QUANTIFICATION OF TOBACCO SPECIFIC NITROSAMINES IN TOBACCO

TOBACCO SCIENCE

By Charles H. Risner and Fred N. Wendelboe[1]

An analytical procedure was developed for the extraction and quantification of tobacco specific nitrosamines (TSNA): *N'*-nitrosonornicotine (NNN), *N'*-nitrosoanatabine (NAT), *N'*-nitrosoanabasine (NAB), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Tobacco was extracted with a citrate-phosphate buffer and the buffer was back-extracted into methylene chloride ($MeCl_2$). The $MeCl_2$ extract was partially evaporated, diluted to a constant volume, and analyzed by gas chromatography with detection by a thermal energy analyzer (TEA). Quantification was performed using an internal standard technique. The chromatographic precision for NNN, NAT, and NAB was about 5% while that for NNK was about 9% due to its poor peak shape. The overall precision using internal standard quantification was found to be better than that based on an external standard and was <8% for NNN, NAT and NNK. The overall precision for NAB was about 25% due to its low concentration in tobacco. The response was linear for TSNA, and recoveries were >80%. Several tobacco types were analyzed for their amounts of TSNA.

Additional key words: ***N'-nitrosoguvacoline, N'-Nitrosopentylipicolyamine.***

INTRODUCTION

Our laboratory staff has performed analyses of nitrosamines in tobacco smoke since 1988 (3), but we had developed no procedure for the determination of nitrosamines in leaf tobacco. Although several procedures exist for tobacco-specific nitrosamines (TSNA) in tobacco (4,6,8), they either do not use a thermal energy analyzer (TEA) for detection (4), or they do not employ a capillary column to separate *N'*-nitrosoanatabine (NAT) and *N'*-nitrosoanabasine (NAB) (6,8). Reported techniques either require extensive (24 hr) extraction times (6,8), or they have rather tedious preparation steps (4). Due to the increased interest in these compounds, a high sample volume procedure was needed for the determination of the major TSNA in tobacco.

A procedure is described herein for the quantification of TSNA in tobacco that requires minimal sample manipulation, provides separation of the NAB-NAT pair, utilizes specific TEA detection, and is capable of performing 12 analyses per day on a variety of tobacco samples.

MATERIALS AND METHODS

Materials

Methylene chloride ($MeCl_2$, HPLC grade) was obtained from Burdick & Jackson (Muskegon, MI). Citric acid, dibasic sodium phosphate, and ascorbic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). Water, 16 meg ohm-cm, was distilled and further purified by passing through a carbon cartridge, two high-capacity mixed-ion exchange cartridges, and a 0.45-µm filter (Barnstead Co., Dubuque, IA). NAT, NAB, *N'*-nitrosonornicotine (NNN), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were obtained from NCI Chemical Carcinogen Repository, Midwest Research Institute (Kansas City, MO). The internal standard (IS), *N'*-nitrosoguvacoline (NG), was a gift from Dr. Mirjana Djordjevic of the American Health Foundation (Valhalla, NY). Chem Elut columns (100 mL water capacity) were obtained from Varian (Harbor City, CA).

Tobacco Sample Preparation

Tobacco samples, such as burley stems and leaves, were ground in a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA) to pass through a 40-mesh screen, and they were not dried. Tobacco cut-filler from cigarettes was used without grinding.

Buffer Preparation

The buffer consisted of a solution of 55 mM citric acid, 90 mM sodium phosphate, dibasic (Na_2HPO_4), and 20 mM L-ascorbic acid in water, and it was the same as that used by other workers (4,7). The buffer was freshly prepared on a weekly basis, it was clear and colorless, and it had a pH of about 4.3.

Tobacco Extraction and Isolation of TSNA

The weights of tobacco samples were about 1.0 g, except for burley stems whose sample weights were about 0.5 g. The samples were weighed into 125-mL Erlenmeyer flasks and the flasks were closed with glass stoppers. Five mL of IS (0.4 µg NG mL^{-1} buffer) and 50 mL of buffer were added to each sample. The samples were sonicated one hour and allowed to cool ten minutes. The water in the sonicator did not exceed 40°C. A Chem Elut column and, below the column, a 300-mL Zymark sampling tube with a 0.5 mL stem (Zymark Corp. Hopkinton, MA) were fastened to a ring stand. The contents of the extraction flask were poured onto the head of the Chem Elut column. The flask was rinsed with 10 mL of buffer, and this was also poured onto the column. After the liquid permeated one-third the length of the column and when it left no liquid standing at the head of the column, a 150 mL portion of $MeCl_2$ was added and allowed to penetrate until no liquid was visible above the solid surface of the column. At this time, a second 150 mL of $MeCl_2$ was poured on the column. The $MeCl_2$ was allowed to pass through the column (about one hour) and it was collected in a 300-mL sampling tube. The elution process was considered complete when the flow of $MeCl_2$ from the column slowed to less than one drop in ten seconds and about 250 mL of $MeCl_2$ had been collected.

Extract Concentration

The sample tube containing the $MeCl_2$ eluent was placed in a TurboVap Concentrator (Zymark Corp., Hopkinton, MA) whose water bath was preheated to 38°C. The pressure of the nitrogen gas flow was increased gradually to create a vortex in the liquid. The sample extract was concentrated to 0.5 mL after which the sampling tube was removed from the apparatus. The contents of the sampling tube were transferred to a 5-mL volumetric flask. Two 2-mL portions of $MeCl_2$ were used to rinse the sampling tube and each rinse was added to the sample. The sample was diluted to a final volume of 5 mL with $MeCl_2$. A portion was pipetted into an autosampler vial and the vial was crimp sealed using a thin Teflon septum (Waters, Milford, MA). Both the 5-mL flask and the sealed sample vial were stored at 4°C before analysis.

Gas Chromatographic System

The gas chromatographic system consisted of a Hewlett-

[1] Senior Research and Development Chemists, R. J. Reynolds Tobacco Co., Bowman Gray Technical Center, Winston-Salem, NC 27102

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Table 1. Sample preparation conditions evaluated for the quantification of tobacco specific nitrosamines (TSNA) in tobacco[a].

Conditions	Average Sample Weight	Buffer	Extraction Procedure	Chem Elut H_2O Capacity	$MeCl_2$[b]	Final Dilution[c]	NNN	NAT	NAB	NNK
	--g--	--mL--		--mL--	--mL--	--mL--	--µg g^{-1}--	--µg g^{-1}--	--µg g^{-1}--	--µg g^{-1}--
1	2.08	50	Stirred	100	200(1st)	5	7.89±0.90[e]	1.50±0.20	nd[f]	2.10±0.28
			Overnight		200 (2nd)	5	0.61	0.14	nd	0.15
2	0.12	50	Stirred	100	300[d](1st)	2	9.25±0.78	1.57±0.07	nd	2.82±0.90
			Overnight		200 (2nd)	2	nd	nd	nd	nd
3	0.12	20	Stirred	50	300[d]	2	8.94±2.26	2.32±0.00	nd	1.69±0.78
			Overnight							
4	1.03	50	Sonicated	100	300[d](1st)	10	8.37±0.10	1.83±0.07	nd	2.26±0.60
			1 hour		100 (2nd)	2	nd	nd	nd	nd
					100 (3rd)	2	nd	nd	nd	nd
5	1.03	50	Stirred	100	300[d](1st)	10	7.36±0.12	1.32±0.08	nd	1.82±0.04
			Overnight		100 (2nd)	2	0.56	0.18	nd	0.15
					100 (3rd)	2	0.24	nd	nd	0.23
6	0.50	50	Stirred	100	200 (1st)	5	10.64±0.16	2.10±0.03	nd	2.93±0.04
			Overnight		200 (2nd)	5	nd	nd	nd	nd
7	0.50	50	Sonicated	100	300[d]	5	10.50±0.22	2.20±0.02	nd	2.86±0.03
			1 hour							

[a] Ground burley stems used as sample, n=2.
[b] Volume of $MeCl_2$ passed through Chem Elut tube (sequence of addition).
[c] Volumetric dilution with $MeCl_2$ of concentrated extract from TurboVap tube.
[d] Two 150-mL portions of $MeCl_2$.
[e] ±Standard deviation.
[f] Below detection limits.

Packard 5890 gas chromatograph, a 7673A autosampler with 10-µL syringe, and a 3393A integrator. The temperature program began at 40°C. This temperature was held for one min, and then it was taken to 50°C at 3°C min^{-1}. The temperature was increased from 50°C to 200°C at 20°C min^{-1}, held isothermal for 15 min, increased to 300°C at 30°C min^{-1}, and held isothermal for 5 min. The program took 35.2 min to run. Including the time for the oven to cool down, it took about 45 min per injection. The carrier gas was helium at 82.8 KPa (12 psi) column head pressure.

The chromatographic column consisted of a 5-m precolumn section (0.53 mm i.d.) of fused silica that was coated with DB-5 phase of 1.5-µm film thickness. The precolumn was connected by means of a glass union (0.53 mm to 0.32 mm) to a 30-m analytical column (0.32 mm i.d.) of fused silica that was coated with DB-5 of 1.0-µm film thickness (J&W Scientific, Folsom, CA). The precolumn was connected to the injection port by means of a column nut, graphite ferrule, megabore insert, and spring (3.0 mm i.d.) that seated against the 5-mm Supelco LB-2 septum (Bellefonte, PA). The autosampler, equipped with a tapered 23-26 gauge syringe, was programmed to inject 2 µL on-column after three sample washes and seven syringe pumps from the sample vial. The viscosity setting on the autosampler was four, and the syringe was washed eight times with $MeCl_2$ after injection.

Table 2. Chromatographic precision of nitrosamine standards used for the quantification of TSNA in tobacco (n = 6).

Compound	Concentration	$\bar{x}$ Area	σ Area	% RSD
	--µg mL^{-1}--			
NG[a]	0.348	60980	2362	3.87
NNN[b]	0.592	90110	4616	5.12
NAT[c]	0.570	86090	3906	4.54
NAB[d]	0.094	15954	861	5.40
NNK[e]	0.228	31640	2786	8.80

[a] Internal standard, N'-nitrosoguvacoline.
[b] *N'*-nitrosonornicotine.
[c] *N'*-nitrosoanatabine.
[d] *N'*-nitrosoanabasine.
[e] 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

Detector

Detection of the TSNA was accomplished with a thermal energy analyzer, TEA Model 543 Analyzer (Thermedetec, Inc., Woburn, MA), whose probe was connected to the analytical column in the GC oven. The pyrolyzer temperature was set at 550°C and the interface temperature at 253°C. The oxygen flow was 69 KPa (10 psi) from a cylinder. Data were acquired from the detector using VAX MULTICHROM (VG Instruments, Danvers, MA).

RESULTS AND DISCUSSION

Sample Preparation Conditions

Ground burley tobacco stems were used to evaluate sample extraction procedures. Burley stems have been reported to contain higher levels of TSNA than other types of tobacco (4). A procedure which removed the largest amount of TSNA from burley stems was considered sufficient for all tobacco samples.

Table 3. Linearity of response of nitrosamine standards used for the quantification of TSNA in tobacco.

Compound	Concentration range[a]	Sensitivity range[b]	R^2	Y-intercept
	--µg g^{-1}-- (µg mL^{-1})	--ng--		--µg--
NG[c]	0.46-11.6 (0.092-2.230)	0 0.18-4.64	0.9999	0.01
NNN[d]	0.37-9.25 (0.07-1.85)	0.14-3.70	0.9996	0.14
NAT[e]	0.71-10.68 (0.14-2.14)	0.28-4.28	0.9996	0.18
NAB[f]	0.47-9.45 (0.09-1.89)	0.18-3.78	0.9994	0.04
NNK[g]	0.38-9.50 (0.08-1.90)	0.15-3.80	0.9982	0.42

[a] Based on 1-g tobacco sample with $MeCL_2$ extract diluted to 5 mL final volume.
[b] Two µL injection volume, on-column.
[c] Internal standard, N'-nitrosoguvacoline.
[d] *N'*-nitrosonornicotine.
[e] *N'*-nitrosoanatabine.
[f] *N'*-nitrosoanabasine.
[g] 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

Table 4. Overall precision of procedure for the quantification of TSNA in tobacco[a].

Compound	IS/ES[b] $\bar{x}$	σ	RSD[c]	MDQ[d]
	--µg g^{-1}--	--µg g^{-1}--	%	µg g^{-1}
NNN[e]	2.55/2.30	0.06/0.12	2.24/5.30	0.04/0.04
NAT[f]	1.50/1.36	0.04/0.09	2.60/6.38	0.04/0.04
NAB[g]	0.08/0.07	0.02/0.02	25.00/28.57	0.04/0.04
NNK[h]	0.98/0.88	0.08/0.10	7.76/10.94	0.04/0.04

[a]Tobacco blend, 1 g extracted in 50 mL buffer.
[b]IS = internal standard; ES = external standard.
[c]RSD = relative standard deviation.
[d]MDQ = minimum detectable quantity at 2 x's signal:noise ratio.
[e]*N'*-nitrosonornicotine.
[f] *N'*-nitrosoanatabine.
[g]*N'*-nitrosoanabasine.
[h]4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

Table 1 lists the sample preparation conditions evaluated in duplicate using aliquots of the same ground burley stem sample. Sample weights tested were 0.1, 1.0 and 2.0 g. Two amounts of buffer were used to extract the sample (20 or 50 mL) and two modes of extraction (sonication or stirring overnight) were evaluated. Two types of Chem Elut tubes (50 or 100 mL water capacity), the amount of $MeCl_2$, and its sequence of addition to the Chem Elut tubes also were evaluated.

When the sample weight was 1 g or greater, one addition of 200 or 300 mL of $MeCl_2$ was insufficient to quantitatively elute TSNA from the Chem Elut tube, especially when the mode of extraction was stirring overnight (conditions 1 and 5, **Table 1**). When the sample size was decreased to 0.1 g and 50 mL of buffer were used (condition 2), all TSNA was removed as indicated by no TSNA being detected in a second elution with $MeCl_2$. Under these conditions, the amounts of TSNA were within the range of that obtained with 20 mL of buffer (condition 3). However, the results were variable. This variation can probably be attributed to the small size of the sample, because a portion of the tobacco remained on the walls of the flask and was not extracted. When the sample size was increased to 1 g, sonication removed more TSNA than did overnight stirring (conditions 4 vs. 5). No TSNA was detected in a second elution with $MeCl_2$ when the sample was sonicated. To ensure more complete extraction, a 0.5 g sample weight was used for burley stems. The amount of extractant remained 50 mL, and sonication was used because it did not require as much time as stirring (condition 7). The sonic bath temperature did not exceed 40°C, and sonication gave equivalent results to overnight stirring (condition 6), indicating no artifact formation was caused by sonication. These values are in the same range as those obtained by other workers (4). The 300 mL of $MeCl_2$ used to elute the Chem Elut tube was the maximum the evaporator collection tube could hold, and it was sufficient for removal of TSNA from the buffer. The 100-mL water capacity Chem Elut tube was used instead of the 50-mL type to ensure sufficient surface for the absorption of water.

Table 5. Standard addition and recovery of nitrosamine standards used for the quantification of TSNA in tobacco from buffer.

Compound[a]	Amount Added	Amount Recovered	% Recovery
	--µg--	--µg--	
NG[b]	1.19	0.99	83.2
	2.38	1.96	82.4
	5.96	5.08	85.2
			Avg. = 83.6
NNN[c]	1.22	1.39	113.9
	2.44	2.52	103.2
	4.88	5.27	108.0
			Avg. = 108.4
NAT[d]	0.91	0.92	101.0
	1.82	1.72	94.5
	2.73	2.61	95.6
			Avg. = 97.0
NAB[e]	0.48	0.42	87.5
	0.96	0.73	76.0
	1.92	1.57	81.8
			Avg. = 81.8
NNK[f]	0.50	0.50	100.0
	1.00	1.15	115.0
	2.00	2.30	115.0
			Avg. = 110.0

[a] Amounts added to 50 mL buffer.
[b] Internal standard, N'-nitrosoguvacoline, R^2 = 0.9999, intercept = 0.080 µg.
[c] *N'*-nitrosonornicotine, R^2 = 0.9988, intercept = 0.100 µg.
[d] *N'*-nitrosoanatabine, R^2 = 0.9995, intercept = 0.080 µg.
[e] *N'*-nitrosoanabasine, R^2 = 0.9934, intercept = 0.002 µg.
[f] 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, R^2 = 0.9968, intercept = 0.280 µg.

Fig. 1. Chromatograms of burley stem extract. Conditions: see Material and Methods section.

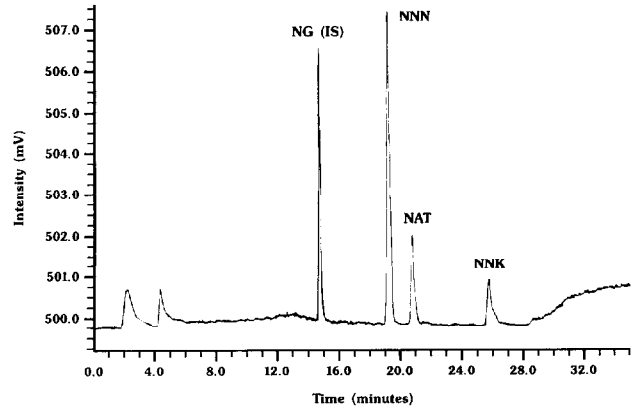
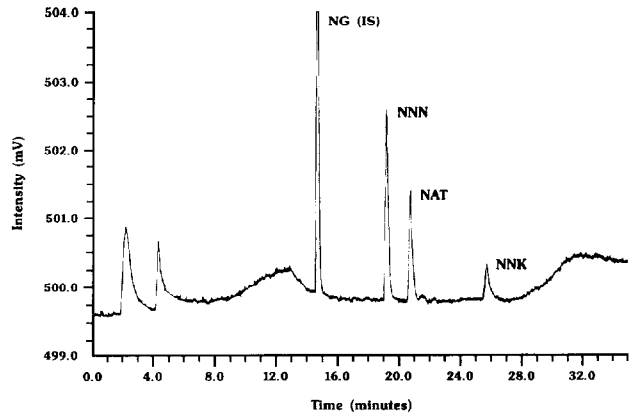


Fig. 2. Chromatograms of tobacco blend extract. Conditions: see Material and Methods section.



Use of Chloroform as Eluent and $MeCl_2$ as Extractant

Because $MeCl_2$ is a potent solvent and has a low boiling point, factors that contribute to the shortening of the septum lifetime and impose limitations on the injection volume due to flash evaporation, we evaluated chloroform as the eluting solvent on the Chem Elut tubes and as the medium for the

TSNA standard. $MeCl_2$ yielded results which were at least 20 percent higher than chloroform. For this reason, the use of chloroform was abandoned.

Because $MeCl_2$ elutes TSNA from the Chem Elut tube efficiently, we extracted the tobacco directly to eliminate the use of buffer. $MeCl_2$ did not remove the TSNA from burley stems when 300 mL was used to extract the stems for one hour with sonication.

Internal Standards

Because *N'*-nitrosopentylpicolyamine (NPePicA) has been used by other workers as an internal standard (6), we evaluated it under the chromatographic conditions described in the Material and Methods section. NPePicA co-eluted with NNN under these conditions, and thus it was not a good choice as an internal standard.

NG has also been used as an internal standard (1,5). It did not interfere with the chromatography of a burley stem extract (**Figure 1**) or with a tobacco blend extract (**Figure 2**). Therefore, NG was chosen as the internal standard.

Chromatographic Precision

The chromatographic precision ranged from 3.9% (NG) to 8.8% (NNK) relative standard deviation (%RSD) (**Table 2**). NNK gave a poor peak shape and this probably contributed to the higher %RSD.

Linearity of Response

Table 3 shows that the TEA detector response was linear over at least a ten-fold concentration range, R^2 being >0.99. The detector was capable of detecting less than a nanogram of TSNA when 2 µL were injected. The y-intercepts were acceptable with the exception of NNK. Again, this was attributed to its poor peak shape.

Overall Precision

Table 4 shows the overall precision obtained by internal and external standards on the same tobacco blend sample. The internal standard quantification yielded higher results for TSNA because it accounted for losses during sample preparation and variations in injection volume. The amounts calculated by using external standard quantification were not greatly different from those found by the internal standard method, and their %RSDs track each other. The %RSD for NAB was quite high due to the small amount of this compound found in tobacco. In over 300 tobacco samples, NAB was always <0.2 µg g^{-1}. NAB was used in the calibration standard, but it was most useful for the purpose of evaluating column deterioration (see below).

Standard Addition/Recovery

Standard addition experiments were performed in buffer only (**Table 5**) and in the presence of a tobacco blend sample extracted with buffer (**Table 6**). This was an effort to show the matrix effect of tobacco on recovery efficiency. A blank (50-mL buffer solution) showed that no TSNA or potential interferences to the TSNA were observed. This is reflected in the overall high percentage recovery of the standards (**Table 5**). Although the amounts added yielded a linear response (R^2 >0.99), the intercepts were not zero (**Table 5**). This may be inherent in the detector because non-zero intercepts were also obtained in **Table 3**. **Table 6** shows that the recoveries

Table 6. Standard addition and recovery of nitrosamine standards used for the quantification of TSNA in tobacco from samples[a].

Compound	Amount Added	Amount Recovered	% Recovery
	--µg--	--µg--	
NG[b]	1.19	1.13	94.8
	2.38	2.26	94.8
	5.96	5.12	85.9
			Avg. = 91.8
NNN[c]	1.22	1.06	86.8
	2.44	2.28	93.4
	4.88	4.67	95.6
			Avg. = 91.9
NAT[d]	0.91	0.78	85.7
	1.82	1.50	82.4
	2.73	2.25	82.4
			Avg. = 83.5
NAB[e]	0.48	0.39	81.2
	0.96	0.78	81.2
	1.92	1.69	88.0
			Avg. = 83.4
NNK[f]	0.50	0.41	82.0
	1.00	0.83	83.0
	2.00	1.73	86.5
			Avg. = 83.8

[a] Added to 50 mL buffer before extracting 1 g tobacco blend.
[b] Internal standard, *N'*-nitrosoguvacoline, R^2 = 0.9998, intercept = 0.14 µg.
[c] µg g^{-1}: standard addition = 2.39, external standard = 2.30, internal standard = 2.55.
[d] µg g^{-1}: standard addition = 1.47, external standard = 1.36, internal standard = 1.50.
[e] µg g^{-1}: standard addition = 0.01, external standard = 0.07, internal standard = 0.08.
[f] µg g^{-1}: standard addition = 0.89, external standard = 0.88, internal standard = 0.98.

Fig. 3. Chromatograms of calibration standard: (A) before to removal of one meter of precolumn; (B) after removal of one meter of the front of the precolumn. Conditions: see Material and Methods section. Concentration (µg mL^{-1}): 0.34 NG (IS), 0.59 NNN, 0.57 NAT, 0.09 NAB, 0.23 NNK.

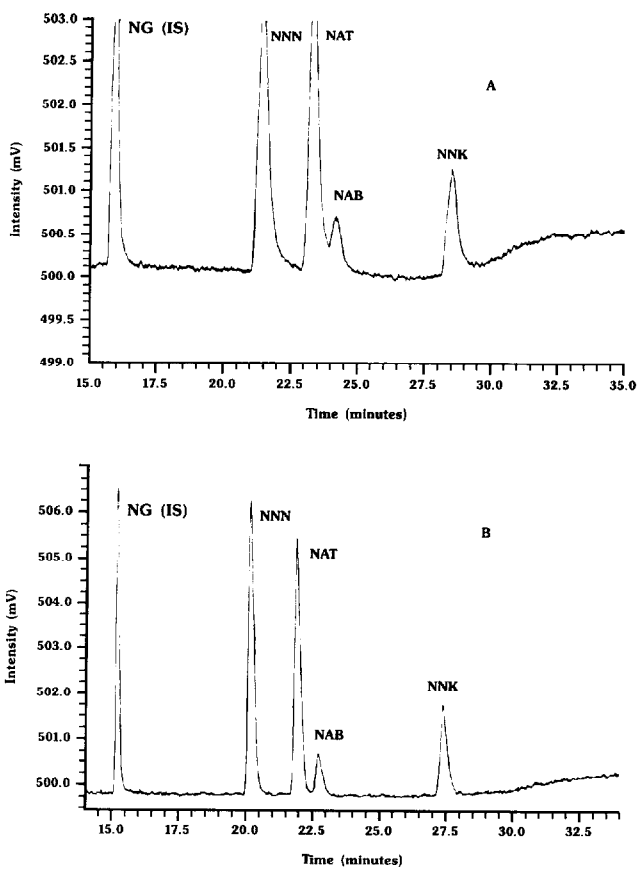


Table 7. Stability and storage condition studies of the internal standard (NG) and TSNA in buffer, tobacco extracts and standards in $MeCl_2$.

Material & Condition	NG	NNN	NAT	NAB	NNK
	----------	----------	μg g^{-1}	----------	----------
Buffer[a]					
Fresh	------	5.80	3.76	0.08	0.38
Two weeks in laboratory	------	5.79	3.81	0.11	0.45
% Change from fresh	------	0.2	1.3	37.5	15.5
Extract[b]					
Fresh	------	2.64	1.56	0.10	1.06
Two Weeks at 2°C	------	2.74	1.62	0.11	1.07
% Change from fresh	------	3.8	3.8	10.0	0.9
	----------	----------	μg mL^{-1}/Area	----------	----------
Standards in $MeCl_2$					
Fresh	0.348/65069	0.592/97373	0.570/92170	0.095/15293	0.228/36766
Two Weeks at 2°C	0.348/64029	0.592/96525	0.570/89364	0.095/14740	0.228/34590
% Area Change from fresh	1.60	0.87	3.40	3.62	5.92
	---μg added/found---				
NG in Buffer[c]					
Fresh	1.788/1.598 (89.4% recovery)				
Two weeks in laboratory	1.788/1.650 (92.2% recovery)				

[a] Burley tobacco extracted with 50 mL buffer, n = 2.
[b] Final 5 mL $MeCl_2$ dilution in volumetric flask, 1 g tobacco blend extracted with 50 mL buffer, n = 2.
[c] Internal standard, *N'*-nitrosoguvacoline.

were greater than 83%, and that the amounts found by standard addition were in the same range as those found by external and internal standard quantification, except for NAB whose amounts approached the detection limit.

Stability, Storage and Column Lifetime

Table 7 reports the changes in the buffer solution, the tobacco extracts, NG, and the standards after two weeks of storage. The two-week-old buffer functioned as well as fresh buffer. NG in buffer changed very little after two weeks storage in the laboratory, as indicated by area count changes and percent recovery. After two weeks at 2°C, the calibration standards in $MeCl_2$ showed slight losses as indicated by reduced area counts. Whether these losses were due to degradation of the standards or whether they were due to changes of the analytical column after use is not known. The final $MeCl_2$ extract of tobacco did not change after two weeks at 2°C. A maximum of 32 injections (a 24-hour run, including standards) should be placed in the autosampler tray. There was evidence of degradation if more injections were queued for analysis.

Table 8. TSNA of various tobacco types.

Sample Description	NNN[a]	NAT[b]	NNK[c]
	--μg g^{-1}--	--μg g^{-1}--	--μg g^{-1}--
Flue cured stems	nd[d]	0.19	nd[d]
Maryland stems	2.85	0.50	0.98
Flue cured, lower stalk	0.62	1.12	1.23
Flue cured, upper stalk	1.26	1.46	1.66
Burley, lower stalk	6.30	3.17	0.50
Burley, mid stalk	4.80	2.49	0.70
Burley, upper stalk	7.35	4.22	0.68
Turkish	0.12	0.18	0.02

[a] *N'*-nitrosonornicotine.
[b] *N'*-nitrosoanatabine.
[c] 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.
[d] Below detection limits.

Over 300 tobacco samples were analyzed using the same analytical column. The chromatographic performance did degrade. This was due to deactivation of the precolumn, caused possibly by compounds with high boiling points in the sample extract, or by the high temperature (20°C below the temperature limit) to which the precolumn was subjected. **Figure 3A** is a typical standard chromatogram where the precolumn functionality had deteriorated to the point where there was no NAB-NAT resolution, and there was a poor peak shape for NNK. This problem was solved by removing a portion (about 1 meter) of the precolumn (**Figure 3B**). Removal of the front section of the precolumn restored resolution, dramatically improved the peak shape, and caused a two-fold increase in response. The retention times decreased slightly due to the decrease in total column length (pre-column plus analytical column), but this change was not detrimental to the chromatography.

Applications

The analytical procedure described herein was applied to a variety of tobacco types and stems. NAB was not reported because its level was near the range of detection limits (<0.2 μg g^{-1}). **Table 8** gives the results for several tobacco types. Nicotine and nornicotine contribute to the formation of NNN (4), and the higher values for NNN relative to the other TSNA in burley tobacco may reflect the higher alkaloid levels found in this tobacco type. However, Burton et al. (2) attributed TSNA to the presence of nitrite.

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A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD TO QUANTIFY PHENOLIC COMPOUNDS IN AN ISOPROPANOL EXTRACT OF MAINSTREAM CIGARETTE SMOKE

By A. Prakash[1] and M. S. Ireland[1]

A high performance liquid chromatographic (HPLC) method was developed to quantify hydroquinone, catechol, and phenol in the isopropanol extract of mainstream cigarette smoke. This method reduces sample preparation, because the same *isopropanol extract is used to determine water, nicotine, and the* major phenolic compounds.

Additional key words: *Nicotiana tabacum*.

INTRODUCTION

Hydroquinone, catechol, and phenol in mainstream cigarette smoke have been quantified by gas chromatography (7), spectrophotometry (2), and high-performance liquid chromatography (HPLC) (1,4,5,8). Tomkins et al. (8) extracted phenol and cresols from Cambridge pads with 1N sodium hydroxide followed by partial neutralization with glacial acetic acid. Gerstenberg & Speck (1) developed a method for the isolation and determination of catechol from smoke condensate. In the published GC and HPLC procedures, phenolic compounds were reported in µg/cigarette. In this paper we describe a new procedure that expresses phenols on the basis of dry particulate matter (DPM). Our procedure requires only one sample, while the published methods require that two sets of cigarettes be smoked, one for the analysis of phenolic compounds and one for the determination of water and nicotine. Our HPLC method quantifies phenols from the isopropanol extract of mainstream cigarette smoke, which has also been used for the determination of water and nicotine.

MATERIALS AND METHODS

A Perkin-Elmer Series 410 LC pump and fluorescence detector LS40 were used for the analysis. A 300-mm µBondapak® phenyl column (Waters, Millipore Corporation, Milford, Mass.) with a 3.9 mm internal diameter, a pore size of 125 Å, and a particle size of 10 µm was used. A 20-µL injection loop was used. The Perkin-Elmer Nelson data system was used to calculate the peak areas.

Kentucky reference, 1R4F cigarettes from the Tobacco and Health Research Institute, Lexington, Ky., were used for this study. Mainstream smoke was collected on a Cambridge pad using the Federal Trade Commission (FTC) procedure (3). Five cigarettes were smoked per Cambridge pad. Each pad was extracted by shaking for 30 min in a Burrell wrist-action shaker with 20 mL of isopropanol containing 0.48% absolute ethanol and 0.048% anethole. Ethanol and anethole are the internal standards for water and nicotine analyses. Water and nicotine were determined by the FTC procedure (6,9). The DPM weight was calculated by subtracting the weight of the water from the wet tar.

The isopropanol extract of mainstream smoke was filtered through 0.2-µm filter and diluted with an equal volume of 6% aqueous acetic acid. The solution was then analyzed for phenols by HPLC.

The chromatographic analysis of phenols was performed under isocratic conditions. Reagent-grade glacial acetic acid (Mallinckrodt), HPLC grade acetonitrile (Burdick & Jackson), and sodium acetate (J. T. Baker) were used for preparation of the mobile phase. The mobile phase for the elution of phenols was a 22 mM acetate-acetic acid buffer in water of pH 3.4 at a flow rate of 1.4 mL/min for 11 min. The column was then washed for 6 min with 89% acetonitrile and 11% buffer at 2.4 mL/min and equilibrated with the mobile phase for 6 min before the next analysis. The total run time was 24 min.

The fluorescence detector was time-wavelength programmed for the analysis of the phenolic compounds. Hydroquinone and catechol were detected at an excitation wavelength of 285 nm, and an emission wavelength of 320 nm. Phenol was detected at an excitation and an emission wavelength of 275 nm and 305 nm, respectively.

Phenols were quantified by an external standard method. Reagent grade phenolic compounds (Aldrich) were used after purification. Phenol was distilled, hydroquinone was recrystallized from benzene-acetone, and catechol from benzene after charcoal treatment.

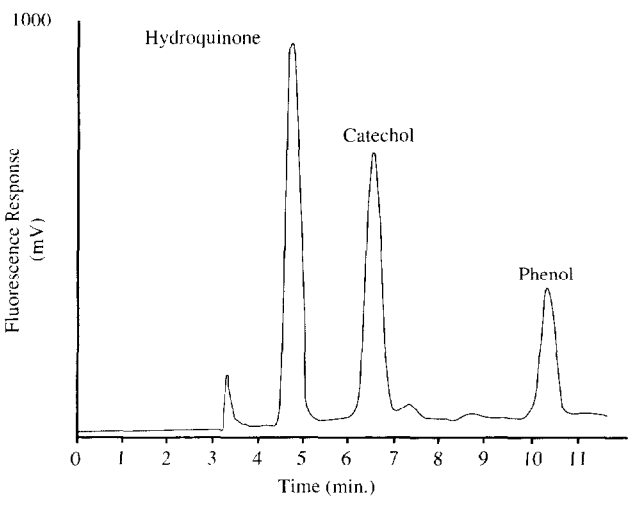
RESULTS AND DISCUSSION

A typical chromatographic profile of the phenolic compounds in a smoke sample is presented in **Figure 1**. Hydroquinone eluted at 4.5 min, catechol at 6.1 min, and phenol at 10.1 min.

The efficiency of isopropanol as an extractant for phenols from the Cambridge pad was studied. A blank Cambridge pad was spiked with 200 µl of isopropanol solution containing 35.8 µg hydroquinone, 34.2 µg catechol, and 48.8 µg phenol. The pads were extracted and analyzed for phenols as described above. Recoveries for hydroquinone, catechol, and phenol were 97%, 100%, and 101%, respectively. The blank pads were also spiked with the phenolic compounds in the range 20-60 µg and their recoveries were above 95%, suggesting isopropanol is an efficient extractant.

Analyses of phenols from spiked sample extract of Kentucky Reference, 1R4F cigarettes were investigated. The mainstream smoke extract of 1R4F cigarettes in isopropanol

Figure. 1 Chromatographic profile of phenolic compounds in the isopropanol extract of mainstream cigarette smoke of reference 1R4F cigarettes



[1] Lorillard Tobacco Co., 420 English Street, Greensboro, NC 27405.

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Table 1. Phenolic Compounds in Standard 1R4F Cigarettes.

Method	Wet Tar	DPM[a]	Hydroquinone		Catechol		Phenol	
	--mg/cig.--		µg/cig.	PPM[b]	µg/cig.	PPM[b]	µg/cig.	PPM[b]
Acetic Acid[c]	10.9	9.2	38	4103	44	4783	9.2	1003
Isopropanol[d]	10.0	9.3	44	4710	43	4602	8.1	877

[a] Dry particulate matter.
[b] Parts per million of dry particulate matter.
[c] Five cigarettes/pad, 20 mL of 1% aqueous acetic acid (5).
[d] Five cigarettes/pad, 20 mL of isopropanol containing ethanol and anethole.

was spiked with 3.1 µg hydroquinone, 3.5 µg catechol, and 2 µg phenol before being analyzed for phenols. Recoveries were 99%, 100%, and 101% for hydroquinone, catechol, and phenol, respectively.

The results of phenolic compounds in the mainstream smoke of 1R4F cigarettes in isopropanol extractant were compared to the results obtained by 1% aqueous acetic acid (5) extractant and are presented in **Table 1**. The DPM value used in the calculation for the 1% acetic acid extraction was an average obtained from a different set of cigarettes.

CONCLUSIONS

A procedure was developed to quantify hydroquinone, catechol, and phenol from the isopropanol extract of mainstream cigarette smoke in less than eleven minutes. Major phenolic compounds, water, and nicotine can be determined from a single isopropanol extract of the same tobacco smoke condensate.

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NINE YEARS CONTINUOUS TOBACCO MONOCROPPING COMPARED WITH ALTERNATIVE CROPPING FREQUENCIES AND SEQUENCES PART 2. EFFECTS ON SOIL CHEMICAL PROPERTIES

TOBACCO SCIENCE

By J. Littlemore[1], P. E. Tonello[2], T. S. Rasmussen[3]

Flue-cured tobacco, *Nicotiana tabacum* L., was grown on a granitic soil (Palestaulf) in Australia for 9 years. Crop rotation treatments for this study were continuous tobacco (1:1), tobacco grown every other year (1:2), and tobacco grown every fourth year (1:4). Soil analyses from treatment plots were compared with those from an adjacent, uncultivated site. After nine years, levels of organic carbon and mineral nitrogen in the 0-30 cm soil profile did not change significantly in any of the crop rotation treatments. In the upper 0-15 cm of the soil compared with the uncultivated site, DTPA-extractable Zn accumulated significantly, Cu and Fe did not change, and Mn declined from 31 to 12 mg/kg. Phosphorous (both acid- and bicarbonate-extractable) accumulated significantly over time in all cropping sequences. Phosphorous rapidly accumulated in the 0-60 cm soil depth and, as the experiment progressed, P moved downward to 60-120 cm. In contrast, K and Ca, which were supplied by the tobacco fertilizer, did not accumulate in the surface profiles. Instead, these cations leached downward to the 45-120 cm depth where they accumulated to 4-5 times that of the uncultivated site. Magnesium, which was only supplied by the irrigation water, accumulated in the 60-120 cm depth to a level twice that of the uncultivated site.

Additional key words: *Nicotiana tabacum*, organic C, mineral N, micro-elements, exchangeable cations, phosphorus.

INTRODUCTION

Tobacco, *Nicotiana tabacum* L., is grown in Australia on sandy soils of granitic origin. These soils have inherently low levels of organic carbon, nitrogen, and phosphorus, and they have a low cation exchange capacity. Tobacco growers control fertilizer in the root zone by irrigating, which leaches out soluble components. Past studies of the soluble fertilizer components in the tobacco root zone include nutrient distribution (10,22) and movement (4,8,21), but these studies did not quantify the movement of nutrients to a depth with repeated fertilizer applications. Osmond & Raper (13) found no difference in root proliferation in a fine sandy loam soil for either banded or broadcast fertilizer placement. They also reported that the bulk of the root development was in the 0-40 cm depth with some roots penetrating below 60 cm. Littlemore et al.(8), in an on-farm study of fertilizer leaching, found quite rapid depletion of N, P, and K in the root zone (0-45 cm) during the grand growth period (between hilling-up and first priming).

Published references on the effects of continuous or rotated tobacco on the nutrient status of soil are lacking or incomplete. Stephens (19), in a comprehensive, long-term rotation study of row crops, briefly mentioned tobacco in the rotation, but he did not report how this affected the nutrient status of the soil. Crop rotation has been extensively studied in other tobacco-producing countries for its effects on soil-borne disease control (11), erosion minimization (3), and improved return per unit area (18,20), but little has been published on soil chemistry. Elliot (3) reported that continuous tobacco and tobacco:rye rotations had no effect on crop value or on the levels of total alkaloids, P, or K in cured tobacco leaves, but they did not publish data on soil chemistry.

There have been studies with other crops which are relevant to our study because they were done with similar soil types and fertilizer additions. Pratt et al. (14,15,16,17) studied the long-term (28 years) effects of fertilizer management in citrus on the accumulation, movement, redistribution, and depletion of P, K, and organic C to a depth of 120 cm in a soil of granitic origin. They found that P accumulated in the 60-90 cm depth, and there was no significant change in P in the 90-120 cm depth (14). The amount of K fixed in the top 30 cm of soil was small in relation to that in the 30-60 and 60-90 cm depths (17). Cover crops increased the organic C and N by small, but statistically significant, amounts (16). Pratt (15) also reported that applications of urea, phosphate, or potash had no effects on the cation exchange capacity of the soil or on the relatively large quantities of K that were fixed in the soil.

The purpose of the study described in this paper was to examine the effects of three cropping frequencies of tobacco on changes in K, Ca, organic C, mineral N, P, and DTPA-extractable Cu, Zn, Mn, and Fe in the soil.

MATERIALS AND METHODS

This experiment was conducted over a nine-year period at the Southedge Tobacco Research Station (16°58'S, 145°21'E, elevation 430 m) in the Mareeba-Dimbulah Irrigation District, Queensland, Australia, on a granitic soil as previously described (9).

Experimental Design

A bedding tobacco crop was grown in 1972 on an experimental site that had been cleared in 1971. In 1973, the following cropping sequence treatments were started: continuous tobacco (1:1), a two-year tobacco rotation (1:2), and a four-year tobacco rotation (1:4) (**Table 1**). The treatments were arranged in a randomized complete block design with three replications for the first 3 years of each 4-year crop cycle. During the fourth year, the plots were arranged in a randomized complete block split plot design with splits consisting of the presence or absence of EDB (ethylene dibromide) fumigation.

The experiment commenced with the planting of soybeans ('Improved Pelican') and peanuts (Virginia-bunch) at commercial rates of planting, and green panic (*Panicum maximum* var. *trichoglume* Eyles). In subsequent plantings, Red Spanish type peanuts were substituted for the Virginia-bunch type. In the second 4-year cycle, cowpeas (*Vigna sinensis*, 'Brandon') were sown at 25 kg/ha, dolichos (*Lablab purpureus*, 'Rongai') at 20 kg/ha, signal grass (*Brachiaria decumbens*) at 5 kg/ha, molopo buffel (*Cenchrus ciliaris*) at 5 kg/ha, and stylo (*Stylosanthus hamata*, 'Verano') at 10 kg/ha. Flue-cured tobacco cultivars were CSIRO 40T from 1973 to 1978, and ZZ 100 in 1979 and 1980. Introduction of ZZ 100 was necessitated because of bacterial wilt infection in the soil.

In the first year following native weeds and grass fallows, tobacco received 34 kg/ha N, 68 kg/ha P, and 168 kg/ha K as a compound fertilizer mixture (3 N, 6.1 P, 15 K, 12 Ca). The same rate of compound fertilizer was applied behind grass and native weeds the second year, but the N rate was increased to 55 kg/ha using sodium nitrate (16% N). In the

[1] Department of Transport, P.O. Box 1412, Brisbane, Queensland, Australia 4001; present address: Quality Assurance Services, Standards Australia, 67 St. Pauls Terrace, P.O. Box 290, Spring Hill, Queensland, Australia, 4004

[2] Dept of Primary Industries, P.O. Box 1054, Mareeba, Queensland, Australia 4880.

[3] Dept of Primary Industries, G.P.O. Box 46, Brisbane, Queensland, Australia 4001.

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Table 1. Tobacco cropping sequences from 1973 to 1980.

	1st 4-year cycle, 1973 to 1976							
	1973		1974		1975		1976	
Plot	Jan.	August	Jan.	August	Jan.	August	Jan.	August
Tobacco every year (1:1)								
C0	Weeds	Tobacco	Weeds	Tobacco	Weeds	Tobacco	Weeds	Tobacco
C1	Green Panic	Tobacco	Green Panic	Tobacco	Green Panic	Tobacco	Green Panic	Tobacco
C2	Soybean	Tobacco	Soybean	Tobacco	Soybean	Tobacco	Soybean	Tobacco
C3	Peanuts	Tobacco	Peanuts	Tobacco	Peanuts	Tobacco	Peanuts	Tobacco
Tobacco every second year (1:2)								
C4	Weeds		Weeds	Tobacco	Weeds		Weeds	Tobacco
C5	Green Panic		Green Panic	Tobacco	Green Panic		Green Panic	Tobacco
C6	Soybean		Green Panic	Tobacco	Soybean		Green Panic	Tobacco
C7	Peanuts		Green Panic	Tobacco	Peanuts		Green Panic	Tobacco
Tobacco every fourth year (1:4)								
C8	Weeds		Weeds		Weeds		Weeds	Tobacco
C9	Green Panic		Green Panic		Green Panic		Green Panic	Tobacco
C10	Soybean		Green Panic		Green Panic		Green Panic	Tobacco
C11	Peanuts		Green Panic		Green Panic		Green Panic	Tobacco

	2nd 4-year cycle, 1977 to 1980							
	1977		1978		1979		1980	
Plot	Jan.	August	Jan.	August	Jan.	August	Jan.	August
Tobacco every year (1:1)								
C0	Weeds	Tobacco	Weeds	Tobacco	Weeds	Tobacco	Weeds	Tobacco
C1	Green Panic	Tobacco	Green Panic	Tobacco	Green Panic	Tobacco	Green Panic	Tobacco
C2	Cowpeas	Tobacco	Cowpeas	Tobacco	Cowpeas	Tobacco	Cowpeas	Tobacco
Tobacco every second year (1:2)								
C3	Dolichos			Tobacco	Dolichos			Tobacco
C4	Weeds			Tobacco	Weeds			Tobacco
C5	Green Panic			Tobacco	Green Panic			Tobacco
C6	Signal Grass			Tobacco	Signal Grass			Tobacco
C7	Molopo Buffel			Tobacco	Molopo Buffel			Tobacco
Tobacco every fourth year (1:4)								
C8	Weeds		Weeds		Weeds			Tobacco
C9	Green Panic		Green Panic		Green Panic			Tobacco
C10	Green Panic/ Stylo cv. Verano							Tobacco
C11	Dolichos		Dolichos		Dolichos			Tobacco

initial 4-year cycle, tobacco crops following leguminous seed crops received zero nitrogen, 68 kg/ha P (as superphosphate), and 168 kg/ha K (as potassium sulphate). All tobacco crops in the final 4 years received 55 kg/ha N and the same rates of P and K. During the years that crops other than tobacco were grown, the plots did not receive commercial fertilizers. Conventional tobacco cultural practices, including seedbed preparation, EDB and pesticide application at recommended rates, and overhead irrigation, were used throughout the duration of the experiment.

Soil Sampling

Initially, composite soil samples were collected at depths of 0-15 and 15-30 cm. This was done before tobacco transplanting and after final harvest each year. As the experiment progressed, the sampling depth was increased to 60 cm in July 1977 and then to 120 cm in Feb. 1980 to measure the nutrients in the lower soil profiles. An uncultivated control site adjacent to the experimental site was sampled in Feb. 1980 & 1981, and July 1981.

Sieved soil samples (<2 mm) were analyzed for acid-extractable P (5), bicarbonate-extractable P (1), and exchangeable Ca, Mg, and K (1N NH_4Cl, pH 7.0). Organic C was determined by the Walkley & Black titration procedure (23), and mineral N was determined by a Kjeldahl digestion followed by steam distillation and titration. The 0-15 cm sample from the final sampling (July 1981) was analyzed for DTPA-extractable Cu, Fe, Mn, and Zn (6).

One physical attribute of this granitic soil type is the high percentage of gravel greater than 2 mm. Results from other experiments conducted at this research station showed that typical percentages of gravel (>2 mm) at various depths in this soil are 21%, 17%, 25%, 59%, and 56% for the 0-15, 15-30, 30-60, 60-90, and 90-120 cm depths, respectively. For these same soil depths, the soil bulk densities are 1.61, 1.85, 1.88, 1.75, and 1.80 g/cc, respectively. Particle size analysis for the 0-15 cm depth showed that this soil is 51% coarse sand, 38% fine sand, 6% silt, and 6% clay.

RESULTS AND DISCUSSION

Organic Carbon

We expected organic C in the surface profile of the soil to decrease in the continuous tobacco treatment and to increase in the 1:2 and 1:4 tobacco rotation treatments. Before treatments were initiated, organic carbon concentrations at the experimental site ranged 0.50-0.66% and 0.44-0.60% for the 0-15 and 15-30 cm depths, respectively. The concentrations at the uncultivated site were 0.80% and 0.48% in February 1980, and 0.61% and 0.32% in July 1981 for the depths 0-15 and 15-30 cm, respectively. There was substantial variation in levels of organic C both between sites and between years. Organic C concentrations changed little over 9 years with any of the cropping sequences (**Table 2**). Also,

Table 2. Percentage of organic carbon in the 0-30 cm soil profile after 1, 5, and 9 years tobacco cropping rotation.

	Date sampled					
	Jan. 1973		Feb 1977		Feb 1981	
	Depth in cm					
Rotation	0-15	15-30	0-15	15-30	0-15	15-30
	%					
1:1	0.56	0.48	0.45	0.37	0.54	0.54
1:2	0.61	0.53	0.53	0.43	0.57	0.61
1:4	0.62	0.50	0.57	0.44	0.74	0.73
LSD(P=0.05)	0.07	0.07	0.06	0.04	0.10	0.10

Table 4. Effect of cropping rotations and soil depth on accumulation of potassium, Feb. 1981.

	Cropping Frequency (yrs)				
Depth (cm)	Uncultivated site	1:1	1:2	1:4	l.s.d. (P=0.05)
	mg/kg				
0-15	41	47	58	54	13
15-30	30	68	81	90	16
30-45	30	123	112	110	20
45-60	26	177	153	158	46
60-90	28	169	149	122	27
90-120	30	142	119	97	26

the concentration of organic C in the 15-30 cm soil depth was similar to that in the surface 0-15 cm.

The tobacco crop residue of roots, stalks, suckers, and flowers has been estimated to be 4,100 kg/ha at a plant density of 15,400/ha (2) and 5,100 kg/ha at a plant density of 19,200/ha (7). For both of these studies, the dry matter residue equates to 266 g/plant. Our results indicate that the organic load of approximately 2,000 kg/ha per year of organic carbon did not accumulate in the soil following decomposition (**Table 2**). Pratt et al. (16) found that organic C and mineral N were not increased by application of alfalfa hay, bean residue, or cereal straw to soils of 0.5% organic C. However, after 28 years of applying manure (14,515 kg/ha), organic C increased from 0.50% to 1.01%, and total N increased from 0.06% to 0.12%. We observed that organic C in the soil under continuous tobacco did not change over the experimental period, even after the addition of approximately 16,330 kg/ha carbon from tobacco residues.

Total Nitrogen

Mineral N was monitored intensively during the first 4-year cycle and twice in the second 4-year cycle (data not shown). Soil concentrations ranged 0.04-0.05% in the surface 0-15 cm and 0.03-0.07% in the 15-30 cm depth. However, there were no significant differences (P>0.05) among treatments for levels of this element or for the C:N ratio which ranged 10-13.

Phosphorus

Pratt & Goulben (17) studied the chemical changes of a sandy loam soil derived from a granitic alluvium after 28 years of irrigation and fertilization. They observed that the soil chemistry of an uncultivated site in their experiment did not change with time. This has relevance to our study. Even though our uncultivated site was not continuously sampled, we are confident that the chemical changes we observed in the soil of our experimental plots were significant compared with the uncultivated site.

Phosphorous levels in the uncultivated site were extremely low at all soil depths (**Table 3**). They varied little over the three sampling dates, so only the Feb. 1981 data are presented. Significant accumulations of P occurred down to 120 cm for the 1:1 and 1:2 rotations and down to 60 cm for the 1:4 rotation. For all three rotations, the bulk of the P was located in the 0-60 cm depth, which is within the root zone of tobacco (13). This indicates that the various crops grown in the different cropping sequences had little effect on P accumulation in the soil. Phosphorus was oversupplied to the crops that have small P requirements. The accumulation of acid-extractable P in the soil to a depth of 120 cm at the completion of the experiment was calculated to be 545, 385, and 169 kg/ha P for the rotations 1:1, 1:2, and 1:4, respectively. If farmers grow continuous tobacco over an extended period or if they rotate with a crop such as potatoes that has a higher P requirement, then they should have a pre-plant soil analysis done to avoid overuse of P fertilizer.

Accumulation, movement, redistribution, and chemical changes of P have been monitored in a similar soil type over a longer period (28 years) than in our study (14). Significant movement of P to the 60-90 cm depth was observed for plots receiving P from fertilizer or manure. In plots receiving no added P, a significant decrease was observed for bicarbonate-extractable P to a depth of 90 cm. In coarse soils of granitic origin and with low clay content, P moves rapidly downward and is lost from the root zone.

Table 3. Effect of tobacco cropping frequency and soil depth on accumulation of phosphorous, Feb. 1981.

	Cropping rotation				
Depth (cm)	Uncultivated site	1:1	1:2	1:4	l.s.d. (P=0.05)
	mg/kg				
Acid extractable					
0-15	2.0	80.1	69.9	48.8	10.5
15-30	1.0	86.4	68.5	48.5	12.1
30-45	0.5	55.8	28.3	19.2	13.9
45-60	1.0	22.8	11.2	7.5	9.3
60-90	1.0	8.8	6.7	4.7	1.9
90-120	1.0	8.9	5.8	4.3	1.9
Bicarbonate extractable					
0-15	1.1	13.9	13.9	11.2	1.7
15-30	0.7	15.9	14.8	11.0	2.8
30-45	0.6	15.8	9.4	4.8	4.7
45-60	0.5	8.4	3.9	2.3	4.5
60-90	0.7	2.5	1.5	1.0	0.7
90-120	0.8	2.1	1.3	0.9	0.4

Exchangeable Cations

There was considerable accumulation of K, Ca, and Mg throughout the soil profiles to a depth of 120 cm for all of the rotation treatments (**Tables 4, 5, 6**). All cations increased in the 45-120 cm depth, which could be due, in part, to leaching. Pratt & Goulben (17) reported that exchangeable K increased with time to depths of 90 cm with little penetration

Table 5. Effect of cropping rotations and soil depth on accumulation of calcium, Feb. 1981.

	Cropping Frequency (yrs)				
Depth (cm)	Uncultivated site	1:1	1:2	1:4	l.s.d. (P=0.05)
	mg/kg				
0-15	332	291	334	364	41
15-30	206	288	318	346	42
30-45	125	262	266	250	43
45-60	99	340	345	295	50
60-90	83	360	380	377	50
90-120	70	319	336	343	60

Table 6. Effect of cropping rotations and soil depth on accumulation of magnesium, Feb. 1981.

Depth (cm)	Uncultivated site	Cropping Frequency (yrs) 1:1	1:2	1:4	l.s.d. (P=0.05)
	mg/kg				
0-15	36	30	37	50	5
15-30	27	24	30	46	5
30-45	28	25	31	34	5
45-60	32	44	56	46	10
60-90	39	78	88	93	17
90-120	43	99	111	115	21

to 100-120 cm. They accounted for 77% of the added K in the top 90 cm of the soil. For the 1:1 rotation, we accounted for 70% of the applied K (1,344 kg/ha) in the top 90 cm of soil. Greater than 100% was accounted for in the 1:2 and 1:4 rotations (**Table 4**). Pratt & Goulben (17) also noted that when K was increased from 450 to 740 kg/ha, the accumulation was rapid in the lower depths.

Exchangeable Ca accumulated to all depths below 15 cm compared with the control. There were few significant differences in Ca accumulation among the rotation treatments (**Table 5**). This is not surprising because there was considerable variation in Ca levels at all depths and rotations, and the uncultivated site had a variation of ±20 mg/kg among samples. Moody & Tonello (12) suggested that Ca accumulation might be due to calcium phosphate precipitates percolating downward, which was aided both by cultivation and by the summer monsoons.

Considerable exchangeable Mg (**Table 6**) accumulated in the 45-120 cm depths, and there were few significant differences in Mg levels between rotations 1:1 and 1:2 for the surface 0-45 cm. In this experiment, Mg was supplied not by the fertilizer but by the irrigation water, which contained small quantities (3-5 mg/L) of Mg. Hence, the accumulation of Mg at lower depths was not as great as was observed for K and Ca. At 45-120 cm, significant differences were found in Mg levels for the 1:2 and 1:4 rotations compared with the 1:1 treatment. This could be partly attributable to the fact that the entire experimental site received irrigation when the tobacco plots were irrigated. The experimental design did not allow for the non-tobacco plots to be isolated from the irrigation system.

In the planning and preparation of this study, several important considerations were overlooked. It would have been useful each year to quantify the nutrients removed in the dry matter from each crop and the nutrients accumulated to the 120 cm depth. This was done in later studies (7,8). One of the benefits of doing this might have been the development of recommendations of fertilizer applications to farmers who grow tobacco without rotation for 8-10 years. This study demonstrates that cations accumulate over time in the soil below the root zone of 0-40 cm (13). This is cause for concern because it does not make economic sense to over-fertilize a tobacco crop with nutrients that leach out of the root zone. Another concern that might be raised by our findings is that these nutrients might eventually move into the ground water supply. Presently, there is little demand for ground water in this tobacco district because it has a surface-irrigation system that provides a cheap and reliable supply of water. Secondly, the ground water moves through a granitic strata, which contaminates the ground water with up to 3,000 mg/L Cl. This renders it unsuitable for either irrigation or domestic use.

Table 7. Accumulation of DTPA-extractable micro-elements after 9 years of tobacco cropping rotations.

Micro-element	Uncultivated Site	Rotation 1:1	1:2	1:4
	mg/kg			
Cu	0.1	0.1	0.1	0.1
Fe	6.0	11.0	10.0	8.0
Mn	31.0	11.0	12.0	15.0
Zn	<0.2	2.1	1.6	1.8

Micro-elements

The DTPA-extractable micro-elements (**Table 7**) in the 0-15 cm soil zone were measured at the completion of the experiment. Significant changes were noted for Fe, Mn, and Zn, while Cu maintained the same value as the uncultivated site (0.1 mg/kg). Zinc accumulated from a deficient level (<0.2 mg/kg) to marginally adequate levels of 1.6-2.3 mg/kg. Mn decreased, and Fe increased slightly from 6 mg/kg to 7-13 mg/kg.

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RESIDUES OF FLUMETRALIN ON GREEN AND FLUE-CURED TOBACCO[1]

TOBACCO SCIENCE

By R. B. Leidy[2], T. J. Sheets[2], and H. Seltmann[3]

Studies were conducted to determine residue levels of flumetralin on green tobacco, *Nicotiana tabacum* L., and to see what effects flue-curing had on residue levels. Immediately after application of flumetralin at 1.35 kg/ha, average residues on green leaves of flue-cured tobacco from the upper stalk position varied from 41.5 ppm at Whiteville, N.C. in 1984 to 117.7 ppm at Reidsville, N.C. in 1985. Residues of flumetralin on green lamina from the upper stalk position were much higher immediately after application than they were on lower stalk samples taken at the same time. The average losses after 4 days for the 1.35 kg/ha application rate were 99.6% for upper stalk leaves and 93.2% for lower stalk leaves. Additional losses occurred during curing. The highest residue levels of flumetralin were found on lower leaves and the lowest amounts were found on middle and upper stalk leaves.

Additional key words: *Nicotiana tabacum*, dinitroanaline herbicide, growth regulator.

INTRODUCTION

Dinitroaniline herbicides have been used for about 25 years to control weeds in cotton (*Gossypium hirsutum* L.), peanuts (*Arachis hypogaea* L.), soybeans (*Glycine max* [L.] Merrill), tobacco (*Nicotiana tabacum* L.), and other broadleaf crops. Most grass species are susceptible to these compounds. Dinitroaniline herbicides persist for several weeks to several months in soils (4,6), and cereals planted in the fall after spring applications of these compounds sometimes are injured. In some instances, residues can persist in soils through the winter months and injure corn planted the next spring (4). Persistence varies according to the chemical structure of the dinitroaniline herbicide, and it is influenced by the type, temperature, and moisture content of the soil, and by the depth of incorporation into the soil (6).

The growth regulator, 2-chloro-*N*-(2,6-dinitro-4[trifluoromethyl]-phenyl)-*N*-ethyl-6-fluorobenzenemethanamine (flumetralin), a dinitroaniline derivative, controls the growth of lateral buds or suckers on tobacco (3). It was registered in 1984 as a sucker control agent. Evaluations of flumetralin used sequentially with 1,2-dihydroxypyridazine 3,6-dione (maleic hydrazide [MH]) and fatty alcohols showed excellent control (7). Jones & Rideout (2) determined the effects of flumetralin on sucker control, yield, and quality of flue-cured tobacco by varying the rate, concentration, and application technique in tank mix combinations with fatty alcohols. Their data indicated that the recommended application rate of 0.8-1.4 kg (AI)/ha could be reduced to 0.42 kg (AI)/ha when used in combination with fatty alcohols. Few data are available on residue levels of flumetralin on green and cured tobacco leaves.

In a continuing effort to increase the data base of pesticide residues on flue-cured tobacco, experiments were conducted at three locations in 1984, 1985, and 1986 to study the disappearance of flumetralin from green leaves under field conditions, and to determine residues remaining on leaves after flue-curing.

MATERIALS AND METHODS

1984 Experiments

Field experiments were conducted at the Border Belt Tobacco Research Station, Whiteville, N.C., and at the Central Crops Research Station, Clayton, N.C. Treatments were repeated four times in a randomized complete-block design. Each plot was 12.3 m long and three rows wide. Rows were 1.2 m apart and each contained 22 competitive plants. Standard cultural practices for each research station, including soil preparation, fertilization, transplanting, and disease and insect control, were performed by research station personnel. Flue-cured tobacco plants of cv. NC 2326 were transplanted April 19 at Whiteville, and plants of cv. McNair 944 were transplanted May 10 at Clayton.

The tobacco was topped at each location, and a 4.0% solution of a fatty alcohol contact sucker control agent (0.5%-C_6, 42%-C_8, 56%-C_{10}, and 1.5%-C_{12}) was applied to all plots at a volume of 470 L/ha.

Two rates of flumetralin were applied with a backpack sprayer equipped with a boom designed to spray one row with three TG-3 nozzles; the rates were 1.35 and 2.70 kg (AI)/ha. MH was applied to nontreated plots to control suckers after topping. In addition, two guard rows separated experimental plots to reduce cross contamination from spray drift.

The first row in each plot was reserved for normal harvesting and curing. Green samples were taken from the second and third rows in each plot. Green leaf samples were removed from control plots immediately before spraying on day 0 (June 25, Whiteville; July 16, Clayton). Samples from treated plants were taken immediately after the spray had dried, about 12 min after spraying. Green samples also were taken on days 1, 2, and 4 after spraying. The tobacco was harvested four or five times, cured, and analyzed. Four leaves were removed from the bottom and four leaves were removed from the top of eight plants. Thus, each top or bottom sample contained 32 leaves. Four disks (5-cm diam) were removed from each leaf. Disks were placed in a polyethylene bag and stored on dry ice in an insulated box for transport to the laboratory. The frozen samples were crushed in the sample bag by hand and mixed for analysis. A 50-leaf sample from each harvest of each plot was flue-cured by conventional methods. Before analysis, the samples were dried for four days at room temperature and then ground in a Wiley mill to pass a screen with 20 mesh/cm.

1985 Experiments

Field experiments were conducted at the Border Belt Tobacco Research Station and at the Upper Piedmont Research Station, Reidsville, N.C. Two treatments (control and flumetralin-treated) were repeated four times in a randomized complete-block design. Following topping and application of a fatty alcohol sucker control agent to all plots as previously described, 1.35 kg (AI)/ha of flumetralin or 2.52 kg (AI)/ha of MH were applied. McNair 944 was used in experiments at Reidsville and NC 2326 was used at Whiteville. Plots that received MH served as the controls.

Green leaf samples were taken from the second row of the plots at 0, 1, 2, and 4 days after application using the sampling methods as in 1984. Control plots were sampled before

[1] The use of trade names does not imply endorsement by the North Carolina Agricultural Research Service of the products named or criticism of similar ones not mentioned.

[2] Pesticide Residue Research Laboratory, North Carolina State University, Raleigh, NC 27695.

[3] Crops Research Laboratory, USDA-ARS, Oxford, NC 27565.

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Table 1. Residues of flumetralin on green leaf lamina from top and bottom stalk positions on tobacco plants at 0, 1, 2, and 4 days after application at three locations in 1984 and 1985.

Rate of application[a]	Stalk position	Time after application	1984 Whiteville[b]	1984 Clayton[c]	1985 Whiteville[b]	1985 Reidsville[c]
--kg/ha--		--days--	ppm[d]			
0.00	Bottom	0	<0.01	0.23	1.61[f]	0.22[f]
		1	0.08[e]	1.46[e]	0.16[f]	0.23[f]
		2	0.01[e]	0.14[e]	0.07[f]	0.14[f]
		4	<0.01[e]	0.07[e]	0.07[f]	0.09[f]
	Top	0	<0.01	0.16	1.70[f]	0.14[f]
		1	0.08[e]	0.20[e]	0.17[f]	0.19[f]
		2	<0.01[e]	0.16[e]	0.08[f]	0.08[f]
		4	0.04[e]	<0.01[e]	0.06[f]	0.04[f]
1.35	Bottom	0	17.30	42.90	20.73	14.16
		1	6.79	11.43	4.04	5.48
		2	2.64	5.30	2.55	2.53
		4	0.83	2.54	0.64	1.90
	Top	0	41.50	64.73	110.30	117.70
		1	11.58	8.23	8.99	11.08
		2	5.17	5.03	3.11	2.20
		4	0.79	1.11	0.43	0.65
2.70	Bottom	0	35.47	61.68	-----	-----
		1	12.76	25.54	-----	-----
		2	12.02	16.70	-----	-----
		4	1.85	7.86	-----	-----
	Top	0	115.30	118.80	-----	-----
		1	25.15	21.93	-----	-----
		2	15.79	15.88	-----	-----
		4	4.36	4.08	-----	-----
--Source of variation--			Significance Level			
Rate			0.02	<0.01		
Stalk position			<0.01	0.02	<0.01	<0.01
Stalk position and time					<0.01	<0.01
Time			<0.01	<0.01	<0.01	<0.01
Rate x stalk position			0.03	0.16		
Rate x time			<0.01	<0.01		
Rate x stalk position x time			<0.01	<0.01		

[a] Flumetralin applied June 25, 1984, at the Border Belt Research Station, Whiteville, N.C.; July 16, 1984, at the Central Crops Research Station, Clayton, N.C.; June 15, 1985, at the Border Belt Research Station; and July 15, 1985, at the Upper Piedmont Research Station, Reidsville, N.C.
[b] Values for Whiteville based on an average moisture content of 76.85% (1984) and 80.72% (1985).
[c] Values for Clayton based on an average moisture content of 81.53% (1984) and 78.18% (1985).
[d] Wet weight basis.
[e] Only one of four samples analyzed.
[f] Values from controls omitted from the analyses of variance as they were composited over replications.

spraying on day 0 (June 17, Whiteville; July 15, Reidsville). Samples were processed as in 1984. The third row in each plot was harvested as the leaves matured. The tobacco was flue-cured in bulk barns and yields were recorded. In addition to individual samples, weighted composite samples of lamina tissue over all stalk positions were saved for analysis.

1986 Experiments

Experiments were conducted at the Upper Piedmont Research Station. Plots, treatments, and cultivars were the same as described for the 1985 experiments. Four replications were used. Tobacco was harvested and flue-cured as described previously.

Analytical Procedure for Flumetralin Residues in Tobacco

The method of analysis by Cheung & Kahrs (1) was modified for this study.

Extraction. Ten grams of dry tobacco were blended for 10 min at low speed with 200 mL of methanol:water (9:1) (v:v) and filtered through a GF/B fiberglass filter (5.5-cm diam) (Whatman International Ltd., Maidstone, England) under reduced pressure. The volume was reduced to about 40 mL under reduced pressure at 40°C and transferred to a 500-mL separatory funnel. One hundred sixty mL of water, 160 mL of saturated sodium chloride solution (317.0 g/L), and 50 mL of methylene chloride were added and shaken gently for 30 sec. The methylene chloride was drained through anhydrous Na_2SO_4 into a 500-mL boiling flask, and the extraction was repeated three times. The Na_2SO_4 was rinsed with an additional 15.0 mL of methylene chloride, and the combined fractions were evaporated under reduced pressure at 40°C just to dryness.

Cleanup. A 30 by 2.5 cm (i.d.) glass chromatography column, containing 25 g of deactivated Florisil (8% water) and topped with 2.5 cm of anhydrous Na_2SO_4, was used to remove coextractives. After prerinsing the column with 50 mL of hexane, 10 mL of hexane were added to the sample flask, the flask was swirled, and its contents were added to the column. This was repeated twice. An additional 30 mL of hexane were added to the column, allowed to drain through, and discarded. Flumetralin was eluted with 150 mL of 10% ethyl ether in hexane, concentrated to 2-3 mL under reduced pressure at 40°C and transferred quantitatively to 12-mL tubes for gas-liquid chromatography.

Analysis. The chromatograph was a Tracor Model 222 equipped with a ^{63}Ni Electron Capture Detector. The column was U-shaped glass (183 by 0.2 cm) packed with 3% SP-2250 on Supelcoport (80/100). Nitrogen was used as the carrier gas at a flow rate of 30 mL/min to the column and 20 mL/min to the detector. Temperatures were as follows: oven, 215°C; detector, 285°C; and inlet, 210°C. Chromatographic data were quantitated by the peak height method using standards of known concentration (5). To determine the accuracy of the method, both green and cured tobaccos from untreated plots were fortified with known amounts of flumetralin and analyzed with each set of experimental samples. Two fortified samples were analyzed with each sample set by adding 1.0 mL of hexane containing varying amounts of flumetralin to 10.0 g of untreated tobacco, mixing thoroughly, and letting the samples sit under a fumehood for two hours before extraction. The values were compared to two tubes containing equivalent amount of flumetralin, which were refrigerated during sample analysis, and then diluted to the same volume as the fortified tobacco extracts.

RESULTS AND DISCUSSION

The analytical method used to determine flumetralin residues was reliable down to a detectable limit of 0.01 ppm. Chromatograms were free of coextractives, and there were no interfering compounds eluting around the flumetralin peak. Recoveries of flumetralin added to green tobacco in amounts of 0.01-100 ppm (45 samples) averaged 94.9%. Recoveries of flumetralin in cured leaf samples fortified at levels of 0.01-3.0

ppm (16 samples) averaged 89.3%. Data from the field experiments were not corrected to reflect extraction efficiencies.

Flumetralin residues on green leaves were, as expected, dependent on the rate of application (**Table 1**). Average residues on leaves immediately after application of flumetralin applied at 1.35 kg/ha averaged 41 and 65 ppm on upper stalk leaves from Whiteville and Clayton, respectively, and they decreased to about 1.0 ppm by day 4. When flumetralin was applied at 2.7 kg/ha, residue levels were about two times higher on upper stalk green leaves compared to residue levels after application of the 1.35 kg/ha rate (**Table 1**). Residues remained higher over the remaining three harvests for leaves treated at the higher rate. Levels generally were higher at Clayton compared to Whiteville, which might have resulted from lower temperatures and humidity at Clayton during and after application.

Residue levels at Whiteville were similar on lower leaves in 1985 and 1984. However, residues on upper leaves at day 0 (1985) were about 2.5 times higher in 1985 than in 1984 (**Table 1**). The day 0 residue levels from Reidsville were similar to those from Whiteville in 1985 (**Table 1**). However, subsequent samplings from both locations in 1985 were similar to data seen in 1984. We have no good explanation for this, but we believe that the difference in residue levels from day 0 samples probably was due to differences in the height between the hand-held spray boom and the top of the plant when flumetralin was applied.

Flumetralin disappeared most rapidly from upper leaves. Residues on the lower leaves at four days after application were similar to those on upper leaves. The average loss of flumetralin over four days for the 1.35 kg/ha rate was 99.6% for upper stalk leaves and 93.2% for lower stalk leaves. This suggests that shading by higher leaves protected flumetralin residues on lower leaves from loss due to volatilization (volatility: 1 x 10^{-6} mm Hg, 20°C) or decomposition by photolysis.

Yields of cured tobacco ranged 3000-3500 kg/ha at Whiteville and 2200-2500 kg/ha at Clayton and Reidsville, but they were unaffected by any of the chemical treatments employed in these experiments.

Residues on cured leaves were low for all harvests in 1984 and 1985 (**Table 2**). Highest flumetralin residues after curing were found on lower stalk leaves (first harvest), and lowest residues were found on middle and upper stalk leaves (fourth or fifth harvests). So that residue levels on green leaves could be compared to those on cured leaves, the data were converted to a common moisture percentage of 13%. Using this conversion, residues on green lower leaves for the 1.35 kg/ha rate at day 4 become 3.04 ppm (Whiteville, 1984), 11.96 ppm (Clayton, 1984), 2.91 ppm (Whiteville, 1985), and 7.56 ppm (Reidsville, 1985). When these values are compared to corresponding values for cured tobacco (**Table 2**) it is evident that additional losses of flumetralin occurred between day 4 and the time of harvest or that the curing process reduced residues. Some loss probably occurred during both periods.

Low residues were detected on control samples in many instances in these experiments. Contamination of samples occurred either from spray drift in the field, or more likely from volatilization from treated samples and deposition on the controls in the curing barns. Because transfer of residues had not been seen in previous studies, nontreated tobacco was placed in separate racks and cured with treated samples.

Residues of flumetralin on composited samples of lamina in 1985 provided measurements of the average residue levels for the whole stalk tobacco. The residues for the 1.35 kg/ha treatment varied from 0.10 to 0.17 ppm. This confirms that flumetralin residues generally are low on flue-cured tobacco when this growth regulator is used at a recommended rate that provides good sucker control.

Residues of flumetralin from Reidsville in 1986 are shown in **Table 2**. The mean residue for the first harvest was 0.79 ppm, and levels decreased progressively from the first to the fourth harvest.

Table 2. Residues of flumetralin on flue-cured tobacco from three locations in 1984, 1985, and 1986.

Rate of application	Harvest number	1984[a] Whiteville[c]	1984[a] Clayton[d]	1985[a] Whiteville[c]	1986[b] Reidsville[e]
--kg/ha--		ppm			
0.00[f]	1	0.03	0.18	0.03	0.13
	2	0.01	0.01	0.02	0.11
	3	0.02	0.07	0.01	0.08
	4	0.02	0.03	<0.01	0.06
	5	<0.01	-----	<0.01	-----
1.35	1	0.24	0.98	0.34	0.79
	2	0.09	0.40	0.09	0.52
	3	0.05	0.20	0.02	0.48
	4	0.05	0.10	0.02	0.29
	5	0.02	-----	0.01	-----
2.70	1	0.25	1.56	-----	-----
	2	0.15	0.79	-----	-----
	3	0.05	0.72	-----	-----
	4	0.03	0.29	-----	-----
	5	0.03	-----	-----	-----
--Source of variation--		Significance Level			
Chemical treatment				0.13	
Chemical x harvest				0.99	
Rate of application		0.70	<0.01		
Harvest number		<0.01	<0.01	<0.01	
Rate x harvest		0.88	0.25		

[a] Residues based on 13.0% moisture level; each value is an average of four replications.
[b] Values from composited samples.
[c] Tobacco harvested 9 July, 2, 9, 15, 22 August, 1984; 2, 23 July, 6, 20, 26 August, 1985.
[d] Tobacco harvested 19, 27 July, 16, 22 August, 1984.
[e] Tobacco harvested 7, 22 August, 9, 29 September, 1986.
[f] Values for the control plots were omitted from the analyses of variance, because they were composited by harvest.

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THE EFFECTS OF A CHITIN-UREA SOIL AMENDMENT ON GLOBODERA TABACUM TABACUM POPULATION CHANGES UNDER SHADE TOBACCO

TOBACCO SCIENCE

By J.A. LaMondia[1]

The preplant application of cottonseed meal-based fertilizer (213 kg N/ha) or chitin-urea amendments (213 or 426 kg N/ha) to soils infested with *Globodera tabacum tabacum* did not affect nematode soil densities or reproduction in shade tobacco over two years of application. Populations of chitinolytic organisms were higher in soil with chitin-urea amendments, but they were not correlated with nematode control or with nonviable *G. t. tabacum* juveniles and eggs. The application of chitin-urea amendments adversely affected leaf quality characteristics in 1989. There were no differences in the fresh weight leaf yields between meal-based fertilizer and chitin-urea amendments.

Additional key words: *Nicotiana tabacum*, tobacco cyst nematode.

INTRODUCTION

The tobacco cyst nematode, *Globodera tabacum tabacum* (Lownsbery & Lownsbery 1954) Behrens 1975 suppresses the growth and yield of Connecticut shade-grown cigar wrapper tobacco (5,8). Currently, *G. t. tabacum* is controlled by preplant soil fumigation with 1,3-dichloropropene (22) or by use of systemic nematicides such as oxamyl (5). Other nematicides have been shown to reduce *G. t. tabacum* densities and increase yields, but these tactics result in reduced leaf quality (22).

Chitin (poly-B-(1-4)-N-acetyl-D-glucosamine) is a polysaccharide component of fungal cell walls, insects (11), and tylenchoid nematode eggs (1). Chitin or chitin-based soil amendments reduce populations of plant parasitic nematodes (6,9,14,17,18), including cyst nematodes such as *Heterodera glycines* Ichinohe (15), *H. avenae* Wollenweber (17), and *H. schachtii* Schmidt (20). A dual mode of action has been proposed for the activity of chitin against nematodes. First, chitin decomposition results in the release of ammonia, which is toxic to nematodes (20). Second, the increase and stimulation of populations of chitinolytic bacteria, actinomycetes, and fungi may increase the biological control activity of amended soils (9,14, 17,18,19,20).

A commercially available chitin-urea amendment (Clandosan 618: 25% chitin amended with organic buffers, urea, and minerals) reduces nematode densities (14). The objectives of this research were: (1) to determine the effects of up to 4.4 metric ton/ha of Clandosan 618 applied in two consecutive years on population densities of *G. t. tabacum* in the soil; and (2) to determine the effects of this chitin-urea amendment on yields of shade tobacco and quality of cigar wrapper tobacco.

MATERIALS AND METHODS

Experiments were conducted in 1989 and 1990 at the Connecticut Agricultural Experiment Station Valley Laboratory in Windsor, Conn. Experimental plots (4.6 X 9.2 m) were established in a cloth-covered shade tent (12) in soil (Entic Haplorthod; 71.8% sand, 23.0% silt, 5.2% clay, pH 6.2, 4.0% organic matter) naturally infested with *G. t. tabacum*. Two weeks before plantings on 18 May 1989, 2.2 or 4.4 ton/ha of Clandosan 618 (10.4-2.3-1.3 N-P-K) or a cottonseed meal-base tobacco fertilizer (5.9-2.8-6.1 N-P-K) at an N rate equivalent to 2.2 ton/ha of Clandosan 618 were broadcast over the plots and incorporated by tilling to 15 cm deep. A preplant application of diazinon (2.2 kg [a.i.]/ha) and metalaxyl (1.1 kg [a.i.]/ha) was incorporated into all plots 24 hours before transplantation (12) according to standard commercial practice. Two-month-old seedlings of the shade tobacco cultivar '0-40' were transplanted on 1 June 1989 to four rows per plot. Plots were one meter apart with 35 cm between plants within rows. All plants were treated identically with respect to sidedress fertilization, cultivation, hand suckering, tying, and harvesting. Tobacco was sidedressed with 28 kg N/ha as cottonseed meal based fertilizer 2, 3, and 4 weeks after transplantation. Foliar insects were controlled by acephate (1 kg [a.i.]/ha) applied to all plots as needed. There were six replicate plots of each treatment.

G. t. tabacum densities in soil were determined by removing and bulking 50 cores (2.5-cm-diam to 15 cm deep) per plot before adding the cottonseed-based fertilizer or the chitin-urea amendment. After harvest and tilling with a disk, soils were sampled again. All soils were air dried and mixed well, and then *G. t. tabacum* cysts were extracted from 250 cm^3 of soil with a modified Fenwick can (5). Cysts were crushed in water and two aliquots of nematodes in suspension were counted to determine the number of free second-stage juveniles (J2) and J2 remaining inside eggs per cm^3 of soil. Viable and nonviable juveniles were visually differentiated and counted separately. Initial *G. t. tabacum* densities ranged 19-86 juveniles per cm^3 of soil. Initial densities averaged 50.1, 45.2, and 44.0 juveniles per cm^3 of soil for the plots that received 0.0, 2.2, and 4.4 ton/ha of the chitin-urea amendment, respectively. Population changes over a season were expressed as the ratio of final (Pf) to initial (Pi) juveniles per cm^3 of soil.

On 22 May 1990, conventional fertilizer or chitin-based nematicide treatments were applied to the same plots as in 1989. Tobacco seedlings (cultivar 0-40) were transplanted on 5 June 1990, and plots were subject to the same conditions and treatments as above.

In both 1989 and 1990, three leaves per plant were harvested on each of six weekly intervals starting on 31 July. The third harvest, typically the best quality, was cured and analyzed for quality characteristics by Culbro, Inc. in a blind test.

G. t. tabacum populations were sampled before treatment and after the final harvest as before. In 1990, populations of chitinolytic organisms were determined by dilution plating three 1-g aliquots of moist soil onto chitin agar (6,7). Serial dilutions of soil suspensions in sterile distilled water were distributed onto the media, spread over the surface with a glass rod, and incubated at ambient temperatures for 10 days. Populations of organisms that formed halos in the media due to chitin degradation were expressed as colony-forming units per gram of soil (oven dry wt equivalent).

Data were analyzed by analysis of variance (ANOVA), and means were separated by the protected least significant difference procedure (16). The ratios of final to initial nematode densities were transformed to arcsines before ANOVA to stabilize variance. Quality rating data from 1989

[1] Associate Scientist, Department of Plant Pathology and Ecology, Connecticut Agricultural Experiment Station, Valley Laboratory, P.O. Box 248, Windsor, Conn. 06095.

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Table 1. Effects of chitin-urea amendments or a conventional fertilizer treatment on changes in viable or nonviable *Globodera tabacum tabacum* population densities over two years.

Treatment	Application rate	Viable Pf/Pi[a]			Nonviable Pf/Pi[a]		
		1989	1990	Total	1989	1990	Total
	-- kg N/ha --						
Cottonseed meal fertilizer	213	3.8	2.1	7.7	2.7	1.3	4.0
Chitin-urea[b]	213	3.5	2.3	7.4	3.0	1.6	4.6
Chitin-urea[b]	426	2.6	2.1	5.3	2.2	1.4	3.1
ANOVA		NS	NS	NS	NS	NS	NS

[a]Pf/Pi (ratio of final to initial populations): total = fall 1990/spring 1989 nematode densities.
[b]Clandosan 618.

Table 3. The influence of two years of chitin-urea amendments or conventional fertilizer application on chitinolytic organisms in soil.

Treatment	Application rate	Chitinolytic organisms
	-- kg N/ha --	-- CFU X 10^5 --
Cottonseed meal fertilizer	213	2.4
Chitin-urea[b]	213	8.6
Chitin-urea[b]	426	12.6
LSD (P=0.05)		5.4

[a]Total chitinolytic organisms (bacteria, actinomycetes, and fungi) per g soil (oven dry weight equivalent).
[b]Clandosan 618.

and 1990 were analyzed by the nonparametric Kruskal-Wallis test (2).

RESULTS

The preplant application of the chitin-urea amendment to soils naturally infested with *G. t. tabacum* did not result in significant differences in nematode Pf/Pi ratios between conventional fertilization and chitin-urea treatments in either 1989 or 1990, or after two years of application (**Table 1**). Numbers of nonviable juveniles and eggs recovered from cysts were not different in 1989, 1990, or after two years of chitin-urea application when compared to conventional applications of cottonseed meal-based fertilizer.

There were no differences in fresh leaf weights between treatments that received the meal-based fertilizer or the chitin-urea amendment (**Table 2**). However, leaf quality characteristics differed among treatments in 1989. Leaf color and body were adversely affected by preplant chitin-urea application compared to conventional fertilizer applications. Leaf size, vein quality (thin, inconspicuous veins for wrapper leaves), burn (rate and color), and taste were not affected. In 1990, there were no differences in leaf quality among treatments; however, leaf quality was poor for all treatments.

After two successive years of treatments to field plots, the number of chitinolytic organisms, primarily bacteria and actinomycetes, was greater in plots receiving the chitin-urea amendment (**Table 3**). There were no significant differences in numbers of chitinolytic organisms between the two rates of chitin-urea application.

DISCUSSION

A chitin-urea soil amendment was not effective in reducing *G. t. tabacum* soil populations or in reducing nematode reproduction in shade tobacco over two years of application in Connecticut. Numbers of chitinolytic organisms in soil were higher after two years of application of the chitin-urea amendment. However, the increase in chitinolytic organisms was not correlated with an increase in nonviable, or potentially parasitized, *G. t. tabacum* juveniles and eggs.

The application of chitin-urea amendments significantly decreased body and color quality characteristics of cured leaves in 1989. Leaf quality was generally poor for all treatments in 1990, probably due to high (80-215 J2/cm^3 of soil) *G. t. tabacum* populations.

Cottonseed meals are the main sources of nitrogen in shade tobacco fertilizer, and they were used as an equivalent nitrogen control in these experiments. The breakdown of cottonseed meal proteins to ammonia usually reaches a maximum within two weeks. The production of nitrites from ammonia by *Nitrosomonas* and conversion of nitrite to nitrate by *Nitrobacter* occurs quickly in the light, well-drained soils where shade tobacco is produced (21). Urea and urea compounds are converted to ammonia and nitrates more quickly than cottonseed meal-based fertilizers (3,6). While urea may be substituted for meal fertilizers in shade tobacco production, rapid decomposition can lead to problems in timing nitrate availability with tobacco growth.

Miller et al. (10) showed that 242 kg N/ha applied as cottonseed meal may temporarily suppress tobacco root

Table 2. Effects of chitin-urea amendments or a conventional fertilizer treatment on leaf yield and quality of shade tobacco.

Treatment	Application rate	Yield[a]	Quality Characteristics[b]					
			Body	Color	Size	Veins	Burn	Taste
	-- kg N/ha --	-- kg/plot --						
		1989						
Cottonseed meal fertilizer	213	30.7	1.6	1.6	1.2	1.9	2.5	2.4
Chitin-urea[c]	213	32.0	2.4	2.0	1.6	2.0	1.8	2.0
Chitin-urea[c]	426	30.5	2.9	2.9	2.1	2.0	2.4	2.1
Kruskal-Wallis		-	*	*	-	-	-	-
		1990						
Cottonseed meal fertilizer	213	-	3.0	3.5	2.8	3.2	1.8	2.0
Chitin-urea[c]	213	-	3.0	3.3	3.0	3.3	2.0	2.2
Chitin-urea[c]	426	-	3.3	3.8	3.3	3.7	2.2	2.2

[a]Fresh weight yield, 18 leaves per plant harvested 3 leaves per occasion over 6 occasions.
[b]Quality ratings done by Culbro, Inc., in a blind test; ratings: 1 = excellent to 5 = poor. Rating data analyzed by the nonparametric Kruskal-Wallis Test.
[c]Clandosan 618.
*Significant at P=0.05. There were no significant differences in 1990 quality characteristics.

infection by *G. t. tabacum*. This effect may have been due to transient ammonia levels suppressive to nematodes. Root infection resumed as ammonia levels decreased (10).

The nematicidal effect of chitin-urea observed in other systems is thought to be due partially to the production of ammonia resulting from the decomposition of urea (13,19,20). Anhydrous ammonia reduces soil populations of a number of nematodes at rates in excess of 150 kg N/ha, and urea suppresses nematodes when applied at rates above 300 kg N/ha (13). Most reports of successful nematode control with chitin-urea amendments resulted from the incorporation of 0.3-4.0% amendments (w/w) in pots or small plots (4,6,15,17,20). These levels are higher than those applied in our experiments.

Studies of nematode control by means of inorganic N fertilizers indicated that effective levels far exceeded those required for crop fertilization, and they were more phytotoxic (13). The rates of cottonseed meal and chitin-urea applied in our experiments were not phytotoxic to tobacco, and this may explain the lack of nematicidal activity. Rates of chitin-urea from 1,093 to 1,868 kg/ha were phytotoxic to Brussels sprouts, but they did not control *Heterodera schachtii* populations (23).

Perhaps, because of the quick conversion of ammonia to nitrate by microbes in tobacco soils (21), and because of apparent losses of significant N fertilizers by leaching of nitrate (3,12) or evaporation of ammonia (21), greater applications of chitin-urea amendments than tested in our experiments may be required for control of *G. t. tabacum*. However, nitrate contamination of ground water is a significant hazard associated with shade tobacco production in Connecticut (12). Nitrate management is an important environmental concern and this would seem to exclude excessive rates of chitin-urea amendments, regardless of efficacy against *G. t. tabacum*.

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DIALLEL ANALYSIS OF CROSSES AMONG VIRGINIA FIRE-CURED TOBACCO CULTIVARS

TOBACCO SCIENCE

By C. A. Wilkinson, J. L. Jones, and W. M. Tilson[1]

An eleven-parent half diallel study of Virginia fire-cured tobacco cultivars was conducted to obtain estimates of combining ability and heterosis for several agronomic traits in a genetically diverse population. The experiment was grown in a randomized complete block design in three environments. Plant height, days to flower, leaf number, leaf length, leaf width, yield (kg ha^{-1}), value (\$ ha^{-1}), average price (\$ kg^{-1}), percent nicotine, and percent nitrogen were determined. Highly significant general combining ability mean squares were observed for all characters. Significant specific combining ability mean squares were observed for yield, middle leaf length, and percent nicotine. Additive and nonadditive gene action appeared to be equally important in the inheritance of yield and middle leaf length, while nonadditive gene action appeared to play a greater role in the inheritance of percent nicotine. Average heterosis values were significant for yield, value, plant height, middle leaf width, top leaf length and width, and percent nicotine. Average heterosis ranged from 6.84% for yield to -1.97% for percent nitrogen. Thirty-eight of the 55 F_1 hybrids outyielded the highest yielding parent. Although development of pure lines may be most important in the commercial production of fire-cured tobacco, F_1 hybrids could be useful in specific situations such as disease resistance.

Additional key words: *Nicotiana tabacum* L., heterosis, combining ability, yield, value.

INTRODUCTION

Estimates of genetic variance, combining ability, inbreeding depression, and heterosis have been determined from diallel studies conducted within and between various types of tobacco (*Nicotiana tabacum* L.) (1,4,5,14,15,17,20,23). Determining the relative merits and feasibility of using F_1 hybrids in commercial tobacco production were the objectives of a considerable portion of that research (2,4,5,15,17,20,21). The development of commercial cultivars in most types of tobacco has been directed toward pure lines with the exception of the burley and cigar-wrapper industries where single-cross hybrids predominate. These hybrids have been developed primarily by crossing publicly developed pure lines. Hybrids permit the breeder to combine multiple characteristics of the parental lines into a single genotype in a short time. These hybrids also generally grow faster early in the season (2,5).

Additive variance is the predominant type of genetic variance reported in flue-cured (7,20,22), burley (15), and type 22 fire-cured (14) tobaccos. However, small but significant values for heterosis and specific combining ability variances have been reported that indicate the presence of nonadditive genetic variance (5,25). Breeding procedures that lead to the development of desirable homozygous cultivars have been suggested because there is a predominance of additive genetic variance and only a small amount of heterosis (17,20,21). Hybrids have been recommended for temporary situations or for specific uses such as disease resistance.

The U.S. Department of Agriculture defines three types of fire-cured tobacco. Types 22 and 23 are grown in Tennessee and Kentucky and type 21 is grown in Virginia. These types are generally not interchangeable in the manufacture of tobacco products. Most type 21 fire-cured tobacco is exported to Europe for pipe or chewing tobacco and roll-your-own cigarettes.

In contrast to most other types of tobacco, yields of Virginia fire-cured tobacco have not been significantly improved in the past 45 years. Jones & Henderson (12) observed midparent heterosis between 2% and 14% when comparing eight Virginia fire-cured cultivars and their hybrids with a male sterile cultivar. A small but significant amount of heterosis for yield has been reported in type 22 and 23 tobaccos (14). The objectives of this research were to obtain estimates of combining ability and heterosis for several agronomic traits in type 21 fire-cured tobacco.

MATERIALS AND METHODS

Eleven fire-cured tobacco genotypes representing the most widely grown cultivars and farmer selections in Virginia were selected as parents. 'VA 309' (8), 'VA 310' (9), 'VA 312' (13), 'VA 331' (10), and 'Sears Special' (9) were released by the Virginia Agricultural Experiment Station (AES). All five of these cultivars are resistant to black root rot (*Thielaviopsis basicola* [Berkeley and Broome] Ferraris), and with the exception of VA 312 and Sears Special, all are resistant to black shank (*Phytophthora parasitica* Dastur var. *nicotianae* [Breda de Haan] Tucker). VA 312 is also resistant to tobacco mosaic virus (TMV). 'KY 170' was released by the Kentucky AES (16), and it was the only type 22 fire-cured tobacco in this study. It is resistant to TMV, fusarium wilt (*Fusarium oxysporum* Schlectend), wildfire (*Pseudomonas syringae* pv. *tabaci* [Wolf and Foster] Young et al.), and black root rot. The remaining parents ('Walker's Broadleaf', 'Lizard Tail Orinoco', 'Nance', 'Brownleaf', and 'Hastings') were farmer selections (18). Walker's Broadleaf and Brownleaf have some resistance to black root rot. The other three farmer selections are susceptible to the common diseases of fire-cured tobacco.

All possible crosses, excluding reciprocals, were made between the 11 parental lines, producing a total of 55 hybrids. Parental selfs and F_1 hybrids were grown in randomized complete block designs in three environments with three replications at each environment. Experiments were conducted for one year at the Southern Piedmont Agricultural Experiment Station (SPAES), Blackstone, Va., and for two years on a private farm (M. Pugh) in Charlotte County, Va. Experiments conducted at SPAES were grown on a Wedowee sandy loam (clayey, kaolinitic, thermic Typic Hapludults) soil. Experiments conducted at Pugh's farm were grown on a Cecil fine sandy loam (clayey, kaolinitic, thermic Typic Kanhapludult) soil. Parents and their F_1 hybrids were grown in single-row, 18-plant plots with 66 cm between plants and 122 cm between rows. Cultural and curing practices were those normally employed for fire-cured tobacco production. Characters measured were days to flower (number of days from transplanting to 50% flower), plant height (measured from ground level to the point at which the inflorescence was removed), number of leaves per plant (the number of harvestable leaves), and length and width of the middle and top leaves. Yield, value, price, percent nicotine, and percent nitrogen were evaluated on the cured leaves. Data were measured on five competitive plants per plot for all characters except yield, value, price, percent nicotine, and

[1] Assistant Professor, Professor, and Research Associate, Southern Piedmont Agricultural Experiment Station, Virginia Polytechnic Institute and State University, Blackstone, Virginia 23824.

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Table 1. Means of parents and hybrids, and average and range of heterosis for characters measured in a Virginia fire-cured tobacco diallel.

Characters	Means		% Heterosis			No. families with F_1 above high parent
	Parents	Hybrids	Avg.	Low	High	
Yield, kg ha^{-1}	1939.48	2072.21	6.84**[a]	-5.18	19.65	38
Value, $ ha^{-1}	3811.36	4063.75	6.62**	-5.50	21.88	35
Price, $ kg^{-1}	1.94	1.95	0.50	-3.60	2.55	14
Plant height, cm	75.70	77.48	2.36*	-4.20	10.86	21
Days to flower	56.15	55.95	-0.36	-5.36	4.43	9
Leaf number	13.62	13.79	1.31	-4.65	9.17	15
Middle leaf length, cm	69.91	70.27	0.51	-4.49	5.83	29
Middle leaf width, cm	30.85	31.49	2.08*	-5.53	13.04	24
Top leaf length, cm	64.70	66.10	2.16*	-4.97	9.54	25
Top leaf width, cm	26.10	26.81	2.70*	-7.76	11.91	26
Nicotine, %	4.94	5.06	2.36*	-12.70	26.59	19
Nitrogen, %	3.11	3.05	-1.97	-12.94	6.32	4

[a]*,**Indicates significance at the 0.05 and 0.01 level of probability, respectively.

percent nitrogen, which were collected on 15 plants per plot. Yield and value of the cured leaves were calculated from the plot weight and average market price of the various grades, respectively. A 30-g cured leaf composite sample from each plot was ground for determination of percent nicotine (3) and percent nitrogen (11).

A half diallel analysis was performed according to Griffing (6) for a fixed effects model with parents included (Method 2, Model 1). Genotypic effects were considered fixed and parental lines comprised the population about which inferences were made. Average heterosis and heterosis for individual hybrids were calculated as previously described (25).

RESULTS

Significant differences were observed among the 55 F_1 hybrids for all agronomic traits except average price and percent nitrogen. Among the parents, significant differences were observed for all traits except yield, value, and percent nitrogen. The lack of interaction effects indicated behavior of the entries across environments was highly consistent. None of the interaction effects were significant with the exception of a general combining ability by location interaction for leaf number and plant height.

Mean squares for parents vs crosses were highly significant for yield, value, leaf length, leaf width, and plant height, indicating the presence of heterosis. The mean of the hybrids exceeded the mean of the parents for all characters except days to flower and percent nitrogen (**Table 1**). The greatest amount of heterosis was observed for yield and value, and the individual hybrids had the largest range in heterosis. Heterosis for yield of individual hybrids ranged from -5.18% to 19.65% with an overall average of 6.84%; heterosis for value of individual hybrids ranged from -5.50% to 21.88% with an overall average of 6.62% (**Table 1**). The F_1 hybrid outyielded the highest yielding parent in 38 of the 55 hybrids and had a value greater than the highest valued parent in 35 of the 55 hybrids. Average heterosis values for plant height, middle leaf width, top leaf length and width, and percent nicotine were low, but significant. The range of heterosis values for individual hybrids for percent nicotine was similar to the range for yield and value; the range for the other characters was smaller.

The parent with the highest yield and value was VA 310, followed by VA 309 and Sears Special; Walker's Broadleaf

Table 2. Parental and hybrid means for agronomic characters of eleven Virginia fire-cured tobacco genotypes crossed in a diallel design.

Cultivar	Yield		Value		Price		Plant Height		Leaf Number		Days to Flower	
	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid
	------ kg ha^{-1} ------		------ $ ha^{-1} ------		------ $ kg^{-1} ------		------ cm ------					
WBL[a]	1755	1959	3366	3837	1.91	1.94	79.11	77.87	13.22	13.51	54.17	56.07
LTO[a]	1955	2121	3844	4176	1.92	1.95	77.67	77.62	14.67	14.08	57.17	55.93
Sears Sp.	1993	2076	4000	4049	1.98	1.93	76.89	76.28	13.22	13.53	54.67	56.12
VA 310	2110	2059	4201	4051	1.97	1.94	63.56	71.52	12.33	13.42	54.83	55.27
VA 331	1799	2082	3525	4107	1.92	1.95	79.22	78.80	14.89	14.17	56.33	56.05
VA 312	1884	2113	3731	4144	1.97	1.95	69.44	76.64	12.89	13.58	57.00	55.70
Nance	1952	2074	3864	4076	1.95	1.94	71.56	76.69	13.22	13.97	56.50	55.48
KY 170	1972	2135	3784	4158	1.90	1.93	86.44	82.57	14.22	14.12	61.50	58.22
VA 309	2053	2092	4052	4098	1.95	1.94	76.89	78.41	13.76	13.93	54.50	56.02
Brownleaf	1973	2051	3881	4017	1.94	1.94	75.89	78.79	13.33	13.87	55.50	55.75
Hastings	1889	2031	3676	3987	1.93	1.94	76.00	77.14	14.00	13.56	55.50	54.87

Cultivar	Mid Leaf Length		Mid Leaf Width		Top Leaf Length		Top Leaf Width		Nicotine		Nitrogen	
	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid
	------ cm ------		------ cm ------		------ cm ------		------ cm ------		------ % ------		------ % ------	
WBL[a]	68.22	70.30	30.44	31.63	67.56	66.36	27.67	27.32	5.31	5.07	3.27	3.18
LTO[a]	69.44	70.12	31.00	31.50	61.78	65.97	23.56	26.23	4.91	5.11	3.04	3.06
Sears Sp.	71.67	70.97	33.11	32.19	67.56	66.89	28.00	27.64	4.27	5.15	2.90	3.06
VA 310	69.44	69.77	32.44	31.40	66.67	65.74	28.89	26.94	5.01	5.12	3.17	3.07
VA 331	67.00	69.89	28.22	30.66	63.11	65.38	25.00	26.04	4.26	4.75	3.38	3.15
VA 312	74.22	73.29	31.56	32.40	68.67	69.88	25.89	27.39	5.02	5.06	2.99	3.02
Nance	66.44	68.71	30.78	31.21	62.11	63.48	26.44	26.23	4.69	4.99	3.09	3.02
KY 170	68.22	70.48	30.89	31.73	61.56	65.48	25.89	27.49	5.25	5.01	3.11	2.99
VA 309	66.56	69.44	31.00	31.63	61.89	64.31	25.11	26.39	4.64	4.85	2.95	3.04
Brownleaf	69.00	69.81	29.89	31.09	66.22	66.91	26.56	26.42	5.44	5.19	3.17	2.99
Hastings	67.78	70.16	30.00	30.96	64.56	66.68	24.11	26.76	5.52	5.29	3.15	2.99

[a]WBL = Walker's Broadleaf, LTO = Lizard Tail Orinoco.

Table 3. General combining ability effects of parental lines for agronomic characters in a Virginia fire-cured tobacco diallel.

Parent	Yield	Value	Price	Plant Height	Leaf Number	Days to Flower	Middle Leaf Length	Middle Leaf Width	Top Leaf Length	Top Leaf Width	Nicotine	Nitrogen
	kg ha^{-1}	\$ ha^{-1}	\$ kg^{-1}	cm			cm	cm	cm	cm	%	%
WBL[a]	-126.13**[b]	-251.82**	0.003	0.42	-0.31**	0.13	0.04	0.16	0.26	0.57	0.02	0.14**
LTOa	54.44*	124.50*	0.011*	0.15	0.32**	-0.02	-0.16	0.01	-0.14	-0.64*	0.06**	0.01
Sears Sp.	4.68	-15.98	-0.009	-1.34*	-0.29*	0.18	0.78	0.78*	0.88	0.93**	0.11**	0.01
VA 310	-14.85	-13.91	0.002	-6.63**	-0.41**	-0.76*	-0.56	-0.10	0.39	0.15	0.08**	0.02
VA 331	10.76	48.36	0.004	1.46*	0.41**	0.11	-0.42	-0.93**	-0.80	-0.85**	-0.34**	0.11**
VA 312	45.22*	89.69*	0.004	-0.93	-0.24*	-0.28	3.36**	1.01**	4.20**	0.65*	0.01	0.04
Nance	2.34	13.98	0.002	-0.88	0.19	-0.52*	-1.73**	-0.31	-2.91**	-0.64*	-0.07**	-0.04
KY 170	69.71*	105.04*	-0.011*	5.65**	0.36**	2.52**	0.23	0.27	-0.69	0.76*	-0.05*	-0.07*
VA 309	22.33	37.59	-0.002	1.03	0.15	0.07	-0.91*	0.16	-1.98**	-0.46	-0.23**	-0.02
Brownleaf	-23.20	-51.85	-0.001	1.45*	0.08	-0.22	-0.51	-0.45	0.90	-0.43	0.15**	-0.06*
Hastings	-45.31*	-85.59	-0.003	-0.38	-0.26*	-1.21**	-0.12	-0.59*	0.65	-0.06	0.26**	-0.07*

[a]WBL = Walker's Broadleaf, LTO = Lizard Tail Orinoco.
[b]*,**Estimate is significantly different from zero at the 0.05 and 0.01 level of probability, respectively.

had the lowest yield and value (**Table 2**). F_1 generation means for yield and value exceeded the midparent for all hybrids except Walker's Broadleaf X VA 310, Sears Special X VA 310, and Nance X VA 309. Nineteen hybrids had higher yields than the highest yielding cultivar, although these differences were not statistically different. All but seven F_1 hybrids exceeded the midparent for top and middle leaf width. F_1 generation means exceeded the midparent for all but nine and ten hybrids for top leaf length and plant height, respectively. The most frequently observed parents in these hybrids were Walker's Broadleaf and Sears Special. F_1 generation means for percent nicotine exceeded the midparent for all but 18 hybrids. Walker's Broadleaf, VA 310, and KY 170 were the parents most frequently observed in these hybrids.

Mean squares for general combining ability (GCA) were highly significant for all characters measured. Significant mean squares for specific combining ability (SCA) were observed for yield, middle leaf length, and percent nicotine. GCA and SCA effects accounted for approximately equal portions of the variation for yield and middle leaf length; however, SCA effects accounted for a greater portion of the variation than GCA effects for percent nicotine. The best parents, based on GCA effects, for improving yield and value should be KY 170, Lizard Tail Orinoco, and VA 312 (**Table 3**). KY 170 should also significantly contribute to increased plant height, days to flower, and leaf number, and decreased nicotine and nitrogen. VA 312 should significantly contribute to increased leaf lengths and widths. In contrast, Walker's Broadleaf and Hastings significantly contributed to decreased yield, value, and leaf number. The three superior crosses in terms of increased yield were Walker's Broadleaf X KY 170, Walker's Broadleaf X VA 312, and Sears Special X VA 312 (**Table 4**). These hybrids did not arise from the highest yielding parents. In contrast, the two poorest crosses were Walker's Broadleaf X Lizard Tail Orinoco and KY 170 X VA 309. Walker's Broadleaf was the lowest yielding parent and its GCA effects indicated it should contribute to decreased yield; however, it contributed to two of the three highest yielding hybrids and to one of the lowest yielding hybrids.

DISCUSSION

Heterotic responses for several agronomic traits have been reported in flue-cured (2,20,23), burley (15,21,25), type 22 fire-cured (14), oriental (17), and cigar-wrapper (5) tobaccos. In general, the reported heterotic responses in flue-cured tobacco were smaller than in the other types. Average heterosis in the fire-cured hybrids from this study was similar to the midparent heterosis of hybrids from type 21 fire-cured cultivars observed by Jones & Henderson (12). In contrast, it was greater than the average heterosis observed by Legg (12) for type 22 fire-cured hybrids, especially for yield. Although Jones & Henderson (12) observed greater heterosis in hybrids involving parents classified as thin-bodied leaf types, we did not observe this in the current study.

General combining ability, a function of additive genetic effects, reflects the average performance of a genotype in hybrid combination. Specific combining ability is detected whenever specific hybrid combinations perform better or worse than expected based on the average performance of parental lines, and it is a function of dominance and other nonadditive genetic effects such as epistasis. Our results indicate that additive gene action plays a significant role in value, price, plant height, leaf number, middle leaf width, top leaf

Table 4. Specific combining ability effects for yield (kg ha^{-1}, above diagonal) and percent nicotine (below diagonal) in a Virginia fire-cured tobacco diallel.

	WBL	LTO	Sears Sp.	VA 310	VA 331	VA 312	Nance	KY 170	VA 309	Brownleaf	Hastings
WBL[a]		-172*[b]	98	-98	26	133*	23	214**	33	68	-58
LTO[a]	-0.12*		-21	24	105	44	-11	-61	25	80	-14
Sears Sp.	-0.65**	0.26**		-74	-115	133*	-22	-46	116	-50	-21
VA 310	-0.20**	0.18**	0.63**		20	66	38	-23	2	12	34
VA 331	0.21**	-0.19**	-0.15*	0.07		-31	17	91	-44	-91	19
VA 312	0.04	0.01	-0.18**	-0.23**	-0.35**		19	-51	-20	27	-56
Nance	-0.16**	-0.45**	0.02	-0.05	0.12*	-0.01		-5	-97	-43	82
KY 170	0.45**	0.50**	-0.09	-0.20**	-0.08	0.17**	0.48**		-140*	32	-11
VA 309	0.15*	-0.35**	-0.05	0.18**	0.19**	-0.08	-0.12*	-0.45**		34	92
Brownleaf	0.33**	0.06	0.28**	-0.33**	-0.07	0.23**	-0.14*	-0.44**	0.36**		-68
Hastings	-0.28**	0.10	-0.05	-0.27**	0.26**	0.39**	0.30**	-0.35**	0.18**	-0.29**	

[a]WBL = Walker's Broadleaf, LTO = Lizard Tail Orinoco.
[b]*,**Estimate is significantly different from zero at the 0.05 and 0.01 level of probability, respectively.

length and width, and percent nitrogen. Both additive and nonadditive gene action appeared to play a significant role in the inheritance of yield, while nonadditive gene action played a significant role in percent nicotine. A predominance of additive genetic variance for most characters and some significant estimates for heterosis and SCA variance suggested the presence of nonadditive gene action in previous diallel analyses in flue-cured and burley tobacco (2,7,15,21,25).

Cured leaves of Walker's Broadleaf, VA 310, VA 331, VA 312, Nance, and Brownleaf are considered thin bodied, while Lizard Tail Orinoco, Sears Special, VA 309, Hastings, and KY 170 have heavy bodied leaves. Jones & Henderson (12) determined that two hybrids, male sterile VA 331 X Nance and male sterile VA 331 X VA 312, warranted further attention. VA 331 X Nance and VA 331 X VA 312 exceeded the high parent for yield in our study, and they had significant positive heterotic values, but other hybrids performed better. Two of the hybrids, Walker's Broadleaf X KY 170 and Sears Special X VA 312, had significant, positive SCA effects, and they also had the highest positive heterotic responses. On the other hand, Walker's Broadleaf X VA 312 had a significant, positive SCA effect for yield, but a much lower heterotic response.

The majority of the parents in this study originated in Virginia. KY 170, a type 22 fire-cured, and Brownleaf originated in Kentucky. KY 170 X Brownleaf exceeded the high parent for yield, value, leaf length, and leaf width. The majority of the hybrids between parents from Virginia and Kentucky also exceeded the high parent for yield and value and their heterotic response was moderately high. Vanderberg & Matzinger (24) and Matzinger & Wernsman (19) suggested that parental lines of diverse origin should be selected because heterosis tends to increase with increasing parental genetic diversity. Crosses between adapted flue-cured cultivars and tobacco introductions have had higher heterosis values for most characters when compared with crosses among adapted flue-cured cultivars (24). Heterosis values greater than normally obtained from hybrids among flue-cured cultivars have been observed in intertype crosses between oriental and flue-cured cultivars (19). Intertype crosses would not be agronomically acceptable or fit into accepted tobacco type classification in most cases.

Overall, the hybrids in this study were taller, had greater leaf lengths and widths, and had greater yields and value than their parents. There was very little difference between the hybrids and their parents for price, leaf number, days to flower, percent nicotine, and percent nitrogen. The combination of significant SCA effects and heterosis values indicated that nonadditive gene action plays a role in the inheritance of yield. The average heterosis for yield (6.84%) reported in this study could result in an additional gross return of \$342 ha^{-1} to the grower.

Development of pure lines has been recommended by previous researchers due to the predominance of additive genetic variance (17,20,21). Production of F_1 hybrids has been suggested for use in temporary or specific situations (20). The presence of additive genetic variance for all agronomic traits measured would suggest breeding procedures for the development of pure lines in fire-cured tobacco would be more productive. However, because both additive and nonadditive gene action play a significant role in the inheritance of yield, high yielding hybrids could be rapidly developed to meet new production problem such as disease resistance. The performance of the F_1 hybrids in this study indicates that evaluation of hybrids rather than parental performance would be necessary in the development of Virginia fire-cured hybrids.

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RESIDUES OF MH, FLUMETRALIN, AND BUTRALIN ON FLUE-CURED TOBACCO[1]

TOBACCO SCIENCE

By T. J. Sheets, H. Seltmann, and F. H. Yelverton[2]

In field experiments conducted during 1990, residues from 2.5 Kg/ha of MH applied to flue-cured tobacco averaged 126 ppm at Clayton, N.C., and 62 ppm at Reidsville, N.C. MH residues were about the same level for all harvests. Residues of flumetralin and butralin were usually highest for first harvest and declined through the fourth harvest. Butralin disappeared more rapidly from tobacco leaves than flumetralin. The highest flumetralin and butralin residues were 0.37 and 0.33 ppm, respectively, for first harvest samples at Reidsville. Tank mixing MH with flumetralin or butralin did not affect the residue levels of any of the three chemicals. The percent reducing sugars and total alkaloids were not affected by MH, flumetralin, or butralin applied alone or as tank mixes. Yields of cured leaves were reduced for treatments that did not effectively control suckers. At Clayton, the stand and vigor of a wheat cover crop planted about 100 days after application of 1.3 Kg/ha of flumetralin was reduced slightly, but no effect on the cover crop was observed for the same treatment at Reidsville. Applications of 3.4 Kg/ha of butralin did not affect wheat stand or vigor at either location.

Additional key words: *Nicotiana tabacum*, maleic hydrazide, sucker control.

INTRODUCTION

Formulated products containing the systemic growth regulator 1,2-dihydropyridazine-3,6-dione, maleic hydrazide (MH), effectively control axillary buds (suckers) of tobacco; but relatively high residues remain on cured leaves after application of the labeled rate (3,7,15). Some understanding of the behavior of MH residues on tobacco leaves has been gained through research (4,12,16). Major efforts by representatives of the U.S. tobacco industry, especially the Flue-Cured Tobacco Cooperative Stabilization Corporation and the Cooperative Extension Services in flue-cured tobacco producing states, have been devoted to reducing residues. In spite of these efforts, residues remain high. The belt-wide average for the 1991 crop was 140 ppm in one survey[3] and 111 ppm in another (14). Both surveys gave an average MH concentration of 115 ppm for the 1992 crop.

The dinitroanaline derivative, 2-chloro-*N*-[2,6-dinitro-4-(trifluoromethyl)phenyl]-*N*-ethyl-6-fluorobenzenemethanamine (flumetralin), is an excellent sucker control agent, and acreage of tobacco treated with this chemical has increased in the last two years. Carryover of flumetralin residues in soil from one season to the next has caused concern in some instances. Potential problems have been circumvented by reducing the rate of application and following improved management practices.

Another dinitroanaline compound, 4-(1,1-dimethylethyl)-*N*-(1-methylpropyl)-2,6-dinitrobenzenamine (butralin), is used in several countries for sucker control in tobacco (13), and it is under development for that purpose in the United States. The government of Spain has set a tolerance of 5.0 ppm for residues of butralin in tobacco (10,13).

Some modifications of the treatment pattern of MH and the two dinitroanaline derivatives have been considered in recent years with the objective of maintaining season-long sucker control and minimizing residues of MH. These treatments included tank mixes and sequential applications of MH and the dinitroanaline compounds (11). In view of the potential success of these approaches, we undertook a study in 1990 to compare residues of MH, flumetralin, and butralin when these growth regulators were applied alone or when the dinitroanaline compounds were applied as a tank mix with MH.

MATERIALS AND METHODS

The experiments were established at the Central Crops Research Station near Clayton, N.C., and at the Upper Piedmont Research Station near Reidsville, N.C. The soil at the Clayton site was a Dothan sandy loam (fine-loamy, siliceous, thermic, Plinthic Paleudult) containing 80% sand, 8% silt, 12% clay, and 0.20% humic matter. This soil had a pH of 6.5 and a cation exchange capacity of 2.02 $cmol_c\ Kg^{-1}$. The soil for the Reidsville site was a Cecil sandy clay loam (fine-loamy, kaolinitic, thermic, Typic Kanhapludult) containing 53% sand, 19% silt, 28% clay, and 0.25% humic matter. It had a pH of 5.5 and a cation exchange capacity of 3.75 $cmol_c\ Kg^{-1}$.

The sites were prepared for tobacco by normal practices used at the research farms. Flue-cured tobacco, cv. McNair 373, was transplanted 23 April at Clayton and 14 May at Reidsville. Rows were 1.14 m apart and plants were spaced 56 cm within the rows. Plots were four rows wide and 12.3 m long. Two applications of a fatty alcohol emulsion (FAE) containing 0.5% C-6, 42% C-8, 56% C-10, and 1.5% C-12 alcohols were made over the entire field at both locations before final topping in preparation for applications of MH, flumetralin, and butralin. The first application of FAE was a 4% concentration and the second was a 5% concentration; each was applied at a volume of 470 L/ha. The two applications of FAE were separated by 6 days at Clayton and by 9 days at Reidsville. Seven to nine days after the second application of the FAE, suckers remaining were removed by hand from all plots; and MH, flumetralin, and butralin treatments were applied. The eight treatments were (a) plants hand suckered each week from topping through the rest of the season, but no chemical sucker control, (b) 2.52 Kg (a.i.)/ha MH, (c) 1.34 Kg (a.i.)/ha flumetralin, (d) 3.36 Kg (a.i.)/ha butralin, (e) 1.26 Kg (a.i.)/ha MH + 0.67 Kg (a.i.)/ha flumetralin as a tank mix, (f) 1.26 Kg (a.i.)/ha MH + 1.68 Kg (a.i.)/ha butralin as a tank mix, (g) 2.52 Kg (a.i.)/ha MH + 1.34 Kg (a.i.)/ha flumetralin as a tank mix, and (h) 2.52 Kg (a.i.)/ha MH + 3.36 Kg (a.i.)/ha butralin as a tank mix. The eight treatments were arranged in randomized block designs with four replications at each location. Four one-row plots adjacent to the experimental area received no additional sucker control chemicals, and no suckers were removed by hand. These plots were used as a topped-but-not-suckered control.

The chemicals were applied with water as the diluent using a one-row, three-nozzle arrangement with a TG-5 tip centered over the row and a TG-3 tip mounted 25 cm to each side of the center nozzle. The two outside nozzles were set at an angle of about 45 degrees to direct the spray to the center of the plants. The height of nozzles was about 35 cm above

[1] Use of trade names in this publication does not imply endorsement of the products named or criticism of similar ones not mentioned.

[2] Professor Emeritus of Toxicology, North Carolina State University, Raleigh, N.C. 27695; Plant Physiologist, USDA-ARS, Crops Research Laboratory, Oxford, N.C. 27565; and Assistant Professor of Crop Science, North Carolina State University, Raleigh, N.C. 27695

[3] Flue-Cured Tobacco Cooperative Stabilization Corporation, Raleigh, N.C., Unpublished data.

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Table 1. Rainfall, dates of chemical applications, and harvests of flue-cured tobacco treated with sucker control agents at Clayton and Reidsville, N.C., 1990.

	Clayton				Reidsville		
Day	June	July	Aug.	Sept.	July	Aug.	Sept.
	cm						
1							
2					0.43	2.54	
3							
4							
5				Har.4[c]			
6		Appl.[a]	0.08 Har.2[c]				
7			3.53			1.63	
8			3.48				
9							0.28
10		2.21	2.77	Count[d]			
11					0.05		0.23
12							Har.3&4[c]
13		0.10			0.05	Har.1[c]	0.28
14		0.15			5.23		0.25
15		0.91	2.01		2.08	0.08	
16	1.50						
17			0.84			1.68	Count[d]
18	1.14	8.13					
19							
20			Har.3[c]				
21							
22						3.18[b]	
23	1.80	Har.1[c]				3.43	
24	1.55		4.04				
25					Appl.[a]		
26							
27					3.18[b]		
28						Har.2[c]	
29					3.18[b]		
30		0.38	0.10		1.90[b]		
31					0.78		

[a]Application of sucker control chemicals.
[b]Overhead irrigation.
[c]Harvest.
[d]Suckers counted and weighed.

the tops of plants. The tractor speed was 3.5 km/hr and the spray pressure was 152 kPa. These settings gave a spray volume of 470 L/ha.

Leaves from the center two rows of each plot were harvested four times. At each harvest, samples that received the same treatment were placed in a bulk-curing rack. At Clayton, the racks were placed in small bulk-curing barns so that treatments with different rates of dinitroanaline compounds were cured separately from one another and from hand-suckered controls. At Reidsville, samples were placed in separate racks and cured in a large curing barn. To reduce cross contamination the racks were separated by at least two racks of untreated tobacco.

Yields were recorded by harvest, and a composite sample containing an amount from each harvest proportional to the weight of the harvest was saved from each plot for analyses of reducing sugars and total alkaloids. These analyses were done in the Tobacco Chemistry Laboratory at North Carolina State University by the method of Harvey et al. (5). A 50-leaf sample from each harvest of each plot was saved for residue analyses. After storage of the samples for two months at ambient temperature, midribs were removed. The samples were air dried, ground to pass through a 1-mm screen, and stored in glass jars.

Five days after the last harvest, green weights of suckers on the middle two rows of each plot were recorded. Percent sucker control was determined by dividing the total weight of suckers on each treated plot by the average total weight of the suckers from the topped-but-not-suckered control plots.

Wheat was planted as a cover crop on 24 Oct., 1990, at Clayton and 1 Nov., 1990, at Reidsville. The time between applications of dinitroanaline compounds and planting of wheat was about 100 days at each location. Experimental areas were disked, wheat was drilled, and fertilizer was applied according to recommended practices at each location. On 29 Jan., 1991, at Clayton and on 5 Feb., 1991, at Reidsville, three persons each subjectively rated the stand and vigor of the wheat plants. Stand ratings were based on a scale of 0-100, with 100 being the best stand and 0 being no plants. Vigor ratings were based on a scale of 0-100, with 0 being no effect of the chemicals and 100 being complete kill of all wheat plants.

Samples from MH-treated plots were analyzed for residues of MH. The official method of analysis adopted by the Association of Official Analytical Chemists (6) and reported by Lane (8) has been modified somewhat and used extensively (2,3,7,15,17). For each set of samples analyzed for MH, one sample of tobacco from an untreated control was fortified with a known amount of the growth regulator. This sample was carried through the procedure to confirm that results fell within the expected range of variation.

Samples from hand-suckered controls and dinitroanaline-treated plots were analyzed for flumetralin or butralin by a method originally supplied by Ciba-Geigy Agricultural Chemicals Company, Greensboro, N.C. (1) and modified in our laboratory for use in these and other studies (9). Samples of untreated tobacco were fortified with different concentrations of flumetralin or butralin, and the spiked samples were carried through the analytical procedure to determine the efficiency of the method.

Table 2. Percent sucker control and yields of cured leaves of flue-cured tobacco treated with MH, flumetralin, and butralin applied alone and as tank mixes.

Treatment	Application rate	Yield		Sucker Control[a]	
		Clayton	Reidsville	Clayton	Reidsville
	Kg/ha	Kg/ha		%	
None (HS)[b]	0	2420	2816		
MH	2.52	2373	3156	85 ab	96 a
Flumetralin	1.34	2254	2958	79 b	84 b
Butralin	3.36	1962	3102	42 c	83 b
MH + flumetralin[c]	1.26 + 0.67	2612	3297	91 ab	97 a
MH + butralin[c]	1.26 + 1.68	2626	3341	78 b	95 a
MH + flumetralin[c]	2.52 + 1.34	2454	3032	92 ab	99 a
MH + butralin[c]	2.52 + 3.36	2636	3277	98 a	99 a
LSD ($\alpha = 0.05$)		306	326		
CV (%)		8.6	7.1	13	3

[a]Means followed by the same letter are not significantly different at the 5% level, according to Duncan's Multiple Range Test.
[b]HS = hand-suckered control.
[c]Tank mix.

Rainfall, application dates, and harvest dates for each location are reported in **Table 1**. Data for yields, sucker control, residue concentrations, wheat stand and vigor, reducing sugars, and total alkaloids were subjected to analyses of variance. The standard LSD or Duncan's Multiple Range Test were used to separate the means. Statistical analyses for residues were performed on weighted averages. A separate weighted average was calculated for each plot of MH, flumetralin, and butralin using yields by harvest as weights.

RESULTS AND DISCUSSION

At Clayton, the yield of cured leaves for the treatment with 3.36 Kg/ha of butralin applied alone was significantly less than that for the hand-suckered control (**Table 2**). No other treatment at Clayton reduced yields. Sucker control was 42% on plots that received butralin alone, 79% on plots that received flumetralin alone, and 78% on plots that received the tank mix of the low rate of MH and butralin (**Table 2**). Sucker control was 85% on plots treated with MH alone, and it was 91 and 92% on plots treated with tank mixes of both rates of MH and flumetralin. Control was 98% on plots treated with the high rate of MH plus butralin.

At Reidsville, yields for four of the treatments were greater than for the hand-suckered control (**Table 2**). None of the treatments significantly reduced yields. Sucker control was 84% and 83%, respectively, on plots treated with flumetralin or butralin alone (**Table 2**). Control on all other plots was excellent (95-99%).

Table 3. MH residues on flue-cured tobacco as affected by tank mixing with flumetralin and butralin at two locations in 1990.

Treatment[a]	Application rate	Harvest number	Clayton	Reidsville
	--------- Kg/ha ---------		---------- ppm ----------	
None (HS)[b]	0	1	<10	<10
		2	<10	<10
		3	<10	<10
		4	<10	<10
		Avg[c]	**<10**	**<10**
MH	2.52	1	104	55
		2	136	47
		3	136	60
		4	130	66
		Avg[c]	**125**	**59**
MH + flumetralin (tank mix)	1.26 + 0.67	1	52	28
		2	75	30
		3	66	23
		4	73	27
		Avg[c]	**67**	**27**
MH + butralin (tank mix)	1.26 + 1.68	1	47	31
		2	58	30
		3	58	25
		4	60	31
		Avg[c]	**56**	**30**
MH + flumetralin (tank mix)	2.52 + 1.34	1	103	60
		2	137	65
		3	131	68
		4	132	71
		Avg[c]	**124**	**67**
MH + butralin (tank mix)	2.52 + 3.36	1	103	64
		2	139	61
		3	139	56
		4	142	64
		Avg[c]	**128**	**61**
LSD (α = 0.05)[d]			19	20
CV (%)			12.5	26.4

[a]*Samples from treatments of flumetralin and butralin applied alone were not analyzed for MH.*
[b]HS = hand-suckered control.
[c]Weighted averages (bold face) were calculated from yields and MH residues by harvests.
[d]The statistical values apply to the weighted averages only.

Residues of MH were about twice as high at Clayton as they were for comparable treatments at Reidsville (**Table 3**). Weighted averages of MH residues for treatments at Clayton that received 1.26 Kg/ha of MH were 67 and 56 ppm; MH residues from the same treatments at Reidsville were 27 and 30 ppm. All of the plots at Clayton that received the full rate of MH (2.52 Kg/ha), including the tank mixes, averaged 126 ppm MH residues; at Reidsville the corresponding value was 62 ppm (**Table 3**). Differences of this magnitude among locations in levels of MH residues are not unusual (7,17). Overall, the poorest sucker control was obtained at Clayton (**Table 2**) where MH residues were highest. Tank mixing MH with flumetralin or butralin did not affect MH residue levels. MH residues did not decline from the first harvest through the third or fourth harvests (**Table 3**), as has been demonstrated in previous research (7).

Table 4. Recoveries of flumetralin and butralin from tobacco samples fortified before extraction with known amounts of these growth regulators.

Compound	Fortification level	Number of samples	Recovery Range	Recovery Average
	-------- ppm --------		---------- % ----------	
Flumetralin	0.02	1	100	100
	0.03	3	87-143	110
	0.05	2	74	74
	0.10	4	86-104	92
	0.20	1	85	85
	0.30	1	88	88
	Avg.	**12**	**86-143**	**93**
Butralin	0.05	2	70-118	94
	0.10	1	100	100
	0.20	1	71	71
	0.50	2	64-79	72
	2.00	1	103	103
	3.00	5	61-92	79
	Avg.	**12**	**61-103**	**83**

Recoveries for flumetralin and butralin are shown in **Table 4**. Except for a high value of 143% for one sample spiked at 0.03 ppm, recoveries fell within the expected ranges. Recovery values for butralin ranged 61-118%. Averages for all recoveries were 93% and 83% for flumetralin and butralin, respectively (**Table 4**). Residue values for dinitroanaline compounds were not corrected for incomplete recovery.

Residues of flumetralin and butralin were usually highest on tobacco from the first harvest, and they declined

Table 5. Residues of flumetralin and butralin in flue-cured tobacco after applications of flumetralin and butralin alone or as tank mixes with MH.

Growth regulator	Treatment[b] number	Application rate	Harvest number	Residues[a] Clayton	Residues[a] Reidsville
		------ Kg/ha ------		---------- ppm ----------	
Flumetralin	3	1.34	1	0.17	0.37
			2	0.12	0.10
			3	0.12	0.10
			4	0.10	0.07
			Avg.	**0.13**	**0.13**
Butralin	4	3.36	1	0.26	0.32
			2	<0.05	0.21
			3	0.05	<0.05
			4	0.06	<0.05
			Avg.	**0.11**	**0.13**
MH + flumetralin (tank mix)	5	1.26 + 0.67	1	0.07	0.18
			2	0.19	0.08
			3	0.08	0.05
			4	0.09	0.03
			Avg.	**0.11**	**0.07**
MH + butralin (tank mix)	6	1.26 + 1.68	1	<0.05	0.21
			2	<0.05	0.07
			3	<0.05	<0.05
			4	<0.05	<0.05
			Avg.	**<0.05**	**0.07**
MH + flumetralin (tank mix)	7	2.52 + 1.34	1	0.30	0.18
			2	0.24	0.10
			3	0.17	0.11
			4	0.16	0.11
			Avg.	**0.22**	**0.13**
MH + butralin (tank mix)	8	2.52 + 3.36	1	0.19	0.33
			2	0.09	0.16
			3	<0.05	<0.05
			4	<0.05	<0.05
			Avg.	**0.10**	**0.12**
Orthogonal Contrasts				Probability of a Greater F	
Treatments 3 and 4 *vs* 7 and 8				0.15	0.54
Treatment 3 *vs* 4				0.68	0.95
Treatment 5 *vs* 6				0.03	0.98
Treatment 7 *vs* 8				<0.01	0.55
CV (%)				44.4	29.9

[a]Flumetralin ranged <0.02-0.03 ppm ($\bar{x}$ = 0.02, n = 6) and butralin ranged <0.05-0.07 ppm ($\bar{x}$ = <0.05, n = 10) from untreated control plots. Samples from treatments 3, 5, and 7 were analyzed for flumetralin and samples from treatments 4, 6, and 8 were analyzed for butralin residues. Weighted averages (bold face) were calculated from yields and residue levels by harvests. Statistical values apply to the weighted averages only.

[b]Samples from the hand-suckered treatment (Treatment 1) and from MH applied alone (Treatment 2) were not analyzed for butralin or flumetralin.

Table 6. Stand and vigor ratings of wheat planted in the fall after application of MH, flumetralin, and butralin for sucker control in tobacco.

Growth regulator	Application rate	Clayton[a] Stand	Clayton[a] Vigor	Reidsville[a] Stand	Reidsville[a] Vigor
	------ Kg/ha ------	---------- % ----------			
None (HS)[b]	0	95 ab	0.6 b	94[c]	0.7[c]
MH	2.52	98 a	0.8 b	91	0.9
Flumetralin	1.34	87 b	1.1 ab	91	1.1
Butralin	3.36	92 ab	0.7 b	92	0.9
MH + flumetralin (tank mix)	1.26 + 0.67	97 a	0.9 ab	91	1.3
MH + butralin (tank mix)	1.26 + 1.68	98 a	0.6 b	90	1.0
MH + flumetralin (tank mix)	2.52 + 1.34	78 c	1.7 a	91	1.3
MH + butralin (tank mix)	2.52 + 3.36	92 ab	1.2 ab	91	1.2
CV (%)		6.3	55.5	5.3	64.7

[a]Within a column means with the same letter are not significantly different at the 5% probability level. Stand ratings were based on a scale of 0-100 with 100 being the best stand and 0 being no plants. The vigor rating scale was 0-100 with 0 being no effect and 100 being complete kill of plants.

[b]HS = hand-suckered control.

[c]No significant differences between treatment means.

through the fourth harvest (**Table 5**). Comparison of weighted averages shows that residues of butralin were about equal to or less than those of flumetralin even though the application rate of butralin was about 2.5 times greater than the application rate of flumetralin. Therefore, we concluded that butralin was less persistent on leaves than flumetralin. The highest average residues for both locations were 0.37 ppm of flumetralin and 0.33 ppm of butralin (**Table 5**). There was no effect of tank mixing flumetralin or butralin with MH on residue levels of these dinitroanaline compounds.

The percent reducing sugars and total alkaloids in cured leaves were not affected by the growth regulator treatments (data not shown).

There was no effect of soil residues of either of the dinitroanaline derivatives on the stand and vigor of wheat at Reidsville (**Table 6**). At Clayton the 1.34 Kg/ha rate of flumetralin reduced stand and vigor of the wheat cover crop, but the reductions were not severe. The clay percentage of the soil at Clayton was less than half that of the soil at Reidsville, and this difference may explain the slightly greater persistence of flumetralin in soil at Clayton. Butralin did not reduce stand or vigor at Clayton.

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FIELD ASSESSMENT OF VIRUS RESISTANCE IN TRANSGENIC *NICOTIANA TABACUM* CV. BURLEY 49 PLANTS EXPRESSING TOBACCO ETCH VIRUS SEQUENCES

TOBACCO SCIENCE

E. B. Whitty[1], R.A. Hill[1], R. Christie[1], J.B. Young[2], J. A. Lindbo[3], and W. G. Dougherty[3,4]

Nicotiana tabacum cv. Burley 49 germplasm, transformed with mutated versions of the tobacco etch virus (TEV) coat protein gene, was tested under field conditions for tolerance and resistance to TEV. Twenty-four independent transgenic lines and eight different mutated versions of the TEV coat protein gene were examined. The test was conducted at the same field location for two successive years. Transgenic plants expressing an mRNA, which was translated into a full length TEV coat protein, became infected when challenged with TEV but then recovered from TEV infection 3 - 4 weeks later. The same response was displayed by plants containing a gene lacking the codons for 29 amino acids at the amino terminus of the TEV coat protein. Plants containing a gene that expressed an antisense RNA version of the TEV coat protein sequence displayed little tolerance to TEV. A similar response to TEV was observed with plants containing only regulatory gene sequences and no TEV-derived sequences, or with plants having no foreign gene sequences at all (Burley 49). Selected entries expressing untranslatable versions of the TEV coat protein mRNA never became infected with TEV, and they displayed a high level of resistance. This study confirms the use of transgenic plants expressing an untranslatable sense RNA as a promising approach in the development of potyvirus-resistant tobacco germplasm.

Additional key words: Potyvirus, transgenic plants, RNA-mediated resistance, Burley tobacco.

INTRODUCTION

The concept of pathogen derived resistance (PDR) suggests that the genome of the pathogen may be a convenient source of resistance genes (30). Pathogen genes could be isolated and altered such that they are expressed in a host organism. Expressing a pathogen's gene at the wrong time, at an elevated level, or in a mutated form could interfere with the highly evolved and coordinated pathogen-host interaction, and this may result in an alteration or abortion of infection.

A number of PDR approaches to control plant viruses and the losses they incur have been tested (1, 3, 11). Many studies demonstrated that the expression of a plant virus coat protein is usually an effective way to generate virus-resistant plants (3). Other approaches have expressed other parts of the viral genome (4, 13, 22) or have expressed non-viral nucleic acids associated with certain virus infections (12, 17). In general, the results of these studies suggest PDR can be implemented in a variety of ways to generate virus-resistant plants.

The resistance generated by these various PDR approaches typically is manifested as a delay in the appearance of symptoms, an attenuation of symptoms, a decrease in virus concentration (24), or in limited cases, a highly resistant state in which no virus can be detected (13, 19, 20). Most of these assessments were made in greenhouse tests under physical confinement. However, over the past five years, a limited number of field studies have been conducted to assess the effectiveness of PDR (18, 29). Many of these studies have confirmed greenhouse results, and they suggest PDR will be an efficient way to generate virus-resistant plants once regulatory concerns are addressed.

Various PDR approaches have been used in attempts to engineer resistance to potyviruses (4, 9, 19, 20, 21, 23, 27, 31). We generated a series of transgenic plants that express various mutated versions of the tobacco etch virus (TEV) coat protein (19, 20). TEV, potato virus Y (PVY), and tobacco vein mottling virus are members of the potyvirus group, and they represent commonly encountered viral pathogens of tobacco (2, 8, 28). Our transgenic plant lines displayed a variety of resistance levels to infection with TEV in greenhouse trials (19). The present study was conducted to answer two questions about the transgenic material: would resistance to TEV be manifested under field conditions and was plant type affected by the transformation, regeneration, and transgene expression? This two-year study suggests excellent virus resistance can be generated, but that plant type can be affected by the expression of selected viral gene products.

MATERIAL AND METHODS

Transgenic Plant Germplasm

Nicotiana tabacum cv. Burley 49 was selected for transformation because of the severe reaction this cultivar displays when inoculated with TEV (16). The construction of the transgenes, transformation procedures and other characteristics of these transgenic plant lines have been described (19). The various transgenes (and their nomenclature) expressed in plants examined in this field study are described in **Figure 1.**

Field Plot Design

In 1992, seeds of 16 different entries were sown on a commercial potting mix. When plants reached a height of 5 - 8 cm, they were transferred to styrofoam trays in a greenhouse float system and grown to transplanting size. Prior to transplanting, each entry was inoculated (as described later) with a local isolate of TEV. Plants were transplanted with a commercial transplanter into a field located on the north side of the University of Florida Agronomy Farm (Green Acres) northwest of Gainesville, FL. Plants were planted 40 cm apart in rows that were 125 cm apart. Each sub-plot consisted of 10 plants.

A split-plot, randomized complete-block design with 4 replicates was used in 1992. Time of inoculation was the main plots and entries were the sub-plots. There were three main plots consisting of inoculation at transplanting, inoculation when plants were 40 cm high, and a control (no inoculation). In 1993, a similar design was followed with two exceptions: 21 entries were examined and the treatment for inoculation when plants were 40 cm high was omitted because TEV spread to uninoculated plants quickly and uniformly. The sixteen entries for 1992 were re-examined along with five additional lines in 1993.

Inoculum Preparation and Procedure

The TEV isolate used was obtained from tobacco grown in a commercial Florida field. Inoculum was prepared using systemically infected leaves from Burley 21 plants. Infected leaves were homogenized in 0.05M Na_2HPO_4 / KH_2PO_4 buffer (pH 7.2) (1 g tissue / 2 mL of buffer) and the homogenate was clarified by expressing through cheesecloth.

[1]Department of Agronomy, University of Florida, Gainesville, FL.
[2]Department of Plant Pathology, North Carolina State University, Raleigh, N.C.
[3]Department of Microbiology and the [4]Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR. 97331-3804.

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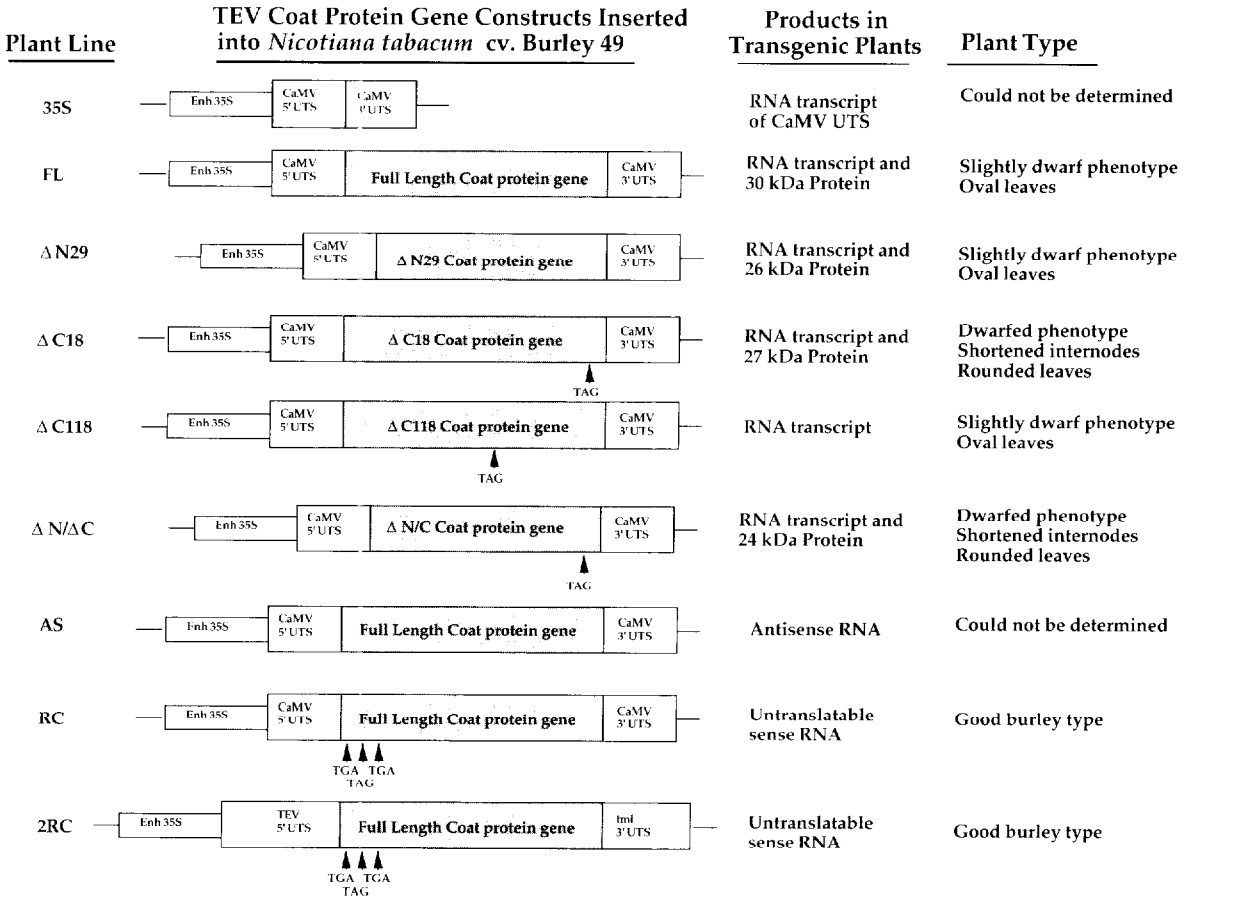
Figure 1. Summary of transgenic plant lines and the transgenes they express. *Nicotiana tabacum* cv. Burley 49 was transformed with 9 different transgenes derived from tobacco etch virus (TEV) sequences. All transgene constructions contain the enhanced cauliflower mosaic virus (CaMV) 35S (Enh 35S) promoter, a 5′ untranslated region (5'UTR) [derived from CaMV or TEV], and a 3′ untranslated region (3'UTR) [derived from CaMV or the *Agrobacterium tumefacians tumor* morphology large gene (tml)]. The nomenclature used to describe the transgenic plant lines is presented along with the gene products detected in those plant lines. In the far right column, burley plant type is given. Abbreviations are as follows: 35S, transgenic plants containing the CaMV 35S promoter and 5′ and 3′ UTS only; FL, ΔN29, ΔC18, ΔC118, ΔN/ΔC, transgenic plants containing the transgene coding for full length TEV CP, TEV CP lacking the amino-terminal 29 amino acids (aa), TEV CP lacking the carboxy-terminal 20 aa, TEV CP lacking the carboxy-terminal 118 aa, or TEV CP lacking both the amino-terminal 29 aa and carboxy-terminal 20 aa, respectively. AS and RC transgenic plants contain the transgene expressed as an antisense form of the TEV CP gene or an untranslatable sense form of the TEV CP gene, respectively. The transgene in the 2RC lines also codes for an untranslatable RNA similar to the RC lines; however, the 5′ and 3′ UTRs are different. Stippled areas represent various forms of the TEV CP nucleotide sequence.



One gram of 600-mesh carborundum was added to each 100 mL of inoculum. The inoculum was stored in a polyethylene bottle in an ice bath, and it was used within 24 h of preparation. Inoculations were made at transplanting by water soaking a small area on two leaves per plant with inoculum. Inoculum soaking was effected with an airbrush (Thayer & Chandler Model E) with a 500 mL reservoir. Inoculum was propelled using carbon dioxide at a pressure of 414 kPa. A young leaf, 8-10 cm in length, near the top of the plant and the next oldest leaf were inoculated on each plant. The inoculated area of each leaf was about 2 cm from the tip of the leaf on the abaxial surface and centered over the mid-vein. The orifice-to-leaf distance of the airbrush was about 1 cm, and this resulted in rapid water soaking of an area of about 1.5 cm in diameter (14).

Plant Evaluation

Plants were visually monitored daily for symptoms associated with TEV infection. Additionally, double diffusion immunoassays (15, 25) and inclusion body analyses (6) were performed on the test entries at selected times. Plant morphology was also noted, and near the end of each growing season, green weights were obtained from three representative plants that were removed from each plot. Each of the three plants was serologically assayed using an enzyme-linked immunosorbant assay (ELISA) for TEV and PVY. Of the seven plants remaining after weighing, four were examined four weeks later for root diseases that may have affected above-ground weight determinations. Although some root galling was observed, it occurred on all entries and it was not believed to be of sufficient severity to affect plant growth before weight determinations. At the end of the test, all transgenic plant material was destroyed.

Cultural Practices

The same experimental site was used for both years. The soil of the experimental area was turned with a bottom plow approximately a month before the tobacco was to be transplanted. In 1992, the herbicides, pendimethalin (1.12 kg [a.i.]/ha) and penulate (4.5 kg [a.i.]/ha); the insecticide, chlorpyrifos (2.2 kg [a.i.]/ha); and the fungicide, metalaxyl (0.56 kg [a.i.]/ha) were applied broadcast and incorporated into the soil with a disk harrow. In 1993, the nematicide, fenamiphos (6.7 kg [a.i.]/ha) was included with the above pesticides. The fumigant nematicide, dichloropropene (56 L/ha) was injected in the row at bedding in 1992, but it was

applied broadcast as the soil was turned in 1993. Thus, the dichloropropene followed the application of other preplant pesticides in 1992 but preceded them in 1993. Two nematicides were used in 1993 because nematodes were expected to be at a high population, and increasing the rate of the fumigant nematicide would increase the risk of phytotoxicity.

Approximately 2,000 L/ha of water was applied during transplanting. Acephate was added to the transplant water to provide 0.8 kg [a.i.]/ha. Also during transplanting, a 6-6-18 fertilizer at the rate of 450 kg/ha was banded to the side of the plants. The experimental area was irrigated by overhead sprinklers after transplanting.

Acephate (0.8 kg [a.i.]/ha) was applied as a foliar spray approximately every two weeks after transplanting. A 6-6-18 fertilizer (860 kg/ha at each of three applications) was side dressed approximately every two weeks after transplanting. When rainfall was insufficient to maintain plant growth, irrigation by overhead sprinklers was used.

RESULTS

Description and plant evaluation of entries

The plant nomenclature, the gene expressed in the transgenic plants, and general plant type are listed in **Figure 1.** Plant growth and morphology were consistent each year and many transgenic lines had a distinct morphology correlated with the transgene expressed. Transgenic plants expressing either a ΔC18 or a ΔN/ΔC version of the TEV coat protein were dwarfed and leaves were rounded in appearance. The internodal distance was shorter than in other entries. Plants expressing the FL, ΔN29, or ΔC118 version of the TEV coat protein were stunted slightly, and the leaves were more typical of Burley tobacco. Transgenic plants expressing the RC or 2RC untranslatable version of the TEV coat protein gene generally possessed a morphology typical of a Burley tobacco plant. Other lines could not be evaluated for plant type because virus infection resulted in severe stunting.

Assessment of Virus Resistance

Different transgenic lines responded to virus infection in an entry-specific manner. The formation of symptoms and the presence of a suspected viral infection was confirmed by cytoplasmic inclusion analysis or by using virus-specific polyclonal antibodies in ELISA. The results are summarized in **Table 1.**

In general, four different plant responses were noted among the different transgenic lines. Some entries became infected with TEV and displayed severe symptoms. These plants were stunted and showed significant etching (necrosis) and extreme chlorosis. All plants of the following entries displayed this response: Burley 49, 35S-4.7, AS-6.1, AS-7.2, and RC-9.1. A second phenotype observed in some lines following TEV infection was characterized by attenuated systemic symptoms, often in the form of localized chlorotic spots on the leaves. Transgenic lines ΔN/ΔC-6.9, ΔN/ΔC-6.14, ΔC18-7.9, and ΔC18-15.7 displayed this attenuated symptom phenotype. The third generalized type of symptomatology was exhibited by all TEV-infected FL- and ΔN29-lines and by some 2RC lines. These transgenic plants initially became infected and displayed typical TEV-induced symptoms, but plants gradually recovered from the infection. As new leaves emerged, symptoms were restricted to interveinal areas. Subsequent leaves possessed less symptomatic tissue until, finally, leaves developed that were totally devoid of symptoms and virus. Once these plants had recovered from TEV infection, the upper leaf tissue was free of virus and symptoms and it never became re-infected with TEV. A fourth response to TEV infection was displayed by selected RC and 2RC lines and one ΔC118 line. Transgenic lines RC-5.02, RC-7.16, 2RC-6.13, 2RC-1.8, and ΔC118-18.1 never displayed any symptoms after infection with TEV via initial mechanical inoculation or subsequent aphid transmission. **Figure 2** shows the dramatic difference in plant growth between infected untransformed Burley 49 plants and highly resistant Burley 49 germplasm expressing an untranslatable RNA molecule (2RC-6.13). Both entries were inoculated with TEV approximately 10 weeks before the photograph was taken.

Table 1. Plant response to infection with tobacco etch virus (TEV).

Entry[a]	Average Symptom Intensity[b]	Average Stunting[c]	Recovered Plant Phenotype[d]
Burley 49	4	4	No
35S-4.7	4	4	No
FL-3.3	3->0	1	Yes
FL-24.3	3->0	1	Yes
ΔN29-2.12	3->0	1	Yes
ΔN29-8.1	3->0	1	Yes
ΔC18-7.9	2	2	No
ΔC18-15.7	2	2	No
ΔN/ΔC-6.9	2	2	No
ΔN/ΔC-6.14	3	2	No
ΔC118-18.1	0	0	N/A
AS-6.1	4	4	No
AS-7.2	4	4	No
RC-5.02	0	0	N/A
RC-7.16	0	0	N/A
RC-9.1	4	4	No
2RC-6.13	0	0	N/A
2RC-1.8	0	0	N/A
2RC-3.3	2->0	0	Yes
2RC-5.2	2->0	0	Yes
2RC-8.13	2->0	0	Yes

[a] Plant entries and their nomenclature are described in Figure 1. Number after line identification (i.e., 4.7 of 35S-4.7) indicates a particular line derived from a single transformed parent plant.

[b] Average symptom intensity was a subjective analysis of TEV-induced symptoms. The rating scale was from 0 to 4, with 0 = no symptoms, 1 = mild chlorosis and mosaic, 2 = moderate mosaic, 3 = severe mosaic and some chlorosis and etching of leaves, 4= severe chlororis and etching of leaves. For plants that displayed the recovered phenotype, ratings are given for initial and final symptoms (i.e., 3->0).

[c] Average stunting was an estimate of how TEV infection impacted plant growth. The rating scale was from 0 to 4 with 0 being no obvious stunting and 4 being severe stunting typical of TEV infection of *N. tabacum* cv. Burley 49.

[d] Some of the transgenic lines displayed a recovery phenotype in which the plant was able to 'outgrow' the infection. Older leaves displayed TEV-induced symptoms while younger leaves possessed no symptoms and virus. N/A, not applicable.

The engineered resistance was TEV specific. None of the lines tested displayed any resistance to PVY or tomato spotted wilt virus, as evidenced by a limited number of plants that were naturally infected with these viruses in the field over the two-year study.

In addition to mechanically transmitted TEV, the test plot was assessed for virus resistance against aphid-vectored TEV (26). No colonizing aphid populations were detected, yet 100% of the uninoculated Burley 49 and susceptible transgenic plants became infected 2-3 weeks after transplanting. Virus movement was likely due to transient, non-colonizing aphid species during this study. Under these conditions, the TEV-resistant RC, 2RC, and ΔC118 lines remained free of TEV.

Green weights of representative plants were also determined as an estimate of virus resistance. In 1992, these measurements were taken when ca. 75% of the plants were flowering. This was two weeks after the highly resistant RC and 2RC lines had flowered. In 1993, green weight measurements were taken when 10% of the plants were flowering. Only plots mechanically inoculated with TEV were weighted. Seven to 10-fold differences in average green

Figure 2. Comparison of tobacco etch virus (TEV) infection of transgenic and untransformed *Nicotiana tabacum* cv. Burley 49 plants. Two entries are shown. Plants of transgenic line 2RC-6.1 are shown in the background. This line expresses an untranslatable version of the TEV coat protein gene. In the foreground are untransformed *N. tabacum* cv. Burley 49 plants. Both lines were mechanically inoculated with a local Florida isolate of TEV concurrent with transplantation into the field. The photograph was taken approximately 10 weeks after transplanting.

weight were readily apparent between protected lines and untransformed Burley 49 tissue. These results are presented in **Table 2**.

DISCUSSION

Results from this field study demonstrate that genetically engineered resistance can be effective in preventing losses incurred by TEV infection.

Two major conclusions were drawn from this study. First, effective virus resistance was displayed in different resistance phenotypes, and resistance could be generated by exploiting different genetic approaches. Second, expression of particular transgenes could alter plant type.

Resistance to TEV was manifested in a variety of phenotypes. Lines producing an untranslatable version of the TEV coat protein gene (RC and 2RC lines) afforded superior protection. Many of these lines never became infected in the two-year field trial. This was in agreement with previous greenhouse studies (19, 20) and was remarkable in view of the inoculum potential provided by endemic TEV and migratory aphid populations. All uninoculated control Burley 49 plants became infected 2 - 3 weeks after transplanting. Therefore, the highly resistant RC and 2RC lines withstood mechanical inoculation and severe aphid-vectored TEV pressure. Transgenic lines expressing these transgenes resulted in green weight difference that were, on average, 10-fold higher than susceptible Burley 49 tissue (**Table 2**).

Another form of resistance manifested itself in a phenotype in which plants recovered. ΔN29, FL, and selected 2RC plant lines initially became infected with TEV but recovered. Newly emerging leaves were free from TEV and TEV-induced symptoms. Of particular interest was the observation that these plants never became re-infected with TEV during the remainder of the field test even under pressure from aphid-transmitted TEV. Laboratory studies suggested that an anti-TEV state is induced in these plants, and therefore, recovered plants are completely resistant to subsequent TEV infection (unpublished observations). These plants also may offer a useful form of resistance. Transgenic plants expressing ΔN/ΔC and ΔC18 forms of the TEV coat protein also displayed resistance to TEV infection, although it was inferior to resistance described above and permitted a mild systemic infection. Our studies also suggested that the 35S and AS transgenic plants did not provide a useful form of resistance to TEV.

The second major conclusion from this study was that the expression of particular transgenes affects plant type. A recent study by Brandle & Miki (5) showed that transformed tobacco plants may have altered agronomic characteristics. In our study, a dwarfed appearance was consistently associated with all plants expressing a ΔC18 or ΔN/ΔC transgene. This phenotype was observed regardless of transgene expression levels, which suggests that the transgene transcript or protein product was responsible for the altered plant morphology. Plants expressing an untranslatable RNA and displaying superior resistance (selected RC and 2RC) had good Burley plant characteristics. These transgenes and their products did not appear to impact Burley growth characteristics.

In summary, extremely effective virus resistance can be generated using a variety of transgenic approaches. Plants expressing an untranslatable sense (or genome sense) RNA will be the transgenic material of choice to deploy. There are several reasons for this conclusion. The virus resistance was superior in these lines. TEV was not detected in these lines over the two- year study. Second, expression of these transgenes did not obviously affect Burley plant type. Third, expression of a gene in the form of an untranslatable RNA molecule may address relevant concerns regarding the

Table 2. Average green weight of plants inoculated with TEV.

Entry[a]	Average green weight (grams/plant)[b] 1992	1993
Burley 49	286	121
35S-4.7	140	91
FL-3.3	1893	-
FL-24.3	1636	-
ΔN29-2.12	1944	-
ΔN29-8.1	1598	-
ΔC18-7.9	2094	-
ΔC18-15.7	1958	-
ΔN/ΔC-6.9	1640	-
ΔN/ΔC-6.14	1682	-
ΔC118-18.1	-	1329
AS-6.1	761	-
AS-7.2	874	-
RC-5.02	1648	1206
RC-7.16	1639	1303
RC-9.1	438	110
2RC-6.13	1487	1123
2RC-1.8	-	1382
2RC-3.3	-	1321
2RC-5.2	-	1121
2RC-8.13	-	848

[a]Plant entry and nomenclature are described in Figure 1.

[b]Three representative plants from each plot were harvested and weighed. The plot was replicated four times and the average green weight for these four replicates is presented. In 1992, green weight was taken after most plants had flowered. The RC and 2RC plants flowered 2 - 3 weeks earlier, presumably because they were free from TEV the entire growing season. Therefore, their green weights are probably lower than the maximum weight achieved. In 1993, green weight measurements were taken as plants began to flower.

potential wide-spread application of PDR practice (7). It has been suggested that transencapsidation of viral RNAs by transgene products may generate virions with expanded host range or new insect vector association. Transencapsidation occurs naturally and recently has been shown to occur in transgenic plants (10). Additionally, RNA recombination between viral genomes and transgene transcripts might increase the possibility of generating new viral entities. Transgenes that express untranslatable RNAs should minimize or essentially negate these concerns. Future questions will focus on the durability of transgenic resistance and the inheritance of this resistance.

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REDUCED POPULATION GROWTH AND DELAYED DEVELOPMENT OF THE CIGARETTE BEETLE (COLEOPTERA: ANOBIIDAE) DUE TO THURINGIENSIN

TOBACCO SCIENCE

By D. W. Keever[1]

Three formulations of thuringiensin, the heat-stable β-exotoxin of *Bacillus thuringiensis*, were incorporated into diet medium (finely ground tobacco) of first instar cigarette beetles, *Lasioderma serricorne* (F.). At 15 and 60 ppm (wt AI/wt diet medium), larval mortality and development, and population growth over one generation were studied. Then the formulation found to be most effective was applied to diet at 15 to 75 ppm. Larvae of all instars were placed on the diet, allowed to develop, and percentage adult emergence was observed. The formulations, all from Abbott Laboratories, were ABG-6211 (1.5% AI [wt/wt] emulsifiable concentrate), ABG-6162A (1.5% AI [wt/wt] emusifiable concentrate) and ABG-6206 (10% AI [wt/wt] wettable powder). Except for ABG-6211-15 and ABG-6162A-15 ("-15" = 15 ppm), all treatments induced larval mortalities of 68 to 100%; control mortality was 17%. Except for ABG-6211-15, all treatments slowed larval development, especially at 60 ppm and especially ABG-6206. After eight weeks of exposure, the sole surviving ABG-6211-60 larva was still a 1st instar, while control larvae had begun pupation. All treatments at 15 ppm significantly ($P<0.05$) reduced population growth, especially ABG-6206-15 (96% reduction); all formulations at 60 ppm caused >99% reduction. In the dose-response test, adult emergence was significantly ($P<0.01$) correlated to the dose and to the interaction between dose and the stadium in which first placed on the diet.

Additional key words: *Nicotiana tabacum*, tobacco, *Lasioderma serricorne*, β-exotoxin.

INTRODUCTION

The cigarette beetle, *Lasioderma serricorne* (F.), is the most important insect pest of all non-farm stored tobacco. Tobacco may be stored for several years, so an acceptable control method may not have to provide total kill immediately. Instead, it might need only to gradually eliminate existing infestations and prevent future infestations. The insect growth regulator, methoprene, is currently used for this purpose.

Almost all cigarette beetle damage to tobacco is done by the larval stage. A fourth instar is more than 100 times heavier than a first instar, and therefore it feeds more and does much more damage (1). Feeding damage would be greatly reduced by killing larvae in the earlier instars.

In farm-stored tobacco, the cigarette beetle is not often a serious problem, but when it is the tobacco farmer has few control options. *Bacillus thuringiensis* Berliner is not effective, and fumigations with methyl bromide or phosphine can be difficult and dangerous. Therefore, it is desirable to find new materials that are effective against the cigarette beetle as well as the tobacco moth, *Ephestia elutella* (Hübner).

Thuringiensin, the heat-stable β-exotoxin of *B. thuringiensis*, has shown significant activity against numerous insect species (2, 5). Thuringiensin also has contact activity against the mites, *Tetranychus urticae*, *T. cinnabarinus*, *T. pacificus*, and *Metaseiulus occidentalis* (3,4). It showed a high degree of residual activity for 14 days against the females of *T. urticae* and *T. cinnabarinus* (4). White & Loschiavo (6) found that *B. thuringiensis* alone (200 to 1000 ppm) had no effect on the stored-product beetles, *Oryzaephilus surinamensis*, *O. mercator*, *Cryptolestes ferrigineus*, and *Tribolium castaneum*. When combined with a high dose of thuringiensin (1% AI), it killed ≥94% of *O. surinamensis* adults and 81% of larvae; it had little effect on the other species (6). However, the preparation and source of the thuringiensin may have varied among published reports.

This is a report on larval mortality and development, population growth over one generation, and dose response of the cigarette beetle when reared on diet medium containing low doses of three formulations of thuringiensin.

MATERIALS AND METHODS

This study was conducted in three experiments. The experiments were to study the effects of thuringiensin on (1) larval mortality and development, (2) one-generation population growth, and (3) adult emergence as a function of treatment dose.

The thuringiensin formulations, all from Abbott Laboratories, were ABG-6211 (1.5% AI [wt/wt] emusifiable concentrate), ABG-6162A (1.5% AI [wt/wt] emusifiable concentrate), and ABG-6206 (10% AI [wt/wt] wettable powder). All test insects were taken from laboratory cultures maintained at 26 ± 0.5°C and 65 to 70% RH on a diet of finely ground flue-cured tobacco. In all incubations during testing, insects were also kept at 26 ± 0.5°C and 65 to 70% RH on a diet of finely ground flue-cured tobacco that was either treated with thuringiensin or untreated (control). Photoperiod was not controlled.

Test materials were mixed with ground tobacco. Being a wettable powder, ABG-6206 was easily shaken into a mixture with ground tobacco. The emulsifiable concentrates, ABG-6211 and ABG-6162A, needed more preparation. For the 60 ppm diets (wt AI/wt ground tobacco), a 2.0 mL aqueous solution containing 400 mg thuringiensin was sprayed onto 100 g of ground tobacco. After drying, the treated tobacco was shaken in a jar to complete the mixing process. The 15 ppm diets were prepared by diluting the 60 ppm diets with more ground tobacco. All treated tobacco was brought to a moisture content of 14.5 to 15%.

Experiment 1 - Larval mortality and development

The purpose of this experiment was to study the extent of larval mortality, the time of mortality, and the delays in development caused by thuringiensin. Thuringiensin retards immature development of some insects (5). Ten 48- to 72-hours-old first instars were placed into 60- by 15-mm petri dishes. The dishes contained 250 mg treated tobacco at either 0, 15, or 60 ppm thuringiensin. The 15 and 60 ppm doses were selected because they are a rough estimate of what might be applied to tobacco in the field under tentative manufacturer's recommendations. Each petri dish constituted a replicate and each of the seven treatments was replicated five times for a total of 35 dishes (30 treatments + 5 controls).

For each treatment, two of the five replicates were microscopically examined six days a week for 13 weeks. Time did not permit examining all replicates daily, but the replicates were examined sequentially and all replicates were

[1] Research Entomologist, United States Department of Agriculture, Agricultural Research Service, Oxford, N.C. 27565-1168. USDA Assistant Professor, Department of Entomology, N.C. State University, Raleigh, N.C. 27695-7613.

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examined the same number of times. Recorded daily were larval mortality and the current developmental stage.

Experiment 2 - Population growth through one generation

Twenty-five unsexed adult cigarette beetles and 30 g treated tobacco (0, 15, or 60 ppm thuringiensin) were placed into 0.47-L jars and incubated. Each jar constituted a replicate and each treatment was replicated five times. The emerging F_1 adults were counted after 10 weeks of incubation, a period that would normally permit emergence of all adults without getting any F_2 adults. The F_1 adults were counted again after 14 weeks of incubation to account for delayed adult emergence due to effects of thuringiensin (5).

Experiment 3 - Effect of dose on adult emergence

This experiment was conducted not to determine an LD_{50} or LD_{95}, but rather to find a dose level that might be optimum for control of the cigarette beetle. Ten larvae were placed into 60- by 15-mm petri dishes containing 250 mg tobacco treated at either 0, 15, 30, 45, 60, or 75 ppm thuringiensin (ABG-6206). To examine the effects of the stadium when there was first exposure to a treatment, larvae were in either the first, second, third, or fourth stadium when first placed into the dish. The first instars were 48- to 72-hours-old and the other instars were 24 to 48 hours post-molting. Each treatment was replicated 10 times. The dishes containing larvae were incubated and counted weekly for adult emergence. Counting continued until adult emergence was complete.

The Student's *t*-test and analysis of variance were used for testing treatment effects.

RESULTS AND DISCUSSION

Experiment 1

Four treatments had significantly more larval mortality over the 13-wk period than did the control (17%) (**Fig. 1**). These treatments were all three formulations at 60 ppm and ABG-6206-15 ("-15" = 15 ppm). ABG-6206-60 was clearly the best treatment because it prevented all larvae from developing to adults with most mortality occurring by the fifth week on the diet. The high control mortality of 17% was probably due to handling of larvae during the experiment.

The same four treatments (all formulations at 60 ppm and

Figure 1. Percentage mortality of cigarette beetle larvae while reared from the first stadium on thuringiensin-treated ground tobacco. All treatments shown gave significantly greater mortality ($P \leq 0.05$) than the final control mortality of 17%; treatments not shown did not. Numerals in legend indicate the concentration (ppm) of formulation used.

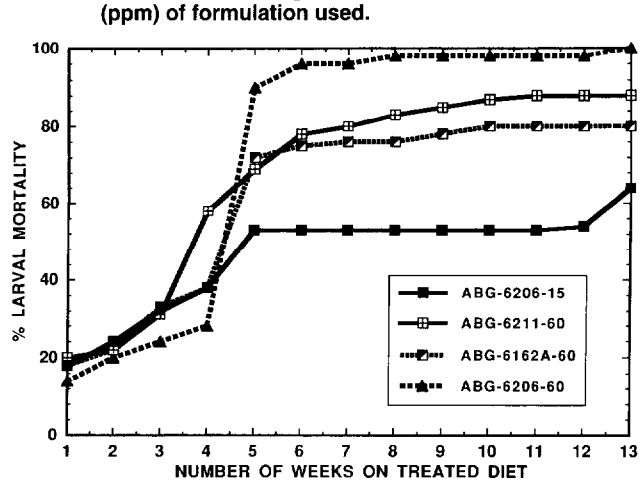
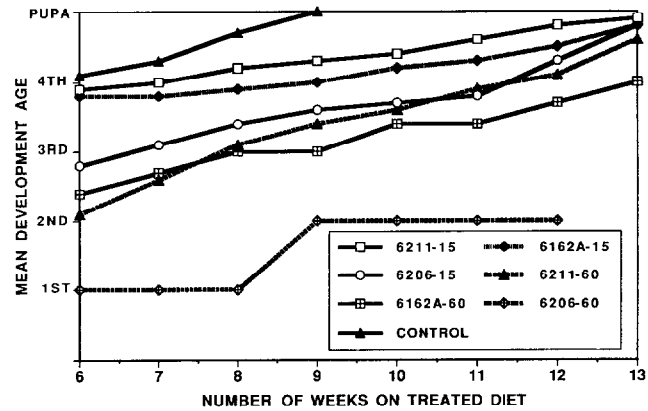


Figure 2. Mean developmental age of cigarette beetle larvae while reared from the first stadium on thuringiensin-treated ground tobacco. Numerals in legend indicate the concentration (ppm) of formulation used. N = 50 larvae per treatment.



ABG-6206-15) retarded development (**Fig. 2**). Larvae treated with ABG-6211-15 or ABG-6162A-15 appeared to develop more slowly than did the controls, which all pupated by the ninth week, but the differences were not significant ($P \geq 0.05$). ABG-6206-60 retarded development so much that the sole surviving larva after eight weeks was still in the first stadium and never developed past the second stadium. Normally all larvae would have developed to the second stadium within two weeks.

Experiment 2

Table 1 shows the number of adult progeny produced within one generation by insects in each treatment. All thuringiensin treatments reduced population growth, but as in Experiment 1, the 60 ppm treatments and ABG-6206 in general were especially effective. Again, ABG-6206-60 prevented all development to the adult stage. It is reasonable to assume that treatment effects were on the immature stage because cigarette beetle adults feed very little, and it is known that thuringiensin is not toxic to adults of many insect species at larvicidal doses (2).

As in Experiment 1, development was greatly delayed. Of the total number of control adults to emerge, 92% did so within the first 10 weeks (**Table 1**). Only 62 and 42% of the ABG-6211-15 and ABG-6162A-15 adult emergence, respectively, occurred within the first 10 weeks. Adult emergence for all of the other treatments occurred after the first 10 weeks.

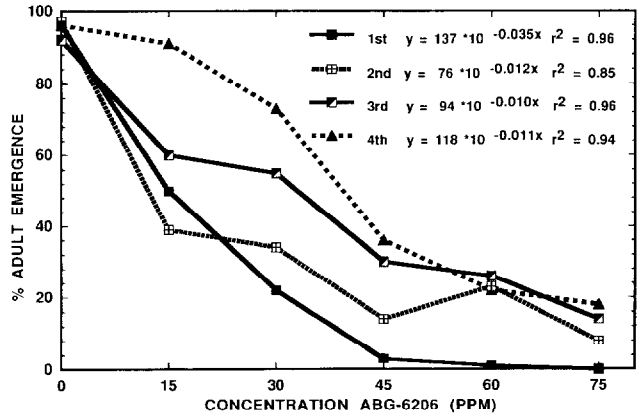
Experiment 3

Significant ($P \leq 0.05$) suppression of adult emergence was achieved at 45 to 75 ppm of ABG-6206 when first exposure of larvae to the treated tobacco was in the first stadium (**Fig. 3**).

Table 1. Mean number F_1 adults to emerge per 25 parent cigarette beetles placed on thuringiensin-treated ground tobacco. Means followed by the same letter do not differ significantly ($P \leq 0.05$).

Formulation	PPM (wt AI/wt tobacco)	Total number of adults emerged by: 10 wks	14 wks
Control	0	627 a	680 a
ABG-6211	15	195 b	314 b
ABG-6262A	15	122 c	292 b
ABG-6206	15	0 d	28 c
ABG-6211	60	0 d	3 d
ABG-6162A	60	0 d	2 d
ABG-6206	60	0 d	0 d

Figure 3. Percentage of cigarette beetle larvae placed on thuringiensin-treated (ABG-6206) tobacco that later emerged as adults. The terms "1st", "2nd", "3rd" and "4th" in legend refer to the stadium in which the larvae were first placed on the treated tobacco.



Also, 75 ppm provided significant suppression of adult emergence when larvae were first exposed while in the other stadia. Analysis of variance showed that adult emergence was significantly correlated ($P<0.001$) to the dose and to the interaction between dose and the instar that was first exposed.

The most important result was not the dose response, but rather the effect of the stadia in which the larvae were first exposed. Significant suppression of adult emergence was achieved only when larvae were first exposed while in the first stadium. This may impact practical application of thuringiensin. This phenomenon was not observed in Experiments 1 and 2 because first exposure was always while larvae were in the first stadium. This indicates that for management of the cigarette beetle with ABG-6206, the population might have to be exposed for at least a full generation.

As in Experiments 1 and 2, ABG-6206 delayed development. Even when first exposed while in the second, third, and fourth stadia, larvae on the 60 and 75 ppm treatments took about 80 and 100% longer, respectively, to emerge as adults than did the controls.

Thuringiensin, especially ABG-6206, greatly suppressed production of cigarette beetle progeny, especially when the larvae were exposed at an early age. Perhaps just as important, thuringiensin retarded immature development. There are several practical implications of this retarded development: (1) smaller larvae do less feeding damage; (2) insect populations with longer developmental cycles have exponentially lower growth rates; and (3) the mouthparts of retarded larvae are usually the first insect structures to be affected by thuringiensin (5). This means that reduced feeding damage to a commodity may begin before mortality is induced.

In addition to thuringiensin's efficacy against the cigarette beetle, a possible advantage in its use involves its thermostability. This thermostability may permit the application of thuringiensin on green tobacco before curing on the farm or on cured tobacco at the processing plant without the destruction of its activity by the heating processes.

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ASSESSING THE QUALITY OF BURLEY TOBACCO PART 1: GRADE INDEX AND ASSOCIATED FACTORS[1]

TOBACCO SCIENCE

By T. C. Bridges, L. R. Walton and J. H. Casada[2]

This study determined an average yearly grade index (GID) from sales data for burley tobacco for the years 1959 - 1990. This index was used as a quality measure for each crop year. Several leaf-group, leaf-color, and seasonal-crop variables were significantly correlated with GID. Within the leaf groups, the leaf and lugs categories had a positive effect on GID, while tips, nondescript, and a combined group (TNM) had negative correlations. A tradeoff existed between these leaf groups in relation to the grade index. For the color groups, the tan groups within the lugs and leaf categories (CF, BF) and the tannish-red leaf group (BFR) had significant positive correlations with GID, while green color in the leaf, tips, and flyings groups had significant negative correlations with GID. Total crop production area had a significant negative correlation with GID, while it was positively correlated with the undesirable leaf groups of tips, nondescript, and TNM. This indicated that market quality was reduced when production area increased. The significantly correlated variables were used to develop a regression equation to predict GID. The equation had a regression coefficient of 0.935, and it predicted GID reasonably well for the 32-year period.

Additional key words: *Nicotiana tabacum*, burley tobacco, quality, grade index.

INTRODUCTION

The quality measure of any burley tobacco crop is impacted by several factors. Among these are the planting date, the growing environment, the harvest date and environment, the type of curing environment, and the manner in which the crop is stripped. In any year these factors vary, and they may combine to affect the market quality of the crop. Often producers make harvesting decisions based on labor supply, which might force them to harvest their tobacco before the crop is mature or during undesirable weather conditions. If the factors that directly affect crop quality could be identified, they might prove beneficial as a predictive tool for both the producer and researcher in terms of better crop quality and improved economic returns.

Burley tobacco is traditionally sold in grades that are indicative of the quality of the crop. Each grade is comprised of a leaf, a color, and a quality group. The leaf group represents stalk position and is somewhat indicative of the leaf maturity. The color group reflects the individual leaf structure and maturity, while the quality group indicates the condition of the tobacco within the leaf and color groups. The tobacco is usually graded on the market or warehouse floor by official graders from the grading service of the United States Department of Agriculture (USDA). The returns to the producer are based on the weight of tobacco in each grade and the price the tobacco companies are willing to pay for it.

There are approximately 110 to 115 grades that may be used each year. Many of these grades are similar in quality considerations, but with the many different combinations it has been difficult to define overall crop quality. Attempts have been made to use average leaf prices for various grades as a quality measure, but this does not always distinguish the amount of high or low quality tobacco present in a given crop because of the effects of supply and demand. Bowman et al. (4) established a grade index for burley tobacco to address this problem. Their procedure was to assign a numerical value to each leaf type as well as to each color and quality group, depending on the desirability of the grade. The index presents a means for measuring the overall quality of an individual crop, and it provides a basis for comparison of the amount of desirable tobacco each year contains.

The objectives for this study were (1) to compute a yearly grade index for burley tobacco and use it as a comparison measure of crop quality between years, (2) to identify and quantify the leaf and color variables that are significantly correlated with the yearly grade index, and (3) to establish a predictive model that will determine yearly grade index as a function of the correlated variables.

MATERIALS AND METHODS

The purpose of this paper was to use gross sales data from 1959 - 1990 by individual color and grade, and to determine which of these factors or combinations of factors had the greatest impact on crop quality. An average yearly grade index (GID) was computed for each year, and it was used as a quality measure of each crop. Factors that affect GID were identified using Pearson correlation coefficients (5), and they were used in developing a mathematical model to predict GID. Because it was not known what factors would affect GID, many combinations were considered. These are detailed in the following discussion along with assumptions made in quantifying the different variables.

The GIDs for 1959 - 1990 were determined using burley sales data from eight states (Ind., Ky., Mo., N.C., Ohio, Tenn., Va., & W.Va.) as reported by the USDA Tobacco Market Review (2). A computer program was written to calculate the numerical value of each grade as defined by Bowman et al. (4). GIDs were determined as follows: First a product was calculated using the percentage of a grade sold in a given year and the numerical value assigned to that grade. Then, the average index was found by summing the products for each grade appearing that year and dividing by 100 percent. Weighting each GID in this manner provided a measure of the crop quality for a given year. For years with a larger proportion of high quality tobacco, the GID was higher than in years where there were more undesirable grades. It was assumed that the tobacco harvested at any location followed the GID for that year.

Eight leaf group variables were considered using the percentage of each leaf category sold per year (2). These were flyings (FLY), cutters or lugs (LUG), leaf (LEA), tips (TIP), mixed (MIX), nondescript (NON), miscellaneous (MIS), and a combined leaf category (TNM) comprised of the sum of the tips, nondescript, and miscellaneous groups. For 1978 and subsequent years, MIS was subdivided into no-grade (NOG), waste, scrap, and unsound groupings. Only NOG contributes to the grade index with a numerical value of one. Prior to 1978 percentages in MIS were all assigned a grade of NOG when calculating GID.

Five major color groups (green, tan, buff, red, tannish-red) were identified by the sales percentage of the color category

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[2]The authors are, respectively: Research Specialist, Professor, and Research Specialist, Department of Agricultural Engineering, University of Kentucky, Lexington, KY 40546-0276.

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sold per year (2). These color categories were further subdivided by leaf group for a total of 15 color-leaf group combinations. For example, greenish color categories were identified to be those leaf groups having the standard color grades of V, VF, VR, G, GF, and GR. The percentage of these color grades were summed within a leaf group and designated as CGR (lugs), BGR (leaf), TGR (tips), and XGR (flyings). This procedure was repeated for each major color category. The percentage of flyings and lugs sold with buff color were identified as XL and CL, respectively. Major tan groupings were summed for flyings, lugs, leaves, and tips and designated as XF, CF, BF, and TF, respectively. Major red groupings included leaves (BR) and tips (TR) with red color. Tannish-red color percentages were also computed for leaves (BFR), tips (TFR), and the mixed group (MFR).

Seasonal crop variables for burley tobacco were also considered as reported by the USDA Crop Reporting Board (1). These were the yearly (1959 - 1990) total harvested crop area (TAREA) in hectares and average crop yield (TYLD) in kg/hectare.

RESULTS AND DISCUSSION

The GIDs had an average value of 59.4 for the 32 years, and they ranged from 68.4 in 1967 to 50.2 in 1983 (**Figure 1**). Several different leaf and color groups were related both to the grade index and to each other, and these will be discussed by the appropriate group.

Leaf Group Variables

Significant correlations were expected between GID and the various leaf categories. **Table 1** presents the correlation coefficients with the GID for the eight leaf groups, as well as the average percentage of each leaf group sold per year. LUG and LEA had a significant positive correlation with GID while TIP, NON, and TNM were significantly correlated in a negative fashion (**Table 1**).

The leaf groups most significantly affecting GID were LUG and TNM. **Figure 1** illustrates this positive correlation between GID and LUG. Lugs are a desirable leaf category, and on the average they constitute about 18% of yearly sales. **Figure 1** also depicts the negative correlation between GID and TNM, and it shows a slight decline in TNM over the 32-year period. The groups comprising the TNM category are less desirable tobacco, and any increase in this group is detrimental to crop quality.

The correlations of LUG and TNM with GID indicate a tradeoff between these groups in relation to the numerical grade index. Further analyses found significant negative correlations between TNM and LUG, and between TNM and LEA (**Table 2**). A possible explanation for these correlations is that where growing conditions are favorable for crop maturity, LUG and LEA would represent a higher percentage of harvest, while TNM would be lower, resulting in a higher quality crop.

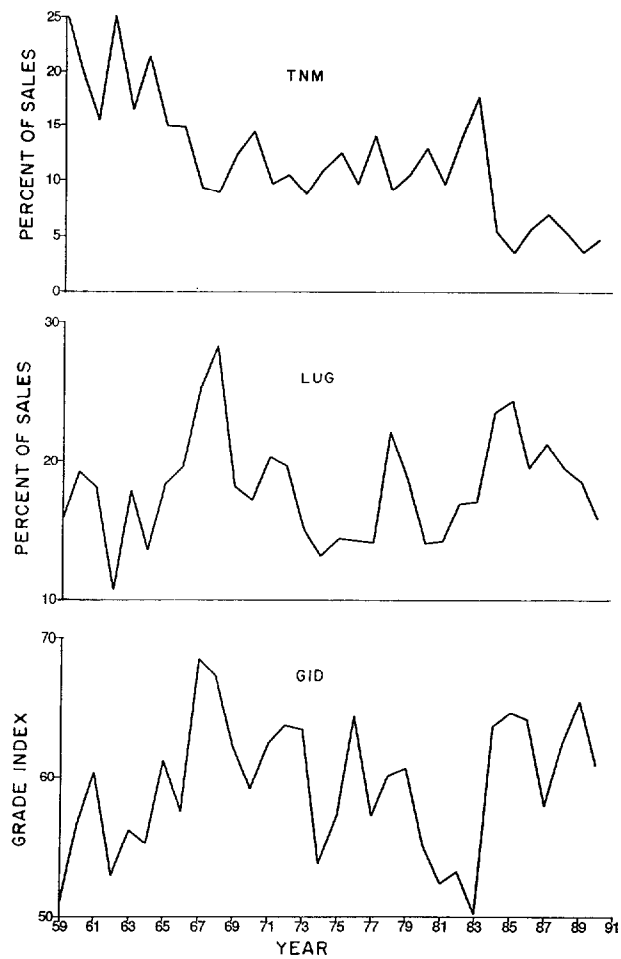
FLY had little effect on the grade index (**Table 1**), but it had a significant positive correlation with TIP, NON, and TNM (**Table 2**). This indicates that for growing seasons where the crop has a higher percentage of TNM, conditions would be favorable for an associated increase in FLY.

Table 1. Correlations for various leaf groups with the yearly grade index (GID) and average group sales per year, 1959 - 1990.

Leaf Group Variable	Correlation Coefficient with GID	Average Sales per Year
		---%---
FLY	0.0131	10.9
LUG	0.6474**[a]	18.0
LEA	0.3731*	48.9
TIP	-0.5313**	4.8
MIX	-0.1423	10.5
NON	-0.5279**	3.3
MIS	-0.3023	3.5
TNM	-0.6747**	11.7

[a] * = correlation significant at the 5% level;
** = correlation significant at the 1% level.

Figure 1. Average yearly grade index (GID), and sales percentages of lugs (LUG) and tips, nondescript, & miscellaneous (TNM), 1959-1990.



Leaf Color Variables

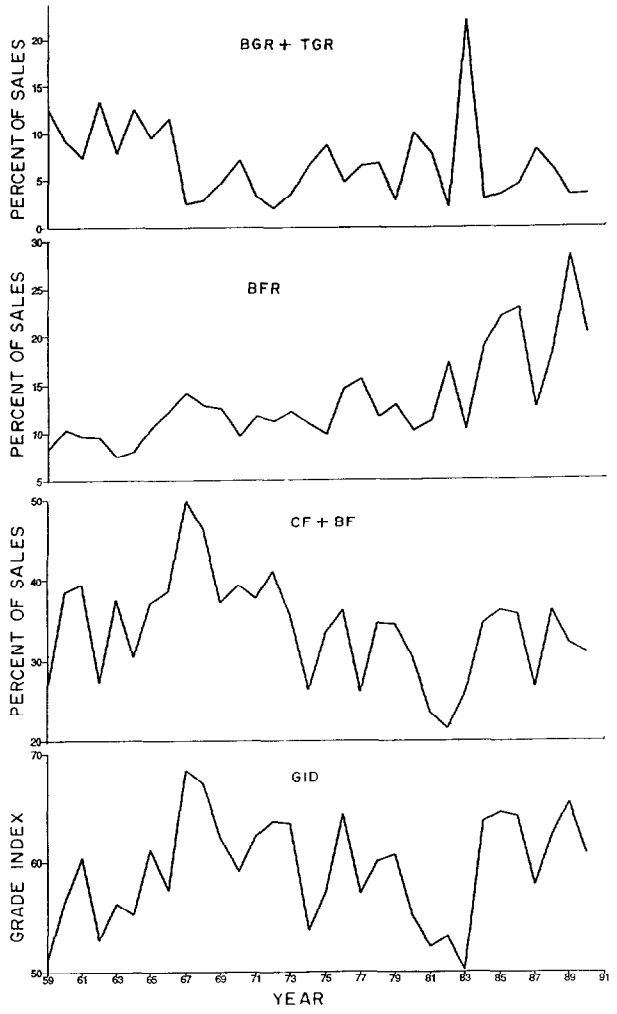
Table 3 presents the correlations of GID with the 15 major color groups. On a percentage basis, the largest color groups sold (**Table 3**) were the tan groupings (CF and BF) within the LUG and LEA categories and the tannish-red group (BFR) also in the LEA category. These are very desirable colors, and each had a significant positive correlation with GID. **Figure 2** shows the positive effect of the sum of the tan groups (CF + BF) as well as that for the tannish-red group (BFR) on GID for

Table 2. Correlations for lugs (LUG), flyings (FLY), and leaves (LEA) with tips (TIP), nondescript (NON), and the sum of tips, nondescript, and miscellaneous (TNM), 1959 - 1990.

Leaf group Variable	Correlation Coefficient with FLY	Correlation Coefficient with LUG	Correlation Coefficient with LEA
TIP	0.5904**[a]	-0.3826*[a]	-0.4630**[a]
NON	0.5566**	-0.3556*	-0.3907*
TNM	0.6202**	-0.4567**	-0.5964**

[a] * = correlation significant at the 5% level;
** = correlation significant at the 1% level.

Figure 2. Average yearly grade index (GID), and sales percentages of tan leaf & lugs (CF+BF), tannish red leaf (BFR), and green leaf & tips (BGR+TGR), 1959-1990.



the 32-year period. BFR had minimal effect on grade index until the mid-1980s when there was a significant increase in sales of this group. Some of the increase in BFR was at the expense of the tan groups, and this was probably the result of a demand for darker colors by the tobacco companies during the last decade.

Three of the four green color categories had significant negative correlation coefficients with GID (**Table 3**). The most significant of these was BGR. LEA generally comprises the largest category sold in a given year (**Table 1**), and when it contains an undesirable color, the quality index would be lower. TGR and TR also showed a significant negative impact on GID, and this was due somewhat to TIP already having a significant unfavorable effect on GID (**Table 1**) (i.e., green tips are worse than tips alone). While the percent of BGR and TGR was small, the negative impact of their sum on GID is clearly demonstrated in **Figure 2**. Clearly, if the factors causing these lower quality and color groupings were identified, this would provide a good measure of crop quality in a given year.

Seasonal Crop Variables

The average crop area harvested (TAREA) was 108.7 x 10^3 hectares, and the average crop yield (TYLD) was 2416.5 kg/ha for the 32 years considered. **Table 4** presents correlations for TAREA and TYLD between GID, the eight leaf groups, and four of the color groups that were

Table 3. Correlations for various leaf color group with the yearly grade index (GID) and average group sales per year, 1959 - 1990.

Leaf Color Variable	Correlation Coefficient with GID	Average Sales Per Year
		---%---
CGR	-0.2314	0.5
BGR	-0.7257**[a]	5.0
TGR	-0.6179**	1.8
XGR	-0.3634*	0.1
BFR	0.5198**	13.3
BR	0.0898	6.4
TFR	0.0890	1.2
TR	-0.3727*	1.5
MFR	-0.2183	2.4
XL	0.2660	1.3
XF	0.0006	8.9
CL	0.2800	0.5
CF	0.7555**	14.0
BF	0.6069**	20.0
TF	-0.2407	0.2

[a] * = correlation significant at the 5% level;
** = correlation significant at the 1% level.

significantly correlated with GID. TAREA showed a significant negative correlation with GID and LUG, while it was positively correlated with TIP, NON, and TNM. **Figure 3** illustrates the inverse correlation between GID and TAREA. TAREA reached maximums of approximately 138 x 10^3 hectares in 1962, 1963, and 1982, and minimums around 98 x 10^3 hectares in 1970, 1971, 1986, and 1987.

The negative correlation between TAREA and GID (**Table 4**) indicated that as more land area was brought into production the overall crop quality was reduced. This conclusion is reinforced by the positive correlations between TAREA and the undesirable leaf groups (TIP, NON, and TNM), and by the negative correlations between TAREA and

Figure 3. Average yearly grade index (GID) and harvested crop area (TAREA), 1959-1990.

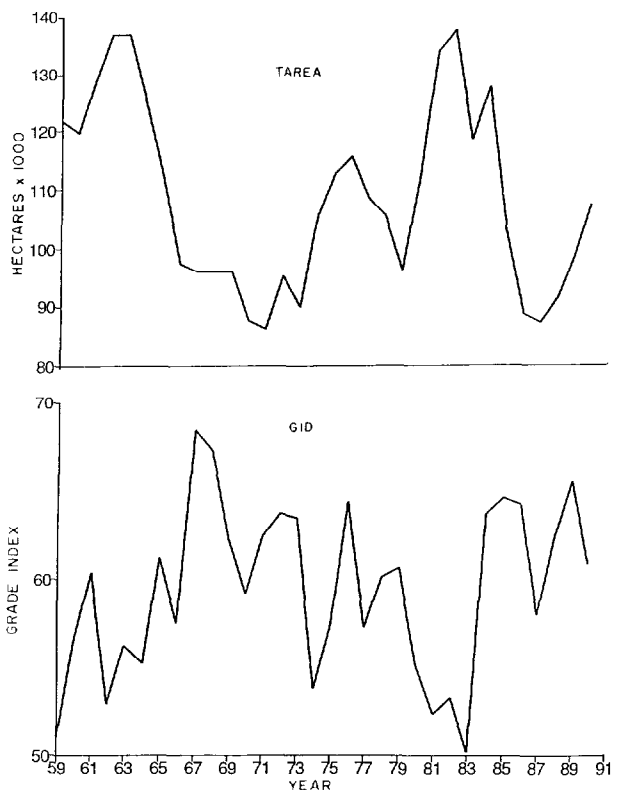


Table 4. Correlations for average crop area (TAREA) and crop yield (TYLD) with the yearly grade index (GID) and various leaf and color group variables, 1959 - 1990.

Variable	Correlation Coefficient with TAREA	Correlation Coefficient with TYLD
GID	-0.5899**[a]	0.3429
Leaf Group		
FLY	0.0748	-0.1285
LUG	-0.4027*	0.1645
LEA	-0.1991	0.0035
TIP	0.5145**	-0.5684**
MIX	-0.0601	0.2276
NON	0.4951**	-0.5331**
MIS	0.0049	0.5857**
TNM	0.5298**	-0.3467
Color Group		
BGR	0.2921	-0.3619*
TGR	0.4261*	-0.6088**
TR	0.5402**	-0.4137*
CF	-0.4772**	0.2978

[a] * = correlation significant at the 5% level;
** = correlation significant at the 1% level.

LUG. In seasons where the crop production area was increased, the tobacco was often grown on marginal land, and it was often harvested at non-optimum maturity because of a longer harvest period. **Table 4** also shows correlations that indicate a tradeoff between the tan and the green or red color groups. While some of the color correlations were due to changes in their respective leaf groups, it appears that as crop area increased, there were higher percentages of the less desirable colors. This may be due in part to overcrowding of the tobacco in available barn space, which contributes to less desirable colors and a lower quality crop.

TYLD was not significantly correlated with GID (**Table 4**), pointing out that yield alone is not a predictor of quality. TIP and NON were negatively correlated with TYLD, while MIS was positively correlated. As one would expect, this indicates that growing seasons with larger percentages of immature and over mature leaves would yield less. No correlation was found between TAREA and TYLD.

Predictive Model for Grade Index

Once the variables having significant correlations with GID had been identified, a stepwise regression (5) was used that maximized the coefficient of determination (r^2) for the resulting five-variable equation:

$$GID = 41.9 + 0.245\ BFR + 0.594\ CF + 0.442\ BF - 0.477\ TNM + 1.79\ TR \quad \textbf{(Equation 1)}$$

where:

GID is the yearly average grade index,

BFR is the yearly percentage of burley sold in the leaf category with tannish-red color,

CF is the yearly percentage of burley sold in the lugs category with tan color,

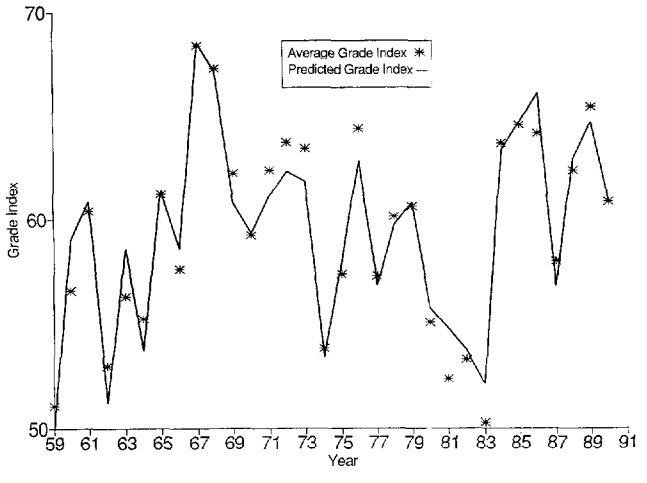
BF is the yearly percentage of burley sold in the leaf category with tan color,

TNM is the sum of the yearly percentage of burley sold as tips, nondescript, and miscellaneous, and

TR is the yearly percentage of burley sold in the tips category with red color.

The r^2 for **Equation 1** was 0.935, and overall it predicted GID well (**Figure 4**). Only for 1960, 1963, and 1981 did the model miss predicting GID by two or more points. The positive benefit for researchers working in this area is the model's ability to predict GID for a given year using easily identified input variables. However, the negative aspect to **Equation 1** is its inability to predict GID for future years. The input variables can be determined only after sales have been complete for a given year, making the equation inappropriate for an ongoing season. If growing and harvest season variables that impact both the grade index and the equation input factors could be identified, the model would be a more useful tool for researchers and producers. These aspects are addressed in Part 2 of this study (3).

Figure 4. Observed average yearly grade index (GID) for 1959-1990, and predicted values according to Equation 1 (see text).



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ASSESSING THE QUALITY OF BURLEY TOBACCO PART 2: ENVIRONMENTAL AND TIMELINESS FACTORS[1]

By T. C. Bridges, L. R. Walton and J. H. Casada[2]

This paper considered growing and harvest season variables that correlated with various leaf and color groups making up the yearly grade index (GID) for burley tobacco for 1959 - 1990. Weekly degree day sums (GDIFF), consisting of temperatures above 22.8°C during the growing season, were positively correlated with the percentage tips and nondescript leaf groups, and they were negatively correlated with crop yield. This indicated that temperatures above 22.8°C are detrimental to crop quality. The color groups of green leaves (BGR) and green tips (TGR) were also positively correlated with GDIFF, indicating that higher growing season temperatures contribute to undesirable green colors. A weekly growing season temperature-rainfall index, computed from GDIFF and weekly rainfall, also had a positive correlation with the percentage of tips, green leaf, and green tips. These correlations were higher than those for GDIFF alone, indicating that lack of rainfall further contributes to undesirable quality groups. Incidence of green color in leaf, lugs, and tips was also positively correlated with both cool, dry and warm, dry curing conditions, indicating low relative humidities are influential for the occurrence of undesirable colors. An equation predicting GID was developed using some of the environmental variables. This equation predicted GID fairly well for the 32-year period.

Additional key words: *Nicotiana tabacum*, burley tobacco, quality, grade index, environmental factors.

INTRODUCTION

Previously, an average yearly grade index was determined from sales data for burley tobacco for the years 1959 - 1990 by Bridges et al. (2). This index was used as a quality measure for each year's crop. Several leaf group, leaf color, and seasonal crop variables were significantly correlated with the average yearly grade index. The lugs category had a positive impact, while tips, nondescript, and the combined category of tips, nondescript, and miscellaneous were negatively correlated with quality. Color groups with negative correlations to quality included green leaves, green tips, green flyings, and red tips. Tannish-red leaves, tan lugs, and tan leaves all had positive correlations with grade index. These variables were used to develop a regression equation that predicted the average yearly grade index for the 32-year period reasonably well (2). While this equation was a reliable predictor of grade index, the input variables could be determined only after burley sales were completed. The focus of the study described herein was to identify and quantify growing and harvest season variables that could be used to predict crop quality factors during a current year.

Many crop and environmental factors are involved in determining the quality or grade assigned to a leaf of tobacco. Very little work has been done to determine factors that may influence crop quality during the growing season and harvest period. Walton & Henson (4) and Walton et al. (5) considered the effects of various temperature and humidity combinations during curing on the quality deterioration of burley tobacco, using support price as a measure. Optimum conditions for curing were defined as 21.1°C and 70% RH. Low relative humidities during the initial curing stage (4 - 6 days) resulted in significant damage to crop quality. Low humidities caused the tobacco to dry too quickly, thus setting an undesirable greenish color in the leaves. However, once the tobacco reached the yellowing stage of curing, little quality loss was seen due to low relative humidities. Damage increased with high humidities above the optimum level and as the period of exposure lengthened. Low temperatures (10°C) during the initial curing stage also caused a green color in the cured leaves, even with humidities as high as 90% RH.

The objectives of this study were (1) to use harvest and weather data to define environmental and timeliness factors associated with the growing and harvest season for each crop year, (2) to identify the environmental and timeliness factors that significantly impact the leaf and color variables used as inputs to the predictive model for the yearly grade index, and (3) to use the significant environmental and timeliness factors to refine the grade index model such that it would be useful for producers and researchers during a current growing season.

MATERIALS AND METHODS

In order to establish factors that might impact crop quality, it was necessary to define the growing season and the harvest period for each crop year. Once these were defined, environmental and timeliness variables were determined for both seasons using a weather data base. The following discussion explains the season and variable definitions and associated assumptions.

Weather Data

In determining the environmental variables that affect crop quality, weather conditions were considered only for a single location, Lexington, Ky. This location represents one of the largest burley tobacco markets in the world, and a significant amount of the crop is raised and sold within 161 km of Lexington each year. The weather data base consisted of mean daily temperatures, relative humidities, and daily rainfall for April 30 through Oct. 31, 1959 - 1990. This database was assumed to be indicative of average environmental conditions existing in the burley growing area in central Kentucky.

Harvest and Growing Seasons

The duration of the harvesting season for each year was determined from data compiled by the Kentucky Crop Reporting Service (1). These data consisted of weekly percentages of burley tobacco harvested across the state for 1959 - 1990. The starting date for harvest (SDH) for each harvest season was assumed to begin seven days prior to the end of the week in which the first harvest percentage was recorded. On the average, there were at least six weeks of recorded harvest percentages each year. When the total recorded harvest percentages did not equal 100% during the first six weeks of harvest, the remaining crop was assumed to have been harvested in the week succeeding the last week recorded by the Kentucky Crop Reporting Service (1). The

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weekly harvest percentages also were assumed to be independent of location.

The growing season also was defined using the recorded harvest period from the Kentucky Crop Reporting Service (1). The seasonal date of planting (SDP) was assumed to be the date 91 days (13 weeks) before SDH. The planting period and the percentage of the crop planted each week also were assumed to equal those of the seven-week harvest distribution. Thus, the percentage of tobacco assumed to be planted in week one of the growing season was the same percentage of tobacco harvested in the corresponding week of harvest. All tobacco was assumed to have the same length of growing season before harvest (91 days) and to have grown under similar weather conditions.

Environmental Variables During Harvest Season

Environmental variables affecting the curing process during harvest were created using temperature, relative humidity, and rainfall data. Temperature and relative humidity variables were based on exposure times to conditions other than the optimum curing values of 21.1°C and 70% RH (4,5). These variables were determined using a degree-day concept for temperature and an analogous concept for relative humidity. For example, if the mean temperature for one day was 26.7°C, then this was considered to be 5.6°C degree days above the optimum temperature. The following temperature variables were considered: DEGDY, the total degree days above and below the optimum temperature; DEGDYA, the degree days above the optimum temperature; and DEGDYB, the total degree days below the optimum temperature. Similar combinations also were considered for relative humidity: RHDY, the total relative humidity days from the optimum; RHDYA, the total relative humidity days above the optimum; and RHDYB, the total relative humidity days below the optimum. Four degree-day cross products (CP1 - CP4) were formed to better describe the type of curing environment in relation to the optimum temperature and relative humidity. CP1 was the product of DEGDYA and RHDYA, representing a warm, moist curing environment. CP2 was the product of DEGDYB and RHDYB, typifying cool, dry conditions. CP3 was the product of DEGDYA and RHDYB, representing a warm, dry curing climate; and CP4 was the product of DEGDYB and RHDYA, depicting a cool, moist curing environment. Rainfall variables consisted of the weekly sums (HR1 - HR7) and the total amount of rain during the harvest curing season (HRTOT).

Walton & Henson (4) showed that the critical exposure period for burley tobacco was generally the first four to six days immediately following harvest. Weekly harvest percentages varied significantly within a given year, possibly subjecting different portions of the crop to different curing conditions. It became necessary to define for each environmental variable an exposure period associated with each weekly harvest percentage. A two-week exposure period was set for each environmental variable (i.e., the week the tobacco was harvested and the week after harvest). Using SDH, the exposure conditions were summed for each two-week period until the entire crop was assumed to be harvested. The final value for each environmental variable was based on a weighted average of the accumulated exposure days accrued for each two-week period and the corresponding harvest percentage of the crop exposed to those conditions.

Timeliness Variables for the Harvest Season

Harvest variables related to timeliness were estimated from the Kentucky Crop Reporting Service (1). These included the following variables: SDH; HD50, the date at which 50% of the harvest occurred for each year; PC31, the percentage of tobacco harvested by Aug. 31; PC15, the percentage of burley harvested by Sept. 15; PCR21, the percentage remaining to be harvested on Sept. 21; and PCDIF, the amount of tobacco harvested in the first two weeks of September (i.e., the percentage harvested on Sept. 15 minus the percentage harvested on Aug. 31). One seasonal variable considered was the average crop yield for Kentucky (KYLD).

Environmental Variables during the Growing Season

The environmental variables for the growing season were created using temperature and rainfall data. Temperature variables were created using a degree-day concept similar to the process used for the harvest season variables. A value of 10°C was used as the base temperature for degree-day calculations during the growing season. Three different degree-day calculations were made for each of the 13 weeks, and they also were summed for the growing season. The three calculations for each week and the seasonal totals were: GRDG1-13 and GRDGT, total degree days above 10°C; G57DG1-13 and G57DGT, total degree days in the range 10°C - 22.8°C; and GDIFF1-13 and GDIFFT, total degree days above 22.8°C. Weekly and seasonal rainfall were designated as GR1-13 and GRTOT. Weekly and seasonal temperature-rainfall indices (TRAIN1-13 and TRAINT) were calculated as (GDIFF1-13)/(GR1-13) and GDIFFT/GRTOT. The exposure period for each variable was determined by the weekly planting percentage and the weather for the 13 weeks after planting. Therefore, the seasonal value for each variable was

Table 1. Correlations for weekly sums of degree days greater than 10°C (GRDG) with sales percentages of tips (TIP), miscellaneous (MIS), average crop yield (KYLD), green leaves (BGR), green tips (TGR), and green flyings (XGR).

Degree day sums by week	Correlation with TIP	Correlation with MIS	Correlation with KYLD	Correlation with BGR	Correlation with TGR	Correlation with XGR
GRDG1	0.1451	0.2088	-0.0305	0.4996**[a]	0.2511	0.3691
GRDG2	0.0677	0.3730	-0.1101	0.4214*	0.1959	0.1972
GRDG3	-0.0107	-0.0151	-0.1240	0.4062*	0.1737	0.2146
GRDG4	0.0526	-0.1250	-0.2051	0.4720**	0.2955	0.2549
GRDG5	0.1775	-0.0828	-0.2718	0.5331**	0.4123*	0.2722
GRDG6	0.3455	0.2391	-0.4421*	0.5069**	0.5424**	0.2969
GRDG7	0.3319	-0.2075	-0.4355*	0.4514**	0.5333**	0.3265
GRDG8	0.3987*	-0.2308	-0.4188*	0.3774*	0.5261**	0.2567
GRDG9	0.3939*	-0.3729*	-0.5412**	0.4417*	0.5845**	0.3540*
GRDG10	0.3906*	-0.4479*	-0.5825**	0.3985*	0.5838**	0.3484
GRDG11	0.4002*	-0.4222*	-0.6065**	0.3721*	0.5763**	0.3135
GRDG12	0.4239*	-0.4421*	-0.6136**	0.2493	0.5433**	0.2319
GRDG13	0.3278	-0.4314*	-0.4971**	0.0968	0.4027*	0.1621
GRDGT	0.3461	-0.2921	-0.4954**	0.5012**	0.5582**	0.3507*

[a] * = correlation significant at the 5% level; ** = correlation significant at the 1% level.

Table 2. Correlations for weekly sums of degree days from 10°C through 22.8°C (G57DG) with sales percentages of tips (TIP), nondescript (NON), miscellaneous (MIS), average crop yield (KYLD), green leaves (BGR), green tips (TGR), tan tips (TF), and buff lugs (CL).

Degree day sums by week	Correlation with TIP	Correlation with NON	Correlation with MIS	Correlation with KYLD	Correlation with BGR	Correlation with TGR	Correlation with TF	Correlation with CL
G57DG1	-0.1005	0.0005	0.0785	0.0520	-0.3027	-0.1583	-0.0076	-0.4587**[a]
G57DG2	-0.0928	0.0521	0.0501	0.0736	-0.2803	-0.1761	0.0115	-0.4285*
G57DG3	-0.1242	0.0138	0.0439	0.1379	-0.3348	-0.2360	-0.0534	-0.3873*
G57DG4	-0.1863	-0.0813	0.0929	0.1954	-0.3720*	-0.2968	-0.0853	-0.2490
G57DG5	-0.2849	-0.2427	0.0593	0.2915	-0.4190*	-0.4028*	-0.2169	-0.1673
G57DG6	-0.4363*	-0.3755*	0.1834	0.4665**	-0.3905*	-0.4974**	-0.4186*	-0.1887
G57DG7	-0.4295*	-0.4556**	0.1904	0.5365**	-0.3850*	-0.5013**	-0.4518**	-0.1181
G57DG8	-0.4865**	-0.4675**	0.2423	0.5222**	-0.2960	-0.4635**	-0.4329*	-0.1710
G57DG9	-0.4691**	-0.4385*	0.4183*	0.6714**	-0.3521*	-0.5302**	-0.4610**	-0.1572
G57DG10	-0.4521**	-0.3713*	0.4485**	0.6918**	-0.3338	-0.5531**	-0.4018*	-0.2602
G57DG11	-0.4785**	-0.4009*	0.3775*	0.7047**	-0.3725*	-0.5718**	-0.4935**	-0.1870
G57DG12	-0.4246*	-0.4119*	0.3060	0.6571**	-0.3730*	-0.5301**	-0.4551**	-0.0859
G57DG13	-0.4137*	-0.4200*	0.1067	0.5691**	-0.4756**	-0.5160**	-0.3873*	-0.0171
G57DGT	-0.4476*	-0.3740*	0.2668	0.5766**	-0.4697**	-0.5540**	-0.3933*	-0.2707

[a] * = correlation significant at the 5% level; ** = correlation significant at the 1% level.

based on a weighted average over the 13 weeks and on the percentage of the crop exposed to those conditions.

Leaf and Color Groups

The environmental variables defined for the growing season and harvest period were tested for significant correlations with the various leaf and color groups that make up the crop quality grade index (GID) (2). Leaf categories considered were flyings (FLY), leaf (LEA), lugs (LUG), miscellaneous (MIS), tips (TIP), nondescript (NON), mixed (MIX), and the combined group of tips, nondescript, and miscellaneous (TNM). Color groups considered were green leaves (BGR), green lugs (CGR), green tips (TGR), green flyings (XGR), red leaves (BR), red tips (TR), tannish-red leaves (BFR), tannish-red tips (TFR), tannish-red mixed (MFR), tan lugs (CF), tan leaves (BF), tan tips (TF), and buff lugs (CL). Significantly correlated variables were identified using Pearson correlation coefficients (3), and the results were discussed by the appropriate environmental variable.

RESULTS AND DISCUSSION

Temperature during the Growing Season

Table 1 shows the significant correlations of weekly sums of degree days above 10°C (GRDG) with several leaf and color groups. Positive correlations were found for weeks 8 - 12 for TIP, weeks 1 - 11 for BGR, and weeks 5 - 13 for TGR. This indicates that there was an increased percentage of these groups as temperatures increased above the base. Negative correlations were found between degree days and MIS and KYLD. This suggests a decrease in these factors with warmer temperatures. The significant correlations were not very definitive, because some of the temperature increases above the arbitrary temperature base were necessary and beneficial for crop growth. A division of degree days between beneficial and harmful portions was needed.

Significant correlations for the G57DG temperature variable are shown in **Table 2**. Negative correlations were found for weeks 6 - 13 for TIP and NON. These two leaf groups negatively impact crop quality (2). These negative correlations suggest that in cooler growing seasons (i.e., where a majority of the temperature in the growing season is within the 10°C - 22.8°C range) the percentages of TIP and NON would be smaller, ensuring a higher grade index. This also was supported by the strong positive correlations between G57DG and KYLD (**Table 2**), suggesting that cooler growing seasons would have increased yields. MIS was positively correlated with G57DG during weeks 9 - 11, indicating that cooler growing conditions during this time period may increase the percentage of the crop in the MIS group.

Several negative correlations (**Table 2**) were found between G57DG and BGR, TGR, and TF. These correlations suggest that cooler growing seasons would have less tobacco with unfavorable (green) colors, which would improve crop quality. The strong negative correlations between G57DG and TGR and TF were due somewhat to the negative correlation between TIP and G57DG (**Table 2**) (i.e., fewer tips

Table 3. Correlations for weekly sums of degree days above 22.8°C (GDIFF) with sales percentages of tips (TIP), nondescript (NON), miscellaneous (MIS), average crop yield (KYLD), green leaves (BGR), green tips (TGR), tan tips (TF), and buff lugs (CL).

Degree day sums by week	Correlation with TIP	Correlation with NON	Correlation with MIS	Correlation with KYLD	Correlation with BGR	Correlation with TGR	Correlation with TF	Correlation with CL
GDIFF1	0.1313	0.0908	0.0639	-0.0449	0.4278*[a]	0.2184	0.0033	0.3677*
GDIFF2	0.0851	-0.0139	-0.0091	-0.0958	0.3659*	0.1953	-0.0358	0.3598*
GDIFF3	0.0635	-0.0264	-0.0315	-0.1356	0.3795*	0.2136	-0.0063	0.3224
GDIFF4	0.1292	0.0294	-0.1109	-0.2060	0.4306*	0.3054	0.0283	0.1933
GDIFF5	0.2443	0.1747	-0.0722	-0.2919	0.4857**	0.4203*	0.1729	0.1111
GDIFF6	0.4097*	0.3007	-0.2155	-0.4716**	0.4578*	0.5356**	0.3887*	0.1420
GDIFF7	0.3999*	0.3556*	-0.2057	-0.5094**	0.4309*	0.5355**	0.4045*	0.0885
GDIFF8	0.4664**	0.3852*	-0.2467	-0.4966**	0.3439	0.5096**	0.4095*	0.1673
GDIFF9	0.4514**	0.3479	-0.4125*	-0.6355**	0.4064*	0.5747**	0.4187*	0.1813
GDIFF10	0.4348**	0.3118	-0.4592**	-0.6583**	0.3719*	0.5808**	0.3677*	0.2879
GDIFF11	0.4538**	0.3491	-0.4069*	-0.6761**	0.3811*	0.5873**	0.4249*	0.2332
GDIFF12	0.4379*	0.3847*	-0.3792*	-0.6579**	0.3273	0.5532**	0.3723*	0.1612
GDIFF13	0.3998*	0.3768*	-0.2835	-0.5739**	0.3140	0.4954**	0.3221	0.1375
GDIFFT	0.4094*	0.3090	-0.2841	-0.5504**	0.4941**	0.5673**	0.3294	0.2611

[a] * = correlation significant at the 5% level; ** = correlation significant at the 1% level.

Figure 1. Degree day sums above 22.8°C (GDIFF) versus week of the growing season for 1967, 1983, and the 32-year average (1959-1990).

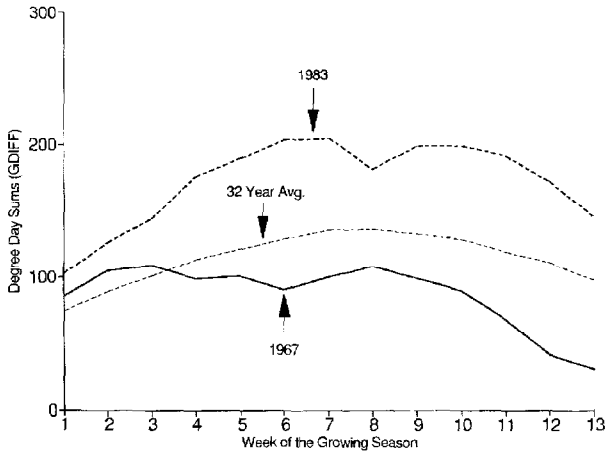
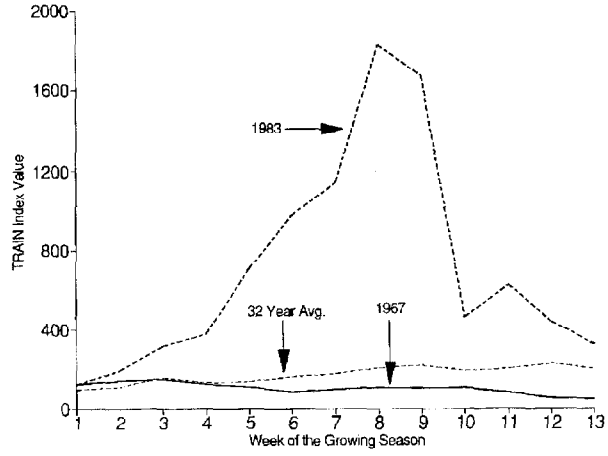


Figure 2. Temperature-rainfall index (TRAIN) versus week of the growing season for 1967, 1983, and the 32-year average (1959-1990).



mean fewer tips with those colors). Buff lugs (CL) also exhibited a negative correlation with G57DG for the first three weeks of the season.

If degree days in the 10 - 22.8°C range are beneficial to crop growth, then temperatures above 22.8°C should be detrimental to crop quality. **Table 3** shows correlations of GDIFF with various leaf and color groups. Generally, these correlations were the opposite of those found for the 10 - 22.8°C range (**Table 2**). One exception was with NON, which had fewer correlated weeks with the GDIFF variable. Average crop yield (KYLD) showed significant negative correlations with GDIFF for weeks 6 - 13 (**Table 3**), indicating that temperatures above 22.8°C may be detrimental to yield. This was also supported by the positive correlations between GDIFF and TIP for the same period. Several weekly sums of GDIFF were positively correlated with BGR and TGR, suggesting that high temperatures contribute to undesirable colors. The strong correlations for GDIFF with TGR and TF were partially due to the overriding significance between GDIFF and TIP.

From the degree-day correlations (**Tables 1 - 3**), it appeared that increased growing temperatures adversely affected crop quality by influencing the production of tips and green color groups. Increased percentages of tips also negatively impacts crop yield (2). The temperature values for this study were arbitrarily chosen, and it is beyond the scope of this paper to more closely define them. However, there appeared to be a temperature value above which maximum crop yield and quality were diminished. This is demonstrated further in **Figure 1** where GDIFF is plotted versus weeks of the growing season for 1967, 1983, and the 32-year average (1959 - 1990). These growing seasons were selected for their extremes in quality, with the 1967 crop having the highest grade index for the 32-year period (68.4), while 1983 had one of the lowest (50.2). **Figure 1** shows that the weekly sums for 1983 were well above the average values for most of the season, and for weeks 4 - 13 they were about twice the amount of those from 1967.

Table 4. Correlations for weekly growing season rainfall (GR) with sales percentages of green leaves (BGR), green tips (TGR), green flyings (XGR), and average crop yield (KYLD).

Rainfall sums by week	Correlation with BGR	Correlation with TGR	Correlation with XGR	Correlation with KYLD
GR1	-0.1214	-0.0276	-0.2512	-0.3016
GR2	-0.0686	0.0438	-0.2589	-0.2567
GR3	-0.1321	-0.0369	-0.3010	-0.1553
GR4	-0.2698	-0.1258	-0.3493*[a]	-0.0834
GR5	-0.3509*	-0.3047	-0.3683*	0.0938
GR6	-0.2816	-0.3962*	-0.3195	0.3392
GR7	-0.3573*	-0.5131**	-0.1896	0.3761*
GR8	-0.4313*	-0.5153**	-0.2845	0.4361*
GR9	-0.2308	-0.3894*	-0.2450	0.3647*
GR10	-0.2900	-0.4056*	-0.2635	0.3884*
GR11	-0.1886	-0.3563*	-0.1932	0.3538*
GR12	-0.1081	-0.3090	-0.0555	0.2592
GR13	-0.1077	-0.2960	0.0449	0.2280
GRTOT	-0.3533*	-0.4228*	-0.3865*	0.2179

[a] * = correlation significant at the 5% level;
** = correlation significant at the 1% level.

Rainfall during the Growing Season

Significant correlations were found between weekly totals of rainfall (GR) and three green leaf groups (BGR, TGR, XGR), and between GR and KYLD (**Table 4**). As expected, rainfall was positively correlated with KYLD for weeks 7 - 11. Negative correlations were found between GR and TGR, BGR, and XGR, indicating that green tobacco was more prevalent in years with low rainfall. Few significant correlations were found for the weekly rainfall totals with other leaf groups (data not shown), indicating rainfall measured in this manner was not a good indicator of crop leaf formation.

Temperature-Rainfall Index during the Growing Season

Because rainfall is vital for crop growth, an index was defined (TRAIN) that included rainfall and temperature as indicators of crop quality. This index was created to be somewhat indicative of crop stress. The correlations between TRAIN and TIP, KYLD, BGR, TGR, XGR, and CL are shown in **Table 5**. These correlations were similar to those found for the temperature variable GDIFF (**Table 3**), but they were somewhat higher. This indicates that a lack of moisture may further increase the negative impact of high temperatures on crop quality. This is also illustrated by the plot of the weekly TRAIN index for 1967 and 1983 (**Figure 2**). For the hot, dry year (1983), the weekly index values were well above the 32-year average. As this index became larger, conditions appeared to be favorable for increased amounts of tobacco with green color. The sum of BGR and TGR exceeded 22% of the total crop in 1983, while in 1967 these groups made up only about 2.4%. This demonstrates the need for irrigation to help reduce unfavorable crop quality factors as well as increase crop yield.

Table 5. Correlations for the weekly temperature-rainfall index (TRAIN) with sales percentages of tips (TIP), average crop yield (KYLD), green leaves (BGR), green tips (TGR), green flyings (XGR), and buff lugs (CL).

Weekly Index	Correlation with TIP	Correlation with KYLD	Correlation with BGR	Correlation with TGR	Correlation with XGR	Correlation with CL
TRAIN1	-0.0979	0.0911	0.2891	0.0382	0.5222**[a]	0.2041
TRAIN2	-0.0942	0.0021	0.2366	0.0450	0.5905**	0.1664
TRAIN3	-0.1405	-0.0294	0.1282	-0.0323	0.5424**	0.0304
TRAIN4	0.1900	-0.2111	0.5235**	0.3946*	0.3128	0.0198
TRAIN5	0.4388*	-0.3858*	0.6316**	0.6671**	0.2519	0.1079
TRAIN6	0.4848**	-0.4703**	0.5749**	0.6982**	0.1764	0.1234
TRAIN7	0.4984**	-0.4891**	0.5791**	0.7243**	0.1322	0.1274
TRAIN8	0.4910**	-0.4611**	0.5836**	0.7108**	0.1325	0.1684
TRAIN9	0.4784**	-0.4869**	0.5839**	0.7146**	0.1680	0.1893
TRAIN10	0.4263*	-0.5160**	0.4241*	0.5502**	0.2968	0.4614**
TRAIN11	0.3514*	-0.5023**	0.3924*	0.5367**	0.2078	0.4475*
TRAIN12	0.1927	-0.3334	0.2039	0.3136	0.1293	0.5707**
TRAIN13	0.1526	-0.3046	0.1481	0.2418	0.0917	0.6591**
TRAINT	0.4781**	-0.5056**	0.6421**	0.7103**	0.3570*	0.2767

[a] * = correlation significant at the 5% level; ** = correlation significant at the 1% level.

Timeliness Variables during the Harvest Season

The correlations for the timeliness variables with the LEA and MIS groups are shown in **Table 6**. A negative correlation was found between LEA and SDH. This suggests that for years when harvest is delayed, the crop would be less mature and the percentage of LEA would be diminished. The positive correlation between SDH and MIS indicates that some tobacco from LEA would be diverted to the MIS category when harvest is delayed. MIS also was positively correlated with HD50 and PCR21, indicating an increase in this group as the length of harvest increased.

Table 6 also shows the correlations between various color groups and the timeliness variables. BGR and CGR were positively correlated with PCR21, indicating that the longer tobacco remains in the field the better chance there is for an increase in this unfavorable color. The BGR, CGR, and XGR color groups were also negatively correlated with PCDIF, suggesting that the first two weeks of September is a favorable harvest period in terms of curing conditions. These correlations indicate that when the percentage of burley harvested during this period increases, there would be less tobacco with a green color and quality would improve. BFR was negatively correlated with SDH, and this may have been partially due to the significance of LEA and SDH (**Table 6**). TFR was positively correlated with SDH, indicating a higher incidence of TFR when harvest is later. This color group also had a positive correlation with PCDIF, suggesting that curing conditions during this period would be favorable for TFR.

Environmental Curing Variables During Harvest

Environmental curing variables during harvest influenced certain color groups as shown in **Table 7**. Strong positive correlations were found for BGR and CGR with DEGDY, which suggests that as temperature variations during the curing period became larger, the percentage of BGR and CGR increased. This is illustrated by **Figure 3**, which shows a trend for increases in the percentage of BGR + CGR when DEGDY increased during the 32-year period. The negative correlation between CF and DEGDY (**Table 7**) indicates a loss of the tan color group as the percentage of green lugs became larger. The temperature influence for green lugs can be attributed to cooler curing seasons as indicated by the positive correlation between CGR and DEGDYB. A temperature influence was also found between TGR and DEGDYA, suggesting that TGR increased during warmer curing periods. BGR, TGR, XL, and XF were positively influenced by RHDYB. These correlations indicate that drier curing seasons were favorable for these color groups. However, BFR and MFR were influenced by RHDYA, suggesting that there was an increase in these darker color groups with higher humidities.

Table 7 also shows the correlations for the cross-product curing variables with various color groups. Strong positive correlations were between CP2 and BGR, and between CP2 and CGR. However, BGR was also influenced by a warm, dry environment (CP3), again indicating that low relative humidities during curing would contribute to undesirable green colors regardless of temperature. TGR and TR were also strongly correlated with CP3. This may be explained, in part, by examining the exposure period. If these conditions occurred early in the curing process (4 - 6 days), low relative humidities might be more instrumental in influencing the greenish color than later in the cure when it appears that a warm, dry environment would tend to enhance a red color in tips. It was also found that the warm, dry climate (CP3) had a strong positive influence on XF. BFR was positively correlated with CP1, again showing the influence of high temperature and humidity with this darker color group. A cool, moist curing season (CP4) had little influence on any of the color groups.

Table 6. Correlations for the timeliness variables with the sales percentages of leaves (LEA), miscellaneous (MIS), green leaves (BGR), green lugs (CGR), green flyings (XGR), red leaves (BFR), and red tips (TFR).

Variable	Correlation with LEA	Correlation with MIS	Correlation with BGR	Correlation with CGR	Correlation with XGR	Correlation with BFR	Correlation with TFR
SDH	-0.5126**[a]	0.4715**	0.0336	-0.0722	-0.3566*	-0.4430*	0.3846*
HD50	-0.1529	0.5250**	0.2238	0.3417	-0.3087	-0.1749	-0.0225
PC31	0.2293	-0.4235*	-0.0915	-0.1146	0.3942*	0.2426	-0.1352
PC15	0.1182	-0.4755**	-0.3580*	-0.4401*	0.1818	0.1492	0.0901
PCR21	-0.0562	0.4896**	0.3936*	0.5566**	-0.0563	-0.0570	-0.1738
PCDIF	-0.2395	0.0102	-0.4268*	-0.5203**	-0.4478*	-0.2127	0.4097*

[a] * = correlation significant at the 5% level; ** = correlation significant at the 1% level.

Table 7. Correlations for the sales percentages of various color groups with the environmental curing variables and cross-products.

Color Group	Correlation with DEGDY	Correlation with DEGDYA	Correlation with DEGDYB	Correlation with RHDYA	Correlation with RHDYB	Correlation with CP1	Correlation with CP2	Correlation with CP3	Correlation with CP4
BGR	0.6260**[a]	0.2523	0.2686	-0.4053*	0.4596**	-0.2412	0.5535**	0.4385*	-0.0247
TGR	0.2398	0.4269*	-0.1430	-0.4184*	0.4356*	-0.1012	0.1597	0.6153**	-0.3144
CGR	0.5093**	-0.0947	0.4418*	-0.0374	0.1396	-0.1330	0.5182**	-0.0452	0.2781
BFR	-0.2488	-0.0735	-0.1266	0.4501**	-0.4185*	0.4466*	-0.3131	-0.3919*	0.0936
BR	-0.1654	0.0591	-0.1646	0.2525	-0.3278	0.2417	-0.3585*	-0.1814	0.0259
TR	-0.0621	0.3371	-0.2963	-0.2864	0.3077	-0.0114	-0.0150	0.4522**	-0.3400
MFR	0.0753	-0.1094	0.1364	0.3871*	-0.3498*	0.2015	-0.2378	-0.2957	0.3337
XL	-0.3042	-0.0258	-0.2026	-0.4420*	0.4180*	0.3689*	0.1453	0.3068	0.3566*
XF	-0.0331	0.1264	-0.1183	-0.4558**	0.5434**	-0.2820	0.3478	0.4860**	-0.3464*
CF	-0.3746*	-0.3749*	0.0059	-0.0258	0.0449	-0.1556	0.0678	-0.3050	-0.1013

[a] * = correlation significant at the 5% level; ** = correlation significant at the 1% level.

Predictive Model for Grade Index

The environmental variables that had significant correlations with the various leaf and color group variables were entered in a stepwise regression (3) to predict GID. The following eight-variable equation resulted:

GID = 94.9 - 0.143 MIX - 0.5 HRTOT - 0.236 TAREA
- 0.0008 (DEGDYB x RHDYB)
- 8.26 (G57GD12/GRDG12) + 0.48 (G57DG13/GR13)
- 0.012 TRAIN11 - 0.02 TRAIN13 **(Equation 1)**

where:

GID is the average yearly grade index,

MIX is the yearly percentage of burley sold as mixed grade,

HRTOT is the total rainfall for the harvest season,

TAREA is the total crop area in hectares,

DEGDYB is the harvest degree days below the optimum curing temperature,

RHDYB is the harvest relative humidity days below the optimum of 70% RH,

G57DG12 is the weekly sum of degree days for 10° - 22.8°C for the 12th week of the growing season,

GRDG12 is the weekly sum of degree days above the base temperature (10°C) for the 12th week of the growing season,

G57DG13 is the weekly sum of degree days for 10° - 22.8°C for the 13th week of the growing season,

GR13 is the weekly rainfall for the 13th week of the growing season,

TRAIN11 is the temperature-rainfall index for the 11th week of the growing season, and

TRAIN13 is the temperature-rainfall index for the 13th week of the growing season.

The coefficient of determination (r^2) for **Equation 1** was 0.886. The ability of **Equation 1** to predict grade index is demonstrated by **Figure 4**, and in general it is an adequate predictor of GID for the 32-year period, except for 1961. Most of the terms in **Equation 1** are negative, suggesting that the input variables are most useful in growing seasons where these factors are detrimental to overall crop quality. TAREA was entered into the equation because it has been shown (2) to be an inverse predictor of crop quality. The negative influence of HRTOT may be indicative of years where crop quality was impacted by harvest delays or by reduction in grades due to wet or muddy tobacco. The negative impact of DEGDYB and RHDYB reflect cool, dry conditions favorable for the occurrence of undesirable green color in the LEA and LUG categories. Weekly temperature and rainfall variables in **Equation 1** indicate that environmental conditions during the last three weeks of the growing season are critical to crop quality. Warmer temperatures during this period can cause the temperature-rainfall index (TRAIN) to increase, adversely affecting crop quality.

Originally, all variables used to predict grade index in Part 1 of this study (2) were excluded from the stepwise regression procedure. However, after several unsuccessful attempts to adequately describe the yearly variation, it was decided that the MIX variable must be included in the regression equation. Unlike other leaf groups, MIX is an indication that the producers have not separated their leaves according to stalk position. All of the other leaf variables are

Figure 3. Harvest degree days above and below 21.1°C (DEGDY) versus percent green leaf and lugs sold (BGR+CGR), 1959-1990.

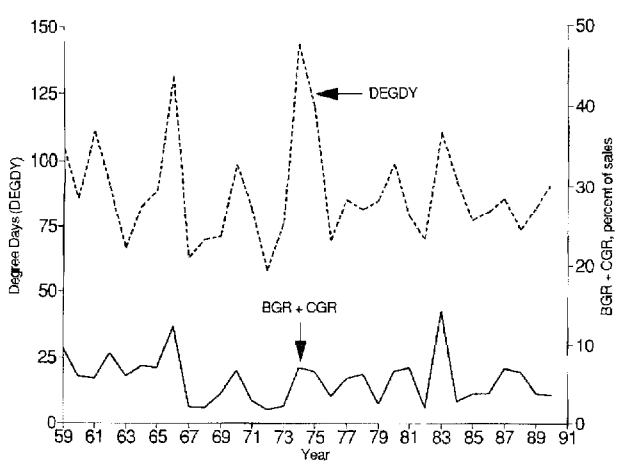
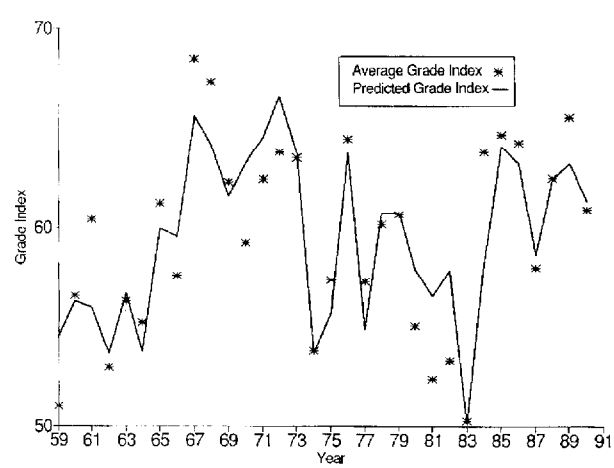


Figure 4. Observed average yearly grade index (GID) for 1959-1990, and predicted values according to Equation 1 (see text).



determined by physical and aromatic characteristics of the leaves, not by a factor determined strictly by the manner in which the producer removes the leaves from the stalk. The use of MIX makes **Equation 1** a reliable predictor of GID. One approach that would preserve the ability of **Equation 1** to predict the quality of tobacco in a current growing season would be to use the percent of MIX from the previous year, or to use an average of the past three years if a trend is desired. The sales percentage of mixed grades is expected to decline because the price for MIX decreased approximately $0.07/kg from 1991 to 1992, while the average price of all grades increased a similar amount for the same time period. The trend in mixed grades is such that the inclusion of the percent of MIX from the previous year should be an acceptable compromise for improving the prediction of the model.

Several factors that affect crop quality were not addressed in this study, and they were beyond the scope of the existing data base. These include the use of fertilizer (especially nitrogen), irrigation, the use of cultural practices such as sucker control materials, and the selection of topping time. **Equation 1** tends to be better at predicting GID after 1971. Regressing **Equation 1** over 1971 - 1990 gave a somewhat better fit of the data (r^2=0.935). The better fit after 1971 may be due to the change in the burley quota system from an acreage to a poundage basis. It also indicates that crop quality has become more of a concern to producers after 1971.

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ANALYSIS OF SCOUTING TECHNIQUE FOR THE TOBACCO BUDWORM ON TOBACCO[1]

By Donald G. Manley[2]

The tobacco budworm, *Heliothis virescens* (F.), is one of the most economically important insect pests of tobacco in South Carolina. Field scouting procedures for this insect on tobacco differ from state to state. Much flue-cured tobacco is planted in a four-row pattern with a skip row, thus having two inside and two outside rows for each four rows of tobacco. This study, conducted using 71 side-by-side comparisons during 1985-91, documents that budworm infestations vary between rows. Although not statistically significant, there was a definite trend toward higher budworm populations in outside rows. These trials demonstrated that 15% of the time a decision to treat depended on whether inside or outside rows were scouted. All of these occurred when budworm populations were at or near the economic threshold level. Thus, it is concluded that when scouting tobacco for tobacco budworms, samples should include an approximately equal number of inside and outside plants. And, if greater accuracy is deemed necessary, additional samples may be taken.

Additional key words: *Nicotiana tabacum*, tobacco, tobacco budworm, *Heliothis virescens*, field scouting, economic thresholds.

INTRODUCTION

The tobacco budworm, *Heliothis virescens* (F.), is one of the most important insect pests of flue-cured tobacco in South Carolina (2,3,8). Combined losses to producers from damage and control costs is approximately two million dollars each year (1,5,6). Field scouting and economic thresholds have become essential components of an integrated pest management system for tobacco designed to reduce dependence on insecticide applications. Both a reasonably accurate economic threshold and a procedure for scouting tobacco in South Carolina have been established (4,7). The economic threshold (treatment level) for the tobacco budworm in South Carolina is 4% of the plants infested during the first four weeks after transplanting, and 10% thereafter, until topping.

Much flue-cured tobacco is planted in a pattern of four rows of tobacco followed by an unplanted skip row. Thus, for every four-row strip, there are two outside rows and two inside rows. If a field scout, or sampler, got far enough inside a field to escape the "edge effect," there was thought to be no difference in budworm infestations between inside or outside rows of a four-row strip. However, preliminary results from scouting in tobacco indicated that certain rows of tobacco were more heavily infested with insect pests than other rows.

Although scouting for insect pests of some crops, such as cotton and soybeans, has long been practiced, scouting procedures for tobacco have only recently been developed. No two states use quite the same scouting techniques and methodology (e.g., the number of plants examined, scouting pattern, rows, etc.). Consequently, tobacco entomologists decided in 1984 that a cooperative study would be undertaken to investigate the possibility of standardizing the scouting techniques of tobacco-growing states. Thus, this study was initiated to determine whether or not row orientation (inside or outside) influences the population density of tobacco budworms, and what impact row orientation has on economic threshold levels.

MATERIALS AND METHODS

Seventy-one side by side comparisons of field scouting data were made during 1985-1991 in flue-cured tobacco fields to determine tobacco budworm population densities. The standard scouting practice in South Carolina is to check 50 plants in a field (10 consecutive plants in five different locations, without regard to inner or outer row) (4). In the multi-state cooperative study, 180 plants were sampled in each field (20 consecutive plants in nine different locations, without regard to inner or outer row). In this study described herein, both sampling techniques were used. However, the number of plants sampled was doubled (i.e., 100 or 360). At each location, plants were sampled from adjacent inside and outside rows. Because all samples were made at least four weeks after transplanting, the 10% threshold level of infested plants was used.

Data were analyzed using a three-factor analysis with the factors being year, field, and row. Fields were nested within year and row in a split-plot effect. Year by row interaction effect was also investigated. The appropriate analysis of variance was accomplished with the use of the general linear models procedure of the Statistical Analysis System (9). Data for each year were combined for statistical analysis.

RESULTS

Both year-to-year and field-to-field variability were statistically significant ($P = 0.0001$), which is expected when sampling arthropod population densities. Infestation percentages for inside row vs. outside row were statistically non-significant ($P = 0.1929$) (**Table 1**).

For 1985, 1989, and for the seven-year mean, whether inside or outside rows were checked would have made a difference in whether or not fields should have been sprayed if the 10% threshold level were strictly adhered to. Using the data from the 71 individual trials (not shown), there would have been a difference in 11 (15%) of the trials. Of those 11 trials, spraying would have been indicated in nine of them (13% of the 71 total) based on scouting outside rows, but it would not have been indicated based on scouting inside rows. The reverse was true for the other two trials.

There were no differences between inside and outside rows, with respect to the number of trials in which an economic threshold was reached, in 1986, 1987, 1990, or 1991. From the data in **Table 1**, it can be seen that 1986 and 1990 were light tobacco budworm years, while there were heavy infestations in 1987 and 1991.

DISCUSSION

Yield loss and control costs are only part of the impact of insect pests on tobacco producers (1,5,6). There are growing concerns of resistance to insecticides by the pests, the possibility of harmful effects that these pesticides may have on the environment, and the health of applicators and field workers. The primary reason for the establishment and use

[1] Technical Contribution No. 3418 of the South Carolina Agricultural Experiment Station, Clemson University.

[2] Department of Entomology, Clemson University, Pee Dee Research and Education Center, Route 1, Box 531, Florence, South Carolina 29501-9603.

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Table 1. Tobacco budworm (TBW) infestation (% of plants infested) in inside versus outside rows of flue-cured tobacco in South Carolina from 1985-1991.[a]

		Inside Rows		Outside Rows	
Year	Trials	TBW/Plants[b]	%	TBWs/Plants	%
1985	5	68/900	7.6	117/900	13.0
1986	5	15/380	3.9	14/380	3.7
1987	7	99/350	28.3	105/350	30.0
1988	16	28/800	3.5	47/800	5.9
1989	18	77/900	8.6	107/900	11.9
1990	11	25/550	4.5	28/550	5.1
1991	9	70/450	15.6	74/450	16.4
TOTAL	71	382/4330	8.8	492/4330	11.4

[a] For each year, there were no statistically significant (P = 0.05) differences in infestation levels between inside rows and outside rows using 2-way Analysis of Variance.

[b] TBWs/Plants equals the number of tobacco budworms found/total number of plants sampled.

of economic thresholds and scouting programs in IPM is to apply insecticides only when necessary. However, scouting techniques must accurately predict the actual insect population density in a given field.

Although the differences were not statistically significant, there was a definite trend toward higher budworm populations in outside rows. Based on these data, a tobacco scout might have erred in telling a producer whether or not to treat for insects 15% of the time. This would not be acceptable in a viable IPM program. The problem of inaccurate recommendations occurs when budworm populations are at moderate levels, that is, when they are near the economic threshold level. When populations are high, as in 1987 and 1991, treatment is clearly necessary, whereas when populations are low, as in 1986 and 1990, treatment is not necessary.

No scouting procedure, short of checking every plant, is totally accurate. In any scouting program there will be a trade-off between efficiency and accuracy. This study, and earlier unpublished studies, indicate that the 50-plant scouting procedure presently being used in South Carolina is acceptable in predicting tobacco budworm population densities under most circumstances. Under research conditions, where greater accuracy is desirable, additional samples may be necessary when populations are near the economic threshold. However, in most field scouting situations, the additional cost of being wrong (spraying when the threshold has not been reached, or not spraying when it has been reached) would probably not justify the time necessary for taking additional samples. It seems advisable that whatever procedure is being used to scout tobacco, that both inside and outside rows be sampled in approximately equal numbers. This eliminates possible bias in determining whether an economic threshold level has been attained, especially if the infestation level is very near the threshold.

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TRANSFORMATION FREQUENCY AND FOREIGN GENE EXPRESSION IN BURLEY AND FLUE-CURED CULTIVARS OF TOBACCO

TOBACCO SCIENCE

By Margaret E. Daub[1], Anne E. Jenns[2], Lori A. Urban[2], and Sara C. Brintle[3]

Three burley and seven flue-cured cultivars of tobacco (*Nicotiana tabacum* L.) were evaluated for transformation frequency and foreign gene expression. Leaf disk explant tissue was transformed with *Agrobacterium tumefaciens* strain LBA4404 carrying the plasmid pBI121, which contains genes encoding kanamycin resistance and β-glucuronidase (GUS) activity. Cultivars were evaluated for time to callus and shoot production, percent of explants producing transformed shoots, and GUS activity of transformed R_1 progeny. All cultivars tested were readily transformed with a frequency of 44-95%, with the exception of the flue-cured cultivar 'K399'. Transformed callus appeared within two weeks of transformation, and shoots were recovered at two to six weeks. Differences among cultivars were found in the ability of transformed R_1 plants to express the GUS gene. Two burley cultivars, 'Burley 21' and 'KY 14', had consistently higher mean GUS activities and a lower percentage of non-expressing plants than the flue-cured cultivars and the burley cultivar 'TN 86'. These cultivars provide an excellent genetic base for genetic engineering studies.

Additional key words: *Nicotiana tabacum*, β-glucuronidase, *Agrobacterium*, GUS.

INTRODUCTION

Genetic transformation of tobacco (*Nicotiana tabacum* L.) for expression of foreign genes has become a routine and widely-available technology. To date, most efforts have focused on the expression of genes that have the potential to improve crop production, such as genes that confer resistance to viral and fungal diseases, insects, or herbicides (5,8,9,10,12,16,17,20,23,24). Increasingly, however, tobacco is also being investigated as a vehicle for the manufacture of novel products such as pharmaceuticals, proteins, enzymes, and antibodies (18). Such efforts hold promise for both efficient production of valuable commodities and economic stability in tobacco-growing regions through the continued demand for efficient tobacco production.

Optimum use of tobacco for production of new products requires that genes be engineered into cultivars that are high-yielding and well adapted to the tobacco-growing regions. To date, however, most studies of tobacco transformation have utilized two varieties, 'Xanthi' (4,11,13,16,23,24) and 'Petite Havana SR1' (8,9,10,12), neither of which is grown commercially in the United States. The purpose of this work was to investigate genetic transformation and foreign gene expression in well-adapted cultivars of flue-cured and burley tobacco in order to determine the suitability of such cultivars for genetic engineering.

MATERIALS AND METHODS

Plant Material

The following commercial cultivars were used in this study: flue-cured cultivars 'Coker 319', 'Coker 371 Gold', 'K326', 'K399', 'McNair 944', 'NC2326', and 'Speight G-28', and the burley cultivars 'Burley 21', 'KY14', and 'TN86'. In addition, the wild species *Nicotiana benthamiana* Domin was tested because of its high susceptibility to viruses and potential usefulness in cross protection studies. Plants were grown in the greenhouse from seed in a 2:1 mixture of Metromix, a commercial growing medium (W.R. Grace and Co., Cambridge, Mass.), and sterile soil containing a 3:9:9 fertilizer and osmocote (a 14:14:14 control-release fertilizer). Seedlings were transplanted after four weeks to 15-cm diameter clay pots containing the same mixture.

Transformation

Plants were transformed by *Agrobacterium*-mediated transformation using the disarmed *A. tumefaciens* vector strain LBA4404 (Clontech Laboratories, Inc., Palo Alto, Calif.). The plasmid used was pBI121 (Clontech Laboratories, Inc.), which contains a gene for kanamycin resistance used for selection of transformants. It also contains a ß-glucuronidase (GUS) gene under the control of the CaMV 35S promoter, which was used for assaying gene expression. Plasmid DNA was introduced into LBA4404 using a freeze/thaw procedure (3). Bacteria were grown in YEP medium (10 g peptone, 10 g yeast extract, 5 g NaCl per liter) containing 25 µg/mL streptomycin and 50 µg/mL kanamycin. Leaf disk explants harvested from fully-expanded leaves of 2-3 month old plants were transformed as described by Bui et al. (7), except that shoots were selected and rooted on medium containing 50 µg/mL kanamycin. Each tobacco cultivar was transformed in two trials. Each trial was carried out at a different time of the year and by a different individual. Within a trial, each cultivar was transformed in two independent experiments, except for K399, which was difficult to transform. In each trial, cultivars were evaluated for the percent of explants producing transformed shoots as well as the time to callus and shoot production. Transformed plants were grown to maturity in the greenhouse, and selfed seed was harvested for analysis of GUS expression.

Gene Epression

GUS gene expression was analyzed in the first generation selfed progeny (R_1 progeny) of transformed plants. Selfed seeds from transformed plants were surface sterilized (60 sec. in 70% ethanol followed by 5 min. in 10% commercial bleach with 0.01% sodium lauryl sulfate) and germinated *in vitro* on hormone-free Murashige & Skoog medium (19) containing 50 µg/mL kanamycin in order to select for transformed seedlings. Seedlings were transferred to fresh kanamycin-containing medium at four weeks. After two additional weeks, kanamycin-resistant seedlings of uniform size (containing cotyledons plus two pairs of leaves) were harvested and stored at -70°C until assayed. Individual, whole seedlings (roots, cotyledons and leaves) were ground in 200 µL buffer, and GUS activity was assayed using a fluorogenic assay as described by Jefferson (15). This assay measures the conversion of 4-methyl umbelliferyl ß-D-glucuronide (MUG) to the fluorescent compound 4-methyl umbelliferone (MU). Reactions were terminated at 0, 5, 10, 15, 20, and 30 minutes, and the amount of MU present quantified using a spectrofluorimeter. GUS enzyme activity was expressed as nmoles MUG converted/minute/mg protein. Protein concentrations were determined by the Bradford method (BioRad Laboratories, Melville, N.Y.). In each trial, 10 seedlings from each of 4-13 initial transformed

[1] Associate Professor, Department of Plant Pathology, North Carolina State University, Raleigh, N.C. 27695-7616
[2] Research Associate, Department of Plant Pathology, North Carolina State University.
[3] Undergraduate Honors Student, North Carolina State University.

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Table 1. Time to callus and shoot formation and percent transformation of leaf explants of flue-cured and burley tobacco cultivars and *Nicotiana benthamiana* following *Agrobacterium*-mediated transformation.

Cultivar	Weeks to callus[a]	Weeks to shoots[a]	% Transformation[b]	
			Trial 1	Trial 2
Coker 319	2	4-6	84	51
Coker 371 Gold	2	4	80	93
K 326	2	2-4	69	56
K 399	-[c]	-[c]	2	0
McNair 944	2	2-4	46	84
NC 2326	2	4-6	95	66
Speight G-28	2	4	50	85
Burley 21	2	4-6	60	91
KY 14	2	4	92	66
TN 86	2-4	6	44	73
N. benthamiana	2	2-4	79	75

[a] Range represents difference between trials.
[b] % of leaf explants producing transformed shoots.
[c] Not determined.

plants of each cultivar were assayed. GUS activity of non-transformed controls ranged from 0.00 to 0.02 nmoles/min/mg protein. Plants with GUS activities of 0.02 or less were classified as having no GUS expression, and are referred to as "non-expressors" even though all expressed resistance to kanamycin.

Statistical Analysis

GUS activity data were subjected to a log transformation to correct for skewness in the data. The transformed activity data were subjected to analysis of variance using the GLM procedure of the SAS system (22). Trial, and plant within cultivar were considered random variables.

RESULTS

Transformation Frequency

With the exception of cultivar K399, large numbers of transformed plants were readily obtained from all the flue-cured and burley cultivars tested (**Table 1**). Transformed callus was readily visible within two weeks of placing transformed leaf explant tissue on selection medium. Shoots appeared at two to six weeks, depending on the cultivar. K326, McNair 944, and *N. benthamiana* produced shoots as early as two weeks, but TN 86 did not produce them until six weeks. With the exception of K399, the percent of explants producing transformed shoots varied greatly from a low of 44% to a high of 95% in the two trials, and there appeared to be no consistent cultivar effect, with greater variation between trials than among cultivars.

K399 was not readily transformed. With explants from this cultivar, growth of the vector bacterium was greatly stimulated and could not be suppressed with antibiotics. Proliferation of the bacteria damaged the explant tissue such that little or no callus was produced. This effect was consistent across six different transformation experiments. With repeated transfers some shoots were recovered, and a total of 11 plants were eventually recovered from the six experiments. GUS activity was determined on R_1 progeny of these 11 plants.

Table 2. Analysis of variance of log-transformed GUS activity.

Trial	Source	df	Mean Square	F	P>F
1	Cultivar	10	115.80	4.38	0.0001
	Plant within cultivar	93	26.47	37.14	0.0001
	Error	915	0.71		
2	Cultivar	9	155.92	3.91	0.0003
	Plant within cultivar	86	39.83	60.87	0.0001
	Error	864	0.65		

Expression of GUS Activity

Analysis of variance of GUS activity of R_1 plants from both trials indicated no significant effect of cultivar or trial, but a highly significant cultivar x trial interaction. When data from each trial were analyzed separately, the variances due to plant within cultivar and due to cultivar were highly significant in each trial (**Table 2**). The variation among R_1 progeny of each cultivar was large (**Figure 1**), ranging from no detectable activity to as high as 20 nmoles/min/mg protein. The majority of the R_1 plants had activities ranging from 1-10 nmoles/min/mg protein. Most cultivars had R_1 plants which were non-expressors (defined in this study as plants with GUS activity of 0.02 nmoles/min/mg protein or less). The proportion of non-expressing plants varied

Figure 1. Expression of GUS activity by R_1 progeny of transformed plants of ten tobacco cultivars and *Nicotiana benthamiana*. GUS activity was assayed by a fluorogenic assay as described in the Materials and Methods. The figure shows percent of progeny with indicated activity ± 0.5.

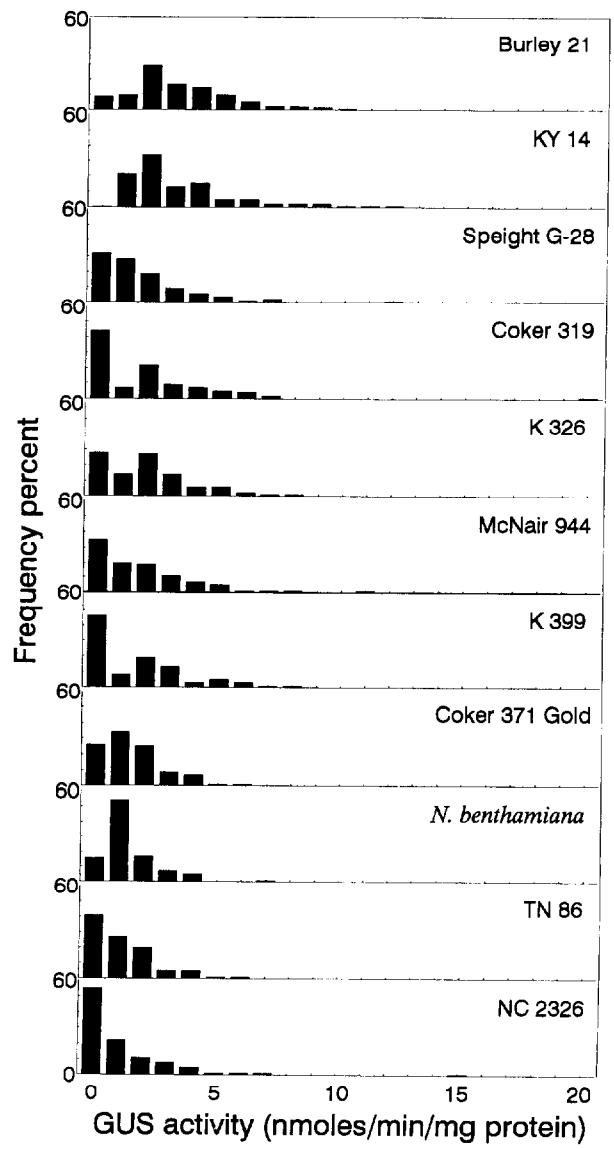

Table 3. Mean GUS activity and percentage of non-expressing plants among offspring of ten transformed tobacco cultivars and *Nicotiana benthamiana.*

	Mean GUS Activity (nmoles/min/mg protein)			Percent of Non-Expressors		
Cultivar	Trial 1	Trial 2	Overall	Trial 1	Trial 2	Overall
Burley 21	3.02	3.25	3.14	9	3	6
KY14	3.79	2.41	3.10	0	0	0
K326	1.67	1.99	1.87	25	22	23
C319	1.00	2.76	1.78	57	12	37
McNair 944	2.64	0.84	1.69	1	50	27
SpG28	2.29	0.90	1.59	2	40	21
K399	1.66	1.51	1.56	55	40	45
C371 Gold	1.28	1.54	1.41	10	10	10
N. benthamiana	1.38	-----	-----	11	---	---
NC2326	1.51	0.72	1.15	12	56	32
TN86	1.17	1.06	1.11	40	25	32

widely from a low of 0% to a high of 67% (**Table 3**).

R_1 progeny of two burley cultivars, Burley 21 and KY14, expressed high GUS activity, and they had the lowest frequency of non-expressing plants in both trials (**Table 3**). Of the flue-cured cultivars, K326 and Coker 319 had the highest activities, and Coker 371 Gold had the lowest percent of non-expressors.

DISCUSSION

The majority of the tobacco cultivars investigated in this study were readily transformed using *Agrobacterium*-mediated transformation. Transformed callus developed within two weeks of placing explants on selection medium, and shoots were obtained at two to six weeks. It was difficult to isolate transformed plants from K399. This difficulty was not due to the inability of the vector bacterium to transform cells of this cultivar. The few shoots that were obtained were stably transformed and they expressed GUS activity equivalent to many of the other flue-cured cultivars. The lack of efficient transformation was due to inhibition of shoot regeneration by vector bacteria, the growth of which was stimulated by the explant tissue. Thus, with this one exception, common flue-cured and burley cultivars appeared to be equally amenable to *Agrobacterium*-mediated transformation, and they provide a viable alternative to the experimental varieties used in many genetic engineering studies.

Differences were found, however, in the ability of the cultivars to express the GUS reporter gene. Progeny of transformed plants of two of the burley cultivars, Burley 21 and KY 14, had higher overall mean GUS activities than did the other burley cultivar TN 86 or any of the flue-cured cultivars. Progeny of the Burley 21 and KY 14 transformants also had the lowest frequency of non-expressing plants. From the 200 plants analyzed each for KY 14 and Burley 21, zero and 12 plants, respectively, were non-expressors compared to the worst cultivar tested, NC2326, where 113 out of the 220 plants assayed were non-expressors.

Lack of uniform gene expression in transformed plants is a consistent problem in genetic engineering studies. Studies generally report large variability in expression of foreign genes even within an individual experiment. Our study was no exception, with values ranging from 1-20 nmoles/min/mg protein in progeny of a single transformed plant. This range in activity has generally been attributed to variation in transcriptional activity of the insertion site of the gene, the so-called "position effect." Support for this hypothesis comes from studies that targeted DNA to specific regions of the chromosome. Allen et al. (1) reported decreased variation and increased expression of the GUS gene in cultured tobacco cells transformed by particle bombardment when the GUS gene was flanked by a DNA sequence from yeast that binds to specific "scaffold" regions on the chromosome. Similar studies with *Agrobacterium*-mediated transformation also reported decreased variation, but with overall lower levels of expression, when a scaffold attachment region was introduced along with the GUS gene (6). Other factors have also been correlated with variations in gene expression. Hobbs et al. (13) determined that low levels of GUS gene expression in transformed tobacco plants were correlated with the presence of multiple T-DNA insertions and with increased methylation of the integrated DNA.

It has been well documented that the chemical composition of the plant plays a major role in induction of the *Agrobacterium* virulence (*vir*) genes and in gene transfer (14). Burley tobacco was originally isolated as a spontaneous mutant of a broadleaf tobacco over 100 years ago (25), and flue-cured and burley cultivars are chemically very different from each other. Thus, it is not surprising that there may be differences in their response to *in vitro* culture and transformation. The difference between TN 86 and the other two burley cultivars, KY 14 and Burley 21, was surprising, particularly since both KY 14 and TN 86 were derived from crosses between Burley 21 and other varieties, and TN 86 has a greater proportion of the Burley 21 genome (R. Rufty, personal communication). All burley cultivars contain two copies of a recessive gene conferring chlorophyll deficiency (25), but TN 86 is the most chlorophyll deficient of the three cultivars tested and is grown commercially only with the application of large amounts of nitrogen fertilizer. In culture, TN 86 was not as vigorous as the other cultivars, requiring up to two weeks longer for callus and shoot production (**Table 1**). Rapid growth of tobacco cells *in vitro* has been positively correlated with transformation frequency (2), and it has been suggested that T-DNA integration is most efficient during host cell replication (21). The slow growth of TN 86 cells *in vitro* may have had a negative impact on gene integration.

Efforts to use tobacco for the production of novel compounds would benefit from cultivars that are well-adapted to the tobacco-growing regions of the United States and that provide uniform expression of genes. Both of these criteria are met by the high-expressing cultivars identified in this study. KY 14 in particular is a high-yielding, widely grown and popular cultivar in the burley-growing regions. Burley 21 is not generally grown, but it remains a quality standard and a component of several hybrid cultivars. The high levels of expression and lack of significant numbers of non-expressing plants should streamline efforts to develop highly-expressing lines for production purposes. These cultivars provide an excellent genetic base and should be utilized for genetic engineering efforts aimed at the use of tobacco for production of novel products.

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ECONOMIC ANALYSIS OF A ONE-PERSON MECHANIZED HARVEST SYSTEM FOR MARYLAND TOBACCO

TOBACCO SCIENCE

By James C. Hanson[1] and Claude G. McKee[2]

The length of time was measured for one person to harvest and barn Maryland tobacco using a partially mechanized system where stalks were cut mechanically, plants were speared onto sticks, sticks were loaded onto beams in the field, and beams were hung in a barn with a cable-hoist system. The average time to complete these tasks was 71.3 hours/hectare. Using this estimate along with historical weather data, it was determined that one person could harvest and barn 4.5 hectares of tobacco three out of every four years without additional help. The length of time required to take down and bundle this tobacco was calculated to be 20.2 hours/hectare. Using a partial budget analysis, it was estimated that the mechanical, beam and cable-hoist system was slightly more expensive than a conventional, all manual labor system. When labor costs were estimated at $7.50/hour, net income for 4.5 hectares of tobacco was reduced by $816 (7% loss) when the mechanical system was used compared to the conventional harvest system. When labor costs were estimated at $9.50/hour, net income was reduced by $462 (4% loss). Many farmers in Maryland have stopped producing tobacco because of the difficulties in obtaining labor for harvesting their crop. In these cases, the reduction in net income by the mechanical harvesting system may be justified if it allows farmers to continue to produce tobacco.

Additional key words: *Nicotiana tabacum*, stalk cutting, labor studies, mechanical harvest.

INTRODUCTION

Maryland tobacco (Type 32), *Nicotiana tabacum* L., is a stalk-cut, air-cured type. Typically, the labor-intensive harvest occurs during the hot, humid period beginning in August and continuing through the third week of September. There has been interest from Maryland growers for a one-person system for harvesting tobacco. Labor for harvesting is difficult to find in most seasons. Also, many workers will not work at heights during the barning process because of the risk of serious injury from falls. Farmers desiring financial protection against such mishaps find the required insurance coverage for workers to be expensive. Another problem is that labor is often available only on weekends, and some growers want to harvest during the week. Other growers, with smaller acreages, are interested in a self-sufficient harvesting system based on one person because it better fits their operations.

McKee et. al. (2) conducted a preliminary labor study that evaluated different crew sizes and harvesting methods for Maryland tobacco. One method involved cutting tobacco with a small blade mounted on a walk-behind tractor and barning the tobacco with a beam and cable-hoist system developed by Duncan & Tapp (1). The ability of one person to efficiently operate this system showed promise. The present study examined the potential for a one-person mechanized harvesting and barning system for Maryland tobacco. This study also estimated the number of hectares one person could harvest in a season, and compared the effect on net income of switching from the conventional harvest method (all manual labor, multi-person crew) to this mechanized system.

MATERIALS AND METHODS

A labor study was conducted for a system in which one person cuts tobacco with a mechanical cutting machine, spears and loads beams with tobacco in the field, and hangs these beams in a barn with the beam and cable-hoist system described by Duncan & Tapp (1) and McKee et al. (2). As opposed to labor studies that evaluate specific operations, this study was designed to emulate the total process of harvesting and barning tobacco by the farmer.

A worker, with one season of experience using the beam and cable-hoist system, timed himself under typical working conditions on five days in August, 1991, when researchers were not present. Each day, 480 plants (96 sticks) were cut. Some plots were six rows of 80 plants, others were 12 rows of 40 plants. Tobacco was planted in rows 1.02-m apart with plants spaced 61-cm apart within rows (16,146 plants/ha.). With this plant spacing, 480 plants occupied 0.03 hectares. The shop building, tobacco field, and curing barn used in this experiment were located in a triangle with approximately 122 m on each side.

Timing began when the worker moved the cutting machine from the machine shop to the field. After cutting and while the plants were wilting, the worker walked back to the shop building and got the tractor. He drove to the barn where he loaded empty beams and the required number of sticks onto two beam-hauling trailers. Each trailer carried one 3.35-m beam that could be loaded with 24 sticks of green tobacco (five plants/stick).

The worker then drove the tractor and trailers to the field. He moved the middle two rows of cut plants to the sides to allow access down the center of the plot (called opening the field). The worker speared the plants on sticks and loaded them onto the beams. The tractor and trailers were moved through the field as necessary for loading, and then they were driven to the barn so that the tobacco could be hung. The hoist units were removed from their holders and put into place, and the two beams were hung at the top and middle beam levels in the barn. Then the worker loaded two more empty beams and sticks onto the trailers and returned to the field for the final load of tobacco. Timing was stopped after the last beam had been hung in the barn and the hoist units had been returned to their holders.

After the tobacco had cured (approximately six weeks later), it was removed from the barn by the same worker. Timing began when the cable-hoist unit was started. Beams were lowered, sticks were removed from the beams, and plants were removed from the sticks and tied into 60-plant bundles. Timing was stopped when the last bundle of plants had been tied, the beams had been stacked, and the hoist units had been returned to their holders. Each day, three beams (360 plants) were taken down and bundled. The three-beam unit was used because the barn was three tiers high. This procedure was repeated on five separate days.

The number of hectares that could be harvested by one person using this mechanical system was estimated using the time it took to harvest and barn tobacco and the number of days available to harvest tobacco in a typical season. Using additional cost and labor data from McKee & Hanson (3) and

[1] Farm Management Specialist, Department of Agricultural and Resource Economics, University of Maryland, College Park, Maryland 20742

[2] Professor, Department of Agronomy, Central Maryland Research and Education Facility, 2005 Largo Road, Upper Marlboro, Maryland 20772

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Table 1. Labor requirements for two methods of harvesting, barning, and taking down from the barn Maryland tobacco.

Operations		Individual[a] operations	Combined operations
		- Hours/Ha -	- Hours/Ha -
Mechanical, beam and cable-hoist system			
Machine cut		2.4	
Open field & load beams		46.2	
Hang beams in barn		8.6	
	Total:	57.2	71.3[b]
Take down beams		5.6	
Bundle tobacco		13.1	
	Total:	18.7	20.2[b]
Conventional, all manual labor system			
Hand cutting		10.6	
Drop sticks		3.4	
Spear and pile		26.0	
Field load wagon		16.7	
Hang in barn		26.6	
	Total:	83.3	104.1[c]
Take down from barn		14.4	
Bundle		10.3	
	Total:	24.7	26.7[d]

[a] From McKee & Hanson (3).
[b] Computed in this study.
[c] Estimated by multiplying 83.3 (conventional individual operations total) by 1.25 (ratio of measured operations for mechanical beam, combined to individual, 71.3/57.2 = 1.25).
[d] Estimated by multiplying 24.7 (conventional individual operations total) by 1.08 (ratio of measured operations for mechanical beam, combined to individual, 20.2/18.7 = 1.08).

the estimate of time required to take down tobacco from the barn and bundle it, a partial budget analysis was conducted to determine the effect on net income of converting from the conventional method of harvesting tobacco (all manual labor, multi-person crew) to a one-person system using the harvesting machine, beams, and cable-hoist unit.

RESULTS

The five recorded times for harvesting and barning 480 plants of tobacco were 2.30, 1.97, 2.05, 2.20, and 2.08 hours for a calculated average of 71.3 hours per hectare. The five recorded times for taking down and bundling 360 plants of tobacco were 0.45, 0.48, 0.39, 0.51, and 0.43 hours for a calculated average of 20.2 hours per hectare (**Table 1**). The ratio of the time required for the combined operations of harvesting and barning tobacco using the beam and cable-hoist system (from this study) to the sum of the times for the individual operations for this system (from McKee & Hanson [3]) was calculated. This ratio (1.25) was multiplied by the sum of the individual operations for the conventional manual-labor system (from McKee & Hanson [3]) to estimate the time it would take if the combined operations of this system had been measured. This estimate (104.1 hours/hectare) reflects a conventional system with rest breaks and with similar locations of the barn, machine shop, and tobacco field. The same procedure was followed for the taking down and bundling operations to estimate the time of the combined operations for the conventional system (26.7 hours/hectare) (**Table 1**).

The number of days available for harvesting tobacco in a typical season was estimated. In Maryland, farmers typically start harvesting tobacco in the first week of August and they continue through the first three weeks in September (53.5 total days). Seven Sundays and 4.6 rain days (39-year average of the number of days when rainfall exceeds five inches at Upper Marlboro, Md., [4]) were subtracted from the total number of available days. An allowance of 2.0 days for wet years was also subtracted (assuming a normal distribution, a one-tailed t value was used to establish a 75% upper confidence limit). Therefore, we calculated that in three out of four years (75% upper confidence level), there were 39.9 days available for harvesting tobacco. Assuming that a farmer worked eight hours per day and that it required 71.3 hours/hectare of his labor, a farmer could harvest and barn 4.5 hectares by himself with the beam and cable-hoist system.

Using a partial budget analysis, the change in net income was calculated for replacing a conventional system (all manual labor) with the beam and cable-hoist system for 4.5 hectares of tobacco (**Table 2**). A partial budget analysis compares the positive effects of changing operations (additional income and reduced costs) with the negative effects (reduced income and increased costs) to determine the change in net income. It was assumed that the farmer could qualify for a loan to finance barn modifications and the purchase of equipment to operate the mechanical system. There would be no effect on income because the price and yield of tobacco would be the same for both systems. Reduced costs were the labor savings from using the harvesting machine, beams, and cable hoist system as compared to the conventional method (**Table 1**). Increased costs were adapted from McKee & Hanson (3) and they reflect the fixed costs of purchasing the harvesting machine and trailers, building the beams, and converting the barn to the cable-hoist system. The other increased costs were attributed to the additional fuel needed to operate the harvesting machine and cable-hoist unit.

When labor costs were valued at $7.50 per hour, then changing to the mechanical system reduced net income by $816 (**Table 2**). When labor costs were valued at $9.50 per hour, net income was reduced by $462. If it is assumed that

Table 2. Partial budget analysis of converting 4.5 hectares of tobacco from a conventional, all manual labor system to a mechanical, beam and cable-hoist system at two different labor costs.

Effects		Labor Costs/Hour $7.50	Labor Costs/Hour $9.50
Positive Effects			
Additional Income:			
No change		$0	$0
Reduced Costs:[a]			
Labor to harvest and barn tobacco		$1,107	$1,402
Labor to take down tobacco and bundle		$219	$278
	Subtotal:	$1,326	$1,680
Negative Effects			
Reduced Income:			
No change		$0	$0
Increased Costs:[b]			
Annual fixed costs for mechanical system		$2,097	$2,097
Variable fuel costs to operate new machinery		$45	$45
	Subtotal:	$2,142	$2,142
Change in net income		-$816	-$462

[a] Per hectare differences are taken from **Table 1** and multiplied by 4.5 and the appropriate labor cost.
[b] Adapted from Table 3 (materials only, excluding construction labor) and Table 4 in McKee & Hanson (3). Beams, trailers, and modifications to the barn were depreciated over 20 years and total annual fixed costs were estimated at 12% of investment costs. The cable hoist system and harvester were depreciated over 10 years and total annual fixed costs were estimated at 17% of investment costs.

average net income per hectare of Maryland tobacco is $2,471, then net income was reduced by 7% and 4%, respectively, for these two scenarios.

DISCUSSION

We have shown that it is possible for a farmer who uses a harvesting machine and beam and cable-hoist system to harvest 4.5 hectares of tobacco without additional labor in three out of four years, but doing so would cause a modest reduction in net income. While not examined in this study, the same farmer would have sufficient time to strip and bale 4.5 hectares of tobacco by him/herself during the winter. The only additional labor required would be at transplanting time; however, locating labor for transplanting is not a problem in Maryland. Another issue not examined was cash flow considerations. It appears that the cash needs associated with converting to the mechanical system would be offset by eliminating wages to other people, although this depends on the amount of non-family labor a grower previously hired.

We project that the estimates presented in this publication understate, not overstate, the value of the beam and cable-hoist system. For example, we assumed that the rest periods for both the manual labor system and the mechanized system were the same. However, it is likely that workers who manually harvest tobacco would rest more, and as a result, the modest reductions in net income might be reduced or eliminated. Also, while tobacco labor is scarce in Maryland, it is available, and during a rainy year (which we estimated to occur 25% of the time) a farmer could hire labor or he could use family members to harvest tobacco. In addition, we did not put a value on the potential liability (career interrupting injury to the farmer or financially liability for a worker) of a serious accident due to a fall in the barn. Finally, because the beam and cable-hoist system reduces much of the physical effort required to harvest tobacco, older farmers may continue to produce tobacco for additional years.

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CONTACT SUCKERCIDE EFFECTIVENESS: A COMPARISON OF THEORETICAL AND ACTUAL RESULTS[1]

TOBACCO SCIENCE

By Heinz Seltmann[2]

Increased concern with residues of maleic hydrazide, the systemic suckercide used on tobacco (*Nicotiana tabacum* L.) in the United States, has renewed interest in chemicals that control suckers (axillary buds) on contact. Contact suckercides when applied with multi-row equipment are generally less effective than when applied manually. Often one of the upper three or four suckers may be missed. To better understand the problem, a roll-out diagram (model) based on the 3/8 phyllotaxy of topped tobacco plants was developed. With the model, it was possible to show the expected track of a contact suckercide when applied to one, two, or three of the three uppermost axils on a topped plant. The model was tested on field-grown plants with 1, 2, and 3 mL of a 4% fatty alcohol formulation applied to the 1st, 2nd, and 3rd leaf axils, in all possible combinations. The model agreed well with the data obtained from the field applications. If the suckercide were applied only to the 1st axil, then the 3rd axil was missed; if applied only to the 2nd axil, then the 1st and 4th axils were missed; if applied only to the 3rd axil, then the 1st, 2nd, and 4th axils were missed. Increased volumes of suckercide reduced the number of suckers missed below the axil of application. To obtain excellent sucker control, the first three leaf axils should not be missed. It was also apparent that the surface configuration of the stalk could direct the contact agent away from a leaf axil.

Additional key words: *Nicotiana tabacum* L., sucker control.

INTRODUCTION

Increased concern in the United States over residues of maleic hydrazide (MH) in tobacco (*Nicotiana tabacum* L.) has renewed interest in chemical sucker control using only contact suckercides. A contact suckercide, to be effective, must flow down the stalk and wet the bud (sucker) in each leaf axil. To accomplish a high degree of sucker control with a contact chemical, stalks must be vertical and straight. Sufficient suckercide must be applied to ensure that no axils are missed, because missed suckers will grow with vigor. Missed suckers on flue-cured tobacco can interfere with leaf harvest and result in reduced yield. Missed suckers on air-cured, stalk-cut types of tobacco also can affect yield, and they must be removed before harvest.

Chemical control of sucker growth on decapitated (topped) tobacco plants was first obtained on a practical scale with mineral oil and its emulsions, where a measured amount was applied to the top of the stalk after topping (2,4,9). The use of mineral oil declined as other contact agents (1,5,6) were found that could be sprayed from above as well as applied down the stalk. These were the methyl esters of C_8 to C_{12} fatty acids, C_8 to C_{12} fatty alcohols, dimethyldodecylamine acetate, chlorpropham, flumetralin, butralin, pendimethalin, and chlorthal dimethyl (DCPA). Only the fatty alcohols (FA) and flumetralin are presently labeled for use on tobacco in the United States.

Contact suckercides can be classified into two types: those that appear to 'burn' the sucker tissue (fatty acids, fatty alcohols, dimethyldodecylamine acetate), and those that appear to control through some systemic action (chlorpropham, flumetralin, butralin, pendimethalin, DCPA). Of the contact suckercides, only the mode of action of the fatty alcohols has been studied. Fatty alcohols work by destroying the semipermeable properties of the plasma membranes (8). This is followed by dessication, which results in a burnt appearance of the sucker.

The development of sprayable contact agents improved production efficiency by eliminating plant-to-plant manipulations. In general, 467 L/ha of the spray solution are applied through a 3-nozzle arrangement (Spraying Systems: TG-3; TG-5; TG-3), with the nozzles being spaced about 25 cm apart on the spray boom that is held about 30 cm above the middle of each row. Nozzle pressure should be low (1.4 kg/cm^2) and tractor speed slow (3.2 km/h). Application before topping, when the plants are in the bud stage of plant development, is more effective because suckers are less developed. Furthermore, at this stage of floral development the uppermost leaves tend to have an acute angle with the stalk. These leaves (top), as well as any floral parts, offer a greater surface upon which more of the spray pattern can be intercepted to flow down the stalk. With the top of the plant removed, it is not unusual for the uppermost leaf to fold over and protect the stalk from the spray. Light breezes during application can increase this tendency, as well as divert the spray pattern.

Leaves of the tobacco plant are arranged in a spiral on the plant stalk. When counting consecutive leaves in an acropetal direction one circles the stalk three times, until the ninth leaf is directly above the first. Botanically, this type of leaf arrangement is designated as a 3/8 phyllotaxy (3). The phyllotaxy may influence how a suckercide will flow down the stalk. Leaves that have an upright attitude act like a funnel and catch the spray. The spray emulsion then flows down the midvein toward the leaf axil and wets the sucker. The excess flows to either side of the leaf insertion of the midvein and down the stalk to a lower axil. The downward flow of the suckercide could be influenced by the winged petioles that clasp the stalk and by the decurrent extensions, if present (7). Thus, it would appear that suckers could be missed not only because of the protection of the upper axils by a leaf, but also because of the flow pattern down the stalk as directed by the external anatomy.

To better understand the flow pattern of a contact suckercide, a theoretical model (diagram) of the leaf insertions and leaf axils based on the 3/8 phyllotaxy of the tobacco plant was developed. The model was tested in the field with an FA suckercide. To determine effectiveness of the suckercide with respect to the placement in a given leaf axil on the plant, small volumes of 1, 2, or 3 mL were used. In a preliminary study, it was found that larger volumes (10-30 mL) masked the subtle differences that were obtained in the present study.

MATERIALS AND METHODS

A "roll-out" diagram or model (**Fig. 1**) was drawn from the average dimensions for stalk height and internode lengths obtained from topped, field-grown tobacco plants of cv. NC2326, which has a 3/8 phyllotaxy. The angle between adjacent leaf insertions according to the phyllotaxy is 135°. The diagram was used to predict the path down the stalk that a

[1] Paper presented at the CORESTA Symposium, Greece, 1990. Cooperative investigation between the Agricultural Research Service of the United States Department of Agriculture and the North Carolina State University, Raleigh, NC 27695. The use of a trade name does not imply endorsement of that product by the U. S. Department of Agriculture or North Carolina State University, nor criticism of similar ones not mentioned.

[2] Plant Physiologist, USDA, ARS, Crops Research Laboratory, Oxford, NC 27565.

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contact suckercide would take when placed into one or more of the upper three leaf axils on a topped plant.

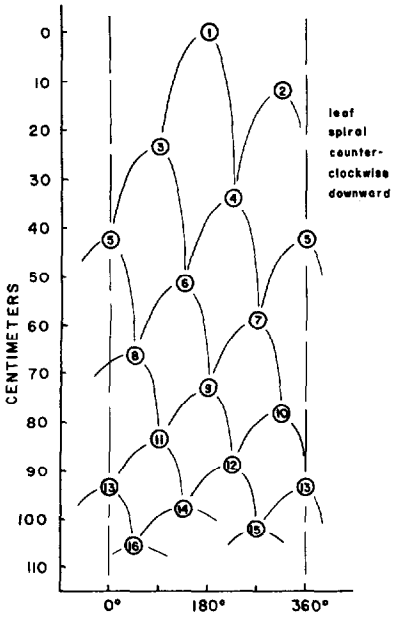
Plants were grown in the field 56 cm in the drill in rows 122 cm apart, according to recommended practices for flue-cured tobacco. After topping in the early (first) flower stage of development, 1, 2, or 3 mL of a 4% emulsion of an FA suckercide (Off-Shoot T) were applied to the axils of the upper three leaves on 20 plants (replicates). The following seven treatment protocols were evaluated: (1) 1st axil (from the top of the plant), (2) 2nd axil, (3) 3rd axil, (4) 1st & 2nd axils, (5) 1st & 3rd axils, (6) 2nd & 3rd axils, and (7) 1st, 2nd, & 3rd axils. An automatic glass syringe with an 8-gauge delivery tube was used to administer the 21 treatments. Two weeks after application, each plant was examined and missed suckers were recorded by position on the stalk. The data are presented as the total number of suckers per plant not contacted per treatment. In addition, bar graphs were drawn to represent the pattern of missed suckers for each of the seven protocols. The values given are the sums of missed suckers from the three different amounts of suckercide applied per protocol.

The data were analyzed by a GLM procedure. The treatment protocols were compared within the number of axils (1 or 2) to which the suckercide was applied. Also, the three axils to which the suckercide was applied were compared to each other.

RESULTS AND DISCUSSION

According to the predicted path from the model diagram (**Fig. 1**), a contact suckercide applied to the 1st axil just below the point of topping, would flow to the 3rd and 4th axils and miss the 2nd axil. All other axils on the plant would be contacted as the suckercide flowed down the stalk. If during application the 1st axil were missed by the suckercide, then the 3rd axil would not receive any suckercide unless it arrived there directly during application. Similarly, if a suckercide were applied to only the 2nd axil, the 1st and 3rd axils would be missed, and if a suckercide were applied only to the 3rd axil, the 1st, 2nd, and 4th axils would be missed. According to the model, if the suckercide were applied to the 1st and 2nd axils, all axils below would be contacted as it flows down the stalk. If the suckercide were applied to the 1st and 3rd axils, the 2nd axil would be missed; if applied to the 2nd and 3rd axils, the 1st axil would be missed. A suckercide applied to all of the upper three axils will contact all axils on the stalk and give the best results.

Fig. 1. Two dimensional model of a tobacco stalk topped to 16 leaves (O) and flow track (-) of a contact suckercide.



The values obtained from the field show that when the contact suckercide was placed only in the 1st axil or only in the 2nd axil, there were fewer suckers per plant then when placed in the 3rd axil (**Table 1**). The average number of suckers not controlled per plant was 2.4, 2.6, and 3.7 for the 1st, 2nd, and 3rd axils, respectively.

When the suckercide was placed in both the 1st & 2nd axils, it was more effective than when placed either in the 1st & 3rd axils or in the 2nd & 3rd axils (**Table 1**). The average number of suckers per plant was 0.3 for applications in the 1st & 2nd axils, 0.6 for applications in the 1st & 3rd axils, and 1.7 for applications in the 2nd & 3rd axils.

Table 1. Average number and percent of suckers per plant that were not controlled when the contact suckercide (1, 2, or 3 mL) was placed in one or more of the top three axils.

Treatment protocol	Axils treated	Amount of suckercide applied	Suckers per plant	Percent not controlled
		----- mL -----		----- % -----
1	1	1	3.4	
		2	2.1	
		3	1.8	
		Average	2.4	15
2	2	1	3.2	
		2	2.1	
		3	2.6	
		Average	2.6	16
3	3	1	4.3	
		2	3.1	
		3	3.7	
		Average	3.7	23
4	1 & 2	1	0.6	
		2	0.2	
		3	0.2	
		Average	0.3	2
5	1 & 3	1	0.8	
		2	0.6	
		3	0.4	
		Average	0.6	4
6	2 & 3	1	2.1	
		2	1.5	
		3	1.4	
		Average	1.7	11
7	1, 2, & 3	1	0.05	
		2	0.05	
		3	0.00	
		Average	0.03	0.2

Treatment Protocol:	Pr > F
1 vs. 2	0.4965
1 vs. 3	0.0006**
2 vs. 3	0.0020**
4 vs. 5	0.3490
4 vs. 6	0.0003**
5 vs. 6	0.0001**

Number of axils:	Pr > F
1 axil vs. 2 axils	0.0001**
1 axil vs. 3 axils	0.0001**
2 axils vs. 3 axils	0.0033**

When suckercide was applied to all three of the upper axils, it was very effective (**Table 1**). Quantities as low as 1 mL in each of the upper three axils were enough to wet all axils below. The data indicate that as the amount of suckercide applied per axil was increased, the average number of suckers missed per plant decreased.

The effectiveness of a contact suckercide when applied according to the seven treatment protocols is shown graphically in **Figures 2-8**.

On field-grown plants, when the suckercide was applied to the 1st axil, no living sucker was found in that axil (**Fig. 2**). Of the 57 plants that received treatment only to the 1st axil, 37 had suckers missed in the 2nd axil, indicating that the major flow of suckercide away from the 1st axil missed the 2nd axil. The greater number of plants with suckers in the 3rd axil (16) than in the 4th axil (3) suggests that more of the suckercide flowed from the 1st axil toward the 4th axil, and that some of the suckercide that flowed around the stem to the 2nd axil may

Fig. 2. Number of suckers not contacted after application in axil #1.

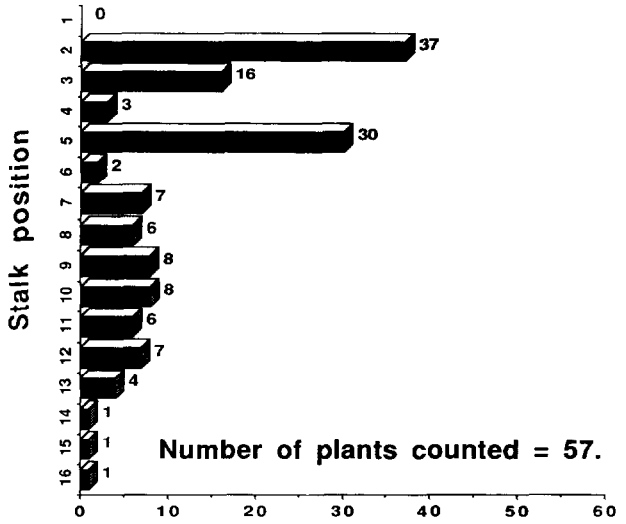
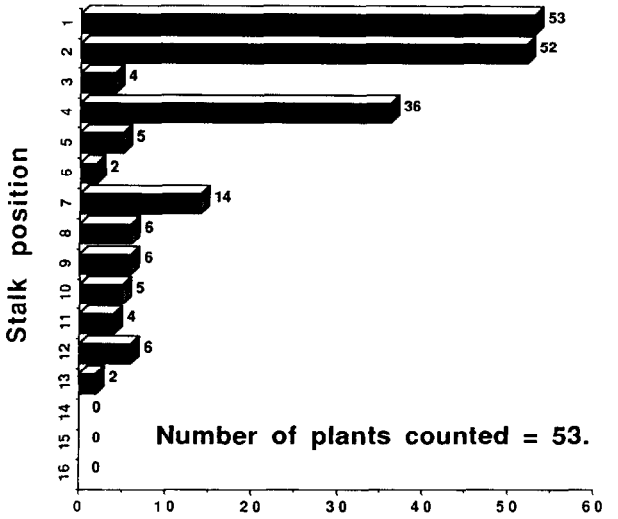


Fig. 4. Number of suckers not contacted after application in axil #3.



have flowed to the 4th axil. The suckercide that flowed from the 1st axil to the 3rd axil would continue, according to the predicted path (**Fig. 1**), to the 5th axil, where 30 plants had suckers. The large number of suckers in the 5th axils can be attributed to a reduced amount of suckercide, that coming only from the 3rd axil and not from the 2nd and 4th axils. The 6th axil, where only two plants had suckers, received suckercide from the 3rd and 4th axils. There was a scattering of suckers missed from the 7th to the 16th axils (**Fig. 2**). Because suckercide was applied to only one axil, the volume per plant (1, 2, or 3 mL) was not enough to wet all axils below.

According to the model prediction (**Fig. 1**), suckercide applied to the 2nd axil would flow to the 4th and 5th axils, and it would miss the 3rd axil. From the 4th axil it would flow to the 6th and 7th axils, and from the 5th axil it would flow to the 7th and 8th axils. Because the 7th axil would receive suckercide from the 4th and 5th axils, it should have fewer suckers than the 8th axil, which would receive suckercide only from the 5th axil. In the field, when suckercide was applied only to the 2nd axil, seven plants had a sucker in that axil (**Fig. 3**). Also, there were six plants where the 1st axil was apparently inadvertently wetted, as evidenced by the fact that 53 of the 59 plants treated had suckers in the 1st axil (**Fig. 3**).

Some of the 3rd axils, which theoretically should not be wetted when applications were to the 2nd axil, were also wetted. Flowability of the emulsion and the configuration of the stalk could account for the wetting of an axil immediately below the one in which application was made. Stem diameters may have an influence. It stands to reason that a stem with a smaller circumference would have leaf axils closer together, measured horizontally, than a stem with a greater circumference. Stem diameters were not taken into account in this study.

According to the model (**Fig. 1**), suckercide applied to the 3rd axil would not wet the 1st, 2nd, and 4th axils. From the 3rd axil, the suckercide would flow to the 5th and 6th axils. In the field experiment, when applications were made only to the 3rd axil, 36 of 53 plants had a sucker in the 4th axil, five plants had a sucker in the 5th axil, and two plants had a sucker in the 6th axil (**Fig. 4**). There also was a tendency for the suckercide to miss the 7th axil.

When suckercide was applied to only one of the top three axils (**Figs. 2-4**), there was a similarity in the pattern of missed suckers downward from the axil of application through the next five axils. The axil that received the application had from zero to only a few suckers, while a large number of suckers were present in the axil immediately below it. Downward from

Fig. 3. Number of suckers not contacted after application in axil #2.

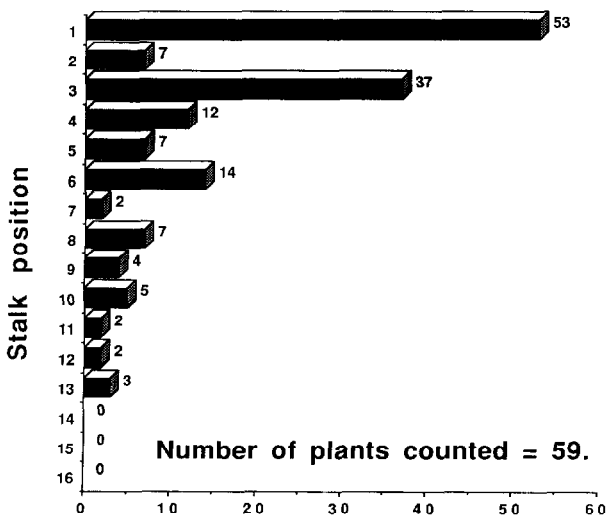


Fig. 5. Number of suckers not contacted after applications in axil #1 and #2.

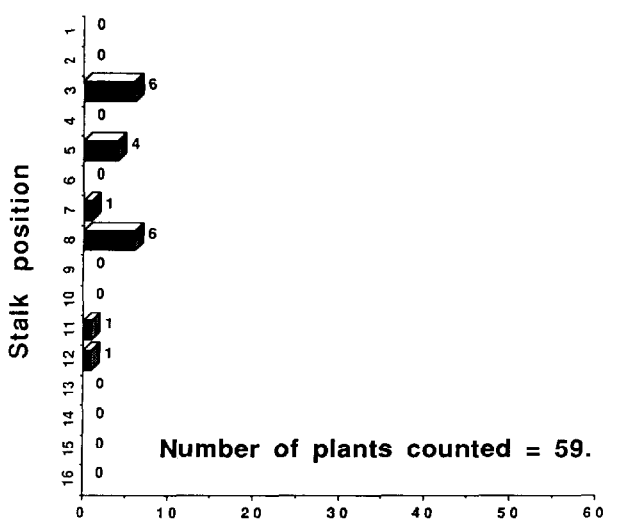


Fig. 6. Number of suckers not contacted after applications in axil #1 and #3.

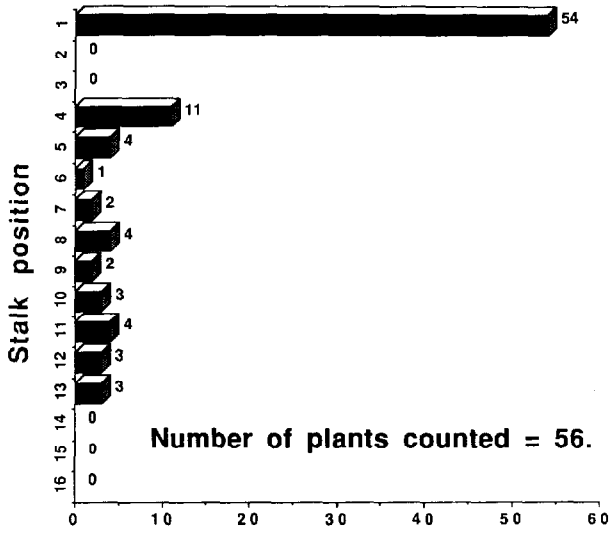
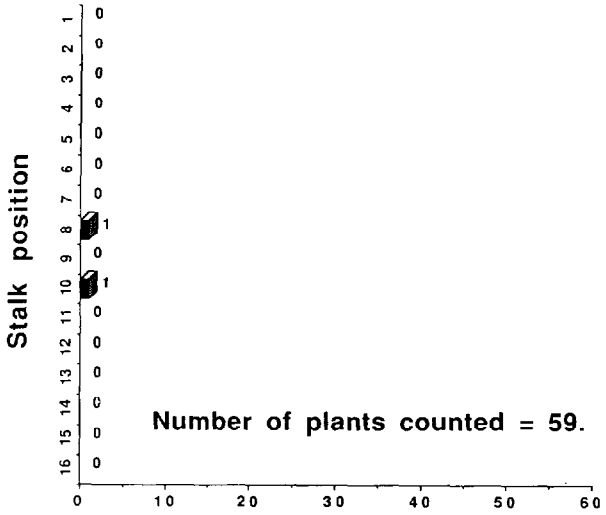


Fig. 8. Number of suckers not contacted after applications in axil #1, #2, and #3.

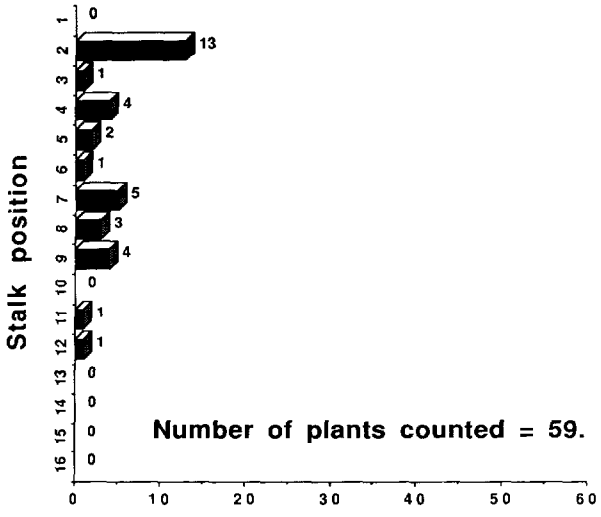


there, the number of suckers missed was small for the next two axils. Many of the fourth axils down from the point of application had a sucker present. This was followed by a decrease in suckers on the axils below. These similarities in the pattern of missed suckers can be explained by the 3/8 phyllotaxy.

According to the model (**Fig. 1**), suckercide placed in both the 1st & 2nd axils would wet all axils below. The results (**Fig. 5**) agreed well with the model. Suckercide applied to both the 1st & 3rd axils would wet all suckers except those in the 2nd axil. The results (**Fig. 6**) showed that 13 of 59 plants had a sucker in the 2nd axil. Apparently, as described above, there can be some movement of the suckercide to axils directly below that are not predicted by the model. Suckercide applied to both the 2nd & 3rd axils would leave suckers in the 1st axil, but none elsewhere on the plant. The results (**Fig. 7**) showed that 54 of 56 plants had suckers in the 1st axil, but no suckers were found in the 2nd or 3rd axils. However, 11 of the 56 plants had suckers in the 4th axil. For this treatment protocol, the 4th axil should receive suckercide only from the 2nd axil. A similar response was obtained when only the 2nd axil received suckercide (**Fig. 3**).

Finally, according to the model (**Fig. 1**), all axils would be wetted if a suckercide were applied to the 1st, 2nd, & 3rd axils. There was good agreement between the model and the results after this treatment protocol (**Fig. 8**).

Fig. 7. Number of suckers not contacted after applications in axil #2 and #3.



It can be concluded that suckercides tend to flow down the stalk of a tobacco plant according to the 3/8 phyllotaxy, theoretical model of the leaf insertions on the stalk surface. If only one of the uppermost three leaf axils below the point of topping receives suckercide, control decreases in the order of 1st axil, 2nd axil, and 3rd axil. If two of the three uppermost leaf axils receive suckercide, control decreases in the order of (1st & 2nd), (1st & 3rd), and (2nd & 3rd). If all three of the uppermost leaf axils receive suckercide, control is better than with any of the other treatment protocols. With all treatment protocols, control increased with increased amounts of suckercide applied per leaf axil. In conclusion, if contact suckercides are to be used effectively for sucker control, 1-3 mL should be applied to each of the upper three leaf axils, the higher amount being preferred.

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TOBACCO TRANSPLANTS. PART 5. PRODUCTION IN POLYETHYLENE COVERED GREENHOUSE TUNNELS

By C.W. Suggs[1], B.M. Lineberger[2], T.R. Seaboch[2], H.B. Peel[3] and J.D. Wheless[3]

It was demonstrated that a temperature-actuated device to open and close V-shaped vents in the polyethylene cover of a greenhouse tunnel controlled heat stress so that excellent tobacco plants could be produced in floating foam-plastic trays. Efforts to store heat in the water used to float the trays were not entirely successful due, at least in part, to the insulation properties of the foam trays. The use of phase change material to store heat during the day did not result in a significant increase in the night temperature of the water or in improved plant growth. Although no heat was added and the attempts to store heat during the day for release at night did not function well, approximately 80% of the tobacco seed germinated and seedlings grew well. In the unventilated check greenhouse, most seedlings did not survive, and those that did survive did not produce acceptable transplants. It was concluded that tobacco seeds could be germinated on floating trays in unheated low-profile, polyethylene-covered greenhouse-row tunnels, and that the seedlings could be grown to transplant size in the tunnels. It was also concluded that the passive temperature-driven actuator system used in these experiments successfully opened and closed vents in the polyethylene-covered greenhouses to control temperature so that plant-damaging heat stress did not occur.

INTRODUCTION

Many tobacco growers in the Southeastern U.S.A. are interested in the production of plants in greenhouses. Approximately 50% of the bright leaf plants already are produced in greenhouses. While greenhouse plants tend to be more expensive to produce than plants grown in field beds, due in large part to the cost of the structure, total costs may be less because costs associated with pulling plants from field beds are eliminated.

Greenhouse plants typically are grown in trays containing cells that are filled with soil media and a single tobacco seed. While tobacco plants produced in these cells tend to suffer less transplant shock than bare-root plants from field beds, crop yield and value are not increased (13). However, greenhouse plants can be produced to meet a planting schedule, and they tend to give a more uniform stand in the field.

While most previous research concerned the use of plastic films on field beds, several researchers have reported on the use of plastic film-covered tunnels approximately 1.2-m wide by 0.6-m high (1,7,10,11,13). Generally, researchers reported that plastic covers improved germination and increased growth rates, with the time to produce field-ready transplants being reduced by as much as three weeks (2,9). The plastic film is a moisture barrier, which maintains a high humidity environment in the enclosed space, thus reducing moisture loss from the soil surface and enhancing seed germination (2,5). The cover is normally sealed to the ground so that air exchange is virtually eliminated. Therefore, much of the incident solar radiation energy is trapped under the cover, sometimes resulting in tunnel temperatures of 40°C or more (6,11). In cool weather the temperature increase is desirable, but in hot weather the resulting temperatures are often detrimental unless controlled.

To prevent temperatures in clear polyethylene-covered seedbeds from becoming excessive, growers may perforate the covers, replace them with a porous material when warm weather begins, or remove them during hot days and replace them during cold nights (4,5,6,8,12). Dean & Davis (5) found that 1.3-cm diameter perforations, 7.6 cm on center in each direction (approximately 2% open space), gave the best overall results. Polyethylene covers give better cold protection than porous covers, such as cheesecloth, with mean soil and air temperatures 3°C to 5°C higher under polyethylene than under cloth (3,6).

Because of the costs of conventional greenhouses, the authors have developed a small, low-clearance greenhouse (row tunnel) that is economical and is suitable for the production of tobacco seedlings. These greenhouse row tunnels contain a water reservoir (pool) in which foam plastic trays are floated to eliminate the need for watering. Greenhouses were located on the Central Crops Research Station near Clayton, North Carolina.

The objective of the research reported here was to evaluate temperature control and plant production in small, unheated, passively ventilated greenhouse row tunnels. Evaluations of plant production included stand, plant mass, stem length, and stem diameter.

METHODS

Greenhouse Description

Greenhouses were 145-cm long by 117-cm wide by 77-cm high, including a 13-cm wooden base, 4-cm thick (**Figure 1**). The cross section was approximately semicircular, and it was formed by 0.6-cm steel rods bent to the desired shape and inserted into holes in the wooden base. A sheet of Styrofoam 1.3-cm thick x 38-cm high was positioned approximately vertically along the north side of each greenhouse to reduce heat loss.

Six-mil black polyethylene film was draped over the wooden base and stapled along the upper edges to form a pool that was filled with water to about 8-cm deep. Foam plastic trays (Carolina Greenhouses, Kinston, N.C.), 35-cm x 70-cm x 7-cm tall, containing a 10 x 20 array of inverted pyramidal cells were filled with soil media (Metromix 220). Each greenhouse held six trays, except treatment 7 where two trays were omitted so that one-third of the water surface was exposed. Trays were planted with pelleted tobacco seed on March 8, 1990, February 28, 1991, and February 26, 1992, and the trays were floated in the pools for germination and growth. The pools were fertilized with 12-6-6 soluble fertilizer at the rate of 100 ppm of nitrogen. Metalaxyl (Ridomil), to control blue mold, was added to the water at the rate of 2 ppm.

Each cell was about 2.5 x 2.5-cm at the top and tapered to form a pyramid-shaped cavity about 7-cm deep. The cell terminated in a 0.6-cm square hole in the bottom of the tray, which was small enough to prevent the loss of soil media but large enough to permit water to enter. The tray initially floated about 1.2-cm deep in the water, but sank somewhat deeper as the weight of the plants increased.

[1] Professor Emeritus, Biological and Agricultural Engineering Department, North Carolina State University, Raleigh, N.C.
[2] Research Engineers, Biological and Agricultural Engineering Department, North Carolina State University, Raleigh, N.C.
[3] Former Research Engineers, Biological and Agricultural Engineering Department, North Carolina State University, Raleigh, N.C.

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Greenhouses were ventilated by means of a series of V-shaped openings in the top, side, or top and side of the 6-mil clear polyethylene covers (Tuflite 801) (**Figures 2 & 3**). Each vent was about 30-cm wide by 15-cm long, and it was formed by cutting along the 22-cm edges of a right isosceles triangle. The apex of each triangle was attached to a cord so that all of these vents in a series could be opened or closed by pulling on the cord. Previously, we showed that passive air flow through V-shaped vents in polyethylene-covered row-tunnels was sufficient to provide adequate ventilation and prevent overheating (14).

The vents were automatically opened and closed by a cylindrical, temperature-actuated motor (Heat Motors, Inc., Sierra Madre, Calif.) having a push rod that extended progressively as the temperature increased (**Figure 3**). An adjusting collar was set to allow the system to start opening the vents at 27°C and to have them fully open at 37°C. A system of pulleys and cords was used to connect the actuator to the vents. A weight on the remote end of the cord closed the vents whenever a decrease in temperature allowed the push rod to retract.

Temperature was measured with three 24-gauge thermocouples in each greenhouse. One was at the soil surface, one was under a shade 2 cm above the soil, and the third was unshaded about 4 cm above the soil surface. Ambient temperature in the shade and the temperature at the center of a 20-cm diameter black-painted metal globe exposed to full sun were also measured. Black globe temperature was measured because it responds to solar radiation and indicates the heat load inside a greenhouse. Recordings were made at hourly intervals in 1990 with an automatic recorder (Campbell Scientific Recorder Model CR5). In 1991 and 1992, recordings were made of each 15 minute average for each of the thermocouples with a different type of automatic recorder (Campbell Scientific Recorder Model CR10). Values were stored in memory and transferred to a computer for analysis. Comparisons of daily temperatures were used to provide a measure of experimental error.

Treatments

In addition to a control greenhouse with no vent (treatment 1, NN), there was one greenhouse with vents only in the top (treatment 2, TN), one with top and side vents (treatment 4, TSN), and in 1991 and 1992 one greenhouse with vents only in the side (treatment 3, SN) (**Table 1**). Four other greenhouses with both top and side vents were fitted with devices designed to store excess heat and release it during cool nights (**Figure 4**). These heat-absorbing devices were a 36-cm high x 140-cm long vertical, south-facing, flat-black metal panel to collect energy and transfer it down into the water (treatment 5, TSA); a row of seven 4-liter (1 gallon), flat-black, water-filled cans (10.5 x 16.8 x 23.7-cm high) positioned along the side of the pool with the lower 2 cm covered with water (treatment 6, TSC); an area of exposed water 35-cm x 145-cm (1/3 of the pool area) with the black plastic liner visible (treatment 7, TSW); and a flat-black painted container (96 x 5 x 23-cm) of $NaSO_4$ $10H_20$ phase-change material (treatment 8, TSP). These devices were positioned along the north side of each greenhouse where they were exposed to the sun without shading the plants. The container of phase-change material was not in contact with the water. These four devices were insulated from the cover film on the north side by the 1.3-cm thick sheet of Styrofoam mentioned above. In addition to storing heat, these devices were designed to release heat back into the greenhouse when the temperature dropped.

Although the water in each pool was about 8-cm deep, it was not free to function as a heat storage system because of the insulating properties of the 7-cm thick trays floating on the surface. Treatments involving the heat-absorbing metal

Fig. 1. Short section of greenhouse tunnel showing temperature-actuated device for opening triangular vents in the top of the structure and a wooden base that held the black plastic film to form a pool for the float trays.

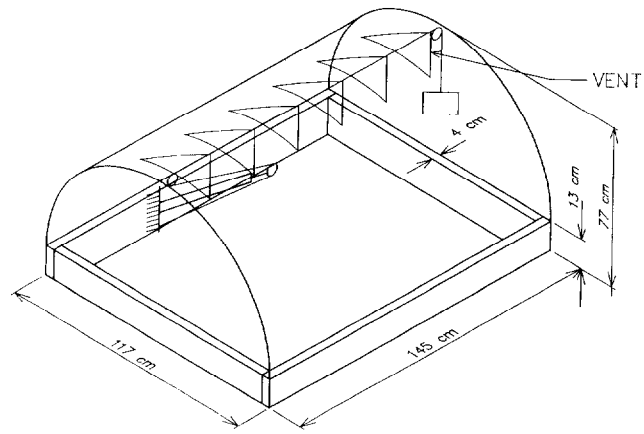


Fig. 2. Details of a temperature-actuated system for opening triangular vents in the top of a greenhouse tunnel.

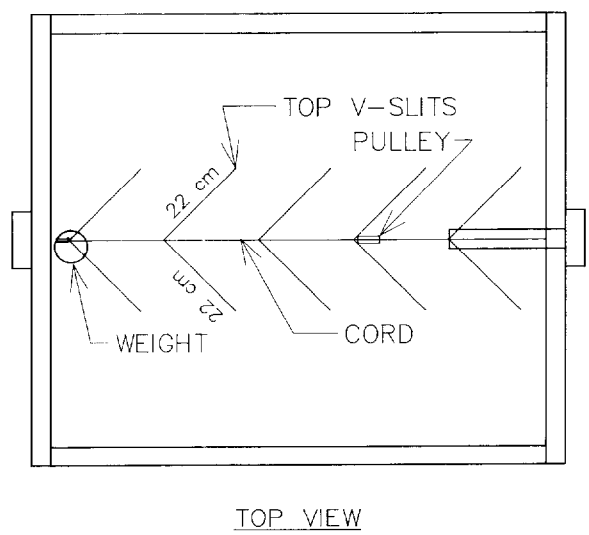


Fig. 3. Details of a temperature-actuated system for opening triangular vents in the side of a greenhouse tunnel.

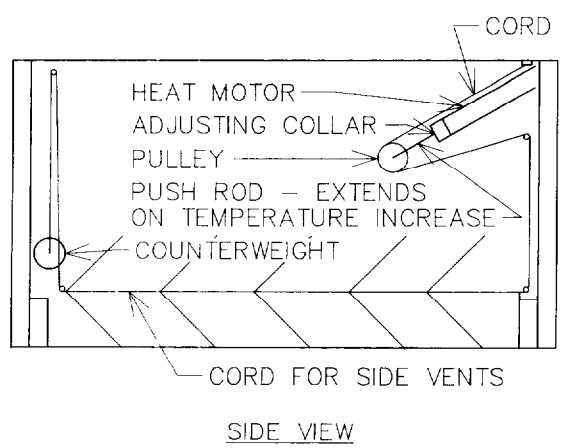


Table 1. Treatment description and stand of tobacco plants seeded into floating trays in small polyethylene covered greenhouses.

Treatment Number	Treatment Designation	Location of Vents	Type of Heat Storage	Plant Stand[a] 1990	1991	1992
				%		
1	NN	None	None	31.2 b	30.0 c	20.0 c
2	TN	Top	None	76.2 a	80.0 ab	81.2 ab
3	SN	Side	None	-	77.5 ab	75.0 ab
4	TSN	Top & Side	None	80.0 a	82.5 a	82.5 ab
5	TSA	Top & Side	Metal Absorber	81.2 a	81.2 ab	81.2 ab
6	TSC	Top & Side	Water Cans	81.2 a	71.2 ab	87.5 a
7	TSW	Top & Side	Exposed Water	82.5 a	66.2 b	81.2 ab
8	TSP	Top & Side	Phase Change	82.9 a	73.8 ab	72.5 b
Mean of vented treatments				**80.7**	**76.1**	**80.2**

[a] Numbers followed by the same letter are not significantly different from each other at the 0.95 probability level according to Duncan's Multiple Range Test.

panel, the cans of water, and the exposed water surface (treatments TSN, TSA, and TSC) were tried in an effort to form a heat-conducting pathway around the insulating trays to the water. The phase-change material was suspended above the trays, and thus it was not in contact with the water.

Plant Measurements

Plant stand was determined approximately 5 weeks after seeding by counting the living plants in each of eight 10-tray-cell replications in each of the greenhouses. Four replications of 10 plants from each greenhouse were evaluated to characterize relative growth and configuration of plants from the different treatments. The measurements were made on May 7, 1990, April 24, 1991, and May 11, 1992, when most of the plants were approximately transplant size. Measurements were made of plant weight, stem diameter, and stem length. Stem diameter was measured at ground level, and stem length was measured from ground level to the base of the bud. Plant weight included the roots. Plant stand, relative growth, and plant configuration data were analyzed as randomized block designs.

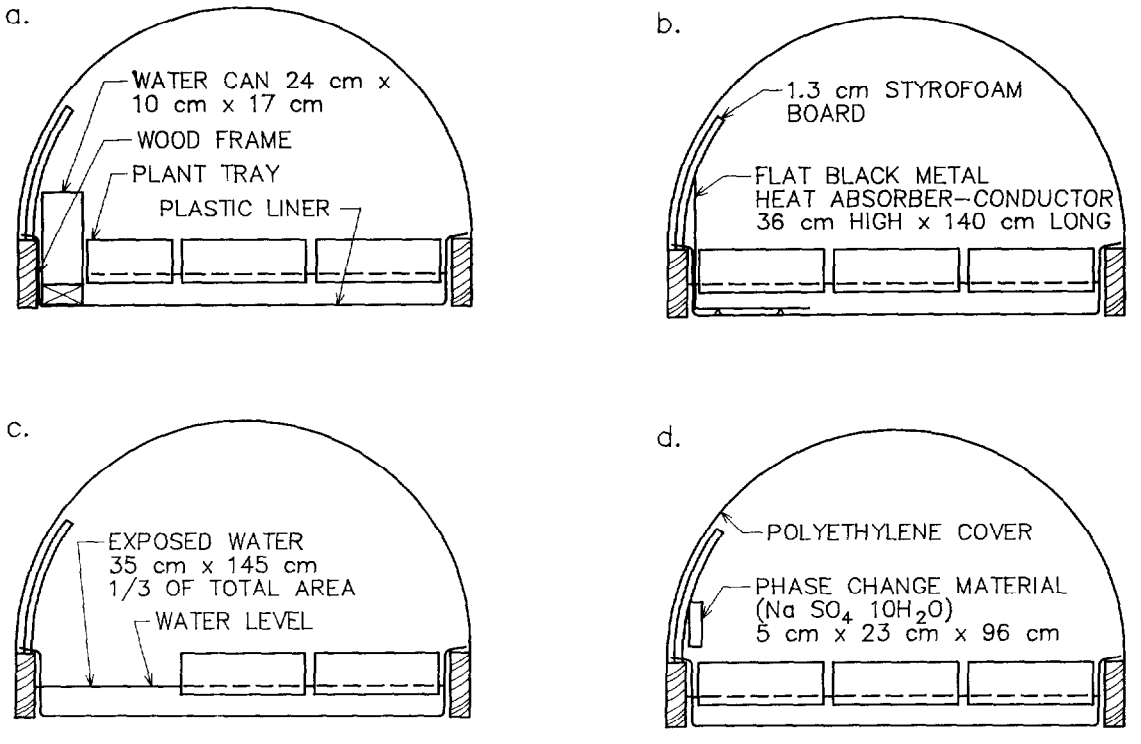
RESULTS AND DISCUSSION

Plant Characteristics

Plant Stand. In 1990, there were no significant differences in plant stands among any of the vented treatments, but all of the vented treatments had significantly better plant stands than the unvented control treatment (**Table 1**). In 1991 and 1992, all of the vented treatments had significantly better plant stands than the unvented treatment; however, among the vented treatments, the highest value for plant stand was significantly different from the lowest value. The best stands numerically were: 1990 TSP, 1991 TSN, and 1992 TSC. The poorest stands in the vented treatments were: 1990 TSN, 1991 TSW, and 1992 TSP. Thus, two of the treatments, TSN and TSP, having the highest numerical values in one year had the lowest values in another year. Germination in the unvented treatment was appreciably better than the stand of plants that was finally established. This was due to many of the plants being killed by excessively high temperatures. The plants that did survive were those closest to the polyethylene cover where convective cooling was greatest. The mean stand density for the vented treatments was 80.7% in 1990, 76.1% in 1991 and 80.2% in 1992.

Weight, Length, and Diameter. In 1990, plants from the unvented control treatment were significantly shorter than

Fig. 4. Heat absorbers and storage devices: (a) a series of water-filled cans, (b) a flat metal plate in contact with the water, (c) exposed water, and (d) a container of phase-change material.



plants from any of the vented treatments, and they weighed significantly less than plants from any of the vented treatments except the metal heat absorber treatment (TSA) (**Table 2**). Stem diameter for the unvented and TSA treatments were smaller than for the other treatments. The largest plants were from TSW, followed by TN and TSN. Stem diameters did not vary nearly as much as did weights and lengths.

In 1991, none of the plants in the unvented greenhouse survived long enough for plant measurements to be made. Plant weight, length, and stem diameter were all largest for plants from TSC, the greenhouse having cans of water as heat absorbers (**Table 2**). This was followed by the exposed-water heat-absorber greenhouse (TSW) and the phase-change greenhouse (TSP). As in 1990, the metal heat absorber (TSA) produced some of the smallest plants of the experiment. However, the top-only vent system (TN), which produced large plants in 1990 and 1992, produced small plants in 1991.

In 1992, a few of the plants in the unvented greenhouse survived until measurements were made, but they were very small (**Table 2**). Plant weights, lengths, and stem diameters were largest for TSP. This was followed by TSW, SN, and TSN. The smallest plants (except for the unvented treatment) were produced in TSA and TSW.

Few trends among the vented treatments were evident. TSW (exposed water), which produced the largest plants in 1990, produced the smallest plants in 1992. However, TSA produced small plants in each of the three years. In each year the largest plants were produced in one of the treatments having a heat absorber, however, they were not significantly larger than plants produced in one of the treatments that did not have an absorber. Thus, on the basis of plant size, it is difficult to justify use of any of the heat absorbers, and it was not possible to select any treatment as superior. All of the vented treatments produced good plants.

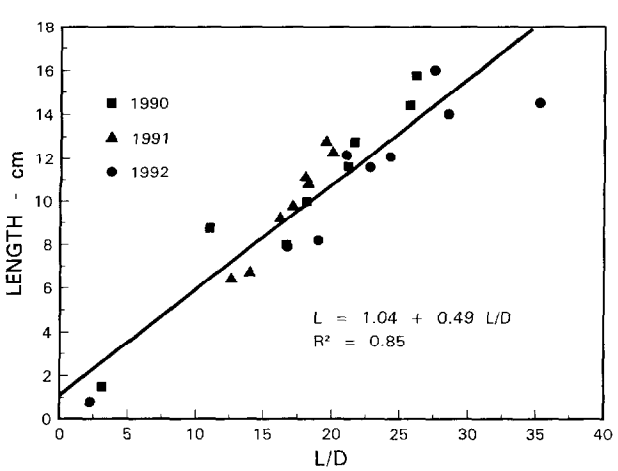
Configuration. Plant shape is largely dependent on stand density and plant size. Because the trays were all the same size and stand was similar for all the treatments except the unvented greenhouse, stand densities were approximately constant. Large plants tended to be relatively slimmer than short plants due to crowding and increased competition (**Table 3**). Regressions of the slenderness ratio (L/D = stem length/stem diameter) versus length (**Figure 5**) showed that as the plants became longer the slenderness ratio increased. After adjusting for plant length, no treatment effects were observed.

Table 2. Characteristics of tobacco plants grown in floating trays in small polyethylene covered greenhouses.

Treatment	1990 Wt	1990 Length	1990 Diam.	1991 Wt	1991 Length	1991 Diam.	1992 Wt	1992 Length	1992 Diam.
	- gm -	- cm -	- mm -	- gm -	- cm -	- mm -	- gm -	- cm -	- mm -
NN	3.6 e[a]	1.5 d	4.8 e	0.0 e[c]	0.0 d	0.0 e	2.2 d	0.9 d	4.0 b
TN	10.7 ab	12.7 ab	5.9 a	4.8 d	6.3 c	5.0 cd	12.4 b	11.6 b	5.1 ab
SN	-[b]	-	-	6.5 c	9.1 b	5.6 bc	13.8 ab	12.2 b	5.8 a
TSN	9.9 ab	14.4 a	5.6 ab	8.4 b	11.2 ab	5.6 bc	12.9 b	14.5 ab	4.1 b
TSA	5.7 de	8.0 c	4.8 e	4.4 d	6.6 c	4.7 d	8.8 c	7.9 c	4.7 ab
TSC	8.6 bc	10.0 bc	5.5 bc	10.3 a	12.7 a	6.5 a	13.7 ab	14.0 ab	4.9 ab
TSW	11.8 a	15.7 a	6.0 a	8.5 b	11.0 ab	6.1 ab	9.7 bc	8.2 c	4.3 b
TSP	7.2 cd	8.7 c	5.2 cd	8.5 b	10.8 ab	5.9 b	15.2 a	16.0 a	5.8 a
Mean of vented treatments	**9.0**	**11.6**	**5.5**	**7.3**	**9.7**	**5.6**	**12.4**	**12.1**	**5.0**

[a]Numbers followed by the same letter are not significantly different from each other at the 0.95 probability level according to Duncan's Multiple Range Test.
[b]Treatment not included.
[c]Plants did not survive.

Fig. 5. Effect of plant length on slenderness ratio (L/D = stem length/stem diameter).



Temperatures

Water Temperatures. The exposed-water treatment (TSW) had a significantly higher average maximum water temperature (20.9°C) than TSA (15.7°C), TSC (14.1°C), or TSP (14.8°C) in 1991 (**Table 4**). In contrast, TSW also had a lower average minimum temperature (6.9°C) than TSP (9.4°C), TSA (9.5°C), or TSC (9.6°C). This large difference (14.0°C) between the minimum and the maximum temperature for TSW indicates that a large amount of energy was stored during the day and released as the air temperature decreased, usually during the night. Small differences between the maximum and minimum water temperatures for the other treatments suggest that the other heat storage systems did not work very well. Some of the treatments without heat storage devices had larger temperature differences than the metal absorber, or the water cans. The phase-change treatment was not expected to store energy in the water. The average maximum water temperature in the treatments without heat storage devices were 14.8°C for top and side vents (TSN), 15.1°C for side vents (SN), 17.9°C for top vents (TN), and 17.8°C for the unvented control (**Table 4**).

Soil-Media Surface Temperatures. Average maximum temperatures at the surface of the soil media in the unvented control treatment was 36.6°C in 1990 (**Table 5**) and 38.8°C in 1991 (**Table 4**). In 1990, all of the other treatments had maximum soil surface temperatures between 26.0 and 29.6°C, and they were not statistically different from each other. In 1991, the values for all treatments except the unvented control fell between 28.2°C and 33.6°C, and they

Table 3. Relationship between treatment, plant length (L) and the slenderness ratio, length/diameter (L/D).

Treatment	1990 L	1990 L/D	1991 L	1991 L/D	1992 L	1992 L/D
NN	1.5	3.1	0	-	0.9	2.3
TN	12.7	21.5	6.3	12.6	11.6	22.8
SN	-	-	9.1	16.3	12.2	21.0
TSN	14.4	25.7	12.2	20.0	14.5	35.2
TSA	8.0	16.7	6.6	14.0	7.9	16.8
TSC	10.0	18.2	12.7	19.5	14.0	28.6
TSW	15.7	26.2	11.0	18.0	8.2	19.1
TSP	8.7	11.0	10.8	18.3	16.0	27.6
Mean of vented treatments	**11.6**	**21.1**	**9.7**	**17.3**	**12.1**	**24.2**

Table 4. Temperatures (°C) observed in small polyethylene greenhouses averaged over 26 days between February 28 and April 26, 1991.

	Average Maximum				Average Minimum				Average Difference			
Treatment	Shaded	Unshaded	Soil Surface	Water	Shaded	Unshaded	Soil Surface	Water	Shaded	Unshaded	Soil Surface	Water
NN	37.3 ab[a]	37.7 ab	38.8 a	17.8 b	2.3 bc	2.0 ab	3.9 b	10.6 a	35.0 a	35.7 ab	34.9 a	7.2 bc
TN	27.5 d	28.4 c	29.4 b	17.9 b	1.9 c	1.6 b	1.9 b	8.5 ab	25.6 bc	26.8 de	27.5 bc	9.4 b
SN	32.9 bc	32.7bc	33.6 b	15.1 c	1.7 c	2.0 ab	2.7 b	9.5 ab	33.1 a	32.7 bc	30.9 b	5.6 c
TSN	29.1 cd	31.5 c	31.4 bc	14.8 c	1.5 c	1.4 b	2.4 b	9.8 a	27.6 b	30.2 cd	29.0 b	5.0 c
TSA	27.8 cd	28.3 c	32.7 bc	15.7 bc	2.2 bc	2.0 ab	2.7 b	9.5 ab	25.6 bc	26.3 de	30.0 b	6.2 bc
TSC	27.5 d	29.4 c	28.2 c	14.1 c	2.3 bc	2.2 ab	2.9 b	9.6 ab	25.2 bc	27.2 de	25.3 c	4.6 c
TSW	27.9 cd	29.4 c	31.2 bc	20.9 a	2.8 bc	2.6 ab	3.5 b	6.9 b	25.1 bc	26.8 de	27.7 bc	14.0 a
TSP	30.2 cd	31.9 c	31.5 bc	14.8 c	2.2 bc	2.1 ab	2.9 b	9.4 ab	28.0 b	29.8 cd	28.6 b	5.4 c
Mean of vented treatments	**29.2**	**30.2**	**31.1**	**16.2**	**2.1**	**2.0**	**2.7**	**9.0**	**27.2**	**28.5**	**28.4**	**7.2**

[a] Numbers followed by the same letter are not significantly different from each other at the 0.95 probability level according to Duncan's Multiple Range Test.

were not statistically different from each other. Many of the values were smaller in 1991 because experiments were initiated and terminated earlier in the year compared to 1990.

Average minimum soil-media surface temperatures were not affected significantly by any of the treatments (**Tables 4 & 5**). In 1990, the average minimum soil surface temperature varied only from 9.9°C to 10.6°C, and no trends were evident (**Table 5**). In 1991, average minimum soil surface temperature varied from 1.9°C to 3.9°C, and none of the treatments were significantly different (**Table 4**).

The difference between average maximum soil surface temperatures and average minimum soil surface temperatures varied from 16.5°C to 26.4°C in 1990, and from 25.3°C to 34.9°C for 1991 (**Tables 4 & 5**). In both years the difference was largest for the unvented greenhouse, 26.4°C in 1990 and 34.9°C in 1991.

Air Temperatures. Average maximum temperatures were highest for the shaded temperature probes in the unvented treatment (40.5°C in 1990 and 37.3°C in 1991) (**Tables 4 & 5**). Values for the unshaded temperature probes in the unvented treatment were not available in 1990 due to an instrumentation problem.

In 1990, the maximum average temperatures for the unshaded probes in the vented greenhouses were not significantly different. However, for shaded temperature probes the greenhouse vented at the top and side (TSN) had a maximum average of 34.0°C, which was higher than for the other vented treatments (**Table 5**).

In 1991, the treatment with the second highest average air temperatures was the side-vented greenhouse (SN), (32.9°C shaded and 32.7°C unshaded). These values suggest that the SN treatment did not allow enough air exchange to adequately cool the greenhouse.

Minimum average air temperatures did not vary significantly over treatments in 1990 or 1991 (**Tables 4 & 5**). Values were 8.3°C to 10.6°C in 1990 and 1.4°C to 2.8°C in 1991. The values were higher in 1990 because the measurement period was later in the year. None of these values were low enough to kill tobacco plants, however, averaging over time does not reflect individual low temperatures. Individual values will be discussed below.

Differences were largest for the shaded and unshaded temperature probes in the unvented treatment (**Tables 4 & 5**). The 1990 values for both shaded and unshaded probes in the vented treatments were not significantly different from each other. In 1991, temperature differences for the side-vent treatment (SN) fell numerically between the unvented treatment and the other vented treatments, corroborating earlier evidence that side vents do not provide enough ventilation to cool the greenhouse as much as top vents plus side vents.

Heat Stress

Because average maximum temperature data tends to mask single high, potentially lethal values, hourly maximum values were examined in 1992. Periods of heat stress varying in length, when the black globe temperature exceeded ambient by at least 15°C, were identified during 25 days between March 1 and May 18, 1992 (**Table 6**). The temperature in the unvented greenhouse reached an extreme of 62.7°C, and maximum temperatures averaged 48.8°C over the 25-day period of heat stress. Extreme values in the vented greenhouses ranged from 31.8°C to 39.6°C (not counting some higher values which occurred before the system was properly adjusted). Average maximum values in the vented greenhouses during the 25-day period ranged from 30.4°C to 34.2°C (**Table 6**). For the vented greenhouses, the highest extreme temperature occurred in the treatment

Table 5. Temperatures (°C) observed in small polyethylene covered greenhouses March 15 to May 7, 1990.

	Average Maximum			Average Minimum			Average Difference		
Treatment	Soil Surface	Unshaded	Shaded	Soil Surface	Unshaded	Shaded	Soil Surface	Unshaded	Shaded
NN	36.6 a[a]	-	40.5 a	10.2 cd	-	10.1 bc	26.4 a	-	30.4 a
TN	27.0 cd	28.4 b	29.9 cd	10.6 cd	8.7 a	9.9 bc	16.5 bc	19.7 bc	20.0 bcd
SN	-	-	-	-	-	-	-	-	-
TSN	29.6 bc	28.0 b	34.0 bc	10.1 cd	8.3 a	9.6 c	19.4 b	19.7 bc	24.5 b
TSA	26.0 cd	28.4 b	27.7 d	10.2 cd	8.9 a	9.6 c	15.8 bc	19.6 bc	18.2 cd
TSC	26.3 cd	25.6 b	29.3 cd	10.1 cd	9.2 a	9.8 c	16.2 bc	16.4 cd	19.5 bcd
TSW	28.2 bc	25.6 b	25.8 d	9.9 d	9.2 a	10.0 bc	18.4 b	16.4 cd	15.8 d
TSP	28.8 bc	26.7 b	25.6 d	9.9 d	9.1 a	9.9 bc	18.9 b	17.6 cd	15.6 d
Mean of vented treatments	**27.6**	**27.1**	**28.7**	**10.1**	**8.9**	**9.8**	**17.5**	**18.2**	**18.9**

[a] Numbers followed by the same letter are not significantly different from each other at the 0.95 probability level according to Duncan's Multiple Range Test.

Table 6. Extreme and average temperatures (°C) during periods of heat and cold stress, 1992.

Treatment	Heat Stress		Cold Stress		Plant Length
	Extreme High	Average	Extreme Low	Average	
					- cm -
NN	62.7	48.8	-4.3	0.8	0.9
TN	34.3	32.7	-4.9	0.6	11.6
SN	33.8	34.2	-4.5	0.9	12.2
TSN	35.3	31.2	-4.5	0.7	14.5
TSA	34.4	32.0	-4.4	0.9	7.9
TSC	31.8	30.9	-4.8	1.3	14.0
TSW	39.6	33.1	-3.6	1.7	8.2
TSP	32.5	30.4	-4.5	0.8	16.0

with the strip of exposed water (TSW). It appears that the vent opening device was slow to operate in TSW in 1992. In previous years, plants in this treatment were among the largest in the experiment. Average daily temperatures varied only by 3.8°C over the 25-day period, and this suggests that all of the vented greenhouses were capable of producing seedlings but that growth rates were variable.

Cold Stress

Hourly temperatures were determined for all periods during which the ambient temperature was less than 4.5°C. The extreme minimum temperatures for all treatments during the 31 cold-stress periods ranged from -4.9°C to -3.6°C (**Table 6**). Thus, there was little evidence to suggest that any of the heat absorbing devices were effective. Also, the extreme minimum temperature in the unvented greenhouse (-4.3°C) was very close to the value for the vented treatments. Average minimum temperatures over the 31 cold-stress periods were also closely grouped. The extreme minimum temperature outside the greenhouse was -4.6°C and the average was 1.5°C. The fact that the average outside temperature during the cold stress periods was higher than the average temperature inside the greenhouses was probably due to air stagnation and to the plastic float trays that insulated the interior from heat rising from the ground.

CONCLUSION

In conclusion, tobacco seedlings can be germinated and grown to transplant size on floating trays in low-profile, polyethylene-covered greenhouse row tunnels. The passive, temperature-driven actuator system successfully opened and closed vents in the polyethylene cover to control temperatures so that plant-damaging heat stress did not occur. There was little response from the heat storage devices with respect to temperature or plant growth. It appears that side vents are not essential, as the greenhouse with only top vents did not overheat.

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THE DETERMINATION OF SCOPOLETIN IN MAINSTREAM TOBACCO SMOKE

By Charles H. Risner[1], Susan V. Parsons, and Lisa S. Winkler

A method was developed that determines the scopoletin content of mainstream cigarette smoke. A Cambridge pad containing the wet total particulate matter (WTPM) of five cigarettes was extracted with 1% acetic acid (HAc), and the extract was subjected to reverse-phase, gradient liquid chromatography using a mobile phase of 1% HAc and acetonitrile (ACN) + 1% glacial HAc. Scopoletin was detected by fluorescence at its characteristic wavelengths of excitation and emission, 342 nm and 464 nm, respectively. The procedure was reproducible with a relative standard deviation of 8.2%. Percent recovery averaged 102% with a detection limit of 1.6 µg. Analyses of several cigarette types indicate that scopoletin varies with the WTPM of mainstream smoke. Scopoletin content was linearly correlated with WTPM (R^2 = 0.87).

Additional key words: Polyphenols, High-Performance Liquid Chromatography, *Nicotiana tabacum*.

INTRODUCTION

Scopoletin, a phenolic compound based on the coumarin skeleton, has been found in sunflowers (11), sweet potatoes (5), oak trees (1), ash trees (6), and more predominantly, in flue-cured tobacco, *Nicotiana tabacum* L. (2,3,12). Reported procedures for analysis of scopoletin in tobacco entail extensive aqueous alcohol extraction with determination by high performance liquid chromatography (HPLC). The methods rely upon absorbance detection at a single wavelength setting that is not selective for scopoletin (2,3,12). Recently, a more selective and sensitive fluorescence detection method has been used for the determination of scopoletin as a particulate marker for environmental tobacco smoke (ETS) in indoor air (9,10). This mode of detection allows at least a ten-fold increase in sensitivity and minimizes interference from other compounds, allowing it to be used in more complicated matrices such as extracts of tobacco tar (8). Because scopoletin is a strongly fluorescing compound, it also has a good potential of being detected in complicated matrices.

This work describes the use of an HPLC procedure using the wavelengths of excitation and emission characteristic of scopoletin for its determination in the mainstream wet total particulate matter (WTPM) of tobacco smoke. Mainstream smoke was collected on a Cambridge pad, and a simple extraction was performed using 1% aqueous acetic acid (HAc). Scopoletin was quantified by reverse-phase chromatography with gradient elution using a mobile phase of HAc and acetonitrile (ACN). Regression analysis of results from the mainstream smoke of 52 cigarette types showed that scopoletin was related to the WTPM of the cigarettes. This is the first procedure reported for the determination of scopoletin in mainstream cigarette smoke and is part of a continuing effort by this laboratory for evaluating particulate markers for cigarette smoke.

MATERIALS AND METHODS

Reagents and Chemicals

Water (17.7 megohm-cm) was distilled and passed through a pre-treatment cartridge, a high capacity cartridge, two ultrapure cartridges, and a 0.45-µm pore size filter (Barnstead Co., Div. of Sybron Corp., Boston, Mass.). Acetic acid (HAc) was reagent grade (Reagents, Inc., Charlotte, N.C.). Acetonitrile (ACN), "distilled in glass," and methanol (MeOH) were chromatography grade obtained from Burdick and Jackson (Muskegon, Mich.). Scopoletin (95%) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.), and it was used as received.

Standard Preparation

A stock scopoletin solution was prepared by weighing 0.009 g to the nearest 0.0001 g in a 100-mL volumetric flask and diluting to the mark with MeOH. The working standards were prepared from the stock solution by serially diluting with 1% HAc to cover a concentration range of 0.25 to 1.28 µg mL^{-1}.

Chromatographic System

The chromatographic system consisted of two Waters (Milford, Mass.) 510 pumps, 717 autosampler, 470 fluorescence detector, and 680 gradient controller. Data were acquired using VG Data Systems VAX Multichrom software (Cheshire, England). A Brownlee (Santa Clara, Calif.) RP-18 Speri-5MPLC guard column refill (30 x 4.6 mm, 5 µm particle size) contained in a 3-cm MPLC holder and a Beckman (San Ramon, Calif.) Ultrasphere-ODS (250 x 4.6 mm, 5 µm particle size) analytical column were used. A two-segment gradient of 1% aqueous HAc and ACN + 1% HAc (**Table 1**) was used at a flow rate of 2.00 mL min^{-1} (8). The injection volume was 10 µL. The wavelength settings of 342 nm excitation and 464 nm emission were selected for detection (**Figure 1**).

Cigarettes

Industry Monitor 14 cigarettes (Philip Morris Corp., Richmond, Va.) were used for method validation. The WTPM value of these standard cigarettes was about 23 mg, the dry tar 17.6 mg, and nicotine 1.19 mg per cigarette.

Sample Collection and Preparation

Mainstream tar was collected on Cambridge pads using the procedure of Pillsbury et al. (7). The pads were extracted on the basis of approximately 1 mL of 1% HAc per mg WTPM on the pad. For example, Industry Monitor 14, which yielded a WTPM of about 115 mg per pad from five cigarettes, was extracted with 100 mL of 1% HAc. The pads were extracted using a wrist action shaker for one-half hour after which a portion of the extract was filtered through a 0.45 µm pore size Autovial (Whatman, Clifton, N.J.) into an autosampler vial.

RESULTS AND DISCUSSION

Precision

The instrument precision was 2.73% relative standard deviation (RSD) based on six injections. The overall precision for scopoletin and WTPM for 18 smoking trials of five cigarettes each (Industry Monitor 14) are shown in **Table 2**. The RSD of the smoking procedure (5.30%) was in the same range as the instrument precision for scopoletin. Even with the contribution of the smoking procedure to the RSD of scopoletin, the overall sample variation of 8.22% was acceptable. The minimum detectable quantity (MDQ) of scopoletin was 15.8 ng mL^{-1} in the 100 mL extract of the

[1] Senior Research and Development Chemist, R. J. Reynolds Tobacco Company, Bowman Gray Technical Center, Winston-Salem, North Carolina 27102.

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Table 1. Mobile phase gradient used for the elution of scopoletin from the extract of mainstream tobacco smoke[a].

Time	Acetonitrile + 1% acetic acid	1% Aqueous acetic acid
----- min -----	----- % -----	-----% -----
0	0	100
10	10	90
40	25	75
41	100	0
56	100	0
57	0	100
65	0	100

[a] Curve 6 (linear) on Waters 680 gradient controller, 25 cm Beckman Ultrasphere ODS column, flow rate of 2.0 mL min^{-1}.

Cambridge pad using a 10-µL injection volume and rather coarse detector settings, conditions which adequately accommodated the sample extracts. The MDQ could be improved to <1 ng mL^{-1} by increasing detector sensitivity and injection volume as done in previous work (10). This detection limit was found to be less than that reported using coulometric array detection (4). Indeed, we found that injection volumes larger than 10 µL saturated the detector when more concentrated (250 µg scopoletin in 100 mL extractant) samples were evaluated. **Figure 2** shows a typical sample chromatogram obtained during the precision study.

In addition to the overall precision determined on for Industry Monitor 14, additional analyses of this cigarette type were conducted to verify the procedure under routine application. The results of 51 smoking trials over a one-month period averaged 82.3 µg scopoletin for five cigarettes (16.5 µg $cigarette^{-1}$) with a standard deviation of 5.00 µg (1.00 µg $cigarette^{-1}$). Although these results were lower than the results in **Table 2** ($\bar{x}$= 92.4 µg, s.d. = 7.60 µg), they overlap considerably.

Linearity

Over the concentration range evaluated, the response for scopoletin was linear (R^2 = 0.9984, **Table 3**). The y-intercept was in the same range as the MDQ (**Table 2**).

Extraction Study

To determine if scopoletin was sufficiently extracted from the tar on the Cambridge pads, an extraction-time study was undertaken. The amounts of scopoletin extracted for extraction times of 15 min to 2 hours were similar (**Table 4**). We concluded that 30 minutes was sufficient for extraction of scopoletin from Cambridge pads used for collection of mainstream smoke.

Figure 1. Excitation and emission spectra of scopoletin: A = excitation, B = emission, 0.70 µg mL^{-1} in 1% HAc.

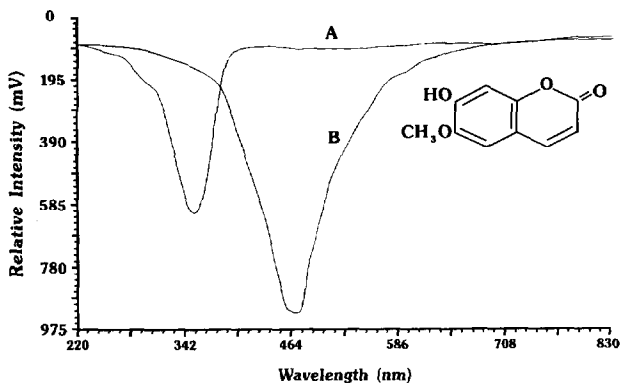

Table 2. Overall precision for scopoletin collection in mainstream tobacco smoke[a].

Sample no.	Scopoletin	WTPM[b]	Sample no.	Scopoletin	WTPM[b]
	----- µg -----	----- g -----		-----µg -----	-----g -----
1	91.08	0.1094	10	83.62	0.1128
2	98.48	0.1163	11	94.03	0.1161
3	107.27	0.1231	12	90.06	0.1203
4	98.01	0.1176	13	88.86	0.1146
5	92.52	0.1112	14	88.43	0.1110
6	107.58	0.1282	15	77.20	0.1073
7	93.02	0.1214	16	90.04	0.1033
8	96.42	0.1202	17	83.45	0.1130
9	93.66	0.1126	18	89.03	0.1198
			$\bar{x}$	92.38	0.1155
			s.d.	7.60	0.0061
			% RSD[c]	8.22	5.30

[a] Five Industry Monitor 14 cigarettes, Cambridge pad extracted with 100 mL 1% HAc for 30 min. Minimum detectable quantity (MDQ) determined as twice the signal-to-noise ratio was 15.8ng mL^{-1} (1.58 µg) in the 100 mL extract.
[b] WTPM = wet total particulate matter.
[c] %RSD = percent relative standard deviation.

Table 3. Linearity of response of scopoletin standards used for the determination of scopoletin in mainstream smoke[a].

Standard concentration range	Sample amount range	Sensitivity range	Linear correlation	Y-intercept
-- µg mL^{-1} --	-- µg[b] --	-- ng[c] --	-- R^2 --	-- µg --
0.252 - 1.275	25.2 - 127.5	2.52 - 12.75	0.9984	1.90

[a] Based on the mainstream smoke from five Industry Monitor 14 cigarettes.
[b] Cambridge pad extracted with 100 mL 1% HAc.
[c] 10 µL injection volume, Waters 470 fluorescence detector using gain = X100 and attenuation = 32.

Standard Addition/Recovery

When scopoletin was added to the pad prior to extraction at three rates (0.5, 1.0, and 2.0 times the amount in Industry Monitor 14), recovery averaged 102.5% (**Table 5**). The amount found by external standard (86.8 µg) compared well to that found by standard addition (91.9 µg).

Stability/Storage of Standards and Sample Extracts

There was about a 15% loss in response for scopoletin when the standards were stored in the laboratory for 4 weeks (**Table 6**). Therefore, we recommend that working standards

Figure 2. Chromatogram of the 100 mL 1% HAc extract of the mainstream tar from five Industry Monitor 14 cigarettes. (See Materials and Methods section for conditions).

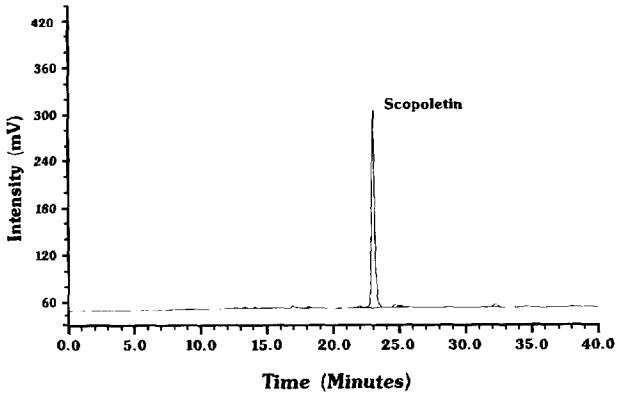

Table 4. Extraction time studies for the removal of scopoletin from mainstream tobacco tar on Cambridge pads[a].

Extraction time	Scopoletin ($\bar{x}$, s.d., % RSD)	WTPM ($\bar{x}$, s.d., % RSD)[b]
--- min ---	--- µg ---	--- g ---
15	87.5, 4.0, 4.56	0.1065, 0.0085, 8.00
30	93.4, 2.0, 2.13	0.1123, 0.0071, 6.32
60	94.1, 9.6, 10.22	0.1077, 0.0030, 2.80
120	96.4, 7.6, 7.90	0.1128, 0.0086, 7.61

[a] Tar obtained from the mainstream smoke of five Industry Monitor 14 cigarettes, 100 mL 1% HAc used to extract pad by wrist action shaking, n = 3.
[b] %RSD = percent relative standard deviation.

Table 5. Recovery and standard addition of scopoletin standard solution added to the mainstream extract[a].

Sample no.	Amount added	Amount recovered[b]	Recovery
	--- µg ---	--- µg ---	--- % ---
1	0.0	86.8	---
2	42.0	130.8	104.8
3	84.0	172.0	101.4
4	168.0	257.0	101.3
			$\bar{x}$ = 102.5

[a] Standard added after smoking, prior to extraction, n = 2.
[b] Amount found (µg) external standard = 86.8, standard addition = 91.9

be prepared from a fresh refrigerated stock standard every week, or when loss of response becomes greater than 5%. The sample extracts were evaluated after 2 weeks storage at room temperature in the light, under dark conditions at room temperature, and under dark conditions at 2°C (**Table 7**). There appeared to be some loss, but the values are within experimental error of 8.22% (**Table 2**). Other components (e.g., hydroquinone) of mainstream tar may be inhibiting the loss of scopoletin in the 1% HAc extract (8).

Applications

The procedure was applied to the mainstream tar of 52 cigarette types, which included 50 commercial brands, Kentucky Reference Cigarette 1R4F (Tobacco and Health Research Institute, Lexington, Ky.), and Industry Monitor 14. Regression analysis of scopoletin as a function of WTPM showed a positive correlation (R2 = 0.87) (**Figure 3**). That this correlation coefficient is not closer to unity may be expected, because scopoletin is higher in flue-cured tobacco (2,12), a varying component in these cigarette blends.

Figure 3. Regression plot of scopoletin versus WTPM. Scopoletin (µg cigarette^{-1}) = WTPM (0.7664) + 1.4641, R^2 = 0.87.

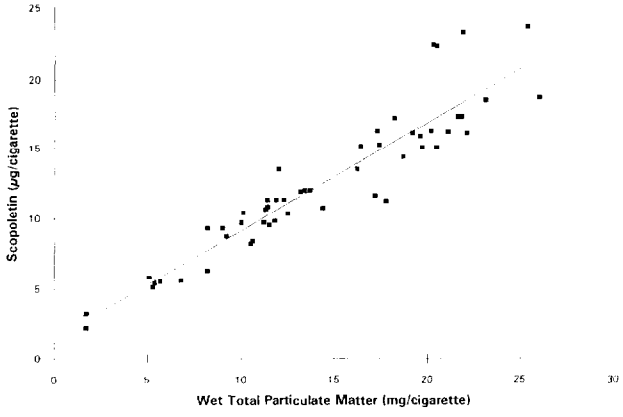

Table 6. Storage and stability of scopoletin standards.

Standards (25°C in light)			
Concentration	Height initially	Height four weeks	Change
--- µg mL^{-1} ---	--- V X 10^{-6} ---	--- V X 10^{-6} ---	--- % ---
0.252	79501	66739	-16.0
0.504	150001	129866	-13.4
0.840	254544	212761	-16.4
1.260	378738	328963	-13.1
			$\bar{x}$ = -14.7

Table 7. Storage and stability of sample extracts[a].

Condition	Initial	After one week, Change	After two weeks, Change
	--- µg ---	--- µg ---,--- % ---	--- µg ---,--- % ---
25°C in Light	94.7	88.9, -6.2	100.1, +5.6
25°C in Dark	102.6	97.0, -5.4	98.8, -3.6
2°C in Dark	100.0	94.0, -6.0	92.2, -7.8

[a] 100 mL extract of 1% HAc in the presence of a Cambridge pad containing tar from the mainstream smoke of five Industry Monitor 14 cigarettes, n = 2.

Previous work showed that the scopoletin content of ETS from a commercially available, 85-mm cigarette containing flue-cured tobacco (10 mg tar and 0.7 mg nicotine per cigarette) was five-times higher than that from a cigarette made entirely from burley tobacco (1755 versus 325 ng m^{-3}) (10). This may also indicate that flue-cured tobacco is a major source of scopoletin in mainstream smoke.

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REPORTED INSECTICIDE USE ON TOBACCO IN KENTUCKY (1989-1992)

By L. H. Townsend and P. M. Dillon[1]

An accurate picture of pesticide-use patterns on crops is needed to increase the effectiveness of grower education programs, to respond to regulatory questions, and to assess needs for pest management. The Kentucky Department of Agriculture, Division of Pesticides, annually surveys approximately 480 retail pesticide dealers to collect sales data. Survey results for the years 1989-1992 showed that growers purchased approximately the same total amount of insecticide active ingredient (2.6 kg/ha) for use on tobacco each year, but there were major shifts in which products were purchased. Total insecticide expenditures for 1992 were calculated to be approximately $5.4 million ($57/ha). Use of strictly soil-applied insecticides declined steadily from 1990 to 1992. An apparent increase in insecticides used in transplant water may be related to the decline in the use of soil insecticides, because growers may have taken advantage of less expensive control measures by adding products to the transplant water instead of the soil. The foliar insecticides acephate and endosulfan represented 60% to 75% of the total insecticide active ingredients applied in 1989-1992. The popularity of these two materials could create future problems. Constant use of an insecticide can favor the development of insecticide resistance in pest populations. Resistance to acephate has already been documented in some aphid populations on flue-cured tobacco. Endosulfan presents another problem, that is, insecticide residues on the crop that exceed tolerance levels. These topics need to be emphasized in educational programs for growers.

Additional key words: ***Nicotiana tabacum.***

INTRODUCTION

Five types of tobacco are grown in Kentucky (3). About 95% of the total area planted is burley, an air-cured tobacco, used primarily in the manufacture of cigarettes. Field production practices, insect pest problems, and pest control strategies generally are the same for all five types. Tobacco flea beetles (*Epitrix hirtipennis* (Melsheimer), tobacco aphids (*Myzus nicotianae* Blackman), tobacco budworms (*Heliothis virescens* [F.]), and tobacco hornworms (*Manduca sexta* [L.]) are key pests, while the black cutworm (*Agrotis ipsilon* [Hufnagel]) sporadically causes economic damage in Kentucky (7). These are the primary pests that growers usually consider when choosing insecticides to use in their fields.

An accurate representation of pesticide-use patterns on crops is essential information that is difficult both to obtain and to assess. It is invaluable, however, in developing pesticide education and pest management programs, accurately answering questions dealing with pesticide impact assessment/risk-benefit analyses, and aiding the industry in evaluating the potential for registration and labeling of new products for crop protection.

Pesticide-use data often are gathered through surveys targeted at a representative sample of growers with the results projected over the entire group. However, growers may be reluctant or unwilling to participate in voluntary surveys or they may lack detailed records upon which to base their responses. Alternatively, expert opinion can be solicited from crop consultants, commercial applicators, or Cooperative Extension personnel, such as specialists and county agricultural extension agents. However, these responses may not reflect practices particularly well and generally cannot be used to develop data on acres treated, control costs, and other factors.

In 1989, the Kentucky Department of Agriculture (KDA), Division of Pesticides, initiated a program that approached the challenge of gathering pesticide-use data from a different source, that is, the retail outlets from which farmers and commercial applicators purchase their products. KDA personnel, who regularly inspect dealer facilities, annually visited all licensed pesticide dealers in the state to collect pesticide sales data for the previous year. This paper reports on the accumulation of four years of survey data. This data allowed for examination for trends and it supplied baseline data to track the use of insecticides on tobacco.

[1] Department of Entomology, University of Kentucky, Lexington, KY 40546-0091.

Contribution received December 27, 1993. Tob. Sci. 38:72-74, 1994.

MATERIALS AND METHODS

Data reported in this survey were collected by Pesticide Inspectors during annual (1989-1992) interviews conducted at approximately 480 licensed retail outlets in the state during January and February. Inspectors were trained before conducting the interviews, and results from the initial survey were used to streamline procedures for subsequent years. Product names, formulations, amounts, and intended crop use were obtained directly from the sales records that are required to be maintained at each store, and they were entered onto standard forms designed for the survey. Sales between dealers were not counted. The information on the forms was entered into a data base using a computer program written by the authors. A second computer program, also written by the authors, was used to convert the formulation volumes to amounts of active ingredient based on the product labels.

Total amounts were calculated for each active ingredient based on the amounts of formulated products sold. Harvested crop areas were taken from annual production reports of the Kentucky Agricultural Statistics Service, Louisville, Ky. Specific product uses (soil application only, transplant-water application only, or foliar application only) were identified from the product labels. For example, chlorpyrifos is registered for preplant-incorporated use only, methomyl is registered solely as a foliar insecticide, etc. The results are discussed in terms of soil, transplant-water, and foliar application. Per hectare use-rates on product labels allowed estimation of potential area treated; information on retail prices of products allowed calculation of expenditures on insecticides for the 1992 growing season.

RESULTS AND DISCUSSION

Survey results showed a relatively stable total amount of insecticide purchased for use on tobacco over the 4-year period, but there appeared to be major shifts in patterns of products chosen (**Table 1**). Probably not all of the products identified by the purchasers for use on tobacco were applied to the crop during that growing season. However, the overall consistency between years, a mean of about 2.6 kg of active ingredient per hectare (range 2.4 to 3.0 kg/ha), reflects a stable purchasing pattern from year to year. Undoubtedly, some tobacco was not harvested each year, but this should have minimal impact on the overall figures. Using an average retail price per kilogram of active ingredient, we calculated a total insecticide expenditure for 1992 of

approximately $5.4 million ($57 per hectare). Acephate and endosulfan together accounted for 58%, 65%, 76%, and 79% of the insecticide volume sold for the crop from 1989 through 1992, respectively.

Soil-applied insecticides

The use of soil insecticides declined steadily from 30% of the active ingredient volume in 1989 to 13% in 1992. Use of carbofuran on tobacco dropped precipitously from a peak in 1990 when approximately 8,822 hectares were treated (assuming use at a labeled rate of 4 pounds of active ingredient per acre [4.5 kg/ha]). Registration of the 15% granular formulation of carbofuran has been dropped for tobacco, as well as for a wide variety of other crops, and this may have had a major impact on its use on burley. Often the granular formulation was applied along with dry fertilizer during field preparation. Because of safety considerations, the flowable formulation would probably be applied as a broadcast spray to the soil surface rather than being incorporated into solid fertilizer. Consequently, use of carbofuran on tobacco may continue to drop unless application alternatives are developed. Carbofuran is labeled for flea beetle control at a rate of 4 pounds of active ingredient per acre (4.5 kg/ha).

The amount of chlorpyrifos purchased for use on tobacco also decreased steadily over the 4-year period. Area treated with chlorpyrifos dropped from a high of about 7,527 hectares to approximately 4,452 hectares. This product is labeled at 2 pounds per acre (2.2 kg/ha) to control cutworm and wireworm infestations.

Disulfoton sales attributed to tobacco peaked at 9,879 kg of active ingredient in 1990. Historically, much of the disulfoton has been used as a pre-seeding treatment for flea beetle control in tobacco plantbeds. Also, it is labeled as a soil-applied preventive treatment for aphids in the field. Serious infestations of the tobacco aphids and grower frustration with foliar sprays may have resulted in additional broadcast pre-plant applications of disulfoton in the field during 1990.

Ethoprop and fonofos sales were low during the entire 4-year period, with no record of ethoprop sales for tobacco during either 1991 or 1992. Ethoprop lacks alternative label uses applicable to Kentucky crops, whereas both carbofuran and chlorpyrifos have broader labels that may make them more attractive to dealers. The large increase in fonofos sales may be due in part to inclusion of this insecticide with the herbicide pebulate in a package mix targeted at tobacco growers. There was a general decline in the use of soil insecticides, which may have resulted from attempts to reduce expenditures for preventive treatments.

Transplant-water insecticides

Transplant-water applications provide less costly early-season control of pests than the soil treatments. Lindane and oxamyl are the only two products labeled for use solely in the transplant water for burley tobacco. Lindane provides protection against wireworms, while the systemic insecticide oxamyl is used for tobacco flea beetle control. Decline in the use of carbofuran and chlorpyrifos may account for the increase in transplant-water applications of lindane and oxamyl seen in 1992. Acephate and diazinon also may be used in the transplant water. However, we could not determine from the survey data the proportions of the latter two insecticides used in the transplant water compared to foliar sprays.

Foliar insecticides

Of the eight products labeled as foliar sprays, applications of carbaryl, dimethoate, methomyl, and methyl parathion were very limited. The use of malathion and diazinon on tobacco remained relatively unchanged. While some diazinon was used as a soil- or transplant-water application, conversations with growers and dealers indicated that much of the use was as a foliar spray, often in combination with acephate for tobacco aphid control. The manufacturers of both malathion (6) and diazinon (Ciba Geigy 1987, personal communication) have stated that they will not support continued labeling of these products for tobacco under the re-registration program.

Acephate and endosulfan together accounted for just over 60% of the total active ingredient of insecticides applied in 1989 and 1990, and at least 75% of the 1991 and 1992 applications. The amount of land treated with these materials during the survey period can be estimated from data in **Table 1** using the label-recommended rate of active ingredient (0.75 lb/acre [0.84 kg/ha] for acephate and 1.0 lb/acre [1.12 kg/ha] for endosulfan). The ratios of acephate:endosulfan from 1989 to 1992 were 11.8:1, 4.5:1, 2.8:1, and 2.7:1, respectively. Acephate sales reported for tobacco averaged 1.3 kg of active ingredient per hectare (range 1.2 to 1.5 kg/ha) during 1989-1992.

The use of endosulfan climbed from 0.13 kg of active ingredient per hectare in 1989 to 0.61 kg/ha in 1992. This resurgence in the use of endosulfan probably stems from grower concern with an apparent lack of control of the red color form of the tobacco aphid with other materials. The red form was first reported from North Carolina in 1985 (5) and subsequently spread throughout areas of the USA where tobacco is grown. An increase in use of this insecticide could result in unacceptably high levels of insecticide residues on the crop. Gibson et al. (2) reported first detecting endosulfan residues in a few burley samples in 1968. They also reported that residues of this insecticide were present in 100% of the samples from the 1970 and 1972 crops and 83% of those from 1971. Studies of endosulfan residues on burley tobacco show that allowing sufficient time between application and harvest results in reduced residues (1). The suggested interval is longer than that stated on the product label, so

Table 1. Insecticide sales (kilograms of active ingredient) for use on tobacco as reported by licensed pesticide dealers in Kentucky, 1989-1992.

Active ingredient	1989	1990	1991	1992
	kg A.I.			
Soil-applied only				
carbofuran	29,397	39,630	24,081	9,280
chlorpyrifos	16,901	15,604	14,197	10,705
disulfoton	4,631	9,879	6,573	7,117
ethoprop	68	980	0	0
fonofos	200	91	435	2,703
Transplant-water only				
lindane	240	363	54	862
oxamyl	163	735	494	848
Foliar only				
Bacillus thuringiensis	-	290	499	472
carbaryl	268	240	386	227
dimethoate	145	23	0	0
endosulfan	9,766	34,995	56,282	57,910
malathion	8,682	7,679	4,182	7,924
methomyl	1,021	222	327	227
methyl parathion	172	431	308	254
Foliar or transplant-water				
acephate	86,573	117,045	118,437	117,662
Soil, transplant-water or foliar				
diazinon	8,183	7,398	4,999	4,985
Total	166,410	235,605	231,254	221,176
Mean kg A.I./ha	2.4	3.0	2.6	2.4

grower and dealer education should continue to emphasize factors to consider for product selection late in the season. This issue is addressed in the annually revised insect control recommendations produced by the University of Kentucky College of Agriculture (8), which recommends that application of endosulfan not be made to the crop after topping.

Extensive and constant use of an insecticide against a pest can result in selection for resistance in the population. Resistance to acephate in *Myzus persicae* (Sulzer) on tobacco was reported from the flue-cured tobacco belt (4). The potential for additional cases of resistance in aphid populations should not be overlooked (5). Educational programs for growers should begin to include information on insecticide resistance and concepts of resistance management.

There was some use of biological insecticides specifically for caterpillars, most notably *Bacillus thuringiensis* Berliner. These products are efficacious against tobacco hornworms and tobacco budworms, but they are ineffective against non-lepidopteran pests. Use of these products may continue to increase as growers look for means to reduce worker exposure to conventional pesticides during mixing, application, and clean-up, and during tasks such as topping, harvesting, and housing that require close human contact with the crop. In addition, growers may be interested in conserving beneficial arthropods.

The survey clearly showed that acephate and endosulfan are the primary insecticides used on tobacco in Kentucky. Consequently, insecticide resistance in populations of the tobacco aphid and unacceptable insecticide residues on the leaf product are possible if these materials are not used judiciously. This information, along with alternative management strategies, is being conveyed to tobacco producers in educational programs in an effort to reduce the potential for such problems to develop. These data also allow estimation of control costs for tobacco pests, and they have been valuable in the reregistration process for chlorpyrifos. Survey programs such as this are valuable and should be continued to provide sound information on which to answer questions on pesticide use.

ACKNOWLEDGEMENTS

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EFFICACY OF WEEKLY AGRIBROM TREATMENTS FOR CONTROL OF ALGAL AND FUNGAL GROWTH IN A TOBACCO TRANSPLANT FLOAT-PRODUCTION SYSTEM[1]

TOBACCO SCIENCE

By Stratford H. Kay[2], W. David Smith[2], and Gordon S. Miner[3]

Two experiments were conducted to determine the effectiveness of Agribrom (1-bromo-3-chloro-5,5-dimethyl-2,4-imidazolidine-dione) for control of algal and fungal slimes on the surface of the growth medium used in the float method for production of tobacco (*Nicotiana tabacum* L.) transplants in greenhouses. In the first experiment, Agribrom was sprayed weekly on the surface of the growth medium at 0, 10, or 20 mg L^{-1} beginning on the date of seeding or at two weeks after seeding. In the second experiment, Agribrom was sprayed weekly at 0, 10, 20, 400, or 800 mg L^{-1} beginning on the date of seeding, or at 10 or 20 mg L^{-1} beginning two weeks after seeding. Both experiments were harvested after five weeks. Agribrom applications had no effect on the number of plants or biomass production in either test. Agribrom also had no significant effects on algal or fungal growth in either experiment at any treatment rate, regardless of the timing of application. These data indicate that spray applications at concentrations up to 800 mg L^{-1} of Agribrom applied at weekly intervals either at seeding or emergence will not control algal or fungal growths in tobacco transplant float-production systems.

Additional key words: *Nicotiana tabacum*, greenhouse transplant culture, algicide, nontarget effects, Chlorophyceae, Cyanophyceae.

INTRODUCTION

Agribrom (1-bromo-3-chloro-5,5-dimethyl-2,4-imidazolidine-dione), a product produced by Great Lakes Chemical Corporation (West Lafayette, Ind.), has been labelled for several years for control of algae in greenhouses (7). Hickman & Viss (3) concluded that Agribrom would effectively eliminate algal growths from greenhouse walkways, under greenhouse benches, and on evaporative cooling system pads. Tayama et al. (9) reported good control of algae and microbial slimes on subirrigation mats and in evaporative cooling systems. There is some evidence that Agribrom will control diseases in greenhouse plug production of ornamental plants, but recent studies of Agribrom for control of diseases of ornamentals suggest that its effectiveness may vary considerably with treatment rate and method of application (1,2,4,6). Some damage to ornamentals has occurred following use of this product for disease control (1,2,5,9).

Many tobacco (*Nicotiana tabacum* L.) growers in North Carolina have converted to greenhouse float systems for production of transplants to reduce the labor requirements for managing plant beds and pulling transplants (4). Dense surface growths of algae, bacteria, and fungi are common in these float systems. A number of growers and commercial producers of tobacco transplants have tried to control these algal and fungal growths by spraying Agribrom onto the surface of the growth medium immediately after planting. The results of this single application generally have not been satisfactory.

The objectives of this study were (1) to evaluate the efficacy of surface-spray applications of Agribrom for control of algal and other microbial slimes in float-production systems using more frequent applications and higher application rates than normally used by tobacco transplant growers; and (2) to determine the effects of Agribrom on the growth of tobacco seedlings.

MATERIALS AND METHODS

Two successive experiments were conducted during the spring of 1991 in a greenhouse using a float system containing 33 x 66-cm polystyrene trays with 200 cells per tray. Each cell had a volume of approximately 27 cm^3. Before seeding, trays were steam-sterilized, filled with Metromix 220® (W. R. Grace & Co., Cambridge, Mass.) soilless growth medium, and dibbled to compact the growth medium. A vacuum-operated seeding device was used to place one pelleted seed (variety Northrup King K-326) into each of the 200 cells. Seeded trays were floated on a solution containing 150 mg N L^{-1} prepared from Peters® water-soluble 20-10-20 fertilizer (Grace Sierra Horticultural Products Co., Milpitas, Calif.). An additional 100 mg N L^{-1} from 20-10-20 was added four weeks after seeding. Additional polystyrene foam was placed between trays to act as separators and to reduce light penetration and algal buildup in the underlying nutrient solution. A maximum-minimum thermometer was used to monitor ambient air temperature at the media surface.

Agribrom usually is applied in greenhouse plug culture by injection into the irrigation system at 20 mg L^{-1} during each mist cycle (or about 10 times per hour during the day). However, mist irrigation systems are not normally used in tobacco transplant float-production systems. Consequently, all Agribrom treatments were applied as surface sprays (60 mL solution per tray) using a backpack sprayer to thoroughly wet the entire surface of the growth medium, leaves of emerged seedlings, and exposed surfaces of the tray. In the first experiment, Agribrom was applied weekly at treatment concentrations of 0, 10, or 20 mg L^{-1}, beginning either at seeding or two weeks after seeding. An additional set of three trays, dibbled, but without plants, was included in this experiment to evaluate algal and fungal growth in the absence of seedlings. In the second experiment, Agribrom was applied at 0, 10, 20, 400, or 800 mg L^{-1} weekly, beginning at seeding, or at 10 or 20 mg L^{-1} beginning two weeks after seeding.

Five weeks after seeding, plants were clipped at the surface of the growth medium, counted to determine plant density, dried for 48 hours at 70°C, and weighed to determine dry-shoot biomass. After plants were removed from the trays, relative densities of algal and fungal growths and a reddish-brown slime resembling oxidized iron were made using a visual, subjective rating scale of 0 to 5, where 0 represented complete absence of growths and 5 represented dense coverage across the entire surface of the tray. Visual ratings were used, because there are no other methods to quantify these growths. Algae were collected from the trays and surface of the nutrient solution and examined microscopically.

Each experiment was established in a randomized complete block design with three replicates per treatment.

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[2] Associate Professors, Department of Crop Science, North Carolina State University, Raleigh, N. C. 27695.

[3] Professor, Department of Soil Science, North Carolina State University, Raleigh, N. C. 27695.

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Seedling dry weight, density, and visual rating data were subjected to an analysis of variance and means were separated using the Duncan's multiple range procedure (8).

Two additional experiments were done to determine whether the source of algal contamination was the clay-coated seed or another component of the float system. To determine if seeds were the source of contamination, either sterilized (autoclaved) or unsterilized seeds were placed in petri dishes on filter paper moistened with nutrient solution. Ten seeds were placed into each dish, and each treatment was replicated three times. Petri dishes were placed in a completely randomized order in a growth chamber. They were incubated at 25 ± 3°C with a 12-h photoperiod for one week and examined daily for appearance of algae. In another experiment, three trays containing Metromix 220 were floated in a small hydroponic system without seeding or dibbling, and they were observed over a two-week period for the appearance of algae. Light, temperatures, and nutrients were similar to those in the initial two greenhouse studies, except that this experiment was conducted in a separate greenhouse to avoid cross-contamination from the other experiments. All trays and other materials were new, and trays were steam-sterilized as described previously.

RESULTS AND DISCUSSION

Algae appeared on the growth medium and trays within one week after seeding in all treatments in both of the initial greenhouse experiments. Algae were distributed over the entire system by the end of the experiments (five weeks). There were no significant differences in algal growth among any of the treatments in either experiment, but densities were slightly lower in the second test than in the first (**Table 1**). Microscopic examination revealed that the dominant forms were the blue-green alga, *Oscillatoria* sp. (Cyanophyceae: Oscillatoriales), and an unidentifiable green alga similar in appearance to *Chlorococcum* sp. (Chlorophyceae: Chlorococales). A flagellated green alga, *Chlamydomonas* sp. (Chlorophyceae: Volvocales), also occurred to a lesser extent on the trays and potting medium, but it was the dominant form found on the surface of the nutrient solution. Dense growths of algae, especially blue-green algae and *Chlamydomonas*, occur commonly in greenhouses whenever suitable temperatures, light, high moisture, and nutrients are present. There is no information available about the sensitivity of any of these algae to Agribrom.

Fungal growth and the reddish-brown slime resembling oxidized iron also appeared about the same time as the algae. The reddish-brown slime occurred on all exposed surfaces of trays, spacers, and potting medium, whereas the fungal growth was limited to surfaces of the potting medium and adjacent upper surfaces of trays. There were no significant differences among treatments in either experiment, with respect to fungal growth. Only in the first experiment did any significant differences occur among treatments, with respect to the reddish-brown slime (**Table 1**). It is uncertain why these differences occurred, as there were no consistent patterns of slime development with treatment. Examination by the soil microbiology laboratory revealed only that the fungal growths and the reddish-brown slime were not pathogens and most likely were saprophytes.

Slightly lower densities of algae, fungi, and reddish-brown slime were observed in the second experiment, which may be a reflection of less-suitable growing conditions. Additionally, the plants were smaller, less dense, and less uniformly distributed within the trays than in the first experiment. Under these conditions, there was less shading of the algae from the seedlings. Light intensities reaching the surface of the growth medium would have been higher, and presumably (not measured), the surface temperature of the growth medium would have been higher during the day than in the first experiment. Very warm temperatures and high sunlight intensities are detrimental to the development of algae and fungi.

Agribrom treatment had no effect on seedling number, total dry matter, or average plant weight in either experiment (**Table 1**). Plant numbers, dry weights, and average weights per plant were significantly lower for the second experiment, compared with the first. Poorer seedling growth in the second experiment may be related to less suitable growing conditions, particularly the higher ambient air temperatures noted in the greenhouse at that time.

Attempts to ascertain the origin of algal, fungal, and reddish-brown slime were inconclusive. No algae, fungi, or

Table 1. Influence of intermittent spray applications of Agribrom on plant growth and the development of surficial algal and fungal slimes in a tobacco transplant float-production system.[a]

Treatment	No. of plants	Dry weight	Avg. plant weight	Rating of surface slimes[b]		
				Algae	Fungi	Brown Deposits[c]
		----- g -----	----- g -----			
Experiment 1						
Control, plants present, no Agribrom	189 NS[d]	14.9 NS	0.07 NS	5.0 NS	4.8 NS	4.7a[e]
No Plants, no Agribrom	-----	-----	-----	5.0	5.0	2.3a
10 mg/L at seeding, 1, 2, 3, and 4 wks	180	14.7	0.08	4.8	4.0	1.7b
10 mg/L at 2, 3, and 4 wks	178	14.8	0.08	5.0	5.0	1.7b
20 mg/L at seeding, 1, 2, 3, and 4 wks	182	14.9	0.08	5.0	5.0	3.0a
20 mg/L at 2, 3, and 4 wks	185	14.6	0.08	5.0	5.0	2.3a
Experiment 2						
Control, plants present, no Agribrom	131 NS[d]	7.0 NS	0.05 NS	4.0 NS	3.7 NS	1.3 NS
10 mg/L at seeding, 1, 2, 3, and 4 wks	133	7.2	0.05	4.3	1.0	1.3
10 mg/L at 2, 3, and 4 wks	134	8.0	0.06	4.3	1.7	1.3
20 mg/L at seeding, 1, 2, 3, and 4 wks	141	9.6	0.07	5.0	3.3	1.0
20 mg/L at 2, 3, and 4 wks	132	8.1	0.06	4.3	1.3	1.3
400 mg/L at seeding, 1, 2, 3, and 4 wks	143	8.1	0.06	4.7	2.0	1.3
800 mg/L at seeding, 1, 2, 3, and 4 wks	132	8.2	0.06	3.7	1.3	2.3

[a] Data are means of three replicates.
[b] Rated using a visual scale of 0 to 5, where 0 represents no accumulation and 5 represents heavy accumulation of slimes.
[c] Brown deposits are assumed to be ferric oxide resulting either from precipitation at the surface of the potting medium or possibly from the activity of iron bacteria.
[d] NS = Nonsignificant treatment means according to analysis of variance.
[e] Means followed by the same letter are not significantly different according to Duncan's New Multiple Range procedure ($\alpha = 0.05$) (8).

reddish-brown slimes appeared in the growth chamber experiment in which autoclaved versus unsterilized seeds were compared. The dense growths of algae, fungi, and reddish-brown slime observed in the two experiments with Agribrom did not appear on untreated trays or the potting medium when the trays were neither seeded nor dibbled, even though conditions were similar to those in the Agribrom tests. Limited growth of *Chalamydomonas* sp. did occur on the surface of the nutrient solution.

These results indicate that weekly spray applications of Agribrom to the surface of the growth medium, even at high rates, may be of little value for controlling algal or fungal growths in float systems used for production of tobacco transplants. Conditions in these experiments were quite different from those occurring in plug production for ornamental plants, however. In the float-production system, watering is provided via subirrigation rather than by regular misting cycles throughout the day. The substrate in float systems is constantly saturated, in contrast to a mist system, which maintains moist but not saturated conditions in the growth medium. Surface evaporation from the float system also tends to concentrate nutrients at the surface of the growth medium. The saturated conditions and surficial accumulation of nutrients enhance algal growth. The failure of Agribrom to control algae and fungi in the float system appears to reflect both the infrequency of treatment (weekly vs. throughout the day with each misting cycle) and more suitable growing conditions for the algae and fungi. Bromine, the active component of Agribrom, is inactivated quickly by organic matter and other readily oxidizable materials, such as ferrous iron. Even at the highest treatment rate (800 mg L^{-1}), the activity of Agribrom probably dissipated rapidly. Also, the infrequency of treatments in this study resulted in application of substantially less Agribrom than would have occurred in a mist system. Only 1,100 mg M^{-2} was applied over the five-week treatment period using weekly applications at 800 mg L^{-1} compared to 5,425 mg M^{-2} using 20 mg Agribrom L^{-1} in the misting system described previously.

The results of these experiments indicate that the primary reasons for the ineffectiveness of Agribrom in commercial tobacco transplant float-production systems may be the combination of conditions highly conducive to algal and fungal growth and the infrequency of Agribrom applications. Further research is needed to determine if more frequent applications of Agribrom, possibly through a mist system, would give improved control of algae and fungi in float-production systems. Examination of other algicides applied to the nutrient solution or as a surface spray also is warranted.

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CURED LEAF SAMPLING OF SMALL TOBACCO RESEARCH PLOTS FOR CHEMICAL ANALYSES

TOBACCO SCIENCE

By D. T. Bowman[1], W. W. Weeks[1], G. S. Miner[2], A. G. Tart[1]

The accuracy of chemical plant analyses from small tobacco research plots is dependent on the sampling method and technique. Typically, a 50-g, half-lamina random leaf sampling method is used to determine reducing sugars and total alkaloids in burley and flue-cured tobaccos. For whole plant samples composited across primings, the fixed 50-g weight causes confusion because contributions from each priming must be calculated based on the contribution of that priming to the total yield and because small errors in weighing can markedly affect the composited mean value. The objective of this study was to examine alternative sampling methods that might simplify this procedure. Sample size, random versus nonrandom leaf selection, whole versus half-lamina leaf selection, a core sampling method, two saw-cut sampling methods, and a percentage sampling method were evaluated. Nonuniform distribution of alkaloids within leaves resulted in an underestimation of alkaloids by the core and saw-cut methods, and random leaf selection improved repeatability over nonrandom leaf selection. The percentage method, whereby a predetermined percentage of each priming was sampled and combined for a whole-plant sample, overcame difficulties encountered in compositing samples by weight. However, this resulted in larger samples for handling, mixing, and grinding. These studies confirmed the validity of the 50-g, half-lamina random leaf method for estimating total alkaloid concentration in flue-cured tobacco. However, there were variety by method interaction effects for reducing sugars when the half-lamina method was compared to whole plot values. The center saw-cut method could be used to sample burley tobacco for nicotine, but one would need to add 1.0% to the values obtained to approximate true means.

Additional key words: *Nicotiana tabacum*, alkaloids, reducing sugars.

INTRODUCTION

Field research with burley and flue-cured tobacco (*Nicotiana tabacum* L.) is usually carried out with replicated one-row plots. Optimum plot size and replicate numbers have been examined for both burley and flue-cured tobacco (1,4,6). Because tobacco is marketed for its flavor and aroma, an accurate description of the chemical composition of the cured leaves is important. Therefore, chemical constituents of cured leaves, usually reducing sugars and/or total alkaloids, are determined as part of routine data collection in field research. Because chemical composition of cured leaves varies by stalk position and within individual leaves, the accuracy of sampling is critical. Thus, reliability of chemical data depends on obtaining representative whole-plant samples for analyses.

Typically, each grade or priming (stalk position) of cured leaves from a given plot is weighed. The weights are summed over all primings and grades. The percentage of each grade or priming for a plot is used to determine the weight of cured leaves to be sampled from each grade or priming to comprise the composited leaf sample. Composited samples typically vary from 50-100 g per plot, with all plots within a particular experiment sampled identically. Because samples are small, slight deviations from calculated weights may cause significant changes in analyses of the chemical constituents. The purpose of this study was to examine alternative sampling methods and consequences of improper sampling.

MATERIALS AND METHODS

Study 1

An N rate-time of harvest study with flue-cured tobacco was used to obtain tobaccos with widely different levels of alkaloids and reducing sugars. These tobaccos were then used to compare various leaf sampling techniques. The specific objectives of this study were (1) to determine the effects of sample size and random versus nonrandom leaf selection within stalk positions on variability of reducing sugars and total alkaloids; (2) to compare whole versus half-leaf lamina selection (minus midrib) on variability of reducing sugar and alkaloid analyses; and (3) to compare a core sampling method (currently used in regional farm tests [2]) to the traditional leaf sampling method, which consists of collecting half-lamina (minus midrib) from randomly selected leaves within a stalk position (priming). These can be composited by weight for a whole-plant sample.

Sampling methods are listed in **Table 1**. Leaf samples were collected from four treatments of the experiment located at the Upper Piedmont Research Station near Reidsville, N. C. Treatments were: low N (43 kg/ha)-early harvest (one week earlier than normal), low N-late harvest (one week later than normal), high N (72 kg/ha)-early harvest, and high N-late harvest. Four replicates of each priming in each treatment were sampled. The intent was to obtain a range in total alkaloid and reducing sugar concentrations among treatments.

All methods were sampled in duplicate to obtain a measure of sampling variability (repeatability). The first sample of nonrandom whole-leaf lamina was taken from sequential leaves on the top of the priming bundles. The bundles were then turned over and the duplicate sample was taken in the same manner. For the random half-lamina samples, leaves were pulled for both duplicate samples from throughout the priming bundle for both the composite and priming samples. A two-inch diameter core sample was taken through each priming bundle near the center, and a duplicate sample was taken about one inch from the first core sample. The core samples included mid-rib as well as lamina tissue. Half-lamina indicates that the lamina from one side of the midrib was removed for inclusion in the sample.

For methods listed under priming, sample weights approximated 30 g (**Table 1**), but because each priming was analyzed separately and composite mean values were calculated based on priming weights, the actual weight had no bearing on the chemical analytical results. Samples composited by weight contained proportional weights of all primings and the analysis represented whole plant alkaloid and sugar concentrations.

Samples were dried at 70°C, ground, thoroughly mixed, and analyzed for total alkaloids and reducing sugars (5). Analytical results for core and random half-lamina samples, analyzed by priming, were combined on a weighted basis to provide whole-plant data that could be compared to composite-sample data. Data were evaluated by an analysis

[1]Crop Science Department, Box 8604, North Carolina State University, Raleigh, N.C. 27695.
[2]Soil Science Department, Box 7619, North Carolina State University, Raleigh, N.C. 27695.

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Table 1. Sampling methods used in Study 1.

Sample weight	Composite sample[a]		Priming[b]	
	Nonrandom whole-lamina	Random half-lamina	Core[c]	Random half-lamina
--- g ---				
30	x	x	x	x
50	x	x	-	-
100	x	-	-	-

[a]Composite samples represent whole-plant samples composited by weight from four primings, with leaves within a priming selected randomly or nonrandomly, and made up of whole or half lamina.
[b]Samples analyzed by priming, and composite mean alkaloid and reducing sugar concentrations were calculated based on priming weights.
[c]Samples included midveins; other samples did not.

of variance (ANOVA) with treatments (N rate-time of harvest) as whole plots and sampling methods as sub plots in a split plot arrangement. Coefficients of variance (C.V.) and sampling errors (between duplicates) were used to compare sampling methods.

Study 2

The specific objective of this study was to devise an efficient, bias-proof method for sampling small plots. A 14-inch table saw was fabricated with four blades attached side-by-side to cut an area approximately 1.9-cm wide. The blades cut sections across the width of each priming bundle and dropped the particles into a tray located directly beneath the blades.

The saw-cut method was compared to the standard half-lamina method by sampling five cultivars of burley tobacco at the Upper Mountain Research Station near Laurel Springs, N. C., and five cultivars of flue-cured tobacco at the Tobacco Research Station near Oxford, N. C., respectively in 1990 and 1991. The treatments were: standard 50-g half-lamina composite samples, composite tip-cut (approximately 1/3 from tip of bundle), composite center-cut, and whole-plot (stemmed).

The whole-plot treatment was used as the control. It consisted of all remaining tobacco from the plot combined across primings, dried at 70°C, ground, mixed, and analyzed for total alkaloids (burley and flue-cured) and reducing sugars (flue-cured only). The whole-plot treatment was subsampled 10 times to provide an accurate estimate of the true mean. Treatments were replicated three times. Data were analyzed by ANOVA with cultivar as the main plot and treatments as subplots. Orthogonal comparisons were made between the check and all other treatments.

Study 3

Research stations have had problems obtaining 50-g half-lamina samples that were composited by weight across stalk positions because the required calculations were confusing. To simplify this sampling procedure, a percentage method was proposed, whereby a certain percentage (suggested 4%) of each priming was weighed and these were combined across primings for a composite plot sample. The priming sample weights varied with yield among plots, but they were proportional to priming weights within plots, so the combined sample was a composite across primings. This greatly simplified mathematical calculations at the research stations. To validate this approach, 10 flue-cured varieties at the Upper Coastal Plain Research Station near Rocky Mount, N. C., were sampled in three ways. For the first method, the standard 50-g randomly selected half-lamina sample was taken with proportionate contributions from each stalk position. For the second method, tobacco was sampled by stemming and combining 4% of each priming by weight, again using randomly selected leaves. The third method consisted of stemming, grinding, and subsampling (10 times) the whole plot to provide a check by which standard and percentage methods were compared. All samples were dried at 70°C, ground, mixed, and analyzed for reducing sugars and total alkaloids. Data were analyzed by ANOVA with cultivar as the main plot and sampling treatment as the subplot.

RESULTS AND DISCUSSION

Study 1

The overall means for total alkaloids and reducing sugars for all methods are shown in **Table 2**. The core method resulted in lower total alkaloids while the random half-lamina method by primings gave significantly higher values. Sugar concentrations were similar among sampling methods, but random half-lamina sampling by priming was slightly higher. Alkaloids are more concentrated near leaf margins (3) so taking cores through the center of priming bundles, which included midrib in many of the leaf segments due to nonalignment of leaves, should give lower alkaloid estimates.

Differences between duplicate samples for the 16 plots (4 treatments X 4 replicates) were averaged to obtain a measure of repeatability (**Table 2**). The larger the mean difference, the greater the variation between duplicate samples. Thus, the core method was the least repeatable by duplicate sampling for total alkaloids but most repeatable for reducing sugars. Apparently, superiority of the core method for sugars was due to uniform distribution of sugars throughout leaf lamina and duplicate cores were taken adjacent to each other. Duplication differences decreased with increasing sample size for nonrandom whole-lamina composite with 50 and 100-g samples giving similar levels of repeatability. Except

Table 2. Statistical comparisons of seven sampling methods for total alkaloids and reducing sugars in flue-cured tobacco.

Statistic	Composite					Priming	
	Nonrandom whole-lamina			Random half-lamina			
	30g	50g	100g	30g	50g	Core	Random half-lamina
Total alkaloids (%)							
Mean concentration[a]	*2.64 bc*	*2.56 c*	*2.62 bc*	*2.70 b*	*2.70 b*	*1.95 d*	*2.80 a*
Avg. duplicate diff.	0.23	0.17	0.15	0.15	0.19	0.26	0.15
C.V. (%)	8.80	7.52	4.76	4.99	6.25	10.61	5.76
Sampling error	0.054	0.037	0.016	0.018	0.028	0.043	0.026
Reducing Sugars (%)							
Mean concentration[a]	16.5 b	16.7 b	15.9 c	16.3 bc	16.5 b	16.2 bc	17.3 a
Avg. duplicate diff.	1.9	1.5	1.4	1.0	1.0	0.4	0.8
C.V. (%)	9.3	7.4	7.4	6.3	5.5	2.7	3.5
Sampling error	2.371	1.533	1.407	1.055	0.812	0.191	0.371

[a]Values within rows not followed by the same letter are significantly different at the 5% level of probability by Duncan's Multiple Range Test.

Table 3. Analyses of certain chemical constituents for flue-cured and burley tobacco using four different sampling methods.

Chemical constituent	Sampling method				Orthogonal comparisons		
	Center-cut	Tip-cut	Whole-plot	Half-lamina	Center vs whole	Tip vs whole	Half-lamina vs whole
	%				P > F		
Flue-Cured							
Reducing sugars	12.3	13.8	12.5	13.5	0.70	0.08	0.15
Variety X sampling interaction					0.66	0.58	0.04
Total alkaloids	3.30	3.67	3.86	3.66	0.0001	0.0023	0.064
Variety X sampling interaction					0.06	0.07	0.28
Burley							
Total alkaloids	3.02	3.50	4.02	3.79	0.001	0.0001	0.0001
Variety X sampling interaction					0.18	0.01	0.02

for the 30-g sample of nonrandom whole-lamina, both nonrandom whole-lamina composites and random half-lamina by priming gave similar levels of repeatability for alkaloids.

Although mean differences between duplicate samplings are indicative of sample repeatability, statistics associated with the analysis of variance are better indicators of sampling performance. For total alkaloids, the magnitude of the C.V.'s generally followed the magnitude of duplication differences among sampling methods (**Table 2**), and those associated with the core method and with the 30 and 50-g samples from the nonrandom whole-lamina method further substantiate that these sampling methods are less repeatable than other methods, particularly for total alkaloid analyses. The duplicate differences, C.V.'s, and sampling errors indicate the core method was best for reducing sugars followed by the random half-lamina by priming. The nonrandom whole-lamina composite methods were least suitable for determining reducing sugar concentrations.

There were significant differences in total alkaloids (P = 0.0001) and reducing sugars (P = 0.048) among methods of sampling. Sugar and alkaloid levels were both significantly higher for the random half-lamina by priming method compared to the other methods (**Table 2**). In general, there were few meaningful differences in sugars and alkaloids between nonrandom whole-lamina and random half-lamina methods, but the core method was inferior to all methods for total alkaloids. Linear regression was used to establish a relationship between the core and random half-lamina by priming methods to determine if total alkaloids could be predicted from core samples. However, the R^2 was only 0.31, and using this regression method to compensate for low alkaloid levels obtained by the core method was unacceptable.

Study 2

The saw-cut method samples every leaf in the bundle and not just the few leaves that are sampled using the current half-lamina method. Particles from the saw were small enough to allow quick drying before grinding. However, the sample from the saw-cut method included midrib as well as lamina. For flue-cured tobacco, there were no significant differences among sampling methods for reducing sugars as revealed in the orthogonal comparisons (**Table 3**). The saw-cut methods did not result in a variety X sampling method interaction for reducing sugars; however, the standard half-lamina method did result in a significant interaction (P = 0.038).

The saw-cut methods significantly underestimated the true level of total alkaloid concentration in flue-cured tobacco, and they nearly created significant variety X sampling method interactions when compared to the whole-plot check (**Table 3**). This was partially due to an unequal distribution of total alkaloids in the cured leaves (3). Also, lamina tissue contains higher concentrations of nicotine than stem and midrib tissue (7).

In burley tobacco, all methods underestimated true means for total alkaloids compared to the whole-plot check (**Table 3**). The half-lamina method gave the closest estimate; however, it also resulted in a significant variety X sampling interaction. The tip cut also created a significant variety X sampling interaction. Even though the center cut did not result in a variety X sampling interaction, it greatly underestimated the true mean for total alkaloids. Regressing center-cut values on whole-plot values resulted in a regression coefficient of 0.98 with an intercept of 1.02 (R^2 = 0.53), which means that by adding 1.0% to the values obtained with the center-cut sampling method, one could avoid any treatment interaction and closely approximate true means.

Table 4. Statistical measurements of reducing sugar and total alkaloid concentrations in flue-cured tobacco using three sampling methods.

Chemical constituent	Sample method	Mean ± SD
Reducing sugar	Standard half-lamina	20.4 ± 2.46
	Percentage	21.0 ± 2.98
	Whole-plot (check)	21.7 ± 3.06
Total alkaloids	Standard half-lamina	2.60 ± 0.53
	Percentage	2.71 ± 0.63
	Whole-plot (check)	2.67 ± 0.60

Study 3

Results from the third study are shown in **Table 4**. It was assumed that whole plots gave the best estimate of the true mean. With this in mind, there were no differences among sampling methods for estimating reducing sugars and total alkaloids. Also, there was no sampling method X cultivar interaction for either chemical constituent. The results suggest that the percentage method is valid; however, the standard half-lamina method is also valid. The percentage method, nevertheless, resulted in sample sizes ranging from 54 g to 150 g based on plot weights compared to only 50-g samples for every plot in the half-lamina method. The smaller sample sizes would require less drying time and less time to grind.

In addition to sampling technique and sample size per priming, accurate weighing also could significantly impact values obtained in chemical analyses. To illustrate the relationship between mean and composite-mean values for reducing sugar concentrations and the effect weighing errors have on the composite-mean, a hypothetical data set was

constructed: priming A - 300 lb/a, 8% sugars; priming B - 600 lb/a, 13% sugars; priming C - 900 lb/a, 18% sugars; and priming D - 1200 lb/a, 16% sugars. Assuming analyses were based on 40-g samples, a mean reducing sugar concentration would require 10 g of sample from each of four stalk positions and mean sugar concentration would be equivalent to the mean across stalk positions (Σ%sugar in all stalk positions/4 = 13.8%). Intuitively, this value is not representative of whole-plant sugar concentration because upper stalk positions contribute much more to total harvest weight that lower stalk positions.

A 40-g sample composited by weight would require 4 g of leaf from priming A (300/3000 X 40 g = 4 g) and 8, 12, and 16 g from primings B, C, and D, respectively. The sugar concentration obtained with this sample would be equivalent to that obtained by taking into account the proportionate contributions from each priming (Σweight X sugar concentrations across 4 primings/40g = 15.2%). This is the correct value on a whole-plant basis. If unknowingly, however only 14 g of priming D is weighed instead of 16 g, the result would be 14.4%. Likewise, weighing 18 g of priming D gives a value of 16.0%. Thus, small weighing errors can greatly affect whole-plot concentrations of reducing sugars and total alkaloids, particularly if weighing errors are made for primings contributing the most weight.

In conclusion, these studies indicate that: (1) No particular method of sampling was superior for both burley and flue-cured tobacco. The standard 50-g, half-lamina method resulted in some significant treatment x sampling method interactions for total alkaloids in burley and reducing sugars in some instances in flue-cured tobacco. (2) The core method and saw-cut method underestimated total alkaloids due to differential alkaloid concentrations within leaves. (3) Random leaf selection gave better repeatability of analyses than did nonrandom leaf selection. (4) Sample sizes of 30 g and 50 g composited by weight across primings with random leaf selection gave concentrations of alkaloids and sugars similar to those obtained by calculation from individual priming analyses and weights. (5) The difficulty of compositing samples by weight (with attendant calculations) was overcome by simply combining a fixed proportion of each priming in a treatment. (6) The 50-g, half-lamina method appeared to be the most appropriate, although not perfect method, for flue-cured tobacco, given that other methods underestimate total alkaloids and some require longer drying times, longer mixing times, and longer grinding times. (7) The center-cut sampling method may be most appropriate for burley tobacco. One would simply need to add 1.0% to the values obtained to approximate true means for total alkaloids.

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EFFECTS OF PLANTING DATES, METALAXYL, AND FENAMIPHOS ON THE TOBACCO BLACK SHANK-ROOT KNOT COMPLEX, TOBACCO QUALITY, AND YIELD

TOBACCO SCIENCE

By A. S. Csinos[1], N. A. Minton[2], M. G. Stephenson[2], and T. P. Gaines[3]

Tobacco transplanted 5 April tended to have a higher incidence of black shank disease than tobacco transplanted 5 May in 1983. The combination of metalaxyl and fenamiphos reduced black shank disease in both plantings over the untreated control. Yields were higher in the 5 May planting than in the 5 April planting, except for one treatment in which there were no differences. In 1984, black shank was reduced by all treatments for the 6 April and 15 May plantings, and by all treatments containing metalaxyl for the 26 April planting. Generally, black shank was reduced for a particular treatment as the transplanting date was delayed. For all three transplanting dates in 1984, root-knot indices were reduced by treatments having fenamiphos. There was a general trend for a reduction in root-knot damage as the transplanting date was delayed. Generally, yields tended to decline as the transplanting date was delayed. Value/ha correlated with yields. The grade index tended to be higher in the 26 April planting compared to the 6 April and 15 May plantings. Nitrogen content of leaves was not different for any treatment. However, total alkaloids and percent reducing sugars differed in some of the chemical treatments.

Additional key words: ***Nicotiana tabacum, Phytophthora parasitica* var. *nicotiana*, *Meloidogyne incognita*, *Meloidogyne javanica*.**

INTRODUCTION

Black shank, which is incited by *Phytophthora parasitica* Dastur var. *nicotianae* (Breda de Haan) Tucker, is one of the most serious fungal diseases of tobacco (*Nicotiana tabacum* L.) in the United States (7). The *Meloidogyne* nematode species *M. incognita* (Kofoid & White) Chitwood, *M. javanica* (Treub.) Chitwood, and *M. arenaria* (Neal) Chitwood also are destructive pathogens of tobacco in the Georgia Coastal Plain (1,3,13). Alone, both pathogens may cause high losses to tobacco, but nematodes also increase losses due to black shank when these pathogens occur together (11). Life cycles of *Meloidogyne* spp. and *P. parasitica* var. *nicotianae* are short in the warm, moist soils of the southeastern USA, and they may go through several cycles in a single season. Damage by one or both pathogens may be dependent on environmental conditions during the season (1,3,8,11). Management practices for black shank and root-knot nematodes include the use of resistant cultivars (9), rotations, and applications of metalaxyl and nematicides (1,2,12,13,15).

Variations in the amount of black shank from year to year have been noted in fields that are infested with *P. parasitica* var. *nicotianae*. This study was initiated to determine the effects of transplanting dates on the severity of tobacco black shank and root-knot nematodes and on the subsequent quality, value, and yield of tobacco treated with metalaxyl and fenamiphos.

MATERIALS AND METHODS

Experiments were established in 1983 and 1984 at the Coastal Plain Experiment Station near Tifton, Georgia, on fuquay, loamy sand (loamy siliceous, thermic Arenic Plinthic Kandiudults; 88% sand, 8% silt, and 4% clay; pH 5.5-6.0; <2% organic matter) in an area infested with *P. parasitica* var. *nicotianae*, *M. incognita*, and *M. javanica*. The tobacco cultivar NC 2326, which has no resistance to nematodes and low resistance to black shank, was used both years. Each plot consisted of two rows that were 10-m long. Plants were spaced 46-cm apart within rows, and rows were 1.2-m apart.

In 1983, tobacco was transplanted on two different dates (5 April = Planting I, and 5 May = Planting II) into adjacent plots within the same field. Each planting date had four chemical treatments and an untreated control arranged in a randomized complete block design with four replications.

In 1984, three transplanting dates and four chemical treatments were included in a 3 x 4 factorial experiment with each treatment combination replicated four times and arranged in a randomized complete block design. The three transplanting dates in 1984 were 6 April (Planting I), 26 April (Planting II), and 15 May (Planting III).

Plot land was prepared for planting by deep turning the soil with a moldboard plow and disc harrowing. In 1983, treatments consisted of (a) metalaxyl (Ridomil®, Ciba Inc.) applied at 2.24 kg (ai)/ha, (b) fenamiphos (Nemacur®, Miles Inc.) at 6.7 kg (ai)/ha, (c) metalaxyl at 2.24 kg (ai)/ha plus fenamiphos at 6.7 kg (ai)/ha preplant incorporated (PPI), and (d) metalaxyl at 1.12 kg (ai)/ha plus fenamiphos at 6.7 kg (ai)/ha PPI and metalaxyl at 0.56 kg (ai)/ha at layby (L). The layby treatment was applied six weeks post-transplanting as a directed spray, and it was incorporated by cultivation. In 1984, chemical treatments consisted of (a) metalaxyl applied at 2.24 kg (ai)/ha PPI, (b) fenamiphos applied at 6.7 kg (ai)/ha PPI, and (c) metalaxyl at 2.24 kg (ai)/ha plus fenamiphos at 6.7 kg (ai)/ha tank mixed and applied PPI. In both years, PPI treatments were applied at 187 L/ha with a boom sprayer mounted in front of a tractor-powered rototiller, which was used to incorporate the chemicals to a depth of 15 cm.

Fertilizer was applied according to the University of Georgia Cooperative Extension Service recommendations based on soil tests. Insecticides (methomyl, chlorpyrifos, and acephate), herbicides (pendemethalin, pebulate, and isoproplin), and growth regulators for sucker control (maleic hydrazide and fatty alcohols) also were used according to Cooperative Extension Service recommendations (9). Rainfall was recorded and supplemented with overhead irrigation as required. Total rainfall in 1983 for Planting I and Planting II was 38 cm and 31 cm, respectively, and each planting received 14 cm of supplemental irrigation. Total rainfall in 1984 for Plantings I, II, and III was 53, 41, and 39 cm, respectively. All plots were irrigated with a total of 5 cm of water in 1984.

The number of living plants in each plot was counted and recorded every two weeks starting four weeks after transplanting, and the percentage of black shank-infected plants (percent disease) and the disease index were calculated. The disease index was calculated using the formula:

$$DI = \frac{\sum_{i=1}^{n} X_i(100-(i-1)\frac{100}{n})}{I}$$

[1] Department of Plant Pathology, Coastal Plain Experiment Station, University of Georgia, Tifton, Georgia, 31793.
[2] USDA-ARS, Coastal Plain Experiment Station, Tifton, Georgia, 31793.
[3] Department of Agronomy, Coastal Plain Experiment Station, University of Georgia, Tifton, Georgia, 31793.

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Table 1. Effects of chemical treatments and planting dates on the incidence of tobacco black shank, disease index, and yield, 1983.

	Chemical treatments						Planting dates[a]		
Variables	C[b]	M[c]	F[c]	M+F[c]	M+F[d]	SE[b]	I	II	SE[b]
Disease (%)	89 a[e]	50 b	85 a	19 c	18 c	5.2	65 a	39 b	2.6
Disease index	45 a	19 b	40 a	5 c	5 c	2.5	27 a	19 b	1.2
Yield (kg/ha)	665 c	2532 b	1052 c	4023 a	4014 a	221.1	1841 b	3073 a	93.7

[a] Planting dates were 5 April (I) and 5 May (II).
[b] C = Untreated control, and SE = Standard error of least-squares means.
[c] Metalaxyl (M) was applied at 2.24 kg ai/ha preplant incorporated (PPI), and fenamiphos (F) was applied at 6.7 kg ai/ha PPI.
[d] Metalaxyl (M) was applied at 1.12 kg ai/ha PPI and 0.56 kg ai/ha at layby, and fenamiphos (F) was applied at 6.7 kg ai/ha PPI. PPI treatments were applied before transplanting and incorporated to a depth of 15 cm. The layby treatment was applied at six weeks post-transplanting as a directed spray and incorporated by cultivation.
[e] Treatments or planting date means within a row followed by the same letter are not significantly different ($P < 0.01$).

where i = ordinal evaluation number, n = number of evaluations (excluding initial stand count), X = number of dead plants since last count, and I = initial number of plants (1). A plant was considered dead when it was permanently wilted and a black lesion was observed on its stem extending from ground level. Nematode determinations were made in 1984 by bulking 20 random soil cores (2.5-cm diam x 25-cm deep) in each plot, mixing the soil, and removing 150 cm^3 for soil assay on three different dates (3 April, 16 July, and 4 September). The centrifugal flotation technique (6) was used to recover nematodes from the soil. In 1984, a final root-knot index was determined by uprooting all living plants after final harvest and rating them for percent of roots galled (1).

In 1983, leaves were hand-harvested beginning on 19 July and 22 July for Plantings I and II, respectively. Remaining harvests were done on 4 August, 17 August, and 22 August for both planting dates, as leaves ripened in 1983. In 1984, leaves were hand-harvested as they ripened on 23 July, 15 August, and 30 August for Planting I; on 1 August, 23 August, and 30 August for Planting II; and on 8 August, 30 August, and 17 September for Planting III. The weight of leaves from each plot was recorded at each harvest. Total fresh weight for each plot was converted to dry weight using a 0.2 conversion factor (1), and yield per hectare was calculated for each plot.

Percent total nitrogen, total alkaloids, and reducing sugars were determined on tobacco leaves of all plantings in 1984. Sample preparation and chemical analyses of tissue were performed as outlined by Gaines (4).

Tobacco was graded after curing by U. S. Department of Agriculture graders. Grade index was a numerical value that ranged from 1 to 99 based on equivalent government grades (16); the higher the number, the higher the grade. Values were calculated by multiplying the average price per kilogram of weight for the grade times the weight of tobacco per hectare.

Data were analyzed using PROC GLM of SAS (14). The statistical model included effects due to chemical treatment, replicate within chemical treatment (main plot error), planting date, and treatment X planting date interaction. Treatment effect was tested using the replicate within treatment mean square. Correlation coefficients (r) between yield and disease traits were computed and presented if significant.

RESULTS

1983

Least-squares means (14) for the 1983 experiments are presented in **Table 1**. The percentage of plants infected with black shank and the disease index were significantly lower and yields were significantly greater in plots treated with both metalaxyl and fenamiphos than they were in plots where these materials were used alone. Metalaxyl alone controlled black shank better and increased yields more than fenamiphos alone.

There were significant differences between the two planting dates for all three variables (**Table 1**). The percentage of diseased plants and the disease index were lower and yields were greater in Planting II than in Planting I.

Yields for Planting I and Planting II were negatively and significantly correlated with the percentage of diseased plants (r = -0.96, P = 0.001; and -0.97, P = 0.001, respectively) and with the disease index (r = -0.98, P = 0.001; and -0.97, P = 0.001, respectively).

1984

Least-squares means combined across planting dates for the variables in the 1984 experiment are presented in **Table 2**. Plots treated with a combination of metalaxyl and fenamiphos had a lower percentage of plants infected with tobacco black shank, a lower disease index, a higher value per hectare, and

Table 2. Effects of chemical treatments and planting date on black shank, root-knot nematode damage, yield, and quality characteristics on tobacco, 1984.

	Chemical treatments (T)					Planting dates[a] (PD)				T X PD
Variables	M[b]+F[b]	M[b]	F[b]	C[c]	SE[c]	I	II	III	SE[c]	Interaction[d]
Disease (%)[e]	3 c[f]	31 b	26 b	57 a	4.0	41 a	20 b	25 b	3.3	NS
Disease index[e]	1 c	8 b	8 b	19 a	1.3	13 a	6 b	8 b	1.0	*
Root-knot index	40 b	85 a	23 b	71 a	7.1	61 a	61 a	42 b	2.6	*
Grade index	34 a	39 a	34 a	35 a	3.1	35 b	48 a	35 b	1.6	NS
Value ($/ha)	18058 a	10806 b	13731 b	6310 c	937.2	12226 a	13052 a	9129 b	468.6	NS
Yield (kg/ha)	4205 a	2751 b	3305 b	1793 c	265.1	3159 a	3300 a	2581 a	121.2	NS

[a] Planting dates I, II, III were on 6 April, 26 April, and 15 May 1984, respectively.
[b] Metalaxyl (M) was applied at the rate of 2.24 kg ai/ha preplant incorporated (PPI), and fenamiphos (F) was applied at the rate of 6.7 kg ai/ha PPI.
[c] C = Untreated control, and SE = Standard error of least-squares means.
[d] * indicates planting date x treatment interaction was significant ($P < 0.05$); NS indicates the interaction was not significant at the 0.05 probability level.
[e] For tobacco black shank.
[f] Treatment or planting date means within a row followed by the same letter are not significantly different ($P < 0.05$).

Table 3. Effects of chemical treatments and planting dates on the numbers of root-knot larvae/150 cm³ soil.

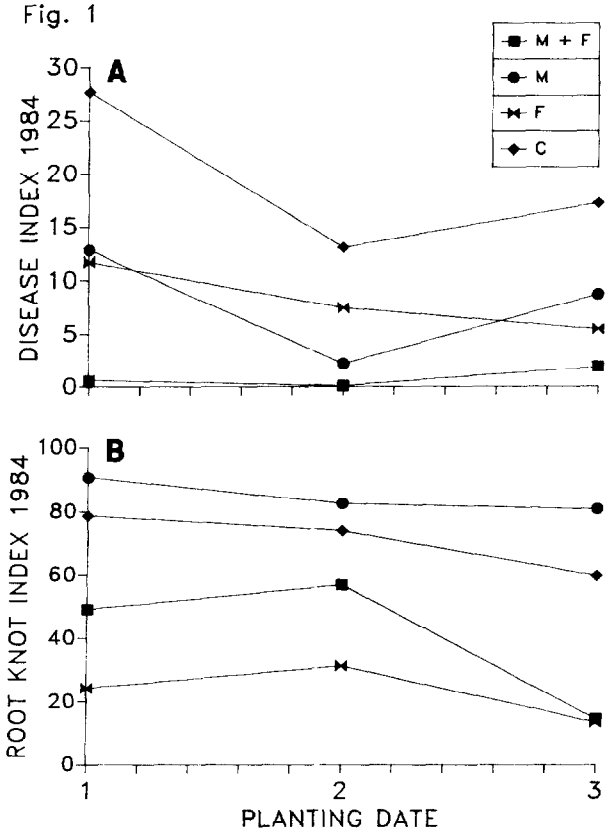
Chemical treatment & Rate (kg [ai]/ha)	3 April Planting I	3 April Planting II	3 April Planting III	16 July Planting I	16 July Planting II	16 July Planting III	4 September Planting I	4 September Planting II	4 September Planting III
Metalaxyl (2.24) + Fenamiphos (6.7)	96 a[a]	94 a	100 a	10 c	5 a	3 a	470 b	2058 ab	785 ab
Metalaxyl (2.24)	40 a	108 a	104 a	113 ab	78 a	8 a	1000 ab	903 b	2755 a
Fenamiphos (6.7)	54 a	70 a	76 a	18 bc	3 a	0 a	1000 ab	3740 a	83 b
Untreated Control	108 a	30 a	148 a	148 a	123 a	3 a	2563 a	268 b	2605 a

[a] Means within a column followed by the same letter are not significantly different ($P < 0.05$). Root-knot juvenile numbers were not significantly different across planting dates.

a higher yield than plots with any other chemical treatment. Treatments with fenamiphos alone or with metalaxyl alone reduced the percentage of diseased plants, reduced the disease index, increased yields, and increased value per hectare over the control plots. Only treatments containing fenamiphos reduced the root-knot index.

The percentage of plants infected with tobacco black shank and the disease index were greater for plots in Planting I than in plots planted later (**Table 2**). Root-knot damage was less for plots in Planting III than for plots planted earlier, and grade index was greatest for plots in Planting II than for the other two planting dates. Although no difference occurred in yields across planting dates, value per hectare was greater for plots in Planting I and Planting II than those in Planting III. Significant interactions occurred between treatment and planting date for disease index and root-knot index (**Figure 1**). For these parameters, the effects of chemical treatments were not similar across planting dates. The efficacies of the chemical treatments were dependent on the planting date. The severity of black shank and root-knot nematode damage in the absence of a chemical treatment tended to be less as planting date was delayed. This trend also was seen for most chemical treatments, except for metalaxyl used alone.

Figure 1. (A) Effect of planting date on black shank (disease index) in tobacco and response to chemical treatments. C = untreated control, F = fenamiphos at 6.7 kg ai/ha, M = metalaxyl at 2.24 kg ai/ha, and M + F = metalaxyl at 2.24 kg ai/ha plus fenamiphos at 6.7 kg ai/ha. (B) Effect of planting date on root-knot nematode damage and response to chemical treatments.



For all three planting dates, yields were negatively and significantly correlated with the percentage of black shank infected plants ($r = -0.89$, $P = 0.0001$; $r = -0.74$, $P = 0.001$; and $r = -0.85$, $P = 0.001$, respectively) and with disease index ($r = -0.86$, $P = 0.0001$; $r = -0.77$, $P = 0.0005$; and $r = -0.78$, $P = 0.0003$, respectively). Yield also was significantly and negatively correlated with root-knot index for Planting III ($r = -0.60$, $P = 0.01$).

Before the 1984 experiment, soil populations of root-knot nematode juveniles were relatively high (30-148/150 cm³ soil) (**Table 3**). Generally, nematodes declined early in the season, but population densities increased to a relatively high level by final harvest. In Planting I, the metalaxyl plus fenamiphos treatment had significantly fewer juveniles than the control plots (2605/150 cm³ soil) by the end of the season. Numbers of nematodes in Planting II did not follow the same trend as in Plantings I and III, and we have no explanation for this variation. Significant differences were not detected among planting dates for any treatment.

Significant differences occurred in total nitrogen, total alkaloids, and reducing sugars among chemical treatments and among planting dates (**Table 4**). Some trends were noted across harvest dates; notably, total nitrogen and total alkaloids tended to increase from first harvest to third harvest.

DISCUSSION

Our results corroborate those of Powell & Nusbaum (11), indicating that an interaction between root-knot nematodes and *P. parasitica* var. *nicotianae* in tobacco occurs. Control of both pests was necessary to obtain maximum tobacco yields. In 1983, the split application (PPI + L) at a total rate of 1.68 kg/ha of metalaxyl was as effective as the single application (PPI) of 2.24 kg/ha.

Under the conditions experienced in North Queensland, O'Brien et al. (10) were able to reduce losses from black shank by planting tobacco during the winter when soil temperatures were cooler. In our experiment during 1983, there was a trend toward a higher level of black shank disease and a lower yield in tobacco planted 5 April than in tobacco planted 5 May. Again, in 1984, disease ratings and nematode indices tended to be greater in the early planting than in the midseason and late plantings. However, yields did not necessarily correlate with these trends. Yields generally were higher the earlier the tobacco was planted in 1984. Variations from this general trend occurred in plots treated with chemicals that affect control of either the fungus or the nematode or both. The use of early planting dates in Australia was probably successful in reducing tobacco black

Table 4. Effects of chemical treatments and planting dates on total nitrogen, total alkaloids, and reducing sugars of cured tobacco for three harvests.

	Chemical treatments					Planting dates[a]			
Variables[b]	M+F[c]	M[c]	F[c]	C[d]	SE[d]	I	II	III	SE[d]
HARVEST 1									
TN	1.83 a[e]	1.80 a	1.90 a	2.05 a	0.12	1.99 a	1.90 ab	1.79 b	0.07
TALK	2.40 b	2.49 ab	2.67 ab	2.86 a	0.14	2.77 a	2.82 a	2.22 b	0.09
RSUG	15.52 a	15.46 a	14.03 a	15.26 a	1.33	15.67 a	16.06 a	13.47 b	0.64
HARVEST 2									
TN	1.86 a	1.86 a	1.96 a	2.01 a	0.08	1.91 a	2.02 a	1.85 a	0.06
TALK	3.24 a	3.30 a	3.36 a	3.35 a	0.19	3.23 b	3.79 a	2.92 b	0.13
RSUG	18.29 a	17.67 ab	17.32 ab	15.84 b	0.78	17.61 a	16.92 a	17.31 a	0.53
HARVEST 3									
TN	2.15 a	2.20 a	2.36 a	2.31 a	0.09	2.37 a	2.15 b	2.24 ab	0.07
TALK	3.58 b	4.02 a	3.56 b	4.25 a	0.13	3.77 b	4.14 a	3.65 b	0.11
RSUG	15.81 a	14.77 ab	15.57 a	13.75 b	0.54	15.39 a	16.58 a	12.95 b	0.43

[a] Planting dates I, II, III were on 6 April, 26 April, and 15 May 1984, respectively.
[b] TN=total nitrogen, TALK=total alkaloids, RSUG=reducing sugars.
[c] Metalaxyl (M) was applied at the rate of 2.24 kg ai/ha preplant incorporated (PPI), and fenamiphos (F) was applied at the rate of 6.7 kg ai/ha PPI.
[d] C = Untreated control, and SE = Standard error of the least squares means.
[e] Treatment or planting date means within a row followed by the same letter are not significantly different ($P < 0.05$).

shank because cooler soil temperatures delay the onset of this disease (8). The average frost-free period for the southern part of Georgia is from 15 March to 15 November. Apparently, soil temperatures and moisture in a given year may influence the infection rate of black shank more than strictly the planting date does. In the tobacco growing belt of Georgia, the acceptable planting window from late March through early May apparently does not provide sufficient climatic variation to prevent losses from either black shank or root-knot nematode by varying planting date alone. Consistent reduction of losses to these parasites was achieved only by the use of a fungicide and a nematicide. Variations in rainfall or soil temperature may be sufficient to increase or decrease losses from black shank from year to year (8), but the unpredictability of these variables in Georgia dictates the use of a fungicide to manage this pest problem. In addition, statistical analysis revealed that the efficacies of chemical treatments were dependent on planting dates.

Nitrogen levels in cured leaves were unaffected by chemical treatments for any of the three harvests, but they tended to be reduced at later planting dates. Alkaloid levels tended to decrease with improved disease control and with delayed planting dates. Reducing sugars tended to increase with improved disease control, and they tended to decrease as planting date was delayed. Under situations of high disease and nematode pressure, subtle reductions on the quality of tobacco may occur (5).

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A FEASIBILITY STUDY OF THE USE OF BAR-CODE TECHNOLOGY BY USDA GRADERS IN U.S. FLUE-CURED TOBACCO AUCTION WAREHOUSES

TOBACCO SCIENCE

By A. Blake Brown and Paul R. Johnson[1]

Each unit (pile) of flue-cured tobacco sold through auction warehouses receives a government grade from an inspector employed by the Agricultural Marketing Service (AMS) of the U.S. Department of Agriculture (USDA). This grade determines the minimum price for that tobacco under the price-support system. Two important uses of these grades are to provide information on grade distribution for calculating the next year's price support structure, and to provide information for producers and others about current market conditions for various grades of tobacco. The present grade-collection system has two potential flaws that affect the users of this information. The information collected by USDA-AMS gives less than 100% coverage of sales, and the data contain resale tobacco. The Agricultural Stabilization and Conservation Service (ASCS) of USDA, the price support agency, would like to have only producers' sale data for more accurate grade distribution calculations. Many segments of the industry would like to have a more complete sample of current price and grade conditions. A pilot program was tested in the 1992 marketing season to see whether a bar-code system of recording grades on tickets could improve the data-collection system. A programmable bar-code printer was used with software developed for the experiment to perform the test. Regular USDA graders were equipped with the devices and they graded some sales in Roxboro, N.C. The technique was feasible, but with the current equipment it was slower than hand grading. Preliminary estimates indicate that the cost of the bar-code system does not exceed the total, allocable costs of the current system.

Additional key words: Grades, price support, bar code, resale tobacco.

INTRODUCTION

The Agricultural Marketing Service (AMS) of the United States Department of Agriculture (USDA) employs inspectors who inspect each pile of flue-cured tobacco that is placed on the tobacco warehouse floor for sale. The inspectors assign a grade to each pile and write the grade on a multiple-copy ticket that accompanies the pile throughout the sale process. Buyers and sellers of flue-cured tobacco pay for the grading services provided by USDA-AMS via an assessment on each pound of tobacco sold. Loyd (2) described the sale process, the grading process, and the tickets used.

USDA-AMS also employs market recorders who, using hand-held computers, record the grades and prices. However, 100% of the grades and prices are not collected. A sample of grades and prices is released daily by USDA-AMS to provide market information to flue-cured producers and buyers. The warehouse operators also record the sale price and weight from each ticket (called "picking up the sale").

UCS, Inc. (3) conducted a feasibility study of various methods of collecting grade and price information. The present study focuses on the design, implementation, and evaluation of one particular method for collecting grade information. Electronic collection of grade and price data at the time of grading (inspection) was evaluated for flue-cured tobacco by using a bar-code system. The successful design and implementation of such a system could result in significant producer and industry benefits, particularly in regard to accuracy of price support level determinations and market reports.

In determining the support prices for different grades of tobacco, the Agricultural Stabilization and Conservation Service (ASCS) of the USDA uses data relating to the amount or percentage of the crop in each government grade. Currently this information is supplied via the USDA-AMS. Due to the current grade-collection process, as discussed above, the grade and price information furnished by USDA-AMS represents a 25-30% sample of each crop. In order to accurately determine the distribution of grade and price, the Commodity Credit Corporation (CCC) of USDA needs data from the first sale of tobacco by producers (producer-first sale data). Currently, USDA-AMS data include resale tobacco, which means that there is some double counting of grades. For example, in 1991, auction resales of burley and flue-cured tobacco amounted to 7.8% and 11.9% of gross sales, respectively.

A bar-code system for grade collection used at the time of inspection has the potential of permitting resale data to be separated from producer first sale data, allowing for more accurate determination of individual grade price support levels. This procedure would give USDA-ASCS a complete set of data on grades and prices for the entire crop, and these data would be for producer-first sales tobacco only. More accurate determinations of price support levels could benefit producers by ensuring that they receive the appropriate support levels on all grades. More accurate determinations of price support levels also could potentially reduce the amount of tobacco purchased by the Flue-Cured Tobacco Cooperative Stabilization Corporation (FCTCSC) (i.e., the amount of tobacco "going under loan"), which could result in savings to producers via decreased assessments.

Another benefit would be that the USDA-AMS (Market News Division) would receive a complete set of data for dissemination. Instead of data from 25-30% of the crop, the USDA-AMS would receive a complete set of producer-first sale data, eliminating bias introduced by inclusion of resale data. Many producers rely on the AMS market reports to compare how their tobacco sells relative to the market. A more accurate market report would enable the producer to make more informed decisions, especially with regard to accepting or rejecting particular sales.

The integrity of the sale process would be easier to monitor with the use of an electronic grade-collection system at the point of inspection. Such improprieties as ticket switching, tampering with tickets (changing of government grades or weights), and unofficial grading would be hampered. Human errors, such as transposition of numbers, poor handwriting, and incorrect reading of the tickets, could potentially be reduced.

Other sectors of the tobacco industry could benefit from research concerning a bar-code system for grade collection. Bar-code systems would allow for complete inventory tracking of each lot of tobacco. Bar codes could increase the efficiency of receiving, shipping, and processing loan tobacco, and they could decrease shipping costs. For the warehouse operator and purchaser, a bar-code system would help with tracking of individual piles of tobacco and their weights. If desired, warehouse operators could record the sale data (i.e., "pick up the sale") using similar scanners, thus decreasing time and reducing costs.

Considering the potential benefits, the Grade Collection

[1] Department of Agricultural and Resource Economics, Box 8109, North Carolina State University, Raleigh, North Carolina 27695-8109.

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Pilot Program Study Committee (GCPPSC) requested that the investigators evaluate the feasibility of inspectors for USDA-AMS using bar-code technology to collect grade data at the point of inspection. The objectives of this study were to develop, implement, and evaluate a bar-code system for collecting, storing, and transmitting the grade, price, and pile identification data. The investigators worked with the USDA-ASCS, the USDA-AMS, the GCPPSC, a computer consultant, tobacco warehousemen, and growers to develop, implement, and evaluate the system.

PROCEDURE

The services of a consulting firm (Taylor Data Systems, Inc. of Florence, S.C.) were subcontracted to help select the appropriate hardware and to help develop software for the project. The principal investigators, certain members of the GCPPSC, and Taylor Data Systems, Inc. developed the specifications for the hardware. Specifications were that the system be able: (1) to retain a minimum of 1,200 records, (2) to be programmable, (3) to allow input by keyboard, wand scanner and laser scanner, (4) to print in bar code and alpha numeric characters, and (5) to have communications capability. The Monarch Pathfinder Plus (a product of Monarch Marking Inc.) was selected because it met the above criteria and because of its flexibility of application.

The investigators, certain members of the GCPPSC, and Taylor Data Systems, Inc. also developed software specifications. The specifications for the software were that it should be capable of: (1) collection and storage of all possible grades, (2) storage and retrieval of support prices, (3) storage of an inspector security code and comparison of this code with one entered by the inspector, (4) collection of ticket information (i.e., the warehouse and ticket number), (5) retention of the current date, and (6) production of a printed label with the grade (in bar code and alpha numeric characters), the price support in alpha numeric characters, the warehouse and ticket numbers in bar code and alpha numeric characters, the inspector's name, the current date, and the number of tickets printed for a particular pile of tobacco. Taylor Data Systems, Inc. designed the software to meet the above criteria. Documentation for this software, as given by Taylor Data Systems, Inc., is available in Brown & Johnson (1). The system was designed to download from a database all grades and support prices, names and social security numbers of all inspectors involved in the study, and the warehouse numbers of the warehouses involved in the study. A USDA-AMS Tobacco Division Training Specialist worked with Taylor Data Systems, Inc. to develop the system. The system was pre-tested at the Bright Leaf Tobacco Warehouse in Darlington, S.C.

Bar-coded tickets were used in the auction warehouses that participated in the study. An example of the front copy of one of the tickets is shown in **Figure 1**. The 10-digit bar code (the ticket number) on the ticket identified the type of tobacco (e.g., flue-cured), the warehouse identification number, and the pile identification number. An inspector began recording the grade information by scanning the bar code on the ticket of a particular pile of tobacco.

A bar-code matrix was designed to allow inspectors to record any grade by using the appropriate combination of bar codes. The matrix was designed such that once an inspector had inspected a pile of tobacco, he or she could input the grade information by separately scanning the appropriate bar code for the group symbol, then for the quality of the tobacco, then for the color, and finally, if necessary, for a special factor. If the bar-code combination scanned was not a valid grade, then INVALID GRADE was displayed and the inspector had to re-enter the data. Once entered, the grade was displayed and the grader had to confirm that the information was correct by pressing ENTER.

The software also allowed the inspector to repeat the grade of the previous pile by scanning one bar code (rather than the combination of three or four bar codes). This feature was added to speed up the grading process where several piles of tobacco of the same grade were in a row on the warehouse floor. Initially, this feature was not included because of concern that it might decrease the incentive to closely inspect contiguous piles that appeared similar.

Once the grade information was entered and the ENTER key pressed, a label was printed with the grade and ticket number in alpha numeric characters and bar code, support price (optional), date, number of labels printed for the ticket number in question, and name of the inspector. Each label was attached via an adhesive backing to the upper left hand corner on the top copy of the ticket where the grade, date, and inspector name would otherwise have been written. Both synthetic and paper labels were tested.

Inspectors also were able to re-inspect (regrade) a pile of tobacco and apply a new label to the ticket. The label also had a number that indicated how many times a pile has been regraded.

The recorder was worn by the inspector in a holster. The inspector used a wand to scan the bar codes on a matrix of grades mounted on a plexiglass board. Then, a label containing the grade and other pertinent information was printed and placed on the ticket for each pile of tobacco.

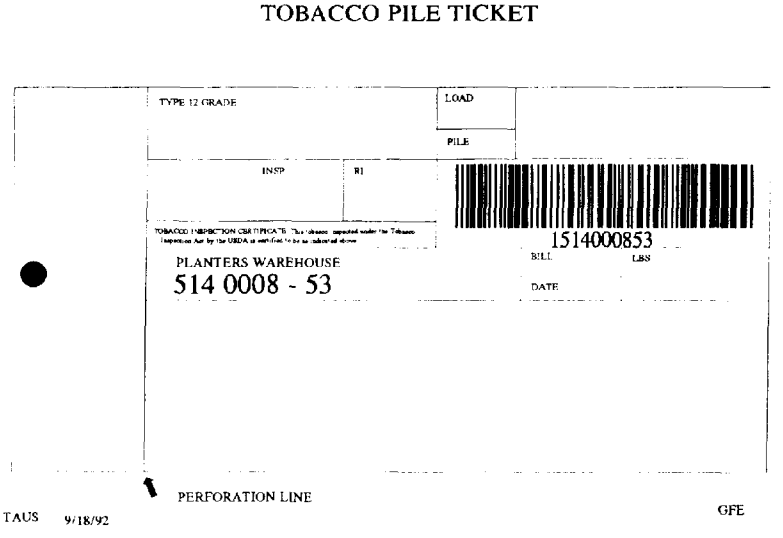
Two software packages were available for the Monarch Pathfinder Plus. Both packages allowed the data to be uploaded to a warehouse or other personal computer, or to a remote computer via a modem. Additional details of the operation of this system were given by Brown & Johnson (1).

The five auction warehouses in the Roxboro, N.C., market were selected for the pilot study. This market uses four USDA-AMS inspectors. A USDA training specialist, who had worked with Taylor Data Systems, trained the inspectors to use the system. The system was tried for five weeks in September and October of 1992.

RESULTS

The bar-code system was able to accurately collect and store, in a retrievable data base, the grade and ticket

Figure 1. Tobacco pile ticket with ticket number in bar code.



information. This data base was in a format that could be transmitted to local or remote computers via the above mentioned communications software furnished by Monarch Marking, Inc. The Monarch Pathfinder Plus had the capacity to retain more than enough records to inspect one day's sales. Both a laser and a wand were tested. The laser had the best first-time read rate but was considered too cumbersome to be practical. Thus, the wand was used throughout the test. Both synthetic and paper labels were tested. Fewer printer problems, particularly printer jams, occurred with the paper labels.

The major problem encountered in the study was that the bar-code collection system significantly slowed the grading process. All involved in the study, including growers, buyers, and warehouse owners, cited lack of speed as the major problem with the system. For example, on the second day of the test, four inspectors graded 502 piles of tobacco in 80 minutes (38 sec/pile) using the bar-code system, and they graded 461 piles of tobacco in 50 minutes (26 sec/pile) using the traditional grading system.

During the second week of the study, several modifications were made in the software in an attempt to speed up the process. Added was the repeat function that enabled a grader to input the same grade for contiguous piles of tobacco of the same grade by scanning one bar code rather than a combination of four bar codes. Also, the requirement that the inspector confirm the grade by pressing ENTER before printing was removed. The scanner/printer then printed a label as soon as a valid grade had been scanned from the grade matrix. The inspectors reported that this worked well and was not a problem since they always checked the label before applying it to the ticket.

One of the more time-consuming aspects of the grade-collection process was printing the label. Processing of the grade information and printing the label took 8-10 seconds. In some instances, one particular grader was able to begin grading the next pile of tobacco while waiting for the label for the previous pile to be printed. However, this made the process quite complicated and the other graders were unable to make productive use of the time during printing. In another attempt to speed up processing, the bar code for the grade was deleted from the label. However, this did not increase the speed of the printing process significantly. Taylor Data Systems, Inc. worked closely with the hardware manufacturer, to increase the speed of processing and printing. However, during the course of the pilot study, they were unable to increase the speed of processing and printing.

The modifications made to the software during the second week of the study significantly increased the speed that graders were able to inspect the tobacco and collect the grade. The time to grade a pile of tobacco also may have decreased because the inspectors became more proficient at using the system. The time required to inspect and grade a pile of tobacco using the scanner was reduced from 38 seconds during the first week of the project to 30-32 seconds during the third and following weeks. This meant that for a sale of 500 piles of tobacco, it took about 15 minutes longer to inspect the tobacco with the bar-code system than it did with the traditional method. By the end of the second sale of the day, the auctioneer and buyers had caught up with the inspectors. If the inspectors used the bar-code system on the third sale, then the sale had to be stopped to let the inspectors move ahead of the sale.

This hold-up in the sale was very undesirable to the buyers, the warehouse owners, and the tobacco growers. Thus, when the auction began catching up with the inspectors, the inspectors immediately switched from the bar-code system to the traditional method. As a result, the bar-code system was used only on the first two sales of each day during most of the study. Taylor Data Systems, Inc. and Monarch Marking were unable to improve the speed of the system during the pilot study.

The second difficulty encountered in the project was that the hardware and peripherals were difficult to handle. The inspectors noted that the plexiglass board with the grade matrix was cumbersome and too large to carry. The inspectors were sometimes hesitant to let the grade matrix board drop by their sides so that they could use both hands to inspect a pile of tobacco. Of course, it was noted that when the inspectors graded a pile of tobacco by the traditional method they often used only one hand to turn over or lift the tobacco in the pile being inspected. The inspectors noted that a smaller, lighter scanner/recorder would be desirable, and that a smaller grade matrix board would be less cumbersome. Reducing the size of grade matrix board was investigated. However, reducing the size of the bar codes made them more difficult to scan and increased the scanning time, so this adaptation was not made.

The inspectors and some farmers and warehouse owners expressed concern that the cumbersomeness of the system, plus the pressure to increase the speed of grading while using the system, detracted from the ability of the inspectors to accurately grade the tobacco. Other farmers and warehouse owners did not believe that the accuracy of the inspection process was compromised by using the bar-code system.

The third area of concern voiced by some warehouse owners, tobacco buyers, and members of the GCPPSC was that the grade information appeared only on the top copy of the pile ticket. The tobacco inspection ticket is the record that follows a pile of tobacco from weigh-in to the destination of the buyer. The top copy of the ticket is the one that serves as the official record through the marketing process. The other copies serve as information records for various parties, particularly the warehouse staff. Self-carbon tickets written by hand show all of the information on all copies. This information includes the weight, price paid, the buyer, and possibly the company grade. Information that might be missing from bottom copies of the ticket using the bar-code system are the government grade, date, and name of the grader. The cost of imprinting more than the top ticket appears prohibitive, but warehouses with computer facilities could easily record any information on a daily basis.

COST

A concern raised by all groups involved was the cost of implementing a bar-code system for grade collection. The initial cost of the scanner/printer and peripherals was about $3,700 per unit. The life of a unit, under constant use, was estimated at five years by Monarch Marking. Because the units would be used only during the tobacco marketing season, this estimate of useful life, in terms of the operability of equipment, was quite conservative. It is more likely that the equipment would become obsolete before it wore out. Using a real discount rate of 4%, the annualized purchase cost was about $800 per unit. A service contract was available for the unit for $250 per year. Thus, the total annual cost of purchase and service was about $1,050. Each inspector would need a unit, plus each market set would need an extra unit as a backup. For example, the annual cost for the Roxboro market, with four inspectors, would be around $5,250 (for 5 units). To implement such a system there also would be considerable training costs. While training costs are difficult to determine, USDA-AMS already has an infrastructure and procedures set up for grader training.

The successful implementation of the bar-code grade collection system would mean that market recorders would no longer be needed to record the grade and price data, which would result in a savings to USDA. To determine whether there would be a net savings to USDA, the cost of

market recorders should be compared to the annualized cost of the bar-code system.

An important consideration is how funds saved from the elimination of market recording would be reallocated. Currently, market recorders are paid by appropriated funds, while the cost of grading services are paid by user fees to growers. Thus, unless the savings to the USDA-AMS (Market News Division) were reallocated to cover the additional costs of adopting the bar-code system, grading fees paid by growers could increase. Of course, if the adoption of the bar-code system resulted in less tobacco being purchased by the FCTCSC (because grade support prices were set more accurately), then adoption could result in savings to buyers and growers in the form of a lower no-net cost assessment. Given the other potential benefits of this system to the industry, successful implementation could be very cost efficient.

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DELAYED TOPPING EFFECTS ON THE YIELD, VALUE, AND LEAF CHEMICAL COMPONENTS OF PHOTOPERIOD-SENSITIVE FLUE-CURED TOBACCO[1]

By G.R. Stocks[2] and E.B. Whitty[3]

The propensity of flue-cured tobacco (*Nicotiana tabacum* L.) cultivars to produce higher yields due to early topping is well documented. However, most studies on time of topping have not documented yield and chemical components by stalk position. This study evaluated the yield and chemical responses of a photoperiod-sensitive flue-cured tobacco cultivar NC 37NF to delayed topping. Six topping times were used. Plants were topped at 20 leaves, and the earliest topping time was when the 20th leaf was 15-20 cm in length. The remaining five topping times were 5, 10, 15, 20, and 30 days after the earliest topping time. Delays in topping decreased yields by an average of 52 kg ha^{-1} day^{-1}, and this decrease was proportionately greater in upper stalk leaves than in lower stalk leaves. Delays in topping did not affect price or visual quality at comparable stalk positions, but it decreased total value by $201 ha^{-1} day^{-1} because it lowered yields. Topping delays also reduced nicotine and reducing sugar concentrations in the uppermost eight leaves. Nitrogen concentration and the N:total alkaloid concentration ratio in the uppermost eight leaves increased with topping delays. The results from this study support the view that timely topping of photoperiod-sensitive flue-cured tobacco cultivars would ensure optimum yields, value, and chemical balance of the cured leaves; whereas delaying apical meristem removal would decrease yields and value and result in cured leaves that may have an undesirable balance of chemical constituents.

Additional key words: *Nicotiana tabacum*, mammoth tobacco, stalk position, topping time.

INTRODUCTION

Topping (removal of the inflorescence or apical meristem) is a basic management practice for production of flue-cured tobacco (*Nicotiana tabacum* L.). It improves the yield, value, and quality of leaves remaining on the plant. The improvement in yield has been attributed to an associated increase in the size of leaf cells (15). Value is enhanced in most part because of higher yields, and cured-leaf quality is improved because leaf chemical components are more favorably balanced.

Delaying topping beyond the button stage (when the inflorescence has formed) reduces yield and value of day-neutral cultivars of flue-cured tobacco (3,4,8,10). Stocks & Whitty (13) also observed yield and value losses associated with delayed topping of a photoperiod-sensitive tobacco cultivar.

Elliot (4) evaluated the effects of delayed topping using the button-stage topping treatment as the standard. He found that for plots with delayed topping, the nicotine concentrations were lower in the upper two primings and the lamina weight was reduced in the last harvest compared to plots topped at the bud stage. Other studies generally have found nicotine concentrations to be lowered and N concentrations to be unaffected by delayed topping (3,4,8,10). Marshall & Seltmann (10) reported that delayed topping lowered reducing sugar concentrations, while Elliot (4) found that it increased reducing sugar concentrations in the last harvest. Stocks & Whitty (13), working with a photoperiod-sensitive tobacco cultivar, reported that delayed topping resulted in an increased N concentration and a higher N:total alkaloid ratio compared to tobacco topped at the earliest stage.

Reductions in yield and value due to delayed topping of both day-neutral and photoperiod-sensitive flue-cured tobacco cultivars are well established. However, with the exception of the study by Elliot (4), no data relate the effects associated with delayed topping to various stalk positions. Therefore, the objective of the present study was to quantify by stalk position the effects of delayed topping on the yield, value, and leaf chemical components of a photoperiod-sensitive, flue-cured tobacco cultivar.

MATERIALS AND METHODS

Field experiments were conducted in 1989 and 1990 at the University of Florida's Green Acres Agronomy Farm near Gainesville, Fla. The photoperiod-sensitive, flue-cured tobacco cultivar NC 37NF was grown on an Arrendondo fine sand (fine-sandy, siliceous, hyperthermic Grossarenic Paleudult) for both years. Transplanting dates were 22 March 1989 and 23 March 1990.

For both years, weed and soil-borne pest management consisted of broadcasting and incorporating by disking 2.26 kg (a.i.) ha^{-1} of chlorpyrifos (insecticide) (O,O-diethyl O-[3,5,6-trichloro-2-pyridinyl]-phosphorothioate), 4.46 kg (a.i.) ha^{-1} of pebulate (herbicide) (S-propyl-butylethylthiocarbamate), and 0.58 kg (a.i.) ha^{-1} of pendimethalin (herbicide) (N-[1-ethylpropyl]-3,4-dimethyl-2,6-dinitrobenzenamine). After these pesticides had been applied, 56.0 L ha^{-1} of 1,3 dichloropropene (nematicide) was injected into the row, which was then bedded. Acephate (O,S-dimethyl acetylphosphoramidothiate) was applied as needed at 0.83 kg (a.i.) ha^{-1} to control foliage-feeding insects. Complete sucker control was achieved by pouring down the stalk at each topping time 25 mL of a solution made up of 20 mL flumetralin (2-chloro-N-[2,6-dinitro-4-(trifluoro-methyl) phenyl]-N-ethyl-6-flouro-benzene-methanamin) and 40 mL fatty alcohols (0.5% hexanol, 42% octonol, 56% decanol, and 1.5% dodecanol) per liter of water.

The experiments were fertilized with 448 kg ha^{-1} of 6-6-18 and 168 kg ha^{-1} of 15-0-14 at transplanting, 448 kg ha^{-1} of 6-6-18 at first cultivation, and 448 kg ha^{-1} of 6-6-18 at last cultivation. All fertilizer was banded along both sides of the plants. Total amounts of primary and secondary nutrients applied were 106 kg N ha^{-1}, 81 kg P_2O_5 ha^{-1}, 266 kg K_2O ha^{-1}, 54 kg Ca ha^{-1}, 54 kg Mg ha^{-1}, and 148 kg S ha^{-1}. In 1990, an additional 112 kg ha^{-1} of 15-0-14 was hand-applied to plots three weeks after the last cultivation to compensate for leaching of N and K.

Leaf position (LP) was defined as the leaves associated with a consecutive group of nodes along the stem. The bottom four leaves were discarded two weeks before initiation of the topping treatments and they were not included in the leaf numbering system. All plants were topped to 20 leaves. Leaves were numbered from the lowest (1) to highest (20) position. Five harvests, each consisting of four leaves per plant, were used to acquire all leaves (i.e., harvest 1 = leaves 1-4, harvest 2 = leaves 5-8, harvest 3 = leaves 9-12, harvest 4 = leaves 13-16, and harvest 5 = leaves 17-20).

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[2] Former Graduate Research Assistant in Agronomy, University of Florida, Gainesville, Fla.; presently Leaf Executive, Rothmans International Services Limited, Denham Place, Village Road, Denham, Uxbridge, Middlesex, UK, UB9 5BL.

[3] Professor of Agronomy, University of Florida, Gainesville, Fla. 32611.

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Table 1. The effects of delayed topping on the leaf position yields of the photoperiod-sensitive flue-cured tobacco cultivar, NC 37NF.

Topping time	Leaves 1-4	Leaves 5-8	Leaves 9-12	Leaves 13-16	Leaves 17-20
	---kg ha^{-1}---	---kg ha^{-1}---	---kg ha^{-1}---	---kg ha^{-1}---	---kg ha^{-1}---
Earliest	619	726	843	984	995
+5 Days	573	692	807	930	982
+10 Days	586	674	769	863	931
+15 Days	580	621	709	771	844
+20 Days	496	497	559	669	725
+30 Days	493	497	540	582	612
Regression Equation[a]	y = 615 - 4.3x r^2 = 0.752	y = 734 - 8.7x r^2 = 0.858	y = 856 - 11.3x r^2 = 0.894	y = 990 - 14.2x r^2 = 0.978	y = 1034 - 13.9x r^2 = 0.953

[a]y = yield (kg ha^{-1}); x = topping time (days after earliest).

Six topping-time treatments were applied each year. The earliest topping time occurred when the 20th leaf averaged 15-20 cm long. The other topping times were 5, 10, 15, 20, or 30 days after the earliest topping time. Four replications of the topping treatments were arranged in a randomized complete block design. There were 15 plants per single-row plot and plants were spaced 41 cm apart within rows that were 121 cm apart.

Cured leaf samples from all treatment harvests were graded by tobacco graders from the United States Department of Agriculture (USDA). These grades were used to determine the value per hectare or weighted average leaf price using the 1989 or 1990 Georgia-Florida flue-cured tobacco (Type 14) market average for the respective grades.

Eight cured leaves were chosen at random from the last two harvests (leaves 13-16 and 17-20) of each plot for chemical analyses. The midveins were removed and the lamina was ground to 1 mm using a Wiley mill. Total N, total alkaloids, and reducing sugar analyses were performed by The American Tobacco Company in Hopewell, Va., using standard industry methods.

Statistical analysis involved an initial analysis of variance (ANOVA) to determine significance of main effects and interactions (6). This ANOVA found no effects for years, but topping time by stalk position interactions were detected. Consequently, data were averaged across years for analysis of stalk position by topping time. Stalk position yields, values, and leaf chemical components were regressed with topping time. All regression equations were significant at the $P \leq 0.05$ level.

RESULTS AND DISCUSSION

Agronomic Characteristics

Yields at all leaf positions (LP) declined as topping time was delayed past the earliest treatment (**Table 1**). Wolf & Gross (15) reported that the more mature a leaf was, the less responsive it was to topping. The highest yields and also the highest yield losses due to delayed topping occurred in the upper two LPs. The first harvest was taken three days after the 15-day delayed topping treatment and before the 20- and 30-day delayed topping treatments. Yields for the first and second harvests (leaves 1-4 and 5-8) of the 20- and 30-day delayed topping treatments were similar. The long delay in topping these treatments allowed the development of 15 to 25 additional leaves that were then removed during the topping procedure.

According to regression analysis, the average yield loss attributable to delaying topping past the earliest topping time was 52 kg ha^{-1} day^{-1} (**Table 2**). Marshall & Seltmann (10) reported that for a day-neutral cultivar yields declined 31 kg ha^{-1} day^{-1} when topping was delayed past the button stage, while Stocks & Whitty (13) found that for a photoperiod-sensitive cultivar yields declined 40 kg ha^{-1} day^{-1}. The differences in the losses observed in these published studies and in our study may be because Marshall & Seltmann (10) evaluated a lower yielding, day-neutral cultivar, and because Stocks & Whitty (13) delayed topping for only 15 days after the earliest topping time. In our study, regression analysis revealed that the yield loss from delaying topping up to 15 days after the earliest topping time was 44 kg ha^{-1} day^{-1}.

For a comparison of the relative yield losses due to delayed topping across various yield regimes, it was necessary to have a common means of comparison. This comparison, which was termed the Daily Relative Yield Loss Percent (DRYL%), was computed by dividing the slope of the yield loss equation by the intercept of that equation. The DRYL% is a predictor of the percentage of the total potential yield that would be lost daily by delays in topping, and it can be used to compare relative yield losses without regard to the magnitude of the maximum yield. DRYL%'s for data from Marshall & Seltmann (10), Stocks & Whitty (13), and our study were 1.20%, 1.01%, and 1.23%, respectively.

All LPs declined in value as topping time was delayed past the earliest stage (**Table 3**). According to regression analysis, the average value lost by delaying topping was $201 ha^{-1} day^{-1} (**Table 2**). Stocks & Whitty (13) found that value declined $169 ha^{-1} day^{-1} for a photoperiod-sensitive cultivar due to delaying topping for 15 days. In the present study, value declined $170 ha^{-1} day^{-1} according to regression analysis performed on topping treatments up to 15 days past the earliest topping time.

Price per kilogram by stalk position did not differ among topping time treatments (data not shown). This result reflected the similar subjective quality appraisals by the

Table 2. The effects of delayed topping on the yield, weighted average price, and value of the photoperiod-sensitive flue-cured tobacco cultivar, NC 37NF.

Topping time	Yield	Price	Value
	---kg ha^{-1}---	---$US kg^{-1}---	---$US ha^{-1}---
Earliest	4,183	3.71	15,528
+5 Days	4,017	3.73	14,967
+10 Days	3,721	3.70	13,752
+15 Days	3,554	3.69	13,096
+20 Days	2,968	3.66	10,848
+30 Days	2,743	3.64	9,984
Regression Equation[a]	y = 4.222 - 52 x r^2 = 0.957	y = 3.73 - 0.003 x r^2 = 0.868	y = 15,705 - 201 x r^2 = 0.957

[a]y = yield, price, or value; x = topping time (days after earliest).

Table 3. The effects of delayed topping on the leaf position values of the photoperiod-sensitive flue-cured tobacco cultivar, NC 37NF.

Topping time	Leaves 1-4	Leaves 5-8	Leaves 9-12	Leaves 13-16	Leaves 17-20
	---$US ha^{-1}---	---$US ha^{-1}---	---$US ha^{-1}---	---$US ha^{-1}---	---$US ha^{-1}---
Earliest	2,012	2,591	3,144	3,861	3,913
+5 Days	1,854	2,440	3,021	3,686	3,896
+10 Days	1,898	2,396	2,798	3,386	3,666
+15 Days	1,874	2,170	2,636	3,018	3,335
+20 Days	1,641	1,730	1,960	2,650	2,878
+30 Days	1,639	1,721	1,886	2,304	2,426
Regression Equation[a]	y = 1985 - 12x r^2 = 0.750	y = 2610 - 33x r^2 = 0.865	y = 3203 - 47x r^2 = 0.886	y = 3891 - 56x r^2 = 0.977	y = 4077 - 54x r^2 = 0.950

[a]y = value ($US ha^{-1}), x = topping time (days after earliest).

USDA graders. However, the price per kilogram as a weighted average across all LPs declined with delayed topping time (**Table 2**). This result was due to relatively higher yield losses in the upper stalk than in the lower stalk (**Table 1**), which caused the less-valuable lower stalk leaves to contribute relatively more to the weighted average price calculation. This is an important observation because growers would not witness any apparent quality losses; however, the lower yields from the upper leaves would reduce the weighted-average price.

Chemical Characteristics

Delayed topping caused reductions in total alkaloid concentrations in the upper two LPs (**Table 4**). No other LP's were evaluated for chemical constituents. This observation supports findings from other studies on delayed topping of tobacco (3,4,10,13). It has been suggested that topping tobacco stimulates root development (12), but another study that evaluated topping and root development did not conclusively prove this postulate (11); however, delayed topping did decrease nicotine concentrations. Whether topping stimulates root development is probably not relevant to the nicotine concentration as affected by topping time, because the decrease in concentration reflects dilution of the nicotine into plant parts that are later discarded. Nicotine is synthesized in the roots and translocated to the shoot region (2). Delayed topping of the photoperiod-sensitive cultivar in the present study allowed development of extra leaves that were discarded at topping time. These extra leaves likely accumulated some nicotine that otherwise would have been destined to the leaves to be harvested. It has been shown that more leaves grown either per plant or per hectare lowers their nicotine concentration (1,5,7,9).

The N concentration of the upper two LP's increased as topping was delayed (**Table 4**). Stocks & Whitty (13) reported an increased N concentration with delayed topping of a photoperiod-sensitive cultivar. Other studies found N concentration to be unaffected or to be slightly reduced with delayed topping (3,4,10). Because delayed topping of a photoperiod-sensitive cultivar allows extra leaves to form above those to be harvested, N concentration was expected to decline, as was found in studies dealing with higher leaf numbers, either on a per-plant or per-hectare basis (1,5,7,9). The reason for this anomaly is not apparent. The increasing N concentration and decreasing total alkaloid concentration with delayed topping resulted in an increasing N:total alkaloid concentration ratio (**Table 4**). An increased N:total alkaloid ratio may reduce the potential smoking quality of cured leaves even when they appear normal (14).

The reducing sugar concentrations declined with delayed topping for the upper two harvests (**Table 4**). Higher leaf N and nicotine concentrations have been associated with lower reducing sugars (1,5,7,9). However, higher leaf populations per plant or per hectare resulted in higher reducing sugar concentrations (1,5,7,9). The decline in reducing sugar concentrations paralleled that of nicotine concentrations. Consequently, the reducing sugar to nicotine concentration ratio was not affected by delayed topping (**Table 4**).

In summary, yield and value were reduced by delays in topping of NC 37NF. The potential yield declined 1.23% each day tops were not removed. There were no visual or price differences in the cured leaves from a respective LP among topping dates. The weighted average price declined with topping delays because yield was reduced at the higher-priced upper stalk leaves. Delayed topping also caused changes in chemical composition of the cured leaves. The

Table 4. Effects of delayed topping on the N, total alkaloid, and reducing sugar concentrations, and the N:total alkaloid and reducing sugar:total alkaloid concentration ratios in the upper two leaf positions of the photoperiod-sensitive flue-cured tobacco cultivar, NC 37NF.

	Nitrogen		Total alkaloids		N:Total alkaloid ratio		Reducing sugars		Reducing sugars: total alkaloid ratio	
Topping Time	Leaves 13-16	Leaves 17-20	Leaves 13-16	Leaves 17-20	Leaves 13-16	Leaves 17-20	Leaves 13-16	Leaves 17-20	Leaves 13-16	Leaves 17-20
	---g kg^{-1}---	---g kg^{-1}---	---g kg^{-1}---	---g kg^{-1}---			---g kg^{-1}---	---g kg^{-1}---		
Earliest	21.6	26.0	25.1	29.4	0.86	0.88	237	162	9.4	5.5
+5 Days	21.6	25.5	21.9	26.4	0.95	0.97	248	189	11.3	7.2
+10 Days	22.5	25.9	22.0	25.6	1.02	1.01	235	168	10.7	6.6
+15 Days	23.1	27.6	20.0	23.7	1.15	1.17	225	178	11.3	7.5
+20 Days	25.4	28.5	19.0	21.3	1.34	1.34	178	103	9.4	4.8
+30 Days	28.4	30.8	14.5	19.8	1.96	1.57	133	92	9.2	4.6
Regression Equation[a]	y=20.3+0.25x r^2 = 0.909	y=25.0+0.18x r^2 = 0.896	y=24.7-0.25x r^2 = 0.958	y=28.6-0.32x r^2 = 0.960	y=0.74+0.036x r^2 = 0.911	y=0.84+0.024x r^2 = 0.977	y=260-3.8x r^2 = 0.856	y=189-3.0x r^2 = 0.650	NS	NS

[a]y = N, total alkaloid, or reducing sugar concentration, or N:total alkaloid concentration ratio; x = topping time (days after earliest).

results of this study support the view that in order to achieve optimum yield, value, and price photoperiod-sensitive flue-cured tobacco cultivars should be topped as soon as possible after the desired number of leaves per plant has been attained. Also, timely topping of photoperiod-sensitive cultivars is crucial for the resulting leaf chemical balance because continued vegetative development acts as a sink for nicotine to the detriment of leaves that are to be harvested.

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