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Variability among plants and their progeny regenerated from protoplasts of *Su/su* heterozygotes of *Nicotiana tabacum*

H. Lörz* and W. R. Scowcroft
CSIRO, Division of Plant Industry, P.O. Box 1600, Canberra, A.C.T. 2601, Australia

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Summary. Mesophyll protoplasts of *Nicotiana tabacum*, heterozygous for the sulfur locus (*Su/su*), were isolated and more than 2,200 calli were cultured. More than 8,000 regenerated shoots were analyzed for leaf colour. Cell culture regimes included media for normal and stressed growth conditions with both short and long culture periods. An additional treatment included N-methyl-N′-nitro-N-nitrosoguanidine mutagenesis. An analysis of the regenerated shoots showed that an extended culture period led to an enhanced frequency of variant colony types, i.e. colonies producing both parental (*Su/su*) and non-parental (*Su/Su* or *su/su*) plants. NNG at 10 mg/l also enhanced the frequency of variant colony types. In some treatments there was also an increase in non-morphogenic colonies but this was independent of genetic changes at the sulfur locus. The frequency of dark green spots and twin spots, presumed to result from somatic crossing-over, was higher in the leaf cells of regenerated plants after both prolonged cell culture and chemical mutagenesis. Genetic analysis of the progeny of selfed regenerants revealed additional tissue culture induced variability with respect to segregation ratios of the different sulfur phenotypes. About two thirds of the lines tested segregated in accordance with a 1:2:1 Mendelian ratio. The remainder deviated from the expected segregation pattern and some lines also showed heterogeneity between progeny families derived from different seed capsules of the same plant. These results demonstrate that genetic changes affecting a specific locus and segregation patterns in progeny of regenerated plants are induced during cell culture.

* Present address: Max-Planck-Institut für Züchtungsforschung, D-5000 Köln 30, Federal Republic of Germany

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Introduction

Genetic variability among plants regenerated from cell culture appears to be a ubiquitous phenomenon. Called somaclonal variation, it is a source of useful genetic variability for plant improvement (Bidney and Shepard 1981; Larkin and Scowcroft 1981; Scowcroft and Larkin 1983). The genetic events which give rise to somaclonal variation are not yet understood. While aneuploidy seems not to be a major contributing factor (Bidney and Shepard 1981; Ogihara 1981), meiotic analysis of regenerated plants has revealed extensive chromosomal rearrangements such as translocations, inversions, subchromatid exchange and partial chromosome loss (Orton 1980; Ogihara 1981; McCoy et al. 1982).

Most documentation of somaclonal variation has occurred in asexually propagated species, such as potato and sugarcane, which are not accessible to detailed genetic analysis (Heinz et al. 1977; Shepard et al. 1980; Thomas et al. 1982; Larkin and Scowcroft 1983). Barbier and Dulieu (1980) established that "mutation rate" during cell culture at each of 2 genetically defined loci affecting leaf colour in tobacco was of the order of 3%. They equivocate, however, on the relative contribution of variation which pre-existed in the explant material and that which arose during cell culture. In maize, progeny analysis of plants regenerated from cell cultures derived from immature embryos showed that on average each regenerated plant carried at least one mutation which segregated in a Mendelian fashion (Edallo et al. 1981). At the molecular level, a restriction endonuclease analysis has revealed presumptive nucleotide sequence changes in mitochondrial DNA of *Drechslera maydis* race T-toxin resistant plants (Kemble et al. 1982)

regenerated from cell cultures of toxin susceptible Texas cytoplasm (Brettell et al. 1980).

We report experiments which utilise the *aurea* mutant, sulfur (*Su*) of *N. tabacum,* to monitor variation in plants regenerated from colonies derived from cultured protoplasts. The semi-dominant, nuclear sulfur mutant segregates in precise Mendelian proportions and all three genotypic classes are phenotypically distinguishable (Burk and Menser 1964). The mutant has been used as a marker to estimate mitotic recombination in plants (Carlson 1974; Evans and Paddock 1976). We have determined the effect of cell culture duration, sub-optimal (stressed) growth conditions, and chemical mutagenesis on somaclonal variation and we demonstrate from progeny analysis of regenerated plants, that cell culture can cause changes which persist in the adult plant and perturb the segregation ratios.

Material and methods

Seeds of the sulfur mutant of *Nicotiana tabacum* were generously provided by Dr. L. G. Burk, North Carolina, and introduced to Australia under quarantine number CPI 89736. The line is maintained by selfing heterozygous plants. Light green heterozygous plants were selected and grown in the glasshouse at 22 to 27 °C with shading during the summer months.

Isolation and culture of protoplasts

Protoplasts were isolated from greenhouse grown plants according to the method described recently (Lörz et al. 1983). A 10 ml enzyme mixture of 4% Cellulase (Onozuka R10, Yakult, Nishinomiya/Japan) 4% Macerase (Calbiochem) and 2% Driselase (Chem. Fabrik Schweizerhall, Basel/Switzerland) previously dialyzed against water for 1 h was mixed with 10 ml of 0.6 M mannitol and 5 ml of hormone free NTK-medium (Scowcroft and Larkin 1980), pH adjusted to 5.8. Protoplasts, liberated after approximately 3 h at 24 °C, were harvested and plated at a density of $8-10 \times 10^4$ protoplasts/ml. The standard protoplast culture medium was NTK (Scowcroft and Larkin 1980) supplemented with 1 mg/l p-chlorophenoxy acetic acid (CPA) and 1 mg/l kinetin, and with incubation at 24 °C in the dark. Three to 4 week old colonies were transferred to medium NTK-MS-1,1 which is a 1 : 1 mixture of NTK and MS-medium (Murashige and Skoog 1962), with the same growth regulators as before. Colonies were cultured at 26–28 °C and a 16 h photoperiod with light intensity of ca. $200\,\mu E \cdot m^{-2} \cdot s^{-1}$. For shoot induction the calli were transferred to MS-medium containing 1 mg/l 6-benzyl-aminopurine (BAP) and 0.5 mg/l indoleacetic acid (IAA). Root induction was achieved by culturing separated shoots on hormone free MS-medium.

Stress was imposed during the cell proliferation phase by culturing protoplast colonies on medium in which inorganic nitrogen was replaced by amino acids (AA medium). This is known to support the growth of tobacco cells at a reduced rate (Müller and Grafe 1978 and our own observations). Stressed culture conditions were achieved by diluting protoplasts cultured for 3 days in liquid NTK medium with an equal volume of nitrate-free medium. Four days later developing colonies were transferred to NTK-AA medium. The amino acid media contained the same combinations and concentrations of growth regulators as the nitrate media. To complete shoot and plant formation the regenerants were transferred to MS-medium without growth regulators and handled as described before.

Mutagenesis

N-methyl-N′-nitro-N-nitrosoguanidine (NNG) was added to the initial protoplast culture medium at concentrations of 5, 7.5 and 10 µg/ml (34, 5 and 69 µM) and left with the cultures.

Colony and plant analysis

Regenerated shoots and plants were phenotypically evaluated from 3 months after culture initiation. Figure 1 describes the colony types derived from the phenotypic classification of regenerated plants: (a) – parental type colonies include those from which all plants were phenotypically light green and classified as heterozygous (*Su/su*); (d and e) – colonies from which all the regenerated plants were either type dark green (*su/su*) or albino (*Su/Su*). These were interpreted as arising from homozygous protoplasts as a consequence of genetic changes in the leaves prior to protoplast isolation and represent pre-existing variation; (b and c) – heterogeneous colonies, which produce both parental (*Su/su*) and at least one phenotypically homozygous (*su/su* and/or *Su/Su*) shoot, arose from genetic events which occurred during cell culture.

In most cases leaf colour phenotypes could be objectively discerned, but for uncertain cases plants were maintained either on hormone free MS-medium or in the glasshouse until they could be classified. Leaf spots, either dark green segments or dark green/yellow twin spots, were counted on fully expanded leaves of glasshouse plants prior to flowering. A $10\,cm^2$ frame was used to make counts of at least 5 areas on the upper surface of leaves chosen at random. Yellow or white spots, other than those appearing as twin spots, could not be reliably counted and have been omitted from the analysis.

Progeny analysis

A number of plants, both phenotypically normal or variant with respect to leaf colour and morphology, were grown to maturity and where possible seeds resulting from self-fertilisation were harvested. Segregation ratios of these plants were determined after germination of seeds on wet cotton wool for 8–12 days in the light. Plants designated R_0 refer to those regenerated from cell culture; their progeny are designated R_1.

Results

Protoplast culture and plant regeneration

Isolation and culture of protoplasts from the aurea mutant of *N. tabacum* was efficient and highly reproducible. Dialysis of the enzyme mixture enhanced protoplast yield and subsequent viability. The average protoplast plating efficiency, i.e. % of cultured protoplasts which divided, was usually over 30% when cultured in NTK-medium containing 1 mg/l each of CPA and kinetin. There were no substantial differences in the initial protoplast culture efficiency of protoplasts isolated from the homozygous mutant, heterozygous

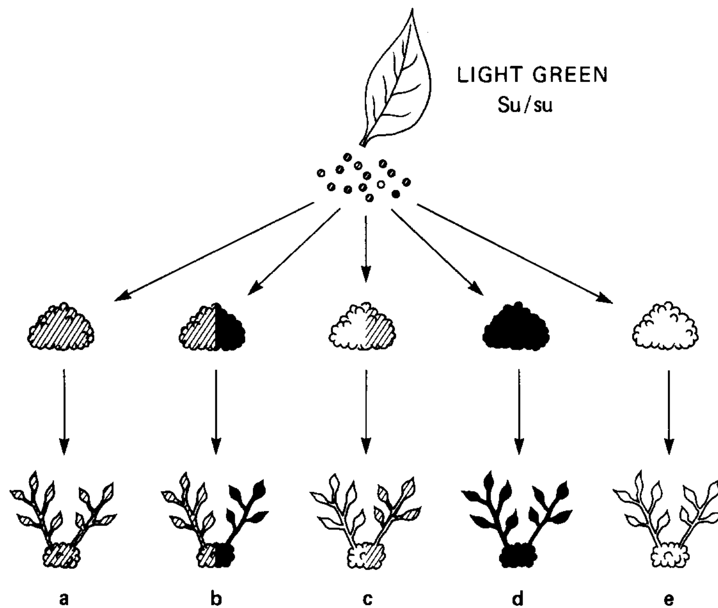

Fig. 1. Plant regeneration pattern from colonies derived from single protoplasts isolated from light green (*Su/su*) leaves. *a* All shoots normal, phenotypically parental type regenerants; *b, c* heterogeneous colonies from which at least one shoot was non-parental, interpreted as cell culture generated variation; *d, e* homogeneous colonies from which regenerants are either non-parental dark green (*d*) or albino (*e*), interpreted as preexisting variation

mutant or wild type genotypes. Plant regeneration was achieved from all genotypes with the same culture conditions. However, the later stages of regeneration, shoot and plant formation, progressed more rapidly in the wild type. These differences between genotypes were considered insufficient to establish possible bias in interpreting subsequent results.

In an attempt to reduce heterogeneity among the population of mesophyll protoplasts, obvious dark green, yellow or twin spots were removed from the leaves prior to protoplast isolation. Frequent microscopic examination of the protoplasts cultured in solidified agar medium gave us confidence that all, (or at least a very high proportion) of the developing colonies were derived from single cells. Under standard conditions protoplast-derived colonies could be transferred 3–4 weeks after culture initiation from NTK-medium to the mixture of NTK + MS medium. About one month later all proliferating calli were transferred to shoot induction medium where they formed multiple shoots readily (Fig. 3a); between one and eight shoots (average 3.8) were induced per callus. Under these culture conditions only 1.3% of the protoplast derived colonies failed to form shoots.

Mutagenesis with NNG at 5 μg/ml, 7.5 μg/ml or 10 μg/ml reduced the initial protoplast plating efficiency to ca. 70, 50 and 40% respectively of the untreated control cultures (Fig. 2). Surviving colonies were handled in the same way as the standard cultures. No abnormality was observed during development but the average time necessary to regenerate shoots and plants was increased.

An extended period of cell culture was achieved by subculturing the calli at least twice (4 week intervals) onto NTK-MS-medium. During this culture phase the undifferentiated calli proliferated rapidly and only segments of each clone were finally used for the induction of shoot morphogenesis. This extension of the callus culture period led to an average increase of non-morphogenic calli to 3.8% or to more than 10% with AA-media (Table 1).

With the amino acid (AA-media) culture regime, the intermediate phase of colony growth was obviously stressful when compared to the standard culture medium. Even after 4 months in culture the diameter of

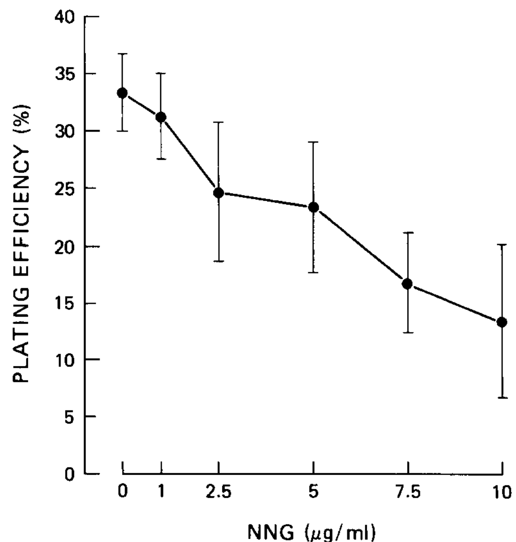

Fig. 2. Inhibitory effect of increasing concentrations of NNG on protoplast plating efficiency. Isolated protoplasts were cultured in the presence of the mutagen in the medium and division frequency was scored 10 days after culture initiation

Table 1. Frequencies of plant regeneration patterns (Fig. 1) from protoplasts cultured under different regimes

Culture treatment	Cell culture period (months)	Plant regeneration pattern				Contingency[a] χ^2
		Parental (a)	Variants		Non-morphogenic colonies	
			Heterogeneous (b+c)	Homogeneous (d+e)		
Standard	3	691	10	1	9	–
Standard	5–7	107	7	2	5	8.48**
AA-media	3	125	3	2	3	0.15
AA-media	5–8	210	8	4	23	3.26
NNG-5	3–6	345	4	2	8	0.01
NNG-7.5	3–4	69	3	2	6	1.54
NNG-7.5	5–7	239	10	4	4	4.79*
NNG-10	3–4	193	10	3	7	7.36**
NNG-10	5–7	98	4	0	1	1.94

[a] Contingency χ^2 with Yate's correction for continuity (Mather 1960) for comparison of parental (a) and heterogeneous variant pattern (b, c) frequencies in the basic standard 3 months culture period with frequencies in the other 8 time/treatment combinations
* $P<0.05$
** $P<0.01$

individual calli was only about 1.5 cm, i.e. the rate of cell division was substantially reduced, and a number of colonies became necrotic.

Analysis of shoot forming colonies

The analysis of the regeneration experiments is summarised in Table 1. From a total of 2,222 protoplast derived colonies induced to regenerate shoots only 66 failed to respond and were classified as non-morphogenic. Figure 1 describes the scheme used to classify the colonies. Colonies cultured for 3–4 months or 5–8 months are referred to as "short" or "long" cell culture duration, respectively. Among 2,156 morphogenic colonies, 79 variant colony types were identified. Twenty of these colonies were phenotypically homogeneous types (Fig. 1 d, e) of which 14 were wild type or dark green plants (presumptive genotype *su/su*) and 6 were albino plants (presumptive *Su/Su*). Conservatively, we must assume that these homogeneous phenotypes arose from protoplasts which were genotypically *su/su* or *Su/Su* as a consequence of genetic changes in the leaves from which the protoplasts were isolated. This we interpret as preexisting variation.

Among the 59 variant colonies which gave rise to phenotypically heterogeneous plants, 31 formed both light green (*Su/su*) and dark green (*su/su*) shoots, 27 light green and albino (*Su/Su*) shoots and one formed both dark green and albino shoots. The frequency of heterogeneous colony types recovered is likely to be a low estimate because of reduced growth rate and regenerative disadvantage of albino tissue. To offset this, those colonies displaying white, presumably *Su/Su* sectors were given special care in that up to 8 shoots per protoplast derived callus were regenerated and scored. Heterogeneous variant colonies which gave shoots of different types are interpreted as resulting from genetic events during the cell culture phase and therefore represent somaclonal variation.

Heterogeneous variant colonies

Short cell culture duration in standard media provides a base level of somaclonal variation. The relative influence of sub-optimal culture media, extended culture time or chemical mutagenesis on somaclonal variation as measured by the relative frequency of heterogenous colonies, can be ascertained by a contingency χ^2 analysis (Table 1). To account for low frequencies in some classes Yate's correction for continuity was applied to the data (Mather 1960).

This analysis revealed that a significant increase in heterogeneous colonies occurred when, a) the cell culture duration was extended under standard media conditions, or b) cells were treated with NNG (10 µg/ml, short duration and 7.5 µg/ml, long duration). The long culture period following NNG treatment with 10 µg/ml did not lead to a statistically enhanced frequency of heterogeneous colony types. This could result from the fewer colonies analysed. Stress conditions (AA-media) did not significantly affect the relative frequency of somaclonal variation. This analysis indicates that both extended cell culture and mutagenesis enhance somaclonal variation.

Table 2. Classification and analysis of segregating progeny from selfed heterozygous (*Su/su*) plants grown from seed, i.e. control plants

	Segregating classes		
Phenotype Genotype	Albino *Su/Su*	Light green *Su/su*	Green *su/su*
Total for 10 families	755	1,519	700
Relative proportions	0.25	0.51	0.24
Heterogeneity χ^2			
	χ^2	*n*	*P*
Deviation	3.41	2	0.2 – 0.1
Heterogeneity	9.40	18	0.95 – 0.90

Non-morphogenic colonies

The relative low frequency of non-morphogenic colony types can also be considered a measure of culture induced variation. The occurrence of non-morphogenic colonies seemed independent of the frequency of heterogeneous variant colonies. A regression analysis showed no significant correlation between relative proportions of non-morphogenic and heterogeneous variant colony types. Thus it would appear that separate genetic events during cell culture were responsible for variation at the *aurea* locus and in the genes involved in morphogenesis.

Variation within leaves of somaclones

The genetic instability which gives rise to somaclonal variation during cell culture appears to "persist" in tissue culture derived plants. Phenotypically heterozygous (*Su/su*) leaves of regenerated plants showed greater variation in the number of single dark green and twin (yellow/dark green) sectors than comparable heterozygous leaves of plants grown directly from seed (Fig. 3 c, d). Both dark green and twin spots can arise in leaves as a consequence of mitotic recombination. Whatever genetic perturbations occurred during cell culture, they can persist through plant regeneration to enhance the frequency of mitotic recombination in developing leaves.

Other morphological abnormalities

Though not analysed to the same extent as variation at the *aurea* locus several other phenotypic variants were observed among the tissue culture derived plants. These included abnormal development of the flower and sexual organs, loss of fertility, abnormal leaf shape and gross plant morphology (Fig. 3 e). Some plants showed extreme yellow/green colour variegation in the leaf which was not consistent with observations in leaves of seed grown plants.

Progeny analysis

Among progeny of plants derived from seeds the *aurea* mutant behaves as a semi-dominant mutant and obeys Mendelian segregation ratios precisely. An average of 300 progeny from each of 10 selfed, seed grown heterozygous plants all segregated in Mendelian proportions (Table 2). A χ^2 analysis showed there was no significant deviation from a 1 : 2 : 1 ratio nor was there any significant heterogeneity between the progeny groups.

Table 3. Classification and analysis of progeny families regenerated from cultured protoplasts of heterozygous *Su/su* leaves

		Segregation classes		
		Conformity with 1 : 2 : 1	Deviation from 1 : 2 : 1	
a	Single capsule progeny	50	10	
	Mean deviation χ^2	1.75 ± 0.24	14.60 ± 3.21 ***	
			Capsules homogeneous	Capsules heterogeneous
b	Multiple capsule progeny	7	11	12
	Mean deviation χ^2	3.29 ± 0.78	26.81 ± 5.15 ***	40.75 ± 12.42 ***
	Mean heterogeneity χ^2	4.05 ± 0.96	4.02 ± 0.30	44.01 ± 9.70 ***
c	Inviable seed (av %)	6.90 ± 1.43	10.33 ± 1.89	8.67 ± 1.73
d	Parental R_0 plant phenotype			
	Normal	42	5	3
	Abnormal[a]	15	16	9

[a] See text "Other abnormalities" for abnormal plant descriptions
*** $P < 0.001$

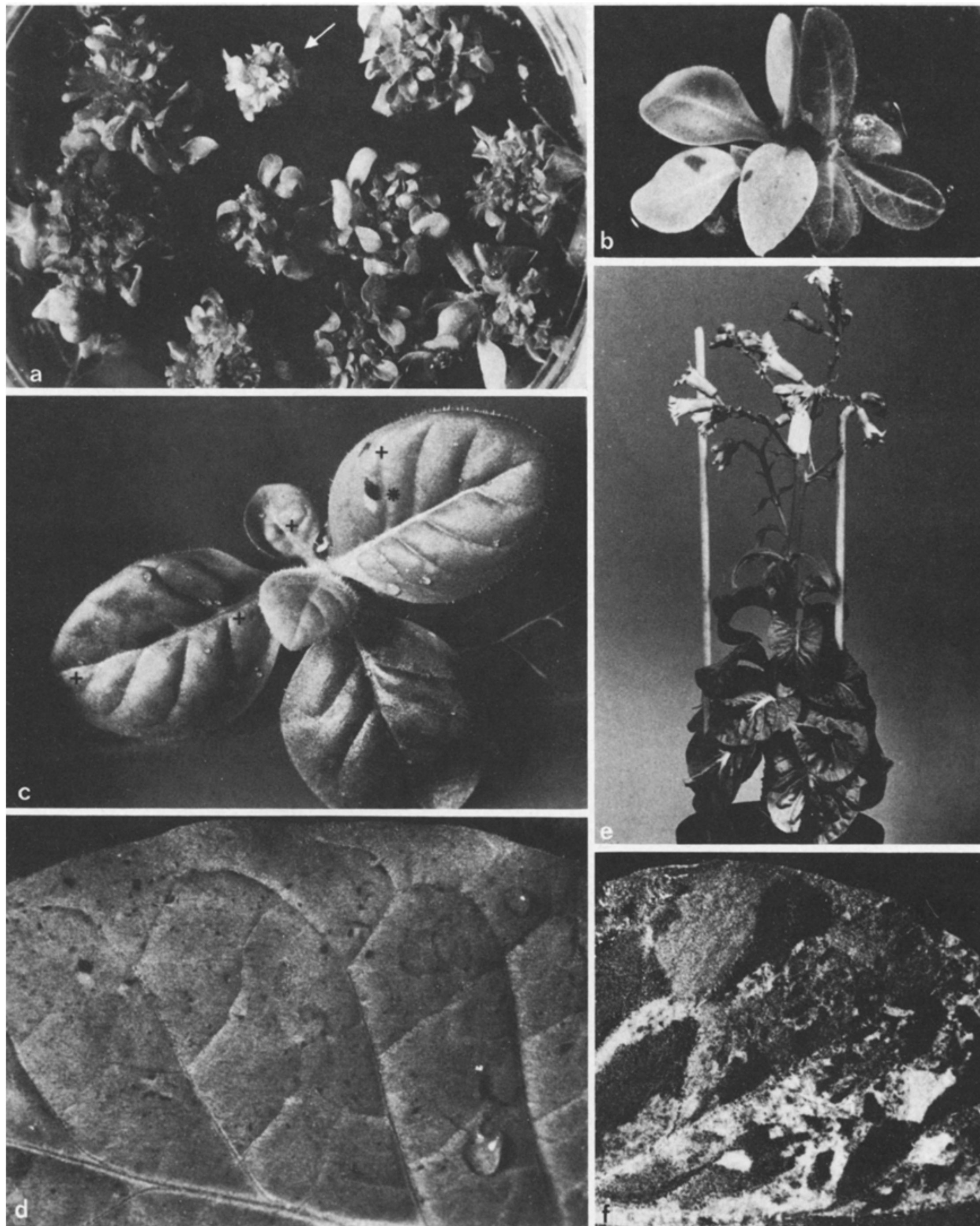

Fig. 3a–f. Somaclonal variation in protoplast derived sulfur tobacco shoots and plants. **a** Shoot formation in calli regenerated from protoplasts. Nine of the colonies are homogeneous light green (*Su/su*) and gave rise to shoots of the parental type. One colony (*arrow*) is a homogeneous variant and formed albino (*Su/Su*) shoots only. **b** Heterogeneous variant colony forming an albino and a green shoot. **c** Dark green (+) and twin spots (*) in a cell culture derived plant at low frequency. **d** Somaclonal variant plant with high frequency of twin spots. Note different size of the yellow/dark green segments. **e** Abnormal variant plant with wrinkled leaves and short, compact flowers. **f** Intensively variegated leaf with white, yellow, light green and dark green sectors resulting from variation in the different cell layers

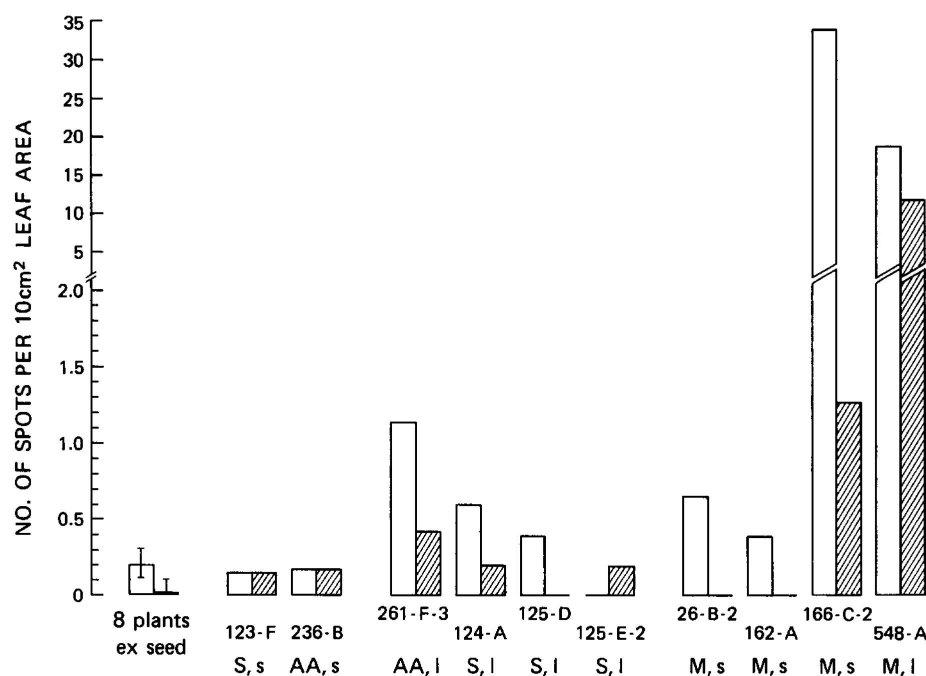

Fig. 4. Variability among protoplast derived plants compared with those derived from seed for the frequency of green and twin sectors. Numbers represent the average number of spots in 10 cm² leaf area of fully expanded leaves counted on plants grown in soil before flowering. Green sectors □, twin sectors ▨. *S* standard and *AA* amino acid media; *M* mutagenesis with NNG; *s* short cell culture duration; *l* long cell culture duration

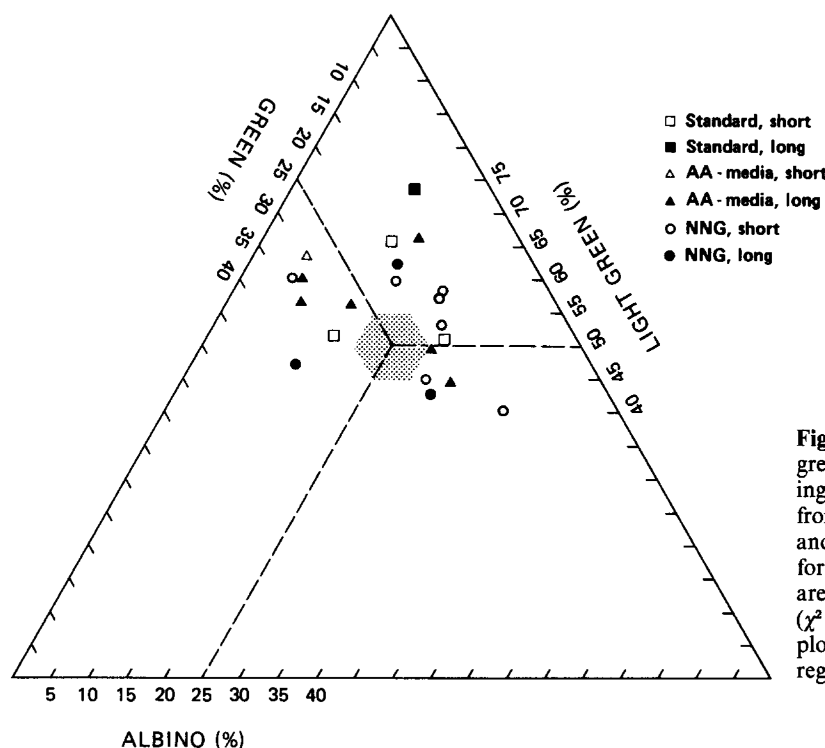

Fig. 5. Segregation of albino, light green and dark green seedlings of sulfur tobacco. Non-germinating seeds were not taken into account. Ten families from seed derived plants were grown as controls and 57 tissue culture derived plants which conformed to a 1 : 2 : 1 ratio plot within the hatched area. 21 plants which showed significant deviation ($\chi^2 > 5.99$) from the expected segregation ratio are plotted. These are identified with respect to culture regimes (Table 1)

Among the plants regenerated from tissue culture 104 were selfed and 96 set seed. Seeds of three of these families failed to germinate. Three of the plants, classified during regeneration as homozygous variants (2 dark green, 1 albino), produced only dark green or albino offspring, respectively. The remaining 90 families for which an average of 175 ± 10 seedlings per family were classified, segregated for all three phenotypes, namely albino (*Su/Su*), light green (*Su/su*) and dark green (*su/su*) (Table 3). For 60 of these 90 plants, seedlings from only one capsule were phenotypically classified and of these 10 gave segregation ratios which deviated significantly from the expected 1:2:1 (Table 3a). From the remaining 30 plants seedlings

from 2 or 3 capsules were independently classified (Table 3b). For 7 of these all capsules gave 1 : 2 : 1 ratio among the seedlings while the multiple capsule data for 21 plants deviated from expectation. These 21 could be further subdivided into 11 where segregation ratios from the several capsules were the same and 12 where the ratios were heterogeneous. Overall the selfed, regenerated plants were classified as producing progeny which either conformed to a 1 : 2 : 1 ratio (57 plants, 63%) or deviated from expectation (33 plants, 37%).

The χ^2 analyses (deviation and heterogeneity χ^2) throw additional light on segregational variability. In the 57 conforming families the goodness of fit was equal to if not better than that for progeny derived from seed produced plants (Table 2). For the 33 families which did not statistically conform to a 1 : 2 : 1 ratio, the mean deviation χ^2's were very large with probability levels less than 0.001. Likewise where capsule heterogeneity was observed, the mean heterogeneity χ^2's were also substantial.

The dispersion of the 21 families (10 single capsule and 11 homogeneous multiple capsule data) which deviated from 1 : 2 : 1 segregation is shown in Fig. 5 along with the ratios for both the conforming families and the progeny of seed derived control plants. There is no significant pattern in the dispersion of the ratios for the deviant families apart from a slight general trend of a reduction in both homozygous offspring classes with a concomitant increase in the heterozygous offspring. There is no reason to suspect seed inviability as a cause of deviant segregation ratios (Table 3c). An analysis of variance of percent non-germinating seeds in the families showed no significant differences between all segregating family classes and seed derived plant progeny.

The R_0 plants whose progeny segregated (Table 3 a, b) included plants from all the culture treatments applied (Table 1). Fifty were normal, i.e. phenotypically indistinguishable from seed derived heterozygotes, and of these only 16% (8) gave progeny ratios which deviated from expectation (Table 3d). In contrast among the 40 R_0 plants classified as abnormal (see earlier results) 62.5% gave segregation ratios which deviated significantly.

Discussion

The semi-dominant sulfur mutant of *N. tabacum* has proved to be a useful genetic marker to monitor somaclonal variation. The use of a protoplast culture system with the heterozygous *Su/su* genotype has enabled regenerated plants to be derived from isolated single cells. The analysis of these plants provide estimates both of variation existing in the leaves prior to protoplast isolation and of variation generated during cell culture.

Among 2,156 morphogenic colonies analysed 79 gave rise to plants some or all of which were non-parental, i.e. *Su/Su* or *su/su* type. Of these 79 colonies, 25% produced only homozygous plants and these we interpreted as representing pre-existing variation resulting from genotypically homozygous leaf protoplasts generated by genetic changes, e.g. mitotic recombination, in the developing leaf. The remaining 75% of colonies were heterogeneous and produced R_0 plants of different phenotypes (light green plus albino or dark green). This represents cell culture generated or somaclonal variation.

Doubling the time in culture during cell proliferation enhanced the proportion of somaclonal variants about twofold. The mutagenic treatment with 10 µg/ml NNG also enhanced the frequency of variants. There was no significant increase of variation in plants regenerated from colonies grown under suboptimal conditions, with organic rather than inorganic nitrogen sources. We suggest this may be due to a slower rate of cell division under these conditions, where it is likely that the rate of DNA synthesis, and therefore the chance of genetic changes, was reduced. The possible mutagenic effect of high nitrate levels in the standard medium cannot be excluded.

Substantial leaf variegation and greatly enhanced occurrence of single or twin spots was observed among regenerated plants. This, and particularly the latter, may reflect genetic events occurring during cell culture which caused enhanced mitotic recombination during leaf development. Compared to seed produced plants, there was a greatly increased frequency of abnormal plants among those regenerated from protoplasts. These included plants with abnormal leaf shape, flower morphology and plant habit.

The segregation patterns among progeny seedlings of 90 selfed regenerated plants revealed a striking effect of somaclonal variation. The segregation ratios among progeny of selfed heterozygous plants, not previously subjected to cell culture, precisely conform to a 1 : 2 : 1 Mendelian pattern. Among plants regenerated from cell culture, 37% gave progeny segregation ratios which deviated significantly from the expected 1 : 2 : 1 ratio. The deviant ratios could not be attributed to selective inviability of seeds. Many of the somaclones which gave deviant segregations were as fecund as those which yielded non-deviating ratios, thus ruling out gross chromosomal abnormalities as a major source of genetic variation. Regenerated heterozygous plants which were judged to be phenotypically abnormal in morphology had a predisposition to produce progeny which deviated from Mendelian expectation. Some 62.5% of such abnormal plants showed such a predis-

position, while only 16% of phenotypically normal heterozygous plants from cell culture gave disparate progeny ratios. This study demonstrates that cell culture does generate phenotypic and, for a defined locus, genetic variability. The consequences of this are apparent in the regenerated plants and in some cases persist to perturb segregation ratios in the progeny of mature plants. The aberrant ratios could be a consequence of genetic changes which affect the development of the sexual organs, disturb meiosis or pollen maturation.

We have not examined the cytogenetic behaviour of the variant plants recovered. Thus any mechanism for the origin of these variants would be entirely speculative. Several mechanisms for the origin of somaclonal variation have been proposed (Larkin and Scowcroft 1981; Scowcroft and Larkin 1983) including ploidy changes, nuclear fragmentation, inter- and intrachromosomal interchanges, somatic crossing-over and sister chromatid exchange, gene amplification and deletion, and possibly transposable genetic elements. It is highly likely that a reasonably balanced genome is necessary for morphogenesis and in particular for fertility of the regenerated plants. In this study less than 3% of calli tested were classed as non-morphogenic and less than 8% of the regenerated plants were sterile. These could result from gross chromosomal changes as well as specific genetic lesions.

Of the genetic mechanisms proposed to account for phenotypic variability the occurrence of ploidy changes, deletions, translocations and inversions have been described in detailed cytological analyses of tissue culture variability in *Hordeum* (Grunewaldt and Malepszy 1975; Orton 1980), *Haworthia* (Ogihara 1981) and *Avena* (McCoy et al. 1982). In the last case the relative frequency of meiotic abnormalities increased with cell culture time and in addition some oat cultivars consistantly gave a higher frequency than others.

This study adds substantial evidence demonstrating that a significant proportion of somaclonal variation is genetic. The application as a potentially useful source of genetic variability in plant improvement is being actively pursued in several economically important crop species. It is important that the origin of somaclonal variation be understood at the genetic and molecular level so that it can be minimised where clonal identity is desired or enhanced and even possibly directed for purposes of plant improvement.

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