Tobacco-Specific *N*-Nitrosamines: Effect of Burley Alkaloid Isolines and Nitrogen Fertility Management

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Tobacco-specific *N*-nitrosamines (TSNA) were quantified in air-cured lamina of Burley tobacco genotypes varying in total alkaloid level and composition. Lamina NO_3^-–N was altered by imposing selected N fertility regimes having different application dates and rates. Levels of isolated TSNA were positively correlated ($P \leq 0.01$) with the levels of nicotine and nornicotine but not with NO_3^- despite a greater than 10-fold difference in NO_3^-–N levels among individual plots. These results indicate that the alkaloid concentration and composition have a greater influence than NO_3^-–N precursor levels on the formation of TSNA in air-cured Burley.

Tobacco alkaloids and nitrite (nitrate derived) are the major precursors of the tobacco-specific *N*-nitrosamines: *N'*-nitrosonornicotine (NNN), *N'*-nitrosoanatabine (NAT), *N'*-nitrosoanabasine (NAB), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). These *N*-nitrosamines, which are present in both tobacco leaf and smoke, are known carcinogens (U.S. Surgeon General, 1982). Approximately 50% of NNN found in tobacco smoke is derived from NNN transferred from the tobacco (Hoffmann et al., 1977).

Recently, Brunnemann et al. (1983) reported that NO_3^- concentrations of 14 commercial tobacco products were not correlated strongly with the levels of NNN ($r = 0.77$). The products contained different tobacco types (e.g., flue-cured, Burley, Maryland, and Turkish) in varying proportions. Cultural practices and the N fertility management of each type of tobacco grown are different. Because of these differences, the total tobacco-specific *N*-nitrosamine (TSNA) content of a product may be related to the proportions of a particular tobacco type in the product.

Compared to flue-cured tobacco, Burley is grown with high rates of N fertilizer to maximize leaf yield. This often results in marked accumulations of NO_3^- in the Burley leaf and higher leaf alkaloid levels than in flue-cured tobacco. A wide range in leaf total alkaloids and nicotine to nornicotine ratios in flue-cured tobacco varieties and experimental crosses was not correlated with cured leaf levels of NNN (Chamberlain and Arrendale, 1983). However, the NO_3^- content in these tobaccos was not reported and may have limited total NNN formation. Burley genotypes accumulating different levels of alkaloids and having an altered alkaloid composition have been selected (Legg and Collins, 1975). There also exists among Burley varieties apparent genotypic differences in lamina NO_3^- accumulation (Ostrem and Collins, 1983), but the genetically determined lamina NO_3^- differential is generally less than lamina NO_3^- differentials obtained by varying N fertilization.

The objective of this study was to determine the relationship of alkaloids and NO_3^- to the levels of TSNA in air-cured Burley. Alkaloid isolines of Burley 21 and a nornicotine converter breeding line that varied in total alkaloids and alkaloid composition were grown in field experiments with selected N fertility regimes to alter accumulations of lamina NO_3^-. Information from these experiments could be useful in developing management systems for lowering leaf levels of TSNA in Burley.

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EXPERIMENTAL SECTION

Growth and Fertilization of Tobacco. Three Burley 21 alkaloid isolines and a nornicotine converter breeding line fertilized with seven different N treatments were grown at Spindletop Research Farm, Lexington, KY, in 1981 and 1982. The Burley 21 isolines used were normal alkaloid (B21), low intermediate alkaloid (LI-B21), and low alkaloid (LA-B21). The nornicotine converter breeding line was KY78-379. The experimental plots were located on a Maury silt loam soil (fine, mixed, mesic, Typic Paleudalf). All plots except the control (no fertilizer N) received 280 kg of N/ha as $Ca(NO_3)_2$. Fertilizer N was banded at transplanting, midseason, or topping in a single application or in split combinations between two of the three application dates. In both years, the experimental design was randomized complete block with three replicates. Recommended cultural practices were followed during the growing season (Atkinson et al., 1976), and the mature tobacco was harvested and air-cured in a manner conventional for Burley tobacco.

Sample Preparation. Air-cured leaf was stripped into four groups (tips, brights, lugs, and flyings). Midribs were removed from the leaves of a composite sample of each of the four leaf groups. Lamina samples ground to pass a 40-mesh screen were stored in plastic bags and kept in containers at room temperature in the darkness prior to analyses. Moisture content of lamina analyzed for TSNA was determined on a subsample at the time of extraction. Other chemical analyses were performed on ground tissue oven-dried to a constant weight at 60 °C stored in darkened containers in a desiccator.

TSNA Analyses. Ground air-cured lamina (10 g) was extracted with 75 mL of citrate buffer (pH 4.5) containing 5.0 mM ascorbic acid (Hoffmann et al., 1979) by shaking stoppered centrifuge tubes on a wrist-action shaker overnight at room temperature. Suspensions were centrifuged at 1000*g* and then decanted. The pellet was washed twice with 25-mL portions of citrate buffer extracting solution by suspending the pellet, centrifuging, and combining each wash with the extract prior to adjusting to pH 5.0 with 1.0 N NaOH. The aqueous extract was partitioned with dichloromethane, concentrated, and chromatographed on 30–40 g of basic alumina (Woelm, activity III) contained in a 30 cm × 2 cm glass column. Impurities were removed with 200 mL of dichloromethane prior to eluting the nitrosamines with 150 mL of acetone and concentrating for analysis.

A Model 502 thermal energy analyzer (TEA) from the Thermo Electron Corp. (Waltham, MA) was used to detect TSNA. The pyrolyzer of the TEA was interfaced directly

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Table I. Effect of Burley Alkaloid Lines on Nicotine, Nornicotine, Total Nitrogen, Nitrate, Nitrite, and Tobacco-Specfic *N*-Nitrosamines (TSNA) in Air-Cured Lamina

Burley lines[a]	nicotine, %	nornicotine, %	total N, %	NO_3^-–N, ppm	NO_2^-–N, ppm	TSNA[b] NNN, ppm	NAT, ppm	NNK, ppm
				1981				
B21	2.96	0.39	4.02	3690	2.44	1.58	0.75	0.19
LI-B21	1.91	0.16	3.74	3450	2.61	0.57	0.46	0.11
LA-B21	0.30	0.07	3.89	4120	2.49	0.36	0.13	0.05
KY78-379	0.41	2.98	4.06	4100	2.55	5.83	0.94	0.23
LSD.05[c]	0.13	0.13	0.10	420	n.s.[d]	0.55	0.19	0.05
				1982				
B21	4.52	0.08	4.07	4420	3.14	7.05	5.85	0.71
LI-B21	2.99	0.03	3.64	4000	3.25	1.73	1.39	0.28
LA-B21	0.71	0.01	3.65	5910	6.01	0.66	0.22	0.08
KY78-379	1.29	1.06	4.10	4500	2.40	13.1	3.11	0.34
LSD.05[c]	0.19	0.04	0.13	590	0.92	2.10	1.29	0.20

[a] Burley 21 alkaloid isolines: normal alkaloid (B21); low intermediate alkaloid (LI-B21); low alkaloid (LA-B21). Burley nornicotine converter breeding line (KY78-379). [b] TSNA abbreviations: NNN (*N'*-nitrosonornicotine), NAT (*N'*-nitrosanatabine), and NNK [4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone]. [c] LSD.05, least significant difference ($P = 0.05$). [d] n.s., not significant.

to a Hewlett-Packard 7620A gas chromatograph (Brunnemann and Hoffmann, 1981). The GC column packed with 10% UCW-982 on Gas-Chrom Q (80–100 mesh) was prepared and conditioned as described by Adams et al. (1983). The GC–TEA conditions were as follows: argon carrier gas flow 34 mL/min, injector temperature 230 °C, oven temperature held at 195 °C for 15 min postinjection, then programmed to 265 °C at 30 °C/min, and held for 8 min, and pyrolyzer temperature 450 °C.

The TSNA standards NNN, NAT, NAB, and NNK provided by Dr. Brunnemann, American Health Foundation, Naylor Dana Institute for Disease Prevention, Valhalla, NY, were prepared according to previously published procedures (Hu et al., 1974; Hecht et al., 1975, 1978). Duplicate analyses of each sample extract were performed. The TSNA levels reported represent isolated amounts.

Other Chemical Analyses. Alkaloids in the air-cured lamina were determined by GC (Andersen et al., 1982). Total nitrogen (including NO_3^-) in Kjeldahl digest (Bradstreet, 1965) was measured spectrophotometrically after reaction of the digest with phenol and hypochlorite (Cataldo et al., 1974). Nitrate was determined on hot-water (97 °C) tissue extracts by a manual adaptation of an automated method employing *Escherichia coli* to reduce NO_3^- to NO_2^- for measurement with a color-developing reagent (Lowe and Gillespie, 1975). Tissue NO_2^- was extracted and determined by a modification of the method described by Sen and Donaldson (1978). Tissue (0.2 g) was extracted with 5.0 mL of 0.5 N NaOH at 50 °C in a Teflon-lined screw-capped test tube. The extract was clarified with 0.425 M $ZnSO_4 \cdot 7H_2O$ and centrifuged before analysis with the color-developing reagent used for NO_3^- determinations. All samples were analyzed in duplicate.

RESULTS AND DISCUSSION

Gas chromatograph–TEA traces for a TSNA standard mixture and a typical air-cured lamina sample are shown in Figure 1. Resolution of NAT and NAB was not achieved. Tissue NAT concentrations reported are the sum of NAT and NAB. Since the ratio of NAT to NAB in commercial tobacco samples containing detectable quantities of NAB generally exceeds 10 (Brunnemann et al., 1983), the reported NAT lamina concentrations represent reasonable estimates for NAT.

For the chemical constituents determined, the interaction between alkaloid genotypes and nitrogen fertility treatment was generally not significant ($P \leq 0.05$) for either the 1981 or 1982 crop (MacKown, 1984). Alkaloid, N, and

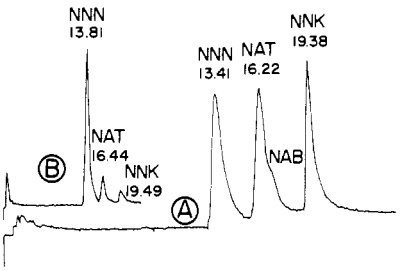

Figure 1. GC–TEA traces of (A) TSNA standard mixture containing 10 ppm each of NNN, NAT, and NNK and 1 ppm of NAB and (B) TSNA in air-cured B21 lamina from plots fertilized with 140 kg of N/ha at transplanting and 140 kg of N/ha at midseason. Numbers indicate the peak retention time in minutes.

TSNA levels of the four Burley lines for 1981 and 1982 are presented in Table I. In both years, the relative rankings of the lines on the basis of nicotine and nornicotine were similar, but the magnitudes varied. Nicotine levels of comparable lines were less (35–70%) in 1981 than in 1982, but nornicotine levels in 1981 were considerably greater (300–700%) than in 1982. Lamina total N (including NO_3^-) concentrations were lower for LI-B21 and LA-B21 than for B21 and KY78-379 in both 1981 and 1982. Despite a lower total N level for LA-B21, the NO_3^-–N concentration of this isoline was consistently greater than either B21 or LI-B21. A consistent genotype effect on the NO_2^-–N level was not evident, whereas TSNA levels were significantly ($P \leq 0.05$) different among the four lines. In 1981 and 1982, the low alkaloid isolines (LI- and LA-B21) had considerably lower levels of NNN and NAT and lower or equivalent levels of NNK than either B21 or KY78-379. Most notable were the much higher (2–20-fold) levels of NNN for the KY78-379 breeding line. These results contrast with the report of NNN in flue-cured tobacco, which varied in nicotine and nornicotine concentrations (Chamberlain and Arrendale, 1983). The N fertility and cultural practices employed for flue-cured tobacco differ markedly from Burley tobacco production [see Akehurst (1981)]. As a consequence of low N fertilizer application and the generally low availability of native soil N to flue-cured tobacco, the levels of NO_3^- in the mature leaf of flue-cured tobacco may limit nitrosamine formation.

The effect of fertilizer treatment on leaf chemical constituents (Table II) was less pronounced than the effect of Burley alkaloid lines. In 1981 a general trend of decreased lamina nicotine level occurred when plots were not fertilized with N (control) or when all or a part of the total

Table II. Effect of Nitrogen Fertility Treatment on Nicotine, Nornicotine, Total Nitrogen, NO_3^--N, NO_2^--N, and Tobacco-Specific N-Nitrosamines (TSNA) in Air-Cured Burley Tobacco Lamina

fertilizer treatment,[a] kg of N/ha	nicotine, %	nornicotine, %	total N, %	NO_3^--N, ppm	NO_2^--N, ppm	TSNA[b] NNN, ppm	TSNA[b] NAT, ppm	TSNA[b] NNK, ppm
				1981				
0 (control)	1.16	0.82	3.33	1610	2.90	1.69	0.38	0.12
280—tran	1.41	0.89	4.24	5330	2.39	2.24	0.64	0.12
280—mid	1.56	0.86	4.02	4260	2.33	2.14	0.49	0.13
280—top	1.33	0.88	3.77	2210	2.61	1.57	0.73	0.20
140—tran/ 140—mid	1.47	0.95	4.08	4790	2.42	2.07	0.57	0.12
140—tran/ 140—top	1.33	1.05	4.07	4810	1.51	2.09	0.58	0.15
140—mid/ 140—top	1.49	0.84	3.99	3950	2.47	2.83	0.61	0.18
LSD.05[c]	0.18	n.s.[d]	0.14	560	0.24	0.73	n.s.	n.s.
				1982				
0 (control)	2.34	0.28	3.17	1810	4.10	4.84	2.63	0.32
280—tran	2.57	0.24	3.95	6640	4.49	6.26	3.26	0.43
280—mid	2.51	0.32	3.93	4270	3.18	6.67	3.54	0.39
280—top	2.34	0.26	3.69	3680	3.81	5.51	2.52	0.34
140—tran/ 140—mid	2.25	0.36	4.16	5870	3.75	5.00	2.00	0.37
140—tran/ 140—top	2.24	0.28	4.06	6140	3.37	5.51	2.25	0.31
140—mid/ 140—top	2.43	0.30	4.14	4870	3.41	5.34	2.37	0.33
LSD.05	n.s.	n.s.	0.17	790	n.s.	n.s.	n.s.	n.s.

[a] Fertilizer treatments were no nitrogen (control) and $Ca(NO_3)_2$ banded at rates of 280 kg of N/ha at transplanting (tran), midseason (mid), or topping (top) and split application of 140 kg of N/ha each at two of the three application dates. [b] TSNA abbreviations: see footnote b of Table I. [c] LSD.05, least significant difference ($P = 0.05$). [d] n.s., not significant.

N fertilizer application was made at transplanting or topping. In 1982 the effect of fertilizer N treatment on nicotine levels was not significant. Nornicotine, NAT, and NNK levels were not altered by N fertility treatments, whereas lamina total N and NO_3^--N concentrations were significantly altered by fertilizer N treatments. Plots receiving no fertilizer N and those receiving all or part of the total fertilizer N after transplanting had lamina with lower total N and NO_3^--N than plots receiving all or a part of the fertilizer N application at transplanting. Accumulation of lamina NO_3^--N and a lamina total N concentration of 3.25% (average over both years) from control plots indicates an ample supply of native soil N. Despite delayed application of the entire amount of N until topping, a significant increase in both lamina total N (15%) and NO_3^--N (70%) occurred during the 3–4 weeks prior to harvesting. Consistent effects of fertilizer N treatment on NO_2^--N and NNN was not evident over both years. However, a trend of lower NNN levels appears to be associated with plots not receiving N.

The markedly higher TSNA levels of the non-nornicotine-accumulating isolines in 1982 may be a consequence of higher nicotine levels in 1982 compared to 1981, since nicotine represents the major alkaloid available for NNN formation (Hecht et al., 1978). However, both nicotine and nornicotine are suitable precursors for NNN. The 1981 KY78-379 crop had lamina nornicotine levels 3-fold greater than in 1982, yet the 1982 crop NNN levels for KY78-379 were 2-fold greater than in 1981 (Table I). An alternative explanation for differences in TSNA levels for the 1981 and 1982 crops may be associated with differences in curing conditions. The TSNA formed during air-curing could depend on the prevailing climatic conditions and curing practices followed. For alkaloid nitrosation to occur, reduction of NO_3^- to NO_2^- is necessary. The proportion of NO_3^- reduced either chemically or via dissimilatory microbial reduction is not known, but both processes would be altered by the curing environment. The prevailing climatic conditions for 3 weeks following harvest are presented in Table III. Temperature highs were similar for both years, but the average low temperature in 1982 (15 °C) was greater than in 1981 (11 °C). More notable were the considerably higher relative humidity extremes of 1982 than 1981 that may have decreased the rate of drying in 1982, thereby resulting in higher levels of TSNA. Definitive experiments designed to determine the effect of curing environment on TSNA formation have not been conducted.

Table III. Weekly Average Weather Data at Curing Barn Site after Tobacco Harvest

	1981 weekly average				1982 weekly average			
	temp, °C		rel humidity, %		temp, °C		rel humidity, %	
week	high	low	high	low	high	low	high	low
1	27	15	85	44	26	16	98	61
	21	10	82	43	26	14	98	58
3	24	9	87	32	27	18	97	67
av	24	11	85	40	26	16	98	62

Table IV. Correlation Coefficients (r) of Tobacco-Specific N-Nitrosamines (TSNA) with Nicotine, Nornicotine, Total Nitrogen, NO_3^--N, and NO_2^--N in Air-Cured Lamina of the Three Burley 21 Alkaloid Isolines

	1981 TSNA[a] NNN	1981 TSNA[a] NAT	1981 TSNA[a] NNK	1982 TSNA NNN	1982 TSNA NAT	1982 TSNA NNK
nicotine	0.61[b]	0.68[b]	0.61[b]	0.71[b]	0.68[b]	0.59[b]
nornicotine	0.74[b]	0.59[b]	0.39[b]	0.29[c]	0.17	0.25[c]
total N	0.26[c]	0.21	0.12	0.40[b]	0.37[b]	0.33[c]
NO_3^--N	−0.10	−0.10	−0.22	−0.14	−0.13	−0.09
NO_2^--N	−0.22	−0.15	−0.08	−0.33[c]	−0.31[c]	−0.30[c]

[a] TSNA abbreviations: see footnote b of Table I. [b] $P \leq 0.01$. [c] $P \leq 0.05$.

Correlation coefficients (r) for TSNA with selected lamina chemical constituents for the three Burley 21 alkaloid

isolines are presented for the 1981 and 1982 crops in Table IV. Nicotine levels were significantly correlated ($P \leq 0.01$) with the levels of each TSNA in both years. The correlation between nicotine and NAT represents a parallel increase of anatabine and nicotine in these genotypes (MacKown, 1984). In 1981, nornicotine was correlated with each TSNA and was more strongly correlated with NNN ($r = 0.74$) than nicotine with NNN ($r = 0.61$). This relationship between nornicotine and nicotine levels was not observed in 1982. Correlations of TSNA with total N or NO_3^-–N were either less strong or not significant compared to correlations with nicotine. All correlations within an alkaloid isoline between TSNA and NO_3^-–N were not significant (MacKown, 1984). These results further support the observation that formation of TSNA in these Burley alkaloid isolines was not enhanced at the higher levels of NO_3^-–N imposed by the N fertility treatments. A consistent relationship between NO_2^- and TSNA was not evident. The NO_2^- formed during air-curing was either involved in nitrosation reactions or possibly further reduced (microbially), thereby eliminating any substantial accumulation of NO_2^-. The rate of NO_2^- formation is probably the limiting step in TSNA synthesis.

The positive correlation of alkaloid concentration and composition with air-cured lamina TSNA level is evident from these studies. Presently, the Regional Burley Tobacco Quality Committee recommends that new varieties contain less than 20% of the total alkaloids as nornicotine. Many of the commonly used Burley cultivars have less than 10% of the total alkaloids as nornicotine. The use of low-alkaloid Burley breeding lines currently available would be useful in lowering leaf TSNA levels. The NO_3^-–N concentration in air-cured lamina from individual plots ranged from 580 to 7430 ppm in 1981 and 430 to 10 700 ppm in 1982. Despite these differences, TSNA in air-cured lamina was not correlated with lamina NO_3^-–N, suggesting that at these levels TSNA in air-cured Burley is not limited by the level of NO_3^-–N precursor. However, these results do not indicate that the level of NO_3^-–N in tobacco products should be ignored. Ample evidence indicates that NO_3^-–N levels are strongly associated with NO formation and the levels of volatile and tobacco-specific *N*-nitrosamines in smoke (Tso et al., 1975; Sims et al., 1979; Brunnemann and Hoffmann, 1982).

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Registry No. NNN, 16543-55-8; NAT, 71267-22-6; NAB, 1133-64-8; NNK, 64091-91-4; nitrate, 14797-55-8; nicotine, 54-11-5; nornicotine, 494-97-3.

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