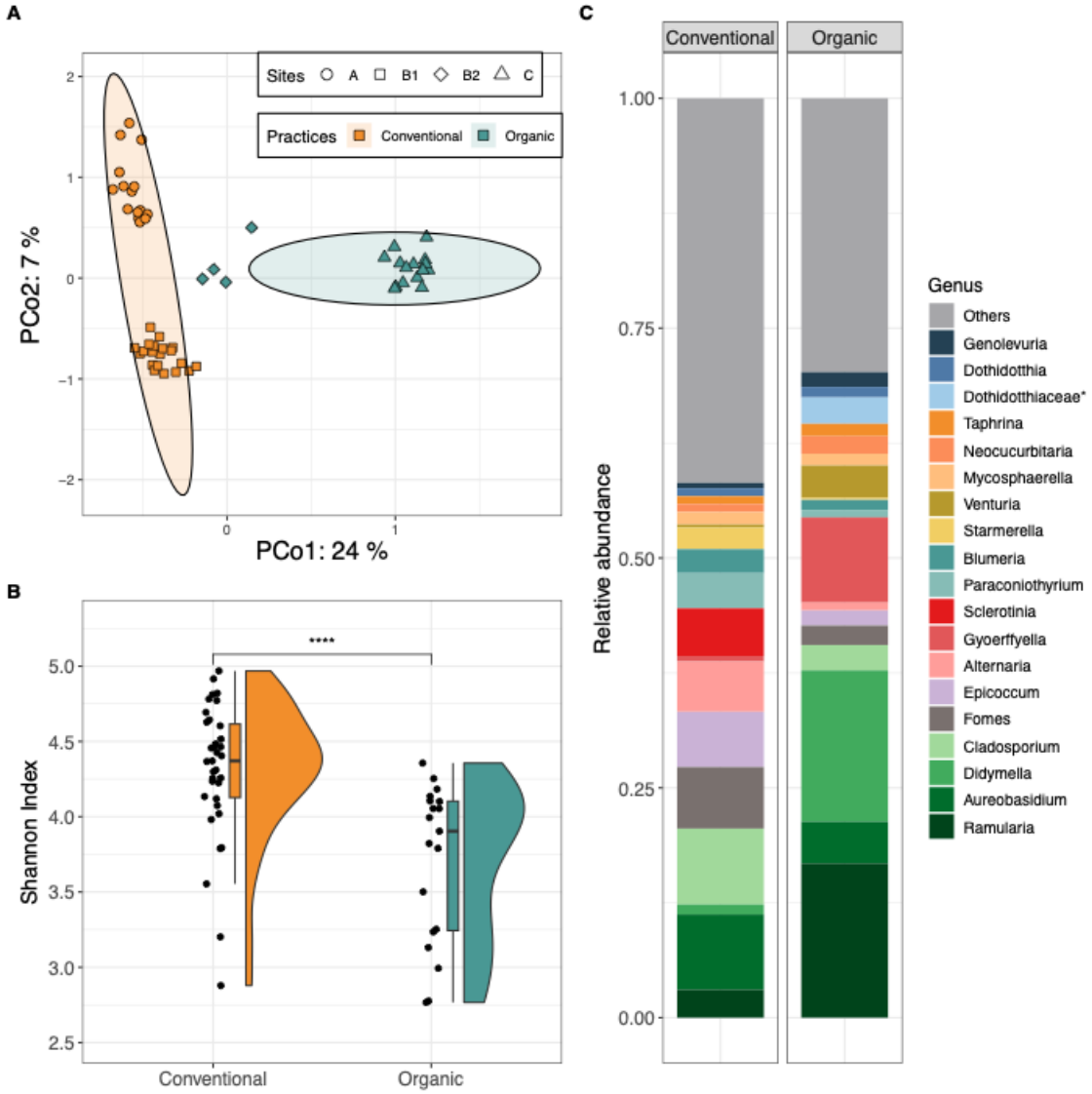
D

	R^2(%)	F	Pr(>F)	
practice	27.5	88.5	0.001	***
cultivar	8.5	3.9	0.001	***
practice:site[*1]	31.1	25.0	0.001	***
practice:cultivar	4.1	2.2	0.003	**
practice:site:cultivar	8.6	2.0	0.001	***
Residual	20.2			
Total explained	79.8			

[*1]Site is nested within practice.

463 **FIGURES**

464



465 **Figure 1.** Fungal composition and diversity of flower samples. **(A)** Principal Coordinates
466 Analysis (PCoA) based on Bray-Curtis dissimilarities among flower samples. Point shapes
467 and colors indicate sampling sites and agricultural practices. Ellipses represent 95%
468 confidence intervals. **(B)** Shannon alpha diversity index across conventional (n =35) and
469 organic (n = 19) sites. Asterisks indicate significance levels from Wilcoxon-Mann-Whitney
470 test (**** $P \leq 0.0001$). **(C)** Average relative abundance of the 20 most abundant fungal
471 genera across flower samples. Taxa not among the top 20 are grouped under "Others." Bar
472 charts are separated by agricultural practice. *Asterisks denote taxa identified at the family*
473 *level but with unknown genus.*

474

Figure 2: Differential abundance analysis of flower communities comparing differences in absolute abundance of genera across agricultural practice, controlling for cultivar. A negative natural log fold difference (LFD) corresponds to an enrichment in the conventional sites (i.e., the reference group in the log-linear model), whereas a positive LFD corresponds to an enrichment in the organic group. Gen. I. s.: *Genus Incertae sedis*.

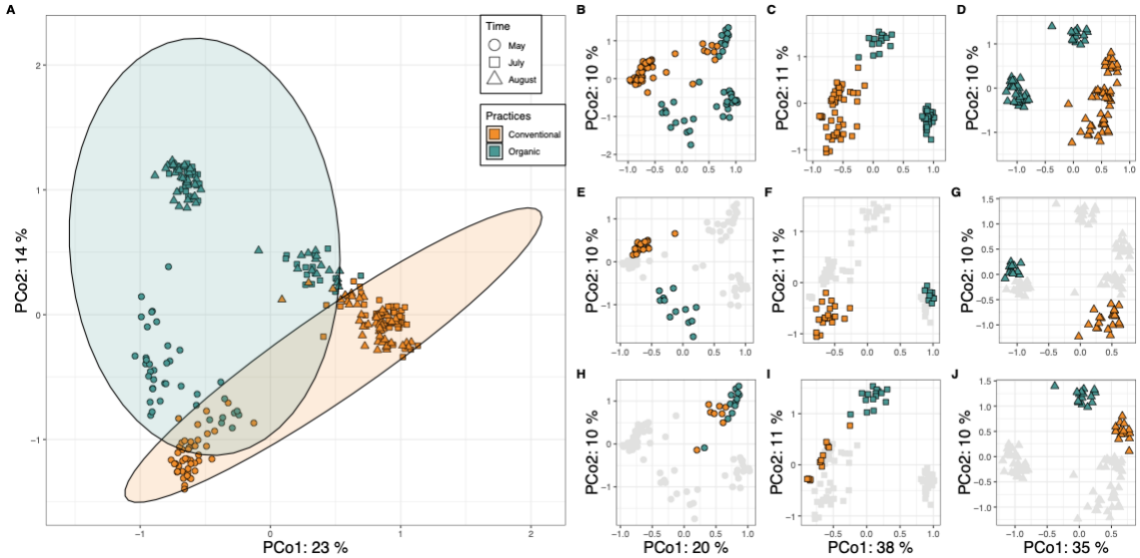


Figure 3. PCoAs on Bray-Curtis dissimilarities performed on leaf samples. **(A)** All leaf samples across sites and sampling months. **(B–D)** Subset of samples collected in May **(B)**, July **(C)**, and August **(D)**, with conventional sites shown in orange and organic sites in green. **(E–G)** Highlighted comparison of sites B1 (conventional) and B2 (organic for May **(E)**, July **(F)**, and August **(G)**; other sites are shown in gray. **(H–J)** Highlighted comparison of sites D1 (conventional) and D2 (organic) for May **(H)**, July **(I)**, and August **(J)**; other sites are shown in gray. Ellipses represent 95% confidence intervals.

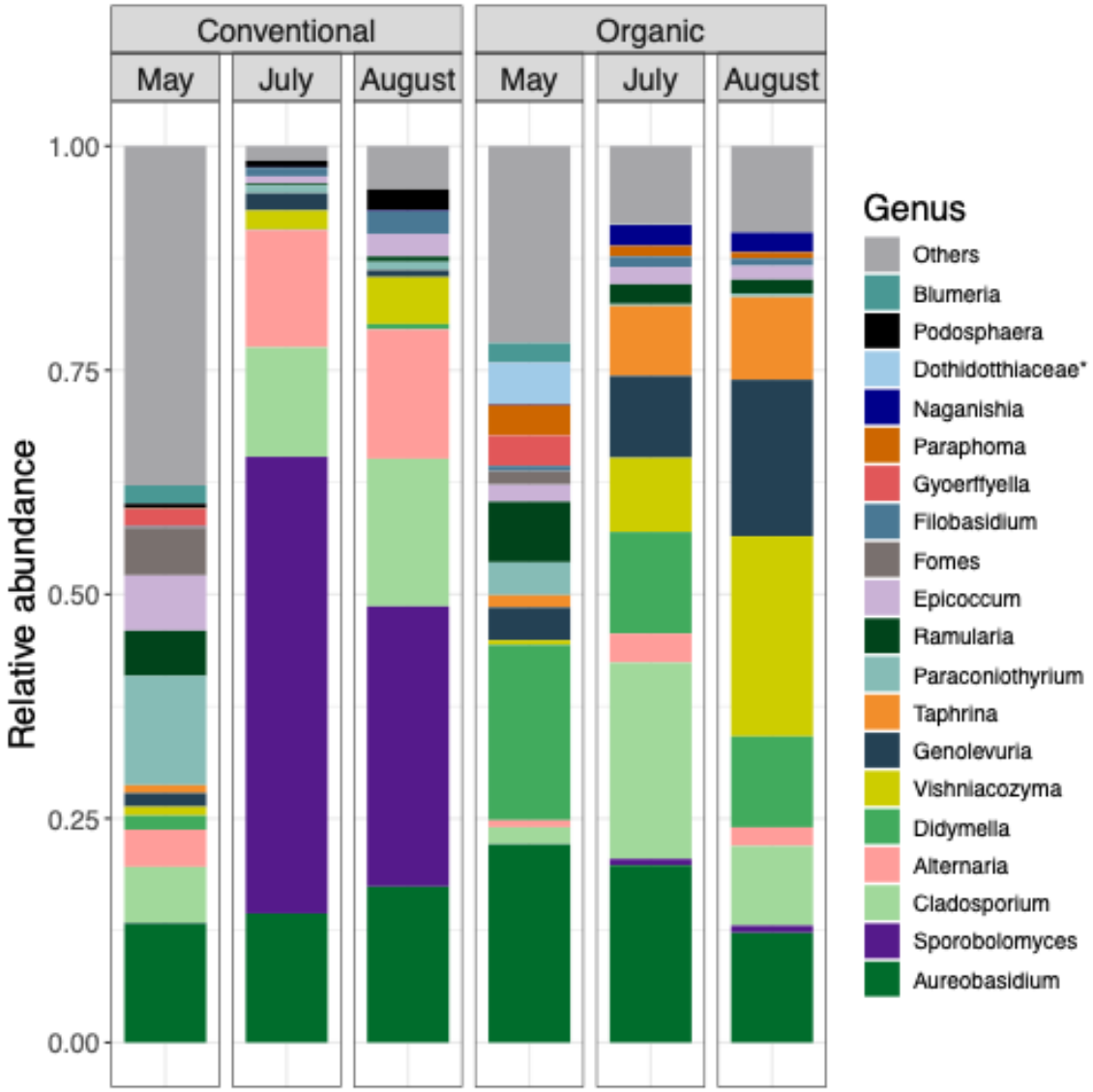
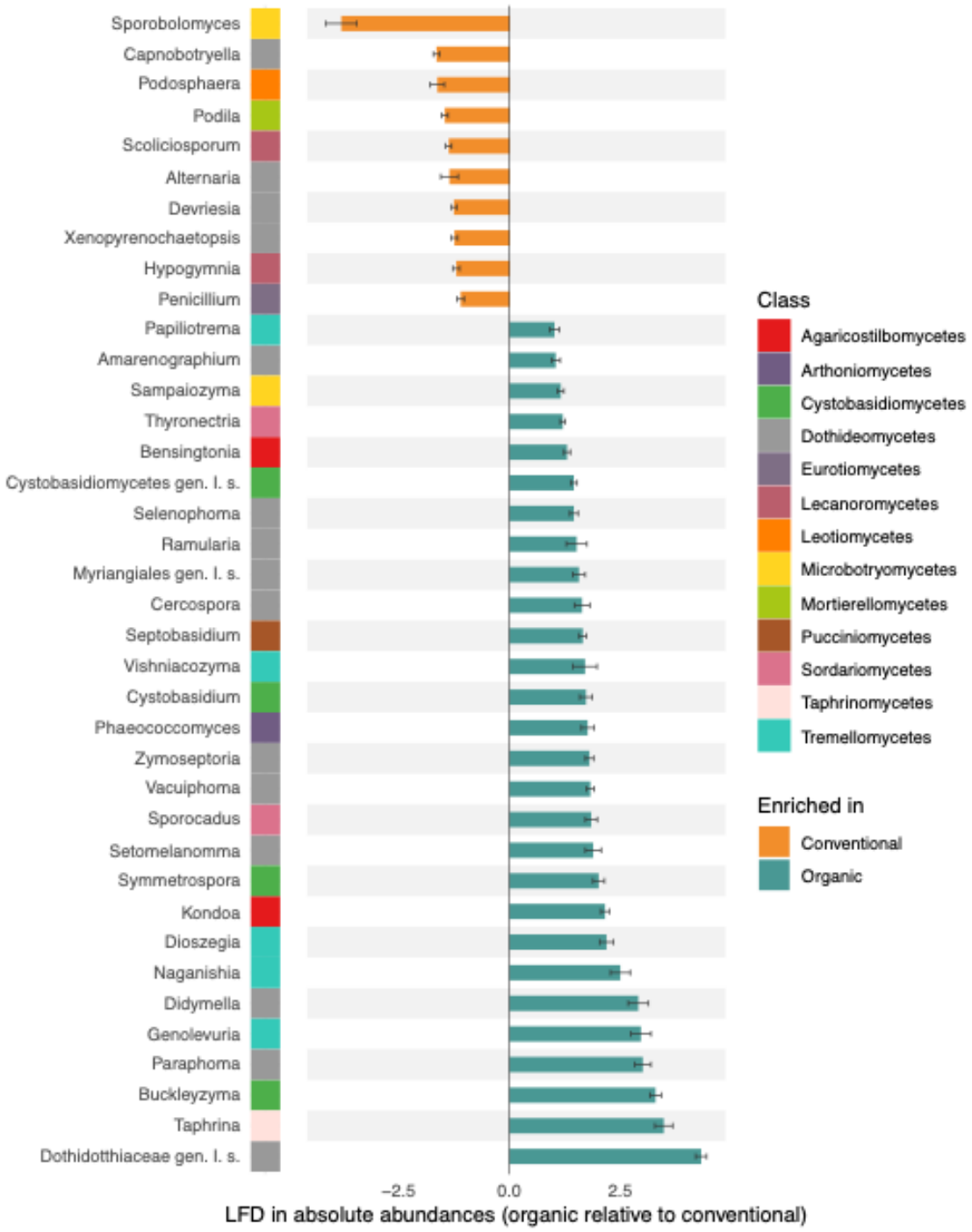


Figure 4. Fungal community composition across all leaf samples (all cultivars). Average relative abundance of the 20 most abundant fungal genera. Taxa not among the top 20 are grouped under "Others." Bar charts are organized by agricultural practice and sampling month (Conventional: n = 45, 48, 54; Organic: n = 40, 44, 44). Asterisks indicate taxa identified at the family level but with unknown genus.

498



499 **Figure 5:** Differential abundance analysis of leaf communities comparing differences in
500 absolute abundance of genera across agricultural practice, controlling for cultivar. A
501 negative natural log fold difference (LFD) corresponds to an enrichment in the
502 conventional sites (i.e., the reference group in the log-linear model), whereas a positive
503 LFD corresponds to an enrichment in the organic group. Gen. I. s.: *Genus Incertae sedis*.

504

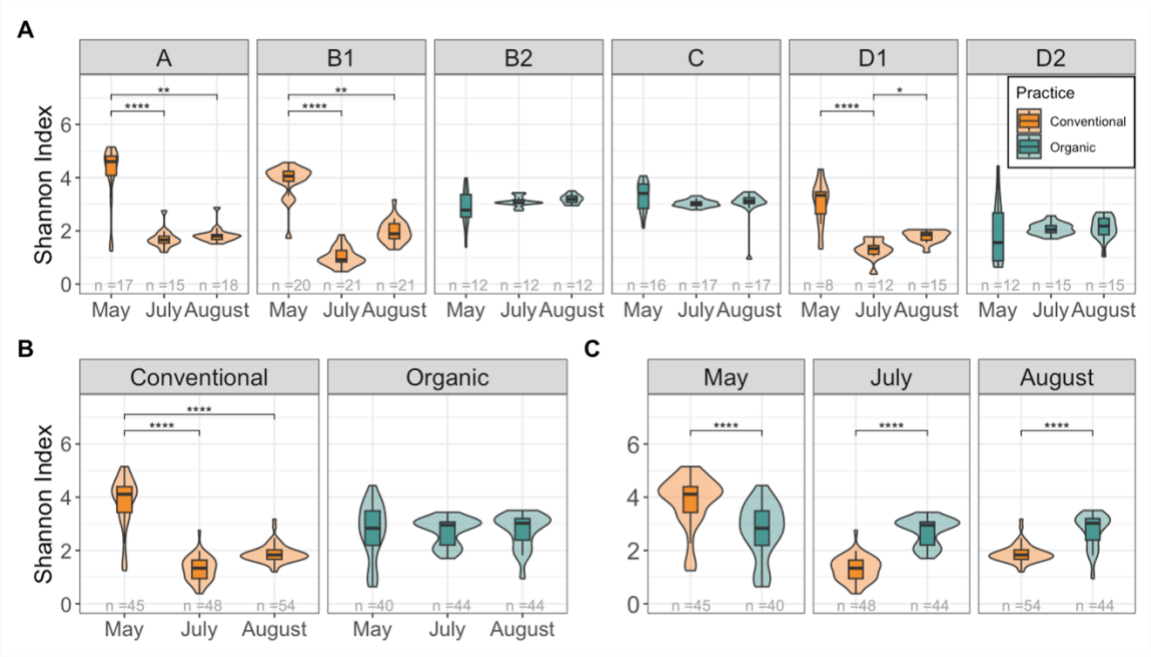


Figure 6. Shannon alpha diversity of leaf samples (all cultivars). **(A)** Alpha diversity across sampling times for each individual site. **(B)** Alpha diversity across sampling times grouped by agricultural practice. **(C)** Comparison of alpha diversity between conventional and organic practices at each sampling time point. Asterisks indicate significance levels from post-hoc Dunn tests following a Kruskal-Wallis test ($P \leq 0.05$; $\mathbf{P} \leq 0.01$; $\boldsymbol{P} \leq 0.001$; $\mathbf{P} \leq 0.0001$).

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Visualization: S.B., J.R-L., A.R. & I.L-L.
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Writing – review & editing: I.L-L., S.B., J.R-L., A.R.

Supplementary materials

Supplementary materials are available at the following GitHub repository: https://github.com/alexis-roy5/orchard_phyllosphere

Conflicts of interest

The authors declare no conflict of interest.

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Data availability

The raw sequences are available on the European Nucleotide Archive under the following accession number: PRJEB96463

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