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Genetic Variability Induced in *Nicotiana sylvestris* by Protoplast Culture

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Summary. Plants were regenerated from axenic plantlets by mesophyll protoplast culture, without mutagenic treatment. Two different lines of *Nicotiana sylvestris* were used: an original line, and a diploid androgenetic line derived from it. The regenerated plants were either diploid and phenotypically similar to their respective protoplast source line, or tetraploid. Genetic studies carried out on several diploid regenerated plants revealed genetic variability. Eight of 13 selfed progenies of plants regenerated from the original line, and 1 of 8 selfed progenies of plants regenerated from the androgenetic line, produced new mutant phenotypes never observed in the protoplast source lines. Two plants regenerated from the same protoplast-derived callus produced different mutations. Selfed progenies without a recognizable mutant phenotype were also different from their respective protoplast source line for quantitative characters; protoplast culture induced a depressive effect on the size of plants derived from protoplasts at younger and older stages of development. The origin of this depression and of the mutations is discussed.

Key words: Induced genetic variability – Genetic studies – *Nicotiana sylvestris* – Protoplast culture

Introduction

Several authors have pointed out the great usefulness of protoplast culture in plant breeding (Bhojwani et al. 1977; Vasil et al. 1979; Shepard 1982): in multiplying plants of various species (Wenzel et al. 1979), in obtaining drug resistance mutants and auxotrophs (Carlson 1970; Bourgin 1978; Maliga 1980), and in somatic hybridization (Belliard and Pelletier 1978; Zelcer et al. 1978; White and Vasil 1979). However these authors did not study the genetics of plants regenerated in the absence of mutagenic treatment or somatic hybridization.

Nicotiana sylvestris was chosen for this study because: (1) its protoplast culture has been perfected (Nagy and Maliga 1976; Bourgin et al. 1976; Durand 1979; Magnien et al. 1980; Negrutiu and Mousseau 1980), (2) the diploid level (2n = 24) of this species facilitates genetic study of regenerated plants, and (3) the phenotypic stability (Goodspeed and Avery 1939) in successive selfed progenies allows one to think that any new phenotypes observed would be induced by protoplast culture. Therefore, the aim of this paper is the analysis of genetic variability found in plants regenerated by mesophyll protoplast culture. Two diploid protoplast source lines were used (Fig. 1): one was the original line, maintained by successive self-pollinations, the other was a doubled haploid line, obtained from the original line after several consecutive cycles of androgenesis (De Paepe et al. 1981). The androgenetic line, theoretically homozygote, was phenotypically different from the original line ("crumpled" phenotype and reduced vigour) as are most lines obtained by androgenesis in *Nicotiana sylvestris* (De Paepe et al. 1981; Prat 1982). We thought it would be interesting to compare the induced variability in protoplast culture of an androgenetic line to that of the original line. Morphological and quantitative characters of diploid plants regenerated from both protoplast source lines were analysed genetically.

Material and Methods

Protoplast Source Lines

The original diploid line of *Nicotiana sylvestris,* supplied by the SEITA of Bergerac, has been maintained by 7 successive self-pollinations in greenhouses at Gif-sur-Yvette. No phenotypic variability was observed in successive selfed progenies.

The androgenetic line was diploid and obtained from the original line by 5 consecutive cycles of pollen culture (De

Fig. 1. Protoplast source lines. Original line (*left*) and androgenetic line (*right*) with crumpled leaves

Paepe et al. 1981) followed by two successive selfings of one regenerated doubled haploid plant. The plants of the androgenetic line were smaller than those of the original line (Fig. 1); their phenotype was also different (De Paepe et al. 1981; Prat 1982) in that they had crumpled leaves. These characteristics of the androgenetic line were not modified by selfing (Prat 1982).

Protoplast Culture and Plant Regeneration

Protoplast source plants were grown in axenic conditions, seeds were sterilized in calcium hypochlorite (at 7%) for 10 min and then sown in Petri dishes (20 cm in diameter) containing 150 ml of Knop's medium (1865) with a sucrose supplement (10 g/l), at pH 5.8. Petri dishes were sealed with Parafilm and kept at 25 °C under continuous light (4,000 lux). Protoplast isolation and culture were achieved by applying the method established by Durand (1979) with some alterations. Leaves from 32 day-old seedlings were cut into fine strips and then put on the isolation medium (Durand 1979). After overnight enzymatic incubation in the dark at 19 °C, the flasks were gently shaken. The protoplast suspension was poured through nylon sieves (150 μ aperture), and centrifuged 7 min at 800 rpm (about 70 g). The protoplast pellet was resuspended in culture medium (Durand 1979) in which we substitute glutamine and asparagine for the conditioned medium, and sorbitol for mannitol. After determination of the protoplast density on a Nageotte haemocytometer, the protoplast suspension was adjusted to 25,000 living (Durand 1979) protoplasts per milliliter and poured into Petri dishes (1.5 ml per dish, 5 cm in diameter) sealed with Parafilm, kept at 23 °C in the dark for one week and then at 25 °C under low light intensity (1,500 lux). Protoplasts developed into calli that were planted out in Petri dishes on solid medium (Bourgin et al. 1979) inducing shoot formation. The shoots were planted out on B medium of Bourgin et al. (1979) induced rooting. Afterwards plantlets were planted in the greenhouse, about 5–6 months after sowing the protoplast source plants.

Chromosome Counts

Mitotic counts were carried out on root tip squashes stained by the Feulgen reaction. Root tips were taken from young plants, immediately treated by 2 mM hydroxyquinoline for 4 h at 8 °C and then fixed in acetic alcohol (1 : 3 v/v). Root tips were hydrolysed in 5 N HCl for 10 min, and then washed in water and stained by Schiff's reagent for 30 min. The chromosome counts were checked at meiosis. Flower buds were taken and fixed in Carnoy's solution (alcohol, chloroform, acetic acid; 6 : 3 : 1; v/v/v), and anthers were stained in aceto-carmin.

Genetic Studies

Morphology. Regenerated plants were compared with their respective protoplast source lines. Selfed progenies of several diploid regenerated plants were also examined. When a previously unknown phenotype was observed in the progenies, self-pollinations, crosses between the new phenotype and its protoplast source line, and then self-pollinations of the obtained hybrids were carried out to study the heredity of the obtained mutations.

Quantitative Genetics. Only diploid regenerated plants were affected by this study. A comparative quantitative test was carried out: some selfed progenies of protoplast-regenerated plants taken at random from each protoplast source line were compared to progenies of plants obtained by selfing the respective protoplast source line, for several characters i.e.: leaf size at different stages of development (rosette plant, after induction of flowering, flowering plant, plant bearing mature seeds), height, and flower size. All compared plants were sown the same day (in culture tubes, on Knop's medium). Forty day-old seedlings were replanted in the greenhouse (photoperiod: 16 h; thermoperiod: 24 °C/17 °C), watered with the nutritive solution of the Phytotron (Nitsch 1969), and then measured the same day or at the same stage of development. Plants were distributed into randomised blocks with the same number of plants for each progeny.

Statistical Tests

Segregations of mutant phenotypes were tested with chi square (Dagnelie 1980). Comparisons of quantitative characters were tested by the contrast method (Dagnelie 1980): the means of selfed progenies of plants regenerated from each

protoplast source line were compared to the means of selfed progenies of plants from the same protoplast source line; the identity of both groups was tested for each protoplast source line and each character independently.

Results

Ploidy of the Regenerated Plants

Both protoplast source lines were similar (Table 1) with respect to the ploidy of plants regenerated from them. One hundred seventy-two plants were regenerated from 82 calli. Seventy-two regenerated plants were diploid (Fig. 2a), while the others were polyploid (generally tetraploid, Fig. 2b). No aneuploids were observed. Thirty-six calli gave diploid plants. Calli from which 2 plants at least were regenerated were classified into three types (Table 2): the first type

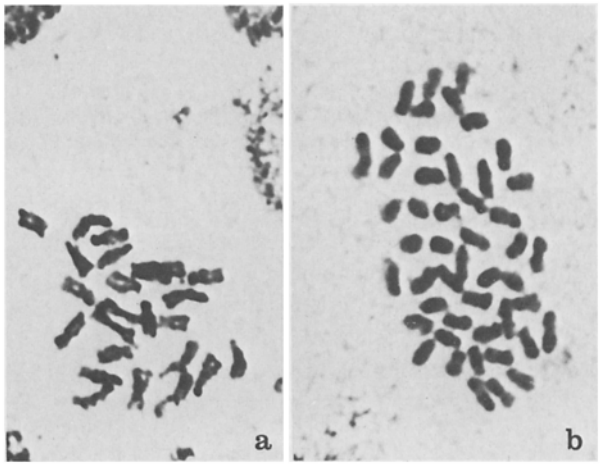

Fig. 2a and b. Metaphases of root tip cells from diploid (**a** 2n = 24) and tetraploid (**b** 4n = 48) regenerated plants

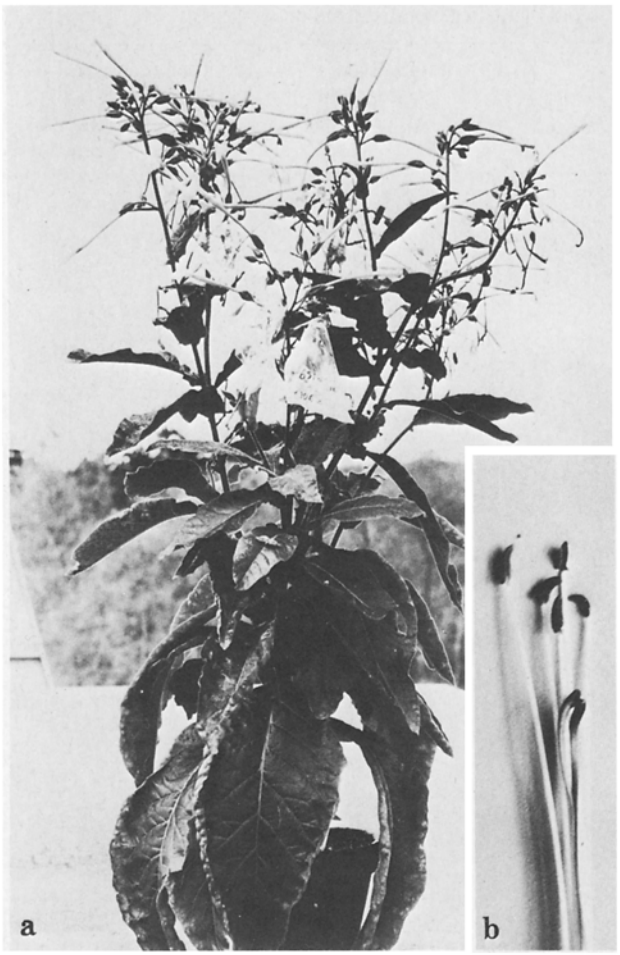

Fig. 3a and b. Mutant plant (**a**) from original line, in flower (selfed progeny 2, callus 35), and (**b**) its abnormal flower (male-sterile) with small anthers and bifid style

Fig. 4. Dwarf plant from original line, in flower (selfed progeny 3, callus 35)

Table 1. Ploidy of regenerated plants obtained from the original and the androgenetic lines by protoplast culture

Line	Number of diploid regenerated plants	Number of tetraploid regenerated plants[a]
Original line	34	47
Androgenetic line	38	53

The two lines are not significantly different: $\chi^2 = 0.00$
[a] A few plants may be 6n or 8n

(12 calli) gave only diploid regenerated plants; the second type (17 calli) gave both diploid and tetraploid regenerated plants; the last type (18 calli) gave only tetraploid regenerated plants. These three types of calli occur at about the same frequency. On the average, about half the regenerated plants were diploid.

Phenotype of the Regenerated Plants

Diploid regenerated plants were not phenotypically distinguishable from their respective source lines. The

Table 2. Ploidy of plants regenerated from calli yielding at least two plants

Line	Number of calli regenerating only diploid plants	Number of calli regenerating both diploid and tetraploid plants	Number of calli regenerating only tetraploid plants
Original line	5	10	9
Androgenetic line	7	7	9

The two lines are not significantly different: $\chi^2 = 0.84$

Table 3. Segregations for mutant phenotypes in selfed progenies of diploid plants regenerated from protoplasts

Line	Callus	Selfed progeny	Mutant phenotype	Observed segregation Mutant : Wild	Proposed theoretical segregation	
					Mutant : Wild	χ^2
Original line	35	1[a]	male-sterile (Fig. 3)	3 : 46	1 : 15	0.02
		2[a]				
		3	dwarf, small leaves, little metaxylem (Fig. 4)	9 : 24	1 : 3[b]	0.09
	36	4	late flowering	4 : 40	1 : 3	0.10
	41	5	–			
		6	rosette leaf necrosis; chlorophyllous variegation (Fig. 5)	6 : 7 : 25	3 : 4 : 9[b]	1.45
	55	7	albino	7 : 16	1 : 3	0.36
		8	wide leaves – and local variegation – and female-sterile	3 : 2 : 1 : 5[d]	?	
	60	9	–			
		10[c]	–			
		11	lethal cotyledon necrosis; first leaf necrosis	42 : 3 : 0	?	
	90	12[c]	–			
	93	13[c]	–			
Androgenetic line	52	14	male-sterile; small non-flowering plant	1 : 1 : 15[d]	?	
		15[c]	–			
	1313	16[c]	–			
	1315	17[c]	–			
		18[c]	–			
	1316	19[c]	–			
		20[c]	–			
	1331	21[c]	–			

[a] Selfed progenies 1 and 2 both produced the same mutant phenotype
[b] Genetic studies of the mutant were carried out, they agree with the proposed theoretical segregation
[c] Selfed progenies having no observable mutant phenotype, compared to selfed progenies of their respective source line for quantitative characteristics
[d] The number of plants that could be studied was limited by greenhouse space

Table 4. Quantitative characteristics of selfed progenies (not producing apparent mutation, cf. table 4) of diploid plants regenerated from protoplasts in comparison to selfed progenies of their respective protoplast source line

Character measured		Original line					Androgenetic line				
		Source line progenies		Regenerated plant progenies		Contrast	Source line progenies		Regenerated plant progenies		Contrast
		n[a]	mean[b]	n[a]	mean[b]		n[a]	mean[b]	n[a]	mean[b]	
Greenhouse input weight	mg	173	367±36	63	305±61	6.40*	156	252±35	140	209±37	5.20*
Leaf length rosette plant	mm	173	144±4.6	63	131±7.7	11.76**	156	102±5.5	138	95±5.8	8.14*
Leaf width rosette plant	mm	173	79±2.5	63	73±4.3	7.25**	156	57±3.0	138	54±3.2	4.22**
Leaf length (after flowering induction)	mm	80	184±5.9	30	189±9.8	1.29	81	183±5.5	72	177±5.8	4.00*
Leaf width (after flowering induction)	mm	80	96±2.6	30	96±4.3	<1	81	88±2.7	72	85±2.9	4.10*
Height flowering plant	cm	80	75±2.6	30	73±4.3	<1	81	59±2.4	72	60±2.5	<1
Leaf length flowering plant	mm	80	345±10.7	30	336±17.9	1.49	81	306±10.2	72	300±10.8	1.20
Leaf width flowering plant	mm	80	157±4.2	30	157±7.0	<1	81	132±4.8	72	128±5.1	2.87
Corolla length	mm	80	89.4±0.5	30	89.5±0.9	<1	81	80.7±0.6	72	79.7±0.6	7.42**
Corolla radius	mm	80	20.4±0.4	30	20.6±0.7	<1	81	19.2±0.4	72	18.6±0.4	8.03**
Height seed-bearing	cm	80	142±3.2	30	135±5.4	9.99**	81	109±3.2	72	107±3.4	2.35
Leaf length seed-bearing	mm	80	361±10.3	30	345±17.2	4.36*	81	310±10.1	72	297±10.7	5.47*
Leaf width seed-bearing	mm	80	171±8.5	30	163±8.5	2.57	81	150±6.0	72	142±6.4	5.92*

[a] Number of plants
[b] Mean ± standard error ($P > 0.99$)
* Significant at 5% level
** Significant at 1% level

Fig. 5. Variegated mutant rosette plant from original line (selfed progeny 6, callus 41)

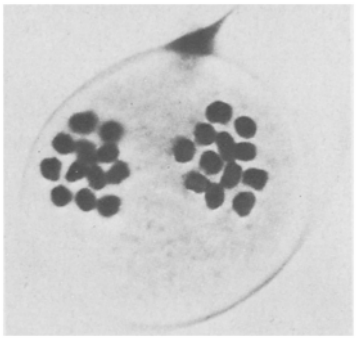

Fig. 6. Metaphase II of pollen meiosis from the variegated mutant (2n = 24)

particular phenotype of the androgenetic line was not affected by protoplast culture. We would like emphasize the fact that in our case, protoplast culture did not have any effect on the phenotype of the diploid plants regenerated from both source lines. Tetraploid regenerated plants had a special phenotype with wider leaves, larger flowers and capsules in both source lines.

Appearance of New Phenotypes in Selfed Progenies of Diploid Regenerated Plants

In the original line, the selfed progenies of 13 plants (regenerated from 7 different calli) were studied (Table 3): 8 progenies showed various mutant phenotypes in segregation never observed in the original line. Their ploidy was normal (2n = 24, Fig. 6). Calli number 35 and 55 gave several regenerated plants and two different mutant phenotypes (Fig. 3 and 4). Some calli (number 41 and 60) gave both mutant (Fig. 5) and non-mutant progenies. Some selfed progenies (number 6 and 8) showed several mutant phenotypes in segregation. Genetic studies of the mutant phenotypes of progenies 3 and 6 showed that they have a monofactorial Mendelian inheritance and that the mutations were recessive.

In the androgenetic line, only one of the 8 selfed progenies of regenerated plants showed mutant phenotypes. Plants of other progenies had the phenotype of the androgenetic line plants.

Quantitative Variability of Selfed Progenies of Diploid Regenerated Plants

A quantitative comparative test was carried out between 8 selfed progenies from each protoplast source line and 8 selfed progenies of plants regenerated by protoplast culture. Only selfed progenies without an apparent mutant phenotype were compared to their respective source line. For some characters (Table 4), the selfed progenies of regenerated plants for both source lines were depressed in comparison to the selfed progenies of their respective source line. These differences were particularly noticeable at young and older stages of development, for the following characters: plantlet weight (at the time they were put in the greenhouse), leaf size of rosette plants, leaf size and plant height at seed maturity. The characters of older plants that showed a significant effect of protoplast culture were not the same for both protoplast source lines. In addition, more characters showed a depressive effect of protoplast culture in the androgenetic line than in the original line.

Discussion and Conclusion

Protoplast culture of *Nicotiana sylvestris* diploid lines led to a large proportion of tetraploid regenerated plants. Faccioti and Pilet (1979) showed that, from haploid protoplasts, no haploid plant was regenerated; they were essentially diploid. Consequently protoplast culture seems to induce, at high frequency, a doubling of the chromosome number, whatever the ploidy of the source plants.

Appearance of heritable, unselected traits has been described in tissue culture (Malepszy et al. 1977) and protoplast-derived plants (Chaleff and Keil 1981). We show as well that various mutations modifying plant morphology and depressing plant vigour may result from protoplast culture. Most selfed progenies of regenerated plants tested were different from their respective protoplast source lines. Mutations affecting plant morphology seem to have occurred at a higher frequency in the original line than in the androgenetic line but the depressive effect induced by protoplast culture was observed in both lines. Protoplast culture induced a wide spectrum of variability in both lines.

In the original line, 8 of the 13 selfed progenies of regenerated plants showed mutations. If mutation events occur independently, this high rate of mutation (61%) may be broken down into a single-mutation rate (47%) and a double-mutation rate (14%), allowing the observation of 6 single-mutant progenies, 2 double-mutant progenies and 5 non-mutant progenies: this is exactly the observed situation. The mutation rate may be different in the androgenetic line. The high frequency of various (non-specific?) mutations raises the problem of the origin of the mutation. The observed mutations are not likely to be due to the cellular differentiation (Thomas et al. 1982) of leaf cells at the origin of the protoplasts: all regenerated plants would then have carried the same mutation (or a few types of mutation), corresponding to the cellular differentiation of the same cells, as may be the case in androgenesis in *Nicotiana sylvestris* (De Paepe et al. 1977; De Paepe

et al. 1981; Prat 1982) where all doubled haploids regenerated from the same type of cell (pollen) had the crumpled phenotype. Mutations were already present in protoplast-derived calli: two plantlets taken from the same callus can produce the same mutation. If mutation takes place at the time of bud induction in calli, each plantlet would have its own mutation, independent of the others. Mutation therefore seems to occur beforehand. There are at least two critical stages between protoplast isolation and shoot induction: (1) induction of the first division of the protoplast, and (2) divisions of the dedifferentiated cells during callus culture. On the one hand Barbier and Dulieu (1980) showed that, for their marked heterozygote genes in *Nicotiana tabacum,* the frequency of mutation significantly increases with the duration of tissue culture. Mutations might take place during callus culture. On the other hand, the protoplast stage shows a particularly high rate of metabolic activity (Robenek and Peveling 1977) with changes in protein synthesis (Fleck et al. 1979) and also in internal DNA precursor pools (Zelcer and Galun 1976). This makes the protoplast a prime candidate for mutation. According to this logic, individual protoplast-derived calli would therefore be homogeneous, but would aggregate during their growth if they are close to each other (as we observed) leading to the formation of chimeric calli. Thus, mutations taking place during either of the two stages would lead to the observed heterogeneous calli yielding plants showing different mutations.

In addition to the mutations affecting plant morphology, the selfed progenies of regenerated plants which did not produce any recognizable mutant phenotype showed a general depressive effect. On the average, these progenies were less vigourous than their respective source lines, for some characters (Table 4). In *Nicotiana,* androgenesis, another system of in vitro culture, also induces nuclear genetic variability (De Paepe et al. 1977; Schnell et al. 1980; De Paepe et al. 1981) affecting essentially quantitative characters, with depressive effects. The depressive effect on the weight of plantlets is large (about – 35%) after one cycle of pollen culture of the original line (De Paepe et al. 1977) of *Nicotiana sylvestris* than after protoplast culture (about – 17%), but the effects are in the same direction. De Paepe et al. (1982) showed that androgenesis induces variations in DNA organisation: highly repetitive sequences seem to be amplified. Are there similar mechanisms inducing mutations in protoplast culture?

Protoplast culture induces a high frequency of recessive mutations affecting qualitative and quantitative characters of plant morphology or plant growth in both lines tested. Some differences appear between the two lines for the frequency of mutations affecting plant morphology, and in the depression of quantitative characters. Protoplast culture induces a wider spectrum of variability for morphological characteristics than androgenesis, which has led only to one particular phenotype (De Paepe et al. 1977; De Paepe et al. 1981). The quantitative variations are higher after androgenesis than after protoplast culture, but they are significantly depressed in both cases. The mutations induced in protoplast culture may restrict its applications in the multiplication of interesting genotypes in breeding programs of the kind proposed by Wenzel et al. (1979), in the case of species that are modified by in vitro culture like *Nicotiana sylvestris.*

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