Effects of Curing on Polyamine Content of Leaves of *Nicotiana tabacum* L. Genotypes with Different Alkaloid Levels

J. P. Madsen, L. P. Bush,* and S. L. Gay

Leaves of tobacco (*Nicotiana tabacum* L.) Burley 21 and low-alkaloid Burley 21 genotypes were evaluated for polyamines (putrescine, spermidine, spermine, cadaverine, norspermidine) and alkaloids (nicotine, anatabine) before, during, and after air curing. Both free and conjugated forms of the polyamines were detected. Putrescine and spermidine were the predominant polyamines in leaves of both genotypes. Leaves of both genotypes contained about 3000 nmol of polyamines/g dry weight before curing. Total polyamine levels were unchanged in leaves of Burley 21 tobacco during curing whereas total polyamine levels doubled in leaves of the low-alkaloid genotype during curing. Greatest change occurred in conjugated polyamines in leaves from the upper stalk position. In both genotypes the portion of polyamines in leaves increased after topping (removal of apical meristem). Nicotine and anatabine levels were much greater in leaves of Burley 21 than in the low-alkaloid genotype. Levels of polyamines in leaves of the two genotypes may indicate differences between the genotypes in the utilization of putrescine for nicotine or polyamine synthesis.

INTRODUCTION

The importance of changes in the nitrogenous component of tobacco leaf during curing is well documented (Leffingwell, 1976; Long and Weybrew, 1981; Smith, 1980). Investigators have reported changes in levels of nitrate, ammonia and amides, and nicotine and other alkaloids, as well as protein and amino acids in leaves during curing. Nitrogenous constituents of tobacco leaf are principal determinants of smoking quality because of their physiological stimulus, flavor, and aroma during smoking. Hydrolysis of protein to amino acids and of starch to reducing sugars during curing precedes the nonenzymatic browning reaction of amino acids with sugars that results in flavorants. Few papers in the literature have reported polyamines in tobacco during development (Hohlt et al., 1970; Irvine and Saxby, 1969; Perdrizet and Prevost, 1981; Smith, 1970; Yoshida, 1969), but polyamine content in cured tobacco leaves is unknown. Polyamines are ubiquitous, naturally occurring products that may be physiologically important in plants. The term polyamines will be used throughout this paper to include both diamines putrescine and cadaverine and the polyamines.

Putrescine is an intermediate in the synthesis of nicotine and is formed from arginine or ornithine in tobacco (Yoshida, 1969). N-methylation of putrescine is an important step in the synthesis of the *N*-methylpyrrolidine ring of nicotine (Dewey et al., 1955; Leete, 1955). Putrescine also is a precursor of spermidine and spermine. Both N-methylation (Mizusaki et al., 1971) of putrescine to *N*-methylputrescine and aminopropylation (Baxter and Coscia, 1973) of putrescine to spermidine require *S*-adenosylmethionine.

Levels of polyamines in tobacco leaves after topping and during curing may be indicative of changes in physiological processes that influence the accumulation of alkaloids in tobacco leaves during maturation and curing. The objective of this research was to compare changes in polyamines and alkaloids during curing of leaves of tobacco genotypes that accumulate different levels of total alkaloids.

MATERIALS AND METHODS

Burley 21 tobacco (*Nicotiana tabacum* L.) of two genotypes near isogenic except at the A and B loci for alkaloid accumulation was used in the study. Genotypes used were the parent Burley 21 and a near-isogenic genotype low-alkaloid Burley 21. These genotypes are designated AABB and aabb, respectively, for alkaloid accumulation. These homozygous combinations were produced by Dr. Glenn Collins from in vitro anther cultures. Plants were grown and cultured in the field using recommended practices for burley tobacco in 1982. Leaf samples were collected on 21 July approximately 3 weeks before topping of the plants, at time of topping on 9 Aug, at harvest on 31 Aug, at end of yellowing on 9 Sept, and as cured leaf on 12 Nov. Samples consisted of leaf tissue from six plants per replicate. The study was replicated three times. All leaf samples were composed of lamina tissue from a 5-cm, midleaf cross section minus the midrib. Samples collected on the first two dates contained tissue from each leaf of the plants. Samples taken after topping were subdivided into four stalk positions from top to bottom, with five leaves per position. Leaf samples were frozen, freeze-dried, and ground before analysis for polyamines and alkaloids.

Polyamine Extraction and HPLC Methodology. Tissue (100 mg dry weight) was homogenized with a Polytron homogenizer in 5 mL of 5% $HClO_4$ in a 15-mL Corex glass centrifuge tube. The homogenate was kept in an ice bath for 1 h before centrifugation at 20000*g* for 20 min at 4 °C. The supernatant containing free polyamines was benzoylated. The pellet was washed twice with 10 mL of 5% $HClO_4$ and once with 10 mL of acetone. The washed pellet was incubated in closed vials containing 2 mL of 6 N HCl at 100 °C for 16 h, after which the solution was centrifuged at 20000*g* for 20 min. The supernatant (1.0 mL) containing polyamines freed from conjugation was mixed with 1.5 mL of HPLC water (Fisher Scientific Co.) and benzoylated.

Benzoylation proceeded by a modified version of methods described elsewhere (Flores and Galston, 1982; Redmond and Tseng, 1979). The supernatant (2.5 mL) was mixed with 5 mL of 2 N NaOH in a 30-mL Corex glass centrifuge tube, and then 0.05 mL of benzoyl chloride was added. The solution was mixed by vortexing for 40 s before heating at 28 °C for 20 min. The solution was added to 10 mL of saturated NaCl in a 50-mL polypropylene centrifuge tube. Diethyl ether (10 mL) was added to extract benzoylamines, and the solvents were mixed well before centrifugation at 5000*g* for 10 min. Five milliliters of the ether phase was transferred to a plastic scintillation vial and dried in a stream of dry air.

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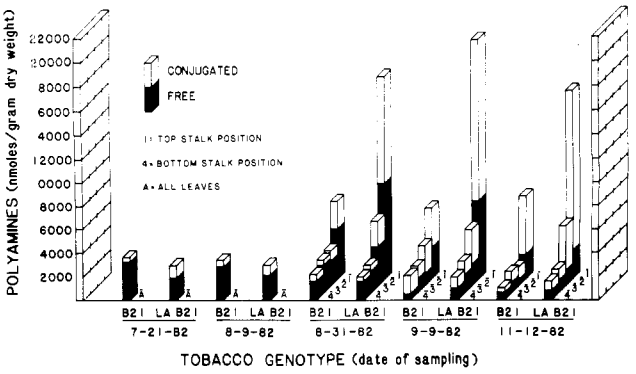

Figure 1. Free and conjugated polyamines in lamina from four stalk positions of Burley 21 (B21) and low-alkaloid Burley 21 (LA B21) genotypes of *Nicotiana tabacum* L. sampled during vegetative growth (7-21-82), at topping (8-9-82), at harvest (8-31-82), at the end of yellowing (9-9-82), and at the ending of curing (11-12-82). LSD values at $p = 0.05$ among free, conjugated, and total forms were 1259, 1409, and 1983 nmol/g dry weight, respectively.

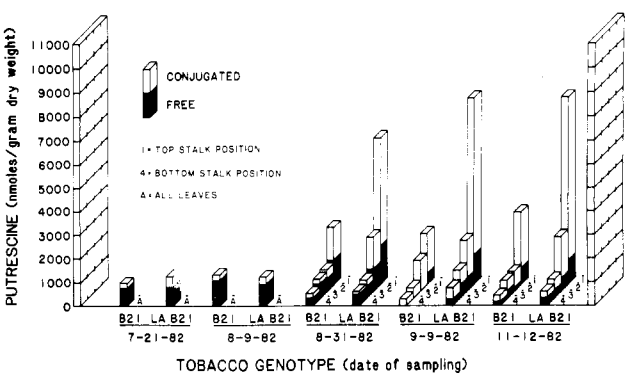

Figure 2. Putrescine, both free and conjugated, in lamina from four stalk positions of Burley 21 (B21) and low-alkaloid Burley 21 (LA B21) genotypes of *Nicotiana tabacum* L. sampled during vegetative growth (7-21-82), at topping (8-9-82), at harvest (8-31-82), at the end of yellowing (9-9-82), and at the end of curing (11-12-82). LSD values at $p = 0.05$ among free, conjugated, and total putrescine were 271, 1069, and 1223 nmol/g dry weight, respectively.

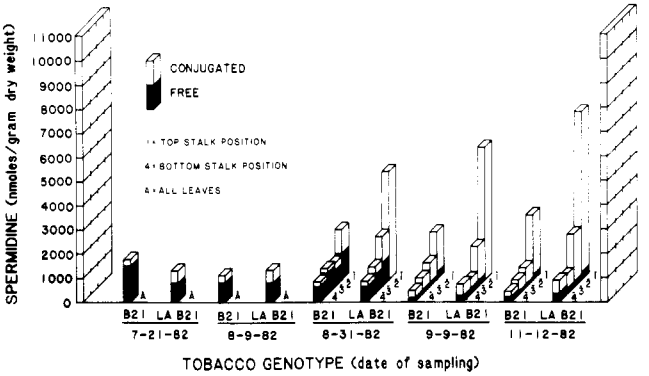

Figure 3. Spermidine, both free and conjugated, in lamina from four stalk positions of Burley 21 (B21) and low-alkaloid Burley 21 (LA B21) genotypes of *Nicotiana tabacum* L. sampled during vegetative growth (7-21-82), at topping (8-9-82), at harvest (8-31-82), at the end of yellowing (9-9-82), and at the end of curing (11-12-82). LSD values at $p = 0.05$ among free, conjugated, and total spermidine were 265, 855, and 943 nmol/g dry weight, respectively.

Benzoylamines were dissolved in 0.12 mL of acetonitrile before injection and separation of a 0.02-mL sample by HPLC. A Varian 5000 liquid chromatograph fitted with a 10 μm silica gel, reversed-phase 30 cm × 4 mm column (C_{18}, MCH-10 N-Cap, Varian) was used. The mobile phase was H_2O at pH 3 (adjusted with H_3PO_4) and acetonitrile with a gradient of 15–100% acetonitrile (85–0% H_2O) over 15 min at a flow of 4 mL/min. Detection occurred at 254 nm. Quantitation was by comparison with authentic standards of known concentration. Polyamines detected were putrescine (Put), spermidine (Spd), spermine (Spm), cadaverine (Cad), and *sym*-norspermidine (*N*-spd).

Alkaloid Extraction and GC Methodology. The extraction method used was essentially the one of Severson et al. (1981). A 37.5-mg sample of tissue was placed in a 2-mL automatic sampling vial with 1.5 mL of 1% (w/v) KOH in methanol with quinoline added as an internal standard. The vial was capped and shaken overnight. Samples were injected on a Varian 8000 series autosampler.

A Varian 3700 GC equipped with a Hewlett-Packard injector and ion-specific detector was used for analysis. Peak integration and data collection were performed with a Varian Vista 401 system. A 50-m WCOT SE-54 fused silica capillary column from Analab was used with a splitless injector. Injector temperature of 220 °C and detector temperature of 230 °C were maintained. Helium was used for the carrier gas. Injector pressure of 3.6 kg/cm^2 resulted in a carrier gas speed of approximately 0.5 m/s.

Oven temperature programming was dependent upon each particular column. An initial 3-min constant temperature was varied from 100 to 140 °C according to column idiosyncracy followed by an increase to 200 °C at 4 °C/min.

RESULTS

Polyamine Changes during Curing. Total free and conjugated polyamines in the two uppermost stalk positions was greater in leaves of the low-alkaloid genotype than in Burley 21 after topping (Figure 1). Total free and conjugated polyamines generally increased in leaves from bottom to top stalk position in both genotypes. Free polyamines decreased, while the conjugated polyamines increased in leaves of both genotypes during curing.

Leaves from the two topmost stalk positions of the low-alkaloid genotype contained more free and conjugated Put than did respective leaves from Burley 21 after topping (Figure 2). Free and conjugated Put generally increased in leaves from the bottom to top of the stalk in both genotypes. Free Put decreased and conjugated Put increased in leaves of both genotypes during curing.

Conjugated Spd in leaves of the low-alkaloid genotype at the top two stalk positions after topping was greater than that in Burley 21 (Figure 3). Free Spd was relatively low and unchanged in leaves from all stalk positions in both genotypes except for an initial decrease during curing. Conjugated Spd increased during curing with the highest concentration in leaves from the top of the stalk.

Spm content was only about 5% that of Put or Spd, and in general the conjugated portion of total Spm was much less than it was for Put and Spd (Figure 4). Spm in leaves of Burley 21 declined initially after topping but increased during curing. Spm in leaves of low-alkaloid Burley 21 generally was greater than that of Burley 21 and declined at all stalk positions by the end of curing. Stalk position effects on Spm were not consistent.

Cad in leaves was almost exclusively in a free form (Figure 5). Cad increased in leaves of Burley 21 before topping and declined throughout the curing process. Cad content in leaves of low-alkaloid Burley 21 increased after topping and continued to increase during curing to levels not different from those of Burley 21 at the end of curing.

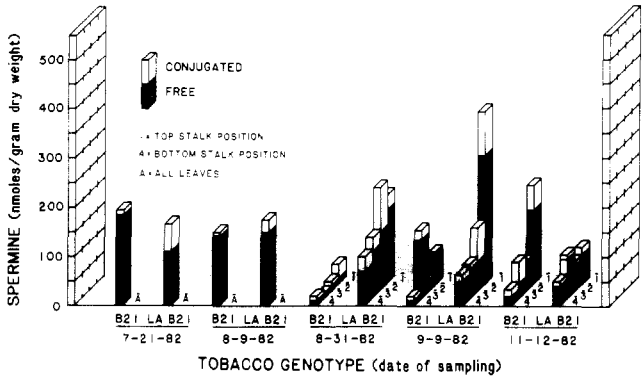

Figure 4. Spermine, both free and conjugated, in lamina from four stalk positions of Burley 21 (B21) and low-alkaloid Burley 21 (LA B21) genotypes of *Nicotiana tabacum* L. sampled during vegetative growth (7-21-82), at topping (8-9-82), at harvest (8-31-82), at the end of yellowing (9-9-82), and at the end of curing (11-12-82). LSD values at $p = 0.05$ among free, conjugated, and total spermine were 86, 47, and 116 nmol/g dry weight, respectively.

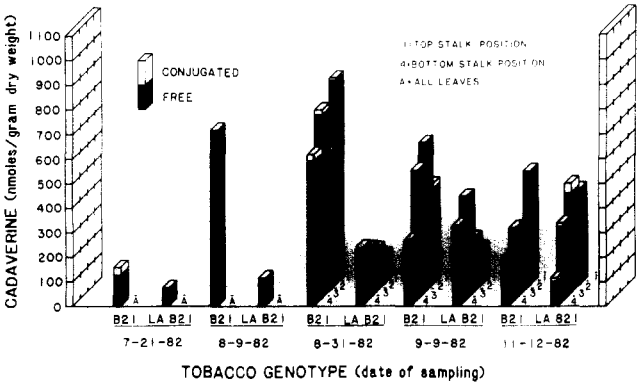

Figure 5. Cadaverine, both free and conjugated, in lamina from four stalk positions of Burley 21 (B21) and low-alkaloid Burley 21 (LA B21) genotypes of *Nicotiana tabacum* L. sampled during vegetative growth (7-21-82), at topping (8-9-82), at harvest (8-31-82), at the end of yellowing (9-9-82), and at the end of curing (11-12-82). LSD values at $p = 0.05$ among free, conjugated, and total cadaverine were 277, 22, and 272 nmol/g dry weight, respectively.

No meaningful differences among stalk positions were measured for Cad.

N-spd content was low in both genotypes before topping (Figure 6). Very little conjugated *N*-spd was found in the leaves of either genotype in this study. Free *N*-spd in leaves of low-alkaloid Burley 21 at the top stalk position was greatly elevated during leaf maturation and through the end of yellowing but declined sharply by the end of curing. Little or no *N*-spd was evident in leaves of either genotype at the end of curing.

Nicotine and Anatabine Changes during Curing. Leaves of Burley 21 contained 7 times more nicotine and 11 times more anatabine than did low-alkaloid Burley 21 (Table I). Nicotine and anatabine content in leaves of Burley 21 increased after topping and during curing. Leaves from the top stalk position of both genotypes contained more nicotine and anatabine compared with leaves lower on the stalk, and nicotine content was much greater than anatabine content at all stalk positions.

DISCUSSION

Cured Burley 21 tobacco leaves contained 0.03% polyamine on a dry weight basis or about 1% the amount of nicotine and 24% the amount of anatabine. Similarly, total free and conjugated polyamines in a cured leaf of

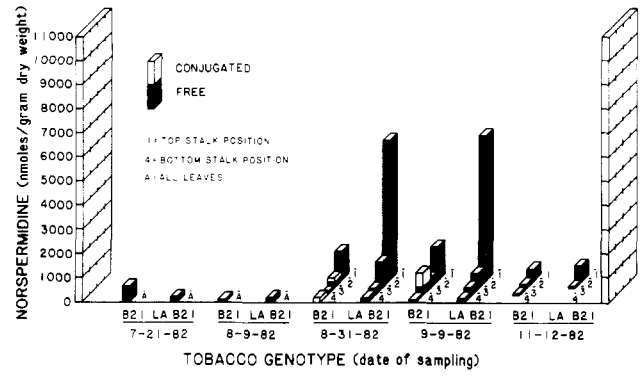

Figure 6. Norspermidine, both free and conjugated, in lamina from four stalk positions of Burley 21 (B21) and low-alkaloid Burley 21 (LA B21) genotypes of *Nicotiana tabacum* L. sampled during vegetative growth (7-21-82), at topping (8-9-82), at harvest (8-31-82), at the end of yellowing (9-9-82), at the end of curing (11-12-82). LSD values at $p = 0.05$ among free, conjugated, and total norspermidine were 1165, 330, and 1208 nmol/g dry weight, respectively.

Table I. Concentration of Nicotine and Anatabine in Lamina from Four Stalk Positions of Burley 21 (B21) and Low-Alkaloid Burley 21 (LA B21) Genotypes of *Nicotiana tabacum* L. during Growth and Curing

sample date (stalk posn)	% dry wt			
	nicotine		anatabine	
	B21	LA B21	B21	LA B21
7-21-82[a]	1.27	0.25	0.035	0.005
8-9-82[a] (topping date)	1.84	0.22	0.050	0.004
8-31-82 (cutting date)				
1[b]	3.26	0.48	0.135	0.009
2	3.20	0.46	0.135	0.012
3	2.40	0.45	0.092	0.010
4	1.98	0.40	0.074	0.008
9-9-82 (end of yellowing)				
1	3.94	0.49	0.142	0.011
2	3.86	0.49	0.139	0.011
3	3.38	0.48	0.116	0.009
4	2.45	0.27	0.083	0.006
11-12-82 (cured)				
1	4.37	0.61	0.152	0.012
2	4.11	0.45	0.151	0.010
3	3.34	0.43	0.114	0.009
4	1.94	0.26	0.077	0.006
LSD = 0.05	0.80	0.12	0.004	0.004

[a] Samples taken on the first two dates consisted of tissue from all leaves of stalk. [b] Stalk position: 1, top; 4, bottom.

low-alkaloid Burley 21 was about 0.05% of the dry weight or about 11% that of nicotine and 550% that of anatabine. On a molar basis the relative value for polyamine with respect to nicotine in Burley 21 becomes slightly larger, but for low-alkaloid Burley 21 the polyamine became approximately 20% of the nicotine. These amounts of polyamines would appear significant in terms of nitrogen metabolism and perhaps sufficient to influence the smoking quality of the cured leaf, particularly leaves at the top stalk position.

Total Put and Spd in leaves of low-alkaloid Burley 21 doubled after topping and during curing, while total Put and Spd in Burley 21 remained the same (Figures 2 and 3). However, all increased levels occurred in leaves from the two top stalk positions. Both genotypes had similar levels of total Put and Spd before and at topping. Increased polyamine levels in low-alkaloid Burley 21 leaves after topping may be due to changes in rates of translocation and/or synthesis and degradation. Total Spd, Spm, and *N*-spd in both genotypes was positively associated with the amount of Put. All correlations were significant at p

< 0.01, and as expected on the basis of the biosynthetic pathway the correlation between Put and Spd was highest, $r = 0.96$.

Changing levels of polyamines in tobacco probably have potential physiological and biochemical implications. It is known that both nicotine and polyamines require Put and *S*-adenosylmethionine during synthesis (Leete, 1955; Baxter and Coscia, 1973). Burley 21 may utilize more Put for nicotine synthesis in roots at the expense of Spd and Spm synthesis. Conversely, greater polyamine levels in low-alkaloid Burley 21 than in Burley 21 may be because of less nicotine synthesis. The greatest increase in polyamine content occurred in upper leaves of low-alkaloid Burley 21, but polyamines increased in the upper leaves of both genotypes. It is known that translocation of nitrogenous compounds from senescing lower leaves to upper leaves occurs during maturation and curing, but the extent of polyamine translocation is not known.

Free Spd in leaves of Burley 21 was inversely correlated with increasing nicotine after topping ($p < 0.01$), an association not evident in the low-alkaloid genotype. However, conjugated Spd increased in both genotypes during curing (Figure 3), which may account partially for the decrease of free Spd in Burley 21.

Results of *N*-spd analysis indicated a low level of *N*-spd in green tobacco leaves (Figure 6). These levels, mostly the free form, remained low in Burley 21 after topping, but free *N*-spd in top leaves of the low-alkaloid genotype increased dramatically from topping to end of yellowing and then virtually disappeared by the end of curing. This response suggests that *N*-spd may be an intermediary in the decomposition of nitrogenous compounds during senescence. The *N*-spd contents further illustrate differences in polyamine metabolism between the two genotypes.

Anatabine is synthesized from two nicotinic acid moieties (Bush, 1981). Differences in anatabine content between Burley 21 and low-alkaloid Burley 21 are a good indication of differences in nicotinic acid metabolism. Quinolinic acid phosphoribosyltransferase (QPT, EC 2.4.4.19) catalyzes the formation of nicotinic acid from quinolinic acid in tobacco roots (Yang et al., 1965). Activity of QPT in tobacco roots is proportional to levels of accumulated nicotine in leaves (Saunders and Bush, 1979). Greater levels of anatabine and total alkaloids in leaves of Burley 21 compared with low-alkaloid Burley 21 are likely the result of greater synthesis of nicotinic acid in roots of Burley 21 after topping. Anatabine levels were correlated with nicotine levels in leaves of Burley 21 ($r = 0.94$, $p < 0.01$) and low-alkaloid Burley 21 ($r = 0.62$, $p < 0.01$). However, anatabine was reduced to a greater extent than nicotine in the low-alkaloid genotype, suggesting that nicotine and anatabine were competing for nicotinic acid moieties for synthesis (Bush, 1981).

It is evident that partitioning of polyamines between free and conjugated forms occurs in tobacco leaves during curing of leaves of both genotypes. Free Put was positively correlated ($p < 0.01$) with conjugated Put, Spd, and Spm. Conjugated Put was positively correlated ($p < 0.01$) with free Put, Spm, and *N*-spd but was negatively correlated ($p < 0.01$) with free Spd. Little is known of the nature of conjugation of polyamines in plants, but polyamines are known to be conjugated with sugars, steroids, phospholipids, and peptides (Ganem, 1982). Increased conjugation during curing may be the result of greater availability of binding sites due to catabolism in senescing leaves.

ACKNOWLEDGMENT

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Registry No. Put, 110-60-1; Cad, 462-94-2; Spd, 124-20-9; Spm, 71-44-3; *N*-spd, 56-18-8; nicotine, 54-11-5; anatabine, 581-49-7.

LITERATURE CITED

Baxter, C.; Coscia, C. J. *Biochem. Biophys. Res. Commun.* **1973**, *54*, 147–154.
Bush, L. P. *Recent Adv. Tob. Sci.* **1981**, *7*, 75–106.
Dewey, L. J.; Byerrum, R. V.; Ball, C. D. *Biochem. Biophys. Acta* **1955**, *18*, 141–142.
Flores, H.; Galston, A. W. *Plant Physiol.* **1982**, *69*, 701–706.
Ganem, B. *Acc. Chem. Res.* **1982**, *15*, 290–298.
Hohlt, H. E.; Maynard, D. N.; Barker, A. V. *J. Am. Chem. Hortic. Sci.* **1970**, *95*, 345–348.
Irvine, W. J.; Saxby, M. J. *Phytochemistry* **1969**, *8*, 473–476.
Leete, E. *Chem. Ind. (London)* **1955**, 537.
Leffingwell, J. C. *Recent Adv. Tob. Sci.* **1976**, *2*, 1–31.
Long, R. C.; Weybrew, J. A. *Recent Adv. Tob. Sci.* **1981**, *7*, 40–74.
Mizusaki, S.; Tanabe, Y; Naguchi, M.; Tamaki, E. *Plant Cell Physiol.* **1971**, *12*, 633–640.
Perdrizet, E.; Prevost, J. *Phytochemistry* **1981**, *20*, 2131–2134.
Redmond, J. W.; Tseng, A. *J. Chromatogr.* **1979**, *170*, 479–481.
Saunders, J. W.; Bush, L. P. *Plant Physiol.* **1979**, *64*, 236–240.
Severson, R. F.; McDuffie, K. L.; Arrendale, R. F. *J. Chromatogr.* **1981**, *211*, 111–121.
Smith, T. A. *Phytochemistry* **1970**, *9*, 1479–1486.
Smith, W. C., Jr. M.S. Thesis, University of Kentucky, Lexington, Ky, 1980.
Yang, K. W.; Gholson, R. K.; Waller, G. R. *J. Am. Chem. Soc.* **1965**, *87*, 4184–4188.
Yoshida, D. *Plant Cell Physiol.* **1969**, *10*, 393–397.

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