Effect of Cercospora nicotianae Infection on Four Major Chemical Constituents in Cured Tobacco Leaves

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ABSTRACT

Cured tobacco leaves from field-grown plants that were heavily infected by *Cercospora nicotianae* averaged 18, 46, and 32% less total alkaloid, reducing sugar, and total phenol, respectively, and 6.5% more total nitrogen than noninfected leaves. Interactions involving presence or absence of disease in leaves from 10 cultivars, two stalk positions, and two curing methods were statistically analyzed for each of the four chemical constituents. Only in flue-cured cultivars, which had the highest inherent levels, were reducing sugar concentrations significantly less in diseased than in healthy leaves. Total alkaloids decreased less and reducing sugars decreased more in diseased leaves from the upper than in those from the lower stalk position. The average nitrogen concentration in infected leaves showed a significant increase only in those from the higher stalk position. Greater reductions occurred in reducing sugars and total phenols in infected flue-cured than in infected air-cured leaves. The results are discussed as they relate to leaf quality and disease physiology.

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Cercospora nicotianae Ell. & Ev. is an endemic pathogen of burley, Maryland, and Florida-grown flue-cured and cigar-wrapper tobacco in the United States. The fungus incites two diseases. Infection of all classes and types of tobacco results in development of frogeye leaf spot before harvest (12, 23). On the Maryland type and usually to a lesser degree on other types, the fungus also incites green spot, which becomes noticeable when the leaves take on a brown or yellow color during curing (12). Although losses can be extensive with severe infection, a small amount of frogeye has long been assumed to be a desirable indication that leaves were mature when harvested (23). Although the presence of some frogeye may increase market value, we know of no previous studies of the postharvest, postcuring effects of *C. nicotianae* infection on the chemical constituents of tobacco leaves.

Percentages of total alkaloids, total nitrogen, total phenols, and, in flue-cured leaves, reducing sugars are commonly measured tobacco constituents relating to leaf quality and ultimate value. Certain levels of these classes of compounds are desirable in the various types of tobacco (1, 6). Perhaps more important than the specific levels of these components are the ratios of total nitrogen to total alkaloids and reducing sugars to total alkaloids (1, 11).

Our first objective was to determine the effect of *C. nicotianae* infection on total alkaloids, reducing sugars, total nitrogen, and phenols. A second objective was to broaden the applicability of the results by determining the influence on any disease-induced changes of ten cultivars, representative of three major kinds of tobacco, two stalk positions, and the two principal methods of curing the crop. An abstract of some of our results has been published (20).

MATERIALS AND METHODS.—Cultivars selected included three burley and three Maryland from the air-cured class and four from the flue-cured class. Burley cultivars included Kentucky 12 (Ky 12), Burley 37 (Bu 37), and Burley 49 (Bu 49). Flue-cured cultivars were Hicks, Coker 254 (C254), Coker 187-Hicks (C187-Hicks), and Coker 139 (C139). The latter cultivar is inherently low in total alkaloids. Maryland cultivars included Maryland 609 (Md 609), Wilson, and Robinson. Nornicotine, rather than the usual nicotine, is the principal alkaloid in Robinson. Most of these 10 cultivars are resistant to one or more of the major tobacco diseases (12), but none is resistant to *C. nicotianae*.

Methods of propagating and transplanting plants to the field, fertilization rates, and soil types were the same as previously described (19). Plants were transplanted to the field on 10 June 1970. The planting was arranged into two five-replicate blocks with six plants of each cultivar/replicate. Randomization of cultivars within replicates was done identically in the two blocks. The plants in one block were not inoculated, and remained essentially free of symptoms. On 9, 14, 22, and 29 July, all plants in the second block were spray-inoculated with *C. nicotianae*. Inoculum was produced in the laboratory under optimal conditions of temperature (22) and nutrition (21), blended, and diluted to 350,000 conidia and mycelial fragments/ml water. Typical frogeye symptoms (12) appeared on inoculated leaves 7 days after the first inoculation. Axillary suckers were removed by hand from all plants beginning on 28 July and at weekly intervals thereafter until harvesting was completed. As plants reached 10% bloom, which occurred between 28 July and 14 August, the stem was cut off (topped) below the first leaf below the inflorescence. Basal inoculated leaves that were killed before harvest were removed and discarded. The same number of leaves was removed from parallel plants in the noninoculated block to ensure that leaves for comparison were similar in age.

On 18 August, the three lowest remaining leaves were harvested from each plant and tied together. These were all low midplant leaves. Three of the six bundles from each replicate of each cultivar were air-cured, and three were flue-cured. Care was taken to insure that leaves for each curing method had similar disease severity. The usual methods were used for curing (1, 6), employing barns at Beltsville, Md., and Oxford, N. C., for air- and flue-curing, respectively. A second harvest, consisting of upper midplant leaves, was made on 1 September, using the same methods. After curing, the occasional, small areas of inoculated leaves damaged by causes other than *C. nicotianae* were removed and discarded. The occasional lesions in noninoculated leaves incited by *C. nicotianae* or other causes were also eliminated. Indices indicating the relative severity of frogeye and green spot on a 0-100 scale were recorded for each diseased leaf. Midribs were then removed and discarded, and leaves were oven-dried at 65 C, ground, and used for chemical analyses.

The percent total alkaloids were determined by the steam distillation procedure (7). Reducing sugars were analyzed with an autoanalyzer employing the Technicon modification of the method of Harvey et al. (10). Percent of total polyphenols was determined by the method of Andersen & Todd (2), spectrophotometrically measuring the polyvinylpyrrolidone precipitated fraction and expressing the total phenol as percent of chlorogenic acid equivalent. Total nitrogen concentrations were determined by the improved Kjeldahl method for nitrate-containing samples (3).

The data for each of the four chemical constituents was statistically analyzed as a split-split-split-plot, using disease versus no disease as the whole-plot, cultivars as the subplot, stalk positions as the subsubplot, and curing methods as the subsubsubplot. Significance of interactions and single comparison main effects was determined by the F test. Least significant differences (LSDs) were calculated for the means involved in all interactions.

RESULTS.—A preliminary, less extensive

TABLE 1. Effects of *Cercospora nicotianae* infection on four chemical constituents in 10 tobacco cultivars

Cultivar	*C. nicotianae*[a]		Chemical constituent[b]: Total alkaloids	Reducing sugars	Total N	Phenols
			%	%	%	%
Bu 37	I		3.20	1.03	4.31	.99
	N		4.64	1.23	4.07	1.26
Bu 49	I		3.09	.99	4.48	1.01
	N		4.01	1.45	4.01	1.36
Ky 12	I		2.76	1.07	3.89	.92
	N		3.88	1.47	4.07	1.31
C139	I		2.43	2.35	3.42	1.92
	N		3.24	4.26	3.14	2.20
C187-Hicks	I		3.25	2.30	3.54	1.96
	N		3.68	5.74	3.22	3.22
C254	I		3.46	2.57	3.27	2.24
	N		4.11	6.37	3.14	3.24
Hicks	I		3.86	1.87	3.87	1.86
	N		4.69	3.32	3.71	2.79
Md 609	I		3.47	1.36	3.97	1.23
	N		3.70	1.76	3.63	2.24
Robinson	I		4.67	1.08	4.20	1.25
	N		5.35	1.47	3.88	1.97
Wilson	I		3.46	1.22	3.85	1.20
	N		3.79	2.00	3.53	1.95
Interaction (disease × cultivar)[c]			**	**	NS	**
LSD between I and N on same cultivar		.05	.54	1.62	.32	.39
		.01	.79	2.34	.45	.58
LSD between cultivars for same disease level		.05	.41	1.32	.27	.26
		.01	.54	1.75	.36	.34
Averages[c]	I		3.37	1.58	3.88*	1.46
	N		4.11**	2.90*	3.64	2.15**

[a] I = *C. nicotianae* infected; N = noninfected.
[b] Each figure is the average over two stalk positions, two curing methods, and five replicates.
[c] ** = F value significant at the 1% level; * = significant at the 5% level; NS = nonsignificant F. The averages given here are from averaging values from all cultivars, stalk positions, and curing methods, and represent the main effects of disease.

examination of the effects of *C. nicotianae* infection of eight cultivars on alkaloids, reducing sugars, and phenols in 1969 produced results similar to those obtained in 1970. Only the 1970 results are given here.

Disease severity.—*Cercospora nicotianae* infection of all harvested, inoculated leaves was severe. Symptom severity was similar on leaves cured by both methods. The disease indices were recorded on a 0-100 scale. Leaves from all cultivars in the first harvest had an average frogeye index of 83.3 and green spot index of 15.7, whereas those from the second harvest decreased to 73.6 and 6.5, respectively. The frogeye index varied little among cultivars or types, being 80.3, 79.5, and 75.8 on burley, flue-cured, and Maryland, respectively, when averaged over both harvests. However, the green spot index averaged 1.8 on burley 12.0 on flue-cured, and 19.5 on Maryland cultivars. Green spot severity differed very little among the three Maryland cultivars. Among flue-cured cultivars, C187-Hicks and Hicks had more green spot than C139 and C254. Not more than a trace occurred on any of the burley cultivars.

Chemical analyses.—Although some interactions among cultivars, stalk positions, and curing methods were significant, results are reported here only for interactions involving disease levels. For brevity, only the data for the main effect involving disease, and the disease X cultivar, disease X stalk position, disease X curing method, and disease X cultivar X curing method interactions are given in tabular form. Tabular data for the main effects of cultivars, stalk positions, and curing methods and for the interactions of disease X cultivar X stalk position and disease X cultivar X stalk position X curing method are omitted for simplicity.

1) *Total alkaloids.*—The total alkaloid content of cured tobacco leaves averaged over all cultivars, stalk positions, and curing methods, was reduced 18% by *C. nicotianae* infection (Table 1). When averaged over all other variables, curing methods had no effect on alkaloid level, but leaves from the upper stalk position had more than those from the lower stalk position. Among cultivars, C139 was lowest and Robinson was highest in alkaloids. The disease X cultivar interaction was significant (Table 1). All cultivars but C187-Hicks, Md 609, and Wilson had significant reductions in percent total alkaloids due to disease. Burley cultivars generally had the greatest and Maryland cultivars the least reduction, the sole major exception being C139, which had a larger percent decrease than Bu 49. All other interactions were not significant.

2) *Reducing sugars.*—Presence of the disease caused a 46% reduction in reducing sugars averaged over all cultivars, stalk positions, and curing methods (Table 1). Reducing sugar levels averaged over all other variables, were 33% higher in flue-cured leaves than in air-cured leaves, and 44% higher in leaves from the upper than in those from the lower stalk position. The four flue-cured cultivars had more than

TABLE 2. Effects of *Cercospora nicotianae* infection on four chemical constituents in tobacco leaves harvested from two stalk positions and cured by two methods

Variable	*C. nicotianae*[a]		Chemical constituent[b]: Total alkaloids	Reducing sugars	Total N	Phenols
			%	%	%	%
Stalk position						
Lower	I		2.06	1.22	3.46	1.36
	N		2.85	2.02	3.36	2.02
Upper	I		4.68	1.95	4.29	1.55
	N		5.37	3.79	3.92	2.28
Interaction (disease X stalk position)[c]			NS	**	**	NS
LSD between I and N at same stalk position		.05	.40	1.09	.20	.23
		.01	.65	1.78	.32	.31
LSD between stalk positions at same disease level		.05	.12	.32	.08	.11
		.01	.16	.42	.11	.14
Curing method						
Air	I		3.35	1.55	3.92	1.44
	N		4.16	2.04	3.69	1.81
Flue	I		3.38	1.62	3.83	1.47
	N		4.06	3.77	3.59	2.50
Interaction (disease X curing method)[c]			NS	**	NS	**
LSD between I and N with same curing method		.05	.43	1.08	.20	.31
		.01	.65	1.77	.32	.51
LSD between curing methods at same disease level		.05	.11	.24	.07	.09
		.01	.15	.32	.09	.12

[a] I = *C. nicotianae* infected; N = noninfected.
[b] Each figure is an average over five replicates, 10 cultivars, and either two curing methods or two stalk positions.
[c] ** = F value for interaction significant at the 1% level; NS = nonsignificant interaction.

twice as much as any of the Maryland or burley cultivars.

All interactions involving presence or absence of disease were significant for reducing sugars except that for disease X stalk position X curing method. The disease-induced decrease in reducing sugars, averaged over stalk positions and curing methods, was significant only for C139, C187-Hicks, and C254 (Table 1). These were the cultivars with the highest reducing sugar content. When averaged over cultivars and either stalk positions or curing methods, the disease-induced reduction was significant solely in flue-cured leaves (57% reduction) or leaves from the upper position (49% reduction), respectively (Table 2).

When averaged over only stalk positions, the

TABLE 3. Effects of *Cercospora nicotianae* infection, curing method, and cultivar on certain chemical constituents averaged over two stalk positions and five replicates

Cultivar	Curing method	*C. nicotianae*[a]		Chemical constituent[b]: Total alkaloids	Reducing sugars	Total N	Phenols
				%	%	%	%
Burley 37	Air	I		3.21	1.07	4.22	1.08
		N		4.65	1.11	4.00	1.19
	Flue	I		3.19	.98	4.40	.91
		N		4.62	1.34	4.14	1.32
Burley 49	Air	I		3.19	1.00	4.54	1.05
		N		4.13	1.29	3.94	1.46
	Flue	I		2.99	.98	4.42	.97
		N		3.89	1.61	4.08	1.26
Ky 12	Air	I		2.81	1.18	4.04	1.04
		N		4.14	1.17	3.99	1.42
	Flue	I		2.71	.96	3.73	.81
		N		3.62	1.77	4.14	1.21
C139	Air	I		2.39	2.30	3.38	2.11
		N		3.25	2.92	3.37	2.09
	Flue	I		2.47	2.40	3.45	1.73
		N		3.23	5.59	2.91	2.32
C187H	Air	I		3.11	2.37	3.64	1.71
		N		3.68	4.55	3.43	2.37
	Flue	I		3.38	2.23	3.44	2.20
		N		3.69	6.92	3.01	4.07
C254	Air	I		3.33	2.01	3.36	1.90
		N		4.03	3.42	3.33	2.31
	Flue	I		3.59	3.12	3.17	2.58
		N		4.19	9.31	2.95	4.17
Hicks	Air	I		3.93	1.66	3.97	1.70
		N		4.61	2.06	3.80	2.11
	Flue	I		3.84	2.08	3.76	2.02
		N		4.77	4.58	3.62	3.48
Md 609	Air	I		3.54	1.50	3.95	1.21
		N		3.88	1.22	3.61	1.74
	Flue	I		3.41	1.22	3.98	1.26
		N		3.52	2.30	3.64	2.73
Robinson	Air	I		4.61	1.08	4.21	1.35
		N		5.47	1.13	3.85	1.72
	Flue	I		4.73	1.08	4.18	1.15
		N		5.23	1.81	3.91	2.22
Wilson	Air	I		3.42	1.28	3.91	1.29
		N		3.75	1.56	3.54	1.70
	Flue	I		3.50	1.15	3.78	1.12
		N		3.83	2.43	3.51	2.21
Interaction (disease X cultivar X curing method)[c]				NS	**	**	**
LSD between I and N with same cultivar and curing method			.05	.61	1.73	.36	.46
			.01	.89	2.49	.51	.68
LSD between cultivars with same disease level and curing method			.05	.48	1.42	.31	.34
			.01	.63	1.89	.42	.45
LSD between curing methods, same cultivar and disease level			.05	.34	.76	.22	.30
			.01	.45	1.00	.29	.40

[a] I = *C. nicotianae* infected; N = noninfected.
[b] Each figure is the average over two stalk positions and five replicates.
[c] ** = F value for interaction significant at the 1% level; NS = nonsignificant interaction.

effect of infection on reducing sugars was significant in the flue-cured leaves of the four flue-cured cultivars, but not in the burley or Maryland cultivars (Table 3). The air-cured leaves of only one cultivar, C187-Hicks, showed a significant reduction. The noninoculated flue-cured leaves of Md 609, Wilson, and the flue-cured cultivars had more reducing sugar than those that were air-cured, but the differences were less in inoculated leaves due to the greater disease-induced reduction in flue-cured leaves.

3) *Total nitrogen.*–*C. nicotianae* infection caused a small but significant increase in total nitrogen when averaged over cultivars, stalk positions, and curing methods (Table 1). Burley cultivars generally had more nitrogen than flue-cured cultivars, whereas the Maryland cultivars were intermediate. No significant interaction for disease and cultivar was present for the nitrogen data.

The disease X curing method interaction was not significant for nitrogen, a 6% increase occurring with each method (Table 2). However, the disease X stalk position interaction was highly significant. The increase due to disease was 9% at the upper stalk position, whereas no significant increase occurred at the lower position (Table 2). When averaged over only curing methods, significant disease-induced increases occurred only in the upper stalk positions of Bu 49, C187-Hicks, and the three Maryland cultivars. When averaged over stalk positions (Table 3), the only significant increases were in air-cured Bu 49 and Wilson and flue-cured C139 and C187-Hicks.

Total nitrogen was the only chemical constituent for which the disease X cultivar X stalk position X curing method interaction was significant. In three of the four flue-cured cultivars, the air-cured leaves from the higher stalk position had significantly more nitrogen than the flue-cured leaves from that position, regardless of disease occurrence. No such general difference between curing methods was apparent in either burley or Maryland cultivars or in the lower stalk position leaves of flue-cured cultivars. Leaves from the upper position had more nitrogen than those from the lower, when compared with other variables at the same levels. However, an outstanding exception to this generalization occurred in flue-cured leaves of flue-cured cultivars, which showed no difference between stalk positions.

4) *Phenols.*–When averaged over all other variables, *C. nicotianae* infection resulted in a 32% reduction in phenols (Table 1). Phenol concentrations were higher in the upper stalk position and in flue-cured leaves than in the lower stalk position or in air-cured leaves. All four flue-cured cultivars, when averaged over other variables, had significantly more than the three Maryland cultivars, and the three Maryland cultivars had more than any of the burley cultivars. The disease caused a significant reduction in all cultivars but Bu 37, Bu 49, Ky 12, and C139 (Table 1). A 41% reduction was caused by the disease in flue-cured leaves, but the disease caused only a 20% reduction in air-cured leaves (Table 2). The percent phenols was significantly reduced by disease in flue-cured leaves of all but the three burley cultivars, but in air-cured leaves of only C187-Hicks and Md 609 (Table 3).

DISCUSSION.–The increase in total nitrogen may be the most adverse effect that we detected of *C. nicotianae* on cured leaf quality, particularly in light of the decrease in total alkaloids. Diseased leaves should thus be expected to have poorer aging capacity (16) and to give a more acrid, irritating smoke than noninfected leaf (1). A qualitative change in the nitrogen fraction is indicated by the simultaneous decrease in total alkaloids. The presence of fungal mycelium in infected leaves could be responsible for part of the additional nitrogen. However, there was a greater increase in nitrogen in diseased leaves from the upper stalk position than in those from the lower. The simultaneous occurrence of less disease in the upper than in the lower stalk position suggests that part of the increased nitrogen was contributed by the host. The higher levels of all the constituents we measured in leaves from the upper stalk position are probably due to greater synthetic activity at the upper than at the lower stalk position at the time of harvest.

Increases in nitrogen have been demonstrated in both brown spot lesions (13) and mosaic infected leaves of flue-cured tobacco (9). Such an increase has also been demonstrated in fungal and virus diseased tissues of other crop plants (17).

The greater reduction in total alkaloids as a result of *C. nicotianae* infection in the upper than in the lower stalk position and in burley than in Maryland cultivars suggests that this reduction is due primarily to the frogeye rather than the green spot type of symptoms. The average frogeye rating for leaves from the second harvest was 11.3 times greater than the green spot rating, but was only 5.3 times greater in the first harvested leaves. Green spot was 10.8 times more severe on Maryland cultivars than on burley cultivars.

Nicotine, the principal alkaloid in nearly all tobacco, is probably the most important chemical constituent of the crop (1, 6). Desirable percentages are in a narrow range. Thus, the decrease in total alkaloids in infected leaf would be of critical importance per se to leaf quality. Furthermore, the ratios of nitrogen and reducing sugars to total alkaloids were both unfavorably affected. The significant reduction in total alkaloids in Robinson, the only cultivar with an appreciable nornicotine content, accompanied by the lack of a significant effect in Md 609 and Wilson, suggest that nornicotine may be affected more by the disease than nicotine. Alternatively, the inherently higher alkaloid concentration in Robinson than in the other Maryland cultivars may be the critical factor.

The decrease in reducing sugar concentrations in flue-cured leaves of flue-cured cultivars should have a depressing effect on quality and value (1, 5). The concentration in flue-cured leaves from cultivars of this class was also diminished by *Alternaria alternata* (13) and tobacco mosaic virus infection (9). Reductions in carbohydrate levels have been reported in several other diseases of other crops (17). Direct utilization by the pathogens could be responsible for much of the reduction in fungal diseases, but reduction in photosynthetic capacity of diseased leaves is also likely to be a contributing factor.

The reduction in phenol concentrations following *C. nicotianae* infection may cause development of undesirable leaf flavor and aroma (15, 18). *C. nicotianae* infection caused significant reductions only in the cultivars with the higher concentrations, although all cultivars showed a similar trend. Flue-cured cultivars have previously been shown to have higher levels of polyphenols than burley cultivars (8, 18), but the intermediate position we found for Maryland cultivars has been less completely documented. The disease-induced reduction was also greater in flue-cured leaves, which had higher concentrations, than in air-cured leaves. In tobacco leaves damaged by ozone, Menser & Chaplin (14) found higher levels of phenols in severely affected leaves than in lightly affected leaves. With an inanimate disease-causing agent, such as ozone, phenol metabolism is apparently stimulated, whereas with *C. nicotianae*, if there is any stimulation it is exceeded by destruction or by inhibited synthesis later.

The levels of the chemical constituents reported here are not necessarily typical of the levels that occur in the usual production areas with the usual production practices for the various cultivars. We had to compromise on agronomic and harvesting methods in order to make the methods uniform for all cultivars tested. However, other than the somewhat low reducing sugar content of flue-cured leaves from flue-cured cultivars, the concentrations of the various constituents that we measured were not out of the generally expected range (1, 4, 5, 8, 11, 16, 18). Concentrations of phenols and alkaloids increase throughout the growing season (8) and concentrations of all the constituents analyzed, except reducing sugars, tend to be higher in leaves from higher than in those from lower stalk positions. Reducing sugar concentrations are usually highest in leaves from midplant stalk positions (1, 4).

The magnitude of the chemical differences that we detected between heavily inoculated and noninoculated leaves is undoubtedly greater than would occur in moderately or lightly infected leaves. It seems reasonable to assume that the changes in concentrations would be in the same directions, but smaller, with less infection. For reducing sugars and phenols, the magnitude of the decrease in concentrations in some cultivars was great enough for a significant effect, probably, to be detectable with moderate or even light infection. Lightly infected leaves would be unlikely to show any change in the nitrogen concentration from that in healthy leaves.

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