



Characteristics of fungal communities and the sources of mold contamination in mildewed tobacco leaves stored under different climatic conditions

Jiayi Zhou¹ · Yu Cheng¹ · Lifei Yu^{1,2} · Jian Zhang¹ · Xiao Zou¹

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Abstract

Tobacco mildew is a common postharvest problem caused by fungal growth. It can directly decrease product quality and cause serious economic loss in the tobacco industry. However, the fungal community characteristics of mildewed tobacco leaves and the related influencing factors remain unknown. Here, next-generation sequencing was used to characterize the fungal communities present in mildewed and healthy tobacco leaves stored under three different climatic conditions. Mildewed leaves showed a higher pH and total nitrogen content as well as a lower carbon nitrogen ratio than healthy leaves. Fungal diversity and richness were significantly lower in the mildewed tobacco leaves than in healthy tobacco leaves, with saprophytic fungi such as *Xeromyces*, *Aspergillus*, and *Wallemia* being the dominant molds. Network analysis showed that the complexity, connectivity, and stability of the fungal network were significantly poorer in heavy mildew tobacco leaves than in healthy leaves. NMDS and PERMANOVA analysis showed that the distribution of fungal communities in warehoused tobacco leaves differed significantly across different regions, and temperature and humidity were the key factors affecting these differences. Mildew-causing fungi were significantly enriched in tobacco leaf samples collected in the period between the completion of flue-curing and the start of pre-re-curing. This study demonstrated that mildew is an irreversible process that destroys the balance of the tobacco ecosystem, and that environmental factors play important roles in shaping fungal communities in tobacco leaves.

Key points

- The diversity and composition of the fungal communities in mildewed tobacco leaves were significantly different from those in healthy tobacco leaves.
- Climatic factors may play an important role in shaping fungal communities in tobacco leaves.
- Tobacco leaves were most vulnerable to mold contamination between the post-flue-curing and pre-re-curing period.

Keywords Tobacco mildew; · Fungal community; · Co-occurrence network; · Climatic factors; · Mold contamination

Introduction

Fungi play an important role in the terrestrial ecosystem as decomposers, mutual symbionts, and pathogens, and they often contribute to nutrient cycling, bioconversion, and energy flow (Treseder and Lennon 2015; Boddy 2016; Tedersoo et al. 2018). However, fungi are the main cause of food spoilage and mildew or decay in stored crops because they secrete extracellular enzymes that can degrade food and help in fungal invasion. Fungal growth reduces the quality of vegetables, fruits, coffee, tobacco, medicinal materials, and crops and thereby cause economic losses (Halt 1998). Mildew

✉ Xiao Zou
xzou@gzu.edu.cn

¹ Department of Ecology/Institute of Fungus Resources, College of Life Sciences, Guizhou University, Guiyang, China

² The Key Laboratory of Plant Resource Conservation and Germplasm Innovation in Mountainous Region (Ministry of Education), Collaborative Innovation Center for Mountain Ecology & Agro-Bioengineering (CICMEAB), Guizhou University, Guiyang, China

is one of the most common fungal diseases, especially in stored crops, and it has caused serious waste and affected human health. Fungal mildew remains a challenge in storage management despite the availability of diverse processing and formulation strategies that allow the control of food-borne microorganisms (Snyder et al. 2019).

Fungal spores act as vehicles for mildew diseases (Dijksterhuis 2017). They exist everywhere, and can be found in air, dust, wood chips, bread factories, and even on seed surfaces (Garcia et al. 2019; Idler et al. 2019; Segura-Medina et al. 2019; Kalia et al. 2020). Tobacco leaves, which are the most important raw material for cigarette production, are easily infected by various fungi and then develop mildew or rot. It has been reported that tobacco leaves have fungal populations as large as 1,528,500 colony-forming units (CFUs) per gram of tobacco (CFUs indicate the number of viable bacterial or fungal cells in a sample) (Welty 1972; El-Ansary et al. 2013). Once tobacco leaves develop mildew, the fungi quickly absorb nutrients from the leaves and destroy their organizational structure while excreting blue, green, and black pigments and releasing an unpleasant odor (Welty and Vickroy 1975; Nyvall 1989). This significantly reduces the quality and marketability of the tobacco leaves and their products (Ogundero 1983), and eventually causes irreversible economic losses, not only to the tobacco industry and tobacco farmers but also to consumers (Yang et al. 2015). Additionally, as fungi grow, they secrete various mycotoxins and allergens into tobacco leaves, including aflatoxins (Pattee 1969), patulin and ochratoxin, sterigmatocystin (Varma et al. 1991), and sphinganine-analog mycotoxins (Chen et al. 2020a). Moreover, large amounts of mold spores are released into the air, causing air contamination and human diseases such as asthma and allergic alveolitis (Kurup et al. 1983; Huuskonen et al. 1984; Lander et al. 1988; Vesper and Wymer 2016). Therefore, tobacco mildew does not only cause serious economic problems but also serious health and social problems. However, the mechanism of fungal mold growth is not yet fully understood. In recent years, with the development of the tobacco industry in China, the scale and quantity of tobacco storage have continuously increased, increasing the risk of mildew (Yang et al. 2016).

Fungi have unique morphological characteristics and have developed diverse metabolic processes through several years of evolution; they thus occupy a variety of ecological niches in the ecosystem (Nilsson et al. 2019). As fungi are heterotrophic organisms, their diversity depends on the diversity of available food sources (Berbee et al. 2017). Currently, approximately 130 genera and 231 species of molds have been identified in mildewed tobacco, with *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, *Trichoderma*, *Cladosporium*, *Alternaria*, and *Fusarium* being the dominant microbiota (Welty and Lucas 1968; Chen et al. 2020c; Verweij et al. 2000). However, traditional microbial isolation and culture methods cannot provide complete information on

the community composition of mold in tobacco leaves because only 1% of the microflora can be isolated from the environment (Li et al. 2020). In addition, stored tobacco leaves constitute an extremely dry micro-habitat (moisture content [MC], 10–13%) (Villemur et al. 2009), and many xerophilic microorganisms, such as *Aspergillus penicillioides*, *Xeromyces bisporus*, and *Eurotium halophilicum*, cannot grow in conventional culture mediums (Stevenson et al. 2017a). Therefore, the fungal community composition in tobacco leaves is not completely understood. Fortunately, next-generation sequencing-based metagenomic analysis can solve this issue (Kumar et al. 2019). Recently, high-throughput sequencing (HTS)-based metagenomic analysis has been used to identify the composition and diversity of microorganisms in tobacco leaves during flue-curing (Chen et al. 2020c; Hu et al. 2021). Nevertheless, to our knowledge, there has been no metagenomic analysis of fungal communities in mildewed tobacco leaves under storage conditions.

In addition, material sources, storage conditions and storage durations can affect the composition of the microbial community (Kalia et al. 2020). Our previous studies have shown that there are significant differences in fungal community structures of tobacco leaves stored in different cities (Zhou et al. 2021). However, the reasons for this phenomenon and whether a similar pattern can be observed in mildewed tobacco leaves remain unclear. Furthermore, previous studies have shown that the majority of fungi invade the tobacco leaves even before the leaves are placed in a warehouse (Welty and Lucas 1969). The majority of fungi are present from the leaf-growth stage to the storage stage; therefore, they are present during harvesting, curing, re-curing, aging, and long-term storage (Pauly and Paszkiewicz 2011). However, the source of mildew-causing fungi in stored tobacco leaves is not clear.

In the present study, we collected 57 tobacco leaf samples from three warehouses in Guizhou, Shanghai and Jilin, China, including 24 healthy and 33 mildewed tobacco leaves. We applied HTS to characterize the fungal community structure in both healthy and mildewed tobacco leaves. Further, to elucidate the impact of storage environment on fungal communities in tobacco leaves, we examined how these community structures vary with climatic factors. Moreover, we performed a comprehensive analysis of the fungal community composition of tobacco leaves during the first four stages of storage to identify the source of fungal tobacco leaf contamination.

Materials and methods

Sample collection

Collection of mildewed tobacco leaves from storage room

Tobacco leaves were collected from storage rooms in Guiyang City, Guizhou Province (GZ); Yanji City, Jilin

Province (JL); and Shanghai City (SH), China, which represent the typical climate types in the country (Supplemental Table S1). Guiyang City, Guizhou, located in southwest China, has a subtropical humid monsoon climate with a high annual average relative humidity (AARH) ($81.18 \pm 0.82\%$). Shanghai, located in eastern China, has a north subtropical monsoon climate and also has the highest average annual temperature (AAT) (18.14 ± 1.33 °C). Yanji City, Jilin, located in northeastern China, has a temperate humid monsoon climate with the lowest AAT (6.77 ± 1.98 °C) and AARH ($62.52 \pm 2.21\%$).

The leaves were sampled between July 2017 and October 2020 (Supplemental Table S1) and were divided into three grades based on the presence of mildew: healthy (H: leaves with no mildew and no musty smell), mild mildew (MM: leaves with obvious mildew that could still be used after removing the damaged part), and heavy mildew (HM: leaves that were noticeably mildewed, had turned black in color, were emitting a strong suffocating musty smell, and had completely lost their use value) (Supplemental Fig. S1). In addition, to investigate if the tobacco source would affect the mold community composition of stored tobacco leaves, we selected tobacco leaves from Guizhou, Hunan, Guangdong and Yunnan provinces which were all stored in Guizhou warehouses. However, due to some inevitable objective and anthropic factors, the number of samples we collected was inconsistent at different sites. Finally, we collected 39 samples from GZ (including 15 H, 15 MM, and 9 HM samples), 6 samples from SH (including 3 H and 3 HM samples), and 12 samples from JL (including 6 H and 6 HM samples) (Supplemental Table S1). Samples were collected using sterile gloves and placed in sterile plastic bags. All samples were transported to the laboratory in a timely manner and stored at -20 °C. Sample details are shown in Supplemental Table S2.

Collection of tobacco leaves during processing

From the green-leaf stage to storage, tobacco leaves undergo processing via several steps such as harvesting, flue-curing, and re-curing, and the latter two involve heating at 30 – 68 °C and 55 – 65 °C, respectively (Supplemental Fig. S2). Samples were collected at different processing stages, including harvesting (stage 1), flue-curing (stage 2), threshing (stage 3), and re-drying (stage 4), from tobacco-growing regions in Zunyi, Anshun and Huishui of Guizhou Province from July to December 2019. Stage 1: samples were collected during harvesting, and 10 medial leaves were harvested randomly from a 20×20 m² field using the five-point method. Stage 2: after baking, five tobacco leaves were randomly selected from the upper, middle, and lower layers of the baking room and mixed together to form one sample. Stage 3: before threshing, 10 tobacco leaves were randomly selected from

the tobacco pile and mixed into one sample. Stage 4: 500 g samples were randomly collected at the end of re-drying. For all tobacco leaf samples, the middle leaf was selected, and the variety was Yunyan 87. A total of 12 samples were obtained, packed in sterile plastic bags, and stored at -80 °C.

Determination of physicochemical parameters and observation of the apparent morphology of tobacco leaves

The MC of tobacco leaves was determined using a moisture meter (WL-760 W, Shenzhen Guanya Moisture Meter Instrument Co., Ltd., China). Further, tobacco pH was determined using a pH meter (PHS-3C, Shanghai INESA Scientific Instrument Co., Ltd., China) (ratio of leaves to distilled water = 1:5, according to the tobacco industry standard YC/T222-2007). The total nitrogen (TN) and total organic carbon (TOC) content of tobacco leaves were determined using the semi-micro Kjeldahl method and the high-temperature oxidation of potassium dichromate volumetric method (Xu et al. 2018), respectively. A saturated potassium sulfate solution was used to control the humidity (97%) and the temperature was maintained at 30 °C to simulate the mildewing of tobacco leaves under high temperature and humidity. Subsequently, the characteristics of tobacco leaves with mildew were observed using scanning electron microscopy (SU8100, Hitachi Ltd., Tokyo, Japan) according to method proposed by Zhang et al. (2020).

DNA extraction and amplicon sequencing of fungal communities

First, 100 g tobacco leaves were weighed out and ground using liquid nitrogen. Fungal DNA was extracted from 10 g of ground tobacco leaves using the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. DNA concentration and quality were determined using the NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA) and 1% (w/v) agarose gel electrophoresis. The fungal internal transcribed spacer (ITS) regions of nuclear ribosomal DNA were amplified using the primers ITS1F (5'-CTTGGTCATTTAGAGGAA GTAA-3') and ITS2R (5'-GCTGCGTTCATCGAT GC-3') (Adams et al. 2013). The PCR products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™ -ST (Promega, Madison, WI, USA). The processed PCR amplicons were sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA) using a paired-end protocol at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Paired-end sequences were merged into a single sequence using FLASH Version 1.2.11 (Magoč and

Salzberg 2011) and then quality-filtered (average quality score > 20) using Trimmomatic version 0.33 (Bolger et al. 2014). The chimeric sequences were subsequently identified and removed using a UCHIME algorithm (Edgar et al. 2011), and effective reads were finally obtained. Operational taxonomic units (OTUs) were identified based on a $\geq 97\%$ similarity cutoff using UParse version 7.0 (Edgar 2013). To reduce spurious OTUs, we removed OTUs represented by less than two sequences. Taxonomy was assigned using the RDP classifier (UNITE version 8.0, ITS database) based on an 80% confidence threshold (Koljalg et al. 2013). For subsequent alpha- and beta- diversity analyses, OTU abundance was normalized using the sequence number corresponding to the sample with the fewest sequences.

Statistical analysis

The alpha-diversity of the fungal communities was calculated using mothur version 1.30.1 (Schloss et al., 2009) to determine the number of observed OTUs. Analyses based on the Chao index, Shannon diversity index, and Simpson evenness index were performed. Partial least square discriminant analysis (PLS-DA) and nonmetric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity or the weighted-UniFrac distance between the relative abundance of OTUs were used to analyze the differences among groups. Analysis of similarity (ANOSIM) was applied to test the differences in the fungal community composition between mildewed and healthy leaves. Permutational multivariate analysis of variance (PERMANOVA) was used to examine the contribution of different factors towards differences between samples (Xiong et al. 2020). To disentangle the relative importance of the environmental variables (physicochemical and climatic factors) for fungal community assembly, redundancy analysis (RDA) and variance partitioning (VPA) were performed using the Bray–Curtis dissimilarity between all healthy and mildewed leaves samples (Jiao et al. 2019). Weighted-UniFrac distance and partitioning around medoids clustering were used to typing of the dominant flora under mildewed and healthy states (Mack et al. 2016). Co-occurrence networks were constructed to evaluate the potential changes in the intensity of interactions among fungal communities and the stability of the tobacco leaf micro-ecosystem. To do this, the Spearman correlation between any two OTUs (considering only OTUs with sequence numbers greater than 2 in at least three samples) was calculated, and only robust (Spearman's $r > 0.6$ or $r < -0.6$) and statistically significant ($p < 0.01$) correlations were retained. Networks were visualized using the interactive Gephi platform (Bastian et al. 2009). Topology characteristics of the network (e.g., average degree, network diameter, average path

length, average clustering coefficient, and neighborhood connectivity) were calculated using the NetworkAnalyzer plugin in Cytoscape (Assenov et al. 2008).

In addition, FUNGuild was applied to predict the function of the fungal communities in tobacco leaves (Nguyen et al. 2016). The community structure and composition of fungi in tobacco leaves at different stages were identified using principal coordinates analysis and heatmaps. Non-parametric statistical tests (Kruskal–Wallis test or Wilcoxon test) were conducted to evaluate the significance of differences in taxonomic composition, alpha-diversity, functional groups, and physicochemical properties among different groups.

Results

Effects of mildew on the fungal community structure in tobacco leaves

Physicochemical and apparent morphological characteristics of tobacco leaves

In order to compare the differences in physicochemical characteristics between mildewed and healthy tobacco leaves, the MC, pH value, TOC content, TN content, and carbon–nitrogen (C/N) ratio were measured (Supplemental Fig. S3). There was no significant difference in MC between the mildewed and healthy tobacco leaves from GZ and JL, but the MC of mildewed tobacco leaves from SH was significantly higher than that of healthy tobacco leaves collected from the same area ($p < 0.05$). pH and TN content were significantly higher in HM tobacco leaves than in H tobacco leaves, while TOC content showed no significant difference between these samples. However, the C/N ratio in HM tobacco leaves was lower than that in H tobacco leaves. These results suggested that carbon metabolism in mildewed tobacco leaves corresponded to that observed in healthy aged tobacco leaves, but the nitrogen metabolism lagged behind. However, MM tobacco leaves seemed to show higher nitrogen consumption.

After 5 days of growth in mildew-promoting conditions (97% relative humidity and 30 °C), tobacco leaves started showing obvious mycelia on their surface. Electron microscopy images showed that the surface of healthy tobacco leaves was very clean and contained obvious stomata. However, in mildewed leaves, the number of mycelium on the leaf surface, density of the mycelium network, and number of fungal spores increased gradually (Supplemental Fig. S4). As expected, the mycelium damage on tobacco leaf surfaces increased with increasing mildew severity.

Differences in fungal community diversity between mildewed and healthy tobacco leaves

After a series of quality control processes, a total of 3,721,388 high-quality sequences were obtained from the 24 healthy and 33 mildewed samples (average number of sequences, 65,287.5 per sample). A total of 1,270 OTUs were obtained across our samples, including 1,065, 546, and 292 OTUs from leaves collected from GZ, JL, and SH, respectively. Unsurprisingly, the number of fungal OTUs in mildewed tobacco leaves was significantly lower than that in healthy leaves (Fig. 1a), and this number decreased with further growth of mildew. In fact, 994, 334, and 101 OTUs were obtained from H, MM, and HM leaves from GZ, respectively. Interestingly, mildewed and healthy tobacco leaves from GZ, JL, and SH shared 58, 65, and 35 OTUs (Supplemental Fig. S5), respectively, indicating that the tobacco leaf itself is an important source of mildew-causing fungi. Similar to the number of OTUs (Sobs index), the Chao and Shannon indices were significantly lower in mildewed tobacco leaves than in healthy ones, while the Simpson index was higher (Fig. 1b–d). As expected, mildew had a greater influence on fungal richness than on fungal diversity.

Analyses of PLS–DA based on Bray–Curtis dissimilarity demonstrated that the fungal communities in mildewed and healthy samples could be divided into two groups (Supplemental Fig. S6a). ANOSIM analysis demonstrated significant differences in the fungal community structure between mildewed and healthy tobacco leaves ($R=0.415$, $p<0.01$). Furthermore, RDA analysis showed that physicochemical

characteristics of tobacco leaves were closely related to fungal community distribution, and MC, pH, and TN content had significant effects on fungal community structure (Supplemental Fig. S6b).

Differences in the composition and function of fungal communities in mildewed and healthy tobacco leaves

A total of six fungal phyla and 460 fungal genera were annotated across the 57 samples. As shown in Fig. 2a, *Ascomycota* and *Basidiomycota* were the dominant fungal phyla in all samples. In leaves from GZ and JL, the relative abundance of *Ascomycota* in mildewed tobacco leaves was significantly higher than that in healthy tobacco leaves (97.84% and 99.35% vs. 40.99% and 79.45%, respectively), while the abundance of *Basidiomycota* was lower (2.15% and 0.02% vs. 54.62% and 19.29%, respectively). In contrast, among samples from SH, while the relative abundance of *Ascomycota* in mildewed tobacco leaves (39.53%) was significantly lower than that in healthy tobacco leaves (48.34%), the reverse was true for *Basidiomycota*. At the genus level, there were 14 taxonomic groups with a relative abundance greater than 1% across all samples, but the dominant genera differed with region (Fig. 2a). In leaves from GZ, *Xeromyces* was the most dominant genus in mildewed tobacco leaves, and its relative abundance was more than 85%, significantly higher than that in healthy tobacco leaves (8.98%). In contrast, *Sampaiozyma* (26.10%) was the dominant genus in healthy tobacco leaves. In leaves from JL, *Xeromyces* and *Aspergillus* were the dominant genera, and their relative

Fig. 1 Alpha diversity of fungal communities in mildewed and healthy tobacco leaves during storage. Diversity indices were calculated using a subset of 40,386 reads per sample. Sobs (a) and Chao (b) indices were used to evaluate community richness, while Shannon (c) and Simpson (d) indices were used to assess community diversity. The statistical significance of differences between mildewed and healthy tobacco leaves was tested using the Wilcoxon rank-sum test. * and ** indicate significance differences at $p<0.05$ and $p<0.01$, respectively

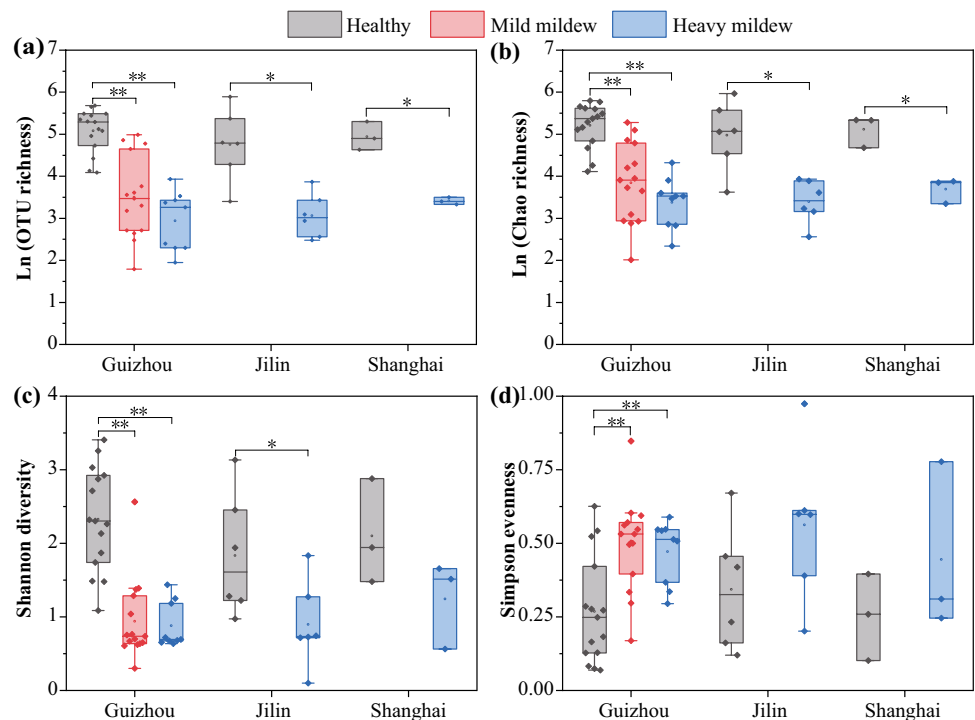
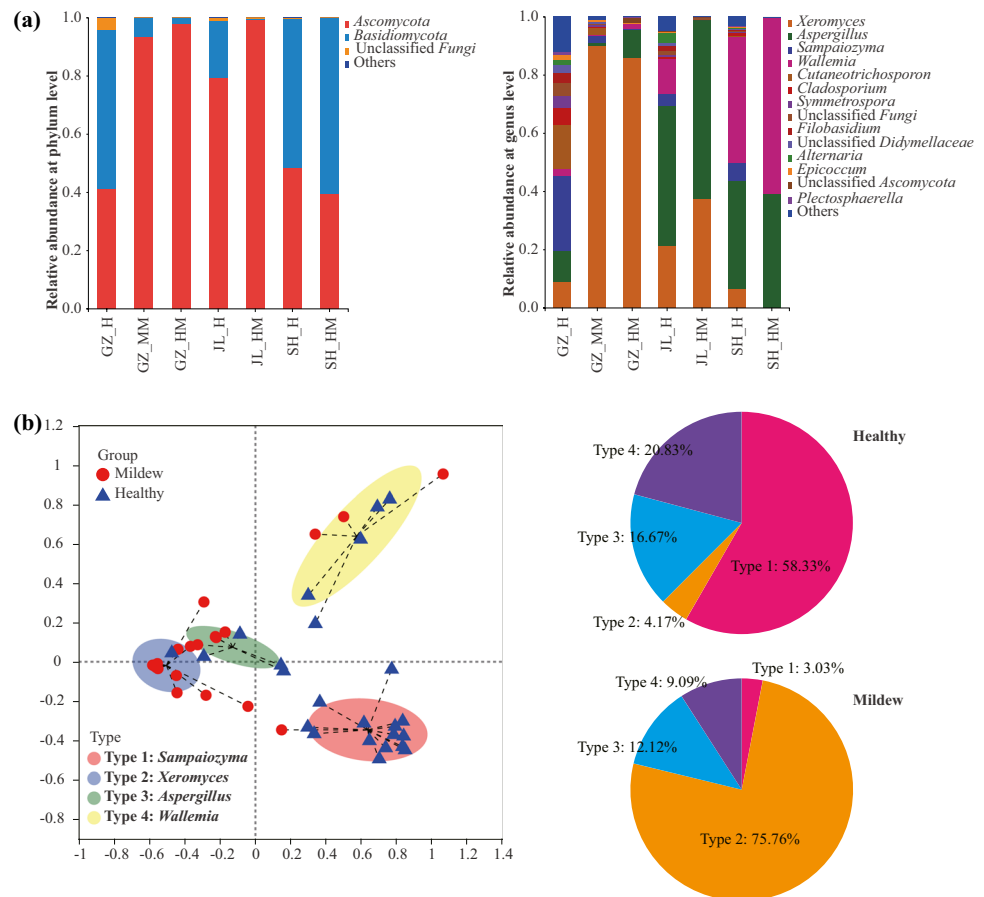


Fig. 2 Community composition and structural typing of fungi in tobacco leaves. **(a)** The relative abundance of fungal communities at the phylum (left) and genus (right) levels. The relative abundance was the average percentage of the total effective fungal sequences in each group. Groups with an average relative abundance less than 1% were designated as “others.” **(b)** Clustering analysis of the fungal community structure in mildewed and healthy tobacco leaves based on weighted-Uni-Frac distances



abundance in HM tobacco leaves (37.25% and 61.69%, respectively) was higher than that in H tobacco leaves (21.30% and 48.19%, respectively). For tobacco leaves from SH, the dominant genera were *Wallemia* and *Aspergillus*, and their relative abundance in HM tobacco leaves was slightly higher than that in H leaves. However, the proportion of most common fungal flora, such as *Cladosporium*, *Alternaria*, *Epicoccum*, *Fusarium*, *Penicillium*, *Rhizopus*, and *Trichoderma*, significantly reduced or even disappeared in mildewed tobacco leaves (Supplemental Fig. S7). In addition, clustering analysis revealed that the fungal community structure in tobacco leaves could be divided into four types (Fig. 2b). Among them, the fungal microbiota dominated by *Sampaiozyma* was representative of healthy tobacco leaves, while that dominated by *Xeromyces*, *Aspergillus*, and *Wallemia* was associated with a potential risk of mildew.

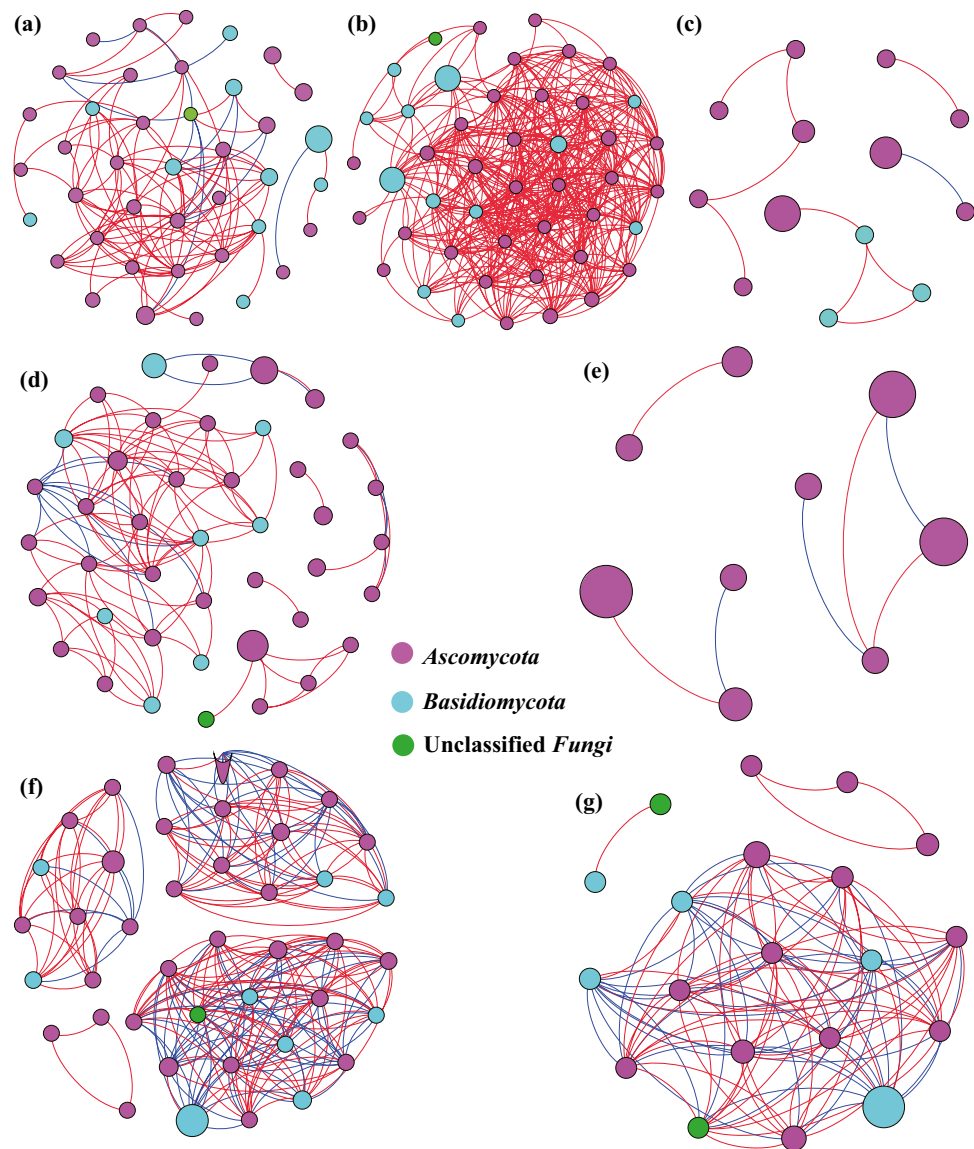
The FUNGuild database was used to classify the identified fungi based on their ecological guild. The results showed that mildew significantly reduced the functional diversity of the fungal community in tobacco leaves (Supplemental Fig. S8a). With the growth of mildew, fungi with pathologic and symbiotic modes of nutrition, including animal pathogens, fungal parasites, plant pathogens, and endophytes, gradually decreased or disappeared (Supplemental

Fig. S8b). However, saprophytic fungi gradually increased in proportion and accounted for more than 90% of all fungi in mildewed tobacco leaves, indicating that saprophytic nutrition was an important characteristic of mildew-causing fungi.

Differences in the network structure of fungal communities between mildewed and healthy tobacco leaves

We further performed network analysis to elucidate the influence of mildew on the ecological functions and co-occurrence patterns of fungal communities. Our results showed that the network in mildewed tobacco leaves was starkly different from that in healthy tobacco leaves, irrespective of which region the leaves came from (Fig. 3). For leaves from GZ, JL, and SH, the number of nodes in the network of H leaves was 3.08, 4.67, and 2.1 times that in the network of HM leaves, and the number of edges was 10.5, 16.14, and 2.32 times higher, respectively, indicating that fungal networks were more complex in H tobacco leaves than in HM tobacco leaves. However, networks in MM leaves seemed to have more nodes and edges than those in H and HM leaves. For example, the fungal networks for H, MM, and HM tobacco leaves from GZ had

Fig. 3 Co-occurrence network among operational taxonomic units (OTUs) in tobacco leaves based on Spearman's correlation analysis ($p < 0.01$). **(a)**, **(b)**, and **(c)** represent healthy (H), mild mildew (MM), and heavy mildew (HM) tobacco leaves from Guiyang, Guizhou Province; **(d)** and **(e)** represent H and HM tobacco leaves from Yanji, Jilin Province; and **(f)** and **(g)** represent H and HM tobacco leaves from Shanghai, respectively. The node colors indicate the phyla to which each OTU belongs, and the node size is proportional to the abundance of taxa. The edges are colored based on interaction types; positive correlations are indicated in red and negative correlations are indicated in blue



40, 46, and 13 nodes and 105, 429, and 10 edges, respectively (Fig. 3a–c). Moreover, the network in HM leaves had a lower average degree, network diameter, average path length, average clustering coefficient and neighborhood connectivity than the network in H leaves (Supplemental Fig. S9), suggesting that mildew could seriously damage the connectivity and stability of the fungal community structure. With increasing mildew growth, the connectivity and stability of the fungal network could deteriorate further, increasing susceptibility to the impact caused by environmental changes. However, to our surprise, the network in MM leaves had the highest average degree, network diameter, average path length, clustering coefficient, and connectivity (Supplemental Fig. S9). This indicated that there was a period of relatively high microbial activity during mildew growth in tobacco leaves, and we speculate that mildew followed a parabolic succession pattern.

Effects of storage environment on fungal community distribution in tobacco leaves

To investigate the influence of the storage environment on the fungal community structure in tobacco leaves, we conducted NMDS ordinations and PERMANOVA analysis and showed that there were significant differences in the fungal community distribution among leaves stored in GZ, JL, and SH ($p < 0.01$, Supplemental Fig. S10). The results showed that the distribution of the fungal community in tobacco leaves was highly heterogeneous. Environmental variation explained 73.5% of the fungal community variability in mildewed tobacco leaves, but it only explained 32.6% of that in healthy tobacco leaves (Supplemental Fig. S10a and 10c). To understand the influence the source of tobacco leaves had on the fungal community structure, we collected tobacco leaves harvested from Guangdong,

Hunan, Yunnan, and Guizhou Provinces that were stored in warehouses in Guiyang (Supplemental Table S2). PER-MANOVA analysis showed that tobacco source had no significant effect on the community structure of stored tobacco leaves ($p > 0.05$), but it had a greater contribution to variability in the fungal community in healthy tobacco leaves than in mildewed tobacco leaves (Supplemental Fig. S10b and 10d). These results indicated that environmental filtration played an important role in shaping the fungal community in tobacco leaves.

Further analysis showed that the AAT and AARH in different regions had significant effects on the fungal community structure in tobacco leaves ($p < 0.05$), and the effect was more significant in mildewed tobacco (Fig. 4a–b). Further, VPA showed that AAT and AARH together explained 8.63% and 26.46% of the variation in the fungal community structure of healthy and mildewed tobacco leaves, respectively, with AARH clearly showing the highest contribution (Fig. 4c–d). As expected, the influence of climate on fungal community assembly was significantly higher in mildewed leaves than in healthy ones. Together, these results indicated that the storage environment had the strongest selection effect on the mildew-causing fungal groups in tobacco leaves.

Investigation of the sources of fungal contamination in tobacco leaves

Tobacco leaves themselves were found to be important sources of fungi, and these fungi primarily caused mildew in stored tobacco leaves. To determine the sources of these fungi, we analyzed the fungal community composition at four important stages of tobacco processing: harvesting, curing, pre-threshing, and post-re-curing. Principal coordinates analysis showed that there were significant differences in the fungal community structure and composition among the four stages ($p < 0.01$, Fig. 5a and 5b). *Sampaiozyma* (44.38%), *Symmetrospora* (21.17%), and *Cladosporium* (4.57%) were the dominant groups in green leaves at stage 1. *Sampaiozyma* (37.90%), *Alternaria* (18.35%), an unclassified *Didymellaceae* (12.87%), and *Cladosporium* (6.42%) were the dominant groups in cured tobacco leaves at stage 2. Further, *Aspergillus* (61.50%) and *Sampaiozyma* (23.36%) were the dominant fungi in tobacco leaves at the pre-threshing stage. After re-curing, the same predominant fungi were observed, but their proportions changed (*Aspergillus* 45.94% and *Sampaiozyma* 25.90%).

Importantly, the two high-temperature stages (flue-curing and re-curing) could kill some fungi, such as *Zymoseptoria*, *Strelitziana*, *Pseudocercospora*, *Golubevia*, and

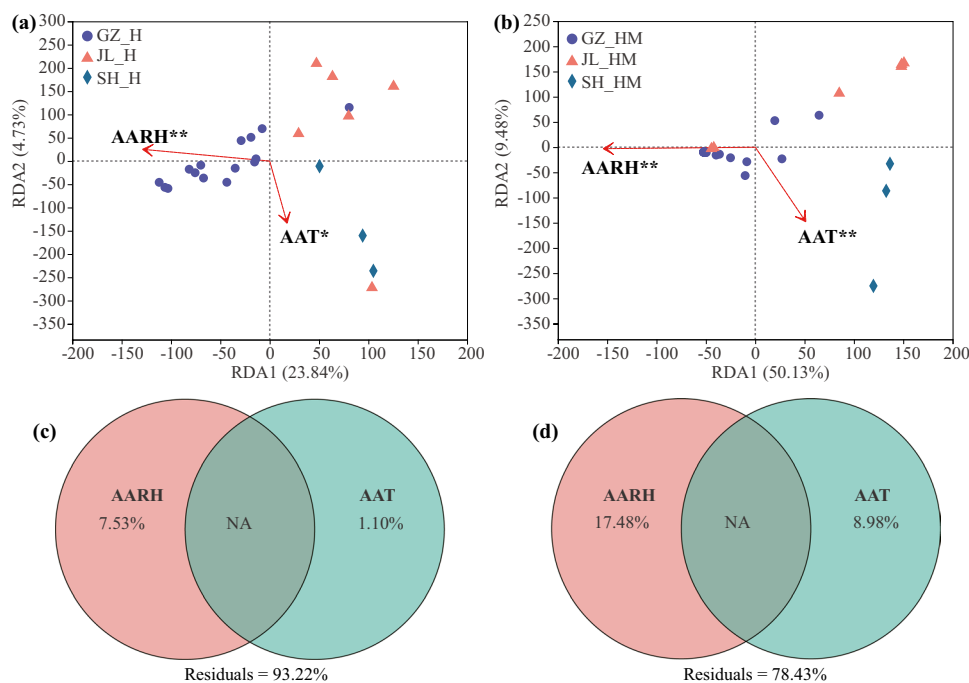
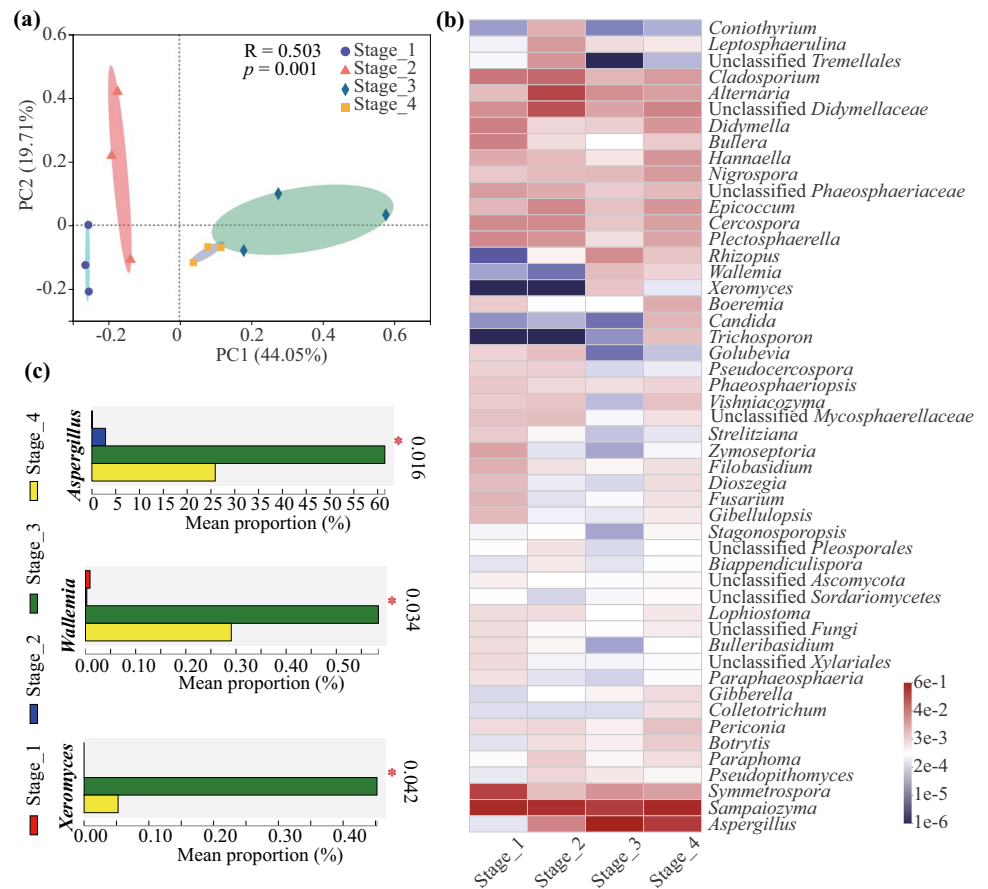


Fig. 4 Effects of climatic factors on fungal communities in tobacco leaves. (a) and (b) Redundancy analysis (RDA) showing the relationship of climatic factors with the fungal community structure in healthy and mildewed tobacco leaves at the operational taxonomic unit level, respectively. (c) and (d) Variation partitioning analysis (VPA) of the relative contributions of climatic variables to variations

in the beta-diversity of fungal communities in healthy and mildewed tobacco leaves, respectively. Dots, triangles, and diamonds represent samples from different regions. AAT and AARH represent the average annual temperature and average relative humidity, respectively. * and ** indicate significance differences at $p < 0.05$ and $p < 0.01$, respectively. NA represents no significant contribution

Fig. 5 Composition of fungal communities at different stages of tobacco leaf processing. **(a)** Principal coordinate analysis based on the Bray–Curtis dissimilarity of the relative abundance of operational taxonomic units. Analysis of similarity was applied to test the differences in the changes in fungal community compositions across the four stages. **(b)** Heat map of the relative abundance of the top 30 taxonomic genus levels during the four main processing periods. **(c)** Changes in the abundance of the four dominant fungi during processing. Kruskal–Wallis tests were performed to test the differences in dominant species among the four stages. * indicates the significance differences at $p < 0.05$. Stages 1, 2, 3, and 4 represent the four stages of tobacco processing: harvesting, flue-curing, pre-threshing, and completion of re-curing, respectively



Coniothyrium. However, even after re-curing, some fungi such as *Phaeosphaeriopsis*, *Cercospora*, *Epicoccum*, *Nigrospora*, *Didymella*, and *Alternaria*, which were plant pathogens, survived. Interestingly, these fungal groups disappeared after mildew development. It is worth noting that some dominant fungi were not present on green tobacco leaves but gradually contaminated the leaves during later stages of processing. For example, *Aspergillus* appeared during flue-curing and its presence increased significantly by stage 3, and *Xeromyces* and *Wallemia* were also significantly enriched at stage 3 (Fig. 5c). The results showed that the fungi that caused mildew largely contaminated the leaves post-harvest, especially between the two drying periods.

Discussion

Mildew damage disrupts the balance of the tobacco microecosystem

Mildew is a plant disease caused by an overgrowth of fungi (molds) and alters leaf quality. As they grow, the mildew-causing fungi secrete various enzymes that break down and digest leaf components and release waste products including

carbon dioxide, mycotoxins, and antimicrobials (Snyder et al. 2019). Li et al. (2014) showed that compared with healthy tobacco leaves, mildewed tobacco leaves have a significantly lower lysine, serine, alanine, leucine, methionine, threonine, proline, arginine, and tyrosine content and a significantly higher phenylalanine, cysteine, aspartic acid, valine, and histidine content. In the present study, the physicochemical properties of mildewed tobacco leaves were found to be different from those of healthy ones. Mildewed leaves showed a higher TN content and a lower C/N ratio (Supplemental Fig. S3). Surprisingly, the pH of mildewed tobacco leaves collected from Guizhou and Jilin was higher than that of healthy tobacco leaves, which was inconsistent with the pH decline observed in normal tobacco leaves (Sun et al. 2011). This could be due to the following reasons. First, this phenomenon may be related to the growth characteristics of mildew-causing fungi in tobacco leaves. The germination and mycelial growth of xerophilic fungi, including *X. bisporus*, *A. penicillioides*, and *E. halophilicum*, have been reported to typically occur at pH ranges of 5.5–7.5 (Stevenson et al. 2017a). Second, this phenomenon may also be related to the decomposition and transformation of tobacco leaf compounds. In this study, the TN content of mildewed tobacco leaves was higher than that of healthy

leaves, indicating that nitrogen-containing compounds may have accumulated in mildewed tobacco leaves. These leaves also contained alkaline compounds, such as nicotine and volatile alkali (Sun et al. 2011). In addition, tobacco leaves are also inhabited by a large number of bacteria, including alkaliphilic *Actinomyces*. Some of these bacteria, such as *Corynebacterium mammoniagenes*, can increase the pH and promote nitrite accumulation through urease activity (Di Giacomo et al. 2007). The results obtained indicated that mildew could significantly change the physicochemical environment in tobacco leaves.

The overgrowth of fungi can change the diversity, composition, and function of fungal communities (Welty and Lucas 1969; Chen et al. 2020b). When comparing the fungal community structure between healthy and mildewed tobacco leaves after curing, Chen et al. (2020b) found that the richness and diversity of the fungal community were higher in healthy tobacco leaves than in mildewed ones, consistent with our finding. In addition, Welty and Lucas (1969) showed that mildew could change the composition of fungal communities in tobacco leaves. They found that *Aspergillus*, *Penicillium*, *Alternaria*, *Cladosporium*, *Fusarium*, and *Rhizopus* are isolated more frequently from healthy tobacco leaves, whereas the most common fungi in mildewed leaves were *Aspergillus repens*, *Aspergillus niger*, *Aspergillus ruber*, and *Penicillium* spp. The present study also showed that mildew changes the fungal community composition of tobacco leaves (Fig. 2), and that *Xeromyces* and *Wallemia*, in addition to *Aspergillus*, are the dominant mildew-causing fungi.

Interactions and resource partitioning may have profound effects on the development of individuals, populations, and communities, thereby affecting food networks and ecosystem functioning (Nunn et al. 2020). FUNGuild functional prediction showed that mildewed tobacco leaves had fewer pathogenic and symbiotic trophic fungi and more saprophytic trophic fungi than did healthy tobacco leaves (Supplemental Fig. S8). Pathogenic and symbiotic fungi obtain nutrition mainly by damaging host cells and exchanging resources with them, whereas saprophytic fungi obtain nutrition by degrading dead host cells (Nguyen et al. 2016). This may be related to the highly resource-selective nature of microorganisms, which use similar but specific resources, such as carbon and nitrogen, and rapidly deplete existing resources (Eldridge et al. 2017). Compared with normal tobacco leaves, mildewed tobacco leaves had significantly lower levels of organic carbon, free amino acids and organic acids (Li et al. 2014), restricting the mold populations that depend on these resources. The network analysis found that the complexity, connectivity, and stability of the network first increased and then decreased with mildew growth, indicating that the interactions between mold communities were not invariable but instead changed dynamically (Fig. 3).

Fungi directly compete with each other for resources by releasing extracellular enzymes into the surrounding environment when the available resources decrease (Magan and Aldred 2007). The present study found that *Xeromyces*, *Aspergillus*, and *Wallemia* were the dominant competitors in mildewed tobacco leaves. These fungi can grow under drought conditions and therefore have an obvious competition advantage (Kralj Kunčič et al. 2013; Stevenson et al. 2017a; Snyder et al. 2019). Material metabolism is also an important approach microorganisms use to engage in effective competition (Machado et al. 2021). On the one hand, molds could improve nutritional utilization by altering their own metabolic regulation, or gain an advantage by exploiting the products synthesized by other microorganisms to reduce gene expression (Bauer et al. 2018). On the other hand, the rapid early production of secondary metabolites, such as mycotoxins, may enable fungi to maintain a spatial competitive advantage over a range of fluctuating conditions (Magan and Aldred 2007). In addition, previous studies have shown that fungal–bacterial interactions play important roles in promoting biochemical cycles, modulating health and disease across plants and animals, and regulating ecosystem biodiversity and stability (de Menezes et al. 2017; Getzke et al. 2019; Ratzke et al. 2020). Therefore, we speculate that they may play a role in the growth of tobacco mildew. However, it must be noted that the activities of these microorganisms were also affected by pervasive abiotic factors such as temperature, water availability, gas balance, and pH (Magan and Aldred 2007).

Water, temperature, oxygen, and pH are determinants of cellular biological activity, biosphere function, and all life processes, and they are important for the germination, growth, and survival of fungi (Stevenson et al. 2017a; Dos Santos et al. 2020). Our study showed that MC, pH, and TN content contribute towards the differences in the fungal community structure between mildewed and healthy tobacco leaves (Supplemental Fig. S6b). Moreover, AARH had a greater contribution towards the variability in the fungal community structure in mildewed tobacco leaves than in healthy tobacco leaves. Fungi can grow in any environment with sustained high humidity for more than 24 h, and their growth rate doubles with every 7.8 °C increase in temperature (Bedard 2016). However, in the present study, we found no significant difference in MC between mildewed and healthy tobacco leaves from Guizhou and Jilin, indicating that other unidentified factors led to mildew development. For example, uneven distribution of humidity, temperature, nutrition, pH, and oxygen levels as well as the contingency of spore colonization and hysteresis of fungal spore germination can all affect the time required for fungal colonies to become visible (Peay et al. 2016; Dijksterhuis 2019).

In conclusion, the diversity and composition of mold communities in mildewed tobacco leaves were significantly

different from those in healthy tobacco leaves. These differences may be related to environmental conditions (such as environmental pH and humidity), availability of resources (such as carbon and nitrogen), and microbial interactions.

Climatic factors play an important role in shaping fungal communities

Fungi have developed unique morphological characteristics and diverse metabolic processes through several years of evolution, and they thus occupy a variety of ecological niches and play roles in shaping ecosystems (Nilsson et al. 2019). The ability of fungal species to colonize and occupy specific ecological niches depends on their ability to effectively compete with other microorganisms that may be present (Magan and Aldred. 2007). From the green-leaf to storage stages (and especially during the two high-temperature processes of flue-curing [38–68 °C] and re-curing [55–65 °C]), tobacco leaves rapidly lose moisture and then remain in a relatively dry state (10–13% moisture) for long durations (Villemur et al., 2009). In this drought-stress environment, mildew-causing fungi usually rely on stress (S-selected) life strategies for survival and reproduction. S-selected species reproduce more slowly, but they can tolerate and thrive in stressful environments. Stress conditions such as high temperatures, low pH, and low water activity favor the growth of fungi capable of surviving under these conditions, such as *Saccharomyces rouxi*, *Eurotium*, *Penicillium*, and *Aspergillus* spp. (Magan and Aldred. 2007; Snyder et al. 2019). Therefore, changes in abiotic and biological factors in the tobacco microenvironment lead to changes in the fungal communities.

The present study showed that the fungal community structure in stored tobacco leaves differs under different climatic conditions, which has been reported previously (Zhou et al. 2021). Our results suggest that environmental factors had an important role in shaping the fungal community structure in tobacco leaves during storage (Supplemental Fig. S9). Environmental factors, such as pH, temperature, and humidity, are important drivers of microbial community assembly (Nemergut et al. 2013). However, fungi are generally more sensitive to regional climate than to pH changes (Peay et al. 2016), and climate can predict the richness and composition of soil microbial communities (Bahram et al. 2012; Tedersoo et al. 2014). For example, precipitation and temperature can directly and indirectly affect the richness, diversity, and metabolism of fungal and bacterial communities (Zhou et al. 2016; Bahram et al. 2018). Previous studies have shown that correlations exist between indoor temperature and humidity conditions and outdoor climate (Nguyen et al. 2014). Microclimates also change with changes in the general climate (Boddy 1999). In the present study, AAT and AARH, and particularly the latter, contributed towards the

differences in fungal community distribution in both healthy and mildewed tobacco leaves (Fig. 4). This was closely related to the production characteristics and high hygroscopicity of tobacco. Per industry norms, tobacco is usually shipped to different countries or regions and stored there for months or years, and storage conditions vary greatly depending on the location (Zhou et al. 2020). Owing to the general characteristics of mycelia, fungi can sense and respond to micron-scale environments while also sharing resources and coordinating activities across the different environments spanned by each mycelium (Boddy 1999; Peay et al. 2016). Furthermore, different fungal groups require different growth conditions. For example, *Xeromyces bisporus* can grow at 0.605 water activity (Pitt and Christian 1968); *A. penicillioides* can germinate at a water activity of 0.585 (Stevenson et al. 2017b); and *Wallemia sebi*, *Wallemia muriae*, and *Wallemia ichthophaga* can grow at 0.69, 0.81, and 0.77 water activity, respectively (Pitt and Hocking 1977; Kralj Kunčič et al. 2013). Therefore, subtle and even instantaneous changes in the tobacco leaf ecosystem can cause changes in the fungal community structure.

Tobacco leaves are most vulnerable to mold contamination during postharvest processing

Fungal spores are important carriers of mildew. Since they are ubiquitous in both outdoor and indoor air, with concentrations typically ranging from 100 to 1000 s of spores per cubic meter of air, any contact with air can result in spore deposition (Dijksterhuis 2019). There is evidence that air in production workshops is the main source of contamination for products such as cheese, dry cured meat and bread (Asefa et al. 2010; Garcia et al. 2019; Kure and Skaar 2019). Our analyses showed that the spores of dominant mildew-causing fungi present in stored tobacco leaves, such as *Xeromyces*, *Aspergillus*, and *Wallemia*, came into contact with the tobacco leaves even before they entered the warehouse. These molds are rapidly enriched and multiply between the post-flue-curing and pre-re-curing period (Fig. 5). Because of the processes involved in grading, trading, and transportation during this period, tobacco leaves are frequently exposed to complex and diverse aerial environments, which increases the probability of fungal spore deposition. Therefore, the cleaning and disinfection of the production environment are essential, and this can reduce the number of fungal spores on tobacco leaves, thus reducing mildew and product spoilage (Kure et al. 2020).

In summary, this study comprehensively analyzed the sources, apparent morphology, and community structure of fungi in stored tobacco leaves from an ecological perspective, and it further identified the factors that drive the heterogenization of these fungal communities. The results showed that mildew significantly disrupts the balance in

tobacco ecosystems, affecting the physicochemical characteristics of tobacco leaves and fungal community diversity, composition, and network structure. The distribution of fungal communities in tobacco leaves differed significantly among different regions, with climatic factors such as temperature and humidity contributing the most to the variations in fungal community distribution in mildewed tobacco leaves. Further, we found that tobacco leaves were vulnerable to contamination by mildew-causing fungi mainly during post-harvest processing. These findings contribute to our understanding of mildew growth in stored materials such as tobacco leaves, tea, Chinese herbal medicine, and crops. Further, they provide a theoretical basis for understanding the mechanism of mildew growth in warehouse-stored plant products and provide clues for the prevention of such mildew growth. In addition, tobacco-related bacteria are believed to be responsible for generating tobacco-specific nitrosamines (TSNAs) (Tyx et al. 2020; Vishwakarma and Verma 2021). However, the role of fungi in TSNA synthesis is not clear, and this should be analyzed in future studies.

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Author contribution JXZ performed the experiments, collected and analyzed the data, and drafted and reviewed the manuscript. YC performed laboratory analyses. LFY, JZ and XZ contributed to study design, protocol development, and reviewed the manuscript. All authors read and approved the final manuscript.

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Data availability All Illumina HiSeq data in this study have been submitted to the NCBI Sequence Read Archive (SRA) database under the BioProject accession number PRJNA723334, <https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA723334>. Other available data can be found in the supplementary materials.

Declarations

Ethics approval This article does not contain any studies with human participants or animals.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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