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Relative Sterol Composition in the Genus *Nicotiana**

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Summary. Stigmasterol, β-sitosterol, campesterol, and cholesterol are the predominant sterols identified by gas-liquid chromatographic techniques in the mature leaves of 50 *Nicotiana* species. The relative composition pattern of the four sterols varies significantly among the subgenera as well as within the subgenus. However, six *N. tabacum* cultivars showed a similar pattern, of which as an average stigmasterol represents the highest proportion (43%) followed by β-sitosterol (30%), campesterol (19%), and cholesterol (8%) in total sterol content. Negative correlations were obtained for the composition of stigmasterol vs. β-sitosterol, cholesterol vs. campesterol, and cholesterol vs. β-sitosterol. Some correlations between geographic distribution of *Nicotiana* species and sterol composition were evident. In evaluating phylogenetic relationship between amphiploid species and the possible diploid progenitors, the results of sterol composition are in favor of *N. undulata* and *N. paniculata* being the ancestors of *N. rustica* and the *N. sylvestris* × *N. tomentosiformis* as the hybrid combination from which *N. tabacum* was evolved.

Introduction

Sterols in higher plants are monohydroxy secondary alcohols with additional methyl and ethyl groups. Stigmasterol, β-sitosterol, campesterol, and cholesterol are the major sterols in tobacco, *Nicotiana tabacum* L., leaf tissues (Stedman, 1968). They are present in free forms or as esters and glycosides. Total sterol content in tobacco leaves may vary depending upon varieties (Cheng et al. 1968; Sheen et al. 1968; Davis et al. 1970), species and plant ontogeny (Cheng et al. 1971a), leaf stalk position (Davis et al. 1970; Cheng et al. 1971b), and cultural practices (Grunwald et al. 1971a). In air-cured burley tobacco sterol content ranged from 1.40 to 2.10 mg/g of dry weight, whereas in flue-cured varieties, a variation of 1.72 to 3.13 mg/g was reported. For individual sterols, the relative composition of cholesterol and campesterol in a given variety and species is not affected by plant ontogeny and environmental variables. On the other hand, a high β-sitosterol accompanying by a low stigmasterol content occurs in young leaves and vice versa in senescent tissues. In studies of the progeny of a *N. tabacum* cross, the leaves in a similar ontogenetic stage showed only slight differences in the relative composition of the above four sterols (Davis et al. 1970).

Varieties of sterols and their relative composition vary considerably among plant species (Kemp et al. 1968; Grunwald, 1970). However, physiological function of sterols in plants remains unknown. Sterols in tobacco leaves have recently been suspected as possible precursors of carcinogens in cigarette smoke (Stedman, 1968). They are transferrable directly from leaves to cigarette smoke (Kallianos et al. 1963; Grunwald et al. 1971b). Decrease in content of sterols and their carcinogenic derivatives in cigarette smoke may be achieved by breeding procedures. We studied the variation of sterol composition in *Nicotiana* cultivars and species with the following objectives: (1) survey the existing *Nicotiana* germplasm desirable for the alteration of sterol composition in tobacco cultivars, (2) correlate individual sterol compositions to understand their biogenetic relationship, and (3) employ sterol composition as a chemotaxonomic means to evaluate the phylogeny and evolution of the genus *Nicotiana*.

Materials and Methods

Ten plants of each of 49 wild *Nicotiana* species, five *N. tabacum* cultivars (Ky Iso 1 Ky 16, Ky Iso 3 Burley 37, Ky Iso 2 Ky 151, Ky Iso 4 Hicks, and Ky Iso 7 Turkish), and one amphidiploid (Ky Iso 5 synthetic, *N. sylvestris* × *N. tomentosiformis* Kostoff; being considered as cultivar herein) were used in this study. The experiment was conducted in a greenhouse during a period from early spring to midsummer. Culture of the plants and greenhouse conditions of 27 °C during the daytime and 18 °C at night with a 16-hour photoperiod were essentially the same as described previously (Sheen, 1970a). Twenty to 25 mature green leaves on middle stalk positions were harvested from five plants of each entry in early July. A second group of five plants for each entry was similarly harvested to provide a replication. Deveined leaf lamina of two replications were separately lyophilized, ground to pass a 40-mesh screen, and stored in amber bottles at −20 °C.

Five grams of leaf powder was extracted with acetone on a Soxhlet apparatus. The extracts were evaporated to dryness and subsequently hydrolyzed in alcoholic H_2SO_4 followed by alcoholic KOH to split free sterols from sterol glycosides and esters. Free sterols were precipitated with a solution of 2% digitonin in 80%

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ethanol. Detailed analytical procedures have been described by Stedman and Rusaniwskyj (1959).

The digitonin-precipitable sterols were cleaved by pyridine and the free sterols were extracted with ether. Acetylation of free sterols and fractionation with gas-liquid chromatographic (GLC) procedures have been described elsewhere (Cheng et al. 1971a). Cholestane was used as an internal standard. Authentic sterol acetates, either purchased from commercial sources or prepared in our laboratory, were run before and after every two or three plant samples for the purpose of sterol identification and the stabilization of the GLC system. Results are expressed on a percentage basis and values for individual sterols were obtained by triangulation of the peaks. For analysis of variance, the percentage data were subjected to an angular transformation according to the method of Bliss (1937).

Table 1. *Relative sterol composition in the green leaves of some Nicotiana tabacum cultivars*

Cultivar	Cholesterol	Campesterol	Stigmasterol	β-sitosterol
Ky Iso 1 Ky 16	8.88	17.00	49.06	25.06
Ky Iso 3 Burley 37	8.33	19.26	41.49	30.91
Ky Iso 4 Hicks	8.14	19.70	44.82	27.34
Ky Iso 2 Ky 151	9.28	22.89	44.84	22.99
Ky Iso 7 Turkish	7.94	18.96	36.43	36.67
Ky Iso 5 Synthetic	8.12	15.26	41.46	35.38
LSD 0.05	NS	NS	NS	NS

Results

The major sterols stigmasterol, β-sitosterol, campesterol, and cholesterol present in *N. tabacum* were also found in the leaves of all wild *Nicotiana* species studied. Their quantities amounted to 95% or more of total sterols measurable with the present extraction and GLC procedures. Among the cultivars representing burley, flue-cured, dark fire-cured, and Turkish types, the relative sterol composition displayed a common pattern that stigmasterol was highest followed by β-sitosterol, campesterol, and cholesterol in the order of decreasing proportion (Table 1). The Turkish strain had approximately an equal proportion of stigmasterol and β-sitosterol. Nevertheless, the variation of individual sterol composition among the six cultivars was statistically nonsignificant. The present results are in agreement with findings recently reported by Grunwald (1970) and Grunwald et al. (1971a). They analyzed the four sterols in mature green leaves of Ky 12, Burley 21, and Coker 139 (a flue-cured type) and found a similar pattern of composition.

A range of variation in relative sterol composition among *Nicotiana* species is present in Table 2. In contrast to *N. tabacum, N. setchellii, N. langsdorffii, N. nudicaulis* had similar or higher cholesterol than campesterol content. *N. repanda* contained similar or less stigmasterol than campesterol. The latter compound was in a comparable quantity as β-sitosterol in *N. glutinosa*. Variance analyses revealed significant differences ($P < 0.01$) among the species for the proportion of individual sterols. Cholesterol content in *N. nudicaulis* and *N. langsdorffii* was approximately 17% of total sterols, whereas *N. glauca, N. sylvestris,* and *N. petunioides* contained only slightly over 3%. The high and low extremes for campesterol were 27.51% in *N. sylvestris* and 10.53% in *N. setchellii*. Stigmasterol in *N. repanda* represented only 18.72% of total sterol content, in contrast, *N. glutinosa* showed as high as 55.73%. The latter species was the lowest (14.6%) for β-sitosterol. The highest percentage of β-sitosterol (58.35%) was found in *N. umbratica*. In studies of correlation coefficients between the four sterols, negative correlations were obtained for stigmasterol vs. β-sitosterol ($r = -0.86$, $p < 0.01$), cholesterol vs. β-sitosterol ($r = -0.43$, $p < 0.01$), and cholesterol vs. campesterol ($r = -0.31$, $p < 0.05$). Their significance bearing on sterol interrelationship will be discussed later.

Patterns of sterol composition may reflect upon the degree of genetic affinity among the species and upon the effect of geographic distribution and ecosystems on sterol metabolism during the course of *Nicotiana* evolution. The variation of sterol pattern is as great between the *Nicotiana* subgenera as within each subgenus. Among the four species of the subgenus *Rustica* studied, *N. glauca* distinguished itself with low percentages of cholesterol and stigmasterol and a high percentage of campesterol. It is worthy to note that *N. glauca* is the only species of this subgenus occurring prevailingly on the eastern slopes of the Peruvian Andes in northwestern Argentina.

Table 2. *Relative sterol composition in the green leaves of Nicotiana species*

Subgenus Section Species	Cholesterol Range[a]	Campesterol Range[a]	Stigmasterol Range[a]	β-sitosterol Range[a]
Rustica				
Paniculatae				
glauca	3.18 –q	22.74 a–e	27.95m–*q*	46.04 a–g
knightiana	7.36 e–o	14.61 g–l	43.67 b–e	34.36 f–l
paniculata	7.93 d–m	10.60 –l	39.46 b–k	42.01 b–j
Rusticae				
rustica	6.48 f–p	12.72 j–l	40.15 b–j	40.66 b–k

Table 2 (continued)

Subgenus Section Species	Cholesterol Range[a]	Campesterol Range[a]	Stigmasterol Range[a]	β-sitosterol Range[a]
Tabacum				
Tomentosae				
otophora	4.98 l–q	13.19 i–l	40.34 b–j	41.49 b–k
tomentosiformis	9.63 c–f	14.82 f–l	44.43 b–d	31.12 i–l
glutinosa	13.34 a–c	16.03 c–l	55.73 a–	14.67 –m
tomentosa	8.04 d–l	19.50 b–i	25.92 n–q	46.53 a–g
setchellii	13.42 a–c	10.53 –l	38.33 b–l	37.72 d–k
Genuinae				
tabacum[b]	8.44 d–j	18.84 b–j	43.01 b–f	29.72 j–l
Petunioides				
Undulatae				
undulata	7.25 e–o	15.48 e–l	46.85 b–	30.42 i–l
wigandioides	5.08 k–q	11.73 k–l	29.88 k–p	53.31 a–b
Trigonophyllae				
trigonophylla	8.45 d–j	15.29 e–l	34.41 d–o	41.96 b–k
palmeri	4.03 p–q	14.77 g–l	31.18 h–p	50.02 a–d
Alatae				
sylvestris	3.23 –q	27.51 a–	40.51 b–i	28.75 k–l
plumbaginifolia	10.78 b–e	14.05 h–l	45.91 b–c	29.26 j–l
alata	9.04 d–i	20.13 a–h	30.32 i–p	40.51 b–k
langsdorffii	16.75 a–	16.61 c–l	30.20 j–k	36.34 e–l
forgetiana	13.80 a–b	12.69 j–l	28.13 l–q	45.38 a–g
bonariensis	10.81 b–e	15.72 c–l	41.88 b–g	31.59 h–l
Repandae				
repanda	10.83 b–e	21.02 a–g	18.72 –q	49.41 a–e
nesophila	9.33 d–g	21.81 a–f	27.34 n–q	41.52 b–k
Noctiflorae				
petunioides	3.15 –q	17.66 b–k	25.93 n–q	53.27 a–b
Acuminatae				
acuminata	6.45 f–p	15.03 f–l	37.65 b–m	40.87 b–k
miersii	4.69 n–q	17.48 b–k	33.75 e–o	44.09 b–i
corymbosa	4.72m–q	15.82 c–l	33.24 f–o	46.22 a–g
angustifolia	6.40 f–p	12.79 j–l	34.79 d–n	45.92 a–g
Bigelovianae				
bigelovii	11.23 b–d	12.79 j–l	33.37 f–o	42.57 b–j
clevelandii	10.19 b–e	15.32 e–l	35.62 c–n	38.87 c–k
Nudicaules				
nudicaulis	17.03 a–	13.88 h–l	28.77 l–q	40.32 b–k
Suaveolentes				
amplexicaulis	8.26 d–k	24.46 a–b	27.04 n–q	40.25 b–k
velutina	5.16 j–q	16.27 c–l	26.70 n–q	51.87 a–c
eastii	4.77m–q	20.17 a–h	30.37 i–p	44.69 b–h
debneyi	4.56 n–q	22.51 a–e	22.88 p–q	50.05 a–d
maritima	7.61 d–n	18.29 b–k	29.36 k–p	44.74 b–h
hesperis	13.39 a–c	13.93 h–l	38.24 b–l	34.44 f–l
occidentalis	4.24 p–q	22.67 a–d	34.30 d–o	38.79 c–k
excelsior	4.47 o–q	15.65 d–l	28.81 l–q	51.04 a–d
suaveolens	7.67 d–n	19.50 b–i	30.64 i–p	42.26 b–j
ingulba	4.20 p–q	18.08 b–k	31.89 g–o	45.83 a–g
exigua	4.61 n–q	21.19 a–g	27.44 n–q	49.76 a–f
goodspeedii	7.63 d–n	15.66 d–l	34.44 d–o	42.28 b–j
benthamiana	8.69 d–i	21.05 a–g	31.21 h–p	39.06 c–k
umbratica	6.56 f–p	13.49 i–l	21.60 p–q	58.35 a–
simulans	8.04 d–l	22.39 a–e	46.16 b–	23.44 l–m
megalosiphon	5.87 h–p	19.75 b–i	39.03 b–k	35.36 f–l
rosulata	6.08 g–p	22.83 a–c	24.42 o–q	46.67 a–f
fragrans	9.33 d–h	16.19 c–l	41.13 b–h	33.10 g–l
gossei	5.14 k–q	16.74 c–k	33.96 e–o	44.29 b–i
rotundifolia	5.73 i–q	20.33 a–h	31.54 h–p	42.40 b–j

[a] Multiple range tests of differences between means were calculated at the 5% level of significance.

[b] Relative percent is an average of six *N. tabacum* cultivars listed in Table 1.

Among the Tomentosae members, *N. glutinosa* and *N. setchellii* contrasted greatly to the remainders in sterol composition. These two species share a common center of frequency at coastal and subcoastal regions in Northern Peru. *N. otophora* together with *N. wigandioides* and *N. sylvestris* of the subgenus *Petunioides* were low in cholesterol composition. All of these grow on the eastern slope of the Andes as does *N. glauca*.

genitor combinations may furnish phylogenetic information. *N. tabacum* (N = 24) and *N. rustica* (N = 24) are two cultivated amphiploids (Goodspeed, 1954). *N. rustica* is known to be a product of hybridization and amphiploidy between *N. undulata* (N = 12) and *N. paniculata* (N = 12). The origin of *N. tabacum* has been debated for years with regard to the progenitor from the Tomentosae section, *N. otophora* (N = 12). *N. tomentosiformis* (N = 12), and

Table 3. *Chi-square tests for goodness of fit for the relative sterol composition between amphiploid Nicotiana species and the possible diploid progenitor combinations*[a]

Species and possible origin	Cholesterol	Campesterol	Stigmasterol	β-sitosterol	P value
N. tabacum	8.44	18.84	43.01	29.72	
N. sylvestris × *N. otophora*	4.11	20.35	40.43	35.12	0.25–0.50
N. sylvestris × *N. tomentosiformis*	6.43	21.17	42.47	29.94	0.75–0.90
N. sylvestris × *N. tomentosa*	5.64	23.51	33.22	37.64	0.05–0.10
N. rustica	6.48	12.72	40.15	40.66	
N. undulata × *N. paniculata*	7.59	13.04	43.16	36.22	0.75–0.90

[a] Relative sterol composition of diploid progenitor combinations is an average of two progenitor species involved.

Species within the section Repandae, Accuminatae and Bigelovii did not vary in relative sterol composition. *N. trigonophylla* and *N. palmeri* are Mexican desert species and have been relegated to synonymy (Wells, 1960). They exhibited a significant contrast in cholesterol content. The same two species showed differences in peroxidase activity (Sheen, 1970b) and peroxidase isozyme pattern (Sheen, 1970a). These results may indicate that *N. trigonophylla* and *N. palmeri* possess at least some degree of genetic divergence. The Australian species of the section Suaveolentes were generally low in cholesterol and stigmasterol composition but high in campesterol and β-sitosterol. *N. suaveolens* and *N. debneyi* were regarded as the modern forms of the original migrants (Goodspeed, 1954) and appeared to be similar in sterol composition pattern. A majority of Australian species had sterol composition comparable to either *N. suaveolens* or *N. debneyi*. Exceptions are high cholesterol composition in *N. hesperis*, high stigmasterol in *N. simulans* and *N. fragrans*, and low β-sitosterol in *N. simulans*. According to Goodspeed (1954) and Burbridge (1960) *N. fragrans* is an endemic species found in certain islands of the South Pacific; *N. simulans* distributes widely in the arid areas of central Australia; and *N. hesperis* occurs in the most western region of that continent.

In tobacco, the total sterol content in F_1 hybrids appeared to be a midparent value (Davis et al. 1970). The levels of sterol composition in a *N. suaveolens* × *N. langsdorffii* hybrid were intermediate between the parental species (Cheng et al. 1971a). If this hereditary pattern also holds true between other species and their hybrids, a comparison of amphiploid *Nicotiana* species with the possible diploid pro-

N. tomentosa (*N.* 12) have been suggested as the possible candidates. The other progenitor is *N. sylvestris* (N = 12) which contributes cytoplasm (Cameron, 1965). Table 3 summarizes the results of chi-square tests for the relative sterol composition between the amphiploids and their possible progenitor combinations. A goodness of fit with a *P* value between 0.75–0.90 is the case of *N. rustica* and its progenitor species. A similar *P* value is obtained between *N. tabacum* and *N. sylvestris* × *N. tomentosiformis* and is greater than those of the combinations involving *N. otophora* or *N. tomentosa*. The results favor the *N. sylvestris* × *N. tomentosiformis* combination as the origin of *N. tabacum*. This is in keeping with the results from genetic analyses (Gerstel, 1960), and isozymic comparisons (Sheen, 1970a; 1972) involving the same species and their hybrids.

Discussion

Sterols together with polyphenols and alkaloids in plants are regarded as allelopathic substances, some of which are repellents or toxins and others are attractants in the adaptation of species and the organization of ecological communities (Whittaker and Feeny, 1971). The composition of allelopathic substances in plant species has therefore been subjected to natural selection, and consequently bears evolutionary impact. Evaluation of plant systematics on the basis of sterol composition has been reported in many plant species (Knights and Berrie, 1971), but not for the genus *Nicotiana*. The present results (Table 1) showed four component sterols occurring in common in *Nicotiana* species. This supports a close relationship of these species with regard to the genetic regulation for sterol production. Furthermore,

the similar pattern of sterol composition among six *N. tabacum* cultivars in the present study and several in other reports (Grunwald, 1970; Grunwald et al. 1971a) substantiates the phylogenetic affinity within this species. Whether or not there are intraspecific variations in *N. tabacum* can only be answered by surveying a large number of cultivars in the future.

The coincidence of geographic distribution with the relative composition of sterols reflects the operation of gene mutation, genetic drift, and adaptation. In a previous study, Sheen (1970a) reported that certain species such as *N. glauca*, *N. sylvestris*, and *N. otophora* in the eastern Andes Mountains resemble one another in peroxidase isozymes. These species appeared to have extremely low cholesterol content in the sterol pool. The modern Australian species consist of a group of aneuploids derived from hybridization and introgression between primitive species *N. debneyi* and *N. suaveolens* (Goodspeed, 1954). With few exceptions possibly because of geographic isolation, a majority of Australian species share a similar sterol pattern. This not only supports their phylogenetic closeness but also suggests that addition and subtraction of individual chromosomes do not drastically affect sterol composition.

The validity of using sterol composition as a criterion for phylogenetic comparison between possible diploid progenitors and tetraploid species rests on several assumptions. First, sterol variation among species should reflect differences in genetic entities. Genetic variants present within a species may bias the phylogenetic information. This necessitates a survey of intraspecific variation for sterol composition. Since wild species are available in limited collections, *N. tabacum* is the only species possible for such investigation. The present results (Table 1) suggest that morphological variation has little effect on sterol composition, and the cultivars studied cannot be atypical in phylogenetic evaluation. Second, sterol composition in interspecific hybrids is an average of the parental plants. This is in part supported by the experimental results which showed a midparent value of sterol content in the F_1 hybrids of intraspecific and interspecific crosses (Davis et al. 1970; Cheng et al. 1971a). Third, following hybridization and chromosome doubling, subsequent differentiation may result in little change. This assumption may be tenable in view of the fact that other allelopathic substances, namely alkaloids and polyphenols, in tobacco leaves retained same concentrations in diploid and autopolyploid plants (Burk et al. 1971; Sheen et al. 1966). Fourth, sterol biosynthesis is governed by a complicated genetic system, and interconversion between structurally related sterols may not be a single enzymatic step controlled by single genes. Up to date, there is no evidence demonstrating a single step of sterol conversion.

Chemically, β-sitosterol differs from stigmasterol by the presence of one double bond at $C_{22}-C_{23}$ (trans). Conversion of the radioactive former to the latter compound was reported (Bennett and Heftmann, 1969) but the recovery of radioactivity was extremely low. A similar situation was observed for conversion of cholesterol into campesterol when *Nicotiana* plants were fed with cholesterol-4-^{14}C (Tso and Cheng, 1971). The structural difference between cholesterol and campesterol is an extra methyl group at C_{24} in campesterol. Therefore, the negative correlation between structurally related sterols merely showed the occurrence of sterol interconversion in a metabolic pool rather than as a single step of enzymatic dehydrogenation or methylation. This is in keeping with the negative correlation between cholesterol and β-sitosterol. These compounds differ from each other not only by an extra methyl group at C_{24} but also by one double bond at $C_{22}-C_{23}$ (trans). Genetic control of sterol composition in relation to metabolic pathway would be a challenging area of future investigation.

From the standpoint of tobacco breeding, the variations of total sterol content within *N. tabacum* (Cheng et al. 1968; Davis et al. 1970) and of relative sterol composition among *Nicotiana* species reported herein assure the existence of desirable germplasm for improvement of sterol quantity and quality in tobacco leaves.

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