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Comparison of *Nicotiana tabacum* and *Nicotiana nesophila* Hybrids Produced by Ovule Culture and Protoplast Fusion

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Summary. Somatic hybrids were produced between *Nicotiana tabacum* and *N. nesophila,* two species incapable of conventional sexual hybridization. Sexual hybrids, though, could be produced between these two species by using ovule culture only when *N. nesophila* was female. Clones of somatic hybrids were compared with sexual hybrids. Statistically significant variation was observed between clones, but not between sexual hybrids, for pollen viability, flower morphology, leaf morphology, and trichome density. As all clones of somatic hybrids have 96 chromosomes, the variability could not be explained by interclonal variation in chromosome number. Variation between somatic hybrids could be the result of cytoplasmic segregation or recombination, mitotic recombination or small chromosomal rearrangements prior to plant regeneration. Variation between clones could be exploited as these interspecies hybrids are now being used to incorporate disease resistance into cultivated tobacco.

Key words: *Nicotiana* – Somatic hybrids – Protoplast fusion – Ovule culture – Genetic variability

Introduction

Somatic hybridization has been suggested as a unique method to transfer useful genetic traits from wild species into cultivated crops. However, most very wide hybrids produced via protoplast fusion are aneuploid and are partially or completely sterile (e.g. the potato-tomato hybrids of Melchers et al. 1978). Amphiploid hybrids between *Nicotiana nesophila* and *N. tabacum* have been obtained following fusion of protoplasts isolated from leaf mesophyll cells of *N. nesophila* and a cell suspension culture of the albino *N. tabacum* Su/Su (Evans et al. 1981). The *N. nesophila* + *N. tabacum* hybrids (NN + Su/Su) are agriculturally important as *N. nesophila* is resistant to several diseases which affect cultivated tobacco (Stavely 1979). Disease resistance has been demonstrated in these somatic hybrids (Evans et al. 1981). By integration of somatic hybrids into a conventional breeding program, desirable traits such as disease resistance can be incorporated into cultivated tobacco. Five NN + Su/Su clones have been identified that have the amphiploid chromosome number.

Despite the potential agricultural applications of somatic hybrids, in no case has a population of somatic hybrids been compared to sexual hybrids recovered in vitro by ovule culture. The NN + Su/Su somatic hybrids represent a unique opportunity to explore the potential applications of somatic versus sexual hybrids, as somatic hybrid clones have been identified with stable amphiploid chromosome number. Although the *N. nesophila* × *N. tabacum* sexual hybrids (NXT) cannot be produced through conventional cross pollination, the sexual hybrid has been recently reported using ovule culture (Reed and Collins 1978). The sexual hybrid can be produced only unidirectionally with *N. nesophila* as the female parent. The availability of both sexual and somatic hybrids for this agriculturally important species combination offers a unique opportunity to compare somatic and sexual hybrids.

Materials and Methods

Plant Material. The semi-dominant chlorophyll deficient mutant of the *N. tabacum* variety 'John Williams Broadleaf' (Su/su) (Burk and Menser 1964) on selfing produces dark green (su/su), light green (Su/su) and albino plants (Su/Su) in the ratio 1 : 2 : 1. Sexual hybrids were made with dark green (su/su) plants. Cell suspension cultures of the albino (Su/Su) were maintained in Murashige and Skoog medium (1962) with vitamins after Gamborg et al. (1968), 4.5 μM 2,4-D and 2.0 grams per liter casein hydrolysate. Chromosome stability of the Su/Su cell suspension culture was verified prior to protoplast fusion. Seed of *N. nesophila* was obtained from Dr. L. G. Burk, Oxford, NC. All plants were raised to maturity in the greenhouse.

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Sexual Hybridization. Sexual hybrids between *N. tabacum* and *N. nesophila* (NXT) were obtained using ovule culture by the technique described earlier (Reed and Collins 1978). Previously reported hybrids were with the 'Burley 21' variety of *N. tabacum*, whereas the hybrids reported here utilized su/su 'John Williams Broadleaf' as the source of *N. tabacum* germplasm. Diploidized NXT plants were obtained by regenerating plants from leaf midrib of NXT plants.

Protoplast Isolation and Fusion. Greenhouse grown *N. nesophila* plants were dark treated for 24 h prior to protoplast isolation. Young plants were used prior to flowering. Mesophyll protoplasts were isolated from young fully expanded leaves in 6 h at 22 – 25 °C in the dark without shaking in enzyme solution containing desalted 0.5% cellulase Onozuka R10 (cellulysin, Calbiochem), 0.5% macerase (macerase, Calbiochem) and 0.25% driselase (Plenum Scientific) dissolved in medium 8p of Kao and Michayluk (1975). Protoplasts of the Su/Su suspension culture were isolated in 6 h at 22 – 25 °C in the dark with shaking at 30 rpm in enzyme solution containing 0.5% cellulase Onozuka R10, 0.25% macerase and 0.25% driselase dissolved in the buffer described previously (Gamborg et al. 1979). Protoplasts of mesophyll *N. nesophila* and suspension cultures Su/Su were mixed 1 : 1. Protoplast fusion was as previously described (Evans et al. 1980). Recovery and verification of the NN + Su/Su somatic hybrids is based on identification of light green shoots (Evans et al. 1981). To conservatively identify independent fusion clones, only one light green shoot was isolated from each fusion experiment.

Classification of Plants. All hybrid plants were grown to maturity in a greenhouse. Chromosome counts were obtained from root tips as described by Evans and Reed (1981). Leaf measurements were obtained from young, fully expanded leaves. Floral measurements were obtained using mature flowers. Mean and standard error was calculated based on four samples of leaves and flowers per plant. Pollen viability was ascertained using both acetocarmine staining, commonly reported for *Nicotiana* hybrids, and in vitro germination. Optimum in vitro germination of fresh pollen was obtained in 2 h in 10% sucrose, 50 mg/l boric acid at 22 – 25 °C at 100% humidity. Statistical comparison was completed on plants derived from five separate protoplast fusion experiments, designated Clones A-E. As each plant was derived from a separate fusion experiment, each plant was designated as a clone and was raised to maturity and maintained in shoot culture and vegetatively propagated in the greenhouse. These clones were compared with 11 embryo hybrids. Each NXT hybrid was derived from a separate fertilization event.

Isoenzyme Analysis. Leaf extracts were prepared using techniques of Wetter (1977). Aspartate aminotransferase and alanylaminopeptidase were separated on 7.5% slab polyacrylamide gels containing 0.5% soluble starch (Brewbaker et al. 1968) for improved band resolution. Gel and electrode buffers described by Wetter (1977) were used. The gels were run at 35 ma for 1 h and then at 70 ma for 2 h or until the bromphenol blue dye marker front migrated off the bottom of the gel. Enzyme activity was detected by employing the staining procedures of Wetter and Kao (1976).

Peroxidase was separated on horizontal 12% hydrolyzed starch gels run at 5 °C. Extraction procedure and buffer solutions previously used for tomato peroxidase were used (Rick et al. 1977). After the gels were run at 150 volts for 30 min, the sample wicks were removed and the gels were then run at 300 volts for 5 1/2 h. Peroxidase stain (Rick et al. 1977) was modified as follows: 10 mg 3-amino-9-ethyl carbazole dissolved in 1.0 ml of N,N-dimethylformamide, 9.0 ml of 0.1 M Na acetate pH 4.5, 0.2 ml of a 1.0 M $CaCl_2$ solution, 0.5 ml of 3% H_2O_2, and 10 ml of 1% Difco Nobel Agar (cooled). Gels were sliced in half horizontally and one half of the stain applied to each slice.

Results

Variability was observed for most morphological traits when the five somatic hybrid clones were compared. Data pertaining to vegetative and floral morphology are summarized in Tables 1 and 2. Preliminary observations suggested that numerous characters were more variable in NN + Su/Su plants than in NXT plants. Leaf shape, quantified as width/length, varied between clones, but was constant between the NXT plants and within NN + Su/Su clones. Leaf petioles were present on *N. nesophila*, absent on *N. tabacum*, and were present to varying degrees on somatic hybrids (Fig. 1). The petioles of the NXT plants, on the other hand, were uniform in design and length (Fig. 1). While variability between somatic hybrid clones was extensive for trichome density, this trait varies with leaf pigment content. As NN + Su/Su plants varied in pigment content, it is not surprising that trichome density is highly variable (range 12.5 – 26.0 trichomes/mm²).

Floral characters have been used extensively in taxonomic studies of the genus *Nicotiana* (Goodspeed 1954) and in most cases, sexual and somatic hybrids contain flowers with morphological traits that are intermediate between the two parental types. Flower color was uniformly light pink in all NXT and amphiploid NXT plants examined. The color of NN + Su/Su plants was variable between clones, while uniform on each individual hybrid plant. While most clones were light

Table 1. Vegetative morphology of clones of somatic and sexual hybrids between *Nicotiana tabacum* and *N. nesophila*

Plants	Leaf width length[a]	Trichome density[b]
Somatic hybrid clones		
Clone A	0.502 ± 0.007	17.0 ± 1.53
Clone B	0.413 ± 0.009	19.5 ± 1.34
Clone C	0.478 ± 0.018	12.5 ± 1.35
Clone D	0.405 ± 0.028	23.5 ± 1.98
Clone E	0.463 ± 0.018	26.0 ± 1.46
Embryo hybrids[c]	0.404 ± 0.006	21.8 ± 1.42
N. tabacum	0.239 ± 0.004	27.8 ± 1.10
N. nesophila	0.559 ± 0.037	38.6 ± 1.00

[a] Mean of four leaves per plant
[b] Trichomes per mm² of lower leaf surface
[c] Mean of 40 leaves on 10 plants

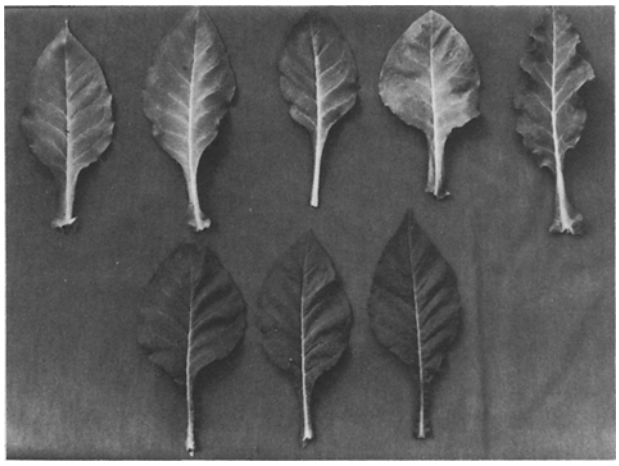

Fig. 1. Sample leaves of clones A through E (left to right) of NN + Su/Su somatic hybrids. Bottom: Three representative NXT sexual hybrids recovered by ovule rescue

pink, Clone A was unique as all flowers were dark pink. A number of floral traits visible in one of the parents were not present in any sexual or somatic hybrid. These traits all behaved as completely recessive characters. One of the five anthers present in *N. tabacum* normally lags in development. No consistent lagger was observed in the NN + Su/Su or NXT flowers. Flowers of *N. nesophila* are vespertine while no hybrid flowers expressed this trait. In addition, the 10-ribbed calyx, a trait unique to *Repandae* species, was not expressed in any hybrid plants. *Nicotiana nesophila* is cleistogamous. Of all NXT or NN + Su/Su hybrid plants only Clone E expressed the cleistogamous character. This trait greatly facilitated self-fertilization of this somatic hybrid as fertilization occurred before the flowers were open. All remaining floral characters are summarized in Table 2.

While floral color and shape was quite uniform within a single embryo hybrid plant, abnormal flowers were present on each somatic hybrid. Variants were observed for both corolla limb shape and flower color. The shape of the corolla limb on all *N. tabacum* and NXT flowers was pentagonal subentire. Flowers of *N. nesophila*, on the other hand, have corolla limbs with four or six lobes in addition to the more frequent five lobes. Among somatic hybrids, similar variants with four to six lobes have been observed as well as flowers without uniform corolla limb shape (Fig. 2). In addition, sectorial variants were commonly observed on flowers of NN + Su/Su plants. These were observed as light pink sectors on dark pink flowers or dark pink sectors on light pink flowers. In addition to the corolla limb, the sectors were often observed inside the corolla tube. No floral sectors were observed on either NXT or amphiploid NXT plants.

In contrast to the morphological variation observed between NXT and NN + Su/Su plants, the cytological and enzymological investigations do not suggest a basis for the variation between NN + Su/Su clones. As all NN + Su/Su clones used in this study had $2n = 96$ chromosomes, no variation was observed in chromosome number between the clones. All NXT plants had 48 chromosomes while the amphiploid NXT had 96 chromosomes. Variation in chromosome number cannot account for the widespread morphological variation. Meiosis of the NN + Su/Su plants was similar to previous reported meiotic analysis of amphiploid NXT (Reed and Collins 1980). Zymograms of all five clones were compared for variation in staining pattern for aspartate aminotransferase, alanylaminopeptidase, and peroxidase. Electrophoretic banding pattern of each of these enzymes could be used to distinguish NXT or NN + Su/Su hybrid plants form the *N. nesophila* or *N. tabacum* parents. No differences in zymograms of alanylaminopeptidase and aspartate aminotransferase were detected between the clones of NN + Su/Su or between NXT and NN + Su/Su hybrids. In addition, differences detected in one band of peroxidase between NN + Su/Su clones were dependent on developmental age and

Fig. 2. Flowers from mature NN + Su/Su somatic hybrid plants. Normal pentagonal flower on right, adjacent to three sample variant flowers frequently observed on NN + Su/Su hybrid plants

Table 2. Floral morphology of clones of sexual and somatic hybrids between *Nicotiana tabacum* and *N. nesophila*[a]

Plants	Flower length	Stigma length	Corolla diameter	Calyx length
Somatic hybrid clones				
Clone A	5.48 ± 0.025	4.90 ± 0.06	3.03 ± 0.025	2.35 ± 0.09
Clone B	4.30 ± 0.09	3.13 ± 0.05	2.65 ± 0.06	1.55 ± 0.03
Clone C	4.98 ± 0.05	3.98 ± 0.06	3.03 ± 0.23	1.95 ± 0.06
Clone D	4.50 ± 0.04	4.00 ± 0.04	3.03 ± 0.03	1.83 ± 0.03
Clone E	4.75 ± 0.12	4.25 ± 0.06	2.90 ± 0.06	2.03 ± 0.03
Embryo hybrids[b]	5.65 ± 0.09	4.52 ± 0.02	2.70 ± 0.06	2.03 ± 0.02
N. tabacum	5.28 ± 0.07	4.03 ± 0.03	2.93 ± 0.08	2.02 ± 0.01
N. nesophila	4.95 ± 0.07	3.40 ± 0.26	2.25 ± 0.09	1.48 ± 0.10

[a] Mean of four flowers per plant; all measurements in cm
[b] Mean of 44 flowers on 5 plants

Table 3. Pollen viability of somatic hybrid clones, *N. tabacum* + *N. nesophila*

NN + Su/Su clone	Stained pollen	Pollen germination
A	69.2	29.0
B	74.6	46.7
C	72.9	14.0
D	70.0	12.2
E	49.8	15.8
NXT hybrid	0	0
Diploidized NXT hybrid	100	72.0
N. tabacum	97.5	95.0
N. nesophila	96.5	85.3

were inconsistent between experiments. Consequently, no isoenzymic basis for interclonal variation in electrophoretic banding pattern could be detected.

Pollen viability was measured in each of the five clones of NN + Su/Su (Table 3). While viability based on acetocarmine stain, normally reported in *Nicotiana* literature, was high, pollen germination in an optimized liquid medium was quite low for most of these clones. Pollen germination was extremely variable within a single plant. Although NXT plants cannot be crossed, amphiploid NXT plants can be backcrossed but cannot be self-fertilized (Reed and Collins 1980). Of the somatic hybrids, Clone A, with highest frequency of pol-

Table 4. Statistical comparison of the means of morphological measurements between NXT hybrids and Clones A – E of NN + Su/Su somatic hybrids of *Nicotiana tabacum* and *N. nesophila*

Character	Mean of NXT plants	Signif >	Same	Signif <
Corolla length	5.65 cm	–	A	B, C, D, E
Flower diameter	2.70 cm	–	A, B, C, D, E	–
Stigma length	4.52 cm	A	E	B, C, D
Calyx length	2.03 cm	A	C, D, E	B
Leaf W/L	0.40 cm	A, B, E	C, D	–

Table 5. Comparison of variation for morphological traits within a population of NXT hybrids versus a population of NN + Su/Su clones

Variable	Population	Mean square	d.f.	F-value
Corolla length	Somatic hybrids	0.8287	4	6.429*
	Sexual hybrids	0.1289	10	
Corolla diameter	Somatic hybrids	0.1063	4	1.839 n. s.
	Sexual hybrids	0.0578	10	
Stigma length	Somatic hybrids	1.6263	4	46.071**
	Sexual hybrids	0.0353	10	
Calyx length	Somatic hybrids	0.3408	4	14.380**
	Sexual hybrids	0.0237	10	
Leaf shape	Somatic hybrids	0.1601	4	4.133 n. s.
	Sexual hybrids	0.0387	10	

*, ** Significant at $p = 0.05$ and 0.01, respectively

len germination, has the greatest flexibility in cross fertilization as it can be used in all possible reciprocal backcrosses to *N. nesophila* and *N. tabacum.* Clone D, on the other hand, has not been successfully backcrossed to either parent, although one viable seed was finally obtained following numerous self-pollinations. Clone E is the only clone that will readily self-fertilize, despite pollen viability that is lower than that of most of the clones. However, as mentioned earlier, Clone E is the only somatic hybrid that is cleistogamous. The value of expression of this variable character is evident as it facilitates recovery of a large F_2 population, otherwise not possible with NN + Su/Su, NXT or amphiploid NXT plants.

Statistical comparison of NN + Su/Su versus NXT hybrids is summarized in Tables 4 and 5. While some characters were constant in all NN + Su/Su and NXT plants that were examined, the mean of certain characters was significantly different between NN + Su/Su and NXT hybrids. While floral diameter is extremely uniform, NN + Su/Su clones have been identified that have longer and shorter stigmas and calyxes than comparable NXT plants (Table 4). These two traits, stigma and calyx length, appear to be most variable in clones of somatic hybrids when compared to NXT plants. Interclonal variation for somatic hybrids was compared with interplant variation for embryo hybrids (Table 5). Variation is greater between NN + Su/Su plants than between NXT plants for the floral characters, corolla length, stigma length, and calyx length.

Discussion

Quantitative variability has been observed between plants produced by protoplast fusion for such traits as plant height (21 – 113 cm; Nagao 1979), leaf petiole length (0 – 55.9 mm; Evans et al. 1980), and pollen viability (0.6 – 74.1%; Nagao 1978). However, in most cases, phenotypic variability has been attributed to variation in chromosome number (Smith et al. 1976). In the comparison of NN + Su/Su clones reported here, the five clones selected, contained uniform chromosome number. The phenotypic variation observed cannot be ascribed to either euploid or aneuploid chromosome changes.

The morphological variation observed is being examined in backcross and self-fertilized progeny of the NN + Su/Su plants, as the genetic basis for this variation is as yet undetermined. Numerous explanations can be advanced to account for this difference in variability between NXT and NN + Su/Su plants. (1) While aneuploidy cannot account for the variability, genetic interchange between the *nesophila* and *tabacum* genomes and small deletions of genetic material could be involved in a cytogenetic basis of this variability. Chromosome rearrangements producing reconstructed chromosmes have frequently been reported in cultured cells (Sacristán 1971). Any intergenomic genetic exchange in NN + Su/Su plants could produce cells of differing phenotypic appearance and altered pollen viability. Evidence for the occurrence of intergenomic recombination in NN + Su/Su plants was reported earlier (Evans et al. 1981) as double spots were frequently observed on the leaves of NN + Su/Su plants. (2) Following protoplast fusion, presence of mixed cytoplasms in somatic hybrids may result in unique nuclear cytoplasmic combinations in somatic hybrids, while the paternal cytoplasmic DNA is routinely excluded from NXT hybrids. Organelle segregation has been frequently observed in somatic hybrids (e.g. Chen et al. 1977). Additionally, recombination of mitochondria (Belliard et al. 1979) and chloroplast (Conde 1981) DNA has been reported in somatic hybrid plants. Preliminary evidence using tentoxin sensitivity suggests that all NN + Su/Su and all NXT hybrids have *nesophila* chloroplasts. (3) Variability has been reported in plants regenerated from mesophyll protoplasts of potato (Shepard et al. 1980). It is possible that some of the phenotypic variability recovered in regenerated plants could be induced by chemicals in the culture medium that was used for plant regeneration. As production of variation in protoplast clones has not yet been genetically characterized or extended to a large number of plant species, it is worth noting that NN + Su/Su plants survived plant regeneration in vitro, while NXT hybrids were recovered on hormone-free culture medium follwing cross pollination. Similarly, it should be noted that the cell suspension culture of *N. tabacum* used for isolation of protoplasts was five months old at the time of protoplast fusion. Any combination of these three factors could be involved in recovery of the phenotypic variability observed in the NN + Su/Su plants. Clearly, if this variability has a genetic basis, it would be extremely valuable in the use of these plants for crop improvement.

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