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Fungal Biodiversity of Apple Bark, Leaves, Stems, and Fruit Under Rain Shelters with Reduced Fungicide Schedule

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Abstract: The use of rain shelters is a promising agronomic practice to protect crops from rainfall, reducing the need for fungicides to control certain pathogens that take advantage of leaf wetness. However, the combined condition of absence of rain and reduced fungicide schedule can affect the fungal populations, possibly favoring biocontrol agents and/or other pathogens. In this study, the effects this practice on epiphytic and endophytic fungal communities associated with barks, leaves, flowers, and fruits of two apple cultivars (Fuji and Golden Delicious) were evaluated across two seasons. Apple plants were grown under two conditions in a commercial-like orchard: (1) covered by rain shelters with reduced fungicide schedule and (2) uncovered with standard integrated pest management (IPM) schedule. The use of rain shelters combined with reduced fungicide applications affects the overall fungal community structure and their abundance of specific taxa. Leaf epiphytes were the most impacted community, and fungal communities also differed between the two apple cultivars. The use of rain shelters helped reduce fungicide input in the orchard, but it increased the abundance of potential pathogens compared to the IPM in open field conditions, such as powdery mildew and apple scab. Understanding how the plant microbiome responds to new practices that help in reducing fungicides can help developing strategies that avoid the build-up of potentially new pathogens.

Keywords: apple microbiome; fungal communities; crop protection; amplicon sequencing

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1. Introduction

Apple (*Malus* × *domestica* Borkh.) is one of the most widely cultivated fruit crops, with approximately 7500 recognized cultivars worldwide [1]. Despite the commercial relevance, most apple cultivars are susceptible to various diseases, mainly caused by fungi and bacteria, such as apple scab, powdery mildew, and fireblight [2]. Among these, apple scab, caused by *Venturia inaequalis* (Cke.) Wint., is one of the most important diseases, impacting fruit quality and leading to considerable economic losses [3]. In particular, when weather conditions are conducive to the disease, the pathogen inoculum is high, and the cultivar is susceptible; numerous fungicide applications are needed to control

apple scab [4]. However, increasing regulatory constraints in the use of pesticides and growing public concern over chemical residues have stimulated the search for alternative methods to reduce reliance on chemical fungicides while ensuring high-quality and pesticide residue-free fruits [2]. The use of nets, which act as a physical barrier to protect the trees against harsh environmental conditions, is increasing because they improve plant health, enhance fruit quality, and reduce the need for plant protection products [5]. Besides protecting plants from abiotic stresses, such as hail, rain, frost, temperature fluctuations, and solar radiation [6], nets also help against biotic agents, such as birds and insects. The combination of rain shelters on the nets has been employed to protect apple trees from rainwater, reducing the incidence of those fungal pathogens that need leaf wetness for the infection, such as apple scab and post-harvest diseases, particularly in regions like South Tyrol and Switzerland [7]. Likewise, research on the use of rain shelters has shown their effectiveness in reducing disease severity and improving profitability across several crops, such as cherries [8], strawberries [9], grapevine [10], and kiwifruit [11]. Based on this evidence, rain shelters can be considered a promising tool to be integrated into integrated pest management (IPM) programs to reduce the use of fungicides. However, despite their benefits in reducing rainfall on plants and leaf wetness duration, rain shelters may also create a microclimate with higher temperatures and increased humidity during nighttime. Such changes in microclimatic conditions can influence plant physiology and the interactions between plants and the associated microorganisms [2,12]. In addition, the reduction in fungicides could theoretically favor secondary pathogens that are commonly controlled by full fungicide schedules [13]. Plant-associated microbial communities play vital roles in promoting plant growth and health [14], and preserving these communities has been proposed as a possible approach of disease control [15]. Despite the recognized importance of these communities, few studies have evaluated if and how rain shelters can impact plant-associated microbial communities. In particular, Zhang et al. [16] found that rain shelters enhanced the metabolism of carbohydrates, amino acids, and organic compounds in pear trees and increased microbial diversity and richness in the rhizosphere. Likewise, when rain shelters were used alongside soil fertilization, they had a significant effect on bacterial structure, promoting the diversity and quantity of rhizosphere bacteria of *Panax notoginseng* [16]. Moreover, Sui et al. [11] found that rain shelters increased the diversity of epiphytic bacterial and fungal communities on kiwifruit compared to open-field cultivation systems. However, Chen et al. [12] found that rain shelters may not always benefit the soil fertility, microbial carbon-source metabolism, organic matter cycling, or rhizosphere microbial diversity of pear trees, suggesting that the effects of rain shelters on plant-associated microbial communities may vary depending on the host plant. On grapevine, a differential effect of biocontrol agents and chemical treatments on the bacterial and fungal communities of leaves protected from rain compared to unprotected ones was reported [17].

Fungal communities associated with apple trees vary according to the plant tissues (e.g., leaf, flower, fruit, and bark), which represent different microhabitats with specific features that host distinct communities [18]. Factors such as tissue age, orchard location, and disease management can influence the composition of fungal and bacterial communities present on apple bark [19], which can act as reservoirs for plant pathogens that can spread to other host tissues through wind or rain splash during the vegetative season [20]. Furthermore, McLaughlin et al. [21] observed that environmental conditions are key drivers of fungal community structure and diversity on apple fruits. To date, no studies have specifically assessed how the integration of rain shelters in IPM programs can affect the biodiversity of fungal communities associated with apple trees. The aim of this study was to investigate the impact of the use of rain shelters with a minimal fungicide application on the composition and dynamics of fungal communities associated with bark, leaf, flower,

and fruit tissues of two apple cultivars (Fuji and Golden Delicious) in a commercial-like orchard. By comparing fungal epiphytic and endophytic communities sheltered from rain with reduced fungicide treatments (IN) and uncovered and left in open-field orchard conditions with standard IPM (OUT) across multiple seasons, we sought to assess how the use of shelters and the related reduced fungicide treatments influence fungal diversity, potential pathogenic fungi, and the overall health of apple trees.

2. Material and Methods

2.1. Experimental Design and Sample Collection

The research was carried out in a commercial-like orchard located in northern Italy (latitude: N 46.273893, longitude: E 11.027136, altitude: 435 m). The orchard was planted in 2017 with two-year-old clonally propagated apple trees of two cultivars (Golden and Fuji) grafted onto M9 rootstock. In 2019, half of the orchard was covered with a rain shelter (Microtex, patent n. 0001422628) to protect plants from rainfall and to allow a reduced fungicide schedule against apple scab, while the other half was left uncovered as a normal open field. A standard IPM program was applied to the orchard (Table 1 and Table S1) and treatments were applied only when the conditions for the infection of the apple pests and pathogens were met, as indicated in the local IPM guidelines (https://www.provincia.tn.it/Documenti-e-dati/Documenti-di-funzionamento/Disciplinare-di-produzione-integrata-edizione-2023; accessed on 10 March 2023). Because of the protection from rainfall and absence of leaf wetness due to the rain shelter, the covered part (IN) of the orchard did not receive fungicide treatments against apple scab (caused by *Venturia inaequalis*), whereas treatments against powdery mildew (caused by *Podosphaera leucotricha*) were normally applied in the covered part (IN) as in the uncovered part (OUT) (Tables 1 and S1). Hourly temperatures, air relative humidity, rainfall, and leaf wetness were recorded by a meteorological station located nearby. Weekly minimum, maximum, and average temperature and relative humidity, accumulated rain, and leaf wetness were calculated (Table S1). Apple scab and powdery mildew incidence on shoots and fruits were recorded for both years for the Golden Delicious plants and expressed as the percentage of infected leaves for each shoot, using the methodology provided by the guidelines of the European Plant Protection Organization (EPPO, https://pp1.eppo.int/standards/; accessed on 1 March 2019; PP1/152(4), PP1/181(4), PP1/005(3), PP1/069(3)).

Plant samples were collected in triplicate (replicates labeled 1 to 3) in two consecutive seasons (2019 and 2020) at three time points per season (on 2 May, T0, full bloom; 4 July, T1, green fruit; and 17 September, T2, ripe fruit, in 2019 and on 23 April, T3, full bloom; 27 July, T4, green fruit; and 17 September, T5, ripe fruit, in 2020). Each replicate consisted of a pool of five randomly selected plants (plant pool) collected according to a split-plot sampling design in the OUT or IN condition, as previously described [20].

Bark samples consisted of a pool of 30 bark curls (0.5 g) collected from five plants using a fire-sterilized scalpel. From the same plant pool, 50 healthy leaves were randomly collected at all time points, 50 flowers were collected at T0 and T3, while 50 fruits were collected at T1, T2, T4, and T5, according to the phenological stage, as previously described [18]. Samples were placed in sterile plastic bags and transported at 10 °C to the laboratory within 1 h.

Table 1. Disease management of apple orchard in the two growth conditions: open field and rain shelter cover, in 2019 and 2020. X indicates where the product was applied.

Product	Aim of Treatment	Treatment Date	Growth Condition	
			Open Field	Rain Shelter
Score (Difenoconazole)	fungicide	10 May 2019	X	
Nando maxi (Fluazinam)	fungicide		X	
Closer (Sulfoxaflor)	insecticide		X	X
Profile plus	hormones		X	X
Nando maxi (Fluazinam)	fungicide	17 May 2019	X	
Uniammin	leaf fertilizer		X	X
Thiocur forte (Myclobutanil)	fungicide		X	X
Brancher	thinning	22 May 2019	X	X
Dirager	thinning		X	X
Delan (Ditianon)	fungicide	24 May 2019	X	
Arius (Quinoxyfen)	fungicide		X	
Thiopron (Sulfur)	fungicide		X	X
Thiopron (Sulfur)	fungicide	30 May 2019	X	X
Score (Difenoconazole)	fungicide		X	
Nando maxi (Fluazinam)	fungicide		X	
Karatane (Meptyldinocap)	fungicide	13 May 2019	X	
Coragen (Chlorantraniliprole)	insecticide		X	X
Delan (Ditianon)	fungicide		X	
Visir pencotec (Penconazole)	fungicide	28 June 2019	X	X
Uniammin	leaf fertilizer		X	X
Topas (Penconazole)	fungicide	23 July 2019	X	X
Captan Arvesta (Captan)	fungicide		X	
Caolin	caolin		X	X
Calcium	leaf fertilizer		X	X
Captan Arvesta (Captan)	fungicide	1 August 2019	X	
Epik (Acetamiprid)	insecticide		X	X
Calcium	leaf fertilizer		X	X
Captan Arvesta (Captan)	fungicide	19 August 2019	X	
Calcium	leaf fertilizer		X	X
Magnesium	leaf fertilizer		X	X
Captan Arvesta (Captan)	fungicide	5 September 2019	X	
Obsthormon 24A	pre-harvest fruit drop control		X	X
Calcium	leaf fertilizer		X	X
Trebon UP (Etofenprox)	insecticide	10 September 2019	X	X
Urea	fungicide, leaf fertilizer	28 October 2019	X	X
Polysulfide	fungicide	29 October 2019	X	
Copper	fungicide	27 March 2020	X	X
Mineral oil	insecticide		X	X
Trebon UP (Etofenprox)	insecticide		X	X
Cidely (Cyflufenamid)	fungicide	10 April 2020	X	X
Phosphonate	fungicide		X	
Teppeki (Flonicamid)	insecticide		X	X
Banjo (Fluazinam)	fungicide	18 April 2020	X	
Cidely (Cyflufenamid)	fungicide		X	
Boron	leaf fertilizer		X	X
Urea	leaf fertilizer		X	X
Banjo (Fluazinam)	fungicide	25 April 2020	X	
Polyram (Metiram)	fungicide		X	
Promalin	thinning		X	X
Banjo (Fluazinam)	fungicide	28 April 2020	X	
Promalin	thinning	1 May 2020	X	X
Banjo (Fluazinam)	fungicide		X	
Sercadis (Fluxapyroxad)	fungicide	9 May 2020	X	
Delan (Ditianon)	fungicide		X	
Closer (Sulfoxaflor)	insecticide		X	X
Delan (Ditianon)	fungicide	14 May 2020	X	

Table 1. *Cont.*

Product	Aim of Treatment	Treatment Date	Growth Condition	
			Open Field	Rain Shelter
Score (Difenoconazole)	fungicide		X	
Cidely (Cyflufenamid)	fungicide		X	X
Thiopron (Sulfur)	fungicide		X	X
Gerlagib	hormone		X	X
Banjo (Fluazinam)	fungicide	18 May 2020	X	
Thiopron (Sulfur)	fungicide		X	X
Sercadis (Fluxapyroxad)	fungicide	22 May 2020	X	
Delan (Ditianon)	fungicide		X	
Banjo (Fluazinam)	fungicide	13 June 2020	X	
Cidely (Cyflufenamid)	fungicide		X	X
Ethrel	hormone		X	X
Score (Difenoconazole)	fungicide		X	
Karathane (Meptyldinocap)	fungicide	25 June 2020	X	X
Affirm (emamectin benzoate)	insecticide		X	X
Banjo (Fluazinam)	fungicide		X	
Merpan (Captan)	fungicide	28 July 2020	X	
Carsol	leaf fertilizer		X	X
Merpan (Captan)	fungicide	7 August 2020	X	
Carsol	leaf fertilizer		X	X
Merpan (Captan)	fungicide	5 September 2020	X	
Obsthormon 24A	pre-harvest fruit drop control		X	X
Carsol	leaf fertilizer		X	X

2.2. DNA Extraction, Amplification, and Sequencing

The method described by [18] was used for the amplicon sequencing analysis of plant-associated fungal communities. Briefly, to analyze fungal epiphytic communities, leaf, flower, and fruit samples were washed in a sterile bag containing 500 mL NaCl 0.85% supplemented with 100 µL/L Tween 80 and homogenized under orbital shaking for 15 min at 120 rpm [18]. Each suspension was filtered with a sterile cheesecloth, collected in 50 mL tubes, centrifuged at 10,000× *g* for 20 min at 4 °C. The resulting pellets of leaf washing, flower washing, and fruit washing were stored at −20 °C until DNA extraction to analyze fungal epiphytic communities. Barks, washed leaves, washed flowers, and peels of washed fruits, which were obtained under sterile conditions using a sterilized scalpel, were frozen in liquid nitrogen to analyze mainly fungal endophytic communities. Each sample (0.5 g) was ground in sterile stainless steel jars containing 2.5 mL of a cold (4 °C) sterile isotonic solution (0.85% NaCl) using a mixer–mill disruptor (MM 400, Retsch, Germany) at 25 Hz for 45 s [18]. Aliquots (500 µL) of the resulting ground bark, leaf, flower, and fruit samples were stored at −20 °C until DNA extraction. The genomic DNA was extracted using the FastDNA spin kit for soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The concentration of the DNA samples was determined using a Qubit dsDNA Quantification Assay Kit (Invitrogn, Thermo Fisher Scientific, Waltham, MA, USA). The quantity of each DNA sample was adjusted to 5.0 ng/µL and fungal internal transcribed spacer 2 (ITS2) was amplified with a nested PCR approach, which is used to limit the amplification of host DNA in amplicon sequencing studies of plant endophytes [22]. Briefly, the first fungal ITS amplification was carried out with the primer ITS1 forward (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and TW13 reverse (5′-GGTCCGTGTTTCAAGACG-3′), which amplifies fungal ITS and part of the ribosomal large subunit [23]. The second PCR amplification was adapted from [24] using the product of the first amplification (3 µL) with equimolar mixes of the ITS3Mix forward primers (5′-CATCGATGAAGAACGCAG-3′, 5′-CAACGATGAAGAACGCAG-3′, 5′-CACCGATGAAGAACGCAG-3′, 5′-CATCGATGAAGAACGTAG-3′, and 5′-CATCGAT

GAAGAACGTGG-3′) [24] and the ITS4Mix reverse primers (5′-TCCTCCGCTTATTGATA TGC-3′ and 5′-TCCTSSSCTTATTGATATGC-3′) to increase coverage of the fungal kingdom [25]. All primers included the Illumina adapters (5′-TCGTCGGCAGCGTCAGATG TGTATAAGAGACAG-3′ and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′ in the forward and reverse primers, respectively). Fungal ITS amplifications were obtained using the FastStart High-Fidelity PCR system (Roche) as described previously [18] with 30 cycles of amplification in the first and second ITS amplification (95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s). All reactions were carried out in duplicate and pooled after amplification.

DNA indexing, quantification, and library preparation for the Illumina MiSeq sequencing (PE300) were carried out as previously described [20], and sequences of the 360 samples across two seasons (2019 and 2020), with three time points per season (T0, T1, and T2 of 2019 season, T3, T4, and T5 of 2020 season), two growth conditions (IN and OUT samples), four ground (bark grinding, leaf grinding, flower grinding, and fruit grinding) and three washed (leaf washing, flower washing, and fruit washing) tissues, and three replicates, were obtained.

2.3. Amplicon-Sequencing Data Processing

Raw reads were processed with MICCA (v.1.7.2) software [26]. The paired end reads were merged using VSEARCH (https://github.com/torognes/vsearch; accessed on 1 Decembre 2020), with a minimum overlap length of 100 and a maximum number of allowed mismatches of 32. Primers were trimmed using Cutadapt v.1.18 [27], and merged reads shorter than 150 bp or with an expected error rate higher than 0.5% were removed. Filtered sequences were clustered into Amplicon Sequence Variants (ASVs) using the UNOISE (https://doi.org/10.1101/081257; accessed on 1 Decembre 2020) algorithm available in MICCA. Taxonomic assignment was carried out using the RDP classifier v. 2.13 [28] against the fungal UNITE + INSD v.8.3 database [29,30].

2.4. Statistical Analysis

The statistical analysis of the sequencing data was performed with the phyloseq R package, ver. 1.44.0 [31], following the protocol by [32]. The sequence reads were rarefied at 90% of the smallest samples, alpha diversity indices were calculated, and multivariate analysis through Principal Coordinates Analysis (PCoA) based on the Bray–Curtis dissimilarity distance matrix was produced. The PERMANOVA test was applied through vegan R package version 2.6-5 and the differentially abundant ASVs were calculated with DESeq2 R package ver. 1.28.1 [33]. The graphs were generated with ggplot2 R package ver. 3.4.4 [34] and microViz ver. 0.11.0 [35].

3. Results

3.1. Weather Conditions and Incidence of Apple Scab and Powdery Mildew

The average temperature varied from 1 °C to 21 °C in 2019 and from 0.7 °C to 20 °C in 2020, whereas relative humidity ranged from 50% to 90% in 2019 and from 58.5% to 85% in 2020. In the OUT condition, the sum of rainfall was 1412.5 mm and 1151 mm in 2019 and 2020, respectively (Table S1). In the IN condition, due to the rain shelter, the rainfall on plants was prevented and the leaf wetness was negligible, and a reduced fungicide schedule was therefore applied (Table 1 and S1).

In 2019, the incidence of apple scab and powdery mildew was similar under OUT and IN conditions (Mann–Whitney test, $p > 0.05$). For apple scab, the incidence on leaves (percentage of infected leaves for each shoot) was 5.15% ± 11.92% and 1.21% ± 4.53% in the IN and OUT conditions, respectively. For powdery mildew, the incidence was

2.91% ± 7.29% and 1.22% ± 3.32% in the IN and OUT conditions, respectively. However, in 2020, the incidence of apple scab and powdery mildew was higher inside the rain shelter compared to outside (Mann–Whitney test, $p \leq 0.05$). The incidence of apple scab was 4.20% ± 8.52% and 0.10% ± 1.00% in the IN and OUT conditions, respectively, while the incidence of powdery mildew was 9.84% ± 23.36% and 0.48% ± 3.09% in the IN and OUT conditions, respectively. No symptoms were observed on fruits in both seasons.

3.2. Fungal Community Composition of Apple Plant Tissues

ITS amplicon sequencing of DNA extracted from bark, flower, leaf, and fruit tissues of apple trees resulted in 54,378,141 raw reads (151,050 ± 33,677 per sample), and 45,300,325 of them remained after filtering and denoising steps (Table S2). A total of 18,624 fungal ASVs (Table S3) belonging to seven phyla were detected (Figure 1). The fungal communities were dominated by Ascomycota (49.98% ± 0.04%), followed by Basidiomycota (17.92% ± 0.02%), and the other phyla had a mean relative abundance lower than 0.10%, such as Chytridiomycota, Entomophtoromycota, Mortierellomycota, Mucoromycota, and Rozellomycota.

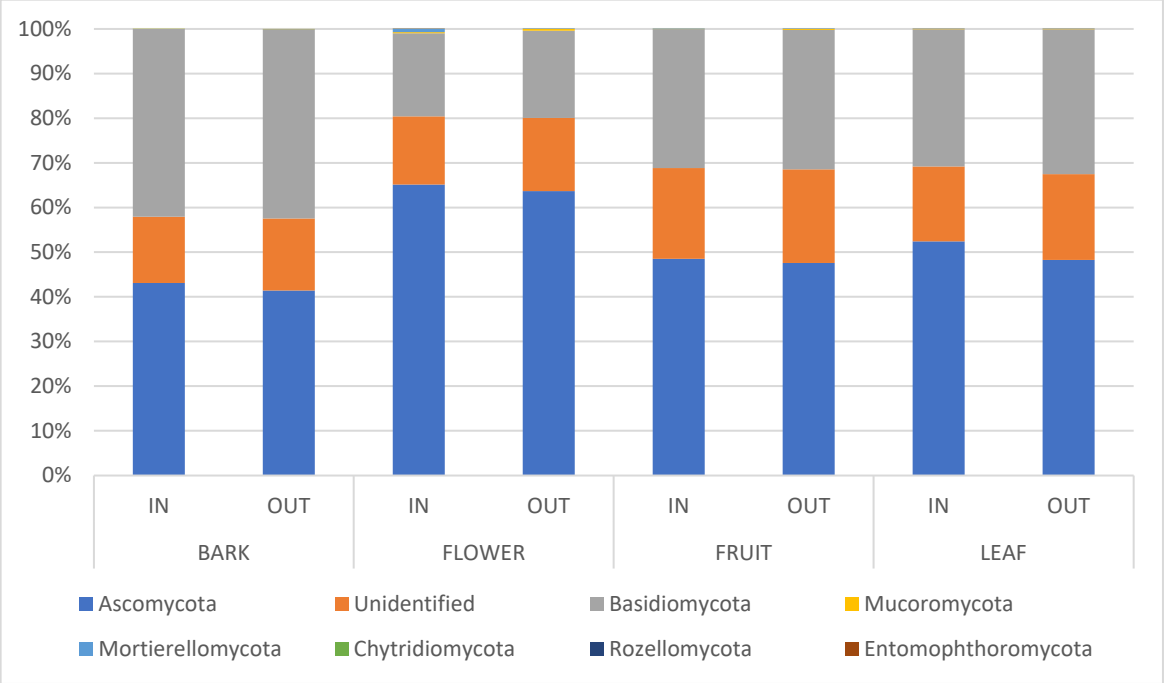

Figure 1. Relative abundances of fungal communities at phylum level for each apple plant tissue of Golden and Fuji cultivars, sampled inside (IN) and outside (OUT) the rain shelter. IN and OUT conditions were treated according to integrated pest management guidelines, with IN condition not receiving fungicide treatments against apple scab.

The phylum Ascomycota was composed mainly of Dothideomycetes (41.88% relative abundance), Leotiomycetes (3.45%), and Sordariomycetes (2.59%). Moreover, Microbotryomycetes (17.83%), Tremellomycetes (4.82%), Cystobasidiomycetes (4.78%), and Agaricomycetes (3.03%) were the main classes of Basidiomycota. The most abundant ascomycete families were Aureobasidiaceae (14.62%), Cladosporiaceae (12.11%), Didimellaceae (5.51%), Pleosporaceae (4.38%), and Erysiphaceae (2.96%), while the most abundant families of basidiomycetes were Sporidiobolaceae (17.18%), Cystobasidiaceae (1.90%), Buckleyzimaceae (1.58%), and Bulleribasidiaceae (1.42%). Considering all the plant samples in all conditions, the most abundant genera were *Aureobasidium* (14.58%), *Cladosporium* (12.11%), *Alternaria* (3.98%), *Podosphaera* (2.89%), *Sporidiobolus* (2.43%), *Cystobasidium* (1.90%), *Buck-*

leyzyma (1.58%), *Rhodotorula* (1.46%), *Vishniacozyma* (1.37%), *Symmetrospora* (1.21%), and *Filobasidium* (1.16%).

The composition of the most abundant fungal families of epiphytes and endophytes varied according to the time of sampling, cultivar, and growth conditions (Figure 2, Table S4). Among the leaf epiphytes, the relative abundance of Erysiphaceae (at T3 and T5) and Aureobasidiaceae (at T4) was higher in the IN condition compared to the OUT condition, while that of Cystobasidiaceae and Buckleyzymaceae was lower the IN condition compared to the OUT condition at T3 and T4 (differential abundance analysis based on the negative binomial distribution, adjusted by false discovery rate, $p < 0.01$; Figure 2, Table S4). Regarding the leaf endophytes, the Pleosporaceae family was more abundant in the IN condition (differential abundance analysis based on the negative binomial distribution, adjusted by false discovery rate, $p < 0.01$; Figure 2, Table S4). Looking at fruit endophytes, the major differences were at T2, when the abundance of Steccherinaceae and Physalacriaceae was higher in the IN condition compared to the OUT condition, while Entylomataceae and Botryosphaeriaceae were more abundant in the OUT condition (differential abundance analysis based on the negative binomial distribution, adjusted by false discovery rate, $p < 0.01$; Figure 2, Table S4).

The presence of the rain shelter and the reduced fungicide application did not affect the alpha diversity (richness and evenness) of fungal endophyte and epiphyte communities in the IN condition across the two years and different tissues, showing no significant difference compared to the OUT condition (Figure 3). However, a significant difference in beta diversity of epiphytes and endophytes between the IN and OUT conditions was observed (PERMANOVA, $p \leq 0.05$; Figure 4 and Table S5).

The differential abundance analysis revealed that 15 ASVs (annotated at genus level and with an abundance > 0.05% in one of the two conditions) were more abundant inside the rain shelter compared to outside (Table 2), and they included *Alternaria*, *Claviceps*, *Cladosporium*, *Lophiostoma*, *Podosphaera*, *Pseudopithomyces*, *Venturia*, and *Vishniacozyma*. Leaf epiphytes were the most impacted community in the IN and OUT conditions, especially during the second year of testing, where their composition significantly differed between the IN and OUT conditions across all three sampling times (T3, T4, and T5; PERMANOVA $p < 0.05$; Table S5). Differential abundance analysis was applied to determine which fungal taxa of the leaf epiphyte community varied in the two growth conditions and identified the genera *Prosthemium* and *Aureobasidium* as more abundant in the OUT condition (0.17% OUT vs. 0.00% IN, and 0.05% OUT vs. 0.02% IN, respectively; according to negative binomial distribution, adjusted $p < 0.05$), while *Claviceps* and *Venturia* were more abundant in the IN condition (0.20% IN vs. 0.17% OUT, and 0.07% IN vs. 0.00% OUT; according to negative binomial distribution, adjusted $p < 0.05$).

Moreover, the fungal community composition of Fuji plants differed from that of Golden Delicious plants (PERMANOVA, $p = 0.001$ Figure 4 and Table S5), and the differential abundance analysis corroborated differences between the two apple cultivars. In particular, *Botrytis*, *Heterobasidion*, *Hygrophorus*, *Pseudopithomyces*, *Starmerella*, *Symmetrospora*, *Tilleptiopsis*, *Venturia*, and *Vishniacozyma* were more abundant in Golden Delicious compared to Fuji, while *Diplodia*, *Fusarium*, and *Podosphaera* were more abundant in Fuji compared to Golden Delicious (Table 3).

Figure 2. Composition of the most abundant fungal families of endophytes and epiphytes of different apple tissues. Taxa composition is reported for each plant cultivar (Golden or Fuji), time of sampling (T0, T1, T2, T3, T4; and T5), and growth condition, such as inside (IN) or outside (OUT) the rain shelter.

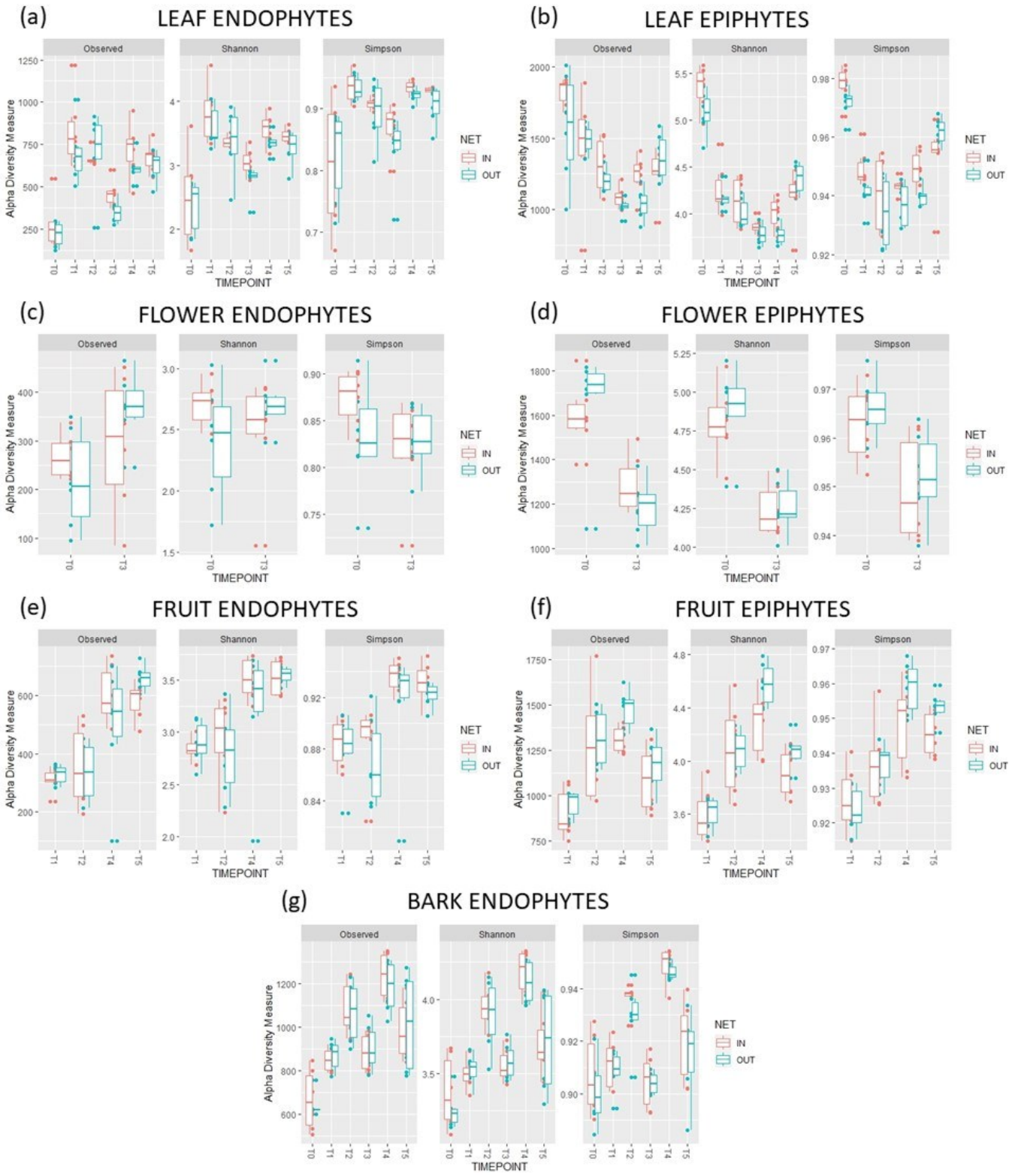

Figure 3. Richness (Observed Species) and diversity indices (Shannon and Simpson) of endophytes and epiphytes of different apple tissues. Taxa composition is reported for each time of sampling (T0, T1, T2, T3, T4, and T5) and growth condition in the IN condition (rain shelter + reduced fungicide applications) and in the OUT condition (open field + standard IPM program).

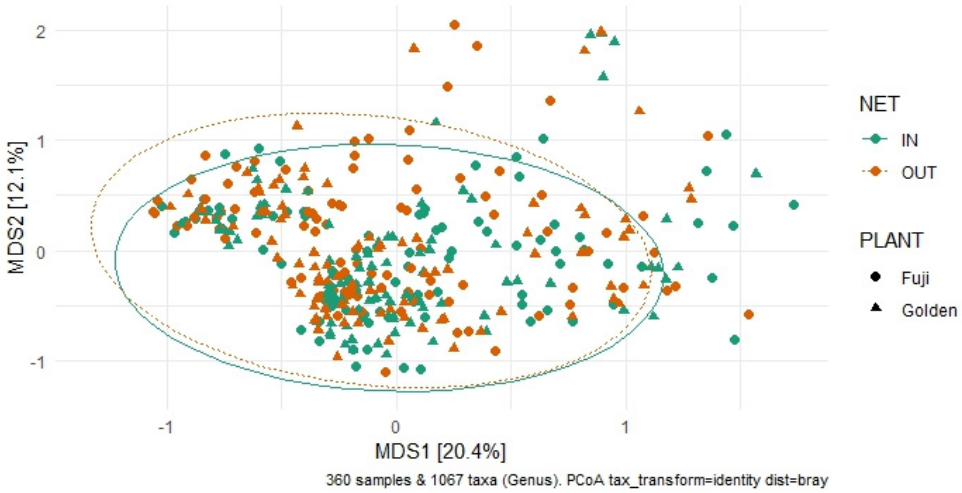

Figure 4. Principal Coordinates Analysis (PCoA) based on the Bray–Curtis dissimilarity distance matrix of Illumina sequencing data of fungal communities in apple tree tissues (Fuji cultivar in round symbol and Golden cultivar in triangle symbol) in the IN condition (rain shelter + reduced fungicide applications; green color) and in the OUT condition (open field + standard IPM program; orange color).

Table 2. List of fungal ASVs with a statistically significant differential abundance (differential abundance analysis based on the negative binomial distribution, adjusted by false discovery rate) between the apple trees in the IN condition (rain shelter + reduced fungicide applications) and in the OUT condition (open field + standard IPM program).

Genus	ASV Code	IN	OUT	*p* Adjusted
Alternaria sp.	DENOVO30	0.82%	0.67%	2.4×10^{-11}
Alternaria sp.	DENOVO11	3.24%	2.70%	2.55×10^{-6}
Cladosporium sp.	DENOVO6	5.25%	4.29%	3.78×10^{-9}
Cladosporium sp.	DENOVO129	0.10%	0.08%	0.000784
Claviceps sp.	DENOVO109	0.07%	0.02%	0.00176
Claviceps sp.	DENOVO107	0.08%	0.01%	0.005313
Lophiostoma sp.	DENOVO124	0.08%	0.01%	1.48×10^{-5}
Pseudopithomyces sp.	DENOVO32	0.41%	0.25%	6.78×10^{-6}
Pseudopithomyces sp.	DENOVO71	0.12%	0.08%	6.78×10^{-6}
Podosphaera sp.	DENOVO90	0.10%	0.07%	0.003134
Podosphaera sp.	DENOVO160	0.07%	0.05%	0.000873
Venturia sp.	DENOVO88	0.14%	0.00%	0.006303
Vishniacozyma sp.	DENOVO37	0.39%	0.23%	0.005462
Vishniacozyma sp.	DENOVO47	0.32%	0.12%	0.004756

Table 3. List of fungal ASVs with a statistically significant differential abundance (differential expression analysis based on the negative binomial distribution, adjusted by false discovery rate) between Fuji cultivar (Fuji) and Golden Delicious cultivar (Golden).

Genus	ASV Code	Fuji	Golden	*p* Adjusted
Botrytis sp.	DENOVO45	0.16%	0.35%	0.00034
Diplodia sp.	DENOVO48	0.40%	0.04%	2.99×10^{-7}
Fusarium sp.	DENOVO79	0.15%	0.05%	0.00578
Heterobasidion sp.	DENOVO189	0.03%	0.06%	0.00876
Hygrophorus sp.	DENOVO28	0.09%	1.09%	4.06×10^{-18}
Podosphaera sp.	DENOVO10	3.03%	1.84%	0.00397
Podosphaera sp.	DENOVO90	0.13%	0.05%	0.00004
Pseudopithomyces sp.	DENOVO32	0.18%	0.48%	0.00018
Starmerella sp.	DENOVO62	0.03%	0.17%	0.00510
Symmetrospora sp.	DENOVO83	0.03%	0.13%	6.18×10^{-7}
Symmetrospora sp.	DENOVO38	0.10%	0.43%	1.41×10^{-6}
Tilletiopsis sp.	DENOVO85	0.02%	0.18%	2.55×10^{-7}
Venturia sp.	DENOVO227	0.01%	0.05%	0.00306
Vishniacozyma sp.	DENOVO47	0.11%	0.33%	0.00356

4. Discussion

The use of rain shelters has become a promising agronomic practice to protect crops from rainfall, thereby reducing the need for fungicides, particularly against diseases that are promoted by rain and long periods of leaf wetness. This practice can be integrated in IPM programs to support sustainable agricultural production [36]. A key aspect of plant protection lies in understanding how agronomic practices in IPM, influence plant microbiome, as microorganisms play a significant role in plant health. As previous studies have shown that agronomic practices can significantly affect the apple tree-associated microbiome [19,37,38], this work specifically investigates how rain shelters in combination with reduced fungicide use impact fungal diversity. In particular, the purpose of our study was to evaluate the combined impact of rain shelter and the reduced fungicide use as part of an IPM program, specifically aimed at controlling pathogens, like apple scab, that are promoted by rain and leaf wetness, with reduced fungicide schedule on two apple cultivars in a commercial-like orchard. Thus, our experimental set-up does consider the separated effects because the reduced fungicide input is a consequence of the rain shelter.

The fungal community composition aligns with previous findings on the apple microbiome from the same region [18] and includes ASVs taxonomically annotated as potential antagonists (e.g., *Aureobasidium* sp.) and potential phytopathogens (e.g., *Alternaria* sp., *Cladosporium* sp., and *Podosphaera* sp.). Among the most abundant taxa, seven yeast genera were identified (*Sporidiobolus*, *Cystobasidium*, *Buckleyzyma*, *Rhodotorula*, *Vishniacozyma*, *Symmetrospora*, and *Filobasidium*), many of them belonging to Basidiomycetes. This is in agreement with previous findings on the prevalence of basidiomycetous yeasts on the phylloplane [39,40] and on apple fruits [41]. Phylloplane yeasts exhibit extensive biodegradative activities; they can assimilate many plant constituents, benefit from plant exudates, compete for nutrients, and protect the plant against phytopathogenic fungi [42]. Among the yeast genera, *Vishniacozyma* was significantly more abundant in the IN condition compared to the OUT condition, and this genus was also found to be more abundant in Golden Delicious compared to Fuji, suggesting its adaptability to specific environmental conditions.

The results indicate that the rain shelter combined with the reduced fungicide applications affects both the overall fungal community structure and the abundance of specific taxa, and the effect could be derived from a combination of the reduced fungicide input and altered microclimate conditions under the rain shelter (e.g., slight increases in temperature and/or humidity, changes in sunlight intensity, leaf wetness, wind intensity, and inoculum spreading). In particular, apple plants in the IN condition showed an increased abundance of ASVs taxonomically annotated as potential fungal pathogens. For example, ASVs of the genus *Podosphaera*, which includes the causal agent of powdery mildew, were more abundant in the IN condition compared to the OUT condition, in agreement with the powdery mildew disease assessment in 2020. The *Podosphaera* abundance was higher in the IN condition compared to OUT condition although fungicides against this pathogen were applied also in the IN condition. Likewise, the incidence of powdery mildew was previously found to be higher under rain shelters, reaching levels over 70% on Golden Delicious plants [2]. Similarly, a study on grapevines reported an increase in powdery mildew severity in plants grown under rain shelters compared to control conditions without fungicide applications [10]. This was attributed to the humid microclimate without reaching leaf wetness under the rain shelters that favored spore germination and infection [2,43]. Additionally, this phytopathogen thrives better in shaded conditions, which are present under rain shelters due to reduced irradiation compared to open-field conditions [10]. In addition, ASVs annotated as *Venturia* sp. were more abundant in the IN condition compared to the OUT condition, in agreement with the higher incidence of apple scab in the IN condition compared to the OUT condition in 2020, indicating that conditions

for apple scab infection, although minimal, can occur also in the IN condition or that the absence of specific fungicides let larger *V. inaequalis* populations develop. Thus, avoiding the application of fungicides against apple scab under rain shelters could be too risky because some leaves close to the net can still become wet during heavy lateral rains and incite infections, although to a limited extent [2]. This suggests that rain shelters can effectively control *Venturia* sp. in commercial cultivation without fungicide applications, although the resulting efficacy may be slightly lower compared to a standard IPM program. In addition, it should be noted that the effectiveness of rain shelters with a reduced fungicide input depends on the apple cultivar susceptibility and the disease pressure in the orchard [2]. Other fungal genera, such as *Cladosporium* and *Alternaria*, were more abundant in the IN condition. Fungi belonging to these genera are associated with core rot, a post-harvest or post-storage internal dry rot of apple fruits [44].

Despite the increase in the relative abundance of ASVs taxonomically annotated as potential phytopathogens in the IN condition, the rain shelter with the reduced fungicides also had positive effects, promoting the abundance of putative antagonists such as *Vishniacozyma* that could include possible biocontrol agents [45]. On the other hand, *Aureobasidium*, a resilient black yeast-like fungus resistant to desiccation and UV radiation [46], was more abundant in the epiphytic community in the OUT condition compared to the IN condition, suggesting that the environmental conditions outside the rain shelters could limit the growth of other fungi but not *Aureobasidium*. Similarly, the genus *Prosthemium*, a common member of the microbiome of hardwood tree branches [47], was more abundant in the OUT condition. *Prosthemium* is considered as endophytic or epiphytic taxa on leaves of Betulaceae [48]. This genus is scarcely found in the apple microbiome and it was observed as an abundant taxa of fungal endophytes in apple replant disease roots [49], although it has not been associated with the cause of the disease.

In our study, the fungal community also differed between the two apple cultivars. The genus *Tilletiopsis*, which includes potential causal agents of the white haze postharvest disorder [50], was more abundant in Golden Delicious compared to Fuji, along with *Botrytis* sp. and *Venturia* sp. On the other hand, *Diplodia*, a genus comprising species reported to cause apple diseases (e.g., frog-eye leaf spot, black rot, dieback, and cankers) [51] was more abundant in Fuji, along with the pathogenic genera *Podosphera* and *Fusarium*. These findings reflect the influence of plant genotype on associated endophytic microbial communities in different environmental conditions and seasons, as observed in other studies on apple rootstock and scion combinations [52,53]. Many reports demonstrating that apple plant genotypes differ in their associated microbiota at the cultivar level [54]. Moreover, certain cultivars of apple showed more diversity of the microbiome than others [55]. In the development of some pathogens, such as *V. inaequalis*, the effects of cultivar susceptibility were observed in experimental orchards without fungicide protection [56,57], classifying the variety Golden Delicious in a broad group considered moderately susceptible to apple scab. Furthermore, commercial apple cultivars have different levels of disease resistance to apple powdery mildew [58]. According to Biggs et al. [59], there is no evidence that resistance to scab is correlated with resistance to powdery mildew; while some scab-resistant cultivars possess additional resistance to powdery mildew, many other cultivars do not.

The ability of nets to modify the environmental conditions with large effects on the light environment, wind speed, and leaf wetness and the tendency for lower air humidity values and higher air temperature [60] might have important effects in the fungal communities exposed to them. In fact, the epiphytic communities that were more exposed were more affected, whereas the endophytic communities that exhibit a more protective habitat in plant tissues were less impacted by the environmental conditions [61].

5. Conclusions

In conclusion, the use of rain shelters combined with reduced fungicide applications in IPM programs can influence the structure and composition of fungal communities on apple trees, primarily affecting epiphytic fungi on leaves. While the use of rain shelters helps in reducing fungicide input in the orchard, it may increase the abundance of ASVs taxonomically annotated as potential pathogens compared to the IPM in open-field conditions, most probably because of the lower fungicide input. Therefore, incorporating additional biological control methods alongside rain shelters could reduce the risk of the build-up of pathogen populations and/or improve disease management without increasing the use of chemical fungicides. Although further studies are needed to quantify the independent effects of rain shelters and the reduction in specific fungicides in various environments, our study demonstrates that the plant microbiome responds significantly to changes in management practices. This finding highlights the importance of including such studies to improve sustainable apple production and the development of optimized pathogen control strategies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture15010017/s1, Table S1: Disease management and climatic data of the 2019 and 2020 seasons; Table S2: Raw reads obtained with ITS amplicon sequencing of DNA extracted from bark, flower, leaf, and fruit tissues of apple trees; Table S3: Fungal ASVs obtained with ITS amplicon sequencing of DNA extracted from bark, flower, leaf, and fruit tissues of apple trees; Table S4: Differential abundance analysis at family level based on the negative binomial distribution, adjusted by false discovery rate between inside the rainshelter and outside the rain shelter for each fungal community, at each timepoint; Table S5: Results from PERMANOVA test (adonis2 function in vegan R package) for the fungal communities between plants inside and outside the rainshelter.

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