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Identification and Evaluation of Diterpenoids from Glandular Trichome Secretions of Air/Sun-Cured Tobacco Germplasm Resources

Chaofan Qi [1,†], Jing Liu [1,†], Yongmei Du [1], Xiao Han [1], Hongbo Zhang [1], Qiujuan Fu [1], Jianhui Zhang [2,*] and Yanhua Liu [1,*]

[1] Plant Functional Ingredient Research Center, Tobacco Research Institute of Chinese Academy of Agricultural Sciences, Qingdao 266101, China; 13020502056@163.com (C.Q.); m17853480108@163.com (J.L.); duyongmei@caas.cn (Y.D.); 46326099@163.com (X.H.); zhanghongbo@caas.cn (H.Z.); fuqiujuan@caas.cn (Q.F.)
[2] Tobacco Science Institute of Sichuan Province, Chengdu 610094, China
* Correspondence: sgg94@126.com (J.Z.); liuyanhua@caas.cn (Y.L.)
† These authors contributed equally to this work.

Abstract: To explore the multipurpose utilization of tobacco germplasm resources, 80 representative air/sun-cured tobacco germplasms were used as experimental materials to determine the composition and content of the diterpenoids of glandular trichome secretions using ultra-performance liquid chromatography (UPLC). Correlation analysis, cluster analysis, and principal component analysis (PCA) were used to comprehensively evaluate 80 air/sun-cured tobacco germplasms. The results are as follows: (1) 11 chromatographic peaks (Peak1–Peak11) were detected by UPLC, and the coefficient of variation of their contents ranged from 26.3 to 143%. (2) Correlation analysis showed that alpha-cembratriene-diol (α-CBT-diol) and beta-cembratriene-diol (β-CBT-diol) had the highest correlation coefficients (0.97), showing a significantly negative correlation with *cis*-abienol and a significantly positive correlation with the other diterpenoids. (3) PCA showed that the cumulative contribution rate of the three principal components was 75.70%, and the diterpenoid-rich germplasms were ranked in the following order: X40 (Maiduo) > X48 (Jianpingpiaoba) > X58 (Mianzhu) > X54 (Shifangpipaliu) > X63 (Xuejia5) > X41 (Tangpeng) > X29 (OLOR) > X73 (Criollo) > X44 (Tiebanqing) > X70 (Nicaragua Changxin). The diterpenoid content of X36 was the lowest. These results provide excellent germplasm for the extraction and exploitation of the diterpenoids and for genetic studies of their metabolism.

Keywords: air/sun-cured tobacco germplasm resources; glandular trichome secretion; diterpenoids; identification and evaluation

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1. Introduction

In 1985, Severson et al. [1] put forward the concept of tobacco surface chemistry for the first time and isolated diterpenoids, sugar esters, waxes, and other chemical components from tobacco glandular trichome secretions. α-cembratriene-diol (α-CBT-diol), β-cembratriene-diol (β-CBT-diol), and *cis*-abienol are the main diterpenoids present in tobacco. The content of α-CBT-diol and β-CBT-diol can account for approximately 60% of the total glandular trichome secretions [2]. These diterpenoids are not only important precursors of the aromatic components of tobacco leaves but also exhibit useful biological effects, such as antibacterial, insecticidal, and anti-tumor activities [2–4]. During tobacco leaf curing, Cembratriene-diol (CBT-diol) is degraded to produce solanone, solanofuran, solandione, and other flavor components. The degradation products of *cis*-abienol are mainly C-16 compounds, including ambroxide and ambreinolide, and other substances that have a strong ambergris (cypress wood) aroma [5]. Menetrez et al. [6] were the first to find that α-CBT-diol and β-CBT-diol have strong inhibitory effects on conidial germination

of tobacco downy mildew. Baraka et al. [7] and Hailat et al. [8] showed that CBT-diol has a strong inhibitory effect on the growth of prostate and breast cancer cells. Seo et al. [9] showed that *cis*-abienol prevents the occurrence of tobacco, tomato, and *Arabidopsis thaliana* (L.) Heynh. wilt disease by inhibiting the growth of bacteria. Mischko et al. [10] indicated that cembratriene-ol (CBT-ol) exhibited bioactivities that specifically target Gram-positive bacteria and displayed insecticidal characteristics in both in vivo and in vitro settings. Thus, the secretions of tobacco glandular trichome have important developmental value.

Previous studies have shown that tobacco glandular trichomes and their secretions are mainly determined by genes. Ju et al. [11] showed that the content of CBT-diol is controlled by two pairs of additive–dominant–epigenetic major genes plus additive–dominant–epigenetic polygenes. The heritability of the major genes for CBT-diol content is 77.55%. Thus, there are significant differences in the composition and content of secretions from different types of tobacco germplasm [12]. CBT-diol content is higher in sun-cured tobacco than in burley, Maryland, oriental, and flue-cured tobacco [13]. *Cis*-abienol is abundant in the glandular trichome secretions of sun/air-cured tobacco, such as oriental and cigar tobacco. However, there is little *cis*-abienol in those of burley, Maryland, and flue-cured tobacco. There are more than 3500 sun/air-cured tobacco germplasm resources in the National Infrastructure for Crop Germplasm Resource (Tobacco; Qingdao) of the Chinese Academy of Agricultural Sciences. These germplasm resources show potential for development in diterpenoids extraction and exploitation. At present, the study of tobacco glandular trichome secretions mainly focuses on the identification and evaluation of CBT-diol content in flue-cured tobacco [2,12,13]. The systematic identification of diterpenoid compounds such as CBT-diol, *cis*-abienol, and CBT-ol in sun/air-cured tobacco germplasm resources is lacking. To explore the use of tobacco germplasm resources in plant-based pesticides and fragrance compounds, we selected 80 representative air/sun-cured tobacco germplasms (including breeding, local, and introduced resources) as experimental materials and analyzed the composition and content of the secretions of ethanol extracts from the flowers of 80 germplasms. The samples were analyzed using ultra-performance liquid chromatography (UPLC). Correlation analysis, cluster analysis, and principal component analysis (PCA) were used to comprehensively evaluate the composition. The selected germplasms rich and lacking in α-CBT-diol, β-CBT-diol, *cis*-abienol, CBT-ol, and other useful components could provide materials for cigarette flavoring, plant-derived pesticides, and genetic studies of diterpenoid compounds.

2. Materials and Methods

2.1. Plant Materials

We tested 80 representative air/sun-cured tobacco germplasms, including 46, 30, and 4 introduced, local, and breeding germplasms, respectively, obtained from the National Infrastructure for Crop Germplasm Resource (Tobacco; Qingdao) of the Chinese Academy of Agricultural Sciences. The accession numbers are X1–X80 (Table 1).

Table 1. Test numbers and sources of 80 air/sun-cured tobacco germplasm resources.

No.	Name	Type	Source	No.	Name	Type	Source
X1	Havana-1	cigar	introduced	X41	Tangpeng	sun-cured	local
X2	Havana No. 1	cigar	introduced	X42	Jiangyouyan	sun-cured	local
X3	Beinhart 1000-1	cigar	introduced	X43	Wushanxiaolanyan	sun-cured	local
X4	Criollo Salteno 11	cigar	introduced	X44	Tiebanqing	sun-cured	local
X5	S-2	cigar	introduced	X45	Meitanshaiyan	sun-cured	local

Table 1. *Cont.*

No.	Name	Type	Source	No.	Name	Type	Source
X6	Havana IIc	cigar	introduced	X46	Hefengheiyan	sun-cured	local
X7	Zrenjanin	cigar	introduced	X47	Liufengmaoba	sun-cured	local
X8	Yinnixuejiabaopi	cigar	introduced	X48	Jianpingpiaoba	sun-cured	local
X9	112–117	cigar	introduced	X49	Dayeziqingyan	sun-cured	local
X10	Bad Geudertheimer Landsorte	cigar	introduced	X50	Lichuanmaoyan	sun-cured	local
X11	Begej	cigar	introduced	X51	Zhushandaliuzi	sun-cured	local
X12	Connecticut Broad Leaf	cigar	introduced	X52	Fengjiedamaoyan	sun-cured	local
X13	Connecticut Shade	cigar	introduced	X53	Shai9118	sun-cured	breeding
X14	E 18	cigar	introduced	X54	Shifangpipaliu	sun-cured	local
X15	Geudetthelmex	cigar	introduced	X55	Shiyan1	sun-cured	local
X16	Hanica	cigar	introduced	X56	Kuiliu	sun-cured	local
X17	Havana 211	cigar	introduced	X57	Bamaoliu	sun-cured	local
X18	Havana 510	cigar	introduced	X58	Mianzhu	sun-cured	local
X19	Manila	cigar	introduced	X59	Bashan1	sun-cured	local
X20	Havana	cigar	introduced	X60	Chongzhoushaiyan2	sun-cured	local
X21	Tuerqixueji	cigar	introduced	X61	Quanyan	sun-cured	local
X22	Conn Shade	cigar	introduced	X62	Zhouyan	sun-cured	local
X23	Dexue 1	cigar	introduced	X63	Xuejia5	sun-cured	introduced
X24	Dexue2	cigar	introduced	X64	New Havana	cigar	introduced
X25	Dexue 3	cigar	introduced	X65	Comstock Spanish	cigar	introduced
X26	Habana92	cigar	introduced	X66	Mont Calme Brun	cigar	introduced
X27	Cubra-Brazil	cigar	introduced	X67	Trapesond 288	cigar	introduced
X28	Kangzhoukuoye	cigar	introduced	X68	CA0705	cigar	breeding
X29	OLOR	cigar	introduced	X69	CA0709	cigar	breeding
X30	Duominijiachangxin	cigar	introduced	X70	Nilajiagua Changxin	cigar	introduced
X31	Duominijiaduanxin	cigar	introduced	X71	Besuki	cigar	introduced
X32	MFPP	cigar	introduced	X72	Ha20	cigar	introduced
X33	MFZS	cigar	introduced	X73	Criollo	cigar	introduced
X34	CP2011	cigar	introduced	X74	Ha12	cigar	introduced
X35	MSCA	cigar	introduced	X75	Ha19	cigar	introduced
X36	CS0708	cigar	breeding	X76	Jilindabaihua	sun-cured	local
X37	Shangzhiyiduohua	sun-cured	local	X77	Mulengdaqingjin	sun-cured	local
X38	Shandongdaye	sun-cured	local	X78	Juanyeshaiyan	oriental	local
X39	Xinbinxiaotuanye	sun-cured	local	X79	Huangmaoyan	sun-cured	local
X40	Liaoduo	sun-cured	local	X80	Baimaoyan	sun-cured	local

2.2. Field Trial Design

Field trials were conducted at the Cigar Scientific Research and Testing Station of Sichuan Province and the Luozhuang Experimental Station of Shandong Province in 2020 and 2021. Seeds were sown in early February and transplanted in mid-April. The field trials were arranged in a randomized block design with three replications. Plants were planted at a density of 25 plants per row with a plant spacing of 45 cm and a row spacing of 110 cm.

2.3. Sampling and Treatment

In the field, five tobacco inflorescences per replicate of each tobacco germplasm were selected at the full bloom stage, and 20 flowers were obtained during the growth period, as shown in the rectangular box in Figure 1. The 20 flowers were divided into two groups of 10 flowers, weighed, and placed in 250 mL conical flasks containing 50 mL 95% ethanol and then shaken for 1 min. Finally, the flowers were removed, and the extract was stored at 4 °C until analysis.

Figure 1. Tobacco flowers at different developmental stages.

2.4. UPLC Analysis

Diterpenoids were detected using the method as follows: the extract (1 mL) was filtered through a 0.22 μm nylon filter membrane (spica, Shanghai Jiayi Biotechnology Co., Ltd., Shanghai, China) and then tested using Waters ACQUITY UPLC with TUV detector (Waters Technologies Ltd., Milford, MA, USA). The sample volume was 5 μL. Analysis was performed using an ACQUITY UPLC BEH C18 column (2.1 mm × 50 mm × 1.7 μm) with the pre-column (Assy, Frit, 0.22 μm, 2.1 mm). The mobile phase consisted of acetonitrile (A) and ultrapure water (B). The elution procedure was as follows: 0–6 min, 20–80% A; 6–11 min, 100% A; 11–16 min, 80–20% A. The flow rate was 0.3 mL/min, column temperature was 35 °C, and detection wavelength was 208 nm.

2.5. UPLC-Q-TOF-MS Analysis

Diterpenoids were identified using ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS). The chromatographic conditions were the same as those used in the UPLC analysis. For mass spectrometry, MS analysis was performed on a UHR-QTOF maXis (Bruker, MA, USA) equipped with an electrospray ionization (ESI) source. The MS data were collected by Hystar and processed by Compass Data Analysis software in positive mode. The ESI source conditions were applied as follows: full-scan data acquisition was performed from m/z 50 to 800; the capillary voltage was 4500 V; end plate offset was −500 V; dry temperature was 180 °C; nebulizer gas pressure was 1.0 bar; and dry gas flow rate was 6 L/min.

2.6. Content Calculation

Peak7, Peak8, Peak10, and Peak11 were identified as α-CBT-diol, β-CBT-diol, *cis*-abienol, and CBT-ol, respectively, using respective standard compounds.

α-CBT-diol, β-CBT-diol, CBT-ol (98% purities, isolated from tobacco by our laboratory), and *cis*-abienol (96% purity, Beijing Bailingwei Technology Co., Ltd., Beijing, China) were dissolved in anhydrous ethanol to prepare a reserve standard solution which was diluted with 60% acetonitrile to prepare the working standard solution.

The standard curve of α-CBT-diol was constructed using concentrations of 12.09, 24.18, 48.36, 72.53, 96.71, 120.89, 160.78, and 183.75 μg/mL. The method's limit of detection and limit of quantification were 0.12 μg/mL and 33.8 μg/g, respectively. The method demonstrated a reproducibility with relative standard deviations of 3.46%.

The standard curve of β-CBT-diol was constructed using concentrations of 3.20, 6.40, 12.80, 19.20, 25.60, 32.00, 44.81, and 51.21 μg/mL. The method's limit of detection and limit of quantification were 0.15 μg/mL and 49.8 μg/g, respectively. The method demonstrated a reproducibility with relative standard deviations of 3.11%.

The standard curve of cis-abienol was constructed using concentrations of 2.08, 3.12, 5.20, 10.40, 20.80, 31.20, 41.60, and 52.00 μg/mL. The method's limit of detection and limit of quantification were 0.11 μg/mL and 13.30 μg/g, respectively. The method demonstrated a reproducibility with relative standard deviations of 3.43%.

The standard curve of CBT-ol was constructed using concentrations of 1.98, 2.97, 4.95, 9.90, 19.80, 29.70, 39.60, and 49.50 μg/mL The method's limit of detection and limit of

quantification were 0.09 μg/mL and 6.34 μg/g, respectively. The method demonstrated a reproducibility with relative standard deviations of 3.09%.

$$C_n = \frac{S_n}{S_{\beta-\text{CBT}-\text{diol}}} \times C_{\beta-\text{CBT}-\text{diol}} \quad (1)$$

where C_n is the content of the Peak$n$ (n = 1, 2. . ., 11); $C_{\beta\text{-CBT-diol}}$ is the content of β-CBT-diol; S_n indicates the peak area of Peakn; and $S_{\beta\text{-CBT-diol}}$ indicates the peak area of β-CBT-diol.

2.7. Data Analysis

IBM SPSS Statistics 23.0 was used for correlation analysis and PCA, while R software was used for cluster analysis. The following formulas were used to comprehensively evaluate and rank the 80 air/sun-cured tobacco germplasm resources.

The relative formula of the comprehensive evaluation method is as follows:

$$U(x_j) = \frac{(x_j - x_{\min})}{(x_{\max} - x_{\min})} \quad (2)$$

$$w_j = \frac{r_j}{\sum_{j=1}^{n} r_j} \quad (3)$$

$$D = \sum_{j=1}^{n} [U(x_j) w_j] \quad (4)$$

where $U(x_j)$ is the membership function value of the comprehensive index of j; x_j indicates the score of the jth comprehensive indicator (j = 1, 2. . ., n); $x_{\max}$ and $x_{\min}$ are the maximum and minimum scores of the jth comprehensive indicator, respectively; w_j, the weight, is the importance of the comprehensive index of j in all the comprehensive indexes; the contribution rate of the comprehensive indicator of j and r_j indicates the contribution rate of the jth comprehensive indicator of the different air/sun-cured tobacco resources; and D is the comprehensive evaluation value of the different air/sun-cured tobacco germplasms [14].

3. Results

3.1. Subsection Diterpenoid Components of Glandular Trichome Secretion

We determined the diterpenoid components and contents of 80 air/sun-cured tobacco germplasms using UPLC. Solvent peaks were removed, and the common chromatographic peaks' limits of quantitation were labelled Peak1–Peak11 from left to right (Figure 2). Peak1–Peak5 were identified as different epimers of hydroperoxyl CBT-diol, Peak6 was identified as epoxy CBT-diol, and Peak 9 was identified as hydroperoxyl CBT-ol through UPLC-Q-TOF-MS; Peak7, Peak8, Peak10, and Peak11 were identified as α-CBT-diol, β-CBT-diol, *cis*-abienol, and CBT-ol, respectively, using respective standard compounds (Figure 3).

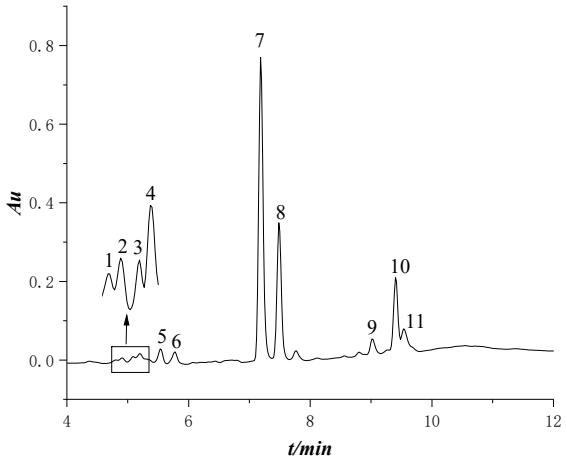

Figure 2. Representative UPLC chromatogram showing the 11 diterpenoids extracted from tobacco glandular trichome secretions. (Numbers 1–11 indicate the Peak1–11).

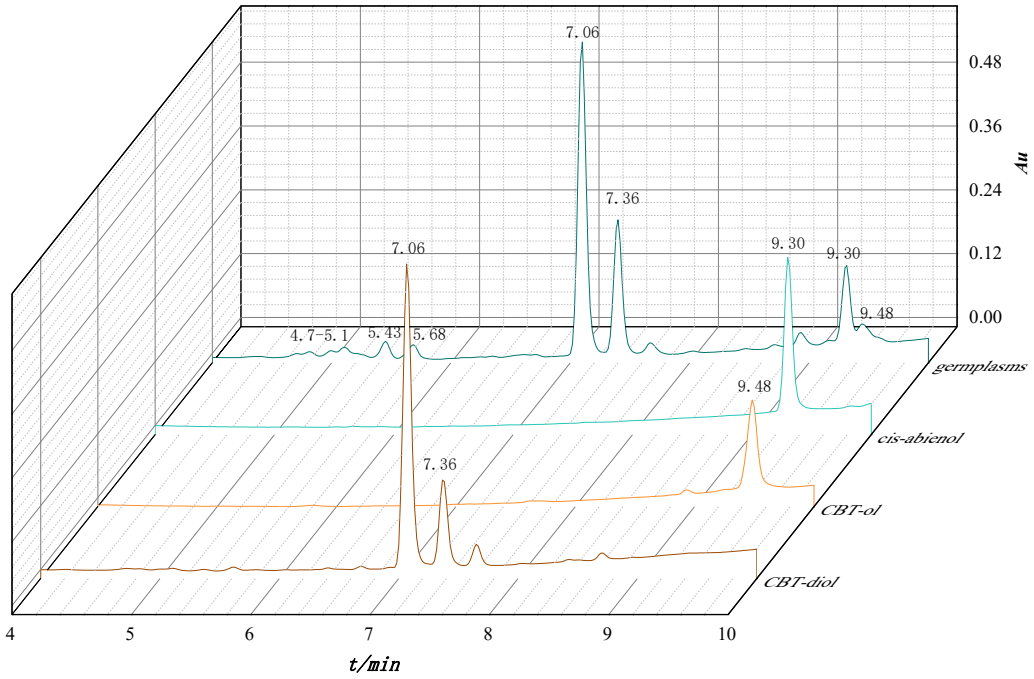

Figure 3. UPLC chromatogram of representative germplasms and standard compounds.

To demonstrate the relative contributions of genotype and year effects on diterpenoids, ANOVA was performed, and the results are shown in Table 2. Both environment and germplasm resources had a significant effect on diterpenoids content. However, the sum of squares of germplasm resources was much larger than that of the environment, illustrating that the discrepancy between germplasm resources was much more obvious than for the environment. The environmental effects are evaluated in Table 3. There were no significant differences between the four environmental variables in Peak1–Peak5, while there were varying degrees of differences among Peak6–Peak11.Therefore, in order to select excellent diterpenoid-rich germplasm resources, we compared and analyzed the average diterpenoids content of the 80 germplasm resources between the four environments, which is presented in the absence of the main effects of location and year and interaction with genotype. The basic data characteristics of the 11 peaks are listed in Table 4. Among these components, the average contents of Peak7 (α-CBT-diol) and Peak8 (β-CBT-diol) were the highest, and the maximum values were 3740.91 and 2732.21 μg/g, respectively. The coefficient of variation of each component ranged from 26.09 to 143.00%, indicating that the contents of diterpenoids had a wide range of variation. Among them, the variation of Peak11 (CBT-ol) was the highest, and that of Peak9 was the lowest.

Table 2. Effects of environment and germplasm resources on diterpenoid content.

	Sum of Squares	DOF	Mean Square	F	P	R^2	Adjust R^2
environment	6141.664	3	11.557	57.074	0.000 **		
germplasm resources	5,897,001.563	79	74,645.589	2081.029	0.000 **	0.999	0.998
proper noun error	8501.085	237	35.87		NaN		

Note: ** indicates an extremely significant difference at the 0.01 level.

Table 3. Contents of diterpenoids in different environments (μg/g).

	Peak1	Peak2	Peak3	Peak4	Peak5	Peak6
1	94.77 ± 66.30 [a]	88.65 ± 63.61 [a]	88.85 ± 43.62 [a]	182.16 ± 69.50 [a]	161.48 ± 65.13 [a]	164.45 ± 61.59 [b]
2	75.99 ± 46.16 [a]	82.11 ± 53.56 [a]	78.30 ± 52.31 [a]	159.97 ± 91.35 [a]	199.98 ± 105.38 [a]	230.60 ± 108.35 [a]
3	83.55 ± 39.96 [a]	78.86 ± 41.64 [a]	81.51 ± 45.03 [a]	166.59 ± 64.95 [a]	183.22 ± 86.29 [a]	206.17 ± 83.10 [ab]
4	79.96 ± 48.70 [a]	74.37 ± 56.96 [a]	81.93 ± 79.13 [a]	157.86 ± 103.30 [a]	192.12 ± 151.52 [a]	229.74 ± 148.62 [b]
	Peak7	Peak8	Peak9	Peak10	Peak11	
1	1674.78 ± 692.89 [b]	1236.21 ± 508.28 [b]	406.78 ± 106.11 [b]	464.46 ± 291.33 [b]	112.36 ± 60.67 [b]	
2	2181.81 ± 1163.86 [ab]	1629.43 ± 96.75 [ab]	950.40 ± 850.20 [a]	868.58 ± 763.87 [a]	325.97 ± 423.77 [a]	
3	2181.83 ± 1060.16 [ab]	1603.29 ± 79.19 [ab]	739.22 ± 405.21 [a]	807.03 ± 625.70 [a]	246.30 ± 198.51 [ab]	
4	2497.54 ± 1793.07 [a]	1816.06 ± 272.88 [a]	892.48 ± 627.62 [a]	799.67 ± 608.21 [a]	264.29 ± 219.05 [a]	

Note: Numbers 1–4 indicate the environment of Shandong (2020), Sichuan (2020), Shandong (2021), and Sichuan (2021), respectively. Different letters (a, b) in the same column indicate significant difference at the level of $p < 0.05$.

Table 4. Basic characteristics of data for 11 chromatographic peaks of glandular trichome secretions.

Fraction	Min.	Max.	Avg.	Median	SD	CV (%)	Skewness	Kurtosis
	μg/g							
Peak1	0.00	294.39	94.77	75.45	66.30	69.96	1.00	0.51
Peak2	0.00	486.96	88.65	75.82	63.61	71.75	3.27	18.63
Peak3	0.00	194.48	88.85	89.08	43.62	49.09	0.06	−0.02
Peak4	5.73	361.71	182.16	174.24	69.50	38.15	0.19	0.21
Peak5	5.76	369.58	161.48	152.24	65.13	40.33	0.74	0.94
Peak6	11.07	353.12	164.45	158.31	61.59	37.45	0.69	1.16
Peak7	123.10	3740.91	1674.78	1675.95	692.89	41.37	0.29	−0.04
Peak8	92.78	2732.21	1236.21	1227.20	508.28	41.12	0.46	−0.04
Peak9	146.84	664.67	406.78	406.89	106.12	26.09	−0.41	0.50
Peak10	0.00	949.81	464.46	522.56	291.33	62.72	−0.37	−0.99
Peak11	0.00	946.32	112.36	64.39	160.67	143.00	3.61	15.86

Star plots were constructed for the 80 air/sun-cured tobacco germplasms based on the composition and content of the 11 peaks (Figure 4). There were evident differences among the 80 glandular trichome secretions. Among them, the contents of CBT-diol in germplasms X48, X51, X54, X58, X63, and X73 were higher than those of the other germplasms. These included four local and two introduced germplasms, with local germplasm X48 showing the highest CBT-diol content. The contents of *cis*-abienol in germplasms X1, X9, X11, X32, X40, and X43 were higher than those of the other germplasms. These also included four introduced and two local germplasms, and the content of the local germplasm X43 was the highest. The diterpenoid content of X36, the breeding germplasm, was the lowest. There was no *cis*-abienol, and the content of CBT-diol was below the detection limit.

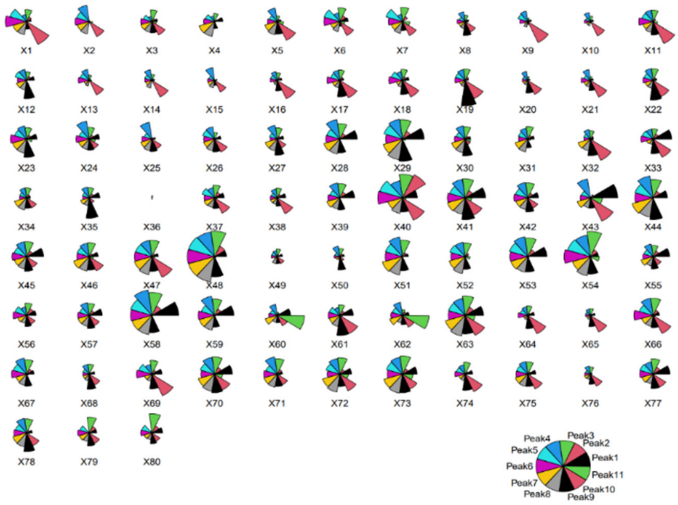

Figure 4. Star plots of 80 air/sun-cured tobacco germplasms based on 11 diterpenoids from the glandular trichome secretions.

3.2. Correlation Analysis

Correlation analysis was performed using SPSS (Table 5). The results showed that Peak1–Peak9 were significantly and positively correlated. Peak7 (α-CBT-diol) and Peak8 (β-CBT-diol) had a correlation coefficient of 0.97. Peak10 (*cis*-abienol) was negatively correlated with Peak1–Peak9, including significant negative correlations with Peak3 and Peak4 and extremely significant negative correlations with Peak7 (α-CBT-diol) and Peak8 (β-CBT-diol). Peak11 (CBT-ol) was significantly and positively correlated with Peak7 (α-CBT-diol) but was not significantly correlated with the other peaks.

Table 5. Correlation analysis of 11 chromatographic peaks.

	Peak1	Peak2	Peak3	Peak4	Peak5	Peak6	Peak7	Peak8	Peak9	Peak10	Peak11
Peak1	1										
Peak2	0.58 **	1									
Peak3	0.50 **	0.75 **	1								
Peak4	0.54 **	0.42 **	0.49 **	1							
Peak5	0.49 **	0.72 **	0.81 **	0.68 **	1						
Peak6	0.45 **	0.70 **	0.77 **	0.66 **	0.95 **	1					
Peak7	0.54 **	0.51 **	0.67 **	0.76 **	0.80 **	0.74 **	1				
Peak8	0.46 **	0.51 **	0.70 **	0.75 **	0.82 **	0.76 **	0.97 **	1			
Peak9	0.41 **	0.38 **	0.56 **	0.41 **	0.41 **	0.38 **	0.47 **	0.46 **	1		
Peak10	−0.17	−0.08	−0.25 *	−0.27 *	−0.17	−0.12	−0.32 **	−0.29 **	−0.11	1	
Peak11	0.17	0.03	−0.06	0.15	0.1	0.1	0.25 *	0.14	−0.04	0.14	1

Note: * indicates a significant difference at the 0.05 level, ** indicates an extremely significant difference at the 0.01 level (a = 0.05, r = 0.2199; a = 0.01, r = 0.2864).

3.3. Cluster Analysis

The pheatmap function in R was used to perform the cluster analysis and design the cluster heatmap according to the Euclidean distance (Figure 5). The 80 air/sun-cured tobacco germplasms were divided into three groups. Group I included 16 germplasms (nine local and seven introduced varieties); group II included 17 germplasms (4 local and 13 introduced varieties); and group III included 47 germplasms (17 local, 26 introduced, and 4 breeding varieties). There was no significant difference in diterpenoid components and the content of glandular trichome secretions between local and introduced tobacco germplasms. This indicated that the diversity of local air/sun-cured germplasms was rich after long-term artificial cultivation and natural selection in China. X40 had the highest Peak2 content, and X48 contained the highest contents of Peak5, Peak6, Peak7 (α-CBT-diol), and Peak8 (β-CBT-diol) in group I. X36, X10, X13, and X14 had the lowest contents of Peak9 in group II. X60 and X62 had the highest contents of Peak11 in group III.

Table 6 illustrates the content of the 11 peaks of the three groups. The average contents of all diterpenoids were the highest in group I, except for Peak10 (*cis*-abienol), which was the lowest (278.58 μg/g). There were significant differences between group II and group III, except for Peak11 (CBT-ol). Group II had the lowest contents, except for Peak10 (*cis*-abienol), which had the highest average content (533.73 μg/g). The contents of all diterpenoids in group III were medium, and there were significant differences compared with group II, except for Peak10 (*cis*-abienol) and Peak11 (CBT-ol). Therefore, the germplasms in group I can be used for the integrated extraction of diterpenoid components, and those in group II can be used for the extraction of *cis*-abienol (Peak10). The germplasms in group III can be used for the extraction of plant fragrances as the components in equilibrium are good for enhancing the aroma and taste of cigarettes.

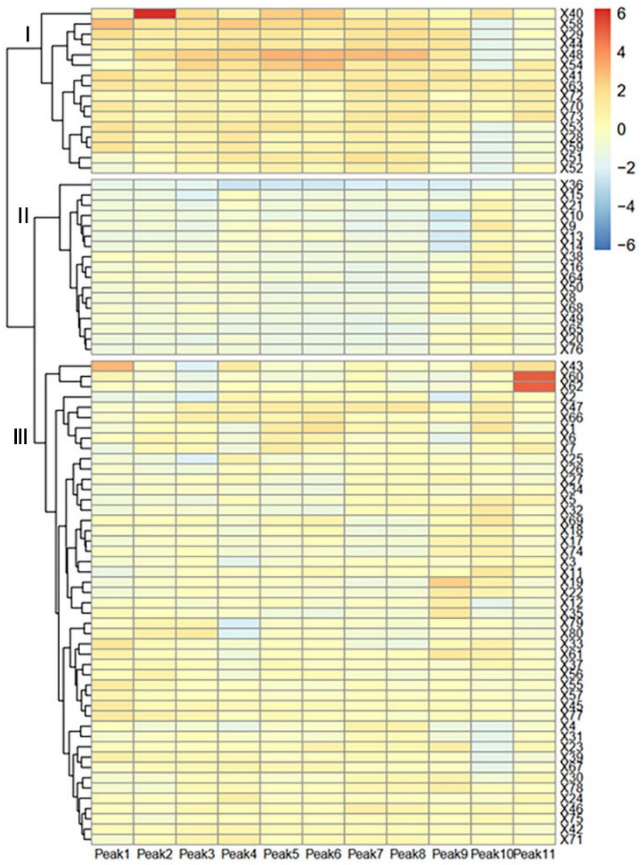

Figure 5. Cluster analysis dendrogram based on the 11 peaks for the contents of 80 air/sun-cured tobacco germplasm resources.

Table 6. The contents of the 11 diterpenoids among the three groups (μg/g).

Group	Peak1	Peak2	Peak3	Peak4	Peak5	Peak6
Group I	165.05 ± 66.56 ^{a}	161.51 ± 94.78 ^{a}	145.97 ± 26.46 ^{a}	275.69 ± 42.77 ^{a}	252.42 ± 53.32 ^{a}	239.46 ± 56.53 ^{a}
Group II	36.93 ± 18.78 ^{c}	36.93 ± 18.78 ^{c}	45.75 ± 21.04 ^{c}	129.59 ± 39.36 ^{c}	88.93 ± 29.16 ^{c}	95.87 ± 29.92 ^{c}
Group III	90.59 ± 55.66 ^{b}	82.56 ± 32.00 ^{b}	85.00 ± 32.38 ^{b}	169.34 ± 54.11 ^{b}	156.76 ± 34.89 ^{b}	163.72 ± 38.27 ^{b}
	Peak7	Peak8	Peak9	Peak10	Peak11	
Group I	2654.41 ± 389.41 ^{a}	1985.34 ± 282.72 ^{a}	489.59 ± 72.14 ^{a}	278.58 ± 364.41 ^{b}	145.78 ± 134.94 ^{a}	
Group II	784.44 ± 242.58 ^{c}	647.72 ± 198.25 ^{c}	309.15 ± 107.22 ^{c}	533.73 ± 221.84 ^{a}	39.38 ± 25.83 ^{a}	
Group III	1663.33 ± 357.37 ^{b}	1194.05 ± 280.95 ^{b}	413.90 ± 87.66 ^{b}	502.68 ± 264.68 ^{a}	127.37 ± 188.61 ^{a}	

Note: a, b, and c indicate significant difference between different groups at the 5% level.

3.4. Principal Component Analysis

We performed PCA on the 11 peaks of 80 air/sun-cured tobacco germplasms using SPSS. The results are presented in Tables 7 and 8. Based on the rule that the eigenvalue is greater than 1, three principal components were selected as comprehensive indicators, which reflected 75.70% of the original components, indicating that the selection of three principal components showed dimensional reduction and comprehensive evaluation [15]. Tables 7 and 8 show that the contribution of the first principal component (PC1) was 55.16%, which mainly reflects the indicators of Peak5, Peak7 (α-CBT-diol), and Peak8 (β-CBT-diol). The contribution of the second principal component (PC2) was 10.88%, reflecting the indicators of Peak11 (CBT-ol). The contribution of the third principal component (PC3) was 9.66%, reflecting the indicators of Peak10 (*cis*-abienol).

Table 7. Eigenvalues of the PCA and rates of contribution of variance.

Principal Component	Eigenvalue	Variance Contribution (%)	Cumulative Contribution (%)
1	6.07	55.16	55.16
2	1.20	10.88	66.04
3	1.06	9.66	75.70
4	0.81	7.33	83.03
5	0.69	6.27	89.29
6	0.51	4.63	93.93
7	0.28	2.54	96.47
8	0.19	1.74	98.21
9	0.13	1.21	99.42
10	0.04	0.40	99.82
11	0.02	0.18	100.00

Table 8. Loadings of principal components.

Component	Principal Components		
	1	2	3
1	0.66	0.10	0.05
2	0.75	−0.05	0.43
3	0.86	−0.23	0.24
4	0.79	0.10	−0.29
5	0.93	0.04	0.12
6	0.89	0.06	0.16
7	0.90	0.13	−0.28
8	0.90	0.04	−0.23
9	0.58	−0.21	0.17
10	−0.29	0.52	0.70
11	0.13	0.89	−0.22

The importance of each factor in the 80 air/sun-cured tobacco germplasm resources was evaluated based on the subordinate function and comprehensive index value of the three principal components (Table 9). The five germplasm resources with the highest PC1 scores among the 80 air/sun-cured tobacco germplasms were X48 > X58 > X40 > X54 > X29, indicating that these germplasms had the highest contents of Peak5, Peak7 (α-CBT-diol), and Peak8 (β-CBT-diol). The five germplasms with the highest PC2 scores were X60 > X62 > X43 > X73 > X72, indicating the highest content of Peak11 (CBT-ol). The five germplasms with the highest PC3 scores were X40 > X69 > X33 > X1 > X80, indicating the highest content of Peak10 (*cis*-abienol). A single principal component could reflect the content of one or several diterpenoids. The comprehensive evaluation (D value) of the diterpenoids of the 80 germplasms, calculated using the three principal components, resulted in the following order: X40 > X48 > X58 > X54 > X63 > X41 > X29 > X73 > X44 > X70.

The highest diterpenoid contents of 10 germplasm resources are listed in Table 10, which shows that the comprehensive ranking and cluster analysis results were consistent. The top 10 germplasms were all clustered in group I, and the total content of diterpenoids was significantly higher than that in groups II and III. X48 (Jianpingpiaoba) with the highest content of CBT-diol (6473.12 μg/g) and hydroperoxyl CBT-diol (369.58 μg/g), X40 (Liaoduoye) with the highest content of *cis*-abienol (909.92 μg/g), and X73 (Criollo) with the highest content of CBT-ol (363.39 μg/g) should be selected for different purposes. X48 and X63 had the highest total diterpenoid content.

Table 9. Comprehensive index value, weight, membership function value, and 11 diterpenoid content ranking for 80 sun/air-cured tobacco germplasm resources.

Tobacco Number	Comprehensive Index Value			Subordinate Function Value			Comprehensive Evaluation (D Value)	Order
	PC1	PC2	PC3	$U(x_1)$	$U(x_2)$	$U(x_3)$		
X1	0.21	0.47	1.55	0.49	0.33	0.57	0.48	19
X2	−0.54	1.08	−1.24	0.35	0.44	0.11	0.33	59
X3	−0.47	0.18	0.22	0.36	0.28	0.35	0.35	52
X4	−0.28	−0.71	−1.63	0.40	0.12	0.04	0.31	62
X5	−0.40	1.13	−0.37	0.38	0.45	0.25	0.37	45
X6	0.02	0.55	0.80	0.45	0.34	0.44	0.44	28
X7	0.01	0.71	0.55	0.45	0.37	0.40	0.43	29
X8	−0.92	−0.36	−0.20	0.28	0.18	0.28	0.27	68
X9	−1.26	0.61	0.57	0.22	0.35	0.41	0.26	70
X10	−1.53	−0.01	−0.12	0.17	0.24	0.29	0.20	79
X11	−0.27	0.72	0.85	0.40	0.37	0.45	0.40	39
X12	−0.15	−1.37	−0.74	0.42	0.00	0.19	0.33	58
X13	−1.17	0.41	−0.27	0.24	0.32	0.27	0.25	71
X14	−1.20	0.42	−0.22	0.23	0.32	0.28	0.25	72
X15	−1.28	0.13	−1.05	0.22	0.27	0.14	0.21	76
X16	−1.15	−0.23	0.94	0.24	0.20	0.47	0.26	69
X17	−0.48	−0.21	0.98	0.36	0.21	0.48	0.35	51
X18	−0.44	0.07	1.09	0.37	0.26	0.49	0.37	46
X19	−0.43	−0.78	1.51	0.37	0.11	0.56	0.36	49
X20	−1.34	−0.48	0.08	0.20	0.16	0.33	0.21	77
X21	−1.05	0.01	0.18	0.26	0.25	0.34	0.27	67
X22	−0.41	−0.31	0.70	0.38	0.19	0.43	0.36	50
X23	0.54	−0.34	−1.17	0.55	0.18	0.12	0.44	24
X24	0.13	0.48	−0.62	0.47	0.33	0.21	0.42	33
X25	−0.55	−0.06	−1.38	0.35	0.23	0.08	0.30	64
X26	−0.32	−0.21	−0.35	0.39	0.21	0.25	0.35	53
X27	−0.22	−0.67	−0.63	0.41	0.12	0.21	0.34	56
X28	0.95	−0.99	−1.17	0.62	0.07	0.12	0.48	18
X29	1.92	−0.72	−0.79	0.80	0.12	0.18	0.63	7
X30	0.10	−0.14	−1.06	0.47	0.22	0.14	0.39	43
X31	−0.13	−0.72	−1.39	0.43	0.12	0.08	0.34	57
X32	−0.58	0.31	0.50	0.34	0.30	0.39	0.34	54
X33	−0.23	0.05	1.55	0.41	0.25	0.57	0.41	36
X34	−0.50	−0.06	−0.65	0.36	0.23	0.20	0.32	60
X35	−0.37	−1.25	−0.19	0.38	0.02	0.28	0.32	61
X36	−2.44	−1.30	−1.15	0.00	0.01	0.12	0.02	80
X37	−0.10	0.07	0.77	0.43	0.26	0.44	0.41	35
X38	−0.89	−0.20	0.57	0.29	0.21	0.41	0.29	65
X39	0.36	−1.08	−0.83	0.52	0.05	0.17	0.41	37
X40	2.24	0.48	4.14	0.86	0.33	1.00	0.80	1
X41	1.47	0.81	0.83	0.72	0.39	0.45	0.64	6
X42	0.36	−0.44	0.09	0.52	0.17	0.33	0.44	23
X43	−0.02	2.94	−0.32	0.45	0.77	0.26	0.47	21
X44	1.78	−1.11	−1.00	0.78	0.05	0.15	0.59	9
X45	0.57	−0.15	0.30	0.55	0.22	0.36	0.48	17
X46	0.31	0.38	−0.22	0.51	0.31	0.28	0.45	22
X47	0.81	0.60	0.34	0.60	0.35	0.37	0.53	12
X48	2.99	−0.82	−0.59	1.00	0.10	0.22	0.77	2
X49	−1.26	−0.79	−0.38	0.22	0.10	0.25	0.21	78
X50	−1.07	−1.08	−0.43	0.25	0.05	0.24	0.22	74
X51	1.15	−0.94	−1.62	0.66	0.08	0.04	0.50	15
X52	0.57	−0.34	−1.60	0.55	0.18	0.05	0.44	27
X53	1.47	−0.90	−0.54	0.72	0.08	0.22	0.57	11
X54	2.11	0.15	−0.76	0.84	0.27	0.19	0.67	4
X55	0.04	−0.27	−0.11	0.46	0.20	0.29	0.40	40
X56	−0.10	−0.71	0.72	0.43	0.12	0.43	0.39	44
X57	0.04	−0.43	0.06	0.46	0.17	0.32	0.40	41
X58	2.40	−0.83	−0.52	0.89	0.10	0.23	0.69	3
X59	1.04	−0.83	−0.98	0.64	0.10	0.15	0.50	14
X60	−0.57	4.24	−1.88	0.35	1.00	0.00	0.40	42
X61	−0.01	0.07	1.18	0.45	0.26	0.51	0.43	32
X62	−0.26	4.04	−1.45	0.40	0.97	0.07	0.44	25
X63	1.59	1.59	0.76	0.74	0.53	0.44	0.67	5
X64	−0.92	−0.10	1.09	0.28	0.23	0.49	0.30	63
X65	−1.40	−0.17	0.57	0.19	0.21	0.41	0.22	73
X66	0.50	0.23	1.01	0.54	0.28	0.48	0.50	16
X67	0.36	−0.90	−0.75	0.52	0.08	0.19	0.41	34
X68	−0.84	−0.65	0.02	0.30	0.13	0.32	0.27	66
X69	−0.12	0.28	1.76	0.43	0.29	0.61	0.43	30
X70	1.08	0.98	0.35	0.65	0.42	0.37	0.58	10
X71	0.33	−0.07	−0.31	0.51	0.23	0.26	0.44	26
X72	0.74	1.27	−0.18	0.59	0.47	0.28	0.53	13
X73	1.26	1.38	−0.11	0.68	0.49	0.29	0.61	8
X74	−0.57	−0.21	1.05	0.35	0.21	0.49	0.34	55
X75	−0.03	0.18	−0.01	0.44	0.28	0.31	0.40	38
X76	−1.26	−0.47	−0.14	0.22	0.16	0.29	0.22	75
X77	0.41	−0.30	1.01	0.52	0.19	0.48	0.47	20
X78	0.26	−0.18	−0.18	0.50	0.21	0.28	0.43	31
X79	−0.33	−1.02	1.25	0.39	0.06	0.52	0.36	48
X80	−0.25	−1.15	1.34	0.40	0.04	0.53	0.37	47

Table 10. Content of diterpenoids in the 10 best-performing air/sun-cured tobacco germplasms (μg/g).

No.	Peak1	Peak2	Peak3	Peak4	Peak5	Peak6	Peak7	Peak8	Peak9	Peak10	Peak11	Ranking
X40	160.20	486.96	179.86	245.14	323.25	322.56	2138.43	1712.40	434.73	909.92	126.82	1
X48	128.41	219.42	194.48	352.08	369.58	353.12	3740.91	2732.21	557.31	3.23	55.11	2
X58	288.94	194.94	174.88	361.71	302.47	280.04	2878.61	2092.68	495.61	4.60	22.40	3
X54	86.06	144.31	192.30	307.94	324.69	342.00	2605.10	2100.89	499.96	3.95	309.26	4
X63	197.90	174.59	148.02	243.57	237.69	222.59	2852.54	2247.44	522.27	812.95	339.22	5
X41	219.75	152.43	144.94	265.80	220.59	234.82	2577.59	1801.52	590.46	703.19	224.92	6
X29	233.20	164.64	147.73	284.44	264.25	222.40	2786.32	2255.71	598.34	0.00	104.58	7
X73	158.33	115.06	139.30	242.54	229.95	202.64	2704.66	2067.63	521.88	609.14	363.39	8
X44	155.15	139.13	140.84	313.10	258.68	240.32	3027.85	2091.26	588.76	14.04	6.58	9
X70	192.57	140.21	131.23	241.74	204.42	181.16	2371.70	1864.34	522.64	644.50	274.78	10

4. Discussion

4.1. Diterpenoid Components in the Glandular Trichome Secretions of Tobacco

Diterpenoids are a class of glandular trichome exudates with biological activities in tobacco. Different diterpenoid components have different biological activities. The differences in diterpenoid content and composition among different tobacco varieties are significant [13]. Wang et al. [16] showed that the antifungal activity of cembranoids is related to the number of hydroxyl groups and double bonds, and that their cytotoxic activity is affected by the type and position of substituents. In this study, we detected 11 chromatographic peaks in ethanol extracts of the glandular trichomes from air/sun-cured tobacco using UPLC, including different epimers of hydroperoxy-CBT-diol, epoxy-CBT-diol, α-CBT-diol, β-CBT-diol, hydroperoxy-CBT-ol, *cis*-abienol, and CBT-ol. β-CBT-diol content was the highest, which is in agreement with the findings of Wang and Wagner [17]. Previous studies on tobacco glandular trichome secretions have mainly focused on α-CBT-diol and β-CBT-diol of flue-cured tobacco, and, therefore, other diterpenoids in the glandular trichome secretions of air/sun-cured tobacco need to be systematically separated and their structure identified and biological activities evaluated.

4.2. Correlation Analysis of Diterpenoid Components

To the best of our knowledge, this study is the first to systematically identify diterpenoids in the glandular trichome secretions of air/sun-cured tobacco resources in China. The results showed that α-CBT-diol, β-CBT-diol, and *cis*-abienol had significant negative correlations. This is due to the metabolic pathways of the compounds. The precursors of CBT-diol and *cis*-abienol are the same and show a competitive relationship [18,19]. In addition, α-CBT-diol and β-CBT-diol were positively correlated with CBT-ol and the other components, which can provide a reference for understanding the cooperative regulation of multiple components in high-quality tobacco breeding.

4.3. Comprehensive Evaluation of Selected Air/Sun-Cured Tobacco Germplasms

Air/sun-cured tobacco germplasms are diverse and abundant in China and have advantages in the development and utilization of plant glandular trichome secretions [20]. In the present study, diterpenoids of the glandular trichome secretions of 80 air/sun-cured tobacco germplasms were identified and evaluated, and the composition and content of diterpenoids differed significantly among the germplasms. The content levels of the germplasms varied from several to several thousand folds [21]. The average CBT-diol content of the local air/sun-cured tobacco germplasms was the highest, and the average *cis*-abienol content of the introduced germplasms was the highest. However, the tobacco germplasms with the highest CBT-diol (X48) and *cis*-abienol (X40) contents were both local resources in China. In this study, we found that 10 germplasm resources rich in hydroperoxy-CBT-diol, α-CBT-diol, β-CBT-diol, *cis*-abienol, or CBT-ol are useful for the

extraction of beneficial active ingredients or can provide germplasms for improving the glandular trichome secretions in high-quality tobacco breeding.

4.4. Obtaining Diterpenoid Components

Traditionally, diterpenoid compounds have been extracted from plants, but the low content has limited their application. Developing better methods to efficiently obtain diterpenoid compounds is expected to overcome this limitation. First, tobacco is a kind of topped crop, and waste inflorescences can be used as a raw material for the extraction of valuable diterpenoids, which can avoid the waste produced by tobacco leaf production and produce more high-value-added products for the development of cigarettes with added fragrance and plant pesticides [22,23]. Therefore, recycling tobacco waste to extract diterpenes is economical and efficient and can help to reduce the pollution and harm caused by agricultural waste. Secondly, tobacco is one of the most abundant plants in nature, and the whole plant is densely covered with glandular trichomes which secrete abundant numbers of secondary metabolites [24]. Furthermore, the tobacco inflorescence can be topped and collected several times such that the 10 selected diterpene-rich germplasms can be used as special resources for the cultivation and extraction of diterpenoids [25]. Finally, biosynthesis is an effective method for obtaining diterpenoids, but the metabolic pathways must be well defined. The total diterpenoid content in the glandular trichome secretions of X36 (CS0708) was the lowest, with some components not detected at all. X36 is therefore an excellent genetic material for the study of the diterpenoid metabolic pathway and gene mining. Moreover, the contents of diterpenoids are also affected by natural environmental factors such as temperature, water, and light. In subsequent experiments, we will also investigate the differences in the contents of glandular trichome secretions of excellent germplasm resources under different environmental conditions to explore the optimal environment for the generation of diterpenoid metabolites [26]. Additionally, it is important to explore potential applications for diterpenoid compounds, such as the development of fragranced cigarettes or plant pesticides.

5. Conclusions

The analysis of the glandular trichome secretions of 80 air/sun-cured tobacco germplasms indicated that the diterpenoid content of glandular trichome secretions, especially CBT-diol and *cis*-abienol, is high in air/sun-cured tobacco germplasms, and the content of each component has a relatively wide range of variation. The contents of α-CBT-diol and β-CBT-diol were the highest and were positively correlated with those of other components, except for *cis*-abienol. Finally, based on PCA, 10 germplasms, namely, X40, X48, X58, X54, X63, X41, X29, X73, X44, and X70, rich in hydroperoxy-CBT-diol, α-CBT-diol, β-CBT-diol, *cis*-abienol, and CBT-ol, were selected. In addition, X36 was identified as an important germplasm for genetic studies of diterpenoid metabolism. Therefore, these air/sun-cured tobacco germplasms will be useful for high-quality tobacco breeding and the development of plant pesticides, botanical perfumes, and medical care, especially the high-performing local germplasms from China.

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Abbreviations

α-CBT-diol, alpha-cembratriene-diol; β-CBT-diol, beta-cembratriene-diol; CBT-diol, Cembratriene-diol; CBT-ol, cembratriene-ol; UPLC, ultra-performance liquid chromatography; PCA, principal component analysis; UPLC-Q-TOF-MS, ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrometry.

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