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GENOTYPIC VARIATION IN NORNICOTINE ACCUMULATION, A PRECURSOR TO HARMFUL CONSTITUTES OF TOBACCO

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ABSTRACT

The study aimed to estimate the content of nicotine and nornicotine levels across twenty-five tobacco genotypes,including FCV, Burley, HD-Burley, Bidi, Chewing, and Lanka types. Chemical analysis showed significant differences for nicotine and nornicotine levels in 25 tobacco genotypes.The highest nicotine content was recorded in FCR-15 (20.8 mg/g), and the lowest nicotine content was observed in FCJ-11 (9.1 mg/g). Among 25 tobacco genotypes, nornicotine content ranges from 7.74 mg/g (Bio Mut-55-1) to 0.0003 mg/g (LT Kanchan). Significant variation was further observed in nicotine-to-nornicotine conversion rates.The Burley genotype Bio Mut 55- 1 exhibited the highest conversion rate (41.7%), followed by Banket A1 (8.453%). Importantly, several genotypes showed negligible nornicotine accumulation. The genotypes with low nornicotine accumulation provide valuable insight for breeding low-TSNA tobacco genotypes, thereby improving the safety profile of tobacco products.

Key words: Nicotine, Nornicotine, TSNAs

INTRODUCTION

Nicotiana tabacum L. (2n = 4x = 48), commonly known as tobacco, is an allotetraploid species with a large genome size of 4.5 Gb. It originated from the hybridization of two diploid progenitor species, *Nicotiana sylvestris* (2.68 Gb) and *Nicotiana tomentosiformis* (2.36 Gb) Globally, tobacco stood as a significant cash crop, cultivated across approximately 3.14 million hectares and yielding a total production of about 5.78 million metric tons annually (FAO STAT, 2023). China led the world in tobacco production, with an area of 1.08 million hectares and production of 2.35 million metric tons, with productivity of 2612.9 kg per hectare. India also played a substantial role in the global tobacco market, with approximately 0.45 million hectares under cultivation, producing 0.8 million metric tons and a productivity of 2,115 kg per hectare (FAO STAT, 2023).

India ranked among the leading global exporters of tobacco, with Flue-Cured Virginia (FCV) and Burley varieties contributing the most to export revenues owing to their superior quality and suitability for cigarette manufacturing. In 2022–23, India earned over Rs.12,005 crores in foreign exchange from tobacco exports, highlighting its economic

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significance (Tobacco Board of India, 2023).Tobacco is predominantly recognized for its role in smoking-related health risks; however, its chemical constituents, particularly alkaloids, have also attracted interest for their potential therapeutic applications. The alkaloids (secondary metabolites) that played a crucial role in plant defence against herbivores and pathogens. (Zenkner *et al.,*2019). In tobacco (Nicotiana spp.), nicotine was the principal alkaloid, accounting for approximately 90 Percent of the total alkaloid content. The remaining 10 percent of the alkaloid pool comprised structurally related compounds, including nornicotine, anatabine, and anabasine. Nornicotine, representing approximately 2–5 Percent of the total alkaloids (Wang *et al*., 2025). It is not only a bioactive compound in its own right but also a metabolic precursor to N-nitrosonornicotine (NNN), a potent carcinogenic tobacco-specific nitrosamine (TSNA). The conversion of nicotine to nornicotine is mediated by N-demethylase enzymes encoded by the cytochrome P450 monooxygenase gene family *CYP82E*, which were active in both leaf and root tissues. Anatabine and anabasine, although present in lower concentrations. Nornicotine formation in Nicotiana species, a key step in the biosynthesis of tobacco-specific nitrosamines (TSNAs), a class of carcinogens formed via nitrosation of alkaloid precursors such as nornicotine, anabasine, and anatabine. Nitrate, acting as a nitrosating agent, significantly contributed to TSNA formation. Thus, reducing both alkaloid precursors and leaf nitrate levels was an effective strategy to mitigate TSNA accumulation, particularly in Burley tobacco, which accumulated high nicotine and nitrate levels. Therefore, the present study was conducted to assess the nicotine and nornicotine levels in 25 genotypes.

MATERIAL AND METHODS

The present experiment was conducted to estimate the nicotine and nornicotine content across the twenty-five genotypes. (Figure 1) These entries were sown in augmented block design (ABD) and replicated twice at the National Institute for Research on Commercial Agriculture. Spacing of 70 cm x 70 cm, which is optimal for proper plant growth and development. Each genotype was sown in two rows. Around 650 individual plants were labelled before harvesting. Leaf harvesting was carried out in three stages. First, the bottom leaves were harvested; after 10 days, the middle leaves were harvested; then, after 10 days, the top leaves were harvested and then put into a curing barn for air curing and sun curing.

Alkaloid measurement

For alkaloid estimation, 1 gram of oven-dried tobacco leaf powder was accurately weighed and transferred into a 50 mL Falcon tube. To this, 10 mL of freshly prepared 2N sodium hydroxide (NaOH) was added. The mixture was homogenized and vortexed for 2 minutes to ensure thorough mixing and alkaline treatment. The sample was then centrifuged at 5000 rpm for 5 minutes to facilitate phase separation. In instances of unclear layer separation, an additional brief centrifugation step was employed. Following centrifugation, 5 mL of the clear aqueous phase from the bottom of the tube was carefully transferred to a new Falcon tube. To this, 25 mL of dichloromethane (DCM) was added, and the tube was vortexed vigorously for 1 minute to extract alkaloids into the organic phase. The resulting mixture was filtered through a funnel lined with cotton and anhydrous sodium sulfate to remove any moisture and particulate matter. The filtered organic extracts were collected into clean tubes. For gas chromatography (GC) analysis, 0.5 mL of the extract was transferred into GC vials. To each vial, 100 µL of *n-heptadecane* was added as an internal standard, followed by 400 µL of an appropriate solvent. (Wu *et al.,* 2002). These prepared samples were then submitted for GC analysis to quantify the concentrations of nicotine, nornicotine. The formula used for calculating percentage nicotine conversion was:

Nicotine Conversion (%) = [% Nornicotine / (% Nicotine + % Nornicotine)] × 100.

This conversion ratio is important for understanding the biochemical transformation of nicotine, which contributes to the formation of TSNAs during curing and storage. If TSNA data (*e.g.,* NNN, NNK) were available across different genotypes or treatments, one-way ANOVA followed by post hoc tests was used to determine statistically significant differences among groups. All statistical interpretations were made in the context of tobacco alkaloid metabolism and TSNA accumulation potential.

RESULTS AND DISCUSSION

Twenty-five genotypes were evaluated for nicotine and nornicotine during the rabi season, 2024. Analysis of variance revealed the statistically significant difference for nicotine and nornicotine content among the genotypes. (Moghbel *et al.,* 2017). The conversion of nicotine to nornicotine across the twenty-five tobacco genotypes, which included Flue-Cured Varieties (FCV) and Non-Flue-Cured Varieties like *Burley, Bidi, Chewing, Lanka and HDBRG* types, shows significant variation, indicating expression of the nicotine demethylase enzyme responsible for this conversion.

The nicotine content among 25 genotypes varied substantially, ranging from 9.1 mg/g to 20.8 mg/g. (Table-1). The highest concentration was recorded in the FCV genotype FCR-15 and the lowest in FCJ-11, indicating substantial intra-group variability within the Flue-Cured Virginia types. Among Burley genotypes, Biomut 66-1 recorded the highest nicotine content (19.2 mg/g), while Biomut 61-2 had the lowest (10.9 mg/g). In the *Bidi* tobacco group, GT-6 showed relatively higher nicotine content (14.1 mg/g), whereas Vedaganga recorded the lowest (9.3 mg/g). The Chewing tobacco varieties also demonstrated notable nicotine variation, with Maragadham recording the highest (14.7 mg/g) and GandakBahar the lowest (10.4 mg/g). In the HD Burley group, intermediate nicotine levels were observed in HDBRG (13.4 mg/g) and Lanka Special (11.6 mg/g), suggesting that these selections maintained a balance between flavor characteristics and nicotine yield.

The highest nornicotine content observed in the genotype revealed considerable variation, ranging from as high as 7.74 mg/g in BioMut 55-1 to as low as 0.00033 mg/g in LT Kanchan (Table 1). Among the Flue-Cured Virginia (FCV) varieties, FCR-15 exhibited the highest nornicotine concentration (0.2833 mg/g), while LT Kanchan showed the lowest (0.0003 mg/g), indicating a relatively limited conversion of nicotine to nornicotine in most FCV types, which is desirable considering the regulatory interest in reducing tobacco-specific nitrosamines (TSNAs), for which nornicotine is a direct precursor (Rodgman and Perfetti, 2009). In Burley genotypes, the nornicotine content was highly variable, BioMut 55-1 recorded the highest level (7.74 mg/g), substantially greater than any other genotype in the study.This suggested the presence of a strong *CYP82E4* allele, known to enhance nicotine demethylation (Xu *et al.,* 2007). In contrast, BioMut 58-1 exhibited negligible nornicotine content (0.0006 mg/g). (Elchamieh *et al*., 2024). GT-4 having the highest content (0.014 mg/g) and GT-6 the lowest (0.001 mg/g). This pattern reflected the traditional selection of *Bidi*

Fig1. Phenology of twenty-five tobacco genotypes at 30 days after transplanting (early vegetative growth) under field conditions.

varieties for mild alkaloid profiles and minimal TSNA precursor accumulation. In the *chewing* tobacco group, Abirami (0.388 mg/g) stood out with the highest nornicotine concentration, whereas DJ-1 and GandakBahar recorded the lowest (0.016 mg/g). The HD Burley genotypes showed moderate levels of nornicotine, with HDBRG at 0.043 mg/g and Lanka Special slightly higher at 0.07 mg/g. (Tayoub *et al.,* 2015), (Tassew *et al.,* 2015). This stark contrast among Burley genotypes aligned with earlier reports indicating that spontaneous or induced mutations in the *CYP82E* genes can result in high or low converter lines, which significantly impact TSNA formation during curing and storage (Lewis *et al*., 2008).

THE PERCENTAGE CONVERSION OF NICOTINE TO NORNICOTINE

The percentage conversion of nicotine to nornicotine is a critical metabolic process in tobacco, primarily mediated by members of the cytochrome P450 monooxygenase family genes specifically *CYP82E4*, *CYP82E5* and *CYP82E10*. These enzymes catalyze the N-demethylation of nicotine to nornicotine, a key precursor in the formation of tobacco-specific nitrosamines (TSNAs) during post-harvest curing and storage (Siminszky *et al.,* 2005; Shoji *et al.,* 2010). The extent of conversion varies widely among tobacco genotypes due to differences in gene expression and allelic variation at the nicotine demethylase loci.

Among the 25 genotypes evaluated, significant variation was observed in nicotine-to-nornicotine conversion rates. The Burley genotype BioMut 55-1 exhibited the highest conversion rate (41.7%), followed by Banket A1 (8.453%) (Table 1). This indicated strong demethylase activity, Conversely, Burley 21 showed minimal conversion (0.123%), suggesting either a recessive or silenced demethylase gene. BioMut 55-1 & Banket A1 (high conversion rates) and Burley 21 (lower conversion rates) may serve as valuable genetic models for dissecting the regulatory pathways of alkaloid transformation and for

Table 1. Mean performance of tobacco genotypes for Alkaloid Traits

S.No.	Genotypes	Nicotine content (mg/g)	Nornicotine content (mg/g)	Conversion of % nicotine to nornicotine
1	SIRI	20.3	0.033	0.145
2	FCJ11	9.1	0.001	0.012
3	FCR-15	20.8	0.2833	1.338
4	FCH-222	9.2	0.002	0.022
5	LT Kanchan	11.1	0.0003	0.003
6	YB22	16.3	0.26	1.565
7	BANKET A1	10.6	0.973	8.453
8	BURLEY 21	19.1	0.023	0.123
9	324C	11.2	0.03	0.298
10	BIO MUT-55-1	10.9	7.74	41.710
11	BIO MUT-58-1	18.6	0.0006	0.003
12	BIO MUT-61-2	10.4	0.6033	5.497
13	BIO MUT-64-2	12.3	0.06	0.482
14	BIO MUT-66-1	19.2	0.17	0.877
15	HDBRG	13.4	0.043	0.323
16	VEDAGANGA	9.3	0.003	0.027
17	GT-6	14.1	0.001	0.007
18	GT-4	10.8	0.014	0.146
19	A119	9.5	0.002	0.023
20	MEENAKSHI	11.7	0.031	0.279
21	GANDAK BAHAR	10.4	0.016	0.171
22	MARAGADHAM	14.7	0.123	0.866
23	DJ-1	13.8	0.016	0.112
24	ABIRAMI	12.5	0.0388	0.329
25	LANKA SPECIAL	11.6	0.07	0.598
	GENERAL MEAN	13.27	0.42	2.53
	MINIMUM	9.1	0.0003	0.003
	MAXIMUM	20.87	7.74	42
	STANDARD DEVIATION	3.72	1.54	8.38

identifying molecular targets in breeding programs aimed at reducing TSNA formation in cured tobacco products.

In Flue-Cured Virginia (FCV) varieties, generally bred for lower TSNA accumulation, FCR-15 exhibited the highest conversion (1.338%), while LT Kanchan had the lowest (0.003%), supported the notion that FCV types are typically low converters due to selection against active *CYP82E* alleles. Song *et al., (*2024),Bidi tobacco genotypes showed comparatively lower conversion, with GT-4 at 0.146% and GT-6 at 0.007% reflecting their mild alkaloid profiles and reduced enzymatic transformation capacity. Among chewing tobacco genotypes, Maragadham showed higher conversion (0.866%) relative to DJ-1 (0.112%), suggesting some diversity in nicotine metabolism within this group. In the HD Burley and Lanka genotypes, HDBRG and Lanka Special recorded conversion rates of 0.23% and 0.598% respectively, indicating intermediate conversion activity. (Hidalgo *et al*., 2020).

CONCLUSION

The analysis of twenty-five tobacco genotypes revealed significant variability in both nicotine and nornicotine content, as well as in the nicotine-to-nornicotine conversion rate. While BIOMUT-55-1 exhibited the highest conversion rate (41.52%), other genotypes such as Banket A1 (8.4%) and several like GT-6, Vedaganga, LT Kanchan and Siri showed extremely low conversion rates despite having moderate to high nicotine levels. This demonstrates that high nicotine content does not necessarily correlate with high nornicotine accumulation or conversion efficiency. These findings are crucial for breeding efforts aimed at reducing tobacco-specific nitrosamines (TSNAs), as genotypes with high nicotine but low conversion rates are ideal candidates for the development of low-TSNA tobacco varieties, contributing to safer tobacco products.

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