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Nicotine Biosynthetic Enzyme Activities in *Nicotiana tabacum* L. Genotypes with Different Alkaloid Levels[1]

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ABSTRACT

Young plants of five *Nicotiana tabacum* L. genotypes were examined for activity of nicotine biosynthetic enzymes. Genotypes near isogenic except at two loci each with two alleles controlling nicotine level were used in a comparison of the four homozygous allelic combinations producing high, high intermediate, low intermediate, and low nicotine levels in a "Burley 21" background. Putrescine *N*-methyltransferase (EC 2.1.1.53) and quinolinic acid phosphoribosyltransferase (EC 2.4.2.19) activities in root tissue of these four genotypes were proportional to leaf nicotine level, whereas *N*-methylputrescine oxidase activity in root tissue differed in proportion and ranking. Quinolinic acid phosphoribosyltransferase activities in leaf tissue were lower than in roots, but no differences were found among the four genotypes. The homozygous recessive alleles at either locus affect levels of all three enzyme activities examined in roots. Each locus seems to be involved in regulation of nicotine metabolism, but whether directly as a regulatory locus or indirectly through the metabolic product of a structural locus is not known.

No difference was observed between enzymic oxidation of putrescine and *N*-methylputrescine by leaf and root extracts of Burley 21 (a high nicotine, low nornicotine genotype) and a high nornicotine cultivar, "Robinson Medium Broadleaf." Putrescine was utilized as a substrate to a greater extent than *N*-methylputrescine by leaf extracts compared with root extracts of both cultivars. It was concluded that genetic differences in levels of nicotine and nornicotine were not due to differences in enzymic oxidation of these two precursors during alkaloid biosynthesis.

Nicotine is the predominant alkaloid in commercial tobacco cultivars, where it is found in concentrations of 2 to 5% of dry weight. Nicotine level as influenced by cultural practices of nitrogen fertilization, plant decapitation, water regime, and axillary bud control has been reviewed recently (2). Low total alkaloid levels are associated with homozygous recessive alleles at both the A and B genetic loci, and intermediate alkaloid levels are found when the homozygous recessive alleles are present at only one of the two loci (11).

Nicotine, composed of single pyridine and *N*-methylpyrrolidine rings linked together, is synthesized primarily in the root (1, 5). Support for the role of ornithine decarboxylase putrescine *N*-methyltransferase (EC 2.1.1.53), and *N*-methylputrescine oxidase in biosynthesis of the *N*-methylpyrrolidine ring is provided by the high level of their activity in tobacco roots but not leaves and the rise in root enzyme activities and nicotine level following plant decapitation (16).

For synthesis of the pyridine ring, quinolinic acid and nicotinic acid are efficient precursors (24). High levels of quinolinic acid phosphoribosyltransferase (EC 2.4.2.19) but not nicotinic acid phosphoribosyltransferase or nicotinamide deamidase in *N. rustica* roots, but not in leaves, suggests that QPT[4] levels may be responsive to changing metabolic demands for the pyridine ring such as likely occurs during nicotine synthesis (12).

In selected tobacco lines the nornicotine levels become greater than those of nicotine. Nornicotine is synthesized primarily by demethylation of nicotine in the leaf. This conclusion is based on nornicotine levels rising "at the expense of" nicotine levels in both green and cured leaf (3, 4, 21, 23), and isotopic labeling studies (10, 19). Other studies have indicated that nornicotine may be synthesized independently of nicotine (8, 9, 22). Putrescine was oxidized 40% as well as *N*-methylputrescine (15), and the enzymic oxidation product of putrescine is 4-aminobutanal, which after spontaneous cyclization to pyrroline might be utilized to synthesize nornicotine. Thus, the role of putrescine oxidation in biosynthesis of nornicotine has not been examined.

In the present study the activities of three enzymes involved in nicotine biosynthesis were measured following decapitation in the four homozygous nicotine level genotypes available at the A and B loci to determine the relationship of these enzymes to the two genetic loci. Relative enzymic oxidation of putrescine and *N*-methylputrescine was determined in extracts of a high and a low nornicotine cultivar of tobacco.

MATERIALS AND METHODS

Plant materials used were Burley 21 tobacco (*Nicotiana tabacum* L.), near isogenic except at the A and B loci where the four combinations of homozygous dominant or recessive alleles were available as the high parent, high intermediate, low intermediate, and low parent. Genetically, these are designated as AABB, AAbb, aaBB, and aabb, respectively. These homozygous allelic combinations were produced by Dr. Glenn Collins through chromosome doubling of haploid tissue from *in vitro* culture of anthers. Seed of a high nornicotine cultivar Robinson Medium Broadleaf was provided by Dr. T. C. Tso.

Seed was germinated in Vermiculite in 45-ml plastic tubes at 26 C and 80% humidity. Seedlings were thinned to one per tube and grown under cool-white fluorescent lighting in dilute Hoagland solution with double KNO_3 and $Ca(NO_3)_2$. For comparison of biosynthetic activity in the four isogenic genotypes, plants at the six- to seven-leaf stage were washed free of Vermiculite and transferred into aerated half-strength nutrient solution in a growth

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[4] Abbreviations: MPO: *N*-methylputrescine oxidase; PMT: putrescine *N*-methyltransferase; QPT: quinolinic acid phosphoribosyltransferase.

chamber with a 14-h day with 26 C and 18 C day-night temperature. After 4 days the plants were transferred to full strength Hoagland solution in continuous light and 26 C in the growth chamber to minimize the confounding of enzyme level response to decapitation with possible diurnal patterns of enzyme activity. Sampling was begun 3 days later when plants were decapitated, trimmed to the two largest leaves, and axillary buds removed. Fresh nutrient solution was provided and axillary buds removed every 48 h thereafter. Plant shoots were dried at 70 C for 24 h and extracted in acidic aqueous methanol for nicotine determination (6, 20).

In the comparison of the high nornicotine (Robinson Medium Broadleaf) and low nornicotine (high parent Burley 21 genotype) lines, plants were started as described above. At the 10- to 12-leaf stage plants were decapitated and transferred to half-strength nutrient solution in continuous light. Leaves and roots were harvested separately 30 h after decapitation.

The temporal pattern of activity of PMT, MPO, and QPT following decapitation was determined separately in roots of the four isogenic genotypes in several experiments with at least four plants at each sampling. Tissue for MPO and QPT was ground in mortar and pestle in the extracting buffer of Mizusaki *et al.* (16). Tissue for PMT assays was ground in 0.5 M Tris-HCl (pH 8.0), 10 mM L-cysteine, 5 mM EDTA, 0.5% (v/v) ascorbic acid, and 0.2 g insoluble polyvinylpolypyrrolidone/g fresh weight tissue. Homogenates were filtered through two layers of cheesecloth and spun down at 20,000*g* for 15 min. Five ml of the supernatant was applied to a 1-cm i.d. column of Sephadex G-25 Fine column previously equilibrated with 25 mM Tris-HCl (pH 7.5), 10 mM mercaptoethanol, and 1 mM EDTA. The eluted protein fraction was used for enzyme assays. All operations were performed at 4 C.

In the four genotype comparison, PMT and MPO were assayed according to Mizusaki *et al.* (16), except that specific radioactivity of *S*-adenosyl-L-[$^{14}CH_3$]methionine was 1.56 μCi/μmol and the specific radioactivity of the *N*-methylputrescine was 2.71 μCi/μmol. QPT was assayed according to the paper chromatographic assay of Mann and Byerrum (12) except that the specific radioactivity of [6-^{14}C]quinolinic acid was 12 μCi/μmol. For the comparison of the high and low nornicotine lines, assay conditions identical to the above MPO assay were used, with either putrescine or *N*-methylputrescine at 2.71 μCi/μmol serving as oxidation substrate. One unit of MPO activity was defined as the formation of 1 μmol of reaction product in 30 min/mg protein. For all enzymes assays, control assays were boiled protein preparations.

RESULTS AND DISCUSSION

A preliminary experiment indicated that leaf nicotine levels of the four genotypes doubled by the 4th day after decapitation under the standard cultural conditions (Fig. 1). Considering the 22-h half-life for nicotine (17), the actual rise in nicotine synthesis may be even greater. Accordingly, the activities of enzymes involved in synthesis of either the pyrrolidine or pyridine moieties of nicotine in the root were followed for 2 to 3 days after decapitation. All three enzymes displayed increased specific activity within 1 or 2 days following decapitation. These increased levels of enzyme activity were not maintained, as also reported by Mizusaki *et al.* (16) and Yoshida (25). Enzyme levels generally ranked the same as nicotine levels for the four genotypes.

QPT activity (Fig. 2) increased in the AABB, AAbb, and aaBB genotypes within 40 h after decapitation. In AABB this amounted to a 3-fold increase in activity relative to undecapitated plants. Increased enzyme activity was less for the intermediate nicotine genotypes and no change was detected for the aabb or low alkaloid parent genotype.

MPO activity (Fig. 3) increased in all genotypes, with a maximum increase of about 30% within 24 h of decapitation. PMT, the other pyrrolidine biosynthetic enzyme examined, showed a similar

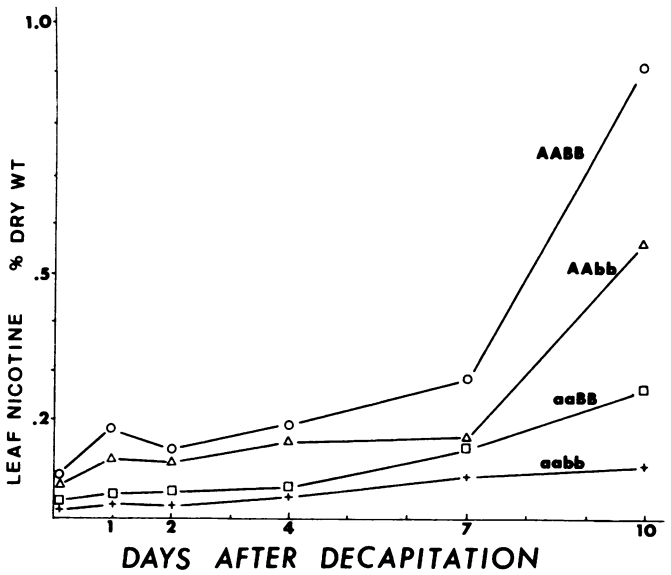

FIG. 1. Changes in leaf nicotine concentration following decapitation for the four genotypes. Each value represents a single measurement from four plants.

pattern of response time and relative activities for the genotypes (Fig. 4).

To visualize better the enzyme activity in the four genotypes, activity levels for all samplings for several experiments for each enzyme were averaged and expressed as percentage of AABB or high alkaloid parent levels. Such averaging is reasonable if, as was the case here, there are no major differences among genotypes for timing of enzyme response to decapitation. Mean leaf nicotine levels for several enzyme experiments are included in the pooled data for comparison (Table I). Relative levels of root QPT and PMT are comparable to leaf nicotine levels of the four genotypes. Root MPO levels differ from leaf nicotine levels in both quantity and ranking.

QPT is the only one of the three enzymes examined in the roots that is thought to be involved in both primary (pyridine nucleotide synthesis) and secondary (nicotine synthesis) metabolism (12). As such, there exists the possibility of both or only one function being associated with the alleles that condition low nicotine level. In an attempt to determine whether the three lower nicotine genotypes are characterized by low primary metabolic QPT activity, enzyme activity was determined in leaves of six- to eight-leaf plants grown under the standard conditions. Total QPT activity in the leaves was found to be low (mean 1.4 nmol nicotinic acid mononucleotide/hr·mg protein), and there were no significant differences among the four genotypes in four samplings. In leaves of AABB (high parent) plants nicotine synthesis as indicated by MPO activity was very low (0.11 units or 1.5% of the activity of comparable roots).

If either the A or B locus is coding for structure or regulation of any single enzymes, we assume that when relative enzyme levels of genotypes are compared, a simple gene-enzyme relationship would be indicated by two groups, each containing one intermediate and one extreme genotype. Members of each group would have in common either the dominant or recessive homozygous allele of the locus being implicated. As no such bimodal grouping was noted for MPO, PMT, or QPT, it appears that none of these enzymes are associated with the A or B locus in a simple manner. A more complex association, perhaps involving some type of coordinate regulation of all three enzymes by one or both loci, is instead suggested by our data. Considering that *N. tabacum* is allotetraploid, present data showing similar effects of the two loci on three enzymes do not exclude the possibility that the A and B loci are homologous.

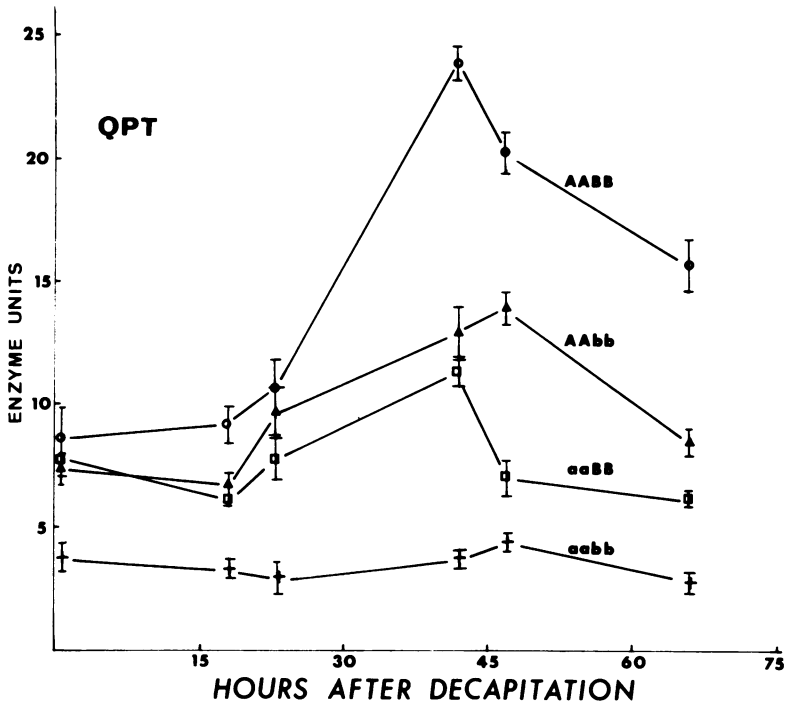

FIG. 2. Changes in root QPT activity in the four genotypes following decapitation. Activity expressed as production of nicotinic acid mononucleotide/h·mg protein. Vertical bars denote 1 SD.

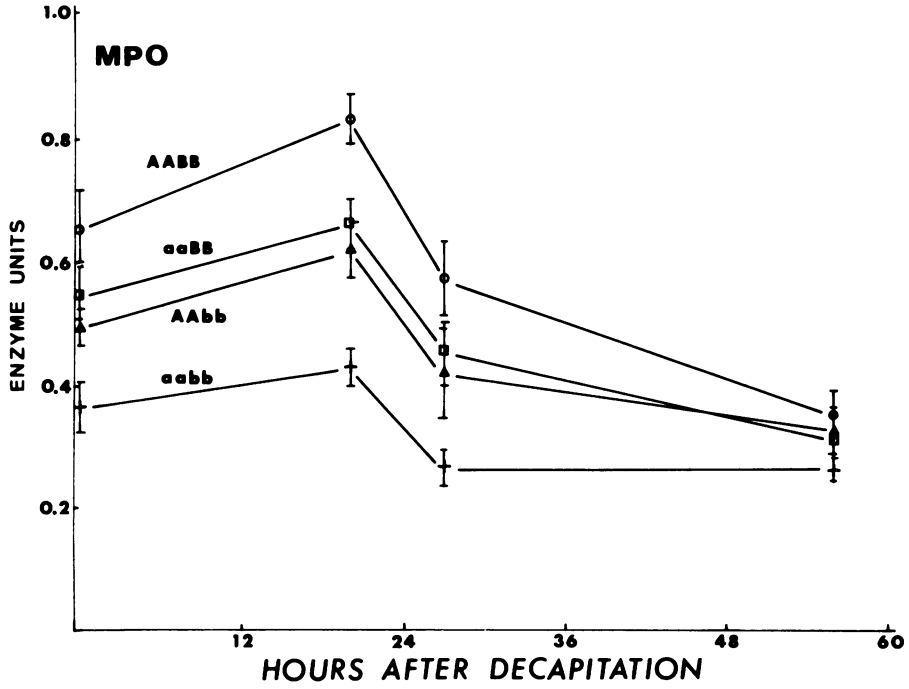

FIG. 3. Changes in root MPO activity in the four nicotine genotypes following decapitation. Activity expressed as nmol *N*-methyl-4-aminobutanal produced/30 min·mg protein. Vertical bars denote 1 SD.

The role of putrescine, *N*-methylputrescine, and *N*-methyl-4-aminobutanal in the biosynthesis of the pyrrolidine ring of nicotine is supported by experiments involving isotope tracers (7, 18), recovery and subsequent incorporation of labeled intermediate (13), isolation of enzymes and determination of substrate specificities (14, 15), and tissue localization and response to decapitation (16). Our data showing that the levels of PMT and MPO are generally proportional to nicotine levels in these four closely related genotypes provides further supportive evidence for the nicotine biosynthetic scheme outlined by Mizusaki *et al.* (15).

Earlier speculation of the role of QPT in nicotine biosynthesis (12), also is supported by present data. The combination of greater activity in roots than leaves, increased activity level in response to plant decapitation, and proportionality to leaf nicotine levels provides strong evidence for the involvement of QPT in nicotine synthesis.

The lower enzyme activity in leaves of nondecapitated plants than in roots was expected because nicotine is synthesized mainly in the root (1, 5). The detection of MPO activity in leaves was contrary to an earlier report (16). The use of *N*-[^{14}C]methylputres-

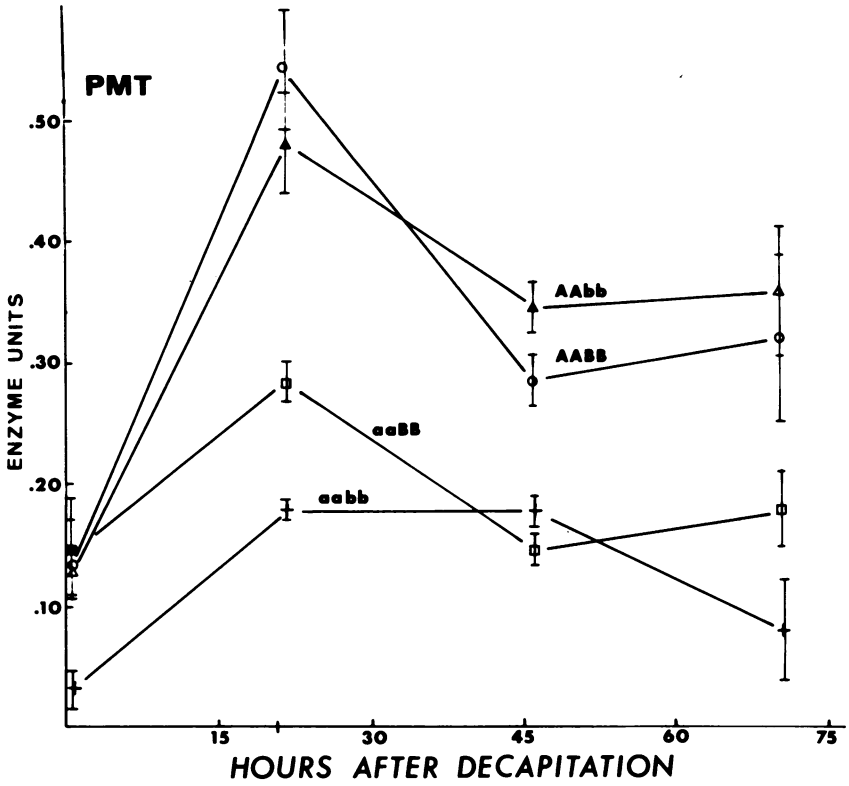

FIG. 4. Changes in root putrescine *N*-methyltransferase in the four nicotine genotypes following decapitation. Activity expressed as production of *N*-methylputrescine/30 min·mg protein. Vertical bars denote 1 SD.

Table I. *Seedling Leaf Nicotine Levels and Enzyme Activities of QPT, MPO, and PMT in Root Tissue Relative to the Level in the High Parent Nicotine Genotype*

Nicotine	Relative Levels[1]				
	Genotype	Leaf nicotine	Root QPT	Root MPO	Root PMT
High parent	AABB	100	100	100	100
High intermediate	AAbb	71	75	84	89
Low intermediate	aaBB	46	50	85	55
Low parent	aabb	26	25	62	28

[1] Average of 16 samplings in three experiments for nicotine and MPO; average of 10 samplings in two experiments for PMT and QPT.

Table II. *Relative Oxidation of Putrescine and N-Methylputrescine by Protein Fractions from Tobacco Cultivars Robinson Medium Broadleaf (RMB) and Burley 21 (B 21)*

Cultivar	Organ	MPO Units[1]	Putrescine[2]
			N-Methylputrescine
RMB	Root	1.24	0.0623
B 21	Root	1.63	0.0650
RMB	Leaf	0.055	0.3048
B 21	Leaf	0.025	0.3058

[1] Average of eight values.

[2] Ratio of dpm/mg protein with putrescine or N-methylputrescine respectively used as substrate.

cine of 25-fold greater specific radioactivity in the present report may explain why MPO activity was detected. MPO activity in leaves of nondecapitated high parent (AABB) plants was 1.5% of the activity in the root. These percentages can be compared to 0.5 to 3% for the estimated proportion of leaf nicotine made in the leaf as determined by reciprocal grafting experiments (5) and to 12 to 16% of leaf nicotine synthesized in the leaf, as calculated from $^{14}CO_2$ fixation data (1).

On a specific activity basis, leaves of nondecapitated AABB plants had about 15% of the QPT activity of roots. Probably only a small fraction of this leaf activity is associated with nicotine biosynthesis. There was no significant difference in QPT activity in the leaves of nondecapitated plants of the four nicotine level genotypes, suggesting that neither the A nor B locus is operative in leaves.

Because the recessive allele at either locus affects levels of all three enzymes in roots, each locus appears to be involved in regulation of nicotine metabolism. Such regulation could be direct, with either or both loci serving as regulatory loci. Alternatively, indirect regulation involving metabolic products of enzymes not assayed here but coded for by the A or B loci also is consistent with present data. Our evidence does not exclude the possibility that either locus codes for the polypeptide sequence of MPO, PMT, or QPT, if the reaction products of one enzyme affect activity of the other two.

QPT activity in roots of nondecapitated aabb plants was low compared to AABB roots and did not rise significantly following decapitation, whereas AABB activity rose 3-fold. Leaf QPT level was 40% of nondecapitated aabb (low parent) root activity and did not differ from those of the other genotypes. If one assumes that fraction I protein is 30% of the protein in our leaf assays and leaf enzyme activities are adjusted by this factor, then the "basal" level of QPT activity in aabb plants is about 2 nmol product/mg protein·h. Thus, only a factor of about 2 for aabb and 12 for AABB would separate basal from maximal QPT activity. This low range in aabb, coupled with the lack of increased activity following decapitation, suggests that a ceiling on QPT activity in aabb plants may limit nicotine synthesis.

When the low nornicotine Burley 21 and the high nornicotine Robinson Medium Broadleaf were compared, no difference was found in relative oxidation of putrescine and *N*-methylputrescine by either leaf or root preparations (Table II). However, putrescine to *N*-methylputrescine ratio as substrate preference was 0.064 and

0.305 for root and leaf extracts, respectively. Because a substrate preference between crude protein preparations from each cultivar is absent, it is unlikely that differences in the levels of nornicotine and nicotine are associated with the diamine oxidation needed for cyclization of the alkaloid precursors.

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LITERATURE CITED

1. ALWORTH WL, RC DESELMS, H RAPAPORT 1964 Biosynthesis of nicotine in *Nicotiana glutinosa* from carbon-^{14}-dioxide. J Am Chem Soc 86: 1608–1616
2. BUSH LP, JW SAUNDERS 1977 Accumulation, manipulation and regulation of nicotine content in tobacco. *In* Recent Advances in the Chemical Composition of Tobacco and Tobacco Smoke. Proc Am Chem Soc Symp, New Orleans, pp 389–425
3. DAWSON RF 1945 An experimental analysis of alkaloid production in *Nicotiana*: the origin of nornicotine. Am J Bot 32: 416–423
4. DAWSON RF 1952 Alkaloid biogenesis: nicotine demethylation in excised leaves of *Nicotiana glutinosa*. Am J Bot 39: 250–253
5. DAWSON RF, ML SOLT 1959 Estimated contributions of root and shoot to the nicotine content of the tobacco plant. Plant Physiol 34: 656–661
6. HARVEY WR, HM STAHR, WC SMITH 1969 Automated determination of reducing sugars and nicotine alkaloids on the same extract of tobacco leaf. Tobacco Sci 13: 13–15
7. KISAKI T, S MIZUSAKI, E TAMAKI 1966 γ-Methylaminobutyraldehyde, a new intermediate in nicotine biosynthesis. Arch Biochem Biophys 117: 677–678
8. KISAKI T, E TAMAKI 1960 The phytochemistry of the tobacco alkaloids. II. Isolation of *d*-form predominant nornicotine from the roots of *Nicotiana tabacum* L. Naturwissenschaften 47: 540–541
9. LADESIC B, TC TSO 1964 Biochemical studies on tobacco alkaloids. VI. Biosynthesis of nicotine through nornicotine. Phytochemistry 3: 541–545
10. LEETE E 1977 Biosynthesis and metabolism of tobacco alkaloids. *In* Recent Advances in the Chemical Composition of Tobacco and Tobacco Smoke. Proc Am Chem Soc Symp, New Orleans, pp 365–388
11. LEGG PD, GB COLLINS 1971 Inheritance of percent total alkaloids in *Nicotiana tabacum* L. II. Genetic effects of two loci in Burley 21 × LA Burley 21 populations. Can J Genet Cytol 13: 287–291
12. MANN DF, RU BYERRUM 1974 Activation of the *de novo* pathway for pyridine nucleotide biosynthesis prior to ricinine biosynthesis in castor beans. Plant Physiol 53: 603–609
13. MIZUSAKI S, T KISAKI, E TAMAKI 1968 Phytochemical studies on the tobacco alkaloids. XII. Identification of γ-methylaminobutyraldehyde and its precursor role in nicotine biosynthesis. Plant Physiol 43: 93–98
14. MIZUSAKI S, Y TANABE, M NOGUCHI, E TAMAKI 1971 The occurrence and properties of putrescine *N*-methyltransferase in tobacco roots. Plant Cell Physiol 12: 633–640
15. MIZUSAKI S, Y TANABE, M NOGUCHI, E TAMAKI 1972 *N*-Methylputrescine oxidase. Phytochemistry 11: 2757–2762
16. MIZUSAKI S, Y TANABE, M NOGUCHI, E TAMAKI 1973 Changes in the activities of ornithine decarboxylase, putrescine *N*-methyltransferase, and *N*-methylputrescine oxidase in tobacco roots in relation to nicotine biosynthesis. Plant Cell Physiol 14: 103–110
17. ROBINSON T 1974 Metabolism and function of alkaloids in plants. Science 184: 430–435
18. SCHUTTE HE, M MAIER, K MOTHES 1966 Methylputrescine as a possible precursor of nicotine in *Nicotiana rustica*. Acta Biochem Polon 13: 401
19. STEPKA W, LJ DEWEY 1961 Conversion of nicotine to nornicotine in harvested tobacco: Fate of the methyl group. Plant Physiol 36: 592–597
20. TECHNICON INDUSTRIAL SYSTEMS 1972 Nicotine in tobacco extracts. Industrial method No. 117-71A, Preliminary
21. TSO TC, RN JEFFREY 1956 Studies on tobacco alkaloids. I. Changes in nicotine and nornicotine content in *Nicotiana*. Plant Physiol 31: 433–440
22. TSO TC, RN JEFFREY1957 Studies on tobacco alkaloids. II. The formation of nicotine and nornicotine in tobacco supplies with N^{15}. Plant Physiol 32: 86–92
23. WADA E 1956 Conversion of nicotine to nornicotine in cherry red tobacco during flue curing. Arch Biochem Biophys 62: 471–475
24. YANG KS, RK GHOULSON, GR WALLER 1965 Studies on nicotine biosynthesis. J Am Chem Soc 87: 4184–4188
25. YOSHIDA D 1973 Mechanisms of alkaloid accumulations in the varieties of tobacco plants containing lower alkaloid. Bull Hatano Tob Exp Sta 73: 245–257