Principal Aliphatic Secondary Amines of Burley Tobacco*

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INTRODUCTION

Investigations on tobacco bases have been mostly confined to nicotine and other alkaloids but recent reports have been concerned with separation and identification of the volatile amines present in tobacco (4, 11). Earlier investigations were hindered by lack of adequate procedures for isolation and detection of amines in plant tissue (9, 10). Richardson (7) reported the use of 1-fluoro-2,4-dinitrobenzene as a quantitative reagent for the determination of simple plant amines. Separation and identification of 17 volatile amines in tobacco were accomplished by use of 4-nitroazobenzene-4'-carboxamide derivatives (6). Among those identified in burley tobacco were dimethylamine, methylethylamine and methylpropylamine. Yasumatsu and Akaike (11) identified 12 amines in tobacco including dimethylamine and diethylamine. In an early survey of volatile amines in flowering plants von Kamienski (10) reported the presence of methylamine and isopentylamine in tobacco. He observed that the amine content of *Mercurialis perennis* was influenced by the location where plant was grown and by the plant tissue assayed. He also reported that the quantities of amines in *M. annua* were less after periods of wilting than in the fresh tissue. Yasumatsu and Akaike (11) demonstrated differences in the amounts of several volatile amines between shoot and root, air-cured and flue-cured leaves, and nitrogen fertilization in Yellow Bright tobacco. Recently, Bush et al. (1) have shown with cured burley tobacco, produced under commercial production conditions, that increased secondary amine content in tobacco tissue was positively correlated with nitrogen content and that secondary amine content was lowest in leaf tissue from the lower stalk positions.

The objectives of these investigations were to determine the influence of different tobacco tissue and tissue processing and of nitrogen fertilization on the volatile aliphatic secondary amines in tobacco cultured under a semi-controlled environment.

EXPERIMENTAL

Plant Culture

All tobacco plants were cultured in the greenhouse at 30° C with a 15 hr. light period and 9 hr. dark period. Plants were watered daily with Hoagland nutrient solution (3). Plants used in the experiments designed to study influence of nitrogen on amines were watered with the standard nutrient solution, or with 2× or 4× the standard amounts of KNO₃ and Ca(NO₃)₂. Plants of *N. tabacum* were harvested at the 15–20 leaf stage (10–11 weeks of age). Other *Nicotiana* spp. were harvested at the time of flower bud development. Oven-dried material was prepared by drying tissue for 24 hrs. in forced draft oven at 70° C. The air-dried leaf tissue was prepared by priming the leaves and drying in the laboratory at room temperature. All dried plant material was ground to pass a 40 mesh screen prior to analysis. Experiments were duplicated 2–4 times with 3 replications per experiment where appropriate.

Fresh tissue was macerated for 3 min. in a blender with 1:2 (w/v) of 3% NaOH and immediately steam distilled. Five-gram samples of dried ground leaf lamina in 50 ml of 3% NaOH were steam distilled. The distillate was collected in 100 ml CH₂Cl₂, 50 ml H₂O and 5 ml conc. HCl. Four hundred ml of distillate were collected, stirred for 30 min. and the aqueous layer washed once with fresh CH₂Cl₂. The 2,4-dinitrophenyl derivatives of the volatile amines were made from the aqueous layer with 1-fluoro-2,4-dinitrobenzene (DNFB) and extracted with cyclohexane. An aliquot of the cyclohexane was concentrated and the individual amine derivatives separated by TLC with Silica Gel G adsorbent and hexane: ether, 70:30 (v/v) solvent system (2). Concentration of individual amines was determined by elution with acetone and spectrophotometric measurement at 355 nm. This procedure will detect free amines and any amines formed during base hydrolysis. More specific details of the procedures are published elsewhere (1, 2).

Individual aliphatic secondary amines — dimethylamine, methylethylamine, and diethylamine mixed with methylpropylamine — were separated and quantified as derivatives of DNFB. On the thin-layer chromatograms N-methyl-n-propylamine and diethylamine had the

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same migration and these amines were estimated together using a diethylamine standard.

Acidic and basic extracts of the tobacco tissue were prepared by homogenizing tissue in 1N HCl or 1N KOH 1:30 (w/v). The homogenate was filtered through Whatman #1 and the filtrate washed three times with chloroform. The DNFB derivatives were made directly from the aqueous fraction after adjusting the pH to approximately 4 before adding the reagents or the aqueous fraction was steam distilled and the derivatives formed. The residue from the filtration and the combined chloroform washings (neutral fraction) were also steam distilled and analyzed. Portions of the aqueous filtrate from the acidic or basic extracts were titrated to pH 3.0 and extracted with n-butanol. The amine derivatives were made on the aqueous fraction of pH 3.0 before and after steam distillation. The butanol fraction was also analyzed for amines.

RESULTS

Method of leaf processing had a pronounced influence on the levels of dimethylamine, but very little influence on the diethylamine fraction (Table 1). Air-drying and oven-drying of the tissue reduced the quantity of dimethylamine by 25% but the quantity of diethylamine was not changed. Freeze-dried tissue contained quantities of all the amines equal to the fresh tissue. Burley tobacco leaf samples were also assayed fresh and after air-curing and in general the curing process did not influence the quantities of the amines studied.

Contents of the principal volatile secondary aliphatic amines differed with plant parts (Table 2). The stem contained the highest quantity of the three amine fractions measured and the leaf midrib contained the lowest amounts. The methylethylamine content was a greater portion of the total amines measured in the seed, stem and roots than in the leaf lamina and leaf midrib. Methylethylamine content in the seed and stem was equal to the dimethylamine content. Many unidentified dinitrophenylamine derivatives were observed on the TL chromatograms from the root samples that were not observed in the other samples. Removing the apical meristem from the growing tobacco plants (topping) prior to harvesting had no influence on the amine content. Also, the age of plants had no consistent influence on the amine content.

Table 1. Influence of drying process on the quantities of dimethylamine, methylethylamine, and diethylamine in burley tobacco leaf.

Drying process	Micrograms per gram dry weight		
	Dimethylamine	Methylethylamine	Diethylamine*
Fresh	43	trace	9
Air-dried	31	trace	8
Oven-dried	33	trace	10
Freeze-dried	42	trace	9

* diethylamine with methylpropylamine.

Not only did the various plant parts differ in amine content but the lamina of different species of *Nicotiana* or varieties of *N. tabacum* also differed in amine content. *N. glutinosa* had similar ratios among the three amines as the *N. tabacum* but the quantities were much lower (Table 3). *N. langsdorffia* and *N. longiflora* contained high total amounts of the amines and most was present as dimethylamine. *N. rustica* and *N. suaveolens* also contained predominantly dimethylamine but the total amount was intermediate. *N. glauca* contained the lowest amounts of the amines of any species analyzed and most was dimethylamine. Very little difference was observed in the aliphatic amines between the burley lines isogenic for nicotine.

The influence of nitrogen fertilization and nitrogen content of tissue on amine content under field conditions has been reported (1). Culturing tobacco in the greenhouse and watering with nutrient solution containing the standard (85 mg N/l), or 2X, or 4X the standard amounts of NO₃-N produced similar results. Data presented in Fig. 1 is the summation of dimethylamine, methylethylamine and diethylamine fractions in leaf lamina. Experiments were run in succession and differences among experiments can only be explained by difference in growth patterns. Within each experiment a linear relationship existed between nitrogen content and amines. The regression line for these data is $Y = 27.2 + 15.16 X$.

Table 2. Dimethylamine, methylethylamine, and diethylamine content in different tissue of oven-dried burley tobacco, *Nicotiana tabacum* var. Burley 21.

Tissue	Micrograms per gram dry weight		
	Dimethylamine	Methylethylamine	Diethylamine*
Seed	6	8	trace
Leaf lamina	14	3	7
Leaf midrib	2	1	4
Stem	14	13	9
Roots	13	7	5

Table 3. Dimethylamine, methylethylamine, and diethylamine content in several *Nicotiana* species.

Species	Micrograms per gram dry weight		
	Dimethylamine	Methylethylamine	Diethylamine*
<i>N. glauca</i>	9	trace	trace
<i>N. rustica</i>	22	trace	trace
<i>N. langsdorffia</i>	75	N.D.**	6
<i>N. longiflora</i>	52	N.D.	2
<i>N. suaveolens</i>	33	trace	trace
<i>N. glutinosa</i>	17	trace	10
<i>N. tabacum</i>			
var. Burley 21	54	3	35
var. Burley 21 low-nicotine	44	trace	33
Ky Iso 7 (Turkish)	32	trace	14

* diethylamine with methylpropylamine.

** not detected.

Figure 1. Influence of total nitrogen on aliphatic secondary amines in tobacco leaf tissue.

Aliphatic secondary amine content was calculated from dimethylamine, methylethylamine, diethylamine and methylpropylamine content of the tissue.

The amines measured in these experiments are readily soluble in aqueous media, thus should be extractable and detectable as the dinitrophenylamine derivatives without steam distillation. Amine analysis following extraction of tissue with 1 N KOH or 1 N HCl indicated very small amounts of these amines were present in the aqueous media (Table 4). The acid fraction represents substances extracted with KOH and the base fraction represents material extracted with HCl. The amines were apparently in both aqueous extracts because if these extracts were steam distilled the amines were found in appreciable amounts in the distillate. These results suggest that the amines in the aqueous extracts were not present as the free bases.

The presence of the amines in the acid, base or neutral fraction was further examined in fresh tobacco leaf tissue. In this experiment all amine determinations were made after steam distillation and for each treatment a new portion of the sample was used. The acid fraction, extracted with 1 N KOH, contained essentially all of the diethylamine fraction and 38% of the dimethylamine (Table 5) compared to the control. The base fraction also contained all of the diethylamine fraction but contained 67% of the dimethylamine fraction. The neutral fraction and the residue (tobacco tissue which had been extracted with both HCl and KOH prior to steam distillation) contained small amounts of the amines. Of the total amines found in the fresh tobacco tissue 58% were removable from the tissue with KOH,

Table 5. Volatile secondary amine content in acidic, basic and neutral extracts from fresh tobacco leaf tissue.

Extract	Micrograms per gram dry weight		
	Dimethylamine	Methylethylamine	Diethylamine*
Tobacco	66	2	40
Acid fraction	25	trace	38
Base fraction	40	trace	40
Neutral fraction	trace	trace	trace
Residue	5	trace	7

* diethylamine with methylpropylamine.

74% were removable from the tissue with HCl, and 11% were not extracted with either solvent.

DISCUSSION

Results of these experiments indicate that the type of tissue, tissue processing, and nitrogen content of the tissue have significant influence on the quantities of steam volatile aliphatic secondary amines in tobacco. Freeze-drying leaf tissue did not reduce amine content compared to amounts extracted from fresh tissue. Air-drying or oven-drying caused a 25% reduction in dimethylamine content but did not alter concentrations of the other amine fractions measured. However, normal air-curing of burley tobacco did not appreciably decrease the amine content and presumably this is because the leaf tissue was cured intact with the stalk whereas in our air-drying process primed leaves were used.

Accumulation of amines was significantly altered by the type of tissue and both the high and low extremes of amine concentrations were found in conducting tissue, stem and the leaf midrib. Because of the carcinogenicity of nitrosamines that may be formed in tobacco smoke from secondary amines and nitrous gases, the observation of low amine concentration in the midrib could have important implications in that more midrib tissue is being used in cigarette manufacturing and thereby lowering the amine content of the tobacco in the cigarette.

Considerable variation existed in the amine content among *Nicotiana* spp. cultured under the same environmental conditions. Based on the observations reported the amine pattern fits the phylogenetic relationships (8) at the subgenus level of *Nicotiana*. *N. langsdorffia*, *N. longiflora* and *N. suaveolens* are all in the subgenus *Petunioides* and all have high dimethylamine content compared to the other amine fractions. *N. glauca* and *N. rustica* are in the subgenus *Rustica* and are low in methylethylamine and diethylamine fractions as the *Petunioides* group but are lower than *Petunioides* in dimethylamine. *N. glutinosa* and *N. tabacum* are in the subgenus *Tabacum* and the diethylamine fraction is 30–40% of the total amines and dimethylamine accounts for most of the remainder.

Amine content of leaf tissue was positively related to nitrogen content and these studies confirm previous work with burley tobacco cultured in the field (1). Within each experiment a linear relationship existed

Table 4. Effect of steam distillation on apparent volatile aliphatic secondary amine content of acid-base extracts of cured tobacco leaf tissue.

Extract	Micrograms per gram dry weight		
	Dimethylamine	Methylethylamine	Diethylamine*
Acid fraction	3	1	1
Acid fraction — steamed	72	15	10
Base fraction	5	0.5	2
Base fraction — steamed	65	8	23

* diethylamine with methylpropylamine.

among nitrogen treatment and amine content, however, reproducibility among experiments was not good. These experiments were replicated in time and the environment in the greenhouse was not the same in all cases. Also, because Ca^{++} and K^+ concentrations were not the same in all nutrient solutions the effect of these cations cannot be ignored. *Krasaesindhu* (5) has suggested an interaction between Ca^{++} and nitrate nitrogen uptake but in the present study the ratio of Ca^{++} and K^+ to NO_3^- was always equal and the Ca^{++} or K^+ content of the tissue was not measured.

The data indicate that the steam volatile amines measured in these investigations were not present as free bases or simple salts because they were only detected in appreciable quantities after steam distillation or base hydrolysis. The compounds giving rise to the amines were present to a greater extent in the base fraction than the acid fraction of fresh tissue and almost totally absent from the neutral fraction. Using an acidic or basic media 89% of the amines could be extracted from the tissue. Separation of amines and amides in the acid or base fraction was done by extraction with butanol at pH 3. The amines should be present in the aqueous layer and the amides in the butanol layer. Of the amines found in the acid fraction 86% remained in the aqueous layer and 14% was in the butanol (present as amides). In the base fraction 94% of the amines detected were present as amines and only 6% were present in the butanol. These results suggest that steam volatile amines are derived from chemically bound amines in the tissue and the amounts detected may vary with type of binding. This conclusion indicates further work is required to define the chemical nature of these compounds and study amine transfer to smoke and to define the influence these amines have on smoke quality.

SUMMARY

Quantity and distribution of the principal aliphatic secondary amines — dimethylamine, methylethylamine, diethylamine, and methylpropylamine — varied within plant tissue and among *Nicotiana* spp. In burley tobacco the stem tissue had the highest content of these amines and the leaf midrib the lowest. Leaf lamina, roots and seed were intermediate in amine content. Among *Nicotiana* spp. there was considerable difference in total amine content as well as among the ratios of the amine fractions measured. Amine content of tissue was positively correlated with nitrogen content of tissue and was altered by the drying process of tissue prior to analysis. The amines were not present as free amines or salts but were detected only following steam distillation from a basic medium.

ZUSAMMENFASSUNG

Quantität und Verteilung der hauptsächlich aliphatischen sekundären Amine — Dimethylamin, Methylethylamin, Diethylamin und Methylpropylamin — waren sowohl innerhalb der pflanzlichen Gewebe als auch

zwischen verschiedenen Spezies von *Nicotiana* unterschiedlich. Beim Burley-Tabak hatte das Stengelgewebe den höchsten und die Mittelrippe der Blätter den geringsten Gehalt an diesen Aminen. Die Blatt-Laminae, Wurzeln und Samen wiesen einen mittleren Amingehalt auf. Beim Vergleich verschiedener *Nicotiana* Spezies ergaben sich beträchtliche Unterschiede sowohl hinsichtlich des Gesamt-Amingehaltes als auch hinsichtlich der Verhältnisse der untersuchten Aminfraktionen. Es zeigte sich eine positive Korrelation zwischen dem Stickstoffgehalt und dem Amingehalt des Gewebes, der sich durch den Trocknungsprozeß des Gewebes vor der Analyse veränderte. Die Amine traten nicht als freie Amine oder Salze auf, sondern wurden erst nach Dampfdestillation aus einem basischen Nährboden festgestellt.

RESUME

La quantité et la répartition des amines aliphatiques secondaires les plus importantes — diméthylamine, méthylethylamine, diéthylamine, méthylpropylamine — varient à l'intérieur des tissus et suivant les espèces de *Nicotiana*. Dans le tabac burley, on a trouvé la concentration la plus élevée de ces amines dans la tige, la moins élevée dans la nervure centrale des feuilles. La concentration était intermédiaire dans les feuilles elles-mêmes, dans les racines et les graines. Suivant les espèces de *Nicotiana*, on a observé des différences importantes des concentrations en amines totales, et dans les proportions des différentes fractions déterminées. La concentration en amine des tissus est en corrélation positive avec leur contenu en azote, et peut varier suivant le procédé de séchage avant l'analyse. Les amines ne sont pas présentes à l'état libre ni comme sels, mais ne purent être détectées qu'après distillation à vapeur d'eau en milieu basique.

REFERENCES

1. Bush, L. P., Sims, J. L., and Atkinson, W. O.: Can. J. Plant Sci. 50 (1970) 289.
2. Day, E. W., Golab, T., and Koons, J. R.: Anal. Chem. 38 (1966) 1053.
3. Hoagland, D. R., and Arnon, D. I.: Calif. AES Circular 347 (1938).
4. Irvine, W., and Saxby, M.: Phytochem. 8 (1969) 473.
5. Krasaesindhu, P.: Master of Science Thesis, University of Kentucky, 1969.
6. Neurath, G., Krull, A., Pirmann, B., and Wandrey, K.: Beitr. Tabakforsch. 3 (1966) 571.
7. Richardson, M.: Nature 197 (1963) 290.
8. Smith, H. H.: Adv. in Genetics 14 (1969) 1.
9. Steiner, M., and Kamienski, E. S. von: Naturwiss. 40 (1953) 483.
10. Kamienski, E. S. von: Planta 50 (1957) 315.
11. Yasumatsu, N., and Akaike, S.: Nippon Noegi. Kagaku. Kaishi. 39 (1965) 347.

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