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Basis and Extent of Genetic Variability Among Doubled Haploid Plants Obtained by Pollen Culture in *Nicotiana sylvestris*

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Summary. All diploid plants (doubled-haploid plants: D.H.) regenerated by androgenesis from binucleated pollen grains in *Nicotiana sylvestris* differ genetically from the original line as far as morphological features and growth rates are concerned. This androgenic variation (A.V.) is under nuclear control and is transmitted continuously by some D.H. for at least four generations of selfing; other D.H. progenies segregate. Further androgeneses carried out on one single D.H. reveal a new variability and increase the drift from the original line. All results cannot be explained by the presence of residual heterozygosity in the original line, and we suggest that most of the A.V. could originate from changes that occur in the DNA of the vegetative pollen grain cell. D.H. resulting from endomitosis of the vegetative cell would be 'homozygous' and stable, whereas D.H. resulting from nuclear fusion between a vegetative and a generative cell would be 'heterozygous' and would segregate in seeds through succeeding generations.

Key words: Androgenesis – Doubled-haploid (D.H.) – Mendelian segregation – Quantitative variation

Introduction

It is possible to regenerate androgenic embryos in a number of species from anthers cultured in vitro (Bourgin and Nitsch 1967; Niizeki and Oono 1968; Kameya and Hinata 1970) and to obtain diploids with the doubling of the pollen genome. If, as assumed, these plants were fully homozygous, they would be of great interest in plant breeding because they would permit the expression of recessive alleles and the immediate fixation of new combinations derived from F1 hybrids.

However, some authors have observed double-haploid (D.H.) lines that differ from their parent plants (Oinuma and Yoschida 1975; Burk and Matzinger 1976; Collins et al. 1974; Devreux and Saccardo 1972; Arcia et al. 1978). Explanations frequently proposed are that either total homozygosity of the D.H. genome leads to inbred depression, or that mutations are induced by the addition of growth substances to the culture medium (d'Amato 1977) or by colchicine used to diploidize the genome (Jensen 1974; Ross 1965). In *Nicotiana sylvestris*, as in *Nicotiana tabacum* (Nitsch 1974) and *Datura innoxia* (Nitsch and Norreel 1973), androgenic plants can easily be regenerated by pollen culture. At variance with Raschid and Stern (1974) and in concordance with McComb and McComb (1977) we obtained the best results when the anthers were put into culture at the binucleated stage: in these conditions, most, if not all, of the embryos derive from the vegetative cell (De Paepe et al. 1977). Diploidization occurs spontaneously during the androgenetic process (McComb and McComb 1977): from our cultures, 18% of the regenerated plants were seen to be spontaneously diploid; 28% became diploid after a colchicine treatment of the flower buds before pollen culture (De Paepe and Pernes 1978). Previous genetic analysis of D.H. regenerated from colchicine treated or non-treated pollen showed all these plants to be genetically modified in both growth rates (De Paepe et al. 1976) and in morphology (De Paepe and Pernes 1978), namely by the crumpled aspect of the leaves, when compared to *N. Sylvestris* original line plants.

In the first generation of selfing, some D.H. with crumpled phenotype (FR) gave homogeneous progenies, comprising only crumpled plants, while other D.H., although apparently normal (N), gave heterogeneous offsprings with variable proportions of 'crumpled' and 'normal' plants. In this report, we extend preceding results to an increased number of D.H. plants. Our purpose is to clarify the genetic nature of D.H. variation by determining nuclear or cytoplasmic inheritance, to test the effects of colchicine upon variation and to determine the behavior of D.H. (N and FR phenotypes) during subsequent selfing or crossing through the fourth generation, as well as the effects of consecutive cycles of androgenesis. Additionally, we suggest that most of the D.H. variation originates from changes occurring in the nuclear DNA of the vegetative cell of the pollen grain.

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Material and Methods

A) Source of N. sylvestris

Pollen cultures were carried out on plants belonging to two related lines of *Nicotiana sylvestris*, Speg. and Comes. One, B 229, was provided by the SEITA of Bergerac; the other, T21, originated from B 229 as a single plant descent from 9 selfings. The two lines were statistically identical in growth characteristics and all plants were phenotypically similar. Several T 21 lines were selfed and crossed successively as control lines, designated here as T lines, for comparison with D.H. progenies.

B) Pollen Isolation and Culture

The method of isolated pollen culture was, with slight modifications, the one previously described (De Paepe and al. 1976). The buds were collected 1-3 days after the first pollen mitosis, when pollen grains are at the binucleated stage. Floral buds were then divided into two groups. In one group the buds were colchicine treated, as described by Nitsch (1977): the petioles were immersed for one night at 4°C in a 0,1% colchicine solution. The 'in vitro' culture was then carried out similarly for the colchicine treated (Col^+) and the non-treated (Col^-) buds. The anthers were excised, sterilised with 7% calcium hypochlorite and placed into sterile Nitsch's liquid culture medium (1974) (macro-elements of Lin and Staba (1961), FeEDTA 37 mg/l, sucrose 20 g/l) for 3 days at 17°C and 3 days at 22°C, in the dark. The pollen was then collected from crushed anthers by filtration through 100 nylon netting in order to eliminate the anther somatic cells and the filtrate was centrifuged at 500 g. The pollen pellet was resuspended in the same liquid medium enriched with 5 g/l inositol, 0.5 g/l glutamine, 0.03 g/l serine and placed on Petri dishes in a culture room (22-25°C, 2000 lux of fluorescent light). After approximately 20 days of culturing several hundred well differentiated plantlets were visible in the dishes.

C) Culture of the Regenerated Plants

A number of plantlets were selected at random, planted on agar medium and placed in rooms maintained at 17-24°C, and a 16

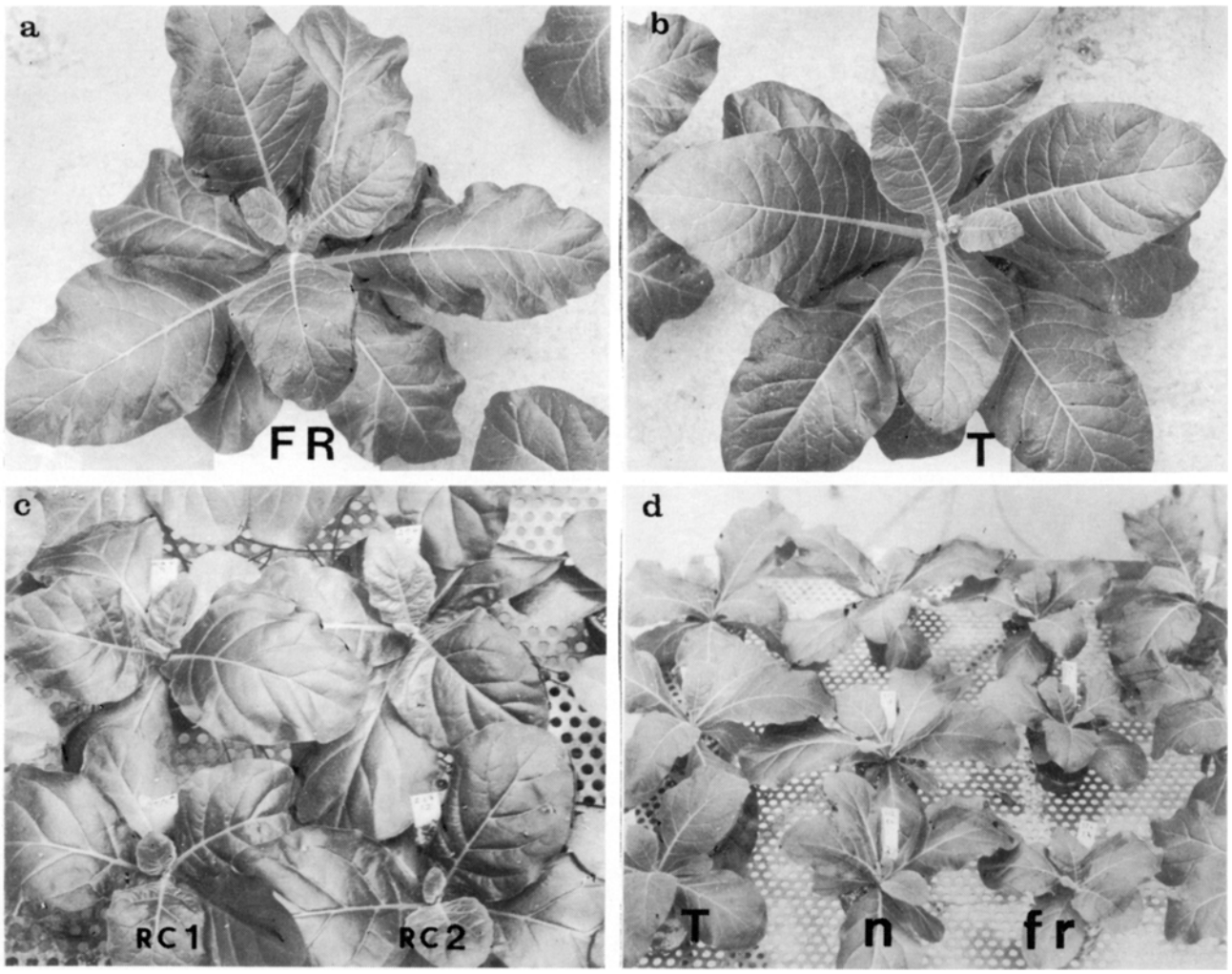

Fig. 1a-d. a, b original line plant (T) and crumpled Doubled-Haploid (FR); c reciprocal crosses (RC1, RC2) between two Fr plants; d homogeneous n and fr lines obtained in second generation of selfing of a N (Col^-) plant (Material and Method E2). On the left: T plants

hour photoperiod at 5000 lux. After the plantlets had developed good roots, they were replanted in the phytotron greenhouses and maintained at 17-24°C with natural light supplementing the 16 hour photoperiod artificial lighting. The plants were fed Nitsch's nutritive solution (1968). Ploidy level was determined cytologically on at least 10 root tip mitosis per plant; the observations were verified during meiosis. Diploid plants arising from original line pollen were called D.H.$^{(1)}$. A second androgenesis cycle carried out with D.H.$^{(1)}$ pollen produces D.H.$^{(2)}$ plants, etc... In this paper we analyse D.H. obtained through out 5 consecutive cycles of androgenesis. Diploid regenerated plants displayed various phenotypes and two classes were defined: D.H. resembling the original line plants were called N (normal), and those evidently modified, namely by a crumpled aspect of the leaves and a lighter coloration, were called FR ('fripé'), whatever the intensity of the modification (Fig. 1).

D) Culture Conditions of the D.H. Progenies, Measurements and Statistical Analysis

Control lines and D.H. progenies were compared in statistical trials. For each progeny, 24 seeds were individually sown in test tubes on sterile medium composed of agar (0.7%), sucrose (1%) and Knop macro-elements (Knop 1865). This procedure avoids both competition among seedlings and early contamination by bacteria or viruses. The tubes were placed in controlled culture rooms (16 h photoperiod, 25-27°C). After 4-6 weeks, according to the experiment, most of the D.H. progenies were placed in Phytotron greenhouses (natural day length plus an artificial lighting of 16 hours, 24°C during the day, 17°C at night). Additionally, in order to study the environmental-genotypic interactions, some progenies were either transferred to open fields located at Gif-sur-Yvette, or placed in Phytotron regulated rooms under varying temperature conditions (ie. 22°, 27°eC) and different photoperiods (ie. 9 or 16 day length). Under greenhouses conditions the plants flowered after 3-5 months, depending on the season, and the following measurements were taken:

– Young stage measurements: fresh weight of the whole plantlet at the end of the tube culture; length and width of the largest leaf after a month of greenhouse culture; height of plants 2 weeks after bolting of the more precocious plant. For these features variability between families was tested by one-way variance analysis. Mean comparisons were done by Scheffé's contrast test (1959).

– Adult stage parameters: time of flowering, height, number of internodes, dimensions of leaves and flowers. In this report these parameters are entered in the principal component analysis.

– Morphological characteristics were noted for each plant after 6 weeks under glasshouse conditions. To distinguish between D.H. regenerated plants and D.H. progeny plants, and to facilitate further analysis and discussion, we use two different notations: (N, FR) for the D.H. regenerated plants themselves (cf material and methods C) and (n, fr) for D.H. progenies.

E) D.H. Progenies Analysed

1 First generation by seeds of D.H.$^{(1)}$ plants. In order to determine the genetic basis of the morphological modifications and to analyse the overall variability of D.H. plants, we selfed most of the D.H.$^{(1)}$ arising from T 21 pollen cultures with (Col^+) or without (Col^-) colchicine treatment: ie. 8N plants from Col^- cultures, 8 N plants and 3 FR plants from Col^+ cultures. We compared these 19 D.H.$^{(1)}$ families with three selfs from the original T21 line (T lines). The characteristics of N and FR ingeritance were studied by means of reciprocal crosses made between 6 D.H.$^{(1)}$, ie. two N plants from Col^-, two N and two FR from Col^+ cultures. We compared the six selfs and the reciprocal crosses made between N plants ($Col^- \times Col^-$, $Col^+ \times Col^+$), FR plants ($Col^+ \times Col^+$), N and Fr plants ($Col^- \times Col^+$), FR (Col^+) and T21 plants.

2 Further generations by seeds of D.H.$^{(1)}$ plants. D.H. characteristics were analysed at successive stages of inbreeding for several N and FR plants derived from T 21 pollen. Two particular schemes of selfing are reported here:

– One N (Col^+) and one FR (Col^+) were selfed continously up to the fourth generation without any external selection for morphological features. A maximum growth rate variability was maintained. We compared on the one hand fourteen N selfs, ie. two S2, four S3, eight S4, and on the other hand twelve FR selfs, ie. two S2, four S3, six S4.

– We selfed one N (Col^-) plant, the first generation of which contained well characterized n and fr plants (see results), up to the third generation, aiming at obtaining stabilized lines for n and fr phenotypes. We compared eight S2 and thirteen S3.

3 Consecutive androgeneses. We analysed the variability which could be induced by consecutive androgeneses carried out from the same D.H.$^{(1)}$. We compared the behavior of D.H.$^{(1)}$ of different origins and phenotypes: one N plant from B 229 pollen, one N and one FR plant from T 21 pollen. Progenies were derived as shown in Table 1.

Table 1. The obtaining of D.H. plants by consecutive androgeneses

Original line	DH$^{(1)}$	DH$^{(2)}$	DH$^{(3)}$	DH$^{(4)}$	DH$^{(5)}$
B229	$\xrightarrow{Col^-}$ (1N) $\xrightarrow{Col^-}$	(2N, 1FR)	–	–	–
	↓	↓			
	1S1	3S1			
T21	$\xrightarrow{Col^-}$ (1N) $\xrightarrow{Col^-}$	(4N)	–	–	–
	↓	↓			
	4S2	4S1			
T21	$\xrightarrow{Col^+}$ (1FR) $\xrightarrow{Col^+}$	(8FR) $\xrightarrow{Col^+}$	(8FR) $\xrightarrow{Col^+}$	(8FR) $\xrightarrow{Col^+}$	(8FR)
	↓	↓	↓	↓	↓
	7S2	8S1	8S1	8S1	8S1

$\Longrightarrow$ Consecutive cycles of androgeneses; N and FR plants are regenerated from either colchicine-treated (Col^+) or no-treated (Col^-) pollen; ⟶ Seed progenies

Results

1 *First Generation by Seeds of D.H.(1) Plants*

Selfings. Table 2 shows the distribution obtained in 22 S1 progenies of N and FR plants, regenerated from col$^-$ and col$^+$ cultures. All the FR selfs are homogeneously crumpled independent of the location they were grown (either fields, greenhouses or Phytotron regulated rooms). However, they differ markedly from each other by the intensity of crumpling and by coloration. On the other hand, the N plants produced heterogeneous progenies in 13 out of 15 cases, with both n and fr plants. In the segregating progenies, the average distribution of n/fr, similar in both col$^-$ and col$^+$, is not statistically different from 3/1 (χ^2 = 1.52). Concerning the growth rates, analysis of variance shows that the families compared are significantly different for all characters although the T group is homogeneous (Table 3). Table 4 shows that the D.H.(1) families have an average growth slower than the T families. Within the D.H.(1) group, the FR families are inferior to the N families; on the other hand, the N (Col$^+$) and the N (Col$^-$) group do not significantly differ, thus the colchicine doesn't have any general effect upon the variation.

Crosses. Crosses between FR plants give only fr homogeneous progenies, with identical phenotypes in the reciprocal crosses. On the other hand, progenies having N plants as parents are heterogeneous: in both (N, N) crosses the

Table 2. Types of progenies obtained in first generation from selfing of N and FR plants

Pollen treatment	Regenerated plants	S1 progenies
Col$^-$	8N	7 heterogeneous 20% fr 1 homogeneous 100% fr
	1FR[a]	homogeneous 100% fr
Col$^+$	7N	6 heterogeneous 18% fr 1 homogeneous 100% fr
	6FR	6 homogeneous 100% fr

[a]Cytomosaic plant (n, 2n), giving a diploid progeny

Table 3. General analysis of variance of T and D.H.(1) plants: F ratios

Source of genetic variation	Weight (mg)	Length (cm)	Width (cm)	Height (cm)
T + D.H.(1)	7.54**	4.06**	4.39**	8.02**
T families only	1.35	< 1	1.87	3.15
D.H.(1) families only	6.44**	3.00**	3.49**	6.45**

Families as described in material and methods E(1): T : 3 original line selfs; D.H.(1): 8N(Col$^-$) selfs, 8N(Col$^+$) selfs, 3 FR (Col$^+$) selfs; 24 plants per family
** $P < 0.01$

Table 4. Mean differences between groups of T and D.H.(1) plants as measured by Scheffé S contrasts

Contrasts	Weight (mg)	Length (cm)	Width (cm)	Height (cm)
T – D.H.(1)	+ 112.4**	+ 3.75**	+ 2.03**	+ 4.99**
N – FR	+ 71.55**	+ 0.88*	+ 0.30*	+ 2.08*
N (Col$^-$) –N(Col$^+$)	– 19.30	– 0.43	– 0.01	– 0.68

Families as for table 3; 24 plants per family
** $P < 0.01$
* $P < 0.05$

Table 5. Comparison of crosses and selfs for five couples of D.H.(1) plants

	Crosses/Selfs				Reciprocal crosses			
Source of genetic variation	Weight (mg)	Length (cm)	Width (cm)	Height (cm)	Weight (mg)	Length (cm)	Width (cm)	Height (cm)
N(Col$^-$), N(Col$^-$)	+149.1**	+1.52**	+0.98**	+0.74	– 66.80	–0.38	–0.05	–0.30
N(Col$^+$), N(Col$^+$)	+ 69.1*	+0.73*	+0.52*	–1.32	– 12.95	+0.76	+0.06	+0.96
FR(Col$^+$), FR(Col$^+$)	+179.3**	+3.83**	+2.95**	+4.33**	+237.8**	–2.27**	–1.46**	–2.02
N(Col$^-$), FR(Col$^+$)	+ 9.5	+1.36**	+0.21	–1.57	+ 61.9	+2.59**	+1.34**	–2.21
N(Col$^+$), FR(Col$^+$)	+ 62**	+0.56	+1.24**	–0.19	– 82.7	–0.38	–0.11	–0.30

For each couple of D.H.(1) are given: differences between mean values of crosses and selfs (+: crosses > selfs), and differences between reciprocal crosses (maternal effect: +; paternal effect: –); 24 plants per family
** $P < 0.01$
* $P < 0.05$

distribution of n/fr was compatible with 3/1, and in both (N, FR) it was compatible with 1/1. There are neither quantitative (i.e. proportions of n and fr plants) nor qualitative (i.e. differences in fr phenotypes) in the reciprocal crosses. (Fig. 1c). The crosses between T 21 and FR plants give homogeneous progenies, all the plants being very similar to the original line plants. Concerning growth rates, Table 5 shows that the D.H.$^{(1)}$ crosses are in most cases superior to the selfs of the same plants. Differences between reciprocal crosses are infrequent and irregular in direction (either maternal or paternal).

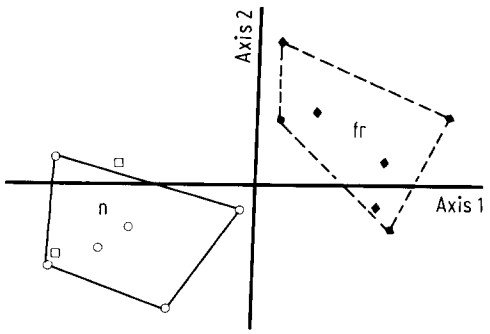

Fig. 2. Segregation amongst S3 progenies derived from one single D.H.$^{(1)}$ plant (N) (see Material and Methods, E 2): projection of the S3 progeny means on the axis (1-2) of principal component analysis. *Axis 1* 38% of total variability, represents mainly precocity and leaf dimensions at young stage (high values on the left). *Axis 2* 21% of total variability, represents mainly leaf dimensions at adult stage (high values down). ○ Six S3 progenies derived from a S2 normal plant (See text), ◆ Seven S3 progenies derived from a S2 crumpled plant; □ T progenies. On axis 1, the contrast between the two groups of S3 progenies is significant at P = 0,01

2 *Further Generations by Seeds of D.H.$^{(1)}$ Plants*

All the FR plants studied remained stable through selfing. Concerning growth characters, Table 6 shows that four consecutive generations of selfing of a FR plant doesn't lead to any creation of variability. On the contrary, the two N plants studied segregate: after the second generation of selfing, differencies between families exist within each generation. Segregation also occurs for the phenotypical changes. From the N (Col$^+$) plant, 4 out of 8 of the S3 families were homogeneously fr, and fixed n lines were obtained in S6. From the N (Col$^-$) plant, we obtained fixed lines immediately after the second generation of selfing: from 8 S2 progenies analysed, 2 were homogeneous for either n or fr (Fig. 1d), 6 others still segregated in a 3/1 ratio. The S3 progenies derived from each of the two homogeneous S2 proved to be stable either for n or fr phenotypes, and the two groups are well separated by a principal component analysis using quantitative measurements only (Fig. 2). The n plants have higher growth rates, greater dimensions at the adult stage and are more precocious than fr plants. A discriminant analysis for n or fr shows that 90% of the S3 plants are correctly classified in one of the two groups by a single quantitative characteristic, such as time of flowering or leaf dimensions at the adult stage. Consequently, the somewhat subjective appreciation of the crumpling can be satisfactorily replaced by numerical data.

3 *Further Cycles of Androgeneses*

In the three cases studied, a second androgenesis from N or FR plants emphasized the D.H. variation in morphology, by either an increase in the proportion of fr plants in

Table 6. Analysis of variance between families within each generation of selfing of D.H.$^{(1)}$ plants: F ratios

Source of genetic variation	Generation	N	Weight (mg)	Length (cm)	Width (cm)
FR (Col$^+$)	S2	2	< 1	1.35	3.20
	S3	4	< 1	< 1	1.04
	S4	8	< 1	< 1	1.45
N (Col$^+$)	S2	2	< 1	< 1	2.25
	S3	4	3.45**	1.10	4.24**
	S4	6	2.39*	1.56	2.77**
N (Col$^-$)	S2	8	5.40**	4.82**	4.25**
	S3	13	4.49**	3.19**	2.52**

Plant material as described in material and methods E(2), N number of selfs per generation; 24 plants per family
**P < 0.01
* P < 0.05

Table 7. Mean differences between D.H.(1) and D.H.(2) families issued from the same D.H.(1) plant

Original D.H.(1) plant	Weight (mg)	Length (cm)	Width (cm)	Height (cm)
T21, N (Col^-)	+ 29*	+ 0.72*	+ 0.35*	+ 0.88
B 229, N (Col^-)	+ 100**	+ 2.47**	+ 0.85**	+ 5.07**
T 21, FR (Col^+)	+ 1	+ 0.90**	+ 0.25**	+ 5.44**

Plant families as described in material and methods E(3).
D.H.(1) > D.H.(2): +
** $P < 0.01$
* $P < 0.05$

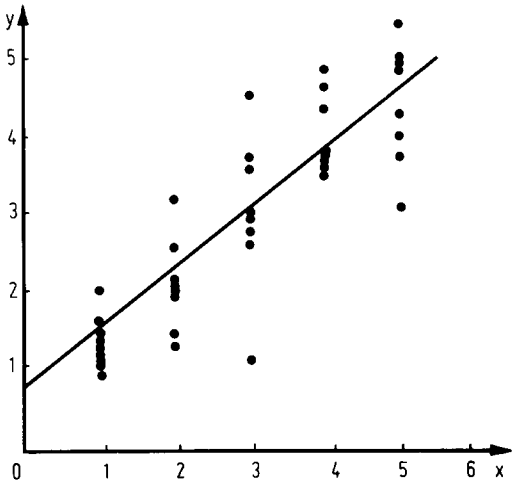

Fig. 3. Evolution through consecutive androgeneses. Graph of the number of D.H. cycles (y) predicted by a multiple linear regression calculated on the family means of young stage measurements, against the actual number of D.H. cycles (x)

D.H.(2) progenies or by accentuation of the crumpling. In each case, a decrease of growth rates is observed from the D.H.(1) to the D.H.(2) group of families (Table 7). The drift opposite the original line values continues up to the fifth cycle of androgenesis for the FR plant studied: in this case, decrease of vigor and creation of variability clearly result from the D.H. process itself and there is a negative correlation between growth rates and D.H. cycles. Fig. 3 shows, for each D.H. progeny, the relation between actual number of androgenesis achieved and the number of androgeneses predicted by a multiple linear regression calculated on growth rate measurements (R = 0.87).

Discussion

The D.H. plants regenerated from *Nicotiana sylvestris* pollen cultures differ genetically from the parent line by a number of features relating to morphology and growth rates, that we call 'D.H. variation'. Each D.H. possesses its own phenotype, which is more or less different from that of the original line plants, that is revealed similarly in all growing conditions (Gif-sur-Yvette greenhouses, Phytotron-regulated rooms or open fields). The variation is not specifically induced by the colchicine sometimes used to enhance the regeneration of diploid plants, and it is inherited without reciprocal effects, ie. on an apparent nuclear mode. Certain D.H., 'crumpled FR', behave as true homozygous, producing S1 homogeneous crumpled progenies (fr plants), and remain stable during successive selfings. Other D.H., more closely related to original line plants, and called 'normal N', in general behave as heterozygous (13 out of 15 cases studied) and produce S1 heterogeneous progenies with n and fr plants. They segregate both for morphology and growth rate into further generations of selfing. The distributions of n/fr plants are compatible with a 3/1 segregation, with n dominant or semidominant over fr. Continuous selfing of a N plant produce stable n and fr lines in addition to families segregating in a 3/1 ratio. This doesn't mean that a unique gene is responsible for the multiple modifications shown by the D.H.. Numerous genes or alleles are probably necessary to account for the variability of expression and intensity of crumpling, and for the almost continuous variation observed for growth rate characteristics. However, the parts of the genome concerned have to be sufficiently closely linked in order to behave as a single unit of segregation and recombination at a first approximation.

Consecutive androgeneses increase the D.H. variation: the D.H.(2) ... D.H.(5) plants derived by consecutive pollen cultures from one single D.H.(1) plant (issued from original line pollen), show such new growth depressive effects and increased morphological abnormalities as reduced dimensions of leaves and flowers.

What mechanisms could account for these unexpected results? Different authors have already described changes in D.H. lines. They generally involve mutations (Oinuma and Yoschida 1975; Burk and Matzinger 1976), cytoplasmic heredity (Truong 1977), or residual heterozygosity in the parent line (Burk and Matzinger 1976). Let us examine these different hypotheses, in view of the facts previously described.

Mutations: The conditions under which the *N. sylvestris* D.H. were obtained by direct embryogenesis on a medium devoid of growth substances, minimise the risk of chromosomal mutations (inversions, translocations, etc.). On the other hand, all the regenerated D.H. differ genetically somewhat from the line of origin, and in the same features, ie. crumpled aspect and slow growth. If we want to use the world 'mutation', it should be systematic mutation, not random mutation, occurring more or less in the same chromosome region, with similar overall effects.

Cytoplasmic variations are generally maternally inherit-

ed. Certain D.H. plants transmit the crumpled feature to all their progeny in selfing and crossing, without any reciprocal effects. Consequently, this second origin of variations would not be a satisfying explanation of the results.

A residual heterozygosity in the original *N. sylvestris* line may lead to some variability among the D.H. selfs, some loss of vigor as an effect of inbreeding, and some 'hybrid' vigor in the families derived from crosses. Indeed we do obtain results in agreement with this, and some concealed variability could exist within our parental line. However this hypothesis alone is not sufficient as it takes into account neither the origin of the crumpled feature (never observed in the original line among several hundred of plants studied), nor the segregations obtained in certain D.H. progenies, nor the new drift after further D.H. processes.

The same arguments go against two other hypotheses:

1) some heterozygosity maintained in the original line by 'zygotic lethal gene combination': in this case, new androgenic rounds from one D.H.(1) plant would not lead to any supplementary variation because the D.H.(1) would be already homozygous for the lethal gene.

2) Part of the diploid regenerated plants come from unreduced microspores, as described by Wenzel et al. (1976), because all D.H. plants obtained are different from the pollen donor plant (original line plant or D.H.): either they are N and look like original line plants, but they give segregating selfed progenies comprising fr plants (such a segregation is never observed in the original line); or they are FR and give stable modified progenies. At last, haploid plants were doubled either by in vitro subcultures or by colchicine treatment of meristematics buds; the diploid plants obtained revealed variation similar to the D.H. plants described in this paper (Ph. Patry, unpublished results).

All the results could be explained if nuclear DNA was in some respect different in generative G and vegetative V pollen cells. Differences in DNA organisation are known to exist between macro- and micro-nuclei of ciliates as *Tetrahymenas* or *Oxytricha* (Lauth et al. 1976). In *Nicotiana sylvestris* pollen, the changes would occur mainly in a limited part of the genome and would be inherited as a single linkage group. Diploid regenerated plants would originate either from the doubling of the V genome, by endomitosis or nuclear fusion, behaving as homozygous FR; or from nuclear fusion between V and G (or derived nuclei), behaving as heterozygous N. Nuclear fusion seems to occur in *Nicotiana tabacum* or *Datura innoxia* pollen cultures having triploid regenerated plants as a possible result (Engvild 1974; Sunderland et al. 1974). We sometimes observed the simultaneous division of V and G nuclei in the same pollen grain. On the other hand, that triploid plants were regenerated does show that some mechanism of nuclear fusion probably takes place. The proposed model could explain others particularities of the D.H. variation. The diploidising agent colchicine should increase the proportion of D.H. resulting from endomitosis, therefore homozygous and crumpled, a fact that we have indeed observed (De Paepe and Pernes 1978). Finally, consecutive androgeneses should emphasize the modifications. Several questions remain open: what could be the origin of DNA differences in V and G nuclei? What parts of the genome are involved? Experiments are now in progress to demonstrate the changes at the DNA molecular level. The first results show that DNA reassociation kinetics are modified in several D.H. families, obtained after, or without, colchicine treatment of the pollen. The extent of zero-time binding sequences on hydroxyapatite column is twice as large in D.H. DNA (De Paepe and Huguet, in preparation).

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