Responses to Agricultural Practices in Apple Orchards: A Field-Based Mycobiome Study of the Phyllosphere

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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. Flower and leaf samples were collected from six sites over three time points during the 2023 growing season. 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 containing known pathogens is observed in organic sites. Alpha diversity declined over time in conventional orchards but remained stable at organic sites. These patterns suggest that shared interventions (e.g., biocontrol) and distinct inputs (e.g., synthetic vs. organic treatments) impose contrasting selective pressures on the phyllosphere mycobiome, thus affecting not only community composition but also diversity. Our findings underscore the ecological relevance of on-farm 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, social demands are high to intensify agriculture productivity. In parallel, pressures on ecosystems biodiversity and functions have never been so strong, and global change is impacting deeply 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 chemical products such as antibiotics, fungicides, and insecticides to protect 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 by the 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 [8] to preserve ecosystem biodiversity and functions. Yet, organic practices also use some treatments (e.g., biocontrol products such as "Blossom Protect" [9] and "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 promotion [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), the phyllosphere [11], an environment that has gained scientific interest in the last two decades.

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 calcinated 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, those that are experimented and dealt with by crop producers. 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.

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.

100 Here, we aim to compare fungal community composition and diversity across
101 conventional and organic agricultural practices on both apple tree flowers and leaves.
102 More precisely, our main objectives are to (1) measure the relative influence of agricultural
103 practices on fungal community composition, (2) characterize how alpha diversity varies
104 across time, sites, and across agricultural practice, and (3) identify differentially abundant
105 taxa within conventional and organic sites. These analyses are performed on a large dataset
106 including four to seven cultivars at each site, as well as a smaller subset of two cultivars
107 present at every sites. Building on previous results from our group [22, 23] and other
108 research groups (**Table S1**), we hypothesize that orchard management practices will drive
109 fungal community composition, but that alpha diversity will be less impacted than
110 composition by the type of practice. We expect selective pressure from agricultural
111 chemicals usage to be higher at conventional sites, leading to stronger alterations in the
112 community composition of dominant fungal taxa, including apple leaf pathogens such as
113 *Venturia inequalis* (the causal agent of apple scab). While this selective pressure is ongoing,
114 we anticipate that alpha diversity (Shannon index) will be reduced at conventional sites.
115 These diverging assemblies across agricultural practices will be shown in the presence of
116 differentially abundant fungal taxa. Finally, we expect to observe spatial (site specific) and
117 temporal variation within fungal community composition and diversity, linked to fungal
118 taxa responding to climatic cues and dispersing from local reservoirs (e.g., soil, land use).

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

127 **Materials and methods**

128 *Sampling*

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

141 *Fungal cells collection and DNA extraction*

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

156 *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 a 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[27] in R v4.4.0 [28] using default parameters unless otherwise stated. Briefly, low complexity sequences and those containing ambiguous nucleotides were discarded (*rm.lowcomplex = TRUE, maxN = 0*). Primers were removed using cutadapt v2.10 [29] with parameters *-n 2 -m 21 -M 300*, then sequences were quality-filtered allowing a maximum expected error of 4 (*maxEE = c(4, 4)*) and a minimum length *minLen = 100*. After learning the error model, sample compositions were inferred using *pool = "pseudo"*. Forward and 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 [30]. 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 S4**). 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 v1.46.0 [31] object 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 *rarefy_even_depth* from the vegan package v2.7-1 [32]. 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.

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 by *estimate_richness* from phyloseq. Beta diversity was estimated by first variance-stabilizing the rarefied count tables using *varianceStabilizingTransformation* from DESeq2 v1.48.1, then computing the Bray-Curtis dissimilarity index using *vegdist* from vegan. Permutational multivariate analyses of variance (PERMANOVA) were applied to the resulting dissimilarity matrices using *adonis2* from vegan with 999 permutations. Homogeneity of group dispersions was verified using the *betadisper* function (**Table S7**). 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. Beta diversity dynamics were tested using the following model: [*Community* ~ *(Practice/Site)* × *Time* + *(Practice/Site)* × *Cultivar*] with 'Site' nested in 'Practice' to reflect the sampling design. Permutations were constrained within individual trees (TreeID) to account for repeated measures. Separate analyses were also conducted for the Honeycrisp (n = 51) and Spartan (n = 53) cultivars to confirm that our results were robust

216 to uneven cultivar identity across sites. To control for temporal effects, we repeated the
217 PERMANOVA and PCoA independently for each sampling time point (May, July, August),
218 using the model: [*Community ~ (Practice/Site) × Cultivar*], allowing to estimate the
219 variance explained by agricultural practices at each time point. To further highlight site-
220 specific patterns, ordinations were visualized to highlight pairs of sites that were spatially
221 closed to one another: (1) for sites B1 and B2 as well as (2) for sites D1 and D2. To visualize
222 the variation in fungal class composition across the PCoA ordination, we performed an
223 *envfit* analysis from the package *vegan* v2.7-1 [32] to test for correlations between the
224 relative abundance of fungal classes and the ordination axes. Significance was assessed
225 using permutation tests, and a Bonferroni correction was applied to account for multiple
226 comparisons.

227 *Leaf fungal alpha diversity*

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

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

236 *Differential abundances analyses*

237 Fold-differences in absolute abundances between organic and conventional sites
238 were estimated using the log-linear model implemented in ANCOM-BC2 [33]. The
239 following procedure was done separately for flower and leaf samples. First, non-rarefied
240 sequence counts were aggregated at the genus level, discarding any ASV with no
241 taxonomic assignment at that level. Genera found fewer than 10% of samples (6 flower
242 and 28 leaf) were not considered. The model controlled for cultivar, as well as sampling
243 month in the case of the leaf samples. *P*-values were manually corrected using the Holm
244 procedure, because ANCOMBC'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. Taxa with an absolute LFC > than 1.5, an adjusted p-value < 0.01, and having passed the pseudo-count sensitivity check are reported as differentially enriched in either group.

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 20% ($P = 0.001$) of the variation observed within those samples (**Table 1**). Site identity was also a driver of fungal community structure, explaining 13% of the variation (**Table 1**). In terms of alpha diversity, flower samples from organic sites had a lower Shannon index than those from conventional sites (conventional = 4.3, organic = 3.7; $P = <0.0001$) (**Figure 1B**). The three main fungal genera observed were *Ramularia*, *Aureobasidium*, and *Didymella* which respectively represent 3%, 8%, and 1% of the total community under conventional practices and 17%, 5%, and 17% under organic practices (**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 genera using a log-linear model, 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. Enriched Lecanoromycetes (n=2 genera) and Eurotiomycetes (n=1 genus) were only associated with conventional sites, while enriched Agaricomycetes (n=11 genera), Phaeococcomyces and Cystobasidiomycetes (n=1 genus each) only associated with organic sites. The most differentially enriched genus, *Exidia*, was estimated to be 12 times more abundant (3.6 ± 0.2 $\log_2$ fold-difference) in organic sites compared to conventional sites.

Overall, we obtained similar results when looking at flower samples from Honeycrisp and Spartan cultivars only. Indeed, samples clustered separately (**Figure S1A**), and the two

275 most relatively abundant genera (*Ramularia* and *Aureobasidium*) (**Figure S1C**) were the
276 same as for the whole dataset. Of note, practices explained an even greater amount of the
277 variation observed within samples (**Table S5**), and the difference in alpha diversity was not
278 statistically significant (conventional = 4.4, organic = 3.9; $P = 0.07$; **Figure S1B**).

279 *Leaf samples across agricultural practices*

280 The impact of agricultural practices was then investigated for leaf fungal communities.
281 In the PCoAs, samples from conventional and organic sites did cluster independently
282 across all sampling time-points (**Figure 3**). When all leaf samples were analyzed together,
283 agricultural practices explained 13% of the variation among samples (**Table 2A**). When the
284 three time-points were evaluated independently, a greater proportion of the variation was
285 explained by the agricultural practices in July (32%) and August (27%) than in May (13%)
286 (**Table 2B-D**). Site identity explained from 15 to 31% of the variation across sampling
287 time-points (**Table 2**). With samples from Honeycrisp and Spartan only, fungal
288 communities from different agricultural practices also clustered separately (**Figure S2**), as
289 agricultural practices explained 14% of the variation (**Table S6**).

290 For leaves, fungal community composition was also explored across sampling time-
291 points (May, July, and August) for both conventional and organic sites. The most relatively
292 abundant fungal genus was *Aureobasidium*, followed by *Sporobolomyces* and
293 *Cladosporium* (**Figure 4**). Similar community profiles were observed when analyzing the
294 data subset including only Honeycrisp and Spartan cultivars. (**Figure S3**).

295 For both practices, there appear to be a shift in fungal communities' composition
296 between May and July, which is confirmed by the change in the absolute abundance of
297 certain taxa. In leaf samples, 10 and 28 genera were found to be differentially enriched in
298 conventional and organic sites, respectively (**Figure 5**). Lecanoromycetes genera (n=2)
299 identified as significantly enriched were again all associated with conventional sites, and
300 both Tremellomycetes (n=5) and Cystobasidiomycetes (n=3) genera were only found to be
301 enriched in organic sites. The most differentially enriched genus was an unnamed
302 Dothidotthiaceae, estimated to be 20 times more abundant (4.32 ± 0.13 $\log_2$ fold-
303 difference) in organic sites compared to conventional.

304 **Discussion**

305 Understanding the dynamics of microbial communities in agroecosystems is essential for
306 developing sustainable agricultural practices. While many observational microbiome
307 studies have focused on spatiotemporal variation in microbial assemblages [34, 35], few
308 have examined these dynamics under *in situ* conditions with direct relevance to producers.
309 In this study, we extended the design of our previous works to assess how fungal
310 communities in the apple phyllosphere respond to agricultural practices (conventional vs.
311 organic) across multiple sites (six orchards) and time points (three sampling months).
312 Our results demonstrate that agricultural practices are associated with alterations in the
313 composition and diversity of fungal communities of the apple tree flowers and leaves, with
314 effects intensifying from May to July before stabilizing in August (**Tables 2-3**; **Figures 1-**
315 **3**). Our work also confirms that both site and month of sampling are strongly correlated
316 with changes in fungal community structure (**Table 1; Table 2A**), reinforcing the idea that
317 orchard-specific conditions, such as microclimate and management practices, contribute to
318 shaping the local phyllosphere mycobiome.

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

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

329 In flower samples, fungal communities from conventional and organic orchards formed
330 distinct clusters in PCoA ordinations (**Figure 1A**), a pattern supported by PERMANOVA
331 showing that agricultural practice explains a significant proportion of the variation in
332 fungal community dissimilarities. Notably, genera such as *Alternaria*, *Didymella*, and
333 *Ramularia*, all of which contain potential plant pathogens species (e.g. *Alternaria mali*,

334 *Ramularia collo-cygni*, *Didymella pomorum*) [36–38], were more relatively abundant in
335 organic orchards (**Figure 1C**). Differential abundance analysis confirmed that *Didymella*
336 and *Ramularia* were significantly enriched at organic sites (**Figure 2**). This dominance of
337 a few potentially pathogenic taxa may contribute to the lower alpha diversity observed in
338 organic flower samples (**Figure 1C**), consistent with previous findings that pathogen
339 proliferation can reduce overall community evenness and richness [39, 40].

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

341 Similar patterns were observed in leaf samples, where agricultural practices associated with
342 fungal composition and diversity. Importantly, the proportion of variance in fungal
343 community composition explained by practice increased over the growing season, from
344 13% of community variation in May to 32% in July and 27% in August. This temporal
345 trend likely reflects the timing of orchard treatments, which may have been applied shortly
346 before or after the May sampling, thus exerting stronger effects later in the season.
347 In conventional orchards, the mid-season shift in community structure was marked by
348 increased relative abundance of *Cladosporium*, *Sporobolomyces*, and *Aureobasidium*,
349 genera often associated with beneficial or commensal roles in the phyllosphere [41–43].
350 Of note, *Aureobasidium* is the active ingredient in the biocontrol product Blossom Protect
351 [9], which is widely used in both organic and conventional orchards to suppress fire blight,
352 potentially contributing to its general abundance across all sites (**Figure 4**).

353 These findings highlight how overlapping and distinct management interventions
354 across organic and conventional regimes impose unique selection pressures on
355 phyllosphere fungal communities, leading to both shared dominant taxa, such as
356 *Aureobasidium*, and divergent overall community structures. Conventional practices
357 appear to promote dominance by potentially beneficial or commensal taxa, while organic
358 practices, show that potentially pathogenic genera are maintained in leaf mycobiomes.
359 Yet, it is important to note that these interpretations are based on relative abundance data
360 derived from metagenomic sequencing, which reflects DNA presence rather than active
361 fungal biomass or viability. In terms of diversity, alpha diversity declined over time in
362 conventional orchards but remained stable in organic sites, a pattern consistent with our
363 previous observations [22]. Interestingly, alpha diversity was higher in conventional sites

364 in May, while organic sites showed greater diversity July and August. This contrasts with
365 patterns observed in bacterial communities, where diversity was lower in organic sites in
366 May but showed no significant differences between practices in July and August [23].
367 These divergent trends suggest that agricultural practices may have distinct and taxon-
368 specific impacts on fungal versus bacterial communities. Taken together, our results
369 provide on-farm information about flower and leaf mycobiome dynamics highlighting that
370 agricultural practices are associated with shifts in fungal community assembly and
371 diversity across the season. By linking fungal community dynamics to orchard practices,
372 we provide actionable insights for growers seeking to optimize plant health and disease
373 resistance through microbiome-informed strategies.

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

375 Consistent with earlier observations by Boutin et al. (2024) [22], cultivar identity explained
376 a small but statistically significant portion of the variation in leaf-associated fungal
377 community structure (**Table 2**). This modest effect aligns with previous studies showing
378 that host species identity tends to exert a stronger influence on microbial communities than
379 cultivar-level differences [34, 44, 45]. Nonetheless, the reproducibility of this pattern
380 across independent datasets underscores that, although this effect is weaker than month of
381 sampling, site identity or agricultural practices, accounting for cultivar identity is relevant
382 to understand the dynamics of the apple phyllosphere mycobiome.

383 *Limitations*

384 While our study provides ecologically relevant insights into the influence of agricultural
385 practices on apple phyllosphere fungal communities, several limitations should be
386 acknowledged. First, working under real field conditions meant that we could not fully
387 control environmental variables or access detailed treatment records for each orchard (e.g.,
388 absence of flowers at sites D1 and D2, not all cultivars present at all sites). As a result,
389 agricultural practices were broadly categorized as conventional or organic, which may
390 mask important nuances in management strategies and input types. Second, our sequencing
391 approach targeted the ITS1 region, which, while widely used for fungal community
392 profiling, has known biases in taxonomic resolution and amplification efficiency across
393 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 metadata will be essential to refine our understanding of how orchard management shapes fungal community structure and function.

Conclusion

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

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

423 **TABLES**

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

426

	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			

427 [*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.

FIGURES

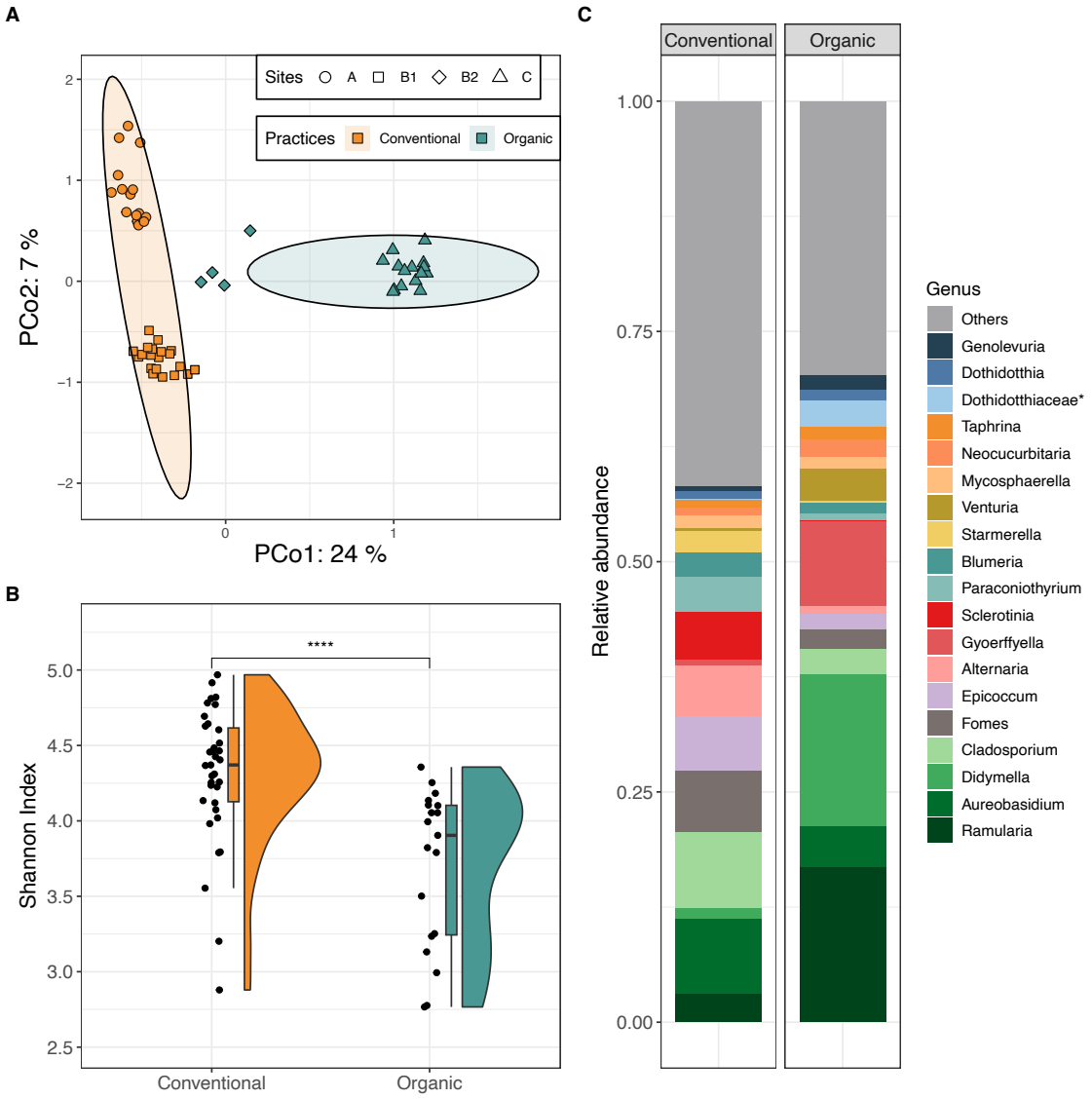

Figure 1. Fungal composition and diversity of flower samples. **(A)** Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarities among flower samples. Point shapes and colors indicate sampling sites and agricultural practices. Ellipses represent 95% confidence intervals. **(B)** Shannon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

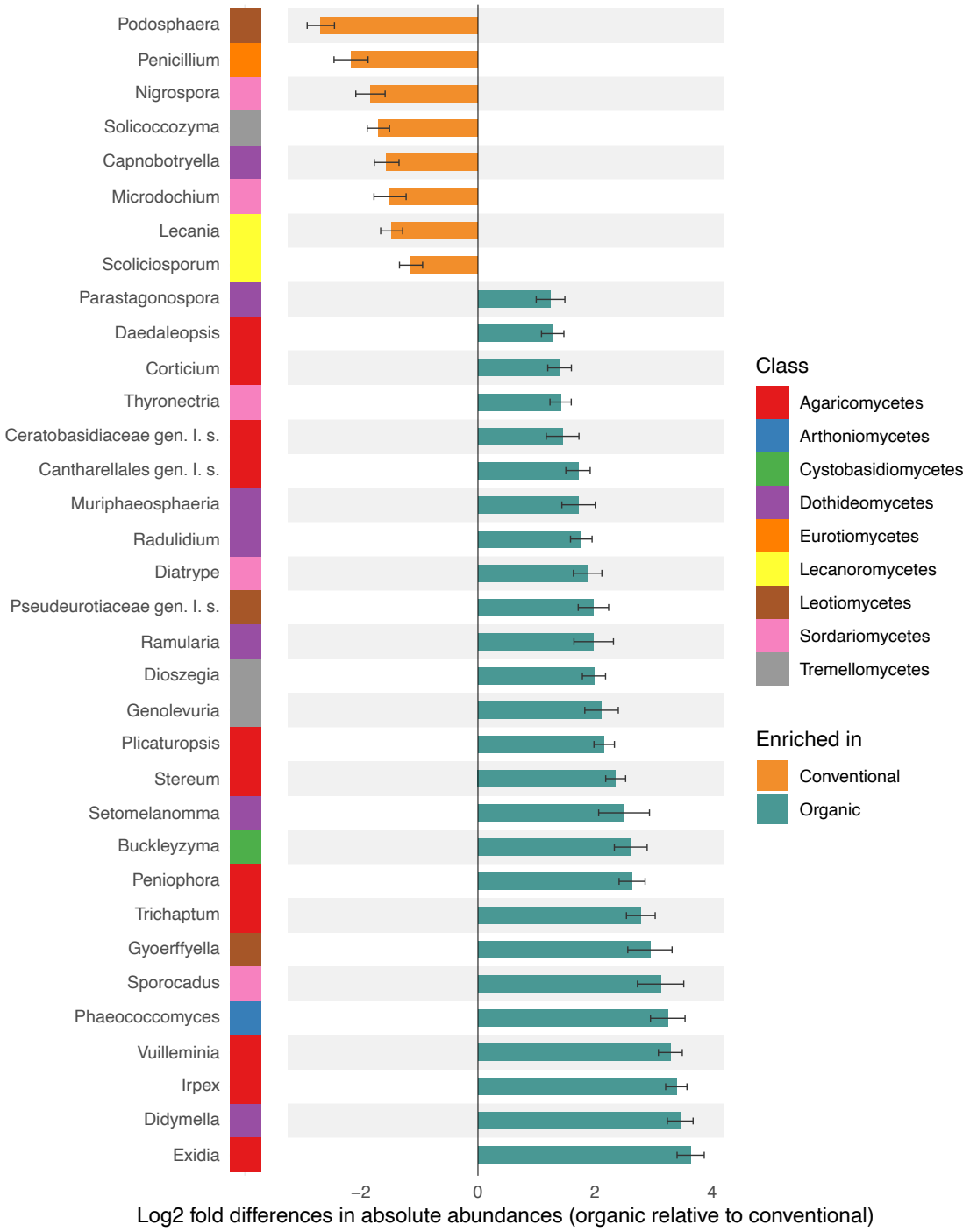

Figure 2: Differential abundance analysis of flower communities comparing changes in absolute abundance of genera across agricultural practice, controlling for cultivar. Only genera with an absolute $\log_2$-fold difference of at least 1.5 and a corrected p-value < 0.01 are shown. Gen. I. s. : Genus Incertae sedis.

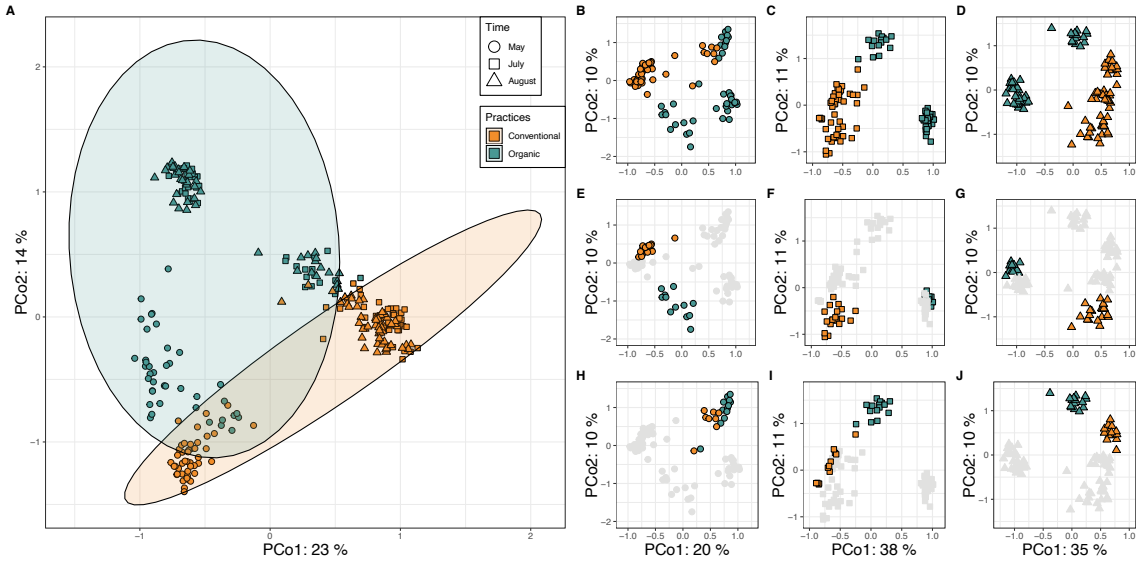

Figure 3. PCoAs on Bray-Curtis dissimilarities performed on leaf samples. **(A)** All leaf samples across sites and sampling months. **(B–D)** Subset of samples collected in May **(B)**, July **(C)**, and August **(D)**, with conventional sites shown in orange and organic sites in green. **(E–G)** Highlighted 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.

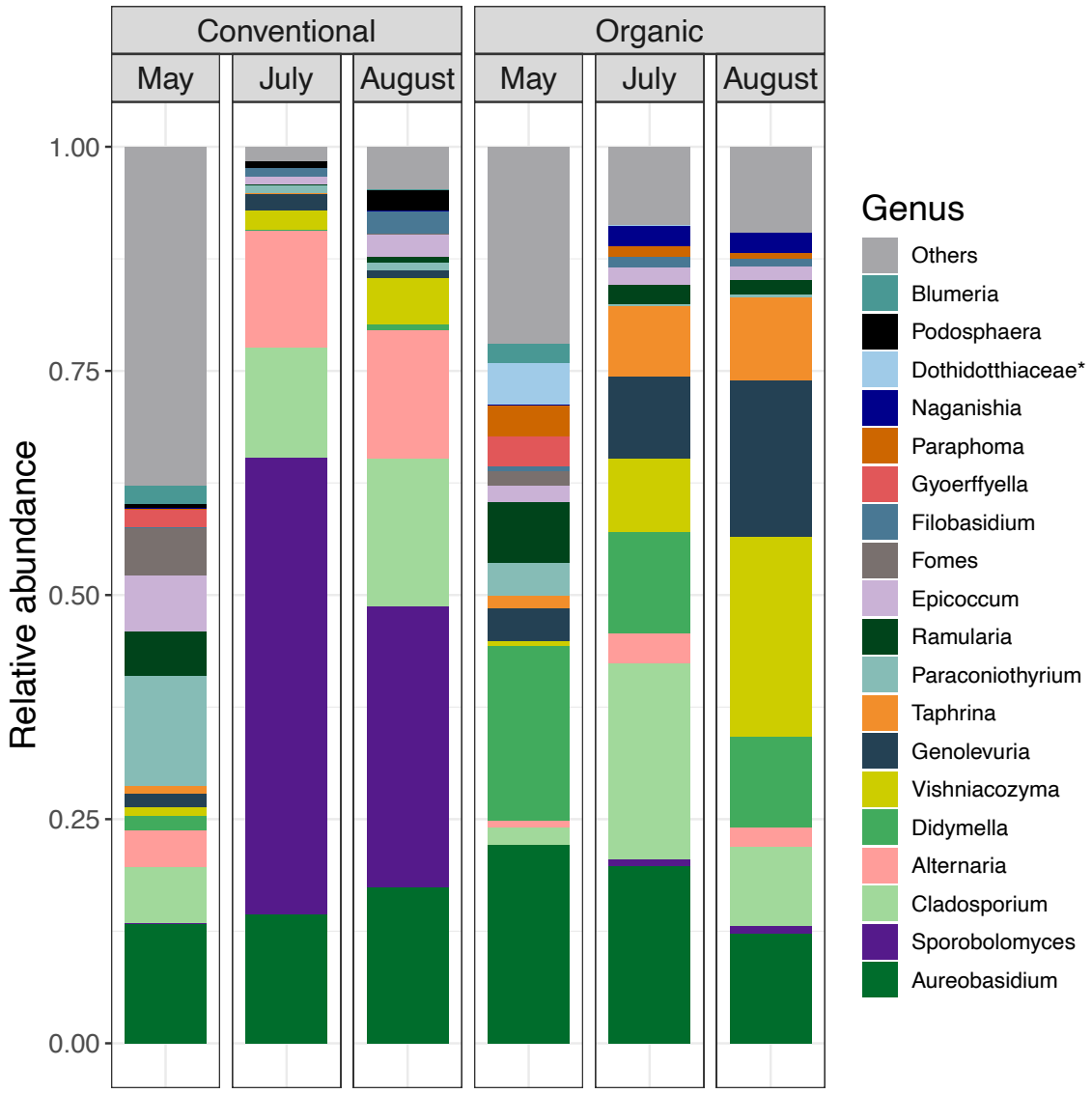

Figure 4. Fungal community composition across all leaf samples (all cultivars). Average relative abundance of the 20 most abundant fungal genera. Taxa not among the top 20 are grouped under "Others." Bar charts are organized by agricultural practice and sampling month (Conventional: n = 45, 48, 54; Organic: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

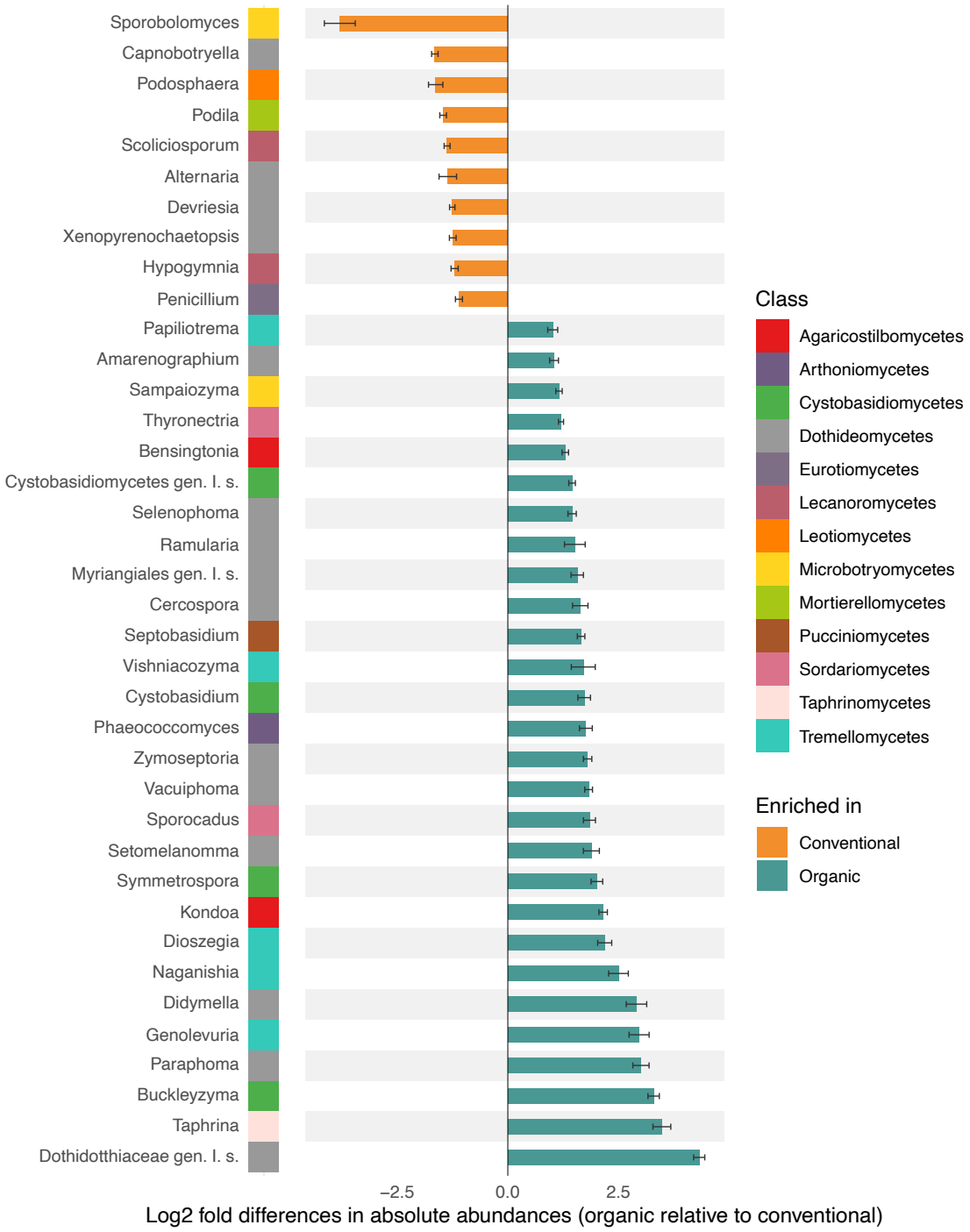

Figure 5: Differential abundance analysis of flower communities comparing changes in absolute abundance of genera across agricultural practice, controlling for cultivar and sampling month. Only genera with an absolute $\log_2$-fold difference of at least 1.5 and a corrected p-value < 0.01 are shown. Gen. I. s. : Genus Incertae sedis.

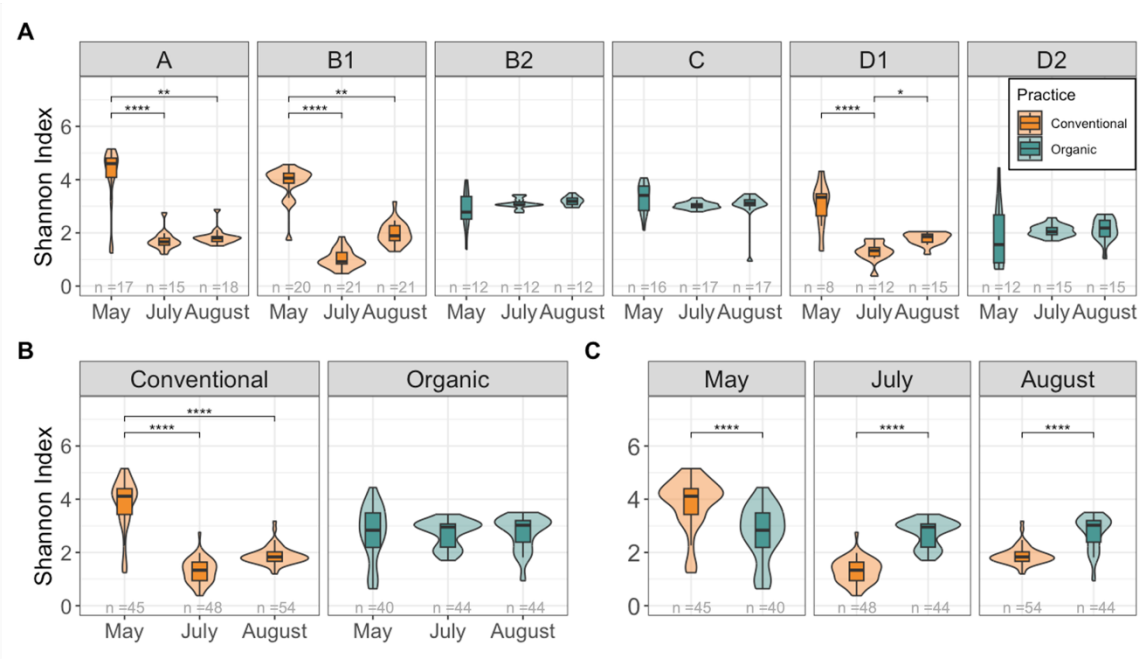

Figure 6. Shannon alpha diversity of leaf samples (all cultivars). **(A)** Alpha diversity across sampling times for each individual site. **(B)** Alpha diversity across sampling times grouped by agricultural practice. **(C)** Comparison of alpha diversity between conventional and organic practices at 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.
Writing – original draft: S.B., J.R-L., A.R. & I.L-L.
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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