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The value of gametoclonal variation in breeding for quantitative traits in flue-cured tobacco (*Nicotiana tabacum* L.)

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Summary. In tobacco (*Nicotiana tabacum* L.), anther-derived doubled haploid populations have been shown to exhibit large amounts of unexpected genetic variation and a severe depression in cured leaf yield when compared to conventionally inbred genotypes from comparable sources. A previous study had predicted that the yield depression observed in a doubled haploid population derived from a near homozygous cultivar, NC95, might be overcome through a recurrent selection program. In the current study, progress from three cycles of full-sib family selection for improved yield in an anther-culture-derived population of NC95 was measured, as well as the remaining genetic variation within the population. A design II experiment was conducted in the population following three cycles of selection. Results indicate that the NC95 yield level has been recovered in the third selection cycle population. Although most of the genetic variation in the population appears to be exhausted, the additive-genetic variance among maternal half-sib families for yield is significant, and it appears that continued yield improvement can be made through recurrent selection. Significant additive-genetic variance for yield was found among maternal half-sib families but was essentially zero among the paternal half-sib families, suggesting that remaining genetic variation is not being transmitted through pollen. One possible explanation results from the phenomenon of DNA amplification that can occur during the anther culture process, and that may enable extraordinary recombinational events and reduce the viability of male gametes.

Key words: Tobacco – Anther culture – Doubled haploids – Recurrent-selection

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Introduction

Plant breeders have historically depended upon naturally occurring genetic variability for their programs. However, with the discovery of large quantities of genetic variation resulting from in vitro tissue culture, breeders have attempted to incorporate this somaclonal variation into their programs. Because qualitative traits can be readily identified and selected, the use of somaclonal variation has been successful in developing these traits within crop species (Chaleff and Keil 1981; Gengenbach et al. 1977). On the other hand, the polygenic nature and complex genic control of quantitative traits makes their identification and selection more difficult. Although the use of somaclonal variation has enabled the production of improved cultivars in some plant breeding programs concerned with qualitative traits, programs studying quantitative traits are still struggling to determine if this variation can be useful.

Park et al. (1976) and Reinbergs et al. (1978) conducted quantitative studies on doubled haploids of barley (*Hordeum vulgare* L.) developed by the *H. bulbosom* L. chromosome elimination method and found no yield inferiority of doubled haploids versus inbred lines. Schaeffer (1982), on the other hand, using anther-derived doubled haploids of rice (*Oryza sativa* L.), found not only a decrease in yield but also a decrease in plant height. This reduced height was found to be genetically stable and usable for development of semidwarf cultivars. Schnell (1984) evaluated an anther-culture-derived doubled haploid population of tobacco derived from the cultivar NC95 and, although a significant amount of genetic variation had been generated, the cost was a 12% reduction in the population mean for cured leaf yield. He predicted it would take at least three cycles of recurrent full-sib selection in this population to recover the

parental yield, and that any remaining genetic variation might be used to further increase the yield beyond that of the parental cultivar, NC95. Using Schnell's original population, this study was undertaken to evaluate progress from three cycles of recurrent full-sib family selection for yield improvement and to determine if sufficient genetic variation remained to further improve yield.

Materials and methods

The base population, C_0, described by Schnell (1984) was derived through anther culture of a single plant of a highly inbred flue-cured tobacco cultivar, NC95. Sixty-four randomly selected anther-derived doubled haploids (DH) of NC95 were randomly assigned to eight sets of eight lines each. Four lines in each set were designated as males and four as females and then intermated to produce 16 full-sib (FS) families. Population C_0 was produced by bulking equal quantities of seed from each of these 128 FS families. The two best yielding families of each set were selected, grown from remnant seed and intercrossed to produce the C_1 population. Two hundred fifty-six plants from the C_1 population were selected at random and used to produce 128 FS families. These families were randomly assigned to eight sets each containing 16 FS families plus the control cultivar NC95. After evaluating the 128 FS families in one environment, the best two families in each of the eight sets were identified. Remnant seed of each FS family was grown and the plants were intercrossed at random. Equal quantities of seed from each cross were composited to from the next selected cycle population. A similar procedure was followed through the third cycle of selection.

In these populations (C_0 to C_2), two replications of each entry in each set were planted at a single location and data were taken for days from transplanting to flowering, plant height (cm), number of leaves per plant, cured leaf yield (kg/ha), cured leaf quality as measured by grade index (Wernsman and Price 1975), and percent total alkaloids and reducing sugars, using methods established by Harvey et al. (1969).

The field plots were seeded in individual beds and plants were transplanted into single-row plots of 22 plants each. The end plants of each row served as competitive border plants and were not included in the data. Fertilization, production, and management practices were those normally used for flue-cured tobacco at the North Carolina state research stations.

Fresh seed of cycles C_0 through C_2 were produced in the field in 1987, by growing each population and randomly intermating 100 plants in 50 FS crosses. This provided seed of equal age to ensure good germination for the 1988 experiments.

The 1988 experiments consisted of two different tests: (1) an evaluation of the base population and three populations resulting from FS family selection and (2) an estimation of the amount of remaining genetic variation for leaf yield in population C_3. The selected population study consisted of five treatments, including the four populations (C_0 through C_3) and the parental cultivar (NC95). Treatments were planted in a randomized, complete block design with eight replications at three locations. The evaluation of selected populations assumed a mixed model for the analysis with fixed populations (treatments). An analysis of variance was conducted on data for all measured quantitative traits. The genetic variance estimation study was planted in two locations in 1988 as a randomized, complete block design of eight sets, with entries replicated twice in each set. The data were analyzed as a NC design II (Comstock and Robinson 1952) using a completely random model. Variation among entries was partitioned into differences between the parental cultivar and the FS families within sets and among FS families within sets for all of the measured traits. NC95 was then dropped from the analysis and the data were reanalyzed for estimation of genetic variance. Genotypic variation was partitioned into that due to males (sets), females (sets), and males × females (sets). Genotype × environment variance was partitioned into environment × females (sets), environment × females (sets), and environment × males × females (sets). Mean squares of these components were tested using the appropriate error terms. The C_3 population was assumed to be noninbred, consequently genetic variance estimates were based on an inbreeding coefficient of $F=0$ (Hallauer and Miranda 1981) and were calculated using the appropriate variance components from the statistical analysis.

Table 1. Mean separation analysis for significant quantitative trait means from combined analysis over locations for the recurrent FS selection study[a]

Entry	Yield[b] (kg/ha)	Days to flower[b]	Grade index[b]
NC95	3136[c]	67.5[e]	51.7[g]
C_0	2994[d]	67.5[e]	49.3[g]
C_1	2939[d]	68.2[e,f]	42.9[h]
C_2	3176[c]	67.9[e]	48.3[g]
C_3	3146[c]	68.9[f]	52.3[g]
Min. sign. diff. 0.05 level	122	0.8	4.5

[a] Mean separation determined by Waller-Duncan K-ratio *t*-test
[b] Means with same letter are not significantly different

Table 2. Analysis of variance for the significant quantitative traits studied in the design II experiment combined over two locations for variance estimation study[a]

Source	*df*	Mean squares		
		Plant height	Total alkaloids	Yield
Males (set)	24	39.62	0.192 **	71,735.50
Females (set)	24	38.80 **	0.093	198,853.25 **
Males × Females (set)	72	14.04	0.079	55,048.86
Location × Males (set)	24	26.36 *	0.058	59,588.38
Location × Females (set)	24	10.26	0.156 *	53,743.16
Location × Males × Females (set)	72	15.20	0.083	44,057.73
Error	240	19.51	0.080	62,791.75

[a] NC95 deleted
*, ** Significant at the 0.05 and 0.01 levels, respectively

Results

The analysis of variance of the data from the populations developed by recurrent selection revealed significant differences among treatments only for cured leaf yield, days to flower, and grade index. There were no significant G × E interactions. A Waller-Duncan *K*-ratio test was conducted for the means of all measured traits for which

Table 3. Estimates of variance components, heritability, and predicted gain of paternal and maternal half-sib families and full-sib family selection for measured quantitative traits combined over locations for the design II experiment

Variance component	Plant height	Leaf number	Days to flower	Grade Index	Total alkaloids	Reducing sugars	Yield
Males (set)	0.90	0.036	−0.021[a]	0.408	0.0086[b]	0.067	72.25
Females (set)	1.86[b]	0.020	–	−0.394[a]	−0.0037[a]	−0.112[a]	8,382.44[b]
Males × Females (set)	−0.29[a]	−0.030[a]	−0.025[a]	1.395	−0.0010[a]	0.090	2,747.78
Additive variance[c]	5.52	0.112	–	0.816	0.0172	0.134	16,909.38
Phenotypic variance[c]	6.03	0.355	0.295	6.160	0.0261	0.891	21,786.77
Heritability[d]	0.915	0.315	–	0.132	0.659	0.150	0.776
Predicted gain[e]	3.43 cm	0.286 lv	–	0.500	0.162 %	0.216%	174.69 kg/ha
% gain	3.4	1.4	–	1.2	4.4	1.6	5.8

[a] Negative variance estimates are assumed to be zero
[b] Estimate exceed twice its standard error
[c] Estimates based on full-sib families
[d] Computed on full-sib family basis
[e] From continued full-sib family selection

the analysis of variance indicated significant differences (Table 1). Linear regression analysis of yield on selection cycle indicated a yield increase of 49 kg/ha per cycle. However, the coefficient of determination, $r^2 = 0.112$, was indicative of wide dispersion in the data. Linear, quadratic, and cubic contrasts of the data revealed that the data most nearly fit a linear trend, indicating a continuing increase in yield with selection cycle.

Since each set in the estimation of remaining variance in population C_3 contained NC95 and there were two replications and two locations, 32 data points were available to estimate the mean yield of NC95 and compare it with the C_3 population mean. The comparison, using single degree-of-freedom contrasts, revealed that the C_3 population and the parental cultivar were significantly different only in plant height and yield. Evaluation of mean yields of the C_3 FS families indicated that the population mean exceeded that of NC95 by 100 kg/ha.

The design II analysis of the data for variance estimation in the C_3 population revealed significant additive-genetic variability in the population for only three traits: total alkaloids among paternal half-sib families and plant height and leaf yields among maternal half-sib families (Table 2). Genetic variances and heritabilities, computed on a full-sib family basis, and expected gain, based on continued full-sib family selection, for the quantitative traits studied are shown in Table 3. Although maternal effects have previously been shown to be unimportant for flue-cured tobacco of doubled haploid origin (Brown and Wernsman 1982), it is interesting to note that the female component of additive-genetic variance for yield in this study is essentially the same as that found in the original study of Schnell (1984), whereas the male component is insignificant.

Discussion

Anther-derived doubled haploids of tobacco have been found to contain increased DNA compared to their parental cultivars (Dhillon et al. 1983; Reed and Wernsman 1989). DePaepe et al. (1982) have also shown DNA amplification in doubled haploids of *Nicotiana sylvestris*. Reasons for this increase have not been established; however, recent studies have indicated that the amplified DNA appears to be distributed throughout the genome of anther-derived doubled haploids (Reed et al. 1989) and is not restricted to specific chromosomes. Reed et al. (1990) observed multivalents occurring at meiosis when DNA-amplified doubled haploid lines were crossed to the cultivar from which they originated, but the DH lines themselves exhibited complete bivalent pairing. Bivalents and multivalents have also been observed at meiosis in tobacco haploids derived from DH lines subjected to four cycles of anther culture (E. A. Wernsman, unpublished results). This may indicate that the increased chromatin in the genome is distributed in such a way as to permit the pairing of homeologous or non-homologous chromosomes within the allotetraploid genome. If pairing of nonhomologous chromosomes results in crossing-over, the production of reciprocal translocations or chromosomal interchanges within the genome would occur.

These altered structures may give rise to unusual pairing relationships in diplotene of meiosis and enable partial pairing of multiple chromosomes, giving rise to multivalent structures as opposed to the normal bivalents observed in DH lines. Depending upon the segregation pattern as the cell goes through anaphase I, there may be a complete set of the genetic material present or duplications or deficiencies of the genetic material may result.

Swanson et al. (1967) note that "deficient chromosomes can survive more readily through the female side in higher plants, perhaps because of the better nutritional circumstances in the ovule." On the other hand, male gametes with chromosome deficiencies rarely survive and those that do cannot successfully compete with normal pollen grains because of their slower growth rate. Swanson et al. (1967) also note that "studies of meiosis in haploid plants, where no pairing would be expected, reveal some unexpected synapsis, which would suggest the presence of duplications." These duplications may be retained for better adaptation to changing environments. Reed and Burns (1989) found pollen mother cells (PMCs) in the F_1 of a cross between a doubled haploid plant and its parental cultivar containing multivalent structures. Pollen grains arising from these PMCs might exhibit reduced viability and pollen tube growth compared to those that arise from PMCs with normal bivalent pairing. Thus, the assumption of "random" transmission of male gametes could be invalidated by an effective screening of pollen grains, resulting in reduced genetic variance of the paternal half-sib families.

The female gamete, on the other hand, is nurtured by the maternal tissues surrounding it and formation of the zygote is independent of its rate of growth. Hence, there may not be screening of the female gamete and variation in the population is transmitted through the egg. This may be the reason for the unexpected, large difference between the estimated additive-genetic variance between males and females in this experiment. Although cytoplasmic and/or maternal effects may have contributed to the differences in additive-genetic variance found between maternal and paternal half-sib families, these effects have generally been found to be unimportant in flue-cured tobacco (Brown and Wernsman 1982). Also, since the entire population originated from a single NC95 plant, any maternal effects must have arisen through the anther culture process. Consequently, cytoplasmic and/or maternal effects were discounted as the reason for the large differences between the maternal and paternal components of additive-genetic variance in this experiment. In view of the data provided by Reed et al. (1990), some of these unexpected responses may result from the additional cycle of random mating used to rejuvenate the seeds in 1987, thus permitting further natural selection among the male gametes, resulting in differences between the C_0 population used in this experiment and the original C_0 population used by Schnell (1984). This raises questions concerning the ability to retain anther-derived genetic variation in a random mating population.

From these studies it is concluded that: (1) genetic variability remaining in population C_3 was apparently not transmitted through pollen; (2) the ability to retain anther-derived gametoclonal variation in a random-mated tobacco population may be questionable; (3) yield of the parental cultivar has been recovered but not exceeded by three cycles of full-sib family recurrent selection; and (4) while additive-genetic variance for yield among maternal half-sib families appears to be sufficient to continue yield improvement in this population using a recurrent selection technique, little overall additive variance remains for other measured traits in the C_3 population.

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