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Genetic Approaches to Varying Chemical Constituents in Tobacco and Smoke*

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Most of the early breeding work on tobacco (*Nicotiana tabacum* L.) was directed toward the incorporation of disease resistance, increasing yields, or improving agronomic characteristics. Geneticists and plant breeders have recently turned their attention to the possibility of altering the chemical constituents of the tobacco leaf and smoke by heritable means. This change in emphasis has been largely brought about by the increasingly important attention that is being given to the health aspects of smoking.

The chemical characters of tobacco smoke may be altered by the manufacturer through blending, changing the filling value, use of filters, changing burn rate, and other technological methods. However, chemical constituents and smoke characteristics may be modified indirectly in the tobacco plant itself. Such changes can be brought about by different cultural practices, postharvest treatments, and by breeding. The development of new cultivars with various levels of chemical constituents may require considerable effort; but the final results, in the long term, are more economical.

Limited research has been done from time to time on the mode of inheritance of several of the more important chemical constituents in cured leaf. However, the literature on these research efforts is not as extensive as reports on studies of total alkaloids (nicotine) and alkaloid quality.

The Agricultural Research Service of the U.S. Department of Agriculture maintains a collection of approximately 1500 foreign tobacco introductions, cultivars, and breeding lines, plus a collection of 62 *Nicotiana* species or close relatives of tobacco. Many of these accessions have been assayed for certain of the more important chemical constituents and considerable variation has been found. The constituents and their ranges in the collection are as follows: total nitrogen, 0.85 to 5.25 %; total alkaloids, 0.20—7.39 %; petroleum ether extracts, 6.5—14.7 %; wax, 0.25—1.73 %; holocellulose, 22.8—42.6 %. Thus, the large variation that exists among the tobacco entries and the *Nicotiana* species provides an opportunity for varying chemical constituents by breeding.

A number of genetic techniques can be used to produce cultivars that possess desirable levels of chemical constituents. In this paper, we propose to discuss the potentials and limitations of some of the older and newer approaches to breeding.

Pedigree Method or Simple Mendelian Principles

Simple Mendelian genetic approaches can be represented as a cross between two chromosomally compatible lines, differing in their levels of one or more specific chemical components. Selections, backcrosses, and selfpollinations in succeeding generations are made until a desired phenotype has been obtained in a homozygous true-breeding form. This method has been followed in tobacco breeding for many years and has resulted in cultivars with different levels of nicotine, sugar, and other constituents.

The development and release of flue-cured cultivars are subject to the limitations and control of a Minimum Standards Program in the United States. In this program, the permissible upper and lower levels of specific components, such as nicotine, sugar, total nitrogen, alpha-amino nitrogen, and total soluble nitrogen, are established in advance and must be strictly adhered to before approval is obtained for the release of a variety. These levels are maintained by simple selection.

Nicotine has probably been studied more than any other constituent in tobacco, and many of the examples used in this presentation will be based on nicotine. However the principles are applicable to other chemical constituents.

At least two systems of genetic control are involved in the quality and level of total alkaloids in the tobacco leaf. A single locus controls the conversion of nicotine to nornicotine (2, 9). Two loci control total alkaloid

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Table 1. Levels of total alkaloids in lines produced by the pedigree method of breeding for alkaloid levels [F_5 ((LA* × NC 95) (BC_5 to NC 95))].

Variety or line	Total alkaloids (%)						
	Plant number						
	1	2	3	4	5	6	Mean
LAFC 53*	0.32	0.22	0.30	0.28	0.26	0.25	0.27
96	1.76	1.98	2.57	2.63	2.37	2.74	2.34
98	2.22	2.41	1.85	2.48	1.38	2.20	2.09
142	4.74	3.97	4.43	5.73	5.62	5.31	4.97
NC 95	3.52	4.63	3.99	4.42	3.11	4.35	4.00

* LAFC / Ref. Chaplin, James F.: Registration of LAFC 53 tobacco germplasm; Crop Sci. 15 (1975) 282.

levels, which may range from a low of around 0.20 through 4.5 percent (8). Although these two genes determine the base level of total alkaloids in tobacco cultivars, these levels are further modified by minor genes or quantitative factors (10), all of which are influenced by environment. Breeding lines with varying levels of nicotine have been produced by the pedigree method (Table 1). The isolation of the double-recessive, low-alkaloid lines is not difficult (Table 2) because they are recovered as one sixteenth of the progeny from self-pollination and are also influenced less by environment (6). However, it is more difficult to establish lines with intermediate levels of total alkaloids.

The principal limitation to the pedigree or Mendelian method is the length of time it takes to fix a breeding line or variety in homozygous form. It has been difficult to identify intermediate alkaloid types because heterozygosity may persist through the F_5+ generation and because of the influence of environment over alkaloid levels. If these problems apply to alkaloids, then we expect that they apply to other chemical characteristics, particularly when the control of a specific trait is polygenic. Despite its acknowledged limitations, the conventional or pedigree system of breeding will probably remain an important part of any breeding program.

Table 2. Comparison of mean of low alkaloid (LA) lines with their recurrent parent for total alkaloids, yield and grade index (6).

Variety or line	Total alkaloids (%)	Yield (kg/ha)	Grade index***
NC 2326	2.56	2567	42
LA lines	0.13*	2387**	41
C-298	2.34	2612	28
LA lines	0.22**	2661	39**
NC 95	2.65	2558	45
LA lines	0.18**	2353**	35**

* Significant difference from zero at 5% level.
** Significant difference from zero at 1% level.
*** The larger the value the better the tobacco.

Table 3. Lines produced by the haploid-diploid method of breeding with varying levels of total alkaloids.

Group or variety	Mean total alkaloids (%)	Range total alkaloids (%)	Range yield (kg/ha)
1	0.32	0.13– .44	1782–3048
2	0.96	0.81–1.20	2171–3308
3	1.57	1.26–1.86	1684–3129
4	2.23	1.97–2.49	2211–3239
5	2.84	2.59–3.09	2026–3102
6	3.39	3.16–3.66	1841–3054
7	3.85	3.73–4.02	1938–2946
NC 95	4.02		2881

Haploid/Diploid Method

The production of haploids and their doubling to dihaploids has been done successfully in many laboratories around the world. We now have the ability to produce several thousand haploid plantlets each month. There are obvious advantages of combining the haploid method with conventional methods of breeding. The haploid is a complete hemizygote, and its doubled dihaploid counterpart is, therefore, a complete homozygote. This ability to produce a homozygous true-breeding line in a single generation offers one of the positive advantages of the haploid-diploid method.

We have used this method to produce tobacco lines with varying nicotine levels (Table 3). In spite of the morphological uniformity of the plants, we encounter considerable plant-to-plant variation for alkaloid content which is environmental. Thus a principal advantage of the haploid/diploid method over the pedigree method is our ability to rapidly establish or fix lines that breed true for different levels of chemical constituents. Entire rows of plants can be sampled, thereby eliminating single plant selections and the identification of heterozygotes in conventional segregating populations.

Our investigations and those of others have pointed up certain limitations to this method. One of these is the lower level of vigor of at least half of the dihaploids obtained from different anther sources. This has been true of dihaploids derived from S_8 and S_{15}++ single plant-to-plant varietal inbreds (1, 3). Dihaploids from a hybrid anther-source have shown less vigor than the hybrid parent with the lower vigor. Increasing the number of dihaploids in a population has also produced progenies that are equal or superior to the anther source. We suspect that haploids are subject to a higher level of mutation than comparable diploids. This may represent a disadvantage from the standpoint of genetic interpretations, but it could offer advantages to the breeder by providing a greater source of variability. We are encouraged by the report from the Iwata Station of their development of the improved breeding line F-211 (12).

\+ F_5 indicates 4th generation of selfing after initial hybridization.
++ S indicates number of selfing generations after the initial cross.

We believe that the ability to rapidly produce homozygous plants makes this method an increasingly important part of the plant breeders procedures. We also believe that this method will be extremely useful in breeding for varying levels of chemical constituents. Because the levels of most chemical constituents are influenced considerably by the environment, the testing of many dihaploid plants has an enormous advantage over testing single plants in the F_2 and succeeding generations for homozygous lines with varying levels of chemical traits.

F_1 Hybrids

The F_1 hybrid provides a rapid means of obtaining a useful variety, particularly when one of the parents is male-sterile. One of the problems with the use of F_1 hybrids is finding parental combinations that will produce a hybrid with desirable chemical or quality characteristics. F_1 hybrids must be made between parents of the same type in order to maintain leaf quality. *Chaplin* (5) studied several chemical constituents from eight flue-cured varieties and their hybrids (Table 4). He concluded that there were variations for each of the constituents and that genetic control of most of these variations was additive. One example of the successful use of hybrids is seen in Burley tobacco, in which the breeding line L8+, known for its undesirable leaf-spotting characteristic can be crossed with other Burley varieties to produce commercial hybrids. The chemical, disease-resistance, or yield traits that one wishes to exploit in an F_1 hybrid must, of course, be dominant. If these conditions are met, then the advantages of the F_1 hybrid, including its phenotypic uniformity, are obvious.

Interspecific Hybridization

Interspecific hybridization and alien substitution, or transfer of useful disease-resistant traits have been amply demonstrated to be useful tools in the improvement of tobacco varieties. These same successes can undoubtedly be duplicated with respect to altering the chemical profile of commercial tobacco. We have a collection of 62 *Nicotiana* species which are relatives of *N. tabacum*. At least 30 of the species have been successfully hybridized with tobacco and the remainder could probably be hybridized by parasexual hybridization. Many of the species have an array of chemical constituents that are not available in commercial tobacco varieties. The species have been surveyed for their alkaloid content (13, 14, 7) and a limited number have been checked for other constituents (15, 7). However, before a program of interspecific hybridization is considered, it will be necessary to carefully assay the *Nicotiana* species for all their chemical constituents and also assay their hybrids with *N. tabacum* for any interactions that may influence the level and quality of these constituents. Such assays would have to be made at all stages of hybridization and trait incorporation. An example is seen in *N. glauca* Grah., which produces anabasine; when it is hybridized with *N. tabacum*, the principal alkaloid is nornicotine (15).

A current project at our laboratory concerns the transfer of the alkaloid mechanism from *N. rustica* into the genome of low-nicotine tobacco. A tetraploid tobacco low in alkaloid (less than 0.20 total alkaloid) was used in the initial cross with *N. rustica* to produce a sesquidiploid. Two additional backcrosses were made to the low alkaloid diploid which was followed by five self-pollinations. Selection among the progeny of this material has produced several lines with at least four different levels of alkaloids, with the principal alkaloid inheritance mechanism derived from *N. rustica*. Inheritance studies on the alkaloid genes from *N. rustica* and chemical studies have yet to be made. This interspecific transfer took six years to complete. Similar transfer of other genetic traits from the *Nicotiana* species have taken considerably longer.

Scientists concerned with interspecific hybridization are well aware of the problems inherent in this method – the many generations involved, cytological and genetic verification of transfer, sterilities and adverse linkages, plus the inadvertent transfer of undesirable genetic material from the foreign species. Although drastic changes in chemical constituents may be brought about by interspecific hybridization, we doubt that it will be an easy method for changing levels of a specific constituent. One should have the presence-absence type of genetic situation to effect a transfer of foreign germplasm. Also, the characteristic that one seeks to establish in the *N. tabacum* genome must be dominant and simply inherited. However, the use of interspecific hybridization may be considered in special situations.

Table 4. Estimates of variances for general and specific combining ability in an 8-variety flue-cured tobacco diallel (5).

Component	Total nitrogen	% Total nitrogen that is soluble	Alpha-amino nitrogen	Nicotine	Reducing sugars	Petroleum ether extract	Water-soluble acids	Non-volatile acids
σ^2_g	0.00191*	0.49356*	0.00008*	0.06377*	0.63179*	0.00699*	0.02682*	0.14032*
σ^2_s	−0.00025	−0.10694	0.00005	0.00657	0.32184	−0.01246	−0.00914	−0.00388

* Significant difference from zero at 1 % level.
g: general combined ability.
s: specific combined ability.

+ L8: A breeding line developed at the University of Kentucky with black shank resistance from *N. longiflora*. (Ref. Collins, G.B., P.D. Legg, C.C. Litton, and J.H. Smiley: Registration of L8 Burley tobacco germplasm; Crop Sci. 11 (1971) 606.)

Parasexual Hybridization

Fusion of diploid protoplasts from *N. tabacum* and a species relative may make it possible in the future to produce allopolyploids that have heretofore not been produced by conventional methods of pollination (4, 11). It may also be possible to fuse protoplasts from species of different genera. However, the success of parasexual hybridization finally depends on the absolute identification of the desired fusions and the ability of the resulting callus to regenerate shoots that are capable of developing into essentially normal plants. Basic genetic research is certainly to benefit from parasexual hybridization, but the practical benefits for the commercial improvement of tobacco are currently in the realm of speculation.

SUMMARY

A vast storehouse of genetic variability is contained in collections of *Nicotiana tabacum* and *Nicotiana* species. Methods and techniques of using this material to alter chemical constituents of commercial tobacco are discussed.

Simple Mendelian procedures that have resulted in improved varieties may also be used to change chemical constituents. Male-sterility permits the rapid production of F_1 hybrids in special situations. Interspecific hybridization allows the transfer of new germplasm. The haploid/diploid method offers instantaneous homozygosity when a haploid is doubled. Any diploidized haploid that shows a favorable change in a chemical trait automatically represents a potentially useful breeding line. Parasexual hybridization is a new technique that involves the fusion of protoplasts. Fusion of protoplasts between diploid tobacco and a species, that cannot be crossed with it by conventional means, provides a valuable new allopolyploid. Thus, conventional breeding methods aided by these new adjunct techniques provide the basis for favorably altering chemical constituents in the leaf and smoke.

ZUSAMMENFASSUNG

In der Gesamtheit von *Nicotiana tabacum* und den anderen *Nicotiana*-Spezies ist ein sehr großes Depot genetischer Variabilität enthalten. Zur Nutzbarmachung dieses Materials für Änderungen chemischer Inhaltsstoffe bei handelsüblichem Tabak werden Methoden und Techniken diskutiert.

Einfache *Mendel*sche Verfahren, die zu einer verbesserten Varietät führen, können auch zur Änderung chemischer Inhaltsstoffe benutzt werden. Männliche Sterilität erlaubt die Schnellproduktion von F_1-Hybriden bei bestimmten Fragestellungen. Interspezifische Hybridisierung gestattet den Übergang von neuem Keimplasma. Die Haploid/Diploid-Methode ergibt unverzüglich Reinerbigkeit durch Verdoppelung des haploiden Chromosomensatzes. Jedes diploidisierte Haploid, das eine günstige Veränderung in einem chemischen Merkmal aufweist, stellt automatisch eine potentiell brauchbare Zuchtlinie dar. Parasexuelle Hybridisierung ist eine neue Technik, die auf der Verschmelzung von Protoplasten beruht. Bei diploidem Tabak und einer Spezies, die mit diesem nicht in herkömmlicher Art gekreuzt werden kann, ergibt diese Protoplastenverschmelzung ein wertvolles neues Allopolyploid. Auf diese Weise bilden herkömmliche Züchtungsmethoden unter Zuhilfenahme neuer Zusatzverfahren die Grundlage für eine günstige Veränderung von chemischen Inhaltsstoffen in Tabakblatt und Tabakrauch.

RÉSUMÉ

Un assortiment des espèces *Nicotiana tabacum* et *Nicotiana* représente un énorme réservoir de variations génétiques. Cette étude discute les méthodes et les techniques qui utilisent ces matériaux pour changer les constituants chimiques du tabac commercial.

Les techniques Mendéliennes dont on se sert ordinairement pour améliorer une espèce, peuvent aussi être utilisées aux fins de changer des constituants chimiques. La stérilité masculine permet la production rapide d'hybrides F_1 dans des situations déterminées. L'hybridisation interspécifique permet le transfert de germplasme nouveau. La méthode haploïde/diploïde permet l'homozygosité instantanée par le doublement des chromosomes haploïdes. Tout haploïde diploïdisé faisant preuve d'un changement chimique favorable représente automatiquement une souche potentiellement utile pour l'élevage. L'hybridisation parasexuelle est une nouvelle technique qui implique la fusion de protoplastes. La fusion de protoplastes de tabac diploïde avec une espèce qui ne peut être croisée avec ce tabac par les méthodes conventionnelles, produit un nouveau allopolyploïde de grande valeur. En conclusion, les méthodes d'élevage conventionnelles enrichies de ces nouvelles techniques fournissent la base de changements favorables de composés chimiques dans la feuille et la fumée de tabac.

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