Nitrogenous and Phenolic Compounds Of Nicotiana Plants. I. Field And Greenhouse Grown Plants

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Mature leaves of various types of field-grown tobacco *(N. tabacum L.)* show total nitrogen to range from about 1.3 to 6% of dry weight, or total nitrogenous compounds from about 7.5 to 34.0% (2). This fraction contains characteristic tobacco components such as alkaloids and other compounds which have important effects on taste of the smoking products (3). Another group of compounds, the phenolic derivatives, are also related to leaf quality and usability (2). Chlorogenic acid, rutin, scopolin, and scopoletin are included in this group.

Since the rate of nitrogen fertilization affects the amino acid composition, and certain amino acids are involved in the biosynthetic pathway of phenolic compounds (6), it is important to examine possible interrelationships among these groups of compounds which occur in tobaccos varying in types, cultures, curing methods, and species.

Materials and Methods

Three types of tobacco were produced in the field under regular farm practices except that different levels of nitrogen fertilization were used. Burley 21 was produced at Greeneville, Tennessee, with three N levels: no nitrogen, 150 lbs/A, and 300 lbs/A. Burley 21 was also produced at Lexington, Kentucky, with four N levels: 60 lbs/A, 120 lbs/A, 180 lbs/A, and 180 lbs/A plus 10 tons manure. Maryland Catterton was produced at Marlboro, Maryland, with three N levels: 80 lbs/A, 120 lbs/A, and 150 lbs/A, and flue-cured Hicks was produced at Tifton, Georgia, with two N levels: 50 lbs/A and 100 lbs/A.

In addition, four *Nicotiana* species were produced at Florence, S. C.: *N. tabacum* var. N. C. 95, *N. rustica, N. glutinosa,* and *N. glauca.* At Waynesville, N. C., Burley 21 and Hicks were grown in the field and samples from both were subjected to air- and flue-curing to compare their respective chemical compositions.

Connecticut Broadleaf tobacco was grown in nutrient solution under greenhouse conditions. McMurtrey's nutrient formulation (5) was used, but with additional nitrate to achieve four N levels: 225 ppm, 450 ppm, 675 ppm, and 1125 ppm.

All chemical determinations were made on cured tobaccos except the solution-cultured Connecticut Broadleaf tobacco, which was analyzed at the matured green stage. Nitrogen fractions in cured tobaccos were determined through the courtesy of the American Tobacco Company (1). Amino acids were determined with a Technicon Amino Acid Analyzer*. The samples were prepared as previously described (8) except the following modifications were made to conform with recommended Technicon* procedures: (A) Ethanol fraction was adjusted to pH 2 for column separation, and (B) Hydrolyzate was prepared with 24 hours digestion of plant tissue in 6N HCl.

Samples for determination of phenolic derivatives were extracted with 70% ethanol for one hour in a steam bath. The extracts were concentrated under reduced pressure and chromatographed on pH 6.75 buffered W-1 paper in a solvent system containing t-amyl alcohol:H_2O (5:1). Reported results are semiquantitative estimation by visual comparison with standards. Confirmation of each compound was carried out by employing other solvents, including 2% acetic acid:N-butanol: acetic acid:H_2O (4:1:5), by Hoepfner spray (4), and by hydrolysis. Results reported are averages of three to five determinations.

Total "polyphenols" were determined on some of these samples. Assays were performed with an autoanalyzer by a spectrophotometric procedure which employed ferricyanide as an oxidizing reagent. Cured samples of tobacco were extracted with boiling water and an aliquot was assayed for total reducing activity. Another aliquot of the same extract was treated with basic lead acetate which precipitates o-dihydroxyphenols and the resultant su-

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* *Mention of a specific trade name is made for identification only and does not imply any endorsement by the U.S. Government or State Experiment Stations.*

Table 1. Nitrogen and phenolic content (%) in samples of various nitrogen fertilization, species, and curing methods

	Nitrogen Fraction			Phenolic Derivates			
	Total	Protein (As Nitrogen)	Nitrate	Chlorogenic Acid	Rutin	Scopolin	Scopoletin	Total
1. Nitrogen Fertilization (lbs/A)								
Tenn. (Burley 21 air-cured)								
0	3.23	1.28	.08	—*	—	—	.01	.01
150	4.46	1.51	.54	—	—	—	.01	.01
300	5.42	1.68	.98	—	—	Tr.	.02	.02
Ky. (Burley 21 air-cured)								
60	4.63	1.76	.39	.01	Tr.	—	.01	.02
120	5.04	1.78	.58	.02	.02	—	.01	.05
180	5.11	1.79	.71	.05	.03	Tr.	.02	.10
180 + 10 ton manure	5.32	1.88	.61	.05	.03	Tr.	.03	.11
Md. (Cotterton air-cured)								
90	2.99	1.37	.17	.05	.03	—	.02	.10
120	3.21	1.37	.31	.05	.03	—	—	.08
150	3.44	1.40	.40	.04	.05	Tr.	—	.09
Georgia (Hicks flue-cured)								
Low N 50	1.53	.73	.02	1.50	1.00	.02	.03	2.55
High N 110	1.98	.79	.02	2.00	1.00	.06	.07	3.13
2. Species (N.C. 95 flue-cured, others air-cured) (S. C.)								
N. tabacum (N.C. 95)	2.55	.91	.03	2.00	.50	.08	.10	2.68
N. rustica	4.45	.86	.02	.20	—	—	.05	.25
N. glutinosa	3.93	.86	.16	.20	—	—	.07	.27
N. grauca	3.01	.84	.05	.30	—	—	.07	.37
3. Curing (Waynesville, N. C.)								
Burley 21 air-cured	4.69	1.74	.53	.10	—	—	.03	.13
flue-cured	4.78	1.99	.53	.50	.20	—	.05	.75
Hicks air-cured	3.83	1.54	.34	.10	Tr.	—	.03	.13
flue-cured	4.22	1.42	.46	1.00	.50	—	.05	1.55

* *Not detected.*

pernatant was assayed in the autoanalyzer. The difference in reducing power is proportional to total polyphenol content.

Results of nitrogen fractions and of total "polyphenols" are expressed on moisture-free basis; "phenolic derivatives" are expressed either on air-dry basis or on fresh weight basis, depending on the condition at which the samples were received.

Results and Discussion

The levels of protein and nitrate nitrogen and of phenolic compounds of tobacco samples from various treatments are shown in **Table I.** Generally, the total nitrogen, protein nitrogen, and nitrate nitrogen in tobacco leaf increased as the rate of nitrogen fertilization increased, except in one case when manure was added the nitrate content decreased. Otherwise, this general pattern holds true in Burley, Maryland, and flue-cured tobaccos studied. The "total phenolic" derivatives, a summation of chlorogenic acid, rutin, scopolin, and scopoletin, appear to be in proportion with total nitrogen content of Hicks and Burley 21 (**Figures 1**

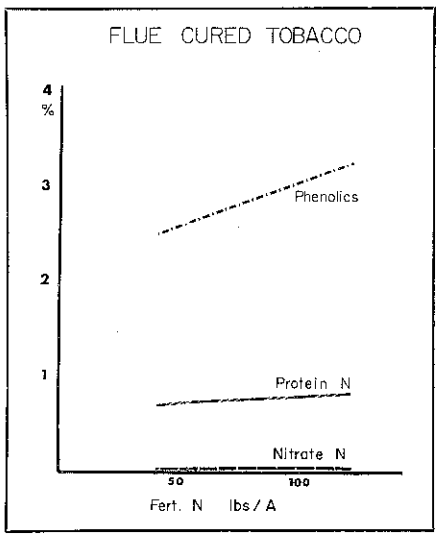

Figure 1. Phenolics, protein N, and nitrate N contents in Hicks tobacco produced at different rates of nitrogen fertilization.

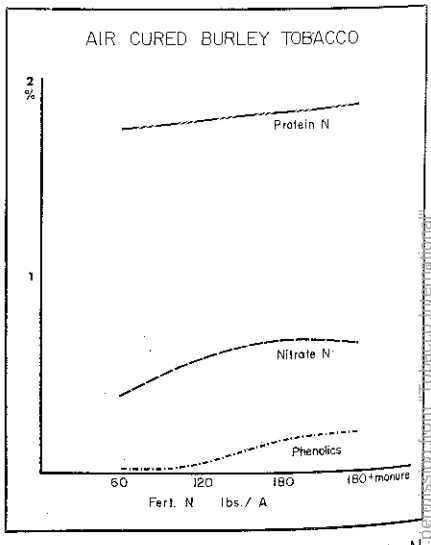

Figure 2. Phenolics, protein N, and nitrate N contents in Burley 21 tobacco produced at different rates of nitrogen fertilization.

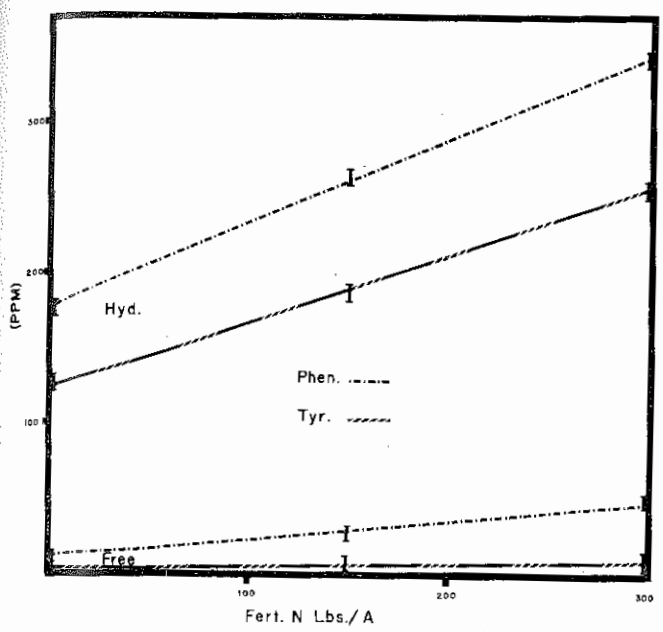

Figure 3. Phenylalanine and tyrosine contents in field-grown Burley 21 produced in Tennessee at different rates of nitrogen fertilization.

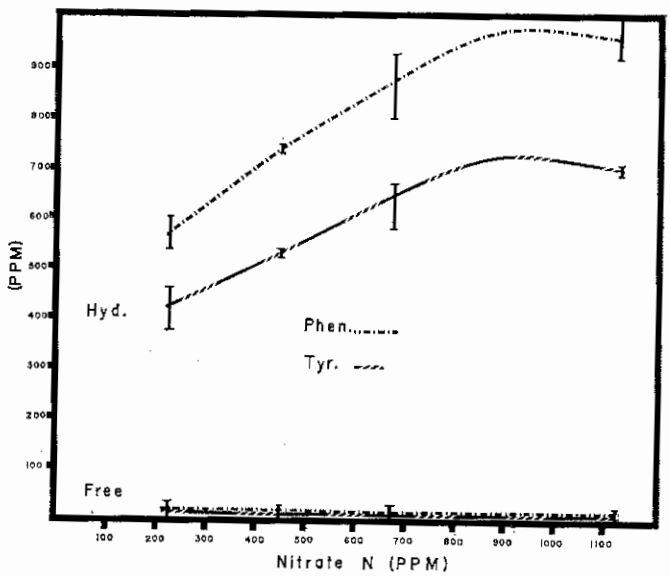

Figure 4. Phenylalanine and tyrosine contents in greenhouse-grown Connecticut Broadleaf tobacco produced at different rates of nitrate nitrogen in solution culture.

and 2) but not of Maryland Catterton.

To examine the possible relationship between nitrogen and phenolic fractions in tobacco, it is necessary to study the biosynthetic pathways of phenolic components in plants. Phenolic compounds, usually considered as secondary metabolites in plants, arise by further metabolism of the aromatic amino acids or from intermediates involved in the synthesis of the aromatic amino acids (6). Through decarboxylation and loss of ammonia, aromatic amino acids are converted to transcinnamic or p-coumaric acids which lead to the formation and accumulation of many phenolic and related compounds.

An air-cured tobacco (Burley 21) and a freshly harvested green tobacco (Connecticut Broadleaf) were used to examine the levels of aromatic amino acids in tobaccos produced under different nitrogen fertilization. Results are shown in **Figures 3 and 4**, respectively. In both cases, phenylalanine and tyrosine, either in free form or from hydrolyzate, increase as the rate of nitrogen fertilization increases. In each case, phenylalanine was always at a higher concentration than tyrosine. Although phenylalanine and tyrosine usually occur in different tobacco varieties, the effectiveness of an operative enzyme system for decarboxylation and deamination may decide the levels of phenolics in that particular plant. The relative effectiveness for utilization of either of these amino acids to form phenolic compounds in tobacco plants is a subject of separate study and will be reported elsewhere.

Method of curing demonstrated prominent effects on phenolic content in cured tobacco leaf (7, 9). The four *Nicotiana* species, as shown in **Table I**, were produced under the same farm practices and fertilizer rates. All components except the protein nitrogen fraction varied widely, especially nitrate nitrogen and phenolic compounds. Although there are differences among species, it should be noted that *N. tabacum*, N.C. 95, was flue-cured, while the other species were air-cured. Within the same group of plants, the effect of curing method on phenolics was clearly indicated. When Burley 21 and Hicks tobacco were grown in the same field, and each was subjected to flue and air curing, most prominent differences were found in phenolic fractions, although the levels of total nitrogen, protein nitrogen, and nitrate nitrogen appeared unaffected. The "total polyphenol" content, calculated as caffeic acid, is 0.5% for air-cured Hicks, and 3.7% for flue-cured Hicks tobacco. These results are in agreement with the data of "phenolic derivatives" as shown in **Table I.**

In green tobacco phenolic compounds are relatively stable. This is due to the presence of components with strong reducing ability in green tobacco and also due to the fact that oxidative enzymes and phenolic compounds are confined in separate leaf structure (2). Oxidation of phenolics takes place when the phenolics and the oxidases were brought into contact resulting from corrosion of cell walls from air curing through partial decomposition of certain components of cell membrane. Similar oxidation, however, was believed not to occur in flue-curing (2) and therefore phenolic compounds present in the green leaf remain unchanged. In addition, decarboxylation and deamination of aromatic amino acids would contribute to further increase of phenolic compounds during flue-curing.

Summary

Experimental tobaccos of various types were produced with different rates of nitrogen fertilization. The amount of phenolic compounds, including chlorogenic acid, rutin, scopolin and scopoletin, appears to be positively correlated to the rate of nitrogen fertilization and therefore nitrogenous fractions in tobacco plants. The levels of phenylalanine and tyrosine in experimental cured and green tobaccos are also proportional to the levels of nitrogen fertilization. These results suggest that aromatic acids are involved in the biosynthetic pathways of phenolic compounds in tobacco

plants and could explain the positive relationship between nitrogen and phenolics in certain tobacco plants when the decarboxylation and deamination enzyme systems are operative. Methods of curing also demonstrated decisive effects on phenolic content in cured tobacco leaf.

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