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Effects of Curing and Fertilization on Nitrosamine Formation in Bright and Burley Tobacco*

by

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SUMMARY

A Bright and a Burley tobacco were grown at four fertilization rates and each tobacco was then both flue-cured and air-cured. Levels of alkaloids and nitrosamines were found to increase with increasing fertilization levels. Levels of alkaloids, *N*-nitrosonornicotine (NNN), and other tobacco-specific nitrosamines (TSNA) were consistently higher in the Burley tobacco than in the Bright tobacco, regardless of curing method. In comparing the effects of curing, it was found that NNN and total TSNA levels were higher in the midrib than in the lamina of the air-cured samples, while just the opposite was found for the flue-cured samples. Flue-curing Bright tobacco produced three times the level of TSNA *vs* air-curing the same tobacco. On the other hand, flue-curing Burley tobacco reduced the alkaloids, but greatly increased the TSNA in the lamina. As midribs from the air-cured Burley leaves had three times the TSNA concentration of the lamina, the use of air-cured midribs in tobacco products should be avoided. It was concluded that lower fertilization levels and careful manipulations of curing parameters could lower nitrosamine levels in cured tobacco.

RESUME

Un tabac blond et un tabac Burley ont été cultivés à quatre taux de fertilisation. Chacun des tabacs a été ensuite séché à l'air chaud (flue-cured) et à l'air naturel. Il est apparu que les teneurs en alcaloïdes et en nitrosamines étaient plus élevées lorsque le taux de fertilisation augmentait. Les teneurs en alcaloïdes : N-nitrosonornicotine (NNN) et autres nitrosamines spécifiques du tabac (TSNA) se sont avérées nettement plus fortes dans le tabac Burley que dans le tabac blond, et ce quelle que soit la méthode de séchage utilisée. En comparant les effets du séchage, on a trouvé que les teneurs en NNN et TSNA totales étaient plus fortes dans les côtes que dans les parenchymes des échantillons séchés à l'air alors que le contraire était observé pour les échantillons «flue-cured». Dans le cas du tabac blond, le séchage à l'air chaud conduit à une teneur en TSNA trois fois plus élevée que le séchage à l'air naturel. D'un autre côté, le «flue-curing» du tabac Burley réduit la quantité d'alcaloïdes mais fait croître considérablement la teneur du parenchyme en TSNA. Comme les côtes des feuilles de Burley séchées à l'air ont une concentration de TSNA qui est le triple de celle du parenchyme, il y a lieu d'éviter d'utiliser dans les produits du tabac des côtes ayant séché de cette manière. En conclusion, il est possible d'abaisser la teneur en nitrosamines du tabac séché en réduisant les taux de fertilisation ainsi qu'en agissant avec précaution sur les paramètres de séchage.

ZUSAMMENFASSUNG

Ein Virgin- und ein Burley-Tabak wurden bei vier Stickstoffkonzentrationen kultiviert und danach röhrensowie luftgetrocknet. Es konnte beobachtet werden, daß der Alkaloid- und Nitrosamingehalt mit zunehmendem Düngegrad ansteigt. Unabhängig von der Trocknungsmethode war der Gehalt an Alkaloiden, N-Nitrosonornikotin (NNN) und anderen tabakspezifischen Nitrosami-

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nen (TSNA) im Burley-Tabak durchweg höher als im Virgin-Tabak. Beim Vergleich der Trocknungsmethoden wurde festgestellt, daß bei den luftgetrockneten Tabaken der NNN- und TSNA-Gehalt in den Rippen größer war als im Blattgut, bei den röhrengetrockneten verhielt es sich fast umgekehrt. Im Virgin-Tabak war der TSNA-Gehalt nach Röhrentrocknung dreimal so hoch wie nach Lufttrocknung. Bei Burley-Tabaken führte Röhrentrocknung zu einer Abnahme des Alkaloidgehalts, der TSNA-Gehalt im Blattgut dagegen stieg stark an. Da der TSNA-Gehalt in den Rippen bei luftgetrockneten Blättern des Burley-Tabaks dreimal so hoch war wie im Blattgut, sollte die Verwendung luftgetrockneter Rippen bei der Herstellung von Tabakprodukten vermieden werden. Aus den Untersuchungen ergibt sich, daß der Nitrosamingehalt durch einen geringeren Düngegrad und umsichtige Handhabung der Trocknungsparameter verringert werden kann.

INTRODUCTION

Since the first report of the presence of nitrosamines in unburned tobacco (1), there has been considerable interest as to how and when the tobacco-specific nitrosamines (TSNA) are formed in the cured tobacco. TSNA include *N*-nitrosonornicotine (NNN), nitrosoanabasine (NAB), nitrosoanatabine (NATB), and 4-methylnitrosoamino-1-(3-pyridyl)-1-butanone (NNK). It has been long established that tobacco alkaloids and the nitrate anion are involved in the formation of TSNA (2). Thus, changes in nitrogen fertilization levels should affect TSNA formation. One study has reported a direct correlation between TSNA formation and nitrate levels in tobacco (3). However, another study has shown no correlation for lamina of Burley tobacco, grown at different fertilization levels (4). The nitrate anion, however, apparently does not react directly with the alkaloids, but is reduced to nitrite anion, which then nitrosates the alkaloids to form the TSNA (5). Recent studies (6–8) have shown a direct relationship between NNN and nornicotine in different genetic lines. Approximately 50 % of the NNN in cigarette smoke is transferred directly from tobacco to smoke, while the other half of NNN is formed during smoking (5). By controlling the curing processes and thereby reducing NNN formation in tobacco, it may be possible to reduce the NNN content of smoke by up to 50 %. Also, reductions in fertilizer should yield tobacco with lower nitrate and alkaloid levels, which would also reduce TSNA in smoke. Current information suggests that tobacco curing conditions may influence TSNA accumulation, as it was shown that the concentration of TSNA increases during post-harvest treatment, curing and aging for both Burley (9–12) and Bright tobacco (13). Recent data (14) have shown that nitrite concentration is positively related to TSNA concentration in cured Burley tobacco and that TSNA increased during curing. Also, one report (11) has shown that higher curing temperatures can cause 2 to 100-fold increases in the levels of TSNA in the air-cured lamina of Burley tobaccos. It was the purpose of this study to determine the effect of fertilization levels and curing methods on the formation of TSNA in lamina and midribs of both Burley and Bright tobaccos, grown and treated under identical conditions. The overall objective is to develop knowledge that leads to production of tobaccos with reduced TSNA levels.

EXPERIMENTAL

Tobaccos were grown at Eden Shale, Kentucky, during the 1986 growing season. Four replicates of both flue-cured G28 tobacco and Burley KY14 tobacco were grown in plots six rows wide and 15 m long, with spacings of 105 cm between rows and 45 cm between plants in a row. The cultivars were grown at four different fertilization rates (0, 75, 150, 300 lbs N/acre*) and all plants were topped 60 days after transplanting. Normal rainfall levels were observed during the growing period. Four treatments were carried out. In treatment I, the cultivars were harvested for air-curing. Plants were cut from rows three and four. Tobacco, hung on eight sticks, with six plants per stick, was air-cured according to usual methods (15). Four leaves from the middle portion of three plants were pulled for sampling. In treatment II, the same two cultivars were grown under conditions identical to treatment I, but tobacco leaves were harvested and then flue-cured, according to standard practice (16). Since a minimum of forty leaves were required for flue-curing, four leaves were taken from the middle portion of each of ten plants. These were then shipped to Oxford, N. C., for flue curing (about 16 hs. elapsed from picking until leaves were placed in the barn for curing). In treatment III, 12 leaves (four leaves each from the middle of three plants) were taken from the high nitrogen plot (300 lbs/acre) and subjected to air-curing. In treatment IV, four leaves from the middle portions of 10 plants, from the high nitrogen plot, were removed, packed in ice for shipment to the curing barn in North Carolina and then flue-cured in the usual manner. Prior to chemical analyses, midveins and lamina were separated, dried in an oven at 85 °C, ground to pass a 20-mesh sieve, and placed in a desiccator. Alkaloids were determined by the gas chromatography (GC) method of Severson et al. (17). The values for total alkaloids represented a sum of the individual alkaloids (nicotine, nornicotine, anabasine, anatabine) obtained in the GC analyses. The determination of the TSNA [NNN, NATB, NAB, and NNK] was conducted as follows: tobaccos were dried and ground to pass a 20-mesh sieve and 3.5-g samples were ultrasonically extracted by a 150-ml volume of aqueous citrate buffer (pH 4.5), containing 3 % ascorbic acid (to prevent artifactual formation of

* To convert from Pound/acre to Kilogramm/hectare multiply by 1.120851.

Table 1.
Effect of nitrogen fertilization of alkaloid and nitrosamine levels in lamina of air-cured and flue-cured tobacco*.

Nitrogen rate	Nicotine (mg/g)		Total alkaloids** (mg/g)		NNN (μg/g)		Total TSNA*** (μg/g)	
lbs/acre	KY 14	G 28	KY 14	G 28	KY 14	G 28	KY 14	G 28
Air-cured								
0	42.82±4.73	14.73±0.82	44.41±4.96	15.14±0.64	0.12±0.01	0.04±0.01	4.28±0.24	1.86±0.31
75	58.61±3.66	19.56±1.46	61.01±3.71	20.39±1.48	0.33±0.03	0.19±0.01	4.16±0.02	2.72±0.24
150	59.24±3.01	23.84±1.75	62.40±3.10	28.15±1.65	0.40±0.04	0.14±0.01	3.05±0.15	3.73±0.12
300	65.77±3.00	26.21±1.41	69.54±3.06	27.92±1.54	0.74±0.05	0.22±0.01	3.72±0.09	3.25±0.17
Flue-cured								
0	30.66±1.56	14.56±0.36	32.83±1.69	15.57±0.41	0.94±0.04	0.37±0.02	7.68±0.60	5.13±0.22
75	32.04±1.12	16.49±0.84	35.24±1.18	18.00±0.96	1.83±0.11	0.69±0.03	9.05±0.50	7.14±0.76
150	35.28±1.13	19.10±0.93	38.65±1.02	21.17±1.06	2.60±0.14	1.35±0.07	9.86±1.00	9.88±1.11
300	33.51±0.55	18.56±0.25	36.57±0.54	20.91±0.02	3.35±0.14	1.99±0.07	11.58±0.41	14.22±2.18

*All values are an average of duplicate runs for each of four replicates (an average of eight values) ± S.E.
**Total of nicotine, anabasine, anatabine, and nornicotine.
***Total of *N*-nitrosonornicotine (NNN), nitrosoanabasine (NAB), nitrosoanatabine (NATB), and 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK).

nitrosamines; see reference 18). The buffer solution was then extracted 3 times with 100-ml volumes of dichloromethane. The organic extract was dried over anhydrous sodium sulfate, concentrated and chromatographed on basic alumina, activity grade II. The first fraction, eluting with methylene chloride and containing TSNA, was concentrated for GC analysis. TSNA analyses were conducted on a Hewlett Packard model 5710A gas chromatograph equipped with a thermionic N-P detector. The standard 5710A gas chromatograph was modified for glass capillary GC analysis, as previously described (19). TSNA analyses were performed in the split mode (100:1) on a 25 m × 0.3 mm i.d. fused silica column coated with OV-17. The oven temperature was programmed from 100 °C to 250 °C at 4 °/min, the flow was 20 cm/sec, and injection port temperature was 280 °C. The thermionic N-P detector was operated under hydrogen and air-flow conditions as recommended by the manufacturer.

RESULTS AND DISCUSSION

In treatment I, we examined the combined effects of air-curing and four fertilization levels on a standard air-cured tobacco, KY14, and on a representative flue-cured tobacco, G28. Although the recommended fertilization rate for Burley tobacco is 300 lbs N/acre, we wanted to see the effects on alkaloids and nitrosamines of lower nitrogen levels. Although increased fertilization levels yielded higher nicotine, total alkaloids, and NNN levels in lamina of KY14 (Table 1), it was surprising to see that TSNA only fluctuated with a slight decreasing trend (these fluctuations were due to changing NATB values). Obviously, doubling fertilizer levels did not double alkaloids or NNN levels. It was interesting to note that decreasing levels of N (such as 150 lbs N/acre) still gave high alkaloid levels in KY14 lamina, but only about half of the NNN and the lowest TSNA value, relative to the recommended 300 lbs** N treatment. Thus, although lower fertilization rates (150 lbs N/acre) will produce lower Burley tobacco yields, the resulting tobacco will have lower NNN and TSNA levels. The values at zero nitrogen fertilization applied do not make sense, but probably reflect the amount of residual nitrogen in the soil, as the field was used for other crops in previous years. Although flue-cured tobaccos are generally grown at about 60 lbs N/acre, we wished to see any unusual effects of excessively large N treatments as well as air-curing. As expected, increasing N levels produced somewhat elevated nicotine, total alkaloid, and TSNA levels, but not NNN levels, in G28 lamina at 150 and 300, compared to the acceptable 75 lbs N/acre level.

When the effects of flue-curing on KY14 and G28 lamina (treatment II) were studied, it was apparent that flue-curing had drastic effects on KY14 (Table 1). Stripping the leaves from the stalk (for flue-curing) reduced the nicotine and alkaloid levels in KY14 lamina by one-half, but increased the NNN or TSNA levels by 300–400 %, compared to air-cured KY14 lamina. Apparently, stripping the leaves from the stalk would stop any possible alkaloid transfer from the stalk to the leaves, as may occur during normal air-curing. Also, the higher temperatures of flue-curing are more conducive to NNN and TSNA formation. Such a temperature effect on TSNA formation was recently observed, when Burley tobacco curing effects at 24 °C and 32 °C were compared (14); these data showed that higher temperatures and relative humidities produced a tobacco with significantly higher TSNA levels.

When the G28 tobacco was flue-cured, it was noted that doubling the fertilization rate (75 to 150) only slightly increased the nicotine and total alkaloid levels, but dou-

** 1 Pound (abbr. lb) = 0.45359237 kg.

Table 2.
Effect of nitrogen fertilization of alkaloid and nitrosamine levels in midribs of air-cured and flue-cured tobacco*.

Nitrogen rate	Nicotine (mg/g)		Total alkaloids** (mg/g)		NNN (μg/g)		Total nitrosamines*** (μg/g)	
lbs/acre	KY 14	G 28	KY 14	G 28	KY 14	G 28	KY 14	G 28
Air-cured								
0	2.74±0.32	2.15±0.09	3 .07±0.35	2.25±0.10	0.31±0.08	0.14±0.11	4.15±0.28	4.32±0.35
75	2.78±0.23	2.04±0.22	3.05±0.23	2.42±2.16	0.66±0.14	0.35±0.14	8.82±1.09	8.20±1.76
150	3.78±0.42	3.23±0.29	4.41±0.35	3.64±0.29	1.13±0.20	1.00±0.11	11.01±2.02	4.02±0.29
300	6.50±0.35	3.65±0.26	7.28±0.36	4.05±0.26	4.89±0.25	1.78±0.36	9.89±1.35	5.10±0.22
Flue-cured								
0	6.46±0.32	3.48±0.21	6.25±0.37	3.69±0.24	0.10±0.01	0.06±0.05	1.21±0.04	2.05±0.23
75	7.03±0.18	4.02±0.35	7.27±0.21	4.14±0.36	0.15±0.01	0.12±0.08	1.11±0.08	1.08±0.05
150	7.41±0.24	4.00±0.07	7.59±0.25	4.07±0.10	0.26±0.04	0.09±0.05	1.02±0.12	0.61±0.08
300	6.78±0.58	3.70±0.06	7.01±0.60	3.79±0.06	0.19±0.10	0.05±0.01	1.00±0.14	0.95±0.18

*All values are an average of duplicate runs for each of four replicates (an average of eight values) ± S.E.
**Total of nicotine, anabasine, anatabine, and nornicotine.
***Total of NNN, NAB, NATB, and NNK (for definitions see Table 1).

bled the NNN level of G28 lamina. It is expected that reducing fertilization levels below 60 lbs N/acre for Bright tobacco will lead to reduced NNN levels; preliminary data on such studies have confirmed this effect. The excessively high rate of 300 lbs N/acre may have had a deleterious effect on plant metabolism, as unexpectedly, both the nicotine and total alkaloid levels decreased somewhat. The most surprising result occurred when comparing NNN values of air-cured and flue-cured G28 lamina. At 75 lbs N/acre, flue-cured lamina contained three times the level of NNN and TSNA as air-cured lamina. Consequently, although flue-curing yields the desirable flavor and aroma effects in bright tobacco, it greatly increases NNN and TSNA levels in the cured leaf. It is suggested that a lowering of flue-curing temperatures be studied, to obtain the best compromise of desirable flavor profiles and low TSNA contents.

As the midribs of the harvested leaves were available, these were also analyzed. As midribs are sometimes used in reconstituted tobacco sheet (RTS) or low tar cigarette blends, their TSNA composition is of importance. It is known and apparent from Table 2 that midribs contain greatly reduced levels of nicotine and alkaloids, relative to lamina. For the air-cured samples, increasing rates of fertilization yielded increased levels of nicotine and alkaloids. As the KY14 lamina has higher alkaloid levels, is was not surprising that its midribs also contained higher NNN and TSNA, compared to air-cured G28 midribs. However, compared to air-cured lamina, the midribs of KY14 had more than three times the levels of NNN and TSNA, at the higher fertilization rates. These high levels of TSNA in the Burley variety midribs indicate that such midribs should not be used in the manufacture of tobacco products. The reason for such high TSNA levels in air-cured Burley midribs may be related to their high nitrate content. Flue-curing KY14 greatly reduced the levels of NNN and TSNA in the midribs.

Just the opposite results were found for the G28 midribs. Air-curing gave higher NNN levels and flue-curing gave lower NNN levels than in the corresponding lamina. TSNA levels in G28 midribs, at 75 lbs N/acre, were one-seventh of those for the corresponding lamina. Therefore, the use of flue-cured tobacco midribs in RTS or low-tar cigarette blends should be encouraged as a means of reducing TSNA.

When data on the individual nitrosamines and the total TSNA are summarized for both lamina and midveins and compared for method of cure or variety, one obtains the data shown in Table 3. The conclusions discussed above are again evident from the average values. Flue-curing *vs* air-curing increased total TSNA in lamina, but decreased TSNA in midribs. Also, KY14 lamina contain more TSNA than G28, as do the KY14 midribs.

The treatment III individually air-cured leaves are compared to stalk-cured leaves of treatment I. Nitrosamine

Table 3.
Average levels of individual nitrosamines by method of cure and variety of lamina and midribs (μg/g).

		N*	NNN**	NAB**	NATB**	NNK**	Total
	Cure						
Lamina	Air	32	0.27	0.15	3.47	0.21	4.18
Lamina	Flue	32	1.81	0.66	4.05	2.77	9.30
Midvein	Air	32	1.53	0.20	4.51	0.69	6.95
Midvein	Flue	32	0.12	0.18	0.52	0.29	1.13
	Variety						
Lamina	G28	32	0.72	0.50	3.24	1.58	6.12
Lamina	KY14	32	1.36	0.31	4.29	1.40	7.35
Midvein	G28	32	0.54	0.19	2.18	0.37	3.29
Midvein	KY14	32	1.11	0.18	2.85	0.62	4.79

*N – Number of observations.
**For definitions see Table 1.

values were higher in both laminas and midribs of the leaves removed from the stalk and air-cured individually. Consequently, stalk-curing Burley tobacco yields lower nitrosamine values. The data for NNN have been selected and compared in Table 4 for all curing regimes for the 300 lb N/acre treatment.

Table 4.
Summary of NNN values from different curing regimes of tobacco grown with 300 lbs/acre nitrogen fertilization.

	NNN (ug/g)	
Tobacco type	Lamina	Midribs
	Air-cured	
KY14	0.74	4.84
G28	0.22	1.78
	Removed from stalk, air-cured	
KY14	1.06	7.49
G28	0.38	3.15
	Flue-cured	
KY14	3.35	0.19
G28	1.49	0.05
	Packed in ice, flue-cured	
KY14	4.70	0.24
G28	1.91	0.06

The last data in Table 4 are for treatment IV, which was designed to evaluate the effects of temporary storage and possible heat build-up prior to flue-curing. Transporting harvested leaves from Kentucky to the curing barn in North Carolina or the piling up of harvested leaves by the farmer on a hot day may produce a temporary heat increase in the pile of green leaves. According to the results, packing the harvested tobacco leaves in ice had no effect on nitrosamine values, compared to uncooled leaves. Therefore, the harvesting process, prior to flue-curing, does not increase leaf TSNA levels.

We have concluded from this study that nitrosamine values can vary widely, particularly in midvein samples of air-cured tobacco, that curing of Burley tobacco leaves under flue-cured conditions would lead to very high NNN levels, that air-curing bright tobacco greatly decreased its TSNA level, and that careful manipulation of curing parameters may be a useful approach to lowering nitrosamine levels in tobacco.

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