STUDIES ON THE REDUCTION OF NITROSAMINES IN TOBACCO

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Three tobacco varieties were grown over a three-year period under three nitrogen fertilization treatments in order to establish any relationships among fertilizer levels, nicotine, and nitrosonornicotine (NNN) levels of the cured tobacco leaves. Generally, we found that higher levels of nitrogen fertilization led to higher levels of alkaloids and NNN. Attempts at blocking the nitrosation of alkaloids in a pre-harvest, field-study were not successful. Based on a converter tobacco study, nornicotine was a more efficient precursor of NNN than nicotine. Compared to normal flue-cured tobacco, air-dried bright tobacco had highly reduced NNN levels.

INTRODUCTION

It is known that N-nitroso derivatives of tobacco alkaloids, such as N'-nitrosonornicotine (NNN) and 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), are powerful environmental carcinogens (4). These nitrosamines are formed in tobacco during curing and processing by the nitrosation of tobacco alkaloids (5). Also, secondary and tertiary amines can be nitrosated with nitrite to yield nitrosamines (11). Thus, nitrosation of secondary amines, such as nornicotine, anabasine and anatabine will give the corresponding nitrosamines (NNN, NAB, and NAT). Interestingly, nitrosation of the tertiary amine, nicotine, produces NNN after cleavage of the $N-CH_3$ bond. NNK or 4-(methylnitrosamino)-4-(3-pyridyl)butanol (NNA) can also be formed directly from nicotine and without demethylation by cleavage of either the 2'-N or 5'-N bond (8). Since the concentration of nicotine in conventional tobacco types is 20-100 times as great as that of nornicotine, nicotine is considered to be the more important precursor of NNN in tobacco and smoke (6). We investigated the effect of nitrogen fertilization (0, 60, 120 lbs/A) on the yield of nicotine and NNN in tobaccos of various nicotine/nornicotine ratios. Since ascorbic acid has been shown to be more than 98% effective in blocking the nitrosation of morpholine and piperazine (10), we attempted to use this blocking agent to decrease NNN formation.

EXPERIMENTAL

Tobaccos were grown at the Coastal Plain Experiment Station, Tifton, Georgia, during 1982, 1983, and 1984. The seeds were provided by Dr. James Chaplin, USDA, Oxford, NC. Three replicates of each tobacco variety were grown in 22 plant plots, with 112 cm between rows and 50 cm between plants, within a row. After topping, the third and eighth leaf were harvested from five plants and leaves were flue-cured in the usual manner. Midveins were separated from lamina and only the lamina were analyzed. For the 1983 crop, one-half of the leaves, grown under the 60 lbs/A fertilization regime, were air-dried, while the other half were flue-cured in the usual manner. In 1982, some harvested leaves were also individually sprayed with ascorbic acid. For the 1984 crop, one-half of each plot was sprayed with a 0.1N solution of ascorbic acid 24 hours prior to the last harvest.

Nicotine and nornicotine were determined by the method of Severson *et. al.* (12). The extraction of NNN was conducted as follows: tobacco samples (2-5 g, ground to pass a 20-mesh sieve) were ultrasonically extracted by a 150-ml volume of aqueous buffer (pH 4.5) (7). The buffer solution was then extracted 3 times with dichloromethane (3 × ml). The organic extract was dried with sodium sulfate, concentrated and chromatographed on a basic alumina (activity grade III) column. The fraction containing NNN was collected and subjected to capillary gas chromatography (2) on fused silica OV-1701 columns.

RESULTS AND DISCUSSION

All cured tobaccos were analyzed for nicotine, nornicotine and NNN. Data for tobaccos grown in the 1982 season have already been reported (3). Significantly, no NNN was found in green tobacco. The 1982 data also suggested a direct relationship between the total amount of alkaloids present and the amount of NNN produced in the cured leaf. The first attempt at blocking the formation of NNN involved the spraying of ascorbic acid solution on harvested NC 2326 leaves. After flue-curing a 76% reduction in the amount of NNN, compared to untreated leaves, was observed.

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1984 Harvest Date	TI 1112 0	TI 1112 60	TI 1112 120	NC 2326 0	NC 2326 60	NC 2326 120	V 445 0	V 445 60	V 445 120
				Nicotine (mg/g)					
7/6	1.06	1.44	2.39	6.50	9.53	7.65	1.28	1.13	1.41
7/20	0.68	1.14	0.91	12.25	18.56	19.06	0.82	0.73	0.76
8/7	1.13	1.37	1.64	30.67	30.17	33.78	1.11	1.03	1.27
				Nornicotine (mg/g)					
7/6	2.69	3.75	4.55	0.11	0.13	0.16	0.54	1.62	1.17
7/20	8.00	9.60	31.02	0.16	0.77	0.47	4.47	7.19	5.33
8/7	12.72	23.63	41.77	0.53	0.75	0.70	5.98	9.26	8.66
				NNN (μg/g)					
7/6	0.00	0.14	0.50	0.00	0.00	0.00	0.00	0.22	0.06
7/20	0.06	0.09	0.17	0.04	0.02	0.00	0.08	0.09	0.09
8/7	7.70	11.50	21.85	1.18	1.25	2.18	2.43	2.81	3.51

Table 1. Effects of Nitrogen Fertilization on Alkaloid and NNN Levels.

Three harvests from the 1982 crop were collected and leaves were flue-cured and analyzed for nicotine, nornicotine, and NNN. For NC 2326, the zero N treatment gave 11.19 mg/g of nicotine, 0.30 mg/g of nornicotine, and 0.53 μg/g of NNN. The high N treatment yielded NC 2326 tobacco containing 13.46 mg/g nicotine, 0.40 mg/g nornicotine, and 1.13 μg/g of NNN. Thus, fertilization caused a marked increase of nicotine and NNN production in the leaves. Although the increase in nicotine levels were not as great as expected, there are many factors that affect alkaloid levels (1,9,13) and produce less than expected results. The results were more dramatic for TI 1112. Zero fertilization produced 0.70 mg/g of nicotine, 3.23 mg/g of nornicotine, and 2.43 μg/g of NNN in the cured tobacco. The 120-lbs fertilization gave tobacco with 0.82 mg/g of nicotine, 9.56 mg/g of nornitotine, and 7.44 μg/g of NNN. Thus, the production of nornicotine and NNN was tripled in TI 1112.

In 1983, it was also of interest to examine the effect of air-drying these tobaccos and comparing the NNN yields with normal flue-cured samples. Unfortunately, most of the air-cured samples were damaged in shipment to the laboratory and only about one-tenth of the leaves could be analyzed. Based on available data, the effect of air-drying was most destructive on NNN. For example, air-dried NC 2326 tobacco from the second harvest yielded only 4% of the NNN of corresponding flue-cured tobacco. For TI 1112, air drying reduced the NNN from 5.76 to 0.22 μg/g, also a 96% reduction. It can be speculated that the lower temperature of air-drying, compared to flue-curing, was responsible for this dramatic reduction. This result is currently being examined further and the effect of air-drying on the quality of bright tobacco will be evaluated.

In 1984, NC 2326, TI 1112, and V 445 were again grown at the three fertilization rates. Alkaloid and NNN levels **(Table 1)** were similar to those found in the first two years, except that NNN levels at the first two stalk positions (first two harvests) were extremely low. At the zero N fertilization level, no NNN was found in the first harvest leaves, even though nornicotine levels were already appreciable or even high (0.27%) for TI 1112. It was interesting to note that for the last harvest, high levels of nornicotine in TI 1112 produced higher levels of NNN, than high levels of nicotine in NC 2326. Thus, for TI 1112 conversion of nornicotine to NNN is a much more facile process than formation of NNN from nicotine, which first requires enzymatic demethylation. In this study, one half of each plot was sprayed with 0.1N ascorbic acid solution 24 hrs before the last harvest with the objective of blocking the formation of NNN. The results of this spraying were disappointing. NNN levels for treated tobaccos were the same or slightly higher than for controls. Thus, the blocking agent had no effect when leaves were still on the plant. Although this was a much more practical approach than spraying already harvested leaves, no reduction of NNN was achieved.

CONCLUSIONS

High levels of nitrogen fertilization generally produced high levels of alkaloids and NNN. Both alkaloids and NNN were accumulated in leaves at the higher stalk positions (second and third harvests). Nornicotine seems to be a more efficient precursor of NNN than nicotine; but because of its abundance in leaf, nicotine is the more important source of NNN. Under certain circumstances, ascorbic acid appears to effectively block the nitrosation reaction; however, in field studies, ascorbic acid was not an effective method for the reduction of NNN. The highly reduced NNN levels in air-dried bright tobacco warrant further study.

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