Impact of Alleles at the *Yellow Burley* (*Yb*) Loci and Nitrogen Fertilization Rate on Nitrogen Utilization Efficiency and Tobacco-Specific Nitrosamine (TSNA) Formation in Air-Cured Tobacco

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ABSTRACT: Tobacco-specific nitrosamine (TSNA) formation in tobacco is influenced by alkaloid levels and the availability of nitrosating agents. Tobacco types differ in their potential for TSNA accumulation due to genetic, agronomic, and curing factors. Highest TSNA concentrations are typically measured in burley tobaccos. One of the main genetic differences between burley and all other tobacco types is that this tobacco type is homozygous for recessive mutant alleles at the *Yellow Burley 1* (*Yb₁*) and *Yellow Burley 2* (*Yb₂*) loci. In addition, burley tobacco is typically fertilized at higher nitrogen (N) rates than most other tobacco types. This study utilized nearly isogenic lines (NILs) differing for the presence of dominant or recessive alleles at the Yb_1 and Yb_2 loci to investigate the potential influence of genes at these loci on TSNA accumulation. Three pairs of NILs were evaluated at three different nitrogen fertilization rates for alkaloid levels, nitrogen physiology measures, and TSNA accumulation after air-curing. As previously observed by others, positive correlations were observed between N application rates and TSNA accumulation. Recessive alleles at Yb_1 and Yb_2 were associated with increased alkaloid levels, reduced nitrogen use efficiency, reduced nitrogen utilization efficiency, and increased leaf nitrate nitrogen (NO_3-N). Acting together, these factors contributed to significantly greater TSNA levels in genotypes possessing the recessive alleles at these two loci relative to those carrying the dominant alleles. The chlorophyll-deficient phenotype conferred by the recessive yb_1 and yb_2 alleles probably contributes in a substantial way to increase available NO_3-N during curing and, consequently, increased potential for TSNA formation.

KEYWORDS: tobacco, tobacco-specific nitrosamines (TSNA), nitrogen utilization efficiency, nitrosating agents

INTRODUCTION

Tobacco-specific nitrosamines (TSNAs) are a class of toxicants present in combustible and smokeless tobacco products. TSNAs are generated through the nitrosation of pyridine alkaloids during the curing, processing, and storage of cured tobacco. They may also be produced during pyrolysis, although the relative importance of this route of formation is unclear.[1−6] *N*-Nitrosonornicotine (NNN), *N*-nitrosoanatabine (NAT), and *N*-nitrosoanabasine (NAB) are derived directly from nitrosation of nornicotine, anatabine, and anabasine, respectively. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is likely derived from nicotine via a nitrosation reaction involving an intermediate, possibly pseudo-oxynicotine.[7]

There is strong motivation to reduce TSNAs in tobacco products because of their implied contribution to unwanted health effects, such as cancer, in users of these products.[8−10] Whereas NAT and NAB are reported to have little to no biological activity,[8−10] NNN and NNK are classified as IARC (International Agency for Research on Cancer) Group I carcinogens.[11] NNN and NNK are present in tobacco smoke and are reported to be the most important carcinogens in smokeless tobacco products.[12,13] The impact on the overall toxicity that might be created by reducing or eliminating these compounds in smoke is unknown. It is expected, however, that the reduction of these compounds in smokeless tobacco would have a significant influence on lowering health risks associated with the use of these products as the TSNAs are by far the most prevalent strong carcinogens in many nonburned products.[10] NNN and NNK in tobacco products have been recommended for regulation by the IARC, the World Health Organization (WHO), and others.[6,14] In addition, the U.S. Food and Drug Administration (FDA) Tobacco Products Scientific Advisory Committee (TPSAC) has placed NNN and NNK on a list of harmful or potentially harmful constituents of tobacco products[15] that may ultimately be regulated in the United States.

To reduce or eliminate TSNAs in cured tobacco leaves, one could (1) reduce or eliminate alkaloid precursors, (2) reduce the availability of nitrosating agents, or (3) cure and store tobacco under conditions not conducive for TSNA formation. Reductions in total TSNAs can be achieved through the use of tobacco lines with reduced genetic potential to accumulate alkaloids, in general.[16,17] Specific reductions in NNN can be achieved by reducing the quantity of the nornicotine precursor through conventional selection,[18] genetic engineering,[19] or introduction of deleterious mutations into genes encoding for enzymes involved in the biosynthesis of this alkaloid.[20] Additional strategies to reduce TSNAs might involve methods to reduce the availability of nitrosating agents. In principle, this

approach might affect all members of the TSNA group. For flue-cured tobaccos, significant TSNA reductions have already been achieved through retrofitting of curing barns with indirect heating systems to reduce gaseous NO_x species that react with alkaloids to form TSNAs.[21,22] In air-cured tobaccos, nitrosating agents are thought to arise from two primary sources: (1) pre-existing nitrite formed via endogenous plant nitrate reductase (NR) activity in curing leaves or (2) nitrite produced via leaf microbe-mediated reduction of leaf nitrate (NO_3-N). The latter source is believed to be the most important nitrosating agent during air-curing.[7]

Great variability is observed among tobacco market classes for their tendency to accumulate TSNAs. TSNA levels are typically highest for burley tobaccos, followed by flue-cured and Oriental tobaccos in decreasing order of potential for accumulation. These differences are probably mostly due to production and curing factors specific to each market type, but genetic differences might also play significant roles. There is great interest in lowering TSNA levels of burley tobacco because of this type's contribution to TSNA levels in American blend cigarettes. Burley tobacco is typically fertilized heavily with applied nitrogen (N) relative to most other tobacco types to get yields comparable to what can be achieved in other types at lower levels of N fertility. Burley tobacco is also typically stalk-cut and cured in rain-protected structures for periods of 7−10 weeks with high temperatures that typically do not exceed 38 °C. This is not the case for flue-cured tobaccos, which are cured using a managed regimen of temperature and humidity control that typically lasts for 5−8 days. Leaves are exposed to temperatures up to 74 °C during flue-curing.

Most likely, there are many genetic differences between tobacco types that contribute to their overall unique quality characteristics. One of the main genetic differences between burley tobacco and other tobacco types is that this tobacco type is homozygous for recessive mutant alleles at both the *Yellow Burley 1* (Yb_1) and *Yellow Burley 2* (Yb_2) loci.[23,24] The $yb_1yb_1yb_2yb_2$ genotype results in a chlorophyll-deficient phenotype that is readily apparent during growth in the field. The $yb_1yb_1yb_2yb_2$ genotype probably plays a role in the increased N fertilization requirement for this market type and may, itself, contribute to the higher potential for TSNA accumulation by causing increased availability of NO_3-N during curing as the result of reduced efficiency of N assimilation.

The influence of recessive yb_1 and yb_2 alleles on N physiology in tobacco has not been evaluated. Likewise, the potential of these alleles to affect cured leaf NO_3-N levels as they relate to potential for TSNA formation has not been studied. Using simple backcrossing methods, nearly isogenic versions of three tobacco cultivars ('NC95', 'Coker 139', and 'SC58') were previously established that differed for the presence or absence of the recessive alleles at the Yb_1 and Yb_2 loci. The first objective of this research was to investigate the influence of recessive alleles at these two loci on N physiology measures (N-uptake efficiency, N-use efficiency, and N-utilization efficiency) and their relationship with NO_3-N availability and TSNA formation in air-cured tobacco. A second objective was to use these genetic materials to study the effect of N fertilization rates on alkaloid levels, NO_3-N, total N, and the above-mentioned N physiology measures. These results were also interpreted with respect to measured TSNA levels in the air-cured leaf.

Table 1. Analysis of Variance for Alkaloids and TSNAs for Six Tobacco Genotypes Evaluated at Three Different N Fertilization Rates in a Split-Plot Design Evaluated in Five North Carolina Environments

source	df	log nicotine (mg g⁻¹)	log nornicotine (mg g⁻¹)	anatabine (mg g⁻¹)	anabasine (mg g⁻¹)	log total alkaloids (mg g⁻¹)	log NNK (ppm)	log NNN (ppm)	log NAT (ppm)	log NAB (ppm)	log total TSNAs (ppm)
						mean squares[a]					
environment	4	0.8957***	2.8015****	36.7188****	0.089802*****	1.044***	56.297**	27.926****	9.473***	52.337	12.183**
rep(environment)	10	0.0433	0.0654	0.3309	0.003032	0.0449	5.886	1.090	0.864	13.779	0.911
N rate	2	0.3447*	1.1856*	0.6328*	0.003072	0.3643*	26.481*	17.819**	11.796**	46.568*	14.124**
N rate × environment	8	0.0532	0.1402	0.0768	0.001204	0.0534	5.625	1.457	1.253	7.241	1.335
pooled error A	4										
genotype	5	3.8576****	5.6192****	10.3985****	0.046461****	3.6344****	26.887*	12.302**	11.170****	8.982*	10.053****
genotype × environment	20	0.0787****	0.1504****	1.1678****	0.003941****	0.0792****	8.005***	1.022****	0.606****	2.839	0.815****
genotype × N rate	10	0.0296*	0.0621	0.0858	0.000566	0.0304*	2.045	0.543	0.234	1.295	0.216
genotype × N rate × environment	40	0.0126	0.0455	0.0543	0.000380	0.1252	3.999	0.382	0.278*	1.876	0.318*
pooled error B	163										

[a]*, **, ***, and **** indicate significance at P = 0.05, 0.01, 0.001, and 0.0001 levels, respectively.

Table 2. Analysis of Variance for Yield, Total N, Leaf Nitrate, and N Physiology Measures for Six Tobacco Genotypes Evaluated at Three Different N Fertilization Rates in a Split-Plot Design Evaluated in Five North Carolina Environments

		mean squares[a]					
source	df	yield (kg/ha)	total N (%)	log NO$_3$-N (ppm)	log N-UPT (kg/kg)	log N-USE (kg/kg)	log N-UTL (kg/kg)
environment	4	24,849,412****	42.388****	10.558****	8.803****	3.180****	2.404****
rep(environment)	10	504,544	0.176	0.063	0.091	0.078	0.012
N rate	2	608,833	10.398**	29.187*	15.477****	23.727****	0.883**
N rate × environment	8	438,048	0.847**	3.848*	0.230	0.074	0.075**
pooled error A	4						
genotype	5	4,239,620****	3.059****	3.640***	0.391***	0.594****	0.234****
genotype × environment	20	380,215****	0.281***	0.558****	0.060***	0.037***	0.022****
genotype × N rate	10	80,418	0.104	0.222	0.020	0.007	0.011
genotype × N rate × environment	40	113,732	0.110	0.230*	0.026	0.015	0.007
pooled error B	163						

[a]*, **, ***, and **** indicate significance at $P = 0.05$, 0.01, 0.001, and 0.0001 levels, respectively.
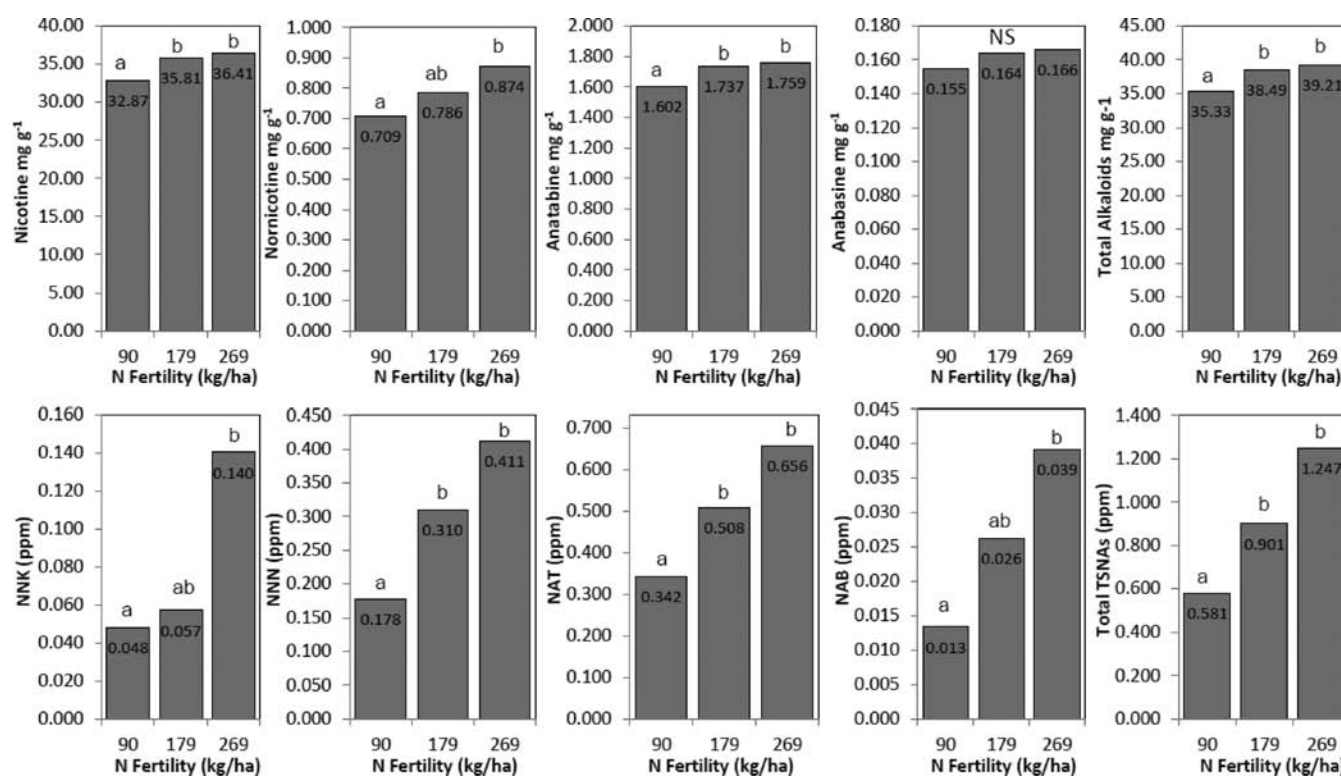

Figure 1. Means based on nontransformed data for three levels of nitrogen fertilization. Statistical tests were performed on transformed data (natural logarithmic transformation) for all measured characters except anatabine and anabasine. Means followed by the same letter are not significantly different from each other at the $P = 0.05$ level of significance. NS = significant differences were not observed among N fertility levels based on an F test.

■ MATERIALS AND METHODS

Plant Materials. Simple backcross breeding was previously used by researchers at North Carolina State University to transfer the recessive yb_1 and yb_2 alleles from a standard burley tobacco cultivar to flue-cured ($Yb_1Yb_1Yb_2Yb_2$) tobacco cultivars 'NC 95', 'SC 58', and 'Coker 139'. Nearly isogenic $yb_1yb_1yb_2yb_2$ versions of these cultivars were in the BC$_8$S$_6$ generation for this study and are estimated to be identical to their respective recurrent parents at >99.6% of the positions across the entire tobacco genome. The double-homozygous recessive ($yb_1yb_1yb_2yb_2$) versions of NC 95, SC 58, and Coker 139 are hereafter referred to as NC 95 yb, SC 58 yb, and Coker 139 yb, respectively.

Experimental Design and Management. Field experiments were conducted at the Lower Coastal Plain Research Station (Kinston, NC) and the Mountain Research Station (Waynesville, NC) during 2007. During 2010, field experiments were carried out at the Lower

Coastal Plain Research Station, the Upper Mountain Research Station (Laurel Springs, NC), and the Upper Piedmont Research Station (Reidsville, NC). A split-plot design with three replicates was used at each location. Main plots consisted of N fertility rates (90, 179, or 269 kg/ha), and subplots consisted of genotypes (NC 95 or NC 95 yb; SC 58 or SC 58 yb; and Coker 139 or Coker 139 yb). At Kinston, plots consisted of two 14-plant rows, with row and intrarow plant spacing of 117.8 and 45.7 cm, respectively. At all other locations, plots consisted of two 12-plant rows, with row spacing of 121.9 cm and intrarow spacing of 45.7 cm. The end plants of each plot served as guard plants and were removed prior to harvest. At each location, 600 kg of 0-0-22 (132 kg of K$_2$O) fertilizer was applied per hectare within a week of transplanting. This was followed by applying half of the designated N for each plot in the form of 30% liquid urea ammonium nitrate (UAN). Fourteen days after the first N application, the remaining
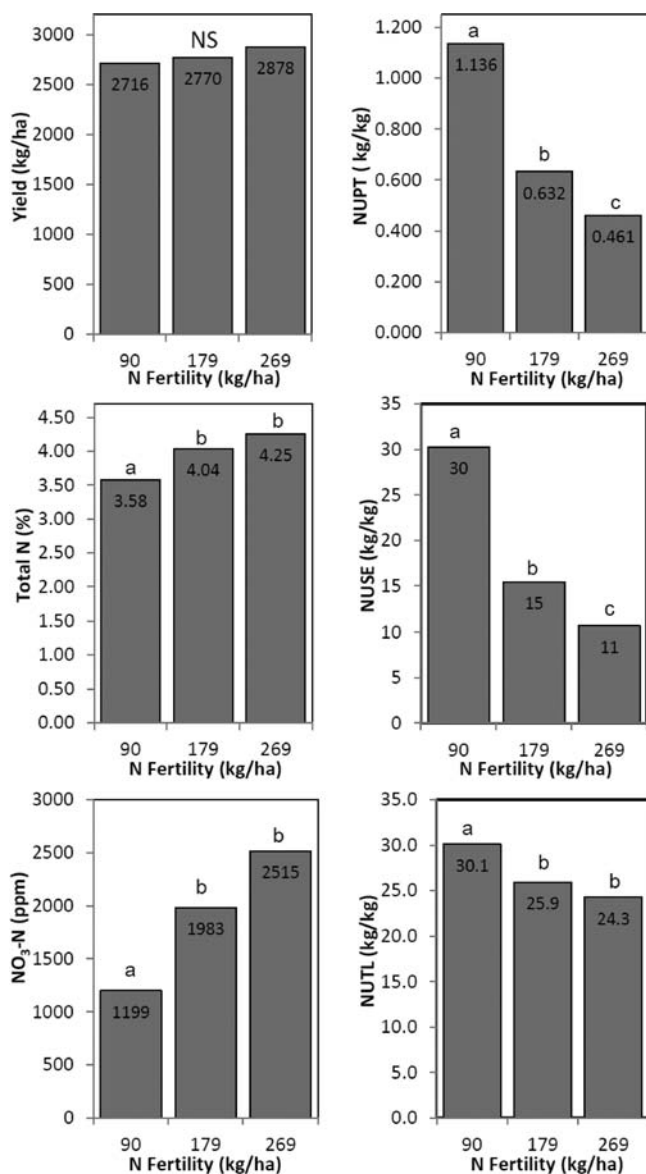
Figure 2. Means based on nontransformed data for three levels of nitrogen fertilization. Statistical tests were performed on transformed data (natural logarithmic transformation) for all measured characters except yield and total N. Means followed by the same letter are not significantly different at the $P = 0.05$ level of significance. NS = no significant differences were observed among N fertility levels based on an F test.

portion of the designated N was applied using 30% UAN. Production practices were consistent with those used for commercial air-cured tobacco production in North Carolina.

At maturity, stalks were cut and plants were hung on sticks or racks and air-cured in rain-protected structures. After curing, the cured leaf for each plot was weighed to estimate plot yield, and the fourth leaf from the top of each plant was collected and processed separately. Leaf midribs were immediately separated from the lamina. For each plot, the collected lamina for these leaves were composited into a single paper bag and allowed to air-dry under ambient laboratory conditions. Samples were subsequently ground to pass through a 1 mm screen and analyzed for nicotine, nornicotine, anatabine, anabasine, NO_3-N, NNK, NNN, NAT, and NAB using procedures described below. Fifty-gram composite cured-leaf samples were also prepared for each plot by compositing cured leaf from each of the four major stalk positions on a weighted-mean basis. Oven-dried composite samples were ground to

pass through a 1 mm screen and analyzed for percent total N using the method outlined below.

Nitrogen Physiology Calculations. N-use efficiency (N-USE), N-uptake efficiency (N-UPT), and N-utilization efficiency (N-UTL) were calculated according to the methods of Moll et al.[25] and Sisson et al.[26] using the following formulas:

N-use efficiency (N-USE, $kg\,kg^{-1}$)

$$= (\text{cured leaf yield, } kg\,ha^{-1}/\text{units N fertilizer, } kg\,ha^{-1})$$

N-uptake efficiency (N-UPT, $kg\,kg^{-1}$)

$$= (\text{N-ACC, } kg\,ha^{-1}/\text{units N fertilizer, } kg\,ha^{-1})$$

where

$$\text{N-ACC} = (\text{cured leaf yield, } kg\,ha^{-1} \times \text{total N concentration, } g\,kg^{-1})$$

and

N-utilization efficiency (N-UTL, $kg\,kg^{-1}$)

$$= (\text{cured leaf yield, } kg\,ha^{-1}/\text{N-ACC, } kg\,ha^{-1})$$

It should be noted that N-UPT and N-UTL are indices based on total N in the leaves only, and not total N for all plant parts. For this study, as in Sisson et al.,[26] the assumption was made that N tissue concentrations for stalk, midribs, and roots are proportional to leaf N concentrations among the genotypes that were tested. Moll et al.[25] suggested that estimates of N accumulation in certain plant parts can provide insight into variation for N physiology components.

Chemical Analyses. Quantitative determinations of nicotine, nornicotine, anabasine, and anatabine in ground fourth-leaf samples were made using a Perkin-Elmer Autosystem XL Gas Chromatograph with Prevent according to analytical methods described for LC Protocol.[18] Ground tobacco samples were treated with aqueous sodium hydroxide followed by extraction with methyl *tert*-butyl alcohol. Quantification was based on chromatographic peak response at the retention times of alkaloid standards. Percent total N determinations were made using the procedure of Nelson and Sommers.[27] NO_3-N was quantified using the methodology of Crutchfield and Grove.[28] Quantifications of NNN, NNK, NAB, and NAT were performed according to method 1 of Morgan et al.[29] Total TSNAs were calculated as the sum of NNK, NNN, NAT, and NAB.

Statistical Analyses. An analysis of variance appropriate for analyzing data from a split block design evaluated over locations[30] was conducted using the PROC GLM procedure of SAS (SAS Institute, Cary, NC). Main plots and subplots were considered as fixed factors and environments as random factors. Censored observations (those below the minimum limit of detection) were replaced by half the detection limit according to Atwood et al.[31] Natural logarithmic data transformations were performed for nicotine, nornicotine, total alkaloids, NNK, NNN, NAT, NAB, total TSNAs, NO_3 N, NUPT, NUSE, and NUTL because of heterogeneous error variances and to approximate normal distributions. Means (averaged over all genotypes) for each of the different levels of N fertilization were compared using Fisher's protected LSD calculated according to the method of Gomez and Gomez.[32] Single degree of freedom linear contrasts were made between genotypic group means ($Yb_1Yb_1Yb_2Yb_2$ vs $yb_1yb_1yb_2yb_2$) using CONTRAST statements in PROC GLM according to method of Steel et al.[33] Pearson correlation coefficients were also calculated using PROC CORR in SAS to describe the relationships, if any, between NO_3-N, alkaloid TSNA precursors, and corresponding TSNAs.

■ RESULTS

Highly significant differences were observed between environments for all measured characters except NAB ($P < 0.01$, Tables 1 and 2). Significant differences were detected among N fertilization rates for all determinations except anabasine and yield ($P < 0.05$, Tables 1 and 2). Significant N rate × environment interactions
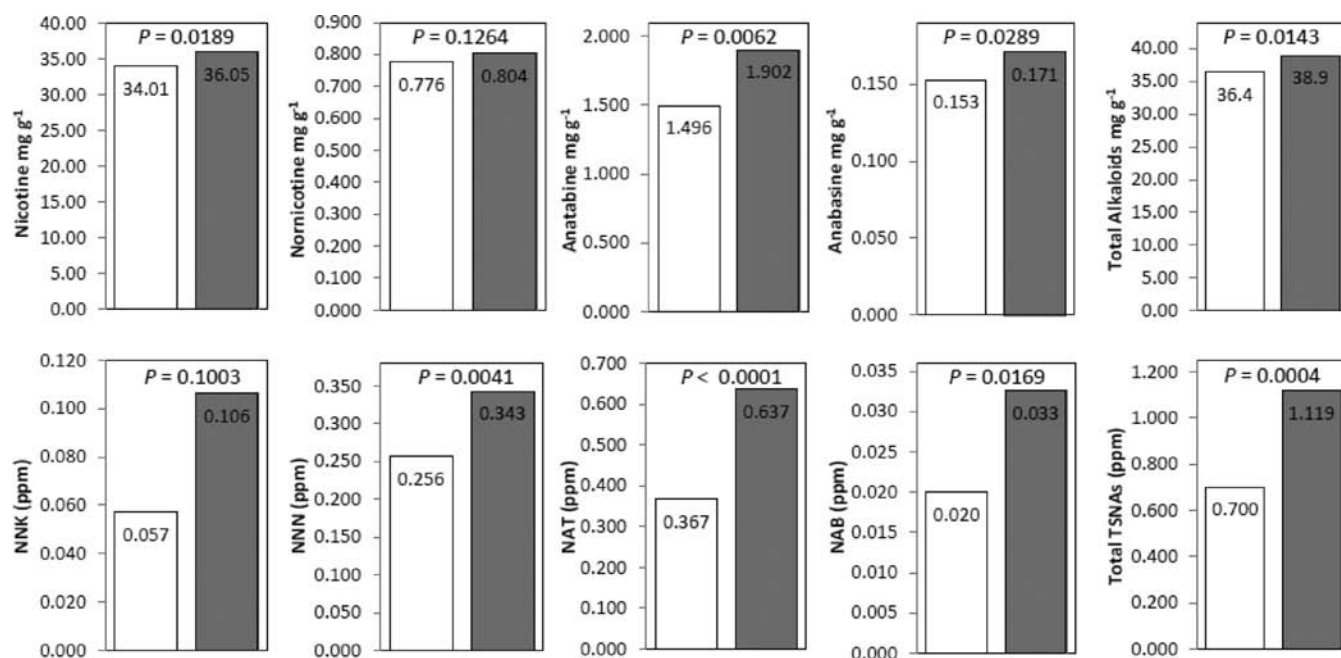
Figure 3. Nontransformed $Yb_1Yb_1Yb_2Yb_2$ group means (white bars) versus $yb_1yb_1yb_2yb_2$ group means (shaded bars) averaged over three levels of N fertilization. P values were generated from single degree of freedom contrasts of group means using PROC GLM of SAS. For statistical tests, natural logarithmic transformations were performed on all measured characters except anatabine and anabasine.

were observed for only total N, NO_3-N, and N-UTL ($P < 0.05$). Because environment was considered as a random factor, means for each N rate (averaged over all genotypes) are presented averaged over environments (Figures 1 and 2). As N fertilization rates increased from the low to the high rate, all alkaloids and their corresponding TSNAs increased significantly ($P < 0.05$), except for anabasine, for which the increase was not signficant (Figure 1). Total N and NO_3-N increased significantly as the N rate increased from the low to the high rate (Figure 2). These determinations were used to calculate N-UPT, N-USE, and N-UTL, all of which were found to decrease significantly as N fertilization rates increased (Figure 2). Decreases in N-USE and N-UTL indicate that as N rates increased, cured leaf weight achieved per unit of N fertilization or unit of accumulated N decreased.

Significant differences were observed between genotypes for every measured trait ($P < 0.05$, Tables 1 and 2). Significant genotype × environment interactions were found for all measured characteristics ($P < 0.001$) except NAB. Slightly significant ($P < 0.05$) genotype × N rate interactions were found for only nicotine and total alkaloids. Because environment was considered as a random factor, and for ease of presentation, means for each genotypic class ($Yb_1Yb_1Yb_2Yb_2$ vs $yb_1yb_1yb_2yb_2$) were presented averaged over environments (Figures 3 and 4). On the basis of single degree of freedom linear contrasts between group means, the $yb_1yb_1yb_2yb_2$ group was significantly higher than the $Yb_1Yb_1Yb_2Yb_2$ group for all of the quantified alkaloids and TSNAs, except for nornicotine and NNK ($P < 0.03$, Figure 3). The $yb_1yb_1yb_2yb_2$ group produced significantly lower yields and exhibited significantly greater levels of total N and NO_3-N than the $Yb_1Yb_1Yb_2Yb_2$ group ($P < 0.0001$ and $P = 0.0002$, respectively) (Figure 4). This translated into significantly lower N-USE and N-UTL for the $yb_1yb_1yb_2yb_2$ group ($P < 0.0001$) (Figure 4).

Pearson correlation coefficients describing the relationships between NO_3-N, alkaloids, and corresponding TSNAs are presented in Table 3. Correlation coefficients between NO_3-N

and each of the TSNAs except NNK were highly significant ($P < 0.0001$). Coefficients between individual TSNAs and their supposed alkaloid precursors were positive in value and varied in their significance. Only the anatabine–NAT and anabasine–NAB coefficients were significant ($P = 0.0002$ and $P = 0.0291$, respectively). The numerical values of r were always higher for NO_3-N–TSNA relationships as compared to the alkaloid–TSNA relationships and exhibited higher levels of statistical significance.

■ DISCUSSION

The objectives of this research were to investigate the impact of N fertilization rates and genotypes at the Yb_1 and Yb_2 loci on TSNA formation and factors that contribute to it. Data from this study indicate that, as N fertilization rates are increased, there is a high tendency for alkaloids, NO_3-N, and total nitrogen to also increase. A strong and undesired correlated tendency is for TSNA levels to also increase with increased N fertilizer. Increased alkaloid accumulation as a result of high N rates has previously been associated with higher TSNA levels in cured tobacco.[7] This unfavorable relationship can also at least partially be attributed to an increase in NO_3-N that occurs under high N rates, for which reductions in N-utilization efficiency were measured. In these cases, accumulated N is likely not being assimilated into N-containing organic molecules that play important roles in plant dry matter accumulation. An increase in the available NO_3-N pool is likely available for reduction to nitrite via microbial activity. The resulting nitrite, in turn, becomes involved in nitrosation reactions with the naturally occurring alkaloids, leading to the formation of nitrosated products.

Significant differences were observed between $Yb_1Yb_1Yb_2Yb_2$ and $yb_1yb_1yb_2yb_2$ genotypic groups for nearly all of the measured characters. Averaged over all N application rates, the $yb_1yb_1yb_2yb_2$ group exhibited significantly higher levels of all alkaloids except nornicotine and accumulated significantly
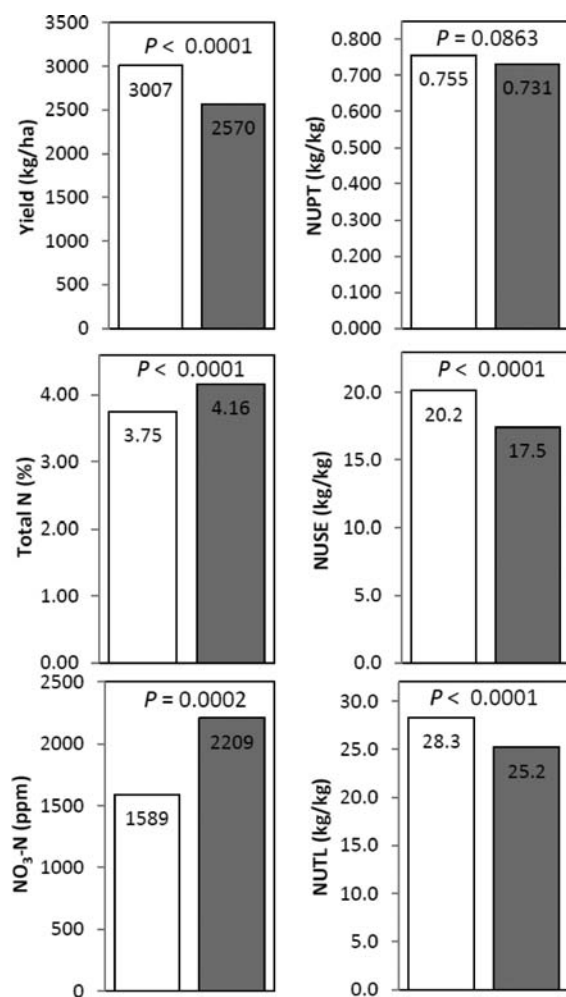
Figure 4. Nontransformed $Yb_1Yb_1Yb_2Yb_2$ group means (white bars) versus $yb_1yb_1yb_2yb_2$ group means (shaded bars) averaged across three levels of N fertilization. P values were generated from single degree of freedom contrasts of group means using PROC GLM of SAS. For statistical tests, natural logarithmic transformations were performed on all measured characters except yield and total N.

higher levels of all measured TSNAs, excluding NNK. The increased TSNA effect was observed at each tested rate of N fertilization (i.e., there was no genotype × N rate interaction).

The $yb_1yb_1yb_2yb_2$ group also exhibited significantly higher NO_3-N, a possible consequence of the observed significantly decreased N-utilization efficiency for this group. The higher TSNA levels in these "burley" genotypes are probably at least partly due to a relatively poor capacity of these tobaccos to assimilate accumulated nitrogen into N-containing organic molecules, such as proteins, involved in plant growth and increases in dry weight. As mentioned above, the pool of free NO_3-N likely becomes available for reduction to nitrite (the primary nitrosating agent for TSNA formation in air-cured tobacco) by plant nitrate reductase enzymes or via microbial activity, with the latter being of probable greater importance.[34] The precise role of the gene(s) at the Yb_1 and Yb_2 loci is not known, but the genetic differences are expressed at the leaf level rather than the root.[35,36]

In the current experiment, the lowest TSNA levels were observed in "green" wild-type tobacco ($Yb_1Yb_1Yb_2Yb_2$) grown at the lowest levels of N fertilization, whereas the highest TSNA levels were observed for "burley" tobacco genotypes ($yb_1yb_1yb_2yb_2$) grown at the highest levels of N fertilization. Both the high fertilization rates that are often used for burley tobacco production and the $yb_1yb_1yb_2yb_2$ genetic constitution of current burley cultivars likely contribute to the high TSNA levels typically observed for this tobacco market class. The relative importance of increases in alkaloids and/or leaf NO_3-N/NO_2-N on TSNA formation has been researched in the past. Several publications have suggested alkaloid levels and their composition to be of greater importance to TSNA formation as compared to NO_3-N levels.[16,17] Burton et al.,[37,38] however, did not observe significant correlations between TSNAs and alkaloids or NO_3-N. Instead, these workers observed greater correlations between TNSA accumulation and levels of nitrite, suggesting that nitrite is the limiting factor in TSNA formation in air-cured tobacco. It seems logical that levels of alkaloids, leaf NO_3-N, and leaf nitrite all play roles in TSNA formation. Data from the current study would seem to indicate that increases in NO_3-N are a bigger contributing factor to higher TSNA levels in air-cured tobacco than increased alkaloid levels. Correlations and their levels of significance were greater between leaf NO_3-N and TSNAs than between individual alkaloids and their derived TSNAs. When $Yb_1Yb_1Yb_2Yb_2$ and $yb_1yb_1yb_2yb_2$ genotypic groups in the genetic backgrounds used in our study were compared, the presence of the recessive alleles contributed to modest percent increases in alkaloid levels

Table 3. Pearson Correlation Coefficients (r) Describing the Relationships between Measured TSNAs and Their Quantified Precursors (NO_3-N and Individual Alkaloids)[a]

	NNK (ppm)	NNN (ppm)	NAT (ppm)	NAB (ppm)	total TSNAs (ppm)
NO_3-N (ppm)	0.1153	0.2696	0.3240	0.2422	0.2909
	(0.0598)	(<0.0001)	(<0.0001)	(<0.0001)	(<0.0001)
% nicotine	0.1062	0.2836	0.2006	0.0845	0.2209
	(0.0833)	(<0.0001)	(0.0010)	(0.1686)	(0.0003)
% nornicotine	−0.0405	0.1037	−0.0193	−0.0682	0.0073
	(0.5098)	(0.0907)	(0.7536)	(0.2671)	(0.9054)
% anatabine	0.1566	−0.1297	0.2258	−0.0903	0.1181
	(0.0104)	(0.0342)	(0.0002)	(0.1410)	(0.0540)
% anabasine	0.0736	0.2118	0.3419	0.1335	0.2683
	(0.2308)	(0.0005)	(<0.0001)	(0.0291)	(<0.0001)
% total alkaloids	0.1110	0.2598	0.2069	0.0706	0.2179
	(0.0701)	(<0.0001)	(0.0007)	(0.2504)	(0.0003)

[a]P values are indicated in parentheses.

(ranging from a 4% increase for nornicotine to a 27% increase for anatabine). The increases in TSNAs, however, were generally much greater in magnitude for materials carrying the double-recessive genotype. Percent increases for NNN, NNK, NAT, and NAB were 34, 86, 74, and 63%, respectively. Total TSNAs were 60% higher for the $yb_1yb_1yb_2yb_2$ genotypic class as compared to the $Yb_1Yb_1Yb_2Yb_2$ genotypic class. Leaf NO_3-N was 39% higher for the double-recessive genotypic class.

The tobacco industry is interested in methodologies that could have large effects on reducing TSNA levels in tobacco products. For smoking products, reductions in TSNAs have been made by retrofitting flue-cured tobacco barns with indirect heating systems[39] and can also be made by reducing the amount of air-cured tobacco used in blended products.[40] Reductions can also be made through the use of tobaccos with modified alkaloid profiles or those produced under lower N fertilization rates.[7] Other strategies have included directed breeding to reduce specific alkaloid precursors[18,20] or stimulation of antioxidant production by root pruning or stalk wounding.[41] The data presented here suggest that for air-cured burley tobacco, very significant reductions in TSNAs can likely be made by substituting the dominant alleles for the current recessive alleles at at least one of the *Yellow Burley* loci. It may be worthwhile to investigate whether or not air-cured tobacco types of the $Yb_1Yb_1Yb_2Yb_2$ genetic constitution could be developed that could substitute for the traditional $yb_1yb_1yb_2yb_2$ burley tobacco types in manufactured tobacco products. Such $Yb_1Yb_1Yb_2Yb_2$ types would likely contribute to reduced TSNA levels in blended tobacco products and also to reduced production costs and environmental impact because of their improved yields and nitrogen use efficiency. The technical feasibility of doing this is very high, as the "burley" phenotype is a simply inherited trait. The possibility of developing $Yb_1Yb_1Yb_2Yb_2$ air-cured tobacco types with quality characteristics similar to conventional burley tobacco cultivars is unknown, however, and any such effort could prove to be unsuccessful. Legg et al.[42] found most of the genetic differences between flue-cured and burley tobacco for a range of cured-leaf chemical characteristics to result from the influence of alleles at the Yb_1 and Yb_2 loci. Nevertheless, in the presence of extreme regulation of TSNA levels in tobacco products, alternative tobacco types and blending approaches may become necessary.

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Notes
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■ ABBREVIATIONS USED

NAB, *N*-nitrosoanabasine; NAT, *N*-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*-nitrosonornicotine; N-UPT, N-uptake efficiency; N-USE, N-use efficiency; N-UTL, N-utilization efficiency; TSNA, tobacco-specific nitrosamine.

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