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Insights into tobacco leaf quality deterioration under long-term storage by investigating dynamic phytochemical and metabolite profile variations

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Abstract

Background Storage conditions affect the metabolome composition and quality of tobacco leaf and, therefore, its economic value. The present study was designed to reveal tobacco leaves' dynamic phytochemical and metabolite profile changes under three different storage conditions: natural (NT), mechanical (MC), and air-conditioning (AC). The 210,003 grade (Hubei Central Tobacco) was selected as the experimental material, and the changes in iodine value absorbance (IV), pH, polyphenols, plastid pigments, conventional chemical compositions, and metabolite profile were analyzed at different times (T0, starting day; T1, three months; T2, five months; and T3, nine months) during storage.

Results The IV significantly increased with the storage duration, while the pH, polyphenols, and stromal pigments had the opposite trends. Lutein, β-carotene, and chlorogenic acid were significantly less degraded under MC and AC than NT. Neoxanthin and chlorophyll b were completely degraded at T3. The nicotine, total sugar, reducing sugar, and chlorine content significantly varied along with the storage duration, reaching their maximum values at T2 under MC and AC. The sugar/base ratio at T3 under MC and AC was 8.53 and 8.44, respectively, higher than in NT (5.85). LC-MS-based untargeted metabolomics analysis identified 124–224, 138–180, and 110–153 significant differential accumulated metabolites (DAMs) under NT, MC, and AC, respectively. Biosynthesis of secondary metabolites was significantly induced under the three storage conditions between T0 and T3. Glutathione metabolism and oxidative phosphorylation were induced under NT. Biosynthesis of terpenoids and steroids was highly induced under AC.

Conclusions Our findings may facilitate the optimization of the storage conditions for better preservation of tobacco leaves.

Clinical trial number Not applicable.

Keywords Tobacco, Mechanical storage, Air-conditioned storage, Chemical composition, Untargeted metabolomics, Preservation

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Background

Tobacco (*Nicotiana tabacum*) is a high-economic value non-food crop widely cultivated in over 100 countries [1]. In China, the top country producer and consumer of tobacco, the tobacco processing industry is vital for the national economic development [2, 3]. Leaves are the most important marketed tobacco organs, and their yield and chemical components influence the economic value of the crop [4]. Tobacco leaves are utilized for diverse applications, such as consumption (snuff), smoking (cigars and cigarettes), etc [5]. After harvesting, tobacco leaves often have defects such as high irritation, heavy green miscellaneous gas, insufficient aroma amount, bitterness, spicy, and astringent, so they must undergo a series of processing before rolling. Storage is a key tobacco processing step that helps significantly reduce the miscellaneous gas and irritation of tobacco leaves and improves the aroma and mellow taste [6]. Tobacco leaf fermentation refers to aging tobacco leaves under natural or artificial intervention conditions after roasting and re-roasting. The fermentation under storage is a critical process linked to improving the quality of smoked food [7–9]. Therefore, it is essential to investigate and understand the dynamic chemical and metabolic changes occurring in tobacco leaves during storage under various conditions, which would favorthe optimization of an ideal storage environment for quality preservation.

Natural fermentation and artificial fermentation are the two main storage processes. Mechanical and modified atmosphere storage are the most commonly used artificial fermentation methods. The temperature and humidity of the storage environments greatly affect the mellowing of tobacco leaves, leading to quality deterioration [8, 10, 11]. The higher the ambient temperature and humidity, the faster the pH, total sugars, and reducing sugars decrease [11–13]. Different oxygen concentrations also significantly impact the storage cycle and quality of tobacco leaves [14]. During tobacco storage, the absorbance of iodine value increases with the increase of storage duration, so the determination of iodine absorbance helps to access and evaluate the storage process [11]. The polyphenol content in tobacco decreases rapidly, reducing the bitterness and astringency of flue-cured tobacco, but excessive degradation will weaken the aroma [11]. The degradation of carotenoids and chlorophyll during storage results in the formation of a variety of aroma components and alcohols [11, 13]. The contents of total sugar, reducing sugar, nicotine, and total nitrogen influence the taste of tobacco leaves, and a moderate increase in nicotine content can enhance the strength and flavor [15, 16]. The reducing sugar content of high-quality flue-cured tobacco ranges between 16.1%~22.1%, and the sugar/base ratio is in the 6–10 range, which can give the smoker psychological and physical satisfaction [17]. Chlorine and potassium contents are related to flammability, and tobacco with a potassium/chlorine ratio higher than 2 is of good flammability [18].

Compared to natural storage conditions, studies have shown that artificial storage conditions can improve the quality of tobacco leaves more effectively [11, 17, 19–21]. For instance, Liu et al. found that the quality of the mellowed tobacco leaves stored under mechanical conditions was superior to that stored in a natural environment [17]. Zheng et al. revealed that 10%~15% oxygen concentrations are suitable for tobacco leaf mellowing [21]. Although these studies show the quality preservation of tobacco leaves under artificial over natural conditions, the major metabolic changes and phytochemical variations are still not well comprehended, notably during long storage durations. Metabolomics technologies play crucial roles in modern systems' biotechnology, allowing a deep assessment of plants' nutritional quality and bioactive substance composition [22–24]. Four main approaches (pseudo-targeted metabolomics, targeted metabolomics, widely-targeted metabolomics, and untargeted metabolomics) are utilized in metabolomics [24–27]. Of these strategies, untargeted metabolomics is the most effective in defining changes in the whole metabolome of plant organs, tissues, or cells under specific conditions and periods [28, 29].

In this study, 210,003-grade tobacco leaves in Hubei Province were selected to investigate dynamic phytochemical and metabolome changes in tobacco leaves under natural, mechanical, and air-conditioning storage conditions. Our objectives were to reveal the impacts of these storage conditions on the quality of tobacco leaves and provide data resources for optimizing an effective storage environment for tobacco quality preservation. Our results may facilitate the sustainable production of high-quality tobacco products.

Results

Dynamic changes in iodine value (IV) absorbance, pH, and pigment composition of tobacco leaf under different storage conditions

The IV of the leaf stored under mechanical (MC) and air-conditioning (AC) significantly increased with the storage duration (Fig. 1A). The highest IV after nine months (T3) of storage of 2.073 was recorded under MC, but there was no significant difference between the IV under NT and AC (Fig. 1A). Compared to modified environments, the IV of leaf under NT significantly increased between T0 and T1, then remained statistically similar (Fig. 1A). As shown in Fig. 1B, the pH of tobacco leaves under NT decreased significantly with the increase in storage time. In contrast, the pH of the leaves under MC and AC decreased significantly and then increased (Fig. 1B). However, the final pH values at T3 were significantly low

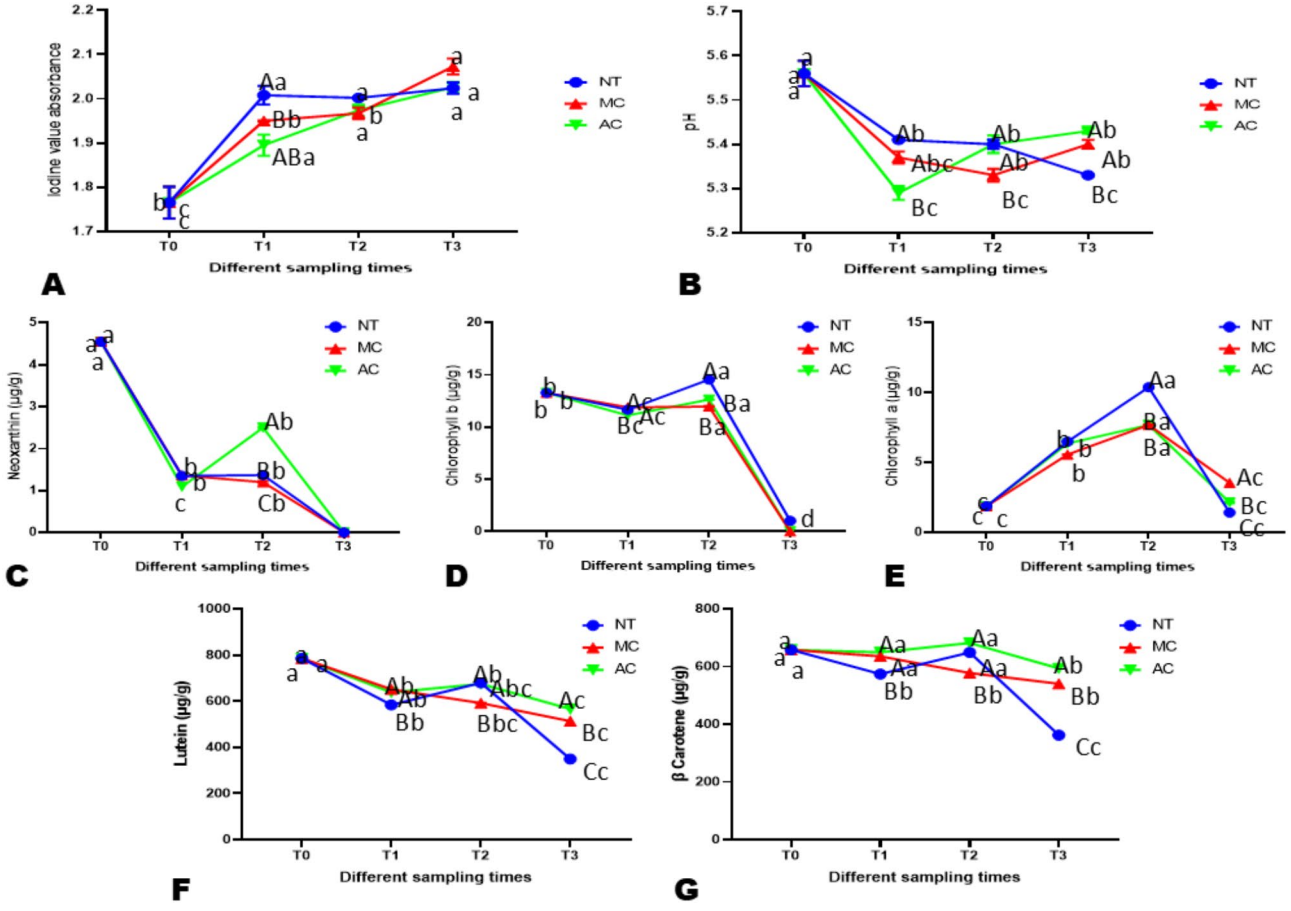

Fig. 1 Effect of different storage conditions on the dynamic changes in iodine value, pH, and pigment content of tobacco leaves. **A**) Iodine value. **B**) and pH. **C**) Neoxanthin. **D**) Chlorophyll b. **E**) Chlorophyll a. **F**) Lutein. **G**) β-carotene. Different uppercase letters in the figure indicate significant differences between different storage conditions ($P < 0.05$), and lowercase letters represent significant differences in sampling time ($P < 0.05$)

Table 1 Effect of different storage conditions on the reduction of pH, polyphenols, and pigments in tobacco leaves

Storage conditions	pH (%)	Polyphenols			Pigmentation				
		chlorogenic acid (%)	Scopolet-in (%)	Rutin (%)	Neoxanthin (%)	Chlorophyll b (%)	Lutein (%)	Chloro-phyll a (%)	β-Carotene (%)
Natural (NT)	4.09	10.66	6.36	20.92	100	92.30	55.54	75.02	44.92
Mechanical (MC)	2.87	6.74	6.19	19.38	100	100	34.58	37.37	17.86
Air-conditioning (AC)	2.33	5.71	12.82	18.95	100	100	27.96	62.22	42.64

compared to T0 (Fig. 1B). In general, the pH dropped by 4.09, 2.87, and 2.33% under NT, MC, and AC, respectively, after nine months (Table 1).

Chlorophyll and carotenoids are the main pigments in tobacco leaves. To reveal the effects of storage conditions on leaf pigment composition, we evaluated the dynamic changes in the contents of neoxanthin, chlorophyll a, chlorophyll b, β-carotene, and lutein (Fig. 1C-G). Neoxanthin and chlorophyll b contents were completely degraded under MC and AC storage conditions after nine months, as well as neoxanthin under NT (Fig. 1C, D; Table 1). The chlorophyll content under both storage conditions significantly increased from T0 to T2 (five months) and then dropped to values statistically similar to those at T0 (Fig. 1E). Lutein and β-carotene contents significantly decreased with the storage duration, and the lowest drops after nine months were recorded under AC (27% for lutein) and MC (17.86% for β-carotene) (Fig. 1F, G; Table 1). Both the pigments were mostly degraded under NT conditions, except for chlorophyll b (Fig. 1C-G; Table 1).

Changes of tobacco leaf polyphenols under different storage conditions

To explore the effects of storage conditions on tobacco leaf polyphenols, we investigated the variation in the

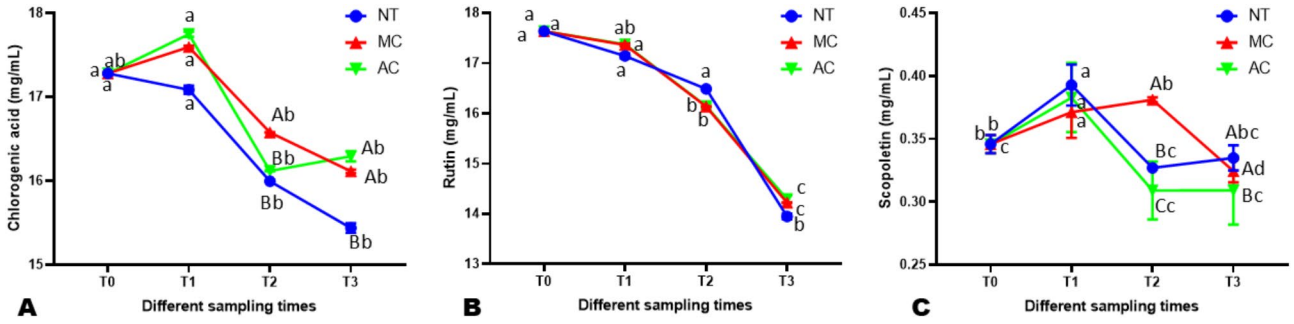

Fig. 2 Effect of different storage conditions on the dynamic changes of polyphenols' content of tobacco leaves. **A**) Chlorogenic acid. **B**) Rutin. **C**) Scopoletin. Different uppercase letters in the figure indicate significant differences between different storage conditions ($P < 0.05$), and lowercase letters represent significant differences in sampling time ($P < 0.05$)

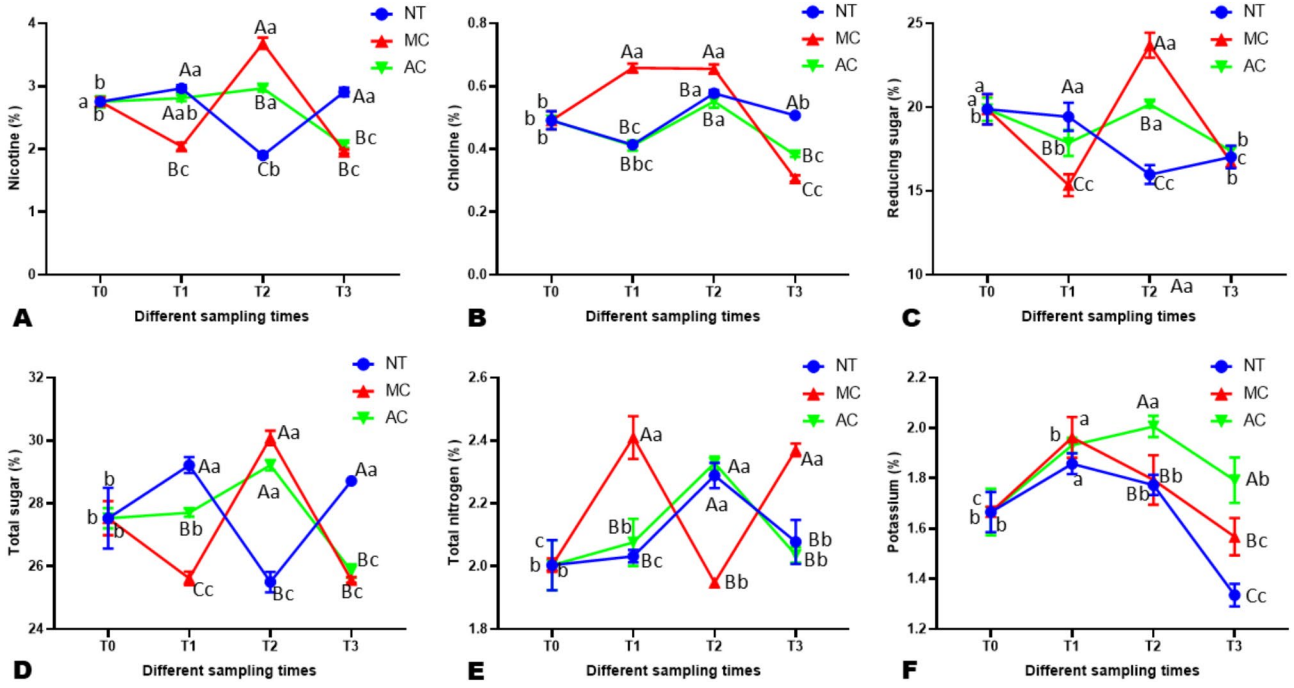

Fig. 3 Effect of different storage conditions on the dynamic changes of conventional chemical composition of tobacco leaves. **A**) Nicotine. **B**) Chlorine. **C**: Reducing sugar. **D**) Total sugars. **E**) Total nitrogen. **F**) Potassium. Different uppercase letters in the figure indicate significant differences between different storage conditions ($P < 0.05$), and lowercase letters represent significant differences in sampling time ($P < 0.05$)

content of chlorogenic acid, rutin, and scopoletin under NT, MC, and AC conditions (Fig. 2; Table 1). The content of chlorogenic acid and scopoletin significantly increased first and then decreased with the extension of storage duration under MC and AC conditions (Fig. 2A, C). The highest decrease in chlorogenic acid content was recorded under NT conditions (10.66%), followed by MC (6.74%) and AC (5.71%) (Table 1). Scopoletin content dropped most under AC (12.82%), followed by NT (6.36%) and MC (6.19%) (Table 1). Regarding the content of rutin, it significantly dropped along with the storage duration, and there was no significant difference in degradation rate between the three storage conditions (Fig. 2B). The decline rate of rutin in the three storage conditions was the fastest between 5 ~ 9 months (Fig. 2B).

Variation in conventional chemical composition of tobacco leaves under different storage conditions

Nicotine, total sugar, reducing sugar, total nitrogen, potassium, and chlorine are key important phytochemicals in tobacco leaves. These traits showed significant variations along with the storage duration under the three conditions, with increasing and decreasing trends (Fig. 3A-F). The content of nicotine, total sugar, reducing sugar, and chlorine reached their maximum values at T2 (5 months of storage) under MC and AC conditions (Fig. 3A-D). The potassium and total nitrogen contents

reached their maximum values at T1 (three months storage) under MC and T2 under AC conditions (Fig. 3E, F). The nicotine, total sugar, reducing sugar, and chlorine contents at T3 under MC and AC conditions were significantly lower than at T0 (Fig. 3A-D). Studies have shown that the sugar/base ratio is between 6 ~ 10 range, and the closer the ratio is to 10, the better the quality of the tobacco leaves [17]. The sugar/base ratio at T3 under MC and AC was 8.53 and 8.44, respectively (Table 2). Under NT conditions, the sugar/base ratio reached 8.41 at T2, then decreased to 5.85 at T3 (Table 2). The potassium/chlorine ratio was between 2 ~ 6 range, with the highest values of 5.11 and 4.7 reached at T3 under MC and AC, respectively (Table 2).

Correlations between traits under the different storage conditions

As shown in Figure S1, the absorbance of iodine value was negatively correlated with pH, chlorogenic acid, rutin, neoxanthin, chlorophyll b, and lutein during the storage. In contrast, it was positively correlated with chlorophyll a, potassium, nitrogen, and total sugar (Figure S1). There was a positive correlation between pH, polyphenols, pigments (except chlorophyll a), and reducing sugar (Figure S1). Potassium was negatively correlated with neoxanthin and lutein (Figure S1). Rutin, neoxanthin, lutein, and chlorogenic acid exhibited significant positive correlations with each other (Figure S1).

Variation of tobacco leaf metabolite profile under different storage conditions

To get more insights into tobacco leaf phytochemical profile variation during storage under NT, MC, and AC conditions, we subjected all samples to LC-MS-based untargeted metabolomics profiling analysis. We detected a total of 4,961 and 4,271 metabolites at the positive and negative electrospray ionization, respectively, with merged total detected metabolites of 7,675 (Table S1). 1,557 metabolites were detected in both positive and negative ionisation modes. Of the merged metabolites, 1,957 were classified into shikimates and phenylpropanoids (30.046%), terpenoids (26.622%), alkaloids (15.738%), fatty acids (15.38%), polyketides (5.518%), carbohydrates (3.372%), and amino acids and peptides (3.321%) (Figure S2, Table S1).

To examine the dynamic metabolite profile changes of tobacco leaves under NT, MC, and AC conditions, we conducted PCA (principal component analysis) and HCA (hierarchical clustering analysis) (Fig. 4A, B). The HCA and PCA revealed considerable changes in the tobacco leaf metabolite profile during storage (Fig. 4A, B). The control samples (CK) clustered separately from others on the PCA and HCA plots, indicating significant metabolism changes occurred in tobacco leaves during storage under NT, MC, and AC conditions (Fig. 4A, B). The metabolite profiles of leaves, respectively, under NT, MC, and AC conditions showed moderate variation from T1 to T3, as PC1 and PC2 scored 12.77% and 8.49%, respectively (Fig. 4A, B).The variation under AC conditions from T1 to T3 was the most noticeable (Fig. 4A, B). These results indicate that major metabolism changes occurred at the early stages of storage (between T0 and T1) and moderate metabolome variation between T1 and T3 under the three storage conditions.

Differential accumulation of metabolites (DAMs) along with the storage duration

DAMs offer exposure to metabolic changes that have occurred in plant organs under a specific condition and period [30]. Therefore, we identified all DAMs in tobacco leaves along with the storage duration under NT, MC, and AC conditions (Fig. 5A, C,E). The volcano plots of DAMs under NT, MC, and AC conditions are shown in Figures S3, S4, S5, respectively. The number of DAMs increased along with the storage duration under NT conditions, while under MC and AC conditions, it increased from T1 up to T2 and then decreased (Fig. 5A, C,E). The highest number of DAMs of 224 (137 up-regulated and 87 down-regulated) was identified at T3 under NT conditions (Fig. 5A). In contrast, the numbers of DAMs at T3 were 158 (99 up-regulated and 59 down-regulated) and 152 (103 up-regulated and 49 down-regulated) under MC and AC conditions, respectively (Fig. 5C, E). There were 68, 60, and 58 overlapped DAMs along with the storage duration under NT, MC, and AC conditions, respectively (Fig. 5B, D,F). These key DAMs may represent metabolic targets for analyzing metabolism changes in tobacco leaves under NT, MC, and AC conditions, respectively (Tables S2-S4).

Table 2 Effect of different storage conditions on sugar/base ratio and potassium/chlorine ratio

Sampling times	Sugar/base ratio			Potassium/chlorine ratio		
	Natural	Mechanical	Air-conditioning	Natural	Mechanical	Air-conditioning
T0	7.22	7.22	7.22	3.39	3.39	3.39
T1	6.55	7.52	6.36	4.49	2.98	4.70
T2	8.41	6.44	6.80	3.07	2.74	3.64
T3	5.85	8.53	8.44	2.63	5.11	4.70

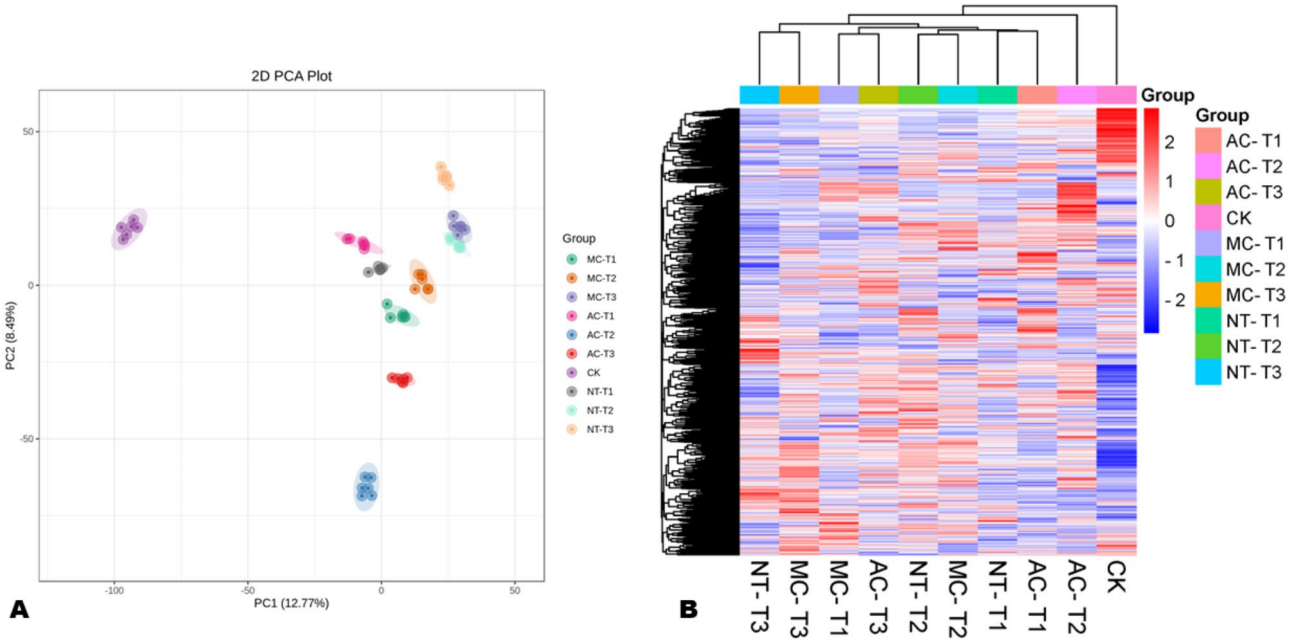

Fig. 4 Principal component analysis (**A**) and hierarchical clustering analysis (**B**) plots, exposing variation in tobacco leaf metabolite profile during storage under natural (NT), mechanical (MC), and air-conditioning (AC) conditions. T0 (CK), T1, T2, and T3 indicate different storage durations

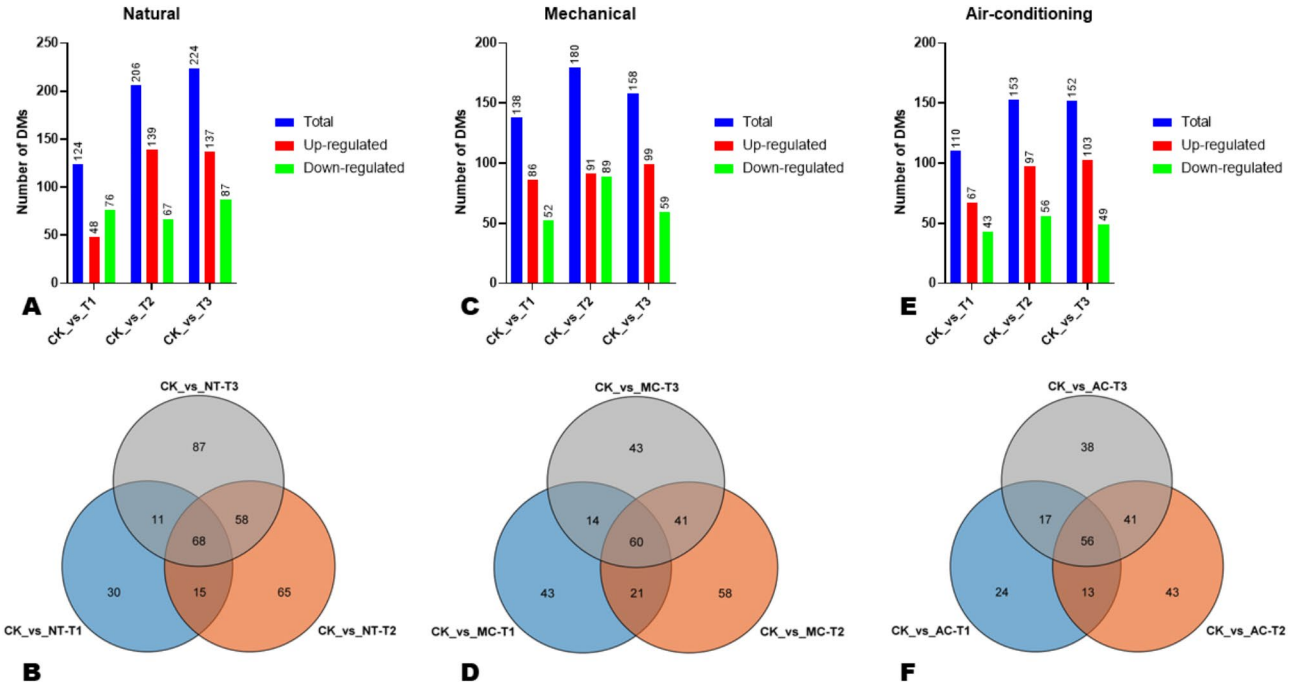

Fig. 5 Differentially accumulated metabolites (DAMs) in tobacco leaves during storage under natural (NT), mechanical (MC), and air-conditioning (AC) conditions. A) and **B**) Number of DAMs and key DAMs, respectively, under NT. **C**) and **D**) Number of DAMs and key DAMs, respectively, under MC. **E**) and **F**) Number of DAMs and key DAMs, respectively, under AC. T0 (CK), T1, T2, and T3 indicate different storage durations

KEGG enrichment analysis of differential metabolites

To reveal major metabolic processes occurring in tobacco leaves under the different storage conditions, we conducted KEGG annotation and enrichment analysis of DAMs between T0 (CK) and T3 (Fig. 6 and S6). The DAMs under NT were mostly assigned to diterpenoid biosynthesis, cell cycle, benzoxazinoid biosynthesis, acarbose biosynthesis, photosynthesis, sulfur metabolism, indole alkaloid biosynthesis, glutathione metabolism, and biosynthesis of secondary metabolites (Fig. 6A).

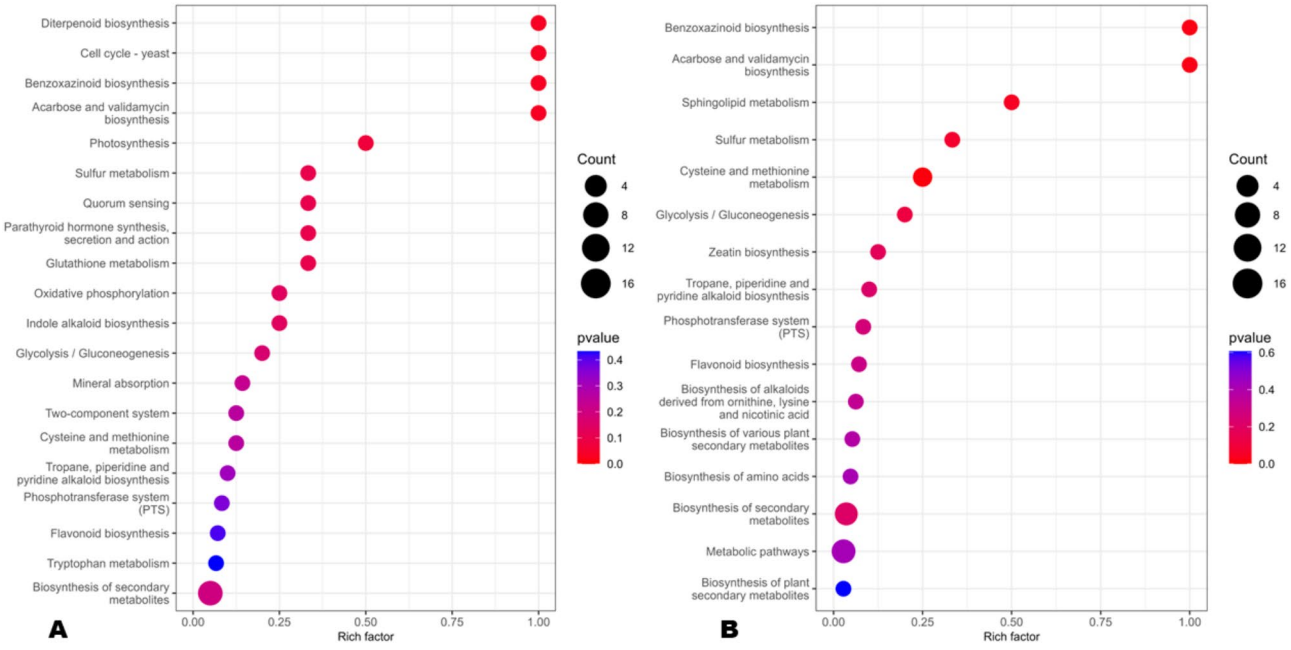

Fig. 6 KEGG annotation and enrichment analysis of DAMs in tobacco leaves during storage between times T0 (CK) and T3 under natural (**A**) and mechanical (**B**) conditions

The main pathways induced under MC were cysteine and methionine metabolism, biosynthesis of secondary metabolites, benzoxazinoid biosynthesis, acarbose biosynthesis, sphingolipid metabolism, glycolysis/gluconeogenesis, and sulfur metabolism (Fig. 6B). Meanwhile, under AC, the DAMs were primarily involved in steroid biosynthesis, diterpenoid biosynthesis, acarbose biosynthesis, sphingolipid metabolism, biosynthesis of secondary metabolites, and flavonoid biosynthesis (Figure S6).

Major differential metabolites

To reveal the major DAMs, we screened out known metabolites with|Log2FC| ≥ 2 between T0 and T3 under the three storage conditions. In total, 16 major DAMs, including seven alkaloids (carbendazim, formylanthranilic acid, pelletierine, isotetrandrine, difloxacin, anhalamine, and vomicine), one flavonoid (aspalathin), three terpenoids (taraxasterol, abietic acid, and sclareol), three polyketides (cis- [8]-shogaol, urushiol II, and bilobol), and two with unknown group (posaconazole and amygdalin) were filtered out, and their Log2FCs are presented in Fig. 7 and S7. In contrast to amygdalin, vomicine, cis- [8]-shogaol, and posaconazole were up-regulated in tobacco leaves under the three storage conditions (Fig. 7A, L and S7A, D). Anhalamine and pelletierine were up-regulated under NT and AC conditions but were down-regulated under MC conditions (Fig. 7B, E). Formylanthranilic acid and carbendazim were significantly up-regulated under AC and NT, respectively (Fig. 7F, G). Aspalathin and abietic acid were significantly down-regulated under AC conditions (Fig. 7H, J). Sclareol and urushiol II were significantly induced under NT and AC conditions (Fig. 7K and S7C).

Discussion

Tobacco industry plays a vital role in the economic development of China and other producing countries. With the improvement of people's living standards, there are many requirements for the quality of tobacco leaves [31]. Tobacco quality is significantly affected by the storage conditions. Thus, this study characterizes dynamic chemical changes occurring in tobacco leaves under NT, MC, and AC conditions.

We found that the IV increased with the storage time, consistent with previous reports [11, 32]. Studies have shown that an IV value in the range of 0.9 ~ 2.5 indicates tobacco quality improvement [11, 32]. The IV values of the tobacco leaves after nine months of storage under the three conditions were close to 2, indicating the quality of the tobacco leaves was maintained. However, the IV under MC was slightly higher, suggesting MC storage conditions were more appropriate for tobacco leaves' quality improvement. It is generally believed that the lower the pH of tobacco leaves, the more mellow the taste and the less irritating the smoke [33]. In this study, the pH decreased with the increase in storage time, and the lowest pH under MC and AC was reached at T1 and T2, respectively. The slight increase in pH at late storage stages under MC and AC may be related to the storage conditions that may have favorited the formation of novel alkali compounds or promoted the accumulation of alkali compounds [33]. As support, KEGG enrichment analysis

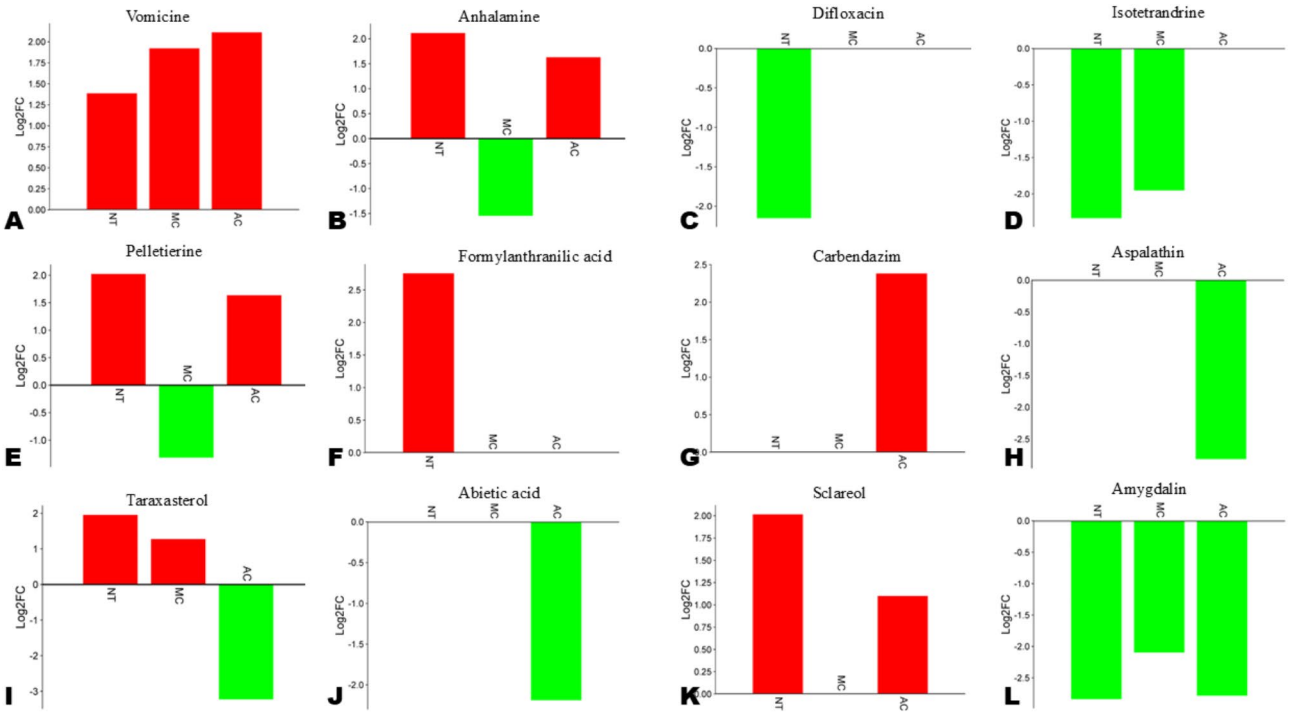

Fig. 7 Log2FCs of major DAMs in tobacco leaves during storage under natural (NT), mechanical (MC), and air-conditioning (AC) conditions. **A**)-G) Alkaloids. **H**) Flavonoid. **I**)-**K**) Terpenoids. **L**) Unknown class

of DAMs revealed that the biosynthesis of secondary metabolites was highly induced under AC, followed by MC and NT at T3. Notably, the biosynthesis pathways of terpenoids, alkaloids, and steroids were significantly enriched under AC at T3. The reduction in nicotine and sugar levels was associated with an increase in nitrogen content and vice versa under the three storage conditions. These results suggest an active central metabolism in tobacco leaves during storage. It has been demonstrated that reduced nitrogen levels are associated with increased carbon levels and carbon-to-nitrogen (C/N) ratios in plants [34, 35]. Further studies are required to decipher central metabolism variation in tobacco leaves during storage.

Polyphenols in tobacco determine the color, taste, alcohol, and smoke of tobacco leaves and are significantly positively correlated with the quality of flue-cured tobacco [36–38]. Herein, we found that the content of rutin, chlorogenic acid, and scopoletin decreased with the increase in storage time, consistent with previous findings [11, 39, 40]. Compared to NT, the chlorogenic acid content decreased less under MC and AC, and the recorded values under these modified atmospheres were higher than 15 mg/g after nine months. These results infer that the sensory quality of tobacco leaves stored under MC and AC conditions may be better than in NT. Supportively, Hao et al. revealed that a chlorogenic acid content of 15 $mg{\cdot}g^{-1}$ in tobacco leaves is associated with improved sensory quality [41]. The degradation of pigments (chlorophyll and carotenoids) is a key processing mechanism for improving the intrinsic quality of tobacco leaves [11, 13]. Concordant with this, we found that the chlorophyll a, chlorophyll b, neoxanthin, lutein, and β-carotene content of the leaves significantly decreased with the storage duration. In particular, neoxanthin and chlorophyll b were completely degraded under MC and AC at T3. Lutein and β-carotene content dropped less under MC and AC than NT, indicating the biological properties of the leaves stored under MC and AC conditions may be significantly pronounced. For instance, lutein is an important carotenoid with recorded pharmacological attributes against eye diseases, neurological disorders, cardiac complications, skin irritation, microbial infections, bone decay, etc [42]. Chlorogenic acid is an effective nutraceutical against diabetes, cancers, inflammation, obesity, etc [43]. These findings indicate that MC and AC storage conditions can enhance the quality of tobacco leaves to a certain extent. The high sugar/base and potassium/chlorine ratios recorded at T3 under MC and AC also supported the better quality of leaves stored in these artificial environments. A higher ratio of potassium/chlorine indicates that tobacco leaves are of good flammability [44]. The closer the sugar-base ratio is to 10, the higher the quality of tobacco leaves [17].

Metabolomics analysis revealed that significant metabolic changes occurred in tobacco leaves under storage

between T0 and T1, while moderate metabolite profile variation were observed between T1 and T3 under the three conditions. The number of DAMs increased with the storage duration under NT, and the highest DAM number of 224 was detected at T3. The number of significant DAMs under MC and AC ranged from 138 (T1) to 180 (T2) and 110 (T1) to 153 (T2), respectively. These results indicate that the longer tobacco leaves are stored under NT, the more their quality will decrease significantly. Moreover, they confirm that storing tobacco leaves under MC and AC conditions may preserve them from quality deterioration over NT. Glutathione metabolism was significantly induced in stored leaves under NT, suggesting the leaves suffered oxidation stress, which might have contributed to the considerable quality loss [45]. Meanwhile, the induction of oxidation phosphorylation under NT indicates that intensive metabolic processes occurred, resulting in high consumption of energy. We identified key overlapped DAMs under the three storage conditions. We also screened out sixteen known major DAMs and their Log2FC after nine months of storage under the three conditions. These DAMs could be targets in future studies to deepen our understanding of mechanisms underlying tobacco leaf quality changes during storage. For instance, amygdalin is a medically important compound, but it presents toxicity risks due to its enzymatic degradation that produces hydrogen cyanide [46, 47]. Interestingly, amygdalin was significantly degraded during storage under the three conditions. Scareol, an essential labdane diterpene used in fragrance and flavor industries, was significantly induced by NT and AC storage conditions [48].

During storage, tobacco leaves respire more actively at higher temperatures. The temperature and oxygen during storage are dependent upon many factors, such as biological reaction rates, the mass transfer process, packing conditions, and varied ambient temperature [49]. To date, many studies have been conducted on the effects of curing temperature and humidity on the quality of tobacco leaves [11, 50, 51]. However, little is known about the effect of oxygen on the quality of aging tobacco leaves. Oxygen is critical for normal fermentation and quality enhancement of tobacco leaves, as it participates in biological reactions, can effectively regulate the aging rate, and can drive shifts in microbial communities that are essential for a smooth aging process [52]. Selecting an ideal oxygen content can improve the aging rate, decrease maintenance costs, promote inventory turnover, and ensure tobacco leaf quality [52]. Although this study found that MC and AC conditions better preserved the quality of aging tobacco leaves, we noticed considerable temperature and oxygen content fluctuations under these conditions. These variations might be due to insufficient sealing of the storage facilities, the influence of the circulating air, and the dynamic metabolic changes occurring in the leaves at different times of storage. Therefore, before promoting the MC and AC conditions for the long-term storage of tobacco leaves, it is essential to further optimize the temperature and oxygen content within the storage boxes. Additionally, the airtightness of the storage environment should be significantly improved to minimize oxygen fluctuations caused by inadequate sealing, thereby ensuring the stability of storage conditions and the consistency of tobacco leaf quality.

Conclusions

In summary, this study revealed changes in tobacco leaf quality characteristics and metabolite profile during storage under natural (NT), mechanical (MC), and air-conditioning (AC) conditions. In general, the quality characteristics of tobacco leaves were more preserved under MC and AC conditions compared to NT. For instance, lutein, β-carotene, and chlorogenic acid were significantly less degraded under MC and AC than NT. The nicotine, total sugar, reducing sugar, and chlorine content significantly varied along with the storage duration, reaching their maximum values under MC and AC after five months of storage. The sugar/base ratio after nine months of storage under MC, AC, and NT was 8.53, 8.44, and 5.85, respectively. There were fewer DAMs under AC and MC than under NT. The quality characteristics of tobacco leaves showed significant correlations under the three storage conditions. Biosynthesis of secondary metabolites was significantly induced in tobacco leaves under the three storage conditions. Glutathione metabolism and oxidative phosphorylation were highly induced under NT, while biosynthesis of terpenoids and steroids was highly induced under AC. Furthermore, we screened out key overlapped DAMs under NT, MC, and AC, as well as major DAMs. Our findings provide guidance for optimizing the storage conditions for better preservation of tobacco leaves.

Materials and methods

Plant materials, storage conditions, and sampling

Flue-cured tobacco graded as 210,003 central tobacco (central Hubei tobacco) was analyzed in this study. It was provided by Hubei China Tobacco Industry Co., Ltd., Wuhan, China. The tobacco leaves were divided into three groups, each containing five replications, for storage under natural (NT), mechanical (MC), and air-conditioning (AC) conditions, respectively, at the Wuhan warehouse of Hubei China Tobacco Industry Co., Ltd. The monthly average temperature, humidity, and oxygen concentration under the three storage conditions are presented in Table 3. For the MC, the leaves were enclosed in boxes, and the environmental conditions were monitored for ideal tobacco mellowing by precisely controlling the

Table 3 Changes in monthly average temperature, humidity, and oxygen concentration under the three storage conditions

Months	Average temperature/℃			Average humidity/%			Average oxygen concentration/%	
	Natural	Mechanical	Air-conditioning	Natural	Mechanical	Air-conditioning	Mechanical	Air-conditioning
2022.8	27.92	28.33	28.85	60.03	58.72	48.33	20.59	7.10
2022.9	25.33	27.23	27.60	59.71	58.38	46.10	20.62	1.20
2022.10	20.70	23.82	24.90	52.55	54.48	47.40	20.66	7.10
2022.11	20.51	21.93	21.30	56.16	55.31	52.85	20.70	0.50
2022.12	13.15	16.88	13.23	38.84	47.18	50.53	20.64	0.50
2023.1	11.01	11.01	12.60	46.51	46.51	52.43	-	15.50
2023.2	10.64	13.07	12.13	50.86	53.32	54.78	20.61	9.05
2023.3	13.59	14.43	12.18	51.77	53.99	56.85	20.63	4.80
2023.4	16.96	17.36	18.35	55.17	54.70	58.70	20.63	0.90

oxygen concentration, temperature, and humidity (Table S5). The oxygen concentration was precisely controlled within the range of 20.59–20.70%, ensuring that the oxygen supply required for the tobacco mellowing process was adequate and stable. The mean value of the box, stack, and inter-bin temperatures was 22.24 °C, 20.14 °C, and 15.98 °C, respectively. The average value of humidity in the stacks was 55.88%. Meanwhile, the average value of humidity between the bins was 47.16%. Similarly, the oxygen, temperature, and humidity in the boxes under AC were maintained between the range of 0.50–15.50%, 11.80 °C to 29.00 °C, and 46.95% and 59.40%, respectively, to prevent the tobacco leaves from premature aging and extend the storage life. Detailed conditions for the AC storage are presented in Table S6.

The 210,003-grade tobacco leaves were sampled on the first day of storage (T0, August 8, 2022) and subsequently on October 8, 2022 (T1), January 8, 2023 (T2), and May 8, 2023 (T3). The five-point sampling method was adopted, and all samples were stored at -20 °C until they were used. The five-point sampling method refers to first determining the center point of the diagonals as the center sampling point, and then selecting four points on the diagonal lines that are equidistant from the center sampling point.

Determination of iodine value absorbance and pH

0.5 g of the sample and 30 mL of distilled water were mixed in a 100 mL triangular flask, sonicated at 40 Hz for 30 min, and then filtered through a qualitative filter paper. Next, 2.5 mL of the supernatant was introduced in a 10 ml EP tube, followed by the addition of 1 mL of KI solution and 1 mL of KIO_3 solution. The mixture was vortexed and kept in darkness for 1 h. The absorbance of the solution was recorded at 460 nm using a UV spectrophotometer (Shanghai Aoyi Instrument Co., Ltd.). The pH was determined using the YC/T 222–2007 pH evaluation method for tobacco and tobacco products [53].

Evaluation of polyphenols, pigments, and conventional chemical composition

The polyphenols (rutin, chlorogenic acid, and scopoletin) and pigments (chlorophyll a, chlorophyll b, neoxanthin, lutein, and β-carotene) were evaluated with reference to tobacco industry standards for p-polyphenols (YC/T 202–2006) and plastid pigments (YC/T 382–2010), respectively [54, 55]. The assay was performed using high-performance liquid chromatography (LC-20 A HPLC, Shimadzu Co., Ltd.).

The contents of nicotine, chlorine, reducing sugars, total sugars, total nitrogen, and potassium were determined using the continuous flow method with reference to tobacco industry standards [56, 57].

Metabolite extraction and UHPLC-MS/MS analysis

Ground leave samples (100 mg) were suspended in 500 µL of prechilled 80% methanol by vortexing. The mixtures were incubated on ice for 5 min, followed by centrifugation (15,000 g at 4 °C for 20 min). Subsequently, the supernatants were collected and diluted with LC-MS grade water to 53% methanol. Each sample was transferred to a novel Eppendorf tube, followed by a second centrifugation. Finally, the supernatants were filtrated using a 0.22 µm micropore membrane (SCAA-104, ANPEL, Shanghai, China), and extracts were subjected to LC-MS/MS untargeted metabolomics analysis [58]. An equal volume of each extract was mixed to generate QC (quality control) samples.

UHPLC-MS/MS (ultra-high-performance liquid chromatography-mass spectrometry) analyses were achieved by a Vanquish UHPLC system (ThermoFisher, Germany) coupled with an Orbitrap Exploris 120 mass spectrometer (Thermo Fisher, Germany) at Bioyi Biotechnology Co., Ltd. Wuhan, China. Each sample was injected into a Hypersil Gold column (100 × 2.1 mm, 1.9 µm) using a 12-minute linear gradient at a 0.2 mL/min flow rate. Eluents A (0.1% formic acid in water) and B (methanol) were used in positive mode. Meanwhile, eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (methanol) were used in the negative mode. We set the solvent

gradient as follows: 2% B, 1.5 min; 2–85% B, 3 min; 85–100% B, 10 min;100-2% B, 10.1 min 2% B, 12 min. Q Exactive TM-HF MS operated in positive/negative modes with a spray voltage of 3.5 kV, capillary temperature of 320 °C, sheath gas flow rate of 35 psi, Aux gas flow rate of 10 L/min, S-lens RF level of 60, and Aux gas heater temperature of 350 °C. Both MS and MS/MS data were acquired at a range of 50–1500 m/z, and the collision energy (CE) of the MS/MS was varied from 0 to 60 V for each target compound.

Metabolomics data processing and metabolite identification

The raw data were treated using CD3.3 software (Compound Discoverer 3.3, Thermo Fisher) to conduct peak alignment, peak picking, and quantification. Operational parameters were: peak area, corrected with QC1; actual mass tolerance, 5 ppm and signal intensity tolerance, 30%; peak width = C (5, 30); mzwid = 0.015; mzdiff = 0.01; and method ="centWave". Next, we normalized peak intensities to the total spectral intensity, and the obtained data served to predict the molecular formula based on spectrum information, retention time, and m/z. Peaks were then matched to the high-quality metDNA2 database constructed from standards [59]. We matched and identified these peaks to maximize the comprehensive information on metabolites. Further, the identified metabolites were confirmed by matching to mzVault, mzCloud (https://www.mzcloud.org/), and MassList databases to yield accurate qualitative and relative quantitative data. Compounds whose coefficient of variation was greater than 30% in QC were discarded.

Data analysis

Metabolites were annotated by mapping to the KEGG database (https://www.genome.jp/kegg/pathway.html), HMDB database (https://hmdb.ca/metabolites), and LIPIDMaps database (http://www.lipidmaps.org/). Level 1-level4 confidence was applied [60, 61]. PCA (principal components analysis) and PLS-DA (partial least squares discriminant analysis) were carried out at metaX [62]. Univariate analysis (t-test) was applied to compute the statistical significance (*P*-value). The metabolites with VIP > 1, *P*-value < 0.05, and fold change ≥ 2 or FC ≤ 0.5 were identified as DAMs (differentially accumulated metabolites). Volcano plots were generated based on log2(FC) and -log10(*P*-value) using ggplot2 in R language (version 4.1.0). For clustering heatmap, normalized data by z-scores of the intensity areas were plotted using the Pheatmap package in R language. KEGG database was used to functionally annotate DAMs. Significantly enriched pathways were selected at $P < 0.05$.

For phytochemical data, SPSS software was used to conduct ANOVA (analysis of variance), with significant differences set at $P < 0.05$. Origin2021 and GraphPad Prism 9 (version 9.01) were used to plot. Three replications were performed for each analysis, and the results are presented as mean ± SD (standard deviation).

Abbreviations

NT	Natural conditions
MC	Mechanical conditions
AC	Air-conditioning conditions
ANOVA	Analysis of variance
UHPLC	MS/MS-ultra-high-performance liquid chromatography-mass spectrometry
DAMs	Differentially accumulated metabolites
IV	Iodine value
PCA	Principal component analysis
HCA	Hierarchical clustering analysis
QC	Quality control
KEGG	Kioto encyclopedia for genes and genomes

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Not applicable.

Author contributions

G.X. Data curation, methodology, writing Original Draft; L.W., T.P., A.X., J.G., and X.T. formal analysis, validation, software, and investigation; L.Y., T.M., M.Z., Z.C., and H.W. visualization, supervision, and Writing Review and Editing; S.S. conceptualization, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Data availability

All other datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The experiments did not involve endangered or protected species. This study used flue-cured tobacco graded (210003), which was given by Hubei China Tobacco Industry Co., Ltd., Wuhan, China. We declare that the collection and use of plant materials in this study comply with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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