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Full Length Research Paper

Nicotine to nornicotine conversion in Chinese burley tobacco and genetic improvement for low conversion hybrids

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Nicotine conversion results in abnormally high levels of nornicotine which lead to high nitrosonornicotine (NNN) content and off-taste of smoke. Nicotine conversion is a worldwide problem in burley tobacco production and has been the focus in burley research in recent years. Nicotine conversion level in Sichuan burley tobacco was the highest with the percent nicotine conversion being 62.4%, followed by Chongqing, Hubei and Yunnan. The leading variety Xuanhan-5NL had serious problem of nicotine conversion, with the proportion of converters being 96% of the total plants. In the populations of hybrid Dabai 1, Dabai 2 and Eyan 1, a substantial amount of converters were also found. Newly developed hybrids Dabai 3 and Dasuo 24 had serious problems of nicotine conversion with almost 100% of the plants being converters. Further study using ethylene early identification method was conducted to investigate the contribution of different parental lines to nicotine conversion in Dabai series hybrids. Both parents of Dabai 1 contributed to the conversion level in the hybrid. The male parent of Dabai 2 was the main contributor of conversion gene to the hybrid. The male parent of Dabai 3, had extremely high converter proportion and conversion level, and was almost the exclusive contributor of conversion gene to Dabai 3. Improved Dabai series hybrids developed by selecting and crossing non-converter parent plants, showed significantly reduced converters in next populations. Genetic improvement for low conversion trait by early non-converter parent selections was very effective.

Key words: Burley, tobacco, nicotine to nornicotine conversion, improvement, hybrid.

INTRODUCTION

Burley tobacco is one of the most important materials for producing blended cigarettes. High quality burley is characteristic with outstanding style, full strength, high aroma and less offensive odor. Nicotine is the most important component in tobacco (*Nicotiana tabacum* L.) and usually accounts for > 94% of the total alkaloid fraction. The content of the secondary alkaloid nornicotine is normally less than 3.5% (Bush, 1981; Bush et al., 1993). In commercial burley varieties, some plants convert a substantial amount of nicotine into nornicotine. Mann et al. (1958) studied inheritance of the conversion of nicotine to nornicotine in varieties of *N. tabacum* and related amphiploids and found that plants that contained high amounts of nornicotine differed from those that did not by a single dominant gene. Siminszky et al. (2005) reported that a cytochrome P450 gene (*CYP82E4v1*) was involved in the conversion of nicotine to nornicotine. Nicotine to nornicotine conversion is undesirable, because high levels of nornicotine directly contribute to the formation of *N*-nitrosonornicotine (NNN), an important tobacco-specific nitrosamine, and other undesirable compounds, such as acylated nornicotines (which decrease the tobacco flavor quality). Harada (1985) compared the smoking quality among burley 21 lines with different levels of nicotine conversion and found an inverse relationship between the nornicotine content and smoking quality that was nearly linear. Tobacco varieties with high levels of nornicotine were characterized with a

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Table 1. Alkaloid contents and percent nicotine conversion in burley tobacco produced in different areas of China.

Areas	Nicotine (mg/g)	Nornicotine (mg/g)	Anabasine (mg/g)	Anatabine (mg/g)	Nicotine + nornicotine (mg/g)	Percent nicotine conversion (%)
Sichuan	22.18	36.81	0.17	1.38	58.99	62.40
Chongqing	31.94	8.53	0.22	1.03	40.47	21.08
Hubei	51.85	5.82	0.43	1.26	57.07	10.09
Yunnan	49.99	1.60	0.23	1.36	57.39	3.10
USA	42.66	1.44	0.19	1.11	44.10	3.27

nornicotine odor and a weak burley aroma. These results were corroborated by Roberts (1988), who studied natural tobacco flavor and found that during pyrolysis nornicotine produced myosmine and substituted pyridine compounds that created objectionable smoke flavors such as an alkaline taste and a mousy aroma. Leaves containing high levels of nornicotine tend to contain high levels of NNN. In 30 plants with different nornicotine levels, there was a significant positive correlation between nornicotine and NNN concentrations (Shi et al., 2000; Bush et al., 2001). A similar correlation was also found in Chinese tobacco (Shi et al., 2000).

Nicotine conversion is a very popular problem in burley tobacco production throughout the world, and has caused extensive attention. In recent years, a lot of efforts have been made to address this issue and to solve this problem by agronomic approach (Shi et al., 2003; Jack et al., 2006; Shi et al., 2007) and biotech approaches (Gavilano et al., 2006; Xu et al., 2007). A great progress has been made in the improvement of US burley varieties and seed production. Chinese burley production is concentrated in four areas, Western Hubei Province, Eastern Sichuan Province, Chongqing City, and Central Yunnan Province. Different producing areas grow different varieties. Former studies revealed that the problem of nicotine to nornicotine conversion in Chinese burley was very severe (Shi et al., 2007) and the improvement of burley varieties for low conversion is in progress. In this study, burley tobacco from different producing areas were collected and analyzed for nicotine conversion. All varieties grown in Sichuan area including parents of some hybrids were determined for nicotine conversion, and the progress in the improvement of Sichuan burley hybrids for low conversion was also reported.

MATERIALS AND METHODS

Burley cured bulk samples of middle stalk position leaves produced in China's four burley producing areas (Hubei, Chongqing, Sichuan, Yunnan) and USA tobacco were provided by Wuhu cigarette manufacturer in 2006. The first three areas in China are all located in the central west of China with high temperature and plenty of rainfall during the growing season and tobacco air-curing. Yunnan area is located in southwestern China with plentiful sunlight and relatively less rainfall during the season. USA tobacco was produced in Kentucky, which is regarded as the high quality burley tobacco producer in the world.

The experiment was set up in Dazhou Tobacco Research Station of Sichuan in 2007 to compare nicotine conversion in cured middle stalk position leaves among all genotypes grown in Sichuan, including Dabai 1, Dabai 2, Dabai 3, Xuanhan 5NL, Xhuanhan 5BL, Eyan 1, TN90, Dasuo 24. All the genotypes are registered cultivars and grown in certain areas. In Sichuan, Xuanhan 5NL was the leading cultivar, while the newly developed Dabai 1, Dabai 2 and Dabai 3 were recommended hybrids due to excellent agronomic characteristics. Eyan 1 was the leading variety both in Hubei and Chongqing, and TN90 was most cultivated in Yunnan. The trial was a randomized block design with 3 replicates. 200 plants were transplanted in each block. Around 100 individual plants for each genotype were labeled before harvesting and then were put into the curing barn for air curing. When the curing process was completed, middle leaves were primed and oven-dried for alkaloid measurement.

In another experiment, 3 promising hybrids (Dabai 1, Dabai 2, and Dabai 3) and their parents were planted. The plants were individually labeled 2 weeks after transplanting, and then one leaf was primed from each labeled plant for early stimulation of nicotine conversion and for identification of converters by using the protocol reported by Shi et al (2001). Non-converters were selected for crossing to produce improved hybrid seeds.

In 2008, an experiment was set up to compare the nicotine conversion in original hybrids and improved hybrids. Three improved hybrids were planted along with 3 original hybrids. Sixty plants for each genotype were labeled 2 weeks after transplanting; one leaf was primed and subjected to early stimulation and evaluation of nicotine conversion.

Alkaloid measurement

Methyl *tert*-butyl ether (MTBE) was used as extraction agent and quinoline as internal standard. For each sample, 100 mg of ground tobacco was accurately weighed into a culture tube, and 0.5 ml of 2 N NaOH was added to moisten the tobacco sample for 15 min. Then 5 ml of extraction solution was added into the tube to extract alkaloids. Samples were shaken in a linear shaker for 2 h with a capped tube. After the solvent and sample were separated, aliquots from the extraction were transferred to a GC vial for alkaloid separation and quantification. An Agilent-7890 (Agilent, Inc., Palo Alto, CA) gas chromatograph equipped with a DB-5 capillary column was used to determine the alkaloid contents, using a modified method of Severson et al. (1981) (Table 1).

Expression of conversion

In the study, percent nicotine conversion was used to express the conversion level of tobacco samples. The formula used for calculating percent nicotine conversion was:

Table 2. Proportion of converters and average percent nicotine conversion in burley from different genotypes.

Genotypes	Proportion of converters (%)			Proportion of non-converters (%)	Average percent nicotine conversion (%)
	Total converters	Low converters	High converters		
Xuanhan 5 (NL)	96.0	20.0	76.0	4.0	44.8
Xuanhan 5 (BL)	97.0	21.0	76.0	3.0	42.6
Dabai 1	25.0	19.0	6.0	75.0	5.2
Dabai 2	20	16.0	4.0	80.0	4.5
Dabai 3	100.0	15.0	85.0	0.0	39.5
Dasuo 24	100.0	14.0	86.0	0.0	35.5
TN90	14.0	14.0	0.0	86.0	3.0
Eyan 1	72.0	60.0	12.0	28.0	11.2

Data presented was the average of 3 replicates.

Nicotine conversion (%) = 100 × nornicotine content / (nicotine content + nornicotine content)

The converter was defined as the tobacco plant having percent nicotine conversion greater than 3%, and high converter as tobacco plant having percent nicotine conversion greater than 20%, and low converters as percent nicotine conversion between 3 and 20%.

RESULTS

Comparison of nicotine conversion among bulk burley samples from different producing areas

There are remarkable differences in nicotine conversion among burley tobaccos produced in different areas. Yunnan tobacco along with USA tobacco had lowest percent nicotine conversion, and Sichuan tobacco had highest conversion level, with the percent nicotine conversion of 62.4%, indicating a lot of converters in the population. Chongqing and Hubei tobaccos also had problems of nicotine conversion. Result showed that the total content of nicotine and nornicotine in Sichuan tobacco was the highest, while the nicotine content was the lowest due to the conversion of nicotine to nornicotine, resulting in higher nornicotine content than nicotine content. This was in line with the fact that Sichuan tobacco had low burley style, less and poor aroma and weaker strength. This finding led to our interest and efforts to further investigate the cause of the problem of nicotine conversion in Sichuan area and the link with tobacco quality (Table 1).

Distribution of nicotine conversion among individual plants for different genotypes

Genotype is one of the key factors affecting tobacco quality and chemistry. Genotypes currently used in China varied with producing areas. In Hubei and Chongqing areas, Eyan 1 was most cultivated in the production, accounting for 70% of the acreage. In Yunnan, TN90 was the most popular variety. In Sichuan, multiple genotypes are used including Xuanhan-5NL, Xuanhan-5BL, Dabai 1, Dabai 2, Eyan 1, TN90 etc, with Xuanhan-5NL being the leading variety accounting for more than 85% of the total acreage.

All 8 genotypes grown in Sichuan were investigated for nicotine conversion by sampling cured leaves of individual plants. Table 2 summarized the proportions of converters and average percent nicotine conversion in cured leaves from different genotypes. Results showed that the dominant variety Xuanhan-5NL and 3 other genotypes (Xuanhan-5BL, Dabai 3, and Dasuo 24) all had more than 96% converters in the populations, and mostly being high converters. The average percent nicotine conversion for Xuanhan-5 NL was 44.8%, and that for Xhuanhan-5BL, Dasbai 3, and Dasuo 24 were 42.6%, 39.5 and 35.5%, respectively, indicating the severe problem of nicotine conversion existing in these genotypes. Considering the poor agronomic performance and low quality potential of Xuanhan-5NL, it was suggested that this variety be replaced by other proved promising varieties. The distribution of percent nicotine conversion in air-cured leaves of individual plants of Xuanhan-5NL was illustrated in Figure 1.

Dabai 1 and Dabai 2 are official varieties showing relatively good agronomic and economic characteristics, but are not cultivated extensively yet. Results showed that the problem of nicotine conversion for these two varieties were much less severe than the former mentioned genotypes. The proportions of total converters were 25 and 20% for Dabai 1 and Dabai 2, respectively, and the proportions of high converters were 6 and 4%, respectively. Figure 2 illustrated the distribution of percent nicotine conversion in individual plants of Dabai 1. Due to the existence of converters in the populations of the two varieties, systemic selections of parent plants and improvement of the varieties were necessary before they are used as leading varieties to ensure high quality tobacco production.

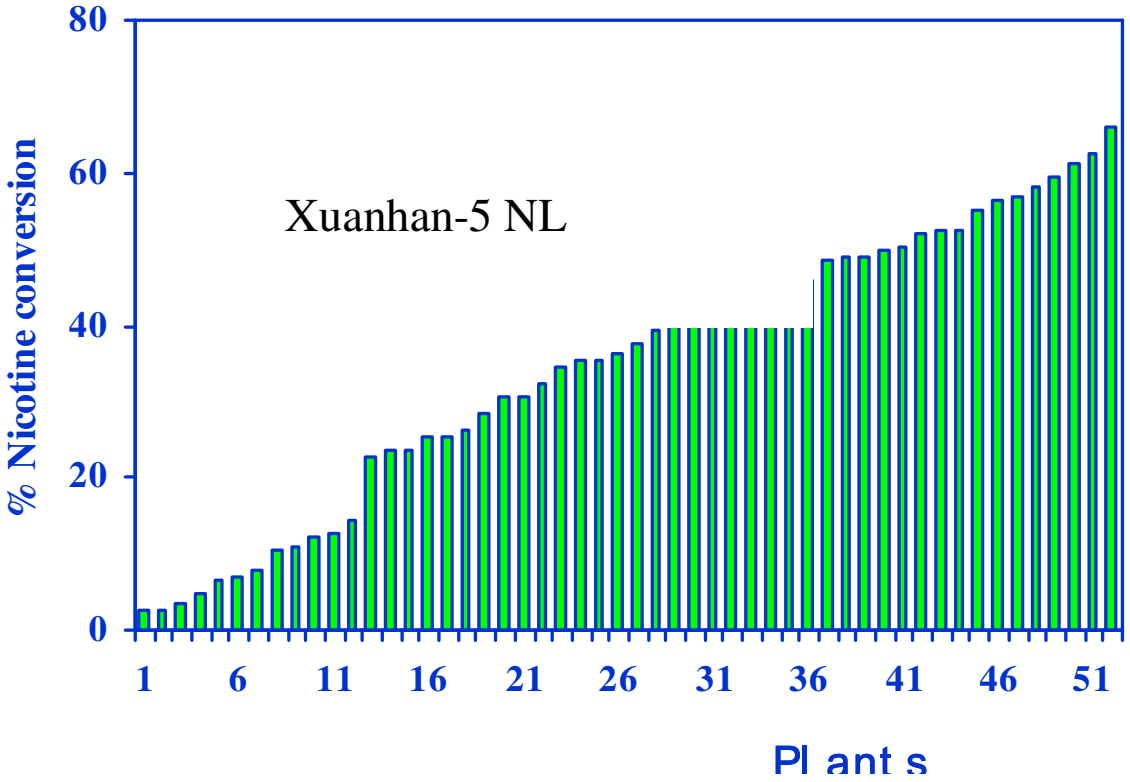

Figure 1. Distribution of percent nicotine conversion in air-cured leaves of individual plants of Xuanhan-5NL.

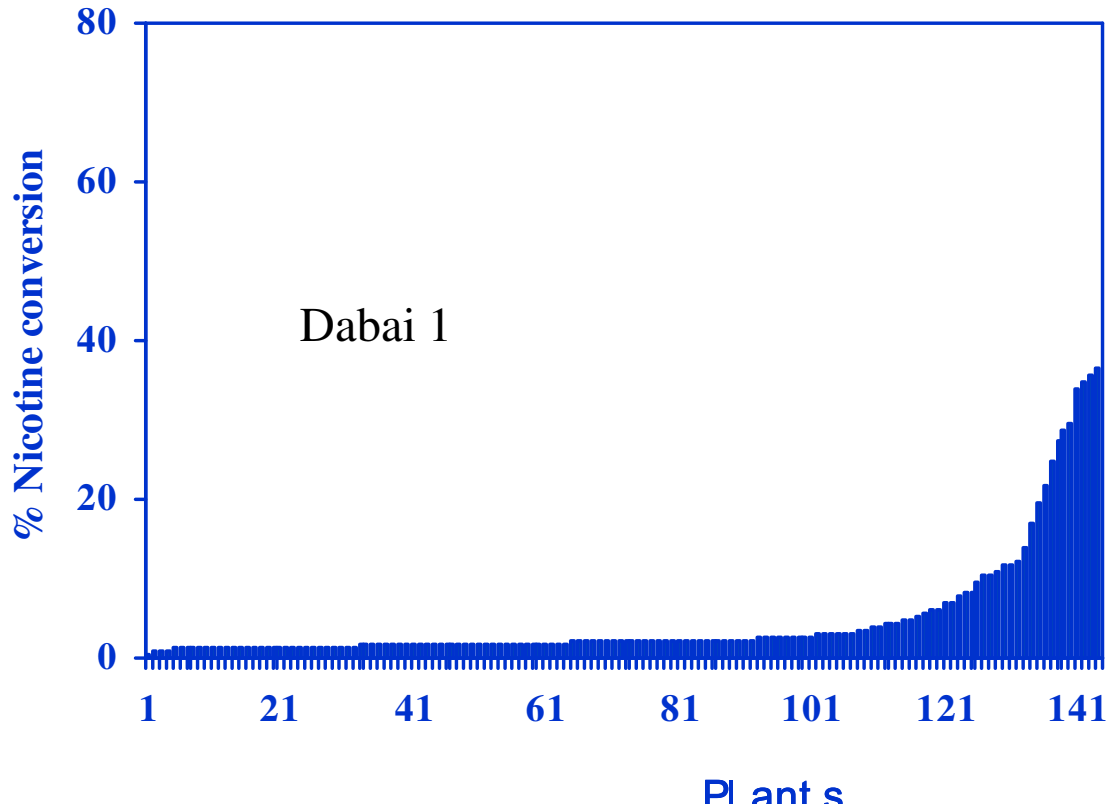

Figure 2. Distribution of percent nicotine conversion in air-cured leaves of individual plants of Dabai 1.

Table 3. Proportion of converters and percent nicotine conversion in the populations of different varieties.

Variety	Population	Proportion of converters (%)			Non-converter Proportion (%)	Average nicotine content (%)	Average nornicotine content (%)	Average nicotine conversion (%)
		Total converters	Low converters	High converters				
	Hybrid	26.9	17.3	9.6	73.1	1.56	0.10	5.94
Dabai 1	MSKY14 (♂)	28.2	19.8	8.4	71.8	1.93	0.08	3.98
	Dasuo 26 (♀)	39.2	27.1	12.1	60.8	1.33	0.12	8.29
	Hybrid	23.7	16.3	7.4	76.3	1.75	0.09	4.89
Dabai 2	MSVA509 (♂)	10.1	10.1	0.0	89.9	1.38	0.03	1.91
	Dasuo 26 (♀)	43.2	20.0	14.1	60.8	1.33	0.12	8.29
	Hybrid	100.0	20.0	80.0	0.0	1.06	0.66	38.30
Dabai 3	MSKY14 (♂)	28.2	19.8	8.4	71.8	1.93	0.08	3.98
	Dasuo 27 (♀)	98.6	2.1	96.6	1.4	0.22	1.05	82.69

Eyan 1 and TN90 made up a small percentage of acreage in Sichuan, although they are dominant varieties in Hubei and Yunnan, respectively. Results showed that in the population of Eyan 1, a substantial amount of converters were found with the proportion of total converters of 72%, although most of them are low converters, indicating that the improvement need to be carried out if the variety continues to be cultivated in this area. TN90 had very few converters in the population. TN90 was an imported American variety. Traditionally it had about 20% converters in the population. In recent years, the systemic selections and purifications had been conducted, which may have contributed to the decrease of nicotine conversion in the variety.

Early identification of nicotine converters in Dabai hybrids and their parents

"Dabai" series hybrids (Dabai 1, Dabai 2 and Dabai 3) were promising varieties and were suggested to be the candidates for replacing Xuanhan 5 NL as the principle varieties. Since these varieties had problems of nicotine conversion at different degrees, the genetic improvement on the trait of nicotine conversion was quite necessary. In this study, early stimulation of nicotine conversion and identification of converters were carried out by using the method of ethylene treatment before flowering. The proportion of nicotine converters of different categories and average percent nicotine conversion for the 3 hybrids and their parents were summarized in Table 3.

For Dabai 1, the maternal parent is MSKY14 and the paternal parent is Dasuo 26. The results showed that both parents of Dabai 1 had substantial amount of converters in the populations, although most of which were low converters (Figures 3 and 4). Therefore, both parents had contributed converting genes into the hybrid, and the parent populations need to be screened to identify converters which should be eliminated thereafter. Hybrid Dabai 2 had MSVA509 as female parent and Dasuo 26 as male parent. Results showed that MSVA509 had very few converters in the population, and all of which were low converters (Figure 5), therefore Dasuo 26 was the principle contributor of converting genes to the hybrid. Hybrid Dabai 3 had extremely high level of nicotine conversion with all the tested plants as converters and most of which were high converters. From the results of conversion measurements in the two parents, maternal MSKY14 and paternal Dasuo 27, it can be drawn that the main contributor of converting genes in Dabai 3 was the male parent Dasuo 27, which had almost exclusively high converters in the population (Figure 6). Obviously, to improve this hybrid, great efforts should be given to finding some non-converters which then be utilized to do the pollination for seed production.

Comparison of nicotine conversion between improved Dabai hybrids with original hybrids

By strictly selecting non-converter maternal and paternal parent plants to do the crossing in seed production, the improved hybrids were produced in 2007. The plants were planted the following year to conduct comparison of nicotine conversion between original hybrids and relevant improved hybrids. The results were shown in Table 4. It can be seen that remarkable improvement was achieved by crossing non-converter plants to produce improved seeds. All improved hybrids showed greatly reduced converters in the populations; especially for Dabai 3, the proportion of total converters reduced from 100% in original hybrid to only 10.9% in improved hybrid, and the average percent nicotine conversion decreased from 40.52 to 2.32%, indicating that the problem of nicotine conversion can be effectively solved by parent selection and control of pollination in the process of seed production.

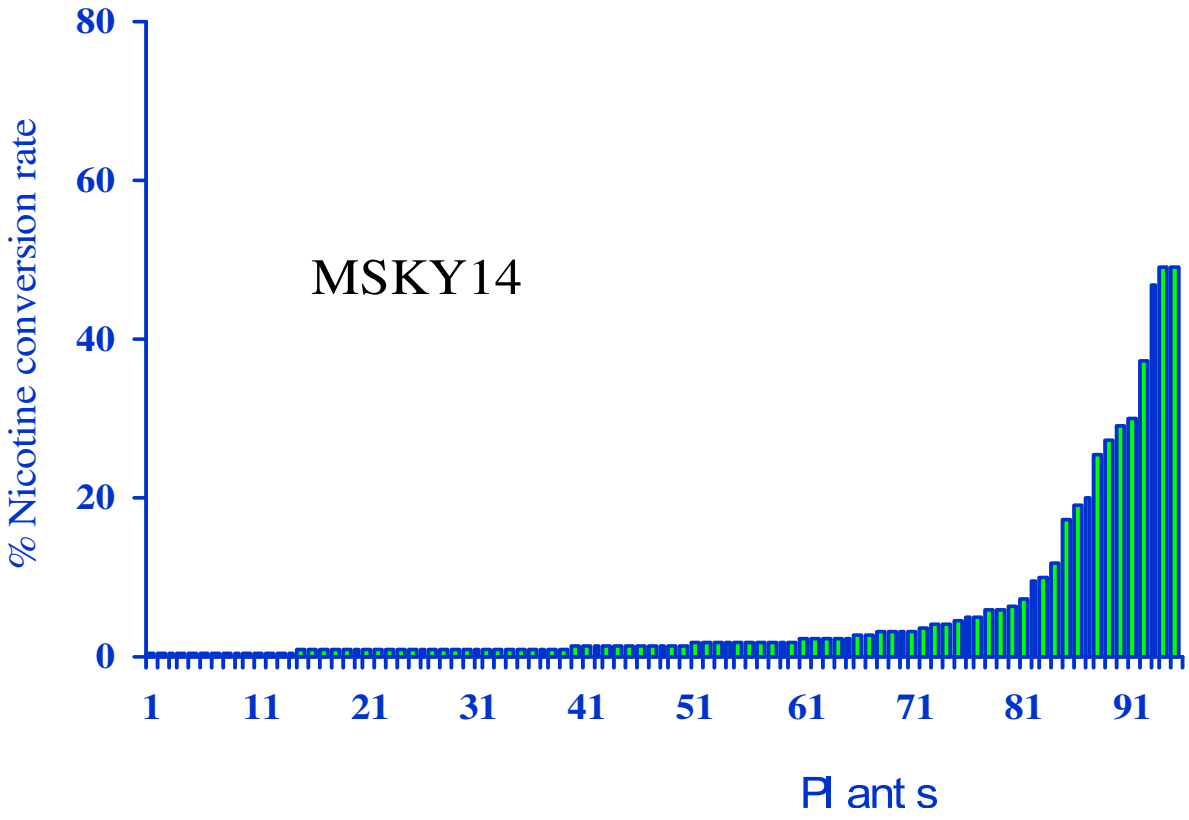

Figure 3. Distribution of percent nicotine conversion in air-cured leaves of individual plants of MSKY14 (the maternal parent for Dabai 1 and Dabai 3).

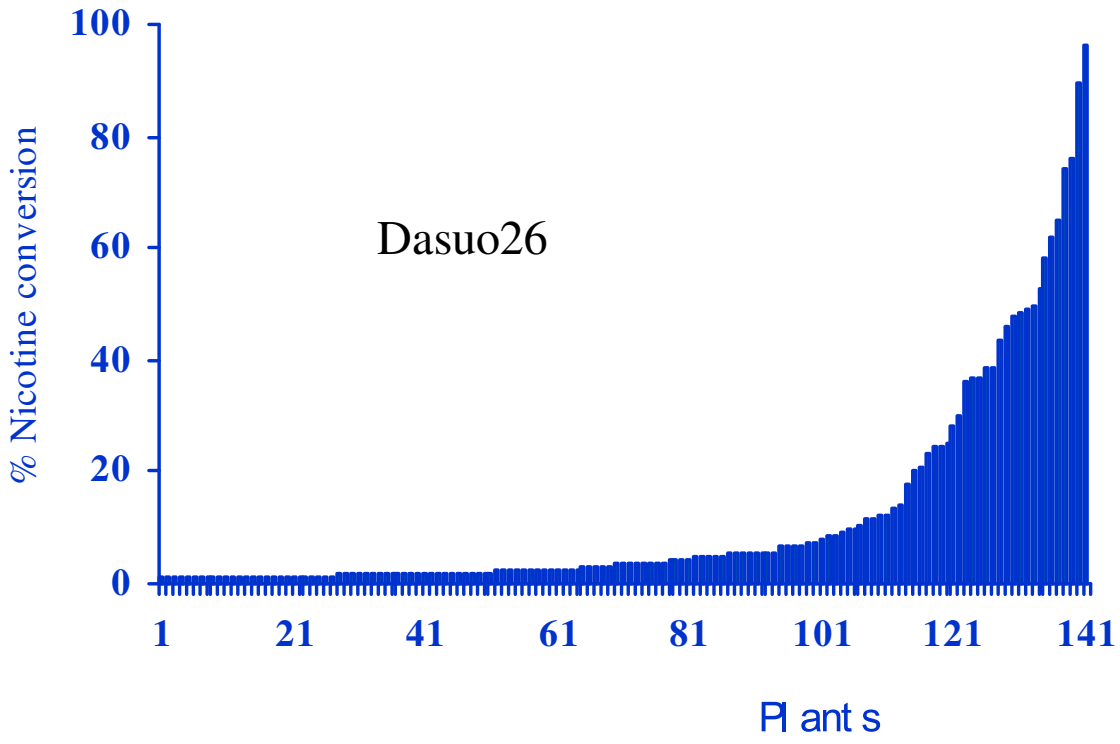

Figure 4. Distribution of percent nicotine conversion in air-cured leaves of individual plants of Dasuo 26 (the paternal parent for Dabai 1 and Dabai 2).

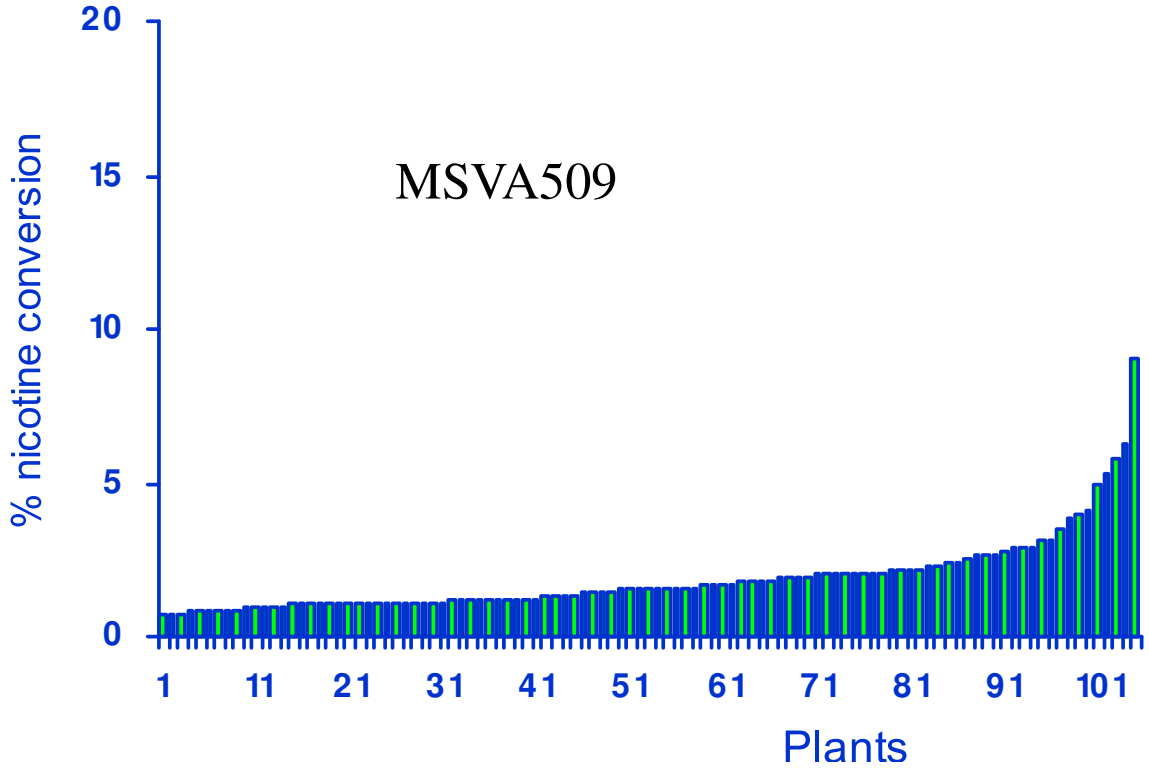

Figure 5. Distribution of percent nicotine conversion in air-cured leaves of individual plants of MSVA509 (the maternal parent for Dabai 2).

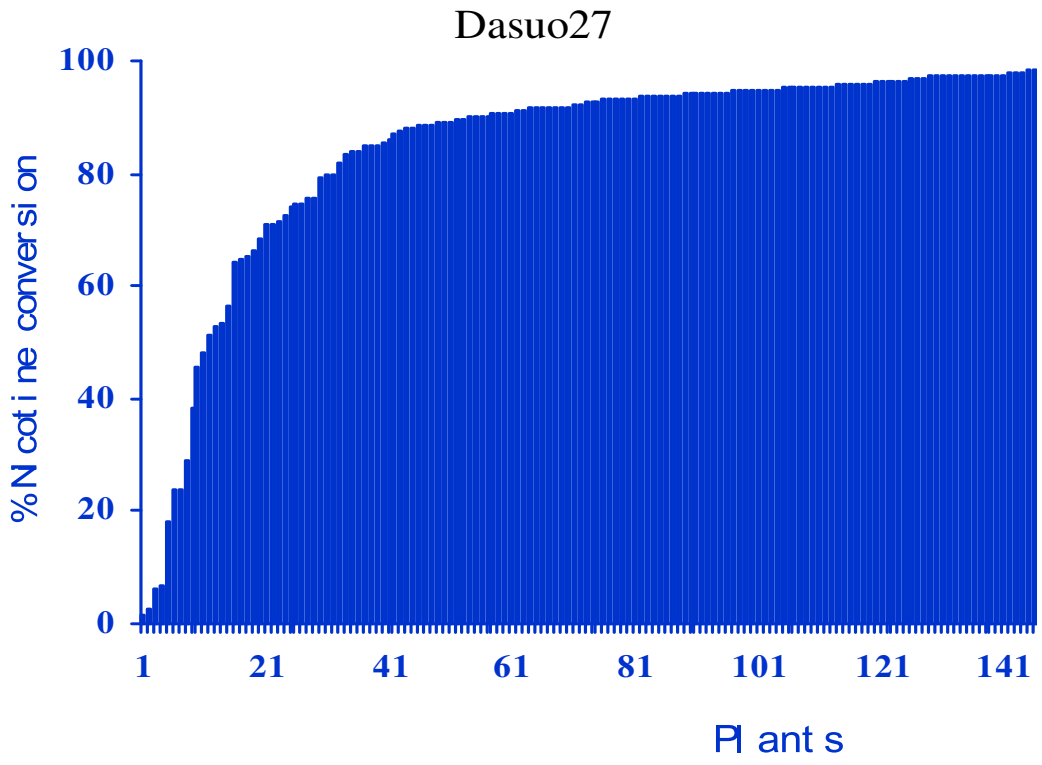

Figure 6. Distribution of percent nicotine conversion in air-cured leaves of individual plants of Dasuo27 (the paternal parent for Dabai 3).

Table 4. Comparison of proportions of converters and percent nicotine conversion between original hybrid and improved hybrid.

Varieties	Population	Proportion of converters (%)			Proportion of non-converters (%)	Average nicotine conversion (%)
		Total converters	Low converters	High converters		
Dabai 1	Original	23.6	16.5	7.1	76.4	5.74
	Improved	8.5	8.5	0	91.5	2.06
Dabai 2	Original	22.4	14.8	7.6	77.6	4.83
	Improved	7.8	7.8	0	92.2	1.93
Dabai 3	Original	100.0	18.0	82.0	0.0	40.52
	Improved	10.9	10.4	0.5	89.1	2.32

DISCUSSION

Nicotine conversion levels were markedly different in burley cured tobaccos from different producing areas. These differences were associated with varied varieties used in different areas. The dominant variety in Sichuan was Xuanhan-5NL which had almost 100% of the plants being converters; it explained that Sichuan tobacco generally had low nicotine content and week burley style and aroma. Since hybrid Dabai 1, Dabai 2, and Dabai 3 had ideal agronomic traits and great quality potential in Sichuan, they have been determined to be the candidates for replacing Xuanhan-5NL as the principle varieties. For Dabai 1, both parents contributed the converting genes to the hybrid due to the fact that the converting trait was controlled by dominant genes (Mann et al., 1958); for Dabai 2, the principle contributor was the male parent Dasuo 26, and for Dabai 3, the main contributor of converting genes was Dasuo 27. Through early stimulation and identification, non-converters were selected and utilized for crossing to produce improved hybrid seeds. It proved that the improved hybrids greatly reduced converters in the population, adding much higher value of utilization in the burley production than the original hybrids. Since the mutation rate from non-converting trait to converting trait was relatively high (Wernsman et al., 2000), continuous selection of non-converters in the parent population was necessary during seed production to ensure maximum non-converters in the hybrid population and to ensure high quality burley production.

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