Effect of Maleic Hydrazide Application on Accumulation of Tobacco-Specific Nitrosamines in Air-Cured Burley Tobacco

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A two year study was initiated to determine the effect of maleic hydrazide application rates (0.5×, 1.0×, 1.5×, and 2.0× the recommended rate) and methods (single, split, and reduced volume application) on accumulation of tobacco-specific nitrosamines (TSNA) and the correlation between TSNA and their precursors in burley tobacco. With increased MH application rates, alkaloids and nitrate levels decreased, whereas nitrite level was not affected. TSNAs were significantly lower (30–50%) in the lamina from the top stalk position than the hand-suckered control. *N'*-Nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N'*-nitrosoanabasine (NAB), and *N'*-nitrosoanatabine (NAT) were lowered by 15–51%, 36–65%, 38–61%, and 30–58%, respectively, at the top stalk position. TSNA content was significantly correlated with the content of alkaloid in the lamina. These results suggest that MH application may alter precursors–TSNA relationship which results in decreases of TSNA in air-cured burley tobacco.

Keywords: *Maleic hydrazide; Nicotiana tabacum L.; alkaloid; tobacco-specific nitrosamines (TSNA); nitrite; nitrate*

INTRODUCTION

The major four tobacco-specific nitrosamines (TSNA) found in tobacco are *N'*-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N'*-nitrosoanabasine (NAB), and *N'*-nitrosoanatabine (NAT). NNN and NNK are known to induce malignant tumors in mice, rats, and hamsters (U.S. Surgeon General, 1982; Hoffmann et al., 1986). The other two TSNA do not exhibit significant tumorigenic activity. Tobacco alkaloids and nitrite are the major precursors of TSNA formation (Bush et al., 1979; Burton et al., 1989; Tso, 1990). Detection of TSNA in cured leaf led to many studies to identify the relationships between TSNA and their precursors. Studies by Djordjevic et al. (1989), MacKown et al. (1984, 1988), and Andersen et al. (1985) indicated that a positive correlation existed between NNN and nornicotine content and between NAT and anatabine content. Burton et al. (1989) reported a significant positive correlation between TSNA and nitrite levels. Peng (1990) and Burton et al. (1989) observed TSNA increased after the end-of-yellowing stage of air-curing burley tobacco. Increased nitrogen fertilization resulted in elevated level of alkaloids and TSNA (Chamberlain and Chortyk, 1992). Flue-curing of bright tobacco produced three times the level of TSNA vs air-curing the same tobacco, while flue-curing of burley tobacco reduced alkaloids, but greatly increased TSNA in the lamina. Effects of agronomic factors such as sucker control, especially chemical sucker control, on TSNA accumulation have not been reported.

Maleic hydrazide (MH), 1,2-dihydro-3,6-pyridazinedione, is a systemic plant growth regulator which has been the most effective and most extensively used sucker control agent for tobacco in the United States (Hawks and Collins, 1983). The mode of action of MH in plants is not clear, but several hypotheses have been proposed and studied (Baker, 1961; Hoffmann and Parups, 1964; Coupland and Pell 1971; Bush and Sims, 1974). Since MH application results in higher equilibrium moisture, decreased nicotine and nitrate, increased sugar and potassium when compared to a conventional hand-suckered control (Chaplin, 1967; Steffens et al., 1969; USDA, 1980; Seltmann and Nichols, 1984; Cui et al., 1995), it may have some effects on TSNA formation. The objectives of this study were to investigate the influence of MH application on TSNA accumulation and the relationship between TSNA and their precursors in air-cured burley tobacco lamina.

MATERIALS AND METHODS

Materials. Burley tobacco (*Nicotiana tabacum* L. cv. KY14) was grown using recommended practices at the Kentucky Agricultural Experiment Station Farm (Spindletop) at Lexington, KY, in 1989 and 1990 according to the previous publication (Crafts-Brandner et al., 1994). Nitrogen fertilization for both years was 168 kg ha^{-1} pretransplant with 112 kg ha^{-1} side-dressed 4–5 weeks after transplant. Potassium was applied pretransplant at 336 kg ha^{-1} and 224 kg ha^{-1} of K_2O in 1989 and 1990, respectively. Plots were two rows wide and 15.25 m long with an untreated border row on each side. Row spacing was 1 m and plant spacing within rows was 45–49 cm, giving plant population 20 000 plants ha^{-1}. Plants were topped when 50% of the plants in the field had one open flower. The potassium salt of MH was applied to the upper leaves as a fine spray with a hand-held CO_2 sprayer one day after topping. The second of the split applications was made 7 days after the first application. Hand-suckering of the control plots was performed twice per week. The following treatments were used in four replications in a completely randomized block design:

(A) Hand-suckered control.

(B) Single applications with recommended volume of water (392 L ha^{-1} or 20 mL $plant^{-1}$): 85 mg $plant^{-1}$ (0.5×, × = recommended rate); 170 mg $plant^{-1}$ (1.0×); 255 mg $plant^{-1}$ (1.5×); 340 mg $plant^{-1}$ (2.0×).

(C) Split application with recommended volume of water (392 L ha^{-1} or 20 mL $plant^{-1}$): 85 mg $plant^{-1}$ + 85 mg $plant^{-1}$

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Table 1. Effects of MH Application Rate and Method on Nitrate-N, Nitrite-N, and Moisture Content in Top Lamina in Air-Cured Burley Tobacco

MH application method	rate (kg ha^{-1})	moisture (%)	NO_3^{-1}-N (mg g^{-1})	NO_2^{-1}-N (μg g^{-1})
hand-suckered		20.1	2.38	2.5
single, water vol 392 L ha^{-1}				
0.5×	1.68	20.7	2.14	2.4
1.0×	3.36	20.8	1.72	2.5
1.5×	5.04	21.2	1.48	2.3
2.0×	6.72	21.5	1.71	2.5
split, water vol 392 L ha^{-1}				
0.5× + 0.5×	3.36	21.1	1.69	2.5
1.0× + 1.0×	6.72	21.2	1.27	2.5
1.5× + 1.5×	10.08	21.6	1.23	2.4
2.0× + 2.0×	13.44	21.9	1.53	2.4
mix, water vol 196 L ha^{-1}				
1.0×	3.36	21.3	1.59	2.4
2.0×	6.72	21.6	2.13	2.5
1.0× + 1.0×	6.72	21.7	1.35	2.4
lsd$_{0.05}$		NS[a]	0.61	NS

[a] NS, not significant.

(0.5× + 0.5×); 170 mg plant^{-1} + 170 mg plant^{-1} (1× + 1×); 255 mg plant^{-1} + 255 mg plant^{-1} (1.5× + 1.5×); 340 mg plant^{-1} + 340 mg plant^{-1} (2.0× + 2.0×).

(D) Application with half the recommended volume of water, high concentration (196 L ha^{-1} or 10 mL plant^{-1}): 170 mg plant^{-1} (1.0×); 340 mg plant^{-1} (2.0×); 170 mg plant^{-1} + 170 mg plant^{-1} (1.0× + 1.0×).

Tobacco plants were stalk cut on the 25th day after topping and air-cured in a conventional barn. Cured leaves were removed from stalks and separated into three leaf position groups (top, middle, and bottom). Ten leaves for each replicate were randomly pulled from the top stalk position for dry matter determination and chemical analysis. Lamina and midribs were separated, dried at 65 °C, weighed to obtain dry weight, ground (850 mm), and stored at ambient temperature in the dark prior to chemical analysis.

Methods. Nitrite-N was determined by using the Griess procedure reported by Crutchfield and Burton (1989). Nitrate nitrogen was measured with an Automated Technicon Analyzer-System II by Griess reaction after Cd-Cu reduction of NO_3^{-1} to NO_2^{-1} (Technician Industrian Method 117-71A, Nov 1975). Individual alkaloids, nicotine, nornicotine, anabasine, anatabine and myosmine were determined by using the gas chromatographic method developed by Severson et al. (1981) and modified by Madsen et al. (1985). The values for total alkaloid represented a sum of the individual alkaloids. Tobacco-specific nitrosamines (TSNA) were determined using the method described by Burton et al. (1988). A Hewlett-Packard 5890 GC in splitless mode equipped with a 50 m DB-5 fused silica capillary column and TEA Model 543 (Thermedics Inc.) were used to detect TSNA (Peng, 1990).

Data analyses were performed as outlined by SAS Institute (1985). Year and replications were combined as blocks. Least significant difference was used to compare the chemical means.

RESULTS AND DISCUSSION

Change of Nitrate-N, Nitrite-N, and Water Content. Nitrate-N content (Table 1) of MH-treated tobacco decreased 5–48% in the top lamina of the plant compared to that of hand-suckered control. The value of NO_3-N ranged from 1.48 to 2.14 and from 1.23 to 1.69 mg g^{-1} for single application and split application, respectively. Douglass et al. (1986b) reported that in vitro nitrate reductase activity was higher in root and lower in leaves of MH-treated seedlings when compared to those of control seedlings. If the same is true in vivo, there would be less nitrate in the leaves, as observed in this experiment. Nitrate uptake rate expressed per gram dry weight of root was equivalent for MH-treated and untreated plants (Douglass et al., 1986a).

Therefore, more reduced nitrogen would be transported to the leaf tissue at the expense of nitrate nitrogen resulting in decreased levels of nitrate in MH treated tobacco lamina in comparison to the untreated control tobacco.

Nitrite-N in top lamina ranged from 2.3–2.5 mg g^{-1} and was not affected by MH treatment. Compared to hand-suckered control, water content in MH treated tobacco increased slightly but not significantly. Since air-cured burley tobacco has nearly no sugar content and flue cured tobacco has 15–25% of sugar after curing, the water content in burley tobacco could be less affected by MH application than that in flue-cured tobacco (Tso, 1990).

Change of Alkaloid Accumulation. Total alkaloid content of MH treated tobacco for top stalk position decreased 9–34% compared to that of the hand-suckered control (Table 2). Alkaloid content of middle and bottom leaves decreased 4–20% and 5–29%, respectively (Cui et al., 1994). When MH application rate was increased within the same application method, alkaloid levels at the top stalk position tended to decrease. However, no significant effects were measured for application method at equal application rate. The changes of nicotine content followed the same pattern as total alkaloids. The minor alkaloids at the top stalk position ranges were 0.48–0.94, 0.21–0.72, 0.74–0.93, and 0.16–0.87 mg g^{-1} for nornicotine, anabasine, anatabine, and myosmine, respectively. However, none of these alkaloids was significantly affected by MH application. Earlier data (Atkinson and Sims, 1973) suggested that the lower nicotine concentration in MH treated tobacco was due to dilution caused by the increased leaf yield. In our study the product of yield (lamina and midrib) and nicotine content indicates 10–25% less nicotine biosynthesis in the MH-treated plant. The inhibition of alkaloid accumulation may be due to suppression of root development (Baker, 1961; Ai et al., 1966) and consequently less alkaloid biosynthesis.

Change of TSNA Accumulation. Measured TSNA in lamina of top stalk position of MH-treated tobacco were significantly lower compared to hand-suckered tobacco (Table 3). Even though no significant differences were observed between different MH application rates, there was a trend of decreased TSNA with increased MH application rate. Since lamina from the top stalk position of the plant generally contains highest

Table 2. Effect of MH Application Rate and Method on Alkaloid Accumulation in Top Lamina of Air-Cured Burley Tobacco

MH application		accumulation (mg g^{-1}) of alkaloids in lamina of top position					
method	rate (kg ha^{-1})	total	nicotine	nornic	anab	anat	myos
hand-suckered		43.8	41.8	0.56	0.44	0.90	0.24
single, water vol 392 L ha^{-1}							
0.5×	1.68	37.9	35.4	0.94	0.47	0.93	0.35
1.0×	3.36	39.1	37.1	0.49	0.51	0.89	0.25
1.5×	5.04	32.5	30.0	0.87	0.46	0.93	0.52
2.0×	6.72	33.4	31.5	0.52	0.40	0.82	0.24
split, water vol 392 L ha^{-1}							
0.5× + 0.5×	3.36	36.0	34.3	0.59	0.21	0.77	0.16
1.0× + 1.0×	6.72	33.1	31.4	0.49	0.35	0.75	0.21
1.5× + 1.5×	10.08	30.3	28.5	0.51	0.45	0.74	0.23
2.0× + 2.0×	13.44	30.7	29.0	0.59	0.19	0.75	0.22
mix, water vol 196 L ha^{-1}							
1.0×	3.36	39.8	37.1	0.61	0.72	0.94	0.87
2.0×	6.72	28.7	26.9	0.48	0.38	0.78	0.19
1.0× + 1.0×	6.72	33.2	30.9	0.63	0.63	0.83	0.35
$lsd_{0.05}$		6.3	6.1	NS[a]	NS	NS	NS

[a] NS, not significant.

Table 3. Effect of MH Application Rate and Method on TSNA Accumulation in Top Lamina of Air-Cured Burley Tobacco

MH application		TSNA accumulation (μg g^{-1}) in lamina of top position				
method	rate (kg ha^{-1})	TSNA	NNN	NAT	NNK	NAB
hand-suckered		24.04	6.90	14.49	2.29	0.70
single, water vol 392 L ha^{-1}						
0.5×	1.68	16.66	5.90	9.47	1.03	0.36
1.0×	3.36	12.86	3.55	8.02	1.10	0.37
1.5×	5.04	11.15	3.45	6.40	1.13	0.34
2.0×	6.72	14.29	4.11	8.75	1.25	0.34
split, water vol 392 L ha^{-1}						
0.5× + 0.5×	3.36	13.76	4.38	7.93	1.29	0.43
1.0× + 1.0×	6.72	11.56	3.49	6.76	1.16	0.31
1.5× + 1.5×	10.08	10.61	3.34	6.12	1.02	0.28
2.0× + 2.0×	13.44	9.34	3.31	4.95	0.81	0.27
mix, water vol 196 L ha^{-1}						
1.0×	3.36	15.77	5.19	8.92	1.49	0.34
2.0×	6.72	14.73	4.31	8.92	1.33	0.33
1.0× + 1.0×	6.72	14.44	4.51	8.23	1.55	0.31
$lsd_{0.05}$		4.37	1.71	2.72	0.66	0.11

level of TSNA (Burton et al., 1989, 1994) and MH application has greatest influence on the upper leaves of burley tobacco (Cui et al., 1995), only the lamina from top stalk position were analyzed to determine the influence of MH on TSNA accumulation. Total TSNA, NNN, NAT, NNK, and NAB decreased 30–50%, 15–51%, 30–58%, 36–65%, and 38–61%, respectively, in the lamina of MH treated tobacco from the top stalk position compared to handsuckered control. Since approximately 95% of the burley tobacco in the United States is treated with MH, it would appear that this agronomic practice has effected a reduction of TSNA in air-cured tobacco. This significant decrease in TSNA was observed for lamina only. TSNA for midrib (data not presented) showed that there was no significant difference between TSNA in untreated control and the MH-treated tobaccos. Therefore, MH only effected a decrease of TSNAs in the lamina and not the midrib.

TSNA and Water Content. Burton et al. (1989) reported that a significant increase in TSNA accumulation occurred during curing of lamina. Qi (1991) demonstrated a negative relationship between TSNA accumulation and leaf moisture content. These results suggest that the leaf moisture content may be closely associated with the microflora or enzyme activities and TSNA formation. In our study the equilibrium moisture content in MH-treated tobacco was not affected and was not correlated with TSNA accumulation ($R = 0.01–0.02$, $n = 96$).

TSNA and Precursor Relationship. Alkaloid and nitrite are the major precursors for nitrosamine formation (Bush et al., 1979; Burton et al., 1989; Tso, 1990) and since nitrite was not affected by MH application in this study, the nitrosamine formation was most highly correlated with the alkaloid content. To show there was a relationship between alkaloid content and individual TSNAs, correlation coefficients were determined for the individual samples (4 replicates × 12 treatments × 2 years = 96). The correlation coefficient of TSNA with nicotine was $R = 0.49^{**}$ ($n = 96$). R values for individual alkaloid precursor and nitrosamines relationships were 0.35^{**} ($n = 96$) for NNK with nicotine, 0.50^{**} ($n = 96$) for NNN with nornicotine, and 0.42^{**} ($n = 96$) for NAT with anatabine. Fischer et al. (1989) reported that nitrate content of tobacco leaf influenced the TSNA level. Other studies by Burton et al. (1989) and MacKown et al. (1984) suggested that TSNA in air-cured lamina was not correlated with nitrate-N. In our study, the R value for nitrate with TSNA was 0.45^{**} ($n = 96$). This may indicate that both nitrate level and reduction of nitrate to nitrite affect TSNA accumulation in MH-treated burley tobacco, since nitrate level and nitrate reductase activity were decreased in MH-treated burley tobacco (Douglass et al., 1986a; Cui et al., 1995). The R value for nitrite with the nitrosamines ranged from 0.08 to 0.1 ($n = 96$) and were not significant. These data suggest that MH may alter the precursor–product relationship and could account for some of the discrep-

ancy reported in the literature about the importance of the alkaloids and nitrite precursors for TSNA formation (Brunnemann et al., 1983; MacKown et al., 1984, 1988; Djordjevic et al., 1985, 1986, 1987; Burton et al., 1989).

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