JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Stimulation of Nicotine Demethylation by $NaHCO_3$ Treatment Using Greenhouse-Grown Burley Tobacco†

HONGZHI SHI, NEWTON E. KALENGAMALIRO,* MARC R. KRAUSS, WALTER P. HEMPFLING,‡ AND FERRUCCIO GADANI

Research Center, Philip Morris USA, Richmond, Virginia 23261

Experiments were conducted in tobacco (*Nicotiana tabacum* L.) to investigate the effect of sodium bicarbonate ($NaHCO_3$) on the conversion of nicotine to nornicotine, a secondary alkaloid that can form the tobacco-specific nitrosamine *N*-nitrosonornicotine (NNN). The results showed that, under optimum conditions, $NaHCO_3$ stimulated nicotine conversion in converter plants to the maximum level predetermined by the genetic background. The conversion level in $NaHCO_3$-treated leaves was 2−3 times higher than that in control leaves. For young seedlings the optimum concentration of sodium bicarbonate was 0.8% aqueous solution, and for adult plants the optimum concentration was 1%. Lower concentrations resulted in partial stimulation, whereas higher concentration damaged leaf tissue and resulted in a lower conversion level. Studies with different temperatures (from 22 to 43 °C) showed that 37 °C was optimal. This temperature allowed the least amount of time, 2−3 days for mature leaves and 4−6 days for green leaves, for the major converters to reach >95% of nicotine conversion. An examination of leaves from different growth stages and stalk positions showed that the amount of time needed for conversion was longer for young leaves and shorter for mature leaves. Treatment of leaves with $NaHCO_3$ affords a rapid and convenient means of identifying and removing nornicotine converter plants during growth in the greenhouse or field.

KEYWORDS: Tobacco; nornicotine; nicotine; conversion; $NaHCO_3$; demethylation

INTRODUCTION

Nicotine is the predominant alkaloid in commercial burley tobacco (*Nicotiana tabacum* L.) and usually accounts for >94% of the total alkaloid fraction. The content of the secondary alkaloid nornicotine is normally <3.5% (*1*, *2*). As early as the middle part of the 20th century, Goodspeed (*3*) and Gerstel (*4*) reported that nornicotine was the major alkaloid in *Nicotiana sylvestris* and *Nicotiana tomentosformis*, progenitor species of tobacco. In certain commercial burley varieties, some plants convert a substantial amount of nicotine into nornicotine. Mann et al. (*5*) studied inheritance of the conversion of nicotine to nornicotine in varieties of *N. tabacum* and related amphiploids and found that plants that contained high amounts of nornicotine differed from those that did not by a single dominant gene. Nicotine to nornicotine conversion is undesirable, because high levels of nornicotine directly contribute to the formation of *N*-nitrosonornicotine (NNN), an important tobacco-specific nitrosamine, and other undesirable compounds, such as acylated nornicotines (which decrease the tobacco flavor quality). Harada (*6*) compared the smoking quality among Burley 21 lines with different levels of nicotine conversion and found an inverse relationship between the nornicotine content and smoking quality that was nearly linear. Tobacco varieties with high levels of nornicotine were characterized with a nornicotine odor and a weak burley aroma. These results were corroborated by Roberts (*7*), who studied natural tobacco flavor and found that during pyrolysis nornicotine produced myosmine and substituted pyridine compounds that created objectionable smoke flavors such as an alkaline taste and a mousy aroma. Leaves containing high levels of nornicotine tend to contain high levels of NNN. In 30 plants with different nornicotine levels, there was a significant positive correlation between nornicotine and NNN concentrations (*8*, *9*). A similar correlation was also found in Chinese tobacco (*10*). The identification and removal of converter plants from the population as early as possible would be of great significance to ensure high-quality tobacco production. Breeding lines and subsequent varieties are tested for nornicotine content during development and subsequent release of breeder's seed.

Harvesting of burley tobacco plants in the United States usually consists of removing the entire plant from the field with one cut 3−4 in. above the ground. Five to six plants are placed on a stick and hung in a barn or outside structure to air cure, which is a slow drying process of the tobacco plant. Complex physical and chemical changes occur during curing of the plants (*11*). Nicotine to nornicotine conversion, which is mediated by nicotine demethylase (*12*−*14*), begins in green leaves and

† Presented in part at the 2002 Congress of the Cooperation Center for Scientific Research Relative to Tobacco (CORESTA), New Orleans, LA, Sept 22−27, 2002.

* Author to whom correspondence should be addressed [telephone (804) 274-1610; e-mail newton.e.kalengamaliro@pmusa.com].

‡ Present address: 9183 Madison Leigh Court, Old Church, VA 23111.

10.1021/jf030136r CCC: $25.00
Published on Web 11/15/2003

Table 1. Difference in Percent Nicotine Conversion between $NaHCO_3$-Treated Leaves and Control Leaves

		$NaHCO_3$ -treated leaves[a]			control leaves[a]		
plant[b]	type	nicotine (%)	nornicotine (%)	% nicotine conversion	nicotine (%)	nornicotine (%)	% nicotine conversion
young plants	nonconverter	0.396	0.012	2.85 (0.42)	0.428	0.011	2.49 (0.39)
	minor converter	0.259	0.150	36.67 (5.82)	0.354	0.032	8.28 (3.25)
	major converter	0.018	0.325	94.75 (4.09)	0.247	0.118	32.42 (5.23)
adult plants	nonconverter	1.132	0.021	1.85 (0.32)	1.089	0.022	1.99 (0.41)
	minor converter	0.801	0.429	34.88 (5.24)	0.885	0.180	16.92 (3.56)
	major converter	0.041	0.596	93.56 (5.59)	0.523	0.399	43.29 (6.18)

[a] All data were the average of 10–15 plants; the data in parentheses are standard deviations. [b] Young plants were 1 week after transplanting, third and fourth leaves from top were detached, one was treated with 0.8% $NaHCO_3$ and the other as control (sprayed with H_2O). Adult plants were 4 weeks after transplanting, fourth and fifth leaves were detached, one treated with 1% $NaHCO_3$ and the other as control. All leaves were cured for 6 days in a chamber at 33 °C and 80% RH.

increases during the postharvest treatment. The conversion activity peaks during the first 3 weeks of air-curing in burley tobacco, when the process of leaf senescence is accelerated (*8*, *15*, *16*). To remove the converter plants from the population while the plants are still growing, stimulation of nicotine conversion is required for identification of converters. Shi et al. (*17*), studying methods for identifying converter plants, reported the stimulating effect of ethylene on nicotine to nornicotine conversion. Miller et al. (*18*) used the treatment with the plant growth regulator ethephon (2-chloroethylphosphonic acid) to stimulate nicotine conversion of green tobacco leaves to identify converters in the breeding process. The application protocol requires a 48 h period before the treated material can be handled safely. To develop a more effective and convenient way to identify nicotine converters at early plant growth stage, a series of chemical compounds were screened as alternatives to ethephon, and $NaHCO_3$ was found to be very effective in stimulating nicotine to nornicotine conversion in green leaves.

The objective of the present study was to investigate the stimulating effect of an aqueous solution of sodium bicarbonate on nicotine to nornicotine conversion and to design a method for the rapid identification of converter plants in the greenhouse and in the field.

MATERIALS AND METHODS

Plants and Sampling. Nonconverter and converter plants of burley variety TN90 were used in the study. Plants were generated from the seeds produced in the previous TN90 generation in which the nontopped plants had been identified as converters and nonconverters using the method of ethephon treatment (*17*). Seeds were germinated in media, and the young seedlings were transplanted to 5 L pots. Hoagland's (*19*) solution (50 mL) was applied every second day. Plants were individually labeled at transplanting. The sampling of the leaves was based upon the objective of each experiment. For the experiments that were aimed at the determination of the stimulating effect of $NaHCO_3$ on nicotine conversion, two adjacent leaves from each plant (converters or nonconverters) were detached, one being thoroughly sprayed with 1% $NaHCO_3$ aqueous solution and the other sprayed with water (H_2O) to be used as the control. For the experiments in which the effect of the leaf position on the stimulation of $NaHCO_3$ on nicotine conversion was evaluated, only converter plants were used, and all of the leaves were detached at the same time. The sampling methods in other experiments (curing temperature and $NaHCO_3$ concentration) will be described subsequently.

Concentration of $NaHCO_3$. Plants were divided into nonconverter [percent nicotine conversion (PNC) <5], minor converter ($5 \leq$ PNC ≤ 20), and major converter (PNC > 20) groups. Experiments were conducted to determine the optimum concentration for maximum stimulation of nicotine conversion on both adult converter plants (4 weeks after transplanting) and young converter plants (1 week after transplanting). For mature plants, the concentrations of $NaHCO_3$ used were 0 (H_2O only), 0.5, 1.0, and 1.5%; for young plants, the concentrations were 0 (H_2O only), 0.5, 0.8, and 1.0%. The third and fourth leaves from the top of each individual plant were detached, thoroughly sprayed with aqueous $NaHCO_3$ solution or water, and cured in conditioned chambers.

Curing Conditions. Different temperature settings were tested to determine the optimum conditions for the stimulation of nicotine demethylation by $NaHCO_3$. Converter leaves of the same age were treated with $NaHCO_3$, divided into five groups, and cured in separate chambers with five different temperature settings. The temperature settings were 23, 30, 37, 40, and 43 °C, respectively. A relative humidity (RH) of 80% was maintained for each treatment to keep leaves from drying too rapidly. Samples were taken at different periods to determine the time course changes in nicotine conversion.

Alkaloid Measurement. Methyl *tert*-butyl ether (MTBE) was used as extraction agent and quinoline as internal standard. For each sample, 100 mg of ground tobacco was accurately weighed into a culture tube, and 0.5 mL of 2 N NaOH was added to moisten the tobacco sample for 15 min. Then 5 mL of extraction solution was added into the tube to extract alkaloids. Samples were shaken in a linear shaker for 2 h with a capped tube. After the solvent and sample were separated, aliquots from the extraction were transferred to a GC vial for alkaloid separation and quantification. An HP-6890 (Agilent, Inc., Palo Alto, CA) gas chromatograph equipped with a DB-5 capillary column was used to determine the alkaloid contents, using a modified method of Severson et al. (*20*).

In the study, percent nicotine conversion was used to express the conversion level of tobacco samples. The formula used for calculating percent nicotine conversion was

$$\%\ \text{nicotine conversion (PNC)} = 100 \times \text{nornicotine content}/(\text{nicotine content} + \text{nornicotine content})$$

RESULTS

Effect of $NaHCO_3$ Treatment on Nicotine to Nornicotine Conversion in Individual Plants. Leaves from young plants (1 week after transplanting) and mature plants before topping (topping is a procedure routinely performed in field production of tobacco that removes the inflorescence from a plant) were tested in separate experiments to determine the effect of $NaHCO_3$ on nicotine to nornicotine conversion. Plants included nonconverter plants and converter plants with different degrees of conversion. The results of this experiment are summarized in **Table 1** and indicate that for nonconverter plants, there was no significant difference in the conversion level between $NaHCO_3$-treated leaves and the control after 6 days of curing. For converter plants, the percent nicotine conversion was generally 2−3 times higher in $NaHCO_3$-treated leaves than in control leaves. It should be noted that the percent nicotine conversion of $NaHCO_3$-treated major converter leaves reached $>93\%$. This indicated that the $NaHCO_3$ treatment was able to

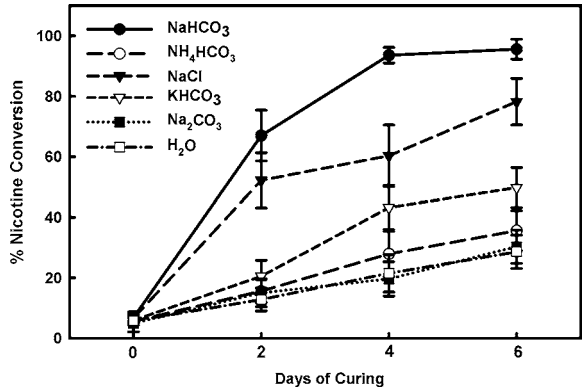

Figure 1. Effect of different salts on nicotine to nornicotine conversion in converter leaves (32 °C, 80% RH).

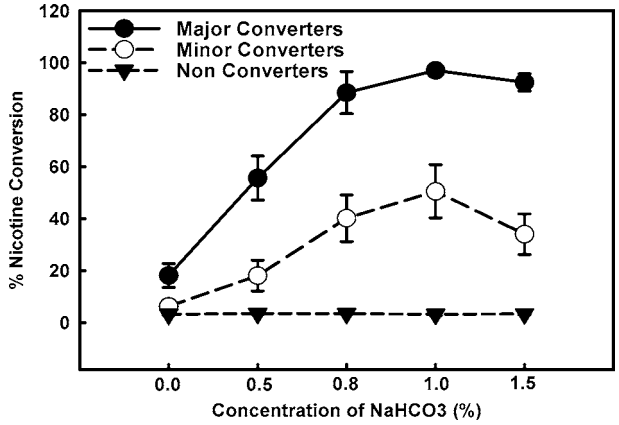

Figure 2. Effect of $NaHCO_3$ concentration on nicotine to nornicotine conversion in adult (4 weeks after transplanting) converter plants (33 °C, 80% RH). Samples were treated with 0.5, 0.8, 1, and 1.5% aqueous solutions of $NaHCO_3$ prior to the start of curing. Each data point is a mean of 12 plants, and error bars (where larger than the symbol) represent two standard deviations of the mean.

stimulate nicotine to nornicotine conversion to the maximum level determined by the genetic background and that it could be effectively used for the identification of nicotine to nornicotine converters.

An experiment was also set up to determine the stimulating effect of other salts on nicotine to nornicotine conversion in major converter leaves, including Na_2CO_3, NH_4HCO_3, $KHCO_3$, and NaCl, with the concentration of 1% for each salt. The results show that NaCl and $KHCO_3$ also exhibit some degree of stimulating effect on nicotine conversion, but much less than $NaHCO_3$. NH_4HCO_3 and Na_2CO_3 had virtually no effect at the concentrations that were tested (**Figure 1**).

Effect of $NaHCO_3$ Concentration on the Stimulation of Nicotine Conversion. The results from experiments with young plants and adult plants showed a great impact of $NaHCO_3$ concentration on the level of nicotine conversion. For leaves from adult plants, the optimum $NaHCO_3$ concentration found to stimulate nicotine conversion was 1%. This was evidenced by the highest conversion level obtained and shortest time required to reach completion. **Figure 2** shows the effect of $NaHCO_3$ concentration on nicotine to nornicotine conversion in adult converter plants. The samples were taken after 4 days of curing after $NaHCO_3$ treatment. The percent nicotine conversion reached >95% after 4 days of treatment with 1% $NaHCO_3$. Lower concentrations of $NaHCO_3$ resulted in partial stimulation and required more time to reach a certain conversion level. Higher concentrations of $NaHCO_3$ tended to destroy the leaf

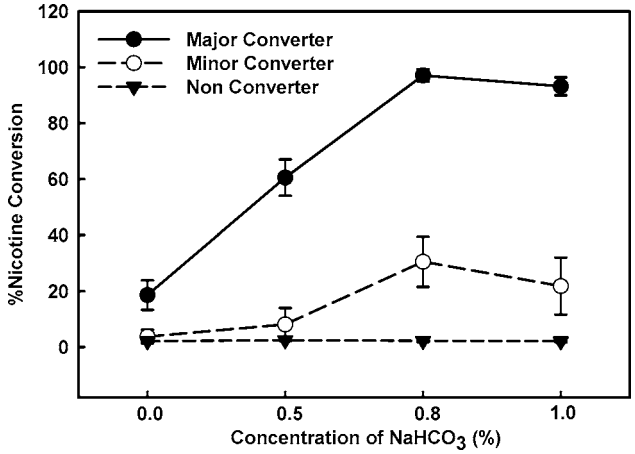

Figure 3. Effect of $NaHCO_3$ concentration on nicotine to nornicotine conversion in young (1 week after transplanting) converter plants (33 °C, 80% RH). Leaves were treated with 0.5, 0.8, 1.0% aqueous solutions of $NaHCO_3$ prior to the start of curing. Each data point is a mean of 12 plants, and error bars (where larger than the symbol) represent two standard deviations of the mean.

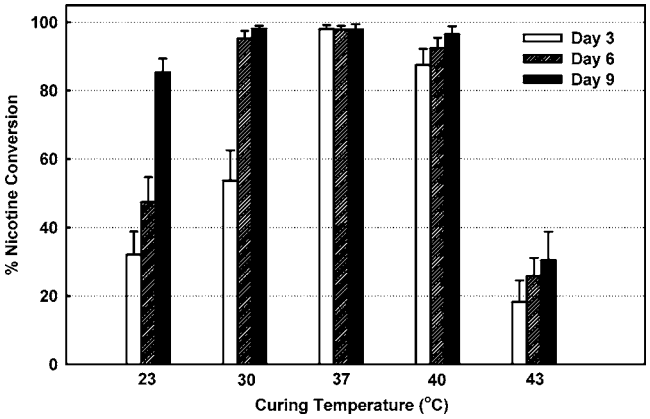

Figure 4. Effect of temperature on nicotine conversion in converter leaves treated with $NaHCO_3$ (85% RH). Burley tobacco leaf samples were drawn at 3, 6, and 9 days from five curing chambers set at 23, 30, 37, 40, and 43 °C, respectively. Each bar is a mean of 12 plants, and the error bars represent two standard deviations of the mean.

tissue and resulted in incomplete nicotine conversion. The stimulation of nicotine conversion after $NaHCO_3$ treatment was accompanied by acceleration of leaf yellowing followed by browning.

Young leaves from seedlings were tender and more sensitive to $NaHCO_3$ treatment. The optimum concentration was determined to be 0.8%. Necrosis of leaf tissue rapidly developed when the $NaHCO_3$ concentration exceeded 0.8%, resulting in decreased conversion levels (**Figure 3**).

Effect of Temperature on Stimulation of Nicotine to Nornicotine Conversion by $NaHCO_3$. The curing temperature after $NaHCO_3$ treatment was crucial for obtaining maximum stimulation of nicotine to nornicotine conversion. In our experiment, 35 converter green leaves of the same age were divided into different groups and cured in chambers with different temperature settings. It was found that there were significant differences in nicotine conversion levels among leaves cured at different temperatures (**Figure 4**). At 37 °C, the conversion level reached the maximum value after 3 days of curing. At decreased temperatures, the conversion level was lower and the time required for conversion was lengthened. At 23 °C, the percent nicotine conversion was only 32% after 3

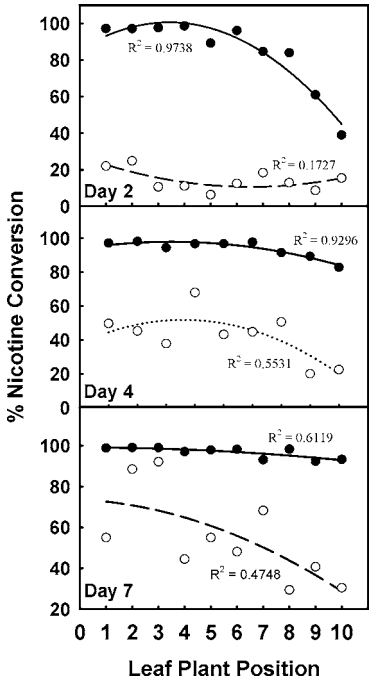

Figure 5. Effect of $NaHCO_3$ treatment (●) [control (○)] on nicotine to nornicotine conversion in burley leaves from different stalk positions. Samples were sprayed with 1% $NaHCO_3$ at harvest and cured in a curing chamber set at 33 °C and 80% relative humidity. Samples were taken at 2, 4, and 7 days. Each data point is a mean of 12 plants.

days of curing. After 9 days at 23 °C, the percent nicotine conversion was still <90%. Temperatures exceeding 37 °C were also unfavorable to the stimulation of nicotine to nornicotine conversion. At 40 °C, dead green spots in the leaves appeared, and at 43 °C almost all of the leaves were rapidly damaged.

Keeping sufficient moisture in the curing environment was also required to prevent leaves from drying too rapidly and thus to ensure the enzymatic conversion of nicotine to nornicotine. We suggest that a relative humidity of >75% be maintained during the curing process after $NaHCO_3$ treatment.

Response of Leaves of Different Ages to $NaHCO_3$ Treatment. All of the leaf samples from mature converter plants were collected 1 week prior to topping to examine the differences in the response of $NaHCO_3$ treatment among leaves at different physiological ages. At this time, the bottom leaves showed a consistent yellow color and were considered to be over-ripe, whereas the top leaves were young, dark green in color, and not fully developed. The results showed that the nicotine conversion in bottom leaves reached maximum level after only 2 days of curing after $NaHCO_3$ treatment (**Figure 5**). As the leaf position shifted to the top of the plant, the conversion level decreased sequentially. By extending the curing time, the differences in the conversion level among leaves from different stalk positions decreased as more and more leaves reached the maximum conversion level. Also, as the curing time increased, the difference in conversion level between $NaHCO_3$-treated leaves and nontreated leaves decreased. The results indicated that young/immature leaves required more time for maximum conversion of nicotine to nornicotine. Thus, the older leaves were more desirable for use in the $NaHCO_3$ treatment to identify nicotine converters.

Consistency of Stimulation of Nicotine Conversion by $NaHCO_3$ at Different Growth Stages. Individual converter plants were labeled, and leaves were taken at different plant growth stages to determine the levels of nicotine to nornicotine conversion. The stages were 1 week after transplanting, 3 weeks after transplanting, at topping, and at harvest. The results showed that the stimulation of nicotine conversion by $NaHCO_3$ treatment before topping was very reliable. Regardless of when the leaf samples were taken, a percent nicotine conversion of >95% in converter leaves could be obtained. We measured lower conversion levels in mature leaves from both major converter plants and minor converter plants sampled at harvest time. We suspect that the lower percent nicotine conversion was related to nicotine accumulation after plants were topped. It should be noted that the total nicotine content in mature leaves was >3.5%, but before topping the total nicotine content was <1.6%. A high concentration of nicotine may increase the competition among nicotine molecules to combine with nicotine demethylase, the amount of which may be limited. The result was coincident with our observation that the percent nicotine conversion in greenhouse-grown tobacco is usually higher than that in field-grown tobacco in which the alkaloid content is higher.

DISCUSSION

These results are similar to the findings of Shi et al. (*8*, *10*), who observed elevated conversion of nornicotine in leaf samples of converter plants treated with ethephon. Ethephon is a systemic plant growth regulator, and when applied to plant tissues, it decomposes into ethylene, which is the active ingredient that leads to leaf senescence and nicotine conversion. From the similarities observed between ethephon and sodium bicarbonate, we speculate that sodium bicarbonate may stimulate ACC oxidase (1-aminocyclopropane-1-carboxylate (ACC) oxidase activity. Kende (*21*) suggested that the final step in ethylene biosynthesis in higher plants is catalyzed by ACC oxidase. From the results and observations made in this work it seems that tobacco ripening is climacteric in nature, characterized by high respiration and ethylene production. Biale and Young (*22*) studied respiration and ripening in fruits and found that ethylene plays a major role in ripening by stimulating autocatalytic production of endogenous ethylene. Current work will explore the mechanism through which sodium bicarbonate stimulates nicotine conversion.

$NaHCO_3$ was found to be effective in stimulating nicotine to nornicotine conversion in green tobacco leaves and can be used to identify nicotine to nornicotine converters while the plants are still growing. Converter plants can thus be removed at early plant growth stages. This is especially useful during the breeding process, when the converters should be identified and removed before flowering to ensure the production of pure nonconverter seeds. Additionally, sodium bicarbonate is environmentally friendly, economical, and convenient to use. It may also provide a possible alternative for other crops and plants to accelerate maturity and senescence.

There are several factors that have significant impact on the effectiveness of identifying nicotine converters by $NaHCO_3$ treatment. The concentration of $NaHCO_3$ used and the curing temperature were crucial for maximum stimulation. Lower $NaHCO_3$ concentrations and lower curing temperatures resulted in partial stimulation and longer periods of time for full expression of conversion; higher $NaHCO_3$ concentrations and higher curing temperatures could destroy leaf tissue or terminate the conversion process mediated by the nicotine demethylase. The stimulating effect, which was accompanied by accelerated yellowing of the leaf, was consistent for plants at different growth stages before topping. Younger leaves required a longer period of time for maximum nicotine conversion than older leaves. Current work in our laboratory is focusing on developing

molecular techniques that may eventually replace the current techniques for screening nicotine converter plants.

ACKNOWLEDGMENT

We express sincere thanks to the following colleagues for the valuable help they provided: Gordon Bokelman, Stephen Haut, Geoffrey Chan, Dale Hill, Jodie Clark, Cheryl Guthrie, Julie May, Jia Wang, and Qinglin (Alan) Li (Philip Morris USA); and Darin Colassaco, Gary Boykin, and Adetoun Adeniji-Adele (Lancaster Laboratories).

LITERATURE CITED

(1) Bush, L. P. Physiology and biochemistry of tobacco alkaloids. *Recent Adv. Tob. Sci.* **1981**, *7*, 75−106.

(2) Bush, L. P.; Fannin, F. F.; Chelvarajan, R. L.; Burton, H. R. Biosynthesis and metabolism of nicotine and related alkaloids. *Nicotine and Related Alkaloids*; Gorrod, J. W., Wahren, J., Eds.; Chapman and Hall: London, U.K., 1993.

(3) Goodspeed, T. H. *The genus Nicotiana*, 1st ed.; Chronica Britanica: Waltham, MA, 1954; p 373.

(4) Gerstel, D. U. Genetic instability of a tobacco mutant. *Tob. Sci.* **1969**, *13*, 94−94.

(5) Mann, T. J.; Weybrew, J. A.; Matzinger, D. F.; Hall, J. L. Inheritance of conversion of nicotine to nornicotine in varieties of *Nicotiana tabacum* and related amphiploids. *Crop Sci.* **1958**, *4*, 349−353.

(6) Harada, T. Studies of breeding of burley tobacco with respect to the nicotine−nornicotine conversion. *Bull. Morioka Tob. Exp. Stn.* **1985**, *19*, 1−80.

(7) Roberts, D. L. Natural tobacco flavor. *Recent Adv. Tob. Sci.* **1988**, *14*, 49−81.

(8) Shi, H.; Fannin, F. F.; Burton, H. R.; Bush, L. P. Factors affecting nicotine to nornicotine conversion in burley tobacco. Presented at the 54th Tobacco Science Research Conference, Nashville, TN, 2000; Abstract 5451.

(9) Bush, L. P.; Cui, M.; Shi, H.; Burton, H. R.; et al. Formation of tobacco-specific nitrosamines in air-cured tobacco. *Recent Adv. Tob. Sci.* **2001**, 55.

(10) Shi, H.; Huang, Y.; Bush, L. P.; et al. Alkaloid and TSNA contents in Chinese tobacco and cigarettes. Presented at the CORESTA Congress, Lisbon, Portugal, 2000; Abstract AP3, p53.

(11) Palmer, G. K.; Pearce, R. C. Light air-cured tobacco. In *Tobacco Production, Chemistry and Technology*; Davis, D. L., Nielson, M. T., Eds.; Blackwell Science: Oxford, U.K., 1999.

(12) Dawson, R. F. Alkaloid biosynthesis: nicotine demethylation in excised leaves of *Nicotiana glutinosa*. *Am. J. Bot.* **1952**, *39*, 250−253.

(13) Chelvarajan, R. L.; Fannin, F. F.; Bush, L. P. Study of nicotine demethylation in *Nicotiana otophora*. *J. Agric. Food Chem.* **1993**, *41*, 858−862.

(14) Hao, D. Y.; Yeoman, M. M. Mechanism of nicotine *N*-demethylation in tobacco cell suspension cultures. *Phytochemistry* **1996**, *41*, 477−482.

(15) Bush, L. P.; Zhan, Y.; Yang, H.; Shi, H.; Fannin, F. F.; Burton, H. R. Time of nornicotine formation in burley tobacco. Presented at CORESTA, Suzhou, China, 1999; Abstract AP37.

(16) Fannin, F. F.; Bush, L. P. Nicotine demethylation in *Nicotiana*. *Med. Sci. Res.* **1992**, *20*, 867−868.

(17) Shi, H.; Fannin, F. F.; Burton, H. R.; Bush, L. P. Identification of nicotine to nornicotine converters in burley tobacco. Presented at the 55th Tobacco Science Research Conference, Greensboro, NC, 2001.

(18) Miller, R. D.; Bush, L. P.; Burton, H. R.; Shi, H. Screening for nornicotine conversion in burley tobacco. Presented at the 55th Tobacco Science Research Conference, Greensboro, NC, 2001.

(19) Hoagland, D. R.; Arnon, D. I. The water culture method for growing plants without soil. *Calif. Agric. Exp. Stn., Circ.* **1950**, *No. 347*.

(20) Severson, R. F.; McDuffie, K. L.; Arrendale, R. T.; Gwynn, G. R.; Chaplin, J. F.; Johnson, A. W. Rapid method for the analysis of tobacco nicotine alkaloids. *J. Chromatogr.* **1981**, *211*, 111−121.

(21) Kende, H. Ethylene biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1993**, *44*, 283−307.

(22) Biale J. B.; Young, R. E. Respiration and ripening in fruits: retrospect and prospect. In *Recent Advances in Biochemistry of Fruits and Vegetables*; Friend, J., Rhodes, M. J. C., Eds.; Academic Press: London, U.K., 1981; pp 1−39.

Received for review February 25, 2003. Revised manuscript received September 16, 2003. Accepted September 22, 2003.

JF030136R