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# Dry-Bulb Temperature-Modulated Curing's Impact on the Carbon and Nitrogen Metabolism of Tobacco Leaves in Clean-Energy Bulk Curing Barns

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**Abstract:**
The optimized curing environment in bulk curing barns can enhance the carbon and nitrogen metabolism of tobacco leaves. The impact of dry-bulb temperature (DBT) treatments during the yellowing stage of the tobacco curing (CK: constant yellowing DBT at 38 °C; T1: DBT fluctuation range ±1 °C, frequency of 3 hours; T2: DBT fluctuation range ±1.5°C, frequency of 5 hours) on the carbon and nitrogen metabolism of tobacco leaves was evaluated. (1) After the yellowing stage, the T1 treatment led to lower levels of starch, total sugars, and soluble proteins, but a higher accumulation of free amino acids in tobacco leaves. (2) Despite differences in magnitudes during tobacco curing, carbon and nitrogen metabolism-related enzymatic activities showed similar trends among different treatments. (3) Treated by T1 method, the expression levels of genes encoding starch enzymes (AMY, BMY-1, and BMY-3) in tobacco leaves rose sharply, while the expression levels of genes encoding soluble starch synthases (SSS-1) and pyrophosphorylase (AGPase-1 and AGPase-2) were at their lowest among all treatments during the same period. In addition, the expression levels of genes involved in nitrogen transfer (GS1-3) were higher, whereas the expression levels of genes related to nitrogen assimilation (NR and GS2) were lower. Finally, based on the constant yellowing DBT at 38 °C, the DBT treatment with T1 can promote thorough degradation and transformation of starch and protein, together with the effective accumulation of free amino acids in tobacco leaves. In this way, chemical composition of tobacco leaves can be better optimized, which vastly affects tobacco leaf quality and reduces ineffective curing energy consumption.

**Keywords:** tobacco curing technology; starch degradation; protein degradation; enzymatic activity; gene expression; optimization of green curing environment.

## INTRODUCTION

The mature leaves of flue-cured tobacco (Nicotiana tabacum L.) are harvested and placed in a bulk curing barn (BCB) for tobacco curing (TC). Carbon and nitrogen metabolism are fundamental material transformation processes in tobacco leaves during bulk curing, closely related to curing conditions and varietal factors [1]. More specifically, carbon metabolism refers to the photosynthetic fixation and assimilation of inorganic carbon, along with monosaccharide conversion and starch accumulation; while nitrogen metabolism involves the absorption, assimilation, transport, utilization, and regulation of nitrogen compounds [2,3]. Duan et al. [4] analyzed the trends in resistant starch content and the expression levels of starch synthesis-related genes in tobacco leaves. According to their research, GBSS1 and SBE2 were potential key genes regulating the content of amylose and amylopectin. In addition, Wu et al. [5] studied post-harvest tobacco leaf drying characteristics, along with dynamic activities and gene expression of enzymes involved in starch metabolism. The results showed that dehydration treatment enabled post-harvest tobacco leaves to have stronger starch degradation and higher sugar content. Referring to Zhang et al. [6], studies have been conducted on the differences and relationships in carbon and nitrogen metabolism, together with the expression of related genes during the senescence period of different tobacco varieties. Finally, varieties with faster leaf senescence demonstrated weak starch synthesis and weak nitrogen assimilation metabolism. Moreover, Zhang et al. [7] examined how growth temperature would affect starch metabolism in tobacco at different growth stages. The results indicated that by affecting the expression of starch metabolism-related genes, temperature could then influence enzymatic activities, together with starch metabolism and transport, ultimately deciding the style of tobacco leaves. According to previous researches, enzymes' activities during the YS of TC depends on the temperature, concentration, and total amount of degraded substrates. Particularly, temperature is highly crucial to carbon and nitrogen metabolism of tobacco leaves. Prolonged exposure to a constant temperature can dull enzymatic activities, which is not conducive to the yellowing of tobacco leaves and the balance of chemical composition.

## OBJECTIVES

The physiological and biochemical changes within tobacco leaves, along with macro-molecules' degradation and transformation are often subject to the dry-bulb temperature (DBT) variations inside the TC environment [8]. In recent years, China uses clean energy such as biomass pellet fuel, air-source heat pumps, or natural gas for TC that can realize effective and precise DBT control during the drying process [9]. Compared to traditional coal direct fired for TC heating, using IoT big data technology to collect data from 2019 to 2021, it was found that the median total curing time of tobacco leaves ranged from 184 to 200 hour with the

time management of the drying process indicated longer extended yellowing stage (YS) [10], which has been extended by a quarter and consumed significantly more curing energy compared to before [11], and tobacco leaves after cured result in problems such as weakened leaf color and stiffened tissue [12]. Due to differences in heating and DBT control methods, the shorter combustion cycle of clean energy instant heating, compared to the longer combustion cycle of coal-fired heating, can cause slight fluctuations in DBT within the BCB [13]. Enzyme activity is greatly influenced by DBT, and under constant DBT conditions, enzymes are prone to deactivation, which impairs their activity [14]. Consequently, the activity and duration of key enzymes within the tobacco leaves are dominated by these fluctuations, thereby affecting the degradation of macro-molecular constituents, such as pigments, proteins and starch, finally deciding tobacco quality after cured [15,16]. In fields, tobacco plants experience monthly and even daily temperature fluctuations, which accompany enzymatic activities during leaf growth and maturation [17]. The precision control of the new energy heating system breaks the traditional YS during TC process, which typically occurs at a constant 38 °C with significant fluctuations, which cannot replicate the temperature fluctuations of tobacco leaves at different times of the day in the field.

With the advancement of clean energy source barns and the growing popularity of precise DBT control in large-scale TC fields, terms such as "DBT and wet-bulb temperature (WBT) control," "field distribution of DBT and WBT," "accurate control of wind speed," and "scientific barn construction" have become key topics in the optimization of the green curing environment in clean-energy bulk curing barns. Based on this, this study designs a DBT control program with tobacco leaves as the experimental subjects. The DBT variation method is used to induce YS during the early stage of the TC process, simulating the DBT changes of the traditional coal-fired heating method. This approach promotes carbon and nitrogen metabolism in tobacco leaves, shortens curing time, and reduces the consumption of ineffective curing energy. In this aspect, final results can serve as references for practical production and optimizing the TC environment.

## METHODS

### Experimental Materials

The experiment was carried at Tuanjie Village, Cangyuan County, Yunnan Province, from July 2023 to September 2024. The village is located at a longitude of 99°34' E and a latitude of 23°17' N, with an elevation of 1,780 m. The experiment used the upper leaves of the tobacco variety “Yunyan 87” with consistent maturity, leaf size, and position as the experimental material, which is known for its good curing characteristics. In particular, the field management measures for tobacco plants followed the technical specifications for regional high-quality tobacco leaf production and all operations finished on the same day. There were three automatic TC chambers with variable DBT control on site. Each chamber had a tobacco loading space of 2.65 m3, accommodating approximately 12-13 layers of upper leaves, with a fresh tobacco leaf weight of approximately 165-195 kg and 1,100-1,300 leaves. To be noted, the harvested upper leaves were fully mature according to corresponding local standards, and all pre-curing operations were completed within one day.

### Experimental Design

The comparative TC experiment was divided by two types: conventional curing and variable DBT curing. The whole TC process was divided into three stages including yellowing, leaf drying and stem drying stage (Figure 1A). There were three specific treatments in YS, as shown in Figure 1B. CK (x=38, n=0, y=0) follows local conventional curing. In terms of T1 (x=38, n=1, y=3), sinusoidal DBT variation based on major YS of 38 °C during the local conventional curing, with a DBT fluctuation within ±1 °C, literally ranging from 37 °C to 39 °C, done by a frequency of 3 hours within YS. The other two stages remained consistent with the local practice. In comparison, T2 (x=38, n=1.5, y=5) also adopted same treatment, but with a DBT fluctuation within ±1.5 °C, or ranging from 36.5 °C to 39.5 °C, which was done by every 5 hours within YS. Also, other curing temperature stages remained consistent with the local practice. In addition, the WBT during the variable DBT experiment was appropriately adjusted through dehumidifying outlet, air inlets, and fan frequency, thus ensuring required curing WBT.

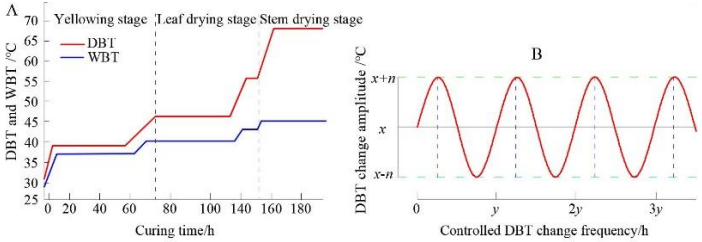



Figure 1. TC technology and experimental treatments settings during the curing process

**Determination of Parameters and Methods**

During TC process, samples were taken from leaves dealt with each treatment at 0 hours, 36 hours, 48 hours, 60 hours, 72 hours, 84 hours, and 96 hours during curing, then immediately stored in liquid nitrogen for later determination of carbon-containing compound content, nitrogen-containing compound content, enzymatic activities related to carbon metabolism, enzymatic activities related to nitrogen metabolism, and key genes related to the carbon and nitrogen metabolism pathway. After sampling, the gaps between tobacco leaves were filled with burlap, so as to prevent bigger gap size and excessive airflow to lower curing effectiveness.

***Determination of moisture content***

In terms of defining the fresh weight and dry weight of leaves and midrib, the method described by Zong et al. [18] is adopted to calculate the total moisture content of the tobacco leaves. Then the rate of moisture loss was calculated according to the method proposed by Wei et al. [19]. The relevant formulas are as follows:

$$\mathrm{m}_f = \frac{(m_l + m_s) - (m_{lt} + m_{st})}{(m_l + m_s)} \times 100\% \quad (1)$$

Where, $m_f$ is the whole leaf moisture content, %; $m_l$ is the leaf fresh weight, g; $m_s$ is the midrib fresh weight, g; $m_{lt}$ is the leaf dry weight, g; $m_{st}$ is the midrib dry weight, g.

$$\mathrm{v} = \frac{m_t - m_c}{t} \quad (2)$$

Where, v is the moisture loss rate, g/h; $m_t$ is the moisture content of tobacco leaves at the previous sampling, g; $m_c$ is the moisture content of tobacco leaves at the current sampling, g; t is the time interval for moisture loss, h.

***Determination of carbohydrate content***

Starch Content: the method described by Gong et al. [20] is adopted. Total Sugar Content: the method proposed by Tang et al. [21] is used.

***Determination of nitrogen-containing compound content***

Soluble Protein Content: following the Coomassie Brilliant Blue method described by Li et al. [22]. Free Amino Acid Content: taking the ninhydrin colorimetric method put forward by Wang et al. [23].

***Determination of enzymatic activities in carbon metabolism***

Approximately 0.1 g of leaf tissue was taken and mixed with 1 ml of extraction solution, then was thoroughly grounded on ice. The subsequent homogenate was centrifuged to obtain the supernatant, which was then kept on ice for further analysis. Meanwhile, the spectrophotometer was preheated for about 30 minutes, and the wavelength was adjusted to match the measurement requirements for the enzymatic activities involved in carbon metabolism. The absorbance values were recorded, and more items were calculated, including the activities of amylase (AL), starch branching enzyme (SBE), soluble starch synthase (SSS), and ADP-glucose pyrophosphorylase (AGP). The enzymatic activity detection kits used in this part were provided by Beijing Solarbio Technology Co., Ltd, with the corresponding model numbers BC2045, BC1865, BC1850, and BC0430, respectively.

***Determination of enzymatic activities in nitrogen metabolism***

Same steps were conducted from weighing about 0.1 g of leaf tissue to wavelength adjustment. The absorbance values were also recorded, and the activities of neutral protease (NP), endopeptidase, glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT) were calculated. The detection kits for measuring nitrogen metabolism-related enzymatic activities were provided by Beijing Solarbio Technology Co., Ltd, with the corresponding model numbers BC2295, YJ602258, BC1565, and BC1555, respectively.

***Determination of genes related to carbon and nitrogen metabolism pathways***

Referring to the methods from Zhang et al. [6] and Zhang et al. [7], total RNA extraction from tobacco leaves was performed using the Plant Total RNA Extraction Kit (Vazyme Biotech Co., Ltd., Model: RC411). Then RNA concentration was quantified

by the NanoDrop™OneC UV-Vis spectrophotometer (Thermo Scientific). Next, reverse transcription and cDNA synthesis were carried out using the TransScript® Fly First-Strand cDNA Synthesis SuperMix (TransGen Corporation, Cat. No. AF301). Moreover, the quantitative analysis of gene expression was performed with the LightCycler480II Real-Time PCR System (Roche), and the data were analyzed using the $2^{-\triangle\triangle CT}$ method. Particularly, tobacco β-actin gene was taken as internal reference.

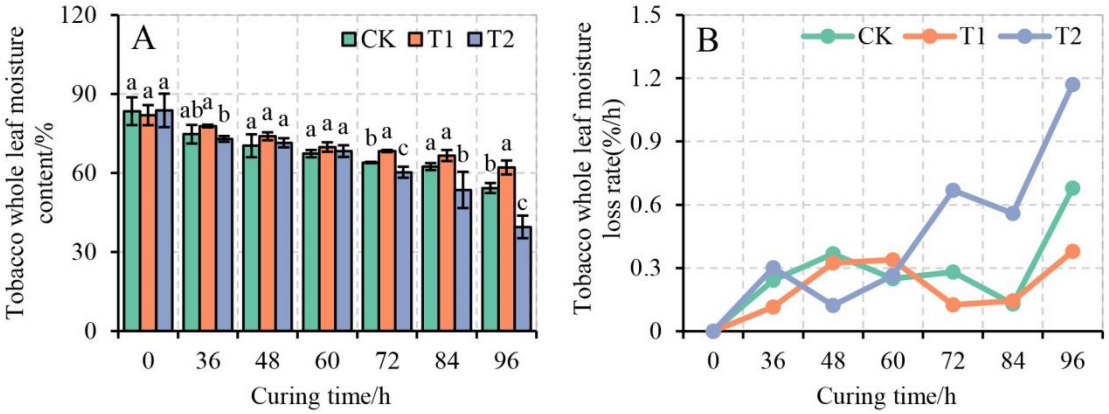
**Data Analysis**

Excel 2016, Origin 2021, DPS 7.0.5, SPSS 26.0, and Photoshop 2021 are utilized for data processing, graphing, and statistical analysis accordingly.

**RESULTS**

**Changes in Leaf Moisture during Curing**

Different DBT treatments can hugely affect moisture content of tobacco leaves during curing (Figure 2). As TC progressed, the moisture content continuously decreased after all three treatments. In particular, there were significant differences in the total moisture content among the three treatments after 72 h and 96 h of curing, with the T1 treatment having the highest moisture content and the T2 treatment having the lowest (Figure 2A). In addition, the rate of moisture loss in tobacco leaves processed by different treatments showed a consistent growing trend (Figure 2B). More specifically, all treatments exhibited a faster and larger amount of moisture loss at the 96 h stage during the YS of TC. From 72 h to 96 h, the T2 treatment showed the highest rate of moisture loss, while T1 having the lowest. In conclusion, the T2 process treatment can better facilitate curing with faster and more moisture loss.




Note: Different lowercase letters indicate significant differences between different treatments in same time at P＜0.05 level. The same as below.

Figure 2. Changes in leaf moisture content during curing

**Indicator Changes of Tobacco Leaves' Carbon Metabolism during Curing**

***Changes in starch and total sugar content during curing***

Similarly, different DBT of TC methods have a significant impact on the starch and total sugar content in tobacco leaves during curing (Figure 3). As TC progressed, starch content decreased after all three processing methods. During the yellowing stage of TC (72-96 hours), the T1 method showed the lowest starch content, while T2 showing the highest (Figure 3A). In addition, as TC dealt with three methods, total sugar content in the tobacco leaves all first increased, then decreased, and finally rose again. However, the leaves after treatment had higher total sugar content compared to that of fresh tobacco leaves. After YS, the T1 method had the significantly lowest total sugar content, while T2 showing the highest (Figure 3B), which indicates that the T1 method can realize more extensive starch degradation in tobacco leaves.

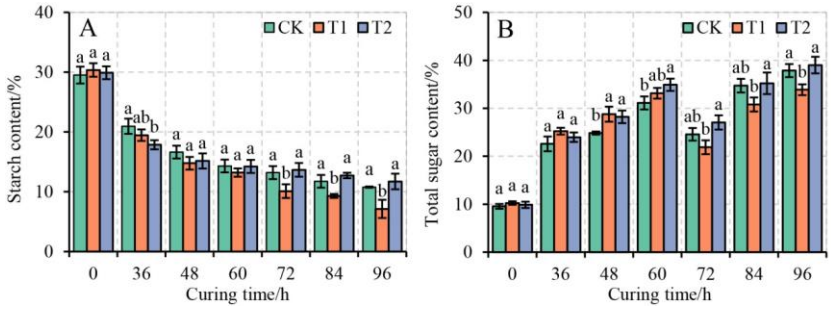



Figure 3. Changes in starch and total sugar content in tobacco leaves during curing

***Changes of enzymatic activities related to carbon metabolism in tobacco leaves during curing***

Different DBT methods demonstrate a significant impact on the AL activity, SSS activity, AGPase activity and SBE activity in tobacco leaves during curing (Figure 4). As TC progressed, the variations in enzymatic activities related to carbon metabolism among the three TC methods all exhibited the trend of "increase-decrease-increase-decrease" throughout curing. After 84 hours of curing, there were huge differences in the AL activities dealt with three treatments. After YS, the highest AL activity was observed in leaves done by T1 treatment, followed by the CK treatment (Figure 4A). In addition, the AL activities after all three treatments exhibited an inverted "V-shaped" trend, or initial increase and then decrease. During YS of TC process, the maximum AL activity was seen at 72 hours, with significant differences among three treatments. After YS, the CK treatment showed the highest AL activity, while T2 exhibiting the lowest (Figure 4B). Throughout YS, both T1 and T2 treatments demonstrated a "increase-decrease-increase" trend of AGPase activity in tobacco leaves, with slight variations after CK treatment. Moreover, after curing at 72 and 84 hours, there were significant differences in the AGPase activity among three treatments (Figure 4C). SBE activities in tobacco leaves after three treatments all followed a "double-peak curve" pattern featuring "increase-decrease". The two peaks occurred at 36 and 84 hours, respectively, with the maximum activities observed in the groups treated by CK and T2 correspondingly. At the end of YS, significantly different SBE activities were observed among these three treatments, with the highest activity done by T2 treatment, followed by the T1 treatment (Figure 4D).

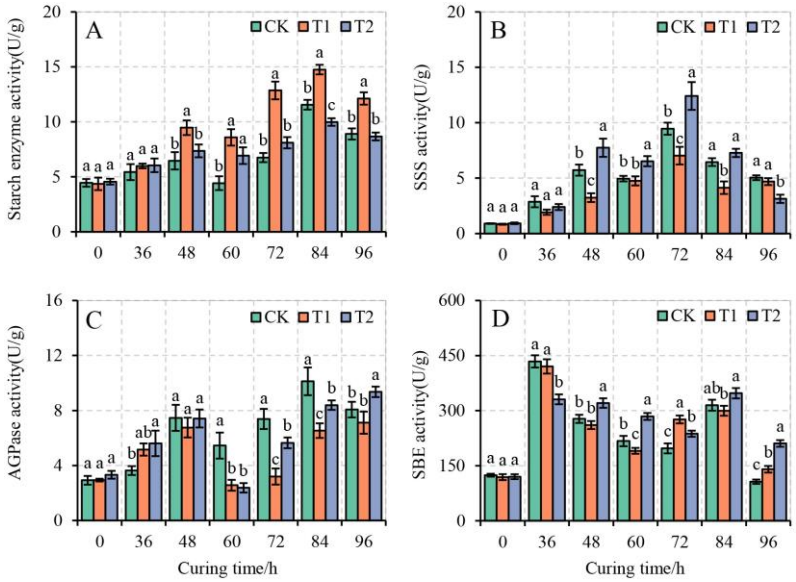



Figure 4. Changes in enzymatic activities related to carbon metabolism in tobacco leaves during curing

***Changes the Levels of Carbon Metabolism-Related Gene Expression in Tobacco Leaves during curing***

(1) Starch enzyme-related genes

Different DBT techniques significantly affect the expression of tobacco leaf AMY, BMY-1, BMY-2, and BMY-3 genes during curing (Figure 5). All three processing methods showed an increasing tendency in the expression levels of genes associated to starch enzymes, such as AMY, BMY-1, BMY-2, and BMY-3, when these genes' expression levels were analyzed in the leaves. Significant variations in the relative expression levels of genes associated to starch enzymes were observed between the approaches at YS. When compared to the other procedures, the T1 processing technique demonstrated noticeably greater relative

expression levels of the AMY, BMY-1, and BMY-3 genes, while the T2 processing technique displayed the lowest relative expression levels. Using the T2 processing method, the BMY-2 gene displayed noticeably higher relative expression levels.

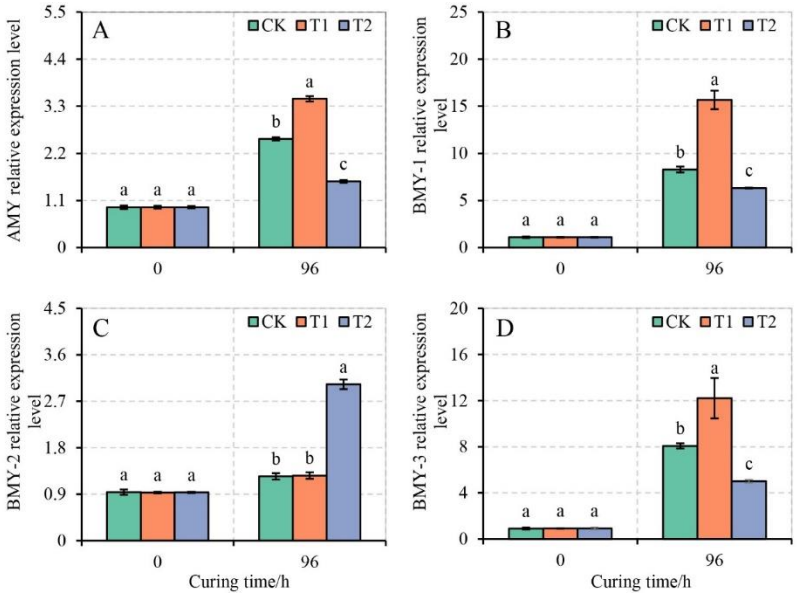



Figure 5. Changes in the gene expression levels related to starch enzyme in tobacco leaves during curing

(2) SSS, AGPase, and SBE-related genes

Different DBT curing techniques have a significant impact on the expression levels of tobacco leaf SSS-1, SSS-2, SSS-3, AGPase-1, AGPase-2, and SBE genes during curing (Figure 6). An rising tendency in the relative expression levels of of SSS, AGPase, and SBE- related genes, including SSS-1, SSS-2, SSS-3, AGPase-1, AGPase-2, and SBE was found by analyzing the expression fluctuations in the leaves, including SSS-1, SSS-2, SSS-3, AGPase-1, AGPase-2, and SBE. On the other hand, a declining trend was observed in the relative expression levels of the SSS-2 and SSS-3 genes. Significant variations in the relative expression levels of SSS, AGPase, and SBE-related genes were noted among the approaches at the conclusion of YS. When compared to alternative procedures, the T1 processing method demonstrated noticeably greater relative expression levels of the SSS-2 and SSS-3 genes. Under the T2 processing method, the SSS-1, AGPase-1, AGPase-2, and SBE genes with the highest relative expression levels were found and there statistically significant differences among the techniques.

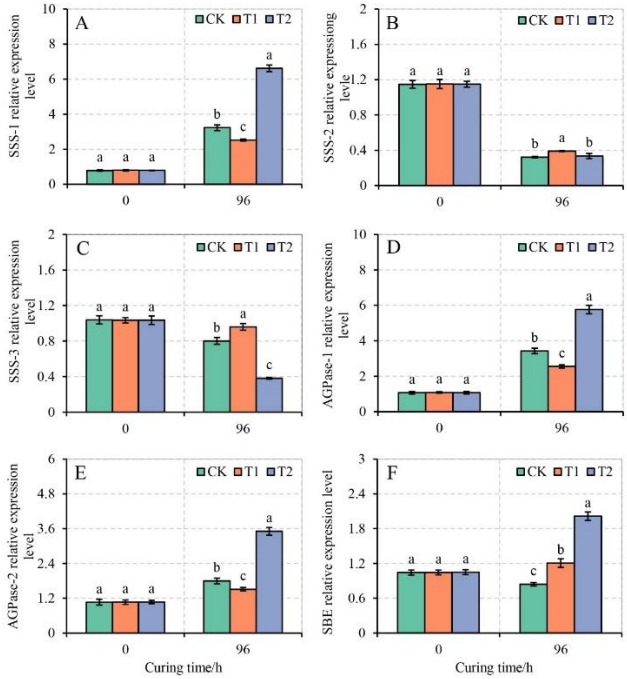



Figure 6. Changes in the gene expression levels related to SSS, AGPase, and SBE in tobacco leaves during curing

**Changes in Nitrogen Metabolism-Related Indicators in Tobacco Leaves during Curing**

***Changes in soluble protein and free amino acid content in tobacco leaves during curing***

The amounts of soluble protein and free amino acids in tobacco leaves during curing are significantly influenced by various DBT methods (Figure 7). The amount of soluble protein in tobacco leaves steadily dropped during curing under all three processing methods. The T1 processing method had the least amount of soluble protein at the conclusion of YS, considerably less than the control (CK). Soluble protein breakdown increased under T1 treatment settings (Figure 7A). Under all three processing methods, the amount of free amino acids in tobacco leaves grew steadily during curing, though at varying rates. The amount of free amino acids in tobacco leaves was significantly impacted by DBT changes during YS. The T1 processing method accumulated the largest content of free amino acids at the conclusion of YS, substantially more than the other methods (Figure 7B). These findings show that the T1 processing method efficiently encourages the breakdown of soluble proteins and makes it easier for free amino acids to build up in tobacco leaves.

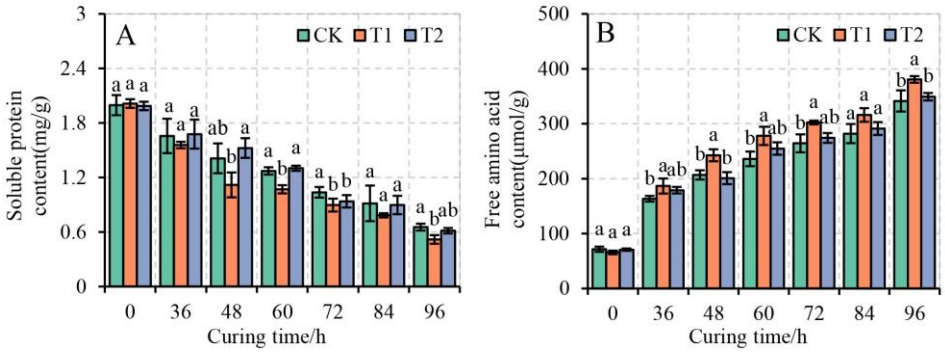



Figure 7. Changes in the content of soluble proteins and free amino acids in tobacco leaves during curing

***Changes in enzymatic activities related to nitrogen metabolism in tobacco leaves during curing***

The activities of NP, endopeptidase, GOT, and GPT in tobacco leaves during curing are greatly influenced by various curing methods (Figure 8). Tobacco leaves' NP activity showed a "increase-decrease" pattern with three peaks under the CK processing method. On the other hand, the "increase-decrease" pattern that resembled a "M" shape was consistently displayed by the T1 and T2 processing methods. The following sequence of NP activities was seen in tobacco leaves at the end of YS: T1 processing method is substantially more active than T2 processing method, with T1 > T2 > CK (Figure 8A). The patterns of NP activity and endopeptidase activity changes during cure were comparable. Under all processing conditions, tobacco leaves' endopeptidase activities increased dramatically between 0 and 36 hours during the early period of YS. The endopeptidase activity in the two DBT-variable treatments peaked at a later time than in the CK, and in both cases, the endopeptidase activity at 36 hours was considerably less than in the CK. The activities of endopeptidase in tobacco leaves at the conclusion of YS were as follows: T1 > T2 > CK, with the T1 processing technique showing noticeably higher activity than both the CK and T2 approaches (Figure 8B). When tobacco leaves were exposed to DBT-variable treatments (T1 and T2), the activities of GOT showed a trend of first increasing, then decreasing, and then increasing again. On the other hand, using the CK processing method, the GOT activity in tobacco leaves displayed a "increase-decrease" pattern that resembled a "M" shape. In general, during the early period of YS, GOT's activities in the DBT-variable treatments were lower than CK's. On the other hand, the enzymatic activity quickly rose after 72 hours. When compared to the other treatments, the T1 DBT-variable treatment had noticeably higher GOT activity at the conclusion of YS (Figure 8C). GOT activity changes and GPT activity changes during curing showed a similar pattern. When YS came to a conclusion, the GPT activities were listed as follows: T1 > T2 > CK, with notable differences observed between the treatments. The T1 DBT-variable treatment maintained comparatively higher overall enzymatic activity as YS of cure advanced (Figure 8D). According to these findings, the T1 DBT-variable processing method significantly affects the activities of nitrogen metabolism-related enzymes in tobacco leaves, encouraging the breakdown of macromolecular protein components and the build-up of free amino acids.

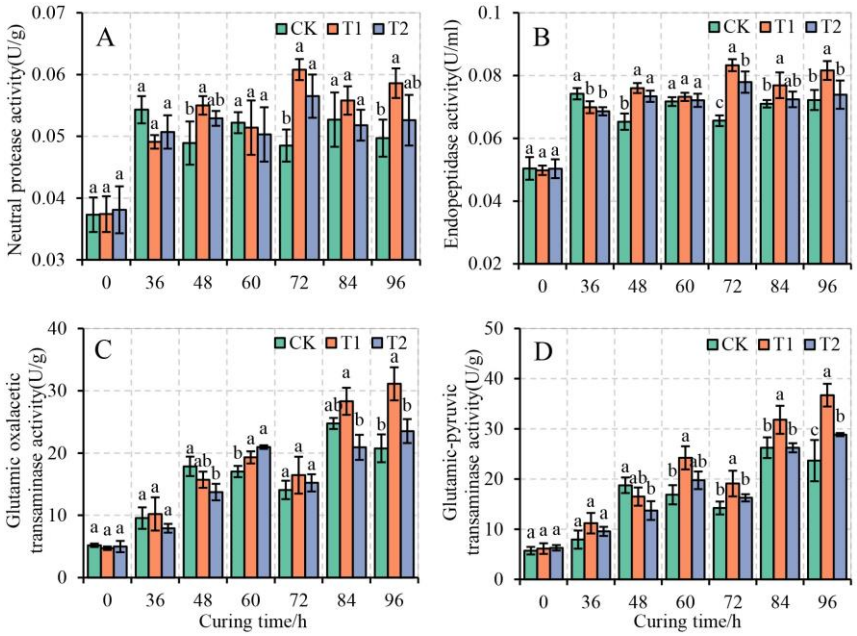



Figure 8. Changes in enzymatic activities related to nitrogen metabolism in tobacco leaves during curing

***Changes in the expression levels of genes related to nitrogen metabolism in tobacco leaves during curing***

Different DBT techniques significantly affect the relative expression levels of NR, GDH1, GS1-3, and GS2 genes in tobacco leaves during curing (Figure 9). The relative expression level of the nitrogen uptake-controlling nitrate reductase gene (NR) increased progressively during the curing process of YS. On the other hand, there was a downward trend in the relative expression levels of the genes GS1-3, which are engaged in nitrogen transfer and recycling, GS2 gene, which is involved in ammonia assimilation, and glutamate dehydrogenase gene (GDH1), which controls nitrogen transport. Significant variations in the relative expression levels of the NR, GDH1, GS1-3, and GS2 genes among the various processing methods were noted at the conclusion of YS. The T1 therapy had the lowest relative expression level of the NR gene, while the CK treatment had the greatest. The GDH1 gene was found to express itself most when using the T1 processing method and least when using the CK treatment. Among the three distinct processing methods, there were no appreciable variations in the relative expression levels of the GS1-3 genes; the T2 treatment showed the greatest level. The GS2 gene's relative expression levels varied significantly between the treatments, with the T2 processing method exhibiting the highest level and the T1 treatment exhibiting the lowest level.

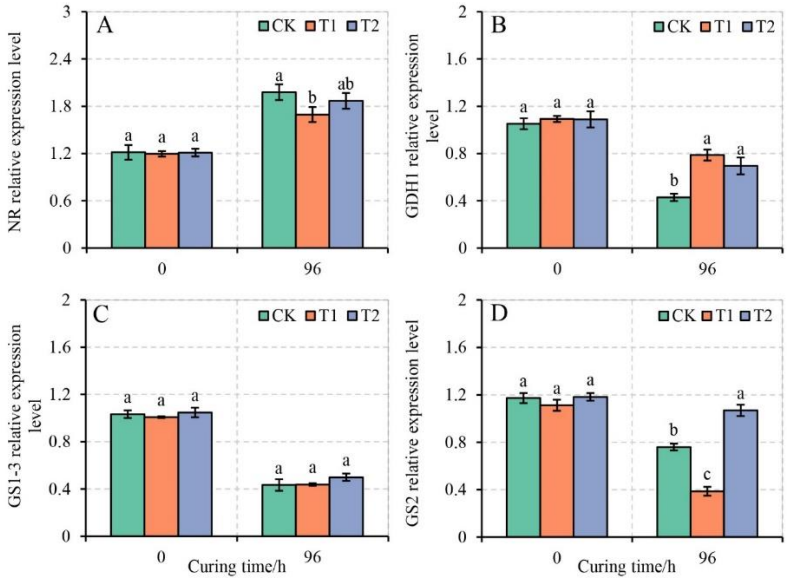
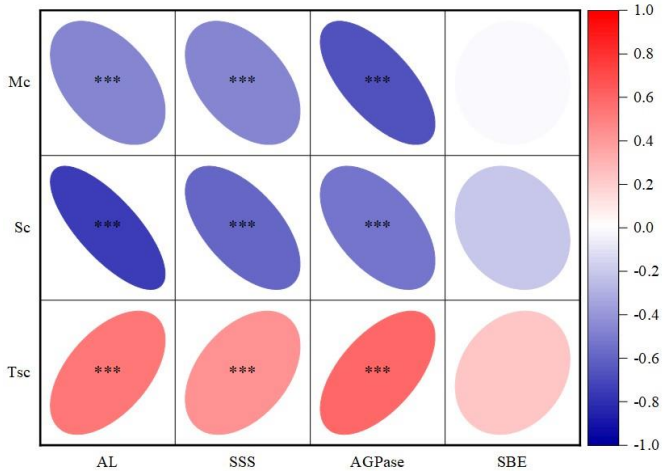



Figure 9. Changes in gene expression levels related to nitrogen metabolism in tobacco leaves during curing

**Synergistic Relationship between Carbon-containing Compounds and Enzymatic Activities Related to Carbon Metabolism during Curing**

***Simple correlation analysis of carbon-containing compounds and enzymatic activities related to carbon metabolism during curing***

From the figure, the leaf moisture content, starch content, and the activities of starch enzyme, SSS, and AGPase exhibit a highly significant negative correlation. Additionally, they are negatively correlated with the activity of SBE. On the other hand, the total sugar content of the leaves shows a highly significant positive correlation with the activities of starch enzyme, SSS, and AGPase. It is also positively correlated with the activity of SBE. These observations indicate a close relationship between leaf moisture, carbon-containing compound content, and enzymatic activities related to carbon metabolism (Figure 10).




Note: Mc: Leaf moisture content; Sc: Starch content; Tsc: Total sugar content; AL: Amylase activity; SSS: Soluble starch synthase activity; AGPase: Pyrophosphorylase activity; SBE: Starch branching enzyme activity. Red ellipses represent positive correlations, blue ellipses represent negative correlations, and the size of the ellipses represents the strength of the correlation.

Figure 10. Heat-map depicting the correlation between carbon-containing compounds and enzymatic activities related to carbon metabolism during curing

***Dynamic monitoring model of carbon-containing compounds and enzymatic activities related to carbon metabolism during curing***

Enzymatic activities linked to carbon metabolism during curing have a close association with molecules containing carbon, according to the findings of a basic correlation analysis (Table 1). This shows that variations in the amount of carbon-containing compounds in tobacco leaves can be accurately reflected in the enzyme activity related to carbon metabolism during curing. By applying the concepts of stepwise regression analysis, a multiple regression equation was created with two carbon-containing compound indicators, starch content (Y1) and total sugar content (Y2), as dependent variables and four enzymatic activity indicators, namely AL activity (X1), SSS activity (X2), AGPASE activity (X3), and SBE activity (X4), as independent variables. Table 1 displays the dynamic monitoring model of carbon-containing chemicals and related enzymatic activity during the curing process. The highly significant F-tests demonstrated that the numerous regression equations for carbon compounds and enzyme activities during curing showed good fitting degrees, indicating great dependability and precision of the developed equations.

Table 1. Dynamic monitoring model of carbon-containing compounds and enzymatic activities related to carbon metabolism during curing

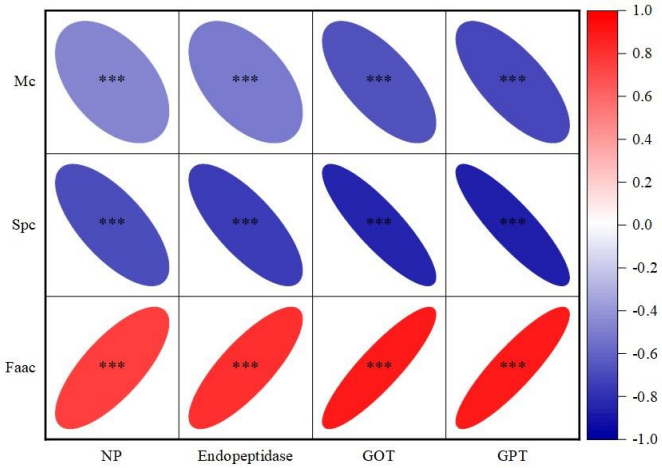
| Indicators | Stepwise regression equation | Mean square | $R^2$ | $F$ value |
|---|---|---|---|---|
| Starch | $Y1=32.875-1.231X1-0.761X2-0.568X3$ | 650.484 | 0.723 | 51.297** |
| Total sugar | $Y2=9.475+1.030X1+1.609X3$ | 1031.890 | 0.446 | 24.120** |

Note: $X1$, $X2$, $X3$, $X4$, $Y1$, and $Y2$ represent AL activity, SSS activity, AGPASE activity, SBE activity, starch content, and total sugar content, respectively. ** denotes correlation at the 0.01 significance level, the same as below.

**Synergistic Relationship between Nitrogen-Containing Compounds and Enzymatic Activities Related to Nitrogen Metabolism during Curing**

***Simple correlation analysis of nitrogen-containing compounds and enzymatic activities related to nitrogen metabolism during curing***

According to the figure, there is a highly significant negative correlation between leaf moisture content, soluble protein content, and the activities of NP, endopeptidase, GOT, and GPT. On the other hand, the content of free amino acids in the leaves exhibited a highly significant positive correlation with the activities of NP, endopeptidase, GOT, and GPT (Figure 11). These observations indicate a close relationship between leaf moisture, nitrogen-containing compound content, and enzymatic activities related to nitrogen metabolism.




Note: Mc: Leaf moisture content; Spc: Soluble protein content; Faac: Free amino acid content; NP: Neutral protease activity; Endopeptidase: Endopeptidase activity; GOT: glutamate oxaloacetate transaminase activity; GPT: Glutamate pyruvate transaminase activity. *** indicates highly significant correlation (P <= 0.001). Red ellipses represent positive correlations, blue ellipses represent negative correlations, and the size of the ellipses represents the strength of the correlation.

Figure 11. Heat-map depicting the correlation between nitrogen-containing compounds and enzymatic activities related to nitrogen metabolism during curing

***Dynamic monitoring model of nitrogen-containing compounds and enzymatic activities related to nitrogen metabolism during curing***

According to the simple correlation analysis, Compounds containing nitrogen and enzyme activity involved in nitrogen metabolism during curing are closely connected (Table 2). This suggests that changes in the amount of nitrogen-containing compounds in tobacco leaves can be accurately reflected by using the enzyme activity linked to nitrogen metabolism during curing. Four indicators of enzymatic activity—NP activity (*X5*), endopeptidase activity (*X6*), GOT activity (*X7*), and GPT activity (*X8*)—and two indicators of nitrogen compounds—soluble protein content (*Y3*) and free amino acid content (*Y4*)—were used as independent variables in the construction of a multiple regression equation using the concepts of stepwise regression analysis. Table 2 displays the dynamic monitoring model of nitrogen-containing chemicals and related enzymatic activity during the nitrogen metabolism curing process. The highly significant *F*-tests demonstrated that the numerous regression equations for nitrogen-containing chemicals and enzyme activities during curing showed good fitting degrees, indicating high reliability and precision of the established equations.

Table 2. Dynamic monitoring model of nitrogen-containing compounds and enzymatic activities related to nitrogen metabolism during curing

| Indicators | Stepwise regression equation | Mean square | $R^2$ | *F* value |
|---|---|---|---|---|
| Soluble protein | $Y3=3.008-16.506X6-0.034X8$ | 5.456 | 0.822 | 138.210** |
| Free amino acid | $Y4=-147.708+3826.484X6+1.100X7+5.423X8$ | 144196.500 | 0.883 | 148.002** |

Note: *X*5, *X*6, *X*7, *X*8, *Y*3, and *Y*4 represent NP activity, endopeptidase activity, GOT activity, GPT activity, soluble protein content, and free amino acid content, respectively.

## DISCUSSION

The primary metabolic activities in tobacco leaves are those involving carbon and nitrogen, and under various DBT and humidity levels, these processes change dramatically during YS [24,25]. Thus, encouraging the well-coordinated and harmonious biochemical changes in flue-cured tobacco requires appropriate curing environment regulation [26]. Consistent with the findings provided by Zhu et al. [27], the study's results show that the moisture content of the tobacco leaves constantly declines as the curing process moves forward. The T2 approach shows the largest rate of moisture loss during the 72–96 hour curing period, whereas the T1 technique shows the lowest rate. This discrepancy could be explained by changes in the WBT and DBT conditions established during the curing process, which would affect the loss of leaves' moisture. The T1 method shows the lowest starch level in the tobacco leaves at the conclusion of YS during curing, indicating a more thorough starch breakdown. The genes encoding the starch enzymes (AMY, BMY-1, and BMY-3) showed a considerable rise in relative expression levels when compared to other genes related to starch metabolism in the tobacco leaves treated with the T1 approach during this time. This rise causes significant starch breakdown in the leaves as well as increased starch enzymatic activity. During the same period, the genes encoding AGPase (AGPase-1 and AGPase-2), which are involved in phosphorylation, and the gene encoding SSS-1, which is involved in phosphorylation, have the lowest relative expression levels when compared to other treatments. The gene that codes for SBE likewise has relatively low relative expression levels and a negligible rise. As a consequence, there is less carbon absorption and less starch synthesis since the activity of SSS, AGPase, and SBE are kept at lower levels. These results are in line with studies by Huang et al. and Niu et al. [28, 29]. The tobacco leaves' overall sugar content lowers throughout the course of 60 to 72 hours of curing. This could be connected to the Maillard reaction's advancement, which uses sugars as substrates. These results are in line with the studies that Zhang et al. [30] presented. In general, the patterns of gene expression match the amounts of starch and the activity of the enzymes involved in carbon metabolism. It is crucial to remember that different genes that encode the same enzyme may not always show high or low expression levels at the same time. Enzyme genes that are different isoforms coordinate their expression levels at particular periods to control enzymatic activity fluctuations and preserve the dynamic balance of starch metabolism [31,32].

The degree of protein breakdown in tobacco leaves is directly determined by changes in enzyme activities linked to nitrogen metabolism during curing [33]. The experiment's findings show that, under different DBT conditions, there are general patterns of changes in soluble proteins, free amino acids, and the enzyme activity linked to nitrogen metabolism. During YS, soluble proteins are continuously degraded. According to studies by Chen et al. and Wang et al. [34, 35], there is a mutually restrictive relationship between free amino acids and soluble protein content, which shows a gradual increase in a consistent pattern. During curing of YS, tobacco leaves treated with the CK approach have a different trend in NP activity than leaves treated with two different DBT techniques. This discrepancy could be explained by regional curing methods, tobacco cultivars, and equipment capability. According to research findings published by He et al. [36], variations in the activity of NP are correlated with changes in the activity of endopeptidases. Overall, there are some similarities between the patterns of GOT and GPT changes during YS. These enzymes' activity follows a "increase-decrease" pattern under the CK curing method, which resembles a "M-shaped" curve. On the other hand, the enzyme activity did not exhibit the characteristic "M-shaped" pattern during the YS under the T1 and T2 DBT-variable treatments. In addition, the enzymes' peak activity happens later than with the CK method. This discrepancy could be explained by the early rise in DBT during the variable-DBT treatments, which causes the tobacco leaves to lose a lot of water, intensify membrane lipid peroxidation, and cause the transaminases to inactivate more quickly. The T1 treatment shows the highest accumulation of free amino acids and the lowest quantity of soluble proteins at the conclusion of the YS, suggesting complete protein breakdown. Rapid nitrogen-containing substance degradation, a decrease in the amount of soluble proteins, and resemblance to the findings reported by Liu et al. [37] result in a higher nitrogen transfer and lower assimilation caused by the relatively high expression levels of GS1-3 genes involved in nitrogen transfer and the low expression levels of NR and GS2 genes associated with nitrogen assimilation. The variations in nitrogen metabolism-related gene expression levels are in good agreement with the patterns found in the composition of substances containing nitrogen. This correlation agrees with what Guo et al. found [38]. The basis of metabolic processes is gene expression, and the genes that encode the enzymes involved in protein metabolism are essential for regulating the synthesis and breakdown of proteins. The activity of these enzymes is closely correlated with the levels of gene expression, and this has an impact on the production and composition of proteins [39, 40]. This study conducted a thorough analysis of the close association that exists during TC of YS between chemicals that contain carbon and the enzyme activity related to carbon metabolism, as well as between compounds that contain nitrogen and the enzymatic activity connected to nitrogen metabolism. Stepwise regression analysis and basic correlation analysis were used to offer a theoretical foundation for cure optimization. The activity of starch enzymes, SSS, and AGPase, and tobacco leaf moisture content, starch and total sugar content, are all highly correlated, according to the results of the basic correlation analysis.

Similarly, there is a strong positive association between the enzyme activity involved in nitrogen metabolism and the moisture, soluble protein, and free amino acid contents of tobacco leaves. These results are consistent with the study carried out by Li et al. [41] and indicate a close relationship between compounds containing carbon and enzymatic activity related to carbon metabolism as well as between compounds containing nitrogen and enzymatic activity related to nitrogen metabolism. According to the stepwise regression analysis, the different moisture content indicators and the tobacco leaves' morphological shrinkage rate during curing suit each other quite well. The established multivariate regression equations appear to be highly precise and dependable, as indicated by the F-test findings, which show a highly significant level.

## CONCLUSION

Two DBT treatments were applied to tobacco leaves in the YS by varying the t DBT range and frequency, based on the convention curing method with a major yellowing DBT of 38 °C. This study indicates that the metabolism of carbon and nitrogen in tobacco leaves can be greatly impacted by the appropriate control of DBT range and frequency in YS in BCBs. It is ideal for the complete breakdown and conversion of protein and starch, as well as the efficient accumulation of free amino acids, under treatment conditions of a DBT range of 1 °C and a frequency of 3 hours. These factors have a major impact on the quality of tobacco leaves. The regression analysis indicates that the regression equations for the enzymatic activity related to carbon metabolism in tobacco leaves during curing are $Y1=32.875-1.231X1-0.761X2-0.568X3$ and $Y2=9.475+1.030X1+1.609X3$. The regression equations for the enzymatic activity related to nitrogen metabolism in tobacco leaves during curing are $Y3=3.008-16.506X6-0.034X8$ and $Y4=-147.708+3826.484X6+1.100X7+5.423X8$. These multivariate regression equations fit the data well and enable the evaluation on how the amount of nitrogen- and carbonaceous-containing chemicals in tobacco leaves changes as the leaves cure.

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